Application to Food Standards Australia New Zealand for the Inclusion of Soybean MON 87708 in Standard 1.5.2 - Food Derived from Gene Technology

Submitted by:

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ABBREVIATIONS AND DEFINTITIONS[1]

~	Approximately
AD_2010	Allergen and gliadin protein sequence database (Release date January 22, 2010)
APHIS	Animal and Plant Health Inspection Service of the United States Department of Agriculture
APVMA	Australian Pesticides and Veterinary Medicines Authority
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
CFR	Code of Federal Regulations
CHT	ceramic hydroxyapatite column
CV	column volume
Da	Dalton
DAP	days after planting
DCGA	2,5-dichloro-3,6-dihydroxybenzoic acid also known as 3,6-dichlorogentisic acid
DCSA	3,6-dichlorosalicylic acid also known as 3,6-dichloro-2-hydroxybenzoic acid
DDI	daily dietary intake
DEEM	dietary exposure evaluation model
Dicamba	3,6-dichloro-2-methoxybenzoic acid
dmo	Coding sequence of the dicamba mono-oxygenase gene from *Stenotrophomonas maltophilia* encoding DMO
DoR	Definition of the Residue
DTT	dithiothreitol
DWCF	dry weight conversion factor
dwt	dry weight of tissue
E. coli	*Escherichia coli*
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
FARRP	Food Allergy Research and Resource Program Database
FASTA	algorithm used to find local high scoring alignments between a pair of protein or nucleotide sequences
FFDCA	Federal Food, Drug and Cosmetic Act
FSANZ	Food Standards Australia New Zealand
fwt	fresh weight of tissue
GenBank	A public genetic database maintained by the National Center for Biotechnology Information at the National Institutes of Health, Bethesda, MD, USA
GI	Gene Identification number
HMGA	3-hydroxy-3-methylglutaric acid
HRP	horseradish peroxidase
IAA	indole acetic acid
IgE	Immunoglobulin E
ILSI	International Life Sciences Institute
kDa	kiloDalton
LB	loading buffer

[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.

LOD	limit of detection
LOQ	limit of quantitation
MALDI-TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MMT	million metric ton
MOE	margin of exposure
MON 87708 DMO	The active form of DMO in MON 87708, a trimer comprised of three monomers. The DMO trimer can be comprised of MON 87708 DMO protein, MON 87708 DMO+27 protein, or a combination of both proteins.
MON 87708 DMO precursor protein	The produced precursor protein from the coding sequence of *dmo* expression cassette, that consists of the MON 87708 DMO protein, a chloroplast transit peptide (CTP), containing the first 24 amino acids from the N-terminus of the mature Rubisco small subunit, and an intervening sequence (IS).
MON 87708 DMO protein	full-length dicamba mono-oxygenase protein
MON 87708 DMO+27 protein	full-length dicamba mono-oxygenase protein with an additional 24 amino acids from the Rubisco small subunit and 3 amino acids from an intervening sequence
MW	molecular weight
MWCO	molecular weight cut off
N/A	not applicable
NADH	nicotinamide adenine dinucleotide
NCBI	National Center of Biotechnology Information at the National Institutes of Health, Bethesda, MD, USA
NFDM	non-fat dried milk
NOAEL	no observable adverse effect level
OSL	over-season leaf
PBS	phosphate buffered saline solution
PBST	phosphate buffered saline solution containing 0.05% (v/v) Tween-20
pea	*Pisum sativum*
PMSF	phenylmethylsulfonyl fluoride
PRT_2010	GenBank protein database, 175.0 (Release date January 22, 2010)
PVDF	polyvinylidene difluoride
RT	room temperature
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
S. maltophilia	*Stenotrophomonas maltophilia*
soybean	Glycine max (L.) Merr.
T-DNA	transfer(ed) DNA
TBS	tris buffered saline
TFA	trifluoroacetic acid
Tm	melting temperature
TMB	3,3',5,5' tetramethyl-benzidine
TOX_2010	Toxin protein sequence database (Release date January 22, 2010)
TSSP	tissue-specific pool
v/v	volume per volume
w/v	weight per volume

Part 1 GENERAL INFORMATION

Applicant Details

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(f) Nature of applicant's business	Technology Provider to the Agricultural and Food Industries
(g) Details of other individuals, companies or organisations associated with the application	-

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 Dicamba tolerant soybean MON 87708 and products containing Dicamba tolerant soybean MON 87708 (hereafter referred to as MON 87708) to the Table to Clause 2 (see below).

Food derived from gene technology	Special requirements
Food derived from Soybean MON 87708	None

Relevant Overseas Approvals

Monsanto has submitted a food and feed safety and nutritional assessment summary for MON 87708 to the United States Food and Drug Administration (FDA) in November 2010 and has also requested a Determination of Nonregulated Status for MON 87708, including all progenies derived from crosses between MON 87708 and other soybean, from the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) in July 2010.

It has been also submitted to Canadian Food Inspection Agency (CFIA) and Health Canada (HC) in November 2010, the European Food Safety Authority (EFSA) in January 2011, Korea Food and Drug Administration (KFDA) for food use in Feburary 2011, and Rural Development Administration (RDA) for feed use in Feburary 2011, and Japan's Ministry of Health, Labour, and Welfare (MHLW) for food use in March 2011.

Regulatory submissions will be made to countries that import significant soybean or food and feed products derived from U.S. soybean 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 and the Ministry of Health, Labour, and Welfare; the Intersectoral Commission for Biosafety of Genetically Modified Organisms, Mexico; as well as to regulatory authorities in other soybean importing countries with functioning regulatory systems. As appropriate, notifications will be made to countries that import significant quantities of U.S. soybean and soybean products and do not have a formal regulatory review process for biotechnology-derived crops.

Furthermore, a regulatory submission has been made to the EPA requesting the establishment of the new, expanded use of dicamba on MON 87708. Dicamba residues on soybean seed (less than 0.07 ppm average residue and less than 0.5 ppm maximum residue) resulting from its application on MON 87708 at the maximum labeled use rate are well below the established 10 ppm soybean seed U.S. pesticide residue tolerance. Therefore, a change to the current U.S. soybean seed tolerance is not needed to support the use of dicamba on MON 87708 in the U.S. Monsanto has requested the establishment of new tolerances for soybean forage and hay, which will allow for the feeding of forage and hay to livestock, however no other revisions to dicamba pesticide residue tolerances are needed including animal products such as meat or milk.

Justificationfor the Application

(a) the need and/or advantages for the proposed change

MON 87708 offers growers an expanded use of dicamba in soybean production from the current preplant and preharvest labeled uses. The tolerance of MON 87708 to dicamba facilitates a wider window of application on soybean, allowing preemergence application of the herbicide up to the day of crop emergence and postemergence in-crop applications through the early reproductive (R1) growth stage. Dicamba provides effective control of over 95 annual and biennial weed species, and suppression of over 100 perennial broadleaf and woody plant species. Dicamba is efficacious on broadleaf weeds that are hard-to-control with glyphosate, such as common lambsquarters, hemp sesbania, morning glory species, nightshade, Pennsylvania smartweed, prickly sida, and wild buckwheat. Additionally, dicamba provides effective control of herbicide-resistant broadleaf weeds, including glyphosate-resistant weeds such as marestail, common ragweed, giant ragweed, palmer pigweed, and waterhemp.

(b) any public health and/or safety issues related to the proposed change e.g. details of target groups and at-risk population groups

Data and information submitted in this submission supports that MON 87708 is as safe and nutritious as conventional soybean. MON 87708 is primarily developed to give growers an expanded use of dicamba in soybean production to provide improved weed control in soybean described above, in order to meet the growing needs of the food, feed, and industrial markets.

(c) any nutrition issues related to the proposed change e.g. nutritional purpose of adding a nutritive substance to each type of food or composition

Not applicable.

(d) if for a food additive or processing aid, its technological function or need

Not applicable.

(e) potential impact on trade

Not applicable.

(f) any consumer choice issues related to the proposed change

Not applicable.

(g) any evidence that the food industry generally or other specific companies have an interest in, or support, the proposed change to the Code (this item is mandatory for applications relating to food additives, processing aids, nutritive substances, novel foods, irradiated foods)

MON 87708 offers growers an expanded use of dicamba in soybean production from the current preplant and preharvest labeled uses. Dicamba is efficacious on broadleaf weeds that are hard-to-control with glyphosate as well as many herbicide-resistant broadleaf weeds, including glyphosate-resistant weeds.

(h) the costs and benefits for industry, consumers and government associated with the proposed change, if available.

Please refer to (g) above.

Assessment Procedure

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

Part 2 SPECIFIC DATA REQUIREMENTS FOR SAFETY ASSESSMENT

A1. Technical information on the GM food

A1(a) A description of the new GM organism

Monsanto Company has developed biotechnology-derived soybean MON 87708 that is tolerant to dicamba (3,6-dichloro-2-methoxybenzoic acid) herbicide. MON 87708 offers growers an expanded use of dicamba in soybean production from the current preplant and preharvest labeled uses. The tolerance of MON 87708 to dicamba facilitates a wider window of application on soybean, allowing preemergence application of the herbicide up to the day of crop emergence and postemergence in-crop applications through the early reproductive (R1) growth stage. Dicamba provides effective control of over 95 annual and biennial weed species, and suppression of over 100 perennial broadleaf and woody plant species. Dicamba is efficacious on broadleaf weeds that are hard-to-control with glyphosate, such as common lambsquarters, hemp sesbania, morning glory species, nightshade, Pennsylvania smartweed, prickly sida, and wild buckwheat. Additionally, dicamba provides effective control of herbicide-resistant broadleaf weeds, including glyphosate-resistant weeds such as marestail, common ragweed, giant ragweed, palmer pigweed, and waterhemp.

MON 87708 will be combined with glyphosate-tolerant soybean MON 89788 (Roundup Ready 2 Yield® soybean) utilizing traditional breeding techniques. The potential use of dicamba or glyphosate herbicides, in addition to the other herbicide options currently labeled for use on soybean, enables an integrated weed management program to control a broad spectrum of grass and broadleaf weed species. Successful integration of MON 87708 into the Roundup Ready® soybean system will; 1) provide growers with an opportunity for an efficient, effective weed management system for control of glyphosate's hard-to-control and resistant broadleaf weeds; 2) provide an easy system for an additional in-crop herbicide mode-of-action in current soybean production practices as recommended by weed science experts to manage future weed resistance development; and 3) continue to provide soybean growers with effective weed control systems necessary for yields to meet the growing needs of the food, feed, and industrial markets.

MON 87708 soybean contains a gene derived from *Stenotrophomonas maltophilia (S. maltophilia)* that expresses a mono-oxygenase that rapidly demethylates dicamba to an inactive metabolite 3,6-dichlorosalicylic acid (DCSA), a well known metabolite of dicamba in soybean and livestock. MON 87708 contains a *dmo* expression cassette that produces a single MON 87708 DMO precursor protein that is post-translationally processed into two forms of the dicamba mono-oxygenase (DMO) protein; referred to as MON 87708 DMO protein and MON 87708 DMO+27 protein. The active form of these proteins, necessary to confer dicamba tolerance, is a trimer comprised of three DMO monomers. In MON 87708, the trimer can be comprised of MON 87708 DMO protein, MON 87708 DMO+27 protein, or a combination of both. Unless specified otherwise in this document, MON 87708 DMO will refer to both proteins and all forms of the trimer, collectively.

The data and information presented in this safety summary demonstrate that the food and feed derived from MON 87708 are as safe and nutritious as those derived from commercially-available, conventional soybean for which there is an established history of

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safe consumption. This safety assessment was conducted utilizing established methods for the evaluation of biotechnology-derived products as articulated in guidelines from the Codex Alimentarius Commission and the Organization for Economic Co-operation and Development (OECD). The food safety of MON 87708 was confirmed through multiple, well established lines of evidence:

1. The safety of the donor organism, *S. maltophilia*, based on its ubiquitous presence in the environment, presence in healthy individuals, and the incidental presence in foods without any adverse safety reports.
2. A detailed molecular characterization of the inserted DNA demonstrated a single, intact copy of the T-DNA stably inserted in a single locus within the soybean genome.
3. A history of safe use has been established for MON 87708 DMO. Data confirmed that MON 87708 DMO is unlikely to be a toxin or allergen based on extensive information collected. MON 87708 DMO was readily digestible in simulated gastric and simulated intestinal fluids, inactivated when exposed to heat, and showed no oral toxicity or cause any adverse effect in mice. Large margins of exposures (MOE) have been demonstrated for human and animal consumption of MON 87708 DMO derived from MON 87708.
4. A compositional assessment of seed and forage confirmed that MON 87708 is compositionally equivalent to conventional soybean.

All data strongly support the conclusion that food and feed derived from MON 87708 will be as safe and nutritious as food and feed derived from conventional soybean.

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" Dicamba tolerant soybean MON 87708 has been assigned the unique identifier MON-877Ø8-9.

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

A commercial trade name for the product has not been determined at the time of this submission and will be available prior to commercial launch of the product.

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

MON 87708 is intended primarily for use as a broad-acre commodity (or field) soybean and not for vegetable, garden, or food-grade soybean that are generally used to produce tofu, soybean sprouts, soymilk, green soybean (*e.g.*, edamame) or other similar food items. Vegetable and food-grade soybean generally have a different size, flavor, texture and other characteristics than field soybean, and are more easily cooked. Other than the introduction of the dicamba tolerance trait, MON 87708 is not materially different from conventional field soybeans and can be processed into a wide variety of food products as described in Section A2(b)(iii). Field soybean is a blended commodity that is highly processed before being consumed by humans. Thus, most food products derived from MON 87708 would likely be blended with those derived from other commercial soybean varieties before entering the human food supply.

A2 Description of donor and host organisms

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

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

The *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

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

The donor organism, *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, chicory, 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 cotton seed, 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 dishwashers, sponges, toothbrushes, flowers, plants, fruits, vegetables, frozen fish, milk, and poultry (Ryan et al., 2009). Strains of *S. maltophilia* have been found in the transient flora of hospitalized 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 colonization 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.

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

As described above, 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 (See Section A2(a)(ii)). 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).

A2(b) Description of the host organism

A2(b)(i) Phenotypic information

The soybean variety used as the recipient for the DNA insertion to create MON 87708 was A3525, a non-transgenic conventional variety developed by Asgrow Seed Company. A3525 is a mid−maturity group III soybean variety with very high yield potential. A3525 has superior yields relative to varieties of similar maturity and has excellent agronomic characteristics (Monsanto Technology, 2006).

Soybean variety A3525 is the near isogenic line to MON 87708 and was used as the conventional soybean comparator (hereafter referred to as the conventional control) in the safety assessment of MON 87708. MON 87708 and the conventional control have similar genetic backgrounds with the exception of the *dmo* expression cassette, thus, the effect of the *dmo* expression cassette and the expressed MON 87708 DMO could be assessed in an objective manner. The commercial reference varieties used at each location for this safety assessment were selected based on their availability and agronomic fit for the respective geographic region.

Cultivated soybean, *Glycine max* (L.) Merr. (*G. max)*, is a diploidized tetraploid (2n=40), which belongs to the family *Fabaceae*, the subfamily Papilionoideae, the tribe Phaseoleae, the genus *Glycine* Willd., and the subgenus *Soja* (Moench) F.J. Herm.

Family	: *Fabaceae*
Subfamily	: Papilionoideae
Tribe	: Phaseoleae
Genus	: *Glycine*
Subgenus	: *Soja* (Moench) F.J. Herm.
Species	: *Glycine max* (L.) Merr.

The genus *Glycine* Willd. is of Asian and Australian origin and is divided into two subgenera, *Glycine* and *Soja* (Moench) F.J. Herm. The subgenus *Glycine* consists of 22 wild perennial species, which are indigenous to Australia, west, central and south Pacific Islands, China, Russia, Japan, Indonesia, Korea, Papua New Guinea, the Philippines, and Taiwan (Hymowitz, 2004). The subgenus *Soja* includes the cultivated soybean, *G. max* and its wild annual relatives from Asia, *Glycine soja* Sieb. and Zucc (*G. soja*). The list of species in the genus *Glycine* Willd. is presented in Table 1.

Glycine soja grows wild in China, Japan, Korea, the Russian Far East, and Taiwan, and is commonly found in fields, hedgerows, roadsides, and riverbanks (Lu, 2004). The plant is an annual, slender in build with narrow trifoliolate leaves. The purple or very rarely white flowers are inserted on short, slender racemes. The pods are short and tawny with hirsute pubescence, producing oval-oblong seeds (Hymowitz, 2004).

Glycine max, the cultivated soybean, is an annual that generally exhibits an erect, sparsely branched, bush-type growth habit with trifoliolate leaves. The leaflets are broadly ovate, and the purple, pink, or white flowers are borne on short axillary racemes or reduced peduncles. The pods are either straight or slightly curved, and one to three ovoid to subspherical seeds are produced per pod (Hymowitz, 2004).

A third and unofficial species named *G. gracilis* is also described within the context of the *Soja* subgenus in addition to *G. soja* and *G. max*. The *G. gracilis* is only found in Northeast China, is somewhat intermediate in morphology between *G. max* and *G. soja*, and is sometimes considered a variant of *G. max*. The three species in the *Soja* subgenus can cross-pollinate, and the hybrid seed can germinate normally and subsequently produce fertile pollen and seed (Singh and Hymowitz, 1989). The taxonomic position of *G. gracilis* has been an area of debate, and neither ILDIS (International Legume Database and Information Service) nor USDA-GRIN (USDA Germplasm Resources Information Network) recognizes *G. gracilis* as a distinct species.

Table 1. List of Species in the Genus Glycine Willd., 2n Chromosome Number, Genome Symbol and Distribution

Genus	2n	Genome[1]	Distribution
Subgenus *Glycine*			
1. *G. albicans* Tind. & Craven	40	I1	Australia
2. *G. aphyonota* B. Pfeil	40	--[2]	Australia
3. *G. arenaria* Tind.	40	HH	Australia
4. *G. argyrea* Tind.	40	A2A2	Australia
5. *G. canescens* F.J. Herm.	40	AA	Australia
6. *G. clandestina* Wendl.	40	A1A1	Australia
7. *G. curvata* Tind.	40	C1C1	Australia
8. *G. cyrtoloba* Tind.	40	CC	Australia
9. *G. dolichocarpa* Tateishi and Ohashi	80	--	Taiwan
10. *G. falcate* Benth.	40	FF	Australia
11. *G. hirticaulis* Tind. & Craven	40	H1H1	Australia
	80	--	Australia
12. *G. lactovirens* Tind. & Craven.	40	I1I1	Australia
13. *G. latifolia* (Benth.) Newell & Hymowitz	40	B1B1	Australia
14. *G. latrobeana* (meissn.) Benth.	40	A3A3	Australia
15. *G. microphylla* (Benth.) Tind.	40	BB	Australia
16. *G. peratosa* B. Pfeil & Tind.	40	--	Australia
17. *G. pindanica* Tind. & Craven	40	H3H2	Australia
18. *G. pullenii* B. Pfeil, Tind. & Craven	40	--	Australia
19. *G. rubiginosa* Tind. & B. Pfeil	40	--	Australia
20. *G. stenophita* B. Pfeil & Tind.	40	B3B3	Australia
21. *G. tabacina* (Labill.) Benth.	40	B2B2	Australia
	80	Complex[3]	Australia, West Central and South Pacific Islands
22. *G. tomentella* Hayata	38	EE	Australia
	40	DD	Australia, Papua New Guinea
	78	Complex[4]	Australia, Papua New Guinea
	80	Complex[5]	Australia, Papua New Guinea, Indonesia, Philippines, Taiwan
Subgenus *Soja* (Moench) F.J. Herm.			
23. *G. soja* Sieb. & Zucc.	40	GG	China, Russia, Taiwan, Japan, Korea (Wild Soybean)
24. *G. max* (L.) Merr.	40	GG	Cultigen (Soybean)

[1] Genomically similar species carry the same letter symbols.
[2] Genome designation has not been assigned to the species.
[3] Allopolyploids (A and B genomes) and segmental allopolyploids (B genomes).
[4] Allopolyploids (D and E, A and E, or any other unknown combination).
[5] Allopolyploids (A and D genomes, or any other unknown combination).
Note: Table is adapted from Hymowitz, (2004).

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

Glycine max, the cultivated soybean, is an annual crop that is planted in late spring from April to May in the north hemisphere, and from November to February in the southern hemisphere. Soybean seed germinates when the soil temperature reaches 10°C and emerges in a 5-7 day period under favourable conditions (OECD, 2000). The system of soybean growth stages divides plant development into vegetative (V) and reproductive (R) stages (Pedersen, 2004). The vegetative stages begin with VE, which designates emergence. V stages continue and are numbered according to how many fully-developed trifoliate leaves are present (i.e., V1, V2, etc.). The reproductive (R) stages begin at flowering (R1) and include pod development and plant maturation. Full maturity is designated R8. The vegetative development phase lasts about 40 days, during which time the root nodules develop slowly, but do not become fully functional. Soybeans grow most rapidly when air temperatures are between 25 and 35°C (Beversdorf, 1993). Pods typically develop in late summer, and harvest occurs in the autumn. The life cycle of soybean is approximately 100 to 160 days, depending on the variety and the region it is cultivated. Harvesting may begin when the plants are completely dry and the seeds are liberated within the pods.

Soybean is a self-pollinated species, propagated by seed (OECD, 2000). The papilionaceous flower consists of a tubular calyx of five sepals, a corolla of five petals, one pistil, and nine fused stamens with a single separate posterior stamen. The stamens form a ring at the base of the stigma and elongate one day before pollination, at which time the elevated anthers form a ring around the stigma (OECD, 2000). The soybean flower stigma is receptive to pollen approximately 24 hours before anthesis and remains receptive for 48 hours after anthesis. The anthers mature in the bud and directly pollinate the stigma of the same flower. Pollination typically takes place on the day the flower opens. The pollen naturally comes in contact with the stigma during the process of anthesis. Anthesis normally occurs in late morning, depending on the environmental conditions. The pollen usually remains viable for two to four hours, and no viable pollen can be detected by late afternoon. Natural or artificial cross-pollination only can take place during the short time when the pollen is viable. As a result, soybean is considered to be a highly self-pollincated species, with cross-pollincation to adjacent plants of other soybean varieties occurring at very low frequency (0 to 6.3%) in adjacent plants (Caviness, 1966).

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

MON 87708 is not materially different from conventional soybean other than the introduction of the dicamba tolerance trait. Soybean has the ability to produce more edible protein per acre of land than any other known crop (Liu, 2004b). On average, dry soybean contains roughly 40% protein and 20% oil (Liu, 2004b). It has the highest protein content among cereals and other legume species, and has the second-highest oil content among all food legumes (Liu, 2004b). Soybean is highly versatile and can be processed into a wide variety of food products. In general, soyfoods can be roughly classified into six major categories (Liu, 2004a):

1. Soybean oil: Soybean oil constitutes approximately 69% of consumption of edible fats and oil in the U.S. (ASA, 2010e), and is the second largest source of vegetable oil worldwide (Soyatech, 2010a). Refined, bleached, and deodorized soybean oil can be further processed to produce cooking oils, shortening, margarine, mayonnaise, salad dressings, and a wide variety of products that are either based entirely on fats and oils or contain fat or oil as a principal ingredient.

2. Traditional soyfoods: Traditional soyfoods are primarily made from whole soybean. The nonfermented traditional soyfoods include soymilk, tofu, soybean sprouts, soymilk film (yuba), soynuts, and green vegetable soybean (*e.g.*, edamame), whereas the fermented soyfoods include soybean paste (miso), soybean sauce, natto, and tempeh.
3. Soybean protein products: Soybean protein products are mostly made from defatted soybean flakes, and include soybean flour, soybean protein concentrate, and soybean protein isolate. Soybean flour has a protein content of approximately 50% and is used mainly as an ingredient in the baking industry. Soybean protein concentrate has a protein content of approximately 70% and is used widely in the meat industry to bind water, emulsify fat, or as a key ingredient of meat alternative products. Soybean protein isolate has a protein content of 90%, and possesses many functional properties such as gelation and emulsification. As a result, it can be used in a wide range of food applications, including processed meats, meat analogs, soup and sauce bases, energy bars, nutritional beverages, infant formula, and dairy replacements. Soybean protein may also be texturized through various mechanisms to achieve a particular structure similar to fiber that is used as a meat analog.
4. Modern soyfoods: Modern soyfoods resulted from modification of traditional soyfoods to suit local tastes in the West. Blending of soybean products and processing techniques have resulted in two major subgroups including meat and dairy alternatives. Examples of modern soyfoods include soy ice cream, soy yogurt, soy cheese, soy burgers, and meatless meatballs.
5. Soybean-enriched foods: Soybean-enriched foods are similar to modern soyfoods, with the exception that soybean is not the main ingredient in soybean-enriched foods. Soybean protein is an ideal choice for increasing the protein content of many common foods. A wide variety of food can be enriched with soybean, including soy bread, soy pastes, and soy cereals.
6. Functional soybean ingredients/dietary supplements: Soybean is a rich source of certain phytochemicals, including lecithin and isoflavones. Often the result of modern processing, these phytochemicals can be recovered and used as a food ingredient or dietary supplements.

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

Soybeans are grown primarily for meal, and oil is a secondary product. There is no food use for unprocessed soybeans, since they contain anti-nutrient factors, such as trypsin inhibitors and lectins (OECD, 2001). Adequate heat processing inactivates these factors. There is no specific processing required for MON 87708 as MON 87708 is not materially different from conventional soybean other than the dicamba tolerance trait.

There are three main methods for processing soybeans; these are hydraulic processing, expeller processing and solvent extraction (SMIC, 2006). The entire description below on soybean processing is adopted from Snyder and Wilson (2003).

Dehulling and flaking soybean

Soybeans are cleaned and cracked into several pieces (meats). The hulls are removed by aspiration and the meats are conditioned by warming and by adding moisture. Conditioning is necessary to make a cohesive flake. The conditioned meats are put through smooth rollers that make flakes of approximately 0.025 cm thickness. Making flakes is advantageous for

uniform penetration of solvent in deep beds (minimal channelling) and for disruption of the soybean tissue, so that solvent can penetrate and dissolve the oil. Additionally, crushers may put the flakes through a cooking extruder to yield collets. This process gives a porous, but still high-density collet, that extracts more readily than flakes. Also, the collet holds less solvent than flake, thereby minimizing the energy needed for removing the solvent.

Oil extraction

The full-fat flakes (or collets) are loaded into the extractor to make beds over which the solvent flows countercurrently to the movement of the beds. The temperature of extraction is about 60ºC to speed up diffusion of solvent and to lower the miscella viscosity, both of which enhance the extraction of oil. Solvent extraction is capable of reducing the residual oil in the soybean flakes to less than 1%.

Removal of solvent and generation of crude oil and meal

Upon completion of the extraction, the solvent will be removed from both the oil and flakes. The full-fat miscella contains 25-30% oil and solvent is removed by two stages of rising-film vacuum evaporators followed by a third-stage stripping column. The flakes are treated in a desolventizer-toaster by direct contact with steam first to remove the solvent and second to heat treat the flakes for trypsin inhibitor destruction.

After leaving the desolventizer-toaster, the flakes are cooled and ground to a meal for use as a high-protein feed ingredient. The protein content is 44% with hulls added or 47.5 - 49% protein without hulls. If flakes are to be used to produce soybean food products, the solvent has to be removed with minimal heat to maintain protein solubility. Flash desolventizers are available in which superheated solvent is used as the heat-transfer medium to evaporate the solvent. With this system, the flakes are kept dry and protein solubility is preserved.

Oil refining

After extraction and removal of solvent, the crude soybean oil needs to be refined to convert it to edible products. Before going to refining, crude oil may undergo alkali refining (to remove free fatty acids), degumming (to remove phospholipids) or bleaching (to lighten the colour of the oil). Hydrogenation and deodorisation made it possible to substitute vegetable oils for animal fats in human diets in the early 1900s. Hydrogenation controlled the texture and stability of the oil, while deodorization improves the flavour of vegetable oils.

The gummy material that is removed during the oil refinement is further processed into lecithin, which is typically used as an emulsifier to keep water and fats from separating in foods such as margarine, peanut butter, chocolate candies, ice cream, and infant formulas.

Soy protein isolate

Defatted white flakes are extracted first with sodium hydroxide at ~pH 9 to remove fibre, and the remaining solution is acidified to ~pH 4 to precipitate the protein. The precipitated protein in slurry form can be spray-dried directly to produce an isoelectric soybean protein isolate (pH 4), or can be neutralized and spray-dried to produce soy proteinate (~pH 7).

Resulting soybean products

- Soybean soil products: after sufficient refining, soybean oil is used to produce shortening, margarines, salad dressings and cooking oils of various types.
- Soybean meal: the majority of the defatted soybean flakes is heated to produce soybean meal as animal feeds.

- Soybean protein products: a variety of food products such as soybean flour, soybean protein concentrates and isolates can be used in a variety of food products including simulating meats, nutrition bars and protein-fortified drinks. See Figure 1.

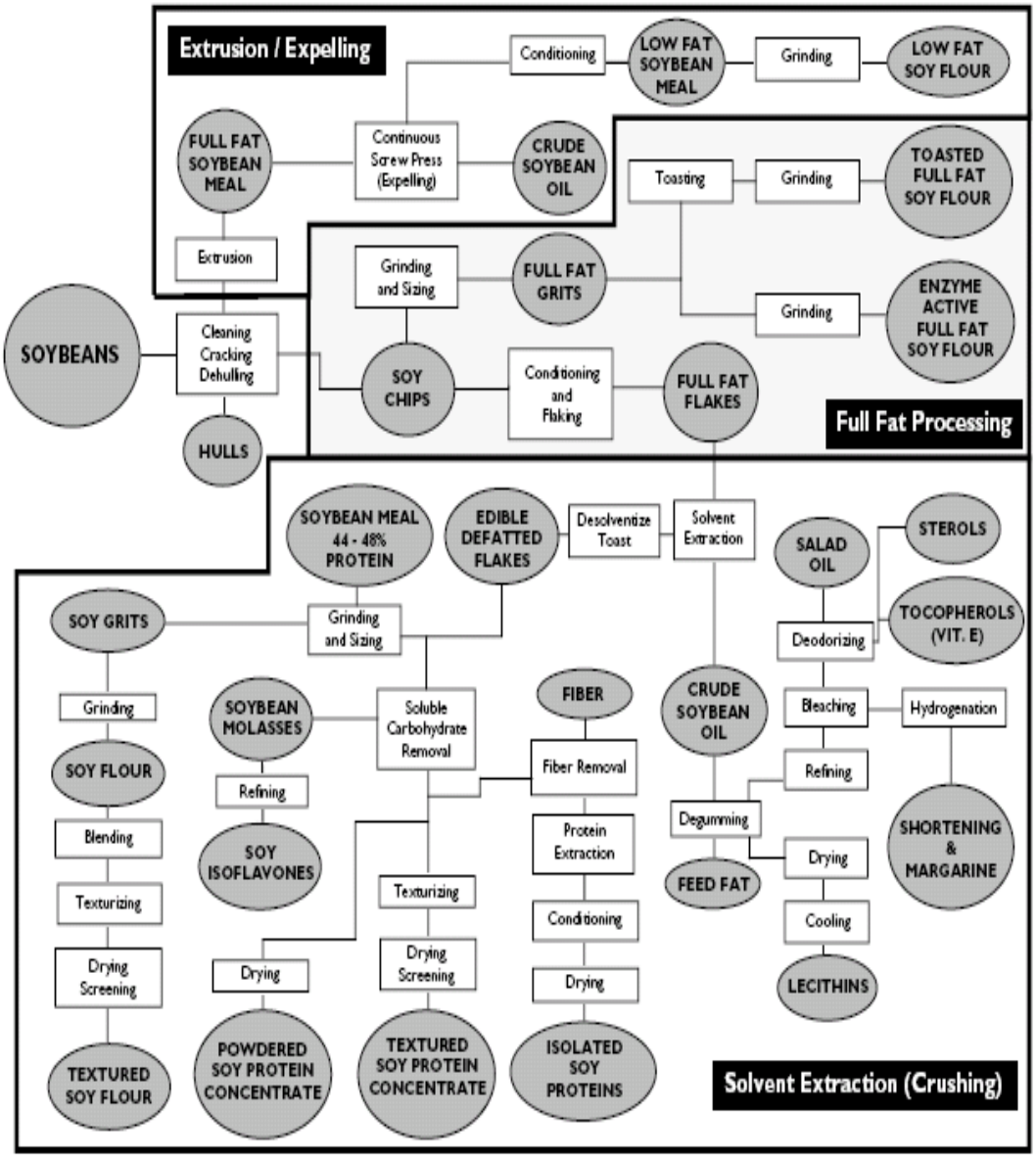


Figure 1. Soybean Processing Using Extrusion or Solvent Extraction

Figure is adapted from Soyatech, 2007.

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

Soybean can be processed into a wide variety of food products, and no changes in human or animal consumption habits are anticipated as a result of MON87708. Therefore, it will be generally consumed in Australia.

Soybean is the most commonly grown oilseed in the world. In 2008/09, approximately 211 MMT of harvested seed were produced, representing 56% of the world's oilseed production (Soyatech, 2010a). Soybean is grown as a commercial crop in over 35 countries. The major producers of soybean are the U.S., Brazil, Argentina, China, India, and Paraguay, which accounted for approximately 94% of the global soybean production in 2008/09 (see below). Approximately one-third of the 2009 world soybean production was in the U.S. (Soyatech, 2010a). The soybean produced in China and India are primarily for domestic use, while a significant portion of that produced in U.S., Brazil, and Argentina is traded globally in the form of soybean harvested seed, soybean meal or soybean oil. Globally, the U.S. was the largest soybean export country (ASA, 2010d), while Argentina led the soybean meal export markets in 2009 (ASA, 2010c).

World Soybean Production in 2008/09

Country	Production (million metric tons)
U.S.	80.5
Brazil	57.0
Argentina	32.0
China	16.0
Other	12.2
India	9.1
Paraguay	3.8

Source: Soyatech, 2010a

Approximately 85% of the world's soybean seed supply was crushed to produce soybean meal and oil in 2009, and the majority of the meal is used in animal feed with a small amount used to prepare protein isolates, and the majority of the oil is consumed as edible vegetable oil (Soyatech, 2010b). Approximately 6% of soybeans are used directly as human food, primarily in Asia (Soyatech, 2010b). Vegetable and food-grade soybean generally have different size, flavor, texture and other characteristics than field soybean. The remainder of the soybean seed produced was used as certified seed, feed, or stocks.

Soybean is used in various food products, including tofu, soybean sauce, soymilk, energy bars, and meat products. A major food use for soybean is purified oil, for use in margarines, shortenings, cooking, and salad oils. Soybean oil generally has a smaller contribution to soybean's overall value compared to soybean meal because the oil constitutes just 18 to 19% of the soybean's weight. Nonetheless, soybean oil accounted for approximately 30% of all the vegetable oils consumed globally, and was the second largest source of vegetable oil worldwide, slightly behind palm oil at approximately 32% market share (Soyatech, 2010a).

Soybean meal is used as a supplement in feed rations for livestock. Soybean meal is the most valuable component obtained from processing the soybean, accounting for roughly 50-75% of its overall value. By far, soybean meal is the world's most important protein feed, accounting for nearly 67% of world protein meal consumption (ASA, 2010a). Industrial

uses of soybean range from a carbon/nitrogen source in the production of yeasts via fermentation to the manufacture of soaps, inks, paints, disinfectants, and biodiesel. Industrial uses of soybean have been summarized by Cahoon (Cahoon, 2003) and the American Soybean Association (ASA, 2010b).

Global soybean plantings reached 96.2 million hectares in 2008/09, about a 15% increase compared to 82.3 million hectares planted in 2002/03 (Soyatech, 2010a). Soybean production has realized, on average, a 6.2% annual growth rate between 1995/96 to 2006/07. Increased planting flexibility, increased yield from narrow-row seeding practices, a higher rate of corn-soybean rotations, and low production costs favored expansion of soybean areas in the mid-1990s, with expansion concentrated in areas where soybean yields were highest.

A3 The nature of the genetic modification

A3(a) Method used to transform host organism

The *Agrobacterium*-mediated soybean transformation used to produce MON 87708 was based on the method described by Martinell et al. (2002), which allows for the generation of transformed plants without utilization of callus. Briefly, meristem tissues were excised from the embryos of germinated conventional seed. After co-culturing with the *Agrobacterium* carrying the vector, the meristems were placed on selection medium containing glyphosate, carbenicillin, cefotaxime, and ticarcillin/clavulanate acid mixture, to inhibit the growth of untransformed plant cells and excess *Agrobacterium*. The meristems were then placed in media conducive to shoot and root development. Rooted plants with normal phenotypic characteristics were selected and transferred to soil for growth and further assessment.

The R_0 plants generated through this transformation were self-pollinated to produce R_1 plants, and the unlinked insertions of T-DNA I and T-DNA II were segregated. A non-lethal dose of glyphosate was applied to R_1 plants and those plants with minor herbicide injury were selected for further analyses, whereas plants showing no injury, indicating that they contained the *cp4 epsps* coding sequence from T-DNA II, were eliminated from further development. Subsequently, plants that were homozygous for T-DNA I were identified by quantitative polymerase chain reaction (PCR) analysis. MON 87708 was selected as the lead event based on superior phenotypic characteristics, dicamba tolerance, and its molecular profile. The major development steps of MON 87708 are depicted in Figure 2. The result of this process was the production of marker-free, dicamba-tolerant soybean MON 87708.

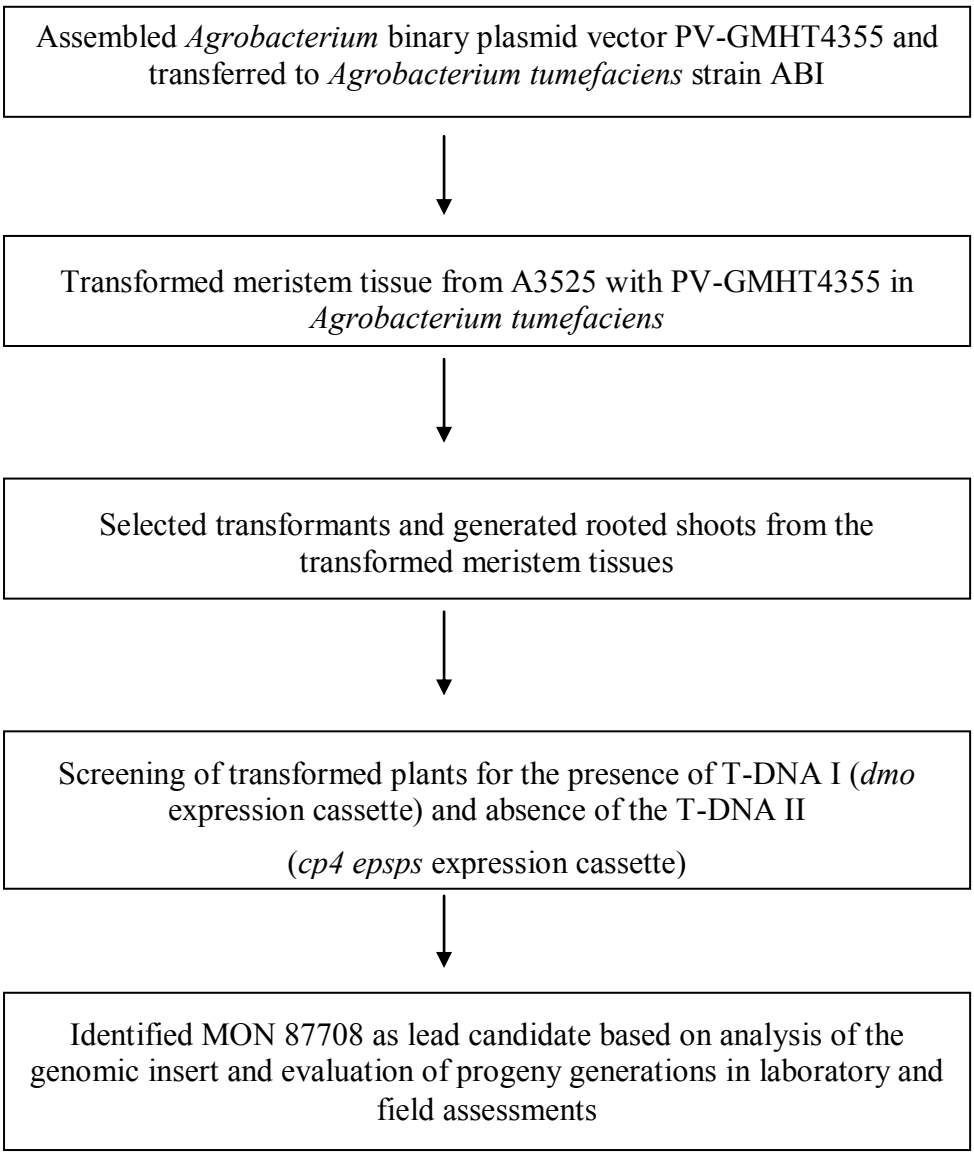


Figure 2. Schematic of the Development of MON 87708

A3(b) Intermediate hosts (eg. bacteria)

A disarmed strain of *Agrobacterium tumefaciens* was the intermediate host used to transfer the T-DNA II containing the full *cp4 epsps* expression cassette from plasmid PV-GMHT4355 into soybean cells to produce MON 87708.

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

Plasmid Vector PV-GMHT4355

Plasmid vector, PV-GMHT4355 was used for the transformation of conventional soybean to produce MON 87708 and is shown in Figure 4; PV-GMHT4355 is approximately 11.4 kb and contains two T-DNAs, each delineated by Left and Right Border regions to facilitate transformation. The first T-DNA, designated as T-DNA I, contains the *dmo* coding sequence under regulation of the peanut chlorotic streak caulimovirus (*PC1SV*) promoter and the pea *E9* 3′ non-translated region. The second T-DNA, designated as T-DNA II, contains the *cp4 epsps* coding sequence under the regulation of the figwort mosaic virus (*FMV*) promoter and the pea *E9* 3′ non-translated region. During transformation, both T-DNAs were inserted into the soybean genome (Section A3(a)) where T-DNA II, containing the *cp4 epsps* expression cassette, functioned as a marker gene for the selection of transformed plantlets. Subsequently, conventional self-pollinated breeding methods and segregation, along with a combination of analytical techniques, were used to isolate those plants that contain the *dmo* expression cassette (T-DNA I) and did not contain the *cp4 epsps* expression cassette (T-DNA II).

The backbone region of PV-GMHT4355 that is outside both of the T-DNAs contains two origins of replication for maintenance of the plasmid vector in bacteria (*ori V*, *ori-pBR322*), a bacterial selectable marker gene (*aadA*), and a coding sequence for repressor of primer (*rop*) protein which is necessary for the maintenance of the plasmid vector copy number in *E. coli*. A description of the genetic elements and their prefixes (*e.g.,* P-, L-, I-, TS-, OR-, B-, CS-, and T-) in PV-GMHT4355 is provided in Table 2.

The *dmo* Coding Sequence and MON 87708 DMO (T-DNA I)

The *dmo* expression cassette (T-DNA I) present in MON 87708 encodes MON 87708 DMO (Figure 3). The *dmo* expression cassette contains the coding region for the DMO from *Stenotrophomonas maltophilia* (Herman et al., 2005; Wang et al., 1997). The presence of MON 87708 DMO confers tolerance to dicamba (refer to Section A3(d)(ii) for more details). MON 87708 produces a MON 87708 DMO precursor protein that is post-translationally processed into two forms of the dicamba mono-oxygenase (DMO) protein; referred to as MON 87708 DMO protein and MON 87708 DMO+27 protein (refer to Section B2(a) for more details). The active form of these proteins, necessary to confer dicamba tolerance, is a trimer comprised of three DMO monomers. In MON 87708, the trimer can be comprised of MON 87708 DMO protein, MON 87708 DMO+27 protein, or a combination of both. Unless specified otherwise in this document, MON 87708 DMO will refer to both proteins and all forms of the trimer, collectively.

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

The *cp4 epsps* expression cassette (T-DNA II), that is not present in MON 87708, encoded a 47.6 kDa CP4 EPSPS protein, consisting of a single polypeptide of 455 amino acids (Padgette et al., 1996). The *cp4 epsps* coding sequence is the codon optimized coding sequence of the *aroA* gene from *Agrobacterium* sp. strain CP4 encoding CP4 EPSPS (Barry

et al., 1997; Padgette et al., 1996). CP4 EPSPS confers tolerance to glyphosate and was used as a selectable marker during the transformation selection process. Through conventional self-pollinated breeding methods and segregation, along with a combination of analytical techniques, plants that did not contain the *cp4 epsps* expression cassette were selected for further development.

Regulatory Sequences

The *dmo* coding sequence in T-DNA I is under the regulation of the *PC1SV* promoter, *TEV* leader, the *RbcS* targeting sequence, and the *E9* 3′ non-translated 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′ non-translated region from the Tobacco Etch virus (Niepel and Gallie, 1999) and is involved in regulating gene expression. The *RbcS* targeting sequence is the sequence encoding the chloroplast transit peptide and the first 24 amino acids of the mature protein of the ribulose-1,5-bisphosphate carboxylase oxygenase gene from pea (*Pisum sativum*) (Fluhr et al., 1986) that directs transport of the DMO precursor protein to the chloroplast. The *E9* 3′ non-translated region is the 3′ non-translated region from the *RbcS2* gene of pea encoding the Rubisco small subunit, which functions to direct polyadenylation of the mRNA (Coruzzi et al., 1984).

T-DNA II contains the *cp4 epsps* coding sequence under the regulation of the *FMV* promoter, *DnaK* leader, the *CTP2* targeting sequence, and the *E9* 3′ non-translated region. The *FMV* promoter is the promoter for the 35S RNA from figwort mosaic virus (Rogers, 2000) that directs transcription in plant cells. The *DnaK* leader is the 5′ non-translated leader sequence from the *Petunia hybrida Hsp70* gene (Rensing and Maier, 1994) that is involved in regulating gene expression. The *CTP2* targeting sequence is the sequence encoding the chloroplast transit peptide region from the *shkG* gene of *Arabidopsis thaliana* encoding EPSPS (Herrmann, 1995; Klee et al., 1987) that directs transport of the CP4 EPSPS precursor protein to the chloroplast. The *E9* 3′ non-translated region is the 3′ non-translated region from the *RbcS2* gene of pea encoding the Rubisco small subunit, which functions to direct polyadenylation of the mRNA (Coruzzi et al., 1984).

T-DNA Borders

PV-GMHT4355 contains Right and Left Border regions (Figure 4 and Table 2) that were derived from *Agrobacterium tumefaciens* (Barker et al., 1983; Depicker et al., 1982; Zambryski et al., 1982). The border regions each contain a 24-25 bp nick site that is the site of DNA exchange during transformation. The border regions separate the T-DNA from the backbone region and are involved in their efficient transfer into the soybean genome. Because PV-GMHT4355 is a 2T-DNA vector, it contains two Right Border regions and two Left Border regions, where one set flanks T-DNA I and the other set flanks T-DNA II.

Genetic Elements Outside the T-DNA Borders

Genetic elements that exist outside of the T-DNA border regions are those that are essential for the maintenance or selection of PV-GMHT4355 in bacteria and are referred to as the plasmid backbone. The *ori V*, derived from the broad host plasmid RK2, is required for the maintenance of the plasmid vector in *Agrobacterium* (Stalker et al., 1981), whereas the *ori-pBR322*, derived from the plasmid vector pBR322, is required for the maintenance of the plasmid vector in *E. coli* (Sutcliffe, 1979). The *rop* is necessary for the maintenance of plasmid vector copy number in *E. coli* (Giza and Huang, 1989). The *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 were not expected to be transferred into the soybean genome. The absence of the backbone sequence in MON 87708 was confirmed by Southern blot analyses (Section A3(d)(ii)).

Table 2. Summary of Genetic Elements in the Plasmid Vector PV-GMHT4355

Genetic Element	Location in Plasmid (bp)	Function (Reference)
T-DNA I (Present in MON 87708)		
B[1]-Right Border Region	8290-8646	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	8647-8691	Sequence used in DNA cloning
P[2]-*PC1SV*	8692-9124	Promoter for the Full-Length Transcript (FLt) of peanut chlorotic streak caulimovirus (Maiti and Shepherd, 1998) that directs transcription in plant cells
Intervening sequence	9125-9144	Sequence used in DNA cloning
L[3]-*TEV*	9145-9276	5′ non-translated region from the Tobacco Etch virus genome (Niepel and Gallie, 1999) that is involved in regulating gene expression
Intervening sequence	9277	Sequence used in DNA cloning
TS[4]-*RbcS*	9278-9520	Sequences encoding the transit peptide and the first 24 amino acids of the mature protein of the *RbcS* gene from *Pisum sativum* (pea) (Fluhr et al., 1986) that directs transport to the DMO precursor protein to the chloroplast
Intervening Sequence	9521-9529	Sequence used in DNA cloning
CS[5]-*dmo*	9530-10552	Coding sequence for the dicamba mono-oxygenase derived from *Stenotrophomonas maltophilia* (Herman et al., 2005; Wang et al., 1997)
Intervening Sequence	10553-10620	Sequence used in DNA cloning
T[6]-*E9*	10621-11263	3′ non-translated region from the *RbcS2* gene of *Pisum sativum* (pea) encoding the Rubisco small subunit, which functions to direct polyadenylation of the mRNA (Coruzzi et al., 1984)
Intervening Sequence	11264-11352	Sequence used in DNA cloning
B-Left Border Region	1-442	DNA region from *Agrobacterium tumefaciens* containing the Left Border sequence used for transfer of the T-DNA (Barker et al., 1983)

Table 2(continued). Summary of Genetic Elements in the Plasmid Vector PV-GMHT4355

Genetic Element	Location in Plasmid (bp)	Function (Reference)
Plasmid Vector Backbone (Not present in MON 87708)		
Intervening Sequence	443-528	Sequence used in DNA cloning
OR[7]-*ori V*	529-925	Origin of replication from the broad host range plasmid RK2 for maintenance of plasmid in *Agrobacterium* (Stalker et al., 1981)
Intervening Sequence	926-1662	Sequence used in DNA cloning
CS-*rop*	1663-1854	Coding sequence for repressor of primer protein derived from the ColE1 plasmid for maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989)
Intervening Sequence	1855-2281	Sequence used in DNA cloning
OR-*ori-pBR322*	2282-2870	Origin of replication from pBR322 for maintenance of plasmid in *E. coli* (Sutcliffe, 1979)
Intervening Sequence	2871-3400	Sequence used in DNA cloning
aadA	3401-4289	Bacterial promoter, coding and 3′ UTR sequences for an aminoglycoside-modifying enzyme, 3″ (9)-O-nucleotidyltransferase from transposon Tn7 (Fling et al., 1985) that confers spectinomycin and streptomycin resistance
Intervening Sequence	4290-4384	Sequence used in DNA cloning
T-DNA II (Not present in MON 87708)		
B-Left Border Region	4385-4795	DNA region from *Agrobacterium tumefaciens* containing the Left Border sequence used for transfer of the T-DNA (Barker et al., 1983)
Intervening Sequence	4796-4809	Sequence used in DNA cloning
T-*E9*	4810-5452	3′ non-translated sequence from *RbcS2* gene of *Pisum sativum* (pea) encoding the Rubisco small subunit, which functions to direct polyadenylation of the mRNA (Coruzzi et al., 1984)
Intervening Sequence	5453-5458	Sequence used in DNA cloning
CS-*cp4 epsps*	5459-6826	Codon optimized coding sequence of the *aroA* gene from *Agrobacterium* sp. strain CP4 encoding CP4 EPSPS (Barry et al., 1997; Padgette et al., 1996)

Table 2(continued). Summary of Genetic Elements in the Plasmid Vector PV-GMHT4355

Genetic Element	Location in Plasmid (bp)	Function (Reference)
TS-*CTP2*	6827-7054	Sequences encoding the chloroplast transit peptide region from the *shkG* gene of *Arabidopsis thaliana* encoding EPSPS (Herrmann, 1995; Klee et al., 1987) that directs transport of the CP4 EPSPS precursor protein to the chloroplast
Intervening Sequence	7055-7063	Sequence used in DNA cloning
L-*DnaK*	7064-7159	5′ non-translated leader sequence from the *Petunia hybrida Hsp70* gene (Rensing and Maier, 1994) that is involved in regulating gene expression
Intervening Sequence	7160-7162	Sequence used in DNA cloning
P-*FMV*	7163-7714	Promoter for the 35S RNA from figwort mosaic virus (Rogers, 2000) that directs transcription in plant cells
Intervening Sequence	7715-7761	Sequence used in DNA cloning
B-Right Border Region	7762-8118	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)
Plasmid Vector Backbone (Not present in MON 87708)		
Intervening sequence	8119-8289	Sequence used in DNA cloning

[1]B -border.
[2]P-promoter.
[3]L- leader.
[4]TS- targeting sequence.
[5]CS-coding sequence.
[6]T- 3' non-translated transcriptional termination sequence and polyadenylation signal sequences.
[7]OR-origin of replication.

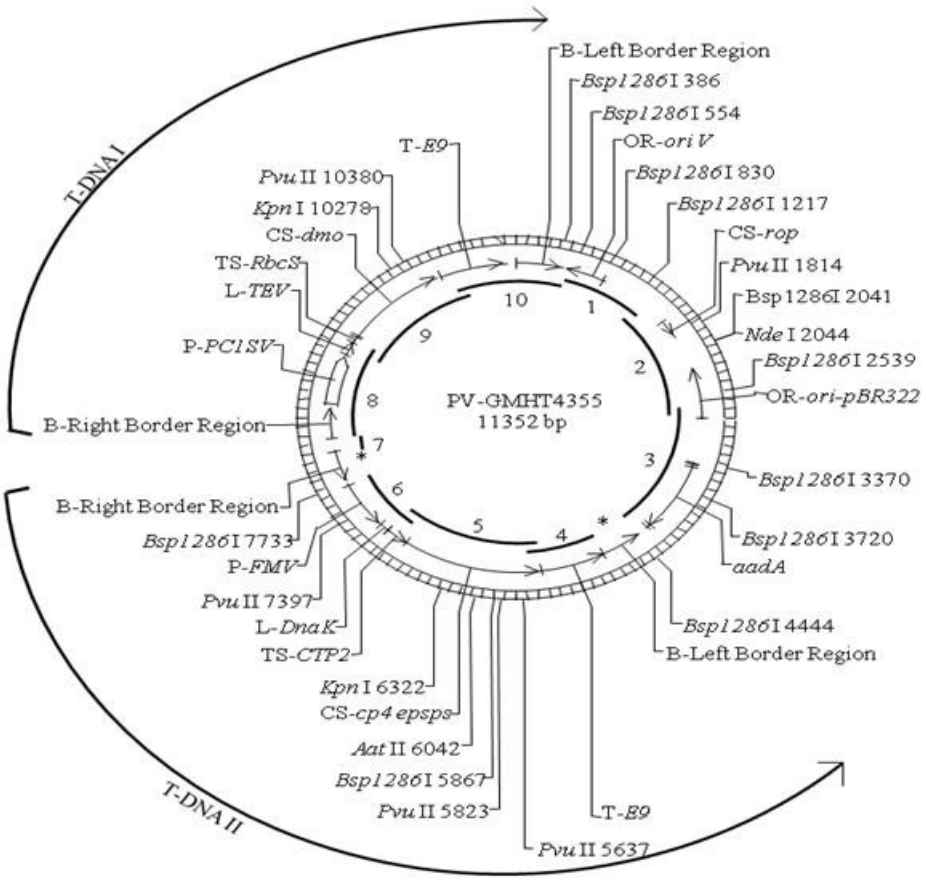
```
1   MASMISSSAV TTVSRASRGQ SAAMAPFGGL KSMTGFPVRK VNTDITSITS NGGRVKCMQV
61  WPPIGKKKFE TLSYLPPLTR DSRAMATFVR NAWYVAALPE ELSEKPLGRT ILDTPLALYR
121 QPDGVVAALL DICPHRFAPL SDGILVNGHL QCPYHGLEFD GGGQCVHNPH GNGARPASLN
181 VRSFPVVERD ALIWICPGDP ALADPGAIPD FGCRVDPAYR TVGGYGHVDC NYKLLVDNLM
241 DLGHAQYVHR ANAQTDAFDR LEREVIVGDG EIQALMKIPG GTPSVLMAKF LRGANTPVDA
301 WNDIRWNKVS AMLNFIAVAP EGTPKEQSIH SRGTHILTPE TEASCHYFFG SSRNFGIDDP
361 EMDGVLRSWQ AQALVKEDKV VVEAIERRRA YVEANGIRPA MLSCDEAAVR VSREIEKLEQ
421 LEAA
```

Figure 3. **Deduced Amino Acid Sequence of the MON 87708 DMO Precursor Protein**

The chloroplast transit peptide and the first 24 amino acids of the mature protein of the *RbcS* elementare underlined and followed by three amino acids of an intervening sequence. The methionine in position 85 is the N-terminus of the MON 87708 DMO protein deduced from the *dmo* element (see Table 2 and Section B2(a), Figure 20 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 Number	Probe Type	Start Position (bp)	End Position (bp)	Total Length (bp)
1	Backbone Probe	443	1328	886
2	Backbone probe	1250	2754	1505
3	Backbone Probe	2625	4384	1760
4	T-DNA II Probe	4796	5637	842
5	T-DNA II Probe	5575	7021	1447
6	T-DNA II Probe	6937	7761	825
7	Backbone Probe	8119	8289	171
8	T-DNA I Probe	8290	9523	1234
9	T-DNA I Probe	9448	10668	1221
10	T-DNA I Probe	10610	442	1185

Figure 4. Circular Map of Plasmid Vector PV-GMHT4355 Showing Probes 1-10

The plasmid vector PV-GMHT4355 containing the T-DNAs used in *Agrobacterium*-mediated transformation to produce MON 87708. Genetic elements and restriction sites for enzymes used in the Southern blot analyses (with positions relative to the size of the plasmid vector) are shown on the exterior of the map. The probes used in the Southern blot analyses (labeled 1-10 on the interior of the map) are detailed in the accompanying table above.

*The Left and Right Border regions of T-DNA II share 100% identity to those of T-DNA I, which were covered by probes 8 and 10 and thus not included in the T-DNA II probes.

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

A multi-faceted approach was taken to characterize the genetic modification that produced MON 87708. The results confirmed that MON 87708 contains a single copy of the *dmo* expression cassette (T-DNA I) that is stably integrated at a single locus and is inherited according to Mendelian principles over multiple generations (Section A3(f)(i)). The results confirmed that no T-DNA II or plasmid vector backbone sequences are detected in MON 87708. These conclusions are based on several lines of evidence: 1) Southern blot analyses to assay the entire soybean genome for the presence of DNA derived from PV-GMHT4355, and to confirm that a single copy of T-DNA I was inserted at a single site and that the insert is stably inherited; 2) DNA sequencing analyses to determine the exact sequence of the inserted DNA and allowed a comparison to the T-DNA I sequence in PV-GMHT4355 to confirm that only the expected sequences were integrated; and 3) a comparison of the DNA flanking T-DNA I to the sequence of the insertion site in conventional soybean to identify any rearrangements that occurred at the insertion site during transformation. Taken together, the characterization of the genetic modification demonstrates that a single copy of the T-DNA I was inserted at a single locus of the genome.

The Southern blot analysis confirmed that T-DNA I reported in Figure 5 represents the only detectable insert in MON 87708. Figure 5 is a linear map depicting restriction sites within the insert as well as within the known soybean genomic DNA immediately flanking the insert in MON 87708. The circular map of PV-GMHT4355 annotated with the probes used in the Southern blot analysis is presented in Figure 4. Based on the linear map of the insert and the plasmid map, a table summarizing the expected DNA segments for Southern analyses is presented in Table 3. The genetic elements integrated in MON 87708 are summarized in Table 4. The generations used are depicted in the breeding history shown in Figure 17.

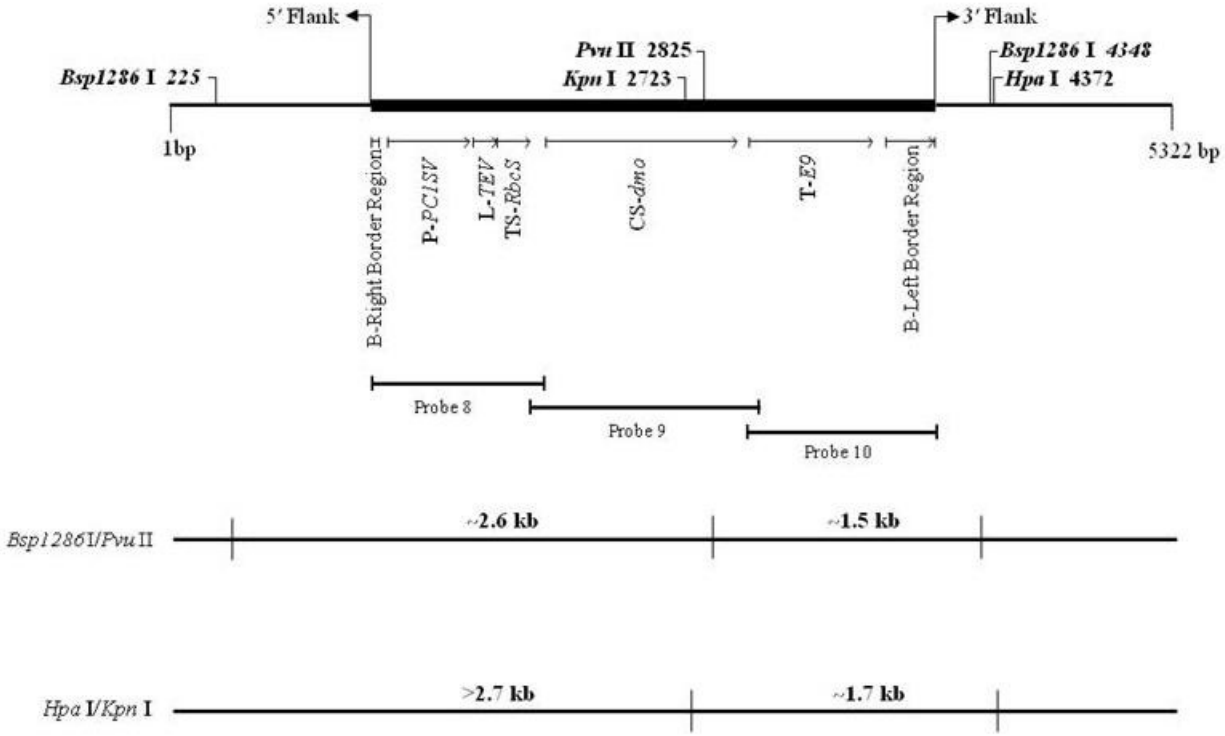


Figure 5. Schematic Representation of the Insert and Genomic Flanking Sequences in MON 87708

A linear map of the insert and genomic DNA flanking the insert in MON 87708 is shown. Identified on the map are genetic elements within the insert, as well as restriction sites with positions relative to the size of the linear map for enzymes used in the Southern analyses. The relative sizes and locations of the T-DNA I probes, which are described in Figure 4, are shown on the middle portion. Shown on the lower portion of the map are the expected sizes of the DNA segments after digestion with respective restriction enzymes. Arrowheads (→) indicate the end of the insert and the beginning of the genomic DNA sequence flanking the 5′ and 3′ end of the insert. The arrows (→) indicate the sequence direction of the elements in MON 87708.

Table 3. Summary Chart of the Expected DNA Segments Based on Hybridizing Probes and Restriction Enzymes Used in MON 87708 Analysis

Southern Blot Figure		Fig 6	Fig 7	Fig 8	Fig 9	Fig 10	Fig 11	Fig 12	Fig 18
Probe Used		8	9	10	4	5	6	1, 2, 3, and 7	9
Probing Target	Digestion Enzyme	Expected Band Sizes (kb) on Each Southern Blot							
Plasmid Vector PV-GMHT4355	*Aat* II/*Nde* I	~7.4	~7.4	~4.0 ~7.4	~4.0 ~7.4	~4.0 ~7.4	~7.4	~4.0 ~7.4	~7.4
Probe Templates[1]	N/A	~~[2]	~~[2]	~~[2]	~~[2]	~~[2]	~~[2]	~0.2 ~0.9 ~1.5 ~1.8	~~[2]
Conventional Control A3525	*Bsp1286* I/*Pvu* II	None	None	None	None	None	None	None	None
	Hpa I/*Kpn* I	None	None	None	None	None	None	None	None
MON 87708	*Bsp1286* I/*Pvu* II	~2.6	~2.6 ~1.5	~1.5	~1.5	None	None	None	~2.6 ~1.5
	Hpa I/*Kpn* I	>2.7*	>2.7* ~1.7	~1.7	~1.7	None	None	None	--[3]

[1] Probe templates were spiked when multiple probes are used in Southern blot analysis.
[2] '~~' indicates that only plasmid template was used since the Southern blot was hybridized with one probe.
[3] '--' indicates that the particular restriction enzyme or the combination of the enzymes was not used in the analysis.
* Southern analysis indicates this segment to be ~5.6 kb.

Table 4. Summary of Genetic Elements in MON 87708

Genetic Element	Location (bp)	Function (Reference)
5′ Flanking Sequences	1-1048	DNA sequence adjacent to the 5′ end of the insertion site
B[1]-Right Border* Region	1049-1091	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	1092-1136	Sequence used in DNA cloning
P[2]-*PC1SV*	1137-1569	Promoter for the Full-Length Transcript (FLt) of peanut chlorotic streak caulimovirus (Maiti and Shepherd, 1998) that directs transcription in plant cells
Intervening sequence	1570-1589	Sequence used in DNA cloning
L[3]-*TEV*	1590-1721	5' non-translated region from the Tobacco Etch virus genome (Niepel and Gallie, 1999) that is involved in regulating gene expression
Intervening sequence	1722-1722	Sequence used in DNA cloning
TS[4]-*RbcS*	1723-1965	Sequences encoding the transit peptide and the first 24 amino acids of the mature protein of the *RbcS* gene from *Pisum sativum* (pea) (Fluhr et al., 1986) that directs transport of the DMO precursor protein to the chloroplast
Intervening Sequence	1966-1974	Sequence used in DNA cloning
CS[5]-*dmo*	1975-2997	Coding sequence for the dicamba mono-oxygenase derived from *Stenotrophomonas maltophilia* (Herman et al., 2005; Wang et al., 1997)
Intervening Sequence	2998-3065	Sequence used in DNA cloning
T[6]-*E9*	3066-3708	3′ non-translated region from the *RbcS2* gene of *Pisum sativum* (pea) encoding the Rubisco small subunit, which functions to direct polyadenylation of the mRNA (Coruzzi et al., 1984)
Intervening Sequence	3709-3797	Sequence used in DNA cloning
B-Left Border* Region	3798-4051	DNA region from *Agrobacterium tumefaciens* containing the Left Border sequence used for transfer of the T-DNA (Barker et al., 1983)
3′ Flanking Sequences	4052-5322	DNA sequence adjacent to the 3′ end of the insertion site

[1]B-border.
[2]P-promoter.
[3]L-leader.
[4]TS- targeting sequence.
[5]S- coding sequence.
[6]T-3' non-translated transcriptional termination sequence and polyadenylation signal sequences.
*These borders are truncated.

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

1) Insert and copy number of T-DNAI in MON 87708

The copy number and insertion site of T-DNA I was assessed by digesting MON 87708 genomic DNA with the restriction enzyme combination *Bsp1286* I/*Pvu* II or *Hpa* I/*Kpn* I and hybridizing Southern blots with probes that span T-DNA I (Figure 4). Each restriction digest is expected to produce a specific banding pattern on the Southern blots (Table 3). Since each detected segment contains flanking genomic DNA, any additional integrated sites would produce a different banding pattern with additional bands.

The restriction enzyme combination *Bsp1286* I/*Pvu* II cuts once within T-DNA I and once within each of the known genomic DNA sequences flanking the 5′ and 3′ ends of T-DNA I (Figure 5). Therefore, if T-DNA I sequences are present at a single integration site in MON 87708, the digestion with *Bsp1286* I/*Pvu* II was expected to generate two border segments with expected sizes of ~2.6 kb and ~1.5 kb (Figure 5, and Table 3). The ~2.6 kb restriction segment contained genomic DNA flanking the 5′ end of T-DNA I, the Right Border, the *PC1SV* promoter, the *TEV* leader, the *RbcS* targeting sequence, and a portion of the *dmo* coding sequence. The ~1.5 kb restriction segment contained a portion of the *dmo* coding sequence, the *E9* 3′ non-translated sequence, the Left Border, and genomic DNA flanking the 3′ end of T-DNA I.

The restriction enzyme combination *Hpa* I/*Kpn* I cuts once within T-DNA I and once within the known genomic DNA flanking the 3′ end of T-DNA I (Figure 5). Therefore, if T-DNA I sequences are present at a single integration site in MON 87708, the digestion with *Hpa* I/*Kpn* I was expected to generate two border segments with expected sizes of ~1.7 kb and greater than 2.7 kb (Figure 5, and Table 3). Since the *Hpa* I/*Kpn* I restriction site in the genomic DNA flanking the 5′ end of the insert lies outside of the known sequence, it was not possible to predict a precise segment size. However, the segment size was determined by Southern blot analyses to be ~5.6 kb (Figure 6 and Figure 7). The ~5.6 kb restriction segment contained genomic DNA flanking the 5′ end of T-DNA I, the Right Border, the *PC1SV* promoter, the *TEV* leader, the *RbcS* targeting sequence, and a portion of *dmo* coding sequence. The ~1.7 kb restriction segment contained a portion of the *dmo* coding sequence, the *E9* 3′ non-translated sequence, the Left Border, and genomic DNA flanking the 3′ end of T-DNA I.

In the Southern blot analyses performed, each Southern blot contained a negative and a positive control. Conventional control genomic DNA digested with either the restriction enzyme combination *Bsp1286* I/*Pvu* II or *Hpa* I/*Kpn* I was used as a negative control to determine if the probes hybridized to any endogenous soybean sequences. As a positive control on the Southern blots, PV-GMHT4355 digested with the restriction enzyme combination *Aat* II/*Nde* I was mixed with predigested conventional control DNA. The positive hybridization control was spiked at 0.1 and 1 genome equivalent to demonstrate sufficient sensitivity of the Southern blot. Individual Southern blots were hybridized with the following probes: probes 8, 9, and 10 (refer to Figure 4 and Table 3). The results of this analysis are shown in Figure 6 through Figure 8.

Probe 8

Conventional control DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II (Figure 6, lanes 1 and 5) or *Hpa* I/*Kpn* I (Figure 6, lanes 3 and 7) and hybridized with probe 8 (Figure 4) produced no detectable hybridization bands as expected for the negative control. PV-GMHT4355, digested with the restriction enzyme combination *Aat* II/*Nde* I and mixed with conventional control DNA predigested with the restriction

enzyme combination *Hpa* I/*Kpn* I (Figure 6, lanes 10 and 11), produced the expected size band at ~7.4 kb (refer to Figure 5 and Table 3). These results indicate that the probe is hybridizing to its target sequence.

MON 87708 DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II and hybridized with probe 8 (Figure 4) produced one unique band at ~2.6 kb (Figure 6, lanes 2 and 6). The ~2.6 kb band is the expected size for the border segment containing the 5′ end of T-DNA I along with the adjacent genomic DNA flanking the 5′ end of T-DNA I (Figure 5).

MON 87708 DNA digested with the restriction enzyme combination *Hpa* I/*Kpn* I and hybridized with probe 8 (Figure 4) produced one unique band at ~5.6 kb (Figure 6, lanes 4 and 8). The ~5.6 kb band is consistent with the expected band being greater than 2.7 kb for the border segment containing the 5′ end of T-DNA I along with the adjacent genomic DNA flanking the 5′ end of T-DNA I (Figure 5).

No additional bands were detected using probe 8. Based on the results presented in Figure 6, it was concluded that T-DNA I sequences covered by probe 8 reside at a single integration locus as one copy in MON 87708.

Probe 9

Conventional control DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II (Figure 7, lanes 1 and 5) or *Hpa* I/*Kpn* I (Figure 7, lanes 3 and 7) and hybridized with probe 9 (Figure 4) produced no detectable hybridization bands as expected for the negative control. PV-GMHT4355, digested with the restriction enzyme combination *Aat* II/*Nde* I and mixed with conventional control DNA predigested with the restriction enzyme combination *Hpa* I/*Kpn* I (Figure 7, lanes 10 and 11), produced the expected size band at ~7.4 kb (refer to Figure 5 and Table 3). These results indicate that the probe is hybridizing to its target sequence.

MON 87708 DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II and hybridized with probe 9 (Figure 4) produced two unique bands at ~1.5 kb and ~2.6 kb (Figure 7, lanes 2 and 6). The ~1.5 kb band is the expected size for the border segment containing the 3′ end of T-DNA I along with the adjacent genomic DNA flanking the 3′ end of T-DNA I (Figure 5). The ~2.6 kb band is the expected size for the border segment containing the 5′ end of T-DNA I along with the adjacent genomic DNA flanking the 5′ end of T-DNA I (Figure 5).

MON 87708 DNA digested with the restriction enzyme combination *Hpa* I/*Kpn* I and hybridized with probe 9 (Figure 4) produced two unique bands at ~1.7 kb and ~5.6 kb (Figure 7, lanes 4 and 8). The ~1.7 kb band is the expected size for the border segment containing the 3′ end of T-DNA I along with the adjacent genomic DNA flanking the 3′ end of T-DNA I (Figure 5). The ~5.6 kb band is consistent with the expected band being greater than 2.7 kb for the border segment containing the 5′ end of T-DNA I along with the adjacent genomic DNA flanking the 5′ end of T-DNA I (Figure 5).

No additional bands were detected using probe 9. Based on the results presented in Figure 7, it was concluded that T-DNA I sequences covered by probe 9 reside at a single integration locus as one copy in MON 87708.

Probe 10

Conventional control DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II (Figure 8, lanes 1 and 5) or *Hpa* I/*Kpn* I (Figure 8, lanes 3 and 7) and hybridized with probe 10 (Figure 4) produced no detectable hybridization bands as expected for the negative control. PV-GMHT4355, digested with the restriction enzyme combination *Aat* II/*Nde* I and mixed with conventional control DNA predigested with the restriction enzyme combination *Hpa* I/*Kpn* I (Figure 8, lanes 10 and 11), produced two bands at ~4.0 kb and ~7.4 kb. Both bands were expected because probe 10 contains *E9* and Left Border regions that hybridized to both the ~4.0 kb and the ~7.4 kb fragments from the digested plasmid (refer to Figure 5 and Table 3). These results indicate that the probe is hybridizing to its target sequence.

MON 87708 DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II and hybridized with probe 10 (Figure 4) produced a unique band at ~1.5 kb (Figure 8, lanes 2 and 6). The ~1.5 kb band is the expected size for the border segment containing the 3′ end of T-DNA I along with the adjacent genomic DNA flanking the 3′ end of T-DNA I (Figure 5).

MON 87708 DNA digested with the restriction enzyme combination *Hpa* I/*Kpn* I and hybridized with probe 10 (Figure 4) produced a unique band at ~1.7 kb (Figure 8, lanes 4 and 8). The ~1.7 kb band is the expected size for the border segment containing the 3′ end of T-DNA I along with the adjacent genomic DNA flanking the 3′ end of T-DNA I (Figure 5).

No additional bands were detected using probe 10. Based on the results presented in Figure 8, it was concluded that T-DNA sequences covered by probe 10 reside at a single integration locus as one copy in MON 87708.

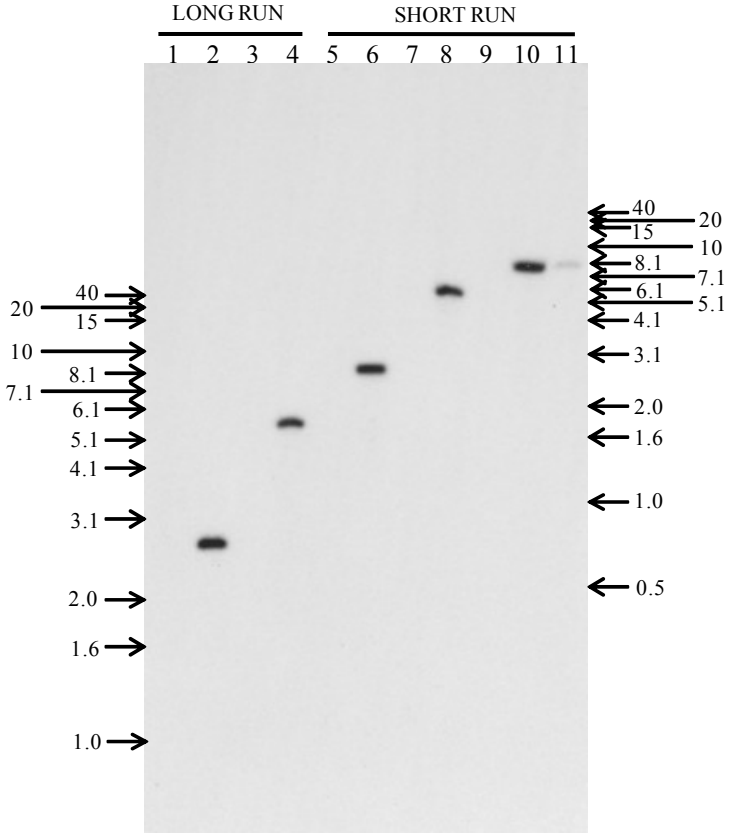


Figure 6. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA I in MON 87708: Probe 8

The blot was hybridized with a ^{32}P labeled T-DNA I probe that spans a portion of the T-DNA I sequence (Probe 8, Figure 4). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane	Description
1.	Conventional control (*Bsp1286* I/*Pvu* II)
2.	MON 87708 (*Bsp1286* I/*Pvu* II)
3.	Conventional control (*Hpa* I/*Kpn* I)
4.	MON 87708 (*Hpa* I/*Kpn* I)
5.	Conventional control (*Bsp1286* I/*Pvu* II)
6.	MON 87708 (*Bsp1286* I/*Pvu* II)
7.	Conventional control (*Hpa* I/*Kpn* I)
8.	MON 87708 (*Hpa* I/*Kpn* I)
9.	Blank
10.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~1 genome equivalent]
11.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~0.1 genome equivalent]

Arrows denote sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

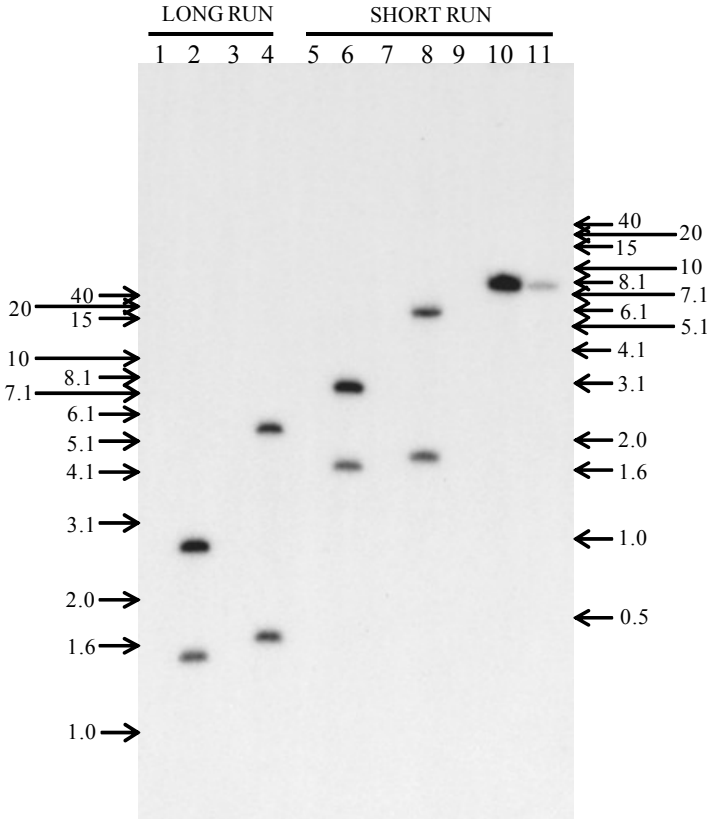


Figure 7. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA I in MON 87708: Probe 9

The blot was hybridized with a ^{32}P labeled T-DNA I probe that spans a portion of the T-DNA I sequence (Probe 9, Figure 4). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane	Description
1.	Conventional control (*Bsp1286* I/*Pvu* II)
2.	MON 87708 (*Bsp1286* I/*Pvu* II)
3.	Conventional control (*Hpa* I/*Kpn* I)
4.	MON 87708 (*Hpa* I/*Kpn* I)
5.	Conventional control (*Bsp1286* I/*Pvu* II)
6.	MON 87708 (*Bsp1286* I/*Pvu* II)
7.	Conventional control (*Hpa* I/*Kpn* I)
8.	MON 87708 (*Hpa* I/*Kpn* I)
9.	Blank
10.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~1 genome equivalent]
11.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~0.1 genome equivalent]

Arrows denote sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

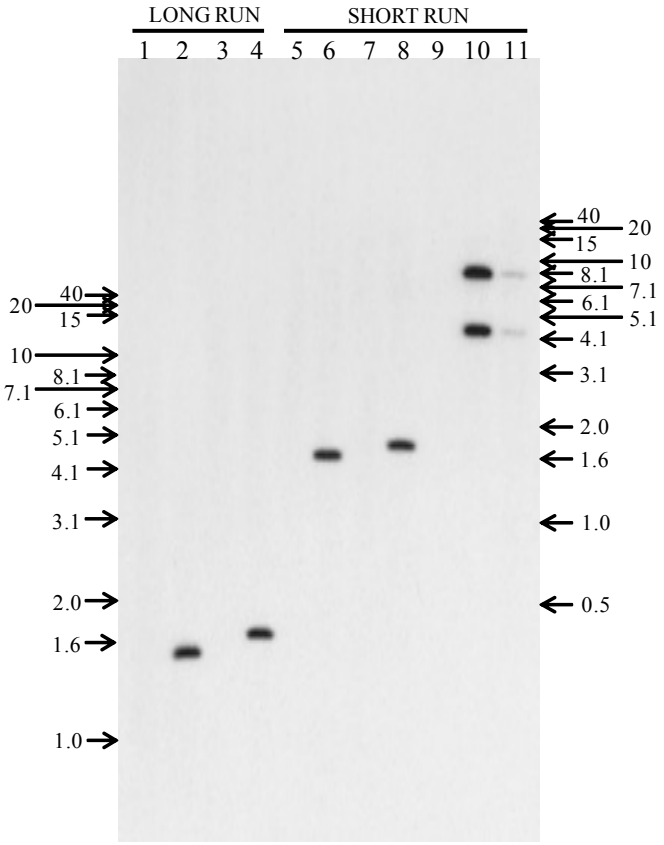


Figure 8. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA I in MON 87708: Probe 10

The blot was hybridized with a ^{32}P labeled T-DNA I probe that spans a portion of the T-DNA I sequence (Probe 10, Figure 4). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane	Description
1.	Conventional control (*Bsp1286* I/*Pvu* II)
2.	MON 87708 (*Bsp1286* I/*Pvu* II)
3.	Conventional control (*Hpa* I/*Kpn* I)
4.	MON 87708 (*Hpa* I/*Kpn* I)
5.	Conventional control (*Bsp1286* I/*Pvu* II)
6.	MON 87708 (*Bsp1286* I/*Pvu* II)
7.	Conventional control (*Hpa* I/*Kpn* I)
8.	MON 87708 (*Hpa* I/*Kpn* I)
9.	Blank
10.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~1 genome equivalent]
11.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~0.1 genome equivalent]

Arrows denote sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

2) Southern Blot Analysis to Determine the Presence or Absence of T-DNA II Sequences in MON 87708

To determine the presence or absence of T-DNA II sequences, MON 87708 and conventional control genomic DNA were digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II or *Hpa* I/*Kpn* I and Southern blots were hybridized with probes that span the T-DNA II sequence (Figure 4). As a positive control on the Southern blots, PV-GMHT4355 digested with the restriction enzyme combination *Aat* II/*Nde* I was mixed with predigested conventional control DNA. The positive hybridization control was spiked at 0.1 and 1 genome equivalent to demonstrate sufficient sensitivity of the Southern blot. Each blot was hybridized with one of three overlapping probes spanning the T-DNA II sequence other than the two border regions that share the same sequences as present in T-DNA I (Probes 4, 5 and 6, Figure 4). If T-DNA II sequences were present in MON 87708, then probing with the T-DNA II sequences should result in unique hybridizing bands. The results of this analysis are shown in Figure 9 through Figure 11.

Probe 4

Conventional control DNA digested with *Bsp1286* I/*Pvu* II (Figure 9, lanes 1 and 5) or *Hpa* I/*Kpn* I (Figure 9, lanes 3 and 7) and hybridized with probe 4 showed no detectable hybridization bands, as expected for the negative control. PV-GMHT4355, previously digested with *Aat* II/*Nde* I and mixed with conventional control DNA predigested with *Hpa* I/*Kpn* I (Figure 9, lanes 10 and 11), produced two bands at ~4.0 kb and ~7.4 kb. Both bands were expected because probe 4 contains *E9* sequence that hybridized to both the ~4.0 kb and the ~7.4 kb fragments from the digested plasmid (refer to Figure 5 and Table 3). These results indicate that the probe is hybridizing to its target sequence.

MON 87708 DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II and hybridized with probe 4 (Figure 4) produced one unique band at ~1.5 kb (Figure 9, lanes 2 and 6). MON 87708 DNA digested with *Hpa* I/*Kpn* I and hybridized with probe 4 (Figure 4) produced one unique band at ~1.7 kb (Figure 9, lanes 4 and 8). Probe 4 contains the *E9* 3′ non-translated region sequence that is also contained in T-DNA I (Figure 4). Therefore, probe 4 was expected to hybridize to the ~1.5 kb and ~1.7 kb fragments (Figure 5) derived from the T-DNA I insert. These bands were also detected by probe 10 (Figure 8, lanes 2 and 6, and lanes 4 and 8). Any T-DNA II sequences other than those associated with T-DNA I would be detected as novel bands. No unexpected bands were detected indicating that MON 87708 contains no detectable T-DNA II elements covered by probe 4.

Probe 5

Conventional control DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II (Figure 10, lanes 1 and 5) or *Hpa* I/*Kpn* I (Figure 10, lanes 3 and 7) and hybridized with probe 5 (Figure 4) showed no detectable hybridization bands, as expected for the negative control. PV-GMHT4355, previously digested with *Aat* II/*Nde* I and mixed with conventional control DNA predigested with *Hpa* I/*Kpn* I (Figure 10, lanes 10 and 11), produced two expected size bands at ~4.0 kb and ~7.4 kb (refer to Figure 5 and Table 3). These results indicate that the probe is hybridizing to its target sequence.

MON 87708 DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II (Figure 10, lanes 2 and 6) or *Hpa* I/*Kpn* I (Figure 10, lanes 4 and 8) and hybridized with probe 5, produced no detectable hybridization bands. These results indicate that MON 87708 contains no detectable T-DNA II elements covered by probe 5.

Probe 6

Conventional control DNA digested with *Bsp1286* I/*Pvu* II (Figure 11, lanes 1 and 5) or *Hpa* I/*Kpn* I (Figure 11, lanes 3 and 7) and hybridized with probe 6 (Figure 4) showed no detectable hybridization bands, as expected for the negative control. PV-GMHT4355 previously digested with *Aat* II/*Nde* I and mixed with conventional control DNA predigested with *Hpa* I/*Kpn* I (Figure 11, lanes 10 and 11) produced one expected size band at ~7.4 kb (refer to Figure 5, and Table 3). These results indicate that the probe is hybridizing to its target sequence.

MON 87708 DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II (Figure 11, lanes 2 and 6) or *Hpa* I/*Kpn* I (Figure 11, lanes 4 and 8) and hybridized with probe 6 produced no detectable hybridization bands. These results indicated that MON 87708 contains no detectable T-DNA II elements covered by probe 6.

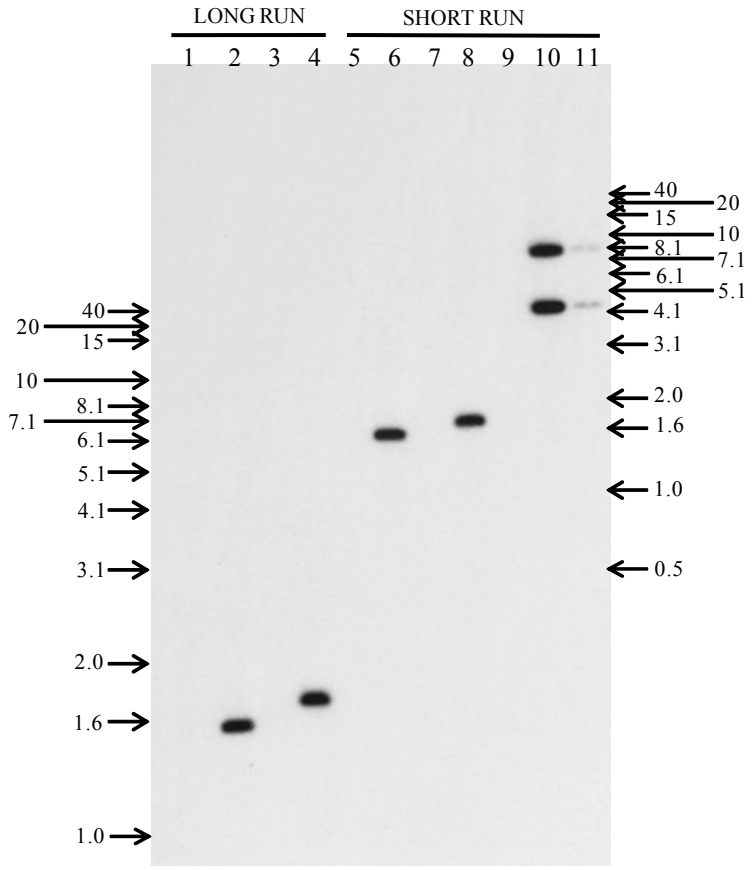


Figure 9. Sothern Blot Analysis to Detect the Presence or Absence of T-DNA II Sequences in MON 87708: Probe 4

The blot was hybridized with a ^{32}P labeled T-DNA II probe that spans a portion of the T-DNA II sequence (Probe 4, Figure 4). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane	Description
1.	Conventional control (*Bsp1286* I/*Pvu* II)
2.	MON 87708 (*Bsp1286* I/*Pvu* II)
3.	Conventional control (*Hpa* I/*Kpn* I)
4.	MON 87708 (*Hpa* I/*Kpn* I)
5.	Conventional control (*Bsp1286* I/*Pvu* II)
6.	MON 87708 (*Bsp1286* I/*Pvu* II)
7.	Conventional control (*Hpa* I/*Kpn* I)
8.	MON 87708 (*Hpa* I/*Kpn* I)
9.	Blank
10.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~1 genome equivalent]
11.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~0.1 genome equivalent]

Arrows denote sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

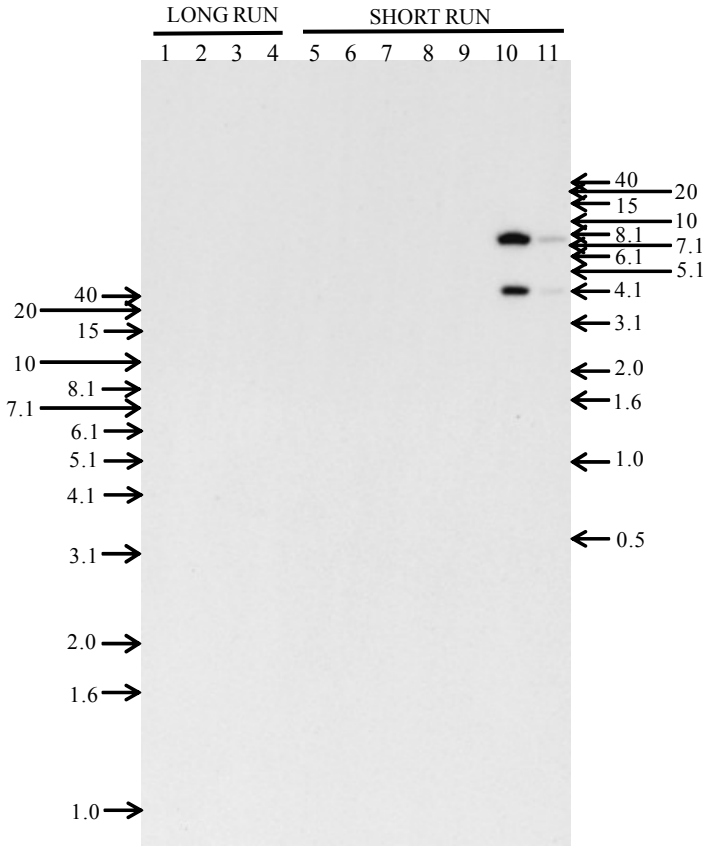


Figure 10. Sothern Blot Analysis to Detect the Presence or Absence of T-DNA II Sequences in MON 87708: Probe 5

The blot was hybridized with a ^{32}P labeled T-DNA II probe that spans the coding region of the T-DNA II sequence (Probe 5, Figure 4). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane	Description
1.	Conventional control (*Bsp1286* I/*Pvu* II)
2.	MON 87708 (*Bsp1286* I/*Pvu* II)
3.	Conventional control (*Hpa* I/*Kpn* I)
4.	MON 87708 (*Hpa* I/*Kpn* I)
5.	Conventional control (*Bsp1286* I/*Pvu* II)
6.	MON 87708 (*Bsp1286* I/*Pvu* II)
7.	Conventional control (*Hpa* I/*Kpn* I)
8.	MON 87708 (*Hpa* I/*Kpn* I)
9.	Blank
10.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~1 genome equivalent]
11.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~0.1 genome equivalent]

Arrows denote sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

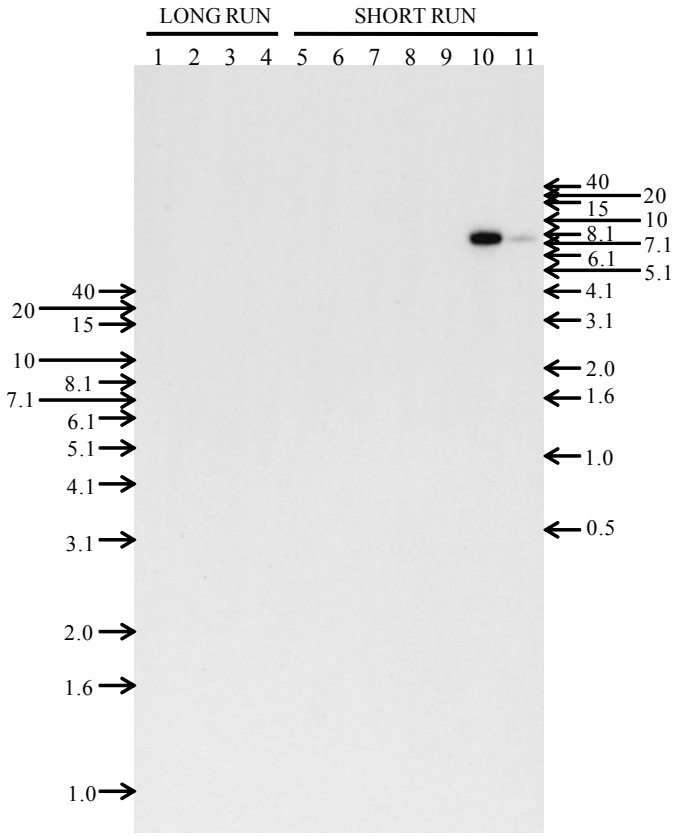


Figure 11. Sothern Blot Analysis to Detect the Presence or Absence of T-DNA II Sequences in MON 87708: Probe 6

The blots were hybridized with a ^{32}P labeled T-DNA II probe that spans a portion of the T-DNA II sequence (Probe 6, Figure 4). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane	Description
1.	Conventional control (*Bsp1286* I/*Pvu* II)
2.	MON 87708 (*Bsp1286* I/*Pvu* II)
3.	Conventional control (*Hpa* I/*Kpn* I)
4.	MON 87708 (*Hpa* I/*Kpn* I)
5.	Conventional control (*Bsp1286* I/ *Pvu* II)
6.	MON 87708 (*Bsp1286* I/*Pvu* II)
7.	Conventional control (*Hpa* I/*Kpn* I)
8.	MON 87708 (*Hpa* I/*Kpn* I)
9.	Blank
10.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~1 genome equivalent]
11.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~0.1 genome equivalent]

Arrows denote sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

3) Southern Blot Analysis to Determine the Presence or Absence of Plasmid Vector PV-GMHT4355 Backbone Sequences in MON 87708

To determine the presence or absence of PV-GMHT4355 backbone sequences, MON 87708 and conventional control genomic DNA were digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II or *Hpa* I/*Kpn* I and Southern blots were hybridized with probes that span the plasmid vector backbone sequence (Figure 4). As a positive control on the Southern blots, digested PV-GMHT4355 and probe templates generated from PV-GMHT4355 were used. Approximately 1 genome equivalent of PV-GMHT4355 digested with the restriction enzyme combination *Aat* II/*Nde* I was mixed with predigested conventional control DNA. As an additional positive control, approximately 0.1 and 1 genome equivalent of probe templates (Figure 4, probes 1, 2, 3, and 7) generated from PV-GMHT4355 were mixed with predigested conventional control DNA. The blot was hybridized with probes 1, 2, 3, and 7 (Figure 4). If backbone sequences are present in MON 87708, then probing with backbone probes should result in hybridizing bands. The results of this analysis are shown in Figure 12.

Plasmid Vector Backbone Probes 1, 2, 3, and 7

Conventional control DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II (Figure 12, lanes 1 and 5) or *Hpa* I/*Kpn* I (Figure 12, lanes 3 and 7) and hybridized simultaneously with the probes 1, 2, 3, and 7 (Figure 4) spanning the entire backbone sequence of PV-GMHT4355 showed no detectable hybridization bands, as expected for the negative control. PV-GMHT4355, previously digested with *Aat* II/*Nde* I and mixed with conventional control DNA predigested with *Hpa* I/*Kpn* I (Figure 12, lane 10), produced two expected size bands at ~4.0 kb and ~7.4 kb (refer to Figure 5 and Table 3). In addition, there are two faint hybridization bands at ~4.5 kb and ~11 kb (Figure 12, lane 10). The ~4.5 kb band was likely due to an artifact that occurred during the electrophoresis, and the ~11 kb band was likely due to undigested plasmid DNA or an artifact that occurred during the electrophoresis. Since these faint bands appeared only in the plasmid spike and the expected bands were observed, they have no negative impact on the conclusions made from this blot. Probe template spikes of probes 1, 2, 3, and 7 (Figure 4) generated from PV-GMHT4355 mixed with conventional control DNA predigested with *Hpa* I/*Kpn* I (Figure 12, lanes 11 and 12) produced the expected size bands at ~0.2 kb, ~0.9 kb, ~1.5 kb, and ~1.8 kb, respectively. The 0.1 genome equivalent copy of the expected ~0.2 kb band was not observed on the exposure of the Southern blot that is reported in Figure 12, lane 12; however, the band was observed on the same blot with a longer exposure. These results indicate that the probes are hybridizing to their target sequences.

MON 87708 DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II (Figure 12, lanes 2 and 6) or *Hpa* I/*Kpn* I (Figure 12, lanes 4 and 8) and hybridized simultaneously with probes 1, 2, 3, and 7 produced no detectable bands. The data indicate MON 87708 contains no detectable backbone sequences from PV-GMHT4355.

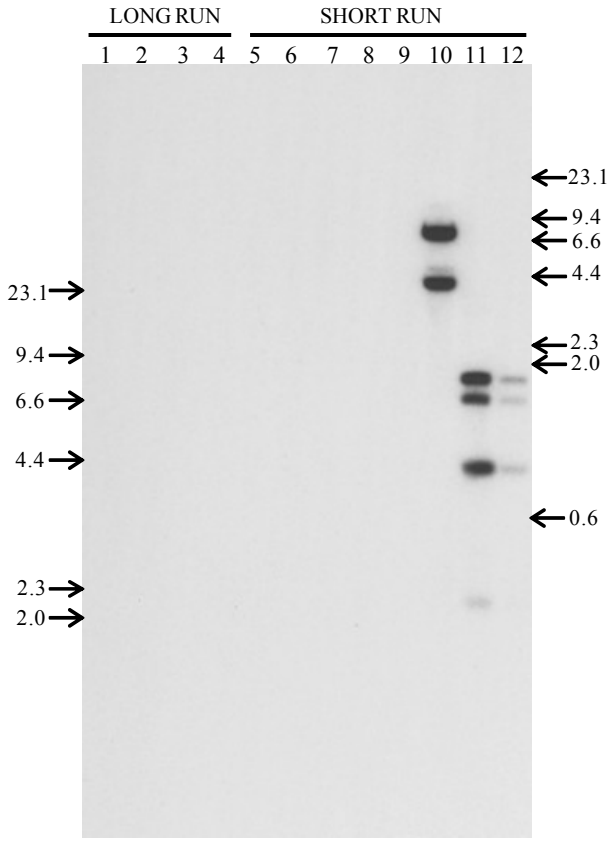


Figure 12. Southern Blot Analysis to Determine the Presence or Absence of PV-GMHT4355 Backbone Sequences in MON 87708: Probes 1, 2, 3, and 7

The blot was hybridized simultaneously with four ^{32}P labeled backbone probes (Probes 1, 2, 3, and 7, Figure 4). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane	Description
1.	Conventional control (*Bsp1286* I/*Pvu* II)
2.	MON 87708 (*Bsp1286* I/*Pvu* II)
3.	Conventional control (*Hpa* I/*Kpn* I)
4.	MON 87708 (*Hpa* I/*Kpn* I)
5.	Conventional control (*Bsp1286* I/*Pvu* II)
6.	MON 87708 (*Bsp1286* I/*Pvu* II)
7.	Conventional control (*Hpa* I/*Kpn* I)
8.	MON 87708 (*Hpa* I/*Kpn* I)
9.	Blank
10.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~1 genome equivalent]
11.	Conventional control (*Hpa* I/*Kpn* I) spiked with probe templates [~1 genome equivalent]
12.	Conventional control (*Hpa* I/*Kpn* I) spiked with probe templates [~0.1 genome equivalent]

Arrows denote sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

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

The organization of the elements within the T-DNA I was confirmed by DNA sequence analyses. PCR primers were designed with the intent to amplify two overlapping regions of the DNA that span the entire length of T-DNA I (Figure 13). The amplified DNA segments were subjected to DNA sequencing analyses. The T-DNA I in MON 87708 is 3003 bp and matches the sequence of plasmid vector PV-GMHT4355, as described in Table 2 and Table 4. Please refer to a report for insert and adjacent DNA sequences in MON 87708 (**CBI**).

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

PCR and sequence analyses were performed on genomic DNA extracted from MON 87708 and a conventional control to examine the insertion sites. The PCR was performed with one primer specific to the genomic DNA sequence flanking the 5′ end of T-DNA I paired with a second primer specific to the genomic DNA sequence flanking the 3′ end of T-DNA I (Figure 14). A sequence comparison between the PCR product generated from the conventional control and the sequence generated from the 5′ and 3′ flanking sequences of T-DNA I in MON 87708 indicates there was an 899 bp deletion and a 128 bp insertion just 5′ of T-DNA I, and a 35 bp insertion just 3′ of T-DNA I. These molecular rearrangements presumably resulted from double stranded break repair mechanisms in the plant during the *Agrobacterium* mediated transformation process process (Salomon and Puchta, 1998).

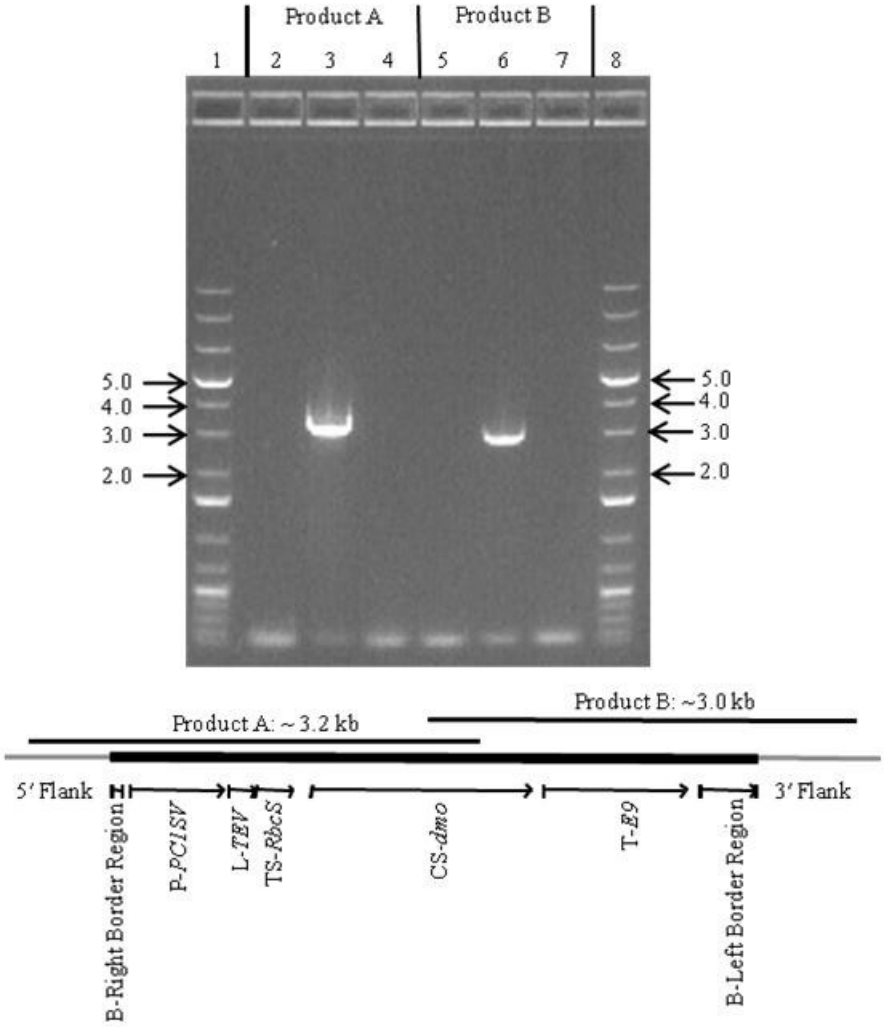


Figure 13. Overlapping PCR Analysis Across the Insert in MON 87708

PCR analyses were performed on MON 87708 genomic DNA extracted from leaf (Lanes 3 and 6). Lanes 2 and 5 contain reactions with conventional control DNA while lanes 4 and 7 are reactions containing no template DNA. Lanes 1 and 8 contain Fermentas GeneRuler™ 1 kb Plus DNA Ladder. Lanes are marked to show which product has been loaded and is visualized on the agarose gel. The expected product size for each amplicon is provided in the illustration of the insert in MON 87708 that appears at the bottom of the figure. Five microliters of each of the PCR products was loaded on the gel. This figure is representative of the data generated; however, the specific bands from this gel were not excised and sequenced.

Lane:

1. GeneRuler™ 1 kb Plus DNA Ladder
2. Conventional control DNA
3. MON 87708 genomic DNA
4. No template DNA control
5. Conventional control DNA
6. MON 87708 genomic DNA
7. No template DNA control
8. GeneRuler™ 1 kb Plus DNA Ladder

Arrows denote sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel

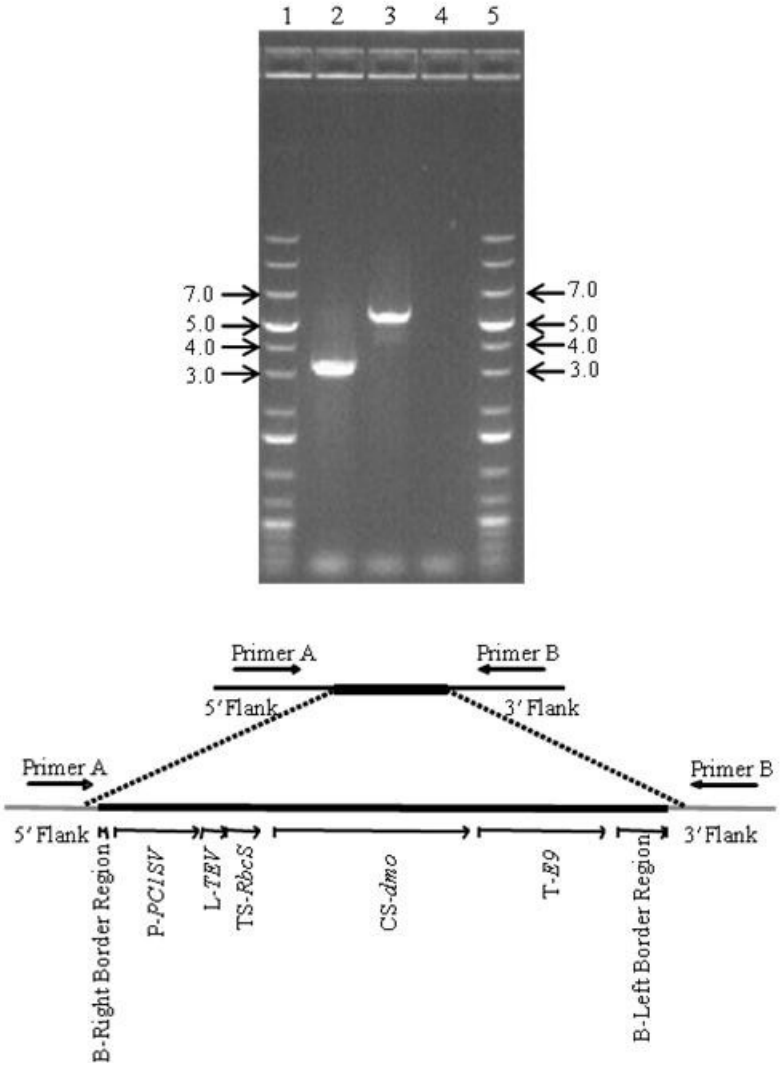


Figure 14. PCR Amplification of the MON 87708 Insertion Site

Depiction of the MON 87708 insertion locus in conventional control and MON 87708. PCR amplification was performed using Primer A in the 5′ flanking sequence and Primer B in the 3′ flanking sequence of the insert in MON 87708 to examine the insertion site in conventional soybean and MON 87708.

Lane	Description
1	GeneRuler™ 1 kb Plus DNA Ladder
2	Conventional control DNA
3	MON 87708 genomic DNA
4	No template DNA control
5	GeneRuler™ 1 kb Plus DNA Ladder

Arrows denote sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

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

The 2003 Codex Alimentarius Commission guidelines for the safety assessment of food derived from biotechnology crops (Codex Alimentarius, 2003) includes an assessment element on the identification and evaluation of "open reading frames within the inserted DNA or created by the insertion with contiguous plant genomic DNA". These assessments examine the potential homology of any putative polypeptides or proteins that could be produced from open reading frames (ORFs) in the insert or at the plant-insert junction to known toxins or allergens. These analyses are conducted even if there is no evidence that such ORFs at the plant-insert junction or alternative reading frames in the insert are capable of being transcribed or translated into a protein. Results from these bioinformatics analyses demonstrate that any putative polypeptides in MON 87708 are unlikely to exhibit allergenic, toxic or otherwise biologically adverse properties.

In addition to the bioinformatic analysis conducted on MON 87708 DMO (Sections B3(a) and B4(b)), bioinformatic analyses were also performed on the MON 87708 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 87708 insert DNA as well as ORFs present in the 5' and 3' inserted DNA-5' and 3' flanking sequence junctions (Table 4 and Figure 15). These various bioinformatic evaluations are depicted in Figure 15. ORFs spanning the 5' flanking sequence DNA-inserted DNA junctions, and 3' flanking sequence DNA-inserted DNA junctions were translated from stop codon to stop codon in all six reading frames (three forward reading frames and three reading frames in reverse orientation). Putative peptides/polypeptides from each reading frame were then compared to toxin, allergen and all proteins databases using bioinformatic tools. Similarly, the entire MON 87708 insert DNA sequence was translated in all six reading frames (three forward reading frames and three reading frames in reverse orientation) and the resulting deduced amino acid sequence was subjected to bioinformatic analyses. There are no analytical data that indicate any putative polypeptides/proteins subjected to bioinformatic evaluation other than the MON 87708 DMO which is part of the insert DNA sequence analysis are produced. Moreover, the data generated from these analyses confirm that even in the highly unlikely occurrence that a translation product other than MON 87708 DMO was derived from frames 1 to 6 of the insert DNA, or the ORFs spanning the insert junctions; they would not share a sufficient degree of sequence similarity with other proteins to indicate they would be potentially allergenic, toxic, or have other safety implications. Therefore, there is no evidence for concern regarding the putative polypeptides for MON 87708 relatedness to known toxins, allergens, or biologically active putative peptides.

Bioinformatics Assessment of Insert DNA Reading Frames

Bioinformatic analyses were performed to assess the potential of toxicity, allergenicity or biological activity of any putative peptides encoded by translation of reading frames 1 through 6 of the inserted DNA in MON 87708 (Figure 15).

The FASTA sequence alignment tool was used to assess structural relatedness between the query sequences and any protein sequences in the AD_2010, TOX_2010, and PRT_2010 databases. Structural similarities shared between each putative polypeptide with each sequence in the database were examined. The extent of structural relatedness was evaluated by detailed visual inspection of the alignment, the calculated percent identity and alignment length as 35% or greater identity in 80 or greater amino acids (to ascertain if alignments

exceeded Codex (Codex Alimentarius, 2003) thresholds for FASTA searches of the AD_2010 database), and the *E*-score. Alignments having 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_2010 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_2010) or toxin (TOX_2010) databases. Furthermore, no short (eight amino acid) polypeptide matches were shared between any of the putative polypeptides and proteins in the allergen database.

When used to search the PRT_2010 database, translations of frames 1-4 yielded alignments with *E*-scores less than or equal to a 1×10^{-5} threshold. Inspections of frame 1, 2, and 4 alignments revealed that they were punctuated with numerous stop codons in the query sequence and required numerous gaps to optimize the alignment. As a result, it is unlikely these alignments reflect conserved structure. When used as a query in a FASTA search of the PRT_2010 database, the translation of frame 3 yielded numerous alignments with *E*-scores less than or equal to the 1×10^{-5} threshold. The top three alignments displayed 99.7% identity over 338 amino acids with an oxygenase from *S. maltophilia*. In addition, a second group of alignments between frame 3 and ribulose 1,5-bisphosphate were observed. These frame 3 alignments positively identify MON 87708 DMO and the associated chloroplast targeting peptide, and the alignments are consistent with the known structure of protein coding sequence contained in the MON 87708 inserted DNA.

Taken together, these data demonstrate the lack of relevant similarities between known allergens or toxins for putative peptides derived from all six reading frames from the inserted DNA sequence of MON 87708. As a result, in the unlikely event that a translation product other than MON 87708 DMO and the associated chloroplast targeting peptide was derived from reading frames 1 to 6, these putative polypeptides are not expected to be cross-reactive allergens, toxins, or display adverse biological activity.

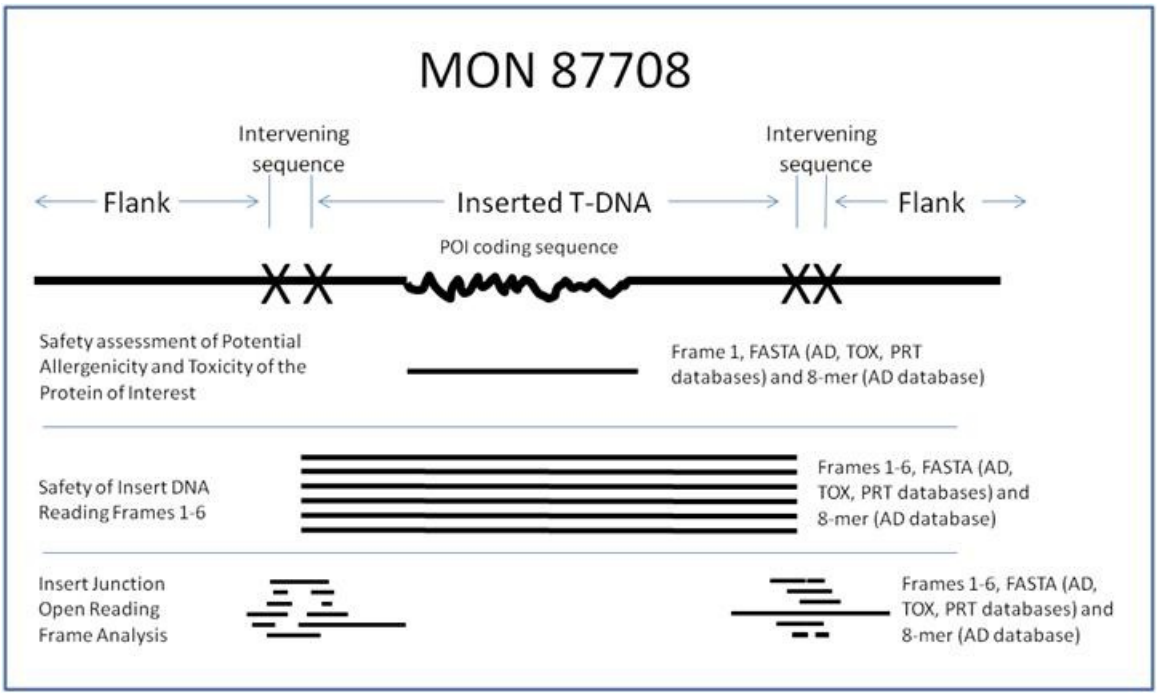
Insert Junction Open Reading Frame Bioinformatics Analysis

Analyses of putative polypeptides encoded by DNA spanning the 5′ and 3′ genomic junctions of the MON 87708 inserted DNA were performed using a bioinformatic comparison strategy (Figure 15). The purpose of the assessment is to evaluate the potential for novel open reading frames (ORFs) that may have homology to known allergens, toxins, or proteins that display adverse biological activity. Sequences spanning the 5′ flanking sequence DNA-inserted DNA and the inserted DNA-3′ flanking sequence DNA (Figure 15) were translated from stop codon (TGA, TAG, TAA) to stop codon in all six reading frames. The resulting putative polypeptides from each reading frame, that were eight amino acids or greater in length, were compared to AD_2010, TOX_2010, and PRT_2010 databases using FASTA and to the AD_2010 database using an eight amino acid sliding window search.

The FASTA sequence alignment tool was used to assess structural relatedness between the query sequences and protein sequences in the AD_2010, TOX_2010, and PRT_2010 databases. Structural similarities shared between each putative polypeptide with each

sequence in the database were examined. The extent of structural relatedness was evaluated by detailed visual inspection of the alignment, the calculated percent identity and the alignment length (to ascertain if alignments exceeded Codex Alimentarius (2003) thresholds for FASTA searches of the AD_2010 database), and the *E*-score. In addition to structural similarity, each putative polypeptide was screened for short polypeptide matches using a pair-wise comparison algorithm. In these analyses, eight contiguous and identical amino acids were defined as immunologically relevant, where eight represents the typical minimum sequence length likely to represent an immunological epitope, and evaluated against the AD_2010 database.

No biologically relevant structural similarity to known allergens or toxins was observed for any of the putative polypeptides. Furthermore, no short (eight amino acid) polypeptide matches were shared between any of the putative polypeptides and proteins in the allergen database. As a result, in the unlikely event that a translation product was derived from DNA spanning the 5′ or 3′ genomic DNA-insert DNA junctions of MON 87708, these putative polypeptides are not expected to be allergens, toxins, or display adverse biological activity.



AD= AD_2010; TOX= TOX_2010 and PRT= PRT_2010 (GenBank release 175): 8-mer = the eight amino acid sliding window search. POI Coding sequence corresponds to the *dmo* coding sequence.

Figure 15. Schematic Summary of MON 87708 Bioinformatic Analyses

A3(e) Family tree or breeding map

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

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

Inheritance of the Genetic Insert in MON 87708

During development of MON 87708, segregation data were generated to assess the heritability and stability of the T-DNA I present in MON 87708. Chi square analysis was performed over several generations to confirm the segregation and stability of T-DNA I in MON 87708. The Chi square analysis is based on testing the observed segregation ratio to the expected segregation ratio according to Mendelian principles.

The MON 87708 breeding path, from which segregation data were generated, is described in Figure 16. The transformed R_0 plant was self-pollinated to produce R_1 seed. An individual plant (#2, designated as MON 87708), that was homozygous for a single copy of the *dmo* expression cassette, was identified from the R_1 segregating population via Invader® and Southern blot analysis. Invader is a non-PCR based assay that can be used to accurately quantify transgene copy number in plant genomes (Gupta et al., 2008).

The selected R_1 MON 87708 plant was self-pollinated to give rise to a population of R_2 plants that were repeatedly self-pollinated through the R_4 generation. At each generation, the fixed homozygous plants were tested for the expected segregation pattern of 1:0 (positive:negative) for the *dmo* expression cassette using the Invader analysis, Southern blot analysis, and/or PCR.

At the R_4 generation, homozygous MON 87708 plants were bred via traditional breeding with a soybean variety that did not contain the *dmo* expression cassette to produce F_1 hemizygous seed. The resulting F_1 plants were then self-pollinated to produce F_2 seed. The F_2 plants were tested for the presence of the *dmo* expression cassette by Invader analysis, and hemizygous F_2 plants were selected and self-pollinated to produce F_3 seed. This process was repeated through the F_4 generation. The heritability and stability of the *dmo* expression cassette in MON 87708 was assessed in the F_2, F_3, and F_4 generations. A total of 2413 out of 3223 plants were positive for the presence of the *dmo* expression cassette in the F_2 generation; however, the zygosity of 200 of those 2413 plants could not be determined from the assay. Exclusion of these *dmo*-positive plants from the analysis likely would have skewed the distribution of homozygous positive: hemizygous positive:homozygous negative plants. Therefore, the segregation assessment in the F_2 generation was based on the presence or absence of the *dmo* expression cassette which was expected to segregate at a 3:1 (positive:negative) ratio according to Mendelian inheritance principles. Subsequently, assessment of segregation in the F_3 and F_4 generations was based on zygosity, and the *dmo* expression cassette was predicted to segregate at a 1:2:1 (homozygous positive:hemizygous positive :homozygous negative) ratio according to Mendelian inheritance principles.

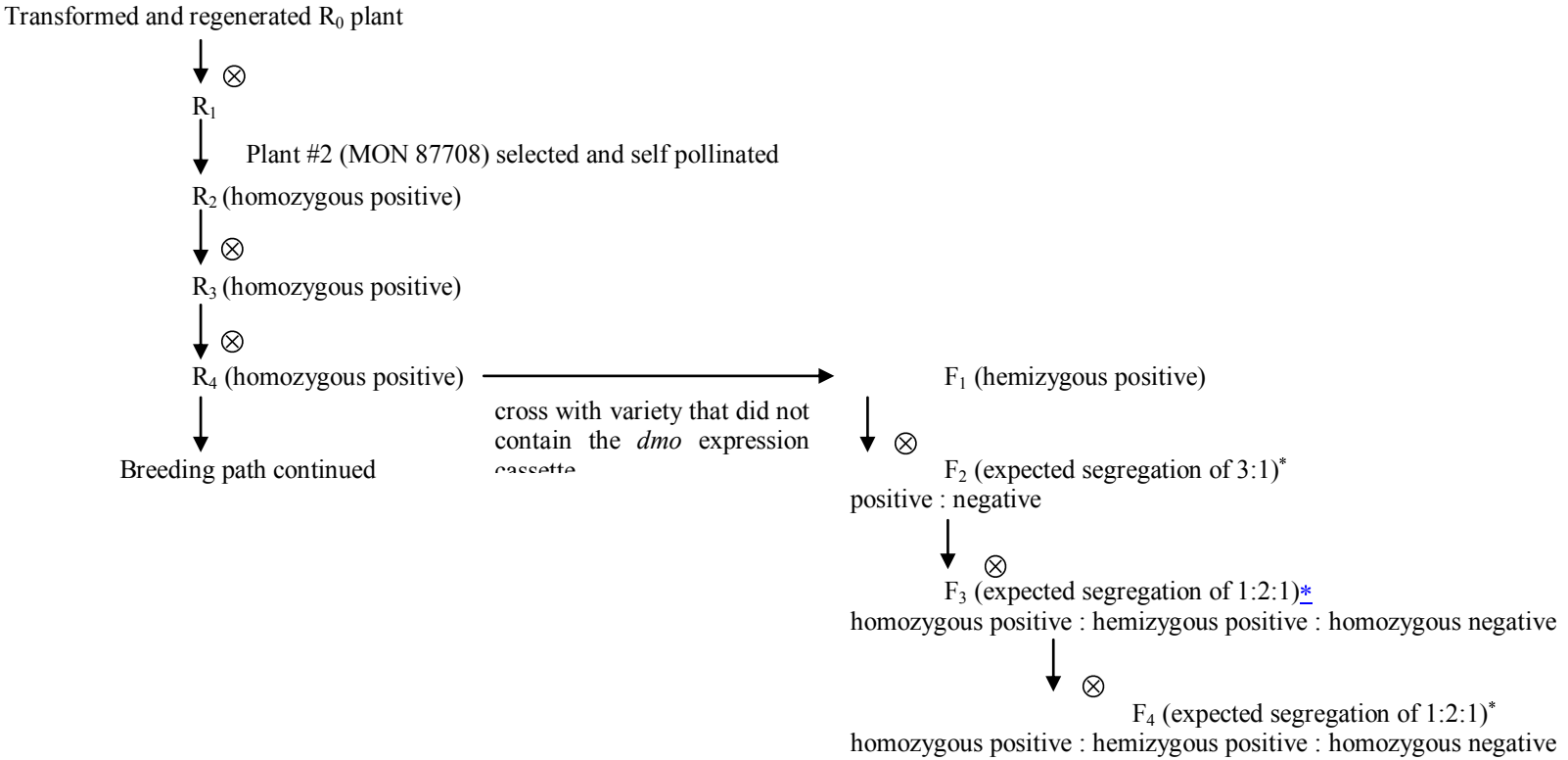
A Chi square ($\chi 2$) analysis was used to compare the observed segregation ratios to the expected ratios according to Mendelian inheritance principles. The $\chi 2$ was calculated as:

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

where o = observed frequency of the phenotype and e = expected frequency of the phenotype. The level of statistical significance was predetermined to be 5%.

The results of the $\chi 2$ analysis of the segregating progeny of MON 87708 are presented in Table 5. The $\chi 2$ value for the F_2, F_3, and F_4 generations indicated no significant difference

between the observed and expected segregation ratios. These results support the conclusion that the *dmo* expression cassette in MON 87708 resides at a single locus within the soybean genome and is inherited according to expected Mendelian inheritance principles. These results are also consistent with the molecular characterization data that indicate MON 87708 contains a single, intact copy of the *dmo* expression cassette that was inserted into the soybean genome at a single locus.



⊗ = Self pollinated

Figure 16. Breeding Path for Generating Segregation Data for MON 87708

* Chi-square analysis conducted on segregation data from the F_2, F_3, and F_4 generations.
Note: Hemizygous positive plants in the F_1, F_2, F_3, and F_4 generations were selected and self-pollinated to produce seed of the subsequent generation.

Table 5. Segregation of the *dmo* Gene During the Development of MON 87708

				3:1 Segregation[3]			
Generation[1]	Total Plants Tested[2]	Observed # Plants Positive	Observed # Plants Negative	Expected # Plants Positive	Expected # Plants Negative	χ^2	Probability
F_2	3223	2413	810	2417.25	805.75	0.03	0.863

					1:2:1 Segregation				
Generation[1]	Total Plants Tested[2]	Observed # Plants Homozygous Positive	Observed # Plants Hemizygous Positive	Observed # Plants Homozygous Negative	Expected # Plants Homozygous Positive	Expected # Plants Hemizygous Positive	Expected # Plants Homozygous Negative	χ^2	Probability
F_3	118	29	52	37	29.5	59	29.5	2.7	0.2534
F_4	343	83	171	89	85.75	171.5	85.75	0.2	0.8991

[1]F_2, F_3, and F_4 progeny were from self-pollinated F_1, F_2, and F_3 plants hemizygous positive for the *dmo* expression cassette, respectively.
[2]Plants were tested for the presence of the *dmo* expression cassette by Invader analysis.
[3]Assessment of segregation in the F_2 generation was based on the presence or absence of the *dmo* expression cassette due to an unacceptable number of *dmo*-positive plants for which zygosity could not be determined from the assay.

Southern Blot Analysis to Examine Insert Stability in Multiple Generations of MON 87708

In order to demonstrate the stability of the T-DNA I insert present in MON 87708 through multiple generations, Southern blot analysis was performed using DNA obtained from five breeding generations of MON 87708. For reference, the breeding history of MON 87708 is presented in Figure 17. The specific generations tested are indicated in the legend of Figure 17. The R_3 generation was used for the molecular characterization analyses shown in Figure 6 through Figure 12. To analyze stability, four additional generations were evaluated by Southern blot analysis and compared to the fully characterized R_3 generation. Genomic DNA, isolated from each of the selected generations of MON 87708 and the conventional control, was digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II (Figure 5) and hybridized with probe 9 (Figure 4). Probe 9 will detect both border fragments generated by the *Bsp1286* I/*Pvu* II digestion. Any instability associated with the T-DNA I insert would be detected as novel bands within the fingerprint on the Southern blot. The Southern blot has the same positive hybridization controls as described in Section A3(d)(ii). The results are shown in Figure 18.

Probe 9

Conventional control DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II produced no hybridization signals (Figure 18, lane 1) as expected for the negative control. PV-GMHT4355, digested with the restriction enzyme combination *Aat* II/*Nde* I and mixed with conventional control DNA predigested with the restriction enzyme combination *Bsp1286* I/*Pvu* II (Figure 18, lanes 8 and 9), produced the expected size band at ~7.4 kb (refer to Figure 5 and Table 3). Additionally, there were two very faint hybridization bands in the ~1 genome equivalent plasmid vector PV-GMHT4355 spike at ~4.3 kb and ~6.5 kb observed in a longer exposure of the Southern blot (data not shown). These bands were likely due to an artifact that occurred during the electrophoresis. Since these faint bands appeared only in the plasmid vector spike and the expected ~7.4 kb band was observed, they do not have any negative impact on the conclusions from this Southern blot analysis. These results indicate that the probe is hybridizing to its target sequence.

Digestion of MON 87708 genomic DNA from multiple generations with the restriction enzyme combination *Bsp1286* I/*Pvu* II and hybridized with probe 9 (Figure 4) produced two bands at ~1.5 kb and ~2.6 kb (Figure 18, lanes 2-6). The ~1.5 kb band is the expected size for the border segment containing the 3′ end of T-DNA I along with the adjacent genomic DNA flanking the 3′ end of T-DNA I (Figure 5). The ~2.6 kb band is the expected size for the border segment containing the 5′ end of T-DNA I along with the adjacent genomic DNA flanking the 5′ end of T-DNA I (Figure 5). The fingerprint of the Southern blot signals from multiple generations, R_2, R_4, R_5, and R_6 (Figure 18, lanes 2, 4, 5, and 6), of MON 87708 is consistent with the fully characterized generation R_3 (Figure 7, lanes 2 and 6; Figure 18, lane 3). No unexpected bands were detected, indicating that MON 87708 contains one copy of T-DNA I that is stably maintained across multiple generations.

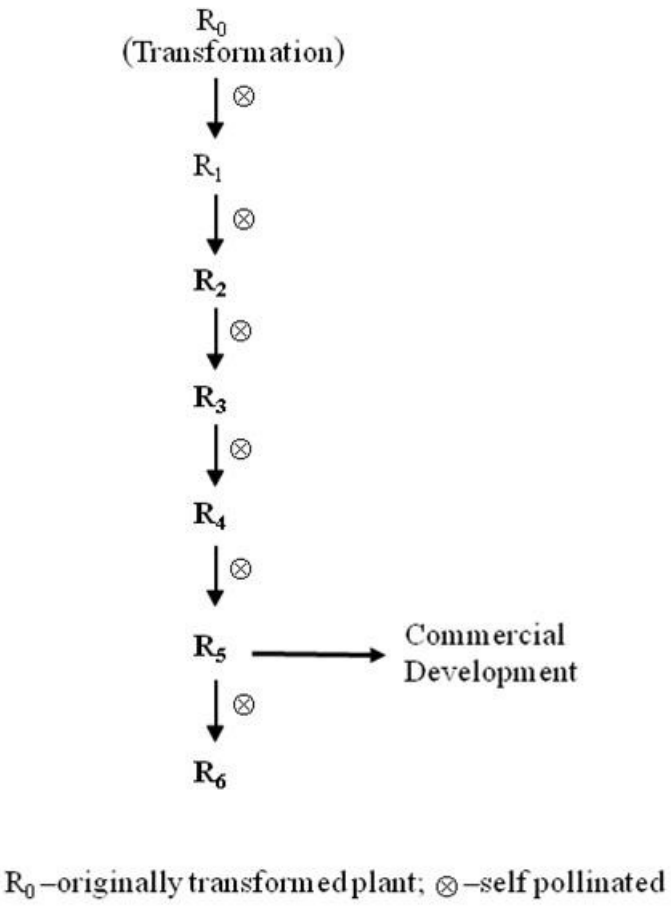


Figure 17. Breeding History of MON 87708

The R_3 generation was used for the molecular analyses reported in Figure 6 through Figure 12 and is referred to as MON 87708 in all Southern blot figures. The R_5 generation was used for development of all commercial products. MON 87708 from generations R_2, R_3, R_4, R_5, and R_6 (bolded in the breeding tree) were used for analyzing the stability of T-DNA I in MON 87708 across generations (Figure 18).

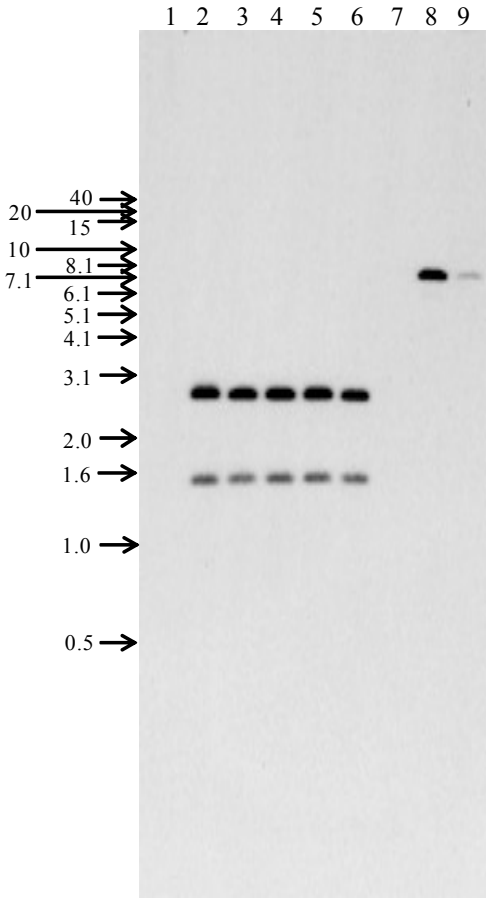


Figure 18. Southern Blot Analysis to Examine Insert Stability in Multiple Generations of MON 87708: Probe 9

The blot was hybridized with a ^{32}P labeled T-DNA I probe that spans the coding region of the T-DNA I (Probe 9, Figure 4). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane	Description
1.	Conventional control (*Bsp1286* I/*Pvu* II)
2.	R_2 generation of MON 87708 (*Bsp1286* I/*Pvu* II)
3.	R_3 generation of MON 87708 (*Bsp1286* I/*Pvu* II)
4.	R_4 generation of MON 87708 (*Bsp1286* I/*Pvu* II)
5.	R_5 generation of MON 87708 (*Bsp1286* I/*Pvu* II)
6.	R_6 generation of MON 87708 (*Bsp1286* I/*Pvu* II)
7.	Blank
8.	Conventional control (*Bsp1286* I and *Pvu* II) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~1 genome equivalent]
9.	Conventional control (*Bsp1286* I and *Pvu* II) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~0.1 genome equivalent]

Arrows denote sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

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

In order to confirm the presence of MON 87708 DMO across multiple generations, western blot analysis of MON 87708 DMO was conducted on leaf tissue collected from generations R_2, R_3, R_4, R_5, and R_6 (Figure 19) of MON 87708, and on leaf tissue of the near isogenic conventional soybean control A3525. As previously described, this document refers to both the MON 87708 DMO protein, the MON 87708 DMO+27 protein, and all forms of the trimer, collectively, as MON 87708 DMO.

MON 87708 DMO was first separated on a denaturing SDS-PAGE and then transferred to a PVDF membrane for immunoblot analysis. The presence of the MON 87708 DMO protein and MON 87708 DMO+27 protein in harvested leaf tissue of the R_2, R_3, R_4, R_5, and R_6 generations of MON 87708 was demonstrated (Figure 19). An *E. coli*-produced DMO standard (10 ng and 5 ng) was used as a reference for the identification of MON 87708 DMO, and the conventional leaf tissue extract was used as the negative control. The presence of MON 87708 DMO in leaf tissue was determined by visual comparison of the bands produced in the multiple generations (Figure 19, lanes 5-9) to the *E. coli*-produced DMO reference standard (Figure 19, lanes 2-3). The MON 87708 DMO protein is clearly observed in all generations migrating to the same position on the immunoblot as the *E.coli*-produced DMO reference standard. The MON 87708 DMO+27 protein is not present in the *E. coli*-produced DMO reference standard for comparison, however, a band was observed for all MON 87708 samples at the expected molecular weight of ~42.0 kDa indicating the presence of the MON 87708 DMO+27 protein in all five generations of MON 87708 harvested leaf tissue samples. A more intense band was observed for the R_3 generation (Figure 19, lane 6) when compared to the other four generations (R_2, R_4, R_5, and R_6; Figure 19, lanes 5, 7-9, respectively). This difference was likely due to the R_3 generation being grown separately from and sampled at a later growth stage than the R_2, R_4, R_5, and R_6 generations. As expected, MON 87708 DMO was not detected in the conventional control leaf tissue (Figure 19, lane 4).

On the western blot an additional band was observed at approximately 50 kDa in both MON 87708 and control leaf tissue samples for all generations (Figure 19, lanes 5-9 and 4, respectively). This is likely the result of non-specific binding of either the primary or secondary antibody to a protein endogenous to soybean leaves. This band appeared more intense in the R_3 generation of MON 87708 and the conventional control (Figure 19, lanes 6 and 4, respectively). This may be explained by the fact that the conventional control and R_3 generation material were from the same greenhouse production, while material from the R_2, R_4, R_5, and R_6 generations was collected from a different greenhouse production. Additional bands are also visible, and it is also likely that they are the result of non-specific binding of either the primary or secondary antibody to a protein endogenous to soybean leaves.

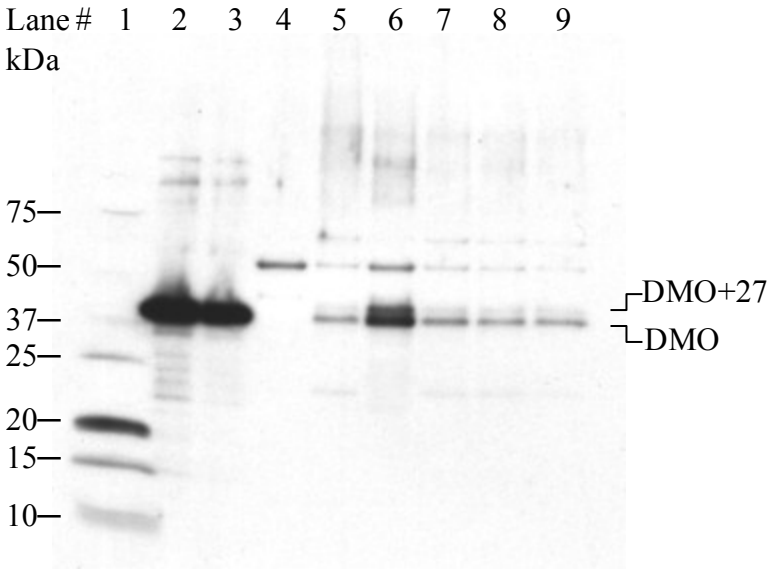


Figure 19. Presence of MON 87708 DMO in Multiple Generations of MON 87708

Aliquots of extracts from five generations of MON 87708 leaf tissues and molecular weight markers were separated by SDS-PAGE and electrotransferred to a PVDF membrane. The membrane was incubated with goat anti-DMO antibody. The image represents a 30 second exposure.

Lane	Description	Amount Loaded on Gel
1	Molecular Weight Marker	-
2	*E. coli*-produced DMO Protein Standard	10ng
3	*E. coli*-produced DMO Protein Standard	5 ng
4	A3525 Conventional Control	20 µl
5	R_2 Generation	20 µl
6	R_3 Generation	20 µl
7	R_4 Generation	20 µl
8	R_5 Generation	20 µl
9	R_6 Generation	20 µl

A4. Information on labelling of the GM food

A4(a) Whether novel DNA is likely to be present in the final food

As explained in section A2(b)(iv), there is no food use for unprocessed soybeans, since they contain anti-nutritive factors such as trypsin inhibitors and lectins (OECD, 2001). Adequate heat exposure as experienced during conventional food processing inactivates these factors as well as the DMO proteins produced in MON 87708. Conventional food processing of soybean would not be expected to remove the presence of soybean DNA from the final food product. It is anticipated that the novel DNA could also be present in food products. Where the novel DNA may be present in the final food product, the product will be labeled in accordance with the requirements of Standard 1.5.2 of the *Australia New Zealand Food Standards Code*.

A4(b) Detection methodology suitable for analytical purposes

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 87708.

B1 Antibiotic Resistance Marker Genes

No genes that encode resistance to an antibiotic marker were inserted into the soybean genome during the development of MON 87708. The backbone of the PV-GMHT4355 plasmid vector contained the *aadA* antibiotic resistant marker gene. Molecular characterization data presented in this application demonstrate the absence of the *aadA* antibiotic resistant marker gene in MON 87708.

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

Not applicable

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

Not applicable

B1(c) Safety of antibiotic protein

Not applicable

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

Not applicable

B2 Characterisation of novel proteins or other novel substances

B2(a) Biochemical function and phenotypic effects of novel protein(s)

Description of MON 87708 DMO

In MON 87708, the introduced DMO proteins are active in the chloroplast, a plastid organelle, where it can interact with other proteins needed for its function (Mode of Action; Behrens et al., 2007). In the construction of the plasmid vector used in the development of MON 87708, PV-GMHT4355, a transit peptide coding sequence (*RbcS,* Table 4) was joined to the *dmo* coding sequence to transport the produced protein to the soybean chloroplast; this coding sequence results in the production of a precursor protein consisting of the DMO protein, a chloroplast transit peptide (CTP), and an intervening sequence (IS), and is referred to as the MON 87708 DMO precursor protein. 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 DMO precursor protein produced in MON 87708.

MON 87708 contains a *dmo* expression cassette that encodes for a single MON 87708 DMO precursor protein targeted to the plant's chloroplasts. The MON 87708 DMO precursor protein contains 84 amino acids at the N-terminus of the protein that were added to target the protein to the chloroplast. These additional amino acids correspond to a 57 amino acid chloroplast transit peptide (CTP) from pea Rubisco small subunit and the first 24 amino acids from the N-terminus of the mature Rubisco small subunit, which are incorporated to improve the targeting of the precursor protein to the chloroplast (Behrens et al., 2007; Comai et al.,

1988). Finally, three amino acids encoded by an intervening sequence were used for cloning purposes (Table 2 and Table 4).

Analysis of mature seed extracts from MON 87708 by western blot demonstrated the presence of two immunoreactive bands (Figure 26). Characterization of these two bands (Section B2(b)) revealed that the precursor protein had been processed into two forms of the protein according to Figure 20. Analysis of these two bands determined that the lower molecular weight band corresponded to the full-length protein that was the result of the removal of the CTP, the 24 amino acids from pea Rubisco small subunit and the amino acids from the intervening sequence (Figure 20 and N-terminal sequence analysis). Additional processing at the N-terminus by methionine aminopeptidase removed the N-terminal methionine residue (N-terminal sequence analysis), a common occurence in all organisms (Arfin and Bradshaw, 1988; Bradshaw et al., 1998). This form of the protein will be referred to as the MON 87708 DMO protein; it has an apparent molecular weight of 39.8 kDa and is a single polypeptide chain of 339 amino acids (Figure 20 and Figure 21). Alternative processing of the MON 87708 DMO precursor protein resulted in the production of a second higher molecular weight (approximately 42 kDa) protein. The 42 kDa protein corresponds to the MON 87708 DMO protein plus 27 amino acids on the N-terminus originating from the pea Rubisco small subunit and intervening sequence that were not cleaved. Since the N-terminal methionine of the MON 87708 DMO protein is not exposed in this processed form of the protein it was not cleaved, resulting in a 367 amino acid polypeptide (Figure 20 and Figure 21). This form of the protein will be referred to as the MON 87708 DMO+27 protein. This alternative processing was not unexpected since alternative processing of a DMO precursor protein was also observed in other dicamba-tolerant plants transformed with a similar cassette containing the *dmo* gene (Behrens et al., 2007).

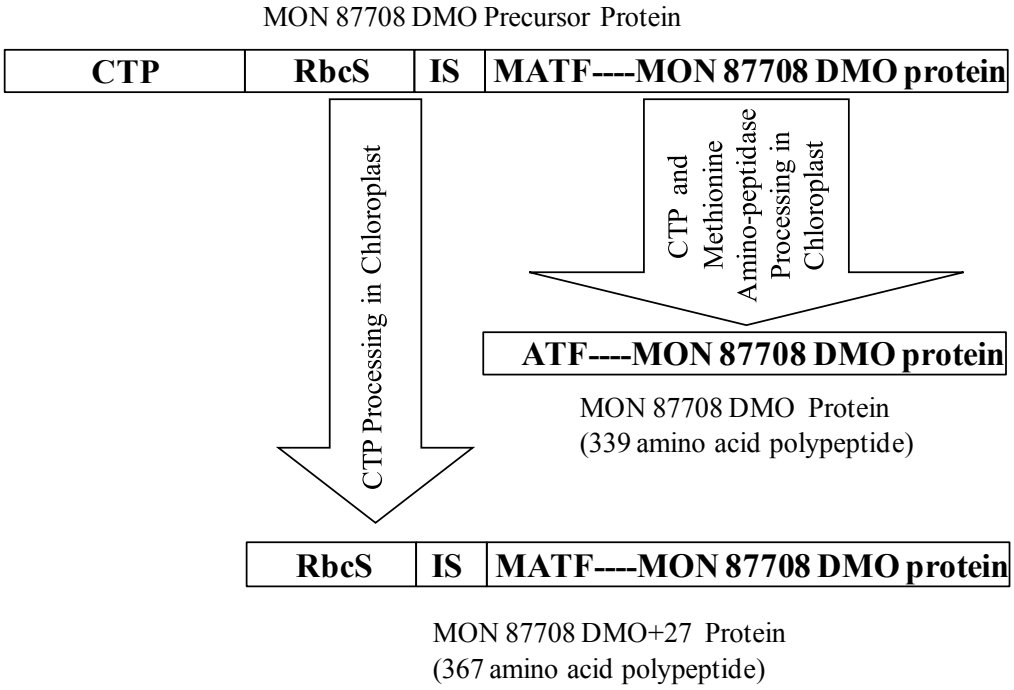


Figure 20. Processing of MON 87708 DMO Precursor Protein

The MON 87708 DMO precursor protein produced in MON 87708 contains a 57 amino acid chloroplast transit peptide (CTP), the first 24 amino acids from the N-Terminus of the small sub-unit of Rubisco (*RbcS*), and three amino acids from an intervening sequence (IS). Processing in the chloroplast removes the *RbcS*, IS, and the N-terminal methionine resulting in the MON 87708 DMO protein (339 amino acids). Alternative processing occurs when only the CTP is cleaved to produce the MON 87708 DMO+27 protein (367 amino acids). MATF represents the N-terminal amino acids of the *dmo* coding sequence. The methionine (M) has been removed in the MON 87708 DMO protein by methionine amino-peptidase.

The MON 87708 DMO protein has an identical sequence to the wild-type DMO protein (Herman et al., 2005), except for an additional alanine in position two added for cloning purposes and a cysteine instead of tryptophan at position 112 (Figure 21). The MON 87708 DMO+27 protein is identical to the MON 87708 DMO protein, and has the same amino acid differences when compared to the wild-type DMO, with the exception of the additional 27 amino acids and the methionine at the N-terminal portion of MON 87708 DMO protein (Figure 21). The differences in the amino acid sequence between the wild-type DMO protein and MON 87708 DMO protein and the MON 87708 DMO+27 protein are not expected to have an effect on structure, activity, or specificity because they are sterically distant from the catalytic site (D'Ordine et al., 2009)

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 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). In MON 87708, the trimer can be comprised of the MON 87708 DMO protein, the MON 87708 DMO+27 protein, or a combination of both. This document will refer to both the MON 87708 DMO protein, the MON 87708 DMO+27 protein, and all forms of the trimer, collectively, as MON 87708 DMO. MON 87708 DMO was purified from soybean of MON 87708 and was characterized (Section B2(b)). Under denaturing conditions, for example when separating on SDS-PAGE, the proteins were analyzed individually (*e.g.*, N-terminal sequencing). In these cases each protein will be referred to individually as either the MON 87708 DMO protein or the MON87708 DMO+27 protein.

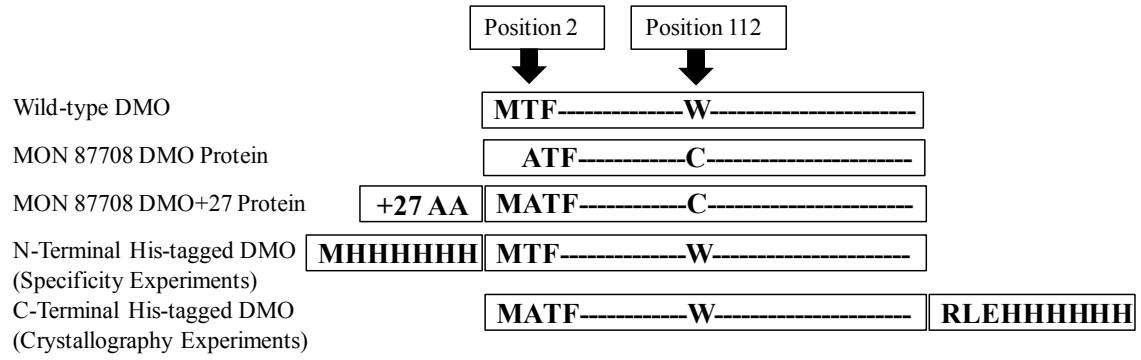


Figure 21. Forms of DMO Protein and Their Relation to the Wild-Type DMO Protein

The diagram represents the various DMO forms described in this dossier. The wild-type DMO form isolated from *S. maltophilia* was the first form sequenced (Herman et al., 2005). MON 87708 DMO was purified from soybean seed of MON 87708 and contained two forms of the protein; the MON 87708 DMO protein and the MON 87708 DMO+27 protein. The purified MON 87708 DMO used in specificity studies was active. The N-terminal histidine-tagged DMO was produced in *E. coli* and was used for *in vitro* specificity studies. The C-terminal histidine-tagged DMO was produced in *E. coli* and was used for crystallography studies described in D'Ordine et al. (2009).

Mode-of-Action and Specificity of DMO

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 catalyzes the demethylation of dicamba to the non-herbicidal compound DCSA and formaldehyde (Chakraborty et al., 2005). DCSA is a known soybean, soil, and livestock 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 thousand 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 nicotinamide adenine dinucleotide (NADH) to oxygen and catalyze the demethylation of an electron acceptor substrate, in this case dicamba (Behrens et al., 2007). This three component redox system is presented in Figure 22.

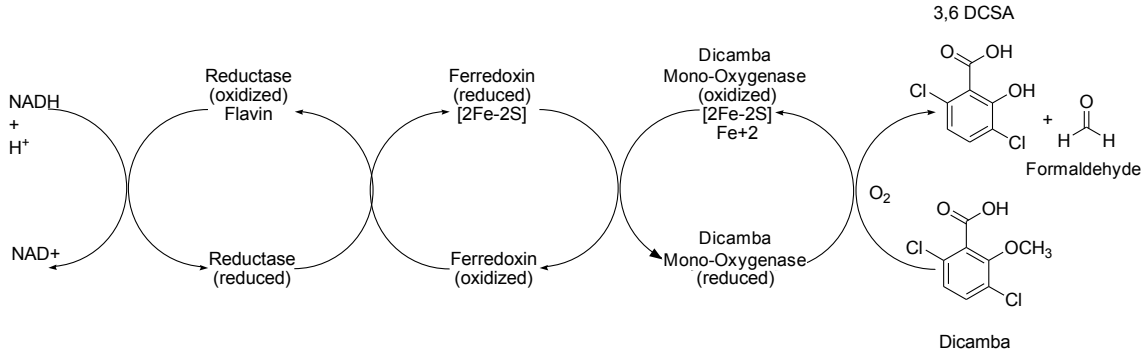


Figure 22. Three Components of the DMO Oxygenase System

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 poly-histidine 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 characterized to determine the fit of dicamba in the site and hypothesize 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 crystallization and is required for electron transport and catalysis (D'Ordine et al., 2009; Dumitru et al., 2009). To catalyze the demethylation of dicamba, electrons transferred from NADH are shuttled through an endogenous reductase and ferredoxin to the terminal DMO (Figure 22). 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 catalyze 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 87708 to be tolerant to dicamba, a functional trimeric MON 87708 DMO must be formed. The active trimeric form of MON 87708 DMO, as purified from MON 87708, confers dicamba tolerance to MON 87708, and its demethylase activity on dicamba was confirmed during characterization (Section B2(b)) supporting the conclusion that the trimer required for functional activity was likely formed in MON 87708.

Specificity of MON 87708 DMO

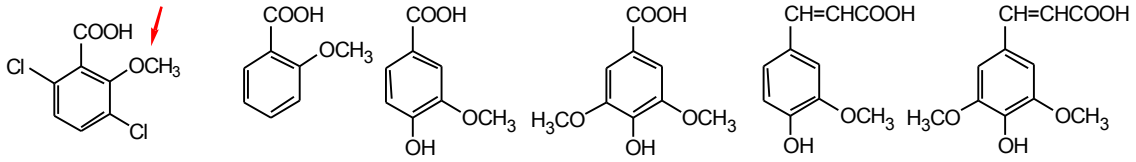
The substrate specificity of MON 87708 DMO was evaluated to understand potential interactions DMO may have with potential substrates present in MON 87708 soybean. As stated previously, this document refers to both the MON 87708 DMO protein, the MON 87708 DMO+27 protein, and all forms of the trimer, collectively, as MON 87708 DMO. DMO catalyzes the o-demethylation reaction (Chakraborty et al., 2005), a common reaction of many mono-oxygenases including P450 enzymes (Barrett et al., 1997; Berg et al., 1995). The specificity of an enzyme is dependent on the structural requirements needed for enzymatic catalysis. 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). Based on crystallography studies, dicamba interacts with amino acids in the catalytic site of DMO through both the carboxylate moiety and the chlorine atoms of dicamba (Dumitru et al., 2009). The carboxylic moiety forms six hydrogen bonds with the amino acids in the catalytic pocket and is a critical binding determinant. The chlorine atoms stabilize the position of the substrate in the proper orientation. These interactions were clearly observed in the crystals of DMO when dicamba was present in the catalytic site and provide evidence that these chemical groups, in addition to the benzene ring, are very important in correctly orienting the substrate for catalysis (D'Ordine et al., 2009; Dumitru et al., 2009).

Based on these interactions, only compounds structurally similar to dicamba (phenyl carboxylic acids containing methoxy moieties) were considered to be potential substrates of DMO. Soybeans contain many phenolic compounds (Janas et al., 2000; Kim et al., 2006), however chlorinated aromatic compounds have not been identified in soybean. Moreover it is known that chlorinated aromatic compounds are of limited existence in plants and other eukaryotes (Gribble, 1998; Gribble, 2004; Wishart, 2010; Wishart et al., 2009). Therefore, only compounds present in plants that contain methoxy and phenyl carboxylic acid moieties were selected for evaluation. 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 23).

The potential for MON 87708 DMO to metabolize endogenous plant substrates was evaluated in *in vitro* experiments using a purified N-terminal histidine tagged DMO that is identical to wild-type DMO, except for a histidine tag at the N-terminus added to aid in protein purification (Figure 21). 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 from those potential substrates in the assay mixture containing DMO were monitored using LC-UV and LC-MS. While dicamba was metabolized by DMO, none of

the tested compounds was metabolized in these *in vitro* experiments using the N-terminal histidine tagged DMO (Figure 24). The differences between DMO protein used for this study and the MON 87708 DMO protein are only the existence of histidine-tag and the amino acid differences at position 112 (tryptophan vs cysteine) and position 2 (the presence of alanine residue at from N-terminus) (Figure 21). The amino acid differences at position 112 and 2, and the existence of histidine-tag should not affect the specificity of the enzyme since these positions in the protein structure are sterically distant from the catalytic site (D'Ordine et al., 2009) and the presence of a histidine-tag does not generally affect the structure of proteins (Carson et al., 2007), therefore would not affect the structure of the active site.

To assess whether MON 87708 DMO has the same specificity as the DMO used in the *in vitro* experiments, MON 87708 DMO isolated from seed of MON 87708 was incubated with *o*-anisic acid, the endogenous compound that has the greatest structural similarity to dicamba. *o*-anisic acid was not metabolized by MON 87708 DMO (Figure 25), indicating the importance of the chlorine atoms in positioning the substrate in the catalytic site as described by D'Ordine et al. (2009). These results demonstrate that DMO, including MON 87708 DMO, is specific for dicamba as a substrate. These results also support that the minor changes in amino acid sequence among the different DMO proteins tested did not affect the specificity of DMO.



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

Figure 23. Dicamba and Potential Endogenous Substrates Tested in *In Vitro* Experiments with DMO

The arrow indicates methyl group removed by DMO.

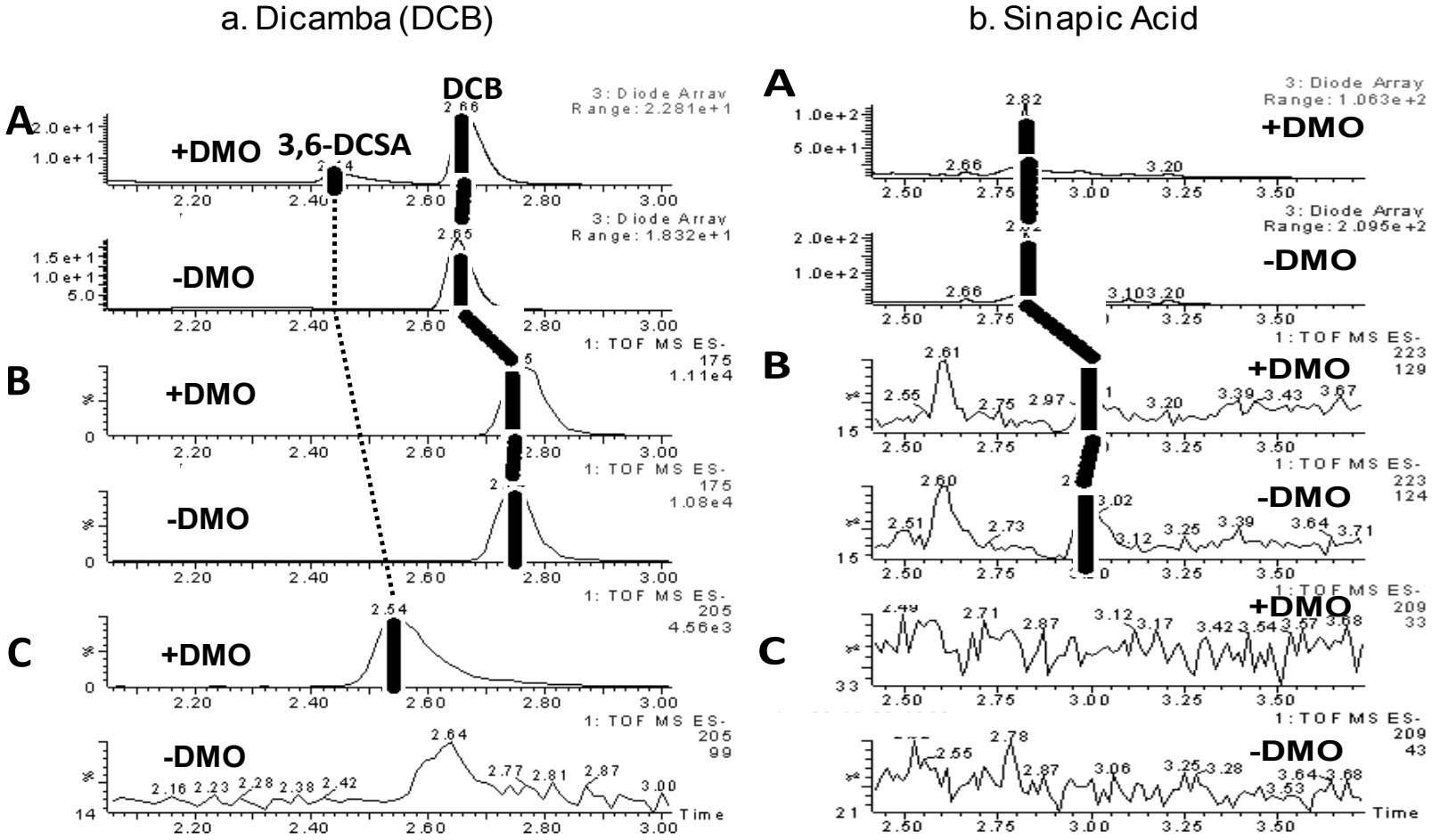


Figure 24. DMO Conversion of Endogenous Substrates

Endogenous substrates, as well as dicamba, were incubated with the N-terminal histidine-tagged DMO, and the formation of predicted oxidative products and the disappearance of each tested compound was monitored by LC-UV (top two chromatograms, A) and LC-MS (bottom chromatograms B and C). The B chromatograms with a 5 µl injection show the expected m/z for the tested compound, while the C chromatograms with a 50 µl injection show the expected m/z for the predicted oxidative product. Dicamba (a) was used as a positive control. Each endogenous compound, sinapic acid (b), ferulic acid (c), *o*-anisic acid (d), syringic acid (e), and vanillic acid (f), was included in a reaction mixture made with (+DMO, upper) and without (-DMO, lower) DMO. The dotted line indicates the migration of the tested compounds (and DCSA in the case of dicamba) in each chromatogram as a result of the UV and MS detectors being connected in series.

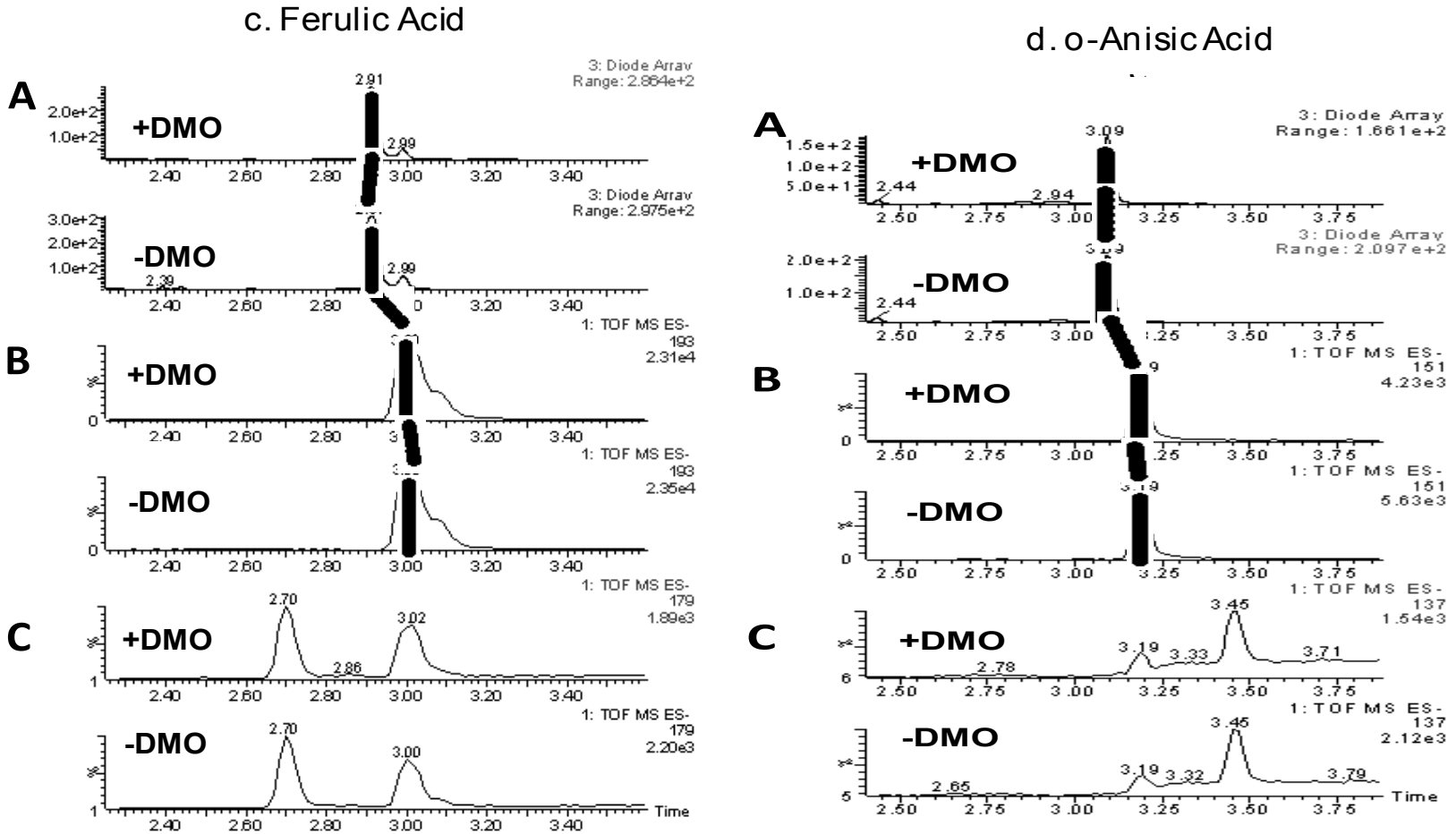


Figure 24 (continued). DMO Conversion of Endogenous Substrates

Endogenous substrates, as well as dicamba, were incubated with the N-terminal histidine-tagged DMO, and the formation of predicted oxidative products and the disappearance of each tested compound was monitored by LC-UV (top two chromatograms, A) and LC-MS (bottom chromatograms B and C). The B chromatograms with a 5 µl injection show the expected m/z for the tested compound, while the C chromatograms with a 50 µl injection show the expected m/z for the predicted oxidative product. Dicamba (a) was used as a positive control. Each endogenous compound, sinapic acid (b), ferulic acid (c), *o*-anisic acid (d), syringic acid (e), and vanillic acid (f), was included in a reaction mixture made with (+DMO, upper) and without (-DMO, lower) DMO. The dotted line indicates the migration of the tested compounds (and DCSA in the case of dicamba) in each chromatogram as a result of the UV and MS detectors being connected in series.

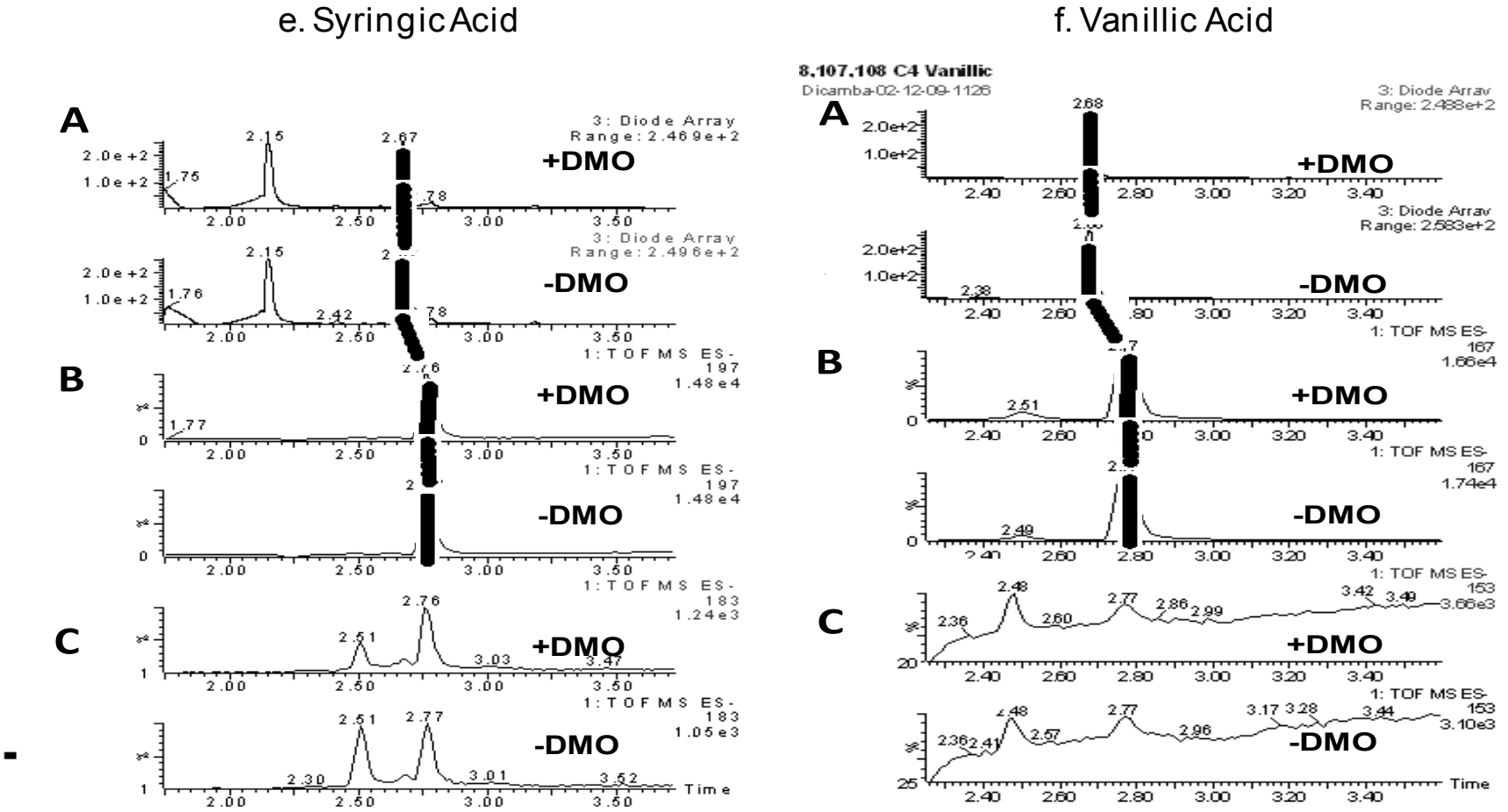


Figure 24 (continued). DMO Conversion of Endogenous Substrates

Endogenous substrates, as well as dicamba, were incubated with the N-terminal histidine-tagged DMO, and the formation of predicted oxidative products and the disappearance of each tested compound was monitored by LC-UV (top two chromatograms, A) and LC-MS (bottom chromatograms B and C). The B chromatograms with a 5 µl injection show the expected m/z for the tested compound, while the C chromatograms with a 50 µl injection show the expected m/z for the predicted oxidative product. Dicamba (a) was used as a positive control. Each endogenous compound, sinapic acid (b), ferulic acid (c), *o*-anisic acid (d), syringic acid (e), and vanillic acid (f), was included in a reaction mixture made with (+DMO, upper) and without (-DMO, lower) DMO. The dotted line indicates the migration of the tested compounds (and DCSA in the case of dicamba) in each chromatogram as a result of the UV and MS detectors being connected in series.

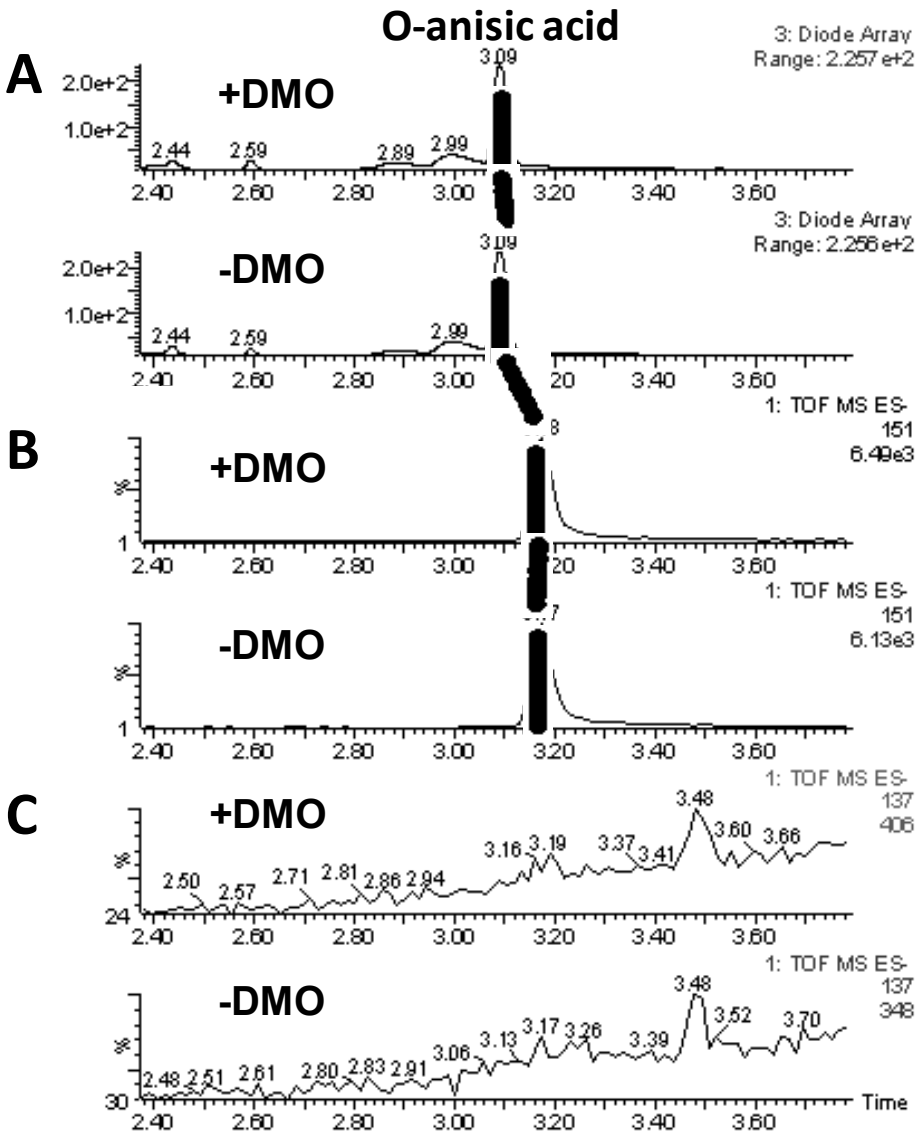


Figure 25. MON 87708 DMO Conversion of *o*-Anisic Acid

200 µM *o*-anisic acid was incubated with MON 87708 DMO and the formation of products and disappearance of substrate was monitored by LC-UV (top two chromatograms, A) and LC-MS (bottom chromatograms, B and C). The B chromatograms with a 5 µl injection show the expected m/z for *o*-anisic acid, while the C chromatograms with a 50 µl injection show the expected m/z for the predicted oxidative product. *o*-Anisic acid was included in a reaction mixture made with (+DMO, upper) and without (-DMO, lower) MON 87708 DMO. The dotted line indicates the migration of the *o*-anisic acid in each chromatogram as a result of the UV and MS detectors being connected in series.

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

The safety assessment of crops derived through biotechnology includes characterization of the functional and physicochemical properties, and confirmation of the safety of the introduced protein. As stated previously, this document refers to both the MON 87708 DMO protein, the MON 87708 DMO+27 protein, and all forms of the trimer, collectively, as MON 87708 DMO. MON 87708 DMO was purified in sufficient quantities directly from the seed of MON 87708 and used in the safety assessment. Typically protein safety assessments are conducted on proteins produced in heterologous expression systems, such as *E. coli* (Harrison et al., 1996). Since the MON 87708 DMO used in the safety assessment was purified directly from MON 87708, equivalence evaluations between plant-produced and bacterial-produced MON 87708 DMO were not necessary. The physicochemical characteristics and functional activity of MON 87708 DMO were determined by a panel of analytical techniques. When the proteins were separated by denaturation (*e.g.*, SDS-PAGE) for analysis each protein will be referred to individually as either the MON 87708 DMO protein or the MON87708 DMO+27 protein. These analytical techniques included: 1) immunoblot analysis to establish identity and immunoreactivity of the MON 87708 DMO protein and the MON 87708 DMO+27 protein using an anti-DMO antibody, 2) N-terminal sequence analysis of the MON 87708 DMO protein and the MON 87708 DMO+27 protein, 3) matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to generate a tryptic peptide map of the MON 87708 DMO protein and the MON 87708 DMO+27 protein, 4) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to establish the apparent molecular weight of the MON 87708 DMO protein and the MON 87708 DMO+27 protein, 5) glycosylation status of the MON 87708 DMO protein and the MON 87708 DMO+27 protein, and 6) MON 87708 DMO activity analysis to demonstrate functional activity. The identities of both the MON 87708 DMO protein and MON 87708 DMO+27 protein produced in MON 87708 were confirmed by western blot, N-terminal sequencing, and MALDI-TOF MS. The antibody specifically detected both the MON 87708 DMO protein and MON 87708 DMO+27 protein on an immunoblot. The N-terminal sequence of the first 15 amino acid residues of both the MON 87708 DMO protein and MON 87708 DMO+27 protein was identical to the predicted amino acid sequence, with the exception of the N-terminal methionine residue. MALDI-TOF MS analyses of the trypsin digested MON 87708 DMO protein and MON 87708 DMO+27 protein yielded peptide masses consistent with their expected sequence. The apparent molecular weights of the MON 87708 DMO protein and MON 87708 DMO+27 protein were 39.8 and 42.0 kDa, respectively and neither were glycosylated. The activity of MON 87708 DMO, in its active trimeric form, was determined by measuring the production of DCSA using dicamba as the substrate, resulting in a specific activity of 62.21 nmoles/min/mg of MON 87708 DMO. Taken together, these data provide a detailed characterization of MON 87708 DMO isolated from the seed of MON 87708.

Immunoblot Analysis

MON 87708 DMO purified from seeds of MON 87708 was analyzed by immunoblot. MON 87708 DMO was first separated under denaturing conditions by SDS-PAGE and then transferred to a PVDF membrane for immuno detection. On the immunoblot, the goat anti-DMO antibody recognized two bands migrating at the expected apparent molecular weights of approximately 39.8 kDa (MON 87708 DMO protein) and 42.0 kDa (MON 87708 DMO+27 protein), respectively (Figure 26). As expected, the intensity of the

immunoreactive bands increased with increasing amount of total protein loaded. No additional immunoreactive bands were observed in MON 87708 DMO. This immunoblot analysis confirmed the identities of the MON 87708 DMO protein and MON 87708 DMO+27 protein.

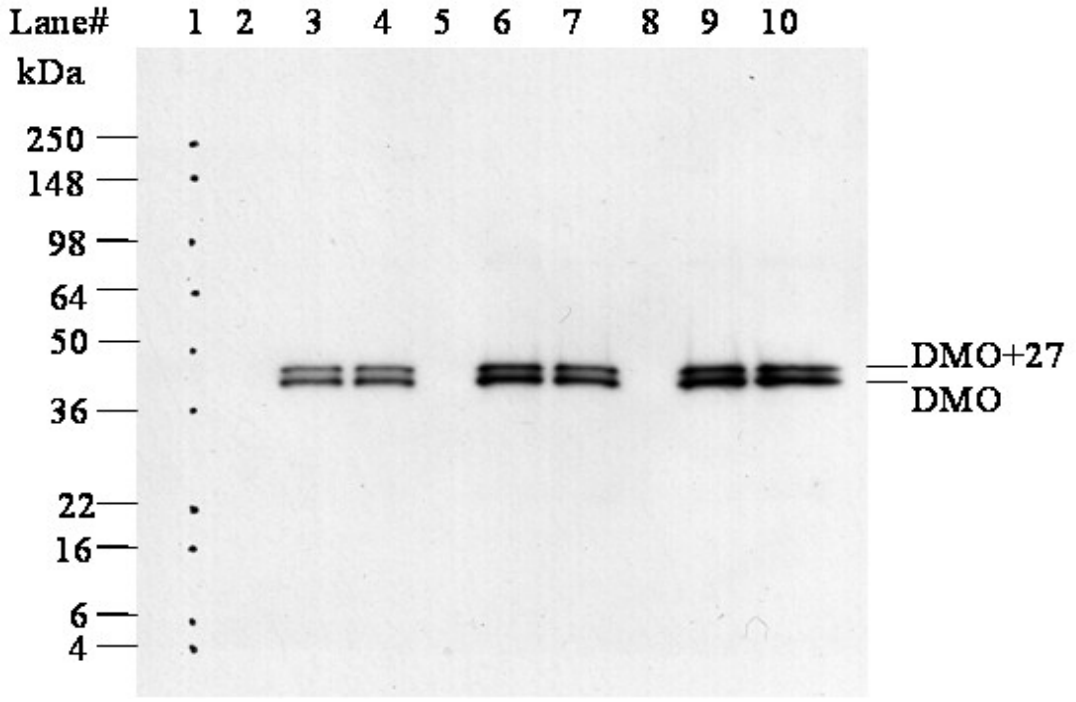


Figure 26. Immunoblot Analysis of the MON 87708 DMO

An aliquot of MON 87708 DMO and molecular weight markers were separated by SDS-PAGE and electrotransferred to a PVDF membrane. The membrane was incubated with goat anti-DMO antibody and immunoreactive bands were visualized using an ECL system. Approximate MWs (kDa) are shown on the left and correspond to the markers loaded in Lane 1. Amount loaded corresponds to total protein. The 20 second exposure is shown.

Lane	Sample	Amount (ng)
1	See Blue Plus2 Pre-Stained MW markers	—
2	empty	
3	MON 87708 DMO	20
4	MON 87708 DMO	20
5	empty	
6	MON 87708 DMO	30
7	MON 87708 DMO	30
8	empty	
9	MON 87708 DMO	40
10	MON 87708 DMO	40

N-Terminal Sequence Analysis

N-terminal sequence analysis of MON 87708 DMO was done by first separating MON 87708 DMO on SDS-PAGE and transferring the protein bands to a PVDF membrane. The two protein bands, similar to those observed in Figure 26, returned a sequence of 15 amino acids per band that matched the expected N-terminal sequences of the MON 87708 DMO protein and the MON 87708 DMO+27 protein (Figure 27 and Figure 28, respectively), which were deduced from the *dmo* and *Rbcs* coding regions present in the seed of MON 87708 (Section A3(c)(i)).

The N-terminal methionine residue in the MON 87708 DMO 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, catalyzed 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). The 15 amino acids correspond to the expected sequence, after the methionine, of the MON 87708 DMO protein.

In the case of the MON 87708 DMO+27 protein, the first cycle of N-terminal sequence analysis resulted in an amino acid that corresponds to a methylated modification of the N-terminal methionine. It is well-known that the N-terminal methionine of the Rubisco small subunit is post-translationally modified to N-methyl methionine *in vivo* in pea and other plant species (Grimm et al., 1997; Whitney and Andrews, 2001). The amino acids identified in the next 14 cycles corresponded to the expected sequence of the N-terminus of the small subunit of Rubisco confirming the alternative processing of the MON 87708 DMO+27 protein.

The N-terminal sequencing results confirm the identity of the MON 87708 DMO protein and MON 87708 DMO+27 protein.

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	A	T	F	V	R	N	A	W	Y	V	A	A	L	P	E
	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Experimental Sequence	-	A	T	F	V	R	N	A	W	Y	V	A	A	L	P	E

Figure 27. N-Terminal Sequence of the MON 87708 DMO Protein

The expected amino acid sequence of the N-terminus of the MON 87708 DMO protein was deduced from the *dmo* coding region present in MON 87708. The experimental sequence obtained from the MON 87708 DMO protein was compared to the expected sequence. (-) indicates the residue was not observed.

Amino acid residue # from the N-terminus →	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Expected Sequence	M	Q	V	W	P	P	I	G	K	K	K	F	E	T	L
	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Experimental Sequence	M*	Q	V	W	P	P	I	G	K	K	K	F	E	T	L

Figure 28. N-Terminal Sequence of the MON 87708 DMO+27 Protein

The expected amino acid sequence of the N-terminus of the MON 87708 DMO+27 protein was deduced from the *dmo* and *RbcS* coding region present in MON 87708. The experimental sequence obtained from the MON 87708 DMO+27 protein was compared to the expected sequence. M* indicates methylated methionine.

Tryptic Peptide Mapping

The identity of the MON 87708 DMO protein and the MON 87708 DMO+27 protein was confirmed by tryptic mapping using MALDI-TOF MS analysis of the fragments produced by trypsin digestion. MON 87708 DMO was first denatured and separated on SDS-PAGE. The protein bands corresponding to the MON 87708 DMO protein and the MON 87708 DMO+27 protein (Figure 31) were excised from the gel, reduced, alkylated and digested with trypsin. The ability to identify a protein using this method is dependent upon matching a sufficient number of observed tryptic peptide fragment masses with predicted tryptic peptide fragment masses. In general, protein identification made by peptide mapping is considered to be reliable if the measured coverage of the sequence is 15% or higher with a minimum of five matched peptides (Jensen et al., 1997).

There were 26 unique peptides identified for the MON 87708 DMO protein that matched the expected masses of the MON 87708 DMO protein trypsin-digested peptides while 29 unique peptides from the MON 87708 DMO+27 protein were found to match the expected masses of the MON 87708 DMO+27 protein trypsin-digested peptides (Table 6 and Table 7, respectively). The identified peptides were used to assemble a coverage map indicating the matched peptide sequences for the entire MON 87708 DMO protein and MON 87708 DMO+27 protein sequences, resulting in 77.4% (263/340) and 82.0% (301/367) coverage of the amino acid sequence, respectively (Figure 29 and Figure 30, respectively). The N-terminal peptides were also identified by MALDI-TOF analysis and confirmed the N-terminal sequencing data that demonstrated the N-terminal methionine was missing in the MON 87708 DMO protein and methylated in the MON 87708 DMO+27 protein (Table 6 and Table 7; Figure 29 and Figure 30). These results confirm the identity of the MON 87708 DMO protein and the MON 87708 DMO+27 protein.

Table 6. Summary of the Tryptic Masses Identified for the MON 87708 DMO Protein Using MALDI-TOF MS

Matrix						Expected[1]	Difference[2]	AAposition[3]	Fragment
α-Cyano		DHB		Sinapinic acid					
Ext.1	Ext.2	Ext.1	Ext.2	Ext.1	Ext.2				
			331.20			331.22	0.02	304-305	RR
		391.34				391.18	0.16	293-295	EDK
		435.38	435.38			435.27	0.11	206-208	FLR
593.61	593.61	593.51	593.53			593.34	0.27	2-6	ATFVR
720.67	720.68	720.60	720.60			720.37	0.30	131-136	VDPAYR
833.78	833.80	833.74	833.77			833.45	0.33	99-105	SFPVVER
856.77	856.78	856.72	856.75			856.43	0.34	242-248	EQSIHSR
914.89	914.91	914.84	914.88			914.53	0.36	296-303	VVVEAIER
1030.96	1030.97	1030.92	1030.92			1030.57	0.39	284-292	SWQAQALVK
1108.93	1108.95	1108.89	1108.94			1108.50	0.43	167-176	ANAQTDAFDR
		1171.08				1170.63	0.45	194-205	IPGGTPSVLMAK
1276.17	1276.20	1276.19	1276.21	1276.19		1275.73	0.44	26-36	TILDTPLALYR
1287.14		1287.19				1286.70	0.44	293-303	EDKVVVEAIER
1429.18	1429.20	1429.23	1429.26	1429.20		1428.69	0.49	209-221	GANTPVDAWNDIR
		1502.35	1502.37	1502.34		1501.79	0.56	180-193	EVIVGDGEIQALMK
1507.27	1507.27	1507.32				1506.73	0.54	167-179	ANAQTDAFDRLER
1578.24	1578.27	1578.32	1578.33	1578.30		1577.73	0.51	270-283	NFGIDDPEMDGVLR
1745.42	1745.51	1745.59		1745.56		1744.93	0.49	225-241	VSAMLNFIAVAPEGTPK
1762.48	1762.48	1762.62		1762.54		1761.90	0.58	37-52	QPDGVVAALLDICPHR
1994.65	1994.67	1994.76		1994.78		1994.03	0.62	150-166	LLVDNLMDLGHAQYVHR
2143.78	2143.84	2143.94		2143.96	2143.95	2143.12	0.66	7-25	NAWYVAALPEELSEKPLGR
2294.97				2294.93		2294.09	0.88	306-326	AYVEANGIRPAMLSCDEAAVR
2398.86	2398.77			2399.15		2398.08	0.78	249-269	GTHILTPETEASCHYFFGSSR
2581.87				2582.22		2582.34	0.47	225-248	VSAMLNFIAVAPEGTPKEQSIHSR
2700.08				2700.31		2699.25	0.83	106-130	DALIWICPGDPALADPGAIPDFGCR
				4218.47[4]		4217.77[4]	0.70	99-136	SFPVVERDALIWICPGDPALADPGAIPDFGCRVDPAYR

[1]Only experimental masses that matched expected masses are listed in the table.
[2]The number represents the difference between the expected mass and the first column, which has the corresponding numbers.
[3]AA position refers to amino acid position within the predicted MON 87708 DMO protein sequence as depicted in Figure 29.
[4]Mass average.

Table 7. Summary of the Tryptic Masses Identified for the MON 87708 DMO+27 Protein Using MALDI-TOF MS

Matrix						ExpectedMass[1]	Difference[2]	AAposition[3]	Fragment
α-Cyano		DHB		Sinapinic acid					
Ext.1	Ext.2	Ext.1	Ext.2	Ext.1	Ext.2				
			331.19			331.22	0.03	331-332	RR
		435.30				435.27	0.03	233-235	FLR
720.55	720.64	720.47	720.55			720.37	0.18	158-163	VDPAYR
795.61	795.71	795.54	795.66			795.42	0.19	27-33	AMATFVR
833.65	833.73	833.59	833.69			833.45	0.20	126-132	SFPVVER
856.64	856.72	856.58	856.64			856.43	0.21	269-275	EQSIHSR
914.74		914.69				914.53	0.21	323-330	VVVEAIER
1030.80	1030.89	1030.75				1030.57	0.23	311-319	SWQAQALVK
1069.78	1069.92					1069.57	0.21	1-9	M*QVWPPIGK
1108.75	1108.90	1108.71				1108.50	0.25	194-203	ANAQTDAFDR
			1170.98			1170.66	0.32	221-232	IPGGTPSVLMAK
1275.97	1276.12	1275.98	1276.14	1275.97		1275.73	0.24	53-63	TILDTPLALYR
1286.95		1286.97				1286.70	0.25	320-330	EDKVVVEAIER
1428.95	1429.10	1429.00	1429.26	1428.93		1428.69	0.26	236-248	GANTPVDAWNDIR
		1470.93		1469.94		1470.63	0.30	164-176	TVGGYGHVDCNYK
				1502.10		1501.79	0.31	207-220	EVIVGDGEIQALMK
1507.01	1507.18	1507.03				1506.73	0.28	194-206	ANAQTDAFDRLER
1565.13	1565.30	1565.22	1565.34			1564.87	0.26	11-23	KFETLSYLPPLTR
1578.02	1578.14	1578.06	1578.29	1578.04		1577.73	0.29	297-310	NFGIDDPEMDGVLR
1693.26	1693.38	1693.29				1692.97	0.29	10-23	KKFETLSYLPPLTR
1745.17	1745.36	1745.28	1745.51	1745.22		1744.93	0.24	252-268	VSAMLNFIAVAPEGTPK
1762.17	1762.37	1762.29				1761.90	0.27	64-79	QPDGVVAALLDICPHR
1994.34	1994.55	1994.48		1994.42		1994.03	0.31	177-193	LLVDNLMDLGHAQYVHR
2143.46	2143.63	2143.57	2143.98	2143.57		2143.12	0.34	34-52	NAWYVAALPEELSEKPLGR
				2294.62		2294.09	0.53	333-353	AYVEANGIRPAMLSCDEAAVR
	2398.72	2398.49		2398.52		2398.08	0.64	276-296	GTHILTPETEASCHYFFGSSR
				2581.78		2582.34	0.56	252-275	VSAMLNFIAVAPEGTPKEQSIHSR
		2699.73		2699.84		2699.25	0.48	133-157	DALIWICPGDPALADPGAIPDFGCR
				4215.70		4215.03	0.67	126-163	SFPVVERDALIWICPGDPALADPGAIPDFGCRVDPAYR

[1]Only experimental masses that matched expected masses are listed in the table.
[2]The number represents the difference between the expected mass and the first column, which has the corresponding numbers.
[3]AA position refers to amino acid position within the predicted MON 87708 DMO+27 protein sequence as depicted in Figure 30.
*Methylated methionine.

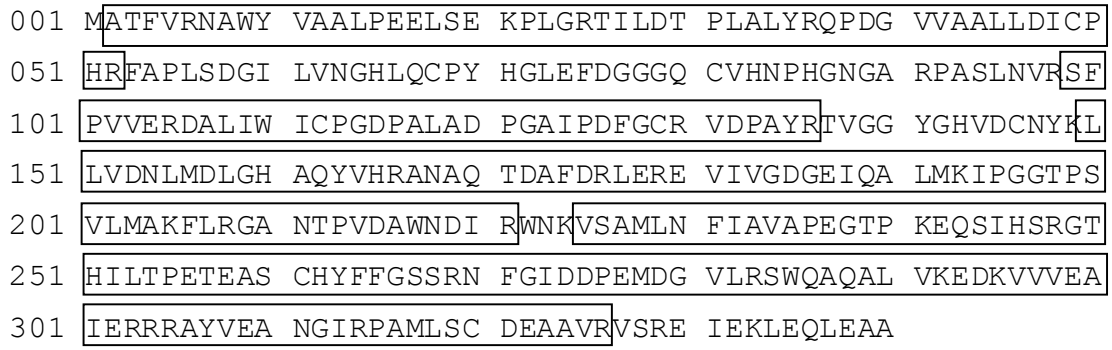


Figure 29. MALDI-TOF MS Coverage Map of the MON 87708 DMO Protein.

The amino acid sequence of the MON 87708 DMO protein was deduced from the *dmo* coding region present in MON 87708. Boxed regions correspond to tryptic peptides that were identified from MON 87708 DMO protein using MALDI-TOF MS. In total, 77.4% (263 of 340 total amino acids) of the expected protein sequence was identified.

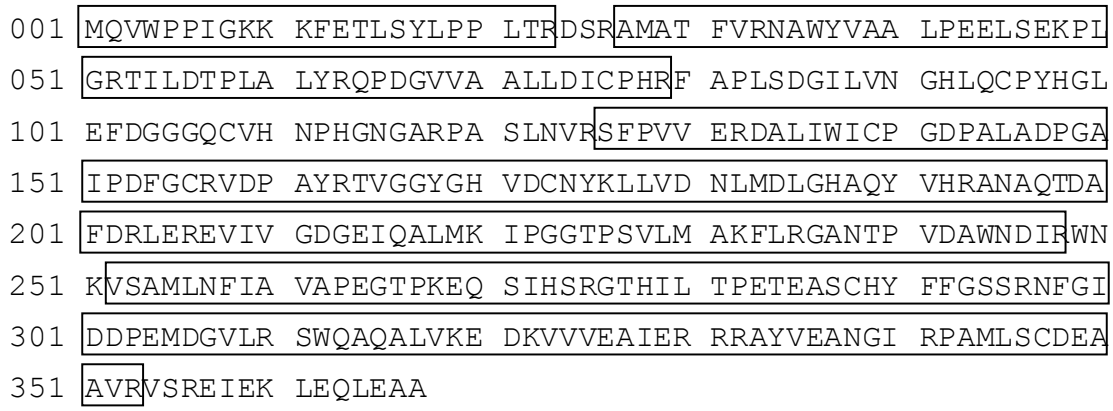


Figure 30. MALDI-TOF MS Coverage Map of the MON 87708 DMO+27 Protein

The amino acid sequence of the MON 87708 DMO+27 protein was deduced from the *dmo* coding region, *RbcS*, and intervening sequence present in MON 87708. Boxed regions correspond to tryptic peptides that were identified from MON 87708 DMO+27 protein using MALDI-TOF MS. In total, 82.0% (301 of 367 total amino acids) of the expected protein sequence was identified.

Molecular Weight and Purity

The apparent molecular weight of MON 87708 DMO protein and the MON 87708 DMO+27 protein was determined by analyzing the proteins using denaturing SDS-PAGE and then staining the gel using Brilliant Blue G Colloidal stain (Sigma). Purity and apparent molecular weight of the MON 87708 DMO protein and MON 87708 DMO+27 protein was determined using densitometric analysis of the gel (Figure 31). As summarized in Table 8, apparent molecular weight values were averaged from duplicated loads of 0.5, 1.0, and 1.5 µg of total protein (Figure 31, lanes 2-7). The predominant bands identified as the MON 87708 DMO protein and MON 87708 DMO+27 protein were estimated to have apparent molecular weights of 39.8 kDa and 42.0 kDa, respectively. Purity of MON 87708 DMO was calculated as the average purity of the MON 87708 DMO protein plus the average purity of the MON 87708 DMO+27 protein. This was done to reflect the purity of the purified MON 87708 DMO, *i.e.*, both processed forms of the proteins and all forms of the trimer. The purity of MON 87708 DMO was 81%.

Table 8. Molecular Weight and Purity of MON 87708 DMO

Total Protein Loaded	Apparent Molecular Weight (kDa)		Purity (%)		
	MON 87708 DMO Protein	MON 87708 DMO+27 Protein	MON 87708 DMO Protein	MON 87708 DMO+27 Protein	MON 87708 DMO[1]
0.5 µg in lane 2	39.2	41.6	34	44	
0.5 µg in lane 3	39.2	41.5	33	43	
1.0 µg in lane 4	39.5	41.7	36	46	
1.0 µg in lane 5	39.8	42.0	34	46	
1.5 µg in lane 6	40.3	42.4	37	47	
1.5 µg in lane 7	40.7	42.8	35	47	
Average	39.8	42.0	35	46	81

[1]Calculated as the sum of the average purity of the MON 87708 DMO protein and the MON 87708 DMO+27 protein.

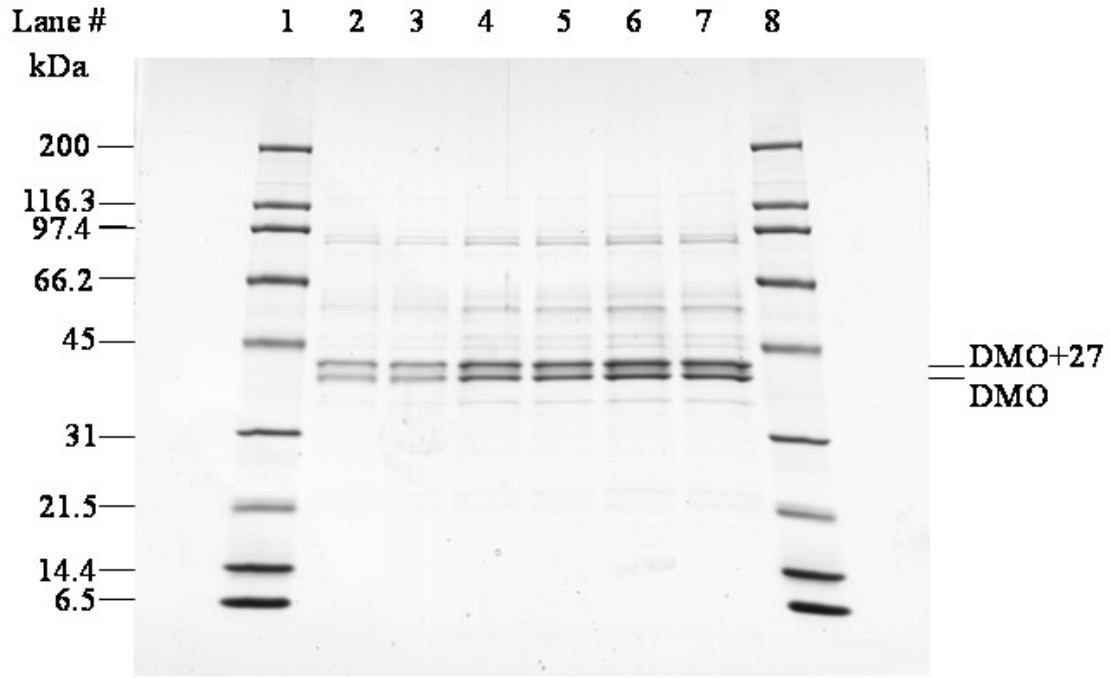


Figure 31. Molecular Weight and Purity Analysis of MON 87708 DMO

An aliquot of MON 87708 DMO was separated on a 4 - 20% Tris glycine polyacrylamide gradient gel and then stained with Brilliant Blue G Colloidal stain. Approximate apparent molecular weights (kDa) are shown on the left and correspond to the markers loaded in Lanes 1 and 8. Amount loaded corresponds to total protein. Empty lanes were cropped.

Lane	Sample	Amount (µg)
1	Broad Range MW markers	4.5
2	MON 87708 DMO	0.5
3	MON 87708 DMO	0.5
4	MON 87708 DMO	1.0
5	MON 87708 DMO	1.0
6	MON 87708 DMO	1.5
7	MON 87708 DMO	1.5
8	Broad Range MW markers	4.5

Functional Activity

MON 87708 DMO functional activity was determined by measuring the production of DCSA. The reaction mixture contained all the necessary compounds required for catalysis including the additional proteins (reductase and ferredoxin) involved in the electron transport from NADH. The specific activity was determined to be 62.21 nmol/min/mg of MON 87708 DMO (Table 9). The value represents an average of three independent assays. This result demonstrates that MON 87708 DMO isolated from the seed of MON 87708 is functionally active.

Table 9. MON 87708 DMO Functional Assay

Assay#	Specific activity (nmol/min/mg)	Average (nmol/min/mg) ±Standard Deviation
1	61.92	
2	51.33	62.21 ± 11.03
3	73.39	

Glycosylation Analysis

To test whether MON 87708 DMO is glycosylated, MON 87708 DMO was first separated on SDS-PAGE and then transferred to a PVDF membrane. The two protein bands, similar to those observed in Figure 26, corresponding to the MON 87708 DMO protein and MON 87708 DMO+27 protein were analyzed for glycosylation using a GE Glycoprotein Detection Module (GE Healthcare). Transferrin, a naturally glycosylated protein, was used as a positive control in the assay. The results of this analysis are presented in Figure 32. The positive control was clearly detected at the expected molecular weight and the bands increased with increasing protein concentration (Figure 32, lanes 2-4). No bands were observed for the MON 87708 DMO protein or MON 87708 DMO+27 protein at their expected molecular weight positions (39.8 and 42.0 kDa) (Figure 32, lanes 5 and 6) indicating that neither are glycosylated.

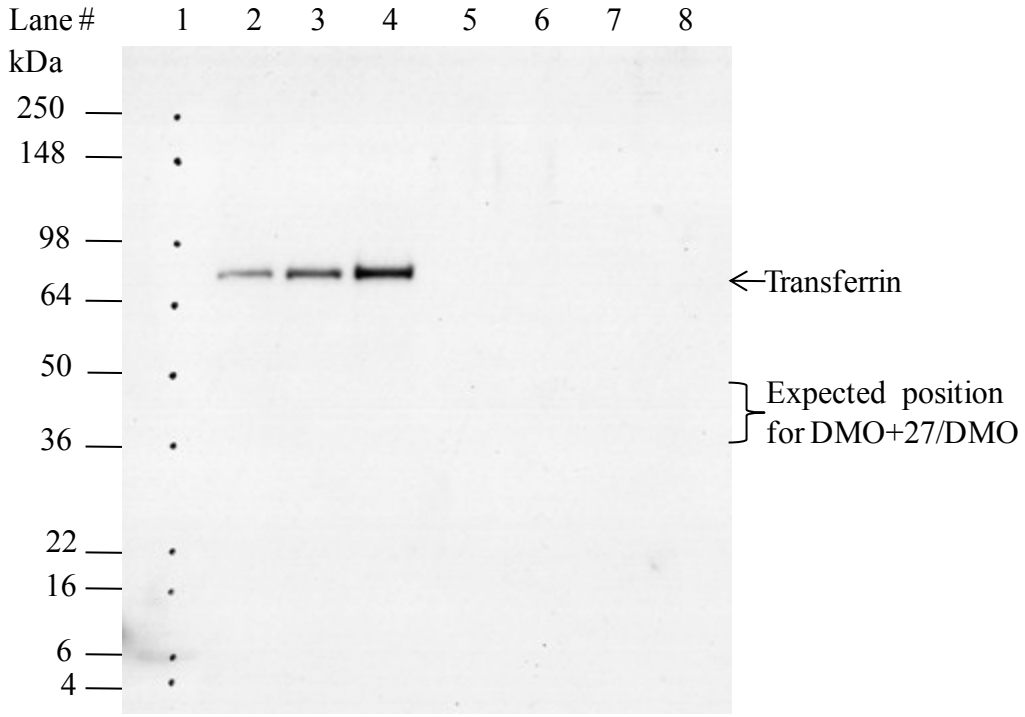


Figure 32. Glycosylation Analysis of the MON 87708 DMO

Molecular weight markers, transferrin (positive control) and an aliquot of MON 87708 DMO was separated by SDS-PAGE and electrotransferred to a PVDF membrane. The image was captured using a Bio-Rad GS800 with Quantity One software (version 4.4.0). Amount loaded corresponds to total protein. The 30 second exposure is shown.

Lane	Sample	Amount (ng)
1	See Blue Plus2 Pre-Stained MW markers	—
2	Transferrin	50
3	Transferrin	100
4	Transferrin	200
5	MON 87708 DMO	100
6	MON 87708 DMO	200
7	Empty lane	
8	Empty lane	

Identification of novel residues and calculation of residue levels

Three studies were conducted to determine the identity and levels of any new metabolites of dicamba produced following the application of dicamba herbicide to MON 87708.

A metabolism study was conducted with [^{14}C] dicamba, in accordance with the requirements of EPA residue chemistry test guideline OPPTS 860.1300, "Nature of the Residue – Plants, Livestock" and the OECD Guideline for the Testing of Chemicals No. 501, "Metabolism in Crops". The purpose of this study was to determine the nature of residues found in/on agricultural commodities of dicamba-tolerant soybean following treatment with dicamba. The dicamba-tolerant soybean event utilized in the metabolism study contained the same dicamba monooxygenase gene expression cassette as MON 87708 soybean.

The study showed that the primary route of metabolism of dicamba in dicamba-tolerant soybean is by demethylation to form 3,6-dichloro-2-hydroxybenzoic acid (DCSA). The DCSA is converted to its glucose conjugate (DCSA glucoside), some of which is further acylated with 3-hydroxy-3- methylglutaric acid (HMGA) on the 6-hydroxyl of the glucose moiety to form DCSA HMGglucoside. As a minor pathway, DCSA is converted by 5-hydroxylation to 2,5-dichloro-3,6-dihydroxybenzoic acid (DCGA) which is present in soybean as its glucose conjugate (DCSA glucoside). Both pathways are similar to common metabolic processes in plants, soil and animals. DCSA is observed as the major aerobic soil metabolite of dicamba; DCGA is also observed in soil as a minor metabolite. Although the glucose conjugate of 5-hydroxydicamba is the major dicamba metabolite in crops such as wheat and grasses, which are naturally tolerant to dicamba, O-demethylation to form DCSA is also observed as a minor pathway in these crops. While the acylation of a xenobiotic or xenobiotic glycoside with HMGA has not, to our knowledge, been previously reported, HMGA is a common plant constituent and there are many plant natural product glycosides, e.g., flavonol glycosides, conjugated with HMGA. Therefore, it was concluded that that the metabolism of dicamba in dicamba-tolerant soybean (e.g., MON 87708) is similar to the metabolism of dicamba in soil and other plant species.

Following the identification of the dicamba metabolites in the metabolism study, a residue study was conducted to determine the levels of residues of dicamba and its principal metabolites in soybean seed after application of dicamba formulations to MON 87708. The study utilized a validated analytical method that quantitated dicamba, DCSA, DCGA and 5-hydroxydicamba. The residue components included in the current EPA Definition of the Residue (DoR) for dicamba in soybean (dicamba, DCSA and 5-hydroxydicamba) comprise the majority of the residues in dicamba-tolerant seed following pre- or postemergence treatment of dicamba-tolerant soybean with dicamba.

The residues of dicamba in soybean seed resulting from application of the maximum anticipated labeled use for dicamba in MON 87708 soybean in the United States were found to be low, with the median total residue at <0.065 ppm and the maximum total residue at 0.471 ppm dicamba acid equivalents of total EPA DoR. These levels are well below the established 10 ppm tolerance for dicamba in or on soybean seed in the United States and Canada.

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

The levels of MON 87708 DMO in various tissues of MON 87708 that are relevant to the risk assessment were determined using a validated enzyme-linked immunosorbent assay (ELISA). Tissues of MON 87708 and the near isogenic conventional soybean control

A3525 were collected during the 2008 growing season from five field sites in the U.S.: Jefferson County, Iowa; Stark County, Illinois; Clinton County, Illinois; Parke County, Indiana; and Berks County, Pennsylvania. These field sites were representative of soybean producing regions suitable for commercial production. At each site, three replicated plots containing MON 87708, as well as the conventional control, were planted using a randomized complete block field design. Over-season leaf (OSL 1 - 4), root, forage, and seed tissues were collected from each replicated plot at all field sites (except for the conventional control for forage tissue from Berks County, Pennsylvania where only two replicates were collected). A description of tissues collected is provided in Table 10.

Table 10. Tissues Collected and Analyzed for MON 87708 DMO

Tissue	Soybean Development Stage[1]	Days After Planting
OSL-1	V3-V4	21-30
OSL-2	V5-V8	31-42
OSL-3	R2-V12	43-58
OSL-4	R5-V16	55-78
Root	R6	70-91
Forage	R6	70-91
Seed	R8	109-147

[1]Soybean plant growth stages described in Soybean Growth and Development (Pedersen, 2004).

The levels of MON 87708 DMO were determined in all seven tissue types as described in Table 10. As previously described, this document will refer to both the MON 87708 DMO protein, the MON 87708 DMO+27 protein, and all forms of the trimer, collectively, as MON 87708 DMO. The ELISA assay detected MON 87708 DMO (*i.e.*, both processed forms of the protein and all forms of the trimer), therefore the levels represent the total MON 87708 DMO. The results obtained from the ELISA analysis are summarized in Table 11. In summary, expression analysis of the samples from the 2008 U.S. field trial showed that MON 87708 DMO was detected in all tissue types across all five sites ranging from 3.9-180 µg/g dry weight (dwt). The mean levels of MON 87708 DMO across the five sites were highest in leaf (ranging from OSL-1 at 17 µg/g dwt, to OSL-4 at 69 µg/g dwt), followed by forage (53 µg/g dwt), seed (47 µg/g dwt), and root (6.1 µg/g dwt). As expected for the conventional control, the ELISA values for MON 87708 DMO were less than the limit of quantitation (LOQ) of the assay in all tissue types.

Table 11. Summary of the Levels of MON 87708 DMO in Leaf, Root, Forage, and Seed from MON 87708 Grown in 2008 U.S. Field Trials

Tissue Type	MON 87708 DMO[1] Mean (SD)[2] (µg/g fwt)[3]	Range[4] (µg/g fwt)	MON 87708 DMO Mean (SD) (µg/g dwt)[5]	Range (µg/g dwt)	LOQ/LOD (µg/g fwt)[6,7]
OSL-1	3.1 (1.9)	0.87 – 6.8	17 (7.7)	6.2 – 29	0.63/0.20
OSL-2	5.2 (2.6)	1.4 – 9.8	31 (13)	12 – 54	0.63/0.20
OSL-3	6.0 (2.2)	3.5 – 11	44 (14)	25 – 71	0.63/0.20
OSL-4	16 (12)	4.6 – 43	69 (46)	23 – 180	0.63/0.20
Root	1.9 (0.73)	1.2 – 3.6	6.1 (2.1)	3.9 – 11	0.031/0.015
Forage	12 (2.5)	7.0 – 17	53 (18)	25 – 84	0.63/0.10
Seed	43 (7.7)	31 – 55	47 (8.7)	34 – 59	1.3/0.21

[1]Represents total for MON 87708 DMO (*i.e.*, both processed forms of the protein and all forms of the trimer).
[2]The mean and standard deviation (SD) were calculated (n=15). The "n" values for the calculated mean and standard deviations represent the number of samples figured into the calculation.
[3]Protein levels are expressed as microgram (µg) of protein per gram (g) of tissue on a fresh weight (fwt) basis.
[4]Minimum and maximum values were determined for each tissue type.
[5]Protein levels are expressed as µg/g dwt. The dry weight values were calculated by dividing the µg/g fwt by the dry weight conversion factors obtained from moisture analysis data.
[6]The limit of quantitation (LOQ) was calculated based on the lowest *E. coli*-produced DMO standard concentration. The "ng/ml" value was converted to "µg/g fwt" using the respective dilution factor and tissue-to-buffer ratio.
[7]The limit of detection (LOD) was calculated as the mean value of a conventional control plus three SD using the data generated with conventional control sample extracts for each tissue type. The LOD value in "ng/ml" was converted to "µg/g fwt" using the respective dilution factor and tissue-to-buffer ratio.

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

Please refer to section B2(b).

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

Not applicable

B2(f) History of human consumption of novel substances or similarity

MON 87708 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 87708 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 87708 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).

As discussed previously, the crystal structure of DMO has been solved (D'Ordine et al., 2009; Dumitru et al., 2009). 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 is 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 87708 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 87708 DMO+27 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 87708 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 26% (Table 12). The highest homology was observed to proteins that are involved in chlorophyll metabolism. Chlorophyllide A oxygenase 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 is also a Rieske-type oxygenase that plays a key role in the overall regulation of chlorophyll degradation in plants (Rodoni et al., 1997). The protein 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 it should have a 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 87708 DMO has occurred through consumption of these crops.

Therefore, MON 87708 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 12. Amino acid sequence identity between the MON 87708 DMO protein, the MON 87708 DMO+27 protein and other proteins present in plants and bacteria

Protein	Accession Number	Source	Sequence Identity (%) MON 87708 DMO Protein	Sequence Identity (%) MON 87708 DMO+27 Protein
Chlorophyllide A oxygenase	ACG42449	Zea mays	26.6	26.2
Rieske iron-sulfur protein Tic55	CAA04157	Pisum sativum	26.2	25.6
Pheophorbide A oxygenase	ABD60316	Brassica napus	25.3	24.8
Lethal leaf spot-1 like protein*	ABA40832	Glycine max	25.3	24.3
Pheophorbide A oxygenase	CAR82238	Pisum sativum	24.3	24.3
Pheophorbide A oxygenase	ACG28057	Zea mays	24.1	23.2
Rieske domain containing protein	ABF99438	Oryza sativa	23.8	22.9
Flavonoid-3-hydroxylase	AAV74195	Sorghum bicolor	20.9	20.2
Choline mono-oxygenase	CAE17617	Oryza sativa	19.1	18.8
Choline mono-oxygenase	AAB52509	Spinacia oleracea	18.2	17.6
Sparse inflorescencel	ACI43576	Zea mays	18.0	17.5
Beta-carotene hydroxylase	AAX45523	Zea mays	15.6	15.5
Rieske domain containing protein	ACG43734	Zea mays	13.1	13.1

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

Protein sequences were extracted from publicly available databases. Each sequence was aligned to the MON 87708 DMO protein and the MON 87708 DMO+27 protein 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).

B3 Assessment of Potential Toxicity

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

Additionally, according to guidelines adopted by the Codex Alimentarius Commission for the assessment of potential toxicity of introduced proteins, the toxic potential of an introduced protein is assessed by comparing the biochemical characteristics of the introduced protein to characteristics of known toxins (Codex Alimentarius, 2003; Codex Alimentarius, 2009).

The assessment of the potential toxicity of an introduced protein is based on comparing the biochemical characteristics of the introduced protein to characteristics of known toxins. These biochemical characteristics are assessed by determining if: 1) the protein has amino acid sequence similarity to known toxins or other biologically-active proteins that could cause adverse effects in humans or animals (B3(a)); 2) the protein is rapidly digested in mammalian gastrointestinal systems (B3(b)); 3) the protein is stable to heat treatment (B3(b)); and 4) the protein exerts any acute toxic effects in mammals (B3(c)). MON 87708 DMO has been assessed for its potential toxicity based on these criteria and was determined to pose no significant toxicological risk. As previously described, this document refers to both the MON 87708 DMO protein, the MON 87708 DMO+27 protein, and all forms of the trimer, collectively, as MON 87708 DMO.

B3(a) Bioinformatic comparison (aa) of novel protein(s) to toxins

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

Potential structural similarities shared between the MON 87708 DMO+27 protein, the MON 87708 DMO protein, and sequences in a protein database were evaluated using the FASTA sequence alignment tool. The FASTA program directly compares amino acid sequences (*i.e.*, primary, linear protein structure) and the alignment data may be used to infer shared higher order structural similarities between two sequences (*i.e.*, secondary and tertiary protein structures). Proteins that share a high degree of similarity throughout the entire sequence are often homologous (Caetano-Anollés et al., 2009; Illergård et al., 2009). Homologous proteins usually have common secondary structures, common three-dimensional configuration, and, consequently, may share similar functions.

FASTA bioinformatic alignment searches using the MON 87708 DMO+27 protein amino acid sequence, that contains the full MON 87708 DMO protein sequence, were performed with the TOX_2010 database to identify possible homology with proteins that may be harmful to human and animal health. The TOX_2010 database is a subset of sequences derived from the GenBank protein database (PRT_2010), release 175 (December, 15, 2009). Sequences were selected using a keyword search and filtered to remove likely non-toxin proteins. The TOX_2010 database contains 8,448 sequences.

An *E*-score acceptance criterion of 1×10^{-5} or less for any alignment was used to identify proteins from the TOX_2010 database with potential for significant shared structural similarity and function with MON 87708 DMO+27 protein. 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 smaller to be considered to have sufficient sequence similarity to infer

homology (Silvanovich et al., 2009). The results of the search comparisons showed that no relevant alignments were observed against proteins in the TOX_2010 database. No FASTA alignment displayed an *E*-score of less than 1×10^{-5}.

The results of the bioinformatic analyses demonstrated that no structurally relevant similarity exists between MON 87708 DMO and any known toxin that would be harmful to human or animal health.

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

The stability of a protein to heat or its degradation in simulated mammalian gastrointestinal fluids is a key consideration in the assessment of its potential toxicity. Exposure to heat during food processing or cooking, and to digestive fluids is likely to have a profound effect on the structure and function of proteins. The effect of heat treatment on the activity of MON 87708 DMO was evaluated using a functional activity assay, and the results show that MON 87708 DMO was completely deactivated by heating at temperatures above 55°C (Section B4(c).2). The digestability of MON 87708 DMO in simulated gastrointestinal fluids was evaluated by incubation with SGF and SIF, and the results show that MON 87708 DMO was readily digested (Section B4(c).1). Therefore, it is anticipated that exposure to functionally active MON 87708 DMO from the consumption of MON 87708 or foods derived from MON 87708 will be negligible.

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

Most known protein toxins act through acute mechanisms to exert toxicity (Hammond and Fuchs, 1998; Pariza and Johnson, 2001; Sjoblad et al., 1992). The primary exceptions to this rule consist of certain anti-nutritional proteins such as lectins and protease inhibitors, that manifest toxicity in a short term (few-week) feeding study (Liener, 1994). The amino acid sequence of the MON 87708 DMO+27 protein is not similar to any of these anti-nutritional proteins or to any other known protein toxins (Section B3(a)). Therefore, an acute oral mouse toxicity study was considered sufficient to evaluate the toxicity of MON 87708 DMO.

MON 87708 DMO, which refers to the MON 87708 DMO protein, the MON 87708 DMO+27 protein, and all forms of the trimer, was administered as a single dose by oral gavage to a group of five male and five female CD-1 mice at a dose level of 140 mg/kg body weight (bw). Additional groups of mice were administered comparable levels of bovine serum albumin (BSA) to serve as a protein control. Following dosing, all mice were observed twice daily for mortality or signs of toxicity. Food consumption was measured weekly. Body weights were measured prior to dosing (study day 0) and on study days 7 and 14. All animals were sacrificed on day 14 and subjected to a gross necropsy. There were no treatment-related effects on survival, clinical observations, body weight, body weight gain, food consumption or gross pathology. Therefore, the No Observable Adverse Effect Level (NOAEL) for MON 87708 DMO was considered to be 140 mg/kg bw.

B4 Assessment of Potential Allergenicity

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

Additionally according to guidelines adopted by the Codex Alimentarius Commission for the assessment of potential allergenicity of introduced proteins, the allergenic potential of an introduced protein is assessed by comparing the biochemical characteristics of the introduced protein to characteristics of known allergens (Codex Alimentarius, 2003; Codex Alimentarius, 2009). The biochemical characteristics are assessed by determining if: 1) the protein is from an allergenic source (B4(a)); 2) the protein shares any amino acid sequence similarities to known allergens (B4(b)); 3) the protein is rapidly digested in mammalian gastrointestinal systems (B4(c).1); and 4) the protein is stable to heat treatment (B4(c).2); and 5) the protein represents only a very small portion of the total plant protein (section B4(e)). In addition, MON 87708 and products derived from MON 87708 do not pose an increased endogenous allergenicity concern to humans over currently consumed soybean foods (B4(d)). MON 87708 DMO has been assessed for its potential allergenicity according to these safety assessment guidelines, and was determined to pose no significant risk of allergenicity.

B4(a) Source of introduced protein

As described in Section A2(a)(ii), 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 (Section A2(a)(ii)). 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 hospitalized 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 colonization 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 mininmal 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.

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

In 2003, the Codex Alimentarius Commission published guidelines for the evaluation of the potential allergenicity of introduced proteins (Codex Alimentarius, 2003). This guideline is based on the comparison of amino acid sequences between introduced proteins and allergens, where potential 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 amino acid sequence similarities between MON 87708 DMO and known allergens, gliadins, and glutenins was assessed using the FASTA sequence alignment tool and an eight-amino acid sliding window search (Codex Alimentarius, 2003; Thomas et al., 2005). The results demonstrated that MON 87708 DMO, which refers to both the MON 87708 DMO protein, the MON 87708 DMO+27 protein, and all forms of the trimer, 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) and the alignment of the data may be used to infer shared higher order structural similarities between two sequences (*i.e.*, secondary and tertiary protein structures). Proteins that share a high degree of similarity throughout the entire sequence are often homologous. Homologous proteins usually have common secondary structures, common three-dimensional configuration, and may share similar functions (Caetano-Anollés et al., 2009; Illergård et al., 2009). The allergen, gliadin, and glutenin sequence database (AD_2010) used for the evaluation was obtained from the Food Allergy Research and Resource Program Database (FARRP, 2010). The AD_2010 database contains 1,471 sequences. The sequence similarity evaluation was conducted using the MON 87708 DMO+27 protein, which contains the MON 87708 DMO protein sequence, and protein sequences contained in AD_2010. A FASTA algorithm, used to search AD_2010, produces an *E*-score (expectation score) which is a statistical measure of the likelihood that the observed similarity could have occurred by chance. 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 need to have an *E*-score of 1×10^{-5} or smaller to be considered to have sufficient sequence similarity to infer homology (Silvanovich et al., 2009). The FASTA analysis yielded no *E*-scores less than or equal to 1×10^{-5}, demonstrating a lack of sequence similarity between the MON 87708 DMO+27 protein (and the MON 87708 DMO protein whose sequence is fully contained in the MON 87708 DMO+27 protein sequence) and sequences in the allergen database. Moreover, no alignment met or exceeded the threshold of 35% identity over 80 amino acids as recommended by Codex Alimentarius (Codex Alimentarius, 2003).

A second bioinformatic tool, an eight-amino acid sliding window search, was used to specifically identify short linear polypeptide matches to known or suspected allergens. It is possible that proteins structurally unrelated to allergens, gliadins, and glutenins may still contain smaller immunologically significant epitopes. An amino acid sequence may be considered to have allergenic potential if it has an exact sequence identity of at least eight linearly contiguous amino acids with a potential allergen epitope (Hileman et al., 2002; Metcalfe et al., 1996). Using a sliding window of less than eight amino acids can produce matches containing significant uncertainty depending on the length of the query sequence (Silvanovich et al., 2006) and are not useful to the allergy assessment process (Thomas et al., 2005). No sequence alignments of eight contiguous amino acids were detected when the MON 87708 DMO+27 protein sequence was compared to the AD_2010 sequence database.

In conclusion, the bioinformatic results demonstrated there were no biologically relevant sequence similarities to allergens when the MON 87708 DMO+27 protein sequence was used as a query for a FASTA search of the AD_2010 database. Furthermore, no short (eight amino acid) polypeptide matches were shared between the MON 87708 DMO+27 protein sequence and proteins in the AD_2010 database. Since the MON 87708 DMO+27 protein sequence contains the MON 87708 DMO protein sequence, these data demonstrate the lack

of both structurally and immunologically relevant similarities between the protein sequences of MON 87708 DMO and known allergens, gliadins, and glutenins.

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

B4(c).1. Digestive Fate of MON 87708 DMO

One characteristic of many protein allergens is their ability to withstand proteolytic digestion by enzymes present in the mammalian gastrointestinal tract (Astwood et al., 1996; Moreno et al., 2005; Vassilopoulou et al., 2006; Vieths et al., 1999). When resistant to digestion, allergens, or their fragments, are presented to the intestinal immune system, which 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 87708 DMO, which refers to both the MON 87708 DMO protein, the MON 87708 DMO+27 protein, and all forms of the trimer, was assessed using assays with both simulated gastric fluid (SGF) containing pepsin and simulated intestinal fluid (SIF) containing pancreatin.

A correlation between the digestibility in SGF and the likelihood of an introduced protein being an allergen has been previously reported with a group of proteins consisting of both allergens and non-allergens (Astwood et al., 1996), but this correlation is not absolute (Fu et al., 2002). The SGF assay protocol has been standardized by ILSI 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 are reproducible when standard protocols were followed. Using this protocol, the pepsin digestion assay was used to assess the susceptibility of MON 87708 DMO to *in vitro* pepsin digestion and a summary of the results is below.

The digestibility of a protein in SIF is used as an independent test system to assess the *in vitro* digestibility of food components (Okunuki et al., 2002; Yagami et al., 2000). The relationship between protein allergenicity and protein stability in the standalone *in vitro* SIF assay is limited, because the protein has not been first exposed to the acidic, denaturing conditions of the stomach, as would be the case *in vivo* (Helm, 2001). Using an established protocol, the pancreatin digestion assay was used to assess the susceptibility of MON 87708 DMO to *in vitro* pancreatin digestion and a summary of the result is below.

Digestibility of MON 87708 DMO in SGF and SIF

Digestibility of MON 87708 DMO in SGF was assessed using SDS-PAGE and immunoblot methods. The extent of MON 87708 DMO digestion was evaluated by visual analysis of stained polyacrylamide gels (Figure 33 and Figure 34) or by visual analysis of western blots (Figure 35 and Figure 36). In both cases MON 87708 DMO was first separated on a denaturing SDS-PAGE and analyzed, or transferred to a PVDF membrane for immunoblot analysis. In each case the degradation of the MON 87708 DMO protein and the MON 87708 DMO+27 protein was evaluated.

Initially, the digestibility of MON 87708 DMO in SGF was evaluated by visual analysis of a colloidal Brilliant Blue G stained Tricine 10-20% polyacrylamide gradient gel where MON 87708 DMO was completely digested within 30 seconds (Figure 33). However, pepsin and the MON 87708 DMO protein migrate to similar positions in this gel system. To further confirm that the MON 87708 DMO protein is being digested and not being masked by pepsin, a visual analysis of a colloidal Brilliant Blue G stained Tris-glycine 8% polyacrylamide gel was also conducted (Figure 34, panel A), confirming the previous results that MON 87708 DMO was completely digested within 30 seconds. The migration of pepsin relative to MON 87708 DMO is different in each gel system. Changes in protein mobility in different gel systems are due to a variety of factors including changes in acrylamide percentage and pH of each gel system (Makowski and Ramsby, 1997).

Due to the improved resolution of pepsin and MON 87708 DMO, a separate Tris-glycine 8% polyacrylamide gel was used to determine the limit of detection (LOD) of the MON 87708 DMO (Figure 34, panel B). The LOD of MON 87708 DMO by Colloidal Brilliant Blue G staining was 0.02 µg or approximately 2% of the total protein loaded (0.02 µg divided by 1.0 µg of total protein loaded in each lane of the gel; Figure 34, panel B, lane 6).

Visual examination of the colloidal Brilliant Blue G stained Tris-glycine 8% gel (Figure 34, panel A) showed that MON 87708 DMO was digested to less than 2% of total protein loaded in SGF within 30 seconds (Figure 34, panel A, lane 5). No fragments corresponding to MON 87708 DMO were observed in the 30 second digestion sample. A diffuse, faint band with an approximate molecular weight of 21 kDa was observed for all time points from 30 seconds to 60 minutes in the colloidal Brilliant Blue stained Tricine 10-20% polyacrylamide gradient gel (Figure 33). The N-terminal sequence of this band was determined and it did not match any of the MON 87708 DMO sequences. It is likely that this fragment originated from soybean proteins that co-purified with MON 87708 DMO.

The digestibility of MON 87708 DMO in SGF was also evaluated by western blot. A two gel system was employed and proteins were separated by SDS-PAGE using a Tricine 10-20% polyacrylamide gradient gel (Figure 35) to determine if any fragments were detected, and a Tris-glycine 8% polyacrylamide gel (Figure 35) to confirm that the MON 87708 DMO protein was digested and not masked due to co-migration with pepsin. In both cases, the results demonstrated that MON 87708 DMO is digested within 30 seconds of exposure to SGF. The western blot of the Tris-glycine 8% polyacrylamide gel (Figure 36, panel A) was run concurrently with a western blot to determine the LOD for MON 87708 DMO (Figure 36, panel B). The LOD was determined to be 0.3 ng or approximately 1.5% of the total protein loaded (0.3 ng divided by 20 ng of the protein loaded in each lane of the gel; Figure 36, panel B, lane 7). Visual examination of the western blot confirmed that MON 87708 DMO was digested to less than 1.5% of the total protein loaded in SGF within 30 seconds (Figure 36, Panel A, lane 5). No fragments corresponding to MON 87708 DMO were observed. A faint band is visible at approximately 25 kDa in the MON 87708 DMO only controls and the SGF T0 sample, which is likely the result of non-specific binding of either the primary or secondary antibody to a protein that co-purified with MON 87708 DMO.

SIF was also used to test digestibility of MON 87708 DMO. The assay was performed according to methods described in the United States Pharmacopeia (USP, 1995). The digestion of MON 87708 DMO in SIF was separated by SDS-PAGE using a Tricine 10-20% polyacrylamide gradient gel and then evaluated by western blot (Figure 37). A western blot to determine the LOD (Figure 37, panel B) of MON 87708 DMO was performed concurrently with the SIF assay (Figure 37, panel A). The LOD was determined to be 1.0 ng

or approximately 5.0% of the total protein loaded (1.0 ng divided by 20 ng of loaded protein loaded in each lane of the gel; Figure 37, panel B, lane 6). Visual examination of the western blot confirmed that MON 87708 DMO was digested to less than 5% of the total protein loaded in SIF within five minutes (Figure 37, panel A, lane 5). No proteolytic fragments of MON 87708 DMO were detected at any digestion time points.

In conclusion, these results show that MON 87708 DMO was readily digestible in SGF and SIF. The rapid digestion of the MON 87708 DMO in SGF and SIF indicates that it is highly unlikely that MON 87708 DMO will pose any safety concern to human and animal health.

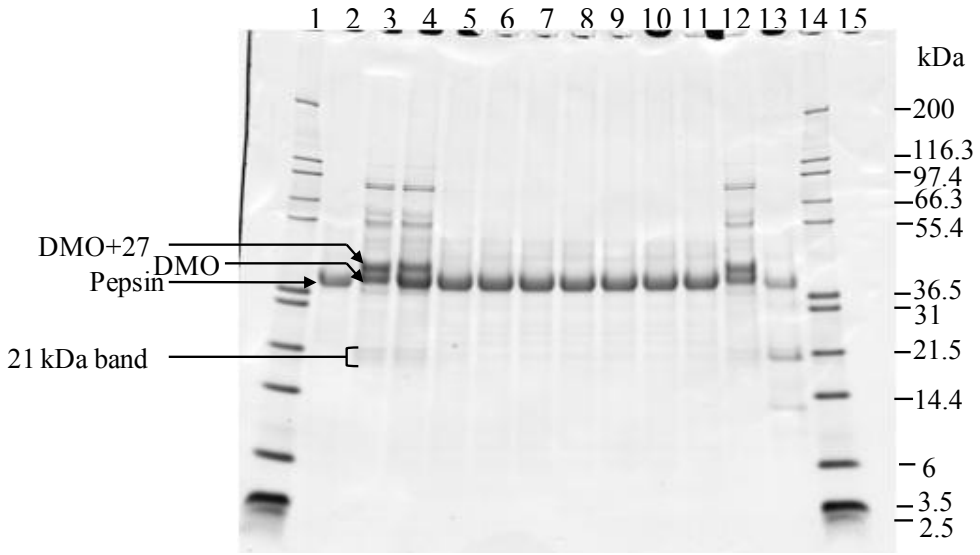


Figure 33. Colloidal Blue Stained 10-20% SDS-PAGE Gel Showing the Digestion of MON 87708 DMO in Simulated Gastric Fluid

Colloidal Brilliant Blue G stained Tricine 10-20% polyacrylamide gel was used to analyze the digestibility of MON 87708 DMO in SGF. Based on pre digestion total protein concentrations, 1.0 µg (total protein) was loaded in each lane containing MON 87708 DMO (SGF T0-SGF T7). Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel. N0 and N7 correspond to control samples that did not contain MON 87708 DMO. P0 and P7 correspond to controls that did not contain pepsin.

Lane	Sample	Incubation Time (min)
1	Mark 12 MWM	-
2	SGF N0	0
3	SGF P0	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	-
15	Blank	-

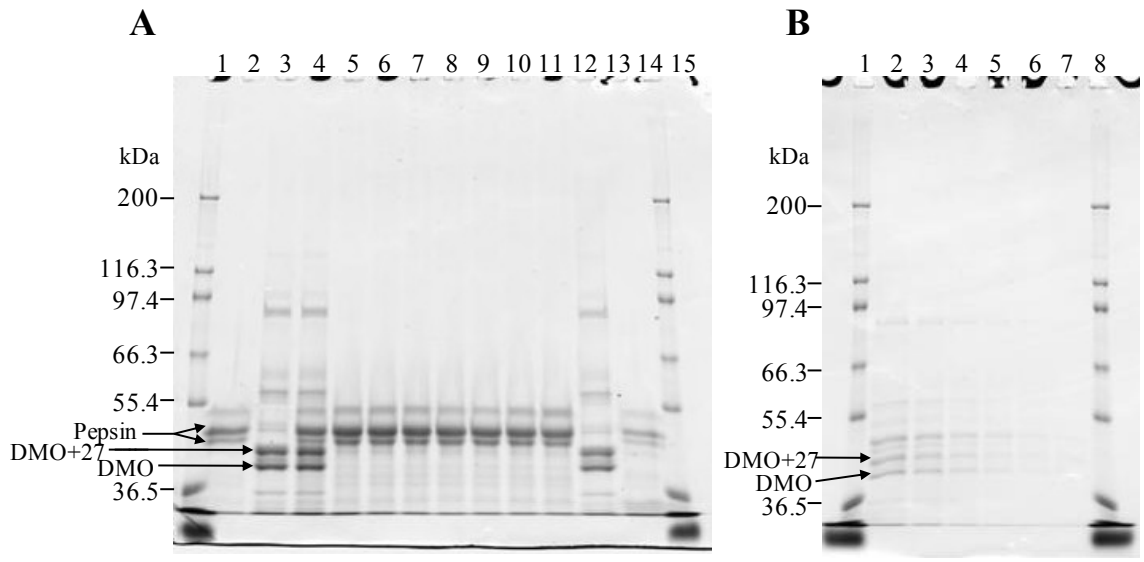


Figure 34. Colloidal Blue Stained 8% SDS-PAGE Gel Showing the Digestion of MON 87708 DMO in Simulated Gastric Fluid

Colloidal Brilliant Blue G stained 8% Tris-glycine polyacrylamide gels were used to analyze the digestibility of MON 87708 DMO in SGF. Panel A corresponds to the MON 87708 DMO digestion in SGF. Based on predigestion protein concentrations, 1.0 µg (total protein) was loaded in each lane containing MON 87708 DMO (SGF T0-SGF T7). Panel B corresponds to the analysis to determine the limit of detection of MON 87708 DMO. The amount loaded corresponds to the total protein amount of MON 87708 DMO. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel. N0 and N7 correspond to control samples that did not contain MON 87708 DMO. P0 and P7 correspond to controls that did not contain pepsin.

Panel A			Panel B		
Lane	**Sample**	**Incubation Time (min)**	**Lane**	**Sample**	**Amount (µg)**
1	Mark 12 MWM	-	1	Mark 12 MWM	-
2	SGF N0	0	2	T0, protein+SGF	0.25
3	SGF P0	0	3	T0, protein+SGF	0.13
4	SGF T0	0	4	T0, protein+SGF	0.06
5	SGF T1	0.5	5	T0, protein+SGF	0.03
6	SGF T2	2	6	T0, protein+SGF	0.02
7	SGF T3	5	7	T0, protein+SGF	0.01
8	SGF T4	10	8	Mark 12 MWM	-
9	SGF T5	20			
10	SGF T6	30			
11	SGF T7	60			
12	SGF P7	60			
13	SGF N7	60			
14	Mark 12 MWM	-			
15	Blank	-			

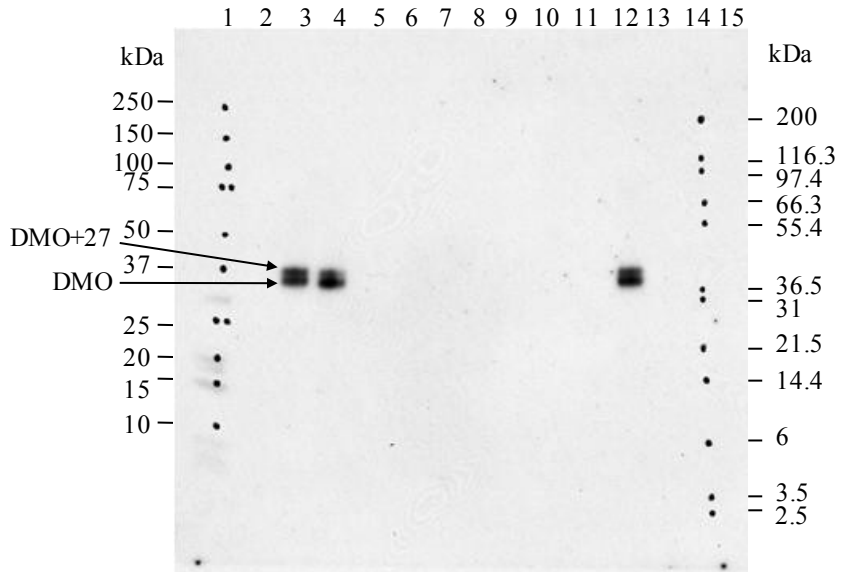


Figure 35. Western Blot Analysis Using 10-20% SDS-PAGE of MON 87708 DMO in Simulated Gastric Fluid

The figure corresponds to MON 87708 DMO digestion in SGF separated by SDS-PAGE using a Tricine 10-20% polyacrylamide gradient gel. Based on pre-digestion total protein concentrations, 20 ng (total protein) was loaded in the lanes containing MON 87708 DMO (SGF T0-SGF T7). Approximate molecular weights (kDa) are shown on the left and right of the blot. A 30 second exposure is shown. N0 and N7 correspond to control samples that did not contain MON 87708 DMO. P0 and P7 correspond to controls that did not contain pepsin.

Lane	Sample	Incubation Time (min)
1	Precision Plus MWM	-
2	SGF N0	0
3	SGF P0	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	-
15	Blank	-

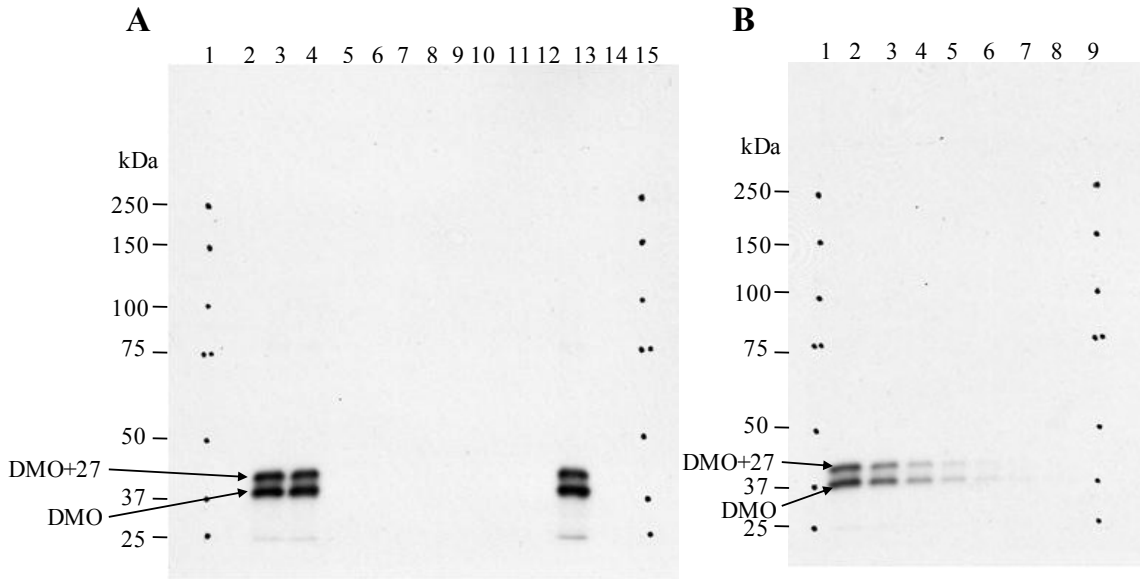


Figure 36. Western Blot Analysis Using 8% SDS-PAGE of MON 87708 DMO in Simulated Gastric Fluid

Panel A corresponds to MON 87708 DMO digestion in SGF separated by SDS-PAGE using Tris-glycine 8% polyacrylamide gels. Based on pre-digestion total protein concentrations, 20 ng (total protein) was loaded in the lanes containing MON 87708 DMO (SGF T0-SGF T7). Panel B corresponds to the analysis to determine the limit of detection of MON 87708 DMO. The amount loaded corresponds to the total protein amount of MON 87708 DMO. The lanes have been cropped and re-numbered. Approximate molecular weights (kDa) are shown on the left, and correspond to the markers loaded in each gel. A 15 second exposure is shown. N0 and N7 correspond to control samples that did not contain MON 87708 DMO. P0 and P7 correspond to controls that did not contain pepsin.

Panel A			Panel B		
Lane	**Sample**	**Incubation Time (min)**	**Lane**	**Sample**	**Amount (ng)**
1	Precision Plus MWM	-	1	Precision Plus MWM	-
2	SGF N0	0	2	T0, protein+SGF	8.0
3	SGF P0	0	3	T0, protein+SGF	4.0
4	SGF T0	0	4	T0, protein+SGF	2.0
5	SGF T1	0.5	5	T0, protein+SGF	1.0
6	SGF T2	2	6	T0, protein+SGF	0.5
7	SGF T3	5	7	T0, protein+SGF	0.3
8	SGF T4	10	8	T0, protein+SGF	0.1
9	SGF T5	20	9	Precision Plus MWM	-
10	SGF T6	30			
11	SGF T7	60			
12	SGF P7	60			
13	SGF N7	60			
14	Precision Plus MWM	-			
15	Blank	-			

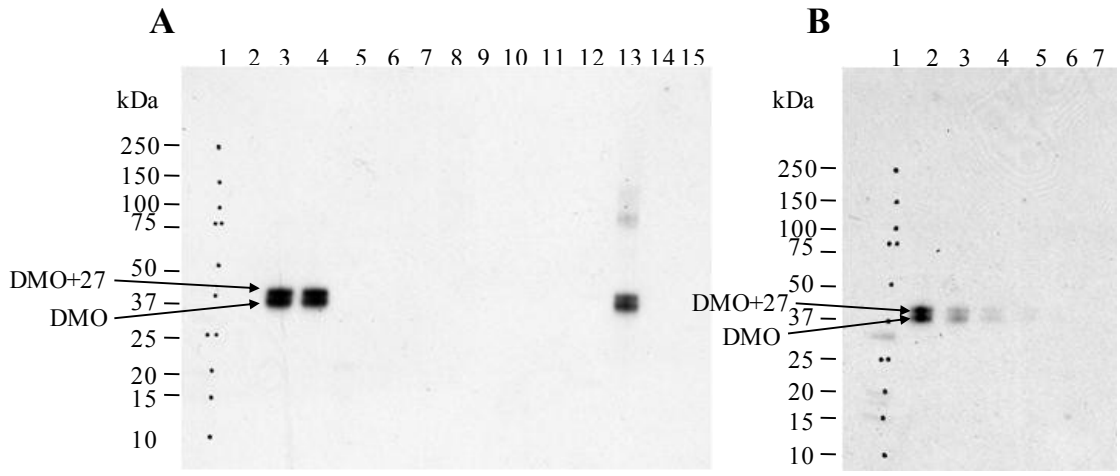


Figure 37. Western Blot Analysis of MON 87708 DMO in Simulated Intestinal Fluid

Panel A corresponds to MON 87708 DMO digestion in SIF. Based on pre-digestion protein concentrations, 20 ng (total protein) was loaded in the lanes containing MON 87708 DMO (SIF T0-SIF T8). Panel B corresponds to the limit of detection of MON 87708 DMO. The amount loaded corresponds to the total protein amount of MON 87708 DMO. The lanes were cropped and re-numbered. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel. A 15 second exposure is shown. N0 and N8 correspond to control samples that did not contain MON 87708 DMO. P0 and P8 correspond to controls that did not contain pancreatin. MWM denotes molecular weight marker.

Panel A			Panel B		
Lane	**Sample**	**Incubation Time**	**Lane**	**Sample**	**Amount (ng)**
1	Precision Plus MWM	-	1	Precision Plus MWM	-
2	SIF N0	0	2	T0, protein+SIF	15
3	SIF P0	0	3	T0, protein+SIF	10
4	SIF T0	0	4	T0, protein+SIF	5
5	SIF T1	5 minutes	5	T0, protein+SIF	2.5
6	SIF T2	15 minutes	6	T0, protein+SIF	1
7	SIF T3	30 minutes	7	Mark 12 MWM	-
8	SIF T4	1 hour			
9	SIF T5	2 hours			
10	SIF T6	4 hours			
11	SIF T7	8 hours			
12	SIF T8	24 hours			
13	SIF P8	24 hours			
14	SIF N8	24 hours			
15	Mark 12 MWM	-			

B4(c).2. Heat Stability of MON 87708 DMO

Temperature can have a profound effect on the structure and function of proteins. Soybean processing involves treatment of soybean with different temperatures for varying periods of time (Lusas, 2000; Lusas and Riaz, 1995). It is reasonable then to assume that the conditions encountered during the processing of soybean from MON 87708 will have an effect on the functional activity and structure of MON 87708 DMO when consumed in different food products.

The effect of heat treatment on the activity of MON 87708 DMO was evaluated using a functional activity assay. Aliquots of MON 87708 DMO were heated to 25, 37, 55, 75, and 95°C for 15 and 30 minutes, while a separate aliquot of MON 87708 DMO was maintained on ice for the duration of the heat treatments to serve as a temperature control. The heated and temperature control MON 87708 DMO samples were denatured and separated and analyzed by SDS-PAGE using Colloidal Blue staining to assess integrity of the MON 87708 DMO protein and the MON 87708 DMO+27 protein.

The effects of heating on the functional activity of MON 87708 DMO are presented in Table 13 and Table 14. The functional activity of MON 87708 DMO was unaffected at 25ºC and 37ºC for 15 and 30 minutes. After incubation at 55 ºC or higher for 15 minutes or more, the functional activity was below the LOQ of the assay, indicating that the majority of the functional activity of MON 87708 DMO had been lost during heating. These results suggest that temperature has a significant effect on the activity of MON 87708 DMO. MON 87708 DMO samples analyzed by SDS-PAGE showed no significant change in band intensity of the heat-treated samples at temperatures up to 55 °C (Figure 38 and Figure 39). Heating at 75 °C for 30 minutes did result in a visually detectable loss of the MON 87708 DMO protein and the MON 87708 DMO+27 protein (Figure 39). Heating at 95 °C for 15 minutes did result in a visually detectable loss of the MON 87708 DMO protein and the MON 87708 DMO+27 protein (Figure 38), and the proteins were almost completely visually undetectable after 30 minutes (Figure 39).

Soybean processing involves treatment of soybean with different temperature regimes, many of which are higher than 55 °C and of variable duration (Lusas, 2000; Lusas and Riaz, 1995). Additionally, many steps, especially deactivation of anti-nutrient components, are carried out at considerably higher temperature (*e.g.*, greater than 100 °C) leading to a loss in active MON 87708 DMO in products such as soybean meal (Lusas, 2000; Lusas and Riaz, 1995). Therefore, it is reasonable to conclude that MON 87708 DMO would not be consumed as an active protein in food products.

Table 13. Specific Activity of MON 87708 DMO Following 15 Minute Heat Treatment

Temperature	Specific Activity[1] (nmole/min/mg MON 87708 DMO)	Activity DMO Remaining (% of control treatment)
0 ºC (Control Treatment)	16.6	100 %[2]
25 ºC	17.9	95 %
37 ºC	17.0	90 %
55 ºC	Below LOQ[3]	<25 %
75 ºC	Below LOQ[3]	<25 %
95 ºC	Below LOQ[3]	<25 %

[1] The specific activity was determined by measuring the production of DCSA. Mean specific activity determined from n=3.
[2]DMO activity of control treatment was assigned 100 % active.
[3] The LOQ is 4.4 nmoles/min/mg MON 87708 DMO.

Table 14. Specific Activity of MON 87708 DMO Following 30 Minute Heat Treatment

Temperature	Specific Activity[1] (nmole/min/mg MON 87708 DMO)	Activity DMO Remaining (% of control treatment)
0 ºC (Control Treatment)	16.6	100 %[2]
25 ºC	17.8	95 %
37 ºC	12.0	85 %
55 ºC	Below LOQ[3]	<25 %
75 ºC	Below LOQ[3]	<25 %
95 ºC	Below LOQ[3]	<25 %

[1] The specific activity was determined by measuring the production of DCSA. Mean specific activity determined from n=3.
[2]DMO activity of control treatment was assigned 100 % active DMO.
[3] The LOQ is 4.4 nmoles/min/mg MON 87708 DMO.

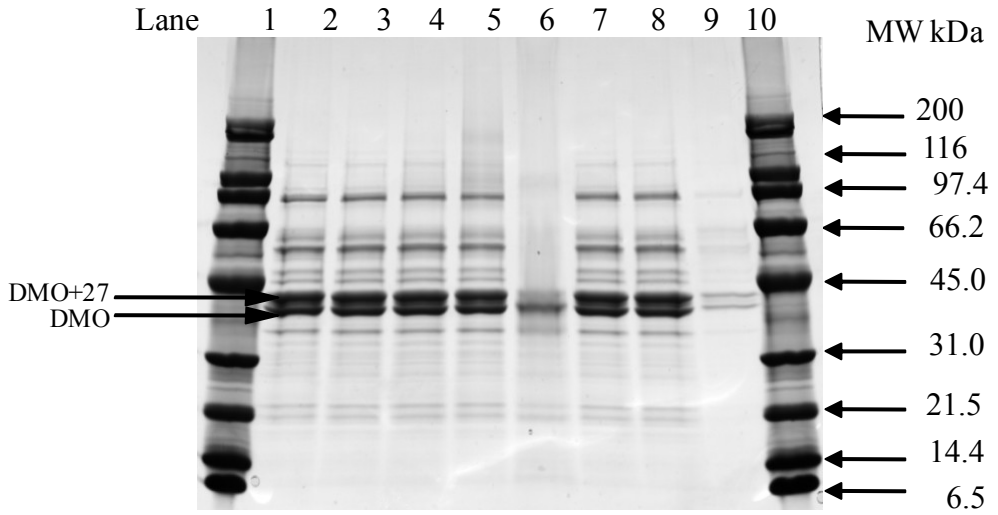


Figure 38. Colloidal Blue Stained SDS-PAGE of MON 87708 DMO Demonstrating the Effect after 15 Minutes at Elevated Temperatures on Protein Structural Stability

Heated samples of MON 87708 DMO (2.8 µg total protein) separated on a Tris-glycine 4-20% polyacrylamide gel under denaturing and reducing conditions. Gels were stained with Brilliant Blue G Colloidal. Approximate molecular weights (kDa) are shown on the right and correspond to molecular weight markers in lanes 1 and 10. Non-treated MON 87708 DMO samples were also mixed with loading buffer and loaded at 2.8 µg (100% equivalence) and 0.28 µg (10% equivalence) total protein.

Lane	Description	Amount (µg)
1	Broad Range Molecular Weight Markers	40.5
2	MON 87708 DMO 25ºC	2.8
3	MON 87708 DMO 37ºC	2.8
4	MON 87708 DMO 55ºC	2.8
5	MON 87708 DMO 75ºC	2.8
6	MON 87708 DMO 95ºC	2.8
7	MON 87708 DMO Temperature Control	2.8
8	MON 87708 DMO Reference 100% Equivalence	2.8
9	MO N87708 DMO Reference 10% Equivalence	0.28
10	Broad Range Molecular Weight Markers	40.5

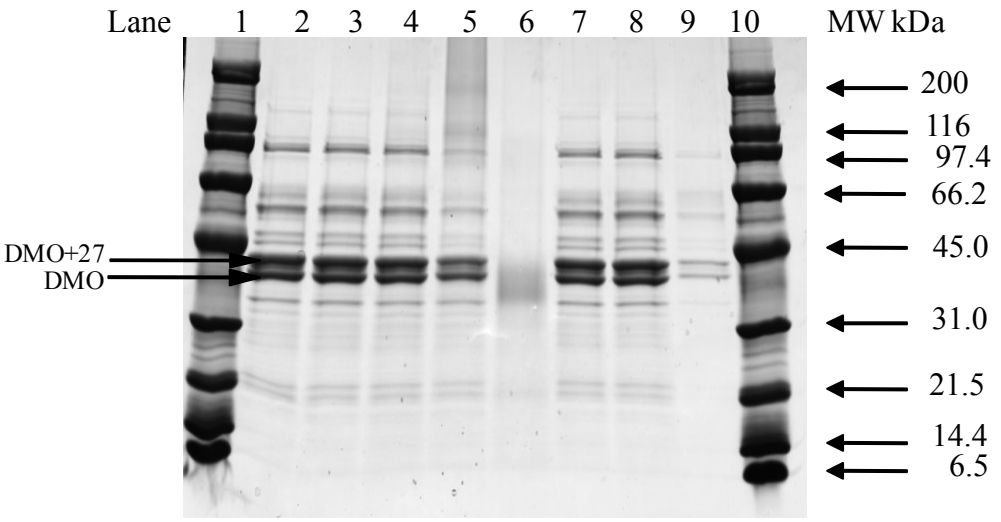


Figure 39. Colloidal Blue Stained SDS-PAGE of MON 87708 DMO Demonstrating the Effect after 30 Minutes at Elevated Temperatures on Protein Structural Stability

Heated samples of MON 87708 DMO (2.8 µg total protein) separated on a Tris-glycine 4-20% polyacrylamide gel under denaturing and reducing conditions. Gels were stained with Brilliant Blue G Colloidal. Approximate molecular weights (kDa) are shown on the right and correspond to molecular weight markers in lanes 1 and 10. Fresh MON 87708 DMO samples were also mixed with loading buffer and loaded at 2.8 µg (100% equivalence) and 0.28 µg (10% equivalence) total protein.

Lane	Description	Amount (µg)
1	Broad Range Molecular Weight Markers	40.5
2	MON 87708 DMO 25ºC	2.8
3	MON 87708 DMO 37ºC	2.8
4	MON 87708 DMO 55ºC	2.8
5	MON 87708 DMO 75ºC	2.8
6	MON 87708 DMO 95ºC	2.8
7	MON 87708 DMO Temperature Control	2.8
8	MON 87708 DMO Reference 100% Equivalence	2.8
9	MO N87708 DMO Reference 10% Equivalence	0.28
10	Broad Range Molecular Weight Markers	40.5

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

Soybean is one of eight allergenic foods that, together, are responsible for approximately 90% of all food allergies (Cordle, 2004). Soybean is less allergenic than other foods in this group and is rarely responsible for severe, life-threatening reactions (Cordle, 2004). Allergy to soybean is more prevalent in children than adults and is considered a transient allergy of infancy/childhood (Sicherer et al., 2000). Since soybean is a known allergenic food crop, there is a need to ensure that the levels of endogenous allergenic proteins in MON 87708 are similar to the levels of these proteins in commercially available soybean varieties that are currently consumed. To determine this, MON 87708 binding values were compared to the binding values observed in commercial soybean varieties. Determining the levels of direct IgE binding using an ELISA has been shown to be an appropriate method to perform such comparisons (Sten et al., 2004), especially when the assay is validated and calibrated prior to the production of the data (Ahlstedt, 2002; Holzhauser et al., 2008).

The purpose of this assessment was to quantitatively evaluate the binding potential of soybean-specific IgE antibody from soybean-allergic subjects to aqueous protein extracts prepared from ground soybean seeds of MON 87708, near-isogenic conventional soybean control A3525, and commercial reference varieties. A quantitative evaluation of soybean-specific IgE provides an estimate of the endogenous allergens present in soybean seed. Protein extracts prepared from ground soybean seeds of MON 87708, conventional control, and 17 commercial reference varieties were evaluated. The commercial reference varieties were used to establish the range of soybean-specific IgE binding using sera from clinically diagnosed soybean allergic individuals and included high protein, high oil, and food-grade (tofu) soybean varieties that are already on the market and are being used for human consumption.

Sera from 13 clinically documented soybean-allergic subjects and five non-allergic subjects were used to assess IgE binding to each soybean extract. Only soybean-allergic subjects with a documented case history of soybean allergy with anaphylaxis and a positive Double-Blind Placebo Controlled Food Challenge (DBPCFC) were included as soybean positive subjects.

Aqueous protein extracts were prepared from the ground soybean seed of MON 87708, the conventional control, and the commercial reference varieties. These extracts were then analyzed for soybean-specific IgE antibody binding using a validated ELISA. Each soybean extract was tested in triplicate. Soybean specific IgE binding was quantified by interpolation against a soybean-specific IgE standard curve and was expressed as ng of IgE/ml of serum. The standard curve was created by loading serial dilutions of human serum PEI 163 that contains a known amount of soybean-specific IgE into wells coated with internal reference soybean extract. The concentration of soybean-specific IgE in serum PEI 163 was 36 kU/l (kilo units per liter) as measured by Capsulated Hydrolic Carrier Polymer-FluoroEnzyme Immunoassay (CAP-FEIA).

The IgE binding values obtained for the 17 commercial reference varieties extracts were used to calculate a 99% tolerance interval for each subject's serum. The IgE binding values obtained for extracts prepared from MON 87708 and the conventional control were compared to the tolerance interval derived for each serum. All of the IgE binding values for MON 87708 and the control were within the commercial reference varieties tolerance limits for each subject's serum (Figure 40). None of the MON 87708, conventional control, or commercial reference varieties showed IgE binding to sera from non-allergic subjects.

The results of this assessment demonstrate that soybean-specific IgE binding to endogenous allergens in MON 87708 and the control are comparable with the IgE binding to commercially available conventional varieties. Therefore, MON 87708 and products derived from MON 87708 do not pose an increased endogenous allergenicity concern to humans over currently consumed soybean foods.

B4(e) MON 87708 DMO as a Proportion of Total Protein

MON 87708 DMO was detected in all plant tissues assayed at a number of time points during the growing season (Table 10). Harvested seed is the most relevant tissue analyzed for an allergenicity assessment because it can be consumed directly. The mean level of MON 87708 DMO in harvested seed is 47 µg/g dwt (Table 11). The mean percent dry weight of total protein in harvested seed from MON 87708 is 40.9% (or 409,000 µg/g) (Table 15). The percent of MON 87708 DMO in harvested seed from MON 87708 is 0.011% and is calculated as follows:

$$(47\ \mu g/g \div 409{,}000\ \mu g/g) \times 100\% = 0.011\%\ \text{of total soybean protein}$$

This low percent of MON 87708 DMO in relation to the total protein reduces the potential for the protein to be an allergen.

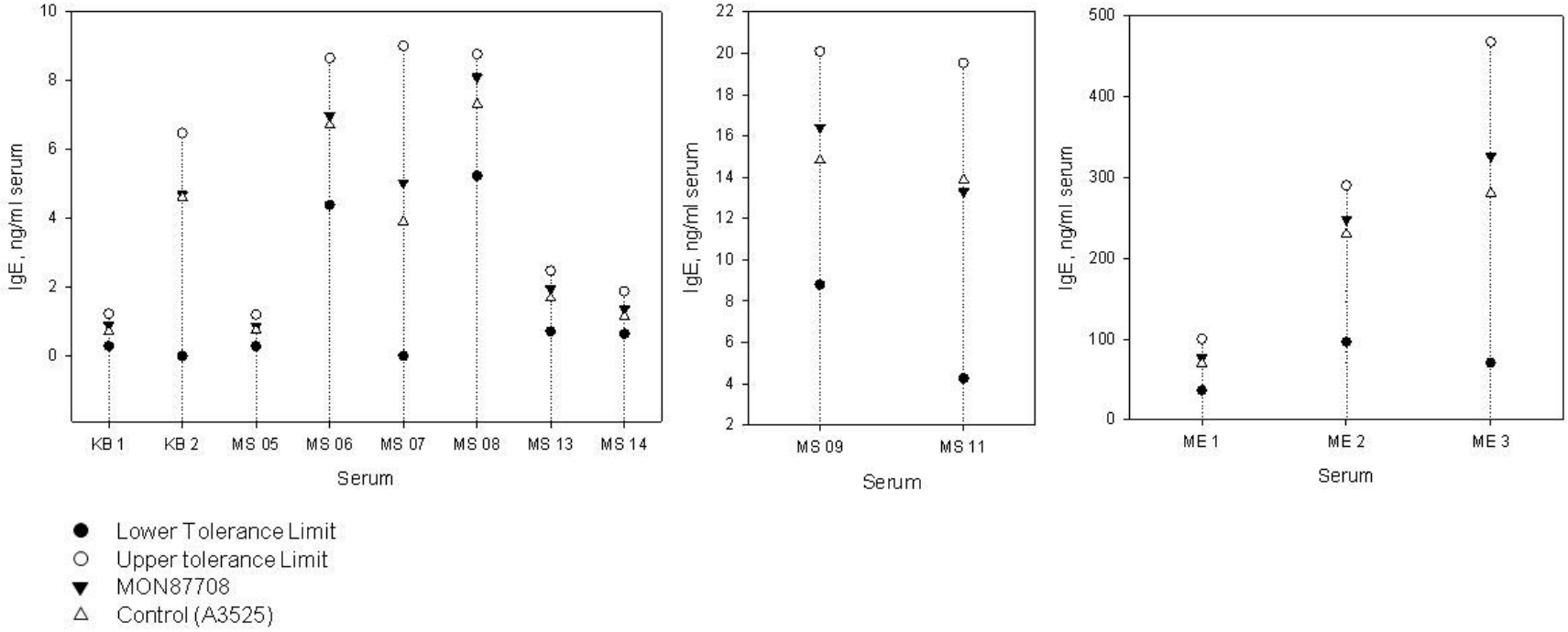


Figure 40. Serum IgE Binding Values for MON 87708, Conventional Control (A3525), and the Tolerance Limits for 17 Commercial Reference Varieties

Only those sera that met the set acceptance criteria were analyzed. The lower and upper tolerance limits for 99 % tolerance intervals with 95 % confidence for each serum are the result of a tolerance interval analysis for 17 commercial reference varieties. Lower limits of the tolerance intervals that were calculated as less than zero were reported as zero in the analysis. Data are presented in three graphs due to the difference in IgE concentration range between sera. Abbreviations KB, MS, and ME are subject designations.

B5 Compositional Assessment

Compositional equivalence between biotechnology-derived and conventional crops provides an "equal or increased assurance of the safety of foods derived from genetically modified plants" (OECD, 2001). The OECD consensus documents emphasize quantitative measurements of essential nutrients and known anti-nutrients. This is based on the premise that such comprehensive and detailed analyses will most effectively discern any compositional changes that imply potential safety and nutritional concerns. Levels of the components in seed and forage of the biotechnology-derived crop are compared to: 1) corresponding levels in a conventional comparator, the non-biotechnology near isogenic line, grown concurrently, under identical field conditions, and 2) natural ranges generated from an evaluation of conventional commercial reference varieties grown concurrently and from data published in the scientific literature.

Analyses of nutrient and anti-nutrient levels in MON 87708 and the near isogenic conventional soybean control A3525 were conducted to assess compositional equivalence. The tissues analyzed included seed and forage harvested from plants grown at five field sites in the U.S. during the 2008 field season. The composition analysis, conducted in accordance with OECD guidelines, also included measurement of nutrients and anti-nutrients in the conventional commercial reference varieties concurrently grown with MON 87708 to provide data on natural variability of each compositional component. All soybean plants including MON 87708, the conventional control, and the conventional commercial reference varieties were treated with maintenance pesticides as necessary throughout the growing season. In addition, MON 87708 plots were treated at the V2-V3 growth stage with dicamba herbicide at the maximum in-crop label rate (0.5 lb acid equivalence [a.e.]/acre).

For MON 87708 the combined-site analysis of both seed and forage showed no statistically significant differences between MON 87708 and conventional control for 21 (42.0%) of the 50 mean value comparisons. Of the statistically significant differences observed, one was from the forage analysis, and 28 were from the seed analysis. Nutrient component differences in seed included mean values for ash, carbohydrates by calculation, protein and 12 amino acids, five fatty acids, ADF, NDF, crude fiber, and vitamin E. In the combined-site analysis, all nutrient component differences in seed between MON 87708 and the conventional control were of small relative magnitude with respect to the conventional control and, whether increased or decreased, ranged from 1.51% to 12.37% for the three proximates, amino acids, fatty acids, and fibers, and 15.13% for vitamin E. Two of the nutrient components in the combined-site analysis (decreased levels of 18:1 oleic acid and increased levels of 18:3 linolenic acid) were also observed to be statistically different at all five individual sites, and one nutrient component (vitamin E) was observed to be increased at four of the five individual sites as in the combined-site analysis. The other combined-site differences occurred at fewer or none of the individual sites. Anti-nutrient component differences in seed were observed in mean values for phytic acid, raffinose, stachyose, and daidzein. In the combined-site analysis, all anti-nutrient component differences in seed between MON 87708 and the conventional control were of small relative magnitude, with respect to the conventional control, and ranged from a 6.14% decrease (phytic acid) to an 11.51% increase (daidzein). None of the anti-nutrient components were observed to be statistically different at more than two of the five individual sites. The only nutrient component difference in forage for the combined-site analysis was observed in ADF and its relative magnitude of difference, with respect to the conventional control, was 10.45%. No differences between MON 87708 and the conventional control ADF mean values were

observed at any of the five individual sites. Mean values of MON 87708 components with statistically significant differences to the conventional control were all within the 99% tolerance interval established from the conventional commercial reference varieties grown concurrently and at the same field sites, as well as ranges in the scientific literature and the ILSI Crop Composition Database.

In summary, a comprehensive evaluation of key nutrients and anti-nutrients in seed and key nutrients in forage supports the conclusion that soybean seed and forage produced from MON 87708 are compositionally equivalent to that of conventional soybean and that neither the dicamba tolerance trait in MON 87708, nor the dicamba herbicide treatment, applied according to maximum in-crop label rates (including the associated dicamba residue levels) have a meaningful impact on the composition and therefore on the food and feed safety or the nutritional quality of MON 87708 compared to conventional soybean.

Compositional Equivalence of MON 87708 Seed and Forage to Conventional Soybean

Seed and forage samples were collected from MON 87708 and the near isogenic conventional soybean control A3525 grown in a 2008 U.S. field production. Four different commercial reference varieties were included at each site of the field production to provide data on natural variability of each compositional component analyzed. The field production was conducted at five sites: Jefferson County, Iowa; Stark County, Illinois; Clinton County, Illinois; Parke County, Indiana; and Berks County, Pennsylvania. Two reference varieties at the site of Parke County, Indiana (Dekalb DKB 34-51 and Pioneer 93M50) are transgenic lines and compositional data from these lines were excluded from the assessment. All soybean plants including MON 87708, the conventional control, and 18 conventional commercial reference varieties were treated with maintenance pesticides as necessary throughout the growing season. In addition, MON 87708 plots were treated at the V2-V3 growth stage with dicamba herbicide at the maximum in-crop label rate (0.5 lb acid equivalence [a.e.]/acre).

Compositional analyses were conducted to assess whether levels of key nutrients and anti-nutrients in MON 87708 were equivalent to levels in the conventional control and within the range of natural variability of the conventional commercial reference varieties. A description of nutrients and anti-nutrients present in soybean is provided in the OECD consensus document on compositional considerations for soybean (OECD, 2001). Nutrients assessed included proximates (ash, carbohydrates by calculation, moisture, protein, and fat), fiber, amino acids (18 components), fatty acids (FA, C8-C22), and vitamin E (α- tocopherol) in seed, and proximates (ash, carbohydrates by calculation, moisture, protein, and fat) and fiber in forage. Anti-nutrients assessed in seed included raffinose, stachyose, lectin, phytic acid, trypsin inhibitors, and isoflavones (daidzein, genistein, and glycitein).

In all, 64 different components were measured (seven in forage and 57 in seed). Of those 64 components, 14 had more than 50% of the observations below the assay limit of quantitation (LOQ) and subsequently were excluded from statistical analysis. Therefore, 50 components were statistically assessed using a mixed-model analysis of variance method. Values for all assessed components were reported on a dry weight basis with the exception of moisture, which was reported as % fresh weight (fwt) and fatty acids, which were reported as % of total FA.

For MON 87708, six statistical comparisons to the conventional control were conducted. One comparison was based on compositional data combined across all five field sites (combined-site analysis) and five separate comparisons were conducted on data from each of the individual field sites. Statistically significant differences were identified at a 5% level of

significance. Data from the conventional commercial reference varieties were combined across all sites and used to calculate a 99% tolerance interval for each compositional component to define the natural variability of each component in soybean varieties that have a history of safe consumption and that were grown concurrently with MON 87708 and the conventional control in the same trial.

For the combined-site analysis, statistically significant differences in nutrient and anti-nutrient components were further evaluated using considerations relevant to the safety and nutritional quality of MON 87708 when compared to the conventional control A3525, the conventional counterpart with a history of safe consumption: 1) the relative magnitude of the difference in the mean values of nutrient and anti-nutrient components of MON 87708 and the conventional control, 2) whether the MON 87708 component mean value is within the range of natural variability of that component as represented by the 99% tolerance interval of the conventional commercial varieties grown concurrently in the same trial, 3) analyses of the reproducibility of the statistically significant combined-site component differences at individual sites, and 4) assessing the differences within the context of natural variability of conventional commercial soybean composition published in the scientific literature and in the ILSI Crop Composition Database (ILSI, 2006[2]; Ridley et al., 2004).

This analysis provides a comprehensive comparative assessment of the levels of key nutrients and anti-nutrients in seed, and of key nutrients in forage of MON 87708 and the conventional control, discussed in the context of natural variability in conventional commercial soybean. Results of the comparison indicate that the composition of the seed and forage of MON 87708 is equivalent to that of the near isogenic conventional soybean control A3525 and within the range of natural variability of the conventional commercial reference varieties.

B5(a) Levels of key nutrients, toxicants and anti-nutrients

Nutrient Levels in Soybean Seed

In the combined-site analysis of nutrient levels in seed, the following components showed no statistically significant differences in mean values between MON 87708 and the conventional control: moisture, total fat, six amino acids (alanine, lysine, methionine, serine, threonine, and tryptophan), and three fatty acids (18:0 stearic acid, 20:0 arachidic acid, and 20:1 eicosenoic acid) (Table 16).

The components that showed statistically significant differences in mean values between MON 87708 and the conventional control in the combined-site analysis were: three proximates (ash, carbohydrates by calculation, and protein), 12 amino acids (arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, phenylalanine, proline, tyrosine, and valine), three types of fiber (acid detergent fiber [ADF], neutral detergent fiber [NDF], and crude fiber), five fatty acids (16:0 palmitic acid, 18:1 oleic acid, 18:2 linoleic acid, 18:3 linolenic acid, and 22:0 behenic acid), and vitamin E (Table 15 and Table 16).

These statistically significant differences in nutrients were evaluated using considerations relevant to the safety and nutritional quality of MON 87708 when compared to the conventional control:

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

1) All nutrient component differences observed in the combined-site analysis, whether reflecting increased or decreased MON 87708 mean values with respect to the conventional control were small. Relative magnitude of differences ranged from 2.65 to 7.91% for amino acids, 1.51 to 8.19% for fatty acids, 15.13% for vitamin E, and 2.41 to 12.37% for proximates and fibers.

2) Mean values for all of these statistically different nutrient components from the combined-site analysis of MON 87708 were within the 99% tolerance interval established from the conventional commercial reference varieties grown concurrently and were, therefore, within the range of natural variability of that component in conventional commercial soybean varieties with a history of safe consumption (Table 15 and Table 16).

3) Assessment of the reproducibility of the combined-site differences at the five individual sites showed: statistically significant differences for carbohydrates by calculation, crude fiber, cystine, and glycine at one site; aspartic acid, phenylalanine, proline, tyrosine, valine, 16:0 palmitic acid, and 18:2 linoleic acid at two sites; protein, arginine, glutamic acid, histidine, isoleucine, leucine, and 22:0 behenic acid at three sites; vitamin E at four sites; and 18:1 oleic acid and 18:3 linolenic acid differed across all five sites. Although they were different in the combined site analysis, no differences were observed for ash, ADF or NDF at any of the individual sites. Individual site mean values of MON 87708 for all nutrient components with statistically significant differences fell within the 99% tolerance interval established from the conventional commercial reference varieties grown concurrently and were, therefore, within the range of natural variability of that component in conventional commercial soybean varieties with a history of safe consumption.

4) With the exception of the minor fatty acid 22:0 behenic acid, all combined-site mean values of MON 87708 for all nutrient components were within the context of the natural variability of conventional commercial soybean composition as published in the scientific literature and/or available in the ILSI Crop Composition Database (ILSI, 2006; Ridley et al., 2004).

Thirteen of the 24 differences between MON 87708 and the conventional control observed in the combined-site data analysis were attributable to small differences in protein and 12 individual amino acids (all expressed as % dwt). The relative magnitude of the difference between the mean protein values for MON 87708 and the conventional control was small (a decrease of 3.65% in the combined-site analysis for MON 87708) and reached statistical significance at only three of the five individual sites. Correspondingly, differences in all amino acids were small and not observed consistently as statistically significant differences at all individual sites. Eleven of the 12 amino acids observed to be different in the combined-site analysis were decreased (2.65 - 7.91%) relative to the conventional control and, as with protein, statistically significant differences were not consistently observed at all individual sites. Cystine showed a relative increase of 3.01% but was statistically significantly different at only one site. Four of the six amino acids (alanine, lysine, serine, and threonine) not observed to be statistically different in the combined-site analysis also showed modest decreases ranging from ~ 1.5 - 2.3% (Table 16) consistent with the directionality of the changes observed in protein content. Overall, observed differences in protein and amino acid levels are not considered to be meaningful from a food and feed safety and nutritional perspective because they were small, and the mean MON 87708 values were within the 99% tolerance interval established by the conventional commercial reference varieties grown concurrently in the same trial.

Five of the combined-site differences between MON 87708 and the conventional control were attributable to fatty acid levels (all expressed as % total FA) in seed, whereas total fat

content was not statistically significantly different. For 18:1 oleic acid and 18:3 linolenic acid, the relative magnitude of differences between the mean values for MON 87708 and conventional control were small in the combined-site analysis (a decrease of 8.19% and an increase of 6.65% compared to the conventional control, respectively) and at the five individual sites (levels were <11% decreased for 18:1 oleic acid and <10% increased for 18:3 linolenic acid at all sites compared to conventional control) (Table 16).

By comparison, the observed differences between MON 87708 and conventional control for 18:1 oleic and 18:3 linolenic acids are markedly less than differences in soybean varieties developed through conventional breeding (Clemente and Cahoon, 2009; Fehr, 2007). The average relative levels of 18:3 linolenic acid in conventional commercial soybean are approximately 10% total FA, while the average relative level of 18:1 oleic acid in conventional commercial soybean is approximately 18-25% total FA. In the compositional analysis presented here, the values of FA components in the conventional control, when assessed as individual replicates across all five individual sites, ranged from 19.6 to 22.4% total FA for 18:1 oleic acid and from 8.4 to 10.1% total FA for 18:3 linolenic acid (Table 16). The values from the conventional commercial reference varieties ranged from 17.9 to 25.3% total FA for 18:1 oleic acid and 7.4 to 10.6% total FA for 18:3 linolenic acid (Table 16). Additionally, literature data from Lundry et al. (2008) and Berman et al. (2009) and the ILSI Crop Composition Database (Berman et al., 2009; ILSI, 2006; Lundry et al., 2008; Ridley et al., 2004) highlight the extensive natural variability in fatty acid levels in soybean, as presented in Table 19. The small relative magnitudes of the differences in 18:3 linolenic acid and 18:1 oleic acid compared to the conventional control as well the broad range of these fatty acids present in conventional commercial soybean varieties, suggest that the differences are not meaningful to food and feed safety and nutritional quality in MON 87708.

The relative magnitudes of differences between the mean values for MON 87708 and the conventional control for the other three fatty acids observed in the combined-site analysis were small (2.29% increase for 16:0 palmitic acid, 1.51% increase for 18:2 linoleic acid and a 4.70% decrease for 22:0 behenic acid). The small magnitude of differences as well as the lack of statistical differences across all individual sites (Table 16) further confirmed that the differences observed in fatty acid composition are not meaningful to food and feed safety and nutritional quality.

One of the combined-site differences observed between MON 87708 and the conventional control was attributable to vitamin E (expressed as mg/100g dwt). The relative magnitude of difference between the mean values of MON 87708 and conventional control for vitamin E in the combined-site analysis was an increase of 15.1% with respect to the conventional control (Table 15).

Levels of vitamin E are known to be affected by environmental growing conditions (E) and germplasm (G) as demonstrated in results from recent assessments on soybean varieties grown at three locations in the U.S. over a period of four years (Britz et al., 2008) and across six environments in Eastern Canada in a single year (Seguin et al., 2009). Britz et al. (2008) showed more than a two-fold variation in levels across their study (units expressed as the ratio of α-tocopherol [vitamin E] to total tocopherol content). Vitamin E values in Seguin et al. (2009) ranged from 0.87 to 3.32 mg/100g dwt. Both assessments showed that G and E effects as well as G × E interaction effects influenced vitamin E content. In the compositional analysis presented here, values of vitamin E in the conventional control, when assessed as individual replicates across all sites, ranged by as much as 0.89 to 2.11 mg/100g dwt (Table 16). Ranges of vitamin E values from the concurrently grown conventional commercial reference varieties were even greater and ranged from 0.69 to 2.91 mg/100g dwt

(Table 16). Literature data from other compositional assessments (Berman et al., 2009; ILSI, 2006; Lundry et al., 2008; Ridley et al., 2004) that further highlight the extensive natural variability in vitamin E levels in soybean are presented in Table 19. Therefore, given this established variability of vitamin E levels in conventional soybean and the fact that soybean is not an important nutritional source of vitamin E in human or animal diets, this increase in vitamin E levels in MON 87708 compared to the conventional control supports the conclusion that this observed difference is not meaningful to food and feed safety and nutritional quality.

The remaining combined-site differences between MON 87708 and the conventional control were attributable to two proximates (ash and carbohydrates by calculation) and three fibers (ADF, NDF, and crude fiber). The relative magnitude of these increases were small (2.41% to 12.37%) and there was no consistency of these combined-site differences at the individual sites (carbohydrates by calculation and crude fiber were different at only one site, whereas ash, ADF and NDF were not different at any of the individual sites). The combined-site mean values for these nutrient components also were within the 99% tolerance interval established from the conventional commercial reference varieties grown concurrently establishing that these differences are not meaningful to food and feed safety and nutrition.

In summary, statistical analyses found no consistent differences across sites in the levels of nutrient components in seed from MON 87708 and the conventional control, except for differences in 18:1 oleic acid, 18:3 linoleic acid, and vitamin E levels that were of small magnitude and were within the natural variability of the concurrently grown conventional commercial soybean varieties. These data support the conclusion that MON 87708 is compositionally equivalent to conventional soybean.

Anti-Nutrient Levels in Soybean Seed

In the combined-site analysis, no statistically significant differences were observed in four of the eight anti-nutrient component comparisons (lectin, trypsin inhibitors, genistein, and glycitein) between MON 87708 and the conventional control. Statistically significant differences were observed between MON 87708 and the conventional control in the other four anti-nutrient components that were measured (Table 15 and Table 17). The differences included decreased mean values for phytic acid, raffinose, stachyose, and an increased mean level of daidzein, compared to the conventional control.

The statistically significant differences in anti-nutrients were evaluated using considerations relevant to the safety and nutritional quality of MON 87708 when compared to the conventional control:

1) All anti-nutrient component differences observed in the combined-site analysis, whether reflecting increased or decreased MON 87708 mean values with respect to the conventional control were small. Relative magnitude of differences in the combined-site analysis for the anti-nutrients that were decreased in MON 87708 ranged from 6.1% (phytic acid) to 7.73% (raffinose). The relative magnitude of difference (increase) in daidzein was 11.5%.

2) MON 87708 mean values for these anti-nutrient components from the combined-site analysis were within the 99% tolerance interval established from the conventional commercial reference varieties concurrently grown in the same trial and, therefore were within the range of natural variability of these components in conventional commercial soybean varieties with a history of safe consumption (Table 15 and Table 17).

3) Assessment of the reproducibility of the combined-site differences at the five individual sites showed no consistent pattern across sites. A statistically significant decrease

was observed for stachyose at one site and phytic acid at two sites, whereas a significant increase was seen for daidzein at two sites. No differences for raffinose were observed at any of the individual sites. Mean values for all of the above anti-nutrient components in MON 87708 at the individual sites were within the 99% tolerance interval established from the concurrently grown conventional commercial reference varieties.

4) All mean values of MON 87708 for all anti-nutrients were within the context of the natural variability of conventional commercial soybean composition as published in the scientific literature and available in the ILSI Crop Composition Database (ILSI, 2006; Ridley et al., 2004).

In summary, statistical analyses found no consistent differences across sites in the levels of anti-nutrient components in seed from MON 87708 and the conventional control. Thus, a comprehensive evaluation of anti-nutrient components in seed support the conclusion that MON 87708 is compositionally equivalent to conventional soybean.

Nutrient Levels in Soybean Forage

In the combined-site analysis of forage, six of the seven nutrient component comparisons did not have a statistically significant difference between MON 87708 and the conventional control (Table 15 and Table 18). The only statistical difference was for the ADF mean value and it was evaluated using considerations relevant to the safety and nutritional quality of MON 87708 when compared to the conventional control.

1) The relative magnitude of difference in ADF, with respect to the conventional control, was small with an increase of 10.45%.

2) The mean value for ADF from the combined-site analysis of MON 87708 was within the 99% tolerance interval established from the conventional commercial reference varieties grown concurrently in the same trial and, therefore within the range of natural variability of that component in conventional commercial soybean varieties with a history of safe consumption (Table 15 and Table 18).

3) Assessment of the reproducibility of the combined-site difference of ADF across the individual sites showed no statistically significant differences at any of the five individual sites.

4) The level of ADF was within the natural variability observed for conventional commercial soybean varieties as published in the scientific literature and available in the ILSI Crop Composition Database (ILSI, 2006; Ridley et al., 2004).

In summary, statistical analyses found no consistent differences across sites in the levels of nutrient components in forage from MON 87708 and the conventional control. Thus, a comprehensive evaluation of nutrient components in forage supports the conclusion that MON 87708 is compositionally equivalent to conventional soybean.

Table 15. Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Mean Difference (MON 87708 minus Control) Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistical Significant Differences Observed in Combined-Site Analysis						
Seed Proximate (% dwt)						
Ash	5.24	5.12	2.41	0.031	4.94 - 5.69	4.75, 6.04
Carbohydrates	37.93	36.64	3.50	0.012	35.65 - 39.21	31.73, 40.38
Protein	40.86	42.41	-3.65	0.016	39.00 - 42.53	35.15, 45.33
Seed Fiber (% dwt)						
Acid Detergent Fiber	13.55	12.86	5.30	0.009	12.45 - 15.57	9.73, 18.36
Crude Fiber	8.29	7.37	12.37	<0.001	6.23 - 9.65	5.71, 10.92
Neutral Detergent Fiber	15.29	14.34	6.63	0.028	13.11 - 17.83	11.03, 19.66
Seed Amino Acid (% dwt)						
Arginine	3.30	3.58	-7.91	0.006	3.09 - 3.50	2.50, 3.88

Table 15. Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control (cont.)

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Mean Difference (MON 87708 minus Control) Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistical Significant Differences Observed in Combined-Site Analysis						
Seed Amino Acid (% dwt)						
Aspartic Acid	4.63	4.78	-3.18	0.016	4.44 - 4.80	4.00, 5.16
Cystine	0.61	0.59	3.01	<0.001	0.58 - 0.63	0.50, 0.67
Glutamic Acid	7.38	7.69	-4.03	0.010	7.05 - 7.73	6.20, 8.36
Glycine	1.76	1.81	-2.65	0.020	1.67 - 1.83	1.51, 1.93
Histidine	1.06	1.09	-3.07	0.017	1.02 - 1.10	0.92, 1.17
Isoleucine	1.88	1.95	-3.58	0.006	1.75 - 1.97	1.63, 2.08
Leucine	3.06	3.17	-3.37	0.008	2.93 - 3.19	2.69, 3.41
Phenylalanine	2.06	2.13	-3.33	0.034	1.92 - 2.18	1.78, 2.31

Table 15. Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control (cont.)

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Mean Difference (MON 87708 minus Control) Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistical Significant Differences Observed in Combined-Site Analysis						
Seed Amino Acid (% dwt)						
Proline	1.99	2.05	-3.24	0.017	1.90 - 2.09	1.62, 2.28
Tyrosine	1.37	1.42	-3.47	0.048	1.28 - 1.46	1.23, 1.51
Valine	1.98	2.06	-3.89	0.006	1.82 - 2.09	1.70, 2.22
Seed Fatty Acid (% Total FA)						
16:0 Palmitic	11.59	11.33	2.29	0.002	11.25 - 12.16	8.44, 12.65
18:1 Oleic	19.20	20.91	-8.19	<0.001	17.85 - 19.94	15.67, 27.49
18:2 Linoleic	54.40	53.59	1.51	0.010	53.42 - 55.67	48.22, 59.63
18:3 Linolenic	10.12	9.49	6.65	<0.001	8.99 - 10.88	5.92, 12.52
22:0 Behenic	0.27	0.28	-4.70	0.001	0.25 - 0.29	0.24, 0.40

Table 15. Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control (cont.)

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Mean Difference (MON 87708 minus Control) Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistical Significant Differences Observed in Combined-Site Analysis						
Seed Vitamin (mg/100g dwt)						
Vitamin E	1.41	1.23	15.13	0.001	1.08 - 2.17	0, 3.61
Seed Anti-nutrient (% dwt)						
Phytic Acid	1.30	1.39	-6.14	0.043	1.08 - 1.51	0.80, 1.93
Raffinose	0.43	0.47	-7.73	0.045	0.32 - 0.59	0.11, 0.73
Stachyose	3.36	3.62	-7.24	0.011	3.07 - 4.02	2.23, 4.11
Seed Isoflavone (µg/g dwt)						
Daidzein	1494.97	1340.71	11.51	0.046	899.83 - 2305.26	0, 2357.53
Forage Fiber (% dwt)						
Acid Detergent Fiber	30.58	27.69	10.45	0.021	23.30 - 45.11	15.60, 42.84

Table 15. Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control (cont.)

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Mean Difference (MON 87708 minus Control) Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistical Significant Differences Observed in Five Individual Sites						
Seed Fatty Acid (% Total FA)						
18:1 Oleic Site IARL	19.38	21.67	-10.58	0.001	19.07 - 19.73	15.67, 27.49
18:1 Oleic Site ILCY	19.74	21.57	-8.46	0.011	19.44 - 19.94	15.67, 27.49
18:1 Oleic Site ILWY	19.52	21.14	-7.66	0.010	19.34 - 19.64	15.67, 27.49
18:1 Oleic Site INRC	18.78	20.19	-6.96	<0.001	18.58 - 18.95	15.67, 27.49
18:1 Oleic Site PAHM	18.58	20.01	-7.13	0.015	17.85 - 19.42	15.67, 27.49
18:3 Linolenic Site IARL	10.64	10.04	5.94	0.033	10.58 - 10.74	5.92, 12.52
18:3 Linolenic Site ILCY	9.07	8.58	5.78	0.007	8.99 - 9.16	5.92, 12.52
18:3 Linolenic Site ILWY	10.54	10.05	4.92	0.026	10.51 - 10.59	5.92, 12.52

Table 15. Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control (cont.)

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Mean Difference (MON 87708 minus Control) Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistical Significant Differences Observed in Five Individual Sites						
Seed Fatty Acid (% Total FA)						
18:3 Linolenic Site INRC	10.03	9.31	7.65	<0.001	9.89 - 10.10	5.92, 12.52
18:3 Linolenic Site PAHM	10.33	9.47	9.02	0.006	9.91 - 10.88	5.92, 12.52
Statistical Significant Differences Observed in Four individual Sites						
Seed Vitamin (mg/100g dwt)						
Vitamin E Site IARL	1.15	0.94	22.25	0.033	1.10 - 1.22	0, 3.61
Vitamin E Site ILCY	2.13	1.86	14.43	0.038	2.10 - 2.17	0, 3.61
Vitamin E Site ILWY	1.18	0.94	24.64	0.011	1.08 - 1.26	0, 3.61
Vitamin E Site PAHM	1.32	1.23	7.90	0.010	1.21 - 1.54	0, 3.61
Seed Proximate (% dwt)						
Protein Site ILCY	40.17	41.72	-3.72	0.047	39.44 - 40.96	35.15, 45.33

Table 15. Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control (cont.)

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Mean Difference (MON 87708 minus Control) Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistical Significant Differences Observed in Three Individual Sites						
Seed Proximate (% dwt)						
Protein Site ILWY	40.88	41.99	-2.64	0.042	40.56 - 41.37	35.15, 45.33
Protein Site PAHM	40.25	43.69	-7.86	0.002	39.00 - 41.05	35.15, 45.33
Seed Amino Acid (% dwt)						
Arginine Site ILWY	3.30	3.57	-7.58	0.002	3.24 - 3.33	2.50, 3.88
Arginine Site INRC	3.44	3.72	-7.37	0.011	3.39 - 3.50	2.50, 3.88
Arginine Site PAHM	3.25	3.88	-16.13	0.001	3.09 - 3.36	2.50, 3.88
Glutamic Acid Site ILCY	7.43	7.61	-2.38	0.032	7.27 - 7.54	6.20, 8.36
Glutamic Acid Site ILWY	7.29	7.51	-2.86	0.002	7.20 - 7.35	6.20, 8.36
Glutamic Acid Site PAHM	7.28	8.00	-9.08	0.003	7.06 - 7.40	6.20, 8.36

Table 15. Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control (cont.)

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Mean Difference (MON 87708 minus Control) Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistical Significant Differences Observed in Three Individual Sites						
Seed Amino Acid (% dwt)						
Histidine Site ILCY	1.06	1.08	-1.84	0.022	1.04 - 1.07	0.92, 1.17
Histidine Site ILWY	1.05	1.07	-1.62	0.019	1.05 - 1.05	0.92, 1.17
Histidine Site PAHM	1.05	1.13	-7.52	0.002	1.02 - 1.06	0.92, 1.17
Isoleucine Site ILCY	1.89	1.97	-3.98	0.010	1.87 - 1.93	1.63, 2.08
Isoleucine Site ILWY	1.87	1.90	-1.22	0.004	1.85 - 1.89	1.63, 2.08
Isoleucine Site PAHM	1.85	2.00	-7.59	0.014	1.79 - 1.90	1.63, 2.08
Leucine Site ILCY	3.09	3.17	-2.42	0.002	3.04 - 3.14	2.69, 3.41
Leucine Site ILWY	3.02	3.10	-2.49	<0.001	3.00 - 3.04	2.69, 3.41

Table 15. Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control (cont.)

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Mean Difference (MON 87708 minus Control) Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistical Significant Differences Observed in Three Individual Sites						
Seed Amino Acid (% dwt)						
Leucine Site PAHM	3.03	3.28	-7.42	0.002	2.96 - 3.09	2.69, 3.41
Seed Fatty Acid (% Total FA)						
22:0 Behenic Site IARL	0.26	0.28	-5.49	0.022	0.25 - 0.27	0.24, 0.40
22:0 Behenic Site ILWY	0.26	0.28	-6.67	0.008	0.26 - 0.27	0.24, 0.40
22:0 Behenic Site INRC	0.28	0.29	-4.85	0.038	0.27 - 0.29	0.24, 0.40
Statistical Significant Differences Observed in Two Individual Sites						
Seed Proximate (% fwt)						
Moisture Site ILWY	6.96	6.16	12.99	0.022	6.80 - 7.17	4.10, 9.78
Moisture Site PAHM	7.84	10.50	-25.30	<0.001	7.38 - 8.47	4.10, 9.78
Seed Amino Acid (% dwt)						
Aspartic Acid Site ILWY	4.59	4.67	-1.90	0.011	4.55 - 4.61	4.00, 5.16

Table 15. Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control (cont.)

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Mean Difference (MON 87708 minus Control) Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistical Significant Differences Observed in Two Individual Sites						
Seed Amino Acid (% dwt)						
Aspartic Acid Site PAHM	4.56	4.94	-7.65	0.002	4.45 - 4.63	4.00, 5.16
Phenylalanine Site ILWY	2.01	2.07	-2.95	0.046	1.96 - 2.06	1.78, 2.31
Phenylalanine Site PAHM	2.04	2.21	-7.96	0.010	2.00 - 2.07	1.78, 2.31
Proline Site ILWY	1.94	2.05	-5.09	0.020	1.93 - 1.96	1.62, 2.28
Proline Site PAHM	1.98	2.10	-5.98	0.016	1.94 - 2.00	1.62, 2.28
Threonine Site ILWY	1.52	1.55	-1.69	0.005	1.51 - 1.53	1.39, 1.69
Threonine Site PAHM	1.55	1.62	-4.23	0.029	1.52 - 1.57	1.39, 1.69
Tyrosine Site INRC	1.38	1.44	-4.49	0.044	1.35 - 1.43	1.23, 1.51

Table 15. Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control (cont.)

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Mean Difference (MON 87708 minus Control) Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistical Significant Differences Observed in Two Individual Sites						
Seed Amino Acid (% dwt)						
Tyrosine Site PAHM	1.35	1.49	-9.43	0.011	1.28 - 1.43	1.23, 1.51
Valine Site ILCY	1.96	2.05	-4.37	0.013	1.94 - 2.01	1.70, 2.22
Valine Site PAHM	1.95	2.13	-8.17	0.012	1.89 - 2.00	1.70, 2.22
Seed Fatty Acid (% Total FA)						
16:0 Palmitic Site IARL	11.49	11.00	4.47	0.001	11.44 - 11.54	8.44, 12.65
16:0 Palmitic Site ILWY	11.26	11.04	2.02	0.017	11.25 - 11.27	8.44, 12.65
18:2 Linoleic Site ILCY	54.54	53.26	2.40	0.021	54.45 - 54.70	48.22, 59.63
18:2 Linoleic Site INRC	54.98	54.43	1.00	0.019	54.80 - 55.14	48.22, 59.63

Table 15. Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control (cont.)

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Mean Difference (MON 87708 minus Control) Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistical Significant Differences Observed in Two Individual Sites						
Seed Anti-nutrient (% dwt)						
Phytic Acid Site IARL	1.36	1.53	-11.28	0.018	1.33 - 1.38	0.80, 1.93
Phytic Acid Site ILWY	1.40	1.55	-9.34	0.030	1.33 - 1.46	0.80, 1.93
Seed Isoflavone (µg/g dwt)						
Daidzein Site ILWY	1458.08	1271.60	14.67	0.004	1416.31 - 1535.98	0, 2357.53
Daidzein Site INRC	1683.50	1419.40	18.61	0.049	1593.24 - 1777.49	0, 2357.53
Glycitein Site ILWY	111.77	79.70	40.23	<0.001	109.88 - 113.86	24.51, 238.51
Glycitein Site INRC	111.51	98.42	13.31	0.016	110.91 - 112.28	24.51, 238.51
Forage Proximate (% dwt)						
Protein Site IARL	25.21	23.00	9.63	0.043	24.71 - 25.52	15.28, 27.10

Table 15. Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control (cont.)

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Mean Difference (MON 87708 minus Control) Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistical Significant Differences Observed in Two Individual Sites						
Forage Proximate (% dwt)						
Protein Site INRC	21.78	23.33	-6.63	0.019	20.99 - 22.51	15.28, 27.10
Statistical Differences Observed in One Individual Site						
Seed Proximate (% dwt)						
Carbohydrates Site PAHM	38.30	35.23	8.71	0.008	37.69 - 38.65	31.73, 40.38
Seed Fiber (% dwt)						
Crude Fiber Site INRC	8.06	6.89	17.03	0.009	7.76 - 8.47	5.71, 10.92
Seed Amino Acid (% dwt)						
Alanine Site PAHM	1.75	1.86	-5.81	0.010	1.74 - 1.77	1.55, 1.92
Cystine Site PAHM	0.62	0.59	4.79	0.024	0.60 - 0.63	0.50, 0.68
Glycine Site PAHM	1.73	1.86	-6.78	0.004	1.69 - 1.75	1.53, 1.92

Table 15. Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control (cont.)

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Mean Difference (MON 87708 minus Control) Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistical Significant Differences Observed in One Individual Site						
Seed Amino Acid (% dwt)						
Lysine Site PAHM	2.60	2.75	-5.39	0.009	2.53 - 2.65	2.31, 2.84
Serine Site ILWY	1.98	2.06	-3.83	0.003	1.97 - 2.00	1.76, 2.27
Tryptophan Site ILCY	0.51	0.48	6.21	0.024	0.49 - 0.53	0.37, 0.52
Seed Anti-nutrient (% dwt)						
Lectin (H.U./mg dwt) Site ILWY	1.10	2.33	-52.88	0.045	0.59 - 1.51	0, 8.11
Stachyose Site INRC	3.14	3.46	-9.18	0.043	3.12 - 3.17	2.23, 4.11
Forage Proximate (% dwt)						
Carbohydrates Site PAHM	70.95	65.81	7.81	0.015	69.23 - 73.31	60.23, 74.00
Moisture (% fwt) Site PAHM	74.27	74.91	-0.86	0.021	73.40 - 75.40	62.12, 90.55

[1]dwt = dry weight; fwt= fresh weight; FA = fatty acid; H.U. = Hemagglutinating Units.
[2]MON 87708 was treated with Dicamba.
[3]Mean = least-square mean.
[4]Control refers to the near isogenic conventional soybean control (A3525).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of conventional commercial reference varieties. Negative limits set to zero.

Table 16. Statistical Summary of Combined-Site Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dwt)						
Ash	5.24 (0.067) (4.94 - 5.69)	5.12 (0.067) (4.73 - 5.47)	0.12 (0.055) (-0.28 - 0.45)	0.011, 0.24	0.031	4.75, 6.04 (5.00 - 5.88)
Carbohydrates	37.93 (0.50) (35.65 - 39.21)	36.64 (0.50) (34.11 - 38.45)	1.28 (0.40) (-0.38 - 4.07)	0.36, 2.20	0.012	31.73, 40.38 (33.82 - 39.26)
Moisture (% fwt)	6.88 (0.65) (5.17 - 8.47)	7.14 (0.65) (5.79 - 10.60)	-0.26 (0.52) (-3.12 - 1.43)	-1.46, 0.94	0.629	4.10, 9.78 (5.50 - 9.23)
Protein	40.86 (0.39) (39.00 - 42.53)	42.41 (0.39) (40.69 - 43.85)	-1.55 (0.51) (-4.84 - 0.088)	-2.73, -0.37	0.016	35.15, 45.33 (37.06 - 43.42)
Total Fat	15.97 (0.59) (14.00 - 18.56)	15.84 (0.59) (14.40 - 18.39)	0.13 (0.31) (-1.90 - 2.37)	-0.58, 0.84	0.691	12.09, 24.56 (15.47 - 21.34)
Fiber (% dwt)						
Acid Detergent Fiber	13.55 (0.40) (12.45 - 15.57)	12.86 (0.40) (11.62 - 14.57)	0.68 (0.25) (-0.71 - 2.13)	0.18, 1.19	0.009	9.73, 18.36 (12.07 - 17.46)

Table 16. Statistical Summary of Combined-Site Soybean Seed Nutrients for MON 87708 vs. Conventional Control(cont.)

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dwt)						
Crude Fiber	8.29 (0.26) (6.23 - 9.65)	7.37 (0.26) (6.05 - 8.64)	0.91 (0.26) (-0.34 - 2.67)	0.40, 1.43	<0.001	5.71, 10.92 (6.35 - 11.31)
Neutral Detergent Fiber	15.29 (0.59) (13.11 - 17.83)	14.34 (0.59) (11.81 - 17.99)	0.95 (0.41) (-1.31 - 4.57)	0.11, 1.79	0.028	11.03, 19.66 (11.66 - 19.45)
Amino Acid (% dwt)						
Alanine	1.76 (0.018) (1.66 - 1.83)	1.80 (0.018) (1.69 - 1.90)	-0.037 (0.017) (-0.16 - 0.042)	-0.075, 0.0018	0.059	1.55, 1.92 (1.59 - 1.86)
Arginine	3.30 (0.069) (3.09 - 3.50)	3.58 (0.069) (3.19 - 3.93)	-0.28 (0.078) (-0.83 - 0.0059)	-0.46, -0.10	0.006	2.50, 3.88 (2.88 - 3.74)
Aspartic Acid	4.63 (0.044) (4.44 - 4.80)	4.78 (0.044) (4.46 - 5.01)	-0.15 (0.050) (-0.56 - 0.12)	-0.27, -0.037	0.016	4.00, 5.16 (4.22 - 4.94)
Cystine	0.61 (0.0049) (0.58 - 0.63)	0.59 (0.0049) (0.56 - 0.62)	0.018 (0.0046) (-0.0071 - 0.053)	0.0085, 0.027	<0.001	0.50, 0.67 (0.53 - 0.64)

Table 16. Statistical Summary of Combined-Site Soybean Seed Nutrients for MON 87708 vs. Conventional Control(cont.)

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dwt)						
Glutamic Acid	7.38 (0.085) (7.05 - 7.73)	7.69 (0.085) (7.12 - 8.14)	-0.31 (0.093) (-1.09 - 0.17)	-0.53, -0.095	0.010	6.20, 8.36 (6.69 - 7.92)
Glycine	1.76 (0.016) (1.67 - 1.83)	1.81 (0.016) (1.70 - 1.89)	-0.048 (0.017) (-0.20 - 0.042)	-0.086, -0.0096	0.020	1.51, 1.93 (1.58 - 1.84)
Histidine	1.06 (0.0095) (1.02 - 1.10)	1.09 (0.0095) (1.02 - 1.14)	-0.033 (0.011) (-0.12 - 0.031)	-0.059, -0.0076	0.017	0.92, 1.17 (0.95 - 1.13)
Isoleucine	1.88 (0.019) (1.75 - 1.97)	1.95 (0.019) (1.79 - 2.04)	-0.070 (0.019) (-0.24 - 0.11)	-0.11, -0.026	0.006	1.63, 2.08 (1.68 - 2.02)
Leucine	3.06 (0.029) (2.93 - 3.19)	3.17 (0.029) (2.96 - 3.32)	-0.11 (0.031) (-0.36 - 0.072)	-0.18, -0.035	0.008	2.69, 3.41 (2.80 - 3.27)
Lysine	2.64 (0.019) (2.53 - 2.71)	2.68 (0.019) (2.54 - 2.77)	-0.041 (0.023) (-0.23 - 0.090)	-0.094, 0.012	0.110	2.31, 2.84 (2.38 - 2.74)

Table 16. Statistical Summary of Combined-Site Soybean Seed Nutrients for MON 87708 vs. Conventional Control(cont.)

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dwt)						
Methionine	0.58 (0.0053) (0.53 - 0.60)	0.58 (0.0053) (0.53 - 0.60)	0.00012 (0.0062) (-0.039 - 0.071)	-0.013, 0.013	0.985	0.50, 0.63 (0.52 - 0.63)
Phenylalanine	2.06 (0.028) (1.92 - 2.18)	2.13 (0.028) (1.95 - 2.27)	-0.071 (0.028) (-0.27 - 0.048)	-0.13, -0.0067	0.034	1.78, 2.31 (1.85 - 2.21)
Proline	1.99 (0.021) (1.90 - 2.09)	2.05 (0.021) (1.89 - 2.13)	-0.067 (0.022) (-0.17 - 0.065)	-0.12, -0.015	0.017	1.62, 2.28 (1.74 - 2.16)
Serine	2.04 (0.023) (1.92 - 2.12)	2.09 (0.023) (1.95 - 2.21)	-0.048 (0.026) (-0.19 - 0.054)	-0.11, 0.013	0.105	1.76, 2.27 (1.90 - 2.18)
Threonine	1.56 (0.015) (1.48 - 1.62)	1.58 (0.015) (1.51 - 1.64)	-0.023 (0.015) (-0.10 - 0.052)	-0.058, 0.012	0.169	1.39, 1.69 (1.47 - 1.64)
Tryptophan	0.47 (0.0085) (0.44 - 0.53)	0.46 (0.0085) (0.43 - 0.50)	0.0070 (0.0097) (-0.035 - 0.064)	-0.015, 0.029	0.494	0.37, 0.52 (0.39 - 0.50)

Table 16. Statistical Summary of Combined-Site Soybean Seed Nutrients for MON 87708 vs. Conventional Control(cont.)

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dwt)						
Tyrosine	1.37 (0.018) (1.28 - 1.46)	1.42 (0.018) (1.34 - 1.52)	-0.049 (0.021) (-0.20 - 0.078)	-0.098, -0.00046	0.048	1.23, 1.51 (1.26 - 1.49)
Valine	1.98 (0.020) (1.82 - 2.09)	2.06 (0.020) (1.90 - 2.17)	-0.080 (0.022) (-0.27 - 0.13)	-0.13, -0.030	0.006	1.70, 2.22 (1.73 - 2.13)
Fatty Acid (% Total FA)						
16:0 Palmitic	11.59 (0.16) (11.25 - 12.16)	11.33 (0.16) (10.92 - 12.08)	0.26 (0.060) (-0.15 - 0.62)	0.12, 0.40	0.002	8.44, 12.65 (9.42 - 11.54)
18:0 Stearic	4.06 (0.10) (3.60 - 4.40)	4.04 (0.10) (3.67 - 4.31)	0.028 (0.049) (-0.19 - 0.42)	-0.085, 0.14	0.584	2.81, 5.23 (3.24 - 4.67)
18:1 Oleic	19.20 (0.30) (17.85 - 19.94)	20.91 (0.30) (19.60 - 22.44)	-1.71 (0.19) (-2.71 - -0.90)	-2.15, -1.27	<0.001	15.67, 27.49 (17.88 - 25.31)
18:2 Linoleic	54.40 (0.37) (53.42 - 55.67)	53.59 (0.37) (52.33 - 54.99)	0.81 (0.24) (-0.59 - 1.68)	0.25, 1.37	0.010	48.22, 59.63 (50.95 - 56.68)

Table 16. Statistical Summary of Combined-Site Soybean Seed Nutrients for MON 87708 vs. Conventional Control(cont.)

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
18:3 Linolenic	10.12 (0.27) (8.99 - 10.88)	9.49 (0.27) (8.42 - 10.14)	0.63 (0.072) (0.36 - 1.20)	0.46, 0.80	<0.001	5.92, 12.52 (7.43 - 10.65)
20:0 Arachidic	0.26 (0.0052) (0.23 - 0.27)	0.26 (0.0052) (0.24 - 0.27)	-0.0012 (0.0031) (-0.013 - 0.020)	-0.0082, 0.0059	0.707	0.19, 0.34 (0.20 - 0.30)
20:1 Eicosenoic	0.093 (0.017) (0.069 - 0.16)	0.090 (0.017) (0.068 - 0.17)	0.0029 (0.0042) (-0.010 - 0.050)	-0.0056, 0.011	0.495	0.015, 0.24 (0.065 - 0.17)
22:0 Behenic	0.27 (0.0038) (0.25 - 0.29)	0.28 (0.0038) (0.27 - 0.30)	-0.013 (0.0029) (-0.023 - 0.0024)	-0.020, -0.0066	0.001	0.24, 0.40 (0.28 - 0.35)
Vitamin (mg/100g dwt)						
Vitamin E	1.41 (0.18) (1.08 - 2.17)	1.23 (0.18) (0.89 - 2.11)	0.19 (0.038) (0.018 - 0.42)	0.098, 0.27	0.001	0, 3.61 (0.69 - 2.91)

[1]dwt = dry weight; fwt = fresh weight; FA = fatty acid.
[2]MON 87708 was treated with Dicamba.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the newar isogenic conventional soybean control (A3525).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of conventional commercial reference varieties. Negative limits set to zero.

Table 17. Statistical Summary of Combined-Site Soybean Seed Anti-Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Anti-nutrient						
Lectin (H.U./mg dwt)	3.17 (0.76) (0.59 - 10.27)	3.16 (0.76) (0.46 - 10.38)	0.013 (0.67) (-4.27 - 8.13)	-1.54, 1.57	0.984	0, 8.11 (0.68 - 8.34)
Phytic Acid (% dwt)	1.30 (0.071) (1.08 - 1.51)	1.39 (0.071) (1.09 - 1.62)	-0.085 (0.035) (-0.29 - 0.15)	-0.17, -0.0034	0.043	0.80, 1.93 (1.00 - 1.64)
Raffinose (% dwt)	0.43 (0.038) (0.32 - 0.59)	0.47 (0.038) (0.36 - 0.60)	-0.036 (0.018) (-0.24 - 0.069)	-0.072, -0.00077	0.045	0.11, 0.73 (0.26 - 0.59)
Stachyose (% dwt)	3.36 (0.078) (3.07 - 4.02)	3.62 (0.078) (3.07 - 4.15)	-0.26 (0.099) (-1.00 - 0.40)	-0.46, -0.062	0.011	2.23, 4.11 (2.50 - 3.94)
Trypsin Inhibitor (TIU/mg dwt)	32.27 (1.40) (26.09 - 39.27)	30.37 (1.40) (25.22 - 34.22)	1.90 (1.79) (-4.76 - 8.72)	-2.23, 6.04	0.319	22.50, 41.37 (23.37 - 44.56)
Isoflavone (µg/g dwt)						
Daidzein	1494.97 (155.94) (899.83 - 2305.26)	1340.71 (155.94) (762.49 - 1729.91)	154.26 (65.62) (-258.27 - 795.19)	2.95, 305.57	0.046	0, 2357.53 (451.33 - 2033.05)

Table 17. Statistical Summary of Combined-Site Soybean Seed Anti-Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Isoflavone (µg/g dwt)						
Genistein	967.01 (90.36) (594.13 - 1496.78)	886.57 (90.36) (588.17 - 1162.01)	80.44 (41.86) (-185.98 - 513.56)	-4.30, 165.19	0.062	25.23, 1945.44 (533.88 - 1726.03)
Glycitein	108.01 (5.24) (77.67 - 119.09)	95.85 (5.24) (68.68 - 122.09)	12.16 (6.91) (-43.86 - 50.41)	-3.77, 28.09	0.116	24.51, 238.51 (73.61 - 231.75)

[1]dw = dry weight; H.U. = Hemagglutinating Units; TIU = Trypsin Inhibitor Units.
[2] MON 87708 was treated with Dicamba.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the near isogenic conventional soybean control (A3525).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of conventional commercial reference varieties. Negative limits set to zero.

Table 18. Statistical Summary of Combined-Site Soybean Forage Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dwt)						
Ash	7.29 (0.54) (5.94 - 9.65)	7.39 (0.54) (6.10 - 10.46)	-0.10 (0.27) (-0.89 - 1.56)	-0.71, 0.51	0.712	3.24, 11.08 (5.20 - 9.81)
Carbohydrates	66.48 (1.03) (62.21 - 73.31)	65.66 (1.04) (62.91 - 67.94)	0.83 (0.96) (-3.95 - 6.90)	-1.40, 3.05	0.414	60.23, 74.00 (62.73 - 71.72)
Moisture (% fwt)	75.63 (1.82) (72.40 - 82.80)	75.55 (1.82) (71.60 - 82.70)	0.081 (0.27) (-1.40 - 1.30)	-0.55, 0.71	0.775	62.12, 90.55 (71.00 - 84.10)
Protein	21.52 (0.95) (15.23 - 25.52)	22.32 (0.95) (20.88 - 24.11)	-0.80 (0.80) (-6.26 - 2.75)	-2.67, 1.07	0.350	15.28, 27.10 (18.50 - 25.86)
Total Fat	4.67 (0.66) (2.00 - 7.34)	4.64 (0.66) (2.01 - 6.72)	0.032 (0.26) (-0.68 - 1.96)	-0.57, 0.63	0.904	0, 10.16 (1.57 - 7.99)
Fiber (% dwt)						
Acid Detergent Fiber	30.58 (1.79) (23.30 - 45.11)	27.69 (1.80) (21.79 - 38.15)	2.89 (1.19) (-4.78 - 16.24)	0.45, 5.34	0.021	15.60, 42.84 (20.98 - 39.23)

Table 18. Statistical Summary of Combined-Site Soybean Forage Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dwt)						
Neutral Detergent Fiber	29.63 (1.68) (24.21 - 38.51)	30.49 (1.70) (23.66 - 39.42)	-0.86 (1.22) (-8.13 - 11.03)	-3.65, 1.94	0.503	20.40, 44.62 (24.81 - 42.80)

[1]dwt = dry weight; fwt = fresh weight.
[2] MON 87708 was treated with Dicamba
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the near isogenic conventional soybean control (A3525).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of conventional commercial reference varieties. Negative limits set to zero.

Table 19. Literature and ILSI Ranges for Components in Soybean Seed and Forage

Tissue Components[1]	Literature Range[2]	ILSI Range[3]
Seed Nutrients		
Proximates (% dwt)		
Ash	4.61 – 6.32[a]; 4.32 – 5.88[b]	3.89 – 6.99
Carbohydrates by calculation	32.75 – 40.98[a]; 29.88 – 43.48[b]	29.6 – 50.2
Moisture (% fwt)	6.24 – 12.10[a]; 5.44 – 11.70[b]	4.7 – 34.4
Protein	34.78 – 43.35[a]; 32.29 – 42.66[b]	33.19 – 45.48
Total Fat	14.40 – 20.91[a]; 15.10 – 23.56[b]; 15.5[4] – 24.7[4]	8.10 – 23.56
Fiber (% dwt)		
Acid Detergent Fiber	9.22 – 26.26[a]; 11.81 – 19.45[b]	7.81 – 18.61
Neutral Detergent Fiber	10.79 – 23.90[a]; 13.32 – 23.57[b]	8.53 – 21.25
Crude Fiber	-	4.12 – 13.87
Amino Acids (% dwt)		
Alanine	1.62 – 1.89[a]; 1.43 – 1.93[b]	1.51 – 2.10
Arginine	2.57 – 3.34[a]; 2.15 – 3.05[b]	2.29 – 3.40
Aspartic acid	4.16 – 5.02[a]; 4.01 – 5.72[b]	3.81 – 5.12
Cystine/Cysteine	0.52 – 0.69[a]; 0.41 – 0.71[b]	0.37 – 0.81
Glutamic acid	6.52 – 8.19[a]; 5.49 – 8.72[b]	5.84 – 8.20
Glycine	1.59 – 1.90[a]; 1.41 – 1.99[b]	1.46 – 2.00
Histidine	0.96 – 1.13[a]; 0.86 – 1.24[b]	0.88 – 1.18
Isoleucine	1.59 – 2.00[a]; 1.41 – 2.02[b]	1.54 – 2.08
Leucine	2.79 – 3.42[a]; 2.39 – 3.32[b]	2.59 – 3.62
Lysine	2.36 – 2.77[a]; 2.19 – 3.15[b]	2.29 – 2.84
Methionine	0.45 – 0.63[a]; 0.39 – 0.65[b]	0.43 – 0.68
Phenylalanine	1.82 – 2.29[a]; 1.62 – 2.44[b]	1.63 – 2.35
Proline	1.83 – 2.23[a]; 1.63 – 2.25[b]	1.69 – 2.28
Serine	1.95 – 2.42[a]; 1.51 – 2.30[b]	1.11 – 2.48
Threonine	1.44 – 1.71[a]; 1.23 – 1.74[b]	1.14 – 1.86
Tryptophan	0.30 – 0.48[a]; 0.41 – 0.56[b]	0.36 – 0.50
Tyrosine	1.27 – 1.53[a]; 0.74 – 1.31[b]	1.02 – 1.61
Valine	1.68 – 2.11[a]; 1.50 – 2.13[b]	1.60 – 2.20
Fatty Acids (% total FA)		
8:0 Caprylic	not available	0.148 – 0.148
10:0 Capric	0.15 – 0.27[b]	not available
12:0 Lauric	not available	0.082 – 0.132
14:0 Myristic	0.063 – 0.11[b]	0.071 – 0.238
14:1 Myristoleic	not available	0.121 – 0.125
15:0 Pentadecanoic	not available	not available
15:1 Pentadecenoic	not available	not available
16:0 Palmitic	9.80 – 12.63[b]	9.55 – 15.77
16:1 Palmitoleic	0.055 – 0.14[b]	0.086 – 0.194
17:0 Heptadecanoic	0.076 – 0.13[b]	0.085 – 0.146
17:1 Heptadecenoic	0.019 – 0.064[b]	0.073 – 0.087
18:0 Stearic	3.21 – 5.63[b]	2.70 – 5.88
18:1 Oleic	16.69 – 35.16[b]	14.3 – 32.2
18:2 Linoleic	44.17 – 57.72[b]	42.3 – 58.8

Table 19. Literature and ILSI Ranges for Components in Soybean Seed and Forage

Seed Tissue Components[1]	Literature Range[2]	ILSI Range[3]
18:3 Gamma Linolenic	not available	not available
18:3 Linolenic	4.27 – 9.90[b]	3.00 – 12.52
20:0 Arachidic	0.35 – 0.57[b]	0.163 – 0.482
20:1 Eicosenoic	0.13 – 0.30[b]	0.140 – 0.350
20:2 Eicosadienoic	0.016 – 0.071[b]	0.077 – 0.245
20:3 Eicosatrienoic	not available	not available
20:4 Arachidonic	not available	not available
22:0 Behenic	0.35 – 0.59[b]	0.277 – 0.595
Vitamins (mg/100g dwt)		
Vitamin E	1.29 – 4.80[a]; 1.12 – 8.08[b]	0.19 – 6.17
Seed Anti-Nutrients		
Lectin (H.U./mg fwt)	0.45 – 10.87[a]; 0.090 – 11.18[b]	0.09 – 8.46
Trypsin Inhibitor (TIU/mg dwt)	20.79 – 59.03[a]; 18.14 – 42.51[b]	19.59 – 118.68
Phytic Acid (% dwt)	0.41 – 1.92[a]; 0.81 – 2.66[b]	0.63 – 1.96
Raffinose (% dwt)	0.26 – 0.84[a]; 0.43 – 1.85[b]	0.21 – 0.66
Stachyose (% dwt)	1.53 – 3.04[a]; 1.97 – 6.65[b]	1.21 – 3.50
Isoflavones	**(µg/g dwt)**	**(mg/kg dwt)**
Daidzein	224.03 – 1571.91[a]; 198.95 – 1458.24[b]	60.0 – 2453.5
Genistein	338.24 – 1488.89[a]; 148.06 – 1095.57[b]	144.3 – 2837.2
Glycitein	52.72 – 298.57[a]; 32.42 – 255.94[b]	15.3 – 310.4

Forage Tissue Components[1]	Literature Range[2]	ILSI Range[3]
Forage Nutrients		
Proximate (% dwt)		
Ash	5.28 – 9.24[a]; 4.77 – 8.54[b]	6.72 – 10.78
Carbohydrates by calculation	62.25 – 72.30[a]; 60.61 – 77.26[b]	59.8 – 74.7
Moisture (% fwt)	68.50 – 78.40[a]; 62.76 – 80.20[b]	73.5 – 81.6
Protein	16.48 – 24.29[a]; 12.68 – 23.29[b]	14.38 – 24.71
Total Fat	2.65[4] – 9.87[a]; 2.96 – 7.88[b]	1.302 – 5.132
Fiber (% dwt)		
Acid Detergent Fiber	23.86 – 50.89[a]; 25.49 – 47.33[b]	not available
Neutral Detergent Fiber	19.61 – 43.70[a]; 30.96 – 54.55[b]	not available

[1]fwt=fresh weight; dwt=dry weight; H.U. = hemagglutinating unit; TIU = trypsin inhibitor unit.
[2]Literature range references; [a]Lundry et al. (2008); [b]Berman et al. (2009).
[3]ILSI 2006. Crop Composition Database Version 3.0. International Life Science Institute, Washington, D.C. http://www.cropcomposition.org/ [Accessed August 12, 2010].

[4]OECD (2001).

B5(b) Levels of other GM-influenced constituents

MON 87708 soybean contains a gene derived from *Stenotrophomonas maltophilia* that expresses a mono-oxygenase that rapidly demethylates dicamba to an inactive metabolite DCSA, a well known metabolite of dicamba in soybean and livestock. In Australia, several dicamba-based products are registered for food use and through the registration process the safety of dicamba use on cereal crops as well as sugar cane has been evaluated by the Australian Pesticides and Veterinary Medicines Authority (APVMA). At the same time, pesticide residue tolerances (MRLs) were established for cereal grains (0.05 ppm) and sugar cane (0.1 ppm) crop commodities. Dicamba is not registered on soybean and dicamba MRLs do not exist for soybean commodities. Consequently, a submission to the Food Standards Australia New Zealand (FSANZ) is planned in 2011 to set an appropriate import MRL to cover residues of dicamba on soybean seed.

B5(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 87708 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 87708 are as safe and nutritious as those derived from commercially-available, conventional soybean for which there is an established history of safe consumption. Therefore, animal feeding studies do not add value to the safety of MON 87708.

Part 3 STATUTORY DECLARATION – AUSTRALIA

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

Signature: _____

Declared before me ……

This …….. day of …….. 2011.

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