FOOD STANDARDS
Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

Supporting Document 1

SAFETY ASSESSMENT (AMENDED)

SUMMARY AND CONCLUSIONS

Background

Drought-tolerant corn line MON 87460 (referred to as MON87460 corn) has been genetically modified for tolerance to agricultural conditions under which water is limited. Tolerance is conferred by expression of a single gene from *B. subtilis*, which encodes cold shock protein B (CSPB). The presence of the trait is expected to reduce yield loss under water-limited conditions. MON87460 corn also contains a second gene, *nptII* (neomycin phosphotransferase type II) from *E. coli* that was used as a selectable marker gene.

MON87460 corn has been developed for commercial cultivation in North America and may therefore enter the Australian and New Zealand food supply as imported, largely processed food products.

In conducting a safety assessment of food derived from drought-tolerant MON87460 corn, a number of criteria have been addressed including: a characterisation of the transferred genes, their origin, function and stability in the corn genome; the changes at the level of DNA, protein and in the whole food; compositional analyses; evaluation of intended and unintended changes; and the potential for the newly expressed proteins to be either allergenic or toxic in humans.

This safety assessment report addresses only food safety and nutritional issues. It therefore does not address any risks related to the environmental release of GM plants used in food production, the safety of animal feed or animals fed with feed derived from GM plants, or the safety of food derived from the non-GM (conventional) plant.

History of Use

Corn is the world's third leading cereal crop, behind wheat and rice, and is grown in over 25 countries. Corn-derived products are routinely used in a large number and diverse range of foods and have a long history of safe use. Products derived from MON87460 corn may include flour, starch products, breakfast cereals and high fructose corn syrup. Corn is also widely used as a feed for domestic livestock.

The source organism of the *cspB* gene, *B. subtilis,* is a ubiquitous soil bacterium that is non-pathogenic and safe for human consumption. Enzyme preparations from *B. subtilis* are used in the food industry and the bacterium itself is commercially available in many countries as a probiotic. *B. subtilis* is also used to make traditional foods, such as natto, which is a fermented soybean product.

The source organism of the *nptII* gene, *E. coli*, is found naturally in the environment by virtue of its presence in the digestive tract of humans and other vertebrate species. Both the gene and donor organism have previously been assessed by FSANZ as posing no safety issues when present in GM food crops.

Molecular Characterisation

Detailed molecular analyses indicated that one copy of the insert containing one copy of the *cspB* and *nptII* genes has been introduced at a single genomic locus in MON87460 corn. The coding region of the *cspB* and *nptII* genes and their regulatory elements are intact and no plasmid backbone sequences are present. The stability of the insert and absence of plasmid backbone sequences were demonstrated through seven generations of breeding.

Characterisation of Novel Proteins

MON87460 corn expresses two novel proteins, CSPB and NPTII. CSPB present in MON87460 corn is nearly identical to that present in *B. subtilis* except for a single amino acid substitution at position 2 (from leucine to valine) necessary for cloning purposes.

CSPB is present in MON87460 corn grain at a mean concentration of 0.041 and 0.33 µg/g fresh weight under well-watered and water-limited conditions, respectively. It conforms in size and amino acid sequence to that expected, is immunoreactive to anti-CSPB antibodies, is not glycosylated and exhibits the expected functional activity.

Bioinformatic analyses of CSPB confirmed the absence of any biologically significant amino acid sequence similarity to known or putative protein toxins or allergens and a digestibility study demonstrated that it would be rapidly degraded in the digestive tract following ingestion, similar to other dietary proteins. An acute oral toxicity study in mice confirmed the absence of toxicity. The safety of CSPB is further supported by the history of safe dietary exposure to this protein and its source organism. The weight-of-evidence indicates that CSPB is unlikely to be toxic or allergenic to humans.

NPTII is expressed at relatively low levels in MON87460 corn grain, with the mean concentration below the limit of quantitation (LOQ). There is an extensive database on NPTII given its history of use in the production of GM crops. An updated bioinformatic analysis confirmed that NPTII has no biologically significant sequence homology with known toxins or allergens. Based on its history of safe use and these confirmatory studies, the presence of NPTII in food derived from MON87460 corn is unlikely to be toxic or allergenic to humans.

Compositional Analyses

Compositional analyses were undertaken to establish the nutritional adequacy of MON87460 corn relative to conventional corn cultivated under well-watered and water-limited conditions. The constituents analysed included proximates, fibre, minerals, vitamins, amino acids, fatty acids, anti-nutrients and secondary metabolites.

No differences of biological significance were observed between MON87460 corn and its conventional counterpart. Statistical differences in some constituents were noted but these reflect the fact that a large number of statistical tests were performed, with 1 in 20 expected to have $p<0.05$ by chance alone. However, the mean levels of all constituents were within the 99% tolerance intervals and literature ranges. Therefore, all the levels of constituents in MON87460 corn are considered to be within the range of natural variation for corn and indicate that the introduction of the *cspB* and *nptII* genes in to MON87460 corn caused no unintended compositional changes. On this basis it can be concluded that food derived from drought-tolerant MON87460 corn is equivalent in composition to conventional corn.

Nutritional Impact

The detailed compositional analysis is considered acceptable to establish the nutritional adequacy of food derived from MON87460 corn. The introduction of MON87460 corn into the food supply would therefore be expected to have little nutritional impact.

Conclusion

No potential public health and safety concerns have been identified in the assessment of drought-tolerant MON87460 corn. On the basis of the data provided in the present Application, and other available information, food derived from MON87460 corn is considered as safe for human consumption as food derived from conventional corn varieties.

1. INTRODUCTION

Monsanto has submitted an application to FSANZ to vary Standard 1.5.2 – Food produced using Gene Technology – in the *Australia New Zealand Food Standards Code* (the Code) to include food from a new genetically modified (GM) corn variety, known as MON87460 corn. The corn has been modified to preserve yield loss under water-limited conditions (i.e. drought).

The drought tolerance trait is achieved through expression in the plant of cold shock protein B (CSPB), which is encoded by the full-length *cspB* gene derived from *Bacillus subtilis*. Cold shock proteins are found naturally in bacteria and allow survival under conditions of low temperature. CSPB functions as an RNA chaperone, facilitating transcription under suboptimal environmental conditions.

Corn is the world's third leading cereal crop, behind wheat and rice, and is grown in over 25 countries (OECD 2002). In 2007, worldwide production of corn was almost 800 million tonnes, with the United States and China being the major producers (~330 and 150 million tonnes, respectively) (FAOSTAT 2009). The majority of grain and forage derived from corn is used in animal feed. Corn grain is also used to produce industrial products, such as ethyl alcohol by fermentation and highly refined starch by wet milling.

Corn is not a major crop in Australia or New Zealand. Domestic production is supplemented by the import of a small amount of corn-based products, largely as high-fructose corn syrup, which is not currently manufactured in either Australia or New Zealand. Such products are processed into breakfast cereals, baking products, extruded confectionery and food coatings. In 2007, Australia imported 529 tonnes of corn, 4613 tonnes of corn flour and 2263 tonnes of corn oil; no forage or silage were imported (FAOSTAT 2009).

Other corn products such as cornstarch are also imported and used by the food industry for the manufacture of dessert mixes and sauces. Corn may also be imported in finished products such as corn chips and canned corn.

MON87460 corn will be grown in North America and is not intended for cultivation in Australia or New Zealand. Therefore, if approved, food from this line may enter the Australian and New Zealand food supply as imported food products.

2. HISTORY OF USE

2.1 Donor organisms

2.1.1 Bacillus subtilis

The source organism of the *cspB* gene, *B. subtilis,* is widely distributed in the environment by virtue of its natural occurrence in soil and is also detectable in water, air and decaying plant material (US EPA 1997). The bacterium is not pathogenic to humans or toxigenic (US EPA 1997) and has been recommended for a qualified presumption of safety (QPS) by the Scientific Committee of the European Food Safety Authority (EFSA 2007a).

FSANZ has previously assessed the safety of *B. subtilis* as the source organism for a number of enzymes used as food processing aids. Standard 1.3.3 of the Code permits the use of the following enzymes derived from *B. subtilis*: α–acetolactate decarboxylase, α- and β–amylase, β–glycanase, hemicellulase endo-1,4- β-xylanase, hemicellulase multicomponent enzyme, maltogenic α –amylase, metalloproteinase, pullulanase and serine proteinase. In the US, enzyme preparations from *B. subtilis* have Generally-Recognised-As Safe (GRAS) status (US FDA 1999).

The bacterium itself is commercially available in many countries as a dietary probiotic intended to improve human health (Duc et al 2004; Henriksson et al 2005). It is also used as an animal feed additive (Fritts et al 2000) and growth promotant in aquaculture (Farzanfar 2006. Strains of *B. subtilis* are used to make fermented soybean products such as natto (Japan) and thua nao (Thailand) (Chantawannakul et al 2002; Hosoi & Kiuchi 2008).

2.1.2 Escherichia coli

The bacterium *Escherichia coli* is the source of the *nptII* gene in MON87460 corn. *E. coli* belongs to the Enterobacteriaceae, a relatively homogeneous group of rod-shaped, Gram-negative, facultative aerobic bacteria. Members of the genus *Escherichia* are ubiquitous in the environment and are normally found in the digestive tracts of vertebrates, including humans (Jefferson et al 1986). The vast majority of *E. coli* strains are harmless to humans, although some strains can cause diarrhoea in travellers and *E. coli* is also the most common cause of urinary tract infections.

More recently, a particularly virulent strain of *E. coli*, belonging to the enterohaemorrhagic *E. coli* group, known as 0157:H7, has come to prominence as a food-borne pathogen responsible for causing serious illness. This particular group of pathogenic *E. coli* are distinct from the strains of *E. coli* (the K-12 strains) that are used routinely in laboratory manipulations. The K-12 strains of *E. coli* have a long history of safe use and are commonly used as protein production systems in many commercial, including pharmaceutical and food ingredient, applications (Bogosian & Kane 1991). For example, Standard 1.3.3 of the Code permits the use of chymosin derived from *E. coli* K-12 strain as a food processing aid.

Both the gene and donor organism have previously been assessed by FSANZ as posing no safety issues when used in the production of GM food crops.

2.1.3 Agrobacterium tumefaciens

The species *Agrobacterium tumefaciens* is a Gram-negative, non-spore forming, rod-shaped bacterium commonly found in the soil. It is closely related to other soil bacteria involved in nitrogen fixation by certain plants.

Agrobacterium naturally contains a plasmid (the Ti plasmid) with the ability to enter plant cells and insert a portion of its genome into plant chromosomes. Normally therefore, *Agrobacterium* is a plant pathogen causing root deformation mainly in sugar beets, pome fruits and viniculture crops. However, adaptation of this natural process has now resulted in the ability to transform a broad range of plant species without causing adverse effects in the host plant.

2.2 Host organism

Corn (*Zea mays* L), otherwise known as maize, is the world's third leading cereal crop, behind wheat and rice, and is grown in over 25 countries worldwide (OECD 2002). In 2007, worldwide production of corn was nearly 800 million tonnes, with the United States and China being the major producers (FAOSTAT 2009).

The majority of grain and forage derived from corn is used as animal feed, however corn also has a long history of safe use as food for human consumption. The grain can be processed into industrial products such as ethyl alcohol (by fermentation), and highly refined starch (by wet-milling) to produce starch and sweetener products. In addition to milling, the corn germ can be processed to obtain corn oil and numerous other products (White & Pollak 1995).

3. MOLECULAR CHARACTERISATION

Submitted studies:
Skipwith A, Feng D, Groat JR, Tian Q & Masucci JD (2007a) Molecular analysis of corn MON87460. Study No. 06-01-B3-15. Study Report: MSL0020487. Lab: Monsanto Company, Production Characterization Center, Biotechnology Regulatory Sciences, St Louis, MO, USA. Sponsor: Monsanto Company, St Louis, MO, USA. Unpublished. [GLP, QA]

Skipwith A, Feng D, Groat JR, Tian Q & Masucci JD (2007b) Amended Report for MSL0020487: Molecular Analysis of Corn MON 87460. Study No. 06-01-B3-15. Study Report: MSL0022131. Lab: Monsanto Company, Production Characterization Center, Biotechnology Regulatory Sciences, St Louis, MO, USA. Sponsor: Monsanto Company, St Louis, MO, USA. Unpublished. [GLP, QA]

Silvanovich A & McClain S (2008) Bioinformatic evaluation of DNA sequences flanking the 5' and 3' junctions of the inserted DNA in corn MON87460: Assessment of putative polypeptides. Study No. REG-08-413. Report No. MSL0021619. Lab & Sponsor: Monsanto Copan, Product Characterization Center, St Louis, MO, USA. Unpublished. . [GLP, QA]

Molecular characterisation is necessary to provide an understanding of the genetic material introduced into the host genome and helps to frame the subsequent parts of the safety assessment. The molecular characterisation addresses three main aspects: the transformation method together with a detailed description of the DNA sequences introduced to the host genome; a characterisation of the inserted DNA including any rearrangements that may have occurred as a consequence of the transformation; and the genetic stability of the inserted DNA and any accompanying expressed traits.

3.1 Description of the introduced genetic elements

A single binary transformation vector, plasmid PV-ZMAP595, was used to generate MON87460 corn (Figures 1 & 2). The genetic elements contained in PV-ZMAP595 are described in Table 1.

The PV-ZMAP595 plasmid contains two expression cassettes. The *cspB* expression cassette contains the coding sequence of the *cspB* gene from *B. subtilis*, which is under the control of the promoter, leader and intron from the rice actin gene, *act1*. The termination signal for the *cspB* gene is provided by the 3' non-translated sequence of the *transcript 7* gene (*tr7*) from *A. tumefaciens.*

The *nptII* expression cassette contains the coding sequence of the *nptII* gene from *E. coli*, which is flanked by *lox*P sites to allow potential excision of the cassette by Cre recombinase. Expression of the *nptII* gene is under the control of the 35S promoter from cauliflower mosaic virus (CaMV), with the termination signal provided by the 3' terminator sequence of the nopaline synthase (*nos*) gene from *A. tumefaciens*.

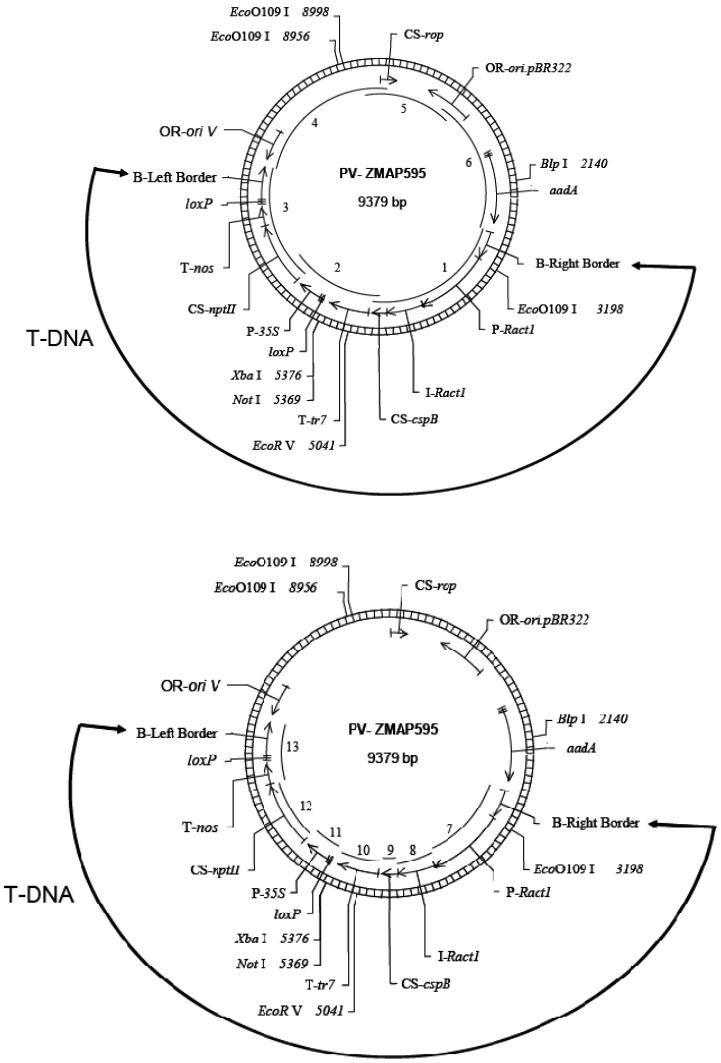


Figure 1: Circular plasmid map for PV-ZMAP595 used to introduce the *cspB* gene into MON87460 corn

The binding sites for Probes 1-6 used in the molecular characterisation experiments are shown in the top map, while the binding sites for Probes 7-13 are shown in the bottom map.

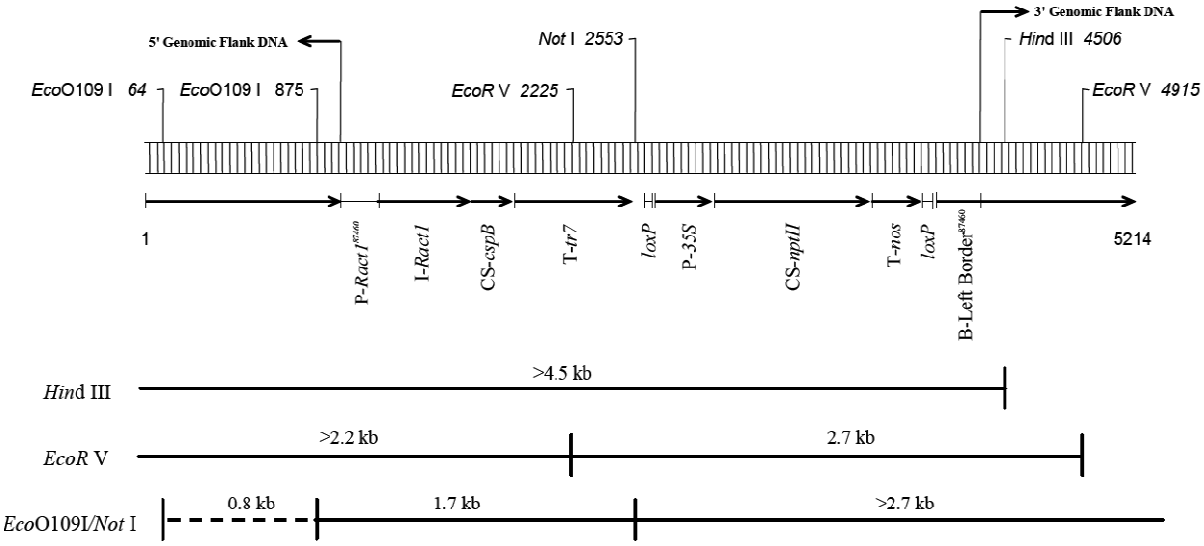


Figure 2: Schematic representation of the insert and flanking sequences in MON87460 corn
Shown are the genetic elements, restriction sites and expected sizes of the restriction fragments. The dotted line indicates the additional DNA fragment that might be present if the partial digestion of the internal EcoO109 I restriction site occurs.

Table 1: Description of genetic elements in plasmid PV-ZMAP595

Genetic element[1]	Location in PV-ZMAP595	Function
PLASMID BACKBONE COMPONENTS		
CS-rop	53-244	Coding sequence for repressor of primer protein for the maintenance of plasmid copy number in *E. coli*
OR-ori.pBR322	672-1260	Origin of replication from plasmid pBR322 for the maintenance of plasmid copy number in *E. coli*
aadA	1791-2679	Bacterial promoter and coding sequence for 3'(9)-O-nucleotidyl transferase from the transposon, Tn7
T-DNA		
RB	2816-3172	Right border sequence from *A. tumefaciens* used for the transfer of T-DNA into MON87460 corn
P-*Ract1*	3205-4128	Promoter and leader from the rice actin gene
I-*Ract1*	4129-4605	Intron from the rice actin gene
CS-*cspB*	4608-4811	Coding sequence of the *cspB* gene from *B. subtilis*
T-*tr7*	4842-5349	Termination signal from the 3' nontranslated sequence of the *tr7* gene from *A. tumefaciens*
loxP	5424-5457	Sequence from *Bacteriophage P1* for the recombination site recognised by Cre recombinase
P-*35S*	5484-5776	Promoter for the 35S RNA of CaMV
CS-*nptII*	5841-6635	Coding sequence from the *Tn5* gene in *E. coli* that encodes neomycin and kanamycin resistance
T-*nos*	6667-6919	Termination signal from the 3' nontranslated sequence of the *nos* gene from *A. tumefaciens*
loxP	6945-6978	Sequence from *Bacteriophage P1* for the recombination site recognised by Cre recombinase
LB	6999-7440	Left border sequence from *A. tumefaciens* used for the transfer of T-DNA into MON87460 corn
PLASMID BACKBONE COMPONENTS		

Genetic element[1]	Location in PV-ZMAP595	Function
OR-ori V	7527-7923	Origin of replication from plasmid RK2 used for the maintenance of plasmid copy number in *A. tumefaciens*

1: Intervening sequences are not shown but were located between the described genetic elements and were used for cloning purposes; CS = coding sequence; OR = origin of replication; RB = right border P = promoter I = intron; T= terminator

3.2 Transformation method

MON87460 corn was produced by *Agrobacterium*-mediated transformation of *Zea mays* line LH59. The *Agrobacterium*-mediated DNA transformation system is the basis of natural plasmid-induced crown-gall formation in many plants and is well understood (Zambryski 1992). The genes of interest were inserted into the plasmid between DNA sequences known as the Left and Right Borders (LB and RB). These border sequences were isolated from the Ti plasmid of *Agrobacterium* and normally delimit the DNA sequence (T DNA) transferred into the plant.

Immature LH59 corn embryos were co-cultivated with *A. tumefaciens* strain ABI carrying PV-ZMAP595. Embryos producing embryogenic callus were transferred to initiation medium containing carbenicillin and paromomycin to select only plants cells containing the T-DNA (carbenicillin and paromomycin eliminate *Agrobacterium* and untransformed plant cells, respectively). Transformed cells were subcultured several times on medium containing kanamycin and regenerated to yield primary transformants (R_0 plants). These plants then underwent further breeding, development and evaluation. The major steps involved in the development of MON87460 corn are summarised in Figure 3.

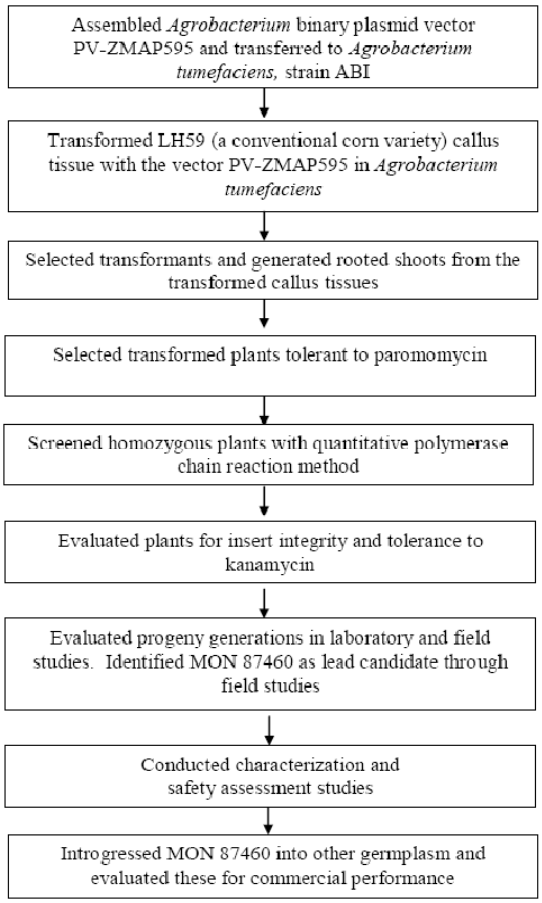
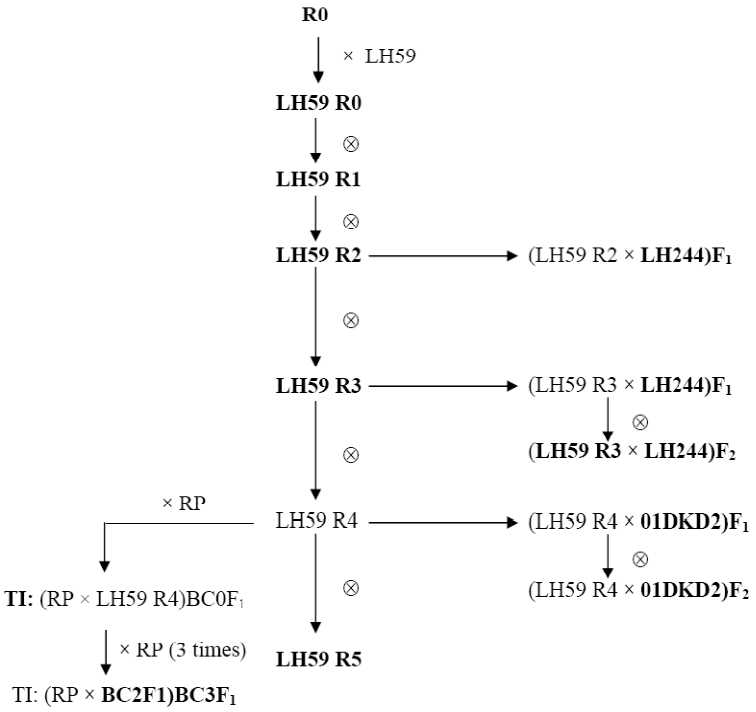


Figure 3:Steps in the development of MON87460 corn

3.3 Breeding history

A pedigree chart showing the breeding history of MON87460 corn is shown in Figure 4. R_0 plants were self pollinated to yield R_1 seed, which was screened for the presence of CSPB, kanamycin tolerance (i.e. presence of NPTII) and homozygosity of the inserted *cspB* gene. Plants meeting these criteria were self pollinated several more times, introgressed into elite corn lines and underwent further evaluation and development. Seed from various generations (highlighted) were used for the molecular, stability, protein and compositional analyses.



R0 = transformed plant; F(#) = filial generation; ⊗ = self-pollination; BC(#) = backcross generation; RP = recurrent parent for commercial seed development; TI = trait integration for commercial seed development.

Figure 4: Breeding history of MON87460 corn

The (LH59 R3 × LH244)F_1 generation was used for the molecular characterization of MON 87460. The (LH59 R2 × LH244)F_1, (LH59 R3 × LH244)F_1, (LH59 R3 × LH244)F_2, LH59 R4, (LH59 R4 × 01DKD2)F_1, (LH59 R4 × 01DKD2)F_2, and (RP×BC2F1)BC3F_1 generations were used for generational stability (indicated in bold). The (LH59 R3 × LH244)F_2 and (LH59 R4 × 01DKD2)F_2 generations were used for the protein expression and compositional analyses.

3.4 Characterisation of the genetic elements introduced into MON87460 corn

The genetic elements introduced into the genome of MON87460 corn were analysed using established molecular biological techniques and included southern blot analysis, polymerase chain reaction (PCR) and DNA sequence analysis, and flanking sequence bioinformatic analysis.

3.4.1 Southern blot analysis

Southern blot analysis was used to analyse the following in MON87460 corn: (1) insert and copy number (2) presence or absence of the plasmid PV-ZMAP595 backbone; (3) copy number of the elements of the *cspB* and *nptII* expression cassettes; and (4) generational stability of the insert, including the presence of the plasmid backbone. The analysis of (1), (2) and (3) was based on seed obtained from the (LH59 R_3 x LH244)F_1 generation, while the analysis of (4) was based on seed obtained from seven generations.

Genomic DNA from MON87460 corn seed was extracted, digested with appropriate restriction enzymes (*Hin*d III, *Eco*R V, *Eco*O109 and *Not* I) separated on agarose gels and transferred to a nylon membrane for Southern analysis. A negative control (DNA isolated from conventional corn with the same genetic background) was included for all analyses, and positive controls consisted of DNA from the transformation vector (plasmid PV-ZMAP595), which was spiked into the negative control DNA. Blots were incubated with one or more of 13 different ^{32}P-labelled DNA probes targeting different positions over the entire PV-ZMAP595 plasmid (Figure 1). Results indicated that MON87460 corn contains: (1) a single insert, integrated at a single site; (2) no DNA sequence(s) from the backbone of plasmid PV-ZMAP595; and (3) no additional genetic elements other than those associated with the intact *cspB* and *nptII* expression cassettes.

To determine the stability of the insert in MON87460 corn over multiple generations, DNA was isolated from seed from the following seven generations: (LH59 R_2 x LH244) F_1; (LH59 R_3 x LH244) F_1; (LH59 R_3 x LH244) F_2; LH59 F_4; (LH59 R_4 x 01DKD2) F_1; (LH59 R_4 x 01DKD2) F_2 and (RP x BC2F_1)BC3F_1 (see Figure 4 for the full breeding history of MON87460 corn). Southern blotting indicated that: (1) the single copy of the insert introduced into MON87460 corn was stable over multiple generations; and (2) multiple generations of MON87460 corn contained no DNA sequence(s) from the backbone of plasmid PV-ZMAP595.

3.4.2 PCR and DNA sequence analysis

PCR and DNA sequence analysis were used to analyse the: (1) organisation and sequence of the insert and adjacent genomic DNA; and (2) insertion site.

Seven overlapping regions spanning the entire insert and flanking genomic DNA were amplified by PCR using genomic DNA isolated from seed of the (LH59 R_3 x LH244)F_1 generation (see Figure 4). Additional PCR analysis was conducted to extend the 5' flanking sequence. Six PCR products (ranging in size from approximately 0.7 to 1.8 kb) generated from MON87460 corn were sequenced then combined to generate a consensus sequence of the insert. The results confirmed the identity and arrangement of all of the genetic elements introduced into MON87460 corn.

Additional analysis was conducted to determine the source of DNA flanking the 5' and 3' ends of the insert within the MON87460 genome. Genomic DNA flanking the 5' and 3' ends of the insert were amplified by PCR using genomic DNA isolated from seed of the (LH59 R_3 x LH244)F_1 generation. The nucleotide sequences of the 5' and 3' PCR products from MON87460 corn were then compared to the nucleotide sequence of the PCR products generated from conventional corn. This comparison revealed a 22 bp deletion in MON 87460 corn as a result of the insertion of the T-DNA from plasmid PV-ZMAP595. The comparison also indicated that the DNA sequences flanking the 5' and 3' ends of the insert were identical to conventional corn.

3.4.3 Flanking sequence bioinformatic analysis

A bioinformatic analysis was undertaken to ascertain the potential toxicity, allergenicity or biological activity of theoretical novel proteins encoded from the DNA sequences at the 5' and 3' insert/corn junctions.

Sequences spanning the MON87460 insert junctions were translated from stop codon to stop codon in all six possible reading frames and any putative proteins greater than eight amino acids compared with the amino acid sequences of known toxic, allergenic or biologically-active proteins contained in the following databases assembled by the Applicant:

- an allergen, gliadin and glutenin (AD8) database [assembled from the FARRP database (2008), University of Nebraska; available online at http://www.farrp.org/)];
- the PROTEIN database (2007) [derived from the GenBank database of the National Centre for Biotechnology Information (NCBI), National Library of Medicine, USA)]; and
- a toxin database (TOXIN6; 2008), which is a subset of 7,176 sequences from the GenBank database selected using a keyword search and filtered to remove proteins that are unlikely to be toxic to humans or animals .

The FASTA algorithm was used to determine the degree of sequence alignment between the theoretical proteins and those contained in the above databases. The degree of structural similarity was determined by examining the alignment of the sequences, percent identity and *E*-score. The latter is a statistical measure of the likelihood that the similarity between two sequences could have occurred by chance alone. An *E*-score of $\leq 1 \times 10^{-5}$ was set as a threshold at which the alignment could represent significant homology between two sequences. The FASTA alignment threshold for potential allergenicity was 35% homology over 80 amino acids, which is consistent with the criterion established by the Codex Alimentarius (2003). The potential allergenicity of the putative proteins was further evaluated using a sliding window search whereby proteins were compared with those contained in the AD8 database for the presence of immunologically-relevant sequences of eight contiguous and identical amino acids.

A total of nine putative proteins greater than eight amino acids in length were identified; five spanning the 5' insert/corn junction and 4 spanning the 3' insert/corn junction. None of the nine putative proteins had any significant sequence homology with any of the allergens in the AD8 database. Only one of the nine proteins, peptide 2 from the 5' junction (designated peptide 5_2) was found to have apparent homology with protein sequences in the TOXIN6 and GenBank protein database. Peptide 5_2 generated an *E*-score of 2.1×10^{-9}, 71% homology and a 38 amino acid overlap with RTX toxins and related calcium-binding protein following a FASTA search of the TOXIN6 database. A FASTA search of the GenBank protein database generated an *E*-score of 7.7×10^{-6}, 51% homology and a 77 amino acid overlap with hypothetical protein GOS8187102. In both cases, it was stated that the homology was due to the alignment entirely of proline residues or to alignments "heavily weighted with proline residues".

On the basis of this amino acid composition bias, the authors concluded that the apparent homology between theoretical peptide 5_2 and single proteins in the GenBank and TOXIN6 databases was unlikely to indicate structural relatedness. It is well recognised that amino acid composition bias may result in the erroneous identification of homology between two protein sequences (Devos & Velencia 2000; Promponas et al 2000; Alba et al 2002) and the generation of high similarity scores (Cymerman et al 2005). On this basis, such a conclusion is considered appropriate.

These results indicated that the theoretical putative proteins encoded by the DNA sequences at the 5' and 3' insert/corn junctions showed no biologically-significant structural or immunological similarity to known toxins, allergens or other biologically-active proteins.

3.4.4 Conclusions

Detailed molecular analyses indicate that one copy of T-DNA, containing one copy of the *cspB* and *nptII* gene expression cassettes have been inserted at a single genomic locus in MON87460 corn. The coding region of the *cspB* and *nptII* genes and their regulatory elements are intact. The molecular analyses also confirmed the absence of plasmid backbone sequences in the GM corn. The stability of the insert and absence of plasmid backbone sequences were confirmed through seven generations of breeding. A small deletion of 22 bp was detected due to the insertion of the T-DNA in to MON87460 corn, while the nucleotide sequences flanking the 5' and 3' ends of the insert were determined to be derived from corn. Bioinformatic analysis indicated that in the highly unlikely event that DNA sequences spanning the 5' and 3' insert/corn junctions were transcribed, the expressed proteins would have no toxic or allergenic potential, and would not be biologically active.

3.5 Stability of genetic changes

Submitted study:
Rosenbaum EW (2008) Assessment of insert segregation for MON 87460. Report No. 07-RA-B3-01. Lab: Monsanto Company, Biotechnology Regulatory Sciences, St Louis, MO, USA. Sponsor: Monsanto Company, St Louis, MO, USA. Unpublished. [GLP, QA]

The segregation and stability of the *cspB* gene in MON87460 corn were examined using genomic DNA isolated from seven generations of self-pollinated or backcrossed plants, which were analysed by PCR to confirm the presence or absence of the *cspB* gene. Chi-square analysis was used to determine whether the observed segregation ratios were consistent with those expected according to Mendelian principles. No significant difference ($p>0.05$) between the expected and observed segregation ratios was determined indicating that the *cspB* gene follows a Mendelian pattern of segregation (Table 2).

Table 2: Segregation frequencies of the *cspB* gene in MON87460 corn

Generation	No. of plants	Observed positives	Observed negatives	Expected positives	Expected negatives	Chi square	P value ($\alpha = 0.05$)
R_1	36	26	10	27	9	0.1481	NS
R_2	89	89	0	89	0	Fixed	-
$BC3F_1$	178	84	94	89	89	0.562	NS
$BC3F_2$	154	124	30	116	39	2.502	NS
$BC3F_3$	474	474	0	474	0	Fixed	-
$BC4F_1$	80	44	36	40	40	0.800	NS
$BC5F_1$	82	44	38	41	41	0.439	NS

NS = not significant

3.6 Horizontal transfer of the *nptII* gene

Horizontal gene transfer is the stable transfer of genetic material from one organism to another without reproduction (reviewed by Keese 2008). It occurs naturally and all genes, irrespective of their origin in an organism, are capable of being transferred to another organism by such a mechanism. Horizontal gene transfer is a rare event; the transfer of DNA from plants to bacteria has only been documented under laboratory conditions and then only between homologous sequences (de Vries et al 2001; de Vries & Wackernagel 2002 Bennet et al 2004).

The human health issues associated with the horizontal transfer of the *nptII* gene from GM plants to gastrointestinal bacteria, such as the spread of antibiotic resistance, have been widely evaluated by regulatory agencies and international bodies. The most recent of these reviews was conducted by the European Food Safety Authority (EFSA 2007b), which concluded that the horizontal transfer of the *nptII* gene from GM plants to bacteria poses negligible health risks.

The Office of the Gene Technology Regulator (OGTR) has evaluated the horizontal transfer of the *nptII* gene from GM crops to other organisms on numerous occasions. In fact the majority of licence applications for the intentional release of GM crops in Australia have involved an assessment of the horizontal transfer of the *nptII* gene [see OGTR (2009) for the most recent evaluation]. The OGTR has consistently concluded that the horizontal transfer of the *nptII* gene from GM plants to other organisms does not pose a risk to human health (or the environment).

FSANZ has previously considered the horizontal transfer of the *nptII* gene as part of its safety assessments of GM cotton (A341, 355 & 379) canola (A372), potato (A382, 383 & 384) and corn (A484). FSANZ (2003) concluded that the transfer of the *nptII* gene from GM plants to microorganisms in the human digestive tract was highly unlikely because of the number and complexity of steps that would need to take place consecutively.

In the highly unlikely event that the *nptII* gene was transferred, the effects on human health were considered negligible because the *nptII* gene is already commonly present in bacteria in the environment, including the human digestive tract (Flavel et al 1992). Further, the antibiotics to which *nptII* confers resistance have very limited clinical use because of their toxic side effects (WHO 1993) and as resistance to these antibiotics is already widespread (Levy et al 1998).

4. CHARACTERISATION OF NOVEL PROTEINS

In evaluating the safety of novel proteins it is important to consider that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects, although a small number have the potential to impair health, for example, because they are allergens or anti-nutrients.

As proteins perform a wide variety of functions, different possible effects have to be considered during the safety assessment including potential toxic, anti-nutritional and allergenic effects. To effectively identify any potential hazards requires knowledge of the characteristics, concentration and localisation of all novel proteins expressed in the organism as well as a detailed understanding of their biochemical function and phenotypic effects. It is also important to determine if the novel protein is expressed as expected, including whether including whether any post-translational modifications have occurred (e.g. glycosylation).

MON87460 corn expresses two novel proteins: CSPB and NPTII. A number of different analyses were done to determine the identity, physiochemical properties, *in planta* expression, bioactivity and potential toxicity and allergenicity of the two proteins. The equivalence of the bacterial-produced proteins to the plant-produced proteins was determined as part of the protein characterisation.

4.1 Function

4.1.1 Function of CSPB

CSPB belongs to the bacterial cold shock protein (CSP) family, which is a group of small, highly conserved proteins that are induced following a sudden decrease in temperature in order to facilitate survival (Horn et al 2007). They are characterised by the presence of a cold shock domain (CSD), which contains the nucleic acid binding motifs, RNA-binding ribonucleoproteins 1 and 2 (RNP1 and RNP2, respectively). These motifs allow CSPs to bind to single stranded RNA and DNA cooperatively but with low sequence specificity (Schindler et al 1999; Zeeb et al 2006). Homologues of the CSD are present in many eukaryotic proteins including Y-box transcription factors and nucleic acid-binding proteins (Schindler et al 1999; Zeeb & Balback 2003; Horn et al 2007).

CSPB is one of three complementary CSPs present in *B. subtilis* and is expressed at both low and normal temperatures (Schindler et al 1999). These CSPs are thermodynamically unstable, unfolding rapidly at 37ºC and being highly susceptible to protease degradation (Schindler et al 1999). Stabilisation is achieved at low temperatures and following nucleic acid binding (Schindler et al 1999). Like other CSPs, CSPB functions as an RNA chaperone at low temperatures, stimulating the transcription of cold shock inducible genes and initiating translation by destabilising RNA secondary structure (Jiang et al 1997; Phadtare et al 2002a & b; Zeeb et al 2006). At normal (i.e. optimal temperatures), CSPB also functions as an RNA chaperone to maintain normal cellular processes (Graumann et al 1997).

Besides prokaryotes, evidence is emerging that cold adaption in plants is also facilitated by the RNA chaperone activity of CSD-containing proteins. Karlson and Imai (2003) reported the widespread occurrence of homologues of the bacterial CSD in nineteen plant genera. They demonstrated that CSD genes in *Arabadopsis* were upregulated at low temperatures, with more recent evidence indicating that freezing tolerance is conferred by a CSD-containing protein, similar to bacterial CSPs (Kim et al 2009). In wheat, a CSD-containing nucleic acid binding protein was shown to function as an RNA chaperone in a similar manner to *E. coli* CSPs under conditions of cold stress (Nakaminami et al 2006).

The ability of MON87460 corn to tolerate drought stress is conferred by expression of the *cspB* gene from *B. subtilis*. While the exact mechanism of drought tolerance in MON87460 corn remains to be defined, it is likely the result of a general tolerance to abiotic stress (not solely drought) due to the RNA chaperone activity of CSPB, which preserves the integrity of protein synthesis (Castiglioni et al 2008). The CSPB present in MON87460 corn is nearly identical to that present in *B. subtilis* except for a single amino acid substitution at position 2 (from leucine to valine) necessary for cloning purposes. This slight difference would not be expected to affect the function of the protein in MON87460 corn plants and indeed the RNA chaperone activity of the protein has been confirmed (see Section 4.2.1).

4.1.2 Function of NPTII

NPTII is a bacterial enzyme that catalyses the transfer of a phosphate group from adenosine 5'-triphosphate (ATP) to a hydroxyl group on the aminohexose moiety of aminoglycoside antibiotics, thereby inactivating them (Davies et al 1986). Many aminoglycosides are phosphorylated by NPTII but NPTII does not inactivate all aminoglycosides because of widely different phosphorylation rates for the different substrates (Redenbaugh et al 1994). NPTII confers resistance to neomycin, kanamycin, geneticin and paromomycin, but not more clinically important aminoglycoside antibiotics such as amikacin and gentamicin B.

The *nptII* gene is widely used as a selectable marker in the transformation of plants and is derived from *E. coli* transposon Tn5 (Beck et al 1982). The gene functions as a dominant selectable marker in the initial, laboratory stages of plant cell selection following transformation (Horsch et al 1984, DeBlock et al 1984). While the *nptII* gene and its encoded protein are present in MON87460 corn plants, it has no function and is simply a remnant from the initial laboratory development of the GM corn line.

4.2 Protein characterisation

4.2.1 CSPB

Submitted study:
Burzio LA, Wang C & Rice EA (2008) Characterization of the *Bacillus subtilis* cold shock protein B (CSPB) purified from the grain of drought tolerant corn MON87460 and comparison of the physicochemical and functional properties of the MON87460-orduced and *E. Coli*-produced CSPB proteins. Study No. REG-07-214. Study Report: MSL0021140. Lab: Monsanto Company, Production Characterization Team, Protein and Molecular Sciences Team, St Louis, MO, USA. Sponsor: Monsanto Company, St Louis, MO, USA. Unpublished. [GLP, QA]

As the level of CSPB in MON87460 was low, it was necessary to express CSPB in *E. coli* in order to obtain sufficient quantities of the protein for analysis.

A range of analytical techniques was used to characterise CSPB from MON87460 corn grain and to establish its equivalence with the CSPB derived from *E. coli*. These techniques included: (1) sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting; (2) N-terminal sequencing; (3) matrix assisted laser desorption ionisation time of flight (MALDI-TOF) mass spectrometry; (4) glycosylation analysis; and (5) a CSPB activity assay.

CSPB was purified from the ground seed (Seed Lot No. GLP-0604-17132-S) of the ($LH59R_3$ x LH244)F_1 and ($LH59R_3$ x LH244)F_2 generations of MON87460 corn (see Figure 4) using ammonium sulphate fractionation and ion exchange, affinity and size exclusion chromatography. It was stated that the identity of the grain was confirmed by PCR. The bacterial-derived CSPB (Lot No. 10000802) was purified from the fermentation of GM *E. coli* and used as a reference standard throughout all phases of the study.

The study authors used a number of criteria to establish the equivalence of CSPB from MON87460 and *E. coli* : the molecular weight should be within $\pm$10%; the immunoreactivity should be within $\pm$35%; the functional activity should be within $\pm$25%; and the protein should not be glycosylated. These criteria were appropriately established by taking into consideration the intrinsic variability of each method.

(1) SDS-PAGE and Western blotting

The concentration, purity and molecular weight of CSPB derived from MON87460 corn were estimated by quantitative densitometry of silver-stained gels, using a standard curve constructed from known concentrations of *E. coli*-derived CSPB. Appropriate molecular weight standards were also used. The concentration of CSPB from MON87460 corn was determined to be 118 µg/mL, the average purity 97% (n=6; range: 94.6-98.6%) and average molecular weight 6.7 kDa (n=6; range: 6.41-6.99 kDa). The difference in molecular weight of CSPB from MON87460 corn and that derived from *E. coli* was 3% (based on the Certificate of Analysis), which was within the acceptance criteria for equivalence.

The identity and immunoreactivity of CSPB from MON87460 corn was confirmed by Western blotting. Following SDS-PAGE, proteins were transferred to nitrocellulose or polyvinylidene fluoride (PVDF) membranes, which were probed with a goat polyclonal antibody to CSPB. Bands were visualised by chemiluminescence-based immunodetection and quantified by densitometry. A single immunoreactive band was visualised, which was consistent with that observed for *E. coli*-derived CSPB.

The stability of CSPB from MON87460 corn was determined by storing it at -80ºC for 14 days and then measuring its purity and molecular weight relative to a day 0 sample. The molecular weight of the stored sample was 6.9 kDa (n=6; range: 6.70-7.01 kDa) *versus* 6.7 kDa in the day 0 sample. Densitometry of the band indicated that the average purity of the stored sample was 99% (n=6; range: 97.4-100%) *versus* 97% in the day 0 sample. Given that the difference in molecular weight (3%) and purity (2%) of the stored sample relative to the unstored sample were within the acceptance criteria of $\pm$10%, the result indicated that CSPB from MON87460 corn is stable when stored at -80ºC for 14 days.

(2) N-terminal sequencing

Following SDS-PAGE and transfer to PVDF, the protein band corresponding to CSPB was excised and subjected to N-terminal sequence analysis for 15 cycles using automated Edman degradation. With the exception of the N-terminal methionine, which was missing due to its likely enzymatic cleavage, the observed sequence of CSPB from MON87460 corn was identical to that predicted from the coding region of the introduced *cspB* gene.

(3) MALDI-TOF

The identity of CSPB from MON87460 corn was confirmed by tryptic peptide mapping. Samples were digested with trypsin, guanidinated and subjected to MALDI-TOF mass spectrometry. Four unique peptide fragments were identified and their masses matched those predicted from the coding region of the introduced *cspB* gene. The masses of these fragments were used to construct a peptide map, which covered 58 of the 66 amino acids in the known sequence of CSPB (Fragment 1: amino acids 7-12; Fragment 2: amino acids 56-64; Fragment 3: amino acids 39-55; Fragment 4 amino acids 13-38). These results confirmed the identity of CSPB in MON87460 corn over 87.9% of the amino acid sequence.

Given the high (97%) purity of CSPB from MON87460 corn, its molecular weight was determined directly by MALDI-TOF mass spectrometry. The average molecular mass was determined to be 7220 Da (n & range unspecified), which was 131 Da less than the expected mass due to the absence of the N-terminal methionine.

(4) Glycosylation analysis

Glycosylation analysis was undertaken to determine whether CSPB from MON87460 contained any carbohydrate moieties. CSPB from MON87460 corn and two positive controls, transferrin and horseradish peroxidise, were separated by SDS-PAGE, transferred to a PVDF membrane and probed for carbohydrates using a commercially-available glycoprotein stain, which reacts with oxidised carbohydrate moieties to create a green fluorescence. No signal was detectable for the test or control CSPB samples indicating that neither protein was glycosylated. In contrast, detectable signals were obtained for the positive control glycoproteins. Staining of PVDF membranes with SYPRO® Ruby Protein Blot Stain confirmed that CSPB proteins had successfully transferred. The absence of carbohydrate moieties in CSPB from MON87460 confirmed its equivalence to its *E. Coli*-derived counterpart.

(5) CSPB activity assay

An *in vitro* functional activity assay (in a microtiter plate format) was used to confirm the ability of CSPB from MON87460 corn to destabilise DNA-hairpin structures. The study authors stated that the assay had previously been validated. CSPB has high affinity for polydeoxythymidylate (poly dT) sequences and therefore the assay involved incubating CSPB with a fluorescently-labelled oligonucleotide DNA probe containing such sequences. The probe consisted of a 6 bp double strand stem containing the fluorophore and a quencher, and a loop of 23 nucleotides (dT) in the middle portion of the molecule. Binding of CSPB to this loop should result in the destabilisation of the double strands resulting in the separation of the fluorophore and quencher, which can then be detected via a microplate reader.

The specific activity of CSPB from MON87460 corn was 0.660 pmoles opened dual labelled probe (DLP)/µg CSPB, which was 12.8% lower than the specific activity of *E. coli*-derived CSPB of 7.57 pmoles opened DLP/µg CSPB. This difference is within the acceptance criteria for equivalence.

Conclusion

The identity of CSPB from MON87460 corn was confirmed in terms of its purity, immunoreactivity, molecular mass, N-terminal sequence, glycosylation status and functional activity. CSPB from MON87460 corn was also determined to be equivalent to CSPB from *E. coli.*

4.2.2 NPTII

Submitted study:
Gu X, Burzio LA & RICE EA (2008) Assessment of the physicochemical equivalence of the plant-produced NPTII protein from the leaf of MON87460 to the *E. Coli*-produced NPTII protein. Study No. REG-07-311. Study Report: MSL0021145. Lab: Monsanto Company, Regulatory Product Characterization Team, Protein and Molecular Sciences Team, St Louis, MO, USA. Sponsor: Monsanto Company, St Louis, MO, USA. Unpublished. [GLP, QA]

There is an already extensive database on NPTII given its widespread use in the production of GM plants. In order to make use of the existing safety and characterisation data on NPTII, the study authors sort to establish the equivalence of NPTII from MON87460 corn to that derived from *E. coli* using Western blotting to compare immunoreactivity and molecular weight.

As undetectable levels of NPTII were present in MON87460 corn grain using the current enzyme-linked immunosorbent assay (ELISA), it was necessary to isolate the protein from corn leaves (plants were grown from seed Lot GLP-0702-18101-S). The presence of the *nptII* gene in leaf of MON87460 corn was confirmed by PCR, with the concentration of NPTII stated as 0.34 µg/g fresh leaf weight. Leaf from conventional corn DM 178 was used as a negative control (plants were grown from seed lot GLP-0702-18108-S); this line was stated as having a similar genetic background to MON87460 corn but does not contain the *nptII* gene. The reference protein was NPTII from *E. coli* (Orion Lot No. 10000766; 96% purity; Molecular weight = 28 kDa).

Protein extracts were prepared from leaf samples of MON87460 and DM178 corn, with some of the latter spiked with a known concentration of the reference protein to serve as an assay control. The leaf extracts, spiked assay control, reference protein and molecular weight markers were subjected to SDS-PAGE, transferred to a nitrocellulose membrane and probed with a rabbit polyclonal antibody to NPTII. Bands were visualised by chemiluminescence-based immunodetection and quantified by densitometry.

The same immunoreactive band of approximately 27 kDa was observed in the leaf extract from MON87460 corn, the spiked assay control and reference protein, but was absent from the leaf extract from DM178 corn. Densitometry of this single band indicated that the average molecular weight of NPTII from MON87460 corn was 27.4 kDa *versus* 27.1 kDa for the *E. coli* derived protein (n & range unspecified). This equates to a 1% difference, which is well within the study author's acceptance criteria for equivalence of ±10%.

The results of this study indicated that NPTII from MON87460 corn is equivalent to that derived from *E. coli* on the basis of similar immunoreactivity and molecular weight.

4.3 Protein expression analysis

4.3.1 Phenotype of MON87460 corn

Corn development is divided into vegetative (V) and reproductive (R) stages, which are summarised in Table 3. The V number corresponds to the number of fully exposed leaves with a visible collar and generally goes up to V18. Corn yield is a function of kernel number (which is dependent on the number of plants per unit area, grained ears/plant and kernels/ear) and kernel weight (which is dependent on the duration and rate of grain fill (Westgate et al 2004).

Table 3: Summary of corn growth stages (Derived from Hanway & Ritchie 1984)

STAGE	DESCRIPTION
Vegetative stage	
VE	Emergence
V1	First fully expanded leaf with a visible leaf collar
V2	Second fully expanded leaf with a visible leaf collar
V(n)	n^{th} fully expanded leaf with a visible leaf collar
VT	Tassel emergence or anthesis
Reproductive stage	
R1	Silking
R2	Blister (early grainfill);10-14 days after silking
R3	Milk (mid grainfill); 18-22 days after silking
R4	Dough (mid grainfill); 24-28 days after silking
R5	Dent (late grainfill); 35-42 days after silking
R6	Physiological maturity 55-65 days after silking

Submitted study:
Eberle M & Whitsel J (2009) Phenotypic evaluation and ecological interactions of drought tolerant corn MON87460 under well-watered and water-limited conditions in Chilean field trials during 2006-2007. Study No. 07-01-B3-19. Report No. MSL0021857. Lab & Sponsor: Monsanto Company, St Louis, MO, USA. Unpublished. [QA]

Experimental: MON87460 corn (Seed Lot No. GLP-0609-17631-S) was cultivated at four Chilean field sites during 2006-2007 under well-watered and water-limited (i.e. drought) conditions to determine the phenotypic characteristics, tissue concentrations of CSPB and NPTII (Section 4.3), and composition relative to conventional corn (Section 5). The four sites were Calera de Tango (CT), Colina (CL), Lumbreras (LUM) and Quillota (QUI), which were chosen because of their low rainfall potential and therefore the relative ease of managing the two treatment regimes.

The conventional corn line, DM1718 (Seed Lot No. GLP-0609-17630-S), was grown concurrently at all sites under the same two treatment conditions. For test and control starting seed, PCR analysis detected NK603 contamination in MON87460 corn seed at a level of ≤1.84%, however, the study director did not consider that this would affect the outcome of the trial. Also grown at each site were four different commercial corn hybrids (see Table 4 for details), which were used to establish the baseline phenotypic characteristics of corn and also to validate the water-limited treatment.

Table 4: Reference varieties of corn grown at the four Chilean sites

Site	Reference Varieties
CL	33D11, BT 6011, Garst 8424, DKC62-30
CT	33NO9, 33K39, BT 6613, DKC63-78
LUM	33N29, Garst 8445, DKC61-50, RX 715
QUI	34N43, BT6610, Garst 8545, DKC60-15

CT = Calera de Tango; CL = Colina; LUM = Lumbreras; QUI = Quillota

Each site was assessed in a step-wise manner to determine whether a differential water treatment was achieved. Each site had to meet three performance criteria: (1) irrigation management practices at each site had to indicate the likelihood that differential water treatments were achieved; (2) analysis of soil moisture content at each site had to confirm that differential water treatments were achieved; and (3) the four reference corn varieties grown at each site had to show at least a 15% yield reduction under water-limited conditions.

The following phenotypic/agronomic characteristics were measured: seedling vigour and early stand count (assessed at V2-V4); days to 50% pollen shed (assessed at pollen shed) days to 50% silking (assessed at silking); stay green, ear height and plant height (assessed at maturity); number of dropped ears, stalk lodged plants, root lodged plants and final stand count (assessed pre-harvest); grain moisture; test weight and yield (assessed at harvest). Signs of stress to various abiotic factors (cold, frost, hail, heat, N deficiency & wind damage), disease damage (due to ear rot, *Fusarium*, grey leaf spot, leaf blight, northern corn leaf blight, root rot, rust, seedling blight, smut & stalk rot) and insect damage (due to aphids, seed corn maggot, thrips & wire worms) were assessed at V2-V4, V10-V15, VT-R5 and R6.

Sites were statistically analysed on an individual and combined site basis, however, one site (QUI) was excluded from the combined site analysis because the four reference varieties of corn grown at the site failed to exhibit the necessary phenotypic response to treatment (i.e. a 15% reduction in yield under water-limited conditions).

Results: There was no significant difference in any of the phenotypic parameters analysed between MON87460 and conventional corn when cultivated under well-watered conditions on both an individual site and combined site basis.

The results of the phenotypic evaluation of MON87460 corn grown under water-limited conditions at CL, CT and LM are summarised in Table 5. Average ear and plant heights were significantly higher ($p<0.05$) in MON87460 corn cultivated at CL compared to the control. In the absence of a similar difference at the other two sites or when data were pooled across sites, this result is not considered biologically significant. Yield was significantly higher ($p<0.05$) in MON87460 corn than the control at CT, with results from CL and LUM also suggesting a higher yield (albeit not significant). The combined site analysis also confirmed a significantly higher ($p<0.05$) yield in MON87460 corn relative to the control (46.3 *versus* 35.1 bu/ha, respectively). On this basis, the increased yield in MON87460 grown under water-limited conditions is considered biologically significant.

Table 5: Phenotypic comparison of MON87460 and control corn grown at CL, CT & LUM under water-limited conditions

Phenotypic characteristic	CL		CT		LUM		Reference Range[1]
	Test	Control	Test	Control	Test	Control	
Seedling vigour[2]	5.0	5.0	5.3	4.7	4.7	4.7	4.0-6.0
Early stand count (No./plot)	75.7	73.0	77.7	76.0	77.0	78.0	67.3-80.7
Days to 50% pollen shed	65.0	65.3	65.3	65.3	72.0	73.7	65.7-75.0
Days to 50% silking	64.3	62.7	63.7	64.0	74.0	73.7	63.3-74.3
Stay green[3]	1.3	1.7	5.7	5.7	6.0	6.7	1.0-7.0
Ear height (cm)[4]	154.9*	130.6	114.0	109.7	96.8	103.9	101.6-153.7
Plant height (cm)[4]	246.9*	201.4	222.3	214.63	170.2	178.8	164.8-245.9
Dropped ears (No./plot)	0	0	0	0	0	0	0
Stalk lodged plants (No./plot)	0	0	0	0	0	0	0
Root lodged plants (No./plot)	0	0	0	0	0	0	0
Final stand count (No./plot)	74.3	71.3	78.0	76.3	77.7	77.7	71.3-80.7
Grain moisture (%)	14.6	16.3	15.5	15.6	28.5	31.9	9.6-25.5
Test weight (kg/bu)[4]	25.9	25.3	27.4	27.4	23.8	23.5	23.3-28.2
Yield (bu/ha)[4]	39.2	26.5	65.1*	50.1	34.7	28.7	22.8-67.8

Results are expressed as means; *p<0.05; 1 = derived from the 3 sites; 2 = rated on a 0-9 scale where 0 is dead and 9 is above average vigour; 3 = rated on a 0-9 scale where 0 is a completely dry plant and 9 is a completely green plant; 4 = converted by FSANZ from imperial to metric units

Under both treatment regimes there was no significant difference between MON87460 and conventional corn with regard to the response to abiotic stressors, disease damage or insect damage.

Conclusion: The results of this field trial indicated that the only phenotypic difference between MON87460 corn and control corn was increased grain yield under water-limited conditions.

A published Monsanto review summarised the results of experiments relating to the development of drought-tolerant corn over a four year period (Castiglioni et al 2008). While this review did not specifically mention MON87460 corn, it demonstrated the growth and yield benefits resulting from the expression of CSBP from *B. subtilis* in corn grown under drought conditions. Relative to control corn, expression of CSPB increased leaf extension rates, chlorophyll content and photosynthesis by up to 24, 4.4 and 8.5%, respectively, under drought conditions. Grain yield was improved by up to 21% (average of 10.5% over 4 years) due to the increased number of plants with kernel-bearing ears and the number of kernels produced per plant.

4.3.2 Protein expression

Submitted study:
Shi L, Chinnadurai P, McClain S & Silvanovich A (2008) Amended report for MSL0021185: Assessment of the CSPB and NPTII protein levels in tissues of drought tolerant corn MON87460 produced in aa2006-2007 Chilean field trial under well watered and water-limited conditions. Study No. REG-07-199. Study Report: MSL0021731. Lab & Sponsor: Monsanto Company, Biotechnology Regulatory Sciences, St Louis, MO, USA. Unpublished. [GLP, QA]

MON87460 corn and conventional corn were grown at four field sites in Chile during 2006-2007 under well-watered and water-limited conditions (see Section 4.3.1 for full details). The concentration of CSPB was measured in over season leaf, root, whole plant, pollen, forage, forage root, senescent root, silk and grain by ELISA. Over season leaf, root and whole plant samples were collected at four different stages of development (V2-V4, V6-V8, V10-V12 and VT stages) to determine whether protein concentrations changed over time. NPTII concentrations were measured in over season leaf, root forage and grain by ELISA. Known concentrations of two reference proteins, *E. coli*-derived CSPB (Lot 20-100099; 100% purity) and NPTII (Lot 20-100075; 96% purity), were used to quantify their counterparts in corn tissue.

The average concentration of CSPB in different tissues from plants grown at the three valid sites (i.e. CL, CT and LUM) are summarised in Table 6. The highest concentration of CSPB was detected in pollen followed by leaf. The concentration in tissues from well-watered and water-limited plants was similar, which is attributable to the constitutive nature of the rice *act1* gene promoter controlling expression of the *cspB* gene in MON87460 corn. Over the growing season, there was a decrease in CSPB concentrations in leaf, root and whole plant. Tissue samples from conventional corn were generally negative although two samples tested positive for CSPB, which the authors attributable to sample contamination.

Table 6: Tissue concentrations of CSPB in MON87460 corn

Tissue	*Well-watered*		*Water-limited*	
	µg/g fwt	µg/g dwt[1]	µg/g fwt	µg/g dwt
Leaf (V2-V4)	0.50±0.19	2.8±1.0	0.50±0.20	2.8±0.95
Leaf (V6-V8)	0.48±0.18	2.6±1.2	0.47±0.15	2.6±1.0
Leaf (V10-V12)	0.13±0.10	0.56±0.48	0.11±0.073	0.45±0.32
Leaf (VT)	0.10±0.041	0.39±0.13	0.11±0.054	0.44±0.17
Root (V2-V4)	0.13±0.029	1.3±0.29	0.14±0.034	1.5±0.43
Root (V6-V8)	0.086±0.025	0.86±0.25	0.10±0.015	0.82±0.092
Root (V10-V12)	0.061±0.012	0.49±0.12	0.054±0.012	0.41±0.13
Root (VT)	0.045±0.012	0.31±0.076	0.058±0.016	0.40±0.087
Whole plant (V2-V4)	0.32±0.11	3.2±0.98	0.30±0.092	2.9±0.84
Whole plant (V6-V8)	0.19±0.036	2.3±0.54	0.18±0.046	2.2±0.61
Whole plant (V10-V12)	0.10±0.042	0.89±0.34	0.091±0.032	0.71±0.25
Whole plant (VT)	0.11±0.026	0.67±0.16	0.13±0.037	0.70±0.16
Forage root	0.0052±0.0018	0.039±0.015	0.011±0.0039	0.076±0.029
Senescent root	0.0040±0.0017	0.031±0.015	0.0067±0.0051	0.076±0.029
Forage	0.026±0.0041	0.11±0.018	0.035±0.0078	0.15±0.040
Stover	0.011±0.0023	0.033±0.0070	0.021±0.010	0.072±0.033
Silk	0.073±0.019	0.82±0.28	0.13±0.048	1.1±0.38
Pollen	18±5.6	25±7.4	18±6.5	27±10
Grain	0.041±0.012	0.048±0.014	0.033±0.0067	0.038±0.0079

Results are expressed as the mean ± 1 standard deviation (SD) (n=9); fwt = fresh weight; dwt = dry weight; The LOQ/LOD (µg/g fwt) were 0.015/0.0069 for leaf, 0.0020/0.0018 for root, 0.0045/0.0043 for whole plant, forage and stover, 0.0075/0.0047 for silk, 0.050/0.045 for pollen and 0.0038/0.0017 for grain

1 = dry weight values were calculated by dividing the fresh weight by a dry weight conversion factor, which was generated by analysing the moisture content of each tissue

The average concentration of NPTII in leaf, root, forage and grain from MON87460 corn grown at the three valid sites is summarised in Table 7. The highest concentration was detected in the leaf, while the concentration in tissues from well-watered and water-limited plants was similar; this again reflects the constitutive nature if the promoter controlling expression of the *nptII* gene. The mean concentration of NPTII in grain across the three sites was below the LOQ (0.0047 µg/g fresh weight).

Table 7: Tissue concentrations of NPTII in MON87460 corn

Tissue	*Well-watered*		*Water-limited*	
	µg/g fwt	**µg/g dwt[1]**	**µg/g fwt**	**µg/g dwt**
Leaf (V2-V4)	0.42±0.23	2.4±1.3	0.46±0.18	2.6±0.98
Root (V2-V4)	0.051±0.0083	0.51±0.083	0.046±0.0075	0.48±0.097
Forage	0.0.037±0.0041	0.16±0.020	0.039±0.048	0.17±0.028
Grain	< LOD	NA	< LOD	NA

Results are expressed as the mean ± 1 standard deviation (SD) (n=9); fwt = fresh weight; dwt = dry weight NA = not applicable; LOD = limit of detection The LOQ/LOD (µg/g fwt) were 0.047/0.0090 for leaf , 0.0075/0.0043 for root, 0.0056/0.0024 for forage and 0.0047/0.0024 for grain

1 = dry weight values were calculated by dividing the fresh weight by a dry weight conversion factor, which was generated by analysing the moisture content of each tissue

4.4 Potential toxicity of novel proteins

While the vast majority of proteins ingested as part of the diet are not typically associated with toxic effects, a small number may be harmful to health. Therefore, if a GM food differs from its conventional counterpart by the presence of one or more novel proteins, these proteins should be assessed for their potential toxicity. The main purpose of an assessment of potential toxicity is to establish, using a weight of evidence approach, that the novel protein will behave like any other dietary protein. The assessment focuses on: whether the novel protein has a prior history of safe human consumption, or is sufficiently similar to proteins that have been safely consumed in food; amino acid sequence similarity with known protein toxins and anti-nutrients; structural properties of the novel protein including whether it is resistant to heat or processing and/or digestion. Appropriate acute oral toxicity studies in animals may also be useful, particularly where results from the biochemical, bioinformatic, digestibility or stability studies indicate a concern.

4.4.1 History of use

CSPB

CSPB from *B. subtilis* has no prior history of intentional food use. However, humans would already be exposed CSPB through contact with *B. subtilis*, which is ubiquitous in the environment. *B. subtilis* is also used to make traditional fermented foods (e.g. natto) and more recently has been used as a probiotic. In addition, the presence of homologues of the bacterial CSD in plants (Karlson & Imai 2003), including food crops (Nakaminami et al 2006), suggests that humans have had a long history of dietary exposure to similar proteins.

NPTII

The *nptII* gene (and its encoded protein) has a considerable history of use as a selectable marker gene in the development of GM plants. Associated with this history of use is a substantial body of evidence to indicate that the presence of NPTII in food derived from GM crops does not pose a significant risk to human health. Further, humans are already exposed to this protein given its widespread environmental presence.

FSANZ has approved food derived from a number of different GM crops containing the *nptII* gene and its encoded protein (Applications A341, 355, 372, 379, 382, 383, 384 & A484) or which were developed using the *nptII* gene but which is absent or not expressed in the final plant (Applications A549 & A595). The conclusion of FSANZ's previous evaluations of NPTII is that its presence in food derived from GM crops poses no toxicity concerns.

Additionally, the toxicity of NPTII has been evaluated by other regulators (e.g. US FDA 1998; EFSA 2007b; OGTR 2009) and considered on numerous occasions in the peer reviewed scientific literature (e.g. Flavell et al 1992; Nap et al 1992; Fuchs et al 1993a; Fuchs et al 1993b).

4.4.2 Similarities with known protein toxins

Submitted study:
Burzio LA, McClain JS & Silvanovich A (2008) Bioinformatics evaluation of the CSPB protein utilizing the AD8, TOXIN6 and PROTEIN databases. Study No. REG-08-091. Report No. MSL-0021261. Lab: Monsanto Company, Regulatory Product Characterization Team, St Louis, MO, USA. Sponsor: Monsanto Company, St Louis, MO, USA. Unpublished. [QA]

Tu H (2009) Updated bioinformatics evaluation of the NPTII protein utilising the AD_2009 and TOX_2009 databases. Study No. REG-09-154. Report No. MSL0021965. Lab & Sponsor: Monsanto Company, Product Characterization Center, St Louis, MO, USA. Unpublished. [QA]

Bioinformatic analyses were undertaken to evaluate the amino acid sequence of CSPB for any similarity with known protein toxins. While the potential toxicity of NPTII has previously been assessed using the bioinformatic approach, an updated search was conducted using more recent versions of protein sequence databases.

CSPB

The FASTA algorithm was used to determine the degree of amino acid sequence alignment between CSPB and proteins contained in the PROTEIN and TOXIN6 databases (see Section 3.4.3 for details). The degree of structural similarity was determined by examining the alignment of the sequences, percent identity and *E*-score. An *E*-score of $\leq 1 \times 10^{-5}$ was set as a threshold at which the alignment could represent significant homology between two sequences.

A total of 5,242 proteins contained in the PROTEIN database had an *E*-score < 1, with 4,291 having an *E*-score < 1×10^{-5}. The majority of these proteins were known or putative cold shock proteins from various bacterial and plant species. The most significant alignment was with a patented stress tolerance protein found in GM plants (100% alignment; *E*-score of 1.8×10^{-26}), while two proteins identical to CSPB were also identified (including CSPB from *B. subtilis*). No significant similarities were detected for any of the 7,176 protein sequences contained in the TOXIN6 database; all had an *E*-score >1. These results indicated that CSPB shares no significant amino acid sequence homology with known protein toxins.

NPTII

An updated bioinformatic analysis was conducted on NPTII using a new toxin sequence database assembled by the Applicant. The database, TOX_2009, is a subset of the most recent (2009) PROTEIN database and comprises 7,651 sequences. No sequence alignments had an *E*-score < 1 or exceeded 35% homology over 80 amino acids. On this basis, it is concluded that NPTII shares no significant structural homology with known or putative protein toxins.

4.4.3 Digestibility

See Section 4.5.3.

4.4.4 Acute oral toxicity study

Proteins which cause toxicity act via acute mechanisms and generally at very low doses (Sjoblad et al 1992). Therefore, when a protein demonstrates no acute oral toxicity at a high dose level using a standard laboratory mammalian test species, this supports the determination that the protein will be non-toxic to humans and other mammals, and will not present a hazard under any realistic exposure scenario, including long-term exposures.

Submitted study:
Smedley JW (2008) An acute toxicity study of cold shock protein B administered by the oral (gavage) route to mice. Lab Study No. EUF00200. Sponsor Study No. CRO-2007-182. Lab: Charles River Laboratories, Spencerville, OH, USA. Sponsor: Monsanto Company, St Louis, MO, USA. Unpublished. [GLP, QA]

As insufficient quantities of CSPB could be purified from MON87460 corn, groups of 10 mice/sex (CD-1 strain; 8 weeks old & 29.8-32.6 g bodyweight; sourced from Charles River Laboratories, St Constant, Quebec, Canada) were administered *E. coli*-derived CSPB (Monsanto Company; Lot No. 10000802; 100% purity) as a single gavage dose of 4.70 mg protein/kg bodyweight (bw). The equivalence of CSPB from *E. coli* and MON87460 corn has previously been established (see Section 4.2.1). It was stated that the dose selection was chosen because it was 3-4 orders of magnitude higher than that expected from human exposure to CSPB. Mice were housed individually under standard conditions. Food and water were available *ad libitum* except 2-3 hours prior to dosing when mice were fasted. A control group of 10 mice/sex was similarly dosed with bovine serum albumin (BSA) (Calbiochem; Lot No. D00019305; >98% purity) at a dose of 3.13 mg protein/kg bw. The concentration, stability and homogeneity of the test and control dosing solutions were established in a separate study (Burzio & Rice 2008).

Mice were observed for 14 days after dosing. Twice daily observations were made for mortalities, with detailed clinical observations made twice on the day of dosing (day 0) and daily thereafter. Bodyweight was recorded prior to fasting, prior to dosing and on day 7 and 14. Food consumption was recorded on day 0, 7 and 14. Survivors were sacrificed on day 14 and necropsied. Bodyweight, bodyweight change and food consumption data were analysed with appropriate statistical tests.

There were no deaths and no treatment-related clinical signs, effects on bodyweight, bodyweight gain or food consumption. There were no treatment-related macroscopic abnormalities detected at necropsy. On the basis of these results, it is concluded that purified CSPB showed no evidence of acute toxicity in mice at a dose of 4.70 mg/kg bw.

4.4.5 Conclusion

The presence of CSPB and NPTII in MON87460 corn poses no toxicological concerns on the basis of the following: it is likely that humans are already exposed to both proteins by virtue of their presence in bacteria commonly encountered in the environment; the presence of NPTII in GM crops has previously been assessed as safe by FSANZ and other regulatory agencies; bioinformatic analyses detected no significant sequence homology between CSPB or NPTII and known protein toxins; and there was no evidence of acute toxicity in mice at a dose of 4.70 mg/kg bw CSPB.

4.5 Potential allergenicity of novel proteins

While the majority of food allergens are proteins, only a small fraction of the many proteins in foods are allergenic and therefore the likelihood of a novel protein being allergenic is small. However, as the use of gene technology can result in additional protein diversity being added to the food supply, the potential allergenicity of any novel protein present in a GM food is considered as part of the safety assessment.

The potential allergenicity of novel proteins is evaluated using an integrated, step-wise, case-by-case approach relying on various criteria used in combination, since no single criterion is sufficiently predictive of either allergenicity or non-allergenicity. The assessment focuses on: the source of the novel protein; any significant amino acid sequence similarity between the novel protein and known allergens; the structural properties of the novel protein, including susceptibility to digestion, heat stability and/or enzymatic treatment; and specific serum screening if the novel protein is derived from a source known to be allergenic or has amino acid sequence similarity with a known allergen. In some cases, such as where the novel protein has sequence similarity to a known allergen, additional *in vitro* and *in vivo* immunological testing may be warranted. Applying this approach systematically provides reasonable evidence about the potential of the novel protein to act as an allergen.

4.5.1 Source of the introduced gene

CSPB

The source of the *cspB* gene, *B. subtilis*, is a ubiquitous soil bacterium widely encountered by humans. Based on its history of food use (see Section 2.1.1), there is no evidence that *B. subtilis* is a food allergen. A search of the Food Allergy Research and Resource Program (FARRP) Protein AllergenOnline database (2009)[1] and the Allergen Database for Food Safety (2009)[2], which contains sequences of known and putative allergens, found no entries for *B. subtilis* or for cold shock proteins.

A search of the published scientific literature identified a number of older journal articles reporting allergic reactions (e.g. asthma, dermatitis) in workers involved in the manufacture of detergents containing the enzyme subtilisin, from *B. subtilis* (reviewed by Chan-Yeung & Malo 1994; US EPA 1997). Such occupational exposure via the respiratory and dermal routes to high levels of detergent dust over prolonged periods of time is not considered relevant to an assessment of food safety. No journal articles were identified relating to the allergenicity of any of the bacterial cold shock proteins.

[1] Available online at http://www.allergenonline.org/
[2] Available online at http://allergen.nihs.go.jp/ADFS/

NPTII

As discussed in Section 4.4.1, FSANZ has previously evaluated the safety of NPTII, including its allergenicity potential. The consolidated conclusion of these assessments is that NPTII is unlikely to be a food allergen on the basis that: there is a history of safe exposure to the protein; it possesses no sequence homology with known or putative allergens; it is heat labile and rapidly digested in mammalian digestive fluid; and is not glycosylated. The allergenicity potential of NPTII has been widely considered by other regulators and independent researchers who concur that there are no human health issues.

4.5.2 Similarity to known allergens

Submitted studies:
Burzio LA, McClain JS & Silvanovich A (2008) Bioinformatics evaluation of the CSPB protein utilizing the AD8, TOXIN6 and PROTEIN databases. Study No. REG-08-091. Report No. MSL-0021261. Lab: Monsanto Company, Regulatory Product Characterization Team, St Louis, MO, USA. Sponsor: Monsanto Company, St Louis, MO, USA. Unpublished. [QA]

Tu H (2009) Updated bioinformatics evaluation of the NPTII protein utilising the AD_2009 and TOX_2009 databases. Study No. REG-09-154. Report No. MSL0021965. Lab & Sponsor: Monsanto Company, Product Characterization Center, St Louis, MO, USA. Unpublished. [QA]

CSPB

Bioinformatic analyses were undertaken to assess CSPB for any amino acid sequence similarity with known or putative allergens. The CSPB amino acid sequence was compared to an allergen, gliadin and glutenin sequence (AD8) database compiled by the Applicant from sequences contained in the FARRP database (2008) (available online at http://www.farrp.org/).

Two different searches against the AD8 Database were performed. Firstly, the database was searched for overall linear sequence identity with CSPB using the FASTA algorithm. Related protein sequences are considered to be potentially cross-reactive if linear identity is 35% or greater in a segment of 80 or more amino acids (FAO/WHO 2001). As CSPB contains only 67 amino acids, none of the sequence alignments would actually meet this criterion. Alignments were also judged based on the *E*-score; an E-score of $\leq 1 \times 10^{-5}$ was set as a threshold at which the alignment could represent significant homology between two sequences. Secondly, searches were performed using a pair-wise comparison algorithm (ALLERGENSEARCH) to identify short amino acid sequences that may represent linear IgE binding epitopes. For this analysis, searches were performed for matches of 8 contiguous amino acids or more.

No significant sequence similarity between CSPB and known or putative allergens in the AD8 Database was identified. Additionally, no short peptide matches, representing putative IgE binding epitopes, were shared between CSPB and proteins in the database.

On the basis of these results, CSPB is considered to possess no amino acid homology of immunological concern with known protein allergens.

NPTII

An updated bioinformatic analysis was conducted on NPTII using a revised allergen database (AD_2009), which contains 1,386 sequences. A FASTA search of this database identified no significant sequence alignments with known or putative allergens and no matches of eight contiguous amino acids. On this basis, NPTII shares no structural or immunological similarity with the protein contained in the AD_2009 database.

4.5.3 In vitro digestibility

Typically, food proteins that are allergenic tend to be stable to enzymes such as pepsin and the acidic conditions of the digestive system, exposing them to the intestinal mucosa and leading to an allergic response (Astwood et al 1996; Metcalfe *et al* 1996). Therefore a correlation exists between resistance to digestion by pepsin and allergenic potential.

As a consequence, one of the criteria for assessing potential allergenicity is to examine the stability of novel proteins in conditions mimicking human digestion. Proteins that are rapidly degraded in such conditions are considered less likely to be involved in eliciting an allergic response.

Submitted study:
Kapadia SA, Goertz BE, Burzio L & Rice EA (2008) Assessment of the *in vitro* digestibility of the cold shock protein B (CSPB) in simulated gastric and simulated intestinal fluids. Study No. REG-07-310. Report No. MSL-0021138. Lab: Monsanto Company, Regulatory Product Characterization Team, St Louis, MO, USA. Sponsor: Monsanto Company, St Louis, MO, USA. Unpublished. [GLP, QA]

SDS-PAGE and Western blotting were used to assess the digestibility of *E. coli*-derived CSPB (Orion Lot No. 10000802; 100% purity; source unspecified) in simulated gastric fluid (SGF) and/or simulated intestinal fluid (SIF). It was stated that CSPB had been characterised by SDS-PAGE (molecular weight = 6.5 kDa) and N-terminal sequencing. The SGF contained the enzyme, pepsin, in an acidic buffer (pH ~1.2), while the SIG contained a mixture of enzymes, called pancreatin, in a neutral buffer. The enzymatic activity of SGF and SIF were verified prior to the commencement of the study.
The digestion of CSPB was analysed at 37ºC under three regimes: (1) In SGF at sampling times of 0, 0.5, 2, 5, 10, 20, 30 and 60 minutes; (2) In SGF at sampling times of 0 and 2 minutes followed by SIF at sampling times of 0, 0.5, 2, 5, 10, 30, 60 and 120 minutes; and (3) In SIF at sampling times of 0, 5, 15 and 30 minutes and at 1, 2, 4, 8, 12 and 24 hours. Control samples were prepared to determine the stability of: CSPB in the SGF and SIF without the respective enzymes present (sampling times of 0 and 60 minutes); and the SGF and SIF without CSPB (sampling times of 0 and 60 minutes).

Samples (test and control) and molecular weight standards were subjected to SDS-PAGE and either stained with colloidal Brilliant Blue G or transferred to nitrocellulose membranes for Western blotting. Blots were probed with a goat polyclonal antibody to CSPB and immunoreactive bands visualised by chemiluminescence. An estimate of the concentration of each band was made based on a densitometric comparison with various dilutions of the SGF and SIF zero time points, which were run concurrently on the gel. The LOD was estimated to be 0.005 µg for SDS-PAGE and 0.1 ng for Western blotting, with these values used to determine the approximate concentration of undigested CSPB.

All control samples gave expected results; i.e. digestion did not occur in the absence of either pepsin or pancreatin, with both enzymes stable in the absence of CSPB.

Both SDS-PAGE and Western blotting indicated that > 99% of full length CSPB was digested within 30 seconds in SGF. However, SDS-PAGE and Brilliant Blue staining detected a transiently stable 2.5 kDa fragment between 30 seconds and 30 minutes, with no fragments detected at 60 minutes. This fragment was not detected by Western blotting. N-terminal sequencing of the 2.5 kDa fragment indicated that it was the N-terminal peptide of CSPB.

Analysis by SDS-PAGE and Brilliant Blue staining of CSPB digested sequentially with SGF followed by SIF indicated that the 2.5 kDa fragment was digested within 30 seconds in SIF. In the absence of pancreatin, the digestion of the 2.5 kDa fragment did not occur. Western blotting confirmed the rapid degradation of CSPB in SGF followed by SIF, with the full length protein only detectable in the 0 minute sample

Western blot analysis indicated that >99% of CSPB was digested in SIF within 5 minutes. The authors did not examine the digestion of CSPB by SIF using SDS-PAGE and Brilliant Blue staining because no stable fragments had been observed during the sequential digestion of CSPB with SGF and SIF.

Conclusion: The results of this experiment suggested that CSPB would be rapidly degraded in the mammalian digestive tract to its constituent amino acids. The very low level (<1%) of a transiently stable 2.5 kDa N-terminal peptide detected at 30 seconds to 30 minutes in SGF is not considered biologically relevant because: (1) digestion was completed between 30-60 minutes; (2) the peptide would not be absorbed from the stomach as the site of amino acid/peptide uptake in mammals is the small intestine (Reviewed by Erickson & Young 1990; Webb 1990); and (3) the peptide was rapidly degraded when CSPB was subjected to sequential treatment with SGF then SIF, which reflects the normal digestive process.

While this experiment examined the *in vitro* digestion of purified CSPB, Schindler et al (1999) demonstrated that the susceptibility of CSPB to *in vitro* trypsin digestion was reduced in the presence of nucleic acid at pH 8.6 and 25°C. However, in the context of the suite of proteases present in the human digestive tract, which have different specificities than trypsin (e.g. pepsin, chymotrypsin, carboxypeptidase), it is highly unlikely that CSPB:nucleic acid complexes could evade digestion. The acidic conditions of the stomach are also likely to promote the dissociation of CSPB:nucleic acid complexes, which are held together non-specifically.

Humans would already be exposed to a variety of CSD-containing proteins in the diet in both the complexed and uncomplexed form, including CSPB, and these would be subject to the same digestive processes as all dietary proteins and nucleic acids. With the exception of the single amino acid substitution at the N-terminus, the sequence of MON87460-derived CSPB is identical to that of the source organism (*B. subtilis*) and therefore its binding to single stranded nucleic acid (via the CSD) would be no more stable (or unstable) to digestion, cooking or processing. On this basis there is no *a priori* reason to consider that CSPB:nucleic acid complexes derived from MON87460 corn would behave any differently to CSPB:nucleic acid complexes already encountered in the diet.

4.5.4 Heat stability

No data were provided on the heat stability of CSPB. As discussed in Section 4.1.1, CSPB is conformationally unstable at 37ºC and as a consequence is highly susceptible to protease degradation (Schindler et al 1999). On this basis it is a reasonable expectation that any processing operations that subject MON87460 corn or corn products to relatively high temperatures would rapidly denature the protein, including complexes with RNA/DNA.

4.5.5 Conclusion on potential allergenicity

There is a history of exposure to *B. subtilis*, which is not recognised as allergenic. While there are some reports of occupational allergy to certain *B. subtilis*-derived proteins, there is no evidence that CSPB or cold shock proteins in general are allergenic. Indeed the widespread presence of CSD-containing proteins in plants would suggest that humans have a history of dietary exposure to similar proteins. A comprehensive bioinformatic analysis indicated that CSPB possesses no structural or immunological similarity to known protein allergens. In addition, CSPB is rapidly digested in simulated gastric and intestinal fluid, and is likely to be labile at high temperatures. The allergenicity potential of NPTII has previously been assessed by FSANZ and other overseas regulatory agencies. An updated bioinformatic analysis confirmed the absence of allergenicity potential for NPTII and on this basis its presence in MON87460 raises no safety concerns.

The weight-of-evidence indicates that the two novel proteins in MON87460 corn are unlikely to be allergenic when present in foods.

4.6 Conclusion from studies on the novel proteins

A variety of experiments and analyses were undertaken to determine the physicochemical properties, concentration, toxicity and allergenicity of the two novel proteins present in MON87460 corn.

CSPB is present in MON87460 corn grain at a mean concentration of 0.041 and 0.33 µg/g fresh weight under well-watered and water-limited conditions, respectively. CSPB from MON87460 corn conforms in size and amino acid sequence to that expected, is immunoreactive to antibodies to CSPB, is not glycosylated and exhibits the expected functional activity. MON87460-derived CSPB was shown to be equivalent to the *E. coli* derived protein. Bioinformatic analyses of CSPB confirmed the absence of any biologically significant amino acid sequence similarity to known protein toxins or allergens and digestibility studies demonstrated that it would be rapidly degraded in the digestive tract following ingestion, similar to other dietary proteins. An acute oral toxicity study in mice using *E. coli*-derived CSPB also confirmed the absence of toxicity. The safety of the CSPB protein is further supported by the long history of safe dietary exposure to this protein and its source organism. Taken together, the evidence indicates that CSPB is unlikely to be toxic or allergenic to humans.

NPTII is expressed at relatively low levels in MON87460 corn grain, with the mean concentration below the LOQ of 0.0047 µg/g fresh weight. There is an extensive database on NPTII given its history of use in the production of GM crops. To make use of this database, the equivalence of NPTII from MON87460 corn leaves to that derived from *E. coli* was demonstrated using Western blotting. An updated bioinformatic analysis confirmed that NPTII has no biologically significant sequence homology with known toxins or allergens. Based on its history of safe use and these confirmatory studies, the presence of NPTII in food derived from MON87460 corn is unlikely to pose any safety concerns.

5. COMPOSITIONAL ANALYSES

The main purpose of compositional analysis is to determine if any unexpected changes in composition have occurred to the food and to establish its nutritional adequacy. Compositional analysis can also be important for evaluating the intended effect where there has been a deliberate change to the composition of food.

The classic approach to the compositional analysis of GM food is a targeted one; rather than analysing every single constituent, which would be impractical, the aim is to analyse only those constituents most relevant to the safety of the food or that may have an impact on the whole diet. Important analytes therefore include the key nutrients, toxicants and anti-nutrients for the food in question (FAO 1996). The key nutrients and anti-nutrients are those components in a particular food that may have a substantial impact in the overall diet. They may be major constituents (fats, proteins, carbohydrates or enzyme inhibitors as anti-nutrients) or minor constituents (minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in an organism, such as compounds whose toxic potency and level may be significant to health (e.g. solanine in potatoes).

In the case of corn, the key components that should be considered in the comparison include protein, fat, carbohydrate, amino acids, fatty acids, vitamins, minerals, and the anti-nutrient phytic acid (OECD 2002).

Submitted study:
Harrigan GG, Miller KD & Sorbet R (2008) Amended report for MSL0021180: Compositional analyses of forage and grain collected from drought tolerant corn MON87460 grown in a 2006/2007 Chile field production. Study No. REG-07-212. Report No. MSL-0021754. Labs: Monsanto Company, Product Safety Center, St Louis, MO, USA; Covance Laboratories Inc, Madison, WI, USA; Certus International Inc, Chesterfield, MO, USA. Sponsor: Monsanto Company, St Louis, MO, USA. Unpublished. [GLP, QA]

5.1 Study design

MON87460 corn plants were cultivated at four sites in Chile during the 2006-2007 growing season under well-watered and water-limited (i.e. drought) conditions (see Section 4.3.1 for full details). The conventional isogenic control corn line, DM1718[3], and 16 different commercial corn hybrids, were also grown at the four sites (see Table 4 for details). The latter were used to assess the validity of the two water treatment regimes and to establish 99% tolerance intervals for the analytes. Forage and grain samples were harvested at an unspecified time and analysed for the constituents listed in Table 8. The composition of MON87460 corn was compared to that of DM1718 corn on an individual site and combined site basis, noting that one of the sites (QUI) was excluded from the combined site analysis because the reference corn varieties failed to show the necessary response to water-limited conditions (refer to Section 4.3.1).

Table 8: Analytes measured in forage and grain from MON87460 corn

Analytes	Forage	Grain
Proximates (protein, carbohydrate, fat, ash & moisture)	Measured	Measured
Fibre (ADF, NDF & TDF)	ADF & NDF only	Measured
Minerals (Ca, Cu, Fe, Mg, P, K, Na & Zn)	Ca & P only	Measured
Vitamins (B1, B2, B6, E, niacin & folic acid)	Not measured	Measured
Amino acids (all)	Not measured	Measured
Fatty acids (C8-C22)	Not measured	Measured
Antinutrients & secondary metabolites (furfural, raffinose, phytic acid, *p*-coumaric acid & ferulic acid)	Not measured	Measured

ADF = acid detergent fibre; NDF = neutral detergent fibre; TDF = total dietary fibre

Results were statistically analysed using a mixed model analysis of variance (AVOVA), with the composition of forage and grain from MON87460 corn grown under well-watered and water-limited conditions compared to the respective controls. Where a statistical difference ($p<0.05$) was identified, means were then compared to the 99% tolerance interval and the published literature and International Life Sciences Institute (ILSI) ranges for conventional corn. Data excluded from the analysis included extreme outliers and analytes having 50% of their values below the LOQ, which included 14 fatty acids, Na and furfural (i.e. 16 of the 77 constituents analysed). A number of analytes in different samples from test, control and reference corn grown at QUI were below the LOQ but were assigned a value of half the LOQ (total fat, raffinose, 22: behenic acid & vitamin E).

[3] DM1718 is a conventional isogenic control and not a null segregant. It was generated by crossing two conventional inbred lines, LH59 and 01DKD2, the latter was crossed with MON87460 corn during the breeding process (Figure 4).

A number of statistically significant differences ($p<0.05$) were calculated between MON87460 corn and the control under well-watered and/or water-limited conditions on an individual and/or combined site basis. An evaluation of these differences proceeds below, but in summary, none are considered biologically significant because the concentration of each constituent was within the 99% tolerance interval established for each site in addition to published literature [Jugenheimer (1976); Watson (1982); Watson (1987); Classen et al (1990); Dowd & Vega (1996); Choi et al (1999); Sidhu et al (2000); OECD (2002); Ridley et al (2002)] and ILSI (2008) ranges for conventional corn. There was also no consistency between sites or between the individual and combined site analyses.

5.2 Forage

Detailed results from the forage analyses were evaluated for the safety assessment but are not presented here. As forage is not consumed by humans the focus of the assessment is primarily on the results obtained from analysis of the grain samples.

Overall, the results of the forage analyses are consistent with those from grain, and do not indicate any significant compositional differences between MON87460 corn and conventional corn grown under the same agricultural conditions.

5.3 Grain

Proximates

There was no significant difference in the level of protein, carbohydrates, ash and moisture in grain from MON87460 corn compared to that from conventional (control) corn. The only significant difference was in the level of total fat in grain harvested from MON87460 corn grown under well-watered conditions at LUM ($p=0.010$) (Table 9). The combined site analysis of this constituent also established a significant difference to the control ($p=0.029$). However, given that a similar difference did not occur with grain harvested at the other three sites or with grain harvested from corn grown under water-limited conditions at any of the sites, and that the levels fell within the 99% TI and published/ILSI ranges (given in Table 9), this statistically significant difference in total fat is not considered biologically significant.

Minerals

There was no significant difference in the level of Ca, Cu, Mn, K, Na and Zn in grain from MON87460 corn compared to that from conventional (control) corn grown at the four sites.

Fe and P levels in grain from MON87460 corn grown under water-limited conditions at CT were significantly lower than the control ($p=0.046$ & 0.027, respectively). There was no such difference at any of the other three sites or in the combined site analysis. The level of Mg in grain from MON87460 corn grown under well-watered conditions at LUM was significantly higher than the control ($p=0.046$); no such difference occurred in grain from any of the other three sites. The combined site analysis also established a significantly higher level of Mg in grain from MON87460 corn relative to the control under well-watered conditions ($p=0.012$).

Given the lack of consistency across sites and that levels of all analytes fell within the respective 99% tolerance interval for each site and the published/ILSI ranges (given in Table 9), none of these statistically significant differences are considered biologically significant.

Vitamins

There was no significant difference in the level of vitamins B1, B2, B6 and niacin in grain from MON87460 corn compared to that from conventional corn grown at the four sites. The combined site analysis found no significant difference in the levels of any of the measured vitamins between test and control grain.

Vitamin E was significantly elevated in MON87460 corn grown at CL under both well-watered and water limited conditions relative to the control (p=0.002 & 0.001, respectively; Table 9). However, this statistical difference was not considered biologically significant as the levels fell within the 99% tolerance interval for the site and the published/ILSI ranges (given in Table 9), and was not observed for any of the other sites. The level of folic acid in grain from MON87460 corn grown at CT under water-limited conditions was significantly higher than the control (p=0.046) but again the level was within the 99% tolerance interval and reference ranges.

Amino acids

The only differences in amino acid levels between test and control corn were increased serine (p=0.025) and threonine (p=0.047) in grain from MON87460 corn grown under well-watered conditions at LUM. As the levels fell within the 99% tolerance interval for this site and the published/ILSI ranges (given in Table 9), and was not observed at the same site under water-limited conditions, or at the other three sites or following the combined site analysis, these differences are not considered biologically significant.

Fatty acids

The levels of several fatty acids in grain from MON87460 corn were significantly different to the control at three sites but displayed no consistency across the sites or between the two treatment regimes (Table 9). At CL, MON87460 grain had a significantly higher (p=0.029) level of 22:0 behenic acid compared to the control when grown under water-limited conditions. At CT, significantly elevated (p=0.048) 18:2 linoleic acid occurred under well-watered conditions, while 20:1 ecosenoic acid was significantly lower (p=0.016) under water-limited conditions. At LUM, the following significant differences were noted: increased 18:0 stearic acid under well-watered conditions (p=0.024); decreased 18:1 oleic acid under water-limited conditions (p=0.030); and decreased 18:3 linolenic acid under well-watered conditions (p=0.042). Given that the levels of all fatty acids analysed were within the 99% tolerance interval established for each site and treatment, and that the levels were within the published reference and ILSI ranges, none of these statistically significant differences are considered biologically meaningful.

For the combined site analysis, there was a significant difference (p=0.042) in 20:1 ecosenoic acid between test and control grain under water-limited conditions. Given that the mean levels of this fatty acid were identical and fell within the 99% TI and reference/ILSI ranges, this difference is considered incidental in nature.

Anti-nutrients and secondary plant metabolites

Corn contains a number of anti-nutrients: phytic acid, raffinose, 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA) and trypsin and chymotrypsin inhibitor. Only phytic acid and raffinose are considered to be biologically relevant (OECD 2002). DIMBOA is present at highly variable levels in corn hybrids and little evidence is available on either its toxicity or anti-nutritional effects and corn contains only low levels of trypsin and chymotrypsin inhibitor, neither of which is considered nutritionally significant.

Phytic acid is considered an important anti-nutrient for animals, especially non-ruminants, since it can significantly reduce the bioavailability of phosphorus in the plant. Feed formulators add the enzyme phytase to pig and poultry diets to improve the utilisation of phosphorus. Raffinose is a non-digestible oligosaccharide and is considered an anti-nutrient because of its gastrointestinal effects (flatulence).

Secondary metabolites are defined as those natural products which do not function directly in the primary biochemical activities which support the growth, development and reproduction of the organism in which they occur. Secondary plant metabolites are neither nutrients nor anti-nutrients but are sometimes analysed as further indicators of the absence of unintended effects of the genetic modification on metabolism (OECD 2002). Characteristic metabolites in corn are furfural and the phenolic acids, ferulic acid and p-coumaric acid.

There are no generally recognised anti-nutrients in corn at levels considered to be harmful, but for the purposes of comparative assessment, the OECD has recommended considering analytical data for the content of the anti-nutrients phytic acid and raffinose, and the secondary metabolites furfural, ferulic acid, p-coumaric acid.

There was no significant difference in the level of raffinose, ferulic acid or p-coumaric acid between test and control corn grain. The level of phytic acid was significantly higher ($p=0.012$) in MON87460 corn grain than the control when grown under water limited conditions at CL (Table 9). No such difference was established at any of the three other sites or following the combined site analysis. As the level of phytic acid in MON87460 corn grain was within the 99% tolerance interval for reference corn grown concomitantly at CT, and as the level fell within the published and ILSI ranges for phytic acid, this significant difference was not considered biologically meaningful.

5.4 Conclusion

The compositional analyses do not indicate any compositional differences of biological significance in forage or grain from MON87460 corn compared to the non-GM control. Several statistically significant differences in key nutrients and other constituents were noted, however, the mean levels observed were within the 99% tolerance interval derived from four reference varieties grown at each site in addition to literature ranges. Therefore, all the levels of constituents noted for MON87460 corn are considered to be within the range of natural variation for corn and indicate that the introduction of the *cspB* and *nptII* genes in to MON87460 corn caused no unintended compositional changes. On this basis it can be concluded that MON87460 corn is equivalent in composition to the conventional corn.

The results of this study have subsequently been published in the scientific literature (Harrigan *et al* 2009). This paper also describes an additional 2006 US field trial where MON87460 was cultivated under typical agricultural conditions (i.e. well-watered) only. The results of this US trial established the compositional equivalence of grain from MON87460 corn to that of the control and several commercial reference varieties.

Table 9: Summary of significant compositional differences in grain between MON87460 and conventional corn

SIte	Analyte	*WELL-WATERED*			*WATER-LIMITED*			Literature range	ILSI range
		MON87460	CONTROL	99% TI	MON87460	CONTROL	99% TI		
CL	Vitamin E[1]	12.46±0.46**	10.50±0.46	0, 19.32	13.34±0.46**	11.16±0.46	0, 22.61	3-12.1[3]; 17-47[4]	1.5-68.7
	22:0 Behenic[2]	0.14±0.019	0.15±0.019	0, 0.48	0.17±0.019*	0.11±0.019	0, 0.32	No data	0.110-0.349
	Phytic acid	0.73±0.047	0.78±0.047	0.28, 1.15	0.87±0.047*	0.69±0.047	0.40, 1.12	0.48-1.12[5]	0.111-1.570
CT	Fe[1]	16.86±0.47	17.37±0.47	10.40, 23.42	17.61±0.47*	18.81±0.47	7.05, 30.38	1-100[3 & 4]	10.42-49.07
	P	0.31±0.0089	0.31±0.0089	0.21, 0.40	0.32±0.0089*	0.35±0.0089	0.25, 0.42	0.26-0.75[3 & 4]	0.1470-0.5330[6]
	18:2 Linoleic[2]	64.64±0.28*	63.82±0.28	49.61, 73.18	64.39±0.28	64.41±0.28	50.63, 72.71	35-70[3]	36.2-66.5
	20:1 Eicosenoic[2]	0.18±0.0047	0.18±0.0047	0.10, 0.36	0.17±0.0047*	0.18±0.0047	0.11, 0.34	No data	0.170-1.917
	Folic acid[2]	0.26±0.017	0.27±0.017	0.11, 0.55	0.30±0.017*	0.25±0.017	0.098, 0.58	0.3[4]	0.0147-0.1464
LUM	Total fat	3.96±0.087*	3.61±0.087	2.47, 4.68	3.97±0.087	3.96±0.087	2.07, 5.10	0.35-3.62[7]; 1.24-4.57[5]	0.296-4.570
	Mg	0.13±0.0048*	0.11±0.0048	0.064, 0.16	0.13±0.0048	0.13±0.0048	0.083, 0.16	0.09-1[3 & 4]	0.0594-0.1940[6]
	Serine	0.50±0.017*	0.44±0.017	0.32, 0.65	0.51±0.017	0.50±0.017	0.36, 0.71	0.37-0.91[8]	0.235-0.7699[6]
	Threonine	0.35±0.011*	0.32±0.011	0.23, 0.42	0.36±0.011	0.35±0.011	0.28, 0.44	0.27-0.49	0.224-0.666[6]
	18:0 Stearic[2]	1.83±0.035*	1.71±0.035	0.60, 2.58	1.79±0.035	1.89±0.035	0.71, 2.57	1-3[3]	1.02-3.40
	18:1 Oleic[2]	20.63±0.015	20.21±0.015	12.40, 36.28	20.38±0.15*	20.89±0.15	12.15, 35.55	20-46[3]	17.4-40.2
	18:3 linolenic[2]	1.20±0.013*	1.23±0.013	0.72, 1.66	1.21±0.013	1.22±0.013	0.67, 1.76	0.8-2[3]	0.57-2.25
Combined	Total fat	3.89±0.082*	3.72±0.082	2.47, 4.68	4.02±0.082	3.96±0.082	2.07, 5.10	0.35-3.62[7]; 1.24-4.57[5]	0.296-4.570
	Mg	0.12±0.0034*	0.11±0.0034	0.064, 0.16	0.13±0.0034	0.13±0.0034	0.083, 0.16	0.09-1[3 & 4]	0.0594-0.1940[6]
	20:1 eicosenoic[2]	0.18±0.0039	0.19±0.0039	0.10, 0.36	0.18±0.0039*	0.18±0.0039	0.11, 0.34	No data	0.170-1.917

Results expressed as the mean ± one standard error (SE) [% dry weight (dw)] unless indicated otherwise; Comb = combined sites (CL + CT + LUM); TI = tolerance interval; * p<0.05; ** p<0.01; ***p<0.001

1 = mg/kg dry weight; 2 = % total fatty acid; 3 = Watson (1982); 4 = Watson (1987); 5 = Ridley et al (2002); 6 = converted from mg/kg dw or mg/g dw to % dw; 7 = Sidhu et al (2000); 8 = OECD (2002)

6. NUTRITIONAL IMPACT

In assessing the safety of a GM food, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and well being. In most cases, this can be achieved through an understanding of the genetic modification and its consequences, together with an extensive compositional analysis of the food.

Where a GM food has been shown to be compositionally equivalent to conventional varieties, the evidence to date indicates that feeding studies using target livestock species will add little to the safety assessment and generally are not warranted (OECD 2003).

If the compositional analysis indicates biologically significant changes to the levels of certain nutrients in the GM food, additional nutritional assessment should be undertaken to assess the consequences of the changes and determine whether nutrient intakes are likely to be altered by the introduction of such foods into the food supply. This assessment should include consideration of the bioavailability of the modified nutrient.

In this case, MON87460 corn is the result of a simple genetic modification to confer drought-tolerance with no intention to significantly alter nutritional parameters in the food. In addition, extensive compositional analyses have been undertaken to demonstrate the nutritional adequacy of MON87460 corn and these indicate it is equivalent in composition to grain from conventional corn hybrids. The introduction of MON87460 corn into the food supply is therefore expected to have little nutritional impact.

7. CONCLUSIONS

On the basis of the available evidence, including detailed studies provided by the Applicant, food derived from drought-tolerant MON87460 corn is considered as safe and wholesome as food derived from other commercial corn varieties.

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