

## Supporting document 1

### Safety Assessment Report – Application A1116

Food derived from Herbicide-tolerant & Insect-protected Corn Line  
MZIR098

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## Summary and conclusions

### Background

A genetically modified (GM) corn line with OECD Unique Identifier SYN-00098-3 (henceforth referred to as MZIR098) has been developed by Syngenta. The corn has been modified to be tolerant to the herbicide glufosinate ammonium (glufosinate) and protected against coleopteran pests, particularly western corn rootworm (*Diabrotica virgifera virgifera*).

Tolerance to glufosinate ammonium is achieved through expression of the enzyme phosphinothricin acetyltransferase (PAT) encoded by the *pat-08* gene (hereafter referred to as *pat*) derived from the common soil bacterium *Streptomyces viridochromogenes*. Protection against coleopteran insect pests is conferred by the expression in the plant of two Cry proteins, a modified Cry3Aa2 protein (mCry3Aa2) encoded by the *mcry3Aa2* gene based on the *cry3Aa2* gene from *Bacillus thuringiensis* ssp. *tenebrionis*, and the eCry3.1Ab protein encoded by a chimeric gene comprising selected sequences of the *mcry3Aa2* gene, and the *cry1Ab3* gene from *B. thuringiensis* ssp. *kurstaki* HD1.

In conducting a safety assessment of food derived from MZIR098, 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**

MZIR098 was generated through *Agrobacterium*-mediated transformation and contains three expression cassettes. Comprehensive molecular analyses of MZIR098 indicate there is a single insertion site containing a single complete copy of each of the *pat*, *mcry3Aa2* and *ecry3.1Ab* genes plus 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 MZIR098 expresses three new proteins, eCry3.1Ab, mCry3Aa2 and PAT. The mean levels of eCry3.1Ab varied considerably between the different plant parts and growth stages, being highest in leaves at the V6 stage and undetectable in pollen; the level in grain was very low. For the mCry3Aa protein, levels were highest in the pollen and lowest in the grain. For PAT, mean levels were low across all plant parts and growth stages but were highest in leaves at the V6 stage and essentially at or below the level of detection in a number of tissues, including the grain.

A range of characterisation studies confirmed the identity of the eCry3.1Ab, mCry3Aa2 and PAT proteins produced in MZIR098 and also their equivalence with the corresponding proteins produced in a bacterial expression system. The plant-expressed eCry3.1Ab, mCry3Aa and PAT proteins have the expected molecular weights, immunoreactivity, lack of glycosylation, amino acid sequence and activity.

There are no concerns regarding the potential toxicity or allergenicity of the expressed proteins. Previous safety assessments of eCry3.1Ab, mCry3Aa2 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.

### **Herbicide Metabolites**

There are no concerns that the spraying of line MZIR098 with glufosinate would result in the production of any novel metabolites that have not been previously assessed.

## **Compositional analyses**

Detailed compositional analyses established the nutritional adequacy of grain from MZIR098 and characterised 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 MZIR098 given two treatments (herbicide-sprayed and unsprayed). The levels were compared to levels in a) an appropriate non-GM hybrid line, 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 13 of the 57 analytes that were reported deviated from the control in a statistically significant manner; for six of these the difference occurred only in one of the MZIR098 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 the differences between these statistically significant means of MZIR098 and the control means were smaller than the variation within the control. It can therefore be concluded that grain from MZIR098 is compositionally equivalent to grain from conventional corn varieties.

## **Conclusion**

No potential public health and safety concerns have been identified in the assessment of MZIR098. On the basis of the data provided in the present Application, and other available information, food derived from MZIR098 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
BLASTP	Basic Local Alignment Search Tool Protein
bp	base pairs
<i>Bt</i>	<i>Bacillus thuringiensis</i>
CaMV	Cauliflower mosaic virus
CFIA	Canadian Food Inspection Agency
CmYLC	Cestrum yellow leaf curling virus
CPB	Colorado potato beetle
Cry	crystal
DNA	deoxyribonucleic acid
T-DNA	transferred DNA
dw	dry weight
ELISA	enzyme linked immunosorbent assay
FAO	Food and Agriculture Organization of the United Nations
FARRP	Food Allergy Research and Resource Program
FASTA	Fast Alignment Search Tool - All
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
MRL	Maximum Residue Level
mw	molecular weight
NCBI	National Center for Biotechnology Information
NDF	neutral detergent fibre
nos	nopaline synthase
OECD	Organisation for Economic Co-operation and Development
OGTR	Australian Government Office of the Gene Technology Regulator
ORF	open reading frame
P or P-value	probability value
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
USA	United States of America
UTR	untranslated region
WHO	World Health Organization

# 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, MZIR098, with OECD Unique Identifier SYN-00098-3 (henceforth referred to as MZIR098). The corn has been modified to be tolerant to the herbicide glufosinate ammonium (glufosinate) and to be protected against coleopteran pests, particularly western corn rootworm (*Diabrotica virgifera virgifera*), northern corn rootworm (*Diabrotica berberis*), and Mexican corn rootworm (*Diabrotica virgifera zea*). These species are serious insect pests of dent corn in the major corn-producing states of the north-central United States of America (USA) and Canada.

Tolerance to glufosinate ammonium is achieved through expression of the enzyme phosphinothricin acetyltransferase (PAT) encoded by the *pat-08* gene (henceforth referred to as *pat*) derived from the common soil bacterium *Streptomyces viridochromogenes*. This protein has been considered in 21 previous FSANZ applications and globally is represented in six major crop species and over 30 approved GM single plant events<sup>1</sup>.

Protection against coleopteran insect pests is conferred by the expression in the plant of a modified Cry3Aa<sup>2</sup> protein designated mCry3Aa<sup>2</sup><sup>3</sup> (based on sequence from the *cry3Aa2* gene from *Bacillus thuringiensis* ssp. *tenebrionis*) and eCry3.1Ab (encoded by a chimeric gene comprising selected sequences of the *mcry3Aa2* gene, and the *cry1Ab* gene from *B. thuringiensis* ssp. *kurstaki*). Both mCry3Aa2 alone and eCry3.1Ab have been considered previously by FSANZ in applications A564 (FSANZ 2006a) and A1060 (FSANZ 2012) respectively. The combination of the two proteins in the same corn hybrid is claimed by the Applicant to offer advantages for insect resistance management since they have two different binding sites in the target insect.

The Applicant has stated that MZIR098 will be crossed by conventional breeding with other approved GM corn lines (a process known as 'stacking'). Thus, another advantage of MZIR098 is that, by combining three traits into a single breeding locus, it will allow simplification of future breeding strategies to obtain other elite lines.

The Applicant states the intention is that any lines containing the SYN-00098-3 event will be grown in North America, and 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 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).

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

<sup>2</sup> For details of unmodified Cry protein nomenclature see [http://www.lifesci.sussex.ac.uk/home/Neil\\_Crickmore/Bt/toxins2.html](http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/toxins2.html)

<sup>3</sup> Note in some figures and tables, this protein (gene) is referred to just as mCry3A (*mcry3A*)

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.

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, ahead of wheat and rice and is grown in over 160 countries (FAOSTAT3 2015). In 2013, worldwide production of corn was over 1 billion tonnes, with the USA 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 USA it is estimated that approximately 93% of all corn planted is GM<sup>4</sup> while in Canada, the estimate of GM corn is approximately 80% of the total corn<sup>5</sup>. No GM corn is currently grown commercially in Australia or New Zealand.

Domestic production in Australia and New Zealand 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 cornstarch. 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. mays* var. *indentata*
- Sweet – *Z. mays* var. *saccharata* & *Z. mays* var. *rugosa*
- Pop – *Z. mays* var. *everta*

Dent corn is the type most commonly grown for grain and silage and is the predominant type grown in the USA (OGTR 2008). The parent line that was transformed to give MZIR098 is an elite Syngenta proprietary conventional inbred corn line, NP2222 (Delzer 2004) that is responsive to *Agrobacterium*-mediated transformation and regeneration in tissue culture. It is a Stiff-Stalk family<sup>6</sup>, yellow dent corn line but could be crossed with other types.

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<sup>4</sup> USDA, Economic Research Service, - <http://www.ers.usda.gov/data-products/adoption-of-genetically-engineered-crops-in-the-us.aspx>

<sup>5</sup> USDA Gain Report, CA14062, 2014 - [http://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual\\_Ottawa\\_Canada\\_7-14-2014.pdf](http://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Ottawa_Canada_7-14-2014.pdf)

<sup>6</sup> 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).



Two main grain processing routes are followed for dent corn (White and Pollak 1995):

- 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.

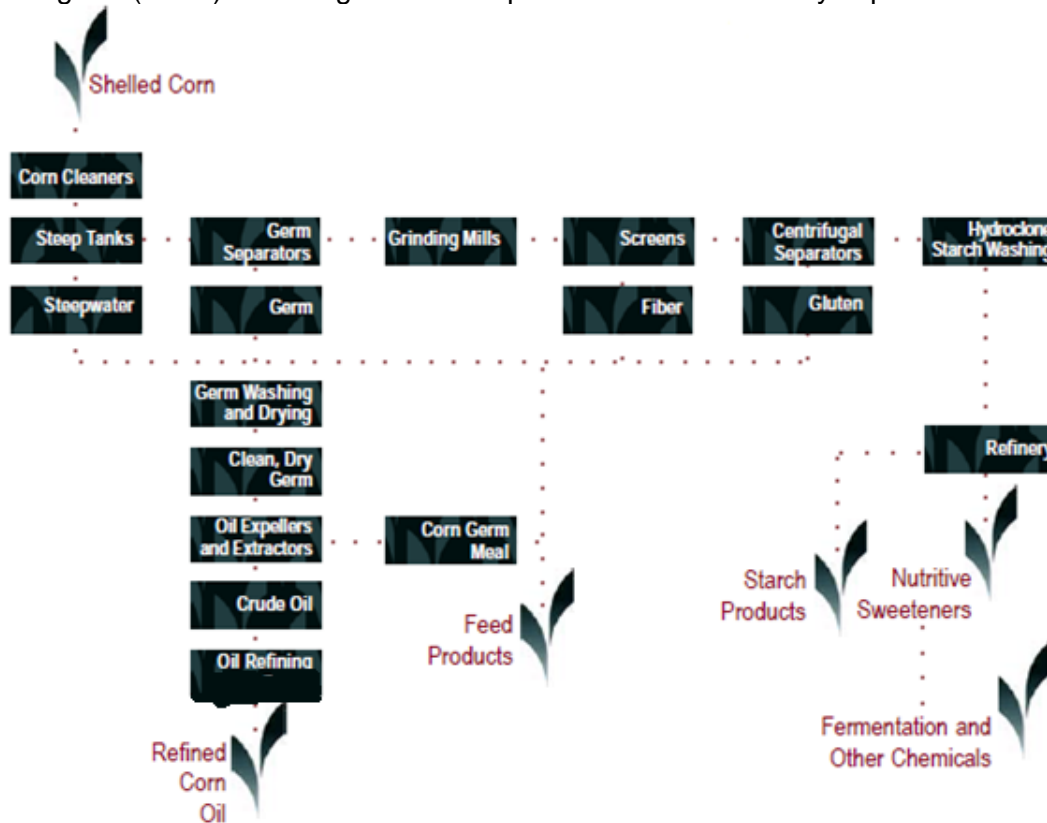


Figure 1: The corn wet milling process (diagram taken from CRA (2006))

## 2.2 Donor organisms

### 2.2.1 *Bacillus thuringiensis*

Many different subspecies of *Bacillus thuringiensis* (*Bt*) have been isolated from dead or dying insects, mostly from the orders Coleoptera, Diptera and Lepidoptera, but many subspecies have also been found in the soil, aquatic environments and other habitats (WHO 1999).

Studies on mammals, particularly laboratory animals, demonstrate that *B. thuringiensis* is mostly non-pathogenic and non-toxic. *B. thuringiensis* has been demonstrated to be highly specific in its insecticidal activity and has demonstrated little, if any, direct toxicity to non-target insects (see NPTN 2000; OECD 2007 and references therein). Infection in humans is unusual although there have been at least two clinical reports, one in the wounds of a soldier (Hernandez et al. 1998) and one in burn wounds (Damgaard et al. 1997), and in both cases impaired immunosuppression was implicated in the cause of the infection. *B. thuringiensis* has also been rarely associated with gastroenteritis (see eg Jackson et al. 1995) but generally, *B. thuringiensis* present in drinking water or food has not been reported to cause adverse effects on human health (WHO 1999; NPTN 2000; OECD 2007).

The effect of *B. thuringiensis* products on human health and the environment was the subject of a critical review by the WHO International Programme on Chemical Safety (WHO 1999). The review concluded that '*B. thuringiensis* products are unlikely to pose any hazard to humans or other vertebrates or the great majority of non-target invertebrates provided that they are free from non-*B. thuringiensis* microorganisms and biologically active products other than the insecticidal proteins'. Approved GM crops incorporating *Bt* Cry proteins have been available for a number of years and have not raised any food safety concerns (Koch et al 2015).

Commercial *Bt* products are powders containing a mixture of dried spores and toxin crystals and have a long history of safe use as a biopesticide for insect control (Siegel 2001; Federici 2005; Narva et al. 2014). Such products are approved for use on crops in Australia<sup>7</sup> and New Zealand<sup>8</sup> and in both countries there is an exemption from maximum residue limits (MRLs) when *Bt* is used as an insecticide<sup>9</sup>.

### 2.2.2 *Streptomyces viridochromogenes*

The source of the *pat* gene is the bacterial species *Streptomyces viridochromogenes*, strain Tü494 (Wohlleben et al. 1988). The *Streptomycetaceae* 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. hygrosopicus* 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.

### 2.2.3 Other organisms

Genetic elements from several other organisms have been used in the genetic modification of MZIR098 (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 Cestrum yellow leaf curling virus (CmYLC), Cauliflower mosaic virus (CaMV) and *Agrobacterium tumefaciens* are not pathogenic in themselves and do not cause pathogenic symptoms in MZIR098.

## 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

<sup>7</sup> <https://portal.apvma.gov.au/pubcris>

<sup>8</sup> <http://www.biosecurity.govt.nz/pests-diseases/forests/white-spotted-tussock-moth/about-btk.htm>

<sup>9</sup> (<http://www.foodsafety.govt.nz/elibrary/industry/nz-mrl-agricultural-compounds-food-standards-07-2014.pdf> ; <http://www.comlaw.gov.au/Details/F2014C00821>).

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

**Studies submitted:**

2015. Event MZIR098 maize: Insert copy number Southern blot analysis. Final report **TK0117497**. Syngenta Seeds, Inc. (unpublished)

2014. Event MZIR098 maize: Insert and flanking sequence analysis. Final report **TK0144270**. Syngenta Seeds, Inc. (unpublished)

2015. Event MZIR098 maize: Genomic insertion site analysis. Final report **TK0117489**. Syngenta Seeds, Inc. (unpublished)

2015. Event MZIR098: Allergenicity and toxicity assessment of start to stop, genome to insert junction open reading frames. Assessment **SSB-131-15**. Syngenta Seeds, Inc. (unpublished)

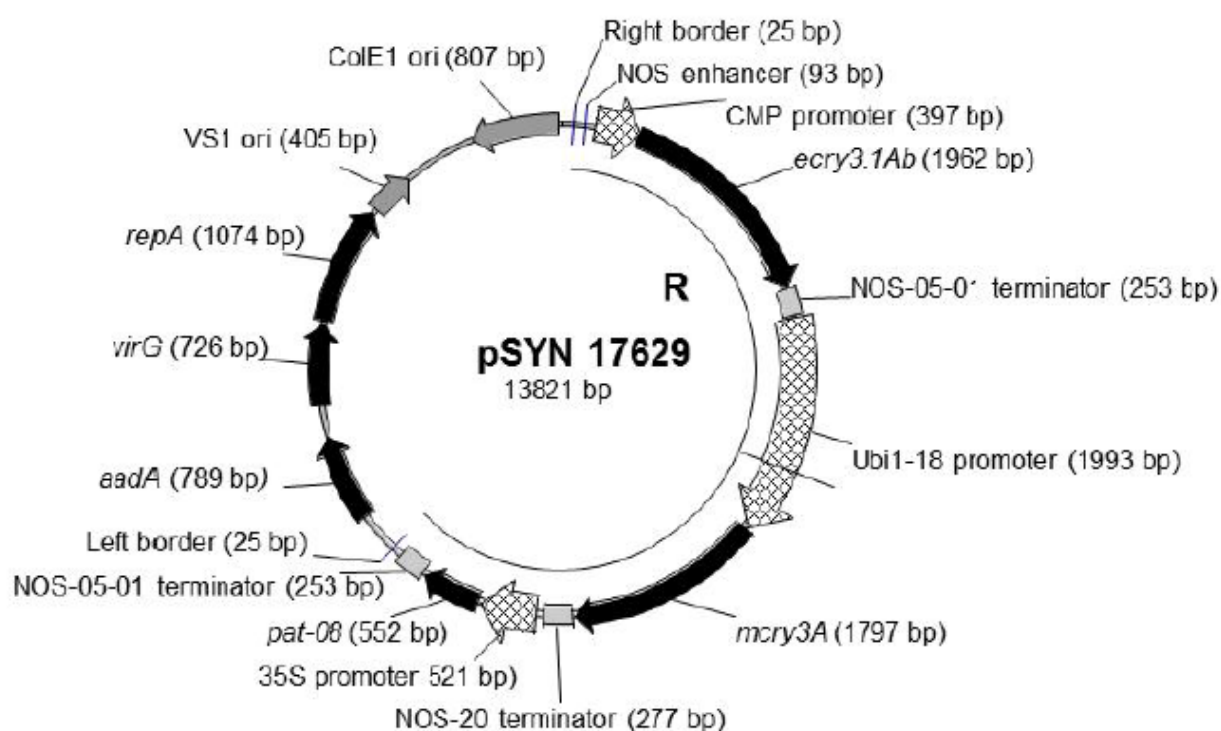
2015. Event MZIR098 maize: Genetic stability analysis of F<sub>2</sub>, F<sub>3</sub>, F<sub>4</sub>, F<sub>5</sub> and F<sub>1</sub> generations. Final report, Amendment 1 **TK0117501** A1. Syngenta Seeds, Inc. (unpublished)

2013. Event MZIR098 maize: Mendelian inheritance analysis. Final report **TK0117487**. 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 pSYN17629 (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<sub>0</sub>) with normal phenotypic characteristics, containing the *ecry3.1Ab*, *mcry3Aa2* and *pat* genes 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). MZIR098 was ultimately chosen as the lead event based on superior agronomic, phenotypic and molecular characteristics.



R = Region intended for insertion.

Figure 2: Genes and regulatory elements contained in plasmid pSYN17629

### 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 three cassettes comprising a total of 8,462 bp located between a 25 bp LB and a 25 bp RB. The complete plasmid is 13,821 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 pSYN17629

Genetic element	Relative bp location on plasmid	Size (bp)	Source	Orient.	Description & Function	References
RIGHT BORDER	1 - 25	25				
<b>ecry3.1Ab cassette</b>						
Intervening sequence	26 - 105	80				
<i>nos-02</i>	106 - 198	93	<i>Agrobacterium tumefaciens</i>	Clockwise	<ul style="list-style-type: none"> <li>Enhancer sequence (NCBI accession # V00087.1)</li> <li>Increases expression of <i>ecry3.1Ab</i></li> </ul>	Bevan et al. (1983)
Intervening sequence	199 - 203	5				
<i>CMP</i>	204 - 600	397	Cestrum yellow leaf curling virus (CmYLC)	Clockwise	<ul style="list-style-type: none"> <li>Constitutive promoter</li> <li>Directs transcription of the <i>ecry3.1Ab</i> gene</li> </ul>	Hohn et al. (2007)
Intervening sequence	601 - 609	9				

Genetic element	Relative bp location on plasmid	Size (bp)	Source	Orient.	Description & Function	References
<b>ecry3.1Ab</b>	610 - 2571	1,962	<i>B. thuringiensis</i> ssp. <i>tenebrionis</i> ; <i>B. thuringiensis</i> ssp. <i>kurstaki</i>	Clockwise	• A chimeric gene combining sequences from <i>cry3Aa</i> and <i>cry1Ab</i> (NCBI accession # GU327680)	Walters et al. (2010)
Intervening sequence	2572 - 2592	21				
<i>nos-05-01</i>	2593 - 2845	253	<i>Agrobacterium tumefaciens</i>	Clockwise	• Terminator region from the <i>nopaline synthase</i> gene (NCBI accession # V00087.1) • Terminates mRNA transcription and induces polyadenylation of the <i>ecry3.1Ab</i> mRNA	Bevan et al. (1983)
<b>mcry3Aa2 cassette</b>						
Intervening sequence	2846 - 2865	20				
<i>Ubi1-18</i>	2866 - 4858	1,993	<i>Zea mays</i> (corn)	Clockwise	• Promoter from the ubiquitin gene (similar to NCBI accession # S94464.1) • Directs constitutive transcription of the <i>mcry3A</i> gene	Christensen et al. (1992)
Intervening sequence	4859 - 4867	9				
<b>mcry3Aa2</b>	4868 - 6664	1,797	<i>B. thuringiensis</i> ssp. <i>tenebrionis</i>	Clockwise	• Synthetic, corn-optimised gene based on native <i>cry3A</i> gene	Walters et al. (2008)
Intervening sequence	6665 - 6683	19				
<i>nos-20</i>	6684 - 6960	277	<i>Agrobacterium tumefaciens</i>	Clockwise	• Terminator region from the <i>nopaline synthase</i> gene (NCBI accession # V00087.1) • Terminates mRNA transcription and induces polyadenylation of the <i>ecry3.1Ab</i> mRNA	Bevan et al. (1983)
<b>pat cassette</b>						
Intervening sequence	6961 - 7019	59				
<i>35S-04</i>	7020 - 7540	521	Cauliflower mosaic virus (CaMV)	Clockwise	• Promoter from the 35S gene • Drives constitutive transcription of the <i>pat</i> gene	Franck et al. (1980); Odell et al. (1985); Pietrzak et al. (1986)
Intervening sequence	7541 - 7564	24				
<i>pat-08</i>	7565 - 8116	552	<i>Streptomyces viridochromogenes</i> strain Tü494	Clockwise	Phosphinothricin acetyltransferase coding sequence (similar to NCBI accession # DQ156557.1)	Wohlleben et al. (1988)
Intervening sequence	8117 - 8147	31				
<i>nos-05-01</i>	8148 - 8400	253	<i>Agrobacterium tumefaciens</i>	Clockwise	• Terminator region from the <i>nopaline synthase</i> gene (NCBI accession # V00087.1) • Directs polyadenylation of the <i>pat</i> gene	Depicket al. (1982)
Intervening sequence	8401 - 8487	87				
LEFT BORDER	8488 - 8512	25				

### 3.2.1 *ecry3.1Ab* expression cassette

The cassette is 2,820 bp in length. The gene *ecry3.1Ab* (Walters et al. 2010) consists of a fusion between the 5' end (Domain I, Domain II and 15 aa of Domain III)<sup>10</sup> of *mcry3Aa2* (see below) and the 3' end [Domain III and Variable Region 6 (Höfte and Whiteley 1989)] of the *cry1Ab* gene. Upstream of the *mcry3Aa2* segment, the *ecry3.1Ab* coding region carries a 67 bp long oligomer extension at its 5' end, which was introduced during the engineering of the variable regions (see Section 4.1.1.1)

The *cry1Ab* gene was originally cloned from *Bacillus thuringiensis* ssp. *kurstaki* strain HD-1 (Geiser et al. 1986). The DNA encoding the protein has been codon-optimised to accommodate the preferred codon usage for corn.

In MZIR098, the *ecry3.1Ab* gene is driven by the constitutive promoter from the CmYLC virus. Transcription is terminated by the polyadenylation signal from the 3' UTR of the *nopaline synthase* (*nos*) gene from *A. tumefaciens*.

### 3.2.2 *mcry3Aa2* expression cassette

The cassette is 4,115 bp in length. The corn-optimised modified *mcry3Aa2* gene encodes a protein of 598 amino acids. The entire coding region of the *mcry3Aa2* gene was synthesised using codons that are preferred in corn (Murray et al. 1989), and to introduce other desired changes to facilitate cloning steps. The synthetic sequence was based on the native Cry3Aa2 protein sequence from *Bacillus thuringiensis* ssp. *tenebrionis* (Sekar et al. 1987). The corn-optimised gene was then modified to incorporate a consensus cathepsin-G protease recognition site within the expressed protein (see Section 4.1.1.2).

The *mcry3Aa2* gene in MZIR098 is regulated by the constitutive promoter from the polyubiquitin gene from *Zea mays*, and the *nos* terminator from *A. tumefaciens*. The *nos* terminator is a variation of the terminator used in the *ecry3.1Ab* cassette and has nucleotide changes to eliminate cross-border unintended open reading frames (ORFs).

### 3.2.3 *pat* expression cassette

The cassette is 1,527 bp in length. The *pat* gene encodes a protein of 183 amino acids 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<sup>11</sup>, 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 *nos* gene of *Agrobacterium tumefaciens*.

## 3.3 Breeding of MZIR098

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

A single T<sub>0</sub> plant was crossed with the parental line NP2222 to generate a hybrid line (designated AF<sub>1</sub> in Figure 3) that was then self-pollinated over several rounds of crossing to obtain seven hybrid generations. MZIR098 is the F<sub>1</sub> (designated CF<sub>1</sub> in Figure 3) result of a cross between the T<sub>7</sub> generation and the Syngenta proprietary, non-GM inbred line NP2391.

<sup>10</sup> For discussion of the structure of Cry proteins with three Domains see Section 4.1.1

<sup>11</sup> 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.

In order to generate hybrid lines to ascertain Mendelian inheritance, hemizygous plants of the F<sub>2</sub> generation were crossed with NP2391. The resulting F<sub>1</sub> generation (designated BF<sub>1</sub> in Figure 3) was backcrossed with the NP2391 recurrent parent to yield the BC<sub>1</sub>F<sub>1</sub> generation. Plants from the BC<sub>1</sub>F<sub>1</sub> generation were backcrossed three more times with NP2391 to yield the BC<sub>2</sub>F<sub>1</sub> and BC<sub>3</sub>F<sub>1</sub> and BC<sub>4</sub>F<sub>1</sub> generations. Only positive hemizygous segregants (determined by tolerance to glufosinate and real-time PCR analysis) were used in each backcross.

Based on fit for various studies, non-GM lines NP2391, and NP2391 x NP2222 were used as controls, in addition to the non-GM parental line NP2222 (see Table 2).

- ⊗ Self-pollinated
- X Crossed with

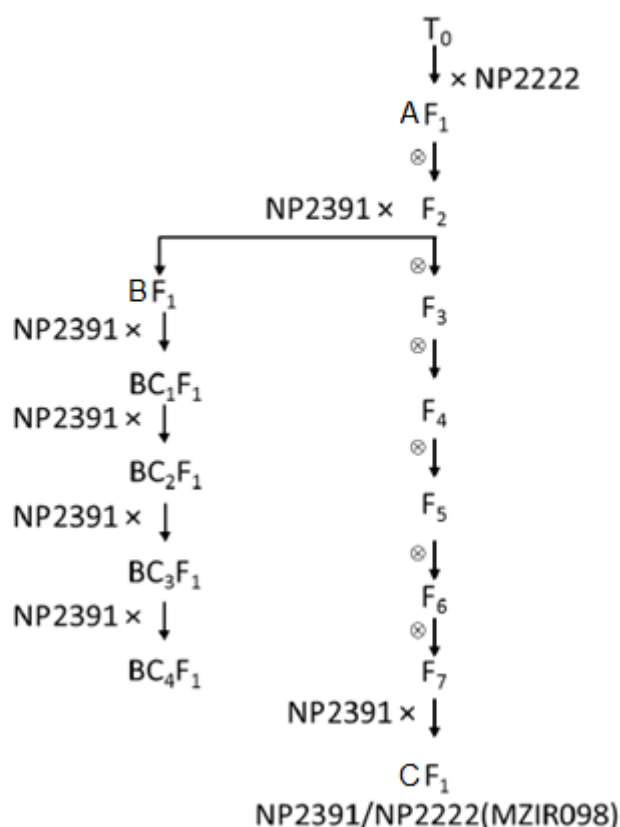


Figure 3: Breeding diagram for MZIR098

Table 2: MZIR098 generations used for various analyses

Analysis	MZIR098 generation used	Control(s) used	Reference comparators
Molecular characterisation (Section 3.4)	AF <sub>1</sub> , F <sub>2</sub> , F <sub>3</sub>	NP2222	
Genetic stability (Section 3.5.1)	F <sub>2</sub> , F <sub>3</sub> , F <sub>4</sub> , F <sub>5</sub> and CF <sub>1</sub>	NP2222, NP2391, NP2391 x NP2222	
Mendelian inheritance (Section 3.5.2)	BC <sub>2</sub> F <sub>1</sub> ; BC <sub>3</sub> F <sub>1</sub> ; BC <sub>4</sub> F <sub>1</sub>	N/A	
Protein expression levels in plant parts (Section 4.1.2)	CF <sub>1</sub>	NP2391 x NP2222	



Analysis	MZIR098 generation used	Control(s) used	Reference comparators
Protein characterisation (Section 4.1.3)	CF <sub>1</sub> -produced protein	N/A	<i>E.coli</i> -produced protein
Compositional analysis (Section 5)	CF <sub>1</sub>	NP2391 x NP2222	6 non-GM commercial reference lines

### 3.4 Characterisation of the genetic modification in the plant

A range of analyses were undertaken to characterise the genetic modification in MZIR098. These analyses focussed on the nature of the insertion of the introduced genetic elements and whether any unintended re-arrangements may have occurred as a consequence of the transformation procedure.

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

Total genomic DNA from verified (real time PCR) pooled leaf tissue of two MZIR098 generations (AF<sub>1</sub> and F<sub>2</sub>) and from the verified negative control (NP2222) was used for Southern blots to determine the number of T-DNA insertions and the sequence integrity of the introduced DNA in line MZIR098, and test for the presence or absence of plasmid vector backbone sequences.

##### 3.4.1.1 Number of integration sites

For determining the number of integration sites in MZIR098 two pairs of restriction enzymes were used (each one cutting once within the MZIR098 insert and at least once within the flanking regions to give two fragments) in combination with three <sup>32</sup>P-labelled probes that covered every base pair of the pSYN17629 T-DNA. 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 points were relevant:

- Any bands in the negative control that are also present in MZIR098 would indicate cross-hybridisation of the T-DNA-specific probe with an endogenous maize sequence.
- Expected hybridisation bands present in MZIR098 and absent in the negative control would indicate the bands are specific to the MZIR098 insert.
- Additional bands present in MZIR098 but not in the negative control would indicate the presence of additional copies of the insert in the MZIR098 genome.
- T-DNA-specific hybridisation bands were not expected in the negative control.
- Each of the T-DNA-specific positive assay controls was expected to result in one hybridisation band.

For all restriction enzyme/probe combinations for the two MZIR098 generations and the positive controls, the expected hybridisation bands were obtained. This indicates that MZIR098 contains a single copy of the insert at a single site.

##### 3.4.1.2 Insert integrity

In this Southern analysis, DNA was doubly digested with two restriction enzymes that recognise the same palindromic DNA sequence, cut twice each in the T-DNA near each border and should generate a fragment of approximately 8.3 kb in MZIR098 samples.



The positive controls should give a different hybridisation band. The same three probes and controls described for the insert number analysis were used. The expected 8.3 kb hybridisation band was present in both MZIR098 generations for all probes (and absent in the NP2222 negative control and the positive controls). The positive controls also gave a band of expected size. The results both confirm the conclusion that there is a single integration site and indicate that the MZIR098 insert is intact.

#### **3.4.1.3 Plasmid backbone**

The presence or absence of sequences from the vector plasmid pSYN17629 was assessed using two backbone-specific probes in combination with three 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 the two MZIR098 generations or the negative control. This confirmed the lack of integration of any plasmid backbone sequences in MZIR098.

#### **3.4.2 Insert organisation and sequence**

Genomic DNA was obtained from verified leaf tissue from the MZIR098 F<sub>3</sub> generation (see Figure 3) and the samples were used to characterise the DNA sequence in the transgene insertion and its flanking border regions.

Five overlapping polymerase chain reaction (PCR) fragments spanning the inserted sequences and border regions in event SYN-00098-3 were amplified and purified. Four of the fragments were then cloned into a bacterial vector. DNA from the vector colonies was randomly selected, confirmed to contain the expected insert and sequenced individually (three separate clones sequenced for each fragment). A discrepancy in a polycytidine region located in the *Ubi1* promoter in the *mcry3Aa2* cassette meant that this fragment was not cloned<sup>12</sup> and was sequenced directly. All sequences were assembled to obtain a consensus sequence using commercially available software (Gene Codes Sequencher®). The MZIR098 sequence thus obtained was then aligned (AlignX® programme from Vector NTI Advance® 11.5.2 software) with the plasmid pSYN17629 T-DNA sequence in order to compare the two.

A total of 10,467 bp of MZIR098 genomic sequence was confirmed (see Figure 4). This comprised 990 bp of the 5' genomic border sequence (including the RB), 1,085 bp of the 3' genomic border sequence (including the LB), and 8,462 bp of inserted T-DNA from pSYN-00098-3. The entire RB was deleted and 10 bp of non-coding sequence adjacent to the RB was deleted. There was a 10 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).

The annotation performed to compare sequence from MZIR098 with the sequence in the T-DNA of plasmid pSYN17629 showed that the 8,462 bp MZIR098 insert is intact with no rearrangements.

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<sup>12</sup> With such a polycytidine region, it is likely that the number of cytosines will vary for each clone sequenced because of polymerase slippage during PCR amplification (Clarke et al. 2001).

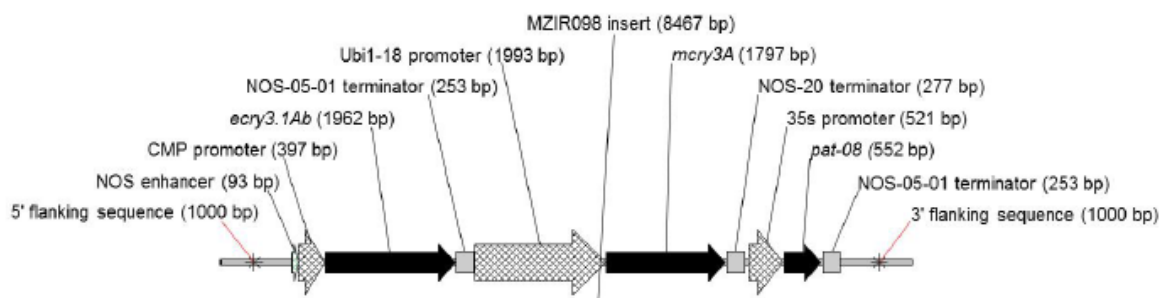


Figure 4: Map of the MZIR098 insert and flanking sequence (intervening sequences not included)

### 3.4.3 Insertion site

In order to assess any changes that may have occurred in the NP2222 genome as a result of integration of the MZIR098 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 MZIR098. 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 MZIR098 T-DNA flanking sequences.

The comparison showed that a 24 bp NP2222 sequence has been deleted at the insertion site in the MZIR098 genome.

### 3.4.4 Open reading frame (ORF) analysis

Sequences spanning the 5' and 3' junctions of the insert in MZIR098 were translated from start codon to stop codon (TGA, TAG, TAA) in all six reading frames using Vector NTI Advance™. Only those encoding sequences of 30 or more amino acids were considered; 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). A single potential ORF comprising 42 amino acids was identified in the 3' junction region of the insert. This putative polypeptide was analysed using a bioinformatics strategy to determine similarity to known protein toxins or allergens (refer to Section 4.1.6)

## 3.5 Stability of the genetic changes in MZIR098

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).

### 3.5.1 Genetic stability

The genetic stability of event SYN-00098-3 was evaluated by Southern blot analysis of genomic leaf DNA from verified plants from five generations (F<sub>2</sub>, F<sub>3</sub>, F<sub>4</sub>, F<sub>5</sub> and CF<sub>1</sub> – see Figure 3). DNA from three non-GM controls – NP2222, NP2391 and NP2222 x NP2391 – was also tested. Two restriction enzyme strategies were used in the analysis.

In the first strategy, the DNA was digested with an enzyme that has a single recognition site in the MZIR098 insert and other sites in the flanking regions; two enzymes were used. In the second strategy the restriction enzyme cut in the flanking sequences to release a single fragment of known size. A single 8.5 kb <sup>32</sup>P-labelled probe covering every base pair of the pSYN17629 T-DNA was used. No insert-specific hybridisation bands were expected in the DNA from the three negative controls. As for the integration analysis described in Section 3.4.1.1, 1 copy and 1/7 copy DNA positive assay controls were included in the analysis and were expected to give a single hybridisation band of known size. The expected hybridisation bands were obtained for all samples. In particular, bands specific to the insert were identical in lanes containing DNA from the MZIR098 generations F<sub>2</sub>, F<sub>3</sub>, F<sub>4</sub>, F<sub>5</sub> and CF<sub>1</sub> thus confirming the stability stable inheritance of the insert.

### 3.5.2 Phenotypic stability

Phenotypic stability was assessed by real-time PCR analysis (using appropriate primers and probes) for the presence of the *ecry3.1Ab*, *mcry3Aa2* and *pat* genes within the DNA from three generations (BC<sub>2</sub>F<sub>1</sub>, BC<sub>3</sub>F<sub>1</sub>, BC<sub>4</sub>F<sub>1</sub> – see Figure 3). Since it was demonstrated that the insert resides at a single locus within the genome of MZIR098, the expectation would be that the genetic material within it would be inherited according to Mendelian principles i.e. *ecry3.1Ab*, *mcry3Aa2* and *pat* would co-segregate and 50% of plants from each generation would be expected to contain all three transgenes while 50% would contain no transgenes (a 1:1 positive:negative ratio).

The results of this analysis are shown in Table 3. The chi-square (X<sup>2</sup>) critical value at significance level α = 0.05 is 3.84 i.e. if the X<sup>2</sup> value is < 3.84 the observed ratio is not significantly different from the expected ratio of 1:1. The X<sup>2</sup> values for all generations were less than 3.84 thereby indicating that the *ecry3.1Ab*, *mcry3Aa2* and *pat* genes are inherited according to Mendelian principles.

**Table 3: Segregation of *ecry3.1Ab*, *mcry3Aa* and *pat* over three generations**

Generation	Total plants	Ratio positive:negative		X <sup>2</sup>	Probability <sup>1</sup>
		Observed	Expected		
BC <sub>2</sub> F <sub>1</sub>	186	1:1.18	1:1	1.38	NS
BC <sub>3</sub> F <sub>1</sub>	140	1:1.02	1:1	0.03	NS
BC <sub>4</sub> F <sub>1</sub>	149	1:0.98	1:1	0.01	NS

<sup>1</sup>NS = not significant - P<0.05 (X<sup>2</sup><3.84)

### 3.6 Antibiotic resistance marker genes

No antibiotic marker genes are present in MZIR098. The insert sequence analysis (Section 3.4.2) showed no plasmid backbone has been integrated into the MZIR098 genome during transformation, i.e. the *aadA* gene, which was used as a bacterial selectable marker gene, is not present in MZIR098.

### 3.7 Conclusion

MZIR098 contains three expression cassettes. Comprehensive molecular analyses indicate there is a single insertion site comprising a single, complete copy of each of the *ecry3.1Ab*, *mcry3Aa2* and *pat* genes with their regulatory elements. The introduced genes 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

The main purpose of the characterisation is to describe the nature of any new substances and their phenotypic and biochemical effects on the organism in which they are expressed, particularly in the parts of the organism consumed as food. Typically, the main focus of the characterisation is on newly expressed (or potentially expressed) proteins, but other (non-protein) substances may need to be considered and in the case of herbicide-tolerant plants, where tolerance is achieved by metabolism of the herbicide, it is possible that one or more novel metabolites may accumulate in the GM plant following the application of herbicide

### 4.1 Newly expressed proteins

In considering the safety of newly expressed 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 newly expressed proteins in the organism as well as a detailed understanding of their biochemical function and phenotypic effects. It is also important to determine if the newly expressed proteins are expressed as expected, including whether any post-translational modifications have occurred.

Two types of proteins were considered:

- The proteins expected to be directly produced as a result of the translation of the introduced gene. A number of different analyses were done to characterise these proteins and determine their *in planta* expression.
- Those that may be potentially translated as a result of the creation of ORFs during the transformation process (see Section 3.4.4).

#### 4.1.1 The Cry proteins

Crystal (Cry) proteins produced by *Bt* are classified by their primary amino acid sequence and more than 500 different *cry* gene sequences have been classified into 74 groups (Cry1–Cry74)<sup>13</sup>. The largest family, with over 50 different Cry groups, comprises the three-domain proteins<sup>14</sup> (3D-Cry) which are subdivided into further groups based on their specificity for target insects; Cry1 (such as Cry1Ab) and Cry2 proteins act specifically on lepidopterans while Cry 3 (such as mCry3Aa2 and eCry3.1Ab) proteins act specifically on coleopterans (Höfte and Whiteley 1989).

The primary action of all Cry toxins is to lyse midgut epithelial cells in the target insect by forming pores in the apical microvilli membrane of the cells, which subsequently leads to ion leakage and cell lysis.

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<sup>13</sup> [http://www.lifesci.sussex.ac.uk/home/Neil\\_Crickmore/Bt/toxins2.html](http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/toxins2.html)

<sup>14</sup> In very basic terms Domain I is a seven  $\alpha$ -helix bundle involved in toxin oligomerisation, membrane insertion and pore formation; Domain II comprises 11 beta sheets and is involved in binding to specific larval mid-gut proteins; and Domain III is a beta sandwich that is also involved in receptor recognition (Bravo et al. 2012).

The crystal inclusions ingested by susceptible larvae dissolve in the alkaline environment of the gut, and the solubilised inactive protoxins are cleaved by midgut proteases yielding 60-70 kDa protease resistant core toxins (Bravo et al. 2007). Toxin activation involves the proteolytic removal of an N-terminal peptide. The activated toxin then binds to specific receptors on the brush border membrane of the midgut epithelium columnar cells (Hofmann et al. 1988; Aronson and Shai 2001) before inserting into the membrane. Toxin insertion leads to formation of lytic pores in microvilli apical membranes (de Maagd et al. 2001; Aronson and Shai 2001) and eventually to cell lysis and disruption of the gut epithelium. The septicaemia that inevitably follows may be mediated by an influx of enteric bacteria into the haemocoel (Broderick et al. 2006).

Approved GM crops incorporating *Bt* Cry proteins have been available for a number of years and have not raised any food safety concerns (Narva et al. 2014; Koch et al. 2015).

#### **4.1.1.1 eCry3.1Ab**

The eCry3.1Ab protein (GenBank accession number GU327680) in MZIR098 is a hybrid mainly comprising mCry3Aa2 (as described below) but with the replacement of Domain III<sup>15</sup> of the mCry3Aa2 with the Domain III variable region from Cry1Ab.

The full-length Cry1Ab is a protein of 1155 amino acids and is derived from *Bacillus thuringiensis* ssp. *kurstaki* HD-1 (Geiser et al. 1986); it has been assessed by FSANZ in a number of different corn applications<sup>16</sup>. The full length protein is normally active against lepidopteran pests.

As described in Walters et al. (2010) the novel insecticidal activity against coleopterans in eCry3.1Ab was generated by exchange of 172 amino acid residues of Cry1Ab sequence with the corresponding region of the original mCry3Aa2 C-terminal sequence. Thus the 653 amino acid eCry3.1Ab protein comprises (from the N-terminus to the C-terminus):

- a peptide cap of 22 amino acid residues (MTSNGRQCAGIRPYDGRQQHRG) introduced during the engineering of the variable regions (see Section 3.2.1)
- amino acids 10 to 468 (459 residues) of mCry3Aa2 (coloured text in Figures 5 and 6)
- amino acids 477 to 648 (172 residues) of Cry1Ab

The resulting unique protein (Figure 5) has a calculated molecular weight (mw) of 74.8 kDa. The portion of Cry1Ab included in eCry3.1Ab has not preserved the activity of Cry1Ab against lepidopterans. Thus the chimeric protein exhibits strong bioactivity against the larvae of western corn rootworm (Coleoptera), a pest species which is not susceptible to either parent protein. FSANZ has assessed the chimeric eCry3.1Ab protein in a previous application<sup>17</sup>.

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<sup>15</sup> A description of the domains fused together to create the chimeric gene is outlined in Section 3.2.1.

<sup>16</sup> A346 (FSANZ 2000), A385 (FSANZ 2001a), and A386 (FSANZ 2001b) and in cotton applications A615 (FSANZ 2009) and A1028 (FSANZ 2010)

<sup>17</sup> Corn application A1060 (FSANZ 2012)

01	MTSNGRQCAG	IRPYDGRQQH	RGLDSSSTTKD	VIQKGISVVG	DLLGVVGFPPF
51	GGALVSFYTN	FLNTIWPSED	PWKAFMEQVE	ALMDQKIADY	AKNKALAELO
101	GLQNNVEDYV	SALSSWQKNP	AAPFRNPHSQ	GRIRELFSQA	ESHFRNSMPS
151	FAISGYEVLV	LTTYAQAANT	HLFLLKDAQI	YGEEWGYEKE	DIAEFYKRQL
201	KLTQEYTDHC	VKWYNVGLDK	LRGSSYESWV	NFNRYRREMT	LTVLDLIALF
251	PLYDVRLYPK	EVKTELTRDV	LTDPVGVN	LRGYGTTFSN	IENYIRKPHL
301	FDYLHRIQFH	TRFQPGYYGN	DSFNYWSGNY	VSTRPSIGSN	DIITSPFYGN
351	KSSEPQVQLE	FNGEKVYRAV	ANTNLAVWPS	AVYSGVTKVE	FSQYNDQTDE
401	ASTQTYDSKR	NVGAVSWDSI	DQLPPETTDE	PLEKGYSHQL	NYVMCFLMQG
451	SRGTIPVLTV	THKSVDFFNM	IDSKKITQLP	LTKSTNLGSG	TSVVKGPGFT
501	GGDILRRSP	GQISTLRVNI	TAPLSQRYRV	RIRYASTTNL	QFHTSIDGRP
551	INQGNFSATM	SSGSNLQSGS	FRTVGFTTTPF	NFSNGSSVFT	LSAHVFNSGN
601	EVIIDRIEFV	PAEVTFEAEY	DLERAQKAVN	ELFTSSNQIG	LKTDVTDYHI
651	DQV				

Figure 5: Amino acid sequence of the eCry3.1Ab protein (coloured text = 459 residues of mCry3Aa2)

#### 4.1.1.2 mCry3Aa2

The mCry3Aa2 protein (598 amino acids and mw = 67.7 kDa – see Figure 6) is based on the native Cry3Aa2 protein sequence from *B. thuringiensis* ssp. *tenebrionis* (Sekar et al. 1987). The native Cry3Aa2 protein (644 amino acids, mw = 73 kDa) is a component of commercial *Bt*-based insecticidal preparations that have been in use for over 50 years. Cry3Aa2 is naturally very active against the Colorado potato beetle (*Leptinotarsa decemlineata*) (Walters et al. 2010), but is not effective for controlling corn rootworm pests (*Diabrotica* spp.). To improve activity, the protein was modified as follows (Walters et al. 2008):

- (i) The original N-terminal region was removed such that the mCry3Aa2 protein commences at a methionine residue in position 48 (Met-48) of the native protein.
- (ii) A cathepsin-G protease cleavage site was added at a location in Domain I known to enhance proteolytic activation. The consensus cathepsin-G site has the sequence alanine-alanine-proline-phenylalanine (green text in Figures 5 and 6), and replaces amino acids valine-155, serine-156 and serine-157 in the native protein (Chen and Stacy 2007)

The addition of the protease recognition site improves binding to corn rootworm midgut membranes; serine protease is the dominant protease in the gut of corn rootworm and the most widely recognised serine recognition site is that of cathepsin-G. Walters et al. (2008) reported that this protease cleavage site was essential to obtain >90% mortality with western corn rootworm larvae.

01	MTADNNTEAL	DSSTTKDVIQ	KGISVVGDLL	GVVGFPPFGGA	LVSFYTNFLN
51	TIWPSEDPWK	AFMEQVEALM	DQKIADYAKN	KALAELOGLQ	NNVEDYVSAL
101	SSWQKNPAAP	FRNPHSQGRI	RELFSAEASH	FRNSMPSFAI	SGYEVLFLLT
151	YAQAANTHLF	LLKDAQIYGE	EWGYEKEDIA	EFYKRQLKLT	QEYTDHCVKW
201	YNVGLDKLRG	SSYESWVNFN	RYRREMTLV	LDLIALFPLY	DVRLYPKEVK
251	TELTRDVLTD	PIVGVNLRG	YGTTFNSIEN	YIRKPHLFDY	LHRIQFHTRF
301	QPGYYGNDSF	NYWSGNYVST	RPSIGSNDII	TSPFYGNKSS	EPVQNLEFNG
351	EKVYRAVANT	NLAVWPSAVY	SGVTKVEFSQ	YNDQTDEAST	QTYDSKRNVG
401	AVSWDSIDQL	PPETTDEPLE	KGYSHQLNYV	MCFLMQGSRG	TIPVLTVTHK
451	SVDFFNMIDS	KKITQLPLVK	AYKLQSGASV	VAGPRFTGGD	IIQCTENGSA
501	ATIYVTPDVS	SQKYRARIH	YASTSQITFT	LSLDGAPFNQ	YYFDKTINKG
551	DTLTYNSFNL	ASFSTPFELS	GNNLQIGVTG	LSAGDKVYID	KIEFIPVN

Figure 6: Amino acid sequence of the mCry3Aa2 protein (coloured text = 459 residues of mCry3Aa2 present in eCry3.1Ab)



FSANZ has assessed a full Cry3Aa4 protein (one amino acid different from Cry3Aa2) and the mCry3Aa2 protein in a number of previous applications<sup>18</sup>.

#### 4.1.2 The PAT protein

The PAT protein encoded by the *pat* gene consists of 183 amino acids (Figure 7), mw = 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.

This protein has been considered in 21 previous FSANZ applications and globally is represented in six major crop species.

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

Figure 7: Amino acid sequence of the PAT protein

#### 4.1.3 Protein expression in the tissues of MZIR098

##### Study submitted:

2014. Quantification of eCry3.1Ab, mCry3A, and phosphinothricin acetyltransferase in event MZIR098 maize tissue. Final Report **TK0117462**. Syngenta Seeds, Inc. (unpublished)

Plants of MZIR098 (generation CF<sub>1</sub>) together with the non-GM NP2391 x NP2222 control were grown from PCR-verified seed lots at four field sites in the USA<sup>19</sup> during the 2013 growing season. These plantings overlapped with the eight plantings used for the compositional analysis described in Section 5.2. At each location, two replicate plots were planted with MZIR098 seed and one plot was planted with the control. One of the MZIR098 replicate plots received a single post-emergent spray application of glufosinate at a nominal rate of 0.46 kg ai/ha<sup>20</sup> when the plants reached the V3/V4 growth stage<sup>21</sup>. 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 MZIR098 plot and a sample from one plant was collected from the control plot. For pollen, a pooled sample was collected from 10 – 15 tassels per plot. Levels of eCry3.1Ab, mCry3Aa2 and PAT were determined for each sample type using a validated enzyme linked immunosorbent assay (ELISA). Detection of all three proteins utilised commercially available kits:

- eCry3.1Ab – Beacon eCry3.1Ab 10 Plate Kit
- mCry3Aa2 – Envirologix QualiPlate™ Kit for Modified Cry3A
- PAT – Envirologix QualiPlate™ ELISA Kit for Liberty Link® PAT/pat.

<sup>18</sup> potato applications A382 (FSANZ 2001c), A383 (FSANZ 2001d) and A384 (FSANZ 2001e) and corn application A564 (FSANZ 2006a)

<sup>19</sup> York, Nebraska; Richland, Iowa; Germansville, Pennsylvania; Delavan, Wisconsin

<sup>20</sup> ai = active ingredient

<sup>21</sup> For information on corn growth stages see e.g. Ransom & Endres (2014)

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 non-GM control for each tissue type was included as an analytical control to test whether there were any performance-inhibiting plant-matrix effects on the ELISA method for each test protein; none was noted.

The results, averaged over all sites, are given in Table 4; there was consistency between the levels of each protein across glufosinate sprayed and unsprayed plants. The mean levels of eCry3.1Ab varied considerably between the different plant parts and growth stages, being highest in leaves at the V6 stage and undetectable in pollen; the level in grain was very low. For the mCry3Aa2 protein, levels were highest in the pollen and lowest in the grain. For PAT, mean levels were low across all plant parts and growth stages but were highest in leaves at the V6 stage and essentially at or below the limit of detection (LOD) in a number of tissues, including the grain.

The reason why the level of mCry3Aa2 protein is relatively high in pollen while the levels of the other proteins are essentially undetectable in pollen, is that the *Ubi1* promoter used to drive expression of the *mcry3Aa2* gene is expressed in pollen tissue whereas the *35S* and *CMP* promoters used to drive *ecry3.1Ab* and *pat* respectively, although regarded as constitutive, are poorly expressed in monocot pollen (Christensen and Quail 1996; Stavalone et al. 2003).



**Table 4: eCry3.1Ab, mCry3Aa2 and PAT protein content of tissue in glufosinate-sprayed and unsprayed MZIR098 at different growth stages (averaged across 4 sites)**

Tissue/Growth stage	Glufosinate-sprayed						No glufosinate applied					
	eCry3.1Ab ( $\mu\text{g/g dw}^1$ )		mCry3Aa2 ( $\mu\text{g/g dw}$ )		PAT ( $\mu\text{g/g dw}$ )		eCry3.1Ab ( $\mu\text{g/g dw}$ )		mCry3Aa2 ( $\mu\text{g/g dw}$ )		PAT ( $\mu\text{g/g dw}$ )	
	Mean $\pm$ SD <sup>2</sup>	Range	Mean $\pm$ SD	Range	Mean $\pm$ SD	Range	Mean $\pm$ SD	Range	Mean $\pm$ SD	Range	Mean $\pm$ SD	Range
Leaf /V6	200.24 $\pm$ 63.04	105.35-322.84	76.41 $\pm$ 12.27	57.38-100.18	7.34 $\pm$ 2.63	3.40-12.54	216.07 $\pm$ 72.13	129.62-333.92	79.51 $\pm$ 14.24	60.65-114.42	7.62 $\pm$ 2.77	6.37 – 11.79
Leaf /R1	95.84 $\pm$ 29.13	42.49-143.64	47.94 $\pm$ 8.22	42.49-143.64	3.36 $\pm$ 1.05	1.77-5.76	107.35 $\pm$ 32.74	61.05-157.55	49.57 $\pm$ 32.74	32.62-61.07	3.57 $\pm$ 1.31	6.21 – 17.03
Leaf /R6	49.59 $\pm$ 21.26	17.83-103.61	41.44 $\pm$ 14.87	30.98-67.07	0.79 $\pm$ 0.49	0.19-1.69	49.7 $\pm$ 19.72	9.68-75.2	35.0 $\pm$ 13.67	7.71-71.74		<LOD – 1.93
Leaf /Senescence	8.54 $\pm$ 4.19	1.10-16.76	13.65 $\pm$ 6.81	12.24-68.51		<LOD – 0.12	9.58 $\pm$ 4.88	2.09-20.12	11.91 $\pm$ 5.07	3.50-22.81		<LOD – 0.13
Root /V6	69.98 $\pm$ 26.21	24.95-128.79	60.92 $\pm$ 18.08	26.49-88.43	1.41 $\pm$ 0.93	0.45-3.36	73.07 $\pm$ 13.68	37.63-96.44	61.63 $\pm$ 18.73	27.11-110.86	1.47 $\pm$ 0.81	0.77 – 3.91
Root /R1	22.17 $\pm$ 8.33	10.35-36.48	31.00 $\pm$ 10.48	14.88-55.42	0.71 $\pm$ 0.21	0.38-1.13	21.76 $\pm$ 11.21	7.82-47.09	24.58 $\pm$ 6.67	13.36-39.51	0.69 $\pm$ 0.31	0.67 – 1.71
Root /R6	11.74 $\pm$ 3.98	5.85-19.49	14.91 $\pm$ 5.05	7.90-22.38		<LOD – 0.61	14.37 $\pm$ 5.74	5.06-23.19	15.53 $\pm$ 5.78	5.47-22.89		<LOD – 0.10
Root /Senescence	9.88 $\pm$ 5.96	2.04-21.18	16.15 $\pm$ 6.0	5.03-25.61		<LOD – 0.48	7.87 $\pm$ 4.60	1.84-18.76	11.86 $\pm$ 4.31	5.07-19.06		<LOD – 0.55
Whole plant /V6	152.53 $\pm$ 39.32	87.02-213.01	73.42 $\pm$ 9.02	58.94-92.79	5.19 $\pm$ 2.18	2.16-9.32	168.14 $\pm$ 40.40	99.40-221.05	71.48 $\pm$ 11.48	49.38-95.09	4.60 $\pm$ 1.73	1.81-6.82
Whole Plant /R1	32.87 $\pm$ 13.67	11.28-62.69	28.48 $\pm$ 8.87	11.87-44.81	1.51 $\pm$ 0.53	0.89-3.07	52.72 $\pm$ 19.31	26.20-98.55	35.43 $\pm$ 8.42	24.48-52.35	1.69 $\pm$ 0.92	0.63-4.60
Whole Plant /R6	7.97 $\pm$ 3.73	2.91-15.07	16.06 $\pm$ 6.32	8.13-30.24		<LOD – 0.37	7.72 $\pm$ 3.48	2.70-18.38	14.49 $\pm$ 4.29	9.20-26.36		<LOD – 0.36
Pollen/R1		<LOD <sup>3</sup>	312.48 $\pm$ 15.84	289.9-326.92		<LOD		<LOD	302.93 $\pm$ 6.37	293.87-308.71		<LOD
Grain/R6	1.88 $\pm$ 1.01	0.76-4.84	14.51 $\pm$ 3.37	8.12-22.38		<LOD	2.42 $\pm$ 1.15	1.28-5.90	14.59 $\pm$ 3.76	8.91-22.83		<LOD – LOQ <sup>4</sup>
Grain/Senescence	1.94 $\pm$ 0.90	0.76-3.72	10.12 $\pm$ 2.58	5.77-14.51		<LOD	2.08 $\pm$ 1.29	0.82-4.52	11.21 $\pm$ 3.41	6.69-19.65		<LOD

<sup>1</sup>dw = dry weight

<sup>3</sup>LOD = Limit of Detection

<sup>2</sup>SD = standard deviation

<sup>4</sup>LOQ = Limit of Quantitation

#### 4.1.4 Characterisation of the proteins produced in MZIR098

It is necessary to confirm that the proteins expressed in MZIR098 have the expected biochemical characteristics. Accordingly, the Applicant used a number of analytical techniques to characterise the eCry3.1Ab, mCry3Aa and PAT proteins in leaf tissue from glasshouse-grown MZIR098 (verified by real-time PCR) CF<sub>1</sub> generation and compare the results with previously characterised eCry3.1Ab, mCry3Aa and PAT (93.3%, 90.3% and 85.9% purity respectively) produced in recombinant *Escherichia coli* systems. It is noted that the microbially-produced eCry3.1Ab protein differed slightly from the protein expected to be produced in MZIR098 in that it contained an additional methionine and six additional histidine residues at the N-terminus; this was an intentional modification that facilitated purification of the protein after microbial production.

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 eCry3.1Ab and mCry3A proteins produced in recombinant *Escherichia coli* with eCry3.1Ab and mCry3A proteins produced in event MZIR098 derived maize plants. Final report **TK0117480**, Syngenta Seeds, Inc. (unpublished)

2015. Comparison of phosphinothricin acetyltransferase (PAT) protein produced in recombinant *Escherichia coli* and PAT protein produced in event MZIR098 derived maize plants. Final report **TK0121937**, Syngenta Seeds, Inc. (unpublished)

##### 4.1.4.1 Molecular weight and immunoreactivity of eCry3.1Ab, mCry3Aa and PAT

This analysis used both crude and immune-purified extracts from MZIR098 and an extract from the non-GM parental corn line NP2222. In addition, as a control for each protein, extract from NP2222 was spiked with the *E.coli*-derived protein; this allowed for comparison of the microbially-produced protein with the plant-produced protein to ascertain whether there was an effect of plant matrix on immunoreactivity.

Following SDS-PAGE, protein bands were transferred to a polyvinylidene fluoride (PVDF) membrane via electroblotting. The membranes for the proteins were then probed with a protein-specific polyclonal goat primary antibody followed by an alkaline phosphatase-conjugated donkey anti-goat secondary antibody. The eCry3.1Ab and mCry3Aa2 proteins were both run on the same Western blot as the primary antibody recognised sequence epitopes common to both proteins. Proteins were visualised following addition of the chromogenic substrate BCIP®/NBT<sup>22</sup>.

For the microbially-produced eCry3.1Ab, NP2222 spiked with microbial eCry3.1Ab and the MZIR098-derived immunopurified eCry3.1Ab samples, a single band was obtained at approximately 74 kDa, which is the calculated size of the eCry3.1Ab protein (see Section 4.1.1.1).

<sup>22</sup> 5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP) and nitroblue tetrazolium (NBT) forms a black-purple precipitate upon reaction with alkaline phosphatase.

Similarly a single band at approximately 68 kDa was obtained for the microbially-produced mCry3Aa2, NP2222 spiked with microbial mCryAa2 and the MZIR098-derived immunopurified mCry3Aa2 samples; this corresponded to the calculated size of the mCry3Aa2 protein (see Section 4.1.1.2). For the crude extract obtained from MZIR098, the two bands described for eCry3.1Ab and mCry3Aa2 were both present along with a prominent band at approximately 55 kDa and a faint band at approximately 35 kDa. These latter two bands are likely to be mCry3Aa2 degradation products that have been noted previously (FSANZ 2006a). Very faint bands at approximately 150 kDa in most samples are likely to represent dimers of eCry3.1Ab or mCry3Aa2. No immunoreactive bands were observed in samples from the NP2222 control.

Analysis of PAT showed, for crude plant extract, NP2222 spiked with microbially-produced PAT, immunoaffinity-purified plant protein, and microbially-produced protein, an immunoreactive band at approximately 17 kDa, which is close to the predicted molecular weight of 21 kDa (see Section 4.1.2). This band was fairly diffuse for both the crude MZIR098 extract and NP2222 spiked with PAT. The applicant states this indicates there was matrix interference from plant tissue. A very faint band at approximately 40 kDa in all four extracts most likely represents a dimer. No immunoreactive bands were observed in samples from the NP2222 control.

The eCry3.1Ab, mCry3Aa2 and PAT proteins expressed in MZIR098 therefore have the expected size and immunoreactivity.

#### **4.1.4.2 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 eCry3.1Ab, mCry3Aa2 and PAT from both microbial and MZIR098 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. eCry3.1Ab, mCry3Aa2 or PAT) protein amino acid sequence.

The collective analysis of the three proteolytic digests for eCry3.1Ab from the microbial and plant sources resulted in coverage of 88.5% and 85.9%, from each source respectively, of the total predicted eCry3.1Ab amino acid sequence. This was adequate to confirm the identity of the protein. In addition, 13 amino acids of the plant-derived N-terminal sequence and 20 amino acids of both the plant and microbial C-terminal 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 in the plant-derived sequence. 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 N-terminal sequence was blocked in the microbially-derived protein.

The collective analysis of the three proteolytic digests for mCry3Aa2 from the microbial and plant sources resulted in coverage of 89.5% and 91.6% from each source respectively and was therefore adequate to confirm the identity of the protein (see Figure 6). In addition, 21 amino acids from the N-terminal sequence of the plant-derived mCry3Aa2 and 16 amino acids from the microbial-derived sequence, and 17 amino acids from the C-terminal sequence of the protein from both sources were identified by peptide mass coverage.

The collective analysis of the three proteolytic digests for PAT from the microbial and plant sources resulted in coverage of 97% and 96%, 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 7). The analysis showed that the N-terminal methionine was missing from the plant-derived sequence.

The eCry3.1Ab, mCry3Aa2 and PAT proteins expressed in MZIR098 therefore have the expected amino acid sequences

#### **4.1.4.3 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<sup>23</sup> predicted six potential sites in the eCry3.1Ab protein, four in the mCry3Aa2 protein and no sites in the PAT protein.

Analysis of plant- and microbially-derived eCry3.1Ab, mCry3Aa2 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. The Schiff reagent stains vicinal diol groups found mainly on peripheral sugars and sialic acids and is used as a general glycoprotein stain.

A dark band was obtained for the positive control (horseradish peroxidase) while the eCry3.1Ab, mCry3Aa2 and PAT proteins from both plant and microbial sources and the negative control (soybean trypsin inhibitor) gave no dark bands. These results support the conclusion that neither microbially- nor MZIR098-derived eCry3.1Ab, mCry3Aa2 and PAT proteins are glycosylated.

#### **4.1.4.4 Protein activity**

##### *eCry3.1Ab and mCry3Aa2*

The insecticidal activity of the proteins was tested in mixtures of the two by feeding the mixtures to first instar Colorado potato beetle (CPB) larvae. Mixtures were sourced from MZIR098 crude extract, microbially-produced eCry3.1Ab + mCry3Aa2, and NP2222 extract spiked with microbially-produced eCry3.1Ab + mCry3Aa2. The mixtures were diluted in buffer and incorporated into CPB diet powder to produce eight diets with final concentrations of 2.84, 2.00, 1.42, 1.00, 0.71, 0.36, 0.18, and 0.09 µg insecticidal protein mixture/ml diet. Negative controls comprised NP2222 extract and plain buffer and were similarly incorporated into CPB diet. The bioassays were conducted in 24-well culture plates with each well containing one freshly-hatched CPB larva and 200 µL diet.

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<sup>23</sup> <http://www.cbs.dtu.dk/services/NetNGlyc/>

Mortality readings were taken periodically starting at 72 hours and continued until 144 hours; LC50<sup>24</sup> determinations, where possible, were assessed.

The results are given in Table 5.

**Table 5: Insecticidal activity of eCry3.1Ab and mCry3Aa2 from various sources**

Concentration (µg/ml of diet)	MZIR098 maize crude extract <sup>a</sup>		Mixture of microbially produced eCry3.1Ab and mCry3A <sup>a</sup>		Fortified nontransgenic maize crude extract <sup>a</sup>	
	CPB dead (no.)	Mortality (%)	CPB dead (no.)	Mortality (%)	CPB dead (no.)	Mortality (%)
0.09	18	25	10 <sup>b</sup>	14	17	24
0.18	24	33	20	28	26	36
0.36	34 <sup>b</sup>	48	35	49	30	42
0.71	43	60	45	63	41	57
1.00	50	69	47	65	44	61
1.42	61	85	53 <sup>b</sup>	75	48	67
2.00	58 <sup>b</sup>	82	51	71	59	82
2.84	66	92	57	79	58	81
Buffer control	6	8	–	–	–	–
Nontransgenic extract control	3	4	–	–	–	–

*N* = 72 except as indicated.

<sup>a</sup>Ratio of eCry3.1Ab to mCry3A = 1.89:1.

<sup>b</sup>*N* = 71.

A relatively high mortality rate was observed in the treatments containing mixtures from MZIR098 crude extract, microbially-produced eCry3.1Ab + mCry3Aa2, and NP2222 extract spiked with microbially-produced eCry3.1Ab + mCry3Aa2. By comparison, low CPB mortality was observed in the negative controls using diet made with buffer (8%), or nontransgenic maize crude extract (4%). The following LC50 rates were calculated:

- MZIR098 crude extract eCry3.1Ab + mCry3Aa2 0.44 µg/ml
- Microbially produced eCry3.1Ab + mCry3Aa2 0.61 µg/ml
- NP2222 fortified with microbially produced eCry3.1Ab + mCry3Aa2 0.58 µg/ml

These data demonstrate that the microbially produced eCry3.1Ab and mCry3A2 proteins are functionally equivalent to the eCry3.1Ab and mCry3A2 proteins produced in MZIR098 maize.

### 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 4.1.2) for 8 min and then the formation of 2-nitro-5-thiobenzoate anion (TNB<sup>2-</sup>) 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. NP2222 extract was included within the enzymatic activity assay as a negative control. A further plant control treatment (non-GM fortified with microbially-produced PAT) was included to account for the effect of extraction procedure and plant matrix on specific activity. Results are given in Table 6.

<sup>24</sup> LC50 = 50% lethal concentration

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

PAT source	Mean PAT specific activity (U/mg PAT) <sup>1</sup>
Microbially-produced PAT	29.26
MZIR098-produced PAT	11.13
Non-GM control spiked with microbially-produced PAT-	14.07
Non-GM control (NP2222)	<LOD

<sup>1</sup> 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<sup>2-</sup> 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 (14.07 and 11.13 U/mg PAT, respectively).

#### 4.1.5 Safety of the introduced proteins

Results presented in Section 4.1.4 have verified the identity of the eCry3.1Ab, mCry3Aa2 and PAT proteins expressed in MZIR098. All three proteins have been considered to be safe by FSANZ on previous applications<sup>25</sup>.

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 updated studies listed below) using an updated (and hence larger) database, the results did not alter conclusions reached previously.

**Table 7: Summary of consideration of eCry3.1Ab, mCry3Aa2 and PAT in previous FSANZ safety assessments**

Consideration	Sub-section	eCry3.1Ab	mCry3Aa2	PAT
Potential toxicity	Amino acid sequence similarity to protein toxins	This application – using search updated in March 2015	This application – using search updated in March 2015	This application - using search updated in April 2015
	<i>In vitro</i> digestibility	A1060 (FSANZ 2012)	A564 (FSANZ 2006a)	A1080 (FSANZ 2013a)
	Stability to heat	A1060 (FSANZ 2012)	A564 (FSANZ 2006a)	A1080 (FSANZ 2013a)
	Acute oral toxicity	A1060 (FSANZ 2012)	A564 (FSANZ 2006a)	A1080 (FSANZ 2013a)

<sup>25</sup> For eCry3.1Ab, this was in A1060 – corn line 5307 (FSANZ 2012); and for mCry3Aa2, this was in A564 (FSANZ 2006a). The PAT protein, encoded by either the *pat* or *bar* genes (Wehrmann et al. 1996), has now been considered in 21 FSANZ safety assessments (A372, A375, A380, A385, A386, A446, A481, A518, A533, A543, A589, A1028, A1040, A1046, A1073, A1080, A1081, A1087, A1094, A1106 and A1112) 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 of the PAT proteins encoded by the two genes (Hérouet et al. 2005).

Consideration	Sub-section	eCry3.1Ab	mCry3Aa2	PAT
Potential allergenicity	Source of the protein	A1060 (FSANZ 2012)	A564 (FSANZ 2006a)	A1087 (FSANZ 2013b)
	Amino acid sequence similarity to allergens	This application – using search updated in March 2015	This application – using search updated in March 2015	This application – using search updated in April 2015

#### Updated Bioinformatics studies submitted

2015. eCry3.1Ab. Assessment of amino acid sequence similarity to known or putative allergens. Assessment **SSB-124-15**. Syngenta Seeds, Inc. (unpublished).

2015. mCry3A. Assessment of amino acid sequence similarity to known or putative allergens. Assessment **SSB-123-15**. 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. eCry3.1Ab. Assessment of amino acid sequence similarity to known or putative toxins. Assessment **SSB-126-15**. Syngenta Seeds, Inc. (unpublished).

2015. mCry3A. Assessment of amino acid sequence similarity to known or putative toxins. Assessment **SSB-125-15**. 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).

#### 4.1.6 Bioinformatic analysis of the ORF created by the transformation procedure

##### Study submitted:

2015. Event MZIR098: Allergenicity and toxicity assessment of start to stop, genome to insert junction open reading frames. Assessment **SSB-131-15**. Syngenta Seeds, Inc. (unpublished)

A bioinformatics analysis was performed to assess the similarity to known allergens and toxins of the putative polypeptide encoded by the single identified ORF in the 3' flanking regions of the MZIR098 insert (see Section 3.4.4).

For sequence similarities towards allergens, a search were done to compare the ORF sequence with known allergens in the Allergen, Gliadin and Glutenin sequence database residing in the 2015 FARRP (Food Allergy Research and Resource Program) dataset within AllergenOnline<sup>26</sup>. The database contained 1,897 non-redundant sequences.

The Fast Alignment Search Tool – All (FASTA) algorithm (Pearson and Lipman 1988), version 3.45 was used to search the database using the BLOSUM50<sup>27</sup> scoring matrix (Henikoff and Henikoff 1992). A separate search was done to find any possible eight amino acid matches.

No alignments with the ORF generated an E-score<sup>28</sup> of  $\leq 1e^{-5}$ , no alignment met or exceeded the Codex Alimentarius (Codex 2009) FASTA alignment threshold for potential allergenicity (35% identity over 80 amino acids) and no alignments of eight or more consecutive identical amino acids (Metcalf et al. 1996) were found.

<sup>26</sup> University of Nebraska; <http://www.allergenonline.org/>

<sup>27</sup> The BLOSUM series of matrices tabulate the frequency with which different substitutions occur in conserved blocks of protein sequences and are effective in identifying distant relationships.

<sup>28</sup> A search generates a parameter known as the E score (see eg Baxevanis 2005). Comparisons between highly homologous proteins yield E-scores approaching zero, indicating the very low probability that such matches would occur by chance. For this search an E-score of  $1 \times 10^{-5}$  was set as an initial high cut-off value for alignment significance.



A toxin similarity search was done for the ORF sequence, using the BLASTP<sup>29</sup> (Basic Local Alignment Search Tool Protein – version 2.2.28+) algorithm (Altschul et al. 1997), and BLOSUM62 scoring matrix, against non-redundant known protein sequences present in a subset (15,588 entries) of the National Center for Biotechnology Information (NCBI<sup>30</sup>) Entrez® Protein database. No significant sequence similarity ( $E$ -value  $\leq 1 \times 10^{-5}$ ) was observed between the translated ORF sequence and any entry in the 2015 toxin database.

#### 4.1.7 Conclusion

Corn line MZIR098 expresses three new proteins, eCry3.1Ab, mCry3Aa and PAT. The mean levels of eCry3.1Ab varied considerably between the different plant parts and growth stages, being highest in leaves at the V6 stage and undetectable in pollen; the level in grain was very low. For the mCry3Aa protein, levels were highest in the pollen and lowest in the grain. For PAT, mean levels were low across all plant parts and growth stages but were highest in leaves at the V6 stage and essentially at or below the LOD in a number of tissues, including the grain.

A range of characterisation studies confirmed the identity of the eCry3.1Ab, mCry3Aa and PAT proteins produced in MZIR098 and also their equivalence with the corresponding proteins produced in a bacterial expression system. The plant expressed eCry3.1Ab, mCry3Aa and PAT proteins have the expected molecular weights, immunoreactivity, lack of glycosylation, amino acid sequence and activity.

There are no concerns regarding the potential toxicity or allergenicity of the expressed proteins. Previous safety assessments of eCry3.1Ab, mCry3Aa and PAT indicate 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.

The glufosinate-tolerance trait is present in lines from 21 previous applications to FSANZ (see Section 4.1.5). 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 2006b).

There are no concerns that the spraying of line MZIR098 with 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.

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<sup>29</sup> The BLASTP algorithm is optimized to identify localized domains or other shared sequence similarities present within the full length query sequence.

<sup>30</sup> NCBI website = <http://www.ncbi.nlm.nih.gov/>



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. 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 minerals. 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 and p-coumaric acid.

## 5.2 Study design and conduct for key components

### Study submitted:

2014. Compositional analysis of forage and grain from MZIR098 maize grown during 2013 in the USA. Report **TK0117452**. Syngenta Seeds, Inc. (unpublished).

The MZIR098 (CF<sub>1</sub> generation – see Figure 3) 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 MZIR098, 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<sup>31</sup> during the 2013 growing season. Three of the plantings were the same as those used for the protein expression analysis (Section 4.1.3). For each treatment there were four replicated blocks at each site, planted in a randomised complete-block design. Maintenance fertiliser and pesticides were applied as needed in order to maintain a relatively weed-free and insect-free environment. One of the two MZIR098 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.

Additionally, a total of six non-GM hybrid lines<sup>32</sup> 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 no analyte level in MZIR098 differed significantly from the level in the control. The mean levels of all analytes measured from both MZIR098 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<sup>33</sup> (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 MZIR098 vs control; unsprayed MZIR098 vs control).

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<sup>31</sup> Richland, Iowa; York, Nebraska; Seymour, Illinois; Bagley, Iowa; Larned, Kansas; Stewardson, Illinois; Wyoming, Illinois; Germansville, Pennsylvania.

<sup>32</sup> Reference lines were H-7191, H-7540, SY SINCERO, NK LUCIUS, CISKO, SY PROVIAL.

<sup>33</sup> SAS website - <http://www.sas.com/technologies/analytics/statistics/stat/index.html>

In assessing the significance of any difference between the mean analyte value for MZIR098 and the control, a P-value of 0.05 was used. This means that approximately 5% of statistically significant differences are expected to occur due to chance alone.

Any statistically significant differences between MZIR098 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<sup>34</sup>. 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 sprayed MZIR098 was significantly higher than that in the control and the mean level of starch in sprayed MZIR098 was significantly lower than that in the control. However, the levels of both analytes were 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 MZIR098 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 MZIR098 and the hybrid control**

Analyte	Control (%dw)	Sprayed MZIR098 (%dw)	Unspray MZIR098 (%dw)	P (sprayed v control)	P (unspray v control)	Reference range (%dw)	Combined literature range (%dw)
Moisture (%fw)	12.8	12.7	12.9			7.99 – 17.4	5.1 – 40.5
Ash	1.42	1.45	1.43	0.310	0.974	1.18 – 1.87	0.62 – 6.28
Protein	10.4	10.4	10.3	0.719	0.416	7.68 – 13.9	5.72 – 17.26
Fat	3.93	3.99	3.96	0.317	0.654	2.39 – 4.41	1.36 – 7.83
Carbohydrate <sup>1</sup>	84.3	84.2	84.3	0.780	0.618	81.3 – 88.0	77.4 – 89.5
ADF	3.90	3.95	3.98	0.477	0.294	2.43 – 4.48	1.41 – 11.34
NDF	11.1	11.5 <sup>2</sup>	11.3 <sup>2</sup>	<b>0.008</b>	0.223	7.42 – 12.2	4.28 – 22.64
Total dietary fibre	16.3	16.5	16.4	0.375	0.726	11.2 – 20.0	8.73 – 35.3
Starch	65.8	63.9 <sup>2</sup>	65.5	<b>0.009</b>	0.657	53.3 – 79.6	26.5 – 83.7

<sup>1</sup> Carbohydrate calculated as 100% - (protein %dw+ fat %dw + ash %dw)

<sup>2</sup> Mauve shading represents MZIR098 mean significantly lower than the control; orange shading represents MZIR098 mean significantly higher 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 9 and can be summarised as follows:

<sup>34</sup> 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 MZIR098 than in the control.
- The mean levels of stearic, oleic and arachidic acids were significantly lower in both sprayed and unsprayed MZIR098 than in the control.
- MZIR098 means for palmitic, palmitoleic, linolenic, eicosenoic and behenic acids did not vary significantly from the control means.
- 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 MZIR098 and the hybrid control**

Fatty acid	Control (%total)	Sprayed MZIR098 (%total)	Unspray MZIR098 (%total)	P (sprayed v control)	P (unspray v control)	Reference range (%total)	Combined literature range (%total)
Palmitic (C16:0)	14.2	14.2 <sup>1</sup>	14.2	0.958	0.493	13.2-17.0	6.81-26.55
Palmitoleic (16:1)	0.131	0.131	0.131	0.732	0.686	0.0876-0.200	<LOQ-0.453
Heptadecanoic (17:0)	0.0821	0.0842 <sup>1</sup>	0.0842 <sup>1</sup>	<b>0.008</b>	<b>0.010</b>	0.0698-0.121	<LOQ-0.203
Stearic acid (C18:0)	2.13	2.09 <sup>1</sup>	2.10 <sup>1</sup>	<b>0.011</b>	<b>0.038</b>	1.59-2.48	1.02 – 3.83
Oleic acid (C18:1)	27.1	26.5 <sup>1</sup>	26.3 <sup>1</sup>	<b>0.001</b>	<b>&lt;0.001</b>	16.5-31.1	17.4 – 42.81
Linoleic acid (C18:2)	53.8	54.5 <sup>1</sup>	54.6 <sup>1</sup>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	47.5-64.1	34.27-67.68
Linolenic acid (C18:3)	1.76	1.77	1.78	0.797	0.219	1.39-2.12	0.55-2.33
Arachidic acid (C20:0)	0.427	0.421 <sup>1</sup>	0.421 <sup>1</sup>	<b>0.015</b>	<b>0.029</b>	0.329-0.485	0.267-0.993
Eicosenoic acid (C20:1)	0.228	0.228	0.227	0.916	0.626	0.178-0.348	<LOQ-1.952
Behenic acid (C22:0)	0.174	0.170	0.174	0.580	0.921	0.0977-0.247	<LOQ-0.417

<sup>1</sup> Mauve shading represents MZIR098 means significantly lower than the control; orange shading represents MZIR098 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 mean for lysine in unsprayed MZIR098 was significantly lower than the level in the control. MZIR098 means for the remaining analytes did not vary significantly from the control means. 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 MZIR098 and the hybrid control**

Amino acid	Control (mg/g dw)	Sprayed MZIR098 (mg/g dw)	Unspray MZIR098 (mg/g dw)	P (sprayed v control)	P (unspray v control)	Reference range (mg/g dw)	Combined literature range (mg/g dw)
Alanine	7.91	7.94	7.85	0.823	0.619	5.42-11.4	4.39-14.80
Arginine	4.91	5.00	4.90 <sup>1</sup>	0.186	0.767	3.47-6.54	1.19-7.08
Aspartate	6.66	6.65	6.42 <sup>1</sup>	0.888	0.277	4.87-8.94	3.35 – 12.08
Cystine	2.03	2.06	2.03	0.279	0.981	1.52-2.59	1.16-5.14
Glutamate	19.1	19.1	19.0	0.967	0.689	12.8-28.9	9.65-35.40
Glycine	3.78	3.80	3.76	0.508	0.637	2.70-4.82	1.84-6.85
Histidine	2.56	2.60	2.58	0.245	0.596	1.95-3.58	1.37-4.56
Isoleucine	3.55	3.55	3.55	0.956	0.982	2.38-5.18	1.79-6.92
Leucine	12.9	12.9	12.8	0.899	0.670	8.30-20.7	6.42-24.92
Lysine	2.97	2.99	2.89 <sup>1</sup>	0.631	<b>0.044</b>	1.88-3.85	1.29-6.68
Methionine	2.16	2.15	2.14	0.708	0.602	1.51-2.49	1.05-4.68
Phenylalanine	5.17	5.18	5.13	0.972	0.595	3.52-7.92	2.44-9.30
Proline	8.97	9.05	8.91	0.542	0.615	5.97-12.6	4.62-17.5
Serine	4.70	4.70	4.57	0.942	0.470	3.33-7.04	1.82-7.69
Threonine	3.57	3.58	3.51	0.911	0.370	2.56-4.74	2.19-6.66
Tryptophan	0.839	0.850	0.843 <sup>1</sup>	0.231	0.704	0.639-1.02	0.271-2.15
Tyrosine	4.02	4.01	4.01	0.919	0.829	2.69-6.09	1.03-7.34
Valine	4.61	4.64	4.61	0.809	0.857	3.27-6.23	2.66-8.55

<sup>1</sup> Mauve shading represents MZIR098 mean significantly lower than the control

### 5.3.4 Minerals

The levels of 10 minerals in grain from MZIR098 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 potassium in both the sprayed and unsprayed MZIR098 samples were significantly higher than the means from the control. For calcium and copper, the means from the unsprayed MZIR098 were significantly higher than the means for the control. For the other minerals, there was no significant difference between the means for the control and the means for both MZIR098 treatments. All means for all treatments fell within both the reference range and literature range.

**Table 11: Mean levels of minerals in the grain of MZIR098 and the hybrid control**

Mineral	Control (mg/kg dw)	Sprayed MZIR098 (mg/kg dw)	Unspray MZIR098 (mg/kg dw)	P (sprayed v control)	P (unspray v control)	Reference range (mg/kg dw)	Combined literature range (mg/kg dw)
Calcium	35.5	36.6	37.8	0.217	<b>0.023<sup>1</sup></b>	27.4-59.1	<LOQ-1010.0
Copper	1.90	1.93	2.02 <sup>1</sup>	0.499	<b>0.013</b>	1.33-3.20	<LOQ-21.20
Iron	19.7	19.7	19.9 <sup>1</sup>	0.901	0.655	13.4-28.8	9.51-191.0
Magnesium	1176	1180	1178	0.830	0.926	867-1400	594.0-1940.0
Manganese	5.93	5.98	5.98	0.739	0.717	3.15-9.10	1.69-14.30
Phosphorus	2989	3060	3030	0.079	0.314	2410-3750	1300-5520

Mineral	Control (mg/kg dw)	Sprayed MZIR098 (mg/kg dw)	Unspray MZIR098 (mg/kg dw)	P (sprayed v control)	P (unspray v control)	Reference range (mg/kg dw)	Combined literature range (mg/kg dw)
Potassium	3549	3670	3680	<b>0.001</b>	<b>&lt;0.001</b>	3170-4640	1810-6030
Zinc	20.4	20.9	22.2	0.597	0.105	12.7-29.3	6.5-42.6

<sup>1</sup> Orange shading represents MZIR098 means significantly higher 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 MZIR098 for the means of thiamine HCl, riboflavin, niacin, pyridoxine HCl and folic acid.
- the means for vitamin A in both sprayed and unsprayed MZIR098 plants were significantly higher than the control mean.
- the mean for  $\alpha$ -tocopherol in sprayed MZIR098 was significantly higher 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 MZIR098 and the hybrid control**

Vitamin	Control (mg/kg dw)	Sprayed MZIR098 (mg/kg dw)	Unspray MZIR098 (mg/kg dw)	P (sprayed v control)	P (unspray v control)	Reference range (mg/kg dw)	Combined literature range (mg/kg dw)
Vitamin A ( $\beta$ -Carotene)	1.45	1.54 <sup>1</sup>	1.54	<b>0.025</b>	<b>0.037</b>	0.64-3.18	0.19 – 49.9
Vitamin B <sub>1</sub> (Thiamine HCl)	3.74	3.79 <sup>1</sup>	3.80	0.450	0.359	2.49-5.06	1.26 – 40.00
Vitamin B <sub>2</sub> (Riboflavin)	2.17	2.33	2.06	0.254	0.437	1.14-3.75	0.50 – 7.3
Vitamin B <sub>3</sub> (Niacin)	20.9	21.1	21.1	0.530	0.453	15.5-41.7	10.37 – 46.94
Vitamin B <sub>6</sub> (Pyridoxine HCl)	5.63	5.62	5.57	0.971	0.580	3.65-9.10	3.68 – 12.14
Vitamin B <sub>9</sub> (Folic acid)	0.441	0.453	0.440	0.337	0.940	0.232-0.640	0.147 – 3.5
Vitamin E ( $\alpha$ -Tocopherol)	12.1	12.7 <sup>1</sup>	12.4	<b>0.044</b>	0.286	7.6-22.21	1.537 – 68.672

<sup>1</sup> Orange shading represents MZIR098 mean significantly higher 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 MZIR098 and the control.

All means also fell within both the reference and literature ranges.

**Table 13: Mean of anti-nutrients in grain from MZIR098 and the hybrid control**

Vitamin	Control (mg/kg dw)	Sprayed MZIR098 (mg/kg dw)	Unspray MZIR098 (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)	0.885	0.876	0.859	0.706	0.282	0.503-1.34	<LOQ-1.570
Raffinose %dw)	0.105	0.109	0.111	0.189	0.076	<LOQ-0.386	<LOQ-0.443
Trypsin inhibitor (TIU/mg)	4.03	4.00	3.94	0.826	0.580	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. No analyte means differed significantly between MZIR098 and the control. 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 MZIR098 and the hybrid control**

Secondary metabolite	Control	Sprayed MZIR098	Unspray MZIR098	P (sprayed v control)	P (unspray v control)	Reference range	Combined literature range
p-coumaric acid (mg/kg)	300	307	304	0.161	0.445	113-435	<LOQ-820.0
Ferulic acid (mg/kg)	3357	3408	3357	0.198	0.987	1700-2920	291.9 – 4397.3
Inositol (ppm)	2520	2565	2505	0.568	0.852	1720-3890	<LOQ-4750.0

### 5.3.8 Summary of analysis of key components

A summary of the statistically significant differences in the analyte levels found between grain of MZIR098 and the control is provided in Table 15.

**Table 15: Summary of analyte levels found in grain of MZIR098 that are significantly (P < 0.05) different from those found in grain of the control**

Analyte	MZIR098 (sprayed) mean <sup>1</sup>	MZIR098 (unsprayed) mean <sup>1</sup>	Control mean	Max difference between MZIR098 & control	Diff between max and min in control	MZIR098 within ref range?	MZIR098 within lit range?
NDF (%dw)	11.5	11.3	11.1	0.4	3.22	yes	yes
Starch (%dw)	63.9	65.5	65.8	1.9	16.9	yes	yes
Heptadecanoic (17:0)	0.0842 <sup>1</sup>	0.0842 <sup>1</sup>	0.0821	0.0021	0.0183	yes	yes
Stearic acid (C18:0) %tot	2.09	2.10	2.13	0.04	0.71	yes	yes
Oleic acid (C18:1) %tot	26.5 <sup>1</sup>	26.3 <sup>1</sup>	27.1	0.8	5.7	yes	yes
Linoleic acid (C18:2) %tot	54.5 <sup>1</sup>	54.6 <sup>1</sup>	53.8	0.8	6.3	yes	yes
Arachidic acid (C20:0) %tot	0.421 <sup>1</sup>	0.421 <sup>1</sup>	0.427	0.006	0.124	yes	yes



Analyte	MZIR098 (sprayed) mean <sup>1</sup>	MZIR098 (unsprayed) mean <sup>1</sup>	Control mean	Max difference between MZIR098 & control	Diff between max and min in control	MZIR098 within ref range?	MZIR098 within lit range?
Lysine (%dw)	2.99	2.89 <sup>1</sup>	2.97	0.08	1.97	yes	yes
Calcium (mg/kg dw)	36.6	37.8 <sup>1</sup>	35.5	2.3	26.5	yes	yes
Copper (mg/kg dw)	1.93	2.02 <sup>1</sup>	1.90	0.12	1.25	yes	yes
Potassium (mg/kg dw)	3670 <sup>1</sup>	3680 <sup>1</sup>	3549	131	1080	yes	yes
Vitamin A (mg/kg dw)	1.54 <sup>1</sup>	1.54 <sup>1</sup>	1.45	0.09	0.85	yes	yes
Vitamin E (mg/kg dw)	12.7 <sup>1</sup>	12.4	12.4	0.3	7.36	yes	yes

<sup>1</sup> mauve shading represents MZIR098 means that are significantly lower than the control means while orange shading represents MZIR098 means that are significantly higher.

## 5.4 Conclusion from compositional analyses

Detailed compositional analyses were done to establish the nutritional adequacy of grain from MZIR098 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 13 of the 57 analytes reported in Tables 8 – 14 deviated from the control in a statistically significant manner; for six of these the difference occurred only in one of the MZIR098 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 the difference between each analyte mean of MZIR098 and the control in Table 15 was smaller than the variation within the control. It can therefore be concluded that grain from MZIR098 is compositionally equivalent to grain from conventional corn varieties.

## 5 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). MZIR098 is the result of a genetic modification designed to provide protection against corn rootworm and tolerance to glufosinate, 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 MZIR098 indicate it is equivalent in composition to conventional corn cultivars. The introduction of food from MZIR098 into the food supply is therefore expected to have little nutritional impact and, as such, no additional studies, including animal feeding studies, are required.



## References<sup>35</sup>

- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25(17):3389–3402
- Aronson AI, Shai Y (2001) Why *Bacillus thuringiensis* insecticidal toxins are so effective: unique features of their mode of action. *FEMS Microbiology Letters* 195:1–8
- Bartholomaeus A, Parrott W, Bondy G, Walker K (2013) The use of whole food animal studies in the safety assessment of genetically modified crops: Limitations and recommendations. *Critical Reviews in Toxicology* 43(S2):1–24
- Baxevanis AD (2005) Assessing Pairwise Sequence Similarity: BLAST and FASTA. Ch 11 In: Baxevanis AD, Ouellette BFF (eds) *Bioinformatics: A Practical Guide to the Analysis of Genes and Proteins*. John Wiley & Sons, Inc., p. 295–324
- Bevan M, Barnes WM, Chilton M-D (1983) Structure and transposition of the nopaline synthase gene region of T-DNA. *Nucleic Acids Research* 11:369–385
- Bradbury JF (1986) *Guide to plant pathogenic bacteria*. Cambridge News Ltd, Aberystwyth
- Bravo A, Gill SS, Soberón M (2007) Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon* 49:423–435
- Bravo A, Gómez I, Porta H, Garcia-Gómez BI, Rodriguez-Almazan C, Pardo L, Soberón M (2012) Evolution of *Bacillus thuringiensis* Cry toxins insecticidal activity. *Microbial Biotechnology* 6:17–26
- Broderick NA, Raffa KF, Handelsman J (2006) Midgut bacteria required for *Bacillus thuringiensis* insecticidal activity. *Proceedings of the National Academy of Sciences* 103:15196–15199
- CFIA (1994) The biology of *Zea mays* L. (corn/maize). BIO1994-11. Canadian Food Inspection Agency, Ottawa.  
<http://www.inspection.gc.ca/plants/plants-with-novel-traits/applicants/directive-94-08/biology-documents/zea-mays-l-eng/1330985739405/1330985818367>
- Chen, E., Stacy, C. (2007) Modified Cry3A toxins. Patent US 7276583 B2.  
<http://www.google.com/patents/US7276583>
- Christensen AH, Quail PH (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Research* 5(3):213–218
- Christensen AH, Sharrock RA, Quail PH (1992) Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. *Plant Molecular Biology* 18(4):675–689
- Clarke LA, Rebelo CS, Gonçalves J, Boavida MG, Jordan P (2001) PCR amplification introduces errors into mononucleotide and dinucleotide repeat sequences. *Journal of Clinical Pathology - Molecular Pathology* 54:351–353
- Codex (2009) *Foods derived from modern biotechnology*, 2nd edition. Codex Alimentarius Commission, Joint FAO/WHO Food Standards Programme, Food and Agriculture Organization of the United Nations, Rome.  
<http://www.codexalimentarius.org/standards/thematic-publications/>

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<sup>35</sup> All website references were current as at 20 November 2015

CRA (2006) Corn oil. 5 ed, Corn Refiners Association, Washington D.C.  
<http://www.corn.org/wp-content/uploads/2009/12/CornOil.pdf>.

D'Halluin K, De Block M, Denecke J, Janssens J, Leemans J, Reynaerts A, Botterman J (1992) The *bar* gene as selectable and screenable marker in plant engineering. *Methods in Enzymology*:415–426

Damgaard PH, Granum PE, Bresciani J, Torregrossa MV, Eilenberg J, Valentino L (1997) Characterization of *Bacillus thuringiensis* isolated from infections in burn wounds. *FEMS Immunology & Medical Microbiology* 18:47–53

de Maagd RA, Bravo A, Crickmore N (2001) How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. *Trends in Genetics* 17:193–199

Delaney B, Astwood JD, Cunny H, Eichen Conn R, Herouet-Guicheney C, MacIntosh S, Meyer LS, Privalle LS, Gao Y, Mattsson J, Levine M, ILSI (2008) Evaluation of protein safety in the context of agricultural biotechnology. *Food and Chemical Toxicology* 46:S71–S97

Delzer, B. (2004) Inbred maize line NP2222. Patent US 6710 233 B2.  
[https://www.lens.org/lens/patent/US\\_6710233\\_B2](https://www.lens.org/lens/patent/US_6710233_B2)

Depicker A, Stachel S, Dhaese P, Zambryski P, Goodman HM (1982) Nopaline synthase: transcript mapping and DNA sequence. *Journal of Molecular and Applied Genetics* 1(6):561–573

FAOSTAT3 (2015) Online database of the Food and Agriculture Organization of the United Nations.  
<http://faostat3.fao.org/browse/T/TP/E>

Fard NA, Minuchehr Z, Mousavi A (2013) Allergenicity study of genetically modified herbicide resistant crops (bioinformatics assessment). *Bulletin of Environment, Pharmacology and Life Sciences* 2(3):24–32

Federici BA (2005) Insecticidal bacteria: an overwhelming success for invertebrate pathology. *Journal of Invertebrate Pathology* 89(1):30–38

Fling ME, Kopf J, Richards C (1985) Nucleotide sequence of the transposon Tn7 gene encoding an aminoglycoside-modifying enzyme, 3''(9)-O-nucleotidyltransferase. *Nucleic Acids Research* 13(19):7095–7106

Franck A, Guilley H, Jonard G, Richards K, Hirth L (1980) Nucleotide sequence of Cauliflower mosaic virus DNA. *Cell* 21(1):285–294

FSANZ (2000) Application A346 - Food derived from insect-protected corn line MON810. Report prepared by Food Standards Australia New Zealand.  
<http://www.foodstandards.gov.au/code/applications/pages/applicationa346/Default.aspx>

FSANZ (2001a) Application A385 - Food derived from insect-protected corn line Bt176. Report prepared by Food Standards Australia New Zealand.  
<http://www.foodstandards.gov.au/code/applications/pages/applicationa385insec939.aspx>

FSANZ (2001b) Application A386 - Food derived from herbicide-tolerant and insect-protected corn line Bt11. Report prepared by Food Standards Australia New Zealand.  
<http://www.foodstandards.gov.au/code/applications/pages/applicationa386insec938.aspx>

FSANZ (2001c) Application A382 - Food derived from insect-protected potato lines BT-06, ATBT04-06, ATBT04-31, ATBT04-36, and SPBT02-05. Report prepared by Food Standards Australia New Zealand.  
<http://www.foodstandards.gov.au/code/applications/pages/applicationa382foodd943.aspx>

FSANZ (2001d) Application A383 - Food derived from insect- and virus-protected potato lines RBMT21-129, RBMT21-350, and RBMT22-82. Report prepared by Food Standards Australia New Zealand.

<http://www.foodstandards.gov.au/code/applications/pages/applicationa383foodp942.aspx>

FSANZ (2001e) Application A384 - Food derived from insect- and virus-protected potato lines RBMT15-101, SEM15-02 and SEM15-15. Report prepared by Food Standards Australia New Zealand.

<http://www.foodstandards.gov.au/code/applications/pages/applicationa384foodp940.aspx>

FSANZ (2006a) Application A564 - Food derived from insect-protected corn line MIR604. Report prepared by Food Standards Australia New Zealand.

<http://www.foodstandards.gov.au/code/applications/pages/applicationa564foodd2972.aspx>

FSANZ (2006b) Application A533: Food Derived from Glufosinate-Ammonium Tolerant Cotton Line LL25. Report prepared by Food Standards Australia New Zealand.

<http://www.foodstandards.gov.au/code/applications/pages/applicationa533foodd2440.aspx>

FSANZ (2009) Application A615 - Food derived from insect-protected cotton line COT67B. Report prepared by Food Standards Australia New Zealand.

<http://www.foodstandards.gov.au/code/applications/pages/applicationa615foodd3783.aspx>

FSANZ (2010) Application A1028 - Food derived from herbicide-tolerant and insect-protected cotton line T304-40. Report prepared by Food Standards Australia New Zealand, available online at.

<http://www.foodstandards.gov.au/code/applications/pages/applicationa1028oid4457.aspx>

FSANZ (2012) Application A1060 - Food derived from insect-protected corn line 5307. Report prepared by Food Standards Australia New Zealand.

<http://www.foodstandards.gov.au/code/applications/pages/applicationa1060food5180.aspx>

FSANZ (2013a) Application A1080 - Food derived from herbicide-tolerant cotton line MON88701. Report prepared by Food Standards Australia New Zealand.

<http://www.foodstandards.gov.au/code/applications/Pages/A1080-Food-derived-from-Herbicide-tolerant-Cotton-Line-MON88701.aspx>

FSANZ (2013b) Application A1087 - Food derived from insect-protected soybean line DAS-81419-2. Report prepared by Food Standards Australia New Zealand.

<http://www.foodstandards.gov.au/code/applications/Pages/A1087-Food-derived-from-Insect-protected-Soybean-Line-DAS-81419-2.aspx>

Geiser M, Schweitzer S, Grimm C (1986) The hypervariable region in the genes coding for entomopathogenic crystal proteins of *Bacillus thuringiensis* nucleotide sequence of the *kurdh1* gene of subsp. *kurstaki* HD1. *Gene* 48(1):109–118

Green Pool (2012) Sugar consumption in Australia. A statistical update. Green Pool Commodity Specialists, Brisbane.

[http://greenpoolcommodities.com/files/8113/4932/3223/121004\\_Sugar\\_Consumption\\_in\\_Australia\\_-\\_A\\_Statistical\\_Update\\_-\\_Public\\_Release\\_Document.pdf](http://greenpoolcommodities.com/files/8113/4932/3223/121004_Sugar_Consumption_in_Australia_-_A_Statistical_Update_-_Public_Release_Document.pdf)

Hammond B, Kough J, Herouet-Guicheney C, Jez JM (2013) Toxicological evaluation of proteins introduced into food crops. *Critical Reviews in Toxicology* 43(S2):25–42

Harrigan GG, Glenn KC, Ridley WP (2010) Assessing the natural variability in crop composition. *Regulatory Toxicology and Pharmacology* 58:S13–S20; doi:10.1016/j.yrtph.2010.08.023

Henikoff S, Henikoff JG (1992) Amino acid substitution matrices from protein blocks. *Proceedings of the National Academy of Sciences* 89:10915–10919

Herman RA, Ekmay R (2014) Do whole-food animal feeding studies have any value in the safety assessment of GM crops? *Regulatory Toxicology and Pharmacology* 68:171–174

- Hernandez E, Ramise F, Ducoureau J-P, Cruel T, Cavallo J-D (1998) *Bacillus thuringiensis* subsp. *konkukian* (serotype H34) superinfection: case report and experimental evidence of pathogenicity in immunosuppressed mice. *Journal of Clinical Microbiology* 36:2138–2139
- Hérouet C, Esdaile DJ, Mallyon BA, Debruyne E, Schulz A, Currier T, Hendrickx K, van der Klis R-J, Rouan D (2005) Safety evaluation of the phosphinothricin acetyltransferase proteins encoded by the *pat* and *bar* sequences that confer tolerance to glufosinate-ammonium herbicide in transgenic plants. *Regulatory Toxicology and Pharmacology* 41:134–149
- Hofmann C, Vanderbruggen H, Höfte H, Van Rie J, Jansens S, Van Mellaert H (1988) Specificity of *Bacillus thuringiensis* d-endotoxins is correlated with the presence of high-affinity binding sites in the brush border membrane of target insect midguts. *Proceedings of the National Academy of Sciences* 85:7844–7848
- Höfte H, Whiteley HR (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiological Reviews* 53:242–255
- Hohn, T., Stovolone, L., De Haan, P. T., Ligon, H. T., Kononova, M. (2007) Cestrum yellow leaf curling virus promoters. Patent US 7,166,770 B2.  
<http://docs.google.com/viewer?url=patentimages.storage.googleapis.com/pdfs/US7166770.pdf>
- ILSI (2014) International Life Sciences Institute Crop Composition Database Version 4.0.  
<http://www.cropcomposition.org/query/index.html>
- Jackson SG, Goodbrand RB, Ahmed R, Kasatiya S (1995) *Bacillus cereus* and *Bacillus thuringiensis* isolated in a gastroenteritis outbreak investigation. *Letters in Applied Microbiology* 21:103–105
- Jensen ON, Podtelejnikov AV, Mann M (1997) Identification of the components of simple protein mixtures by high-accuracy peptide mass mapping and database searching. *Annals of Chemistry* 69(23):4741–4750
- Kim S-I, Veena, Gelvin SB (2007) Genome-wide analysis of *Agrobacterium* T-DNA integration sites in the Arabidopsis genome generated under non-selective conditions. *The Plant Journal* 51:779–791
- Koch MS, Ward JM, Levine SL, Baum JA, Vicini JL, Hammond BG (2015) The food and environmental safety of Bt crops. *Frontiers in Plant Science* 6:283
- Kützner HJ (1981) The Family Streptomycetaceae. In: Starr MP, Stolp H, Trüper HG, Ballows A, and Schlegel HG (eds) *The Prokaryotes: A Handbook on Habitats, Isolation and Identification of Bacteria*. Springer Verlag, Berlin, p. 2028–2090
- Lebrun, M., Sailland, A., Freyssinet, G., DeGryse, E. (2003) Mutated 5-enolpyruvylshikimate-3-phosphate synthase, gene coding for said protein and transformed plants containing said gene. Patent US 6 566 587 B1.  
<http://www.google.com/patents/US6566587>
- Metcalf DD, Astwood JD, Townsend R, Sampson HA, Taylor SL, Fuchs RL (1996) Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Critical Reviews in Food Science and Nutrition* 36 Suppl:S165–S186
- Miletich JP, Broze Jr. GJ (1990) b Protein C is not glycosylated at asparagine 329. *The Journal of Biological Chemistry* 265:11397–11404
- Murray EE, Lotzer J, Eberle M (1989) Codon usage in plant genes. *Nucleic Acids Research* 17(2):477–498
- Narva KE, Storer NP, Meade T (2014) Discovery and development of insect-resistant crops using genes from *Bacillus thuringiensis*. Ch 4 In: Dhadialla TS, Gill SS (eds) *Advances in insect physiology*. Volume 47: Insect midgut and insecticidal proteins. Academic Press, London, p. 177–248

Negrotto D, Jolley M, Beer S, Wenck AR, Hansen G (2000) The use of phosphomannose-isomerase as a selectable marker to recover transgenic maize plants (*Zea mays* L.) via *Agrobacterium* transformation. *Plant Cell Reports* 19:798–803

NPTN (2000) *Bacillus thuringiensis* (Technical Fact Sheet)., National Pesticide Telecommunications Network, Oregon State University & U.S. Environmental Protection Agency.  
<http://npic.orst.edu/factsheets/BTtech.pdf>

Odell JT, Nagy F, Chua N-H (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature* 313:810–812

OECD (2002) Consensus document on compositional considerations for new varieties of maize (*Zea mays*): Key food and feed nutrients, anti-nutrients and secondary plant metabolites. Organisation for Economic Co-operation and Development, Paris.  
[http://www.oecd.org/officialdocuments/displaydocumentpdf?cote=env/jm/mono\(2002\)25&doclanguage=en](http://www.oecd.org/officialdocuments/displaydocumentpdf?cote=env/jm/mono(2002)25&doclanguage=en)

OECD (2003) Considerations for the safety assessment of animal feedstuffs derived from genetically modified plants. ENV/JM/MONO(2003)10. Organisation for Economic Co-operation and Development, Paris.  
[http://www.oecd.org/officialdocuments/displaydocumentpdf?cote=ENV/JM/MONO\(2003\)10&doclanguage=en](http://www.oecd.org/officialdocuments/displaydocumentpdf?cote=ENV/JM/MONO(2003)10&doclanguage=en)

OECD (2007) Consensus document on safety information on transgenic plants expressing *Bacillus thuringiensis* - derived insect control proteins. ENV/JM/MONO(2007)14. Organisation for Economic Co-operation and Development.  
[http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO\(2007\)14&docLanguage=En](http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2007)14&docLanguage=En)

OGTR (2008) The biology of *Zea mays* L. ssp *mays* (maize or corn). Prepared by the Office of the Gene Technology Regulator, Australia.  
<http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/riskassessments-1>

Orlando R, Yang Y (1998) Analysis of Glycoproteins. Ch 9 In: Larsen BS, McEwen CN (eds) *Mass Spectrometry of Biological Materials*. 2nd ed, Marcel Dekker, p. 216–246

Pearson WR, Lipman DJ (1988) Improved tools for biological sequence comparison. *Proceedings of the National Academy of Sciences* 85(8):2444–2448

Pietrzak M, Shillito RD, Hohn T, Potrykus I (1986) Expression in plants of two bacterial antibiotic resistance genes after protoplast transformation with a new plant expression vector. *Nucleic Acids Research* 14:5857–5868

Polevoda B, Sherman F (2000) Na-terminal Acetylation of Eukaryotic Proteins. *Journal of Biological Chemistry* 275(47):36479–36482

Ransom J, Endres GJ (2014) Corn growth and management quick guide. A-1173, North Dakota State University Extension Service.  
<http://www.ag.ndsu.edu/publications/landing-pages/crops/corn-growth-and-management-quick-guide-a-1173>

Ridley WP, Harrigan GG, Breeze ML, Nemeth MA, Sidhu RS, Glenn KC (2011) Evaluation of compositional equivalence for multitrait biotechnology crops. *Journal of Agricultural and Food Chemistry* 59:5865–5876

Sekar V, Thompson DV, Maroney MJ, Bookland RG (1987) Molecular cloning and characterization of the insecticidal crystal protein gene of *Bacillus thuringiensis* var. *tenebrionis*. *Proceedings of the National Academy of Sciences* 84:7036–7040

- Siegel JP (2001) The mammalian safety of *Bacillus thuringiensis*-based insecticides. *Journal of Invertebrate Pathology* 77(1):13–21
- Stavolone L, Kononova M, Pauli S, Ragozzino A, de Haan P, Milligan S, Lawton K, Hohn T (2003) Cestrum yellow leaf curling virus (CmYLCV) promoter: a new strong constitutive promoter for heterologous gene expression in a wide variety of crops. *Plant Molecular Biology* 53:703–713
- Thompson CJ, Movva NR, Tizard R, Crameri R, Davies JE, Lauwereys M, Botterman J (1987) Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus*. *The EMBO Journal* 6:2519–2523
- Tzfira T, Li J, Lacroix B, Citosky V (2004) *Agrobacterium* T-DNA integration: molecules and models. *Trends in Genetics* 20(8):375–383
- van Heerwaarden J, Hufford MB, Ross-Ibarra J (2012) Historical genomics of North American maize. *Proceedings of the National Academy of Sciences* 109(31):12420–12425
- Walling LL (2006) Recycling or regulation? The role of amino-terminal modifying enzymes. *Current Opinion in Plant Biology* 9(3):227–233
- Walters FS, Stacy CM, Lee MK, Palekar N, Chen JS (2008) An engineered chymotrypsin/cathepsin G site in domain I renders *Bacillus thuringiensis* Cry3A active against western corn rootworm larvae. *Applied and Environmental Microbiology* 74(2):367–374
- Walters FS, deFontes CM, Hart H, Warren GW, Chen JS (2010) Lepidopteran-active variable-region sequence imparts coleopteran activity in eCry3.1Ab, an engineered *Bacillus thuringiensis* hybrid insecticidal protein. *Applied and Environmental Microbiology* 76(10):3082–3088
- Wehrmann A, Van Vliet A, Opsomer C, Botterman J, Schulz A (1996) The similarities of *bar* and *pat* gene products make them equally applicable for plant engineers. *Nature Biotechnology* 14:1274–1278
- White PJ, Pollak LM (1995) Corn as a food source in the United States: Part II. Processes, products, composition, and nutritive values. In: *Cereal Foods World* 40. p. 756–762
- WHO. Microbial pest control agent *Bacillus thuringiensis*. (217). 1999. Geneva, World Health Organization. Environmental Health Criteria.
- Wohlleben W, Arnold W, Broer I, Hillemann D, Strauch E, Punier A (1988) Nucleotide sequence of the phosphinothricin N-acetyltransferase gene from *Streptomyces viridochromogenes* Tü494 and its expression in *Nicotiana tabacum*. *Gene* 70:25–37
- Zhou J, Harrigan GG, Berman KH, Webb EG, Klusmeyer TH, Nemeth MA (2011) Stability in the composition equivalence of grain from insect-protected maize and seed from glyphosate-tolerant soybean to conventional counterparts over multiple seasons, locations, and breeding germplasms. *Journal of Agricultural and Food Chemistry* 59:8822–8828