Application
to
Food Standards Australia New Zealand
for the Inclusion of
Cotton MON 88701
in Standard 1.5.2 - Food Derived from Gene Technology

Submitted by:

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UNPUBLISHED REPORTS BEING SUBMITTED

[redacted]

[redacted] 2011. Stability of the DNA Insert and Expression of MON 88701 DMO and PAT (*bar*) Proteins in MON 88701. MSL0023322. Monsanto Company.

[redacted] 2011. Segregation Analysis of the Coding Sequences Present in Herbicide Tolerant Cotton MON 88701 Across Multiple Generations. RPN-2011-0089. Monsanto Company.

[redacted] 2011. Bioinformatics Evaluation of the Transfer DNA Insert in MON 88701 Utilizing the AD_2011, TOX_2011 and PRT_2011 Databases. MSL0023565. Monsanto Company.

[redacted] 2012. Amended Report for MSL0023585: Bioinformatics Evaluation of DNA Sequences Flanking the 5' and 3' Junctions of Inserted DNA in MON 88701: Assessment of Putative Polypeptides. MSL0024371. Monsanto Company.

[redacted] 2012. Characterization of Dicamba Mono-oxygenase (DMO) Protein Purified from the Cottonseed of MON 88701 and Comparison of the Physicochemical and Functional Properties of the Plant-Produced and *Escherichia coli*-Produced DMO Proteins. MSL0023517. Monsanto Company.

[redacted] 2012. Characterization of Phosphinothricin N-Acetyltransferase (*bar*) Protein Purified from the Cottonseed of MON 88701 and Comparison of the Physicochemical and Functional Properties of the Plant-Produced and *Escherichia coli*-Produced PAT (*bar*) Proteins. MSL0023428. Monsanto Company.

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[redacted] 2012. Bioinformatics Evaluation of the DMO Protein in MON 88701 Utilizing the AD_2011, TOX_2011 and PRT_2011 Databases. MSL0023516. Monsanto Company.

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[redacted] [redacted] 2011. Effect of Heat Treatment on the Functional Activity of *E. coli*-Produced MON 88701 DMO Protein. MSL0023606. Monsanto Company.

[redacted] [redacted] 2011. The Effect of Heat Treatment on the Functional Activity of *E. coli*-Produced Phosphinothricin N-acetyltransferase (*bar*) Protein. MSL0023584. Monsanto Company.

[redacted] [redacted] 2012. Summary of the Magnitude of Residues of Dicamba in Undelinted Cotton Seed after Application to MON 88701. MSL0024066. Monsanto Company.

[redacted] [redacted] 2012. Amended Report for MSL0024393: Compositional Analyses of Cottonseed Collected from MON 88701 treated with Dicamba and Glufosinate Grown in the United States during 2010. MSL0024606. Monsanto Company.

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ABBREVIATIONS AND DEFINITIONS[1]

symbol or abbrev.	definition
~	Approximately
α-Cyano	α-Cyano-4-hydroxycinnamic acid
a.e.	acid equivalent
AA	Amino Acid
AAbA	α-aminobutyric acid
ADF	Acid Detergent Fiber
AD_2011	Allergen, gliadin, and glutenin protein sequence database (Release date February 18, 2011)
APHIS	Animal and Plant Health Inspection Service of the United States Department of Agriculture
bar	Bialaphos Resistance Gene from *Streptomyces hygroscopicus*
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
CFR	Code of Federal Regulations
CHT	Ceramic hydroxyapatite
CoA	Coenzyme A
CTAB	Hexadecyltrimethylammonium bromide
COA	Certificate of Analysis
DAP	Days After Planting
Da	Dalton
dCTP	Deoxycytidine triphosphate
DEAE-	Diethylaminoethyl-
DHB	2,5-dihydroxybenzoic acid
DCSA	3,6-dichlorosalicylic acid
DDI	Daily Dietary Intake
dicamba	3,6-dichloro-2-methoxybenzoic acid
dmo	Mono-oxygenase gene from *Stenotrophomonas maltophilia*
DMO	Dicamba mono-oxygenase
DNA	Deoxyribonucleic acid
DTNB	5,5'-dithio-bis (2-nitrobenzoic acid)
DTT	Dithiothreitol
dw	Dry weight
DWCF	Dry weight conversion factor
ECL	Enhanced Chemiluminescence
E. coli	*Escherichia coli*
E.coli-produced MON 88701 DMO	DMO protein produced from *E. coli* with the same sequence as MON 88701 DMO
ELISA	Enzyme-linked Immunosorbent Assay
EPA	Environmental Protection Agency

[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.

E-Score	Expectation score
FA	Fatty Acid
FARRP	Food Allergy Research and Resource Program
FASTA	Algorithm used to find local high scoring alignments between a pair of protein or nucleotide sequences
FDA	Food and Drug Administration (U.S.)
FFDCA	Federal Food, Drug and Cosmetic Act (U.S.)
FT	Flow through
fw	Fresh weight
glufosinate	butanoic acid, 2-amino-4-(hydroxymethylphosphinyl)
GLP	Good Laboratory Practice
g	Gram
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Peroxidase
HU	Hemagglutinating Unit
ILSI	International Life Sciences Institute
kb	Kilobase
kDa	Kilodalton
kg	Kilogram
LB	Laemmli buffer
LOD	Limit of Detection
LOQ	Limit of Quantitation
MALDI-TOF-MS	Matrix Assisted Laser Desorption Ionization - Time of Flight Mass Spectrometry
µg	Microgram
mg	Milligram
MOE	Margin of Exposure
MON 88701 DMO	DMO protein produced in MON 88701
MW	Molecular Weight
MWCO	Molecular Weight Cutoff
N-acetyl glufosinate	2-acetamido-4-methylphosphinico-butanoic acid
NADH	Nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information at the National Institutes of Health, Bethesda, MD, USA
NDF	Neutral Detergent Fiber
NFDM	Non-fat Dried Milk
NOAEL	No Observable Adverse Effect Level
OECD	Organisation for Economic Co-operation and Development
ORF	Open Reading Frame
OSL	Overseason Leaf
p	Probability from PRESS
PAT	Phosphinothricin N-acetyltransferase
PAT (*bar*)	PAT protein produced by the *bar* gene
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline containing Tween-20
PCR	Polymerase Chain Reaction
PI	Prediction Interval

ppm	parts per million
PPT	Phosphinothricin
PRESS	Predicted Residual Sum of Squares
PRT_2011	GenBank protein database, 181.0 (Released December 18, 2010)
PTH	Phenylthiohydantoin
PVDF	Polyvinylidene difluoride
PVP	Polyvinyl pyrrolidone
RBD	Refined, Bleached, and Deodorised
RED	Reregistration Eligibility Decision
RT	Room temperature
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SE	Standard Error
SGF	Simulated Gastric Fluid
S. hygroscopicus	*Streptomyces hygroscopicus*
SIF	Simulated Intestinal Fluid
Sinapinic Acid	3,5-dimethoxy-4-hydroxycinnamic acid
S. maltophilia	*Stenotrophomonas maltophilia*
SOP	Standard Operating Procedure
TBA	Tris-borate buffer with L-ascorbic acid
TBS	Tris Buffered Saline
TCEP	Tris(2-carboxyethyl)phosphine
T-DNA	Transfer DNA
TDF	Total Dietary Fiber
TFA	Trifluoroacetic Acid
TFE	2,2,2,-trifluoroethanol
TIU	Trypsin Inhibitor Unit
Tm	Melting temperature
TNB	5-thio-nitrobenzoate
TOX_2011	Toxin protein sequence database (Release date February 18, 2011)
V	volts
v/v	volume to volume ratio
w/v	weight to volume ratio

Part 1 GENERAL INFORMATION

1.1 Applicant Details

(a) Applicant's name/s	[redacted]
(b) Company/organisation name	Monsanto Australia Limited
(c) Address (street and postal)	12 / 600 St Kilda Road, Melbourne, Victoria, 3004 PO Box 6051, St Kilda Road Central, Victoria, 8008
(d) Telephone and facsimile numbers	[redacted]
(e) Email address	[redacted]
(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

1.2 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 MON 88701 cotton and products containing MON 88701 cotton (hereafter referred to as MON 88701) to the Table to Clause 2 (see below).

Food derived from gene technology	Special requirements
Food derived from herbicide tolerant cotton line MON 88701	None

1.3 Justification for the Application

1.3(a) The need for the proposed change

Monsanto Company has developed dicamba and glufosinate-tolerant cotton, MON 88701, that will permit in-crop applications of dicamba and glufosinate herbicide. Both herbicides provide a unique mode-of-action for effective weed management, including the control of glyphosate-resistant weeds. The in-crop use of dicamba and glufosinate herbicides, when used in combination with glyphosate herbicide, provides new weed management options in cotton, to control a broad spectrum of grass and broadleaf weed species and effective control of weeds resistant to several herbicide families.

1.3(b) The advantages of the proposed change over the status quo, taking into account any disadvantages

MON 88701 will be combined, through traditional breeding methods, with other approved herbicide-tolerant (*i.e.* glyphosate) events. MON 88701 may also be combined, through traditional breeding methods with previously approved insect-protected cotton events. Successful integration of MON 88701 into the glyphosate-tolerant cotton system will: 1) provide growers with an opportunity for an efficient, effective weed management system for hard-to-control and herbicide resistant weeds; 2) provide a flexible system for two additional in-crop herbicide modes-of-action in current cotton production practices as recommended by weed science experts to manage future weed resistance development; and 3) provide cotton growers with additional weed management tools to enhance weed management systems necessary to maintain yield and quality to meet the growing needs of the food, feed, and industrial markets.

1.4 Regulatory Impact Information

1.4(a) Costs and benefits

If the draft variation to permit the sale and use of food derived from MON 88701 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 cotton products.
- There is unlikely to be any significant increase in the prices of foods if manufacturers are able to use comingled cotton products.

- Consumers wishing to do so will be able to avoid GM cotton products as a result of labeling requirements and marketing activities.

Industry and business in general:

- There are benefits to cotton growers through the option to use new weed management options in cotton to control a broad spectrum of grass and broadleaf weed species and effective control of weeds resistant to several herbicide families.
- The cotton industry (including Monsanto) will benefit through implemention of a system which will enhance the sustainability of existing cotton products and promote good stewardship in the area of weed management.
- Sellers of processed foods containing cotton derivatives would benefit as foods derived from cotton MON 88701 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 cotton products or imported foods manufactured using cotton derivatives.
- Possible cost to food industry as some food ingredients derived from cotton MON 88701 would be required to be labelled.

Government:

- Benefit that if cotton MON 88701 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 cotton MON 88701 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.

1.4(b) Impact on international trade

If the draft variation to permit the sale and use of food derived from MON 88701 was rejected it would result in the requirement for segregation of any cotton derived products containing MON 88701 from those containing approved cotton, which would be likely to increase the costs of imported cotton derived foods.

It is important to note that if the draft variation is approved, cotton MON 88701 will not have a mandatory introduction. The consumer will always have the right to choose not to use/consume this product.

1.5 Assessment Procedure

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

1.6 Exclusive Capturable Commercial Benefit

This application is likely to result in an amendment to the Code that provides exclusive benefits and therefore Monsanto intends to pay the full cost of processing the application.

1.7 International and Other National Standards

1.7(a) International standards

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* (Codex Alimentarius, 2009).

In addition, the composition analysis is conducted in accordance with OECD guidelines and includes the measurement of OECD-defined cotton nutrients and anti-nutrients based on conventional commercial cotton varieties (OECD, 2009).

1.7(b) Other national standards or regulations

Monsanto has submitted a food and feed safety and nutritional assessment summary for MON 88701 to the United States Food and Drug Administration (FDA) and has also requested a Determination of Nonregulated Status for MON 88701, including all progenies derived from crosses between MON 88701 and other cotton, from the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA).

Applications have also been submitted to Canadian Food Inspection Agency (CFIA) and Health Canada (HC), Korea Food and Drug Administration (KFDA) for food, and Rural Development Administration (RDA) for feed use, and Japan's Ministry of Health, Labour, and Welfare (MHLW) for food use.

Regulatory submissions will be made to countries that import significant cotton or food and feed products derived from countries where MON 88701 cotton will be grown and have functional regulatory review processes in place. This will result in submissions to a number of additional governmental regulatory agencies including, but not limited to Ministry of Agriculture, People's Republic of China; Japan's Ministry of Agriculture, Forestry, and Fisheries, the European Food Safety Authority (EFSA), Mexico; as well as to regulatory authorities in other cotton importing countries with functioning regulatory systems.

Part 2 SPECIFIC DATA REQUIREMENTS FOR SAFETY ASSESSMENT

A. TECHNICAL INFORMATION ON THE GM FOOD

A1 Nature and Identity of the genetically modified food

A1(a) A description of the new GM organism

Monsanto Company has developed dicamba and glufosinate-tolerant cotton, MON 88701, that will permit in-crop applications of dicamba herbicide for the control of broadleaf weeds from pre-emergence to seven days pre-harvest and glufosinate herbicide for broad spectrum weed control from emergence through early bloom growth stage. Both herbicides provide a unique mode-of-action for effective weed management, including the control of glyphosate-resistant weeds. MON 88701 will be combined, through traditional breeding methods, with other approved herbicide-tolerant (*i.e.* glyphosate) events. MON 88701 may also be combined, through traditional breeding methods with previously approved insect-protected cotton events. The in-crop use of dicamba and glufosinate herbicides, when used in combination with glyphosate herbicide, provides new weed management options in cotton, to control a broad spectrum of grass and broadleaf weed species and effective control of weeds resistant to several herbicide families.

MON 88701 contains a demethylase gene from *Stenotrophomonas maltophilia* that expresses a dicamba mono-oxygenase (DMO) protein to confer tolerance to dicamba herbicide and a bialaphos[2] resistance (*bar*) gene from *Streptomyces hygroscopicus* that expresses the phosphinothricin N-acetyltransferase (PAT) protein to confer tolerance to glufosinate herbicide. DMO protein rapidly demethylates dicamba to the herbicidally inactive metabolite 3,6-dichlorosalicylic acid (DCSA). DCSA has been previously identified as a metabolite of dicamba in cotton, soybean, livestock and soil. PAT (*bar*) protein acetylates the free amino group of glufosinate to produce the herbicidally inactive metabolite 2-acetamido-4-methylphosphinico-butanoic acid (N-acetyl glufosinate).

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 88701 has been assigned the unique identifier MON-887Ø1-3.

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

Cotton containing the transformation event MON 88701 will be produced in North America and Australia. There are currently no plans to produce this product in New Zealand. A

[2] Bialaphos is a bacterial tripeptide composed of L-phosphinothricin (PPT) plus two alanines. *In vivo* the alanines are removed to produce L-PPT, a naturally occurring glutamate analogue with herbicidal activity through the inhibition of glutamine synthetase. Glufosinate is a synthetically produced racemic mixture of D and L-PPT.

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

Other than the introduction of the dicamba and glufosinate tolerant trait, MON 88701 is not materially different from conventional cotton and can be processed into four major products: oil, meal, hulls and linters. Only cottonseed oil and linters are utilised as food sources. For a further description of food uses and processing of oil and linters, refer to Section A2(b)(iii) and Section A2(b)(iv).

A2. History of Use of the Host and Donor Organisms

A2(a) Description of all donor organism(s)

A2(a)(i) Common and scientific names and taxonomic classification

The *dmo* gene is derived from the bacterium *Stenotrophomonas maltophilia* strain DI-6, isolated from soil at a dicamba manufacturing plant (Krueger et al., 1989). *S. maltophilia* was originally named *Pseudomonas maltophilia*, and then transferred to the genus *Xanthomonas* before it was given its own genus (Palleroni and Bradbury, 1993). The taxonomy of *S. maltophilia* is (Palleroni and Bradbury, 1993; Ryan et al., 2009):

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Xanthomonadales

Family: Xanthomonadaceae

Genus: Stenotrophomonas

The *bar* gene is derived from the bacterium *Streptomyces hygroscopicus* (Thompson et al., 1987). The taxonomy of *S. hygroscopicus* is (Waksman and Henrici, 1943):

Kingdom: Bacteria

Phylum: Actinobacteria

Class: Actinobacteria

Order: Actinomycetales

Family: Streptomycetaceae

Genus: Streptomyces

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

Strains of *S. maltophilia* have been found in the transient flora of hospitalised patients as a commensal organism (Echemendia, 2010). *S. maltophilia* can be found in healthy individuals without causing any harm to human health (Denton et al., 1998) and infections in humans caused by *S. maltophilia* are extremely uncommon (Cunha, 2010). Similar to the indigenous bacteria of the gastrointestinal tract, *S. maltophilia* can be an opportunistic pathogen (Berg, 1996). As such, *S. maltophilia* is of low virulence in immuno-compromised patients where a series of risk factors (severe debilitation, the presence of indwelling devices such as ventilator tubes or catheters, for prolonged periods of time and prolonged courses of antibiotics) must occur for colonisation by *S. maltophilia* in humans (Ryan et al., 2009). Therefore, infections by *S. maltophilia* almost exclusively occur in hospital settings, in which case they are only present in a minimal percentage of infections (Ryan et al., 2009). Finally, *S. maltophilia* has not been reported to be a source of allergens.

The ubiquitous presence of *S. maltophilia* in the environment, the presence in healthy individuals without causing infections, the incidental presence in foods without any adverse safety reports, and the lack of reported allergenicity establishes the safety of the donor organism.

S. hygroscopicus is a saprophytic, soil-borne bacterium with no known safety issues. *Streptomyces* species are widespread in the environment and present no known allergenic or toxicity issues (Kämpfer, 2006; Kutzner, 1981), though human exposure is quite common (Goodfellow and Williams, 1983). *S. hygroscopicus* is not considered pathogenic to plants, humans or other animals (Cross, 1989; Goodfellow and Williams, 1983; Locci, 1989). *S. hygroscopicus* history of safe use is discussed in Hérouet et al., (2005) and this organism has been extensively reviewed during the evaluation of several glufosinate-tolerant events with no safety or allergenicity issues identified by a number of regulatory agencies, including FSANZ[3].

The ubiquitous presence of *S. hygroscopicus* in the environment, the widespread human exposure without any adverse safety or allergenicity reports, and the successive reviews of several glufosinate-tolerant events by regulators have identified no safety or allergenicity issues further establishes the safety of the donor organism.

[3]A372, A533, A589, A1028, A1040: http://www.foodstandards.gov.au

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

S. maltophilia is an aerobic, environmentally ubiquitous gram negative bacterium commonly present in aquatic environments, soil, and plants. *S. maltophilia* is ubiquitously associated with plants and has been isolated from the rhizosphere of wheat, maize, grasses, beet, cucumber, potato, strawberry, sugarcane, and rapeseed (Berg et al., 1996; Berg et al., 1999; Berg et al., 2002; Denton et al., 1998; Echemendia, 2010; Juhnke and des Jardin, 1989; Juhnke et al., 1987; Lambert et al., 1987). *S. maltophilia* has also been isolated from cottonseed, bean pods, and coffee (Nunes and de Melo, 2006; Swings et al., 1983); thus, *S. maltophilia* can be found in a variety of foods and feeds. *S. maltophilia* is also widespread in the home environment and can be found around sponges, flowers, plants, fruits, vegetables, frozen fish, milk, and poultry (Berg et al., 1999; Denton and Kerr, 1998; Echemendia, 2010).

S. hygroscopicus is a saprophytic, soil-borne bacterium with no known safety issues. *Streptomyces* species are widespread in the environment and present no known allergenic or toxicity issues (Kämpfer, 2006; Kutzner, 1981), though human exposure is quite common (Goodfellow and Williams, 1983). *S. hygroscopicus* is not considered pathogenic to plants, humans or other animals (Cross, 1989; Goodfellow and Williams, 1983; Locci, 1989).

The ubiquitous presence of *S. hygroscopicus* in the environment, the widespread human exposure without any adverse safety or allergenicity reports, and the successive reviews of several glufosinate-tolerant events by regulators have identified no safety or allergenicity issues and further establishes the safety of the donor organism.

A2(b) Description of the host organism

A2(b)(i) Phenotypic information

Cotton is a dicot, with production generally carried out with seeds. Germination occurs when appropriate temperature and moisture conditions have been met. Additional cotton biological characteristics are listed below as defined by Oosterhuis and Jernstedt (1999), unless otherwise cited. The plant sprouts by emergence of the radicle through the micropyle of the seed. The primary root develops from the radical. The hypocotyl elongates, carrying the cotyledons upward and out of the soil.

The cotton plant has a taproot which, according to the age of the plant and soil characteristics, may reach a depth of around three meters in appropriate conditions. Numerous lateral roots grow from the main root which, in turn, branch out.

Under agricultural production practices, the cotton plant typically reaches approximately 1 to 1.5m in height (OECD, 2008). Initial above-ground growth is slow, but following the attainment of 4 to 5 expanded internodes, the plant grows vigourously. The number of branches varies depending on variety and environmental conditions. The plant has a prominent, upright main stem, monopodial in growth, which bears the branches. Leaves are arranged in a spiral around the main axis of the stem and branches. Phylotaxis is 3/8 of a turn around the stem or branch relative to the last leaf.

Two types of branches are produced: monopodial, usually at lower nodes only, and sympodial. Like the main stem, monopodial branches produce flowers and fruit only on attached sympodial branches. Sympodial branches nodes adopt a more horizontal position than vegetative branches. Sympodial branches have a slightly zig-zagging appearance. Fruits are borne only on sympodial branches.

Most cotton leaves above the lower main stem nodes have 3 to 5 lobes with the degree of indentation highly variable according to genotype. Their surface contains a large number of pores or stomata, located mostly on the under-surface (OECD, 2008). The typical petiole length is as long as leaf, and its point of insertion in the stem or branch is flanked by two small stipules (OECD, 2008).

Each sympodial branch produces flower buds. Three triangular bracts occur outside of the flower (OECD, 2008). The structure of the cotton flower consists of five mostly fused sepals that form the calyx, enclosing the five petals of the sympetalous corolla. A fused staminal column surrounds the style. The pistil is composed of the ovary, containing 3 to 5 carpels; the style; and the stigma. Each carpel defines one locule and contains multiple ovules. Each carpel may contain approximately 8 to 15 ovules occurring in two rows. Once fertilised, the fruit of the cotton plant is called a boll (OECD, 2008). Bolls are spherical or ovoid, varying in shape and size in each species (OECD, 2008). Compared to *G. hirsutum*, *G. barbadense* bolls tend to appear darker green and have more oleiferous glands (OECD, 2008). Cotton fibers are single-celled hairs arising from the epidermis of the fertilised ovule, or seed (OECD, 2008). The embryo cells contain cottonseed oil in their cytoplasm (OECD, 2008).

Gossypol-containing pigment glands occur in most parts of the cotton plant, including the seeds, roots, leaves, and flower buds (OECD, 2008).

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

Cotton is the leading plant fibre crop produced in the world. Most of the world's cotton production is grown in China (30.50 million bales), India (26.40 million bales), United States (18.1 million bales), Brazil (9.00 million bales), Pakistan (8.64 million bales), Australia (4.20 million bales) and Uzbekistan (4.10 million bales) (USDA-FAS, 2010). Cotton is grown primarily for the value of the fibre with cottonseed being a by-product. Cotton fibre accounts for almost 50% of the world textile fibres, with approximately 80% of the cotton fibre market made up of apparel and household products. Commercial cotton has been extensively characterised and has a long history of agricultural production (OECD, 2008; USDA-AMS, 2001). A short review of the biology and growth and development of cotton is available in the literature (Brubaker et al., 1999; Lee, 1984; OGTR, 2008). *Gossypium hirsutum* is the predominant species globally (Lee, 1984; OECD, 2008). Cotton fibre is used for cordage and other non-woven products, as well as for textiles.

Cottonseed is processed into four major products as follows: oil, meal, hulls, and linters. Processing of cottonseed typically yields (by weight): 16% oil, 45% meal, 26% hulls, and 9% linters, with 4% lost during processing (Cherry, 1983). Cottonseed oil has been used safely for human food for more than 100 years in most cotton producing countries as a major contributor to edible oil (NCPA, 1993). Cottonseed oil is premium quality oil used for a variety of food uses, including frying oil, salad, and cooking oil, mayonnaise, salad dressing, shortening, margarine, and packing oil. Previous studies have shown that the resulting oil contains no detectable protein (Reeves and Weihrauch, 1979). Cottonseed meal and hulls from the seed are not used for human consumption, but principally are sold as feed for livestock. The presence of gossypol and cyclopropenoid fatty acids in cottonseed also limits its use as a protein supplement in animal feed except for cattle, which are unaffected by these components. Inactivation or removal of these components during processing enables the use of some cottonseed meal for ruminants, where most other farm animals (monogastric animals) are not fed cottonseed meal to any appreciable level. Cottonseed meal is principally sold as feed for livestock (NCPA, 2002).

An additional by-product of cotton production is linters, which are the short fibers on the cottonseed. Linters consist of nearly pure (*i.e.*, >99%) cellulose (NCPA, 2002; Nida et al., 1996); after extensive processing at alkaline pH and temperatures >100 ºC (AOCS, 2009), the linters can be used as a high fiber dietary product. Food uses include fiber supplements, casings for processed meats, binders for solids in the pharmaceutical industry, and the improvement of viscosity in products such as salad dressings (NCPA, 2002).

See also Section A2(b)(iii) and Section A2(b)(iv).

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

After ginning to remove fibres for textile manufacturing, cottonseed is processed into four major products: oil, meal, hulls, and linters. Processing of cottonseed typically yields (by weight): 16% oil, 45% meal, 26% hulls, and 9% linters, with 4% lost during processing (Cherry, 1983). Only cottonseed oil and linters are utilised as food sources, both are further discussed below.

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

Cottonseed is highly processed during the production of oil and meal. After hulling, the cottonseed is flaked by a rolling process to facilitate oil removal. Prior to oil extraction, the flakes are heated at temperatures of 88 ºC to greater than 130 ºC to break down the cell walls, reduce the viscosity of oil, inactivate proteins, and detoxify gossypol (Harris, 1981; NCPA, 1993). After heating, oil is typically removed from the meal by direct solvent extraction with hexane. Crude cottonseed oil is further processed with refining, bleaching and deodorisation steps to produce high purity vegetable oil. Temperatures up to 230 ºC are used in the deodorisation process (Harris, 1981; NCPA, 1993).

Further processing (refining) for all the uses of cottonseed oil includes deodorisation and bleaching. Deodorisation greatly reduces the cyclopropenoid fatty acid content of the oil due to extreme pH and temperature conditions (NCPA, 1993). A winterisation step is added to produce cooking oil, whereas for solid shortening a hydrogenation step is added to transform the liquid oil into a solid fat. Previous studies have shown that the resulting oil contains no detectable protein (Reeves and Weihrauch, 1979). Cottonseed oil is traded as premium quality oil that is used for a variety of food uses, including frying oil, salad and cooking oil, mayonnaise, salad dressing, shortening, margarine, and packing oil.

The material left after the extraction of the crude cottonseed oil is the cottonseed meal. The gossypol levels in the meal after extraction are reduced by approximately half.

Linters are the short fibres on American upland cottonseed that remain after the long fibres have been removed at the ginning process for textile manufacturing. Linters consist of nearly pure (*i.e.*, >99%) cellulose (NCPA, 2002; Nida et al., 1996) and after extensive processing at alkaline pH and temperatures >100 ºC (AOCS, 2009), the linters can be used as a high fibre dietary product. Food uses include fibre supplement, casings for processed meats, binder for solids in the pharmaceutical industry, and to improve viscosity in products such as toothpaste, ice cream, and salad dressings (NCPA, 2002). The highest grade linters can also be used in the manufacturing of absorbent cotton, medical pads, and gauze (NCPA, 2002), however as mentioned earlier these would consist of nearly pure cellulose, with negligible amounts of protein.

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

It is anticipated that MON 88701will be generally consumed in cotton products in Australia and New Zealand. As described in Section A2(b)(iv), cotton can be processed into food products including oil used for frying, cooking, salad dressing, mayonnaise, margarine and packing or linters used in processed meats, toothpaste, ice cream and salad dressings.

A3 The Nature of the Genetic Modification

A3(a) Method used to transform host organism

MON 88701 was developed through *Agrobacterium*-mediated transformation of PV-GHHT6997 (Figure 4) into cotton hypocotyls, based on published methods (Duncan, 2010; Duncan and Ye, 2011). In summary, hypocotyl segments were excised from dark grown seedlings of germinated Coker 130 seed. After co-culturing with the Agrobacterium carrying the vector, the hypocotyl segments were placed on a sequence of media for callus growth containing carbenicillin and cefotaxime to inhibit the growth of excess Agrobacterium and glufosinate to inhibit growth of untransformed cells. The somatic embryos developing on the culture medium were then placed on medium that contained plant growth regulators conducive to shoot regeneration, but no antibiotics or glufosinate. Rooted plants (R0) with normal phenotypic characteristics were selected and transferred to soil for growth and further assessment.

The R0 plants generated through the *Agrobacterium*-mediated transformation were self-pollinated to produce R1 seed. R0 and R1 plants were evaluated for tolerance to dicamba and glufosinate and screened for the presence of the T-DNA (dmo and bar expression cassettes) and absence of plasmid vector backbone (oriV). Subsequently, the dmo and bar homozygous positive R1 plant was self-pollinated to give rise to R2 plants. Homozygous positive R2 plants containing only a single T-DNA insertion, were identified by a combination of analytical techniques including dicamba and glufosinate sprays, polymerase chain reaction (PCR), and Southern blot analysis, resulting in production of dicamba and glufosinate-tolerant cotton MON 88701. MON 88701 was selected as the lead event based on superior phenotypic characteristics and its molecular characteristics. Studies on MON 88701 were initiated to further characterise the genetic insertion and the expressed proteins, and to establish the food, feed, and environmental safety relative to conventional cotton. The major steps involved in the development of MON 88701 are depicted in Figure 1.

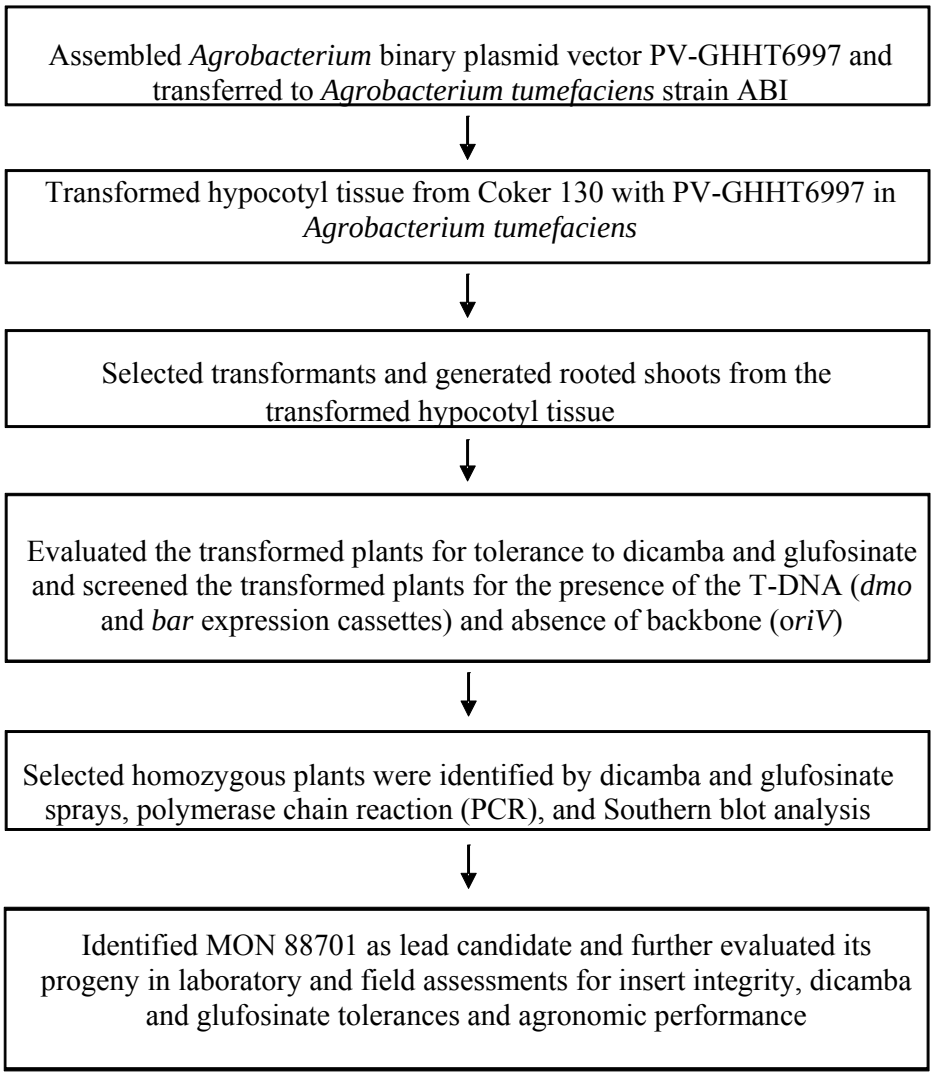


Figure 1 Schematic of the Development of MON 88701

A3(b) Intermediate hosts (*e.g.* bacteria)

A disarmed strain of *Agrobacterium tumefaciens* was the intermediate host used to transfer the T-DNA containing the *dmo* and *bar* expression cassettes from plasmid PV-GHHT6997 into cotton cells to produce MON 88701.

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

PV–GHHT6997

PV–GHHT6997 was used in the transformation of cotton to produce MON 88701 and its plasmid map is shown in Figure 4. The elements included in this plasmid vector are described in Table 1. PV-GHHT6997 is approximately 9.4 kb and contains one T-DNA that is delineated by Left Border and Right Border regions. The T-DNA contains the *dmo* and *bar* expression cassettes. The *dmo* expression cassette is regulated by the peanut chlorotic streak caulimovirus (*PC1SV*) promoter, the tobacco etch virus (*TEV*) 5′ leader sequence, and the 3' untranslated sequence of the *E6* gene from *Gossypium barbadense*. The chloroplast transit peptide CTP2 directs transport of the DMO protein to the chloroplast in MON 88701 and is derived from the *CTP2* target sequence of the *Arabidopsis thaliana shkG* gene (Herrmann, 1995; Klee et al., 1987). The *bar* expression cassette is regulated by the *e35S* promoter from the 35S RNA of cauliflower mosaic virus (CaMV), the heat shock protein 70 (*Hsp70)* leader, and the nopaline synthase (*nos)* 3′ untranslated region.

The backbone region of PV–GHHT6997, located outside of the T-DNA, contains two origins of replication for maintenance of plasmid vector in bacteria (*oriV* and *ori-pBR322*), a bacterial selectable marker gene (*aadA*), and a coding sequence for repressor of primer (*rop*) protein for maintenance of plasmid vector copy number in *Escherichia coli (E. coli)*. A description of the genetic elements and their prefixes (*e.g.*, B-, P-, L-, TS-, CS-, T-, and OR-) in PV–GHHT6997 is provided in Table 1.

The *dmo* Coding Sequence and MON 88701 DMO Protein

The *dmo* expression cassette encodes a ~39 kDa MON 88701 DMO precursor protein consisting of a single polypeptide of 416 amino acids (Figure 2). The *dmo* coding sequence is the codon optimised coding sequence from *Stenotrophomonas maltophilia* that encodes the DMO protein (Herman et al., 2005; Wang et al., 1997). The presence of MON 88701 DMO protein confers dicamba tolerance.

The *bar* Coding Sequence and PAT (*bar*) Protein

The *bar* expression cassette encodes a ~21 kDa PAT (*bar*) protein consisting of a single polypeptide of 183 amino acids (Thompson et al., 1987) (Figure 3). The *bar* coding sequence is from *Streptomyces hygroscopicus* and encodes the phosphinothricin N-acetyltransferase (PAT) protein (Thompson et al., 1987). The presence of PAT (*bar*) protein confers glufosinate tolerance.

Regulatory Sequences

The *dmo* coding sequence in MON 88701 is under the regulation of the *PC1SV* promoter, the *TEV* 5′ leader, and the *E6* 3′ untranslated region. The *PC1SV* promoter is the promoter for the Full-Length Transcript (FLt) of peanut chlorotic streak caulimovirus (Maiti and Shepherd, 1998) that directs transcription in plant cells. The *TEV* leader is the 5′ untranslated region from the tobacco etch virus (Niepel and Gallie, 1999) and is involved in regulating gene expression. The chloroplast transit peptide CTP2 directs transport of the DMO protein to the chloroplast in MON 88701 and is derived from *CTP2* target sequence of the *Arabidopsis thaliana shkG* gene (Herrmann, 1995; Klee et al., 1987). The *E6* 3′ non-translated region is the 3′ untranslated region from the *E6* gene of *Gossypium barbadense* encoding a fibre protein, which functions to direct polyadenylation of the mRNA (John, 1996).

The *bar* coding sequence in MON 88701 is under the regulation of the *e35S* promoter, the *Hsp70* leader, and the *nos* 3′ untranslated region. The *e35S* promoter is the promoter for the 35S RNA of cauliflower mosaic virus (CaMV) (Odell et al., 1985) containing the duplicated enhancer region (Kay et al., 1987) that directs transcription in plant cells. The *Hsp70* leader is the 5' untranslated region from the *DnaK* gene from *Petunia hybrida* (Rensing and Maier, 1994; Winter et al., 1988) and is involved in regulating gene expression. The *nos* 3′ untranslated region is the 3′ untranslated region from the nopaline synthase (*nos*) gene of *Agrobacterium tumefaciens* encoding NOS that directs polyadenylation of the mRNA (Bevan et al., 1983; Fraley et al., 1983).

T-DNA Border Regions

PV–GHHT6997 contains Right Border and Left Border regions (Figure 4 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 of T-DNA into the cotton 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–GHHT6997 in bacteria. The origin of replication, *oriV,* 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 cotton genome. The absence of detectable backbone sequence in MON 88701 has been confirmed by Southern blot analyses (see Section A3(d)(ii)).

Table 1. Summary of Genetic Elements in PV–GHHT6997

Genetic Element	Location in Plasmid Vector	Function (Reference)
T-DNA		
B[1]-Right Border Region	1-331	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	332-433	Sequence used in DNA cloning
P[2]-*PC1SV*	434-866	Promoter from the Full-Length Transcript (FLt) of peanut chlorotic streak caulimovirus (*PC1SV*) that directs transcription in plant cells (Maiti and Shepherd, 1998)
Intervening Sequence	867-872	Sequence used in DNA cloning
L[3]-*TEV*	873-1004	5' UTR leader sequence from the RNA of tobacco etch virus (TEV) (Niepel and Gallie, 1999) that is involved in regulating gene expression
Intervening Sequence	1005-1005	Sequence used in DNA cloning
TS[4]-*CTP2*	1006-1233	Targeting sequence of the *ShkG* gene from *Arabidopsis thaliana* encoding the EPSPS transit peptide region that directs transport of the protein to the chloroplast (Herrmann, 1995; Klee et al., 1987)
CS[5]-*dmo*	1234-2256	Codon optimised coding sequence for the dicamba mono-oxygenase (DMO) protein of *Stenotrophomonas maltophilia* that confers dicamba tolerance (Herman et al., 2005; Wang et al., 1997)
Intervening Sequence	2257-2310	Sequence used in DNA cloning
T[6]-*E6*	2311-2625	3' UTR sequence of the *E6* gene from *Gossypium barbadense* (cotton) encoding a fibre protein involved in early fibre development (John, 1996) that directs polyadenylation of mRNA
Intervening Sequence	2626-2637	Sequence used in DNA cloning
P-*e35S*	2638-3249	Promoter from the 35S RNA of cauliflower mosaic virus (CaMV) (Odell et al., 1985) containing the duplicated enhancer region (Kay et al., 1987) that directs transcription in plant cells
Intervening Sequence	3250-3252	Sequence used in DNA cloning

Table 1. Summary of Genetic Elements in PV–GHHT6997 (continued)

Genetic Element	Location in Plasmid Vector	Function (Reference)
L-*Hsp70*	3253-3348	5' UTR leader sequence of the *DnaK* gene from *Petunia hybrida* that encodes heat shock protein 70 (*HSP70*) (Rensing and Maier, 1994; Winter et al., 1988) that is involved in regulating gene expression
Intervening Sequence	3349-3354	Sequence used in DNA cloning
CS-*bar*	3355-3906	Coding sequence for the phosphinothricin N-acetyltransferase (PAT) protein of *Streptomyces hygroscopicus* that confers glufosinate tolerance (Thompson et al., 1987)
Intervening Sequence	3907-3911	Sequence used in DNA cloning
T-*nos*	3912-4164	3' UTR sequence of the nopaline synthase (*nos*) gene from *Agrobacterium tumefaciens* pTi encoding NOS that directs polyadenylation (Bevan et al., 1983; Fraley et al., 1983)
Intervening Sequence	4165-4183	Sequence used in DNA cloning
B-Left Border Region	4184-4625	DNA region from *Agrobacterium tumefaciens* containing the Left Border sequence used for transfer of the T-DNA (Barker et al., 1983)
Plasmid Vector Backbone		
Intervening Sequence	4626-4711	Sequence used in DNA cloning
OR[7]-*oriV*	4712-5108	Origin of replication from the broad host range plasmid RK2 for maintenance of plasmid in *Agrobacterium* (Stalker et al., 1981)
Intervening Sequence	5109-6616	Sequence used in DNA cloning
CS-*rop*	6617-6808	Coding sequence for repressor of primer protein from the ColE1 plasmid for maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989)
Intervening Sequence	6809-7235	Sequence used in DNA cloning
OR-*ori-pBR322*	7236-7824	Origin of replication from plasmid pBR322 for maintenance of plasmid in *E. coli* (Sutcliffe, 1979)

Table 1. Summary of Genetic Elements in PV–GHHT6997 (continued)

Genetic Element	Location in Plasmid Vector	Function (Reference)
Intervening Sequence	7825-8354	Sequence used in DNA cloning
aadA	8355-9243	Bacterial promoter, coding sequence, and 3' UTR for an aminoglycoside-modifying enzyme, 3"(9)-*O*-nucleotidyltransferase from the transposon *Tn7* (Fling et al., 1985) that confers spectinomycin and streptomycin resistance
Intervening Sequence	9244-9379	Sequence used in DNA cloning

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

```
  1   MAQVSRICNG VQNPSLISNL SKSSQRKSPL SVSLKTQQHP RAYPISSSWG
 51   LKKSGMTLIG SELRPLKVMS SVSTACMLTF VRNAWYVAAL PEELSEKPLG
101   RTILDTPLAL YRQPDGVVAA LLDICPHRFA PLSDGILVNG HLQCPYHGLE
151   FDGGGQCVHN PHGNGARPAS LNVRSFPVVE RDALIWIWPG DPALADPGAI
201   PDFGCRVDPA YRTVGGYGHV DCNYKLLVDN LMDLGHAQYV HRANAQTDAF
251   DRLEREVIVG DGEIQALMKI PGGTPSVLMA KFLRGANTPV DAWNDIRWNK
301   VSAMLNFIAV APEGTPKEQS IHSRGTHILT PETEASCHYF FGSSRNFGID
351   DPEMDGVLRS WQAQALVKED KVVVEAIERR RAYVEANGIR PAMLSCDEAA
401   VRVSREIEKL EQLEAA
```

Figure 2. Deduced Amino Acid Sequence of the MON 88701 DMO Precursor Protein

The amino acid sequence of the MON 88701 DMO precursor protein was deduced from the full-length coding nucleotide sequence present in PV–GHHT6997 (see Table 1 for more detail). The chloroplast transit peptide (CTP2) and the first 76 amino acids of the precursor protein are underlined. CTP2 targets MON 88701 DMO protein to the chloroplast. The CTP2 is cleaved in the chloroplast producing the mature 349 amino acid MON 88701 DMO protein that begins with the valine at position 68 (See Section B.1). The double underline shows the nine amino acids from CTP2 that are at the N-terminus of the mature MON 88701 protein.

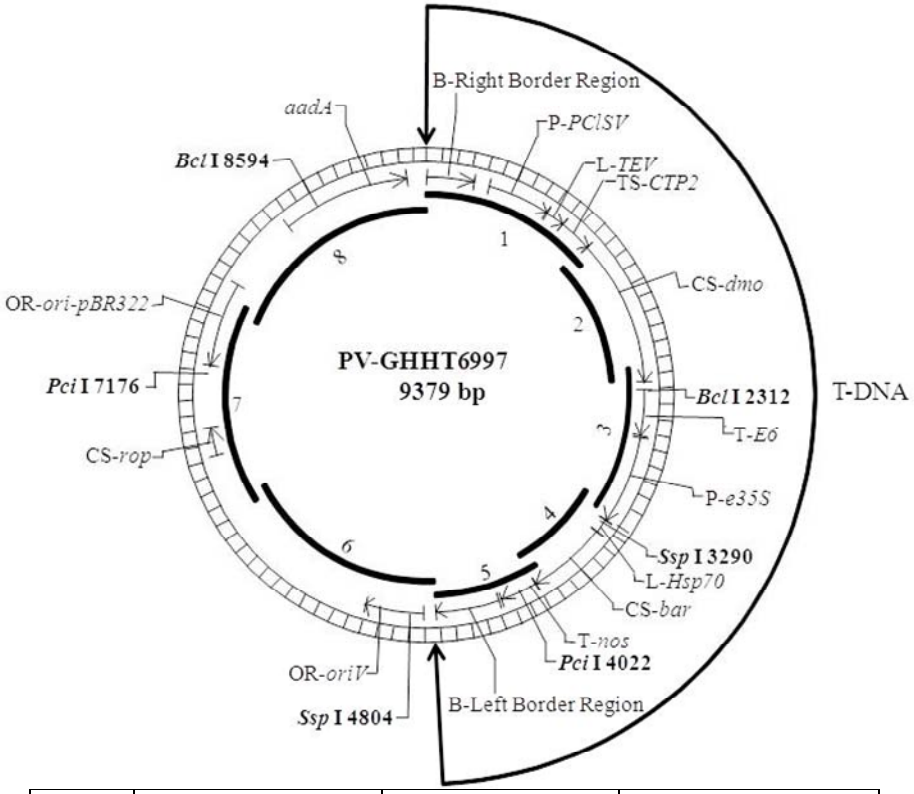
```
  1   MSPERRPADI RRATEADMPA VCTIVNHYIE TSTVNFRTEP QEPQEWTDDL
 51   VRLRERYPWL VAEVDGEVAG IAYAGPWKAR NAYDWTAEST VYVSPRHQRT
101   GLGSTLYTHL LKSLEAQGFK SVVAVIGLPN DPSVRMHEAL GYAPRGMLRA
151   AGFKHGNWHD VGFWQLDFSL PVPPRPVLPV TEI
```

Figure 3 Deduced Amino Acid Sequence of the MON 88701-produced PAT (*bar*) Protein

The amino acid sequence of the MON 88701-produced PAT (*bar*) protein was deduced from the full-length coding nucleotide sequence present in PV–GHHT6997 (see Table 1 for more detail).

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

Plasmid map with locations of genetic elements are shown in Figure 4.



Probe	Start Position (bp)	End Position (bp)	Total Length (~kb)
1	1	1310	1.3
2	1223	2241	1.0
3	2142	3252	1.1
4	3153	3914	0.8
5	3832	4625	0.8
6	4626	6282	1.7
7	6204	7708	1.5
8	7630	9379	1.8

Figure 4. Circular Map of PV–GHHT6997 Showing Probes 1-8

A circular map of PV–GHHT6997 used to develop MON 88701 is shown. PV–GHHT6997 contains a single T-DNA. 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.

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

Characterisation of the DNA insert in MON 88701 was conducted by Southern blot, PCR and DNA sequence analyses. The results of this characterisation demonstrate that MON 88701 contains a single copy of the *dmo* and *bar* expression cassettes, lacks plasmid backbone, the T-DNA 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 cotton genome for the presence of the T-DNA and absence of the plasmid backbone sequences derived from PV-GHHT6997, and demonstrated that only a single copy of the T-DNA was inserted at a single genomic site and that the insert is stably inherited; 2) DNA sequence analyses to determine the exact sequence of the inserted DNA and the DNA sequences flanking the 5' and 3' ends of the insert, allowing a comparison to the T-DNA sequence in the plasmid vector to confirm that only the expected sequences were integrated; 3) DNA sequences flanking the 5' and 3' ends of the insert were compared to the sequence of the insertion site in conventional cotton to identify any rearrangements that occurred at the insertion site during transformation. Taken together, the characterisation of the genetic modification demonstrates that a single copy of the T-DNA was stably integrated at a single locus of the cotton genome and that no plasmid backbone sequences are present in MON 88701.

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 cotton 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 characterise 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 hybridisation pattern. This strategy also maximises the possibility of detecting an insertion elsewhere in the genome that could be overlooked if that band co-migrated on the gel with an expected band.

To determine the number of copies and insertion sites of the T-DNA, and the presence or absence of the plasmid vector backbone sequences, duplicated samples that consisted of equal amounts of digested DNA were run on the agarose gel. One set of samples was run for a longer period of time (long run) than the second set (short run). The long run allows for greater resolution of large molecular weight DNA, whereas the short run allows 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 6 through Figure 9). Southern blot analyses determined that a single copy of the T-DNA was inserted at a single locus of the cotton genome, and no additional genetic elements, including backbone sequences, from PV–GHHT6997 were detected in MON 88701.

The PCR and DNA sequence analyses complement the Southern analyses. PCR and DNA sequence analyses performed on MON 88701 determined the complete DNA sequence of the insert and flanking genomic DNA sequences in MON 88701, confirmed the predicted organisation of the genetic elements within the insert, and determined the sequences flanking the insert. In addition, DNA sequence analyses confirmed that each genetic element (except for the border regions) in the insert is intact and the sequence of the insert is identical to the corresponding sequence in PV–GHHT6997 (Figure 10). Furthermore, genomic organisation at the MON 88701 insertion site was determined by comparing the sequence flanking the 5' and 3' ends of the insert to the sequence of the insertion site in conventional cotton (Figure 11).

The stability of the T-DNA present in MON 88701 across multiple generations was demonstrated by Southern blot fingerprint analysis (Figure 15). Genomic DNA from five generations of MON 88701 (Figure 13) was digested with one of the enzyme sets used for the insert and copy number analyses and was hybridised 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 88701. Results from this analysis demonstrated the inheritance and stability of the insert was as expected across multiple generations (Figure 14, Table 4 and Table 5), which corroborates the molecular insert stability analysis and establishes the genetic behaviour of the T-DNA at a single chromosomal locus.

The Southern blot analyses confirmed that the T-DNA reported in Figure 5 represents the only detectable insert in MON 88701. A circular map of PV–GHHT6997 annotated with the probes used in the Southern blot analysis is presented in Figure 5 and the genetic elements within the MON 88701 insert are summarised in Table 3. A linear map depicting restriction sites within the insert as well as within the DNA immediately flanking the insert in MON 88701 is shown in Figure 1. Based on the plasmid map and the linear map of the insert, a table summarising the expected DNA segments for Southern analyses is presented in Table 2. The results from the Southern blot analyses are presented in Figure 6 through Figure 9. PCR amplification of the MON 88701 insert and the insertion site in the conventional control 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 13 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 14 and the results for the segregation analysis are presented in Table 4 and Table 5.

Please also refer to [redacted]., 2011(MSL0023280), [redacted] 2011 (MSL0023322) and [redacted], 2011 (RPN-2011-0089).

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

Insert and Copy Number of T-DNA in MON 88701

The numbers of copies and insertion sites of the T-DNA sequences in the cotton genome were evaluated by digesting MON 88701 and conventional control genomic DNA samples with the restriction enzyme *Bcl* I or the restriction enzyme *Ssp* I and hybridising Southern blots with probes that span the T-DNA (Figure 4). 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 *Bcl* I cleaves once within the inserted T-DNA and within the known genomic DNA flanking the 3' end of the insert (Figure 5). Therefore, if T-DNA sequences were present as a single copy at a single integration site in MON 88701, the digestion with *Bcl* I was expected to generate two border segments with expected sizes of ≥3.1 kb and ~2.4 kb (Figure 5 and Table 2). The restriction enzyme *Ssp* I cleaves once within the inserted T-DNA and within the known genomic DNA flanking the 5' and 3' ends of the insert (Figure 5). If T-DNA sequences were present as a single copy at a single integration site in MON 88701, the digestion with *Ssp* I was expected to generate two border segments with expected sizes of ~3.4 kb and ~1.2 kb (Figure 5 and Table 2).

The Southern blots were hybridised with T-DNA probes that collectively span the entire inserted DNA sequence (Figure 4 and Figure 5, Probe 1, Probe 2, Probe 3, Probe 4, and Probe 5). Conventional control genomic DNA digested with the restriction enzyme *Bcl* I and spiked with either probe templates and/or digested PV–GHHT6997 DNA served as positive hybridisation controls. The positive hybridisation control was spiked at approximately 0.1 and 1.0 copies of 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 6 through Figure 8.

T-DNA Probes 1 and 5

Conventional control genomic DNA digested with *Bcl* I (Figure 6, Lane 1 and Lane 8) or with *Ssp* I (Figure 6, Lane 3 and Lane 10) and simultaneously hybridised with Probe 1 and Probe 5 (Figure 4 and Figure 5) produced no detectable hybridisation bands as expected for the negative control in the reported exposure shown in Figure 6. In a longer exposure of the blot, faint endogenous hybridisation bands were present in both the *Bcl* I digest and the *Ssp* I digest in the conventional control genomic DNA (data not shown). Conventional control genomic DNA digested with *Bcl* I and spiked with probe templates of Probe 1 and Probe 5 (Figure 4) produced the expected bands at ~1.3 kb and ~0.8 kb (Figure 6, Lane 5 and Lane 6).

Conventional control genomic DNA digested with *Bcl* I and spiked with the PV–GHHT6997 DNA, previously digested with the restriction enzyme *Pci* I (Figure 4), produced two bands at ~6.2 kb and ~3.2 kb (Figure 6, Lane 7), as expected. Detection of the positive controls indicates that the probes hybridised to their target sequences.

MON 88701 DNA digested with *Bcl* I and simultaneously hybridised with Probe 1 and Probe 5 (Figure 4 and Figure 5) produced the expected bands at ~3.5 kb and ~2.4 kb (Figure 6, Lane 2 and Lane 9) which is consistent with the expected ≥3.1 kb and ~2.4 kb bands (Figure 5 and Table 2), respectively. MON 88701 DNA digested with the restriction enzyme *Ssp* I and hybridised with Probe 1 and Probe 5 (Figure 4 and Figure 5) produced two bands at ~3.4 kb and ~1.2 kb (Figure 6, Lane 4 and Lane 11), as expected.

The results presented in Figure 6 indicate that the sequences covered by Probe 1 and Probe 5 reside at a single detectable locus of integration in MON 88701.

T-DNA Probes 2 and 4

Conventional control genomic DNA digested with *Bcl* I (Figure 7, Lane 1 and Lane 8) or with *Ssp* I (Figure 7, Lane 3 and Lane 10) and simultaneously hybridised with Probe 2 and Probe 4 (Figure 4 and Figure 5) produced no detectable hybridisation bands as expected for the negative control. Conventional control genomic DNA digested with *Bcl* I and spiked with probe templates of Probe 2 and Probe 4 (Figure 4) produced the expected bands at ~1.0 kb and ~0.8 kb (Figure 7, Lane 5 and Lane 6). Conventional control genomic DNA digested with *Bcl* I and spiked with the PV–GHHT6997 DNA, previously digested with the restriction enzyme *Pci* I (Figure 4), produced one band at ~6.2 kb (Figure 7, Lane 7), as expected. Detection of the positive controls indicates that the probes hybridised to their target sequences.

MON 88701 DNA digested with *Bcl* I and simultaneously hybridised with Probe 2 and Probe 4 (Figure 4 and Figure 5) produced the expected bands at ~3.5 kb and ~2.4 kb (Figure 7, Lane 2 and Lane 9) which is consistent with the expected ≥3.1 kb and ~2.4 kb bands (Figure 5 and Table 2), respectively. MON 88701 DNA digested with the restriction enzyme *Ssp* I and hybridised with Probe 2 and Probe 4 (Figure 4 and Figure 5) produced two bands at ~3.4 kb and ~1.2 kb (Figure 7, Lane 4 and Lane 11), as expected.

The results presented in Figure 7 indicate that the sequences covered by Probe 2 and Probe 4 reside at a single detectable locus of integration in MON 88701.

T-DNA Probe 3

Conventional control DNA digested with *Bcl* I (Figure 8, Lane 1 and Lane 7) or with *Ssp* I (Figure 8, Lane 3 and Lane 9) and hybridised with Probe 3 (Figure 4 and Figure 5) produced endogenous hybridisation signals that were present in all lanes (Figure 8, Lane 1 through Lane 10). The same hybridisation band was produced in conventional control and MON 88701 DNA lanes when digested with the same enzyme.

When digested with *Bcl* I and hybridised with Probe 3 hybridisation bands of ~1.9 kb and ~1.7 kb were produced with conventional control genomic DNA and MON 88701 DNA

(Figure 8, Lane 1, Lane 2, and Lanes 5–8). When digested with *Ssp* I and hybridised with Probe 3, a hybridisation band of ~2.5 kb was produced with conventional control genomic DNA and MON 88701 DNA (Figure 8, Lane 3, Lane 4, Lane 9, and Lane 10). Since these bands are present in both control and test substances, these signals are considered to be weak hybridisation of probes to endogenous *E6* sequences and are not specific to the inserted DNA in MON 88701.

Conventional control genomic DNA digested with *Bcl* I and spiked with the PV–GHHT6997 DNA, previously digested with the restriction enzyme *Pci* I (Figure 4), produced one band at ~6.2 kb (Figure 8, Lane 5 and Lane 6), as expected. Detection of the spiked controls indicates that the probe hybridised to its target sequence.

MON 88701 DNA digested with *Bcl* I and hybridised with Probe 3 (Figure 4 and Figure 5) produced two expected bands at ~3.5 kb and ~2.4 kb, which is consistent with the expected ≥3.1 kb and ~2.4 kb bands (Figure 5 and Table 2), and is in addition to the endogenous hybridisation bands discussed above (Figure 8, Lane 2 and Lane 8). The ~3.5 kb band is less intense than the ~2.4 kb band. The difference in band intensity is likely due to hybridisation of a smaller portion of Probe 3 to the ~3.5 kb fragment. The ~3.5 kb band represents the 5′ end of the inserted DNA and the adjacent DNA flanking the 5′ end of the insert; this correlates with the expected border fragment size of ≥ 3.1 kb. The ~2.4 kb band represents the 3′ end of the inserted DNA and the adjacent DNA flanking the 3′ end of the insert. MON 88701 DNA digested with *Ssp* I (Figure 8, Lane 4 and Lane 10, Figure 5, and Table 2) and hybridised with Probe 3 produced one expected band at ~3.4 kb in addition to the endogenous hybridisation bands discussed above. The ~3.4 kb band represents the 5′ end of the inserted DNA and the adjacent DNA flanking the 5′ end of the insert.

The results presented in Figure 8 indicate that the sequence covered by Probe 3 resides at a single detectable locus of integration in MON 88701.

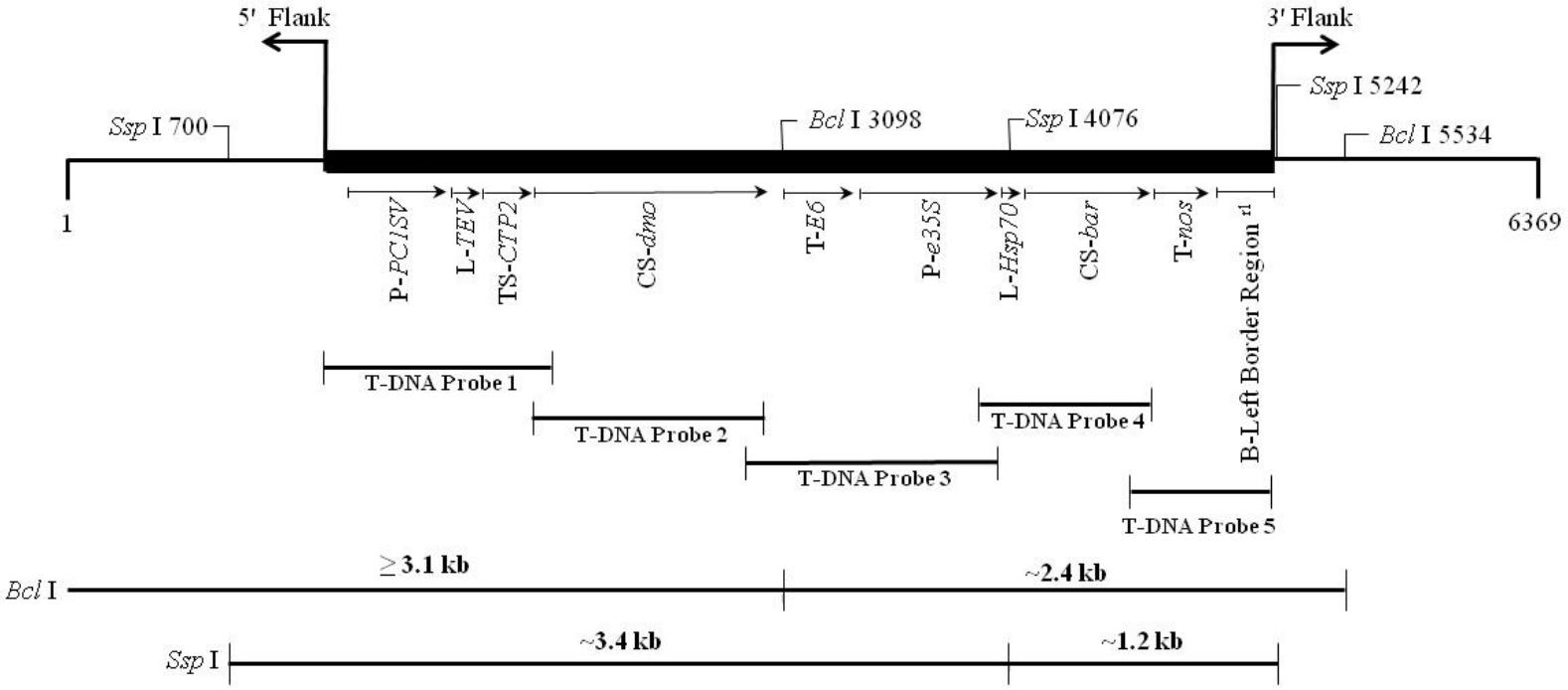


Figure 5. Schematic Representation of the Insert and Flanking DNA in MON 88701

A linear map of the insert and DNA flanking the insert in MON 88701 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 Table 1. [r1]Superscript in Left Border Region indicates that the sequence in MON 88701 was truncated compared to the sequences in PV–GHHT6997.

Table 2. Summary Chart of the Expected DNA Segments Based on Hybridising Probes and Restriction Enzymes Used in MON 88701 Analysis

Southern Blot Analysis		**T-DNA**			**Backbone**
Figure		Figure 6	Figure 7	Figure 8	Figure 9
Probe(s) Used		1,5	2,4	3	6, 7, 8
Probing Target	**Digestion enzyme**	**Expected Band Sizes on each Southern Blot**			
PV–GHHT6997	*Pci* I	~6.2 kb ~3.2 kb	~6.2 kb	~6.2 kb	~6.2 kb ~3.2 kb
Probe Templates[1]	N/A	~1.3 kb ~0.8 kb	~1.0 kb ~0.8 kb	~~[2]	~1.5 kb ~1.7 kb ~1.8 kb
MON 88701	*Bcl* I	≥3.1 kb ~2.4 kb	≥3.1 kb ~2.4 kb	≥3.1 kb ~2.4 kb	None
	Ssp I	~3.4 kb ~1.2 kb	~3.4 kb ~1.2 kb	~3.4 kb	None

[1]Probe template spikes were used as positive hybridisation controls in Southern blot analyses when multiple probes were hybridised to the blot simultaneously.

[2]' ~~' indicates that probe template was not used.

Table 3. Summary of Genetic Elements in MON 88701

Genetic Element	Location in Sequence	Function (Reference)
5′ Flank	1-1126	Cotton genomic DNA
Intervening Sequence	1127-1219	Sequence used in DNA cloning
P[1]-*PC1SV*	1220-1652	Promoter from the Full-Length Transcript (FLt) of peanut chlorotic streak caulimovirus (*PC1SV*) that directs transcription in plant cells (Maiti and Shepherd, 1998)
Intervening Sequence	1653-1658	Sequence used in DNA cloning
L[2]-*TEV*	1659-1790	5' UTR leader sequence from the RNA of tobacco etch virus (TEV) (Niepel and Gallie, 1999) that is involved in regulating gene expression
Intervening Sequence	1791-1791	Sequence used in DNA cloning
TS[3]-*CTP2*	1792-2019	Targeting sequence of the *ShkG* gene from *Arabidopsis thaliana* encoding the EPSPS transit peptide region that directs transport of the protein to the chloroplast (Herrmann, 1995; Klee et al., 1987)
CS[4]-*dmo*	2020-3042	Codon optimised coding sequence for the dicamba mono-oxygenase (DMO) protein of *Stenotrophomonas maltophilia* that confers dicamba tolerance (Herman et al., 2005; Wang et al., 1997)
Intervening Sequence	3043-3096	Sequence used in DNA cloning
T[5]-*E6*	3097-3411	3' UTR sequence of the *E6* gene from *Gossypium barbadense* (cotton) encoding a fibre protein involved in early fibre development (John, 1996) that directs polyadenylation of mRNA
Intervening Sequence	3412-3423	Sequence used in DNA cloning
P-*e35S*	3424-4035	Promoter from the 35S RNA of cauliflower mosaic virus (CaMV) (Odell et al., 1985) containing the duplicated enhancer region (Kay et al., 1987) that directs transcription in plant cells

Table 3. Summary of Genetic Elements in MON 88701 (continued)

Genetic Element	Location in Sequence	Function (Reference)
Intervening Sequence	4036-4038	Sequence used in DNA cloning
L-*Hsp70*	4039-4134	5' UTR leader sequence of the *DnaK* gene from *Petunia hybrida* that encodes heat shock protein 70 (*HSP70*) (Rensing and Maier, 1994; Winter et al., 1988) that is involved in regulating gene expression
Intervening Sequence	4135-4140	Sequence used in DNA cloning
CS-*bar*	4141-4692	Coding sequence for the phosphinothricin N-acetyltransferase (PAT) protein of *Streptomyces hygroscopicus* that confers glufosinate tolerance (Thompson et al., 1987)
Intervening Sequence	4693-4697	Sequence used in DNA cloning
T-*nos*	4698-4950	3' UTR sequence of the nopaline synthase (*nos*) gene from *Agrobacterium tumefaciens* pTi encoding NOS that directs polyadenylation (Bevan et al., 1983; Fraley et al., 1983)
Intervening Sequence	4951-4969	Sequence used in DNA cloning
B[6]-Left Border Region [r1]	4970-5231	DNA region from *Agrobacterium tumefaciens* containing the Left Border sequence used for transfer of the T-DNA (Barker et al., 1983)
3′ Flank	5232-6369	Cotton genomic DNA

[1]P, Promoter
[2]L, Leader
[3]TS, Targeting Sequence
[4]CS, Coding Sequence
[5]T, Transcription Termination Sequence
[6]B, Border
[r1]Superscript in Left Border Region indicates that the sequence in MON 88701 was truncated compared to the sequences in PV–GHHT6997.

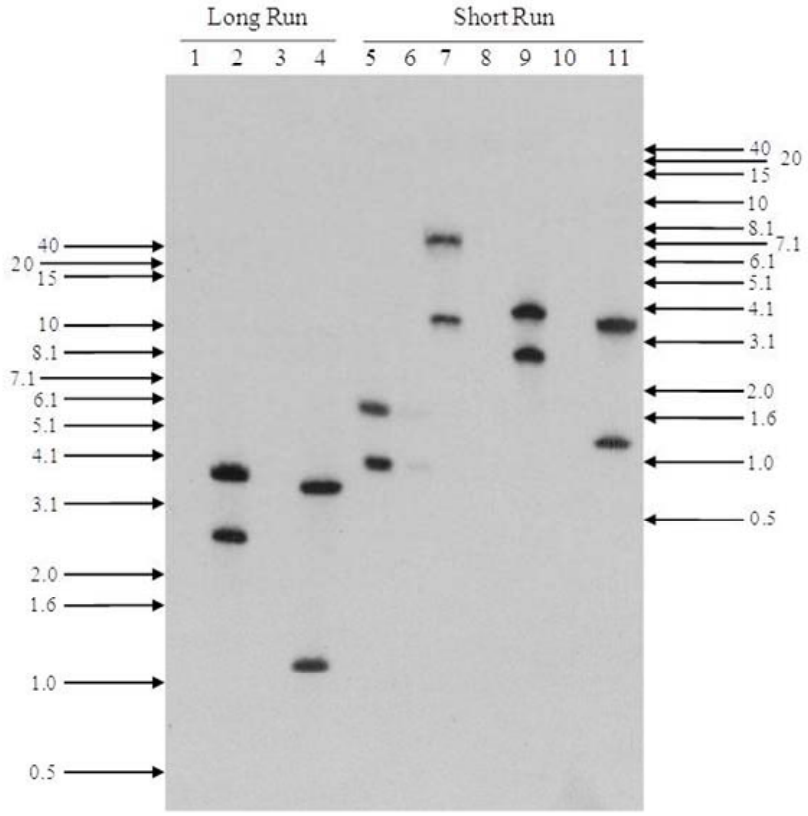


Figure 6. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA in MON 88701: Probes 1 and 5

The blot was simultaneously hybridised with two ^{32}P-labeled probes that span a portion of the T-DNA sequence (Figure 4, Probe 1 and Probe 5). 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 (*Bcl* I)
2 MON 88701 (*Bcl* I)
3 Conventional Control (*Ssp* I)
4 MON 88701 (*Ssp* I)
5 Conventional Control (*Bcl* I) spiked with Probe 1 and Probe 5 template [~1.0 genome equivalent]
6 Conventional Control (*Bcl* I) spiked with Probe 1 and Probe 5 template [~0.1 genome equivalent]
7 Conventional Control (*Bcl* I) spiked with PV–GHHT6997 (*Pci* I) [~1.0 genome equivalent]
8 Conventional Control (*Bcl* I)
9 MON 88701 (*Bcl* I)
10 Conventional Control (*Ssp* I)
11 MON 88701 (*Ssp* I)

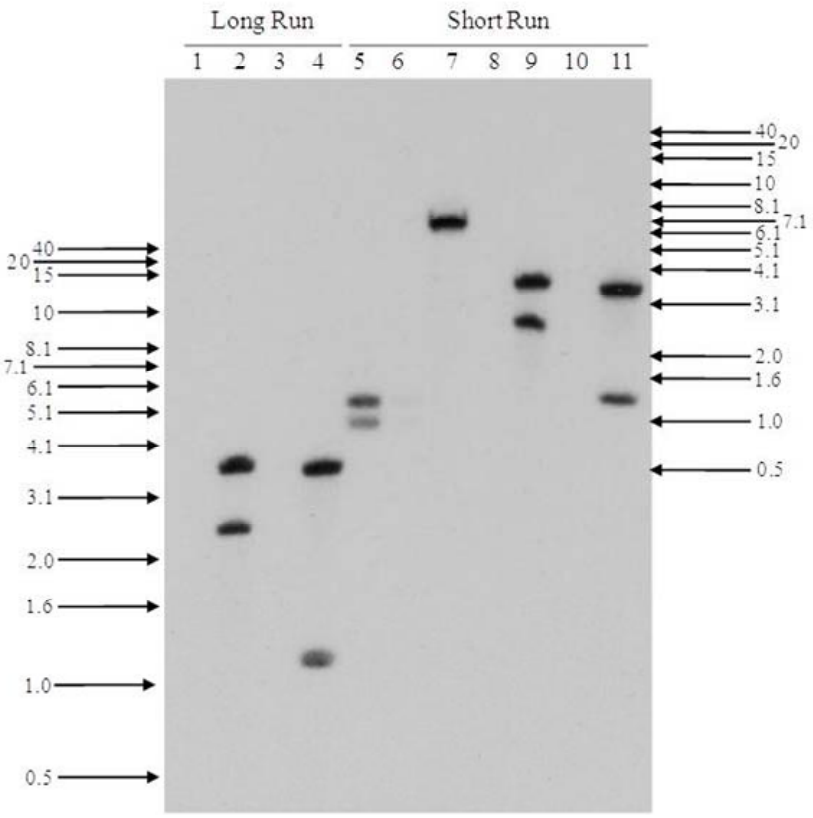


Figure 7. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA in MON 88701: Probes 2 and 4

The blot was simultaneously hybridised with two ^{32}P-labeled probes that span a portion of the T-DNA sequence (Figure 4, Probe 2 and Probe 4). 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 (*Bcl* I)
2 MON 88701 (*Bcl* I)
3 Conventional Control (*Ssp* I)
4 MON 88701 (*Ssp* I)
5 Conventional Control (*Bcl* I) spiked with Probe 2 and Probe 4 template [~1.0 genome equivalent]
6 Conventional Control (*Bcl* I) spiked with Probe 2 and Probe 4 template [~0.1 genome equivalent]
7 Conventional Control (*Bcl* I) spiked with PV–GHHT6997 (*Pci* I) [~1.0 genome equivalent]
8 Conventional Control (*Bcl* I)
9 MON 88701 (*Bcl* I)
10 Conventional Control (*Ssp* I)
11 MON 88701 (*Ssp* I)

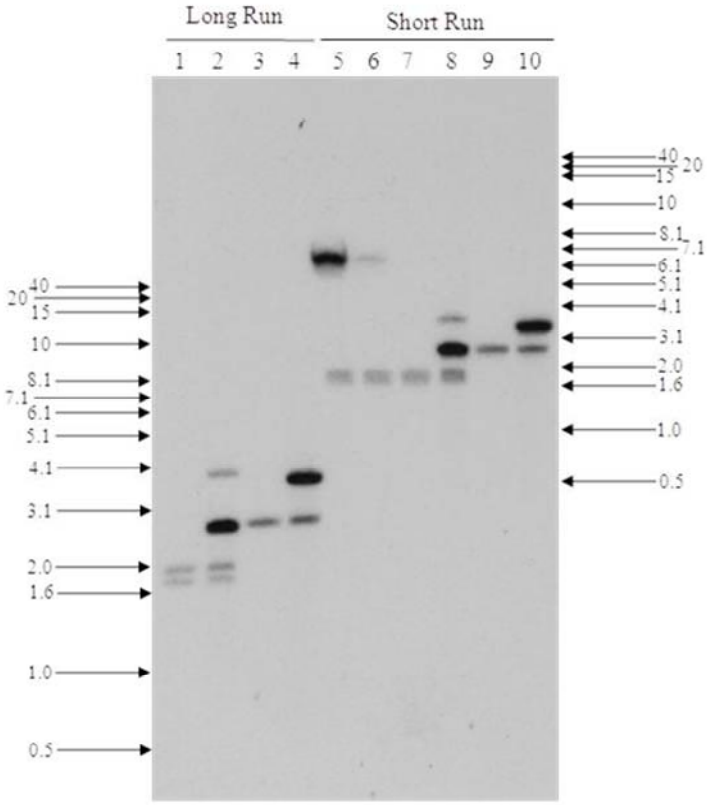


Figure 8 Southern Blot Analysis to Determine Insert and Copy Number of T-DNA in MON 88701: Probe 3

The blot was hybridised with a ^{32}P-labeled probe that spans a portion of the T-DNA sequence (Figure 4, 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 (*Bcl* I)
2	MON 88701 (*Bcl* I)
3	Conventional Control (*Ssp* I)
4	MON 88701 (*Ssp* I)
5	Conventional Control (*Bcl* I) spiked with PV–GHHT6997 (*Pci* I) [~1.0 genome equivalent]
6	Conventional Control (*Bcl* I) spiked with PV–GHHT6997 (*Pci* I) [~0.1 genome equivalent]
7	Conventional Control (*Bcl* I)
8	MON 88701 (*Bcl* I)
9	Conventional Control (*Ssp* I)
10	MON 88701 (*Ssp* I)

Southern Blot Analysis to Determine the Presence or Absence of PV–GHHT6997 Backbone Sequences in MON 88701

To determine the presence or absence of the PV–GHHT6997 backbone sequences, MON 88701 and conventional control genomic DNA were digested with the restriction enzyme *Bcl* I or restriction enzyme *Ssp* I, and hybridised with the three backbone probes that collectively span the entire backbone sequences (Figure 4, Probe 6, Probe 7, and Probe 8). If backbone sequences are present in MON 88701, then probing with backbone probes should result in hybridising bands. Conventional control genomic DNA digested with the restriction enzyme *Bcl* I and spiked with probe templates and with digested PV–GHHT6997 DNA served as positive hybridisation controls. The positive hybridisation control was spiked at approximately 0.1 and 1.0 copies of 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 9.

Backbone Probes 6, 7, and 8

Conventional control DNA digested with *Bcl* I (Figure 9, Lane 1 and Lane 10) or the restriction enzyme *Ssp* I (Figure 9, Lane 3 and Lane 12) and hybridised with Probe 6, Probe 7, and Probe 8 (Figure 4) produced no detectable hybridisation bands as expected for the negative control.

Conventional control genomic DNA digested with *Bcl* I and spiked with probe templates of Probe 7 and Probe 8 (Figure 4) produced the expected bands at ~1.5 kb and ~1.8 kb (Figure 9, Lane 5 and Lane 6). Conventional control genomic DNA digested with *Bcl* I and spiked with probe template of Probe 6 (Figure 4) produced the one expected band at ~1.7 kb (Figure 9, Lane 7 and Lane 8). Conventional control DNA digested with *Bcl* I and spiked with the PV–GHHT6997 DNA, previously digested with the restriction enzyme *Pci* I (Figure 4), produced two bands at ~6.2 kb and ~3.2 kb (Figure 9, Lane 9), as expected. Detection of the positive controls indicates that the probe hybridised to its target sequence.

MON 88701 DNA digested with *Bcl* I (Figure 9, Lane 2 and Lane 11) or the restriction enzyme *Ssp* I (Figure 9, Lane 4 and Lane 13) and hybridised with Probes 6, 7, and 8 produced no detectable bands.

The results presented in Figure 9 indicate that MON 88701 contains no detectable backbone sequences covered by Probes 6, 7, and 8.

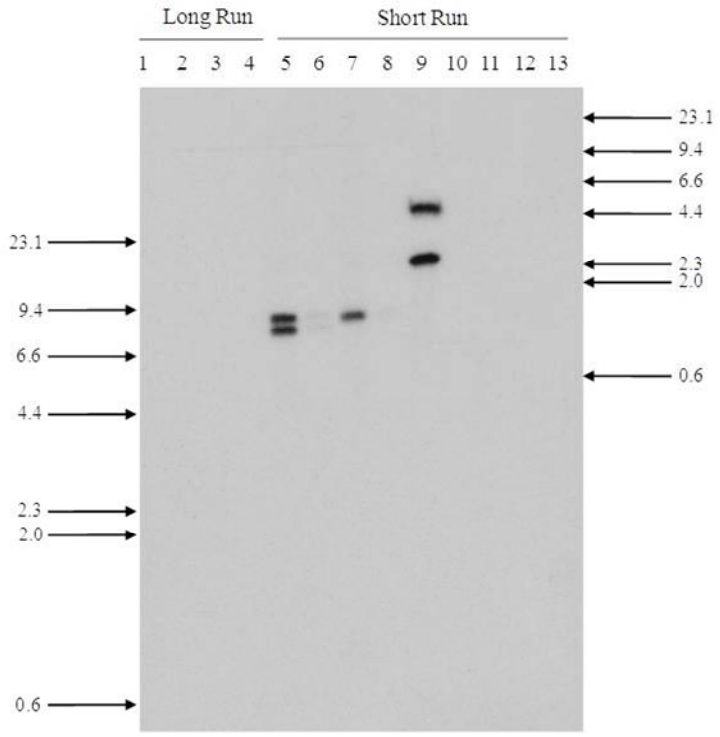


Figure 9. Southern Blot Analysis to Determine the Presence or Absence of PV–GHHT6997 Backbone Sequences in MON 88701: Probes 6, 7, and 8

The blot was hybridised with three ^{32}P-labeled probes that spans the plasmid vector backbone sequences (Figure 4, Probe 6, 7, and 8). 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 (*Bcl* I)
2 MON 88701 (*Bcl* I)
3 Conventional Control (*Ssp* I)
4 MON 88701 (*Ssp* I)
5 Conventional Control (*Bcl* I) spiked with Probe 7 and Probe 8 template [~1.0 genome equivalent]
6 Conventional Control (*Bcl* I) spiked with Probe 7 and Probe 8 template [~0.1 genome equivalent]
7 Conventional Control (*Bcl* I) spiked with Probe 6 template [~1.0 genome equivalent]
8 Conventional Control (*Bcl* I) spiked with Probe 6 template [~0.1 genome equivalent]
9 Conventional Control (*Bcl* I) spiked with PV–GHHT6997 (*Pci* I) [~1.0 genome equivalent]
10 Conventional Control (*Bcl* I)
11 MON 88701 (*Bcl* I)
12 Conventional Control (*Ssp* I)
13 MON 88701 (*Ssp* I)

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

Organisation and Sequence of the Insert and Adjacent Genomic DNA in MON 88701

The organisation and sequence of the elements within the MON 88701 insert was confirmed by DNA sequence analysis. PCR primers were designed with the intent to amplify three 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 88701 insert is 4105 bp long (Table 3) and is identical to the corresponding T-DNA sequence of PV–GHHT6997 as described in Table 1. Please also refer to [redacted] 2011 (MSL0023280).

PCR and DNA Sequence Analyses to Examine the MON 88701 Insertion Site

PCR and sequence analyses were performed on genomic DNA extracted from MON 88701 and the conventional control to examine the MON 88701 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 88701 insert were separately performed to determine the integrity and genomic organisation of the insertion site in MON 88701. The alignment analyses indicated a 123 base pair deletion from the conventional genomic DNA occurred upon T-DNA insertion in MON 88701. Minor deletions and/or insertions of DNA due to double-strand break repair mechanisms in the plant during *Agrobacterium*-mediated transformation process are not uncommon (Salomon and Puchta, 1998). Please also refer to [redacted] 2011 (MSL0023280).

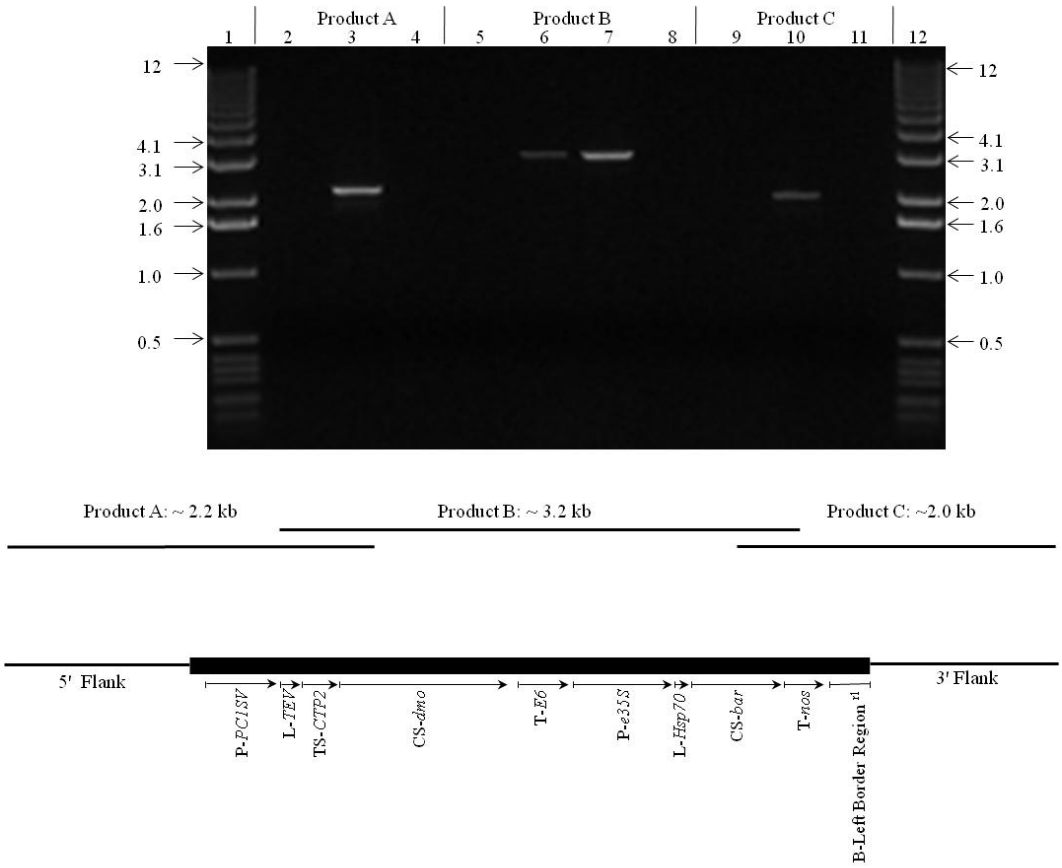


Figure 10. Overlapping PCR Analysis across the Insert in MON 88701

PCR was performed on both conventional control genomic DNA and MON 88701 genomic DNA using three pairs of primers to generate overlapping PCR fragments from MON 88701 for sequence analysis. Approximately five microliters of each of the PCR reactions was loaded on the gel. The expected product size for each amplicon and an illustration of the insert in MON 88701 is provided 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 88701
4	No template DNA control
5	Conventional Control
6	MON 88701
7	PV–GHHT6997
8	No template DNA control
9	Conventional Control
10	MON 88701
11	No template DNA control
12	1 Kb DNA Ladder

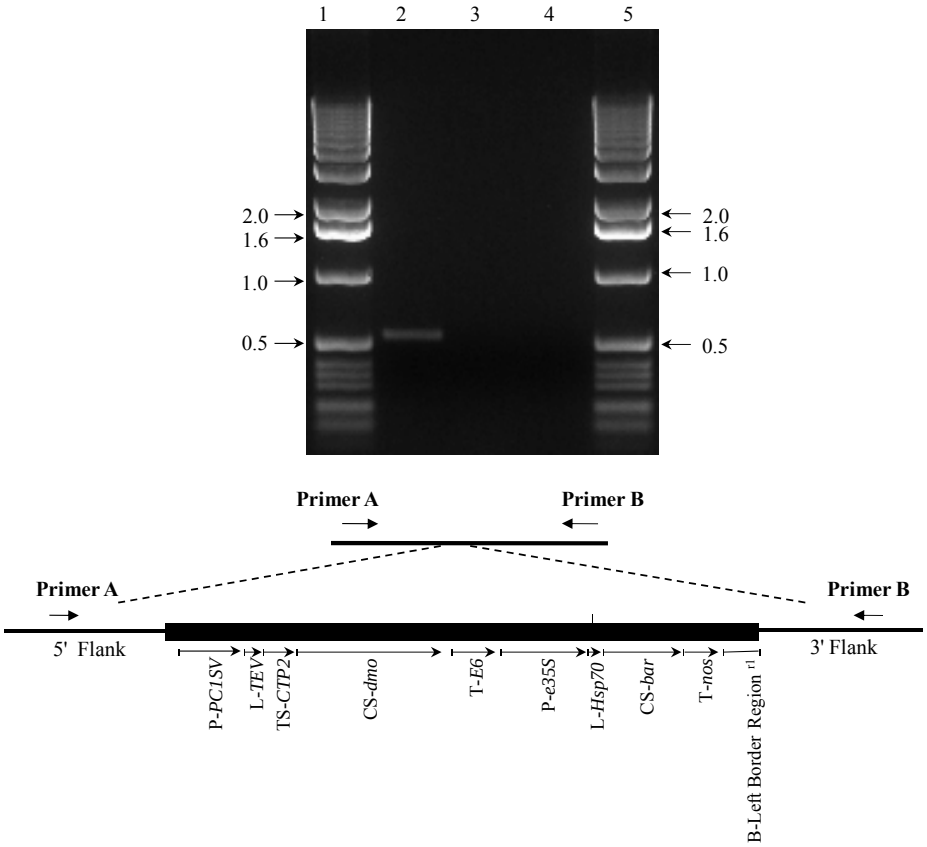


Figure 11. PCR Amplification of the MON 88701 Insertion Site in Conventional Control

PCR was performed on both conventional control genomic DNA and MON 88701 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 88701, to generate DNA fragments for sequence analysis. The insertion site in the conventional control (top) and MON 88701 (bottom) are illustrated at the bottom of the figure. Approximately 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 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 88701
4 No template DNA control
5 1 Kb DNA Ladder

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

PCR and DNA sequence analyses performed on MON 88701 and the conventional control determined the organisation of the genetic elements within the insert as given in Figure 5 and Table 4.

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

Bioinformatic Assessment of Putative Open Reading Frames (ORFs) of MON 88701 Insert and Flanking Sequences

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 bioinformatics analyses demonstrate that any putative polypeptides in MON 88701 are unlikely to exhibit allergenic, toxic or otherwise biologically adverse properties.

In addition to the bioinformatic analyses conducted on MON 88701 DMO and PAT (*bar*) protein sequences, bioinformatic analyses were also performed on the MON 88701 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 88701 insert DNA, as well as, ORFs present in the 5' and 3' flanking sequence junctions (Table 3). These various bioinformatic evaluations are depicted in Figure 12. ORFs spanning the 5' 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 orientation)[4]. Polypeptides of eight amino acids or greater from each reading frame were then compared to toxin, allergen and all proteins databases using bioinformatic tools. Similarly, the entire MON 88701 insert DNA sequence was translated in all six reading frames and the resulting deduced amino acid sequence was subjected to bioinformatic analyses. There are no analytical data that indicate any putative polypeptides subjected to bioinformatic evaluation other than the MON 88701 DMO and PAT (*bar*) proteins, which are 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 88701 DMO and PAT (*bar*) proteins

[4] An evaluation of sequence translated from stop codon to stop codon represents the most conservative approach possible for flank junction analysis as it does not assume that a start codon is necessary for the production of a protein sequence.

were 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 relatedness of the putative polypeptides for MON 88701 to known toxins, allergens, or biologically active putative peptides.

Bioinformatics Assessment of Insert DNA Reading Frames

Bioinformatic analyses were performed to assess the potential of toxicity, allergenicity or biological activity of any putative peptides encoded by translation of reading frames 1 through 6 of the inserted DNA in MON 88701 (Figure 12).

The FASTA sequence alignment tool was used to assess structural relatedness between the query sequences and any protein sequences in the AD_2011, TOX_2011, and PRT_2011 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 to ascertain if alignments exceeded Codex (Codex Alimentarius, 2009) thresholds for FASTA searches of the AD_2011 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 (Ladics et al., 2007). In addition to structural similarity, each putative polypeptide was screened for short polypeptide matches using a pair-wise comparison algorithm. In these analyses, eight contiguous and identical amino acids were defined as immunologically relevant, where eight represents the typical minimum sequence length likely to represent an immunological epitope (Silvanovich et al., 2006) and evaluated against the AD_2011 database.

The results of the search comparisons showed that no relevant structural similarity to known allergens or toxins were observed for any of the putative polypeptides when compared to proteins in the allergen (AD_2011) or toxin (TOX_2011) 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 query the PRT_2011 database, translations of frame 2, 3, 5 and 6 yielded alignments with *E*-scores less than or equal to 1×10^{-5}. Inspection of the two alignments with frame 2 revealed that they contained a stop codon within the aligning region and required numerous gaps to optimise the alignment. As a result, it is unlikely these alignments reflect conserved structure. Translation of frame 3 positively identified the DMO protein (GI# 314865630) disclosed in a patent filed by Monsanto. The alignment obtained showed 100% identity over 340 amino acids with an *E*-score of 4.4×10^{-149}. In addition, alignment with frame 3 also positively identified PAT (*bar*) protein (GI# 32265028) with an alignment that displayed 100% identity in 183 amino acids and an *E*-score of 9.8×10^{-77}. Translation of frame 5 yielded one alignment with an *E*-score less than or equal to 1×10^{-5} when used to search the PRT_2011 database. The alignment obtained showed 97.9% identity over 49 amino acids with an *E*-score of 5.9×10^{-10} with GI-3327940. Inspection of the alignment revealed that there was one stop codon in the query sequence and it is unlikely the alignment reflects conserved structure. Translation of frame 6 yielded five alignments with *E*-scores

less than or equal to 1×10^{-5} when used to search the PRT_2011 database. Inspection of the top five alignments revealed that they were all with an unnamed protein product derived from the translation of the reverse complement strand of the *bar* coding sequence in a synthetic protein construct. These alignments are not unexpected because the identified *bar* gene is contained in MON 88701. Taken together, these data demonstrate the lack of relevant similarities between known allergens or toxins for putative peptides derived from all six reading frames from the inserted DNA sequence of MON 88701. As a result, in the unlikely event that a translation product other than the DMO or PAT (*bar*) protein sequences were derived from reading frames 1 to 6, these putative polypeptides are not expected to be cross-reactive allergens, toxins, or display adverse biological activity. Please also refer to [redacted] 2011 (MSL0023565).

Insert Junction Open Reading Frame Bioinformatics Analysis

Analyses of putative polypeptides encoded by DNA spanning the 5' and 3' genomic junctions of the MON 88701 inserted DNA were performed using a bioinformatic comparison strategy (Figure 12). 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' and 3' flanking sequence DNA-inserted DNA junctions (Figure 12) were translated from stop codon (TGA, TAG, TAA) to stop codon in all six reading frames. Putative polypeptides from each reading frame of eight amino acids or greater in length, were compared to AD_2011, TOX_2011, and PRT_2011 databases using FASTA and to the AD_2011 database using an eight amino acid sliding window search.

The FASTA sequence alignment tool was used to assess structural relatedness between the query sequences and protein sequences in the AD_2011, TOX_2011, and PRT_2011 databases. Structural similarities shared between each putative polypeptide with each sequence in the database were examined. The extent of structural relatedness was evaluated by detailed visual inspection of the alignment, the calculated percent identity and the alignment length to ascertain if alignments exceeded Codex (Codex Alimentarius, 2009) thresholds for FASTA searches of the AD_2011 database, and the *E*-score. In addition to structural similarity, each putative polypeptide was screened for short polypeptide matches using a pair-wise comparison algorithm. In these analyses, eight contiguous and identical amino acids were defined as immunologically relevant, where eight represents the typical minimum sequence length likely to represent an immunological epitope, and evaluated against the AD_2011 database.

No biologically relevant structural similarity to known allergens or toxins, or proteins that display adverse biological activity was observed for any of the putative polypeptides. Furthermore, no short (eight amino acid) polypeptide matches were shared between any of the putative polypeptides and proteins in the allergen database. As a result, in the unlikely event that a translation product was derived from DNA spanning the 5' or 3' genomic DNA insert junctions of MON 88701, these putative polypeptides are not expected to be allergens, toxins, or display adverse biological activity. Please also refer to [redacted] 2012 (MSL0024371).

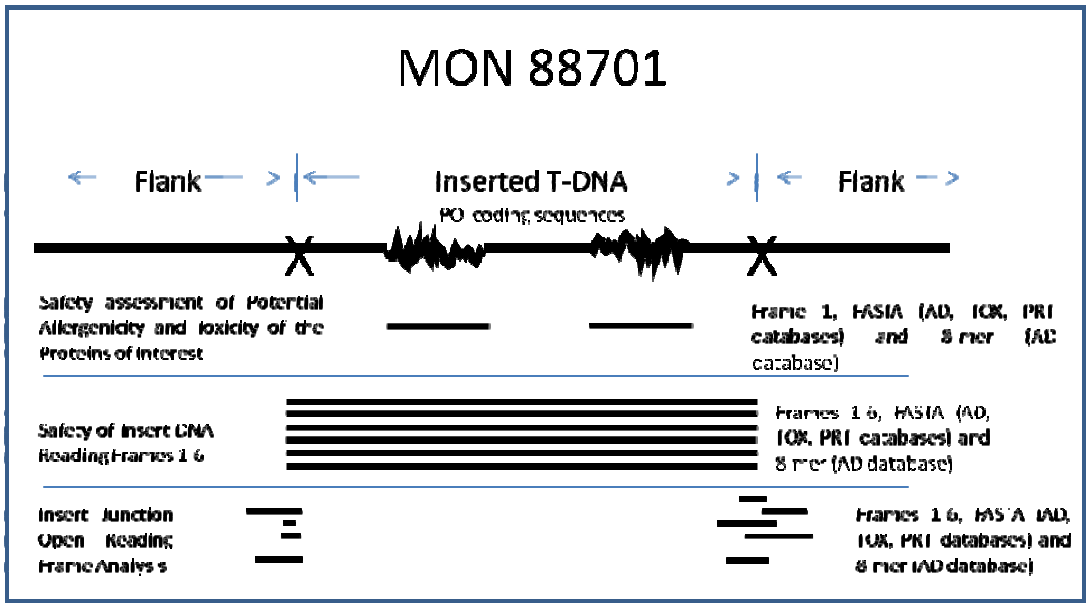


Figure 12. Schematic Summary of MON 88701 Bioinformatic Analyses

A3(e) Family tree or breeding process

MON 88701 was developed through *Agrobacterium*-mediated transformation of PV-GHHT6997 into cotton hypocotyls, based on published methods (Duncan, 2010; Duncan and Ye, 2011). The R_0 plants generated through the *Agrobacterium*-mediated transformation were self-pollinated to produce R_1 seed. R_0 and R_1 plants were evaluated for tolerance to dicamba and glufosinate and screened for the presence of the T-DNA (*dmo* and *bar* expression cassettes) and absence of plasmid vector backbone (o*riV*). Subsequently, the *dmo* and *bar* homozygous positive R_1 plant was self-pollinated to give rise to R_2 plants. Homozygous positive R_2 plants containing only a single T-DNA insertion, were identified by a combination of analytical techniques including dicamba and glufosinate sprays, polymerase chain reaction (PCR), and Southern blot analysis, resulting in production of dicamba and glufosinate-tolerant cotton MON 88701.

The R_3 generation was used for the molecular characterisation and commercial development of MON 88701. R_2, R_3, R_4, R_5, and R_6, generations were used for insert stability analysis and the R_4, generation was used for compositional analysis. See Figure 13.

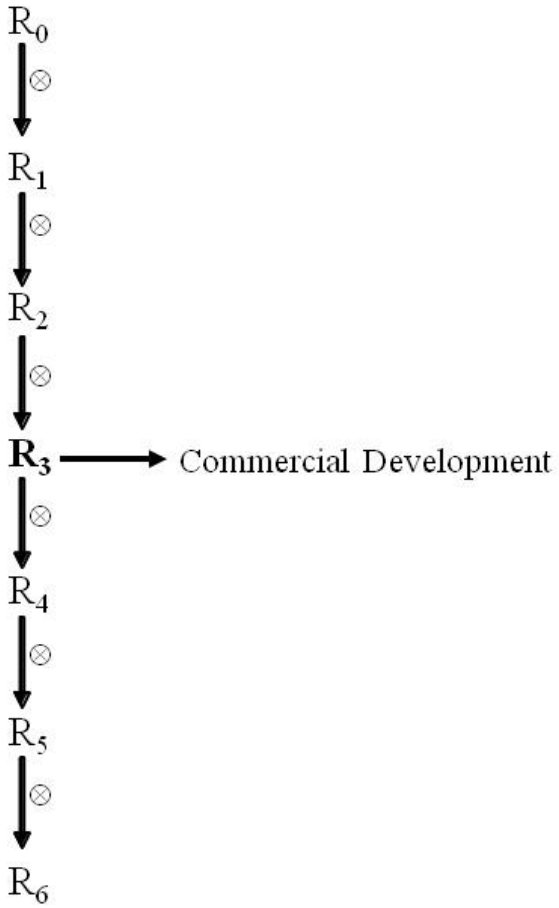


Figure 13. Breeding History of MON 88701

R_0 corresponds to the original transformed cotton plant. ⊗ designates self-pollination. The R_3 generation was used for the molecular characterisation and commercial development of MON 88701. The R_2, R_3, R_4, R_5, and R_6 generations of MON 88701 were used to analyse the stability of the insert across generations. The R_5 and R_6 generations were used for expression analysis and composition analysis (plant tissue other than seed= R_5, seed= R_6).

A3(f) Evidence of the stability of the genetic changes

A3(f)(i) Pattern of inheritance of insert and number of generations monitored

Inheritance of the Genetic Insert in MON 88701

The MON 88701 T-DNA resides at a single locus within the cotton genome and is inherited according to Mendelian principles of inheritance. During development of MON 88701, phenotypic and genotypic segregation data were recorded to assess the inheritance and stability of the MON 88701 T-DNA using Chi-square (χ^2) analysis over several generations. The χ^2 analysis is based on comparing the observed segregation ratio to the expected segregation ratio according to Mendelian principles.

The MON 88701 breeding path for generating pollinated segregation data is described in Figure 14. The transformed R_0 plant was self-pollinated to generate R_1 seed. The segregating R_1 generation was assessed using Real-Time TaqMan analysis for the *dmo* coding region. A single homozygous positive R_1 plant was selected and self-pollinated to give rise to R_2 plants that were self-pollinated to produce R_3 seed. Phenotypic and genotypic assays confirmed the lack of segregation in these self-pollinated generations.

Homozygous positive R_3 plants were crossed to a Monsanto proprietary cotton inbred, which does not contain the *dmo* or *bar* coding sequence, via traditional breeding techniques to produce hemizygous F_1 seed. The F_1 plants, hemizygous for the dicamba and glufosinate tolerant trait, were crossed with a Monsanto proprietary cotton inbred, which does not contain the *dmo* or *bar* coding sequence, to produce $BC1F_1$ seed. The $BC1F_1$ generation was assessed using a glufosinate herbicide application to select for plants containing the MON 88701 T-DNA. The plants that survived the herbicide application were confirmed to be hemizygous for the MON 88701 T-DNA by End-Point TaqMan analysis. The hemizygous $BC1F_1$ plants were self-pollinated to produce the $BC1F_2$ plants. For the $BC1F_2$ generation, the plants were assessed using a glufosinate herbicide application and the surviving plants were assessed by End-Point TaqMan analysis for the MON 88701 T-DNA.

The inheritance of the MON 88701 T-DNA was assessed in the R_1, $BC1F_1$, and $BC1F_2$ generations. At the $BC1F_1$ generation, the MON 88701 T-DNA was predicted to segregate at a 1:1 ratio (hemizygous: homozygous negative) according to Mendelian inheritance principles. At the R_1 and $BC1F_2$ generations, the MON 88701 T-DNA was predicted to segregate at a 1:2:1 ratio (homozygous positive: hemizygous: homozygous negative) according to Mendelian inheritance principles.

A Chi-square (χ^2) analysis was used to compare the observed segregation ratios of the MON 88701 T-DNA to the expected ratios. The Chi-square (χ^2) analysis used the statistical program R Version 2.12.0 (2010-10-15).

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 88701 segregating progeny are presented in Table 4 and Table 5. The χ^2 value in the $BC1F_1$ generation indicated no statistically significant difference between the observed and expected 1:1 segregation ratio (hemizygous: homozygous negative) of the MON 88701 T-DNA. The χ^2 value for the R_1 and $BC1F_2$ generations indicated no statistically significant difference between the observed and expected 1:2:1 segregation ratio (homozygous positive: hemizygous: homozygous negative) of MON 88701 T-DNA. These results support the conclusion that the MON 88701 T-DNA resides at a single locus within the cotton genome and is inherited according to Mendelian principles of inheritance. These results are also consistent with the molecular characterisation data indicating that MON 88701 contains a single intact copy of the *dmo* and *bar* expression cassettes inserted at a single locus in the cotton genome. Please also refer to [redacted] 2011 (RPN-2011-0089).

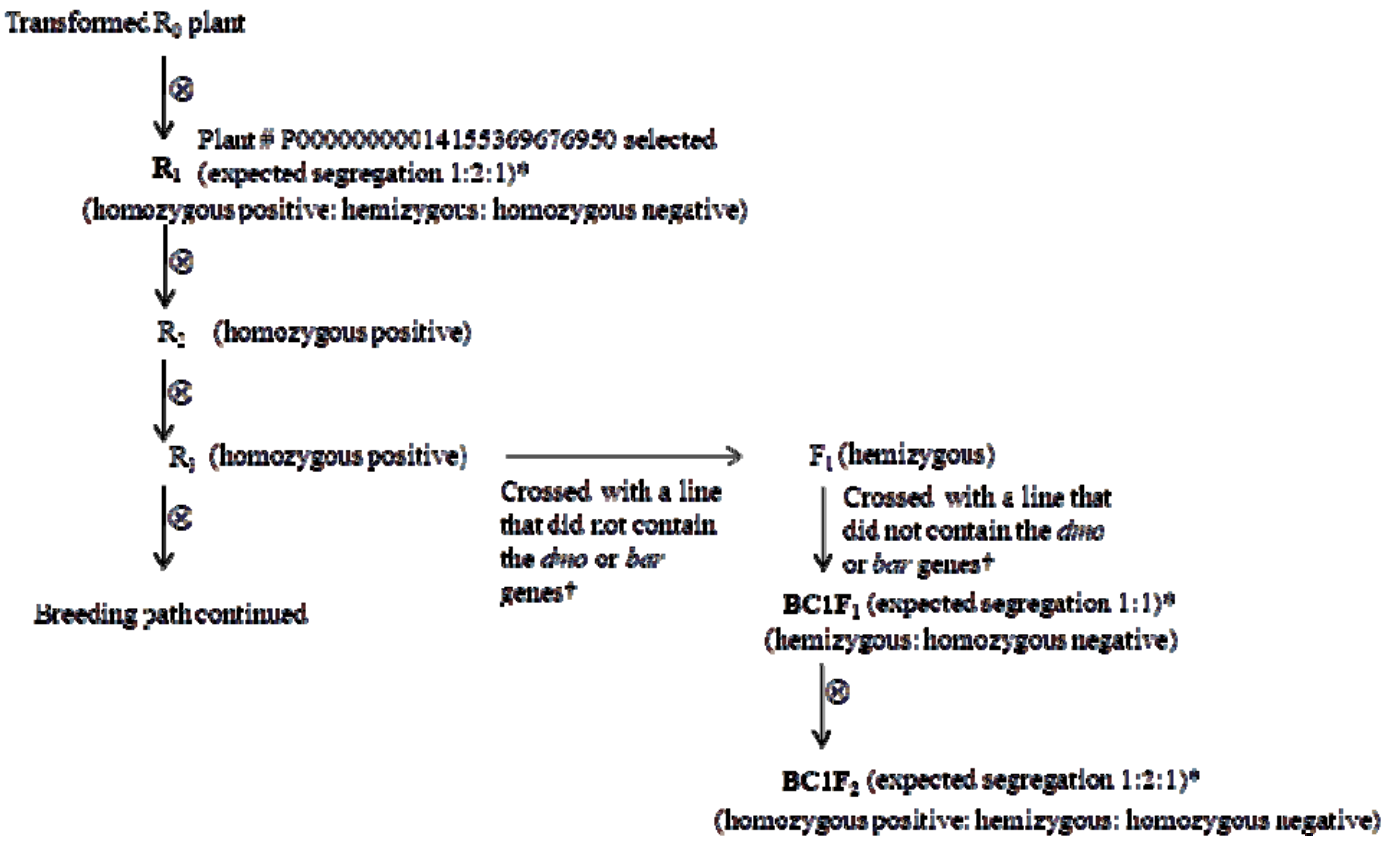


Figure 14. Breeding Path for Generating Segregation Data for MON 88701

*Chi-square analysis was conducted on segregation data from the R_1, $BC1F_1$, and $BC1F_2$ generations (bolded text).
†The cotton line used in the cross that did not contain the *dmo* or *bar* genes is a Monsanto proprietary cotton inbred.
⊗=Self- Pollinated

Table 4. Segregation of the T-DNA During the Development of MON 88701: 1:1 Segregation

				1:1 Segregation			
Generation	**Total Plants**	**Observed # Plants Hemizygous**	**Observed # Plants Homozygous Negative**	**Expected # Plants Hemizygous**	**Expected # Plants Homozygous Negative**	**χ^2**	**Probability[2]**
$BC1F_1$[1]	261	123	138	130.5	130.5	0.862	0.3532

[1] Segregation was evaluated using a glufosinate herbicide application followed by End-Point TaqMan analysis for the MON 88701 insert.

[2] Chi-square analysis was performed to analyse the segregation ratios ($p \leq 0.05$).

Table 5. Segregation of the T-DNA During the Development of MON 88701: 1:2:1 Segregation

					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[3]**
R_1[1]	173	33	99	41	43.25	86.50	43.25	4.353	0.1135
$BC1F_2$[2]	118	36	56	26	29.50	59.00	29.50	2.000	0.3679

[1] Segregation was evaluated using Real-Time TaqMan analysis for the *dmo* coding region.

[2] Segregation was evaluated using a glufosinate herbicide application followed by End-Point TaqMan analysis for the MON 88701 insert.

[3] Chi-square analysis was performed to analyse the segregation ratios ($p \leq 0.05$).

Southern Blot Analysis to Examine Insert Stability in Multiple Generations of MON 88701

In order to demonstrate the stability of the insert in MON 88701, Southern blot analysis was performed using genomic DNA extracted from leaf tissues from five breeding generations of MON 88701. For reference, the breeding history of MON 88701 is presented in Figure 13. The specific generations tested are indicated in the legend of Figure 13. The R_3 generation was used for the molecular characterisation analyses shown in Figure 6 through Figure 9. To analyse insert stability, four samples from four additional generations of MON 88701 were evaluated by Southern blot analysis and compared to the R_3 generation. Genomic DNA, isolated from each of the selected generations of MON 88701, was digested with the restriction enzyme *Bcl* I and simultaneously hybridised with Probe 2 and Probe 4 (Figure 4 and Figure 5), which was designed to detect both fragments generated by the *Bcl* 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)(ii). Please also refer to [redacted] 2011 (MSL0023322).

T-DNA Probes 2 and 4

Conventional control genomic DNA digested with restriction enzyme *Bcl* I and simultaneously hybridised with Probe 2 and Probe 4 (Figure 4 and Figure 5) produced no hybridisation signals (Figure 15, Lane 1) as expected for the negative control. Conventional control genomic DNA digested with *Bcl* I and spiked with the PV–GHHT6997 DNA, previously digested with the restriction enzyme *Pci* I (Figure 4 and Figure 5), produced one expected band at ~6.2 kb (Figure 15, Lane 2). Conventional control genomic DNA digested with *Bcl* I and spiked with probe templates of Probe 2 and Probe 4 produced the expected bands at ~1.0 kb and ~0.8 kb (Figure 15, Lane 3 and Lane 4). Detection of the positive controls indicates that the probes hybridised to their target sequences.

MON 88701 genomic DNA digested with *Bcl* I and hybridised with Probe 2 and Probe 4 (Figure 4 and Figure 5) is expected to produce a Southern fingerprint with two bands at ~3.5 kb and ~2.4 kb (Figure 4 and Figure 5). Southern fingerprints produced from multiple generations (Figure 15, Lane 5 and Lanes 7-9) of MON 88701 are consistent with the one produced from the fully characterised generation R_3 (Figure 7, Lane 2 and Lane 9, and Figure 15, Lane 6), indicating that MON 88701 contains one copy of the T-DNA insert that is stable across multiple generations.

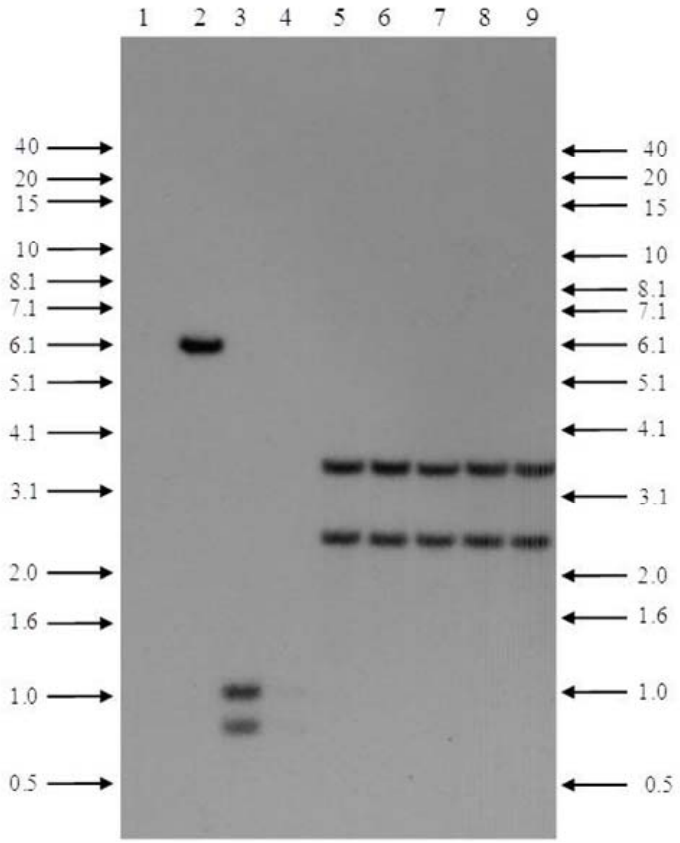


Figure 15. Southern Blot Analysis to Examine Insert Stability in Multiple Generations of MON 88701: Probes 2 and 4

The blot was simultaneously hybridised with two ^{32}P-labeled probes that span a portion of the T-DNA sequence (Figure 4, Probe 2 and Probe 4). 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 (*Bcl* I)

2 Conventional control (*Bcl* I) spiked with PV–GHHT6997 (*Pci* I) [~1.0 genome equivalent]

3 Conventional control (*Bcl* I) spiked with Probe 2 and Probe 4 template [~1.0 genome equivalent]

4 Conventional control (*Bcl* I) spiked with Probe 2 and Probe 4 template [~0.1 genome equivalent]

5 MON 88701 (R_2) (*Bcl* I)

6 MON 88701 (R_3) (*Bcl* I)

7 MON 88701 (R_4) (*Bcl* I)

8 MON 88701 (R_5) (*Bcl* I))

9 MON 88701 (R_6) (*Bcl* I)

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

In order to assess the presence of the MON 88701 DMO and PAT (*bar*) proteins in MON 88701 across multiple generations, western blot analysis of MON 88701 was conducted on leaf tissue collected from generations R_2, R_3, R_4, R_5, and R_6 (Figure 13) of MON 88701, and on leaf tissue of the conventional control.

MON 88701 DMO Protein Generational Stability

The presence of the MON 88701 DMO protein in harvested leaf tissue of the R_2, R_3, R_4, R_5, and R_6 generations of MON 88701(Figure 13) was demonstrated (Figure 16). An *E coli*-produced MON 88701 DMO standard (1 ng) was used as a reference for the identification of the MON 88701 DMO protein. The presence of MON 88701 DMO protein in MON 88701 leaf tissue samples was determined by visual comparison of the bands produced in multiple breeding generations (Figure 16, Lanes 3–7) to the MON 88701 DMO reference standard (Figure 16, Lane 1). As shown in Figure 16, MON 88701 DMO protein was present in multiple generations of MON 88701 tissue samples and migrated with a mobility indistinguishable from that of the *E. coli*-produced protein standard analysed on the same western blot. As expected, the MON 88701 DMO protein was not detected in the conventional control extract (Figure 16, Lane 8). Please also refer to [redacted] 2011 (MSL0023322).

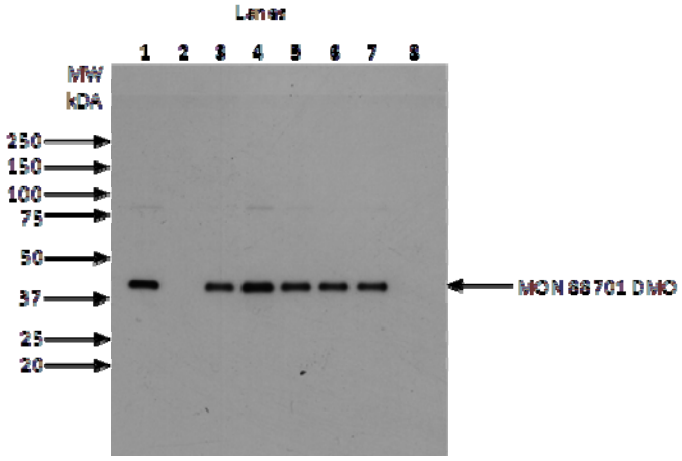


Figure 16. Presence of MON 88701 DMO Protein in Multiple Generations of MON 88701

Extracts from five generations of MON 88701 leaf tissues, conventional control leaf tissues, *E. coli*-produced MON 88701 DMO protein standard, and molecular weight markers were subjected to SDS-PAGE and electrotransferred to a nitrocellulose membrane. The membrane was incubated with goat anti-DMO antibody and immunoreactive bands visualised through the use of ECL reagents. The image represents a 20 sec exposure. Arrows denote the size of the protein, in kiloDaltons (kDa), obtained from the Precision Plus Protein Dual Color Standards (Bio-Rad) transferred to the western membrane. Lane designations are as follows:

Lane	Sample	Amount (µl)
1	*E. coli*-produced MON 88701 DMO protein	20 (1 ng)
2	Empty	-
3	R_2 Generation	20
4	R_3 Generation	20
5	R_4 Generation	20
6	R_5 Generation	20
7	R_6 Generation	20
8	Conventional Control – Coker 130	20

MON 88701-produced PAT (*bar*) Protein Generational Stability

The presence of the PAT (*bar*) protein in harvested leaf tissue of the R_2, R_3, R_4, R_5, and R_6 generations of MON 88701 was demonstrated (Figure 17). An *E. coli*-produced PAT (*bar*) standard (0.5 ng) was used as a reference for the identification of the PAT (*bar*) protein. The presence of PAT (*bar*) protein in MON 88701 leaf tissue samples was determined by visual comparison of the bands produced in the multiple breeding generations (Figure 17, Lanes 3–7) to the PAT (*bar*) reference standard (Figure 17, Lane 1). As shown in Figure 17, PAT (*bar*) protein was present in multiple generations of MON 88701 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 PAT (*bar*) protein was not detected in the conventional control extract (Figure 17, Lane 8). Please also refer to [redacted], 2011 (MSL0023322)

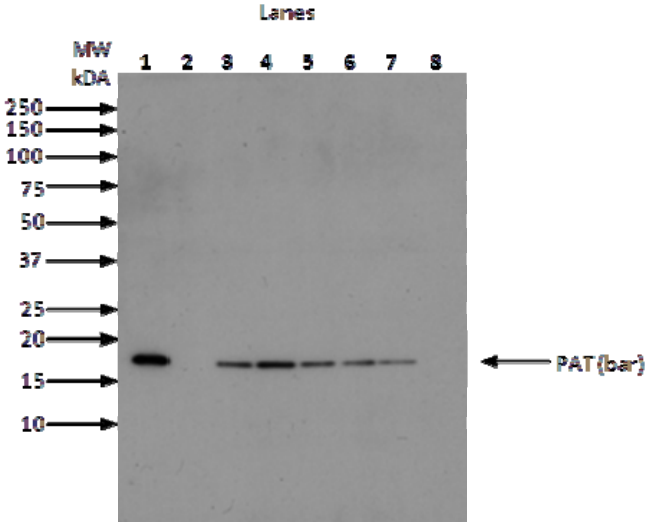


Figure 17. Presence of PAT (*bar*) Protein in Multiple Generations of MON 88701

Aliquots of extracts from five generations of MON 88701 leaf tissues, conventional control leaf tissues, *E. coli*-produced PAT (*bar*) protein standard, and molecular weight markers were subjected to SDS-PAGE and electrotransferred to a nitrocellulose membrane. The membrane was incubated with goat anti-PAT (*bar*) antibody and immunoreactive bands visualised through the use of ECL reagents. The image represents a 20 sec exposure. Arrows denote the size of the protein, in kiloDaltons (kDA), obtained from the Precision Plus Protein Dual Color Standards (Bio-Rad) transferred to the Western membrane. Lane designations are as follows:

Lane	Sample	Amount (µl)
1	*E. coli*-produced PAT (*bar*) protein	20 (0.5 ng)
2	Empty	-
3	R_2 Generation	20
4	R_3 Generation	20
5	R_4 Generation	20
6	R_5 Generation	20
7	R_6 Generation	20
8	Conventional Control – Coker 130	20

A4. Analytical method for detection

The T-DNA insert can be detected by isolating genomic DNA from leaf tissue and digesting with appropriate restriction endonucleases. Southern Blot analysis following digestion of genomic DNA with the appropriate restriction endonucleases will produce banding patterns consistent with the presence of the insert in MON 88701.

B. Information Related to the Safety of the GM Food

B1 Equivalence Studies

MON 88701 DMO Protein Identity and Equivalence

The safety assessment of crops derived through biotechnology includes characterisation of the physicochemical and functional properties of and confirmation of the safety of the introduced protein(s). For the safety data generated using *E. coli*-produced MON 88701 DMO to be applied to MON 88701 DMO protein produced in MON 88701, the equivalence of the plant- and *E. coli*-produced proteins must be assessed. To assess the equivalence between MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins, a small quantity of the MON 88701 DMO protein was purified from MON 88701 cottonseed. The MON 88701 DMO protein was characterised and the equivalence of the physicochemical characteristics and functional activity between the MON 88701 DMO and the *E. coli*-produced MON 88701 DMO proteins was assessed using a panel of six analytical tests as shown in Table 6. Taken together, these data provide a detailed characterisation of the MON 88701 DMO protein and establish the equivalence of MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins.

Please also refer to [redacted] 2012 (MSL0023517).

MON 88701-produced PAT (bar) Protein Identity and Equivalence

The safety assessment of crops derived through biotechnology includes characterisation of the physicochemical and functional properties of and confirmation of the safety of the introduced protein(s). For the safety data generated using *E. coli*-produced PAT (*bar*) protein to be applied to PAT (*bar*) protein produced in MON 88701, the equivalence of the plant- and *E. coli*-produced PAT (*bar*) proteins was assessed. To assess the equivalence between MON 88701- and *E. coli*-produced PAT (*bar*) proteins, a small quantity of the PAT (*bar*) protein was purified from MON 88701 cottonseed. The MON 88701-produced PAT (*bar*) protein was characterised and the equivalence of the immunoreactive and physicochemical characteristics and functional activity between the MON 88701-produced PAT (*bar*) and the *E. coli*-produced PAT (*bar*) proteins was assessed using a panel of six analytical tests as shown in Table 11. Taken together, these data provide a detailed characterisation of the MON 88701-produced PAT (*bar*) protein and establish the equivalence of MON 88701- and *E. coli*-produced PAT (*bar*) proteins.

Please also refer to [redacted] 2012 (MSL0023428).

B2 Antibiotic Resistance Marker Genes

No genes that encode resistance to an antibiotic marker were inserted into the cotton genome during the development of MON 88701. The backbone of the PV-GHHT6997 plasmid vector contained the *aadA* antibiotic resistant marker gene. Molecular characterisation data presented in in this application demonstrate the absence of the *aadA* antibiotic resistant marker gene in MON 88701.

B2(a) Clinical importance of antibiotic that GM is resistant to (if any)

Not applicable.

B2(b) Presence in food of antibiotic resistance protein (if any)

Not applicable.

B2(c) Safety of antibiotic protein

Not applicable.

B2(d) If GM organism is micro-organism, is it viable in final food?

Not applicable.

B3 Characterisation of novel proteins or other novel substances

B3(a) Biochemical function and phenotypic effects of novel protein(s)

Description of MON 88701 DMO Protein

In MON 88701, the introduced DMO protein is active in the chloroplast, a plastid organelle, where it can interact with other proteins needed for its function (Behrens et al., 2007). In the construction of the PV–GHHT6997 plasmid vector used in the development of MON 88701 a transit peptide coding sequence (*CTP2,* Table 3) was joined to the *dmo* coding sequence. This coding sequence results in the production of a precursor protein consisting of the DMO protein and an additional 76 amino acids at the N-terminus of the protein. These additional amino acids correspond to the chloroplast transit peptide (CTP) from *Arabidopsis thaliana* EPSPS (CTP2), which is incorporated to improve the targeting of the precursor protein to the chloroplast (Herrmann, 1995; Klee et al., 1987). Typically, transit peptides are precisely removed from the precursor protein following delivery to the targeted plastid (Della-Cioppa et al., 1986) resulting in the full-length protein. However, there are examples in the literature of alternatively processed forms of a protein targeted to a plant's chloroplast, where part of the transit peptide remains (Behrens et al., 2007; Clark and Lamppa, 1992). Such alternative processing is observed with the MON 88701 DMO protein produced in MON 88701.

Analysis of cottonseed extracts from MON 88701 determined that the expressed protein had an apparent molecular weight of 39.5 kDa and corresponded to the DMO protein with nine amino acids on the N-terminus originating from the EPSPS chloroplast transit peptide. The resulting 349 amino acid polypeptide is referred to as MON 88701 DMO. Alternative processing of DMO precursor proteins has been observed in other dicamba-tolerant plants containing the *dmo* gene (Behrens et al., 2007).

Except for the nine amino acids derived from the CTP2 and an additional leucine at position two, the MON 88701 DMO protein has an identical sequence to the wild-type DMO protein from the DI-6 strain of *S. maltophilia* (Herman et al., 2005). The differences in the amino acid sequence between the wild-type DMO protein and MON 88701 DMO protein are not expected to have an effect on structure, activity, or specificity because the N-terminus and position two are sterically distant from the catalytic site (D'Ordine et al., 2009; Dumitru et al., 2009). The DMO protein produced in MON 88701 is hereinafter referred to as MON 88701 DMO protein. Accordingly, the DMO protein produced from *E. coli* with the same sequence as MON 88701 DMO is referred to as *E. coli*-produced MON 88701 DMO protein.

MON 88701 DMO was purified from cottonseed of MON 88701 and its activity was confirmed during characterisation.

MON 88701 DMO Mode-of-Action

DMO is an enzyme classified as a mono-oxygenase. Mono-oxygenases are enzymes that incorporate a single oxygen atom as a hydroxyl group with the concomitant production of water and oxidation of nicotinamide adenine dinucleotide (NADH) (Harayama et al., 1992) and are found in diverse phyla ranging from bacteria to plants (Ferraro et al., 2005; Schmidt and Shaw, 2001). The active form of DMO, necessary to confer dicamba tolerance, is a trimer comprised of three DMO monomers (Chakraborty et al., 2005; D'Ordine et al., 2009; Herman et al., 2005). The formation of a trimer is required because the electron transport that culminates in the demethylation of dicamba occurs from one monomer to another in the native conformation of the enzyme (D'Ordine et al., 2009).

Wild-type DMO was initially purified from the *S. maltophilia* strain DI-6 that was isolated from soil at a dicamba manufacturing plant (Krueger et al., 1989). DMO is an enzyme that catalyses the demethylation of dicamba to the non-herbicidal compound DCSA and formaldehyde (Chakraborty et al., 2005). DCSA is a known cotton, soy, livestock, and soil metabolite of dicamba whose safety has been evaluated and deemed safe (reasonable certainty of no harm as defined by FFDCA) by the EPA (U.S. EPA, 2009). Formaldehyde is routinely produced in plants and is present at levels up to several hundred parts per million (ppm) across those different plants (Adrian-Romero et al., 1999). Thus, neither DCSA nor formaldehyde generated by the action of DMO on dicamba pose a significant food or feed safety risk.

DMO is a Rieske-type non-heme iron oxygenase, that forms part of a three component system comprised of a reductase, a ferredoxin, and a terminal oxygenase, in this case a DMO. These three proteins work together in a redox system similar to many other oxygenases to transport electrons from NADH to oxygen and catalyse the demethylation of an electron acceptor substrate, in this case dicamba (Chakraborty et al., 2005) This three component redox system is presented in Figure 18.

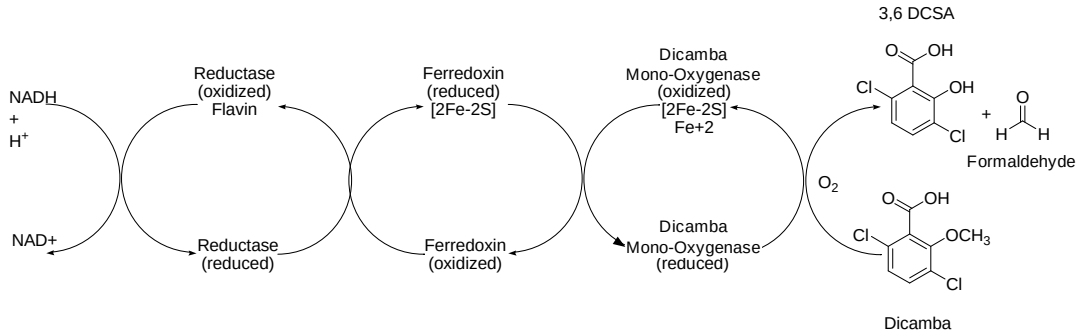


Figure 18. Three Components of the DMO Redox System

Depicted is the electron transport chain that starts with NADH and ends with DMO resulting in the demethylation of dicamba to form DCSA.

The crystal structure of DMO has been solved using a C-terminal histidine tagged DMO (D'Ordine et al., 2009; Dumitru et al., 2009), which is identical to wild-type DMO, except for containing an additional alanine at position two, which was added for cloning purposes, and a histidine tag at the C terminus. The addition of a polyhistidine tag fused to the N or C terminus of a protein of interest is a common tool used to aid in protein purification (Hochuli et al., 1988). The crystal structure of DMO was determined to be a trimer comprised of three identical DMO monomers (D'Ordine et al., 2009; Dumitru et al., 2009). Each monomer contains a Rieske [2Fe-2S] cluster domain and a non-heme iron center domain (D'Ordine et al., 2009; Dumitru et al., 2009) that are typical of all Rieske-type mono-oxygenases and are the key domains involved in electron transport (Ferraro et al., 2005). The catalytic site in each monomer was characterised to determine the fit of dicamba in the site and hypothesise the reaction mechanism of dicamba demethylation (D'Ordine et al., 2009; Dumitru et al., 2009).

The trimeric quaternary structure of DMO was the native form of the enzyme observed during crystallisation and is required for electron transport and catalysis (D'Ordine et al., 2009; Dumitru et al., 2009). To catalyse the demethylation of dicamba, electrons transferred from NADH are shuttled through an endogenous reductase and ferredoxin to the terminal DMO (Figure 18). The electrons are received from ferredoxin by the Rieske [2Fe-2S] cluster on one of the DMO monomers of the trimer and transferred to the non-heme iron center at the catalytic site of an adjacent monomer (D'Ordine et al., 2009; Dumitru et al., 2009), where it reductively activates oxygen to catalyse the final demethylation of dicamba. For this electron transfer to occur between adjacent monomers of DMO, a trimeric structure is required with precise spacing and orientation between the three monomers (D'Ordine et al., 2009). Electron transport from the Rieske [2Fe-2S] cluster domain to the non-heme iron center domain cannot occur within a monomer since the distance it too vast (D'Ordine et al., 2009; Dumitru et al., 2009).

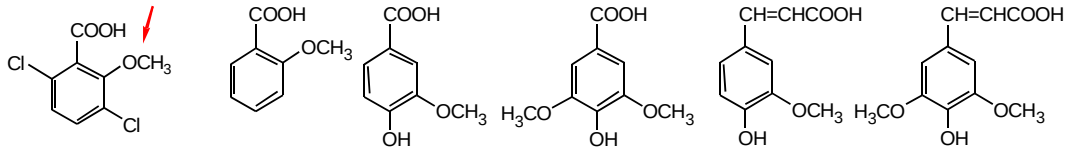
Therefore, in order for MON 88701 to be tolerant to dicamba, a functional trimeric MON 88701 DMO must be formed. The active trimeric form of MON 88701 DMO, as purified from MON 88701, confers dicamba tolerance to MON 88701, and its demethylase activity on dicamba was confirmed during characterisation supporting the conclusion that the trimer required for functional activity was formed in MON 88701.

MON 88701 DMO Specificity

The substrate specificity of MON 88701 DMO was evaluated to understand potential interactions DMO may have with potential substrates present in MON 88701 cotton. The literature indicates the specificity of DMO for dicamba is due to the specific interactions that occur at the catalytic site (D'Ordine et al., 2009; Dumitru et al., 2009). Dicamba interacts with amino acids in the catalytic site of DMO through both the carboxylate moiety and the chlorine atoms of dicamba, which are primarily involved in orienting the substrate in the catalytic site. These chlorine atoms are required for catalysis (D'Ordine et al., 2009; Dumitru et al., 2009). Given the limited existence of chlorinated compounds with structures

similar to dicamba in plants and other eukaryotes (Wishart, 2010; Wishart et al., 2009), it is unlikely that MON 88701 DMO will catalyse the conversion of other endogenous substrates.

The potential for MON 88701 DMO to metabolise endogenous plant substrates was evaluated in *in vitro* experiments using a purified N-terminal histidine tagged DMO that was identical to wild-type DMO, except for a histidine tag at the N-terminus added to aid in protein purification. A set of potential endogenous substrates was selected for evaluation based on structural similarity of the compounds to dicamba and their presence in cotton, corn and soybean (Buchanan et al., 2000; Janas et al., 2000; Lege et al., 1995; Schmelz et al., 2003). The potential substrates tested were *o*-anisic acid (2-methoxybenzoic acid), vanillic acid (4-hydroxy-3-methoxybenzoic acid), syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid), ferulic acid [3-(4-hydroxy-3-methoxy-phenyl) prop-2-enoic acid] and sinapic acid [3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid] (Figure 19). The assay mixture included NADH, reductase, ferredoxin and DMO. Dicamba was first used as a positive control to demonstrate that the assay system was functional. The disappearance of potential substrates and the formation of potential oxidation products were monitored using LC-UV and LC-MS. None of the tested substrates, except dicamba, were metabolised by the histidine tagged DMO in these *in vitro* experiments. To assess whether MON 88701 DMO protein has the same specificity as the histidine tagged DMO used in the *in vitro* experiments, the *E. coli*-produced MON 88701 DMO protein, shown to be equivalent to the plant produced MON 88701 DMO protein, was incubated with *o*-anisic acid, the endogenous compound that has the greatest structural similarity to dicamba. Again dicamba was used as a positive control to demonstrate the assay system was functional. This analysis demonstrated that *o*-anisic acid was not metabolised by the *E. coli*-produced MON 88701 DMO protein, but dicamba was. These results indicate that DMO, including the MON 88701 DMO protein, is specific for dicamba as a substrate.



Dicamba o-Anisic Acid Vanillic Acid Syringic Acid Ferulic Acid Sinapic Acid

Figure 19. Dicamba and Potential Endogenous Substrates Tested in *In Vitro* Experiments with DMO

The arrow indicates methyl group removed by DMO.

Description of PAT (bar) Protein

Phosphinothricin N-acetyltransferase (PAT) proteins conferring tolerance to glufosinate herbicide (2-amino-4-(hydroxymethylphosphinyl) butanoic acid) have been isolated from two separate species of *Streptomyces*, *S. hygroscopicus* (Thompson et al., 1987) and *S. viridochromogenes* (Wohlleben et al., 1988). The PAT protein isolated from *S. hygroscopicus* is encoded by the *bar* gene, and the PAT protein isolated from *S. viridochromogenes* is encoded by the *pat* gene. These PAT proteins are made up of 183 amino acids with 85% identity at the amino acid level. Based on previous studies (Wehrmann et al., 1996) that have extensively characterised PAT proteins produced from *bar* and *pat* genes, OECD recognises both proteins to be equivalent with regard to function and safety (OECD, 1999). The safety of PAT proteins present in biotechnology-derived crops has been extensively assessed (Hérouet et al., 2005; ILSI-CERA, 2011).

The PAT protein produced in MON 88701 is from the *bar* gene, and for clarity, the PAT protein produced in MON 88701 will be referred to as PAT (*bar*). Analysis of cottonseed extracts from MON 88701 determined that the expressed protein corresponded to the 183 amino acid polypeptide, resulting in a 24.1 kDa PAT (*bar*) protein. The activity of the PAT (*bar*) protein purified from MON 88701 cottonseed was confirmed during characterisation.

PAT (bar) Mode-of-Action

The mode-of-action for PAT protein has been extensively assessed, as a number of other glufosinate-tolerant products have been reviewed by FSANZ[5] PAT, including the PAT (*bar*) protein produced in MON 88701, is an enzyme classified as an acetyltransferase which acetylates glufosinate to produce non-herbicidal N-acetyl glufosinate. Glufosinate is a racemic mixture of the D- and L- forms of phosphinothricin. The herbicidal activity of glufosinate results from the binding of L-phosphinothricin to glutamine synthetase (OECD, 1999; 2002). Glutamine synthetase is responsible for the assimilation of ammonia generated during photorespiration. The binding of L-phosphinothricin to glutamine synthetase results in the inactivation of glutamine synthetase and a subsequent toxic build-up of ammonia within the plant, resulting in death of the plant (Manderscheid and Wild, 1986; OECD, 1999; 2002; Wild and Manderscheid, 1984).

The PAT (*bar*) protein produced in MON 88701 acetylates the free amine group of L-phosphinothricin form of glufosinate to produce non-herbicidal N-acetyl glufosinate. The acetylated glufosinate is unable to bind to glutamine synthetase and therefore does not disrupt photorespiration and avoids the build-up of ammonia. MON 88701 confers tolerance to glufosinate herbicide via the detoxification by phosphinothricin, as described above.

[5] A372, A533, A589, A1028, A1040: heep://www.foodstandards.gov.au

PAT (bar) Specificity

The PAT proteins, including PAT (*bar*), are highly specific for glufosinate in the presence of acetyl-CoA (Thompson et al., 1987; Wehrmann et al., 1996). While the herbicidal activity of glufosinate comes from the L-amino acid form, other L-amino acids are unable to be acetylated by PAT protein, and competition assays containing glufosinate, high concentrations of other amino acids and PAT showed no inhibition of glufosinate acetylation (Wehrmann et al., 1996). Furthermore, L-glutamate, an analogue of glufosinate, also showed no inhibition of glufosinate acetylation in competition assays (Wehrmann et al., 1996). In addition, the PAT (*bar*) protein has more than 30-fold higher affinity towards L-phosphinothricin over other plant analogues (Thompson et al., 1987). Thus, the PAT (*bar*) protein has high substrate specificity for L-phosphinothricin, the herbicidal component of glufosinate, and it is unlikely to affect the metabolic system of MON 88701 cotton. Numerous glufosinate-tolerant products including those in cotton, corn, soy, canola, sugarbeet and rice have been reviewed by FSANZ and other regulatory authorities with no concerns identified (ILSI-CERA, 2011).

B3(b) Identification of novel substances (e.g. metabolites), levels and site

B3(b)(i)Characterisation of the Protein from MON 88701

Characterisation of the MON 88701 DMO Protein from MON 88701

The safety assessment of crops derived through biotechnology includes characterisation of the physicochemical and functional properties of and confirmation of the safety of the introduced protein(s). For the safety data generated using *E. coli*-produced MON 88701 DMO to be applied to MON 88701 DMO protein produced in MON 88701, the equivalence of the plant- and *E. coli*-produced proteins must be assessed. To assess the equivalence between MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins, a small quantity of the MON 88701 DMO protein was purified from MON 88701 cottonseed. The MON 88701 DMO protein was characterised and the equivalence of the physicochemical characteristics and functional activity between the MON 88701 DMO and the *E. coli*-produced MON 88701 DMO proteins was assessed using a panel of six analytical tests as shown in Table 6. Taken together, these data provide a detailed characterisation of the MON 88701 DMO protein and establish the equivalence of MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins.

Please also refer to [redacted] 2012 (MSL0023517).

Table 6. Summary of MON 88701 DMO Protein Identity and Equivalence

Analytical Test Assessment	Analytical Test Outcome
N-terminal sequence analysis of the MON 88701 DMO protein to assess identity	The identity could not be confirmed by N-terminal sequence analysis MALDI-TOF MS[1] analysis of peptides derived from tryptic digested MON 88701 DMO established the N-terminal sequence of MON 88701 DMO
MALDI-TOF MS[1] analysis of peptides derived from tryptic digested MON 88701 DMO protein to assess identity	MALDI-TOF MS[1] analysis yielded peptide masses consistent with the expected peptide masses from the theoretical trypsin digest of the MON 88701 DMO sequence
Western blot analysis using anti-DMO polyclonal antibodies to assess identity and immunoreactive equivalence between MON 88701 DMO and the *E. coli*-produced MON 88701 DMO proteins	MON 88701 DMO protein identity was confirmed using a western blot probed with antibodies specific for DMO protein Immunoreactive properties of the MON 88701 DMO and the *E. coli*-produced MON 88701 DMO proteins were shown to be equivalent
SDS-PAGE[2] to assess equivalence of the apparent molecular weight between MON 88701 DMO and the *E. coli*-produced MON 88701 DMO proteins	Electrophoretic mobility and apparent molecular weight of the MON 88701 DMO and the *E. coli*-produced MON 88701 DMO proteins were shown to be equivalent
Glycosylation analysis of the MON 88701 DMO protein to assess equivalence between the MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins	Glycosylation status of MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins were shown to be equivalent
DMO enzymatic activity analysis to assess functional equivalence between MON 88701 DMO and the *E. coli*-produced MON 88701 DMO proteins	Functional activity of the MON 88701 DMO and the *E. coli*-produced MON 88701 DMO proteins were shown to be equivalent

[1] MALDI-TOF MS = Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry

[2] SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis

Results of the N-Terminal Sequencing Analysis

N-terminal sequencing reaction was performed on MON 88701 DMO protein. The reaction did not yield any observable sequence, presumably because the N-terminus was blocked. Although this analysis did not yield N-terminal sequence data, the N-terminus of the MON 88701 DMO protein was determined using MALDI-TOF tryptic mass map analysis.

Results of MALDI-TOF Tryptic Mass Map Analysis

The identity of the MON 88701 DMO protein was confirmed by MALDI-TOF MS analysis of peptide fragments produced from tryptic digestion of the MON 88701 DMO 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 ≥ 40% of the protein sequence was identified by matching experimental masses observed for the tryptic peptide fragments to the expected masses for the fragments (Biron et al., 2006; Krause et al., 1999).

There were 19 unique peptides identified that corresponded to the masses expected to be produced by tryptic digestion of the MON 88701 DMO protein (Table 7). The identified masses were used to assemble a coverage map of the entire MON 88701 DMO protein (Table 7). The experimentally-determined mass coverage of the MON 88701 DMO protein was 66.5% (232 out of 349 amino acids). This analysis serves as identity confirmation for the MON 88701 DMO protein.

To identify the N-terminus, the experimentally-determined masses of the peptides produced from tryptic digestion of the MON 88701 DMO protein were examined for the presence of a mass that matched the theoretical mass expected from the MON 88701 DMO protein deduced from the *dmo* gene present in MON 88701. A mass was identified that corresponded to the predicted mass of an acetylated peptide with nine amino acids from CTP2 followed by the MON 88701 DMO protein deduced from the *dmo* gene present in MON 88701. The additional nine amino acids of CTP2 resulted from the alternative processing of CTP2. Alternative processing of DMO precursor proteins has been observed in other dicamba-tolerant plants containing the *dmo* gene (Behrens et al., 2007). Hence, the MON 88701 DMO protein was designated to have an N-terminal end as shown in Figure 20.

Table 7. Summary of the Tryptic Masses[1] Identified for the MON 88701 DMOProtein Protein Using MALDI-TOF MS

α-cyano	DHB	Sinapinic acid	Expected Mass	Diff.[2]	Fragment[3]	Sequence
720.40			720.37	0.03	140-145	VDPAYR
833.51	833.45		833.45	0.06	108-114	SFPVVER
856.49			856.43	0.06	251-257	EQSIHSR
914.60			914.53	0.07	305-312	VVVEAIER
	1030.58		1030.57	0.01	293-301	SWQAQALVK
1108.61	1108.59		1108.50	0.11	176-185	ANAQTDAFDR
1275.87	1275.83		1275.73	0.14	35-45	TILDTPLALYR
1286.83			1286.70	0.13	302-312	EDKVVVEAIER
1428.84	1428.83		1428.69	0.15	218-230	GANTPVDAWNDIR
	1470.74		1470.63	0.11	146-158	TVGGYGHVDCNYK
	1501.91		1501.79	0.12	189-202	EVIVGDGEIQAALMK
	1506.86		1506.73	0.13	176-188	ANAQTDAFDRLER
	1577.89	1577.80	1577.73	0.16	279-292	NFGIDDPEMDGVLR
		1731.92	1731.80	0.12	1-15	VMSSVSTACMLTFVR +42 Da (N-acetylation)
	1745.09	1744.99	1744.93	0.16	234-250	VSAMLNFIAVAPEGTPK
	1994.30	1994.23	1994.03	0.27	159-175	LLVDNLMDLGHAQYVHR
		2143.35	2143.12	0.23	16-34	NAWYVAALPEELSEKPLGR
	2398.37	2398.35	2398.09	0.28	258-278	GTHILTPETEASCHYFFGSSR
		2724.72	2724.31	0.41	115-139	DALIWIWPGDPALADPGAIPGCR

[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 MON 88701 DMO sequence as depicted in Figure 20.

DHB = 5-dihydroxybenzoic acid matrix, α-cyano = α-cyano-4-hydroxycinnamic acid matrix, Sinapinic acid = 3, 5-dimethoxy-4-hydroxycinnamic acid matrix.

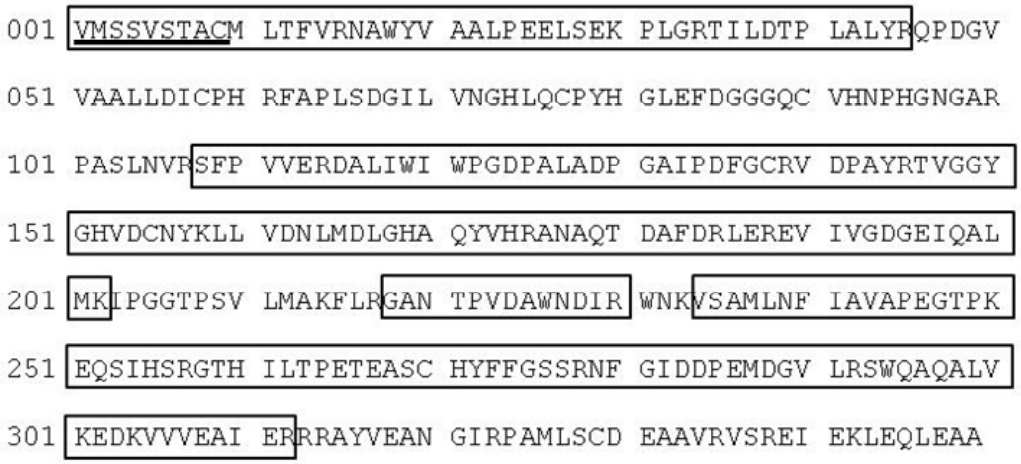


Figure 20. MALDI-TOF MS Coverage Map of the MON 88701 DMO Protein

The amino acid sequence of the MON 88701 DMO protein was deduced from the *dmo* gene present in MON 88701. Boxed regions correspond to regions covered by tryptic peptides that were identified from the MON 88701 DMO protein sample using MALDI-TOF MS. Underlined region corresponds to the nine amino acids from CTP2 retained at the N-terminus of the MON 88701 DMO. In total, 66.5% (232 of 349 total amino acids) of the expected protein sequence was covered by the identified peptides.

Results of Western Blot Analysis of the MON 88701 DMO Protein Isolated from the Cottonseed of MON 88701 and Immunoreactivity Comparison to *E. coli*-produced MON 88701 DMO

Western blot analysis was conducted using goat anti-DMO polyclonal antibodies to 1) assess the identity of the MON 88701 DMO protein isolated from the cottonseed of MON 88701; and 2) to determine the relative immunoreactivity of the MON 88701 DMO and the *E. coli*-produced MON 88701 DMO proteins. The results demonstrated that the anti-DMO antibodies recognised the MON 88701 DMO protein that migrated to the same position on the blot as the *E. coli*-produced MON 88701 DMO protein (Figure 21). Furthermore, the immunoreactive signal increased with increasing amounts of MON 88701 DMO protein loaded. Two other bands, one migrating at ~75 kDa and the other at ~17 kDa were also observed. These bands were prominent in lanes with higher load amounts (Figure 21, Lanes 3-6), and may represent products of aggregation and degradation of DMO, respectively.

Densitometric analysis was conducted to compare the immunoreactivity of MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins. The mean signal intensity (OD × mm^2) from the MON 88701 DMO bands and from the *E. coli*-produced MON 88701 DMO bands at each amount of protein analysed was calculated and then overall mean signal intensity was calculated (Figure 21). The immunoreactivity was considered equivalent if the overall mean signal intensity of all MON 88701 DMO protein bands was within ±35% of the overall mean signal intensity of *E. coli*-produced MON 88701 DMO protein bands across all loading levels.

The overall mean signal intensity of the *E. coli*-produced MON 88701 DMO bands was 6.500 OD × mm^2 and the overall mean signal intensity of the MON 88701 DMO bands was 4.440 OD × mm^2. Because overall mean signal intensity of the MON 88701 DMO protein bands was between 4.225 and 8.775 (between -35% and +35% of the *E. coli*-produced MON 88701 DMO bands), the MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins were determined to have equivalent immunoreactivity.

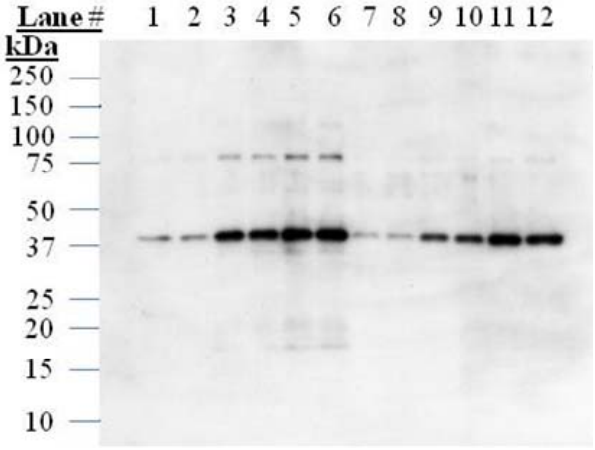


Figure 21. Western Blot Analysis of the MON 88701 DMO and *E. coli*-produced MON 88701 DMO Proteins

Aliquots of the MON 88701 DMO protein and the *E. coli*-produced MON 88701 DMO protein were subjected to SDS-PAGE and electrotransferred to a nitrocellulose membrane. The membrane was incubated with anti-DMO antibodies and immunoreactive bands were visualised using an ECL system (GE Healthcare, Piscataway, NJ). Approximate molecular weights (kDa) are shown on the left. Lanes loaded with molecular weight markers were cropped, and lanes were renumbered relative to the original gel loading. The 6 min exposure is shown. Lane designations are as follows:

Lane	Sample	Amount (ng)
1	*E. coli*-produced MON 88701 DMO protein	0.5
2	*E. coli*-produced MON 88701 DMO protein	0.5
3	*E. coli*-produced MON 88701 DMO protein	2
4	*E. coli*-produced MON 88701 DMO protein	2
5	*E. coli*-produced MON 88701 DMO protein	6
6	*E. coli*-produced MON 88701 DMO protein	6
7	MON 88701 DMO protein	0.5
8	MON 88701 DMO protein	0.5
9	MON 88701 DMO protein	2
10	MON 88701 DMO protein	2
11	MON 88701 DMO protein	6
12	MON 88701 DMO protein	6

Table 8. Comparison of Immunoreactive Signals between MON 88701 DMO and *E. coli*-produced MON 88701 DMO Proteins

Mean Signal intensity from MON 88701 DMO[1] (OD × mm^2)	Mean Signal intensity from *E. coli*-produced MON 88701 DMO (OD × mm^2)	Preset Acceptance limits for MON 88701 DMO[1] (OD × mm^2)
4.440	6.500	4.225 – 8.775

[1]The acceptance limits for MON 88701 DMO are based on the interval between +35% (6.500 × 1.35) and -35% (6.500 × 0.65) of the overall mean of the *E. coli*-produced MON 88701 DMO signal intensity across six loads.

Results of the MON 88701 DMO Protein Apparent Molecular Weight and Purity Analysis

The molecular weight and purity of the MON 88701 DMO protein was determined to be 39.5 kDa and 97%, respectively. To assess molecular weight (MW) and purity, the MON 88701 DMO protein was subjected to SDS-PAGE. The gel was stained with Brilliant Blue G Colloidal stain and analysed by densitometry (Figure 22). *E. coli*-produced MON 88701 DMO protein was loaded in a single lane for reference (Figure 22, Lane 2). The MON 88701 DMO protein (Figure 22, Lanes 3-8) had an apparent molecular weight of 39.5 kDa (Table 9). The apparent molecular weight of the *E. coli*-produced MON 88701 DMO protein as reported on its Certificate of Analysis was 38.7 kDa (Table 9). Because the apparent MW of MON 88701 DMO protein was within the preset acceptance limits for equivalence (Table 9), the MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins were determined to have equivalent apparent MWs.

The purity of the MON 88701 DMO protein was calculated based on the six loads on the gel (Figure 22, Lanes 3-8). The average purity was determined to be 97%.

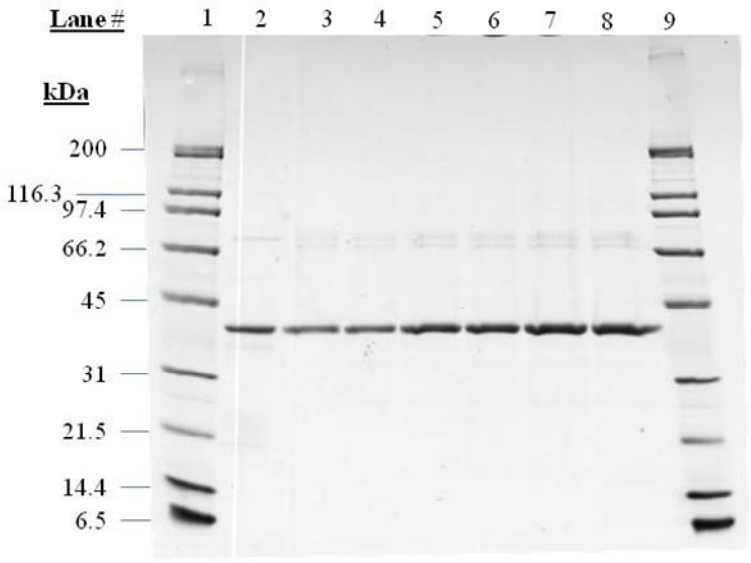


Figure 22. Molecular Weight and Purity Analysis of the MON 88701 DMO Protein

Aliquots of the MON 88701 DMO and the *E. coli*-produced MON 88701 DMO proteins were separated by SDS-PAGE 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. Empty lane was partially cropped. Lane designations are as follows:

Lane	Sample	Amount (µg)
1	Broad Range Molecular Weight Markers	4.5
2	*E. coli*-produced MON 88701 DMO protein	0.5
3	MON 88701 DMO protein	0.5
4	MON 88701 DMO protein	0.5
5	MON 88701 DMO protein	1
6	MON 88701 DMO protein	1
7	MON 88701 DMO protein	1.5
8	MON 88701 DMO protein	1.5
9	Broad Range Molecular Weight markers	4.5

Table 9. Molecular Weight Comparison Between the MON 88701 DMO and *E. coli*-produced MON 88701 DMO Proteins Based on SDS-PAGE

Apparent MW of MON 88701 DMO[1] (kDa)	Apparent MW of E. coli-produced MON 88701 DMO[2] (kDa)	Preset Acceptance Limits for MON 88701 DMO[3] (kDa)
39. 5	38.7	38.5-39.7

[1] The reported value is the mean molecular weight across all six loads.

[2]The molecular weight of the *E. coli*-produced MON 88701 DMO protein as reported on its Certificate of Analysis.

[3]See [redacted] 2012 (MSL0023517).

MON 88701 DMO Glycosylation Equivalence

Some eukaryotic proteins are post-translationally modified by the addition of carbohydrate moieties (Rademacher et al., 1988). To test whether DMO protein was glycosylated when expressed in the cottonseed of MON 88701, the MON 88701 DMO protein was analysed 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 88701 DMO and *E. coli*-produced MON 88701 DMO proteins, the *E. coli*-produced MON 88701 DMO protein was also analysed. The positive control was clearly detected at expected molecular weight (~80 kDa) and the band intensity increased with increasing concentration (Figure 23, Panel A, Lanes 1-2). In contrast, signals were not observed in the lanes containing the MON 88701- or *E. coli*-produced protein at the expected molecular weight for the MON 88701 protein (Figure 23, Panel A, Lanes 7-8 and Lanes 4-5, respectively). To assess that sufficient MON 88701 DMO and *E. coli*-produced MON 88701 DMO 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 23 Panel B). Both the MON 88701- and *E. coli*-produced MON 88701 DMO proteins were clearly detected (Figure 23 Panel B, Lanes 7-8 and Lanes 4-5, respectively). These data indicate that the glycosylation status of MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins are equivalent and that neither is glycosylated.

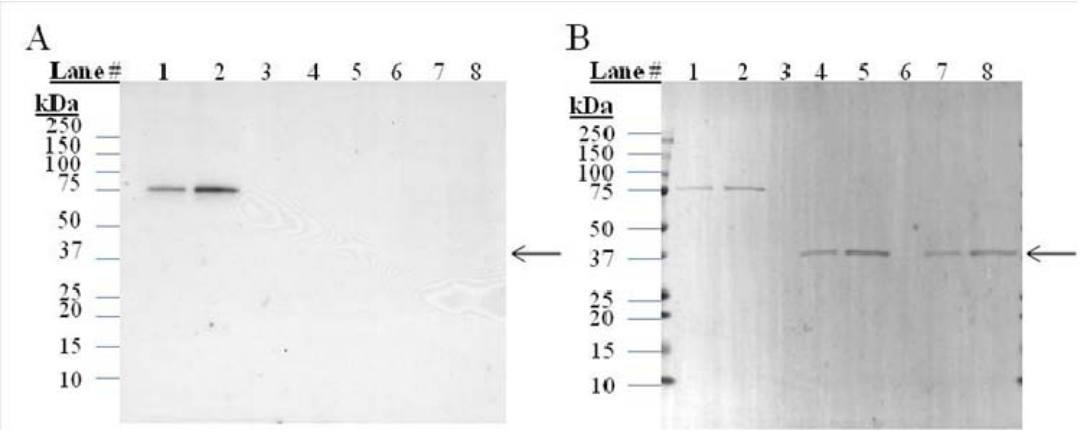


Figure 23. Glycosylation Analysis of the MON 88701 DMO Protein

Aliquots of the transferrin (positive control), *E. coli*-produced MON 88701 DMO protein and MON 88701 DMO protein were subjected to SDS-PAGE and electrotransferred to PVDF membranes. Panel A corresponds to detection of the labeled carbohydrate moieties, where present, using the ECL-based system with exposure to Hyperfilm. A 6 min exposure is shown. Panel B corresponds to Coomassie Blue R250 staining of an equivalent blot 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). Lanes loaded with molecular weight markers were partially cropped, and lanes were renumbered relative to the original gel loading. Arrows indicate the expected migration MON 88701 DMO protein. Lane designations are as follows:

Lane	Sample	Amount (ng)
1	Transferrin (positive control)	50
2	Transferrin (positive control)	100
3	Empty	-
4	*E. coli*-produced MON 88701 DMO (negative control)	50
5	*E. coli*-produced MON 88701 DMO (negative control)	100
6	Empty	-
7	MON 88701 DMO	50
8	MON 88701 DMO	100

MON 88701 DMO Functional Activity

The functional activities of the MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins were determined by quantifying the conversion of dicamba to DCSA using HPLC separation and fluorescence detection. In this assay, protein-specific activity is expressed as nmol DCSA × minute^{-1} × mg^{-1} of DMO.

The experimentally-determined specific activities for the MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins are presented in Table 10. The specific activities of MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins were 5.48 and 7.23 nmol DCSA × minute^{-1} × mg^{-1} of DMO, respectively. Because the mean specific activities of the MON 88701-produced and *E. coli*-produced MON 88701 DMO proteins fall within the preset acceptance criterion (Table 10), the MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins were determined to have equivalent functional activity.

Table 10. MON 88701 DMO Functional Activity

MON 88701 DMO1 **(nmol DCSA × minute-1 × mg-1)**	***E. coli-produced MON 88701 DMO1*** **(nmol DCSA × minute-1 × mg-1)**	***Preset Acceptance Limits for MON 88701 DMO2*** **(nmol DCSA × minute-1 × mg-1)**
5.48 ± 1.3	7.23 ± 2.1	1.69-20.74

[1]Value refers to mean and standard deviation calculated based on n = 5.

[2]See [redacted] 2012 (MSL0023517).

MON 88701 DMO Protein Identity and Equivalence Conclusion

The MON 88701 DMO protein purified from cottonseed of MON 88701 was characterised and the equivalence of the physicochemical and functional properties between the MON 88701 DMO and the *E. coli*-produced MON 88701 DMO proteins was established using a panel of analytical tests: 1) the identity could not be confirmed by N-terminal sequence analysis; however, MALDI-TOF MS analysis of peptides derived from tryptic digested MON 88701 DMO established the N-terminal sequence of MON 88701 DMO; 2) MALDI-TOF MS analysis yielded peptide masses consistent with the expected peptide masses from the theoretical trypsin digest of the MON 88701 DMO sequence; 3) MON 88701 DMO protein was detected on a western blot probed with antibodies specific for DMO protein and the immunoreactive and physiochemical properties of the MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins were shown to be equivalent; 4) the electrophoretic mobility and apparent molecular weight of the MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins were shown to be equivalent; 5) glycosylation status of MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins were determined to be equivalent; and 6) functional activity of the MON 88701 DMO and the *E. coli*-produced MON 88701 DMO proteins were demonstrated to be equivalent.

Taken together, these data provide a detailed characterisation of the MON 88701 DMO protein and establish the equivalence of the MON 88701 DMO and the *E. coli*-produced MON 88701 DMO protein. This equivalence justifies the use of the *E. coli*-produced MON 88701 DMO as a test subtance in the protein safety studies.

MON 88701-produced PAT (*bar*) Protein Identity and Equivalence

The safety assessment of crops derived through biotechnology includes characterisation of the physicochemical and functional properties of and confirmation of the safety of the introduced protein(s). For the safety data generated using *E. coli*-produced PAT (*bar*) protein to be applied to PAT (*bar*) protein produced in MON 88701, the equivalence of the plant- and *E. coli*-produced PAT (*bar*) proteins was assessed. To assess the equivalence between MON 88701- and *E. coli*-produced PAT (*bar*) proteins, a small quantity of the PAT (*bar*) protein was purified from MON 88701 cottonseed. The MON 88701-produced PAT (*bar*) protein was characterised and the equivalence of the immunoreactive and physicochemical characteristics and functional activity between the MON 88701-produced PAT (*bar*) and the *E. coli*-produced PAT (*bar*) proteins was assessed using a panel of six analytical tests as shown in Table 11. Taken together, these data provide a detailed characterisation of the MON 88701-produced PAT (*bar*) protein and establish the equivalence of MON 88701- and *E. coli*-produced PAT (*bar*) proteins.

Please also refer to [redacted] 2012 (MSL0023428).

Table 11. Summary of MON 88701-produced PAT (*bar*) Protein Identity and Equivalence

Analytical Test Assessment	Analytical Test Outcome
N-terminal sequence analysis of the MON 88701-produced PAT (*bar*) protein to assess identity	The identity was confirmed by N-terminal sequence analysis
MALDI-TOF MS[1] analysis of peptides derived from tryptic digested MON 88701-produced PAT (*bar*) protein to assess identity	MALDI-TOF MS[1] analysis yielded peptide masses consistent with the expected peptide masses from the theoretical trypsin digest of the MON 88701 PAT (*bar*) sequence
Western blot analysis using anti- PAT (*bar*) polyclonal antibodies to assess identity and immunoreactive equivalence between MON 88701- and the *E. coli*-produced PAT (*bar*) proteins	MON 88701-produced PAT (*bar*) protein identity was confirmed using a western blot probed with antibodies specific for PAT protein Immunoreactive properties of the MON 88701- and the *E. coli*-produced PAT (*bar*) proteins were shown to be equivalent
SDS-PAGE[2] to assess equivalence of the apparent molecular weight between MON 88701- and the *E. coli*-produced PAT (*bar*) proteins	Electrophoretic mobility and apparent molecular weight of the MON 88701- and the *E. coli*-produced PAT (*bar*) proteins were shown to be equivalent
Glycosylation analysis of the PAT (*bar*) protein to assess equivalence between the MON 88701- and the *E. coli*-produced PAT (*bar*) proteins	Glycosylation status of MON 88701- and the *E. coli*-produced PAT (*bar*) proteins were shown to be equivalent
PAT (*bar*) enzymatic activity analysis to assess functional equivalence between MON 88701- and the *E. coli*-produced PAT (*bar*) proteins	Functional activity of the MON 88701- and the *E. coli*-produced PAT (*bar*) proteins were shown to be equivalent

[1] MALDI-TOF MS = Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry

[2] SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis

Results of the N-Terminal Sequencing Analysis

N-terminal sequencing of the first 15 amino acids was performed on MON 88701-produced PAT (*bar*). The expected sequence for the PAT (*bar*) protein deduced from the *bar* gene present in MON 88701 was observed. The data obtained correspond to the deduced PAT (*bar*) protein beginning at amino acid positions 2 and 3 (Figure 24, Experimental Sequence 1 and 2, respectively). The N-terminal methionine residue in the PAT (*bar*) protein was not observed, indicating that it was removed during post-translational processing of the precursor protein. This result is expected as removal of the N-terminal methionine, catalysed by methionine aminopeptidase, is common in many organisms and has no effect on protein structure or activity (Arfin and Bradshaw, 1988; Bradshaw et al., 1998; Polevoda and Sherman, 2000). Hence, the sequence information confirms the identity of the PAT (*bar*) protein isolated from the cottonseed of MON 88701.

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

Figure 24. N-Terminal Sequence of the MON 88701-produced PAT (*bar*) Protein

The expected amino acid sequence of the N-terminus of PAT (*bar*) protein was deduced from the *bar* coding region present in MON 88701. The experimental sequences obtained from the MON 88701-produced PAT (*bar*) protein were compared to the expected sequence. The single letter IUPAC-IUB amino acid code is M, methionine; S, serine; P, proline; E, glutamic acid; R, arginine; A, alanine; D, aspatic acid; I, isoleucine; and T, threonine. X indicates that the residue was not identifiable; (-) indicates the residue was not observed.

Results of MALDI-TOF Tryptic Mass Map Analysis

The identity of the MON 88701-produced PAT (*bar*) protein was also confirmed by MALDI-TOF MS analysis of peptide fragments produced from tryptic digestion of the MON 88701-produced PAT (*bar*) 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 ≥ 40% of the protein sequence was identified by matching experimental masses observed for the tryptic peptide fragments to the expected masses for the fragments (Biron et al., 2006; Krause et al., 1999).

There were 10 unique peptides identified that corresponded to the masses expected to be produced by tryptic digestion of the PAT (*bar*) protein (Table 12). The identified masses were used to assemble a coverage map of the entire MON 88701-produced PAT (*bar*) protein (Figure 25). The experimentally determined mass coverage of the MON 88701-produced PAT (*bar*) protein was 84.7% (155 out of 183 amino acids). This analysis serves as additional identity confirmation for the MON 88701-produced PAT (*bar*) protein.

Table 12 Summary of the Tryptic Masses[1] Identified for the MON 88701-produced PAT (*bar*) Protein Using MALDI-TOF MS

α-cyano		DHB		Sinapinic acid		Expected Mass	Diff.[2]	Fragment[3]	Sequence
Extract 1	Extract 2	Extract 1	Extract 2	Extract 1	Extract 2				
		879.65				879.46	0.19	113-120	SLEAQGFK
1144.65	1144.75	1144.84				1144.56	0.09	136-145	MHEALGYAPR
1403.93	1404.03	1404.12	1404.18			1403.79	0.14	100-112	TGLGSTLYTHLLK
1523.02	1523.13	1523.14	1523.19	1522.93		1522.86	0.16	121-135	SVVAVIGLPNDPSVR
1843.07	1843.18	1843.27		1842.98	1843.19	1842.85	0.22	38-52	TEPQEPQEWTDDLVR
1859.06	1859.22	1859.22		1858.98	1859.18	1858.86	0.20	81-96	NAYDWTAESTVYVSPR
				2391.45	2391.64	2391.20	0.25	57-78	YPWLVAEVDGEVAGIAYAGPWK
2676.67				2676.64	2676.88	2676.35	0.32	55-78	ERYPWLVAEVDGEVAGIAYAGPWK
				2840.62		2840.32	0.30	13-37	ATEADMPAVCTIVNHYIETSTVNFR
3353.14	3353.36			3353.17	3353.48	3352.73	0.41	155-183	HGNWHDVGFWQLDFSLPVPPRPVLPVTEI

[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 PAT (*bar*) sequence as depicted in Figure 25.

DHB = 5-dihydroxybenzoic acid matrix, α-cyano = α-cyano-4-hydroxycinnamic acid matrix, Sinapinic acid = 3, 5-dimethoxy-4-hydroxycinnamic acid matrix.

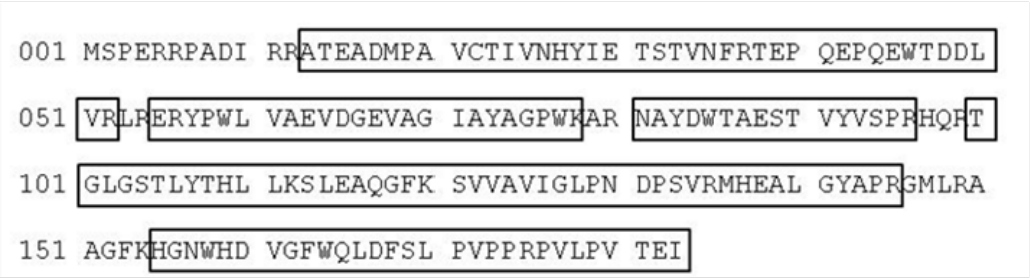


Figure 25. MALDI-TOF MS Coverage Map of the MON 88701-produced PAT (*bar*) Protein

The amino acid sequence of the PAT (*bar*) protein was deduced from the *bar* gene present in MON 88701. Boxed regions correspond to regions covered by tryptic peptides that were identified from the MON 88701-produced PAT (*bar*) protein sample using MALDI-TOF MS. In total, 84.7% (155 out of 183 amino acids) of the expected protein sequence was covered by the identified peptides.

Results of Western Blot Analysis of the PAT (*bar*) Protein Isolated from the Cottonseed of MON 88701 and Immunoreactivity Comparison to *E. coli*-produced PAT (*bar*) Protein

Western blot analysis was conducted using goat anti- PAT (*bar*) polyclonal antibodies to 1) assess the identity of the PAT (*bar*) protein isolated from the cottonseed of MON 88701; and 2) to determine the relative immunoreactivity of the MON 88701- and the *E. coli*-produced PAT (*bar*) proteins. The results demonstrated that the anti-PAT (*bar*) antibodies recognised the MON 88701-produced PAT (*bar*) protein that migrated to an identical position on the blot as the *E. coli*-produced PAT (*bar*) protein (Figure 26). Furthermore, the immunoreactive signal increased with increasing amounts of PAT (*bar*) protein loaded.

Densitometric analysis was conducted to compare the immunoreactivity of MON 88701- and the *E. coli*-produced PAT (*bar*) proteins. The mean signal intensity (OD × mm^2) from the MON 88701-produced PAT (*bar*) bands and from the *E. coli*-produced PAT (*bar*) bands at each amount of protein analysed was calculated and then overall mean signal intensity was calculated (Table 13). The immunoreactivity was considered equivalent if the overall mean signal intensity of all MON 88701-produced PAT (*bar*) protein bands was within ±35% of the overall mean signal intensity of all *E. coli*-produced PAT (*bar*) protein bands.

The overall mean signal intensity of the *E. coli*-produced PAT (*bar*) bands was 4.669 OD × mm^2 and the overall mean signal intensity of the MON 88701-produced PAT (*bar*) bands was 4.167 OD × mm^2. Because overall mean signal intensity of the MON 88701-produced PAT (*bar*) protein bands was between 3.035 and 6.303 OD × mm^2 (between -35% and +35% of the *E. coli*-produced PAT (*bar*) bands), the MON 88701-produced and *E. coli*-produced PAT (*bar*) proteins were determined to have equivalent immunoreactivity.

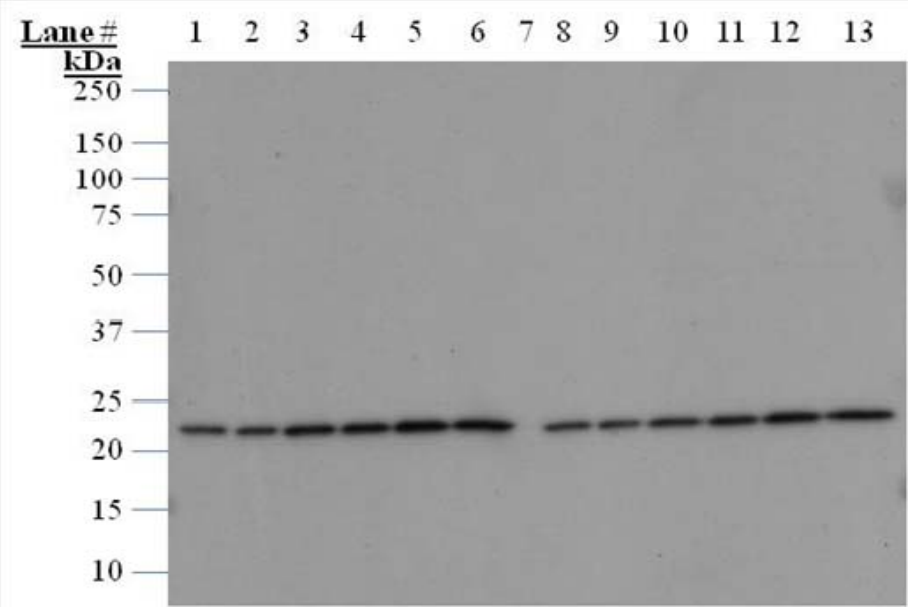


Figure 26. Western Blot Analysis of the MON 88701- and *E. coli*-produced PAT (*bar*) Proteins

Aliquots of the MON 88701-produced PAT (*bar*) protein and the *E. coli*-produced PAT (*bar*) protein were subjected to SDS-PAGE and electrotransferred to a PVDF membrane. The membrane was incubated with anti-PAT (*bar*) antibodies and immunoreactive bands were visualised using an ECL system (GE Healthcare, Piscataway, NJ). Approximate molecular weights (kDa) are shown on the left. Lanes loaded with molecular weight markers were cropped, and lanes were renumbered relative to the original gel loading. The 1 min exposure is shown. Lane designations are as follows:

Lane	Sample	Amount (ng)
1	*E. coli*-produced PAT (*bar*) protein	2
2	*E. coli*-produced PAT (*bar*) protein	2
3	*E. coli*-produced PAT (*bar*) protein	4
4	*E. coli*-produced PAT (*bar*) protein	4
5	*E. coli*-produced PAT (*bar*) protein	6
6	*E. coli*-produced PAT (*bar*) protein	6
7	Empty	-
8	MON 88701-produced PAT (*bar*) protein	2
9	MON 88701-produced PAT (*bar*) protein	2
10	MON 88701-produced PAT (*bar*) protein	4
11	MON 88701-produced PAT (*bar*) protein	4
12	MON 88701-produced PAT (*bar*) protein	6
13	MON 88701-produced PAT (*bar*) protein	6

Table 13. Comparison of Immunoreactive Signals between MON 88701- and *E. coli*-produced PAT (*bar*) Proteins

Mean Signal Intensity from MON 88701-produced PAT (bar) (OD x mm^2)	Mean Signal Intensity from *E. coli*-produced PAT (bar) (OD x mm^2)	Preset Acceptance Limits for MON 88701-produced PAT (bar)[1] (OD x mm^2)
4.167	4.669	3.035 – 6.303

[1]The acceptance limits for the MON 88701-produced PAT (*bar*) are based on the interval between +35% (4.669 × 1.35) and -35% (4.669 × 0.65) of the overall mean of the *E. coli*-produced PAT (*bar*) signal intensity across all six loads .

Results of the MON 88701-produced PAT (*bar*) Protein Apparent Molecular Weight and Purity Analysis

The molecular weight and purity of the PAT (bar) protein was determined to be 24.1 kDa and 99%, respectively. To assess apparent molecular weight (MW) and purity, the MON 88701-produced PAT (bar) protein was subjected to SDS-PAGE. The gel was stained with Brilliant Blue G Colloidal stain and analysed by densitometry (Figure 27). *E. coli*-produced PAT (bar) protein was loaded in a single lane for reference (Figure 27, Lane 2). The MON 88701-produced PAT (bar) protein (Figure 27, Lanes 3-8) had an apparent MW of 24.1 kDa (Table 14). The apparent molecular weight of the E. coli-produced PAT (bar) protein as reported on its Certificate of Analysis was 25.0 kDa (Table 14). Because the apparent MW of MON 88701-produced PAT (bar) protein was within the preset acceptance limits (Table 14), the MON 88701-produced and E. coli-produced PAT (bar) proteins were determined to have equivalent apparent MWs.

The purity of the MON 88701-produced PAT (*bar*) protein was calculated based on the six loads on the gel (Figure 27, Lanes 3-8). The average purity was determined to be more than 99%.

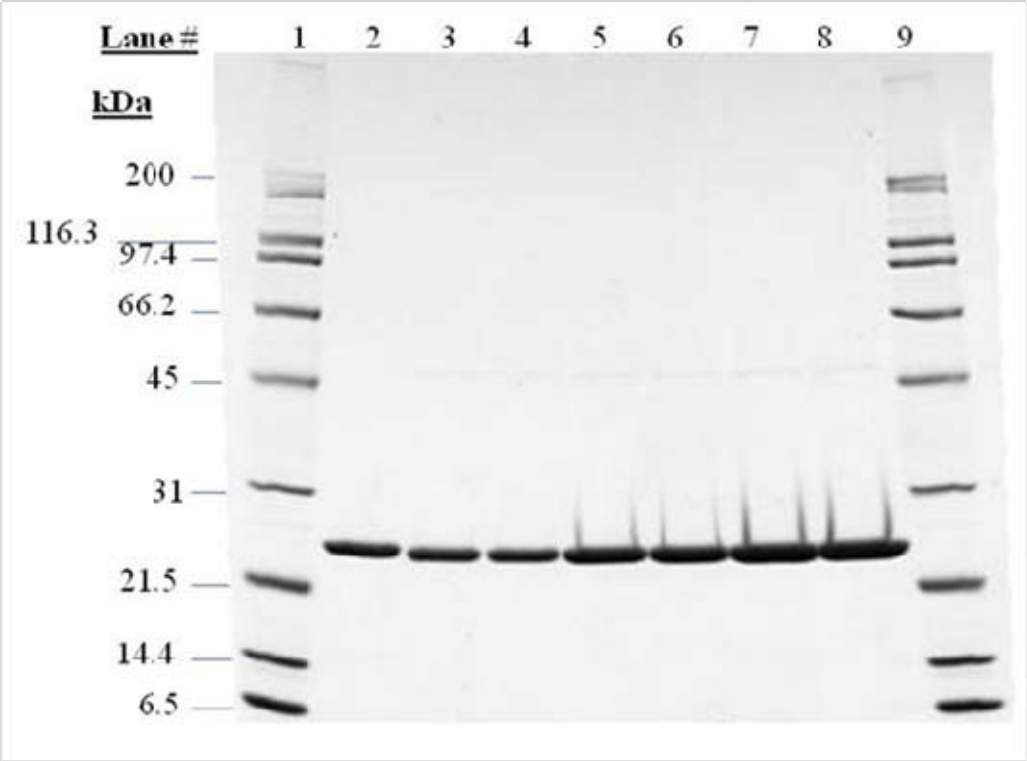


Figure 27. Molecular Weight and Purity Analysis of the MON 88701-produced PAT (*bar*) Protein

Aliquots of the MON 88701-produced and the *E. coli*-produced PAT (*bar*) proteins were subjected to SDS-PAGE 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. Empty lane was partially cropped. Lane designations are as follows:

Lane	Sample	Amount (µg)
1	Broad Range Molecular Weight Markers	4.5
2	*E. coli*-produced PAT (*bar*) protein	1
3	MON 88701-produced PAT (*bar*) protein	1
4	MON 88701-produced PAT (*bar*) protein	1
5	MON 88701-produced PAT (*bar*) protein	2
6	MON 88701-produced PAT (*bar*) protein	2
7	MON 88701-produced PAT (*bar*) protein	3
8	MON 88701-produced PAT (*bar*) protein	3
9	Broad Range Molecular Weight markers	4.5

Table 14. Molecular Weight Comparison Between the MON 88701- and *E. coli*-produced PAT (*bar*) Proteins Based on SDS-PAGE

Apparent Molecular Weight of MON 88701-produced PAT (bar)[1] (kDa)	Apparent Molecular Weight of *E. coli*-produced PAT (bar)[2] (kDa)	Preset Acceptance Limits for MON 88701-produced PAT (bar)[3] (kDa)
24.1	25.0	23.9-25.4

[1]The reported value is the mean molecular weight across all six loads.

[2]The molecular weight of the *E. coli*-produced PAT (*bar*) protein as reported on its Certificate of Analysis.

[3]See [redacted] 2012 (MSL0023428).

PAT (*bar*) Glycosylation Equivalence

Some eukaryotic proteins are post-translationally modified by the addition of carbohydrate moieties (Rademacher et al., 1988). To test whether PAT (*bar*) protein was glycosylated when expressed in the cottonseed of MON 88701, the MON 88701-produced PAT (*bar*) protein was analysed 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 88701- and *E. coli*-produced PAT (*bar*) proteins, the *E. coli*-produced PAT (*bar*) protein, was also analysed. The positive control was clearly detected at the expected molecular weight (~80 kDa) and the band intensity increased with increasing concentration (Figure 28, Panel A, Lanes 1-2). In contrast, signals were not observed in the lanes containing the MON 88701- or *E. coli*-produced protein at the expected molecular weight for the PAT (*bar*) protein (Figure 28 Panel A, Lanes 7-8 and Lanes 4-5, respectively). To assess whether the MON 88701- and *E. coli*-produced PAT (*bar*) proteins were loaded appropriately for glycosylation analysis, a second membrane (with identical loadings and transfer times) was stained with Coomassie Blue R250 for protein detection (Figure 28 Panel B). Both the MON 88701- and *E. coli*-produced PAT (*bar*) proteins were clearly detected (Figure 28 Panel B, Lanes 7-8 and Lanes 4-5, respectively). These data indicate that the glycosylation status of MON 88701-produced PAT (*bar*) protein and *E. coli*-produced PAT (*bar*) protein are equivalent and that neither is glycosylated.

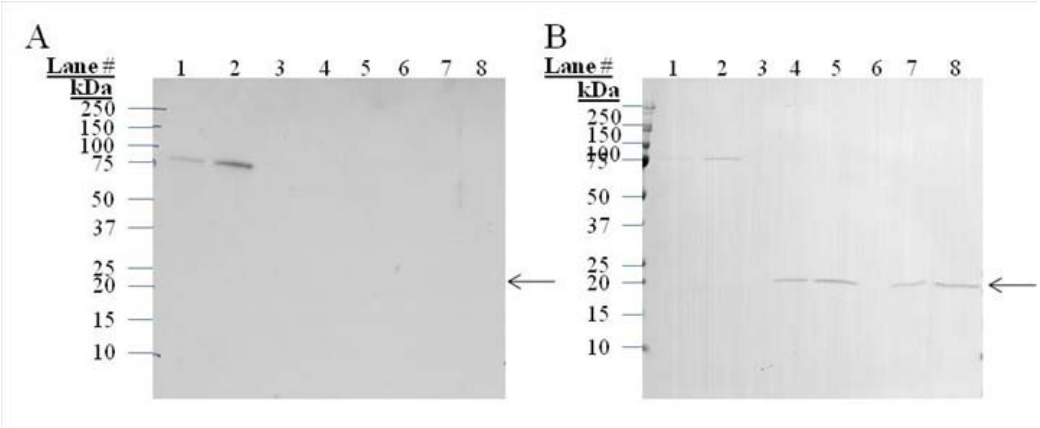


Figure 28. Glycosylation Analysis of the MON 88701-produced PAT (*bar*) Protein

Aliquots of the transferrin (positive control), *E. coli*-produced PAT (*bar*) protein and MON 88701-produced PAT (*bar*) protein were subjected to SDS-PAGE and electrotransferred to PVDF membranes. Panel A corresponds to detection of labeled crabohydrate moieties, where present, using the ECL-based system with exposure to Hyperfilm. A 7 min exposure is shown. Panel B corresponds to Coomassie Blue R250 staining of an equivalent blot 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). Lanes loaded with molecular weight markers were cropped, and lanes were renumbered relative to the original gel loading. Arrows indicate the expected migration of PAT (*bar*) protein. Lane designations are as follows:

Lane	Sample	Amount (ng)
1	Transferrin (positive control)	50
2	Transferrin (positive control)	100
3	Empty	-
4	*E. coli*-produced PAT (*bar*) (negative control)	50
5	*E. coli*-produced PAT (*bar*) (negative control)	100
6	Empty	-
7	MON 88701-produced PAT (*bar*)	50
8	MON 88701-produced PAT (*bar*)	100

PAT (*bar*) Functional Activity

The functional activities of the MON 88701- and *E. coli*-produced PAT (*bar*) proteins were assessed using a colorimetric assay that measures PAT (*bar*) catalysed release of coenzyme A (CoA) from acetyl-CoA upon transfer of an acetyl-group to phosphinothricin. In this assay, protein-specific activity is expressed as µmol × minute^{-1} × mg^{-1} of PAT enzyme.

The experimentally-determined specific activities for the MON 88701- and *E. coli*-produced PAT (*bar*) proteins are presented in Table 15. The specific activities of MON 88701- and *E. coli*-produced PAT (*bar*) proteins were 36.4 and 46.2 µmol × minute^{-1} × mg^{-1} of PAT (*bar*), respectively. Because the specific activities of the MON 88701-produced and *E. coli*-produced PAT (*bar*) proteins fall within the preset acceptance criterion (Table 15), the MON 88701- and *E. coli*-produced PAT (*bar*) proteins were determined to have equivalent functional activity.

Table 15. PAT (bar) Functional Activity

MON 88701-produced PAT (bar)[1] (µmol × minute^{-1} × mg^{-1})	***E. coli*-produced PAT (bar)[1]** (µmol × minute^{-1} × mg^{-1})	**Preset Acceptance Limits for MON 88701-produced PAT (bar)[2]** (µmol × minute^{-1} × mg^{-1})
36.4 ± 1.3	46.2 ± 2.1	30.17 - 51.70

[1]Value refers to mean and standard deviation calculated based on n = 5.

[2]See [redacted] 2012 (MSL0023428).

MON 88701-produced Protein PAT (*bar*) Protein Identity and Equivalence Conclusion

The MON 88701-produced PAT (*bar*) protein purified from cottonseed of MON 88701 was characterised and the equivalence of the immunoreactive and physicochemical characteristics and functional activity between the MON 88701- and the *E. coli*-produced PAT (*bar*) proteins was established using a panel of analytical tests: 1) N-terminal sequence analysis of the MON 88701-produced PAT (*bar*) protein established identity; 2) MALDI-TOF MS analysis yielded peptide masses consistent with the expected peptide masses from the theoretical trypsin digest of the MON 88701-produced PAT (*bar*) sequence; 3) MON 88701-produced PAT (*bar*) protein was detected on a western blot probed with antibodies specific for PAT (*bar*) protein and the immunoreactive properties of the MON 88701-produced and *E. coli*-produced PAT (*bar*) proteins were shown to be equivalent; 4) the electrophoretic mobility and apparent molecular weight of the MON 88701-produced and *E. coli*-produced PAT (*bar*) proteins were shown to be equivalent; 5) glycosylation status of MON 88701- and *E. coli*-produced MON 88701 PAT (*bar*) proteins were determined to be equivalent; and 6) functional activity of the MON 88701- and *E. coli*-produced PAT (*bar*) proteins were demonstrated to be equivalent.

Taken together, these data provide a detailed characterisation of the MON 88701-produced PAT (*bar*) protein and establish the equivalence of the MON 88701-produced and the *E. coli*-produced PAT (*bar*) proteins. This equivalence justifies the use of protein safety studies conducted in which the *E. coli*-produced PAT (*bar*) protein was used as a test substance.

B3(c) Site of expression of all novel substances and levels

Expression Levels of MON 88701 DMO and PAT (*bar*) Proteins in MON 88701

MON 88701 DMO and PAT (*bar*) protein levels in various tissues of MON 88701 relevant to the risk assessment were determined by a validated enzyme-linked immunosorbent assay (ELISA). Tissues of MON 88701 were collected from four replicate plots planted in a randomised complete block field design during the 2010 growing season from the following eight field sites in the U.S.: Arkansas (ARTI), Georgia (GACH), Kansas (KSLA), Louisiana (LACH), North Carolina (NCBD), New Mexico (NMLC), South Carolina (SCEK) and Texas (TXPL). MON 88701 plots were treated at the 3-5 leaf stage with glufosinate herbicide at the label rate (0.5 lbs active ingredient [a.i.]/acre) and at the 6-10 leaf stage with dicamba herbicide at the proposed label rate (0.5 lbs acid equivalent [a.e]./acre). The field sites were representative of cotton producing regions suitable for commercial production. Seed, pollen, root, and over-season leaf (OSL-1 through OSL-4) tissue samples were collected from each replicated plot at all field sites, except OSL1 at TXPl and OSL4 at LACH. Please also refer to [redacted] 2012 (MSL0024523).

MON 88701 DMO Expression Levels

MON 88701 DMO protein levels were determined in all seven tissue types. The results obtained from ELISA analyses are summarised in Table 16. Due to a limited amount of tissue, moisture content was not measured for pollen; therefore, pollen is reported on a fresh weight (fw) basis only. MON 88701 DMO protein levels in MON 88701 across tissue types ranged from <LOD to 410 µg/g dw. The mean MON 88701 DMO protein levels were determined across eight sites, with the exception of OSL-1 (7 sites) and OSL-4 (7 sites). Samples <LOD were not included in mean determinations. The mean MON 88701 DMO protein levels were highest in leaf (ranging from OSL-2 and OSL-3 at 240 µg/g dw, OSL-4 at 230 µg/g dw to OSL-1 at 180 µg/g dw), followed by root at 43 µg/g dw, seed at 21 µg/g dw, and pollen at 14 µg/g fw.

Table 16. Summary of MON 88701 DMO Protein Levels in Tissues from MON 88701 Grown in 2010 U.S. Field Trials

Tissue[1]	Development Stage[2]	Days After Planting (DAP)	MON 88701 DMO Mean (SD) Range (µg/g fw)[3]	MON 88701 DMO Mean (SD) Range (µg/g dw)[4]	LOD/LOQ[5] (µg/g fw)
OSL-1	2-4 leaf	14-25	27 (7.6) 13 – 42	180 (52) 110 – 280	0.168/0.313
OSL-2	4-7 leaf	25-37	41 (12) 19 – 65	240 (69) 110 – 380	0.168/0.313
OSL-3	9 leaf - Full flower	35-99	52 (17) 24 – 97	240 (75) 91 – 410	0.168/0.313
OSL-4	Full flower – Cutout	70-121	57 (18) 0.70 – 91	230 (59) 2.8 – 310	0.168/0.313
Root	50% open flower – Full flower	62-99	14 (3.7) 8.2 – 21	43 (12) 26 – 72	0.136/0.313
Pollen	50% open flower – Full Flower	68-99	14 (28) 0.31 – 110	NA (NA) NA	0.043/0.125
Seed	Maturity	148-183	20 (4.6) 8.2 – 29	21 (5.0) 8.9 – 33	0.059/0.313

[1]OSL= over-season leaf. Seed = black seed (ginned and delinted).

[2]The crop development stage each tissue was collected (Ritchie et al., 2007).

[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 (n=32, except OSL-3 n=31 due to one sample <LOD, OSL-1 and OSL-4 n=28 due to missed sample collections, and pollen n=29 due to two samples expressing <LOD and one being inconclusive).

[4]Protein levels are expressed as µg/g on a dry weight (dw) basis. The dry weight values were calculated by dividing the µg/g fw by the dry weight conversion factors obtained from moisture analysis data. NA= Not Applicable.

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

MON 88701-produced PAT (bar) Expression Levels

PAT (*bar*) protein levels were determined in all seven tissue types. The results obtained from ELISA are summarised in Table 17. Due to a limited amount of tissue, moisture content was not measured for pollen; therefore, pollen is reported on a fresh weight (fw) basis only. PAT (*bar*) protein levels in MON 88701 across tissue types ranged from <LOQ to 10 µg/g dw. The mean PAT (*bar*) protein levels were determined across eight sites, with the exception of OSL-1 (7 sites) and OSL-4 (7 sites). Samples <LOD were not included in mean determinations. The mean PAT (*bar*) protein levels were highest in seed at 6.6 µg/g dw, followed by leaf (ranging from OSL-2 at 6.4 µg/g dw, OSL-1 at 5.5 µg/g dw, OSL-3 at 4.8 µg/g dw to OSL-4 at 3.2 µg/g dw), root at 1.8 µg/g dw, and pollen at 0.56 µg/g fw.

Table 17. Summary of PAT (*bar*) Protein Levels in Tissues from MON 88701 Grown in 2010 U.S. Field Trials

Tissue[1]	Development Stage[2]	Days After Planting (DAP)	PAT (*bar*) Mean (SD) Range (µg/g fw)[3]	PAT (*bar*) Mean (SD) Range (µg/g dw)[4]	LOD/LOQ[5] (µg/g fw)
OSL-1	2-4 leaf	14-25	0.84 (0.21) 0.46 – 1.4	5.5 (1.5) 3.7 – 9.1	0.162/0.188
OSL-2	4-7 leaf	25-37	1.1 (0.26) 0.68 – 1.6	6.4 (1.4) 3.8 – 9.4	0.162/0.188
OSL-3	9 leaf – Full flower	35-99	1.0 (0.34) 0.34 – 1.7	4.8 (2.0) 1.3 – 10	0.162/0.188
OSL-4	Full flower – Cutout	70-121	0.78 (0.29) 0.42 – 1.7	3.2 (1.2) 2.0 – 6.7	0.162/0.188
Root	50% open flower – Full flower	62-99	0.56 (0.18) 0.27 – 0.89	1.8 (0.75) 0.93 – 3.3	0.096/0.188
Pollen	50% open flower – Full flower	68-99	0.56 (0.24) 0.27 – 0.90	NA (NA) NA	0.021/0.188
Seed	Maturity	148-183	6.1 (0.95) 4.8 – 8.8	6.6 (1.1) 5.2 – 9.6	0.032/0.188

[1]OSL= over-season leaf. Seed = black seed (ginned and delinted).

[2]The crop development stage each tissue was collected (Ritchie et al., 2007).

[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 (n=32, except OSL-1 n=28 due to missed sample collections, OSL-4 n=27 due to missed sample collections and one sample expressing <LOD, OSL-3 n=31 due to one sample expressing <LOD, and pollen n=6 due to 26 samples expressing <LOQ).

[4]Protein levels are expressed as µg/g on a dry weight (dw) basis. The dry weight values were calculated by dividing the µg/g fw by the dry weight conversion factors obtained from moisture analysis data. NA= Not Applicable.

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

B3(d) Post-translational modifications to the novel protein(s)

Please refer to section B3(b).

B3(e) Evidence of silencing, if silencing is the method of modification

Not applicable.

B3(f) History of human consumption of novel substances or similarity to substances previously consumed in food

History of Safe Use of MON 88701 DMO Protein

As described below, MON 88701 DMO is homologous to proteins that are common in the environment and in the diets of animals and humans. Given the extensive exposure of humans and animals to these homologous oxygenase proteins, it can be concluded that the oxygenase proteins have a history of safe use. When determining the homology among proteins both the linear amino acid sequence of the protein, as well as the higher order structure of the proteins should be taken into account. Higher order structures are a relevant measure of homology since structure is more conserved than amino acid sequence. Changes in amino acid sequence are, evolutionarily, mostly conservative, meaning that the changes do not affect the structure which also determines function (Caetano-Anollés et al., 2009; Illergård et al., 2009). This conservation of structure is predominant within important functional and structural domains of proteins in similar classes (Illergård et al., 2009). Therefore, it is necessary to understand the different levels of protein structure to properly assess homology and determine if homologues of MON 88701 DMO are widely distributed in nature or are present in sources that have been consumed by humans and animals.

As noted earlier, DMO is classified as an oxygenase. Oxygenases are enzymes that incorporate one or two oxygen atoms into substrates, and are widely distributed in many universal metabolic pathways (Harayama et al., 1992). Within this large enzymatic class are mono-oxygenases that incorporate a single oxygen atom as a hydroxyl group with the concomitant production of water and oxidation of NADH (Harayama et al., 1992). Non-heme iron oxygenases, where iron is involved in the catalytic site, are an important class of oxygenases. Within this class are Rieske non-heme iron oxygenases, which contain a Rieske iron-sulfur [2Fe-2S] cluster. All Rieske non-heme iron oxygenases contain two catalytic domains, a non-heme iron domain (nh-Fe) that is a site of oxygen activation, and a Rieske [2Fe-2S] domain which functions by transporting electrons from ferredoxin to the non-heme iron domain (Ferraro et al., 2005). MON 88701 DMO belongs to this class of oxygenases which are found in diverse phyla ranging from bacteria to plants consumed by humans and animals (Ferraro et al., 2005; Schmidt and Shaw, 2001).

The crystallography results demonstrated that the quaternary structure of DMO is a trimer, where each individual monomer is in a precise orientation that allows for electron transport between two conserved domains; the Rieske and the non-heme iron domain. Similar to all Rieske non-heme iron oxygenases, DMO monomers contain these two catalytically important and highly conserved domains (D'Ordine et al., 2009; Dumitru et al., 2009; Ferraro et al.,

2005). The primary structure of these domains are highly conserved, leading to secondary and tertiary structural domains that result in the correct spatial orientation of the non-heme iron and the Rieske [2Fe-2S] domains in DMO monomers to ensure electron transport from ferredoxin and between the monomers of DMO (D'Ordine et al., 2009; Ferraro et al., 2005).

Rieske domains are ubiquitous in numerous bacterial and plant proteins such as the iron-sulfur protein of the cytochrome bc_1 complex, chloroplast cytochrome b_6-*f* complex in spinach, and choline mono-oxygenases (Breyton, 2000; Darrouzet et al., 2004; Gray et al., 2004; Hibino et al., 2002; Rathinasabapathi et al., 1997; Russell et al., 1998). The presence of two conserved domains, a Rieske [2Fe-2S] domain and a non-heme iron domain, suggests that all Rieske type non-heme iron oxygenases share the same reaction mechanism, by which the Rieske domain transfers electrons from the ferredoxin to the non-heme iron to allow catalysis (Chakraborty et al., 2005; Dumitru et al., 2009; Ferraro et al., 2005). The conservation of these important structural domains required for enzymatic activity is further evidence of the evolutionary relatedness of all Rieske non-heme iron oxygenases to each other (Nam et al., 2001; Rosche et al., 1997; Werlen et al., 1996). Therefore, enzymes with structural and functional homologies to MON 88701 DMO have been described in plants and bacteria and have been extensively consumed.

Additionally, a FASTA alignment search of publicly-available databases using the MON 88701 DMO protein sequence as a query yielded homologous sequences from many different species, predominantly bacteria, with amino acid sequence identity ranging up to approximately 42%. Alignments of MON 88701 DMO with plant proteins revealed homologous oxygenases present in crops, such as canola (*Brassica napus*), corn (*Zea mays*), pea (*Pisum sativum*), rice (*Orysa sativa*), and soy (*Glycine max*), which were determined to have sequence identities up to approximately 27% (Table 18). The highest homology was observed to proteins that are involved in chlorophyll metabolism. Chlorophyllide A oxygenase (Accession number: ACG42449) is a Rieske-type oxygenase that is required for the formation of chlorophyll *b,* which is present in all plants (Tanaka et al., 1998). Pheophorbide A oxygenase (Accession number: ABD60316) is also a Rieske-type oxygenase that plays a key role in the overall regulation of chlorophyll degradation in plants (Rodoni et al., 1997). Pheophorbide A oxygenase is constitutively present in all green tissues and, at slightly lower levels, in etiolated and non-photosynthetic tissues including seeds (Yang et al., 2004). As a Rieske-type oxygenase, Pheophorbide A oxygenase is expected to have high degree of secondary and tertiary structure homology to similar structural elements in DMO as described above. The presence of these conserved structural domains in these plant proteins is further evidence that exposure to a structural homolog of MON 88701 DMO has occurred through consumption of these crops.

Therefore, MON 88701 DMO shares homologies across all levels of protein structure (*i.e.*, primary, secondary, tertiary) with a wide variety of oxygenases present in bacteria and plants widely prevalent in the environment and consumed, establishing that animals and humans are extensively exposed to these structural homologs without any reports of adverse effects due to the protein.

Table 18. Amino Acid Sequence Identity between MON 88701 DMO and Other Proteins Present in Plants

Protein	Accession Number[1]	Scientific Name	Common Name	Sequence Identity (%)[2]
Chlorophyllide A oxygenase	ACG42449	*Zea mays*	Corn	27.3
Pheophorbide A oxygenase	ABD60316	*Brassica napus*	Canola/Oilseed Rape	26.0
Lethal leaf spot-1 like protein*	ABA40832	*Glycine max*	Soybean	25.7
Rieske iron-sulfur protein Tic55	CAA04157	*Pisum sativum*	Pea	25.4
Pheophorbide A oxygenase	CAR82238	*Pisum sativum*	Pea	24.6
Pheophorbide A oxygenase	ACG28057	*Zea mays*	Corn	24.3
Rieske domain containing protein	ABF99438	*Oryza sativa*	Rice	23.7
Flavonoid-3-hydroxylase	AAV74195	*Sorghum bicolor*	Sorghum	21.1
Sparse infloroscence1	ACI43576	*Zea mays*	Corn	17.8
Choline mono-oxygenase	AAB52509	*Spinacia oleracea*	Spinach	17.6
Beta-carotene hydroxylase	AAX45523	*Zea mays*	Corn	15.8
Rieske domain containing protein	ACG43734	*Zea mays*	Corn	14.5
Choline mono-oxygenase	CAE17617	*Oryza sativa*	Rice	12.6

*Later identified as Pheophorbide A Oxygenase (Yang et al., 2004).

[1]The accession numbers shown are from the GenBank database.

[2]Protein sequences were utilised from publicly available databases. Each sequence was aligned to the MON 88701 DMO protein by Clustal W method and sequence identity was calculated using the MegAlign function of the Lasergene suite of sequence analysis software [version 8.0.2 (13)] (DNASTAR, Inc. Madison, Wisconsin).

History of Safe Use of PAT (bar) Protein

The PAT (*bar*) protein expressed in MON 88701 is identical to the wild type protein produced in *S. hygroscopicus* and is analogous to the PAT proteins in other previously assessed genetically modified crops. Based on studies characterising the kinetic and chemical mechanisms of PAT proteins (Wehrmann et al., 1996), OECD recognises PAT proteins produced from different genes to be equivalent with regard to function and safety (OECD, 1999).

A comprehensive study on the safety of PAT proteins present in biotechnology-derived crops (Hérouet et al., 2005) demonstrated structural similarity only with other acetyltransferase known not to cause adverse effects after consumption, lack of sequence homology to know allergens and toxins, lack of glycosylation sites, rapid degradation in gastric and intestinal fluids and no adverse effects in mice treated with high doses of PAT proteins. Hérouet et al. (2005) concluded that there is a reasonable certainty of no harm resulting from the inclusion of PAT proteins in human food or animal feed.

The history of safe use of PAT is supported by the lack of any documented reports of adverse effects related to this protein since the introduction of glufosinate-tolerant crops in 1995 (Duke and Powles, 2009). Since then, approvals have been issued by regulatory agencies of 11 different countries for the environmental release of greater than 38 transformation events, including 8 different species of plants expressing the PAT protein (ILSI-CERA, 2011).

B4 Assessment of Potential Toxicity

The assessment of the potential toxicity of an introduced protein is based on comparing the biochemical characteristics of the introduced protein to characteristics of known toxins. A protein is not likely to be associated with toxicity if: 1) the protein lacks any structural similarity to known toxins or other biologically-active proteins that could cause adverse effects in humans or animals; 2) the protein is rapidly digested in mammalian gastrointestinal systems; and 3) the protein is unstable to heat treatment. The lack of any effects in an acute oral mammalian toxicity study performed at dose levels substantially greater than anticipated human exposure levels can provide further confirmation that an introduced protein is unlikely to pose a significant risk to human or animal health. The MON 88701 DMO and PAT (*bar*) proteins in MON 88701 have been assessed for their potential toxicity based on these criteria.

B4(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 MON 88701 DMO and PAT (*bar*) proteins with sequences in a protein database were evaluated using the FASTA sequence alignment tool. The FASTA program directly compares amino acid sequences (*i.e.*, primary, linear protein structure) and the alignment data may be used to infer shared higher order structural similarities between two sequences (*i.e.*, secondary and tertiary protein structures). Proteins that share a high degree of similarity throughout the entire sequence are often homologous. Homologous proteins often have common secondary structures, common three-dimensional configuration, and, consequently, may share similar functions (Caetano-Anollés et al., 2009; Illergård et al., 2009).

FASTA bioinformatic alignment searches using the MON 88701 DMO amino acid sequence and the PAT (*bar*) amino acid sequence were performed with the toxin database to identify possible homology with proteins that may be harmful to human and animal health. The toxin database, TOX_2011, is a subset of sequences derived from the PRT_2011 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_2011 database contains 10,570 sequences.

An E-score acceptance criteria of $1x10^{-5}$ or less for any alignment was used to identify proteins from the TOX_2011 database with potential for significant shared structural similarity and function with MON 88701-produced DMO and PAT (*bar*) proteins. 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_2011 database.

The results of the bioinformatic analyses demonstrated that no structurally relevant similarity exists between the MON 88701 DMO or PAT (bar) proteins and any sequence in the TOX_2011 database, as no alignments displaying an E-score < 1×10^{-5} were observed. This is comparable with previously published safety assessments of PAT (*bar)* protein (Hérouet et al., 2005).

Please also refer to [redacted] 2012 (MSL0023516) and [redacted] 2011 (MSL0023528).

B4(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 factor in the assessment of its potential toxicity. The digestibility of MON 88701 DMO and PAT (*bar*) proteins were evaluated by incubation with simulated gastric fluid and simulated intestinal fluid, and the results show that both MON 88701 DMO and PAT (*bar*) proteins were readily digested. 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 MON 88701 DMO and PAT (*bar*) proteins was evaluated using functional assays 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 MON 88701 DMO protein was completely deactivated by heating at temperatures 55 ºC or higher and PAT (*bar*) protein was substantially deactivated by heating at temperatures 75 ºC or above. In addition, RBD oil and linters are processed fractions that contain undetectable or negligible amounts of protein, respectively and minimal, if any, dietary exposure to MON 88701 DMO and PAT (*bar*) proteins is expected from consumption of foods derived from MON 88701. Therefore, it is anticipated that exposure to functionally active MON 88701 DMO or PAT (*bar*) protein from the consumption of MON 88701 or foods derived from MON 88701 is unlikely. Please also refer to [redacted] 2011 (MSL0023579) and [redacted] 2011 (MSL0023567).

B4(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 both MON 88701 DMO and PAT (*bar*) proteins produced in MON 88701 are not similar to any of these anti-nutritional proteins or to any other known protein toxins. Further, MON 88701 DMO and PAT proteins have a history of safe use, are functionally inactivated at temperatures below those used in processing, and are digested in gastric and intestinal model systems. In additional the safety of PAT proteins has been previously demonstrated and there is a long history of safe use (Hérouet et al., 2005; ILSI-CERA, 2011; Wehrmann et al., 1996). These assessments satisfy the criteria described in

the Codex guidelines specific to the safety assessment of biotechnology-derived plants; thus, it was not necessary to conduct an acute toxicity assay with MON 88701 DMO and PAT (*bar*) proteins. Further, there is no anticipated human exposure to these proteins because only highly processed cotton products (oil and linters) containing non-detectable or negligible amounts of protein are consumed by humans. Nevertheless, an acute gavage assay was conducted with MON 88701 DMO and PAT (*bar*) proteins to provide additional support for the primary toxicity assessment studies.

The *E. coli*-produced MON 88701 DMO protein and *E. coli*-produced PAT (*bar*), in independent studies, were administered as a single dose by oral gavage to 10 male and 10 female CD-1 mice. The MON 88701 DMO protein was administered at a dose level of 283 mg/kg body weight (bw) and PAT (*bar*) was administered at a dose level of 1086 mg/kg bw. The dose levels were selected based on the risk assessment principles of hazard identification and margin of exposure. The selected doses are sufficiently high for potential hazard identification in light of the lack of anticipated human exposure and the other evaluations of safety described above. Each study contained an additional group of 10 male and 10 female mice to serve as a concurrent control. These control groups in each study were administered an amount of bovine serum albumin (BSA) comparable to the amount of test substance (i.e., MON 88701 DMO or PAT (*bar*)) administered to the test group in each study on a mg/kg bw basis. The BSA was suspended in the appropriate buffer at a volume comparable to that received by the test substance group. Following dosing, all mice were observed twice daily throughout the study for general health, mortality and moribundity. A detailed clinical observation was performed on each animal once prior to and twice following treatment on the day of dosing (*i.e.,* three times on the day of dosing) and daily thereafter. Food consumption was measured weekly. Body weights were measured prior to dosing (day 0) and on study days 3, 7, 10 and 14. All animals were euthanised on day 14 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 Effect Levels, adverse or otherwise, (NOELs and NOAELs) for MON 88701 DMO and PAT (*bar*) were considered to be 283 mg/kg bw and 1086 mg/kg bw, respectively. Please also refer to [redacted] 2012 (CRO-2011-035) and [redacted] 2012 (CRO-2011-007).

B5 Assessment of Potential Allergenicity

B5(a) Source of introduced protein

The *dmo* gene is derived from the bacterium *Stenotrophomonas maltophilia* (Palleroni and Bradbury, 1993). *S. maltophilia* is ubiquitous in the environment and is found associated with the rhizosphere of plants. *S. maltophilia* can be found in a variety of foods and feeds, and is widespread in the home environment. Exposure to *S. maltophilia* is incidental to its presence in food. It has been isolated from "ready to eat" salads, vegetables, frozen fish, milk, and poultry (Qureshi et al., 2005; Ryan et al., 2009). *S. maltophilia* can be found in healthy individuals without causing any harm to human health (Denton et al., 1998) and infections caused by *S. maltophilia* are extremely uncommon (Cunha, 2010). Strains have been found in the transient flora of hospitalised patients as a commensal organism (Echemendia, 2010) and, similar to the indigenous bacteria of the gastrointestinal tract, *S. maltophilia* can be an opportunistic pathogen (Berg, 1996). As such, *S. maltophilia* is of low virulence in immuno-compromised patients where a series of risk factors (severe debilitation, the presence of indwelling devices such as ventilator tubes or catheters, for prolonged periods of time and prolonged courses of antibiotics) must occur for colonisation by *S. maltophilia* in humans (Ryan et al., 2009). Therefore, infections by *S. maltophilia* almost exclusively occur in hospital settings, in which case they are only present in a minimal percentage of infections (Ryan et al., 2009). Finally, *S. maltophilia* has not been reported to be source of allergens.

The ubiquitous presence of *S. maltophilia* in the environment, the presence in healthy individuals without causing infections, the incidental presence in foods without any adverse safety reports, and the lack of reported allergenicity establishes the safety of the donor organism.

The *bar* gene is derived from the bacterium *Streptomyces hygroscopicus* (Thompson et al., 1987). The ubiquitous presence of *S. hygroscopicus* in the environment, the widespread human exposure without any adverse safety or allergenicity reports, and the successive reviews of several glufosinate-tolerant events with no safety or allergenicity issues identified establishes the safety of the donor organism.

B5(b) Bioinformatic comparison (aa) of novel protein(s) to allergens

Structural Similarity of MON 88701 DMO to Known 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 MON 88701 DMO protein sequence 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 MON 88701 DMO protein does not share amino acid sequence similarities with known allergens, gliadins, or glutenins.The FASTA program directly compares amino acid sequences (*i.e.*, primary, linear protein structure). This alignment data may be used to infer shared higher order structural similarities between two sequences (*i.e.*, secondary and tertiary protein structures). Proteins that share a high degree of similarity throughout the entire sequence are often homologous. 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_2011) was obtained from Food Allergy Research and Resource Program Database (FARRP, 2011) and was used for the evaluation of sequence similarities shared between the MON 88701 DMO protein and all proteins. The AD_2011 database contains 1,491 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 meaningful homology. Results indicate that the MON 88701 DMO protein sequence does not share meaningful 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 meaningful 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 considerable 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 MON 88701 DMO protein sequence was compared to the proteins in the AD_2011 sequence database.

The bioinformatic results demonstrated there were no biologically relevant sequence similarities to allergens when the MON 88701 DMO protein sequence was used as a query for a FASTA search of the AD_2011 database. Furthermore, no short (eight amino acid) polypeptide matches were shared between the MON 88701 DMO protein sequence and proteins in the allergen database. These data show that the MON 88701 DMO protein

sequence lacks both structurally and immunologically relevant similarities to known allergens, gliadins, and glutenins.

Please also refer to [redacted] 2012 (MSL0023516).

Structural Similarity of PAT (*bar*) to Known Allergens

The scientific justification and methodology for these analyses are provided above. The results are provided below.

The FASTA analysis (as described above) indicated that the PAT (*bar*) protein sequence does not share significant similarity with sequences in the allergen database. No alignment met or 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}.

The bioinformatic anlaysis of the eight-amino acid sliding window search demonstrated that there were no biologically-relevant sequence similarities to allergens when the PAT (*bar*) protein sequence was used as a query for a FASTA search of the AD_2011 database. Furthermore, no short (eight amino acid) polypeptide matches were shared between the PAT (*bar*) protein sequence and proteins in the allergen database. These data show that the PAT (*bar*) protein sequence lacks both structurally and immunologically relevant sequence similarities to known allergens, gliadins and glutenins.

Please also refer to [redacted] 2011 (MSL0023528).

B5(c) Structural properties, including digestion by pepsin, heat treatment

B5(c)(i) Digestive Fate of Protein

Digestibility of Proteins

Proteins introduced into commercial crops using biotechnology are evaluated for their safety for human and animal consumption. Proteins are an essential dietary component for humans and animals, and most are rapidly degraded to the component amino acids for nutritional purposes (Hammond and Jez, 2011). Therefore, evaluating a protein's intrinsic sensitivity to proteolytic digestion with enzymes of the gastrointestinal tract is a key aspect to understanding the safety of any introduced proteins in GM crops. One characteristic of protein toxins and many allergens is their ability to withstand proteolytic digestion by enzymes present in the gastrointestinal tract (Astwood et al., 1996; Moreno et al., 2005; Vassilopoulou et al., 2006; Vieths et al., 1999). Allergenic proteins or their fragments, when presented to the intestinal immune system, can lead to a variety of gastrointestinal and systemic manifestations of immune-mediated allergy. The complete enzymatic degradation of an ingested protein by exposure to gastric pepsin and intestinal pancreatic proteases makes it highly unlikely that either the intact protein or protein fragment(s) will reach the absorptive epithelial cells of the small intestine where antigen processing cells reside (Moreno et al., 2005). To reach these cells, protein or protein fragment(s) must first pass through the stomach where they are exposed to pepsin and then the duodenum where they are exposed to

pancreatic fluid containing a mixture of enzymes called pancreatin. Therefore, the digestive fate of MON 88701 DMO and PAT (*bar*) proteins were assessed using assays with both simulated gastric fluid (SGF) containing pepsin and simulated intestinal fluid (SIF) containing pancreatin.

A correlation between protein digestibility in simulated gastric fluid (SGF) and the likelihood of the protein being an allergen has been previously reported (Astwood et al., 1996), but this correlation is not complete (Fu et al., 2002). The SGF assay protocol has been standardised based on results obtained from an international, multi-laboratory ring study (Thomas et al., 2004). The study showed that the results of *in vitro* pepsin digestion assays were reproducible when a standard protocol was followed. The susceptibility of the MON 88701 DMO and PAT (*bar*) proteins to pepsin digestion was assessed using this standardised *in vitro* pepsin digestion protocol that utilised a physiologically relevant acidic pH to simulate conditions in a stomach.

Digestibility of MON 88701 DMO in SGF

The digestibility of *E. coli*-produced MON 88701 DMO in SGF was assessed using two methods: visual analysis of a Brilliant Blue G Colloidal stained SDS-PAGE gel and visual analysis of a western blot probed with an anti-DMO polyclonal antibody.

Digestibility of *E. coli*-produced MON 88701 DMO in SGF was evaluated over time by analyzing digestion mixtures incubated for targeted time intervals. For SDS-PAGE analysis, approximately 1 µg of total protein was analysed for each time point (Figure 29). The controls, SGF N0 and SGF N7 (Figure 29, Lanes 2 and 13), which evaluate the stability of the pepsin in the test system (SGF) lacking the MON 88701 DMO protein, demonstrated that the pepsin was observed as a stained protein band at ~38 kDa throughout the experimental phase.

Visual examination of SDS-PAGE data showed that the intact *E. coli*-produced MON 88701 DMO protein was completely digested within 0.5 min of incubation in SGF (Figure 29, Lane 5). The pepsin (~38 kDa) and the MON 88701 DMO (~39 kDa) protein migrated to similar positions in this gel system. However, the intensity of the stained protein band at SGF time zero (T0) (Figure 29, Lane 4 pepsin plus DMO) appears to be the combination of the intensity of both proteins when they are each run separately (Figure 29, Lane 2, pepsin alone and Lane 3, DMO alone). After 0.5 min digestion (SGF T1), the intensity of the ~38 kDa band was reduced to approximately the same level as observed for pepsin alone (SGF N0) (Figure 29, compare Lanes 2 and 5) suggesting that the intact MON 88701 DMO protein was digested. In addition, no fragments of the MON 88701 DMO protein were observed at 0.5 min of digestion or thereafter.

No change in the *E. coli*-produced MON 88701 DMO protein band intensity was observed in the absence of pepsin in the controls SGF P0 and SGF P7 (Figure 29, Lanes 3 and 12) indicating that the digestion of the MON 88701 DMO protein was due to the proteolytic activity of pepsin present in SGF and not due to instability of the protein while incubated at pH ~1.2 at ~37 °C for 60 min.

For the SDS-PAGE analysis, the LOD of the MON 88701 DMO protein was not determined because intact MON 88701 DMO protein and pepsin were not separated in this gel system. Therefore, the percent digestion of intact MON 88701 DMO protein was not estimated on Brilliant Blue G Colloidal stained gel. Digestion of MON88701 DMO protein was confirmed and the LOD was calculated using western blot analysis (Figure 30). In summary the results from visual analysis of a Brilliant Blue G Colloidal stained SDS-PAGE gel show that *E coli*-produced MON 88701 DMO protein is rapidly digested in SGF.

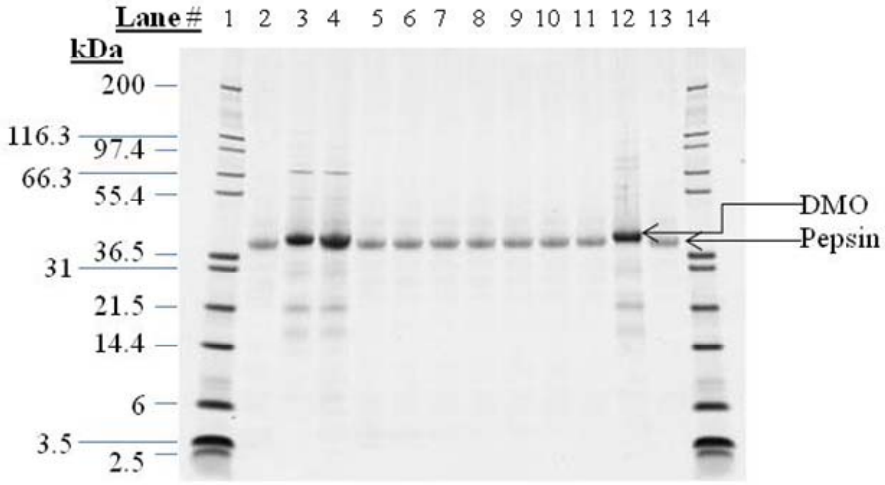


Figure 29. Colloidal Blue Stained SDS-PAGE Gel Showing the Digestion of Purified *E. coli*-produced MON 88701 DMO Protein in Simulated Gastric Fluid

Brilliant Blue G Colloidal stained SDS-PAGE gels were used to assess the digestibility of MON 88701 DMO in SGF. Proteins were subjected to SDS-PAGE and detected by staining with Brilliant Blue G Colloidal stain. Total protein was loaded at 1 µg per lane based on pre-digestion concentrations. Approximate molecular weights (kDa) are shown on the left of each gel, and correspond to the markers loaded. Empty lanes on the gel were cropped from the image. T = time. Lane designations are as follows:

Lane	Sample	Incubation Time (min)
1	Mark 12 MWM	-
2	SGF N0 (No DMO control)	0
3	SGF P0 (No pepsin control)	0
4	SGF - T0	0
5	SGF T1	0.5
6	SGF T2	2
7	SGF T3	5
8	SGF T4	10
9	SGF T5	20
10	SGF T6	30
11	SGF T7	60
12	SGF P7 (No pepsin control)	60
13	SGF N7 (No DMO control)	60
14	Mark 12 MWM	-

For the western blot analysis of MON 88701 DMO digestibility in SGF, the blot used to assess the stability of the MON 88701 DMO protein to pepsin digestion (Figure 30 Panel A) was run concurrently with a blot to estimate the LOD of the intact MON 88701 DMO protein (Figure 30 Panel B). Twenty ng of total protein was analyzed by western blot for each time point. No immunoreactive bands were observed in controls SGF N0 and SGF N7 (Figure 30 Panel A, Lanes 2 and 13). This result indicates that non-specific interactions between the test system components and the DMO-specific antibody did not occur under these experimental conditions.

Western blot analysis demonstrated that the MON 88701 DMO protein was digested below the LOD within 0.5 min of incubation in SGF (Figure 30 Panel A, Lane 5). The LOD of the MON 88701 DMO protein was visually estimated to be 0.2 ng (Figure 30 Panel B, Lane 7). The LOD estimated for the MON 88701 DMO protein was used to calculate the maximum amount of intact MON 88701 DMO protein that could remain visually undetected after digestion. This corresponded to approximately 1.0% of the total MON 88701 DMO protein loaded. Based on the western blot LOD for the MON 88701 DMO protein, it can be concluded that within 0.5 min more than 99% (100% - 1.0% = 99%) of the intact MON 88701 DMO protein was digested and no other fragments were observed.

No change in the MON 88701 DMO protein band intensity was observed in the absence of pepsin in the controls SGF P0 and P7 (Figure 30 Panel A, Lanes 3 and 12). This result reaffirms that the MON 88701 DMO protein was stable in the test system without pepsin.

As indicated on the LOD blot, 2 ng of intact MON 88701 DMO was readily detected by the antibody and blotting methods used for this analysis (Figure 30, Panel B, Lane 4). Thus, the 20 ng per lane loaded to assess digestibility in SGF represented a heavy loading of the MON 88701 DMO protein for western blot analysis; this amount of MON 88701 DMO protein was applied to increase the probability that any intact protein or protein fragments of MON 88701 DMO would be visible. Under those loading conditions, minor aggregation and breakdown products of the MON 88701 DMO protein were observed in the absence of digestion (Figure 30, Panel A, Lanes 3, 4, and 12).

In summary, the western analysis demonstrate that greater than 99% of the *E coli*-produced MON 88701 DMO protein was digested in SGF within 0.5 min. and other immunoreactive bands were not detected.

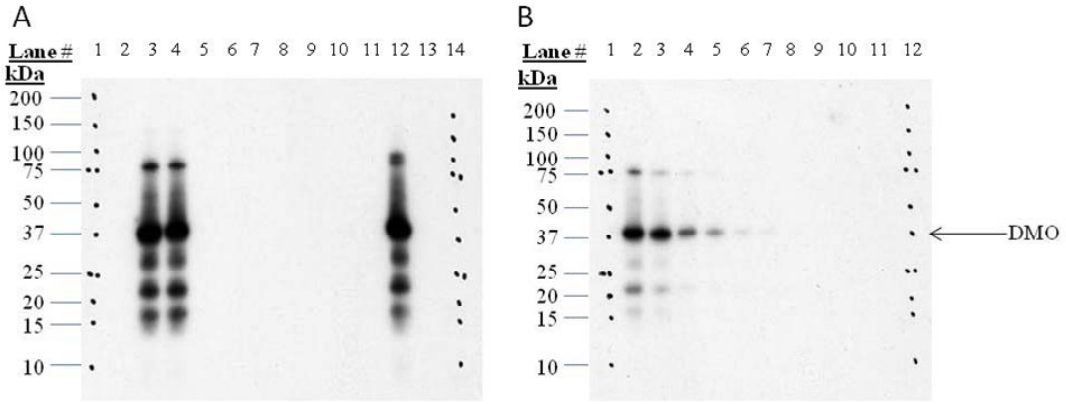


Figure 30. Western Blot Analysis of Purified *E. coli*-produced MON 88701 DMO Protein in Simulated Gastric Fluid

Western blots probed with an anti-DMO antibody were used to assess the digestibility of MON 88701 DMO in SGF. Approximate molecular weights (kDa) are shown on the left of each gel, and correspond to the markers loaded. Empty lanes on the gels were cropped from the images. A 0.5 min exposure is shown. T = time. Panel A corresponds to *E. coli*-produced MON 88701 DMO protein digestion in SGF. Based on pre-digestion protein concentrations, 20 ng of total protein was loaded in each lane containing DMO protein (SGF T0-SGF T7). Panel B corresponds to the analysis to determine LOD of *E. coli*-produced MON 88701 DMO. Indicated amounts of the DMO protein from the SGF T0 sample were loaded to estimate the LOD of the protein. Lane designations are as follows:

Panel A		
Lane	**Sample**	**Incubation Time (min)**
1	Precision Plus MWM	-
2	SGF N0 (No DMO control)	0
3	SGF P0 (No Pepsin Control)	0
4	SGF T0	0
5	SGF T1	0.5
6	SGF T2	2
7	SGF T3	5
8	SGF T4	10
9	SGF T5	20
10	SGF T6	30
11	SGF T7	60
12	SGF P7	60
13	SGF N7	60
14	Precision Plus MWM	-

Panel B		
Lane	**Sample**	**Amount (ng)**
1	Precision Plus MWM	-
2	T0, protein+SGF	10.0
3	T0, protein+SGF	4.0
4	T0, protein+SGF	2.0
5	T0, protein+SGF	1.0
6	T0, protein+SGF	0.4
7	T0, protein+SGF	0.2
8	T0, protein+SGF	0.1
9	T0, protein+SGF	0.05
10	T0, protein+SGF	0.02
11	T0, protein+SGF	0.01
12	Precision Plus MWM	-

Digestibility of MON 88701 DMO in SIF

The digestibility of the *E. coli*-produced MON 88701 DMO protein in SIF was assessed by western blot (Figure 31). The western blot used to assess the *in vitro* digestibility of the MON 88701 DMO protein in SIF (Figure 31, Panel A) was run concurrently with a western blot used to estimate the LOD (Figure 31 Panel B) of the intact MON 88701 DMO protein in this assay. The gel used to assess the digestibility of the MON 88701 DMO protein in SIF by western blot was loaded with 20 ng total protein (based on pre-digestion protein concentrations) for each of the incubation time points. No immunoreactive bands were observed in controls SIF N0 and SIF N8, which represent the SIF test system without *E. coli*-produced MON 88701 DMO protein (Figure 31, Panel A, Lanes 2 and 14). This result demonstrates the absence of non-specific antibody interactions with the SIF test system.

Western blot analysis demonstrated *E. coli*-produced MON 88701 DMO protein was digested to a level below the LOD within 5 min of incubation in SIF (Figure 31 Panel A, Lane 5), the first time point assessed. The LOD of the MON 88701 DMO protein was visually estimated to be 0.2 ng (Figure 31, Panel B, Lane 7). This LOD was used to calculate the maximum amount of MON 88701 DMO protein that could remain visually undetected after digestion, which corresponded to approximately 1% of the total protein loaded. Therefore, based on the LOD, more than 99% (100% - 1% = 99%) of the MON 88701 DMO protein was digested in SIF within 5 min. A faint immunodetectable band of less than ~12 kDa was observed at the 5 min time point in SIF, but was gone by 15 min. No other immunoreactive bands were detected in any other digestion specimens.

Comparison of the signal for *E. coli*-produced MON 88701 DMO protein in the controls SIF P0 and SIF P8 (Figure 31 Panel A, Lanes 3 and 13), which represent the test system without pancreatin, suggests that MON 88701 DMO showed some tendency to aggregate when incubated in the test system buffer at 37 °C for 24 h. However, the MON 88701 DMO protein is still readily observed in the SIF P8 sample, indicating that the lack of MON 88701 DMO in the SIF T1 sample is due to pancreatin activity rather than protein aggregation.

In summary, the results from this analysis demonstrate that greater than 99% of the *E coli*-produced MON 88701 DMO protein was digested in SIF within 5 min.

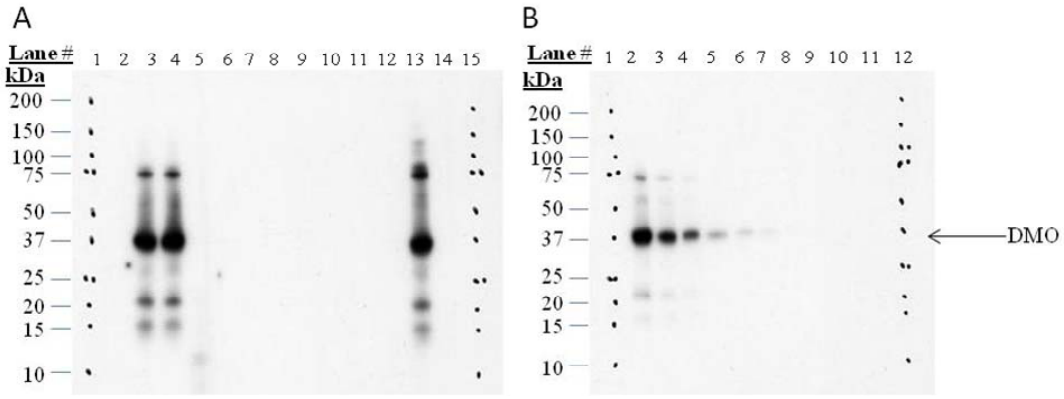


Figure 31. Western Blot Analysis of Purified *E. coli*-produced MON 88701 DMO Protein in Simulated Intestinal Fluid

Western blots probed with an anti-DMO antibody were used to assess the digestibility of MON 88701 DMO in SIF. Approximate molecular weights (kDa) are shown on the left of each gel, and correspond to the markers loaded. A 0.5 min exposure is shown. T = time. Panel A corresponds to E. coli-produced MON 88701 DMO protein digestion in SIF. Based on pre-digestion protein concentrations, 20 ng of the total protein was loaded in each lane containing DMO protein. Panel B corresponds to the analysis to determine LOD of E. coli-produced MON 88701 DMO. Indicated amounts of the DMO protein from the SIF T0 sample were loaded to estimate the LOD of the protein. Empty lanes on the blot were cropped from the image. Lane designations are as follows:

Panel A		
Lane	**Sample**	**Incubation Time**
1	Precision Plus MWM	-
2	SIF N0 (No DMO control)	0
3	SIF P0 (No pancreatin control)	0
4	SIF T0	0
5	SIF T1	5 min
6	SIF T2	15 min
7	SIF T3	30 min
8	SIF T4	1 hr
9	SIF T5	2 hr
10	SIF T6	4 hr
11	SIF T7	8 hr
12	SIF T8	24 hr
13	SIF P8	24 hr
14	SIF N8	24 hr
15	Precision Plus MWM	-

Panel B		
Lane	**Sample**	**Amount (ng)**
1	Precision Plus MWM	-
2	T0, protein+SIF	10
3	T0, protein+SIF	4.0
4	T0, protein+SIF	2.0
5	T0, protein+SIF	1.0
6	T0, protein+SIF	0.4
7	T0, protein+SIF	0.2
8	T0, protein+SIF	0.1
9	T0, protein+SIF	0.05
10	T0, protein+SIF	0.02
11	T0, protein+SIF	0.01
12	Precision Plus MWM	-

Digestibility of MON 88701 DMO - Conclusions

Digestibility of the MON 88701 DMO protein was evaluated in SGF and SIF. The results of the study demonstrate that greater than 99% of the *E coli*-produced MON 88701 DMO protein was digested in SGF within 0.5 min, when analysed by Brilliant Blue G Colloidal stained SDS-PAGE and by western blot using a DMO-specific antibody. Additionally, at least 99% of the MON 88701 DMO protein was digested within 5 min during incubation in SIF.

Results from the digestibility experiments show that *E coli*-produced MON 88701 DMO protein is rapidly digested in the *in vitro* model gastrointestinal digestive system. Rapid digestion of the *E coli*-produced MON 88701 protein in SGF and SIF support the conclusion that the MON 88701 DMO protein is highly unlikely to pose a safety concern to human and animal health.

Please also refer to [redacted] 2011 (MSL0023579).

Digestibility of PAT (bar) in SGF

The digestibility of *E. coli*-produced PAT (*bar*) in SGF was assessed using two methods: visual analysis of a Brilliant Blue G Colloidal stained SDS-PAGE gel and visual analysis of a western blot probed with an anti-PAT (*bar*) polyclonal antibody. For this assessment, a separate SDS-PAGE gel containing dilutions of the pre-digestion test sample was run concurrently to estimate the limit of detection (LOD) of the undigested *E. coli*-produced PAT (*bar*) protein.

Digestibility of *E. coli*-produced PAT (*bar*) in SGF was evaluated over time by analysing digestion mixtures incubated for targeted time intervals. For SDS-PAGE analysis, approximately 1 µg of total protein was analyzed for each timepoint (Figure 32 Panel A). The controls, SGF N0 and SGF N7 (Figure 32 Panel A, Lanes 2 and 13), which evaluate the stability of the pepsin in the test system (SGF) lacking the PAT (*bar*) protein, demonstrated that the pepsin was observed as a stained protein band at ~38 kDa throughout the experimental phase.

No change in the PAT (*bar*) protein band intensity was observed over time in the absence of pepsin (compare SGF P0 to SGF P7; Figure 32 Panel A, Lanes 3 and 12) indicating that the digestion of the PAT (*bar*) protein was due to the proteolytic activity of pepsin present in SGF and not due to instability of the protein while incubated at pH ~1.2 at ~37 °C for 60 min.

Visual examination of SDS-PAGE data showed that the intact PAT (*bar*) protein was completely digested within 0.5 min of incubation in SGF (Figure 32 Panel A, Lane 5). For the SDS-PAGE analysis, the LOD of the PAT (*bar*) protein was visually estimated to be 13 ng, or 0.013 µg (Figure 32 Panel B, Lane 6). This LOD used to calculate the maximum amount of intact PAT (*bar*) protein that could remain visually undetected after digestion, which corresponded to approximately 1.3% of the total protein loaded. Based on that LOD, more than 98.7% (100% - 1.3% = 98.7%) of the intact PAT (*bar*) protein was digested within

0.5 min of incubation in SGF. This is comparable with previously published safety assessments of PAT (*bar*) protein (Hérouet et al., 2005).

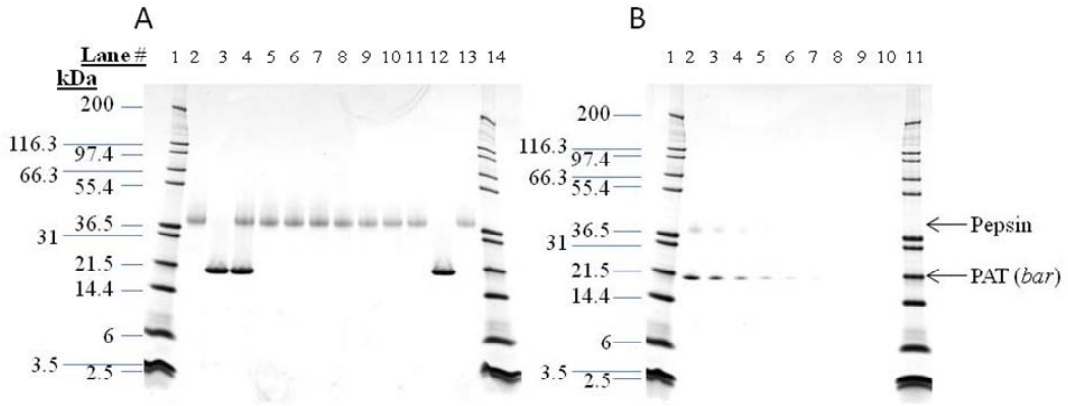


Figure 32. Colloidal Blue Stained SDS-PAGE Gel Showing the Digestion of Purified *E. coli*-produced PAT (*bar*) Protein in Simulated Gastric Fluid

Brilliant Blue G Colloidal stained SDS-PAGE gels were used to assess the digestibility of PAT (*bar*) in SGF. Proteins were subjected to SDS-PAGE and detected by staining with Brilliant Blue G Colloidal stain. Approximate molecular weights (kDa) are shown on the left of each gel, and correspond to the markers loaded. Empty lanes on the gels were cropped from the images. T = time. Panel A corresponds to PAT (*bar*) protein digestion in SGF. *E. coli*-produced PAT (*bar*) protein was loaded at 1 µg per lane based on pre-digestion concentrations. Panel B corresponds to the analysis to determine Limit of Detection (LOD) of PAT (*bar*). Sample amount indicates the amount of *E. coli*-produced PAT (*bar*) protein in the SGF T0 sample loaded to estimate the LOD of the PAT (*bar*) protein. Lane designations are as follows:

Lane	Sample	Incubation Time (min)
1	Mark 12 MWM	-
2	SGF N0 (No PAT (*bar*) control)	0
3	SGF P0 (No pepsin control)	0
4	SGF T0	0
5	SGF T1	0.5
6	SGF T2	2
7	SGF T3	5
8	SGF T4	10
9	SGF T5	20
10	SGF T6	30
11	SGF T7	60
12	SGF P7	60
13	SGF N7	60
14	Mark 12 MWM	-

Lane	Sample	Amount (ng)
1	Mark 12 MWM	-
2	T0, protein+SGF	200
3	T0, protein+SGF	100
4	T0, protein+SGF	50
5	T0, protein+SGF	25
6	T0, protein+SGF	13
7	T0, protein+SGF	6.3
8	T0, protein+SGF	3.1
9	T0, protein+SGF	1.6
10	T0, protein+SGF	0.8
11	Mark 12 MWM	-

For the western blot analysis of PAT (*bar*) digestibility in SGF, the blot used to assess the stability of the PAT (*bar*) protein to pepsin digestion (Figure 33 Panel A) was run concurrently with a blot to estimate the LOD of the PAT (*bar*) protein (Figure 33 Panel B). Ten ng of total protein was analysed by western blot for each time point. No immunoreactive bands were observed in controls SGF N0 and SGF N7 (Figure 33 Panel A, Lanes 2 and 13). This result indicates that non-specific interactions between the test system components and the PAT (*bar*) -specific antibody did not occur under these experimental conditions.

No change in the intact PAT (*bar*) protein band intensity was observed in the absence of pepsin (compare SGF P0 to P7) (Figure 33 Panel A, Lanes 3 and 12). This result reaffirms that the PAT (*bar*) protein was stable in the test system without pepsin.

Western blot analysis demonstrated that the *E. coli*-produced PAT (*bar*) protein was digested below the LOD within 0.5 min of incubation in SGF (Figure 33 Panel A, Lane 5). The LOD of the PAT (*bar*) protein was visually estimated to be 0.16 ng (Figure 33 Panel B, Lane 7). The LOD estimated for the PAT (*bar*) protein was used to calculate the maximum amount of PAT (*bar*) protein that could remain visually undetected after digestion, which corresponded to approximately 1.6% of the total PAT (*bar*) protein loaded. Based on the western blot LOD for the PAT (*bar*) protein, the conclusion was that more than 98.4% (100% - 1.6% = 98.4%) of the intact PAT (*bar*) protein was digested within 0.5 min. This is comparable with previously published safety assessments of PAT (*bar*) protein (Hérouet et al., 2005).

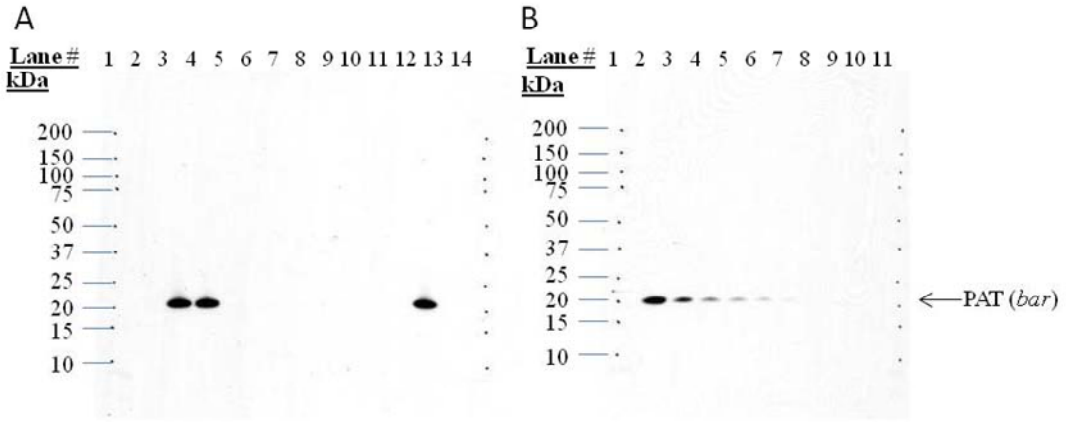


Figure 33. Western Blot Analysis of Purified *E. coli*-produced PAT (*bar*) Protein in Simulated Gastric Fluid

Western blots probed with an anti-PAT (*bar*) antibody were used to assess the digestibility of PAT (*bar*) in SGF. Approximate molecular weights (kDa) are shown on the left of each gel, and correspond to the markers loaded. Empty lanes on the gels were cropped from the images. A 0.5 min exposure is shown. T = time. Panel A corresponds to *E. coli*-produced PAT (*bar*) protein digestion in SGF. Based on pre-digestion protein concentrations, 10 ng of total protein was loaded in each lane containing PAT (*bar*) protein (SGF T0-SGF T7). Panel B corresponds to the analysis to determine LOD of E. coli-produced PAT (*bar*) protein. Indicated amounts of the PAT (bar) protein from the SGF T0 sample were loaded to estimate the LOD of the protein. Lane designations are as follows:

Panel A

Lane	Sample	Incubation Time (min)
1	Precision Plus MWM	-
2	SGF N0 (No PAT (*bar*) control)	0
3	SGF P0 (No pepsin control)	0
4	SGF T0	0
5	SGF T1	0.5
6	SGF T2	2
7	SGF T3	5
8	SGF T4	10
9	SGF T5	20
10	SGF T6	30
11	SGF T7	60
12	SGF P7	60
13	SGF N7	60
14	Precision Plus MWM	-

Panel B

Lane	Sample	Amount (ng)
1	Precision Plus MWM	-
2	T0, protein+SGF	5
3	T0, protein+SGF	2.5
4	T0, protein+SGF	1.3
5	T0, protein+SGF	0.63
6	T0, protein+SGF	0.31
7	T0, protein+SGF	0.16
8	T0, protein+SGF	0.08
9	T0, protein+SGF	0.04
10	T0, protein+SGF	0.02
11	Precision Plus MWM	-

Digestibility of PAT (bar) in SIF

The digestibility of the PAT (*bar*) protein in SIF was assessed by western blot (Figure 34). The western blot used to assess the *in vitro* digestibility of the PAT (*bar*) protein in SIF (Figure 34 Panel A) was run concurrently with a western blot used to estimate the LOD (Figure 34 Panel B) of the intact PAT (*bar*) protein in this assay. The gel used to assess the digestibility of the PAT (*bar*) protein in SIF by western blot was loaded with 10 ng total protein (based on pre-digestion protein concentrations) for each of the incubation time points. No immunoreactive bands were observed in controls SIF N0 and SIF N8, which represent the SIF test system without *E. coli*- -produced PAT (*bar*) protein (Figure 34 Panel A, Lanes 2 and 14). This result demonstrates the absence of non-specific antibody interactions with the SIF test system.

No change in PAT (*bar*) protein band intensity was observed in the controls SIF P0 and SIF P8 (Figure 34 Panel A, Lanes 3 and 13), which represent the test system without pancreatin. This result reaffirms that PAT (*bar*) was stable in the test system without pancreatin.

Western blot analysis demonstrated that a band corresponding to the PAT (*bar*) protein was digested to a level below the LOD within 5 min of incubation in SIF (Figure 34 Panel A, Lane 5), the first time point assessed. The LOD was visually estimated to be 0.16 ng (Figure 34 Panel B, Lane 7). This LOD was used to calculate the maximum amount of PAT (*bar*) protein that could remain visually undetected after digestion, which corresponded to approximately 1.6% of the total protein loaded. Therefore, based on the LOD, more than 98.4% (100% - 1.6% = 98.4%) of the PAT (*bar*) protein was digested in SIF within 5 min. A faint immunodetectable band of less than 10 kDa was observed at the 5 and 15 min time point in SIF, but was gone by 30 min. No other immunoreactive bands were detected in any other digestion specimens. This is comparable with previously published safety assessments of PAT (*bar*) protein (Hérouet et al., 2005).

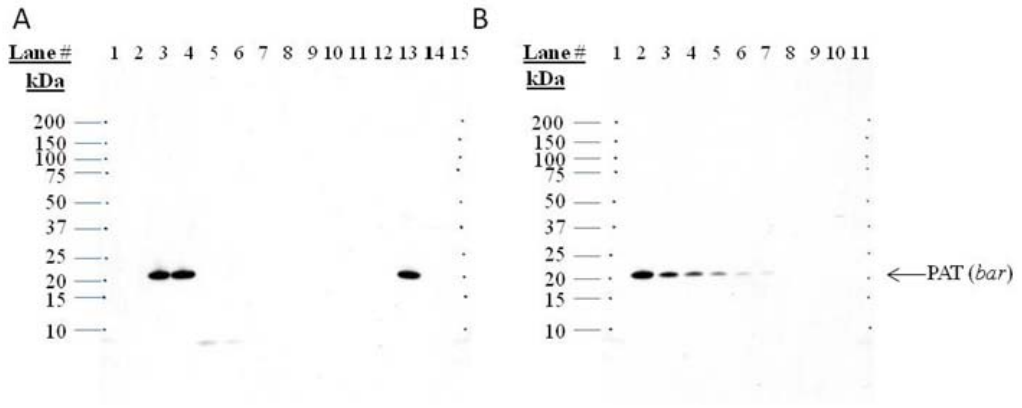


Figure 34. Western Blot Analysis of Purified *E. coli*-produced PAT (*bar*) Protein in Simulated Intestinal Fluid

Western blots probed with an anti- PAT (*bar*) antibody were used to assess the digestibility of PAT (*bar*) in SIF. Approximate molecular weights (kDa) are shown on the left of each gel, and correspond to the markers loaded. A 1 min exposure is shown. T = time. Panel A corresponds to *E. coli*-produced PAT (*bar*) protein digestion in SIF. Ten ng of total protein was loaded per lane based on pre-digestion concentrations. Panel B corresponds to the analysis to determine Limit of Detection (LOD) of *E. coli*-produced PAT (*bar*) protein. Indicated amounts of the PAT (*bar*) protein from the SIF T0 sample were loaded to estimate the LOD of the protein. Empty lanes on the blot were cropped from the image. Lane designations are as follows:

Panel A

Lane	Sample	Incubation Time
1	Precision Plus MWM	-
2	SIF N0 (No PAT (*bar*) control)	0
3	SIF P0 (No pancreatin control)	0
4	SIF T0	0
5	SIF T1	5 min
6	SIF T2	15 min
7	SIF T3	30 min
8	SIF T4	1 hr
9	SIF T5	2 hr
10	SIF T6	4 hr
11	SIF T7	8 hr
12	SIF T8	24 hr
13	SIF P8	24 hr
14	SIF N8	24 hr
15	Precision Plus MWM	-

Panel B

Lane	Sample	Amount (ng)
1	Precision Plus MWM	-
2	T0, protein+SIF	5
3	T0, protein+SIF	2.5
4	T0, protein+SIF	1.3
5	T0, protein+SIF	0.63
6	T0, protein+SIF	0.31
7	T0, protein+SIF	0.16
8	T0, protein+SIF	0.08
9	T0, protein+SIF	0.04
10	T0, protein+SIF	0.02
11	Precision Plus MWM	-

Digestibility of PAT (bar) - Conclusions

Digestibility of the PAT (*bar*) protein was evaluated in SGF and SIF. Comparable to previously published safety assessment data on PAT (*bar*) protein (Hérouet et al., 2005), the results of the present studies demonstrate that greater than 98.4% of the *E. coli*-produced PAT (*bar*) protein was digested in SGF within 0.5 min, when analysed by Brilliant Blue G Colloidal stained SDS-PAGE and by western blot using a PAT (*bar*)-specific antibody. Additionally, at least 98.4% of the PAT (*bar*) protein was digested within 5 min during incubation in SIF.

Results from the digestibility experiments show that the PAT (*bar*) protein is rapidly digested in the *in vitro* model gastrointestinal digestive system. Rapid digestion of the *E. coli*-produced PAT (*bar*) protein in SGF and SIF supports the conclusion that the PAT (*bar*) protein is highly unlikely to pose a safety concern to human and animal health.

Please also refer to Wang et al., 2011 (MSL0023567).

B5(c)(ii) Heat Stability of the Purified Protein

Heat Stability of the Purified MON 88701 DMO Protein

Temperature can have a profound effect on the structure and function of proteins. Cottonseed processing involves treatment of cottonseed for hours with temperatures from 88 ºC to greater than 130 ºC for meal processing and up to 230 ºC for deodorisation of the oil (Harris, 1981; NCPA, 1993). In addition the processing of linters involves processing at temperatures greater than 100 ºC (AOCS, 2009). Therefore it is reasonable to assume that the conditions encountered during the processing of cottonseed and linters from MON 88701 will have an effect on the functional activity and structure of MON 88701 DMO protein when consumed in different feed products derived from MON 88701 and in human food products in the unlikely event protein is present.

The effect of heat treatment on the activity of *E coli*-produced MON 88701 DMO protein was evaluated using purified protein. Heat-treated samples and an unheated control sample of *E coli*-produced MON 88701 DMO protein were analysed: 1) using a functional assay to assess the impact of temperature on the enzymatic activity of MON 88701 DMO protein; and 2) using SDS-PAGE to assess the impact of temperature on protein integrity.

Aliquots of *E coli*-produced MON 88701 DMO were heated to 25, 37, 55, 75, and 95 °C for 15 and 30 min, while a separate aliquot of *E coli*-produced MON 88701 DMO was maintained on ice for the duration of the heat treatments to serve as a temperature control. The effect of heat treatment on the activity of MON 88701 DMO was evaluated using a functional activity assay. The effect of heat treatment on the integrity of the MON 88701 DMO protein was evaluated using SDS-PAGE analysis of the heated and temperature control MON 88701 DMO samples.

The effects of heating on the functional activity of *E coli*-produced MON 88701 DMO are presented in Table 19 and Table 20. The functional activity of MON 88701 DMO was unaffected at 25 ºC and 37 ºC for 15 and 30 min. The functional activity of MON 88701

DMO was below the LOQ of the assay following incubation at 55 ºC or higher for 15 min or more, indicating that the majority of the functional activity of MON 88701 DMO had been lost during heating. These results suggest that temperature has a considerable effect on the activity of MON 88701 DMO.

Analysis by SDS-PAGE stained with Brilliant Blue G Colloidal demonstrated that the MON 88701 DMO control treatment and reference standard contain a major band at ~38 kDa, corresponding to the MON 88701 DMO protein (Figure 35 and Figure 36, Lanes 2 and 8). No apparent decrease in the intensity of this band was observed in heat-treated MON 88701 DMO at any temperatures at 15 or 30 min (Figure 35, Lanes 3–7 and Figure 36, Lanes 3–7).

These data demonstrate that *E. coli*-produced MON 88701 DMO remains intact, but is deactivated at temperatures 55 ºC and above. Therefore, in the unlikely event that processed cottonseed oil or linters contain protein (NCPA, 2002; Nida et al., 1996; Reeves and Weihrauch, 1979), it is reasonable to conclude that MON 88701 DMO protein would not be consumed as an active protein in food or feed products due to standard processing practices that include heat treatment.

Please also refer to [redacted] 2011 (MSL0023606).

Table 19. Activity of *E. coli*-produced MON 88701 DMO Protein after 15 Minutes at Elevated Temperatures

Temperature	Functional Activity (nmol DCSA × minute^{-1} × mg^{-1})[1]	Relative Activity[2] (% of unheated control)
Unheated Control (0 ºC)	6.74	100%
25 ºC	6.29	93%
37 ºC	7.48	111%
55 ºC	Below LOQ[3]	<22%
75 ºC	Below LOQ[3]	<22%
95 ºC	Below LOQ[3]	<22%

[1] Mean specific activity determined from n=3.

[2] Relative activity = [activity of sample/ activity of unheated control] × 100; DMO protein activity of unheated control was assigned 100%.

[3] The LOQ is 1.5 nmol DCSA×min^{-1}×mg^{-1} of *E. coli*-produced MON 88701 DMO protein.

Table 20. Activity of *E. coli*-produced MON 88701 DMO Protein after 30 Minutes at Elevated Temperatures

Temperature	Functional Activity (nmol DCSA × minute^{-1} × mg^{-1})[1]	Relative Activity (% of unheated control)
Unheated Control (0 ºC)	6.74	100%
25 ºC	7.36	109%
37 ºC	7.28	108%
55 ºC	Below LOQ[3]	<22%
75 ºC	Below LOQ[3]	<22%
95 ºC	Below LOQ[3]	<22%

[1] Mean specific activity determined from n=3.

[2] Relative activity = [activity of sample/ activity of unheated control] × 100; DMO protein activity of unheated control was assigned 100%.

[3] The LOQ is 1.5 nmol DCSA×min^{-1}×mg^{-1} of *E. coli*-produced MON 88701 DMO protein.

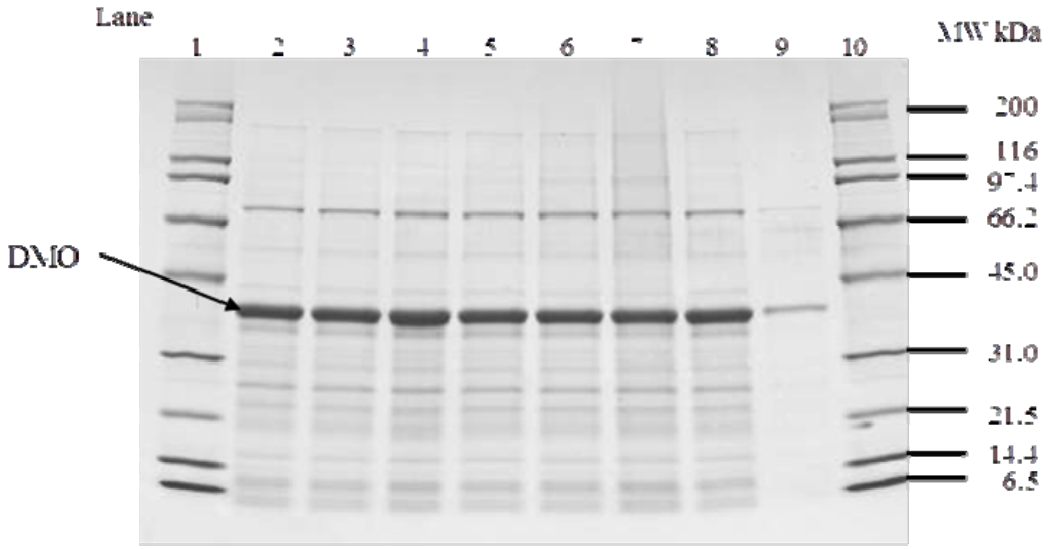
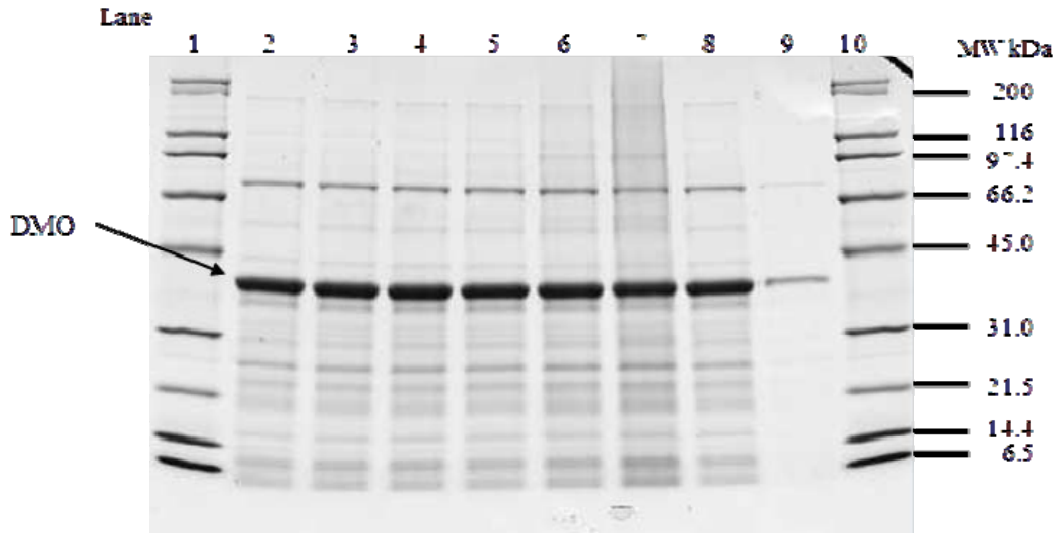


Figure 35. SDS-PAGE of *E. coli*-produced MON 88701 DMO Protein Following Heat Treatment for 15 Minutes

Heat-treated samples of *E. coli*-produced MON 88701 DMO (3.3 µg total protein) subjected to SDS-PAGE and 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 designations are as follows:

Lane	Description	Amount (µg)
1	Broad Range MWM	5.0
2	*E. coli*-produced MON 88701 DMO Unheated Control (0 ºC)	3.3
3	*E. coli*-produced MON 88701 DMO 25 ºC	3.3
4	*E. coli*-produced MON 88701 DMO 37 ºC	3.3
5	*E. coli*-produced MON 88701 DMO 55 ºC	3.3
6	*E. coli*-produced MON 88701 DMO 75 ºC	3.3
7	*E. coli*-produced MON 88701 DMO 95 ºC	3.3
8	*E. coli*-produced MON 88701 DMO Reference	3.3
9	*E. coli*-produced MON 88701 DMO Reference	0.33
10	Broad Range MWM	5.0



Figure

Figure 36. SDS-PAGE of *E. coli*-produced MON 88701 DMO Protein Following Heat Treatment for 30 Minutes

Heat-treated samples of *E. coli*-produced MON 88701 DMO (3.3 µg total protein) subjected to SDS-PAGE and 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 designations are as follows:

Lane	Description	Amount (µg)
1	Broad Range MWM	5.0
2	*E. coli*-produced MON 88701 DMO Unheated Control (0 ºC)	3.3
3	*E. coli*-produced MON 88701 DMO 25 ºC	3.3
4	*E. coli*-produced MON 88701 DMO 37 ºC	3.3
5	*E. coli*-produced MON 88701 DMO 55 ºC	3.3
6	*E. coli*-produced MON 88701 DMO 75 ºC	3.3
7	*E. coli*-produced MON 88701 DMO 95 ºC	3.3
8	*E. coli*-produced MON 88701 DMO Reference	3.3
9	*E. coli*-produced MON 88701 DMO Reference	0.33
10	Broad Range MWM	5.0

Heat Stability of the Purified PAT (*bar*) Protein

Temperature can have a profound effect on the structure and function of proteins. Cottonseed processing involves treatment of cottonseed for hours with temperatures from 88 ºC to greater than 130 ºC for meal processing and up to 230 ºC for deodorisation of the oil (Harris, 1981; NCPA, 1993). In addition, the processing of linters involves processing at temperatures greater than 100 ºC (AOCS, 2009). Therefore is reasonable to assume that the conditions encountered during the processing of cottonseed and linters from MON 88701 will have an effect on the functional activity and structure of MON 88701 PAT (*bar*) protein when consumed in different feed products derived from MON 88701 and in human food products in the unlikely event protein is present.

The effect of heat treatment on the activity of *E. coli*-produced PAT (*bar*) protein was evaluated using purified protein. Heat-treated samples and an unheated control sample of *E. coli*-produced PAT (*bar*) protein were analysed: 1) using a functional assay to assess the impact of temperature on the enzymatic activity of PAT (*bar*) protein; and 2) using SDS-PAGE to assess the impact of temperature on protein integrity.

Aliquots of *E. coli*-produced PAT (*bar*) were heated to 25, 37, 55, 75, and 95 °C for 15 and 30 min, while a separate aliquot of *E. coli*-produced PAT (*bar*) was maintained on ice for the duration of the heat treatments to serve as a temperature control. The effect of heat treatment on the activity of PAT (*bar*) was evaluated using a functional activity assay. The effect of heat treatment on the integrity of the PAT (*bar*) protein was evaluated using SDS-PAGE analysis of the heated and temperature control PAT (*bar*) samples.

The effects of heating on the functional activity of PAT (*bar*) are presented in Table 21 and Table 22. The functional activity of PAT (*bar*) was unaffected at 25 ºC and 37 ºC for 15 and 30 min. The functional activity of PAT (*bar*) heated to 55 ºC demonstrated a substantial reduction in *E. coli*-produced PAT (*bar*) activity with 40% activity remaining at the 15 min incubation time and 24% activity remaining after the 30 min incubation. The functional activity of PAT (*bar*) heated to 75 ºC or higher for 15 min or more demonstrated a >90% loss of functional activity relative to the temperature control PAT (*bar*) sample. These results suggest that temperature has a considerable effect on the activity of functional activity of PAT (*bar*).

Analysis by SDS-PAGE stained with Brilliant Blue G Colloidal demonstrated that the PAT (*bar*) control treatment and reference standard contain a major band at ~25 kDa, corresponding to the PAT (*bar*) protein (Figure 37 and Figure 38, Lanes 2 and 8). No apparent decrease in the intensity of this band was observed in heat-treated PAT (*bar*) at all temperatures at 15 or 30 min (Figure 37, Lanes 3–7 and Figure 38, Lanes 3–7). There was a slight visible appearance of higher molecular weight species at heat treatments of 75 ºC and 95 ºC, presumably due to protein aggregation.

These data demonstrate that PAT (*bar*) remains intact, but is deactivated at temperatures of 75 ºC and above. This is comparable with what has been previously published on the safety assessment of PAT (*bar*) protein (Hérouet et al., 2005; Wehrmann et al., 1996). Therefore,

in the unlikely event that cottonseed oil contains protein (Reeves and Weihrauch, 1979), it is reasonable to conclude that PAT (*bar*) protein would not be consumed as an active protein in food or feed products due to standard processing practices that include heat treatment.

Please also refer to [redacted] 2011 (MSL0023584).

Table 21. Activity of *E. coli*-produced PAT (*bar*) Protein after 15 Minutes at Elevated Temperatures

Temperature	Functional Activity (µmol × minute^{-1} × mg^{-1})[1]	Relative Activity (% of unheated control)[2]
Unheated Control (0 ºC)	27.2	100%
25 ºC	22.1	81%
37 ºC	22.4	82%
55 ºC	10.9	40%
75 ºC	2.3	8%
95 ºC	2.4	9%

[1] Mean specific activity determined from n = 3.

[2] Relative activity = [activity of sample/ activity of unheated control] × 100; PAT (*bar*) protein activity of unheated control was assigned 100%.

Table 22. Activity of *E. coli*-produced PAT (*bar*) Protein after 30 Minutes at Elevated Temperatures

Temperature	Functional Activity (µmol × minute^{-1} × mg^{-1})[1]	Relative Activity (% of unheated control)[2]
Unheated Control (0 ºC)	27.2	100%
25 ºC	21.5	79%
37 ºC	24.1	89%
55 ºC	6.6	24%
75 ºC	2.3	8%
95 ºC	2.4	9%

[1] Mean specific activity determined from n = 3.

[2] Relative activity = [activity of sample/ activity of unheated control] × 100; PAT (*bar*) protein activity of unheated control was assigned 100%.

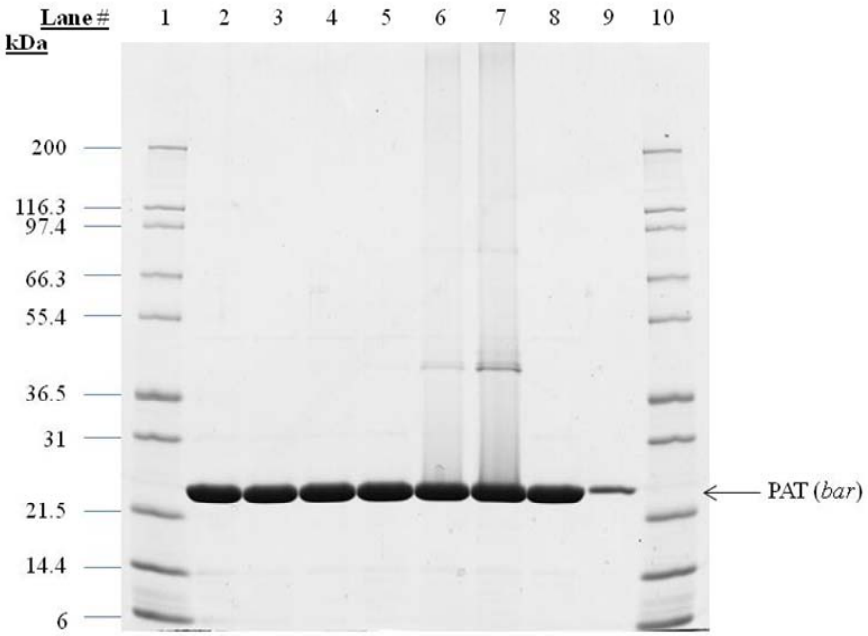


Figure 37. SDS-PAGE of *E. coli*-produced PAT (*bar*) Protein Following Heat Treatment for 15 Minutes

Heat-treated samples of PAT (*bar*) (3.0 µg total protein) subjected to SDS-PAGE and 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 designations are as follows:

Lane	Description	Amount (µg)
1	Mark 12 MWM	-
2	*E. coli*-produced PAT (*bar*) Unheated Control (0 ºC)	3.0
3	*E. coli*-produced PAT (*bar*) 25 ºC	3.0
4	*E. coli*-produced PAT (*bar*) 37 ºC	3.0
5	*E. coli*-produced PAT (*bar*) 55 ºC	3.0
6	*E. coli*-produced PAT (*bar*) 75 ºC	3.0
7	*E. coli*-produced PAT (*bar*) 95 ºC	3.0
8	*E. coli*-produced PAT (*bar*) Reference	3.0
9	*E. coli*-produced PAT (*bar*) Reference	0.3
10	Mark 12 MWM	-

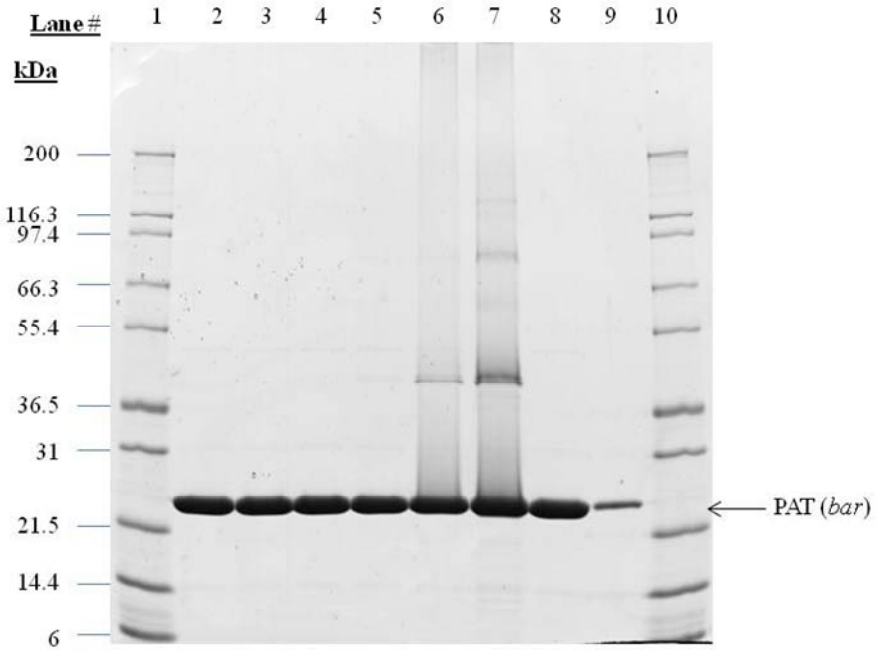


Figure 38. SDS-PAGE of *E. coli*-produced PAT (*bar*) Protein Following Heat Treatment for 30 Minutes

Heat-treated samples of PAT (*bar*) protein (3.0 µg total protein) subjected to SDS-PAGE and 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 designations are as follows:

Lane	Description	Amount (µg)
1	Mark 12 MWM	-
2	*E. coli*-produced PAT (*bar*) Unheated Control (0 ºC)	3.0
3	*E. coli*-produced PAT (*bar*) 25 ºC	3.0
4	*E. coli*-produced PAT (*bar*) 37 ºC	3.0
5	*E. coli*-produced PAT (*bar*) 55 ºC	3.0
6	*E. coli*-produced PAT (*bar*) 75 ºC	3.0
7	*E. coli*-produced PAT (*bar*) 95 ºC	3.0
8	*E. coli*-produced PAT (*bar*) Reference	3.0
9	*E. coli*-produced PAT (*bar*) Reference	0.3
10	Mark 12 MWM	-

B5(d) Specific serum screening if protein from allergenic source

Not applicable.

B5(e) Protein as a Proportion of Total Protein

The MON 88701 DMO Protein as a Proportion of Total Protein

The MON 88701 DMO protein was detected in all plant tissues assayed, at a number of time points during the growing season (Table 16). Concerns for assessing potential allergenicity are less relevant to MON 88701 since the only human food currently produced from cottonseed is refined, bleached, and deodorised (RBD) oil, and to a smaller extent, linters. RBD oil contains undetectable amounts of protein (Reeves and Weihrauch, 1979) and linters are a highly processed product composed of nearly pure (*i.e.*, >99%) cellulose (NCPA, 2002; Nida et al., 1996). Because RBD oil and linters are processed fractions that contain negligible amounts of total protein an allergenicity assessment is primarily considered a theoretical assessment. However, since cottonseed is the source of cottonseed oil and linters, cottonseed is the most appropriate tissue to assess the potential food allergenicity of MON 88701. The mean level of MON 88701 DMO protein in cottonseed of MON 88701 is 21 µg/g dw. The mean percent dry weight of total protein in seed of MON 88701 is 27.91% (or 279,100 µg/g; Table 27). The percentage of MON 88701 DMO protein in MON 88701 seed is calculated as follows:

(21 µg/g ÷ 279,100 µg/g) × 100% ≈ 0.008% or 80 ppm of total cottonseed protein

Therefore, the MON 88701 DMO protein represents a very small portion of the total protein in the cottonseed of MON 88701 and due to the harsh conditions used in cottonseed processing is most likely absent in the oil and linters that are used for food production.

The MON 88701-produced PAT (*bar*) Protein as a Proportion of Total Protein

The MON 88701-produced PAT (*bar*) protein was detected in all plant tissues assayed, at a number of time points during the growing season (Table 17). Concerns for assessing potential allergenicity are less relevant to MON 88701 since the only human food currently produced from cottonseed is refined, bleached, and deodorised (RBD) oil, and to a smaller extent, linters. RBD oil contains undetectable amounts of protein (Reeves and Weihrauch, 1979) and linters are a highly processed product composed of nearly pure (*i.e.*, >99%) cellulose (NCPA, 2002; Nida et al., 1996). Because RBD oil and linters are processed fractions that contain negligible amounts of total protein an allergenicity assessment is primarily considered a theoretical assessment. However, since cottonseed is the source of cottonseed oil and linters, cottonseed is the most appropriate tissue to assess the potential food allergenicity of MON 88701. The mean level of MON 88701-produced PAT (*bar*) protein in seed of MON 88701 is 6.6 µg/g dw. The mean percent dry weight of total protein in seed of MON 88701 is 27.91% (or 279,100 µg/g; Table 27). The percentage of MON 88701-produced PAT (*bar*) protein in MON 88701 seed is calculated as follows:

(6.6 µg/g ÷ 279,100 µg/g) × 100% ≈ 0.002% or 20 ppm of total cottonseed protein

Therefore, the MON 88701-produced PAT (*bar*) protein represents a very small portion of the total protein in the cottonseed of MON 88701 and due to the harsh conditions used in cottonseed processing is most likely absent in the oil and linters that are used for food production.

B6 Toxicity of novel herbicide metabolites in GM herbicide-tolerant plants

MON 88701 contains a demethylase gene from *S. maltophilia* that expresses a dicamba mono-oxygenase (DMO) protein to confer tolerance to dicamba herbicide and a bialaphos resistance (*bar*) gene from *S. hygroscopicus* that expresses the phosphinothricin N-acetyltransferase (PAT) protein to confer tolerance to glufosinate herbicide. DMO is an enzyme that catalyzes the demethylation of dicamba to the non-herbicidal compound 3,6-dichlorosalicylic acid (DCSA) and formaldehyde (Chakraborty et al., 2005). PAT (*bar*) protein acetylates the free amino group of glufosinate to produce non-herbicidal N-acetyl glufosinate, a well known metabolite in glufosinate tolerant plants (OECD, 2002).

DCSA is a known soybean, soil and livestock metabolite whose safety has been evaluated by the US EPA (U.S. EPA, 2009). DCSA is also the primary degradate in soil from dicamba aerobic soil metabolism and is therefore not new to the environment; it is not persistent in the environment and has low potential for leaching to ground water (EFSA, 2007a). DCSA has been evaluated for its toxicity to organisms in the environment. Based on studies using Rainbow trout (*Oncorhynchus mykiss*), *Daphnia*, green algae (*Selenastrum caprinornutum*), *Lemna* and earthworm (*Eisenia fetida*), it was concluded that "the metabolite 3,6-dichlorosalicylic acid (DCSA) was not found to give rise to unacceptable risks" (EFSA, 2007b).

Formaldehyde is ubiquitous in the environment and atmosphere from a variety of biogenic (*e.g.* plant and animal) and anthropogenic (*e.g.* automotive or industrial emissions) sources. Formaldehyde also degrades rapidly in environmental compartments (air, soil and water)(U.S. EPA, 2008). The rapid degradation of formaldehyde in the environment combined with the understanding that formaldehyde is widely used by living organisms as a 1C source, support a conclusion that any environmental effects of formaldehyde, including effects on other plants and NTO's, resulting from dicamba treatment, would be negligible.

In Australia, dicamba has been registered for use on cereal crops as well as sugar cane by the Australian Pesticides and Veterinary Medicines Authority (APVMA) with maximum residue limits (MRLs) established for cereal grains (0.05 ppm) and sugar cane (0.1 ppm) crop commodities. Dicamba is not registered for use in or on cotton in Australia and dicamba MRLs do not exist for cotton commodities in Australia. Additionally, there is no import MRL set in Standard 1.4.2 for residues of dicamba in cottonseed. The MRL for dicamba in cottonseed in the United States is set at 0.2 ppm and the Codex MRL for dicamba is set at 0.04 ppm. Petition to establish new tolerances for cotton undelinted seed and cotton gin by-products were submitted in the United States and will be submitted to Codex. Consequently, a request will be submitted concurrently with this application to establish an import MRL to cover residues of dicamba on cottonseed into Australia.

In Australia, both the import MRL set by FSANZ and the MRL set by the APVMA for glufosinate in cottonseed is set at 3 ppm. The use pattern and rate of glufosinate on MON 88701 will follow the existing glufosinate-tolerant cotton uses outlined on the glufosinate herbicide label and the glufosinate residues in MON 88701 treated with commercial glufosinate rates are below the established pesticide residue tolerances for

cottonseed. Monsanto will not pursue any changes in the established tolerances for its use on MON 88701 cotton and because glufosinate and glufosinate metabolite residues have been previously assessed in relation to application A533 (Food Derived from Glufosinate-Ammonium Tolerant Cotton Line LL25), additional data on the identity and levels of herbicide and any metabolites are not given in this application.

Magnitude of Dicamba Residues and Metabolites in Undelinted MON 88701 Cottonseed after Dicamba Application

The residues of dicamba in undelinted cottonseed were determined after application of dicamba formulations to MON 88701. The test system used in the treated and control plots was MON 88701 cotton that is tolerant to dicamba (3,6-dichloro-2-methoxybenzoic acid) herbicide and glufosinate-ammonium (2-amino-4-(hydroxymethylphosphinyl) butanoic acid, monoammonium salt) herbicide. The formulation used in the treatments described in this report was MON 54140, which is the Monsanto designation for the Clarity®[6] formulation containing the diglycolamine salt of dicamba.

The 2010 field study was conducted at thirteen field sites across eight states in the U.S. including Arkansas (Region 4 – 1 trial), California (Region 10 – 2 trials), Georgia (Region 2 – 1 trial), Louisiana (Region 4 – 1 trial), Missouri (Region 4 – 1 trial), Oklahoma (Region 8 – 2 trials), South Carolina (Region 2 – 1 trial), and Texas (Region 6 – 1 trial and Region 8 – 3 trials). The sites were typical of the major cotton producing regions of the United States. Control and treated plots were established at each site. The treated plots were 800 to 10,000 square feet in size (planted area). The minimum distance between the control plot and any treated plot was 60 feet. The minimum distance between the treated plots was 30 feet. The maximum labeled rate and timing for applications of dicamba to MON 88701 in the United States are summarised in Table 23. Treatments 3 and 4 represent the two treatment options expected to provide the maximum residues under the proposed label.

[6] Clarity® is a registered trademark of BASF (US registered product, EPA Reg. No. 7969-137). Clarity® is not registered in Australia by the APVMA.

Table 23. Applications of Dicamba to MON 88701

Treatment	Application Rate lb/a (kg/ha)				
	Pre-Emergence	6 Leaf	1st White Flower + 15 Days	First Open Boll	7 Days Preharvest
1	—	—	—	—	—
3	1.0 lb/a (1.12 kg/ha)	—	—	0.50 lb/a (0.56 kg/ha)	0.50 lb/a (0.56 kg/ha)
4	—	0.50 lb/a (0.56kg/ha)	0.50 lb/a (0.56 kg/ha)	0.50 lb/a (0.56 kg/ha)	0.50 lb/a (0.56 kg/ha)

*Treatment #2 and 5 were removed from the dossier and the report. Treatment 2 was removed because the residue level is expected to be lower than those from treatments #3 and 4. Treatment 5 was removed because a formulation which will not be registered was used in treatment.

Dicamba, formulated as MON 54140 (Clarity®) was applied at a total rate of 2.0 lb a.e./a (2.24 kg a.e./ha) in sequential broadcast applications. The target spray volume for all applications was 20 ± 1 gal/a (190 L/ha). Spray solutions for all applications also contained a non-ionic surfactant (80% minimum active) at a target concentration of 0.125 liters per 100 liters of spray solution. Additionally, ammonium sulfate was added to all spray solutions at a rate of 2 kg per 100 liters of spray solution.

Residue analysis quantified DCSA and DCGA along with dicamba and 5-hydroxydicamba using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Analytes were then corrected for matrix effects using ^{13}C-labelled internal standards.

The dicamba analytical method was validated down to a lower limit of method validation (LLMV) of 0.02 ppm for dicamba, 5-hydroxydicamba, DCSA and DCGA in cotton undelinted seed.

Table 24 summarises the median and range of residues in seed for each analyte and the total residue for cottonseed as per the proposed definition of residue for dicamba in MON 88701 (dicamba, DCSA and 5-hydroxydicamba). Individual residues are expressed as the concentration of the analyte *per se*. Individual analyte values below the LLMV are listed as <0.02, and are included in the total as 0.02. Total residues are expressed as dicamba acid equivalents, and include the analytes in the proposed residue definition: dicamba, 5-hydroxydicamba, and DCSA. None of the residues are corrected for background or recovery.

Treated MON 88701 seedcotton for processing was obtained from Treatment 4 plots treated at 2 lb a.e./a (2.24 kg a.e./ha). Both control and treated seed were collected at two locations, and were processed using small-scale commercial-type equipment. The residues in the meal were compared to the residues in the undelinted cottonseed prior to processing to determine a concentration factor and are shown in Table 25.

Please also refer to [redacted] 2012 (MSL0024066).

Table 24. Summary of Dicamba Residues in MON 88701 Seed

Analyte	Treatment 3, ppm			Treatment 4, ppm		
	Mean	Median[a]	Range[b]	Mean	Median[a]	Range[b]
DCGA	0.03	0.02	<0.02-0.09	0.05	0.03	0.02-0.14
DCSA	0.05	0.03	<0.02-0.16	0.08	0.06	0.02-0.27
Dicamba	0.64	0.65	0.06-1.38	0.61	0.47	0.12-1.42
5-Hydroxy-dicamba	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Total[c,d]	0.72	0.71	0.10-1.57	0.71	0.54	0.17-1.72

[a] Median of residues across all sites in this treatment.
[b] Range of residues (minimum/maximum) across all sites.
[c] Individual analyte residues are expressed as each analyte *per se.*
[d] Total residues are expressed as dicamba acid equivalents. Total residue (ppm) = [Dicamba] + ([5-hydroxydicamba] x 0.933) + ([DCSA] x 1.068)

Table 25. Summary of Cotton Undelinted Seed Concentration Factors in Treatment 4 Processed Fractions

Process Fraction	Analyte/Residues (ppm)[1]					
	DCGA	DCSA	Dicamba	5-Hydroxy-dicamba	Total[2,3]	Conc. Factor[4]
Undelinted Seed	0.04	0.04	0.65	<0.02	0.71	-
Meal	<0.02	<0.02	0.11	<0.02	0.15	0.22

[1] Average of individual residues in undelinted cottonseed processed fraction sample analyses across two sites.
[2] Total Residue (ppm) = [Dicamba] + ([5-hydroxydicamba] x 0.933) + ([DCSA] x 1.068) as per proposed residue definition.
[3] Total residues are expressed as dicamba equivalents. Individual analytes are expressed as each analyte per se. Note: The lower limit of method validation of dicamba, 5-hydroxydicamba, DCSA and DCGA in cotton processed fractions is 0.02 ppm. Values below 0.02 are shown as <0.02 and included in the totals as 0.02.
[4] Concentration factor based on total residues calculated as proposed definition of residue.

B7 Compositional Assessment

Compositional analyses comparing MON 88701 treated with dicamba and glufosinate herbicides to the conventional control variety (Coker 130) and commercial reference varieties demonstrated that MON 88701 is compositionally equivalent to conventional cotton. Samples of acid-delinted cottonseed were collected from MON 88701 and the conventional control grown in a 2010 U.S. field production. Nine unique conventional cotton varieties, known as reference substances, were included across all sites of the field production with four varieties per site to provide data on natural variability of each compositional component analyzed. The field production was conducted at eight sites: Arkansas (ARTI), Georgia (GACH), Kansas (KSLA), Louisiana (LACH), North Carolina (NCBD), New Mexico (NMLC), South Carolina (SCEK) and, Texas (TXPL). The sites were planted in a randomised complete block design with four blocks per site. All cotton plants including MON 88701, the conventional control, and the reference varieties were grown under normal agronomic field conditions for their respective geographic regions, including maintenance pesticides as needed. In addition, MON 88701 plots were treated at the 3-5 leaf stage with glufosinate herbicide at the label rate (0.5 lb a.i. /acre) and at the 6-10 leaf stage with dicamba herbicide at the proposed label rate (0.5 lb a.e. /acre).

Compositional analyses were conducted to assess whether levels of key nutrients and anti-nutrients in MON 88701 were equivalent to levels in the conventional control and also comparable to the composition of conventional reference varieties. A description of nutrients and anti-nutrients present in cotton is provided in the OECD consensus document on compositional considerations for cottonseed (OECD, 2009). Nutrients assessed in this analysis included proximates (ash, calories and carbohydrates by calculation, fat, moisture, and protein), acid detergent fiber (ADF), neutral detergent fiber (NDF), crude fiber (CF), total dietary fiber (TDF), amino acids (AA, 18 components), fatty acids (FA, C8-C22), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium and zinc) and vitamin E. The anti-nutrients assessed in this analysis included gossypol and cyclopropenoid fatty acids (dihydrosterculic, malvalic and sterculic).

In all, 65 different analytical components were measured. Due to statistical constraints, in order to proceed with the statistical analysis of any component in this study, at least 50% of the observed values for that analyte needed to be greater than the assay limit of quantitation (LOQ). Of the 65 components measured, 13 had more than 50% of the observations below the assay LOQ and were excluded from statistical analysis. 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 88701, nine sets of statistical comparisons to the conventional control were conducted. One comparison was based on compositional data combined across all eight field sites (the combined-site analysis) and eight separate comparisons to the control were conducted on data from each of the eight individual field sites. Statistically significant differences were identified at a 5% level of significance ($p < 0.05$). Compositional data from the reference substances, grown concurrently in the same trial as MON 88701 and the

conventional control Coker 130, were combined across all sites and used to calculate a 99% tolerance interval for each component to define the natural variability in cotton varieties that have a history of safe consumption.

For the combined-site analysis, statistically significant differences ($p < 0.05$) in nutrient and anti-nutrient components were evaluated further using considerations relevant to the safety and nutritional quality of MON 88701 when compared to the conventional control. The evaluation included: 1) the relative magnitude of the significant difference in the mean values of nutrient and anti-nutrient components of MON 88701 compared to the conventional control; 2) whether the MON 88701 component mean value is within the range of natural variability of that component as represented by the 99% tolerance interval of conventional reference varieties grown concurrently in the same trial; 3) analyses of the reproducibility of the significant combined-site component differences at individual sites; and 4) assessing the combined-site statistically significant differences and reproducible individual site significant differences within the context of natural variability of commercial cottonseed composition published in the scientific literature and/or in the International Life Sciences Institute Crop Composition Database (Table 29)(ILSI, 2011).

This analysis provides a comprehensive comparative assessment of the levels of key nutrients and anti-nutrients in cottonseed of MON 88701 and the conventional control discussed in the context of natural variability in composition of commercial cotton. Results of the comparison indicate that the composition of the cottonseed of MON 88701 is equivalent to that of conventional cotton.

Please also refer to [redacted] 2012 (MSL0024606).

B7(a) Levels of key nutrients, toxicants and anti-nutrients

Nutrient Levels in Cottonseed

In the combined-site analysis of nutrient levels in cottonseed, the following components had no statistically significant differences ($p < 0.05$) in mean values between MON 88701 and the control: one proximate (protein), one type of fiber (crude fiber), 15 amino acids (alanine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, serine, threonine, tryptophan, tyrosine and valine), seven fatty acids (16:0 palmitic acid, 16:1 palmitoleic acid, 18:0 stearic acid, 18:1 oleic acid, 18:3 linolenic acid, 20:0 arachidic acid and 22:0 behenic acid), and four minerals (copper, iron, phosphorus and sodium) (Table 27).

The components that had significant differences in mean values between MON 88701 and the conventional control in the combined-site analysis were: five proximates (ash, calories, carbohydrates, moisture and total fat), three types of fiber (ADF, NDF and TDF), three amino acids (arginine, methionine and proline), two fatty acids (14:0 myristic acid and 18:2 linoleic acid), five minerals (calcium, magnesium, manganese, potassium and zinc) and vitamin E (Table 26).

The statistical significant differences in nutrients were further evaluated using the four previously described considerations relevant to the safety and nutritional quality of MON 88701 when compared to the conventional control:

All nutrient component differences observed in the combined-site statistical analysis, whether reflecting increased or decreased MON 88701 mean values with respect to the conventional control, were 14.09% or less. The relative magnitudes of the differences were : 0.66 to 5.00% for proximates, 4.08 to 5.72% for fibers, 2.61 to 4.82% for amino acids, 0.69 to 2.69% for fatty acids, 4.94 to 14.09% for minerals and 6.70% for vitamin E.

With the exception of methionine, mean values for all significantly different nutrient components from the combined-site analysis of MON 88701 were within the 99% tolerance interval established from the conventional commercial reference varieties grown concurrently in the same trial.

Assessment of the reproducibility of the combined-site differences at the eight individual sites showed significant differences for: NDF, methionine, proline and 18:2 linoleic acid at one site; carbohydrates, total fat, ADF, manganese and zinc at two sites; TDF, arginine, 14:0 myristic acid, potassium, and vitamin E at three sites; magnesium at four sites, ash at six sites and calcium at seven sites. Moisture and calories were not affected at any site. With the exception of methionine, arginine and zinc, all individual site mean values of MON 88701 for all nutrient components with significant differences were within the 99% tolerance interval established from the conventional commercial reference varieties grown concurrently in the same trial.

All combined-site mean values and individual mean values of MON 88701 for all nutrient components, including those that were significantly different, were within the context of the

natural variability of commercial cotton composition as published in the scientific literature and/or available in the ILSI Crop Composition Database (ILSI, 2011).

Five of the 19 cottonseed nutrient statistically significant differences between MON 88701 and the conventional control that were observed in the combined-site data analysis were attributable to small differences in proximates (ash, carbohydrates, total fat expressed as % dw, calories expressed as kcal/ 100g % dw, and moisture expressed as % fw). For ash, calories and total fat the relative magnitude of the differences between the mean value for MON 88701 and the conventional control were all small increases (5.00% for ash, 0.66% for calories, and 3.71% for total fat). The differences for carbohydrates and moisture between the mean value for MON 88701 and the conventional control were both small decreases (2.60% for carbohydrates and 4.51% for moisture). All of the nutrient mean values for MON 88701 observed in the combined-site analysis for proximates were within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial. Significant differences for most proximate mean values between MON 88701 and the conventional control were not consistently observed among individual sites. Total fat was increased at two sites ranging from 6.74 to 8.46% and carbohydrates were decreased at two sites, with decreases ranging from 4.33 to 5.08%. There were no significant differences at any of the individual sites for calories or moisture. Although ash was increased in MON 88701 when compared to the conventional control at six sites, increases ranged from 4.95 to 11.50%, which was less than the variability for the control samples (range 3.46 to 4.29, a relative difference of 24.0%, Table 26). Overall, observed differences in proximate values between MON 88701 and the conventional control were not considered to be meaningful from a food and feed safety or nutritional perspective because the magnitudes of combined-site differences were 5.00% or less, most were not consistently reproduced across the individual sites, and the mean MON 88701 combined-site values were within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial, and were within the context of the natural variability of commercial cotton composition as published in the scientific literature and/or available in the ILSI Crop Composition Database (ILSI, 2011).

Three of the 19 cottonseed nutrient statistically significant differences between MON 88701 and the conventional control observed in the combined-site data analysis were attributable to small differences in fiber (ADF, NDF and TDF all expressed as % dw). All relative magnitudes of the differences for fiber between the mean values for MON 88701 and the conventional control were small decreases (4.94% for ADF, 5.72% for NDF and 4.08% for TDF). All of the nutrient mean values for MON 88701 observed in the combined-site analysis for fiber were within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial. Significant differences for fiber mean values between MON 88701 and the conventional control were not consistently observed among individual sites. TDF and ADF were decreased at three and two sites, respectively, with decreases ranging from 4.55 to 8.15% for TDF and 9.27 to 9.86% for ADF. NDF was significantly different at one site with a small decrease of 7.40%. Overall, observed differences in fiber values between MON 88701 and the conventional

control were not considered to be meaningful from a food and feed safety or nutritional perspective because they were small in magnitude, not consistently reproduced across the individual sites, and the mean MON 88701 combined-site values were within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial, and were within the context of the natural variability of commercial cotton composition as published in the scientific literature and/or available in the ILSI Crop Composition Database (ILSI, 2011).

Three other combined-site nutrient statistically significant differences between MON 88701 and the conventional control observed in the combined-site analysis were attributed to small differences in amino acids (arginine, methionine and proline expressed as % dw). For both arginine and proline, the relative magnitude of the differences between the mean values for MON 88701 and the conventional control were small decreases (3.80% for arginine and 2.61% for proline). Methionine was increased 4.82% when MON 88701 was compared to the conventional control. With the exception of methionine, the nutrient mean values for MON 88701 observed in the combined-site analysis for amino acids were within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial. The combined-site difference value for methionine was within the context of natural variation of methionine found in commercial cotton as published in the scientific literature or as found in the ILSI Crop Composition Database (ILSI, 2011). Significant differences for amino acid mean values between MON 88701 and the conventional control were not consistently observed at all eight individual sites. Arginine and proline were decreased at three sites and one site, respectively, with decreases ranging from 6.10 to 8.35% for arginine and 6.16% for proline. Methionine was increased 12.03% at only one site. Overall, observed differences in amino acid values between MON 88701 and the conventional control were not considered to be meaningful from a food and feed safety or nutritional perspective because they were small in magnitude, not consistently reproduced across the individual sites, and with the exception of methionine, the mean MON 88701 combined-site values were within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial. In addition, all MON 88701 amino acid values were within the context of the natural variability of commercial cotton composition as published in the scientific literature or available in the ILSI Crop Composition Database (ILSI, 2011).

Two of the combined-site nutrient statistically significant differences between MON 88701 and the conventional control were attributed to the fatty acids 14:0 myristic acid and 18:2 linoleic acid (expressed as % total FA). The relative magnitudes of the differences between the mean fatty acid values for MON 88701 and the conventional control in the combined-site analysis were small decreases (2.69% for 14:0 myristic acid and 0.69% for 18:2 linoleic acid). The nutrient mean values for MON 88701 observed in the combined-site analysis for both 14:0 myristic acid and 18:2 linoleic acid were within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial. Significant differences for fatty acid mean values between MON 88701 and the conventional control were not consistently observed among individual sites. 14:0 myristic

acid was decreased at three sites, while 18:2 linoleic acid was decreased at one site with differences ranging from 4.43 to 8.36% for 14:0 myristic acid and 1.93% for 18:2 linoleic acid. Overall, observed differences in fatty acid values between MON 88701 and the conventional control were not considered to be meaningful from a food and feed safety and nutritional perspective because they were small, not consistently reproduced across the individual sites, and the mean MON 88701 values were within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial and were within the context of the natural variability of commercial cotton composition as published in the scientific literature and/or available in the ILSI Crop Composition Database (ILSI, 2011).

Five of the 19 cottonseed nutrient statistically significant differences between MON 88701 and the conventional control observed in the combined-site analysis were attributed to small differences in minerals (calcium, magnesium, and potassium expressed as % dw and manganese and zinc expressed as mg/kg dw). For calcium, magnesium, potassium and manganese the relative magnitudes of the differences between the mean values for MON 88701 and the conventional control were increases of (14.09% for calcium, 5.63% for magnesium, 9.20% for manganese, and 4.94% for potassium). The relative magnitude of the difference for zinc between the mean value for MON 88701 and the conventional control was a decrease of 6.39%. All of the nutrient mean values for MON 88701 observed in the combined-site analysis for minerals were within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial. Significant differences for mineral mean values between MON 88701 and the conventional control were not consistently observed among individual sites. Although calcium was significantly different at seven sites, with increases ranging from 6.92 to 22.70%, this was less than the variability observed for the control samples (range 0.091 to 0.18, a relative difference of 97.8%, Table 26).

Magnesium, potassium, and manganese were significantly different at four, three and two sites, respectively with increases ranging from 5.54 to 9.36% for magnesium, 8.01 to 16.37% for potassium and from 16.52 to 20.59% for manganese. Zinc was significantly different at two sites with decreases ranging from 7.68 to 17.66%. Overall, observed differences in mineral values between MON 88701 and the conventional control were not considered to be meaningful from a food and feed safety or nutritional perspective because they were small in magnitude, not consistently reproduced across the individual sites (with the exception of calcium), and the mean MON 88701 combined-site values were within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial and within the context of the natural variability of commercial cotton composition as published in the scientific literature and/or available in the ILSI Crop Composition Database (ILSI, 2011).

The last nutrient statistically significant difference observed in the combined-site analysis between MON 88701 and the conventional control was attributed to vitamin E (expressed as mg/kg dw). The relative magnitude of the difference between the mean vitamin E value for MON 88701 and the conventional control in the combined-site analysis was a small increase

of 6.70%. The nutrient mean value for MON 88701 observed in the combined-site analysis for vitamin E was within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial. Significant differences for vitamin E mean values between MON 88701 and the conventional control were not consistently observed among individual sites, with significant increases ranging from 7.78 to 13.28% observed at three sites. Overall, the observed differences in the vitamin E values between MON 88701 and the conventional control in the combined-site analysis were not considered to be meaningful from a food and feed safety and nutritional perspective because they were 13.28% or less, not consistently reproduced across the individual sites, and the mean MON 88701 values were within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial and were within the context of the natural variability of commercial cotton composition as published in the scientific literature and/or available in the ILSI Crop Composition Database (ILSI, 2011).

In summary, statistical analyses found no consistent differences between the levels of nutrient components in cottonseed from MON 88701 and the conventional control. Differences were observed for calcium and ash in combined analyses and most individual sites, but the magnitudes of differences for these nutrients were less than the variability for the control samples, and values were within the range of natural variability for cottonseed. These findings support the conclusion of compositional equivalence of MON 88701 to conventional cotton.

Anti-Nutrient Levels in Cottonseed

Cottonseed was analysed for five anti-nutrients and in the combined-site analysis of anti-nutrient levels in cottonseed no statistically significant differences ($p < 0.05$) in mean values between MON 88701 and the conventional control: for two cyclopropenoid fatty acids (malvalic and sterculic) (Table 28).

The components that showed statistically significant differences in mean values between MON 88701 and the conventional control in the combined-site analysis were the cyclopropenoid fatty acid dihydrosterculic, free gossypol, and total gossypol (Table 26).

The statistically significant differences in anti-nutrients were further evaluated using the four previously described considerations relevant to the safety and nutritional quality of MON 88701 when compared to the conventional control.

All anti-nutrient component differences observed in the combined-site statistical analysis, which reflected an increase in MON 88701 mean values with respect to the conventional control, were small in magnitude. The relative magnitude of the differences for dihydrosterculic acid, free gossypol and total gossypol were 9.59%, 6.23% and 6.75%, respectively.

Mean values for all significantly different anti-nutrient components from the combined-site analysis of MON 88701 were within the 99% tolerance interval established from the conventional commercial reference varieties grown concurrently in the same trial.

Assessment of the reproducibility of the combined-site differences at the eight individual sites showed significant differences for: dihydrosterculic at one site; free gossypol at two sites; and total gossypol at three sites. All individual site mean values of MON 88701 for all anti-nutrient components with significant differences were within the 99% tolerance interval established from the conventional commercial reference varieties grown concurrently in the same trial.

All combined-site mean values of MON 88701 for all anti-nutrient components including those that were significantly different were within the context of the natural variability of commercial cotton composition as published in the scientific literature and/or available in the ILSI Crop Composition Database (ILSI, 2011).

The three cottonseed anti-nutrient statistically significant differences between MON 88701 and the conventional control observed in the combined-site data analysis were attributed to small differences in one cyclopropenoid fatty acid (dihydrosterculic expressed as % total fatty acid), free gossypol and total gossypol (expressed as % dw). For dihydrosterculic acid, free gossypol and total gossypol, the relative magnitude of the differences between the mean values for MON 88701 and the conventional control were increases of 9.59% for dihydrosterculic acid, 6.23% for free gossypol and 6.75% for total gossypol. These anti-nutrient differences between MON 88701 and the conventional control observed in the combined-site analysis were within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial. Significant differences for the three anti-nutrient mean values between MON 88701 and the conventional control

were not consistently observed across all eight individual sites. Dihydrosterculic acid, free gossypol, and total gossypol were significantly different at one, two and three sites respectively, with an increases of 28.35% for dihydrosterculic acid, and ranging from 12.69 to 22.32% for free gossypol and 9.54 to 15.53% for total gossypol. Overall, observed differences in anti-nutrient values between MON 88701 and the conventional control were not considered to be meaningful from a food and feed safety or nutritional perspective because they were generally small, not consistently reproduced across the individual sites, and the mean MON 88701 values were within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial and within the context of the natural variability of commercial cotton composition as published in the scientific literature and/or available in the ILSI Crop Composition Database (ILSI, 2011).

In summary, statistical analyses found no consistent statistically significant differences between the levels of anti-nutrient components in cottonseed from MON 88701 and the conventional control and mean values for anti-nutrients were within the natural variability found for cottonseed. These findings supported the conclusion of compositional equivalence of MON 88701 to conventional cotton.

Table 26. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control) Mean Difference (% of Control)	Mean Difference (MON 88701 minus Control) Significance (p-Value)	MON 88701 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in Combined-Site Analysis						
Cottonseed Proximate (% dw)						
Ash	4.31	4.11	5.00	0.001	3.77 - 4.74	3.42, 4.65
Calories (Kcal/ 100g)	498.50	495.24	0.66	0.013	482.46 - 517.46	457.61, 527.56
Carbohydrates	44.64	45.83	-2.60	<0.001	41.40 - 48.89	40.26, 56.45
Moisture (% fw)	7.15	7.48	-4.51	0.005	5.93 - 9.67	4.79, 9.92
Total Fat	23.14	22.31	3.71	0.001	19.79 - 26.78	15.01, 28.51
Cottonseed Fibre (% dw)						
Acid Detergent Fibre	25.27	26.58	-4.94	0.002	23.26 - 27.74	22.24, 31.96
Neutral Detergent Fibre	30.73	32.59	-5.72	<0.001	25.13 - 34.42	27.03, 42.49

Table 26. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control)		MON 88701 Range	Commercial Tolerance Interval[5]
			Mean Difference (% of Control)	Significance (p-Value)		
Statistical Differences Observed in Combined-Site Analysis						
Cottonseed Fibre (% dw)						
Total Dietary Fibre	39.44	41.12	-4.08	<0.001	36.91 - 42.13	34.52, 52.58
Cottonseed Amino Acid (% dw)						
Arginine	3.03	3.15	-3.80	0.002	2.33 - 3.60	2.38, 3.47
Methionine	0.40	0.38	4.82	0.026	0.35 - 0.46	0.32, 0.38
Proline	1.00	1.03	-2.61	0.037	0.82 - 1.21	0.83, 1.08
Cottonseed Fatty Acid (% Total FA)						
14:0 Myristic	0.77	0.79	-2.69	0.009	0.66 - 0.95	0.16, 1.37
18:2 Linoleic	55.77	56.15	-0.69	0.026	54.24 - 58.22	47.49, 63.18

Table 26. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control)		MON 88701 Range	Commercial Tolerance Interval[5]
			Mean Difference (% of Control)	Significance (p-Value)		
Statistical Differences Observed in Combined-Site Analysis						
Cottonseed Mineral						
Calcium (% dw)	0.15	0.13	14.09	<0.001	0.10 - 0.22	0.058, 0.21
Magnesium (% dw)	0.40	0.38	5.63	<0.001	0.35 - 0.44	0.28, 0.47
Manganese (mg/kg dw)	12.81	11.73	9.20	0.001	10.18 - 14.81	9.07, 17.33
Potassium (% dw)	1.12	1.07	4.94	0.021	0.98 - 1.24	0.92, 1.21
Zinc (mg/kg dw)	37.58	40.14	-6.39	0.005	27.31 - 46.74	27.27, 44.95
Cottonseed Vitamin (mg/kg dw)						
Vitamin E	140.14	131.33	6.70	<0.001	86.23 - 179.34	41.91, 205.89
Cottonseed Cyclopropenoid Fatty Acid (% Total FA)						
Dihydrosterculic Acid	0.15	0.14	9.59	0.003	0.11 - 0.19	0.078, 0.25

Table 26. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control)		MON 88701 Range	Commercial Tolerance Interval[5]
			Mean Difference (% of Control)	Significance (p-Value)		
Statistical Differences Observed in Combined-Site Analysis						
Cottonseed Gossypol (% dw)						
Free Gossypol	0.94	0.89	6.23	0.016	0.80 - 1.18	0.099, 1.57
Total Gossypol	1.04	0.97	6.75	<0.001	0.84 - 1.24	0.064, 1.76
Statistical Differences Observed in More than One Individual Site						
Cottonseed Mineral - 7 Sites						
Calcium (% dw) Site ARTI	0.15	0.12	22.70	0.010	0.14 - 0.16	0.058, 0.21
Calcium (% dw) Site GACH	0.13	0.11	17.57	<0.001	0.13 - 0.13	0.058, 0.21
Calcium (% dw) Site KSLA	0.20	0.18	14.74	0.007	0.19 - 0.22	0.058, 0.21
Calcium (% dw) Site NCBD	0.15	0.14	6.92	0.007	0.14 - 0.15	0.058, 0.21
Calcium (% dw) Site NMLC	0.15	0.13	16.83	0.003	0.14 - 0.15	0.058, 0.21

Table 26. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control)		MON 88701 Range	Commercial Tolerance Interval[5]
			Mean Difference (% of Control)	Significance (p-Value)		
Statistical Differences Observed in More than One Individual Site						
Cottonseed Mineral - 7 Sites						
Calcium (% dw) Site SCEK	0.11	0.091	17.98	0.027	0.10 - 0.11	0.058, 0.21
Calcium (% dw) Site TXPL	0.16	0.14	15.31	<0.001	0.16 - 0.16	0.058, 0.21
Cottonseed Proximate (% dw) - 6 Sites						
Ash Site GACH	4.53	4.21	7.56	<0.001	4.45 - 4.57	3.42, 4.65
Ash Site KSLA	4.53	4.29	5.64	0.027	4.25 - 4.66	3.42, 4.65
Ash Site LACH	4.35	4.12	5.56	0.013	4.23 - 4.47	3.42, 4.65
Ash Site NCBD	4.34	4.14	4.95	0.033	4.29 - 4.40	3.42, 4.65
Ash Site SCEK	4.11	3.74	9.95	0.010	3.99 - 4.28	3.42, 4.65

Table 26. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control)		MON 88701 Range	Commercial Tolerance Interval[5]
			Mean Difference (% of Control)	Significance (p-Value)		
Statistical Differences Observed in More than One Individual Site						
Cottonseed Proximate (% dw) - 6 Sites						
Ash Site TXPL	3.85	3.46	11.50	0.001	3.77 - 3.92	3.42, 4.65
Cottonseed Fatty Acid (% Total FA) - 5 Sites						
18:0 Stearic Site ARTI	2.68	2.51	6.70	0.019	2.65 - 2.72	1.98, 2.95
18:0 Stearic Site LACH	2.68	2.52	6.04	0.001	2.64 - 2.73	1.98, 2.95
18:0 Stearic Site NCBD	2.50	2.34	6.85	0.036	2.39 - 2.64	1.98, 2.95
18:0 Stearic Site NMLC	2.51	2.64	-5.13	<0.001	2.47 - 2.56	1.98, 2.95
18:0 Stearic Site TXPL	2.35	2.46	-4.67	0.006	2.30 - 2.43	1.98, 2.95
Cottonseed Mineral - 4 Sites						
Magnesium (% dw) Site GACH	0.41	0.38	6.92	<0.001	0.40 - 0.41	0.28, 0.47

Table 26. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control)		MON 88701 Range	Commercial Tolerance Interval[5]
			Mean Difference (% of Control)	Significance (p-Value)		
Statistical Differences Observed in More than One Individual Site						
Cottonseed Mineral - 4 Sites						
Magnesium (% dw) Site KSLA	0.43	0.40	6.85	0.002	0.41 - 0.43	0.28, 0.47
Magnesium (% dw) Site SCEK	0.39	0.36	9.36	0.005	0.37 - 0.41	0.28, 0.47
Magnesium (% dw) Site TXPL	0.35	0.34	5.54	0.003	0.35 - 0.37	0.28, 0.47
Cottonseed Fiber (% dw) - 3 Sites						
Total Dietary Fiber Site KSLA	38.32	40.14	-4.55	0.034	37.62 - 38.75	34.52, 52.58
Total Dietary Fiber Site LACH	39.82	43.35	-8.15	0.002	39.02 - 40.86	34.52, 52.58
Total Dietary Fiber Site NMLC	39.16	41.10	-4.73	0.016	37.46 - 40.44	34.52, 52.58
Cottonseed Amino Acid (% dw) - 3 Sites						
Arginine Site GACH	2.95	3.21	-8.35	0.008	2.87 - 3.02	2.38, 3.47

Table 26. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control) Mean Difference (% of Control)	Mean Difference (MON 88701 minus Control) Significance (p-Value)	MON 88701 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in More than One Individual Site						
Cottonseed Amino Acid (% dw) - 3 Sites						
Arginine Site KSLA	3.02	3.28	-7.87	0.013	2.95 - 3.10	2.38, 3.47
Arginine Site NMLC	3.48	3.71	-6.10	0.005	3.42 - 3.60	2.38, 3.47
Cottonseed Fatty Acid (% Total FA) - 3 Sites						
14:0 Myristic Site KSLA	0.68	0.72	-5.33	0.007	0.66 - 0.71	0.16, 1.37
14:0 Myristic Site NCBD	0.68	0.75	-8.36	0.002	0.66 - 0.70	0.16, 1.37
14:0 Myristic Site NMLC	0.93	0.98	-4.43	0.001	0.92 - 0.95	0.16, 1.37
Cottonseed Mineral - 3 Sites						
Potassium (% dw) Site GACH	1.21	1.12	8.01	<0.001	1.17 - 1.24	0.92, 1.21
Potassium (% dw) Site SCEK	1.13	1.02	10.88	0.042	1.11 - 1.17	0.92, 1.21

Table 26. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control)		MON 88701 Range	Commercial Tolerance Interval[5]
			Mean Difference (% of Control)	Significance (p-Value)		
Statistical Differences Observed in More than One Individual Site						
Cottonseed Mineral - 3 Sites						
Potassium (% dw) Site TXPL	1.01	0.87	16.37	0.004	0.98 - 1.06	0.92, 1.21
Cottonseed Vitamin (mg/kg dw) - 3 Sites						
Vitamin E Site GACH	151.03	140.12	7.78	0.025	148.34 - 154.95	41.91, 205.89
Vitamin E Site LACH	169.88	149.96	13.28	0.001	163.34 - 175.33	41.91, 205.89
Vitamin E Site TXPL	114.39	103.66	10.35	0.033	107.81 - 118.39	41.91, 205.89
Cottonseed Gossypol (% dw) - 3 Sites						
Total Gossypol Site KSLA	1.13	1.01	12.00	0.049	1.00 - 1.24	0.064, 1.76
Total Gossypol Site NMLC	0.92	0.80	15.53	0.026	0.84 - 0.97	0.064, 1.76
Total Gossypol Site SCEK	1.17	1.07	9.54	0.017	1.13 - 1.23	0.064, 1.76

Table 26. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control)		MON 88701 Range	Commercial Tolerance Interval[5]
			Mean Difference (% of Control)	Significance (p-Value)		
Statistical Differences Observed in More than One Individual Site						
Cottonseed Proximate (% dw) - 2 Sites						
Carbohydrates Site SCEK	46.56	48.67	-4.33	0.031	45.10 - 47.48	40.26, 56.45
Carbohydrates Site TXPL	44.03	46.39	-5.08	0.010	42.73 - 45.99	40.26, 56.45
Total Fat Site NCBD	23.04	21.59	6.74	0.024	21.89 - 23.76	15.01, 28.51
Total Fat Site SCEK	25.65	23.65	8.46	0.019	24.23 - 26.78	15.01, 28.51
Cottonseed Fibre (% dw) - 2 Sites						
Acid Detergent Fibre Site ARTI	24.81	27.53	-9.86	0.007	24.44 - 25.20	22.24, 31.96
Acid Detergent Fibre Site LACH	25.72	28.35	-9.27	0.005	24.16 - 27.08	22.24, 31.96
Cottonseed Amino Acid (% dw) - 2 Sites						
Phenylalanine Site GACH	1.40	1.49	-5.89	0.039	1.37 - 1.43	1.12, 1.58

Table 26. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control)		MON 88701 Range	Commercial Tolerance Interval[5]
			Mean Difference (% of Control)	Significance (p-Value)		
Statistical Differences Observed in More than One Individual Site						
Cottonseed Amino Acid (% dw) - 2 Sites						
Phenylalanine Site KSLA	1.44	1.53	-5.88	0.025	1.40 - 1.46	1.12, 1.58
Cottonseed Fatty Acid (% Total FA) - 2 Sites						
16:0 Palmitic Site LACH	24.48	24.04	1.81	0.014	24.37 - 24.55	16.54, 30.55
16:0 Palmitic Site SCEK	24.74	24.39	1.43	0.029	24.59 - 24.94	16.54, 30.55
16:1 Palmitoleic Site NCBD	0.46	0.48	-3.88	0.019	0.44 - 0.47	0.39, 0.70
16:1 Palmitoleic Site NMLC	0.53	0.54	-2.27	0.014	0.52 - 0.53	0.39, 0.70
18:3 Linolenic Site ARTI	0.14	0.13	11.92	0.012	0.14 - 0.15	0.060, 0.24
18:3 Linolenic Site NMLC	0.16	0.14	8.12	0.009	0.15 - 0.16	0.060, 0.24

Table 26. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control)		MON 88701 Range	Commercial Tolerance Interval[5]
			Mean Difference (% of Control)	Significance (p-Value)		
Statistical Differences Observed in More than One Individual Site						
Cottonseed Mineral - 2 Sites						
Iron (mg/kg dw) Site NCBD	43.21	48.04	-10.05	0.025	41.96 - 44.44	47.30, 97.12
Iron (mg/kg dw) Site TXPL	60.47	79.02	-23.47	0.039	56.94 - 66.50	47.30, 97.12
Manganese (mg/kg dw) Site GACH	13.41	11.51	16.52	0.003	12.79 - 14.14	9.07, 17.33
Manganese (mg/kg dw) Site TXPL	10.91	9.04	20.59	0.007	10.18 - 11.37	9.07, 17.33
Zinc (mg/kg dw) Site NCBD	40.79	49.54	-17.66	0.006	40.28 - 41.37	27.27, 44.95
Zinc (mg/kg dw) Site NMLC	45.63	49.43	-7.68	0.009	44.12 - 46.74	27.27, 44.95
Cottonseed Gossypol (% dw) - 2 Sites						
Free Gossypol Site KSLA	1.07	0.95	12.69	0.014	1.03 - 1.10	0.099, 1.57

Table 26. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control)		MON 88701 Range	Commercial Tolerance Interval[5]
			Mean Difference (% of Control)	Significance (p-Value)		
Statistical Differences Observed in More than One Individual Site						
Cottonseed Gossypol (% dw) - 2 Sites						
Free Gossypol Site NMLC	0.85	0.69	22.32	0.011	0.83 - 0.88	0.099, 1.57
Statistical Differences Observed in One Site						
Cottonseed Proximate (% dw)						
Protein Site TXPL	29.43	28.48	3.33	0.017	29.06 - 30.14	22.30, 29.41
Cottonseed Fibre (% dw)						
Crude Fiber Site KSLA	16.43	17.67	-7.04	0.019	16.06 - 17.24	16.93, 22.68
Neutral Detergent Fibre Site TXPL	29.75	32.12	-7.40	0.006	28.74 - 30.56	27.03, 42.49
Cottonseed Amino Acid (% dw)						
Alanine Site LACH	1.07	1.03	3.73	0.030	1.00 - 1.11	0.86, 1.11
Aspartic Acid Site GACH	2.31	2.45	-6.03	0.019	2.24 - 2.36	1.94, 2.57

Table 26. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control)		MON 88701 Range	Commercial Tolerance Interval[5]
			Mean Difference (% of Control)	Significance (p-Value)		
Statistical Differences Observed in One Site						
Cottonseed Amino Acid (% dw)						
Glutamic Acid Site GACH	4.57	4.96	-7.95	0.010	4.35 - 4.77	3.74, 5.28
Isoleucine Site GACH	0.90	0.94	-4.21	0.034	0.90 - 0.91	0.75, 0.96
Leucine Site GACH	1.51	1.58	-4.32	0.024	1.49 - 1.54	1.25, 1.62
Lysine Site LACH	1.26	1.18	7.01	0.023	1.17 - 1.31	1.01, 1.30
Methionine Site LACH	0.42	0.38	12.03	0.013	0.37 - 0.44	0.32, 0.38
Proline Site GACH	0.98	1.05	-6.16	0.033	0.97 - 0.99	0.83, 1.08
Threonine Site GACH	0.85	0.90	-5.14	0.049	0.83 - 0.88	0.72, 0.89
Tryptophan Site SCEK	0.35	0.38	-6.70	0.023	0.33 - 0.38	0.34, 0.42

Table 26. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control)		MON 88701 Range	Commercial Tolerance Interval[5]
			Mean Difference (% of Control)	Significance (p-Value)		
Statistical Differences Observed in One Site						
Cottonseed Amino Acid (% dw)						
Tyrosine Site GACH	0.80	0.84	-4.30	0.037	0.79 - 0.82	0.67, 0.84
Valine Site GACH	1.21	1.26	-4.19	0.017	1.19 - 1.23	1.00, 1.28
Cottonseed Fatty Acid (% Total FA)						
18:1 Oleic Site LACH	14.70	14.29	2.89	0.021	14.48 - 15.01	11.38, 20.64
18:2 Linoleic Site LACH	55.53	56.63	-1.93	0.001	55.15 - 55.99	47.49, 63.18
20:0 Arachidic Site LACH	0.31	0.29	6.78	0.033	0.31 - 0.32	0.17, 0.38
22:0 Behenic Site ARTI	0.14	0.15	-9.92	0.008	0.13 - 0.14	0.070, 0.21
Cottonseed Mineral						
Sodium (% dw) Site KSLA	0.022	0.0080	178.30	0.020	0.019 - 0.025	0, 0.066

Table 26. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control)		MON 88701 Range	Commercial Tolerance Interval[5]
			Mean Difference (% of Control)	Significance (p-Value)		
Statistical Differences Observed in One Site						
Cottonseed Cyclopropenoid Fatty Acid (% Total FA)						
Dihydrosterculic Acid Site GACH	0.15	0.12	28.35	0.022	0.14 - 0.16	0.078, 0.25

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2]MON 88701 was sprayed with dicamba and glufosinate.
[3]Mean = least-square mean.
[4]Control refers to the non-biotechnology derived, conventional control (Coker 130).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

Table 27. Statistical Summary of Combined-Site Cottonseed Nutrients for MON 88701 vs. Conventional Control

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control)			Commercial Tolerance Interval[5] (Range)
			Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	
Proximate (% dw)						
Ash	4.31 (0.11) (3.77 - 4.74)	4.11 (0.11) (3.34 - 5.00)	0.21 (0.052) (-0.49 - 0.61)	0.094, 0.32	0.001	3.42, 4.65 (3.18 - 4.68)
Calories (Kcal/ 100g)	498.50 (1.65) (482.46 - 517.46)	495.24 (1.71) (487.70 - 512.65)	3.26 (1.29) (-14.30 - 18.37)	0.70, 5.82	0.013	457.61, 527.56 (466.09 - 509.91)
Carbohydrates	44.64 (0.56) (41.40 - 48.89)	45.83 (0.57) (42.14 - 50.30)	-1.19 (0.32) (-5.19 - 2.45)	-1.82, -0.56	<0.001	40.26, 56.45 (43.28 - 54.90)
Moisture (% fw)	7.15 (0.26) (5.93 - 9.67)	7.48 (0.27) (6.15 - 9.19)	-0.34 (0.11) (-1.82 - 0.79)	-0.56, -0.11	0.005	4.79, 9.92 (6.05 - 10.50)
Protein	27.91 (0.77) (22.71 - 31.47)	27.79 (0.77) (23.53 - 31.27)	0.13 (0.31) (-1.99 - 3.73)	-0.53, 0.78	0.685	22.30, 29.41 (20.58 - 29.28)
Total Fat	23.14 (0.31) (19.79 - 26.78)	22.31 (0.33) (20.71 - 25.20)	0.83 (0.26) (-2.89 - 3.86)	0.32, 1.34	0.001	15.01, 28.51 (16.58 - 25.25)

Table 27. Statistical Summary of Combined-Site Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control)			Commercial Tolerance Interval[5] (Range)
			Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	
Fibre (% dw)						
Acid Detergent Fibre	25.27 (0.34) (23.26 - 27.74)	26.58 (0.35) (22.08 - 29.58)	-1.31 (0.35) (-5.42 - 1.77)	-2.06, -0.57	0.002	22.24, 31.96 (23.42 - 31.62)
Crude Fibre	18.17 (0.37) (15.97 - 21.66)	18.54 (0.38) (16.06 - 21.70)	-0.38 (0.32) (-3.36 - 4.75)	-1.02, 0.27	0.246	16.93, 22.68 (16.92 - 23.32)
Neutral Detergent Fibre	30.73 (0.51) (25.13 - 34.42)	32.59 (0.53) (28.87 - 35.89)	-1.86 (0.41) (-6.95 - 1.16)	-2.68, -1.05	<0.001	27.03, 42.49 (29.27 - 40.63)
Total Dietary Fibre	39.44 (0.39) (36.91 - 42.13)	41.12 (0.41) (39.05 - 44.37)	-1.68 (0.36) (-5.34 - 1.09)	-2.45, -0.91	<0.001	34.52, 52.58 (37.29 - 48.60)
Amino Acid (% dw)						
Alanine	1.06 (0.020) (0.91 - 1.14)	1.05 (0.020) (0.88 - 1.17)	0.0026 (0.0091) (-0.13 - 0.12)	-0.017, 0.022	0.775	0.86, 1.11 (0.83 - 1.22)
Arginine	3.03 (0.10) (2.33 - 3.60)	3.15 (0.10) (2.41 - 3.77)	-0.12 (0.033) (-0.47 - 0.39)	-0.19, -0.049	0.002	2.38, 3.47 (2.30 - 3.55)

Table 27. Statistical Summary of Combined-Site Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control)			Commercial Tolerance Interval[5] (Range)
			Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	
Amino Acid (% dw)						
Aspartic Acid	2.39 (0.062) (1.94 - 2.64)	2.41 (0.062) (1.92 - 2.74)	-0.015 (0.027) (-0.29 - 0.29)	-0.072, 0.042	0.575	1.94, 2.57 (1.79 - 2.72)
Cystine	0.41 (0.0091) (0.32 - 0.47)	0.40 (0.0094) (0.31 - 0.46)	0.0096 (0.0070) (-0.063 - 0.082)	-0.0043, 0.023	0.174	0.31, 0.45 (0.29 - 0.47)
Glutamic Acid	4.76 (0.13) (3.80 - 5.38)	4.84 (0.14) (3.66 - 5.70)	-0.079 (0.072) (-0.78 - 0.79)	-0.23, 0.077	0.295	3.74, 5.28 (3.39 - 5.45)
Glycine	1.10 (0.020) (0.93 - 1.19)	1.09 (0.020) (0.91 - 1.20)	0.0014 (0.011) (-0.13 - 0.14)	-0.021, 0.024	0.896	0.90, 1.14 (0.85 - 1.23)
Histidine	0.74 (0.019) (0.58 - 0.85)	0.75 (0.019) (0.61 - 0.84)	-0.0014 (0.0073) (-0.062 - 0.091)	-0.017, 0.014	0.854	0.59, 0.81 (0.57 - 0.84)
Isoleucine	0.91 (0.018) (0.75 - 1.01)	0.92 (0.018) (0.77 - 1.03)	-0.0066 (0.0079) (-0.077 - 0.096)	-0.023, 0.010	0.421	0.75, 0.96 (0.72 - 1.03)

Table 27. Statistical Summary of Combined-Site Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control)			Commercial Tolerance Interval[5] (Range)
			Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	
Amino Acid (% dw)						
Leucine	1.53 (0.032) (1.29 - 1.70)	1.54 (0.032) (1.28 - 1.69)	-0.0018 (0.013) (-0.14 - 0.16)	-0.029, 0.026	0.892	1.25, 1.62 (1.20 - 1.72)
Lysine	1.24 (0.025) (1.05 - 1.38)	1.23 (0.025) (1.06 - 1.39)	0.0069 (0.015) (-0.11 - 0.15)	-0.026, 0.039	0.658	1.01, 1.30 (0.99 - 1.44)
Methionine	0.40 (0.0079) (0.35 - 0.46)	0.38 (0.0084) (0.32 - 0.46)	0.018 (0.0081) (-0.066 - 0.12)	0.0023, 0.035	0.026	0.32, 0.38 (0.29 - 0.49)
Phenylalanine	1.43 (0.039) (1.14 - 1.66)	1.46 (0.039) (1.15 - 1.66)	-0.022 (0.014) (-0.18 - 0.19)	-0.052, 0.0084	0.144	1.12, 1.58 (1.10 - 1.63)
Proline	1.00 (0.029) (0.82 - 1.21)	1.03 (0.029) (0.81 - 1.25)	-0.027 (0.012) (-0.12 - 0.10)	-0.052, -0.0018	0.037	0.83, 1.08 (0.79 - 1.17)
Serine	1.08 (0.025) (0.90 - 1.23)	1.09 (0.026) (0.86 - 1.24)	-0.0036 (0.015) (-0.18 - 0.16)	-0.035, 0.028	0.807	0.83, 1.21 (0.81 - 1.24)

Table 27. Statistical Summary of Combined-Site Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control)			Commercial Tolerance Interval[5] (Range)
			Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	
Amino Acid (% dw)						
Threonine	0.87 (0.016) (0.74 - 0.94)	0.86 (0.016) (0.73 - 0.95)	0.0057 (0.0083) (-0.10 - 0.10)	-0.012, 0.023	0.504	0.72, 0.89 (0.67 - 0.96)
Tryptophan	0.41 (0.0092) (0.33 - 0.52)	0.42 (0.0095) (0.37 - 0.52)	-0.0061 (0.0066) (-0.081 - 0.078)	-0.019, 0.0071	0.361	0.34, 0.42 (0.31 - 0.46)
Tyrosine	0.81 (0.017) (0.67 - 0.92)	0.81 (0.018) (0.67 - 0.91)	-0.0011 (0.0083) (-0.074 - 0.12)	-0.019, 0.017	0.898	0.67, 0.84 (0.63 - 0.91)
Valine	1.21 (0.027) (1.00 - 1.40)	1.23 (0.027) (1.00 - 1.40)	-0.012 (0.011) (-0.090 - 0.12)	-0.036, 0.012	0.296	1.00, 1.28 (0.97 - 1.36)
Fatty Acid (% Total FA)						
14:0 Myristic	0.77 (0.030) (0.66 - 0.95)	0.79 (0.031) (0.71 - 0.98)	-0.021 (0.0071) (-0.077 - 0.047)	-0.036, -0.0060	0.009	0.16, 1.37 (0.45 - 1.04)
16:0 Palmitic	23.95 (0.30) (22.34 - 25.28)	23.80 (0.30) (22.69 - 25.05)	0.15 (0.076) (-0.68 - 0.76)	-0.016, 0.31	0.073	16.54, 30.55 (19.11 - 26.73)

Table 27. Statistical Summary of Combined-Site Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control)			Commercial Tolerance Interval[5] (Range)
			Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	
Fatty Acid (% Total FA)						
16:1 Palmitoleic	0.50 (0.0094) (0.44 - 0.54)	0.50 (0.0094) (0.45 - 0.54)	0.0022 (0.0038) (-0.025 - 0.039)	-0.0060, 0.010	0.572	0.39, 0.70 (0.44 - 0.67)
18:0 Stearic	2.54 (0.058) (2.29 - 2.85)	2.47 (0.058) (2.15 - 2.76)	0.068 (0.036) (-0.16 - 0.24)	-0.0091, 0.14	0.079	1.98, 2.95 (1.98 - 2.97)
18:1 Oleic	15.10 (0.26) (14.15 - 16.45)	14.96 (0.26) (14.06 - 16.44)	0.14 (0.070) (-0.48 - 0.75)	-0.0049, 0.29	0.057	11.38, 20.64 (13.71 - 18.39)
18:2 Linoleic	55.77 (0.39) (54.24 - 58.22)	56.15 (0.40) (54.04 - 57.93)	-0.39 (0.16) (-1.42 - 0.80)	-0.72, -0.053	0.026	47.49, 63.18 (49.78 - 59.61)
18:3 Linolenic	0.18 (0.022) (0.14 - 0.34)	0.17 (0.022) (0.12 - 0.30)	0.011 (0.0068) (-0.0073 - 0.052)	-0.0038, 0.025	0.136	0.060, 0.24 (0.10 - 0.29)
20:0 Arachidic	0.29 (0.0086) (0.23 - 0.32)	0.28 (0.0087) (0.23 - 0.32)	0.0044 (0.0047) (-0.027 - 0.046)	-0.0057, 0.015	0.364	0.17, 0.38 (0.20 - 0.36)

Table 27. Statistical Summary of Combined-Site Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control)			Commercial Tolerance Interval[5] (Range)
			Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	
Fatty Acid (% Total FA)						
22:0 Behenic	0.15 (0.0051) (0.12 - 0.19)	0.15 (0.0051) (0.13 - 0.21)	-0.0035 (0.0029) (-0.049 - 0.032)	-0.0098, 0.0029	0.260	0.070, 0.21 (0.051 - 0.19)
Mineral						
Calcium (% dw)	0.15 (0.0093) (0.10 - 0.22)	0.13 (0.0093) (0.081 - 0.19)	0.018 (0.0022) (-0.012 - 0.038)	0.013, 0.023	<0.001	0.058, 0.21 (0.081 - 0.18)
Copper (mg/kg dw)	8.90 (0.70) (5.22 - 11.91)	8.93 (0.70) (5.40 - 11.92)	-0.025 (0.16) (-2.59 - 1.29)	-0.34, 0.29	0.875	2.97, 12.86 (4.46 - 11.62)
Iron (mg/kg dw)	67.21 (4.40) (41.96 - 83.17)	71.33 (4.48) (45.03 - 95.10)	-4.12 (2.74) (-38.15 - 12.79)	-9.96, 1.71	0.153	47.30, 97.12 (39.49 - 114.34)
Magnesium (% dw)	0.40 (0.0083) (0.35 - 0.44)	0.38 (0.0084) (0.33 - 0.44)	0.021 (0.0032) (-0.036 - 0.054)	0.015, 0.028	<0.001	0.28, 0.47 (0.31 - 0.46)
Manganese (mg/kg dw)	12.81 (0.47) (10.18 - 14.81)	11.73 (0.48) (8.61 - 14.11)	1.08 (0.28) (-1.95 - 2.54)	0.48, 1.68	0.001	9.07, 17.33 (9.07 - 17.14)

Table 27. Statistical Summary of Combined-Site Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control)			Commercial Tolerance Interval[5] (Range)
			Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	
Mineral						
Phosphorus (% dw)	0.72 (0.031) (0.56 - 0.84)	0.72 (0.031) (0.54 - 0.87)	0.0081 (0.0067) (-0.087 - 0.11)	-0.0053, 0.021	0.230	0.49, 0.87 (0.48 - 0.87)
Potassium (% dw)	1.12 (0.028) (0.98 - 1.24)	1.07 (0.028) (0.79 - 1.27)	0.053 (0.020) (-0.12 - 0.27)	0.0089, 0.097	0.021	0.92, 1.21 (0.90 - 1.26)
Sodium (% dw)	0.034 (0.0095) (0.018 - 0.12)	0.029 (0.0096) (0.0053 - 0.10)	0.0045 (0.0046) (-0.065 - 0.030)	-0.0053, 0.014	0.346	0, 0.066 (0.0054 - 0.077)
Zinc (mg/kg dw)	37.58 (2.01) (27.31 - 46.74)	40.14 (2.02) (28.22 - 52.95)	-2.57 (0.77) (-11.57 - 3.27)	-4.22, -0.91	0.005	27.27, 44.95 (25.07 - 48.49)
Vitamin (mg/kg dw)						
Vitamin E	140.14 (9.87) (86.23 - 179.34)	131.33 (9.88) (91.78 - 162.98)	8.80 (2.07) (-6.54 - 26.36)	4.39, 13.22	<0.001	41.91, 205.89 (84.07 - 162.76)

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2]MON 88701 was sprayed with dicamba and glufosinate.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the non-biotechnology derived, conventional control (Coker 130).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

Table 28. Statistical Summary of Combined-Site Cottonseed Anti-nutrients for MON 88701 vs. Conventional Control

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control)			Commercial Tolerance Interval[5] (Range)
			Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	
Cyclopropenoid Fatty Acid (% Total FA)						
Dihydrosterculic Acid	0.15 (0.0034) (0.11 - 0.19)	0.14 (0.0037) (0.11 - 0.17)	0.013 (0.0044) (-0.026 - 0.068)	0.0044, 0.022	0.003	0.078, 0.25 (0.038 - 0.23)
Malvalic Acid	0.39 (0.015) (0.20 - 0.55)	0.37 (0.016) (0.26 - 0.49)	0.013 (0.015) (-0.16 - 0.16)	-0.016, 0.043	0.371	0.23, 0.54 (0.11 - 0.59)
Sterculic Acid	0.22 (0.0067) (0.13 - 0.29)	0.21 (0.0072) (0.17 - 0.27)	0.0067 (0.0081) (-0.085 - 0.078)	-0.0096, 0.023	0.412	0.17, 0.27 (0.061 - 0.34)
Gossypol (% dw)						
Free Gossypol	0.94 (0.037) (0.80 - 1.18)	0.89 (0.037) (0.68 - 1.20)	0.055 (0.020) (-0.086 - 0.20)	0.012, 0.099	0.016	0.099, 1.57 (0.50 - 1.41)
Total Gossypol	1.04 (0.037) (0.84 - 1.24)	0.97 (0.037) (0.74 - 1.10)	0.066 (0.017) (-0.021 - 0.23)	0.031, 0.10	<0.001	0.064, 1.76 (0.56 - 1.61)

[1]dw = dry weight; FA = fatty acid.
[2]MON 88701 was sprayed with dicamba and glufosinate.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the non-biotechnology derived, conventional control (Coker 130).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

Table 29. Literature and ILSI Ranges for Components in Cottonseed

Cottonseed Tissue Components[1]	Literature Range[2]	ILSI Range[3]
Cottonseed Nutrients		
Proximates (% dw)		
Ash	3.87 – 5.29[a]; 3.7 – 4.2[d]	3.761 – 5.342
Carbohydrates by calculation	45.28 – 53.62[a]	39.0 – 53.6
Calories by calculation (Kcal/100g)	471.34 – 506.95[a]	
Moisture (% fw)	2.25 – 7.49[a]	2.3 – 9.9
Protein	24.54 – 30.83[a]; 21.2 – 25.9[b]	21.48 – 32.97
Total Fat	17.37 – 25.16[a]; 14.4 – 16.9[d]	17.201 – 27.292
Fiber (% dw)		
Acid Detergent Fiber	21.10 – 34.8[a]; 37.6 – 40.5[d]	19.74 – 38.95
Neutral Detergent Fiber	32.92 – 45.83[a]; 50.0 – 53.6[d]	25.56 – 51.87
Crude Fiber	13.85 – 17.94[a]	13.86 – 23.10
Total Dietary Fiber	not available	33.69 – 47.55
Amino Acids	**(% total AA)**	**(% dw)**
Alanine	4.16 – 4.41[a]; 3.6 – 4.2[b]	0.80 – 1.22
Arginine	11.28 – 12.51[a]; 10.9 – 12.3[b]	2.06 – 3.72
Aspartic acid	9.73 – 9.99[a]; 8.8 – 9.5[b]	1.82 – 2.94
Cystine/Cysteine	1.60 – 1.92[a]; 2.3 – 3.4[b]	0.35 – 0.56
Glutamic acid	20.76 – 21.61[a]; 20.5 – 22.4[b]	3.91 – 6.72
Glycine	4.44 – 4.58[a]; 3.8 – 4.5[b]	0.83 – 1.32
Histidine	3.00 – 3.12[a]; 2.6 – 2.8[b]	0.57 – 0.91
Isoleucine	3.10 – 3.67[a]; 3.0 – 3.4[b]	0.62 – 1.05
Leucine	6.27 – 6.65[a]; 5.5 – 6.1[b]	1.14 – 1.86
Lysine	4.85 – 5.37[a]; 4.2 – 4.6[b]	0.94 – 1.46
Methionine	1.46 – 1.88[a]; 1.3 – 1.8[b]	0.30 – 0.47
Phenylalanine	5.56 – 5.77[a]; 5.0 – 5.6[b]	1.02 – 1.72
Proline	4.06 – 4.28[a]; 3.1 – 4.0[b]	0.75 – 1.23
Serine	4.45 – 4.86[a]; 3.9 – 4.4[b]	0.91 – 1.35
Threonine	3.26 – 3.59[a]; 2.8 – 3.2[b]	0.55 – 0.92
Tryptophan	0.97 – 1.21[a]; 1.0 – 1.4[b]	0.194 – 0.319
Tyrosine	2.65 – 2.92[a]; 2.8 – 3.3[b]	0.53 – 0.84
Valine	4.76 – 5.14[a]; 4.3 – 4.7[b]	0.87 – 1.49
Fatty Acids (% total FA)		
8:0 Caprylic	not available	not available
10:0 Capric	not available	not available
12:0 Lauric	not available	not available
14:0 Myristic	0.55 – 2.40[a]; 0.6 – 1.5[b]	0.455 – 2.400
14:1 Myristoleic	not available	not available
15:0 Pentadecanoic	0.050 – 0.17[a]	0.103 – 0.481
15:1 Pentadecenoic	not available	not available
16:0 Palmitic	21.23 – 27.9[a]; 17.6 – 24.8[b]	15.11 – 27.90
16:1 Palmitoleic	0.55 – 1.16[a]	0.464 – 1.190
17:0 Heptadecanoic	not available	0.092 – 0.119

Table 29. Literature and ILSI Ranges for Components in Cottonseed (continued)

Cottonseed Tissue Components[1]	Literature Range[2]	ILSI Range[3]
17:1 Heptadecenoic	not available	not available
18:0 Stearic	1.99 – 3.11 [a]; 2.0 – 2.5 [b]	0.20 – 3.11
18:1 Oleic	13.90 – 20.10 [a]; 15.0 – 20.7 [b]	12.8 – 25.3
18:2 Linoleic	46.00 – 56.88 [a]	46.0 – 59.4
18:3 Gamma Linolenic	0.050 – 0.25 [a]	0.097 – 0.232
18:3 Linolenic	0.050 – 0.25 [a]	0.11 – 0.35
20:0 Arachidic	0.25 – 0.33 [a]	0.186 – 0.414
20:1 Eicosenoic	not available	0.095 – 0.098
20:2 Eicosadienoic	not available	not available
20:3 Eicosatrienoic	not available	not available
20:4 Arachidonic	not available	not available
22:0 Behenic	0.13 – 0.17 [a]	0.104 – 0.295
Vitamins	**(mg/kg fw)**	**(mg/kg dw)**
Vitamin E	99 – 224[c]	70.825 – 197.243
Minerals (% dw)		
Calcium	0.10 – 0.33[a]	0.10323 – 0.32581
Copper (mg/kg dw)	3.54 – 11.14[a]	3.13 – 24.57
Iron (mg/kg dw)	40.58 – 56.54 [a]	36.71 – 318.38
Magnesium	0.37 – 0.46 [a]	0.34709 – 0.49312
Manganese (mg/kg dw)	11.06 – 18.31 [a]	10.69 – 21.96
Phosphorus	0.60 – 0.84 [a]	0.48254 – 0.99157
Potassium	0.98 – 1.24 [a]	0.98345 – 1.44835
Sodium	0.0054 – 0.74 [a]	0.01118 – 0.73548
Zinc (mg/kg dw)	30.21 – 47.75 [a]	27.0 – 59.5
Cottonseed Anti-Nutrients		
Gossypol, Total (% dw)	0.57 – 1.42[a]; 0.55 – 0.77[d]	0.547 – 1.522
Gossypol, Free (% dw)	0.53 – 1.20[a]	0.454 – 1.399
Cyclopropenoid Fatty Acids (% total FA)		
Dihydrosterculic	0.13 – 0.24 [a]	0.075 – 0.310
Malvalic	0.33 – 0.58 [a]	0.229 – 0.759
Sterculic	0.21 – 0.56 [a]	0.190 – 0.556

[1]fw=fresh weight; dw=dry weight
[2]Literature range references; [a](Hamilton et al., 2004); [b](Lawhon et al., 1977); [c](Smith and Creelman, 2001); [d](Bertrand et al., 2005).
[3](ILSI, 2011).

Compositional Assessment of MON 88701: Summary and Conclusion

Detailed comparisons were conducted on nutrient and anti-nutrient levels in MON 88701 cottonseed and compared to levels in the conventional control. The analytes evaluated are consistent with those identified by the OECD as important to understanding the safety and nutrition of new varieties of biotechnology-derived cotton (OECD, 2009). These compositional comparisons were made by analyzing the acid-delinted cottonseed harvested from plants grown at each of eight field sites in the U.S. during the 2010 field season. Composition analysis of all samples was conducted in accordance with OECD guidelines and included analysis for nutrients including proximates (ash, calories and carbohydrates by calculation, fat, moisture, and protein), ADF, NDF, CF, TDF, amino acids, fatty acids (C8-C22), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc), and vitamin E. The anti-nutrients assessed in this analysis included total and free gossypol and cyclopropenoid fatty acids (dihydrosterculic, malvalic and sterculic). These analyses also included measurements of the same nutrients and anti-nutrients in conventional cotton varieties, known as reference substances, to provide data on natural variability of each compositional component analysed. All cotton plants including MON 88701, the conventional control, and the commercial reference varieties were treated with maintenance pesticides as necessary throughout the growing season. In addition, MON 88701 plots were treated at the 3-5 leaf stage with glufosinate herbicide at the label rate (0.5 lb a.i. /acre) and at the 6-10 leaf stage with dicamba herbicide at the proposed label rate (0.5 lb a.e. /acre).

For MON 88701 compared to the conventional control, the combined-site analysis of cottonseed showed no statistically significant differences ($p < 0.05$) between nutrient and anti-nutrient components of MON 88701 and the control for 30 (57.7%) of the 52 mean value comparisons. Combined-site statistical differences for nutrients in cottonseed included mean values for five proximates (ash, calories, carbohydrates, moisture and total fat), three types of fiber (ADF, NDF and TDF), three amino acids (arginine, methionine and proline), two fatty acids (14:0 myristic acid and 18:2 linoleic acid), five minerals (calcium, magnesium, manganese, potassium and zinc), and vitamin E. Combined-site statistical differences for anti-nutrients in cottonseed included mean values for dihydrosterculic acid, free gossypol and total gossypol. All nutrient and anti-nutrient component statistical differences observed in the combined-site statistical analysis, whether reflecting increased or decreased MON 88701 mean values with respect to the conventional control, were 14.09% or less. Mean values for all significantly different nutrient and anti-nutrient components from the combined-site analysis of MON 88701, with the exception of methionine, were within the 99% tolerance interval established from the conventional commercial reference varieties grown concurrently in the same trial. All combined-site mean values including methionine and individual site mean values of MON 88701 for all nutrient and anti-nutrient components were within the context of the natural variability of commercial cotton composition as published in the scientific literature and/or available in the ILSI Crop Composition Database (Table 29)(ILSI, 2011).

For MON 88701 compared to the conventional control, most of the combined-site differences were not reproducible among the individual sites with the exception of ash and calcium; however, all of the combined-site component values were within the range of values reported in the scientific literature and/or in the ILSI Crop Composition Database. Based on the results of this composition analysis it is concluded that cottonseed from MON 88701 is compositionally equivalent to conventional cotton and therefore the food and feed safety and nutritional quality of this product is comparable to that of the conventional cotton.

Conventional cotton processing is described in this document in Section A2(b). The processing of MON 88701 cotton is not expected to be any different from that of conventional cotton. As described in this section, detailed compositional analyses of key components of MON 88701 have been performed and have demonstrated that MON 88701 is compositionally equivalent to conventional cotton. Additionally, the modes-of-action of MON 88701 DMO and PAT (*bar*) proteins, as described in Section B3(a), is well understood, and there is no reason to expect interactions with endogenous toxicants or important nutrients that may be present in cotton. Therefore, when MON 88701 and its progeny are 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 cotton.

B7(b) Levels of other GM-influenced constituents

Not applicable.

B7(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 88712 as described in section B5(a).

C2 Data from an animal feeding study, if available

The data and information presented in this submission demonstrate that the food and feed derived from MON 88701 are as safe and nutritious as those derived from commercially-available, conventional cotton for which there is an established history of safe consumption. Therefore, animal feeding studies do not add value to the safety of MON 88701.

Part 3 STATUTORY DECLARATION – AUSTRALIA

Statutory Declarations Act 1959

I, [redacted] Monsanto Australia Limited, make the following declaration under the *Statutory Declarations Act 1959:*

1. The information provided in this application fully sets out the matters required
2. The information provided in this application is true to the best of my knowledge and belief
3. No information has been withheld that might prejudice this application, to the best of my knowledge and belief

I understand that a person who intentionally makes a false statement in a statutory declaration is guilty of an offence under section 11 of the *Statutory Declarations Act 1959*, and I believe that the statements in this declaration are true in every particular.

Signature: ..... [redacted] .....

Declared at Monsanto Australia, Level 12 / 600 St Kilda Road, Melbourne VIC 3004 on ..... 16 ..... of January 2013.

Declared before me .. [redacted] .....

... [redacted] .....

..... [redacted] .....

..... [redacted] .....

[Full name, qualification and address of person before whom the declaration is made]

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