Supporting document 1

Safety Assessment Report (at Approval) – Application A1112

Food derived from Herbicide-tolerant Corn Line MZHG0JG

Summary and conclusions

Background

A genetically modified (GM) corn line with OECD Unique Identifier SYN-000JG-2 (henceforth referred to as MZHG0JG) has been developed by Syngenta. The corn has been modified to be tolerant to the herbicides glyphosate and glufosinate ammonium (glufosinate).

Tolerance to glyphosate is achieved through expression of the enzyme 5-enolpyruvyl-3-shikimatephosphate synthase (mEPSPS) encoded by a modified *epsps* gene (*mepsps-02,* hereafter referred to just as *mepsps*) derived from corn (*Zea mays*). Functionally similar EPSPS proteins have been considered by FSANZ in 18 previous applications and an identical EPSPS protein has been considered in four previous applications.

Tolerance to glufosinate is achieved through expression of the enzyme phosphinothricin acetyltransferase (PAT) encoded by the *pat-09* gene (hereafter referred to just as *pat*) derived from the common soil bacterium *Streptomyces viridochromogenes.* This protein has been considered in 20 previous FSANZ applications and globally is represented in six major crop species and over 30 approved GM single plant events.

In conducting a safety assessment of food derived from MZHG0JG, a number of criteria have been addressed including: a characterisation of the transferred gene sequences, their origin, function and stability in the corn genome; the changes at the level of DNA, and protein in the whole food; compositional analyses; and evaluation of intended and unintended changes.

This safety assessment report addresses only food safety and nutritional issues of the GM food *per se*. It therefore does not address:

- environmental 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
- the safety of food derived from the non-GM (conventional) plant.

History of Use

In terms of production, corn is the world's dominant cereal crop, ahead of wheat and rice and is grown in over 160 countries. It has a long history of safe use in the food supply. Sweet corn is consumed directly while corn-derived products are routinely used in a large number and diverse range of foods (e.g. cornflour, starch products, breakfast cereals and high fructose corn syrup). Corn is also widely used as a feed for domestic livestock.

Molecular Characterisation

MZHG0JG was generated through *Agrobacterium*-mediated transformation and contains two expression cassettes. Comprehensive molecular analyses indicate there is a single insertion site comprising a single, complete copy of each of the *mepsps*, and *pat* genes together with their regulatory elements. The introduced genetic elements are stably inherited from one generation to the next. There are no antibiotic resistance marker genes present in the line and no plasmid backbone has been incorporated into the transgenic locus.

Characterisation and safety assessment of new substances

Newly expressed proteins

Corn line MZHG0JG expresses two new proteins, mEPSPS and PAT. The mean levels of both proteins in both herbicide-sprayed and non-sprayed plants were highest in leaves at the R1 stage and lowest (bordering on undetectable) in senescent leaves and the pollen. For PAT, mean levels were also equally low in the whole plant at R6 and grain at both stages. The mean level of mEPSPS in the grain at harvest maturity (stage R6) was approximately 60 µg/g dw.

A range of characterisation studies confirmed the identity of the mEPSPS and PAT proteins produced in MZHG0JG and also their equivalence with the corresponding proteins produced in a bacterial expression system. The plant-expressed mEPSPS and PAT proteins have the expected molecular weights, immunoreactivity, lack of glycosylation, amino acid sequence and enzyme activity.

There are no concerns regarding the potential toxicity or allergenicity of the expressed proteins. Previous safety assessments of both mEPSPS and PAT indicate that the proteins would be rapidly degraded in the stomach following ingestion and would be inactivated by heating. Additionally, updated bioinformatic studies considered in this assessment confirm the lack of any significant amino acid sequence similarity to known protein toxins or allergens.

Herbicide Metabolites

The spraying of line MZHG0JG with glyphosate and/or glufosinate does not result in the production of any novel metabolites that have not been previously assessed.

Compositional Analyses

Detailed compositional analyses were done to establish the nutritional adequacy of grain from MZHG0JG and to characterise any unintended compositional changes. Analyses were done of proximates, fibre, minerals, amino acids, fatty acids, vitamins, secondary metabolites and anti-nutrients in grain taken from MZHG0JG given two treatments (herbicide-sprayed and unsprayed). The levels were compared to levels in a) an appropriate non-GM hybrid line, N2319 x N2222 b) a reference range compiled from results taken for six non-GM hybrid lines grown under the same conditions and c) levels recorded in the literature.
Only 16 of the 58 analytes that were reported deviated from the control in a statistically significant manner; for five of these the difference occurred only in one of the MZHG0JG treatments. However, the mean levels of all of these analytes fell within both the reference range and the historical range from the literature. It is also noted that, with the exception of vitamin A, the differences between these statistically significant analytes means of MZHG0JG and the control means were smaller than the variation within the control.

It can therefore be concluded that grain from MZHG0JG is compositionally equivalent to grain from conventional corn varieties.

Conclusion

No potential public health and safety concerns have been identified in the assessment of MZHG0JG. On the basis of the data provided in the present Application, and other available information, food derived from MZHG0JH is considered to be as safe for human consumption as food derived from conventional corn varieties.

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List of Abbreviations

ADF	acid detergent fibre
ai/ha	active ingredient per hectare
BLAST	Basic Local Alignment Search Tool
bp	base pairs
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
CaMV	Cauliflower mosaic virus
CFIA	Canadian Food Inspection Agency
CTP	Chloroplast transit peptide
DNA	deoxyribonucleic acid
T-DNA	transferred DNA
dw	dry weight
ELISA	enzyme linked immunosorbent assay
FAO	Food and Agriculture Organization of the United Nations
FMV	Figwort mosaic virus
FSANZ	Food Standards Australia New Zealand
GM	genetically modified
kDa	kilo Dalton
LB	Left Border of T-DNA
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantitation
NDF	neutral detergent fibre
OECD	Organisation for Economic Co-operation and Development
OGTR	Australian Government Office of the Gene Technology Regulator
ORF	open reading frame
PAT	phosphinothricin acetyltransferase
PCR	polymerase chain reaction
PPT	phosphinothricin
PVDF	polyvinylidene fluoride
P or P-value	probability value
RB	Right Border of T-DNA
RNA	ribonucleic acid
mRNA	messenger RNA
SAS	Statistical Analysis Software
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TMV	Tobacco mosaic virus
U.S.	United States of America
USDA	United States Department of Agriculture

1   Introduction

Syngenta Australia Pty Ltd, on behalf of Syngenta Crop Protection LLC, has submitted an application to FSANZ to vary Schedule 26 in the *Australia New Zealand Food Standards Code* (the Code) to include food from a new genetically modified (GM) corn line, MZHG0JG, with OECD Unique Identifier SYN-000JG-2 (henceforth referred to as MZHG0JG). The corn has been modified to be tolerant to the herbicides glyphosate and glufosinate ammonium (glufosinate).

Tolerance to glyphosate is achieved through expression of the enzyme 5-enolpyruvyl-3-shikimatephosphate synthase (mEPSPS) encoded by a modified *epsps* gene (*mepsps-02,* hereafter referred to as *mepsps*) derived from corn (*Zea mays).* FSANZ has considered an identical EPSPS (designated mEPSPS or 2mEPSPS) in four previous applications. The applicant has specifically nominated corn line GA21 (Spencer et al. 2000), considered in Application A362 (FSANZ 2000a) as expressing the same mEPSPS protein as found in MZHG0JG.

Tolerance to glufosinate is achieved through expression of the enzyme phosphinothricin acetyltransferase (PAT) encoded by the *pat-09* gene (hereafter referred to as *pat*) derived from the common soil bacterium *Streptomyces viridochromogenes.* This protein has been considered in 20 previous FSANZ applications and globally is represented in six major crop species and over 30 approved GM single plant events[1]. The applicant has specifically nominated corn line Bt11, considered in Application A386 (FSANZ 2001), as expressing the same PAT protein as found in MZHG0JG.

The Applicant states that MZHG0JG will offer growers increased flexibility in weed control and that, with dual modes of herbicide tolerance at a single locus, it will also be of value in conventional breeding by permitting a reduction in the time in which these traits can be combined with other valuable traits via the conventional crossing of MZHG0JG with other elite genotypes.

The Applicant states the intention is that any lines containing the MZHG0JG event will be grown initially in the United States of America (U.S.) and Canada. Approval for cultivation in Australia or New Zealand is not being sought; therefore, if approved, food derived from this line may enter the Australian and New Zealand food supply as imported food products.

2   History of use

2.1   Host and donor organism

Mature corn (*Zea mays*) plants contain both female and male flowers and usually reproduce sexually by wind-pollination. This provides for both self-pollination and natural out-crossing between plants, both of which are undesirable since the random nature of the crossing leads to lower yields (CFIA 1994). The commercial production of corn now utilises controlled cross-pollination of two inbred lines (using conventional techniques) to combine desired genetic traits and produce hybrid varieties known to be superior to open-pollinated varieties in terms of their agronomic characteristics.

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[1] FAO GM Foods Platform - http://www.fao.org/food/food-safety-quality/gm-foods-platform/browse-information-by/commodity/en/

This inbred-hybrid concept and resulting yield response is the basis of the modern corn seed industry and hybrid corn varieties are used in most developed countries for consistency of performance and production.

In terms of production, corn is the world's dominant cereal crop (2015 forecast = 1,007 MT[2]) ahead of wheat (723 MT) and rice (499 MT) and is grown in over 160 countries (FAOSTAT3 2015). In 2013, worldwide production of corn was over 1 billion tonnes, with the United States and China being the major producers (~353 and 217 million tonnes, respectively) (FAOSTAT3 2015). Corn is not a major crop in Australia or New Zealand and in 2013, production was approximately 506,000 and 201,000 tonnes respectively (FAOSTAT3 2015). In the U.S. it is estimated that approximately 93% of all corn planted is GM[3] while in Canada, the estimate of GM corn is approximately 80% of the total corn[4]. No GM corn is currently grown commercially in Australia or New Zealand.

Domestic production is supplemented by the import of corn grain and corn-based products, the latter of which are used, for example, in breakfast cereals, baking products, extruded confectionery and food coatings. In 2011, Australia and New Zealand imported, respectively, 856 and 5,800 tonnes of corn grain, 10,600 and 306 tonnes of frozen sweet corn and 8,427 and 900 tonnes of otherwise-processed sweet corn (FAOSTAT3 2015). Corn product imports to Australia and New Zealand included 6,050 and 2,096 tonnes respectively of corn flour and 3,455 and 13 tonnes respectively of corn oil (FAOSTAT3 2015). Corn is a major source of crystalline fructose and high fructose corn syrup, both of which are processed from corn-starch. Approximately 3,000 tonnes of crystalline fructose, but negligible high fructose corn syrup, were imported into Australia in 2011 (Green Pool 2012); neither Australia nor New Zealand currently produce fructose (either crystalline or as high fructose corn syrup).

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. There are five main types of corn grown for food:

- Flour – *Zea mays* var. *amylacea*
- Flint – *Z. mays* var. *indurata*
- Dent – *Z. may*s var. *indentata*
- Sweet – *Z. mays* var. saccharata & *Z. mays* var. *rugosa*
- Pop – *Z. mays* var. *everta*

Dent corn is the most commonly grown for grain and silage and is the predominant type grown in the U.S. (OGTR 2008). The parent line that was transformed to give MZHG0JG is an elite Syngenta proprietary conventional inbred corn line designated NP2222 (Delzer 2004) that is responsive to *Agrobacterium*-mediated transformation and regeneration in tissue culture. It is a Stiff-Stalk family[5], yellow dent corn line but could be crossed with other types. Two main grain processing routes are followed for dent corn (White and Pollak 1995):

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[2] FAO Cereal Supply & Demand Brief, http://www.fao.org/worldfoodsituation/csdb/en/
[3] USDA, Economic Research Service, - http://www.ers.usda.gov/data-products/adoption-of-genetically-engineered-crops-in-the-us.aspx
[4] USDA Gain Report, CA14062, 2014 -
http://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Ottawa_Canada_7-14-2014.pdf
[5] Maize inbreds have a complex history, having been derived from multiple open-pollinated varieties and crosses among the inbreds themselves. In the late 1950s, there was the inception of three so-called heterotic groups which today constitute genetically distinct breeding pools providing superior hybrid performance. Iowa Stiff Stalk Synthetic was one of the groups (Lebrun et al. 2003; van Heerwaarden et al. 2012).

- Dry milling that gives rise to food by-products such as flour and hominy grits.
- Wet milling (CRA 2006), that involves steeping the grain, coarse and fine grinding, centrifugation and evaporating the steep, to yield food by-products such as starch (for cornstarch, corn syrup and individual sweeteners such as dextrose and fructose) and germ (for oil) – see Figure 1. Corn products are used widely in processed foods.
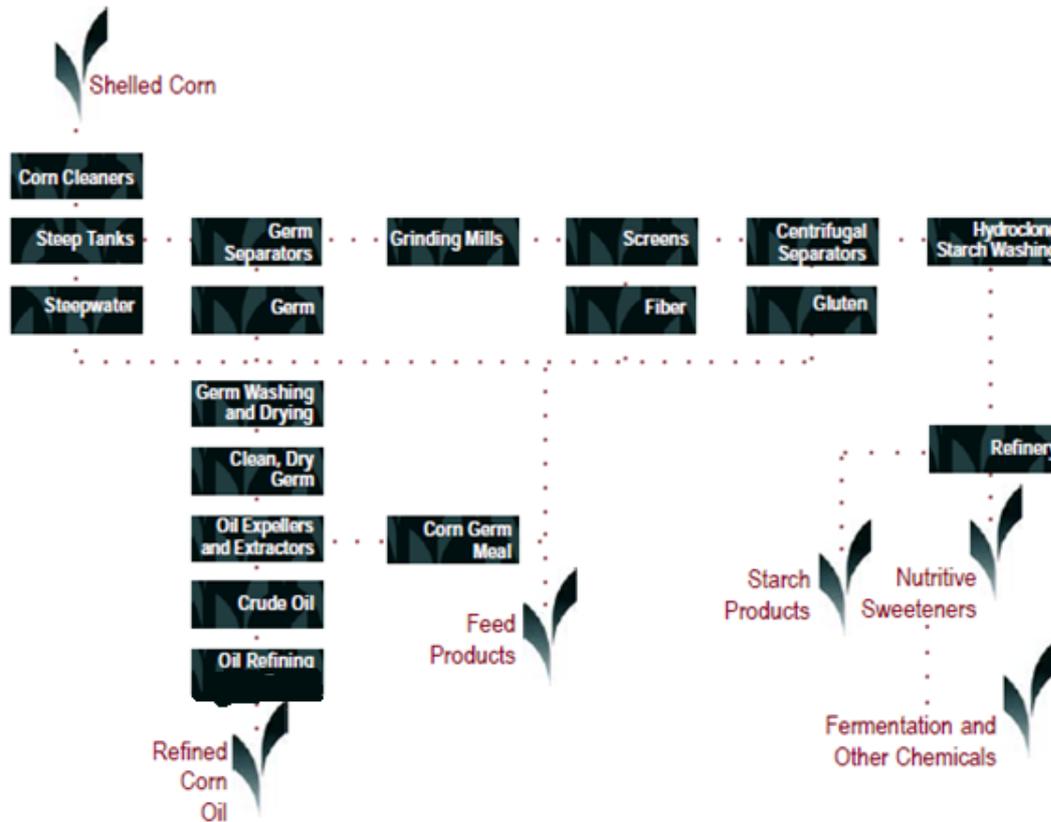
Figure 1: The corn wet milling process (diagram taken from CRA (2006))

The *mepsps* gene used to confer glyphosate tolerance is modified from an endogenous corn *epsps* gene.

2.2   Other donor organisms

2.2.1   *Streptomyces viridochromogenes*

The source of the *pat* gene is the bacterial species *Streptomyces viridochromogenes*, strain Tü494 (Wohlleben et al. 1988). The *Streptomycetae* bacteria were first described in the early 1900's. These organisms are generally soil-borne, although they may also be isolated from water. They are not typically pathogenic to animals including humans, and few species have been shown to be phytopathogenic (Kützner 1981; Bradbury 1986).

Although these organisms are not used in the food industry, the *pat* gene from S. *viridochromogenes*, has been used to confer glufosinate ammonium-tolerance in a range of food producing crops. The *bar* gene from the closely related *S. hygroscopicus* produces a protein that is structurally and functionally equivalent to the protein encoded by the *pat* gene (Wehrmann et al. 1996) and has similarly been used widely for genetic modification of crop species.

2.2.2   Other organisms

Genetic elements from several other organisms have been used in the genetic modification of MZHG0JG (refer to Table 1). These non-coding sequences are used to drive, enhance or terminate expression of the novel genetic material. None of the sources of these genetic elements is associated with toxic or allergenic responses in humans. The genetic elements derived from the plant pathogens Figwort mosaic virus (FMV), Cauliflower mosaic virus (CaMV), Tobacco mosaic virus (TMV) and *Agrobacterium tumefaciens* are not pathogenic in themselves and do not cause pathogenic symptoms in MZHG0JG.

3   Molecular characterisation

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
- the genetic stability of the inserted DNA and any accompanying expressed traits.

Studies submitted:

2015. Event MZHG0JG maize: Southern analyses. Final report **TK0062613.** Syngenta Seeds, Inc. (unpublished)

2015. Event MZHG0JG maize: Insert and flanking sequence analysis. Final report **TK0117253.** Syngenta Seeds, Inc. (unpublished)

2015. Event MZHG0JG maize: Genomic insertion site analysis. Final report **TK0062609.** Syngenta Seeds, Inc. (unpublished)

2015. Event MZHG0JG maize: Basic local alignment search tool for translated nucleotides (BLASTX) analyses of maize genomic sequences flanking the insert. Assessment **TK0062616**. Syngenta Seeds, Inc. (unpublished)

2014. Event MZHG0JG maize: Mendelian inheritance analysis. Final report **TK0062603.** Syngenta Seeds, Inc. (unpublished)

3.1   Method used in the genetic modification

Immature embryos from line NP2222 were aseptically removed from 8 – 12 day post-pollination ears and transformed, using a disarmed strain (LBA4404) of *Agrobacterium tumefaciens,* with the T-DNA from plasmid vector pSYN18857(see Figure 2) following the method of Negrotto et al (2000).

After co-culturing with the *Agrobacterium* carrying the vector, the embryos were placed on selection medium containing ticarcillin and silver nitrate, to inhibit the growth of excess *Agrobacterium*, and to permit the development of callus tissue. Resulting callus was then placed in a medium that contained glufosinate as a selection agent and potentially transformed callus was then transferred to a medium that supported plantlet regeneration and also contained cefotaxime in order to ensure that *A. tumefaciens* was cleared from the transformed tissue. Rooted plants (generation T_0) with normal phenotypic characteristics, containing both the glyphosate (*mepsps*) and glufosinate (*pat*) expression cassettes and not containing the spectinomycin/streptomycin resistance gene (*aadA*) (Fling et al. 1985) from the plasmid backbone - as shown by polymerase chain reaction (PCR) - were selected for further assessment and development (see Section 3.3). MZHG0JG was ultimately chosen as the lead event based on superior agronomic, phenotypic and molecular characteristics.
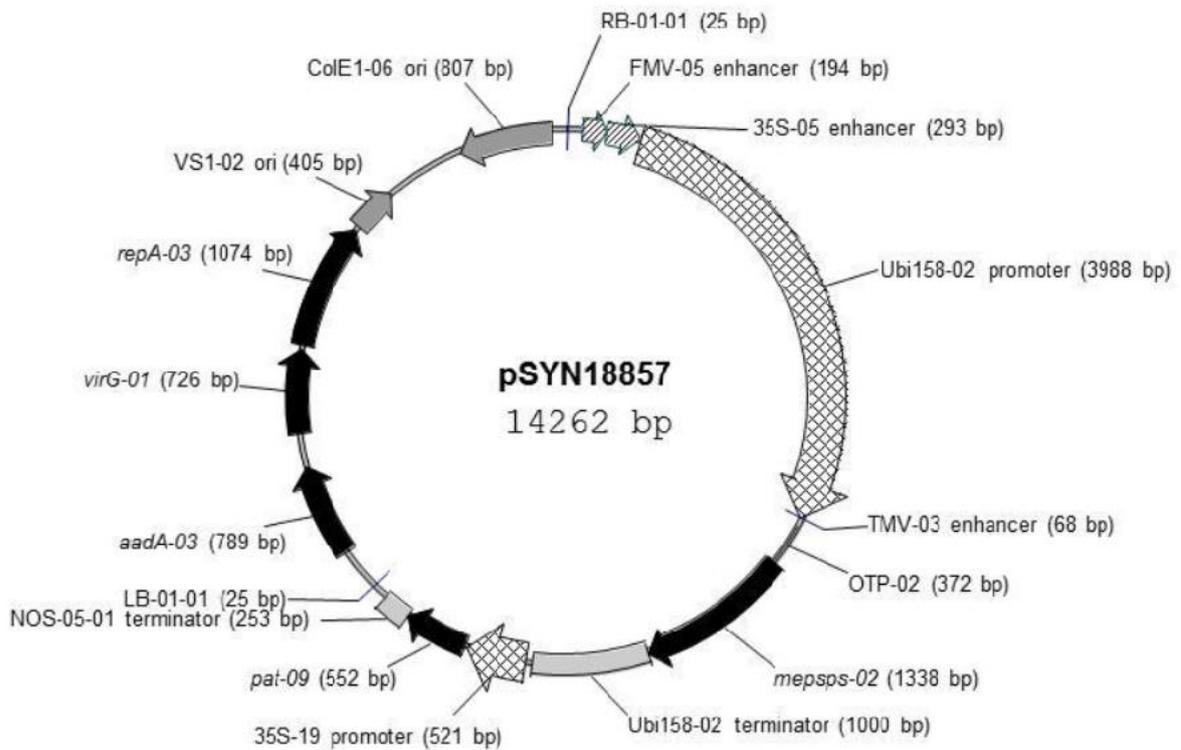
Figure 2: Genes and regulatory elements contained in plasmid pSYN18857

3.2   Function and regulation of introduced genes

Information on the genetic elements in the T-DNA used for transformation is summarised in

Table *1*. There are two cassettes comprising a total of 8,903 bp located between a 25 bp left border (LB) and a 25 bp right border (RB). The complete plasmid is 14,262 bp in size (i.e. the vector backbone comprises 5,309 bp).

Table 1: Description of the genetic elements contained in the T-DNA of pSYN18857

Genetic element	Relative bp location on plasmid	Size (bp)	Source	Orient.	Description & Function	References
RIGHT BORDER		25				
mepsps cassette						
Intervening sequence	26 - 127	102				
FMV-05	128 - 321	194	FMV	clockwise	• Enhancer region (similar to NCBI accession # X06166.1) • Increases expression of *mepsps*	Maiti *et al.* (1997)
Intervening sequence	322 - 327	6				
35S-05	328 - 620	293	CaMV	clockwise	• 35 S enhancer region • Activates the Ubi 158-02 promoter	Ow *et al.* (1987)
Intervening sequence	621 - 630	10				
Ubi158-02	631 - 4618	3988	*Zea mays* (corn)	clockwise	• Promoter based on the ubiquitin gene (similar to NCBI accession # S94466.1) • Directs constitutive transcription of the *mepsps* gene	Christensen *et al.* (1992)
TMV-03	4619 - 4686	68	TMV	clockwise	• 5' non-coding leader sequence • Translational enhancer	Gallie *et al.* (1987); Gallie (2002)
OTP-02	4687 - 5058	372	*Helianthus annuus* (sunflower) and *Zea mays* (corn)	clockwise	• Optimised chimeric chloroplast transit peptide (CTP). • Directs transport of mEPSPS to the chloroplast	Lebrun *et al.* (1996)
mepsps-02	5059 - 6396	1338	*Zea mays* (corn)	clockwise	• Modified coding sequence of the *epsps* gene	Lebrun *et al.* (2003)
Intervening sequence	6397 - 6403	7				
Ubi158-02	6404 - 7403	1000	*Zea mays* (corn)		• 3' untranslated region from the ubiquitin gene (similar to NCBI accession # S94466.1) • Terminates mRNA transcription and induces polyadenylation of the *mepsps* gene	Christensen *et al.* (1992)
pat cassette						
Intervening sequence	7404 - 7460	57				
35S-19	7461 - 7981	521	CaMV	clockwise	• Promoter from the 35S gene • Drives constitutive transcription of the *pat* gene	Franck *et al.* (1980); Odell *et al.* (1985); Pietrzak *et al.* (1986)
Intervening sequence	7982 - 7994	13				
pat-09	7995 - 8546	552	*Streptomyces viridochromogenes*	clockwise	Phosphinothricin acetyltransferase coding sequence (similar to NCBI accession # DQ156557.1)	Wohlleben *et al.* (1988)
Intervening sequence	8547 - 8550	4				
nos-05-01	8551 - 8803	253	*Agrobacterium tumefaciens*	clockwise	• Terminator region from the *nopaline synthase* gene (NCBI accession # V00087.1) • Directs polyadenylation of the *pat* gene	Depicket et al. (1982)
Intervening sequence	8804 - 8928	125				
LEFT BORDER		25				

3.2.1   *mepsps* expression cassette

The *mepsps* gene was produced by cloning the wildtype gene from corn in a plasmid and introducing specific changes to the DNA using standard *in vitro* techniques (Padgette et al. 1991). The resulting protein differs from the wildtype by two amino acid substitutions.

The *mepsps* coding region in MZHG0JG is 1,338 bp in length and is regulated by the constitutive corn ubiquitin *Ubi158* promoter and terminator. Sequences from FMV, CaMV and TMV enhance expression of *mepsps*. Natural EPSPS enzyme is located within chloroplasts, the site of aromatic amino acid biosynthesis in plant cells. As for many proteins with subcellular locations, newly synthesised pre-proteins are directed to a particular organelle by a transit peptide usually at one end of the mature protein. Following delivery to the organelle, the short transit peptide is cleaved from the mature protein and is rapidly degraded (Della-Cioppa et al. 1986). To direct the mEPSPS protein to the chloroplast of MZHG0JG cells, the *mepsps* gene is fused to an optimised synthetic chimeric CTP (designated OTP) derived from sequences from sunflower and corn.

3.2.2   *pat* expression cassette

The *pat* coding region is 552 bp in length and is driven by the constitutive CaMV 35S promoter. The gene encodes the same amino acid sequence as that of a synthetic gene obtained from AgrEvo[6], Germany but with several nucleotide changes made to remove a cryptic splice site, a restriction site and unintended open reading frames (ORFs). Transcription is terminated by the polyadenylation signal from the nopaline synthase (*nos*) gene of *Agrobacterium tumefaciens*.

3.3   Breeding of MZHG0JG

The breeding pedigree for the various generations is given in Figure 3.

From a single T_0 plant, produced from line NP2222, several rounds of self-pollination and seed bulking proceeded to produce specific generations that were used in characterisation and analysis (as indicated in Table 2). At the T_3 and T_5 generations, the transformation events were crossed with elite conventional proprietary inbred lines plants and the progeny were used to generate information for regulatory requirements and/or to assess field performance.

In order to generate hybrid lines to ascertain Mendelian inheritance, hemizygous MZHG0JG corn plants of the T_3 generation were crossed with non-GM line NP2681. The resulting F_1 generation (designated MF_1 in Figure 3) was backcrossed with the non-GM recurrent parent (NP2681) to yield the BC_1F_1 generation. Plants from the BC_1F_1 generation were backcrossed two more times with NP2681 to yield the BC_2F_1 and BC_3F_1 generations. Only positive hemizygous segregants (determined by tolerance to glyphosate) were used in each backcross.

MZHG0JG is the F_1 result of a cross between the T_5 generation and the non-GM inbred line NP2391 (Delzer 2007). Based on fit for various studies, non-GM lines NP2391, and NP2391 x NP2222 were used as controls, in addition to the parental line NP2222 (see Table 2).

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[6] AgrEvo was a joint venture company in existence from 1995–1999. In 1999, its majority shareholder, Hoechst, merged with the French pharmaceutical and chemical company Rhône-Poulenc to become Aventis CropScience. In 2002, Aventis was acquired by Bayer (now Bayer CropScience). AgrEvo had particular interest in the herbicide glufosinate ammonium and the biotechnology associated with herbicide resistance.
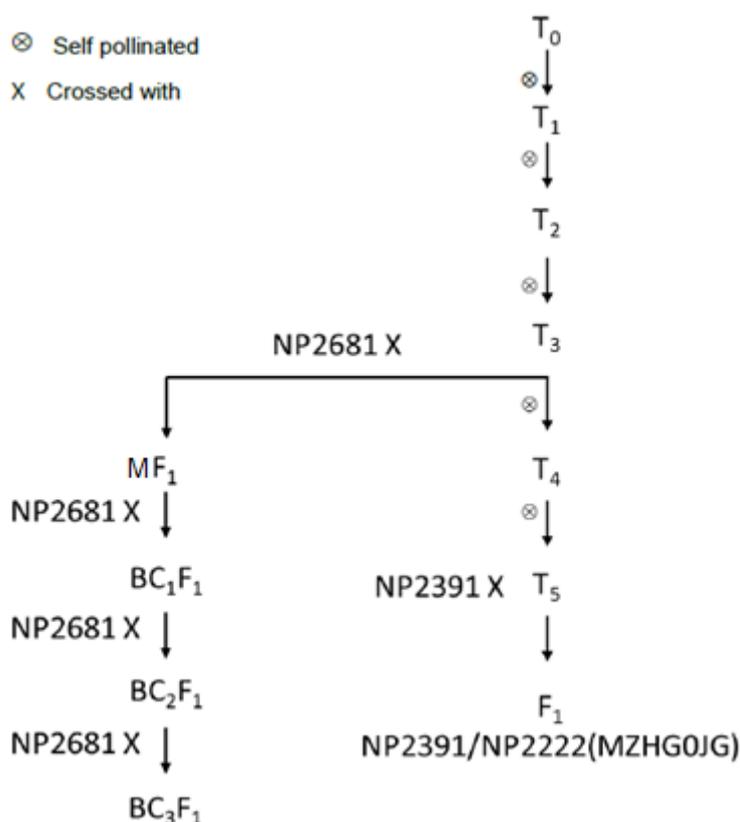
Figure 3: Breeding diagram for MZHG0JG

Table 2: MZHG0JG generations used for various analyses

Analysis	MZHG0JG generation used	Control(s) used	Reference comparators
Molecular characterisation (Section 3.4)	T_3	NP2222, NP2391, NP2391 x NP2222	
Genetic stability (Section 3.5)	T_2, T_3, T_4, T_5, F_1	NP2222, NP2391, NP2391 x NP2222	
Mendelian inheritance (Section 3.5)	BC_1F_1; BC_2F_1; BC_3F_1	N/A	
Protein expression levels in plant parts (Section 4.1.2)	F_1	NP2391 x NP2222	
Protein characterisation (Section 4.1.3)	F_1-produced protein	N/A	E.coli-produced protein
Compositional analysis (Section 5)	F_1	NP2391 x NP2222	6 non-GM commercial reference lines

3.4 Characterisation of the genetic modification in the plant

A range of analyses was undertaken to characterise the genetic modification in MZHG0JG. These analyses focussed on the nature of the insertion of the introduced genetic elements and whether any unintended genetic re-arrangements may have occurred as a consequence of the transformation procedure.

3.4.1   Southern analyses: Insert copy number, insert integrity and plasmid backbone

Total genomic DNA from verified (real time PCR) pooled leaf tissue of a number of MZHG0JG generations (T_2 - two samples from ear 4 and ear 35 stages, T_3, T_4, T_5, and F_1) and from verified negative controls (NP2222, NP2391 and NP2391 x NP222) was used for Southern blots to determine the number of T-DNA insertions and the sequence integrity of the introduced DNA in line MZHG0JG, and test for the presence or absence of plasmid vector backbone sequences.

Insert number

For the determination of insert number in MZHG0JG a total of six different restriction enzymes were used (each one cutting once within the MZHG0JG insert) in combination with three ^{32}P-labellled probes that covered every base pair of the pSYN18857 T-DNA i.e. each probe was used with two restriction enzyme digests. Three positive assay controls were included in the analysis; these were 1 copy and 1/7 copy DNA fragments of known size that corresponded to each of the three T-DNA probes.

In analysing the results, the following assumptions were relevant:

* Any bands in the negative controls that are also present in MZHG0JG would indicate cross-hybridisation of the T-DNA-specific probe with an endogenous maize sequence.
* Expected hybridisation bands present in MZHG0JG and absent in the negative controls would indicate the bands are specific to the MZHG0JG insert.
* Additional bands present in MZHG0JG but not in the negative controls would indicate the presence of additional copies of the insert in the MZHG0JG.genome.
* T-DNA-specific hybridization bands were not expected in any of the negative controls.
* Each of the T-DNA-specific positive assay controls was expected to result in one hybridisation band.

For all restriction enzyme/probe combinations and all MZHG0JG generations the expected hybridization bands were obtained, indicating that MZHG0JG contains a single insert. In some restriction enzyme/probe combinations, an additional hybridization band was detected in MZHG0JG because of sequence similarity between the *35S* enhancer in the *mepsps* cassette and the *35S* promoter in the *pat* cassette (see Table 1). Apart from this explained anomaly, no additional bands were detected in MZHG0JG, indicating that the MZHG0JG genome contains no extraneous DNA fragments of the insert.

Insert integrity

In this Southern analysis, DNA was digested with two restriction enzyme combinations with sites located at the extremities of the insert that therefore released a fragment of predictable size (around 8.8 kb) encompassing most of the insert. The same three probes and controls described for the insert number analysis were used. The expected 8.8 kb hybridisation band was present in all MZHG0JG generations and absent in the negative and positive assay controls for all probes, thereby indicating the intactness of the MZHG0JG insert.

Plasmid backbone

The presence or absence of sequences from the vector plasmid pSYN18857 was assessed using two backbone-specific probes in combination with two restriction enzyme digestions. The positive assay controls were 1 copy and 1/7 copy DNA fragments of known size corresponding to the two backbone probes and one hybridisation band was expected.

The expected band was obtained for the positive assay controls and no hybridisation bands were detected for either MZHG0JG (all generations) or the negative controls. This confirmed the lack of integration of any plasmid backbone sequences in MZHG0JG.

3.4.2 Insert organisation and sequence

Genomic DNA was obtained from verified leaf tissue from the MZHG0JG T_3 generation (see Figure 3) and the samples were used to characterise the DNA sequence in the transgene insertion and its flanking border regions. Two sequencing strategies, giving three fragments were followed:

- Two overlapping polymerase chain reaction (PCR) fragments spanning the inserted sequences and border regions in event SYN-000JG-2 were amplified, purified and then cloned into a bacterial vector. For each fragment, the DNA from vector colonies was randomly selected, confirmed to contain the expected insert and sequenced individually. The sequences were aligned to obtain a consensus sequence using commercially available software (Lasergene DNAStar®).

- A discrepancy in a stretch of 18 cytosines located in the *Ubi158* promoter occurred in the initial sequencing. This result was most likely due to polymerase slippage during PCR amplification of the region (Clarke et al. 2001) and not to variance in the plant genomic DNA. To investigate the discrepancy, a clone containing the *Ubi158* promoter fragment was sequenced using primer extension sequencing with multiple primers. A final consensus sequence for the clone was then obtained using Gene Codes Sequencher® 5.2.3 analysis.

Finally, the consensus sequences for all three fragments was assembled using Gene Codes Sequencher® 5.2.3

A total of 10,903 bp of MZHG0JG genomic sequence was confirmed (see Figure 4). This comprised 1,000 bp of the 5′ genomic border sequence (including the RB), 1,000 bp of the 3′ genomic border sequence (including the LB), and 8,903 bp of inserted T-DNA from pSYN-000JG-2. There was a 22 bp deletion from the RB and a 21 bp deletion from the LB; this truncation of the border sequences is not uncommon for *Agrobacterium*-mediated transformation events (Tzfira et al. 2004; Kim et al. 2007).

In addition to sequencing, annotation was performed using Vector NTI Advance® 11.5.2 software to compare sequence from MZHG0JG with the sequence in the T-DNA of plasmid pSYN18857. This showed that the 8,903 bp MZHG0JG insert is intact with no re-arrangements.
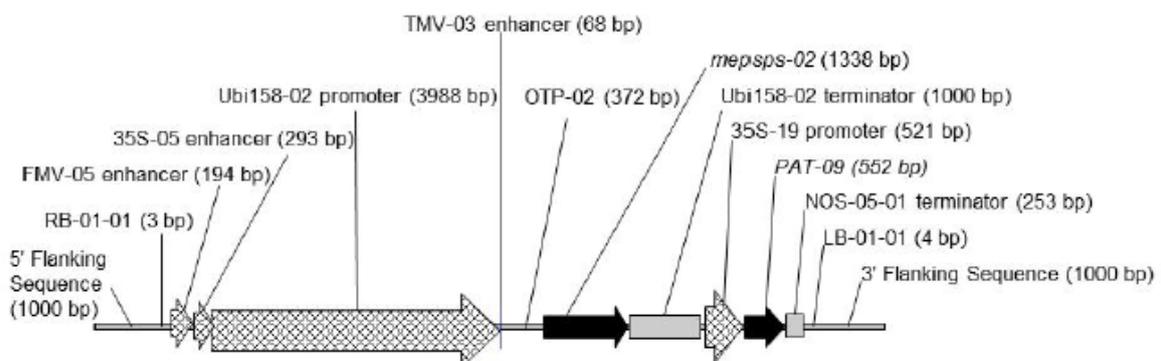

Figure 4: Map of the MZHG0JG insert and flanking sequence (intervening sequences not included)

3.4.3   Insertion site

To confirm that the flanking sequences of the MZHG0JG insert are of *Zea mays* origin, the sequences of the 1,000 bp 5' and 3' genomic borders were screened for similarity to translated DNA sequences in the National Center for Biotechnology (NCBI) non-redundant protein database[7] comprising entries from several public reference databases. The similarity searches used the BLAST (Basic Local Alignment Search Tool) algorithm (Altschul et al. 1990). BLAST is now frequently applied for searching for similarities in protein sequences by performing local alignments of domains or short sequence similarities. The analyses showed there were seven alignments for the corn genomic sequence flanking the 5' region of the MZHG0JG insert and two alignments for the sequence flanking the 3' region of the MZHG0JG insert. This indicated that the flanking sequences are of *Zea mays* origin.

In order to assess any changes that may have occurred in the NP2222 genome as a result of integration of the MZHG0JG insert, PCR amplification and sequencing of the insertion locus from line NP2222 was carried out. The design of a forward and reverse primer was based on genomic sequences in the 5' and 3' borders of MZHG0JG. Fragments were cloned and colonies selected and sequenced as described in Section 3.4.2. The resulting consensus sequence of the NP2222 non-transgenic locus was then compared to the MZHG0JG T-DNA flanking sequences.

The comparison showed that:

•   a 22 bp NP2222 sequence has been deleted in the MZHG0JG genome.

•   a total of 43 bp of DNA has been inserted comprising 4 bp at the 5' junction and 39 bp at the 3' junction. The 39-bp insertion is the reverse complement of a DNA sequence in the 5' flanking region, and appears to be a duplication of this sequence. Such insertions are often observed during Agrobacterium tumefaciens–mediated transformation and have been called "filler" DNA (Windels et al. 2003; Tzfira et al. 2004).

3.4.4   Open reading frame (ORF) analysis

Sequences spanning the 5' and 3' junctions of the insert in MZHG0JG were translated from start codon to stop codon (TGA, TAG, TAA) in all six reading frames, and encoding sequences of 30 or more amino acids. ORFs shorter than 29 amino acids were not evaluated since a minimum 35% identity requires at least a match of 29 amino acids over 80 amino acids. The 35% identity is a recommended criterion for indicating potential allergenicity (Codex 2009). No potential ORFs were found.

3.5   Stability of the genetic change in MZHG0JG

The concept of stability encompasses both the genetic and phenotypic stability of the introduced trait over a number of generations. Genetic stability refers to maintenance of the modification (as produced in the initial transformation event) over successive generations. It is best assessed by molecular techniques, such as Southern blot analysis. Phenotypic stability refers to the expressed trait remaining unchanged over successive generations. It is often quantified by a trait inheritance analysis to determine Mendelian heritability via assay techniques (chemical, molecular, visual).

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[7] NCBI Proteins - http://www.ncbi.nlm.nih.gov/guide/proteins/

The genetic stability of event SYN-000JG-2 was shown in the Southern analyses described in Section 3.4.1. In all MZHG0JG generations (i.e. T_2 - two samples from ear 4 and ear 35 stages, T3, T4, T5, and F1) for all restriction enzyme/probe combinations the expected hybridisation patterns were obtained.

Phenotypic stability was assessed by determining the segregation of the *mepsps* and *pat* genes within three generations (BC_1F_1, BC_2F_1, BC_3F_1 – see Figure 3). Since it was demonstrated that the insert resides at a single locus within the genome of MZHG0JG, the expectation would be that the genetic material within it would be inherited according to Mendelian principles i.e. *mepsps* and *pat* would co-segregate and 50% of plants from each generation would be expected to contain both transgenes while 50% would contain no transgenes (a 1:1 ratio).

Leaf discs from individual plants from each generation were analysed by real-time PCR for the presence of the *mepsps* and *pat* genes and the results are shown in Table 3. The chi-square (X2) critical value at significance level α = 0.05 is 3.84 i.e. if the X^2 value is < 3.84 the observed ratio is not significantly different from the expected ratio of 1:1. The X^2 values for all generations were less than 3.84 thereby indicating that the *mepsps* and *pat* genes are inherited according to Mendelian principles.

Table 3: Segregation of *mepsps* and *pat* over three generations

Generation	Total plants	Ratio positive:negative		X^2	Probability[1]
		Observed	Expected		
BC_1F_1	220	1:0.91	1:1	0.455	NS
BC_2F_1	216	1:1.16	1:1	1.185	NS
BC_3F_1	177	1:0.82	1:1	1.633	NS

[1]NS = not significant

3.6   Antibiotic resistance marker genes

No antibiotic marker genes are present in MZHG0JG. The insert sequence analysis (Section 3.4.1) showed that no plasmid backbone has been integrated into the MZHG0JG genome during transformation, i.e. the *aadA* gene, which was used as a bacterial selectable marker gene, is not present in MZHG0JG.

3.7   Conclusion

MZHG0JG contains two expression cassettes: Comprehensive molecular analyses indicate there is a single insertion site comprising a single, complete copy of each of the *mepsps,* and *pat* genes together with their regulatory elements. The introduced genetic elements are stably inherited from one generation to the next. There are no antibiotic resistance marker genes present in the line and no plasmid backbone has been incorporated into the transgenic locus.

4 Characterisation and safety assessment of new substances

4.1 Newly expressed proteins

In considering the safety of novel proteins it is important to note 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, e.g., because they are allergens or anti-nutrients (Delaney et al. 2008). 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 any post-translational modifications have occurred.

It is noted, from Section 3.4.4, that no potential ORFs have been created in MZHG0JG i.e. the only novel proteins that need to be considered are the mEPSPS and PAT proteins.

4.1.1 The mEPSPS and PAT proteins

EPSPS proteins are universally present in plants and microorganisms and, although their sequences are variable, their chemical function is highly specific and conserved (Mclean 2011). They are involved in the shikimate pathway. Specifically, they catalyse the transfer of the enolpyruvyl group from phosphoenol pyruvate (PEP) to the 5-hydroxyl of shikimate-3-phosphate (S3P), to yield inorganic phosphate and 5-enolpyruvylshikimate-3-phosphate the latter being the penultimate product of the shikimate pathway (Alibhai and Stallings 2001). Shikimate is a substrate for the biosynthesis of aromatic amino acids (phenylalanine, tryptophan and tyrosine) as well as many secondary metabolites, such as tetrahydrofolate, ubiquinone, and vitamin K.

Glyphosate acts as a herbicide by inhibiting endogenous EPSPS thereby leading to deficiencies in aromatic amino acids in plant cells and eventually to the death of the whole plant. The shikimate biochemical pathway is not present in animals.

It is the PEP binding site of wild type EPSPS that confers specificity, and substitution of amino acids in this site can confer glyphosate tolerance (Padgette et al. 1991). The modified mEPSPS coded for by *mepsps* in pSYN18857 has an approximate molecular weight of 47.4 kDa and comprises 445 amino acids (Figure 5). It differs from the wild type corn enzyme by two amino acid substitutions – threonine replaced by isoleucine (I) at position 103, and proline replaced by serine (S) at position 107. These two amino acid changes result in a protein with greater than 99% identity to the native corn EPSPS protein. However the modification confers a decreased binding affinity for glyphosate thus allowing the protein to maintain an adequate level of enzymatic activity in the presence of the herbicide. Plants expressing the modified corn enzyme, therefore, are able to continue to function in the presence of the herbicide. In terms of chemical structure, mEPSPS has little similarity (only 26%) to the more commonly used CP4 EPSPS protein. Despite different chemical structures all EPSPS proteins conferring glyphosate tolerance have identical functionality.

```
  1  MAGAEEIVLQ PIKEISGTVK LPGSKSLSNR ILLLAALSEG TTVVDNLLNS
 51  EDVHYMLGAL RTLGLSVEAD KAAKRAVVVG CGGKFPVEDA KEEVQLFLGN
101  AGIAMRSLTA AVTAAGGNAT YVLDGVPRMR ERPIGDLVVG LKQLGADVDC
151  FLGTDCPPVR VNGIGGLPGG KVKLSGSISS QYLSALLMAA PLALGDVEIE
201  IIDKLISIPY VEMTLRLMER FGVKAEHSDS WDRFYIKGGQ KYKSPKNAYV
251  EGDASSASYF LAGAAITGGT VTVEGCGTTS LQGDVKFAEV LEMMGAKVTW
301  TETSVTVTGP PREPFGRKHL KAIDVNMNKM PDVAMTLAVV ALFADGPTAI
351  RDVASWRVKE TERMVAIRTE LTKLGASVEE GPDYCIITPP EKLNVTAIDT
401  YDDHRMAMAF SLAACAEVPV TIRDPGCTRK TFPDYFDVLS TFVKN
```

Figure 5: Amino acid sequence of the mEPSPS protein

The PAT protein encoded by the *pat* gene consists of 183 amino acids (Figure 6), has a molecular weight of 21 kDa, and exhibits a high degree of enzyme specificity; recognising only one substrate. PAT functions by detoxifying phosphinothricin (PPT), the active constituent of glufosinate ammonium herbicides. PPT acts by inhibiting the endogenous enzyme glutamine synthetase, an enzyme involved in amino acid biosynthesis in plant cells. By inhibiting this enzyme, PPT causes rapid accumulation of ammonia in the plant cell, leading to plant death. In transformed corn plants, the introduced PAT enzyme chemically inactivates the PPT by acetylation of the free ammonia group, giving rise to herbicide tolerance in the whole plant.

```
  1  MSPERRPVEI RPATAADMAA VCDIVNHYIE TSTVNFRTEP QTPQEWIDDL
 51  ERLQDRYPWL VAEVEGVVAG IAYAGPWKAR NAYDWTVEST VYVSHRHQRL
101  GLGSTLYTHL LKSMEAQGFK SVVAVIGLPN DPSVRLHEAL GYTARGTLRA
151  AGYKHGGWHD VGFWQRDFEL PAPPRPVRPV TQI
```

Figure 6: Amino acid sequence of the PAT protein

4.1.2   mEPSPS and PAT expression in the tissues of MZHG0JG

Study submitted:

2015. Quantification of double mutated 5-enol pyruvylshikimate-3phosphate synthase and phosphinothricin acetyltransferase in event MZHG0JG maize tissues; Final report TK0062594. Syngenta Seeds, Inc. (unpublished)

Plants of MZHG0JG (generation F_1) were grown from verified seed lots at four field sites in the U.S.[8] during the 2013 growing season. These plantings overlapped with the eight plantings used for the compositional analysis described in Section 7.2. Controls, comprising crude extracts from the non-GM line from the cross NP2391 x NP2222 spiked with either mEPSPS or PAT reference protein, were used to confirm either the minimum dilution factor for each tissue (in the case of mEPSPS) or the efficiency of the method used to extract each protein from the crude extract (in the case of PAT).

At each location, two replicate plots were planted with MZHG0JG seed and one plot was planted with the control. One of the MZHG0JG replicate plots received a single post-emergent spray application of glufosinate at a nominal rate of 0.46 kg ai/ha[9] when the plants reached the V3/V4 growth stage[10].

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[8] York, Nebraska; Kimballton, Iowa; Richland, Iowa; Germansville, Pennsylvania
[9] ai = active ingredient
[10] For information on corn growth stages see e.g. Ransom & Endres (2014)

The same plot also received a single post-emergent spray application of glyphosate at a nominal rate of 0.88 kg ai/ha when the plants reached the V5 growth stage. It is noted that the R6 stage is physiological maturity but that any grain harvested at this stage will require artificial drying to be stored safely.

Five replicate samples of each tissue type (Table 4) except pollen were collected from each plot. For pollen, a pooled sample was collected from 10–15 tassels per plot. Levels of mEPSPS and PAT were determined for each sample type using a validated enzyme linked immunosorbent assay (ELISA). Detection of both proteins utilised commercially available kits – AgraQuant® EPSPS Plate Test Kit and Envirologix QualiPlate™ ELISA Kit for Liberty Link® PAT/*pat*. Plates were analysed on a microplate spectrophotometer, and commercial software (SoftMax® Pro GxP, ver 6.3, Molecular Devices) was used to convert optical density values to protein concentration

The results, averaged over all sites, are given in Table 4. The mean levels of both mEPSPS and PAT in both herbicide-sprayed and non-sprayed plants were highest in leaves at the R1 stage and lowest (bordering on undetectable) in senescent leaves and the pollen. For PAT, mean levels were also equally low in the whole plant at R6 and grain at both stages.

Table 4: mEPSPS and PAT protein content of tissue in herbicide-sprayed and unsprayed MZHG0JG at different growth stages (averaged across 4 sites)

Tissue/Growth stage	Herbicide-sprayed				No herbicide applied			
	mEPSPS µg/g dw[1]		PAT µg/g dw		mEPSPS µg/g dw		PAT µg/g dw	
	Mean±SD[2]	Range	Mean±SD	Range	Mean±SD	Range	Mean±SD	Range
Leaf /V6	1198±365	583 - 1799	8.52±1.62	5.32 – 11.33	1421±410	697 - 2059	8.39±1.44	6.37 – 11.79
Leaf /R1	1816±856	940 - 3507	10.22±3.37	6.75 – 16.87	1934±678	920 -3203	9.95±3.02	6.21 – 17.03
Leaf /R6	1103±798	197 - 2685	2.26±2.02	0.38 – 6.53	954±686	138 - 2760	2.15±1.95	0.11 – 6.07
Leaf /Senescence		<LOD[3] –44.38		<LOD - 0.42		<LOD - 182		<LOD - 0.71
Root /V6	404±258	162 - 1212	1.94±1.27	0.75 – 5.09	357±149	124 - 707	1.68±1.08	0.77 – 3.91
Root /R1	412±118	246 - 655	1.11±0.26	0.73 – 1.44	367±103	176 - 529	1.08±0.30	0.67 – 1.71
Root /R6	322±91.96	131 - 486	1.29±0.90	0.58 – 3.64	294±87.89	147 - 396	1.24±0.72	0.62 – 2.69
Root /Senescence	156±105	30.72 - 467	0.80±0.42	0.20 – 1.91	153±57.72	57.58 - 261	0.80±0.25	0.37 – 1.32
Whole plant /V6	1293±400	857 - 2262	7.01±0.98	5.17 – 8.54	1496±445	734 - 2251	6.70±1.46	3.13 – 9.76
Whole Plant /R1	1438±315	830 - 2066	4.33±1.54	2.54 – 7.88	1468±398	948 - 2347	4.48±1.64	2.23 – 8.00
Whole Plant /R6	298±220	85.66 - 934		<LOD – 1.97	329±213	94.78 - 753		<LOD – 2.43
Pollen/R1		<LOQ[4]		<LOD		<LOQ		<LOD
Grain/R6	61.14±15.49	34.82 –93.06		<LOD – 0.04	58.2±14.87	30.37-86.15		<LOD – 0.02
Grain/Senescence	42.20±8.78	28.33 – 60.30		<LOD	36.9±10.06	19.94-56.54		<LOD

[1] dw = dry weight   [3] LOD = Limit of Detection
[2] SD = standard deviation   [4] LOQ = Limit of Quantitation

4.1.3 Characterisation of the proteins produced in MZHG0JG

It is necessary to confirm that the proteins expressed in MZHG0JG have the expected biochemical characteristics. Accordingly, the Applicant used a number of analytical techniques to characterise mEPSPS and PAT proteins in leaf tissue from glasshouse-grown MZHG0JG (verified by real-time PCR) F_1 generation and compare the results with previously characterised mEPSPS and PAT (79.2% and 85.9% purity respectively) produced in recombinant *Escherichia coli* systems

The techniques used were:

* Western blot analysis following sodium dodecyl polyacrylamide gel electrophoresis (SDS - PAGE)
* Peptide mass mapping and N- and C-terminal sequencing
* Glycosylation analysis
* Enzymatic activity analysis

Studies submitted:

2015. Comparison of double mutated maize 5-enolpyruvylshikimate-3-phosphate synthase (mEPSPS) protein produced in recombinant *Escherichia coli* and mEPSPS protein produced in event MZHG0JG derived maize plants. Final report **TK0062573.** Syngenta Seeds, Inc. (unpublished)

2015. Comparison of phosphinothricin acetyltransferase (PAT) protein produced in recombinant Escherichia coli and PAT protein produced in event MZHG0JG derived maize plants. Final report **TK0062575.** Syngenta Seeds, Inc. (unpublished)

4.1.3.1 *Molecular weight and immunoreactivity of mEPSPS and PAT*

Following SDS-PAGE, protein bands were transferred to a polyvinylidene fluoride (PVDF) membrane via electroblotting. For mEPSPS. the membrane was probed with a monoclonal mouse anti-EPSPS primary antibody followed by an alkaline phosphatase-conjugated anti-mouse secondary antibody. The protein was visualised following addition of the chromogenic substrate BCIP®/NBT[11]. For PAT a similar procedure was used except the primary antibody was a polyclonal goat anti-PAT and the secondary antibody was an alkaline phosphatase-conjugated anti-goat.

The Western blot analysis of crude plant extract, immunoaffinity-purified plant protein, and microbially-produced protein for mEPSPS showed a prominent immunoreactive band at approximately 47 kDa which is close to the predicted molecular weight of 47.4 kDa. Fainter immunoreactive bands of higher molecular weights (approximately 94 and 141 kDa) were also observed in the plant-derived protein and probably represent a dimer and trimer of the mEPSPS. A faint lower molecular weight band at approximately 35 kDa in the plant-derived protein probably represents a breakdown product of mEPSPS.

Analysis of crude plant extract, immunoaffinity-purified plant protein, and microbially-produced protein for PAT showed a prominent immunoreactive band at approximately 17 kDa, which is close to the predicted molecular weight of 21 kDa. A very faint band at approximately 40 kDa most likely represents a dimer.

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[11] 5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP) and nitroblue tetrazolium (NBT) forms a black-purple precipitate upon reaction with alkaline phosphatase.

The mEPSPS and PAT proteins expressed in MZHG0JG therefore have the expected size and immunoreactivity.

4.1.3.2 MALDI-TOF tryptic mass fingerprint and N- and C-terminal sequencing

Protein identification by peptide mass fingerprinting is considered reliable if the measured coverage of the sequence is 15% or higher (Jensen et al. 1997).

Peptide mass coverage analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed on the trypsin-, chymotrypsin- and endoproteinase Asp-N-digested protein samples of mEPSPS and PAT from both microbial and MZHG0JG sources. Each acquired MS/MS spectrum was submitted to the Mascot search engine (Matrix Science, version 2.2.06) to obtain the peptide identities, searching against a database containing the relevant (i.e. mEPSPS or PAT) protein amino acid sequence.

The collective analysis of the three proteolytic digests for mEPSPS from the microbial and plant sources resulted in coverage of 92% and 88%, from each source respectively, of the total predicted mEPSPS amino acid sequence. This was adequate to confirm the identity of the protein. In addition 26 amino acids of the plant-derived N-terminal sequence (24 of the *E. coli*-derived sequence) and 16 amino acids of the plant C-terminal sequence (15 of the *E. coli*-derived sequence) were identified by peptide mass coverage and were found to be consistent with the predicted sequence (see Figure 5) except that the N-terminal methionine was missing. This is not unexpected since, following translation, the terminal methionine is often cleaved from nascent proteins by methionine aminopeptidase (Polevoda and Sherman 2000; Walling 2006).

The collective analysis of the three proteolytic digests for PAT from the microbial and plant sources resulted in coverage of 97% and 90%, from each source respectively, of the total predicted PAT amino acid sequence. This was adequate to confirm the identity of the protein. In addition 15 amino acids of the plant-derived N-terminal sequence (16 of the *E. coli*-derived sequence) and 19 amino acids of the plant C-terminal sequence (24 of the *E. coli*-derived sequence) were identified by peptide mass coverage and were found to be consistent with the predicted sequence (see Figure 6). The analysis showed that the N-terminal methionine was missing from the plant-derived sequence.

4.1.3.3 Glycosylation status

Many eukaryotic proteins are glycoproteins that have been post-translationally modified by the addition of carbohydrate moieties (glycans) covalently linked to the polypeptide backbone.

N-glycosylated proteins are glycosylated on an asparagine residue and commonly contain an asparagine-X-serine/threonine sequence (N-X~(P)-[S/T], where X~(P) indicates any amino acid except proline (Orlando and Yang 1998). Although rare, the sequence asparagine-X-Cysteine (N-X-C) can also be an N-glycosylation site (Miletich and Broze Jr. 1990). A basic search using NetNGlyc[12] predicted two potential sites in the mEPSPS protein and no sites in the PAT protein.

Analysis of plant- and microbially-derived mEPSPS and PAT proteins was done using a commercial kit (Sigma® Glycoprotein Detection Kit) following SDS-PAGE. The kit is designed to selectively stain glycoproteins on a nitrocellulose membrane using a modification of the Periodic Acid-Schiff (PAS) method. Staining of sugar moieties of glycoproteins yields magenta bands with a colorless background.

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[12] http://www.cbs.dtu.dk/services/NetNGlyc/

The Schiff reagent stains vicinal diol groups found mainly on peripheral sugars and sialic acids and is used as a general glycoprotein stain.

A visible band was obtained for the positive control (horseradish peroxidase) while the mEPSPS and PAT proteins from both plant and microbial sources and the negative control (soybean trypsin inhibitor) gave no visible bands. These results support the conclusion that neither microbially- nor MZHG0JG-derived mEPSPS or PAT proteins are glycosylated.

4.1.3.4 Enzymatic activity

mEPSPS

EPSPS catalyses the transfer of the enolpyruvyl moiety of phosphoenolpyruvate to the 5-OH hydroxyl group of shikimate 3-phosphate and releases inorganic phosphate. The amount of inorganic phosphate released in the reaction is measured spectrophotometrically (660 nm) using a malachite green dye method (Lanzetta et al. 1979) and is directly related to the specific activity of the enzyme.

It was predicted that the extraction procedure and plant matrix may have an effect on the activity of the plant-derived mEPSPS and to test this, the activity of a crude extract from the control (NP2391 x NP2222) fortified with microbially-produced mEPSPS from which the mEPSPS was then extracted was also tested. The results are given in Table 5.

Table 5: Specific activity of mEPSPS from various sources (mean of 3 replicates)

mEPSPS source	Mean EPSPS specific activity (U/mg mEPSPS)[1]
Microbially-produced mEPSPS	6172
MZHG0JG-produced mEPSPS	3444
Non-GM control fortified with microbially-produced mEPSPS-	3699
Non-GM control (NP2391 x NP2222)	<LOD

[1] One unit (U) of mEPSPS activity is defined as the amount of enzyme required to produce 1 nmol of phosphate per minute.

Correcting for the effect of the extraction procedure and the plant matrix, the detected specific activities of the microbially- and plant-produced mEPSPS are comparable (3699 and 3444 U/mg mEPSPS, respectively).

PAT

The activity of plant- and microbially-derived PAT was measured using a continuous spectrophotometric assay (Thompson et al. 1987; D'Halluin et al. 1992) with minor modifications. PAT was incubated with PPT (see Section 4.1.1) for 8 min and then the formation of 2-nitro-5-thiobenzoate anion(TNB^{2-}) was monitored over 5 min with readings at 412 nm every 12 s. These readings can be converted into the molar acetylation of PPT by PAT. Nontransgenic (NP2391 x NP2222) extract was included within the enzymatic activity assay as a negative control. As for the mEPSPS assay, a further plant control treatment was included to account for the effect of extraction procedure and plant matrix on specific activity. Results are given in Table 6.

Table 6: Specific activity of PAT from various sources (mean of 3 replicates)

PAT source	Mean PAT specific activity (U/mg PAT)[1]
Microbially-produced PAT	28.06
MZHG0JG-produced PAT	7.81
Non-GM control fortified with microbially-produced PAT-	9.62
Non-GM control (NP2391 x NP2222)	<LOD

[1] One unit of PAT activity is defined as the amount of enzyme required to acetylate 1 µmol of phosphinothricin per minute (equivalent 1 µmol TNB^{2-} produced per minute) under the described reaction conditions.

Correcting for the effect of the extraction procedure and the plant matrix, the detected specific activities of the microbially- and plant-produced PAT are comparable (9.62 and 7.81 U/mg PAT, respectively).

4.1.4   Safety of the introduced proteins

Results presented in Section 4.1.3 have verified the identity of the mEPSPS and PAT proteins expressed in MZHG0JG. Both proteins have been assessed as safe by FSANZ in previous applications. The mEPSPS protein, also referred to as 2mEPSPS, has been considered in four previous applications: A362 – corn line GA21 (FSANZ 2000a), A614 – cotton line GHB614 (FSANZ 2009), A1051 – soybean line FG72 (FSANZ 2011) and A1073 – soybean line DAS-44406-6 (FSANZ 2013a). Results in the literature also support the safety of 2mEPSPS (see e.g. Fard et al. 2013; Hammond et al. 2013). The PAT protein, encoded by either the *pat* or *bar* genes (Wehrmann et al. 1996), has now been considered in 20 FSANZ safety assessments (A372, A375, A380, A385, A386, A446, A481, A518, A533, A543, A589, A1028, A1040, A1046, A1073, A1080, A1081, A1087, A1094 and A1106) as well as being accepted in the literature as having neither toxicity nor allergenicity concerns (see e.g. Hérouet et al. 2005; Delaney et al. 2008; Fard et al. 2013; Hammond et al. 2013). There is 85% similarity (29 amino acids difference) in the amino acid sequences, and structural and functional equivalence, of the PAT proteins encoded by the two genes (Wehrmann et al. 1996; Hérouet et al. 2005)

A summary of these previous characterisations is provided in Table 7. For information, a reference is provided to the application in which the most recent detailed study or information was considered by FSANZ and is available on the FSANZ website. For the bioinformatic studies, which analyse sequence similarity to known protein toxins and allergens, where the Applicant provided searches (see provided studies listed below) using an updated (and hence larger) database, the results did not alter conclusions reached previously. The Applicant also provided *in vitro* digestibility and thermolability studies (see provided studies listed below) for each protein that confirmed the conclusions reached by FSANZ in the relevant previous applications listed in Table 7. The digestibility studies suggest the proteins would be rapidly degraded in the stomach following ingestion; and thermolability studies show that both proteins are inactivated by heating.

Taken together, the evidence indicates that neither mEPSPS nor PAT are likely to be toxic or allergenic to humans.

Table 7: Summary of consideration of mEPSPS and PAT in previous FSANZ safety assessments

Consideration	Sub-section	mEPSPS	PAT
Potential toxicity	Amino acid sequence similarity to protein toxins	This application – using search updated in March 2015	This application – using search updated in April 2015
	In vitro digestibility	A1073 (FSANZ 2013a)	A1080 (FSANZ 2013b)
	Stability to heat	A1073 (FSANZ 2013a)	A1080 (FSANZ 2013b)
	Acute oral toxicity	A1051 (FSANZ 2011)	A1080 (FSANZ 2013b)
Potential allergenicity	Source of the protein	A362 (FSANZ 2000a)	A1087 (FSANZ 2013c)
	Amino acid sequence similarity to allergens	This application – using search updated in March 2015	This application – using search updated in April 2015

Provided studies

1.   Digestibility studies

2005. *In vitro* digestibility of double-mutated maize 5-enol pyruvylshikimate-3-phosphate synthase (mEPSPS) test substances GA21-0104 and IAPGA21-0105 under simulated mammalian gastric conditions. Amended report #1, **SSB-007-05 A1**. Syngenta Biotechnology, Inc. (unpublished).

2012. *In vitro* digestibility of phosphinothricin acetyltransferase (PAT) under simulated mammalian gastric conditions. Final report, **TK0062551**. Syngenta Seeds, Inc. (unpublished).

2014. *In vitro* digestibility of double-mutated maize 5-enol pyruvylshikimate-3-phosphate synthase (mEPSPS) protein under simulated mammalian intestinal conditions. Final report **TK0179518**. Syngenta Seeds, Inc. (unpublished).

2010. *In vitro* digestibility of phosphinothricin acetyltransferase (PAT) under simulated mammalian intestinal conditions. Final report, **TK0030220**. Syngenta Biotechnology, Inc. (unpublished).

2.   Thermolability studies

2007. Effect of temperature on the immunoreactivity of the double-mutated maize 5-enol pyruvylshikimate-3-phosphate synthase (mEPSPS) enzyme. Final report, **SSB-021-07** Syngenta Biotechnology, Inc. (unpublished).

2013. Effect of temperature on the immunoreactivity of the phosphinothricin acetyltransferase (PAT). Final report, **TK0062552** Syngenta Seeds, Inc. (unpublished).

3.   Bioinformatics studies - updated

2015. mEPSPS. Assessment of amino acid sequence similarity to known or putative allergens. Assessment **SSB-114-15**. Syngenta Seeds, Inc. (unpublished).

2015. PAT (pat): Assessment of amino acid sequence similarity to known or putative allergens. Assessment **SSB-116-15**. Syngenta Seeds, Inc. (unpublished).

2015. mEPSPS. Assessment of amino acid sequence similarity to known or putative toxins. Assessment **SSB-115-15**. Syngenta Seeds, Inc. (unpublished).

2015. PAT (pat): Assessment of amino acid sequence similarity to known or putative toxins. Assessment **SSB-117-15**. Syngenta Seeds, Inc. (unpublished).

4.1.5   Conclusion

Corn line MZHG0JG expresses two new proteins, mEPSPS and PAT. The mean levels of both proteins in both herbicide-sprayed and non-sprayed plants were highest in leaves at the R1 stage and lowest (bordering on undetectable) in senescent leaves and pollen. For PAT, mean levels were also equally low in the whole plant at R6 and grain at both stages. The mean level of mEPSPS in the grain at harvest maturity (stage R6) was approximately 60 µg/g dw.

A range of characterisation studies confirmed the identity of the mEPSPS and PAT proteins produced in MZHG0JG and also their equivalence with the corresponding proteins produced in a bacterial expression system. The plant mEPSPS and PAT proteins have the expected molecular weight (approximately 47 kDa and 20 kDa respectively), immunoreactivity, lack of glycosylation, amino acid sequence and enzyme activity.

There are no concerns regarding the potential toxicity or allergenicity of the expressed proteins. Previous safety assessments of both mEPSPS and PAT indicate that the proteins would be rapidly degraded in the digestive system following ingestion and would be inactivated by heating. Additionally, updated bioinformatic studies considered in this assessment confirm the lack of any significant amino acid sequence similarity to known protein toxins or allergens.

4.2   Herbicide metabolites

As part of the safety assessment it is important to establish whether the expression of a novel protein(s) is likely to result in the accumulation of any novel metabolites. If such substances are found to occur as a result of the genetic modification, then it is important to determine their potential toxicity.

As discussed in Section 4.1.1 mutant EPSPS proteins that confer tolerance to glyphosate may vary in amino acid sequence but function in the same way, namely, they have a decreased binding affinity for the herbicide. This means, therefore, that metabolites are unlikely to be formed as a result of the direct interaction between an EPSPS protein and the herbicide. It does appear, however, that at least some plants may possess an endogenous mechanism (glyphosate oxidoreductase = GOX) for degrading, to greater or lesser degrees, glyphosate to aminomethylphosphonic acid (AMPA) and glyoxylate (Duke 2011). AMPA and glyoxylate are therefore metabolites that may accumulate in both GM and non-GM plants sprayed with glyphosate. AMPA is further degraded by microorganisms and glyoxylate is broken down in plant cells via the glyoxylic pathway for metabolism (FSANZ 2000b).

The glufosinate-tolerance trait is present in lines from 20 previous applications to FSANZ (see Section 4.1.4). The enzyme activity of PAT results in the acetylation of the free amino group of glufosinate to produce the non-herbicidal N-acetyl glufosinate. This is a well-known metabolite in glufosinate-tolerant plants and was previously considered in detail by FSANZ in cotton line LL25 (FSANZ 2006).

There are no concerns that the spraying of line MZHG0JG with glyphosate and/or glufosinate would result in the production of any novel metabolites that have not been previously assessed.

5   Compositional analysis

The main purpose of compositional analysis is to determine if, as a result of the genetic modification, any unexpected changes have occurred to the food. These changes could take the form of alterations in the composition of the plant and its tissues and thus 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 the food.

The classic approach to the compositional analysis of GM food is a targeted one. Rather than analysing every single constituent, 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.

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 such 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).

5.1   Key components

For corn there are a number of components that are considered to be important for compositional analysis (OECD 2002). As a minimum, the key nutrients of corn grain appropriate for a comparative study include the proximates (crude protein, fat, ash, acid detergent fibre and neutral detergent fibre), amino acids and fatty acids. In addition, mineral and vitamin levels may be considered and international guidance also suggests that levels of the key anti-nutrients and secondary metabolites could be determined for new varieties of corn.

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.

5.2   Study design and conduct for key components

Study submitted:

2014. Compositional analysis of forage and grain from MZHG0JG maize grown during 2013 in the USA. Report **TK0062583**. Syngenta Seeds, Inc. (unpublished).

The MZHG0JG (F_1 generation) was used for compositional analysis. Ideally, the comparator in compositional analyses should be the near isogenic parental line grown under identical conditions (OECD 2002). In the case of MZHG0JG, the control was the hybrid NP23291 x NP2222 since this represents the closest non-GM genetic line for the purposes of comparison.

The test and control lines were grown from verified (real-time PCR) seed lots at eight field sites across the US corn belt[13] during the 2013 growing season. Five of the plantings were the same as those used for the protein expression analysis (Section 4.1.2). For each treatment there were four replicated blocks at each site planted in a randomised complete-block design. Maintenance fertilizer and pesticides were applied as needed in order to maintain a relatively weed-free and insect-free environment. One of the MZHG0JG treatments received a single post-emergent spray application of glufosinate at a nominal rate of 0.46 kg ai/ha when the plants reached the V3/V4 growth stage. The same plot also received a single post-emergent spray application of glyphosate at a nominal rate of 0.88 kg ai/ha when the plants reached the V5 growth stage.

Additionally, a total of six non-GM hybrid lines[14] were also grown as reference lines at each location in order to generate tolerance ranges for each analyte and hence to aid in the determination of the normal variation found in corn analyte levels.

For the grain samples, 15 ears from each plot were harvested at R6 and a pooled sample of 500 g was collected and used for analysis. Samples were analysed for proximates, starch, fibre (acid detergent fibre – ADF; neutral detergent fibre – NDF; total dietary fibre), fatty acids, amino acids, minerals, vitamins, anti-nutrients and secondary metabolites.

Key analyte levels (proximates, fibre, calcium and phosphorus) for forage (five plants harvested at R4) were also obtained but are not reported here; it is noted, however, that in the combined site analysis the level of carbohydrate was significantly higher in MZHG0JG than in the control and the level of phosphorus was significantly lower. The mean levels of all analytes from both MZHG0JG and the control fell within the reference range and the literature range.

Methods of composition analysis were based on internationally recognised procedures (e.g. those of the Association of Official Analytical Chemists), methods specified by the manufacturer of the equipment used for analysis, or other published methods.

5.3   Analyses of key components in grain

In total, 72 analyte levels were measured and carbohydrate was calculated rather than being measured i.e. there was a total of 73 analytes considered. Fifteen analytes had a significant number of the observations below the assay limit of quantitation (LOQ) and were excluded from the statistical analysis. Moisture contents of each sample were measured for conversion of components to dry weight but were not statistically analysed. The data for 57 analytes were therefore analysed. Statistical analyses were performed using Statistical Analysis Software[15] (SAS) v. 9.4. For each analyte, 'descriptive statistics' (mean and standard error) were generated. A mixed model Analysis of Variance was used for both across-location and within-location comparisons. The results summarised in Tables 8 – 14 are for the across-location comparisons. The results of the within-location comparisons were consistent with those obtained for the across-location comparisons. For each analyte, t-tests were used to assess the statistical significance of the two comparisons of interest (herbicide-sprayed MZHG0JG vs control; unsprayed MZHG0JG vs control). In assessing the significance of any difference between the mean analyte value for MZHG0JG and the control, a P-value of 0.05 was used.

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[13] Richland, Iowa; York, Nebraska; Seymour, Illinois; Bagley, Iowa; Larned, Kansas; Stewardson, Illinois; Wyoming, Illinois; Germansville, Pennsylvania.
[14] Reference lines were H-7191, H-7540, SY SINCERO, NK LUCIUS, CISKO, SY PROVIAL.
[15] SAS website - http://www.sas.com/technologies/analytics/statistics/stat/index.html

This means that approximately 5% of statistically significant differences are expected to occur due to chance alone.

Any statistically significant differences between MZHG0JG and the NP23291 x NP2222 control have been compared to the results of the 6 reference lines and to a combined literature range for each analyte, compiled from published literature for commercially available corn[16]. It is noted, however, that information in the published literature is limited and is unlikely to provide a broad reflection of the natural diversity that occurs within corn (Harrigan et al. 2010; Zhou et al. 2011; Ridley et al. 2011). Therefore, even if means fall outside the published range, this is unlikely to raise a concern.

5.3.1   Proximates and fibre

Results of the proximate, starch and fibre analysis are shown in Table 8. Moisture levels were not compared statistically. The mean level of NDF in unsprayed MZHG0JG was significantly lower than that in the control but was within both the reference range and literature range. For all other analytes, there was no significant difference between the mean level in either sprayed or unsprayed MZHG0JG and the control; all means were also within both the reference and literature ranges.

Table 8: Mean percentage dry weight (%dw) of proximates, starch and fibre in grain from MZHG0JG and the hybrid control

Analyte	Control (%dw)	Sprayed MZHG0JG (%dw)	Unspray MZHG0JG (%dw)	P (sprayed v control)	P (unspray v control)	Reference range (%dw)	Combined literature range (%dw)
Moisture (%fw)	12.4	12.4	12.1			7.99 – 17.4	5.1 – 40.5
Ash	1.41	1.43	1.42	0.755	0.902	1.18 – 1.87	0.62 – 6.28
Protein	10.5	10.1	10.2	0.115	0.218	7.68 – 13.9	5.72 – 17.26
Fat	3.85	3.81	3.80	0.566	0.560	2.39 – 4.41	1.36 – 7.83
Carbohydrate[1]	84.3	84.7	84.6	0.109	0.187	81.3 – 88.0	77.4 – 89.5
ADF	4.06	3.94	3.98	0.212	0.408	2.43 – 4.48	1.41 – 11.34
NDF	11.5	11.1	11.0[2]	0.083	***0.031***	7.42 – 12.2	4.28 – 22.64
Total dietary fibre	16.5	16.1	16.0	0.140	0.094	11.2 – 20.0	8.73 – 35.3
Starch	65.4	66.3	65.0	0.332	0.608	53.3 – 79.6	26.5 – 83.7

[1] Carbohydrate calculated as 100% - (protein %dw+ fat %dw + ash %dw)
[2.]Mauve shading represents MZHG0JG mean significantly lower than the control

5.3.2   Fatty Acids

The levels of 22 fatty acids were measured. Of these, the following 12 had many observations below the LOQ and were therefore excluded from analysis - C8:0 caprylic, C10:0 capric, C12:0 lauric, C14:0 myristic, C14:1 myristoleic, C15:0 pentadecanoic, C15:1 pentadecenoic, C17:1 heptadecenoic, C18:3 gamma linolenic, C20:2 eicosadienoic, C20:3 eicosatrienoic, and C20:4 arachidonic. Results for the remaining 10 fatty acids are given in Table 8 and can be summarised as follows:

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[16] Published literature for corn incorporates references used to compile listings in the ILSI Crop Composition Database Version 4 (ILSI 2014).

- The mean levels of heptadecanoic and linolenic acids were significantly higher in both sprayed and unsprayed MZHG0JG than in the control.
- The mean level of palmitic acid was significantly lower in sprayed MZHG0JG than in the control.
- All means for all fatty acids and all treatments fell within the reference and combined literature ranges.

Table 9: Mean percentage composition, relative to total fat, of major fatty acids in grain from MZHG0JG and the hybrid control

Fatty acid	Control (%total)	Sprayed MZHG0JG (%total)	Unspray MZHG0JG (%total)	P (sprayed v control)	P (unspray v control)	Reference range (%total)	Combined literature range (%total)
Palmitic (C16:0)	14.3	14.1[1]	14.1	*0.010*	0.051	13.2-17.0	6.81-26.55
Palmitoleic (16:1)	0.129	0.129	0.130	0.962	0.430	0.0876-0.200	<LOQ-0.453
Heptadecanoic (17:0)	0.0834	0.0858[1]	0.0866[1]	*0.019*	*0.004*	0.0698-0.121	<LOQ-0.203
Stearic acid (C18:0)	2.12	2.14	2.13	0.297	0.439	1.59-2.48	1.02 – 3.83
Oleic acid (C18:1)	26.8	26.7	26.6	0.500	0.359	16.5-31.1	17.4 – 42.81
Linoleic acid (C18:2)	54.0	54.2	54.3	0.196	0.191	47.5-64.1	34.27-67.68
Linolenic acid (C18:3)	1.78	1.82	1.81	*0.012*	*0.045*	1.39-2.12	0.55-2.33
Arachidic acid (C20:0)	0.427	0.426	0.425	0.887	0.580	0.329-0.485	0.267-0.993
Eicosenoic acid (C20:1)	0.229	0.227	0.227	0.107	0.096	0.178-0.348	<LOQ-1.952
Behenic acid (C22:0)	0.182	0.175	0.182	0.141	0.876	0.0977-0.247	<LOQ-0.417

[1] Mauve shading represents MZHG0JG mean significantly lower than the control; orange shading represents MZHG0JG means significantly higher than the control;

5.3.3 Amino acids

Levels of 18 amino acids were measured. Since asparagine and glutamine are converted to aspartate and glutamate respectively during the analysis, levels for aspartate include both aspartate and asparagine, while glutamate levels include both glutamate and glutamine.

The results in Table 10 show that the means for arginine, aspartate, lysine and tryptophan were significantly lower (in one or both of the MZHG0JG treatments) than the control mean. For the remaining 14 amino acids there was no significant difference between the means of the control and MZHG0JG. All means for all amino acids and treatments were also within both the reference range and the literature range.

Table 10: Mean weight of amino acids in grain from MZHG0JG and the hybrid control

Amino acid	Control (mg/g dw)	Sprayed MZHG0JG (mg/g dw)	Unspray MZHG0JG (mg/g dw)	P (sprayed v control)	P (unspray v control)	Reference range (mg/g dw)	Combined literature range (mg/g dw)
Alanine	8.05	7.67	7.73	0.062	0.110	5.42-11.4	4.39-14.80
Arginine	4.95	4.79[1]	4.80[1]	*0.018*	*0.021*	3.47-6.54	1.19-7.08
Aspartate	6.69	6.38[1]	6.42[1]	*0.020*	*0.038*	4.87-8.94	3.35 – 12.08

Amino acid	Control (mg/g dw)	Sprayed MZHG0JG (mg/g dw)	Unspray MZHG0JG (mg/g dw)	P (sprayed v control)	P (unspray v control)	Reference range (mg/g dw)	Combined literature range (mg/g dw)
Cystine	2.02	2.07	2.01	0.059	0.793	1.52-2.59	1.16-5.14
Glutamate	19.4	18.6	18.6	0.098	0.109	12.8-28.9	9.65-35.40
Glycine	3.80	3.73	3.77	0.107	0.505	2.70-4.82	1.84-6.85
Histidine	2.60	2.51	2.52	0.083	0.106	1.95-3.58	1.37-4.56
Isoleucine	3.57	3.41	3.44	0.069	0.127	2.38-5.18	1.79-6.92
Leucine	13.1	12.5	12.5	0.104	0.114	8.30-20.7	6.42-24.92
Lysine	2.96	2.85[1]	2.89	*0.010*	0.095	1.88-3.85	1.29-6.68
Methionine	2.19	2.24	2.22	0.240	0.431	1.51-2.49	1.05-4.68
Phenylalanine	5.26	4.98	4.98	0.058	0.055	3.52-7.92	2.44-9.30
Proline	9.08	8.70	8.70	0.054	0.055	5.97-12.6	4.62-17.5
Serine	4.75	4.56	4.57	0.070	0.086	3.33-7.04	1.82-7.69
Threonine	3.61	3.50	3.51	0.081	0.123	2.56-4.74	2.19-6.66
Tryptophan	0.859	0.838[1]	0.835[1]	*0.019*	*0.009*	0.639-1.02	0.271-2.15
Tyrosine	4.09	4.06	4.02	0.784	0.414	2.69-6.09	1.03-7.34
Valine	4.66	4.46	4.48	0.057	0.074	3.27-6.23	2.66-8.55

[1] Mauve shading represents MZHG0JG mean significantly lower than the control

5.3.4 Minerals

The levels of 10 minerals in grain from MZHG0JG and the hybrid control were measured. For selenium and sodium, levels below the LOQ precluded calculation of the means and statistical comparisons across locations. Results for the remaining eight analytes are given in Table 11 and show that the means for copper and calcium in both the sprayed and unsprayed MZHG0JG samples were significantly lower than the means from the control; however, the means were within both the reference and literature ranges. For the other minerals, there was no significant difference between the means for the control and the means for both MZHG0JG treatments.

Table 11: Mean levels of minerals in the grain of MZHG0JG and the hybrid control

Mineral	Control (mg/kg dw)	Sprayed MZHG0JG (mg/kg dw)	Unspray MZHG0JG (mg/kg dw)	P (sprayed v control)	P (unspray v control)	Reference range (mg/kg dw)	Combined literature range (mg/kg dw)
Calcium	36.0	35.3	34.8	0.339	0.086	27.4-59.1	<LOQ-1010.0
Copper	2.06	1.76[1]	1.75[1]	*<0.001*	*<0.001*	1.33-3.20	<LOQ-21.20
Iron	19.3	18.7[1]	18.5[1]	*0.028*	*0.008*	13.4-28.8	9.51-191.0
Magnesium	1177	1183	1161	0.734	0.305	867-1400	594.0-1940.0
Manganese	6.02	5.81	5.73	0.229	0.111	3.15-9.10	1.69-14.30
Phosphorus	3033	3071	3012	0.432	0.649	2410-3750	1300-5520
Potassium	3593	3570	3517	0.594	0.089	3170-4640	1810-6030
Zinc	20.9	20.8	20.4	0.728	0.152	12.7-29.3	6.5-42.6

[1] Mauve shading represents MZHG0JG means significantly lower than the control

5.3.5 Vitamins

Levels of seven vitamins were measured. The results in Table 12 show:

- there was no significant difference between the control and MZHG0JG for the means of riboflavin, niacin and folic acid.
- the means for vitamin A in both sprayed and unsprayed MZHG0JG plants were significantly higher than the control mean.
- the means for thiamine HCl, pyridoxine HCl and vitamin E were significantly lower (in one or both of the MZHG0JG treatments) than the control mean.
- all means for all vitamins in all treatments fell within both the reference and literature ranges.

Table 12: Mean weight of vitamins in grain from MZHG0JG and the hybrid control

Vitamin	Control (mg/kg dw)	Sprayed MZHG0JG (mg/kg dw)	Unspray MZHG0JG (mg/kg dw)	P (sprayed v control)	P (unspray v control)	Reference range (mg/kg dw)	Combined literature range (mg/kg dw)
Vitamin A (β-Carotene)	1.45	1.78[1]	1.69[1]	*<0.001*	*<0.001*	0.064-0.318	0.19 – 49.9
Vitamin B_1 (Thiamine HCl)	3.77	3.57[1]	3.70	*0.011*	0.291	0.249-0.506	1.26 – 40.00
Vitamin B_2 (Riboflavin)	2.20	2.02	2.04	0.182	0.255	0.114-0.375	0.50 – 7.3
Vitamin B_3 (Niacin)	20.4	20.5	21.1	0.806	0.065	1.55-4.17	10.37 – 46.94
Vitamin B_6 (Pyridoxine HCl)	5.52	5.31	5.21[1]	0.112	*0.030*	0.365-0.910	3.68 – 12.14
Vitamin B9 (Folic acid)	0.43	0.439	0.459	0.668	0.120	0.0232-0.0640	0.147 – 3.5
Vitamin E (α-Tocopherol	12	11.6[1]	11.7[1]	*0.003*	*0.013*	0.76-2.221	1.537 – 68.672

[1] Orange shading represents MZHG0JG means significantly higher than the control; mauve shading represents MZHG0JG means significantly lower than the control

5.3.6 Anti-nutrients

Levels of three key anti-nutrients were measured. Results in Table 13 show that none of the means differed significantly between MZHG0JG and the control. All means also fell within both the reference and literature ranges.

Table 13: Mean of anti-nutrients in grain from MZHG0JG and the hybrid control

Vitamin	Control (mg/kg dw)	Sprayed MZHG0JG (mg/kg dw)	Unspray MZHG0JG (mg/kg dw)	P (sprayed v control)	P (unspray v control)	Reference range (mg/kg dw)	Combined literature range (mg/kg dw)
Phytic acid (%dw)	0.883	0.876	0.840	0.783	0.108	0.503-1.34	<LOQ–1.570
Raffinose %dw)	0.113	0.116	0.116	0.444	0.482	<LOQ-0.386	<LOQ-0.443
Trypsin inhibitor (TIU/mg)	3.87	4.04	4.05	0.322	0.291	1.67-6.09	<LOQ-8.42

5.3.7 Secondary metabolites

The levels of four secondary metabolites were measured (see Table 14), Measurements for furfural were below the LOQ and were excluded from analysis.

For p-coumaric acid and inositol the means were significantly higher (in one or both of the MZHG0JG treatments) than the control mean. There was no significant difference between the means of the control and MZHG0JG for ferulic acid. All means for all three secondary metabolites in all treatments fell within both the reference and literature ranges.

Table 14: Mean level of three secondary metabolites in grain from MZHG0JG and the hybrid control

Secondary metabolite	Control	Sprayed MZHG0JG	Unspray MZHG0JG	P (sprayed v control)	P (unspray v control)	Reference range	Combined literature range
p-coumaric acid (mg/kg)	303	340[1]	347[1]	*<0.001*	*<0.001*	113-435	<LOQ-820.0
Ferulic acid (mg/kg)	3387	3431	3409	0.180	0.501	1700-2920	291.9 – 4397.3
Inositol (ppm)	2528	2757[1]	2481	*0.045*	0.659	1720-3890	<LOQ-4750.0

[1] Orange shading represents MZHG0JG means significantly higher than the control;

5.3.8   Summary of analysis of key components

A summary of the statistically significant differences in the analyte levels found between grain of MZHG0JG and the control is provided in Table 15.

Table 15: Summary of analyte levels found in grain of MZHG0JG that are significantly (P < 0.05) different from those found in grain of the control

Analyte	MZHG0JG (sprayed) mean[1]	MZHG0JG (unsprayed) mean[1]	Control mean	Max difference between MZHG0JG & control	Diff between max and min in control	MZHG0JG within ref range?	MZHG0JG within lit range?
NDF (%dw)	11.1	11.0	11.5	0.5	4.78	yes	yes
Palmitic acid (% total)	14.1	14.1	14.3	0.2	3.8	yes	yes
Heptadec. Acid (% total)	0.0858	0.0866	0.0834	0.0032	0.051	yes	yes
Linolenic acid (% total)	1.82	1.81	1.78	0.04	0.73	yes	yes
Arginine (%dw)	4.79	4.80	4.95	0.16	3.07	yes	yes
Aspartate (%dw)	6.38	6.42	6.69	0.31	4.07	yes	yes
Lysine (%dw)	2.85	2.89	2.96	0.11	1.97	yes	yes
Tryptophan (%dw)	0.838	0.835	0.859	0.024	0.381	yes	yes
Copper (mg/kg dw)	1.76	1.75	2.06	0.31	1.87	yes	yes
Iron (mg/kg dw)	18.7	18.5	19.3	0.8	15.4	yes	yes
Vitamin A (mg/kg dw)	1.78	1.69	1.45	0.33	0.254	yes	yes
Vitamin B$_1$ (mg/kg dw)	3.57	3.70	3.77	0.20	0.257	yes	yes
Vitamin B$_6$ (mg/kg dw)	5.31	5.21	5.52	0.31	0.545	yes	yes
Vitamin E (mg/kg dw)	11.6	11.7	12	0.4	1.461	yes	yes
p-Coumaric acid (mg/kg)	340	347	303	44	322	yes	yes

Analyte	MZHG0JG (sprayed) mean[1]	MZHG0JG (unsprayed) mean[1]	Control mean	Max difference between MZHG0JG & control	Diff between max and min in control	MZHG0JG within ref range?	MZHG0JG within lit range?
Inositol (ppm)	2757	2481	2528	229	2,170	yes	yes

[1] mauve shading represents MZHG0JG means that are significantly lower than the control means while orange shading represents MZHG0JG means that are significantly higher.

5.4   Conclusion from compositional analyses

Detailed compositional analyses were done to establish the nutritional adequacy of grain from MZHG0JG and to characterise any unintended compositional changes. Analyses were done of proximates, fibre, minerals, amino acids, fatty acids, vitamins, secondary metabolites and anti-nutrients. The levels were compared to levels in a) an appropriate non-GM hybrid line, N2319 x N2222 b) a reference range compiled from results taken for six non-GM hybrid lines grown under the same conditions and c) levels recorded in the literature. Only 16 of the 57 analytes reported in Tables 8 – 14 deviated from the control in a statistically significant manner; for five of these the difference occurred only in one of the MZHG0JG treatments. However, the mean levels of all of these analytes fell within both the reference range and the historical range from the literature. It is also noted that, with the exception of vitamin A (red text in Table 14), the difference between each analyte mean of MZHG0JG and the control in Table 15 was smaller than the variation within the control. It can therefore be concluded that grain from MZHG0JG is compositionally equivalent to grain from conventional corn varieties.

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.

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.

Where a GM food has been shown to be compositionally equivalent to conventional varieties, the evidence to date indicates that feeding studies will add little to the safety assessment and generally are not warranted (see e.g. OECD 2003; Bartholomaeus et al. 2013; Herman and Ekmay 2014). MZHG0JG is the result of a genetic modification designed to provide tolerance to two herbicides with no intention to significantly alter nutritional parameters in the food. In addition, the extensive compositional analyses of grain that have been undertaken to demonstrate the nutritional adequacy of MZHG0JG indicate it is equivalent in composition to conventional corn cultivars. The introduction of food from MZHG0JG into the food supply is therefore expected to have little nutritional impact and as such no additional studies, including animal whole food feeding studies, are required.

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