

## **Supporting document 1**

### **Safety assessment report (at Approval)**

#### **Application A1060**

#### **Food derived from Insect-protected Corn Line 5307**

### **Summary and conclusions**

#### **Background**

Insect-protected corn line 5307 (Syngenta Seeds Pty Ltd) is a genetically modified (GM) line expressing a novel insecticidal protein, eCry3.1Ab, which was engineered from selected portions of two naturally occurring crystal (Cry) proteins found in the common soil bacterium *Bacillus thuringiensis* (Bt). The protein is a chimera derived from modified Cry3A (mCry3A) and Cry1Ab, which have been used individually as insecticidal proteins in GM crops previously approved for use in Australia and New Zealand.

The eCry3.1Ab chimeric protein is active against the larvae of Western corn rootworm (*Diabrotica virgifera virgifera* Le Conte), Northern corn rootworm (*D. longicornis barberi* Smith and Lawrence) and Mexican corn rootworm (*D. virgifera zea* Krysan and Smith). These coleopteran pests cause significant damage annually to corn crops grown in the United States of America (U.S). The engineered Cry protein binds specifically to receptors in midgut epithelial cells of target insects, leading to paralysis of the gut, cessation of feeding, and eventual death of the insect larvae.

The *pmi* gene (also known as *manA*) from *Escherichia coli* strain K-12 was used as a plant selectable marker during development of corn line 5307. The gene encodes phosphomannose isomerase (PMI), an enzyme that enables plants to utilise mannose as a carbon source.

Corn line 5307 has not been developed for commercial cultivation in Australia or New Zealand. Consequently, the Applicant has not applied to the Office of the Gene Technology Regulator (OGTR), or to the Environmental Risk Management Authority (ERMA) for a licence to grow corn line 5307 in Australia or New Zealand. Subject to regulatory approvals in relevant countries, it is intended that plant breeders would use corn line 5307 in conventional breeding programs with other insect-protected corns to generate products with a broad spectrum of insect control.

#### **History of Use**

Maize (field corn) is the world's third most important cereal crop, behind wheat and rice, and is grown in over 25 countries. Corn-derived products are routinely used in a large number and diverse range of foods and have a long history of safe use. Food products include flour, breakfast cereals, high fructose corn syrup and other starch derivatives.

*B. thuringiensis* has been used extensively as a microbial pest control agent for many decades. The organism itself has been extensively studied and commercially exploited in forestry, agriculture, including organic food production systems, as well as home gardens. Members of the large family of *Bt* Cry proteins have been used in the control of mosquitoes that are vectors of human diseases, and in the development of transgenic insect protected plants. Cry proteins are typically highly specific to their target insect, are innocuous to humans, vertebrates and plants, and are completely biodegradable.

### **Molecular Characterisation**

Molecular analysis of corn line 5307 demonstrated that one intact and functional copy of the two-gene cassette was inserted into the corn genome. The absence of plasmid backbone sequences in the original transformed line, and the stability of the insert over multiple generations were confirmed following phenotypic and genotypic analyses of plants generated from a series of conventional crosses with other selected corn lines.

### **Characterisation of Novel Proteins**

The identity and physicochemical properties of eCry3.1Ab and PMI as expressed in corn line 5307 were confirmed in several laboratory studies. The proteins conformed in size and amino acid sequence to that expected from the gene sequence, there was no evidence of glycosylation, and both proteins exhibited the expected functional activity in specialised assays.

The eCry3.1Ab and PMI proteins are expressed at low levels in grain from corn line 5307, with mean levels of 4.9 µg/g and 1.3 µg/g respectively. After processing of the grain, the levels of the two proteins were highest in germ, a product of the dry-milling process. The mean level of eCry3.1Ab in germ was 19.3 µg/g, and of PMI was 4.0 µg/g. This is consistent with higher extracted protein levels overall in dry-milled fractions. Germ is subsequently used for oil extraction.

Further studies provided evidence that the eCry3.1Ab and PMI proteins are unlikely to be either toxic or allergenic in humans. Separate bioinformatic analyses confirmed the absence of any biologically significant amino acid sequence similarity to known or putative protein toxins or allergens. Digestibility studies demonstrated that these proteins would be degraded through normal digestive processes. Separate oral toxicity studies in mice with eCry3.1Ab and PMI also confirmed the absence of acute toxicity.

The protein components of eCry3.1Ab are already approved for use in other GM crops, and the source organism *Bt* is already in the food supply. Similarly, PMI proteins are found widely in nature, including in food products. This history of dietary exposure further supports the safety of both novel proteins in corn line 5307.

### **Compositional Analyses**

Compositional analyses of grain were undertaken to establish the nutritional adequacy of corn line 5307 compared with its conventional counterpart. The constituents analysed included proximates, fibre, minerals, vitamins, amino acids, fatty acids, anti-nutrients and secondary plant metabolites.

No differences of biological significance were observed between corn line 5307 and the near-isogenic line used as a comparator. A small number of statistical differences in some analytes were noted, but these occurred randomly across trial sites. Differences such as these simply reflect the fact that a large number of tests were performed, and approximately 5% of these would be expected to show statistically significant differences by chance alone.

The mean levels of all constituents measured in corn line 5307 were within the commercial reference range reported in the ILSI crop composition database for corn. Some constituents in particular, showed a very broad natural range. Based on these results, the genetic modification in corn line 5307 did not result in any unintended changes in composition.

### **Nutritional Impact**

The detailed comparison of corn line 5307 with its conventional counterpart did not show any unintended changes in the nutrient profile of the GM line and confirmed that it is compositionally equivalent to conventional corn varieties. Its introduction into the food supply would therefore be expected to have little nutritional impact. As additional confirmation, a feeding study in broiler chickens was conducted to establish the nutritional adequacy of corn line 5307. This study confirmed that a diet containing grain from corn 5307 was equivalent to a diet containing conventional corn grain in supporting typical growth and nutritional well-being in rapidly growing broiler chickens.

### **Conclusion**

No potential public health and safety concerns have been identified in the assessment of insect protected corn line 5307. On the basis of the data and information currently available, food derived from corn line 5307 is as safe for human consumption as food derived from conventional corn varieties.

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## LIST OF KEY ABBREVIATIONS

ADF	acid detergent fibre
BLAST	Basic Local Alignment Search Tool
bp	base pairs
Bt	<i>Bacillus thuringiensis</i>
CMP	cestrum yellow leaf curling virus promoter
Cry1Ab	Cry1Ab insecticidal protein from <i>Bacillus thuringiensis</i>
Cry3A	Cry3A insecticidal protein from <i>Bacillus thuringiensis</i>
DNA	deoxyribonucleic acid
dw	dry weight
<i>ecry3.1Ab</i>	chimeric gene engineered from <i>mcry3A</i> and <i>cry1Ab</i> genes
eCry3.1Ab	protein encoded by <i>ecry3.1Ab</i>
ELISA	enzyme linked immunosorbent assay
FAO	Food and Agriculture Organization of the United Nations
FSANZ	Food Standards Australia New Zealand
GC	gas chromatography
GM	genetically modified
IgE	immunoglobulin E
ILSI	International Life Sciences Institute
kb	kilo base
kDa	kilo Dalton
LOQ	limit of quantitation
LOD	limit of detection
MALDI-MS	matrix assisted laser desorption ionization mass spectrometry
mCry3A	modified Cry3A protein
NHANES	National Health and Nutrition Examination Survey (U.S.)
NH&MRC	National Health & Medical Research Council (Australia)
NCBI	National Center for Biotechnology Information
NDF	neutral detergent fibre
NUTTAB	Nutrient Tables (Australian Food Composition Tables)
OECD	Organisation for Economic Co-operation and Development
ORF	open reading frame
<i>ori</i>	(bacterial) origin of replication
PCR	polymerase chain reaction
mRNA	messenger RNA
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
T-DNA	transferred DNA
U.S.	United States of America
UTR	untranslated region
WHO	World Health Organisation

## 1. Introduction

Insect-protected corn line 5307, a product of Syngenta Seeds Pty Ltd, is a genetically modified (GM) corn that is protected from feeding damage caused by the larvae of a number of insect pest species. Protection is conferred by expression in the plant of a chimeric insecticidal protein derived from two naturally occurring crystal (Cry) proteins found in *Bacillus thuringiensis* (Bt), a common soil bacterium. The Cry proteins exert their effect by causing lysis of midgut epithelial cells, which leads to gut paralysis, cessation of feeding and eventual death of the insect larvae. The lysis of the epithelial cells is mediated by the binding of the activated Cry protein to specialised receptors on the cells of the insect midgut.

The gene introduced into corn line 5307, *ecry3.1Ab*, encodes a chimeric protein engineered from selected portions of the modified Cry3A (mCry3A) and Cry1Ab proteins. The chimeric eCry3.1Ab protein is insecticidally active against the larvae of Western corn rootworm (*Diabrotica virgifera virgifera* Le Conte), Northern corn rootworm (*D. Longicornis barberi* Smith and Lawrence) and Mexican corn rootworm (*D. Virgifera zea* Krysan and Smith). These coleopteran species cause significant damage to corn crops grown in North American regions.

Corn line 5307 also contains the bacterial *pmi* gene, also known as *manA*, which is derived from *Escherichia coli* strain K-12. Expression of the PMI protein in plants allows growth on mannose as a carbon source. This was used as a selectable marker to assist with identification of transformed corn cells in the early stages of plant development.

Corn is not a major crop in Australia or New Zealand. Corn line 5307 is intended for use mainly in conventional plant breeding programs to generate agricultural products suitable for commercial cultivation in the United States and Canada, and is not intended for cultivation in Australia or New Zealand. If approved, food from this line may enter the Australian and New Zealand food supply as imported food products.

This safety assessment report addresses food safety and nutritional issues. It does not address potential environmental risks related to the environmental release of GM plants used in food production, or the safety of animal feed or food products derived from animals fed with GM feed.

## 2. History of use

### 2.1 Donor organisms

#### 2.1.1 *Bacillus thuringiensis*

The two Cry proteins used to construct the novel insecticidal protein in corn line 5307 are found naturally in *B. thuringiensis*, a ubiquitous soil organism. Bt is a facultative anaerobic, Gram-positive, spore-forming bacterium that produces characteristic insecticidal proteins, as parasporal crystals, during spore formation. The Cry proteins are highly specific to a narrow range of target insect species, but innocuous to humans, other vertebrates and plants, and are completely biodegradable (Bravo et al 2007). Bt has been used as an effective insect control agent in agriculture over many decades, including in organic food production systems. Target insects belong to the orders *Coleoptera*, *Lepidoptera* and *Diptera* (flies). Bt demonstrates little if any direct toxicity to non-target insects.

The widespread use of *B. thuringiensis* products in the field can result in considerable aerosol and dermal exposure in humans. With the exception of case reports on ocular and dermal irritation, no adverse health effects have been documented after occupational exposure to *B. thuringiensis* products (McClintock et al 1995).

Studies with human volunteers who ingested and inhaled large quantities of a Btk formulation (*B. thuringiensis* subspecies *kurstaki*) did not demonstrate any adverse effects (Fisher & Rosner 1959). Similarly, *B. thuringiensis* present in drinking water or food has not been reported to cause adverse effects on human health (WHO 1999).

FSANZ has previously noted the long-term uses of the Bt organism as a microbial pest control agent on food crops, and as a source of a number of genes for transgenic expression in plants. More than 200 different *cry* genes have been isolated and many of the Cry proteins have been studied in detail. Three major applications of Cry proteins are: 1) the control of insect pests in forestry, 2) the control of mosquitoes that are vectors of human diseases, and 3) the development of transgenic insect-protected plants, including food crops (Soberon *et al.* 2007).

### 2.1.2 *Escherichia coli*

The bacterium *Escherichia coli* is the source of the selectable marker gene, *pmi* (also known as *manA*), which encodes the enzyme phosphomannose isomerase (PMI). *E. coli* belongs to the Enterobacteriaceae, a relatively homogeneous group of rod-shaped, Gram-negative, facultative aerobic bacteria. Members of the genus *Escherichia* are ubiquitous in the environment and are normally found in the digestive tracts of vertebrates, including humans (Jefferson *et al* 1986). The vast majority of *E. coli* strains are harmless to humans, although some strains can cause diarrhoea and occasionally urinary tract infections.

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

The *pmi* gene from *E. coli* K-12 has previously been assessed by FSANZ and no food safety concerns were identified when used in the production of GM food crops.

### 2.1.3 *Agrobacterium tumefaciens*

The species *Agrobacterium tumefaciens* is a Gram-negative, non-spore forming, rod-shaped bacterium commonly found in the soil. It is a natural plant pathogen causing root deformation mainly in sugar beets, pome fruits and viticulture crops.

*Agrobacterium* is closely related to other soil bacteria involved in nitrogen fixation by certain plants. It normally contains a plasmid (the Ti plasmid) with the ability to enter plant cells and insert a portion of its genome into plant chromosomes. This natural process has been adapted for use in the development of transgenic crops. It is now possible to transform a broad range of plant species without causing adverse effects in the host plant.

## 2.2 Host organism

Corn (*Zea mays* L), also known as maize or field corn, is the world's second food crop behind sugarcane, and is grown in over 25 countries worldwide (OECD 2002).

In 2009, worldwide production of corn was nearly 900 million tonnes, with the United States and China being the major producers (FAOSTAT 2011). Corn is not a major crop in Australia or New Zealand, with annual production of grain at approximately 300,000 tonnes and 150,000 tonnes respectively.

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

Corn plants usually reproduce sexually by wind-pollination, which provides for natural out-crossing between plants. In the field, open pollination of hybrids leads to the production of grain with properties derived from different lines and, if planted, would produce lower yields. Outcrossing of corn does however present an opportunity for plant breeders to manipulate the pollination process in order to produce higher-performing hybrid seed. Controlling the cross-pollination of inbred lines from chosen genetic pools (using conventional techniques) enables the production of a hybrid line with a combination of desired genetic traits, and results in improved agronomic performance and increased yields. The concept of using an inbred-hybrid plant system and resulting yield response is the basis of the commercial seed industry in several food commodities, including corn.

### 3. Molecular characterisation

#### Submitted studies:

##### New, S. (2010) –

1. Event 5307 Maize: Copy Number Functional Element Southern Blot Analysis. Report No.: SSB-189-10 A1 (unpublished)
2. Event 5307 Maize: Insert Sequence Analysis. Report No.: SSB-159-10 A1 (unpublished)
3. Event 5307 Maize: Flanking Sequence Determination. Report Nos.: SSB-160-10 (unpublished)
4. Event 5307 Maize: Genetic Stability Analysis. Report No.: SSB-184-10 A1 (unpublished)
5. Event 5307 Maize: Genome to Insert Junction Analysis for Translated Open Reading Frames with a Minimum Size of 30 Amino Acids: Assessment of Amino Acid Sequence Similarity to Known or Putative Toxins. Report Nos.: SSB-187-10 (unpublished)
6. Event 5307 Maize: Genome to Insert Junction Analysis for Translated Open Reading Frames with a Minimum Size of 30 Amino Acids: Assessment of Amino Acid Sequence Similarity to Known or Putative Allergens. Report No.: SSB-188-10 (unpublished)
7. Event 5307: Genomic Insertion Site Analysis. Report No.: SSB-202-10 A1 (unpublished)

##### New, S. (2011) –

8. Event 5307 Maize: Mendelian Inheritance Analysis. Report No.: SSB-203-10 A1 (unpublished)

The data used for molecular characterisation provide a detailed understanding of the genetic material introduced into the host genome. The molecular characterisation addresses three main aspects: the transformation method including a description of the DNA sequences introduced into the host genome; a characterisation of the inserted DNA including any rearrangements that may have occurred as a consequence of the transformation; and the genetic stability of the inserted DNA and phenotype. This characterisation frames the subsequent parts of the safety assessment.

#### 3.1 Transformation method

Insect-protected corn line 5307 was produced by *Agrobacterium*-mediated transformation of a proprietary *Zea mays* line NP2222. This DNA transformation system is well documented, and the basis for insertion of DNA sequences between the Left and Right borders of plasmid vectors is well understood (see Zambryski 1992).

Immature embryos from the proprietary maize line were co-cultivated with *A. tumefaciens* strain LBA4404 and the transforming plasmid pSYN12274 (see Section 3.2). After removal of excess *A. tumefaciens*, expression of the *pmi* gene was used as a means of identifying transformed cells by transferring the embryonic callus to a cell culture medium containing mannose, as described in Negrotto *et al.* 2000. Transformed tissue was subsequently transferred to selective media containing 500 mg/l of the broad-spectrum antibiotic cefotaxime, and grown for four months to ensure that any remaining *A. tumefaciens* was cleared from the transformed tissue.

Regenerated plantlets were tested for the presence of *pmi* and *ecry3.1Ab*, and for the absence of the spectinomycin resistance gene (*spec*) on the vector backbone (ie outside of the Left and Right border sequences) by real-time polymerase chain reaction (PCR) analysis (Ingham *et al.* 2001). Plants positive for *pmi* and *ecry3.1Ab* and negative for *spec* were transferred to the greenhouse for further propagation.

### 3.2 Description of the introduced genetic elements

Plasmid pSYN12274 contained two expression cassettes between the Left and Right border sequences (see Figure 1). The *Bt* expression cassette consisted of the CMP (Cestrum Yellow Leaf Curling Virus) promoter driving expression of the chimeric *ecry3.1Ab* gene (1962 bp), followed by the NOS terminator sequence. The selectable marker cassette consisted of the promoter region of the corn polyubiquitin gene controlling expression of the bacterial *pmi* gene, followed by the NOS terminator sequence. The genetic elements in the transforming plasmid pSYN12274 are described in more detail in Table 1.

#### 3.2.1 Description of the chimeric *Bt* gene

The gene *ecry3.1Ab* consists of a fusion between the 5' end (Domain I, Domain II and 15 aa of Domain III) of a modified Cry3A gene (*mcry3A*) and the 3' end [Domain III and Variable Region 6 (Hofte and Whiteley 1989)] of a synthetic *cry1Ab* gene (see below). Upstream of the *mcry3A* 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 and is translated into the following 22 amino acid residues: MTSNGRQCAGIRPYDGRQQHRG. Immediately following this sequence, the DNA encodes 459 amino acid residues which are identical to those of mCry3A, followed by 172 amino acid residues of Cry1Ab.

Details relating to the *mcry3A* gene have been published. The entire coding region of the *mcry3A* gene was synthesised using codons that are preferred in maize (Murray *et al.* 1989), and to introduce other desired changes to facilitate cloning steps. The synthetic sequence was based on the native Cry3A protein sequence from *Bacillus thuringiensis* subsp. *tenebrionis* (Sekar *et al.* 1987). The maize-optimised gene was then modified to incorporate a consensus cathepsin-G protease recognition site within the expressed protein. The amino acid sequence of the encoded mCry3A corresponds to that of the native Cry3A, except that (i) the N-terminus corresponds to methionine-48 of the native protein, and (ii) a cathepsin-G protease recognition site has been introduced, beginning at residue 155 of the native protein (Walters *et al.* 2008) (see Section 4.1.1).

The *cry1Ab* gene was originally cloned from *Bacillus thuringiensis* subsp. *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.

#### 3.2.2 Description of the selectable marker gene

The promoter region from the maize polyubiquitin gene, which contains the first intron, provides constitutive expression in corn line 5307 of the *pmi* gene from *E. coli*.

This gene encodes phosphomannose isomerase and catalyses the isomerisation of mannose-6-phosphate to fructose-6-phosphate.

**Table 1: Description of the genetic elements in the T-DNA of plasmid pSYN12274. Protein coding regions are in bold type.**

Genetic element	Nucleotides in plasmid	Source and Function
Right border (RB)	1 - 25	Right border region of T-DNA from the nopaline synthase Ti-plasmid of <i>Agrobacterium tumefaciens</i> . This is a short direct repeat sequence that flanks the T-DNA and is required for the transfer of the T-DNA into the plant genomic DNA (Wang <i>et al.</i> 1984).
Intervening sequence	26 - 228	Polylinker region with restriction enzyme sites use for cloning purposes.
CMP promoter	229 - 574	Cestrum Yellow Leaf Curling Virus promoter region (Hohn <i>et al.</i> 2007) providing constitutive expression in corn.
Intervening sequence	575 - 583	Sequence for DNA cloning purposes.
<b>ecry3.1Ab</b>	584 - 2545	An engineered gene encoding a chimeric Cry protein. Because Cry proteins share structural similarities, chimeric <i>cry</i> genes can be engineered via the exchange of domains that are homologous between different <i>cry</i> genes (Walters <i>et al.</i> 2010)(see Section 3.2.1).
Intervening sequence	2546 - 2575	Sequence for DNA cloning purposes.
nos	2576 - 2828	Termination signal from the 3' untranslated region of the <i>nos</i> gene from <i>A. tumefaciens</i> . This 3' regulatory element directs termination of transcription and polyadenylation of the mRNA of the introduced genes.
Intervening sequence	2829 - 2853	Sequence for DNA cloning purposes.
ZmUbilnt promoter	2854 - 4846	Promoter region from the maize polyubiquitin gene which contains the first intron (Christensen <i>et al.</i> 1992), and allows constitutive gene expression.
Intervening sequence	4847 - 4858	Non-coding sequence.
<b>pmi</b>	4859 - 6034	Also known as <i>manA</i> , this gene from <i>E. coli</i> encodes phosphomannose isomerase, an enzyme that catalyses the isomerisation of mannose-6-phosphate to fructose-6-phosphate (Negrotto <i>et al.</i> 2000).
Intervening sequence	6035 - 6094	Sequence for DNA cloning purposes.
nos	6095 - 6347	Termination signal from the 3' untranslated region of the <i>nos</i> gene from <i>A. tumefaciens</i> (as above)
Intervening sequence	6348 - 6435	Sequence for DNA cloning purposes.
Left border (LB)	6436 - 6460	Left border region of T-DNA from the nopaline synthase Ti-plasmid of <i>Agrobacterium tumefaciens</i> . This is a short direct repeat sequence that is required for the transfer of the T-DNA into the plant cell (Zambryski <i>et al.</i> 1982).

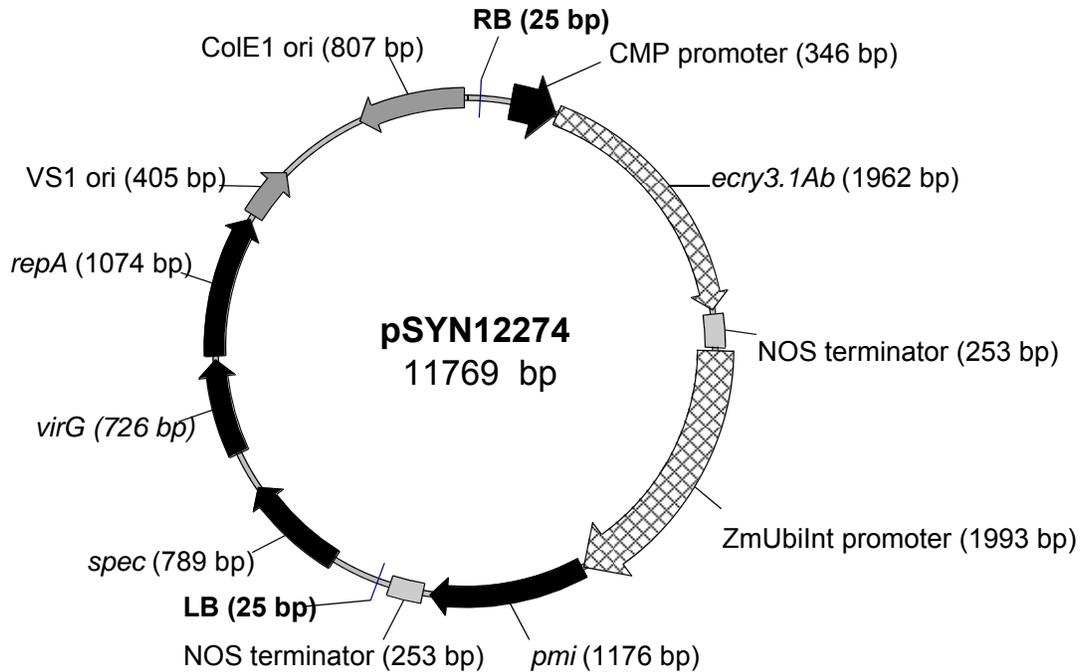


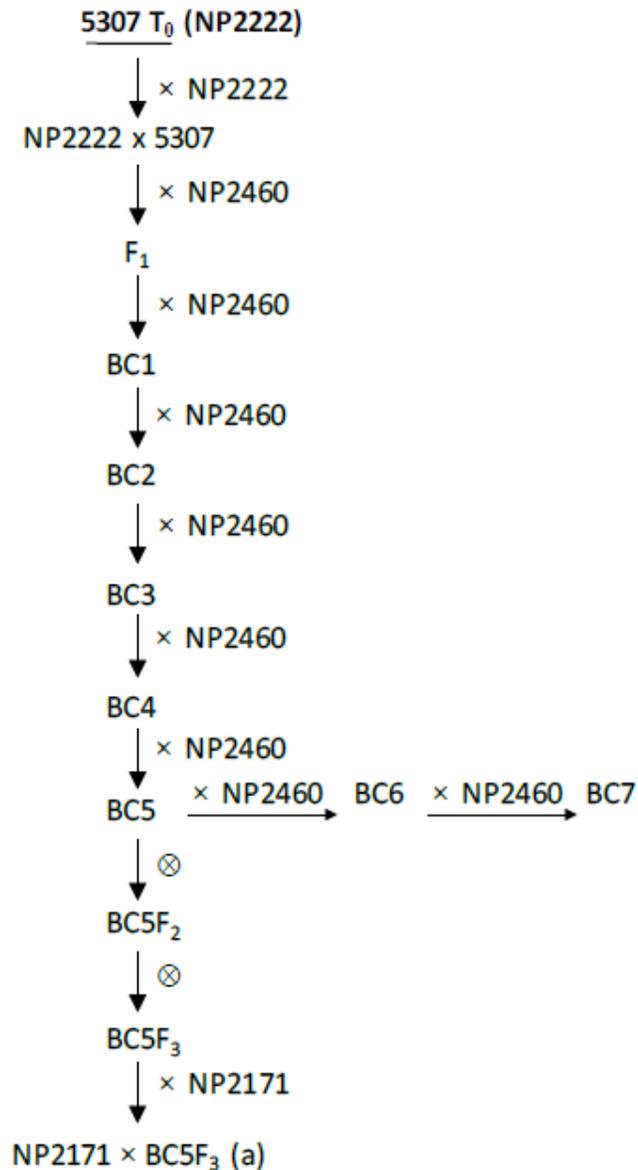
Figure 1: Circular plasmid map of pSYN12274 showing size and orientation of the elements in the two-gene expression cassette (T-DNA) used in the transformation of corn.

### 3.3 Characterisation of the genes in the plant

Southern blots were used to determine the number of insertion sites within the corn genome, the copy number of each functional element, and the presence or absence of plasmid backbone sequences. DNA sequencing was also performed to unambiguously determine the organisation and integrity of introduced genetic elements in corn line 5307, and to characterise the junction regions and flanking corn genomic DNA.

A number of breeding steps involving the original transformed line occurred to generate test material with the appropriate genetic background for analysing the 5307 corn event. As indicated in Figure 2, for molecular characterisation purposes, the transformed test material used in the Southern blots was selected as 5307 NP2171 x BC5F<sub>3</sub>. Four non-transformed lines were used as controls: NP2171/NP2460 (near isogenic line to the transgenic line), NP2222 (original parent line), NP2460 and NP2171. The additional controls were necessary because the corn 5307 generation analysed in the Southern blots was created by crossing with these lines, as depicted in Figure 2. The test and control substances were verified by real-time PCR analysis to confirm their identity and purity.

Genomic DNA used for Southern blot analyses was isolated from pooled leaf tissue obtained from plants of the verified genotype grown in the greenhouse. Extracted DNA was digested with a range of restriction endonucleases for 8 to 16 hours. The positive control for the Southern blots was digested DNA from transforming plasmid pSYN12274. Element-specific probes were generated by radiolabelling of the corresponding PCR products derived from plasmid pSYN12274. The probes detected *ecry3.1Ab* (1962 bp), *pmi* (1176 bp), CMP promoter (346 bp), ZmUbilnt promoter (1993 bp), NOS terminator (253 bp), and the plasmid backbone (5312 bp).



(a) = Southern blot analyses  
T<sub>0</sub> = original transformant  
× = cross  
BC = backcross  
⊗ = self-pollination

Figure 2: Pedigree history in the development of corn line 5307, indicating the generations used in the molecular characterisation studies.

### 3.3.1 Copy number

Assuming a single T-DNA insertion event, the theoretical number and size of bands expected to be observed in the Southern blots was calculated for each restriction enzyme digest or combination digest. This provided a reference for comparison with the results obtained from both the transgenic line (corn 5307) and the non-transgenic control lines used as genetic comparators.

The Southern blot analyses with the *ecry3.1Ab*- and *pmi*-specific probes detected respectively only one hybridisation band of the expected size, for each restriction enzyme digest tested. No unexpected bands were detected. These results indicated that (i) corn line 5307 contains a single copy of *ecry3.1Ab* and *pmi*, and (ii) no extraneous DNA fragments of the introduced genes are present in the corn genome.

Similarly, the results of the Southern blots using the CMP-specific probe indicated the presence of one copy of the CMP promoter sequence which is consistent with the insertion of a single *ecry3.1Ab* expression cassette in the genome of corn line 5307.

Given that the promoter sequence used in the selectable marker cassette (ZmUbilnt) is endogenous to the corn genome, additional bands were expected in the Southern blots as a result of cross-hybridisation of the ZmUbilnt promoter-specific probe with the endogenous maize polyubiquitin promoter sequence. The expected pattern of hybridisation bands obtained from the 5307 NP2171 x BC5F<sub>3</sub> was compared with that observed in the control plant material NP2171/NP2460 and additional control substances NP2171, NP2222 and NP2460 for a number of particular restriction enzyme combinations. A unique band of the theoretically calculated size was observed in the transgenic 5307 line, indicating the presence of a single copy of the introduced ZmUbilnt promoter sequence in corn line 5307.

The Southern blot analysis with genomic DNA digested with a single restriction enzyme and probed with the NOS termination element-specific probe showed two hybridisation bands in DNA extracted from 5307 NP2171 x BC5F<sub>3</sub> as expected. These hybridisation bands were absent in the DNA extracted from the tissue of control plants. One hybridisation band was observed in the double restriction digest of 5307 NP2171 x BC5F<sub>3</sub>, and no corresponding bands were observed in the non-transgenic control samples. One hybridisation band was observed in the positive pSYN12274 control. These results indicate the presence of two copies of the NOS terminator element in corn line 5307, which is consistent with the presence of one copy of the *ecry3.1AB* cassette and one copy of the *pmi* cassette.

No hybridisation bands were detected in the Southern blots probed with the pSYN12274 plasmid backbone-specific element, in either the transgenic or non-transgenic corn genomic samples.

### *Conclusion*

Southern blot analyses of corn 5307 (generation 5307 NP2171 x BC5F<sub>3</sub>) demonstrated that this line contains a single copy of *ecry3.1Ab*, *pmi*, the CMP and ZmUbilnt promoters, and two copies of the NOS termination sequence, as would be expected from the insertion of a single copy of the T-DNA from plasmid pSYN12274. There was no evidence of insertion of extraneous T-DNA fragments elsewhere in the corn genome and no indication of the presence of fragments derived from the backbone of the transforming plasmid pSYN12274.

### 3.3.2 *DNA sequence*

PCR and DNA sequence analysis were used to confirm the organisation of genetic elements introduced into corn line 5307, and the integrity of the nucleotide sequence of the insert with respect to the T-DNA, as well as the adjacent plant genomic DNA.

Two overlapping regions spanning the entire insert were amplified by PCR using genomic DNA isolated from leaf tissue of the same generation as used for the Southern blot analyses, 5307 (NP2171 x BC5F<sub>3</sub>) (see Figure 2 ). Three individual clones of each PCR amplicon were sequenced individually to generate a consensus sequence for each region. The consensus sequence data for corn 5307 insert was compared to sequence of the transforming plasmid pSYN12274.

This comparison showed that the insert was intact and the organisation of functional elements comprising the insert corresponded exactly with the order in pSYN12274.

Several details in the sequencing results were noted. One nucleotide change (G to T) was identified 48 bp upstream of the CMP promoter in the non-coding region (intervening sequence) of the insert in corn line 5307. In addition, truncations at the 5' and 3' ends of the insert were revealed. The entire right border (25 bp) and three bp of non-coding sequence at the 5' end of the insert, and eight bp of the left border were deleted. These deletions and the single nucleotide change had no effect on the functionality of the genes in the insert. It is not uncommon for such truncations to occur in *Agrobacterium*-mediated transformations and, where gene elements are intact and retain their expected functionality, the deletions do not represent a safety concern.

### **3.4 Integration site analysis**

Further DNA sequencing was carried out to determine the genomic flanking regions adjacent to the insert in corn line 5307, and to characterise the wildtype integration site. The 5' and 3' ends of the insert were amplified by PCR using genomic DNA extracted from leaf tissue of the 5307 NP2171 x BC5F<sub>3</sub> generation, the same test material as that used in the other molecular characterisation studies. The PCR products were cloned and multiple clones were sequenced to generate 1000bp of consensus sequence from each flanking region. The test material was obtained from plants whose identity and purity had been verified by real-time PCR analysis.

The genome sequence of the non-transgenic NP2222 corn (parental line) at the site of integration was also determined using a similar PCR analysis followed by DNA sequencing. A comparison of this specific genomic region pre- and post-integration of the T-DNA provides information about possible changes that could have occurred at the insertion site as a result of the transformation. The sequence data show that 33 bp of corn genomic DNA were deleted when the T-DNA integrated into the corn genome. Using BLAST (Basic Local Alignment Search Tool) to compare the flanking sequence in corn line 5307 with sequences in public databases, the Applicant determined that the inserted DNA did not disrupt any known endogenous corn gene.

#### *3.4.1 Flanking sequence bioinformatic analysis*

While there is no experimental evidence to suggest that unexpected novel proteins are encoded by DNA at the 5' and 3' insert-to-plant junction regions in corn 5307, bioinformatic analysis was undertaken to ascertain whether the sequence in these regions contained any open reading frames (ORFs) that could translate into a peptide sequence of at least 30 amino acids. ORFs were defined as any DNA sequence between a putative start codon (ATG) and a putative stop codon (TAG, TAA or TGA) in the same reading frame. The NCBI Entrez® Protein Database (containing over 10 million sequences) was then used to screen the derived amino acid sequence for similarity to known or putative allergens or toxins, using the BLASTP algorithm (version 2.2.19).

The bioinformatic analysis identified one putative ORF (243 bp) spanning the junction between the corn genomic sequence and the 3' region of the insert in corn line 5307. This putative ORF translated into a putative peptide of 81 amino acids. The BLASTP analysis identified alignment with a hypothetical protein from *Agrobacterium tumefaciens*. The region of similarity was the left border sequence from *A. tumefaciens* and insert sequence adjacent to the left border, which is consistent with the original biological source of the sequence in this region. The hypothetical *Agrobacterium* peptide is not a known or putative toxin.

To determine possible similarity to known or putative allergens, the Applicant conducted two different searches against the Food Allergy Research and Resource Program (FAFFP) AllergenOnline database (version 10.0), which contained 1,471 amino acid sequences of known and putative allergens. The first search looked for greater than 35% shared sequence identity, over 80 successive amino acid residues, of the translated ORF when compared with the sequences in the database. The second search looked for the presence of immunologically-relevant sequences of eight contiguous and identical amino acids in common with any of the known or putative allergen sequences in the database. The FASTA algorithm was used to determine the degree of sequence alignment between the theoretical protein and those contained in the protein databases.

The results of the bioinformatic analyses indicated that the translated sequence of amino acids deduced from the ORF at the 3' insert-to-plant junction has no structural similarity with any known or putative toxin, and shows no biologically relevant amino acid sequence similarity to any known or putative protein allergens.

### **3.5 Stability of genetic changes**

Southern blot analysis was used to investigate genetic stability of the insert in four separate generations. The four generations of corn 5307 tested were F<sub>1</sub>, BC6, BC7 and the generation used for the main molecular analyses, 5307 NP2171 x BC5F<sub>3</sub> (see Figure 2). This latter generation is 10 conventional breeding steps beyond the original transformed line (T<sub>0</sub>), as depicted in Figure 2. The four control substances for these experiments were nontransgenic corn seed NP2171/NP2460 (near isogenic line), as well as NP2222 (parent line), NP2460 and NP2171 which were used in the conventional breeding steps. Control material from NP2460 and NP2171 was required because the corn 5307 generations tested were created by crossing with these non-GM corn lines.

Seed of each test and control line was harvested from glasshouse grown plants. Following verification of the plants' identity by real-time PCR analysis, leaf tissue from each line was collected and used to prepare genomic DNA for the Southern blots. For these experiments, a full-length T-DNA-specific probe and a pSYN12274 backbone-specific probe were generated by radiolabelling of PCR products. The full-length T-DNA probe contains sequence of the polyubiquitin promoter (ZmUbilnt) native to corn, and therefore cross-hybridised to genomic DNA from the non-transgenic control lines. The pattern of hybridising bands obtained for each of the control lines served as a reference for the detection of the endogenous promoter element, and facilitated interpretation of the results for the four transgenic generations.

Data from these analyses demonstrated that the genetic elements introduced into the corn genome were integrated into a single locus which is maintained in subsequent generations, including when crossed with a non-GM elite breeding line. These results indicate that the insert in corn line 5307 is stably inherited from one generation to the next.

#### **3.5.1 Mendelian inheritance**

Individual plants from four generations were tested for the presence of the introduced genes *ecry3.1Ab* and *pmi* by real-time PCR analysis, and the results used to determine the segregation ratios for these genes. Ninety-two plants from the F<sub>1</sub> seed, 91 plants from the BC6 seed, 90 plants from the BC7 seed and 100 plants from 5307 NP2171 x BC5F<sub>3</sub> seed were grown in the greenhouse. Leaf discs were sampled from each individual plant for PCR and statistical analysis.

Chi-square analysis of the segregation data for three of the generations was performed to test the hypothesis that the genomic insert in corn line 5307 is inherited in a predictable manner, according to Mendelian principles.

The segregation data aligned with the expected segregation ratio for *ecry3.1Ab* and *pmi*, indicating that the genomic insert in corn line 5307 is inherited in subsequent generations according to Mendelian principles.

Plants from 5307 NP2171 x BC5F<sub>3</sub> generation were grown from seed that was produced by crossing a nontransgenic maize line (NP2171) with plants that were homozygous for *ecry3.1Ab* and *pmi*. The progeny were all expected to be positive for the two transgenes, and this was observed. Although Chi-square analysis was not appropriate in this case, this result supports the conclusion that the 5307 insert is inherited according to Mendelian patterns of inheritance.

### 3.6 Conclusions

Based on the molecular analyses of corn line 5307, a single copy of the T-DNA from plasmid pSYN12274, which contains the *ecry3.1Ab* and *pmi* gene expression cassettes, inserted into the corn genome at a single nuclear genomic locus. *Ecry3.1Ab* encodes an engineered insecticidal protein created from selected domains of the *cry1Ab* and modified *cry3A* genes, both derived from *Bacillus thuringiensis*. The bacterial *pmi* gene encodes phosphomannose isomerase and was used as a laboratory selectable marker. The regulatory elements and coding region of both genes are intact and functional, and the absence of plasmid backbone sequences was confirmed. Bioinformatic analysis of the insert-to-plant junction regions indicated that a putative ORF, identified at the 3' end of the insert, showed homology with *Agrobacterium* derived sequence and would not encode a peptide with structural similarity to toxins or known protein allergens. The stability of the insert in corn line 5307 was demonstrated through testing of multiple generations that were the result of conventional breeding of the transgenic line with selected non-GM corn lines.

## 4. Characterisation of novel proteins

In considering the safety of novel proteins it is important to consider that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects. Only a small number of dietary proteins have the potential to impair health, for example, because they are allergens or anti-nutrients (Delaney *et al* 2008b). 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, quantity and localisation of all novel proteins expressed in the GM plant, as well as a detailed understanding of their biochemical function and phenotypic effects. It is also important to determine if the novel protein is expressed as expected in the plant, including whether any post-translational modifications have occurred.

The two novel proteins expressed in corn line 5307 as a result of the genetic modification are the insecticidal protein eCry3.1Ab and the bacterial enzyme phosphomannose isomerase (PMI). Because expression of the introduced proteins *in planta* is usually too low to allow purification of sufficient quantities for safety assessment studies, a specific microbial expression system is generally used to produce larger amounts of individual proteins. It is necessary therefore to establish the equivalence of the bacterially-produced proteins to the plant-produced proteins through additional characterisation studies.

### 4.1 Function of Cry proteins

The World Health Organisation has produced a comprehensive review of the biology of *Bacillus thuringiensis* and its delta-endotoxins (International Programme on Chemical Safety series, WHO 1999).

In addition, there is a wealth of published literature on the insecticidal activity and specificity of a large number of Cry proteins found in *B. thuringiensis*. The Cry proteins are active against lepidopteran, dipteran or coleopteron insects.

During sporulation, *B. thuringiensis* produces cytoplasmic inclusions containing one or more of the insecticidal crystal proteins. Most proteins are synthesised intracellularly as inactive protoxins that spontaneously form small crystals, approximately 1 µm in size. Upon ingestion by susceptible insects, the highly alkaline pH of the insect midgut promotes solubilisation of the protoxin-containing crystals. The protoxin is then activated by trypsin-like gut proteases, which cleave off domains from the carboxy- and amino- termini, leaving a protease resistant core, which is the active toxin. The active toxin binds to a highly specific glycoprotein receptor on the surface of midgut epithelial cells in the insect. Aggregation of the core toxins results in the formation of a pore through the cell membrane. These cells eventually swell and burst causing loss of gut integrity and resulting in larval death within 1 to 2 days (Hofte and Whiteley, 1989; Schnepf *et al.*, 1998).

One distinguishing feature of Cry proteins is their high specificity for particular target insects. Phylogenetic analyses has established that the diversity of the Cry family of proteins evolved by the independent evolution of three structural domains, and by swapping of domains between toxins. Similarities in the structural domains make it possible to engineer novel chimeric proteins in the laboratory *via* the exchange of homologous DNA domains between different *cry* genes. The aim of this research is to improve the specific activity, or to broaden the spectrum of insecticidal activity of the encoded Cry proteins.

#### 4.1.1 Development of eCry3.1Ab

##### Published Studies:

Walters FS, Stacy CM, Kyong Lee M, Palekar N and 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 and 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.

The eCry3.1Ab protein (GenBank accession number GU327680) in corn line 5307 was generated following exchange of variable-regions of two crystal proteins – a modified Cry3A (mCry3A) and Cry1Ab<sup>1</sup>. This chimeric protein exhibits strong bioactivity against the larvae of western corn rootworm (*Diabrotica virgifera virgifera* LeConte), a pest species which is not susceptible to either parent protein sequence.

The coleopteran-active eCry3.1Ab protein is comprised of (from the N-terminus to the C-terminus) a peptide cap of 22 amino acid residues, amino acids 10 to 468 (459 residues) of mCry3A, followed by amino acids 477 to 648 (172 residues) of Cry1Ab. This unique protein has a calculated molecular mass of 74.8 kDa.

FSANZ has assessed the safety of mCry3A and its parent Cry3A, in addition to Cry1Ab in previous applications. Cry3Aa was used as the insect control protein in GM potatoes (Application A382), and mCry3A was used in insect-protected corn line MIR604 (Application A564). The Cry1Ab protein is present in Bt176<sup>2</sup> and Bt11, two previously assessed GM corn lines (Applications A385 and A386).

*mCry3A*

<sup>1</sup> A description of the domains fused together to create the chimeric gene is outlined in *Section 3.2.1*

<sup>2</sup> Bt 176 is now a discontinued product.

The mCry3A protein (598 amino acids) was based on the native Cry3A protein sequence from *B. thuringiensis* subspecies *tenebrionis*. The native Cry3A protein is a component of commercial Bt-based insecticidal preparations that have been in use for over 50 years. Cry3A is naturally very active against the Colorado potato beetle (*L. decemlineata*) (Walters *et al.* 2010), however was not useful for controlling corn rootworm pests until modified with a chymotrypsin/cathepsin G protease recognition site, as described below.

The amino acid sequence of mCry3A corresponds to the native Cry3A sequence, except for the following changes:

- (i) The original N-terminal region was removed and the mCry3A protein commences at a methionine residue in position 48 (Met-48) of the native protein. Naturally, *B. thuringiensis* produces both the full-length Cry3A protein and the truncated version, and both are active;
- (ii) A cathepsin-G protease cleavage site begins at amino acid residue 155 of the native protein. The consensus cathepsin-G site has the sequence alanine-alanine-proline-phenylalanine, and has replaced amino acids valine-155, serine-156 and serine-157 in the native protein (Chen and Stacy 2007).

A consensus cathepsin-G protease recognition site was incorporated into the protein to broaden its spectrum of biological activity. Serine protease is the dominant protease in the gut of corn rootworm and the most widely recognised serine recognition site was 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.

### *Cry1Ab*

The full-length Cry1Ab is a protein of 1155 amino acids and is derived from *Bacillus thuringiensis* subsp. *kurstaki* HD-1. The native protein is moderately active against Lepidopteran pests (Walters *et al.* 2010). Truncated versions of Cry1Ab are the active insecticidal proteins in corn lines Bt11 and Bt176, both previously assessed by FSANZ. The truncated versions are proteins of 615 and 648 amino acids respectively.

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 Cry3A C-terminal sequence.

## **4.2 Function of phosphomannose isomerase**

Plants transformed with the *pmi* (*manA*) gene (from *E. coli*) can utilise mannose as a source of carbon. The expression of the PMI enzyme in plant cells is therefore useful as a selectable marker to assist with identification of transformed cells. Phosphomannose isomerase catalyses the interconversion of mannose 6-phosphate and fructose 6-phosphate and is present in some plants. Plants lacking this enzyme are unable to survive on culture media containing mannose.

Mannose, a hexose sugar, is taken up by plants and converted to mannose-6-phosphate by hexokinase. This product cannot be further utilised in many plants as they lack the PMI enzyme. The accumulation of mannose-6-phosphate inhibits phosphoglucose isomerase, causing a block in glycolysis. It also depletes cells of orthophosphate required for the production of ATP. Therefore, while mannose has no direct toxicity on plant cells, it causes growth inhibition (Negrotto *et al.*, 2000).

The PMI enzyme has been assessed previously as a novel protein in corn lines MIR604 (Application A564) and MIR162 (Application A1001). The PMI enzyme in MIR604 has two

amino acid changes from the intended sequence (V61A and Q210H) however these changes did not affect the function of the enzyme.

### 4.3 Equivalence of test material

#### 4.3.1 eCry3.1Ab

**Submitted studies:**

- Nelson, A (2008) Characterisation of Test Substance ECRY3.1AB-0208 and Certificate of Analysis. Syngenta Study No. 5307-08-13, Report No. SSB-010-08 (Unpublished)
- Nelson, A (2009) Comparison of eCry3.1Ab Protein Produced in Event 5307-Derived Maize Plants and eCry3.1Ab Protein Produced in Recombinant *Escherichia coli*. Syngenta Study No. 5307-08-05, Report No. SSB-002-09 (Unpublished)

The amount of eCry3.1Ab protein that could be obtained from corn line 5307 was insufficient for all required safety analyses. The protein was therefore produced in the laboratory from a recombinant *E. coli* overexpression system. Several techniques were used to determine whether the eCry3.1Ab protein from the plant and microbial sources were biochemically and functionally equivalent.

Transgenic and control corn plants were grown under standard greenhouse conditions. Young leaves from greenhouse-grown 5307 maize (pedigree NP2171 x BC5F3) and near-isogenic, non-GM plants (pedigree NP2171 x NP2460) were collected 4-6 weeks after emergence. Extracts of the harvested leaf material were used in Western Blot and glycosylation analyses and insect bioassays. In order to investigate whether the plant matrix affected the mobility and immunoreactivity of eCry3.1Ab, a known amount of the microbially-produced protein was added to the near-isogenic control leaf extract. This sample allowed comparison of the microbially-produced eCry3.1Ab protein and the plant produced form in the same background matrix.

The ELISA kit used to quantify the proteins was a double-antibody sandwich assay, using a monoclonal anti-mCry3A antibody and a rabbit polyclonal anti-Cry1Ab antibody.

The eCry3.1Ab protein in leaf tissue extracts was purified using an affinity column with bound mouse anti-mCry3A antibody. The purified plant-produced eCry3.1Ab was used in Western Blots, glycosylation analysis, N-terminal sequencing and peptide mass mapping analysis.

The eCry3.1Ab protein produced in *E. coli* contains seven additional amino acids at the N-terminus, compared with the plant-produced form. The additional methionine and six histidine residues (histidine tag) facilitated purification of the protein from the bacterial system. The concentration of protein was determined spectrophotometrically to be 92.4%. The identity of the protein was confirmed by examining the apparent molecular weight, immunoreactivity and total mass of the protein. Following densitometric analysis, the purity of the eCry3.1Ab preparation was determined to be 89.6% by weight. The predicted molecular weight of 74.83 kDa was confirmed by mass spectrometry.

- *Immunoreactivity and molecular weight:* On Western Blots, the eCry3.1Ab samples from plant and microbial sources revealed immunoreactive bands consistent with their predicted molecular weights (73.7 kDa and 74.8 kDa, respectively), based on the presence of the His tag in the protein from the microbial source.
- *Insecticidal activity:* Near-isogenic control leaf extract was fortified with the microbially-produced eCry3.1Ab to provide a more meaningful comparison of insecticidal activity in the same background matrix. In three independent insect bioassays, the transgenic

leaf-extracted eCry3.1Ab protein showed LC50<sup>3</sup> values comparable with the microbially-produced protein added into near-isogenic control leaf extract.

- *N-terminal Amino Acid Sequence:* As expected, N-terminal sequencing of the microbially-produced protein revealed the histidine tag and the next few amino acid residues of the eCry3.1Ab protein, as predicted from the gene sequence: Met-His-His-His-His-His-His-Met-Thr-Ser. The majority of the protein purified from leaf material from corn line 5307 was N-terminally blocked. However, the N-terminal peptide from the plant-produced protein was identified by peptide mass mapping.
- *Peptide mass mapping:* Analysis of the eCry3.1Ab protein purified from extract of corn line 5307 leaf tissue yielded coverage equivalent to 76% of the total predicted eCry3.1Ab amino acid sequence, as shown in Figure 3. Similar analysis of the microbially-produced protein yielded coverage equivalent to 87% of the total predicted eCry3.1Ab amino acid sequence, as shown in Figure 4. These results confirmed the identification of the purified proteins from both the plant- and microbial- sources as eCry3.1Ab. In addition, the peptide mass analysis showed that the N-terminal methionine was removed in the plant-derived protein, leaving a threonine residue at the N-terminus. Removal of the N-terminal methionine is known to be a common process during translation of proteins. The analysis also suggested the presence of an additional acetyl group at the primary amino group of the N-terminal threonine, which explained the block encountered during N-terminal sequencing of the plant-produced eCry3.1Ab protein. This is a common modification known for plant-expressed proteins (Martinez *et al.* 2008).
- *Glycosylation analysis:* Following polyacrylamide gel electrophoresis and staining, visual comparisons between the microbially-produced and immunopurified plant-produced eCry3.1Ab proteins and a positive control glycoprotein, transferrin, indicated that plant-produced eCry3.1Ab is not glycosylated. The absence of detectable carbohydrate moieties associated with eCry3.1Ab derived from corn line 5307 confirmed the equivalence of the proteins from the plant- and microbial- sources.

The identity of eCry3.1Ab from corn line 5307 was confirmed in terms of its molecular mass, immunoreactivity, peptide mass over a large proportion of the protein, and functional insecticidal activity. The microbially-produced eCry3.1Ab was also shown to be structurally and functionally equivalent to the plant-produced protein.

#### 4.3.2 PMI

**Submitted study:**

Nelson, A (2009) Comparison of Phosphomannose Isomerase Produced in Event 5307-Derived Maize Plants and Phosphomannose Isomerase Produced in Recombinant *Escherichia coli*. Syngenta Study No. 5307-08-07, Report No. SSB-003-09 (Unpublished)

Corn line 5307 and non-transgenic control plants were grown under standard greenhouse conditions. Protein extracts were prepared from young leaves from corn 5307 (pedigree NP2171 x BC5F3) and near-isogenic control plants (pedigree NP2171 x NP2460) collected 4-6 weeks after emergence. The near-isogenic corn tissue was used as the negative control sample in these analyses.

<sup>3</sup> LC50 is the concentration of protein associated with mortality in at least 50% of Colorado potato beetle larvae.

**MTSNGRQCAGIRPYDGRQQHRGLDSSTTKDVIQKGISVVGDLLGVVGFPPFGGALVSFYT**  
**NFLNTIWPSEDPWKAFMEQVEALMDQKIADYAKNKALAELOGLQNNVEDYVSALSSWQK**  
**NPAAPFRNPHSQGRIRELFSQAESHFRNSMPSFAISGYEVLFLTTYAQAANTHLFLKDAQIYGE**  
**EWGYEKEDIAEFYKRQLKLTQEYTDHCVKWINVGLDKLRGSSYESWVNFNRYR**  
**REMTLTVLDLIALFPLYDVRLYPKEVKTELTRDVLTDPIVGVNNLRGYGTTFSNIENYI**  
**RKPHLFDYLRHQFHTRFQPGYYGNDSFNYWSGNYVSTRPSIGSNDIITSPFYGNKSSE**  
**PVQNLFEFNGEKVYRAVANTNLAVWPSAVYSGVTKVEFSQYNDQTDEASTQTYDSKRNVG**  
**AVSWDSIDQLPPETTDEPLEKGYSHQLNYVMCFMQGSRGTIPVLTWTHKSVDFNMD**  
**SKKITQLPLTKSTNLGSGTSVVKGPFGFTGGDILRRTSPGQISTLRVNITAPLSQRYRVR**  
**IRYASTTNLQFHTSIDGRPINQGNFSATMSSGSNLQSGSFRTVGFSTPPNFNSNGSSVFT**  
**LSAHVFNSGNEVYIDRIEFVPAEVTFEAEYDLERAQKAVNELFTSSNQIGLKTVDVTDYH**  
**IDQV**

Figure 3: Predicted amino acid sequence of eCry3.1Ab and sequence identified by peptide mass mapping analysis of eCry3.1Ab purified from extract of leaf material from corn line 5307. Identified eCry3.1Ab protein fragments are in bold type.

**MHHHHHMTSNGRQCAGIRPYDGRQQHRGLDSSTTKDVIQKGISVVGDLLGVVGFPPFGG**  
**ALVSFYTNFLNTIWPSEDPWKAFMEQVEALMDQKIADYAKNKALAELOGLQNNVEDYVS**  
**ALSSWQKNPAAPFRNPHSQGRIRELFSQAESHFRNSMPSFAISGYEVLFLTTYAQAANT**  
**HLFLKDAQIYGEWGYEKEDIAEFYKRQLKLTQEYTDHCVKWINVGLDKLRGSSYESW**  
**VNFNRYRREMTLTVLDLIALFPLYDVRLYPKEVKTELTRDVLTDPIVGVNNLRGYGTTF**  
**SNIENYIRKPHLFDYLRHQFHTRFQPGYYGNDSFNYWSGNYVSTRPSIGSNDIITSPF**  
**YGNKSSEPVQNLFEFNGEKVYRAVANTNLAVWPSAVYSGVTKVEFSQYNDQTDEASTQTY**  
**DSKRNVGAVSWDSIDQLPPETTDEPLEKGYSHQLNYVMCFMQGSRGTIPVLTWTHKSV**  
**DFNMDSKKITQLPLTKSTNLGSGTSVVKGPFGFTGGDILRRTSPGQISTLRVNITAPL**  
**SQRYRVRIRYASTTNLQFHTSIDGRPINQGNFSATMSSGSNLQSGSFRTVGFSTPPNFNS**  
**NGSSVFTLSAHVFNSGNEVYIDRIEFVPAEVTFEAEYDLERAQKAVNELFTSSNQIGLK**  
**TDVTDYHIDQV**

Figure 4: Predicted amino acid sequence of eCry3.1Ab and sequence identified by peptide mass mapping analysis of microbially-produced eCry3.1Ab. Identified eCry3.1Ab protein fragments are in bold type.

In order to investigate whether the plant matrix affected the mobility and immunoreactivity of PMI, a positive control sample was generated by adding a known amount of the microbially-produced protein to the non-transgenic, near-isogenic control leaf extract.

The concentration of PMI in the plant extracts was quantified by ELISA, using a polyclonal rabbit antibody generated against the PMI protein, a mouse monoclonal anti-PMI antibody, and a rabbit anti-mouse IgG conjugated with horseradish peroxidase (HRP). Extracts of the harvested leaf material were examined in Western Blots and enzyme activity assays.

PMI was produced in the laboratory using a recombinant *E.coli* over-expression system. The purified material was determined to be 89.5% PMI by weight.

- Immunoreactivity and molecular weight:** On Western Blots, the PMI samples from plant and microbial sources revealed a dominant immunoreactive band consistent with the predicted molecular weight of PMI of 42.8 kDa. As expected, no corresponding immunoreactive band was observed in the negative plant extract sample from the non-transgenic, near-isogenic plants. The Western blot also revealed a very faint protein band with a molecular weight of approximately 30 kDa, only in the microbially-produced PMI sample. The study authors considered this to be a degradation product of PMI, since it was detected by the anti-PMI antibody.

This band was not visible in the control plant extract fortified with a known amount of microbially-produced PMI, due to the effects of the matrix.

- **Enzyme activity.** The enzymatic activity of PMI was measured in triplicate using a continuous coupled spectrophotometric assay. The average PMI activity in the triplicate corn 5307 plant extract samples was 455.7 U/mg PMI, and in the microbially-produced material was 526.3 U/mg PMI. The non-transgenic, near-isogenic leaf extract fortified with microbially-produced PMI had a specific activity of 518.8 U/mg PMI. As expected, no PMI activity was detected in the near-isogenic control samples.

In summary, PMI derived from corn line 5307 plants and from a recombinant *E. coli* expression system exhibited the same mobility and immunoreactivity in Western blots, and showed an apparent molecular weight consistent with the predicted molecular weight of approximately 42.8 kDa. The plant- and microbially-produced PMI protein preparations demonstrated similar enzyme activity. These results indicate that microbially-produced PMI is biochemically and functionally equivalent to PMI in corn line 5307.

#### 4.4 Levels of novel proteins in corn line 5307

**Submitted study:**

Bednarcik M (2010) Quantitation of eCry3.1Ab and Phosphomannose Isomerase in Key Processed Fractions Prepared from Event 5307 Maize Grain. Syngenta Study No: TKRS0000035, Report No: SSB-004-10. Additional Performing Laboratory: Food Protein R&D Center, Texas A&M University, TX, USA.

The concentrations of eCry3.1Ab and PMI proteins were measured in key wet- and dry-milled grain fractions. Grain samples obtained from corn 5307 and the near-isogenic (non-GM) control line were processed into commercially representative food and feed fractions: gluten, starch and germ fractions were produced from wet-mill processing procedures, and flour and germ fractions from dry-mill procedures. These fractions are those most likely to enter the food and feed chain. The novel proteins were quantified by their respective enzyme-linked immunosorbent assays (ELISAs). Sample extracts were quantified in triplicate. The results from these assays are presented in Table 2. The limit of detection (LOD) for each protein was estimated based on the lowest concentration of reference protein that could be distinguished from the background signal of non-GM sample extracts.

**Table 2: Concentrations of eCry3.1Ab and PMI in 5307 corn grain, and processed fractions, mean of three replicate analyses**

Sample	eCry3.1Ab (µg/g) Mean ± SD	PMI (µg/g) Mean ± SD
<b>Grain</b>	4.98 ± 0.36	1.31 ± 0.05
<b>Wet-milled fractions</b>		
Gluten	< LOD <sup>a</sup>	< LOD <sup>d</sup>
Starch	< LOD <sup>b</sup>	< LOD <sup>e</sup>
Dried germ	< LOD <sup>c</sup>	< LOD <sup>f</sup>
<b>Dry-milled fractions</b>		
Flour	1.06 ± 0.03	0.20 ± 0.01
Germ	19.33 ± 2.08	3.97 ± 0.32

<sup>a</sup>LOD = 0.048 µg/g sample

<sup>b</sup>LOD = 0.048 µg/g sample

<sup>c</sup>LOD = 0.024 µg/g sample

<sup>d</sup>LOD = 0.003 µg/g sample

<sup>e</sup>LOD = 0.002 µg/g sample

<sup>f</sup>LOD = 0.003 µg/g sample

The concentrations of eCry3.1Ab and PMI are highest in the dry-mill corn germ, which is consistent with the distribution of total protein within the kernel. Corn germ accounts for only 10% of the total dry weight of mature kernel. Approximately 18% of corn germ is protein, whereas protein comprises only 8-10% of intact kernel. The results are also consistent with the fact that more protein is extracted during dry-milling compared with wet-milling (Parris *et al.* 2006). Flour produced from the dry-milling process also contained quantifiable levels of the novel proteins.

#### **4.5 Potential toxicity of the novel proteins in corn line 5307**

The vast majority of plant, animal and microbial proteins ingested as part of a typical diet are not associated with toxic effects, however a small number may be harmful to health. Where a GM food differs from its conventional counterpart by the presence of one or more novel proteins, these proteins should be assessed for their potential toxicity.

The main purpose of an assessment of potential toxicity is to establish, using a weight of evidence approach, that the novel protein will be digested like any other dietary protein. The assessment focuses on whether the novel protein: has a prior history of safe human consumption, or is similar to proteins that have been safely consumed in food; has amino acid sequence similarity with known protein toxins and anti-nutrients; has structural properties that make it resistant to heat, processing and/or digestion. In some circumstances, acute oral toxicity studies in animals may also be useful, particularly where results from the biochemical, bioinformatic, digestibility or stability studies indicate the need for further investigations.

##### *4.5.1 History of use*

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

A number of different commercial Bt formulations have been registered worldwide as an insecticide for use on agriculture and forestry crops, vegetable foliage, soil, water and in food storage facilities. While the Bt spores or vegetative cells may persist in the environment for weeks, months or years, the Cry protein toxins become inactive within hours or days.

As eCry3.1Ab is an engineered chimeric protein, there is no prior history of human consumption. There has however been previous dietary exposure to the mCry3A and Cry1Ab proteins, from which the C- and N-terminal domains of eCry3.1Ab are derived.

The native Cry3A protein is a component of microbial pesticides containing spore preparations of *Bacillus thuringiensis* subsp. *tenebrionis* which have a long history of safe use as organic pesticides. In addition, the safety for human consumption of the Cry3A protein was previously determined in the assessment of NewLeaf™ potatoes (Application A382). Similarly, the safety of mCry3A was evaluated by FSANZ in 2006, in the assessment of insect-protected corn line MIR604 (Application A564). Cry1Ab is derived from *Bacillus thuringiensis* subsp. *kurstaki* HD-1, and truncated versions of this protein have been previously assessed as the novel protein in other approved insect-protected corn lines, Bt11 and Bt176 (Applications A385 and A386). The Bt proteins used to construct eCry3.1Ab have therefore all been studied previously, do not raise food safety concerns, and are already present in currently approved GM food crops.

#### **PMI**

PMI proteins are ubiquitous in nature. Current data and information support a history of safe human consumption of PMI proteins through plant foods such as walnut, *Brassica* species, and the seeds of soybean and other legumes.

Small amounts of PMI proteins are thus likely to have always been in the food supply. Genes encoding likely PMI proteins have also been identified in diverse species including bacteria, yeast, rats, pigs and humans.

FSANZ has approved food derived from a number of different GM crops containing bacterial PMI. The PMI in corn line 5307 is currently found in approved commercially available products such as MIR604 and MIR162 insect-protected corn lines (Applications A564 and A1001 respectively). The conclusion from FSANZ's previous evaluations is that the use of PMI as a selectable marker in particular GM crops poses no food safety concerns.

#### 4.5.2 Similarities with known protein toxins

**Submitted studies:**

McClain S (2011) eCry3.1Ab (Entrez® Accession Number ADC30135): Assessment of Amino Acid Sequence Similarity to Known or Putative Toxins. Report No. SSB-165-11.

Harper B (2011) Phosphomannose Isomerase (Entrez® Accession Number AAA24109): Assessment of Amino Acid Sequence Similarity to Known or Putative Toxins. Report No. SSB-145-11.

Bioinformatic analyses were undertaken to evaluate the sequence of eCry3.1Ab protein for amino acid similarity with known protein toxins. While the potential toxicity of PMI has previously been investigated using a bioinformatic approach, an updated search was conducted using more recent versions of protein sequence databases.

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

The Basic Local Alignment Search Tool for Proteins program (BLASTP) was used to search the National Center for Biotechnology Information (NCBI, 2011) Entrez® Protein Database. The search was used to identify firstly, whether eCry3.1Ab showed significant similarity with any proteins in the database and secondly, whether any of the identified proteins were known or putative toxins. The degree of structural similarity was determined by examining the alignment of the sequences, percentage identity and *E*-score. An *E*-score of  $\leq 0.1$  was set as a threshold at which significant alignments between two sequences were identified.

The search identified 550 sequences with significant similarity to the amino acid sequence of eCry3.1Ab, however none of these were associated with known toxicity in humans or other mammals. Of the total, 505 proteins were known or putative insecticidal crystal (Cry) proteins (delta-endotoxins), including a number of synthetic Cry protein constructs. The remaining proteins identified in the alignment search revealed proteins of unknown function from six species, additional *Bacillus thuringiensis* proteins, and a small number of signal proteins from other species. None of these proteins with varying degrees of structural similarity with eCry3.1Ab are known or putative toxins.

#### **PMI**

The NCBI protein database (2011) was searched using the same bioinformatic parameters as outlined above for eCry3.1Ab. In this case, the bioinformatic analysis of the PMI amino acid sequence was an update on database searches conducted in previous years.

Of a total of 1384 proteins identified with significant amino acid similarity to PMI, 1208 alignments were to proteins from 587 different species and were identified as known or putative PMI enzymes, or proteins involved in carbohydrate/sugar metabolism or transport. The remaining alignments were to proteins of unknown function from 128 species, but none were identified as known or putative toxins.

Based on these results, it is concluded that neither eCry3.1Ab nor PMI share significant structural homology with known or putative protein toxins.

#### 4.5.3 Toxicity studies

Proteins which cause toxicity act generally at very low doses via acute mechanisms (Sjoblad *et al.* 1992). A protein that demonstrates no acute oral toxicity at a high dose level using a standard laboratory mammalian test species is not considered likely to be toxic in humans and other mammals, particularly under a more realistic exposure scenario, including long-term exposures.

##### **Submitted studies:**

Korgaonkar CK (2009) ECRY3.1AB-0208: Single-Dose Oral (Gavage) Toxicity Study in Mice with a 14-Day Observation Period. WIL Research Laboratories, LLC, Ashland, OH, USA. Study and Report No. WIL-639031. Sponsor: Syngenta Crop Protection, Inc., Greensboro, NC, USA.

Korgaonkar CK (2009) PMI-0105: Single-Dose Oral (Gavage) Toxicity Study in Mice with a 14-Day Observation Period. WIL Research Laboratories, LLC, Ashland, OH, USA. Study No. WIL-639011. Sponsor: Syngenta Crop Protection, Inc., Greensboro, NC, USA.

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

The test substance eCry3.1Ab (microbially-produced) was administered to five mice/sex (CD-1 strain; approximately 9 weeks old; sourced from Charles River Laboratories, Inc., Raleigh, NC) at a single dose level of 2230 mg/kg bw (89.6% w/w purity) by gavage. This dose level was selected because it is a limit dose for this type of study (OECD 2001; U.S. EPA 2002). A control group was treated similarly with an equal volume of water containing 0.5% carboxymethylcellulose. Mice were housed individually under standard conditions. Food and water were available *ad libitum* except for a fasting period of three hours prior to dosing. A normal diet was resumed approximately 1-2 hours after dosing.

Clinical signs were monitored at dose administration, approximately 1-2 hours later, and again 4-5 hours after dosing, and finally twice daily thereafter. Individual body weights were recorded weekly during the pre-test period, at the random allocation stage, just prior to dosing (after three hours of fasting) and daily during the observation period. Mean body weights and mean body weight changes were calculated for the corresponding intervals. Food consumption was recorded daily during the study period. A complete necropsy was conducted on all animals. This included a detailed macroscopic examination of organs *in situ* followed by collection of selected tissues and organs for microscopic examination.

There were no deaths or treatment-related clinical signs. No treatment-related reductions in bodyweight gain or food consumption were found over the course of the study. There were no adverse treatment-related macroscopic or microscopic abnormalities detected at necropsy. On the basis of these results, it was concluded that eCry3.1Ab showed no evidence of acute toxicity in CD-1 mice.

#### **PMI**

Phosphomannose isomerase (purity 89.5% w/w) was administered by gavage to five mice/sex (CD-1 strain; approximately 9 weeks old; sourced from Charles River Laboratories, Inc., Raleigh, NC) at a single dose level of 2230 mg/kg bw. This dose level was selected because it was a limit dose for this type of study (OECD 2001; U.S. EPA 2002). A control group was treated similarly with an equal volume of deionised water. Mice were housed individually under standard conditions. Food and water were available *ad libitum* except for a fasting period of three hours prior to dosing. A normal diet was resumed approximately 1-2 hours after dosing.

Clinical signs were monitored at dose administration, approximately 1-2 hours later, and again 4-5 hours after dosing, and finally twice daily thereafter. Individual body weights were recorded weekly during the pre-test period, at the random allocation stage, just prior to dosing (after three hours of fasting) and daily during the observation period. Mean body weights and mean body weight changes were calculated for the corresponding intervals. Food consumption was recorded daily during the study period. Blood samples were collected for a range of clinical pathology evaluations (haematology and serum chemistry) from all animals immediately prior to the scheduled necropsy on day 14 of the study. A complete necropsy was conducted on all animals. This included a detailed macroscopic examination of organs *in situ* followed by collection of selected tissues and organs for microscopic examination. All statistical analyses were performed using appropriate computing devices or programs.

There were no deaths or treatment-related clinical signs during the observation period. No treatment-related reductions in bodyweight gain or food consumption occurred over the course of the study.

The clinical pathology results showed no statistically significant differences between the control and test-group mice in any haematology parameters measured in this study, however there were some differences found in serum chemistry parameters. In test-group males, there was a statistically significant increase in urea nitrogen (n=4), and alkaline phosphatase (n=5) levels, compared with controls (n=4 and n= 5 respectively). In all females in the PMI-treated group (n=5), there was a statistically significant increase in alanine aminotransferase levels, compared with controls (n=5). These serum chemistry changes were reported to be related to administration of the test substance.

There were no treatment-related macroscopic abnormalities detected at necropsy. Microscopic examination of relevant tissues revealed some common histological changes, but these were considered to be incidental rather than test substance-related. Prior to the histological studies, selected organs were weighed and relative-to-body and relative-to-brain weights calculated. In test-group animals, the testes and epididymides of males were significantly lower than controls, and in females the adrenal glands were significantly higher than controls in mean absolute, relative-to-body and relative-to-brain weights. These organ weight changes were reported to be related to administration of the test substance.

#### 4.5.3.1 Previous evaluation of potential toxicity of PMI

FSANZ assessed the potential toxicity of the *E. coli*-derived PMI protein in 2006 (Application A564), and reviewed the data in 2008 (Application A1001). An acute oral toxicity study in mice was evaluated as follows:

##### **Study submitted with Applications A564 and A1001:**

Kuhn, J.O. (1999) Phosphomannose isomerase (sample PMI-098): Acute oral toxicity study in mice. Stillmeadow Inc Study No. 4708-98.

Test material:	Phosphomannose isomerase preparation from <i>E. coli</i> (60% phosphomannose isomerase enzyme)
Vehicle:	0.5% w/v aqueous carboxymethylcellulose
Test Species:	Hsd:ICR (CD-1®) mice (seven males and six females)
Dose:	5050 mg/kg bw (equivalent to 3080 mg/kg bw PMI protein) in two gavage doses, 1 hour apart.
Control:	vehicle only

The mice received a gavage dose of 3080 mg/kg bw PMI and were observed for two weeks. Parameters evaluated included body weights and detailed clinical observations.

One male in the control group and two in the test group died shortly after dosing or were in distress after dosing and subsequently died. Necropsy revealed a perforated oesophagus in each animal due to gavage error, which was not related to the test substance. One replacement animal was available for each group and dosed in the same manner on day 0.

No clinical signs of toxicity were observed in either group and there was no test substance-related mortality during the study. At the end of the study all animals were killed and examined post mortem. Brain, liver, kidneys and spleen were weighed. There were no test-substance related effects on body weight, organ weights or gross pathology.

Under the conditions of this study, it was concluded that the acute oral LD<sub>50</sub> of the PMI protein in mice is greater than 3080 mg/kg bw.

#### 4.5.3.2 Discussion

Differences in three serum chemistry parameters (urea nitrogen, alkaline phosphatase and alanine aminotransferase) and certain organ weights reported in the 2009 study were identified by statistical analysis. In view of a lack of corroborating histopathology in relevant tissues, and in consideration of the results from the previous acute oral study of the PMI protein completed in 1999, the likelihood for an adverse treatment-related effect in the latest study is not compelling.

In terms of the serum chemistry results, group mean values for urea nitrogen were within the appropriate historical control range (WIL version 2.5), noting that the level of urea nitrogen in one male exceeded the historical control range by a small margin. Group mean as well as individual animal values for alkaline phosphatase and alanine aminotransferase (ALT) for males and females in the test group were all within WIL historical control ranges (see Table 3). In addition, there were no corresponding macroscopic or microscopic changes observed in either the kidney or liver of test-group animals.

**Table 3: Serum Chemistry Parameters (Urea nitrogen, alkaline phosphatase and alanine aminotransferase) in Control- and Test-Group Mice and WIL Historical control ranges (Version 2.5, Age 9-12 weeks).**

	Control group (Mean)	2000 mg/kg bw PMI group (Mean)	Historical Control Data	
			Mean	Reference range
<b>MALES</b>				
Urea nitrogen (mg/dL)	19.5	27.5*	23.3	17.4 – 30.3
Alkaline phosphatase (U/L)	65	92*	86	49 – 141
<b>FEMALES</b>				
Alanine aminotransferase (U/L)	30	41*	48	24 – 122

\* Significantly different from the control group mean value at 0.05 using Dunnett's test

Similarly, there were no observed macroscopic or microscopic changes in the testes and epididymides of test-group male mice, despite calculated lower mean testicular and epididymal weights compared with those of the control mice. In test-group females, the statistically significant slight increase in adrenal gland weights compared with the control group did not correlate with distinct histological changes in the organs, and mean adrenal weights were within the WIL Research Laboratories' historical control data. In addition, a reverse trend was noted in test-group males where adrenal gland weights were slightly lower than in the control group (not statistically significant).

All additional test results were within normal parameters for CD-1 mice. FSANZ agrees with the authors of the study that the biochemical changes are not sufficient to identify kidney or liver as a target organ for toxic effects.

On the basis of the results obtained in both studies of PMI, the statistical differences reported in the latest study (2009) are likely to be due to chance alone and do not indicate a test substance-related effect.

#### 4.5.4 Conclusion

Based on the totality of studies investigating potential toxicity, there is no evidence that eCry3.1Ab or PMI are likely to be toxic if consumed by humans. In addition, because the proteins are expressed at low levels in the plant, this would mean only minimal dietary exposures from the consumption of corn products derived from corn line 5307.

### 4.6 Potential allergenicity of novel proteins in corn line 5307

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

#### 4.6.1 Source of the introduced genes

Both novel proteins present in corn line 5307 are derived from bacterial sources, *B. thuringiensis* and *E.coli*, which have been assessed previously for any known or potential allergenicity. Neither of these organisms is associated with food allergies in humans.

#### 4.6.2 Similarity to known allergens

**Submitted studies:**

Harper, B (2011) eCry3.1Ab (Entrez® Accession Number ADC30135): Assessment of Amino Acid Sequence Similarity to Known or Putative Allergens. Report No. SSB-112-11.

Harper, B (2011) Phosphomannose Isomerase (Entrez® Accession Number AAA24109): Assessment of Amino Acid Sequence Similarity to Known or Putative Allergens. Report No. SSB-102-11.

#### eCry3.1Ab

Bioinformatic analysis was undertaken to assess the eCry3.1Ab amino acid sequence for structural similarity with known or putative allergens. The eCry3.1Ab protein sequence was compared to sequences in the Food Allergy Research and Resource Protein (FARRP) Allergen database, version 11.0 (available online at <http://www.farrp.org>), which contained 1491 amino acid sequences of known or putative allergens.

Two different bioinformatic searches were performed. Firstly, the FARRP database was searched for overall linear sequence identity with eCry3.1Ab using the FASTA algorithm.

This search aimed to identify any protein sequences with 35% or greater identity over a segment of 80 or more amino acids (FAO/WHO 2001). Secondly, searches were performed to identify short amino acid sequences that may represent linear IgE binding epitopes. For this analysis, searches aimed to identify exact matches of 8 or more contiguous amino acids between eCry3.1Ab and allergenic proteins in the database.

No significant sequence similarity between eCry3.1Ab and known or putative allergens in the FARRP Online Database was observed. Additionally, no short oligopeptide matches, representing putative IgE binding epitopes, were identified between eCry3.1Ab and proteins in the database. On the basis of these results, eCry3.1Ab shares no structural or immunological similarity to known or putative protein allergens in this database.

### PMI

Using the same bioinformatic parameters, an updated *in silico* analysis was conducted on the PMI amino acid sequence to search for any similarity with known allergens. A full-length sequence search using FASTA identified no significant sequence alignments with known or putative allergens. A search for exact matches of eight or more contiguous amino acid residues revealed a single match with  $\alpha$ -parvalbumin from frog (*Rana* species CH-2001). This match was also reported in previous assessments. To investigate this match in greater detail, immunological testing was undertaken using serum IgE antibody screening. Serum from a single individual with demonstrated IgE-mediated allergy to this specific  $\alpha$ -parvalbumin from *Rana* species, did not react with any portion of PMI. Based on the updated bioinformatic analysis, there is no indication that PMI shares structural similarity with known or putative protein allergens that would raise concerns about potential allergenicity.

#### **4.7 In vitro digestibility**

Typically, food proteins that are allergenic tend to be stable to proteolytic enzymes such as pepsin, and to the acidic conditions of the digestive system. This resistance allows the protein to be exposed to the intestinal mucosa, which potentially can lead to an allergic response in certain sensitised individuals (Astwood et al 1996; Metcalfe *et al* 1996). Therefore some degree of correlation exists between resistance to digestion and potential allergenicity.

One of the criteria for assessing potential allergenicity is to examine the stability of novel proteins in conditions mimicking human digestion. Proteins that are rapidly degraded in simulated digestive fluids are considered less likely to elicit an allergic response.

#### **Submitted studies:**

Song S (2010) *In vitro* Digestibility of eCry3.1Ab Protein under Simulated Mammalian Gastric Conditions. Syngenta Study No. TK0028111.

Seastrum L (2009) *In vitro* Digestibility of eCry3.1Ab Protein as Contained in Test Substance ECRY3.1AB-0208 Under Simulated Mammalian Intestinal Conditions. Syngenta Study No. 5307-08-12 Report No. SSB-015-09 A1.

Nelson A (2009) *In vitro* Digestibility of Phosphomannose Isomerase (PMI) as Contained in Test Substance PMI-0105 Under Simulated Mammalian Gastric Conditions. Study No. PMI-07-03, Report No. SSB-034-07 A1.

Nelson A (2009) *In vitro* Digestibility of Phosphomannose Isomerase (PMI) as Contained in Test Substance PMI-0105 Under Simulated Mammalian Intestinal Conditions. Syngenta Study No. PMI-07-04, Report No. SSB-036-07.

#### 4.7.1 eCry3.1Ab

##### SGF

The susceptibility to digestion of the eCry3.1Ab protein in simulated gastric fluid (SGF) was investigated by SDS-PAGE and Western blotting. The reaction mixture was incubated at 37°C, over 15 minutes with samples taken at 0, 15, 30 and 45 seconds, 1 minute, 1 minute 15 seconds, 1 minute 45 seconds, and 2, 3, 5, 10 and 15 minutes, for analysis. The reactants were in the ratio of 1 µg eCry3.1Ab to 10 pepsin activity units (Thomas *et al*, 2004). The time-zero sample served as an undigested control to which all other samples were compared. Two other controls were used – eCry3.1Ab incubated for 15 minutes with SGF without pepsin, and SGF alone without eCry3.1Ab.

All control samples yielded the expected results. Degradation of pepsin over the time course was negligible (SGF alone), and the eCry3.1Ab control without pepsin showed no significant degradation after incubation for 15 minutes. Compared with the zero-time point, a very faint band corresponding to undigested eCry3.1Ab (74.8 kDa) remained visible after 15 seconds exposure to pepsin, however this band was no longer visible at 30 seconds. Two very faint and diffuse bands, with molecular weights of approximately 4 kDa and 5 kDa, were visible at 15 seconds. These two bands diminished in intensity with time and were no longer visible after 10 minutes incubation time. The lowest amount of eCry3.1Ab that could be visually detected in this gel system was 0.025 µg. The densitometry analysis of the Coomassie® blue-stained gel was consistent with digestion of eCry3.1Ab occurring in less than 30 seconds in SGF. These results were also confirmed in the Western blot analysis. No immunoreactive bands corresponding to intact eCry3.1Ab or eCry3.1Ab-derived fragments were detected in the sample at 30 seconds and longer time points.

##### SIF

The susceptibility to proteolytic degradation (at 37°C) of the eCry3.1Ab protein in simulated intestinal fluid (SIF) containing the enzyme pancreatin was investigated using SDS-PAGE and Western blotting. The reaction mixture was sampled at the following timepoints: 0, 1, 2, 5, 10, 30 and 60 minutes and 2, 3, 6, 24 and 48 hours incubation time. SIF without pancreatin (intestinal fluid control solution) was prepared for use in negative control samples. These were (i) a sample of eCry3.1Ab added to SIF without pancreatin, used to monitor the stability of the test protein over the 48 hour time course in the absence of pancreatin, and (ii) SIF alone (with pancreatin), used to monitor the stability of the pancreatin over 48 hours.

After 1 minute incubation with SIF, no intact eCry3.1Ab was detected, either by SDS-PAGE or Western blot analysis. The visibility of several lower molecular weight protein bands on the gel gradually diminished over the time course of 48 hours. There was significant proteolysis observed in the control sample containing SIF only (with pancreatin) at 2 hours and, by 48 hours, some bands had disappeared. Because of the overlap with SIF degradation products on the gel, the Western blot analysis shows more clearly the pattern of degradation of eCry3.1Ab over the course of the experiment.

An immunoreactive band corresponding to a molecular weight of approximately 56 kDa appeared at 1 minute and persisted over the timecourse. A small number of immunoreactive bands of variable molecular weight less than the intact eCry3.1Ab protein were faintly visible on the Western blot at various times over the incubation period. One of these bands, corresponding to a molecular weight of approximately 40 kDa, was observed in the 1 hour sample and increased in intensity over the timecourse. An immunoreactive band, corresponding to a molecular weight of approximately 6 kDa, appeared on the Western blot in the 1 minute sample and gradually diminished over time until it was no longer detectable in the 1 hour sample.

Another immunoreactive band, corresponding to a molecular weight of approximately 5 kDa, appeared after 5 minutes incubation with SIF, and remained visible over the remaining timecourse. As all of these bands cross-reacted with the anti-eCry3.1Ab antibodies, they were identified as degradation products of eCry3.1Ab protein.

### Conclusion

Digestibility studies demonstrated that the combined effects of normal gastric and intestinal digestion would almost completely degrade the eCry3.1Ab protein. This susceptibility to digestion means that there would be virtually no systemic exposure to intact eCry3.1Ab from the consumption of corn line 5307.

#### 4.7.2 PMI

### SGF

The susceptibility to proteolytic degradation of phosphomannose isomerase in simulated gastric fluid (SGF) was investigated by SDS-PAGE and Western blotting. The reaction mixture was incubated at 37°C, over a 60 minute time course. Samples were taken at 0, 1, 2, 5, 10, 30 and 60 minutes for analysis. The reactants were in the ratio of approximately 10 pepsin activity units per µg of PMI (Thomas *et al*, 2004). The time-zero sample served as an undigested control, to which all other test samples were compared. An aliquot of the test protein solution added to SGF without pepsin was prepared to monitor the stability of PMI over the 60 minute time course. In addition, a sample without PMI was prepared to monitor the stability of the SGF containing pepsin for the duration of the experiment. Both of these controls were sampled at 0 and 60 minutes.

There was no intact PMI remaining in the reaction mix at the 1 minute time point, as visualised on SDS-PAGE and on Western blot. Faint and diffuse low molecular weight bands (approximately 4 kDa) visible on the gel at 1 minute, diminished rapidly and were undetected at 5 minutes. These bands did not cross-react with anti-PMI antibody. On the Western blot, intact PMI was only detected at time 0. No immunoreactive bands corresponding to the intact PMI or lower molecular weight degradation products could be detected at 1 minute or subsequent time points. The negative control showed no digestion of PMI after 60 minutes incubation in the absence of pepsin. The pepsin control showed that the enzyme was stable over the course of the experiment. These results demonstrate that PMI is readily digested in SGF.

### SIF

The susceptibility to proteolytic degradation at 37°C of the PMI protein in simulated intestinal fluid (SIF) was investigated using SDS-PAGE and Western blotting. The reaction mixture was sampled at the following time points: 0, 1, 2, 5, 15, 30 and 60 minutes and 2, 3, 6, 24 and 48 hours incubation time. Controls used in the experiment were (i) PMI incubated with SIF minus pancreatin, used to monitor the stability of the test protein over the 48 hour time course in the absence of pancreatin, and (ii) SIF alone (with pancreatin), used to monitor the stability of the pancreatin over 48 hours.

As noted in the previous section, incubation of the SIF control solution alone (containing pancreatin) resulted in significant progressive degradation of the pancreatin itself, which was observed at 2 hours and at 48 hours. The number of pancreatin degradation products co-migrating with other protein bands on the gel made accurate interpretation of the digestibility of PMI difficult. For this reason, the Western blot analysis was considered more informative.

The Western blot showed negligible degradation of PMI in the absence of pancreatin, even after incubation for 48 hours, however in SIF with pancreatin, there were no immunoreactive bands visualised in samples beyond the 5 minute time point. These results indicate that intact PMI is readily degraded in SIF.

### Conclusion

Digestibility studies demonstrated that the bacterial PMI protein is readily susceptible to mammalian digestive processes. The combined effects of gastric and intestinal digestion would result in almost complete proteolysis of PMI. As a result, there would be virtually no systemic exposure to intact PMI from the consumption of corn line 5307.

## **4.8 Heat stability**

Food processing operations that subject corn products to relatively high temperatures would rapidly denature plant proteins, including complexes with nucleic acids. Studies were conducted on both eCry3.1Ab and PMI to investigate the effect of temperature on the activity of the protein in each case.

### **Submitted studies:**

Nelson A (2010) Effect of Temperature on the Bioactivity of eCry3.1Ab Protein as Contained in Test Substance ECRY3.1AB-0208. Syngenta Study No. TKRS0000041, Report No. SSB-014-09 A1

Mims G (2009) Effect of Temperature on Phosphomannose Isomerase as Contained in Test Substance PMI-0105. Syngenta Study No. PMI-07-05, Report No. SSB-023-09

### *4.8.1 Insecticidal activity of eCry3.1Ab*

The eCry3.1Ab protein expressed in corn line 5307 is an engineered chimera of mCry3A and Cry1Ab proteins and has insecticidal activity against significant plant pests including some corn rootworm (*Diabrotica*) species and Colorado potato beetle (*Leptinotarsa decemlineata*). The effects of temperature on the bioactivity of eCry3.1Ab was investigated by incubating duplicate aliquots of the protein at 25°C, 37°C, 65°C and 95°C for 30 minutes. Any loss of insecticidal activity at these temperatures was determined by comparison with a control sample in an insect bioassay using the freshly hatched larvae of *L. decemlineata*. The control sample of eCry3.1Ab was incubated at 4°C for 30 minutes.

Compared to the activity of the control sample, eCry3.1Ab retained insecticidal activity after temperature treatment at 25°C, 37°C and 65°C, as measured by LC<sub>50</sub> values (the concentration of protein causing death in 50% of the test larvae). In contrast, eCry3.1Ab samples incubated at the highest temperature (95°C) did not retain insecticidal activity, as determined by a low mortality of larvae at all concentrations tested. This study supports the conclusion that eCry3.1Ab is denatured and therefore inactivated after heat treatment at 95°C for 30 minutes.

### *4.8.2 Activity of PMI*

PMI catalyzes the reversible inter-conversion of mannose-6-phosphate and fructose-6-phosphate, and is used as a selectable marker in corn line 5307. Plant cells that have been transformed with the *E. coli manA* gene and express PMI, are able to utilise mannose as a carbon source. The effects of temperature on the enzyme activity of PMI was investigated by exposing triplicate aliquots of the protein to various temperatures (4°C, 25°C, 37°C, 65°C and 95°C) for 30 minutes. PMI activity was measured by the production of NADPH in a coupled reaction containing phosphoglucose isomerase (PGI) and glucose-6-phosphate dehydrogenase (G6PDH), based on the method described by Gracy and Noltmann (1968)

and Gill *et al.* (1986), and validated for use by the Applicant (Nelson, 2009. Syngenta Regulatory Science Report No. SSB-003-08).

The results of the enzyme assays show that the incubation of PMI at temperatures up to 37°C for 30 minutes did not decrease specific enzyme activity. Exposure to higher temperatures resulted in a loss of PMI enzyme activity below the limit of quantitation of the assay. This study supports the conclusion that PMI is labile to heat at 65°C and above, which results in loss of enzymatic activity.

#### **4.9 Summary and conclusion from studies on the novel proteins**

A series of laboratory and *in silico* analyses was conducted to determine the level and physicochemical properties of the two novel proteins present in corn line 5307, and to investigate whether either of the proteins was likely to be toxic or allergenic in humans. Both novel proteins were detectable in grain of corn line 5307 at relatively low levels – eCry3.1Ab at 4.98 µg/g, and PMI at 1.31 µg/g.

The novel proteins expressed in corn line 5307 conform to the expected size and amino acid sequence of eCry3.1Ab and PMI respectively, based on the genes inserted into the corn, and are not glycosylated. In addition, they are detected by specific antibodies raised against the proteins, and exhibit the required functional activity in each case. Updated bioinformatic analyses confirmed the absence of any biologically significant amino acid sequence similarity to known protein toxins or allergens. Digestibility studies demonstrated that these proteins would be rapidly degraded under normal digestive conditions, similar to other dietary proteins. A separate acute oral toxicity study in mice for each protein using equivalent *E. coli*-derived material confirmed the absence of acute toxicity. The safety of the eCry3.1Ab protein was further supported by the previous history of safe use of mCry3A and Cry1Ab in other crops, and PMI also has a history of use as a selectable marker protein in transgenic food crops. Taking all available evidence into consideration, the presence of eCry3.1Ab and PMI as novel proteins in corn line 5307 does not raise food safety concerns.

### **5. Compositional analyses**

Compositional analyses are important for evaluating whether the genetic modification has introduced any unexpected changes to the composition of the food, and to establish its nutritional adequacy. Where there has been a deliberate change in composition brought about by the genetic modification, compositional analyses are primarily important for evaluating the intended effect.

The classic approach to the compositional analysis of a GM food is to target key constituents, rather than analyse every single constituent, which would be impractical and not necessarily of value to a safety assessment. The aim is to analyse only those components most relevant to the safety of the food, or that may have a substantial impact on the whole diet, including major nutrients, toxicants and anti-nutrients for the food in question. The key nutrients and anti-nutrients are typically major constituents (fats, proteins, carbohydrates or enzyme inhibitors as anti-nutrients) or quantitatively more minor constituents (minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in an organism, such as compounds whose toxicity and level may be significant to health (e.g. solanine in potatoes).

#### **5.1 Growth of corn**

Corn development is divided into vegetative (V) and reproductive (R) stages, which are summarised in Table 4. The V number corresponds to the number of fully exposed leaves with a visible collar and generally goes up to V18.

Corn yield is a function of kernel number (which is dependent on the number of plants per unit area, grained ears/plant and kernels/ear) and kernel weight (which is dependent on the duration and rate of grain fill) (Westgate *et al* 2004).

**Table 4: Summary of corn growth stages** (Derived from Hanway & Ritchie 1984)

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

## 5.2 Key components

For the purposes of a comparative analysis with conventional varieties, key constituents of corn include protein, fat, carbohydrate, amino acids, fatty acids, vitamins, minerals, and the anti-nutrients trypsin inhibitors, phytic acid and raffinose (OECD 2002). Phytic acid is considered an anti-nutrient because it causes chelation of mineral nutrients (including calcium, magnesium, potassium, iron and zinc) thereby making them unavailable to monogastric animals including humans. Trypsin inhibitors interfere with digestion of protein and can inhibit animal growth, but are generally labile to heat-processing. Raffinose is a low molecular weight carbohydrate that is not completely digested in humans, and is associated with gastrointestinal symptoms.

## 5.3 Study design

### Submitted study:

Launis K (2011) Compositional Analysis of Forage and Grain from Event 5307 Hybrid Maize Grown During 2008 in the USA. Report No. SSB-170-09 A2 (Volumes 1 & 2).

Hybrid corn line 5307 (pedigree NP2171/NP2460) and the corresponding non-GM, near-isogenic hybrid line were grown at six locations in the USA during 2008. Trial sites located in the States of Minnesota, Wisconsin, Indiana, Illinois and Missouri were selected as representative of typical agricultural regions for hybrid corn cultivation. Plants of each genotype were grown in a randomised complete block design, with three replicate plots for each genotype per site. Conventional agronomic practices were used to plant, maintain and harvest plants – both 5307 corn and the non-GM control line were treated with conventional pesticides to maintain optimal plant health. Plants were self-pollinated by hand, and the developing ears were bagged to prevent cross-pollination.

For plants of both genotypes, ears were collected from 15 plants from each replicate plot at each location. Ears were harvested at physiological maturity (R6 stage), and then mechanically dried to approximately 9-12% moisture content. Each sample consisted of the grain shelled from the ears of the 15 plants harvested from each plot. Plants were also harvested for forage analysis, however the results from the forage analysis are not presented in this report.

## 5.4 Analyses of key components in grain

Analyses were carried out at Covance Laboratories, Inc. according to methods published and approved by AOAC International (AOAC or other industry-standard analytical methods). Compositional analyses of corn grain samples included proximates (protein, fat, ash, moisture, carbohydrates and starch), acid detergent fibre (ADF), neutral detergent fibre (NDF), total dietary fibre (TDF), amino acids, fatty acids, vitamins, minerals, secondary metabolites and anti-nutrients (phytic acid, raffinose and trypsin inhibitor). Moisture content of grain was not statistically analysed because the samples had been mechanically dried.

The data for each analyte were subjected to appropriate statistical analyses using a mixed model analysis of variance (ANOVA). Significantly different results were identified at  $P < 0.05$ . The mean levels of each component for each location and across locations were calculated and compared with means and ranges for forage and grain composition published in the ILSI Crop Composition Database (2008). This database was considered by the Applicant to represent the most comprehensive and current source of compositional data available for most nutritional components in corn, and was used as the commercial reference range.

### 5.4.1 Proximates, fibre and starch

The combined results of the proximate, fibre and starch analysis of corn grain harvested from line 5307 and the near-isogenic control line grown at all sites are shown in Table 5, together with the commercial ranges from the ILSI reference database. There were no significant differences in any of these analytes in grain from 5307 corn compared to that from conventional (control) corn. This was also reflected at the individual site level for the majority of analytes.

**Table 5: Combined Site Results of Proximates (Protein, Fat, Ash, Moisture and Carbohydrates) and Fibre (Acid Detergent Fibre, Neutral Detergent Fibre, Total Dietary Fibre) and Starch Analysis in Corn Grain – Statistical Summary<sup>1</sup>**

Analyte (% DW <sup>3</sup> )	Corn line 5307 Mean (S.E.) [Range]	Control Mean (S.E.) [Range]	p-Value <sup>2</sup>	ILSI database Mean (N) [Range]
<b>Protein</b>	10.86 (0.375) [9.12 – 12.60]	10.92 (0.375) [9.20 – 13.00]	0.737	10.30 (1434) [6.15 – 17.26]
<b>Fat</b>	4.54 (0.067) [3.85 – 4.93]	4.72 (0.067) [4.43 – 5.09]	0.053	3.55 (1174) [1.74 – 5.82]
<b>Ash</b>	1.46 (0.044) [1.22 – 1.60]	1.40 (0.044) [1.09 – 1.67]	0.138	1.44 (1410) [0.62 – 6.28]
<b>Moisture (%FW)</b>	10.13 [9.54 – 11.40]	10.18 [9.21 – 12.20]	-	11.3 (1434) [6.10 – 40.50]
<b>Carbohydrate</b>	83.1 (0.44) [81.0 – 85.3]	83.0 (0.44) [80.7 – 84.7]	0.515	84.6 (1410) [77.4 – 89.5]
<b>ADF</b>	2.74 (0.069) [2.23 – 3.34]	2.85 (0.069) [2.47 – 3.48]	0.281	4.05 (1350) [1.82 – 11.34]
<b>NDF</b>	8.85 (0.128) [7.68 – 9.52]	8.83 (0.128) [7.79 – 10.2]	0.930	11.23 (1349) [5.59 – 22.64]
<b>TDF</b>	11.8 (0.19) [10.8 – 13.4]	11.7 (0.19) [10.6 – 13.5]	0.700	16.43 (397) [8.85 – 35.31]
<b>Starch</b>	69.4 (1.21) [62.0 – 73.7]	70.3 (1.21) [63.1 – 77.3]	0.589	57.7 (168) [26.5 – 73.8]

<sup>1</sup> DW – dry weight; FW – fresh weight; S.E. – standard error; N – number of ILSI values

<sup>2</sup> Probability values  $< 0.05$  are statistically significant.

<sup>3</sup> Moisture Units %FW

**Table 6: Mineral Analysis, Combined Site Results – Statistical Summary<sup>1</sup>**

Analyte (mg/kg DW)	Corn line 5307 Mean (S.E.) [Range]	Control Mean (S.E.) [Range]	p-Value <sup>2</sup>	ILSI database Mean (N) [Range]
<b>Ca</b>	43.9 (1.28) [38.6 – 49.3]	44.0 (1.28) [40.3 – 50.1]	0.891	46.4 (1344) [12.7 – 208.4]
<b>Cu</b>	1.52 (0.253) [0.89 – 4.20]	1.89 (0.253) [1.02 – 4.36]	0.058	1.75 (1249) [<LOQ – 18.5]
<b>Fe</b>	23.7 (0.85) [21.2 – 28.0]	23.3 (0.85) [20.3 – 28.1]	0.308	21.81 (1255) [10.42 – 49.07]
<b>Mg</b>	1323 (21.2) [1150 – 1430]	1336 (21.2) [1220 – 1450]	0.401	1193.8 (1257) [594.0 – 1940.0]
<b>Mn</b>	5.65 (0.249) [4.69 – 6.61]	5.43 (0.249) [4.43 – 6.38]	0.131	6.18 (1256) [1.69 – 14.30]
<b>P</b>	3228 (94.7) [2620 – 3520]	3307 (94.7) [2650 – 3600]	0.110	3273.5 (1349) [1470.0 – 5330.0]
<b>K</b>	3758 (81.0) [3400 – 4010]	3776 (81.0) [3240 – 4150]	0.707	3842 (1257) [1810.0 – 6030.0]
<b>Se</b>	- [<LOQ – 0.36]	- [<LOQ – 0.40]	-	0.2 (89) [<LOQ – 0.75]
<b>Na</b>	- [<LOQ]	- [<LOQ]	-	31.75 (223) [<LOQ – 731.54]
<b>Zn</b>	23.0 (0.78) [19.5 – 26.9]	23.4 (0.78) [20.5 – 27.9]	0.355	21.6 (1257) [6.5 – 37.2]

<sup>1</sup> DW – dry weight; FW – fresh weight; S.E. – standard error; N – number of ILSI values excluding those <LOQ

<sup>2</sup> Probability values <0.05 are statistically significant.

#### 5.4.3 Amino Acids

The levels of 18 amino acids were measured in the GM and control grain, and the analysis of results across all locations is presented in Table 7. No statistically significant differences in the level of any amino acid were observed in this data set. Several amino acids were found to be significantly different in the GM corn compared with the non-GM control at one of the six trial locations only. The differences however were very small, and all amino acid values were within the ranges reported in the ILSI database.

#### 5.4.2 Minerals

There were no significant differences in the levels of 10 mineral compounds (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, selenium, sodium and zinc) measured in grain from the GM and non-GM corns, as shown in the combined site results presented in Table 6. In the comparisons between the GM and non-GM corn at individual locations, the levels of magnesium, manganese, phosphorus and zinc were significantly different at one location. Mineral levels at all locations were within the ranges reported in the ILSI database.

**Table 7: Comparison of Amino Acids in Corn Grain – Combined Site Analysis<sup>1</sup>**

Amino Acid (% DW)	Corn line 5307 Mean (S.E.) [Range]	Control Mean (S.E.) [Range]	p-Value <sup>2</sup>	ILSI database Mean (N) [Range]
Alanine	8.21 (0.345) [6.64 – 9.97]	8.24 (0.345) [7.06 – 10.0]	0.846	7.90 (1350) [4.39 – 13.93]
Arginine	4.81 (0.144) [3.72 – 5.56]	4.82 (0.144) [4.20 – 5.32]	0.892	4.33 (1350) [1.19 – 6.39]
Aspartic acid	6.93 (0.236) [5.82 – 8.15]	6.88 (0.236) [6.00 – 8.20]	0.625	6.88 (1350) [3.35 – 12.08]
Cystine	2.33 (0.043) [2.07 – 2.50]	2.36 (0.043) [2.14 – 2.59]	0.284	2.21 (1350) [1.25 – 5.14]
Glutamic acid	20.4 (0.90) [16.4 – 24.9]	20.6 (0.90) [17.4 – 25.3]	0.715	20.09 (1350) [9.65 – 35.36]
Glycine	3.95 (0.087) [3.52 – 4.39]	3.97 (0.087) [3.61 – 4.35]	0.761	3.85 (1350) [1.84 – 5.39]
Histidine	2.99 (0.088) [2.57 – 3.44]	3.01 (0.088) [2.57 – 3.43]	0.684	2.96 (1350) [1.37 – 4.34]
Isoleucine	3.92 (0.163) [3.19 – 4.77]	3.91 (0.163) [3.23 – 4.71]	0.947	3.68 (1350) [1.79 – 6.92]
Leucine	13.8 (0.66) [10.8 – 17.1]	13.8 (0.66) [11.5 – 17.3]	0.789	13.41 (1350) [6.42 – 24.92]
Lysine	3.10 (0.059) [2.76 – 3.36]	3.09 (0.059) [2.74 – 3.38]	0.902	3.15 (1350) [1.72 – 6.68]
Methionine	2.29 (0.049) [1.97 – 2.51]	2.36 (0.049) [2.08 – 2.56]	0.102	2.09 (1350) [1.24 – 4.68]
Phenylalanine	5.50 (0.239) [4.34 – 6.68]	5.52 (0.239) [4.73 – 6.70]	0.883	5.25 (1350) [2.44 – 9.30]
Proline	9.23 (0.375) [2.03 – 2.19]	9.24 (0.375) [7.84 – 10.9]	0.973	9.51 (1350) [4.62 – 16.32]
Serine	5.14 (0.203) [4.10 – 6.10]	5.17 (0.203) [4.44 – 6.28]	0.736	5.12 (1350) [2.35 – 7.69]
Threonine	3.80 (0.123) [1.54 – 1.65]	3.79 (0.123) [1.49 – 1.62]	0.908	3.75 (1350) [2.24 – 6.66]
Tryptophan	0.57 (0.03) [0.38 – 0.70]	0.56 (0.03) [0.38 – 0.70]	0.722	0.63 (1350) [0.27 – 2.15]
Tyrosine	3.18 (0.153) [1.57 – 4.18]	3.26 (0.153) [1.67 – 3.98]	0.711	3.36 (1350) [1.03 – 6.42]
Valine	5.12 (0.179) [4.33 – 6.08]	5.13 (0.179) [4.28 – 6.08]	0.877	4.90 (1350) [2.66 – 8.55]

<sup>1</sup> DW – dry weight; N – number of ILSI values; S.E. – standard error

<sup>2</sup> Probability values <0.05 are statistically significant

#### 5.4.4 Fatty Acids

Grain samples from corn line 5307 and the control line were analysed for 22 fatty acids. Of these, the levels of caprylic (C8:0), capric (C10:0), lauric (C12:0), myristic (C14:0), myristoleic (C14:1), pentadecanoic (C15:0), pentadecenoic (C15:1), heptadecanoic (C17:0), heptadecenoic (C17:1), eicosadienoic (C20:2), eicosatrienoic (C20:3) and arachidonic (C20:4) were less than the limit of quantitation (LOQ) in all replicates at all locations. Eight fatty acids were quantifiable and were used in the combined-site statistical comparison. As shown in Table 8, palmitic, stearic, linolenic and eicosenoic acids were significantly different in corn line 5307 compared with the control line, although the magnitude of the difference for each fatty acid component was small.

The results at individual locations indicated that the differences occurred randomly. For all quantifiable fatty acids, mean levels across locations and for each location were within the ILSI reference range.

**Table 8: Comparison of Fatty Acids in Corn Grain – Combined Site Analysis<sup>1</sup>**

Fatty Acid (% total FA)	Corn line 5307 Mean (S.E.) [Range]	Control Mean (S.E.) [Range]	p-Value <sup>2</sup>	ILSI database Mean (N) [Range]
Palmitic acid (C16:0)	15.7 (0.07) [15.1 – 16.1]	15.2 (0.07) [14.6 – 15.9]	<0.001	11.50 (1344) [7.94 -20.71]
Stearic acid (C18:0)	1.74 (0.059) [1.50 – 2.04]	1.81 (0.059) [1.54 – 2.17]	0.038	1.82 (1344) [1.02 – 3.40]
Oleic acid (C18:1)	24.5 (0.54) [22.0 – 27.0]	24.9 (0.54) [22.6 – 26.4]	0.108	25.8 (1344) [17.4 – 40.2]
Linoleic acid (C18:2)	55.6 (0.60) [53.2 – 58.1]	55.7 (0.60) [53.8 – 58.4]	0.599	57.6 (1344) [36.2 – 66.5]
Linolenic acid (C18:3)	1.60 (0.017) [1.48 – 1.71]	1.50 (0.017) [1.40 – 1.57]	<0.001	1.20 (1344) [0.57 – 2.25]
Arachidic acid (C20:0)	0.392 (0.009) [0.353 – 0.453]	0.387 (0.009) [0.361 – 0.437]	0.186	0.412 (988) [0.279 – 0.965]
Eicosenoic acid (C20:1)	0.250 (0.003) [0.238 – 0.265]	0.242 (0.003) [0.232 – 0.261]	<0.001	0.297 (987) [0.170 – 1.917]
Behenic acid (C22:0)	0.220 (0.005) [0.186 – 0.252]	0.213 (0.005) [0.194 – 0.247]	0.243	0.176 (924) [<LOQ – 0.349]

<sup>1</sup> S.E. – standard error; N – number of ILSI values excluding those <LOQ

<sup>2</sup> Probability values <0.05 are statistically significant.

#### 5.4.5 Vitamins

The results of vitamin analysis of grain from corn line 5307 and the control line are presented in Table 9. On a statistical basis, the levels of vitamins A, B<sub>6</sub> and B<sub>9</sub> were significantly different between the two lines, although the magnitude of the difference was small in each case. It is noted that the levels of vitamin B<sub>9</sub> in corn are naturally at least five times lower than the levels of other B vitamins, showing that corn is not a major source of this vitamin. Moreover, all mean levels in the combined sites and at each location were within the range reported for vitamins in the ILSI database.

#### 5.4.6 Antinutrients and secondary plant metabolites

There are no generally recognised antinutrients 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 antinutrients phytic acid and raffinose, and the secondary metabolites furfural, ferulic acid and p-coumaric acid (OECD 2002). The levels of trypsin inhibitor are also generally too low to be regarded as nutritionally significant.

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

Secondary metabolites are defined as those natural substances which do not function to 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.

A range of antinutrient compounds were measured in grain from corn line 5307 and the control line, as shown in Table 10. There were no significant differences between the lines in any of these analytes, and all values were within the range reported in the ILSI database. The levels of furfural in all samples from both corn line 5307 and the control line from across the trial sites were below the LOQ and were not included in this report.

**Table 9: Comparison of Vitamin Levels in Corn Grain – Combined Site Analysis<sup>1</sup>**

Nutrient (mg/100g DW)	Corn line 5307 Mean (S.E.) [Range]	Control Mean (S.E.) [Range]	p-Value <sup>2</sup>	ILSI database Mean (N) [Range]
<b>Vitamin A (β-carotene)</b>	0.155 (0.0049) [0.133 – 0.185]	0.176 (0.0049) [0.155 – 0.216]	<b>&lt;0.005</b>	0.684 (276) [0.019 – 4.681]
<b>Vitamin B<sub>1</sub> (Thiamine)</b>	0.449 (0.0126) [0.399 – 0.511]	0.458 (0.0126) [0.408 – 0.518]	0.146	0.530 (894) [0.126 – 4.000]
<b>Vitamin B<sub>2</sub> (Riboflavin)</b>	0.198 (0.0096) [0.156 – 0.264]	0.198 (0.0096) [0.152 – 0.318]	0.941	0.125 (704) [0.050 – 0.236]
<b>Vitamin B<sub>3</sub> (Niacin)</b>	3.13 (0.104) [2.53 – 4.11]	3.18 (0.104) [2.51 – 3.70]	0.674	2.376 (415) [1.037 – 4.694]
<b>Vitamin B<sub>6</sub> (Pyridoxine)</b>	0.692 (0.0167) [0.587 – 0.769]	0.737 (0.0167) [0.621 – 0.815]	<b>0.005</b>	0.644 (415) [0.368 – 1.132]
<b>Vitamin B<sub>9</sub> (Folate)</b>	0.0397 (0.00199) [0.0305 – 0.0460]	0.0382 (0.00199) [0.0289 – 0.0463]	<b>0.031</b>	0.0651 (895) [0.0147 – 0.1464]
<b>Vitamin E<sup>3</sup> (α-tocopherol)</b>	0.0093 (0.00055) [0.00719 – 0.0111]	0.0090 (0.00055) [0.00607 – 0.0110]	0.074	0.0103 (863) [0.0015 – 0.0687]

<sup>1</sup> DW – dry weight; S.E. – standard error; N – number of ILSI values

<sup>2</sup> Probability values <0.05 are statistically significant

<sup>3</sup> Vitamin E units – mg/g

## 5.5 Conclusion

The compositional analyses do not indicate any differences of biological significance between the grain from corn line 5307 and the non-GM control. Statistically significant differences were noted in a small number of constituents, however in each case the individual site results demonstrated that the differences were minimal and occurred randomly. Almost all mean values were within the appropriate reference range reported in the ILSI database, which demonstrated a naturally broad range of values for numerous analytes in conventional corn.

**Table 10: Secondary Metabolite & Antinutrients in Corn Grain – Combined Site Analysis<sup>1</sup>**

Analyte (Units)	Corn line 5307 Mean (S.E.) [Range]	Control Mean (S.E.) [Range]	p-Value <sup>2</sup>	ILSI database Mean (N) [Range]
<b>Ferulic acid (mg/kg DW)</b>	1906 (52.4) [1670 – 2190]	1889 (52.4) [1620 – 2090]	0.691	2201.1 (817) [291.9 – 3885.8]
<b>p-Coumaric acid (mg/kg DW)</b>	186 (9.1) [153 – 229]	186 (9.1) [148 – 226]	0.926	218.4 (817) [53.4 – 576.2]
<b>Inositol (ppm DW)</b>	2510 (86.1) [0.156 – 0.264]	2504 (86.1) [0.152 – 0.318]	0.951	1331.5 (504) [89.0 – 3765.4]
<b>Phytic acid (% DW)</b>	0.910 (0.0261) [0.671 – 1.03]	0.942 (0.0261) [0.729 – 1.06]	0.216	0.745 (1196) [0.111 – 1.570]
<b>Trypsin inhibitor (TIU/mg DW)</b>	3.34 (0.118) [0.587 – 0.769]	3.46 (0.118) [0.621 – 0.815]	0.393	2.73 (696) [<LOQ – 7.18]
<b>Raffinose (% DW)</b>	0.156 (0.0087) [0.115 – 0.199]	0.163 (0.0087) [0.199 – 0.188]	0.066	0.132 (701) [<LOQ – 0.320]

<sup>1</sup> DW – dry weight; S.E. – standard error; N – number of ILSI values

<sup>2</sup> Probability values <0.05 are statistically significant

## 6. Nutritional impact

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

If the compositional analysis indicates biologically significant changes to the levels of certain nutrients in the GM food, additional nutritional assessment should be undertaken on a case-by-case basis. However, where a GM food has been shown to be compositionally equivalent to conventional varieties, the evidence indicates that feeding studies using target livestock species will add little value to the safety assessment (OECD 2003; FSANZ 2007; EFSA 2008).

In this case, corn line 5307 is resistant to certain insect pests as a result of a simple genetic modification. This trait was not expected to significantly alter nutritional parameters in the food. Moreover, it has been determined that corn line 5307 is equivalent in composition to the conventional control line. The introduction of corn line 5307 into the food supply is therefore expected to have negligible nutritional impact on human diets.

### 6.1 Feeding studies

Although not essential information for safety assessment purposes, the Applicant conducted a feeding study in broiler chickens. Feeding studies are not designed to test strict toxicological endpoints but rather to determine whether the GM food is able to support typical growth and well-being in rapidly growing animals, preferably using a species that naturally consumes the food in question. Chickens (*Gallus domesticus*) consume large quantities of field corn grain in commercial poultry feeds.

Broiler chickens in particular also consume relatively high amounts of maize because commercial feeding regimens have been designed to provide maximal body weight gain in the shortest amount of time, between 42-49 days.

### 6.1.1 Broiler feeding study

**Study submitted:**

Evaluation of Event 5307 Transgenic Maize Grain in a Broiler Chicken Feeding Study. Principal Investigator: J.T. Brake; Performing Laboratory: Department of Poultry Science, North Carolina State University, Raleigh, NC, USA; Report number: SSB-211-10. (Study completed: 12 November 2010)

This study compared the nutritional performance of grain from transgenic corn line 5307, the conventional near-isogenic line, and a commercial reference corn (NCSU 2007) when incorporated into the diet of broiler chickens over a period of 49 days. The parent bird stock was a commercial strain of Heritage broiler breeders grown and maintained at the North Carolina Department of Agriculture and Consumer Services Piedmont Research Station under the supervision of Professor John T. Brake.

Prior to the study, all three lines of corn were analysed for proximates, amino acids and the presence of mycotoxins. This enabled a calculation for metabolisable energy for each type of grain. The final standardised formulations for each maize grain source resulted in three broiler chicken diets prepared to meet the nutritional requirements of the birds at each stage of their growth: starter (1-15 days), grower (16-34 days) and finisher (35-49 days) diets.

Equal numbers of one day old male and female chickens were assigned to one of the three different corn diets (180 birds per treatment). There were six pens each with 15 birds of the same sex, for each dietary treatment. Feed and drinking water were provided *ad libitum* up until 12 hours before slaughter.

Birds were observed daily for overall condition and health, including overt clinical signs, injuries and mortalities. Total pen body weight data were collected at hatch (day 1), 16 days, 35 days and 49 days of age. Feed consumption per pen was determined in order to calculate feed conversion ratios and adjusted feed conversion ratios (if some birds had died). Body weight, feed consumption, feed conversion and survival data were analysed to determine statistical differences between the three corn diets, and between males and females. The performance endpoints measured in this study were body weight, survival, feed consumption, feed conversion ratios and carcass yield.

As expected in broiler studies, there was a low incidence of mortality in all groups (approximately 2%), with a higher number of deaths in male birds over the course of the study. In survivors, there were no statistically significant differences in body weights between animals fed corn 5307, the control, or the NCSU 2007 reference corn diet. Noting that male chickens consumed significantly more feed than females, there were no overall differences in feed consumption between the three test diets over the length of the study.

The feed conversion ratio is an indicator of how efficiently a bird converts feed to live body weight. Improved efficiency occurs through gastrointestinal and/or general metabolic processes and is reflected in lower feed conversion ratios. The unadjusted and adjusted feed conversion ratios were calculated for 16, 35 and 49 days of age. As expected, male birds showed a lower adjusted feed conversion ratio relative to females, indicating significantly improved growth efficiencies. Broilers fed corn 5307 had improved feed conversion (both unadjusted and adjusted) during the grower period (16-35 days), as well as cumulatively from 0-35 days, compared with broilers fed the NCSU 2007 reference diet.

Cumulative feed conversion ratios over the duration of the study (0-49 days) were 1.73 (corn 5307), 1.76 (non-transgenic control) and 1.77 NCSU reference corn, which was not a statistically significant difference between groups.

In terms of carcass yields, there were no statistically significant differences in the carcass portions (on an absolute weight basis) for male and female broilers between the three diet regimens.

#### Discussion and conclusion

Although the diets made from the three different maize sources were formulated to contain similar levels of nutrients, the authors noted that the diets were not identical. Despite some nutritional variation, the results show no differences in the growth performance of broiler chickens fed corn line 5307 and those fed the non-transgenic control. A poultry diet prepared with corn line 5307 is able to support rapid growth in broiler chickens at low mortality rates and with no adverse effects, when compared with a diet containing conventional corn.

## **7. Conclusion**

On the basis of the evidence presented in this report, food derived from insect-protected corn line 5307, which contains bacterially-derived genes encoding the novel proteins eCry3.1Ab and PMI, is as safe for human consumption as that derived from other commercial corn varieties.

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