SYNGENTA SEEDS PTY LTD

Type of Submission:	**Amendment to Standard 1.5.2 – Food Produced Using Gene Technology**
Product:	**Foods derived from insect-protected corn containing the *ecry3.1Ab* and *pmi* genes – Line 5307**
Volume:	**Volume 1 of 6**
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A. General information on the application

1. Purpose of the application

The applicant is seeking an amendment to Standard 1.5.2 – Food produced using Gene Technology, in the Australia New Zealand Food Standards Code (the Code), to approve food derived from insect-protected corn line 5307.

2. Justification for the application

This part includes general statements addressing:

a) The advantages of the genetically modified food.

GM corn 5307 produces the eCry3.1Ab and PMI proteins. The gene *ecry3.1Ab* encodes the eCry3.1Ab protein, a chimeric protein engineered from portions of modified Cry3A (mCry3A) and Cry1Ab proteins which are derived from *Bacillus thuringiensis*. The 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. Vigifera zeae* Krysan and Smith). These important coleopteran pests cause significant damage to U.S. corn crops annually. The *pmi* gene, also known as *manA*, is derived from *Eschericha coli* strain K-12 and encodes the PMI protein, which was utilised as a selectable marker during the development of 5307 corn. GM corn 5307 in breeding stack combinations with other insect-resistant corn products will allow growers to have optimal broad-spectrum insect control.

b) The safety of the genetically modified food.

No intended compositional changes are expected as a result of the genetic modification in 5307 corn. Line 5307 corn is as safe and nutritious as food and feed derived from conventional corn varieties. Section C below contains detailed information supporting the safety of the genetically modified corn.

c) The potential impact on trade.

GM corn 5307 is not intended for commercialisation in Australia or New Zealand, therefore, it is not expected that there will be any undue prejudice to trade with other countries as a result of approval of 5307 corn in Australia or New Zealand. Authorisation of 5307 corn for food and feed uses, import and processing will be sought in all major importing corn countries.

d) The costs and benefits for the industry, consumers and government associated with the use of the genetically modified food.

Food manufacturers may gain broader market access and increase choice in sourcing raw materials as a result of approval of this corn. Importers of processed food could benefit from knowing that imported corn products that may contain 5307 corn would be compliant with the Code.

A possible cost to the industry may be that some food ingredients may require labelling as genetically modified.

Consumers may benefits from approval of GM corn 5307 in the potentially wider range of imported corn products.

The government would benefit from approval of 5307 corn in that if this corn was ever detected in corn imports, that these products would be compliant with the Code and that there would be little potential for trade disruptions on regulatory grounds.

B. Technical information on the genetically modified food

1. Nature and identity of the genetically modified food

This part includes all of the following:

a) A description of the GM organism from which the new GM food is derived. The description must include the nature and purpose of the genetic modification.

Syngenta has developed a GM corn known as Line 5307 that produces the eCry3.1Ab and PMI proteins. The eCry3.1Ab protein is a chimeric protein engineered from portions of modified Cry3A (mCry3A) and Cry1Ab proteins, which are derived from *Bacillus thuringiensis*. The 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. Vigifera zeae* Krysan and Smith). These important coleopteran pests cause significant damage to U.S. corn crop annually. The *pmi* gene, also known as *manA*, is derived from *Escherichia coli* strain K-12 and encodes the PMI protein, which was utilised as a selectable marker during the development of 5307 corn. GM corn 5307 in breeding stack combinations with other insect-resistant corn products will allow growers to have optimal broad-spectrum insect control.

b) The name, number or other identifier of each of the new lines or strains of GM organism for the food is derived.

The designation of the GM corn is Line 5307.

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

Not applicable. Syngenta does not intend to cultivate 5307 corn in either Australia or New Zealand. Corn containing 5307 has been developed for cultivation in breeding stack combinations, mainly for the United States and Canada.

Food derived from the 5307 corn will therefore likely enter the Australian and New Zealand food supply as only as imported and largely processed products.

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

GM corn 5307 will be grown for the same uses as current commercially available corn (maize) in the US. In 2009, there were more than 87 million acres planted to corn in the US producing over 10 billion bushels of grain. Corn grown in the US is predominantly of the yellow dent type, a commodity crop largely used to feed domestic animals, either as grain or silage. The remainder of the crop is exported or processed, such as by wet or dry milling to yield products such as high fructose corn syrup and starch or oil, grits and flour. These processed products are used extensively in the food industry. For example, corn starch serves as a raw material for an array of processed foods, and in industrial manufacturing processes. Since the early 1980's, a significant amount of grain has also been used for fuel ethanol production. The by-products from these distilling processes are often used in animal feeds.

Domestic production of corn in Australia and New Zealand is supplemented by the import of a small amount of corn-based products, largely as high-fructose corn syrup, which is not currently manufactured in either Australia or New Zealand. These products are used in the manufacture of breakfast cereals, bakery products, confectionery and food coatings.

Corn starches are also imported and used in the manufacture of dessert mixes and sauces.

Corn may also be imported in finished products such corn chips and canned corn, or dry milled goods such as cornflour.

2. History of use of the host and donor organisms

This part includes all of the following:

a) A description of all the donor organisms from which the genetic elements are derived, including:

i. Common and scientific names and taxonomic classification;
ii. Information about any known pathogenicity, toxicity or all allergenicity of relevance to the food; and
iii. Information about the history of use of the organism in the food supply or history of human exposure to the organism through other intended food use (e.g. as a normal contaminant).

GM corn 5307 contains the *ecry3.1Ab* gene which was engineered using portions of a modified *cry3A* (*mcry3A*) gene and a *cry1Ab* gene, derived from *Bacillus thuringiensis (B.t.). B.t. is* from the Family Bacillaceae, Genus *Bacillus*; and is a gram-positive spore forming rod. *B.t.* is an ubiquitous soil and plant bacterium. The World Health Organisation (WHO) International Program on Chemical Safety (IPCS) report on environmental health criteria for *B.t.* concludes that '*B.t.* has not been documented to cause any adverse effects on human health when present in drinking water or food' (IPCS, 1999).

GM corn 5307 also contains the *pmi* gene which was initially isolated from *Escherichia coli* strain K-12, a non-pathogenic strain. *E. Coli* is from the family Enterobacteriaceae, Genus *Escherichia*; and is a gram-negative, motile, facultatively anaerobic rod. Certain serotypes are enteropathogenic and are known to cause diarrhoea in infants. Some strains also cause diarrhoea in adults. *E. coli* is a normal inhabitant of the intestinal flora of humans and animals, where it doesn't normally cause disease.

b) A description of the host organism into which the genes were transferred and its history of safe use for food, including:

i. Any relevant phenotypic information;
ii. How the organism is typically propagated for food use;
iii. What part of the organism is typically used as food
iv. Whether special processing is required to render food derived from the organism safe to eat; and
v. The significance to the diet in Australia and New Zealand of food derived from the host organism.

Zea is a genus of the family Graminae (Poaceae), commonly known as the grass family. Corn (*Zea mays* L.) is a tall, monecious annual grass with overlapping sheaths and broad conspicuously distichous blades. Plants have staminate spikelets in long spike-like racemes that form large spreading terminal panicles (tassels) and pistillate inflorescences in the leaf

axils, in which the spikelets occur in 8 to 16 rows, approximately 30 cm long, on a thickened, almost woody axis (cob). The whole structure (ear) is enclosed in numerous large foliaceous bracts and long styles (silks) protrude from the tip of the ear as a mass of silky threads. Pollen is produced entirely in the staminate inflorescence and eggs, entirely in the pistillate inflorescence. Corn is wind-pollinated and both self- and cross-pollination are usually possible. Shed pollen usually remains viable for 10 to 30 minutes, but can remain viable for longer durations under favourable conditions. Cultivated corn is presumed to have been derived from teosinte (*Z. mexicana*) and is thought to have been introduced into the old world in the sixteenth century. Corn is cultivated worldwide and represents a staple food for a significant proportion of the world's population. No significant native toxins are reported to be associated with the genus *Zea*.

Propagation for food use:

Corn plants usually reproduce sexually by wind-pollination. This provides for natural out-crossing between plants, but it also presents an opportunity for plant breeders to produce hybrid seed by controlling the pollination process. Open pollination of hybrids in the field leads to the production of grain with properties derived from different lines and, if planted, would produce lower yields. Instead, by controlling the cross-pollination of inbred lines from chosen genetic pools (using conventional techniques), the combining of desired genetic traits into a controlled hybrid line results in improved agronomic performance and increased yields. This inbred-hybrid concept and resulting yield response is the basis of the modern seed industry in several food commodities including corn.

Part(s) of organism used for food:

Immature corn can be eaten fresh as a vegetable (sweet corn) either as corn-on-the cob or as processed sweet corn.

Processing:

Corn is either dry milled or wet milled. In dry milling, the germ of the corn kernel is removed and the endosperm milled to produce grits, flour, meal and hominy feed. Corn meal is used to make tortillas. In wet milling, the protein and starch are separated. The starch is used in packaged foods. It is also hydrolysed to produce dextrose, corn syrup solids or glucose for use in food production. Fibre and protein by-products of wet milling are used as animal feed.

The germ is used as raw material for corn oil production by mechanical pressing or by extraction methods.

More than 60% of corn produced in the USA is used to produce a wide range of food ingredients, industrial products, animal feeds and alcoholic beverages.

Significance in Australian and New Zealand diet:

In Australia, corn is not a major crop, with only about 300,000 tonnes produced annually from about 100,000 hectares. Of the total production, approximately 200,000 tonnes is used for stockfeed, and 70,000 to 80,000 tonnes is utilised for human consumption. The remainder of the crop is exported.

New Zealand produces approximately 65,000 hectares of field corn, and 6,000 hectares of sweet corn or 108,000 tonnes of product. From the field corn, New Zealand produces 150,000 tonnes of grain and 1,250,000 tonnes of silage, equalling 1,400,000 tonnes of product.

3. The nature of the genetic modification

This part includes all of the following:

a) A description of the method used to transform the host organism.

Transformation of *Z. mays* to produce 5307 corn was accomplished using immature embryos of a proprietary corn line *via Agrobacterium tumefaciens*-mediated transformation. Using this method, DNA within a left border (LB) and right border (RB) elements of a transformation plasmid, referred to as the transferred DNA (T-DNA), is integrated into the genome of infected cells, while genetic elements outside of the plasmid borders are not.

Immature embryos were excised from 8-12 day old corn ears and rinsed with fresh medium in preparation for transformation. Embryos were mixed with a suspension of *A. tumefaciens* strain LBA4404 harbouring plasmid pSB1 and the transformation plasmid pSYN12274, vortexed for thirty seconds, and allowed to incubate for an additional five minutes. Excess *A. tumefaciens* suspension was aspirated, and embryos were moved to plates containing a non-selective culture medium. Embryos were co-cultured with the remaining *A. tumefaciens* at 22°C for 2-3 days in the dark. Embryos were then transferred to culture medium supplemented with ticarcillin (200 mg/l) and silver nitrate (1.6 mg/l), and incubated in the dark for ten days. The *pmi* gene was used as a selectable marker during the transformation process. Embryos producing embryogenic callus were transferred to cell culture medium containing mannose. After initial incubation with *A. tumefaciens*, transformed tissue was transferred to selective media containing 500 mg/l of the broad-spectrum antibiotic cefotaxime and grown for four months, ensuring that the *A. tumefaciens* was cleared from the transformed tissue. Cefotaxime has been shown to kill *A. tumefaciens* at this concentration. Regenerated plantlets were tested for the presence of both the *pmi* and the *ecry3.1Ab* genes, and for the absence of the spectinomycin (*spec*) resistance gene present on the vector backbone, by real-time PCR analysis. This screen allows for the selection of transgenic events that carry the T-DNA and are free of vector

backbone DNA. Plants positive for both the *pmi* and the *ecry3.1Ab* genes and negative for the *spec* gene were transferred to the greenhouse for further propagation.

5307 corn transformation employed a binary vector system. Plasmid pSYN12274, which contained the *pmi* gene from *E. coli* and the coding sequence for *ecry3.1Ab* between the right and left borders of the T-DNA, was placed into *A. tumefaciens* strain LBA4404.

b) Information about the intermediate host organisms (e.g. bacteria) used for all laboratory manipulations prior to transformation of the host organism.

Agrobacterium tumefaciens is a ubigquitous soil borne pathogen. It is a gram negative, motile, rod shaped bacterium which is non sporing, and is closely related to the N-fixing rhizobium bacteria which form root nodules on leguminous plants. The bacterium is surrounded by a small number of peritricious flagella. Virulent bacteria contain one or more large plasmids, one of which carries the genes for tumour induction and is known as the Ti (tumour inducing) plasmid. The Ti plasmid also contains the genes that determine the host range and the symptoms, which the infection will produce. Without this Ti plasmid, the bacterium is described as being non virulent and will not be able to cause disease on the plant.

A. tumefaciens is most well known for its ability to integrate a small part of the Ti plasmid into the host plant genome and it is this process which gives *A. Tumefaciens* its potential to be used as a tool for plant transformation.

c) A description of the gene construct and the transformation vectors used, including:
    i. The size, source and function of all the genetic components including marker genes, regulatory and other elements; and

Event 5307: Copy Number Functional Element Southern Blot Analysis, SSB-189-10 A1 – Appendix 1

Table 1 contains a description of each of the constituents of the pSYN12274 backbone, including the size in base pairs (bp) and the position within the plasmid.

Table 1. Plasmid pSYN12274 backbone: each genetic element and its description.

Genetic element from pSYN12274	**Size (bp)**	**Position**	**Description**
Left border (LB)	25	6436 to 6460	Left border region of T-DNA from *Agrobacterium tumefaciens* nopaline Ti-plasmid (Entrez® Accession Number J01825 [NCBI 2010]). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell.
Intervening sequence	349	6461 to 6809	Intervening noncoding sequence with restriction sites used for cloning
spec	789	6810 to 7598	Streptomycin adenylyltransferase, aadA gene from *Escherichia coli* transposon Tn7 (similar to Entrez® Accession Number X03043 [NCBI 2010]). Confers resistance to streptomycin and spectinomycin and is used as a bacterial selectable marker.
Intervening sequence	299	7599 to 7897	Intervening noncoding sequence with restriction sites used for cloning
virG	726	7898 to 8623	The VirGN54D gene (virG) from pAD1289 (similar to Entrez® Accession Number AF242881 [NCBI 2010]). The N54D substitution results in a constitutive virG phenotype. VirG is part of the two-component regulatory system for the virulence (*vir*) regulon in *Agrobacterium tumefaciens.*
Intervening sequence	29	8624 to 8652	Intervening noncoding sequence with restriction sites used for cloning
repA	1074	8653 to 9726	Gene encoding the pVS1 replication protein from *Pseudomonas aeruginosa* (similar to Entrez® Accession Number AF133831 [NCBI 2010]), which is a part of the minimal pVS1 replicon that is functional in Gram-negative, plant-associated bacteria.
Intervening sequence	42	9727 to 9768	Intervening noncoding sequence with restriction sites used for cloning

Table 1. Plasmid pSYN12274 backbone: each genetic element and its description. *(Continued)*

Genetic element from pSYN12274	**Size (bp)**	**Position**	**Description**
VS1 ori	405	9769 to 10173	Consensus sequence for the origin of replication and partitioning region from plasmid pVS1 of *Pseudomonas aeruginosa* (Entrez® Accession Number U10487 [NCBI 2010]). Serves as origin of replication in *Agrobacterium tumefaciens* host.
Intervening sequence	677	10174 to 10850	Intervening noncoding sequence with restriction sites used for cloning
ColE1 ori	807	10851 to 11657	Origin of replication (similar to Entrez® Accession Number V00268 [NCBI 2010]) that permits replication of plasmids in *Escherichia coli*.
Intervening sequence	112	11658 to 11769	Intervening noncoding sequence with restriction sites used for cloning
Right border (RB)	25	1 to 25	Right border region of T-DNA from *Agrobacterium tumefaciens* nopaline Ti-plasmid (Entrez® Accession Number J01826 [NCBI 2010]). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell.

The selectable marker cassette contains the ZmUbiInt promoter sequence, *pmi*, and the NOS terminator sequence. Table 2 contains a description of each of the constituents of the pSYN12274 selectable marker cassette, including the size in bp and the position within the plasmid.

Table 2. Plasmid pSYN12274 selectable marker cassette: each genetic element and its description.

Genetic element from pSYN12274	**Size (bp)**	**Position**	**Description**
Intervening sequence	25	2829 to 2853	Intervening noncoding sequence with restriction sites used for cloning
ZmUbiInt promoter	1993	2854 to 4846	Promoter region from the corn polyubiquitin gene which contains the first intron (Entrez® Accession Number S94464 [NCBI 2010]). Provides constitutive expression in monocots
Intervening sequence	12	4847 to 4858	Intervening noncoding sequence with restriction sites used for cloning
pmi	1176	4859 to 6034	*Escherichia coli* gene *pmi* encoding the enzyme phosphomannose isomerase (PMI) (Entrez® Accession Number M15380 [NCBI 2010]); this gene is also known as *manA*. Catalyses the isomerisation of mannose-6-phosphate to fructose-6-phosphate
Intervening sequence	60	6035 to 6094	Intervening noncoding sequence with restriction sites used for cloning
NOS terminator	253	6095 to 6347	Terminator sequence from the nopaline synthase gene of *Agrobacterium tumefaciens* (Entrez® Accession No. V00087 [NCBI 2010]). Provides a polyadenylation site
Intervening sequence	88	6348 to 6435	Intervening noncoding sequence with restriction sites used for cloning

The active ingredient cassette contains the CMP promoter sequence, *ecry3.1Ab*, and the NOS terminator sequence. Table 3 contains a description of each of the constituents of the pSYN12274 active ingredient cassette, including the size in bp and the position within the plasmid.

Table 3. Plasmid pSYN12274 active ingredient cassette: genetic elements and descriptions.

Genetic element from pSYN12274	**Size (bp)**	**Position**	**Description**
Intervening sequence	203	26 to 228	Intervening noncoding sequence with restriction sites used for cloning
CMP promoter	346	229 to 574	Cestrum Yellow Leaf Curling Virus promoter region. Provides constitutive expression in corn.
Intervening sequence	9	575 to 583	Intervening noncoding sequence with restriction sites used for cloning
ecry3.1Ab	1962	584 to 2545	An engineered Cry gene active against certain corn rootworm (*Diabrotica*) species (Entrez® Accession No. GU327680 [NCBI 2010]). As an engineered chimeric protein, eCry3.1Ab has similarities to other well characterized Cry proteins. Because Cry proteins share structural similarities, chimeric Cry genes can be engineered *via* the exchange of domains that are homologous between different Cry genes. 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 of a synthetic Cry1Ab gene (see descriptions of *mcry3A* and *cry1Ab* below). Upstream of the *mcry3A* domain, the gene *ecry3.1Ab* 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. The next 459 amino acid residues are identical to those of mCry3A, followed by 172 residues of Cry1Ab. Description of *mcry3A*: a corn-optimized *cry3A* was synthesized to accommodate the preferred codon usage for corn. The synthetic sequence was based on the native Cry3A protein sequence from *Bacillus thuringiensis* subsp. *tenebrionis*. The corn-optimized 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 (1) its N-terminus corresponds to methionine-48 of the native protein and (2) a cathepsin G protease recognition site has been introduced, beginning at amino acid residue 155 of the native protein. This cathepsin-G recognition site has the sequence alanine-alanine-proline-phenylalanine, and has replaced the amino acids valine-155, serine-156, and serine-157 in the native protein

Table 3. Plasmid pSYN12274 active ingredient cassette: each genetic element and its description *(Continued)*

Genetic element from pSYN12274	Size (bp)	Position	Description
			Description of *cry1Ab*: the gene *cry1Ab* was originally cloned from *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1. Its amino acid sequence has been codon-optimize to accommodate the preferred codon usage for corn.
Intervening sequence	30	2546 to 2575	Intervening noncoding sequence with restriction sites used for cloning
NOS terminator	253	2576 to 2828	Terminator sequence from the nopaline synthase gene of *Agrobacterium tumefaciens* (Entrez® Accession No. V00087 [NCBI 2010]). Provides a polyadenylation site

ii. A detailed map of the location and orientation of all the genetic components contained within the construct and vector, including the location of relevant restriction sites.

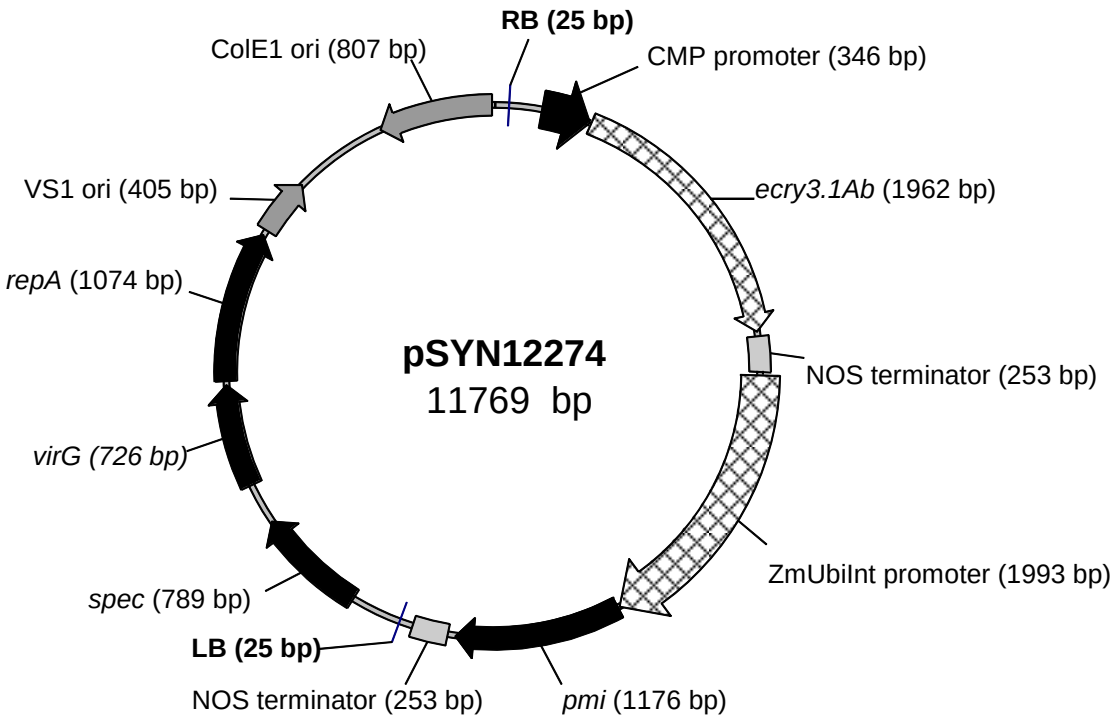


Figure 1. Plasmid map for vector pSYN12274

d) A full molecular characterisation of the genetic modification in the new organism, including:

i. Identification of all transferred genetic material and whether it has undergone any rearrangements;
ii. A determination of the number insertion sites, and the number of copies at each insertion site;
iii. Full DNA sequence data of each insertion event, including junction regions with the host DNA, sufficient to identify any substances expressed as a consequence of the inserted material, or where more appropriate, other information such as analysis of transcripts or expression products to identify any new substances that may be present in the food;
iv. A map depicting the organization of the inserted genetic material at each insertion site, and
v. The identification and characterization of any unexpected open reading frames within the inserted DNA or created by insertion with contiguous genomic DNA, including those that could result in fusion proteins or unexpected protein expression products.

Characterisation of the genetic modification is summarised below and covers data presented in the following reports:

- *Event 5307: Copy Number Functional Element Southern Blot Analysis, SSB-189-10 A1 – Appendix 1*
- *Event 5307 Maize: Insert Sequence Analysis, SSB-159-10 A1 Appendix 2*
- *Event 5307 Maize: Flanking Sequence Determination, SSB-160-10 Appendix 3*
- *Event 5307 Maize: Genetic Stability Analysis, SSb-184-10 A1 – Appendix 4*
- *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, SSB-187-10 – Appendix 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 Allergens, SSB-188-10 – Appendix 6*
- *Event 5307 Maize: Genomic Insertion Site Analysis, SSB-202-10 A1 – Appendix 7*

The Introduced DNA

Southern blot analyses demonstrate that 5307 corn (1) contains, at a single locus within the corn (*Z. mays*) genome, a single copy each of the gene *ecry3.1Ab*, its CMP promoter sequence, the marker gene *pmi* (also known as *manA*), its ZmUbiInt promoter sequence, and the two expected copies of the NOS polyadenylation sequence, one NOS polyadenylation sequence regulating *ecry3.1Ab* and one NOS polyadenylation sequence regulating *pmi*; (2) does not contain any extraneous deoxyribonucleic acid (DNA) fragments

of the functional elements inserted elsewhere in the corn genome; (3) and does not contain backbone sequence from the transformation plasmid, pSYN12274.

Nucleotide sequence analysis of the entire 5307 insert confirms that the insert is intact and that the organization of the functional elements within the insert is identical to their organization within pSYN12274. One nucleotide change compared to the sequence of pSYN12274 was identified 48 base pairs (bp) upstream of the CMP promoter in a non-coding region of the insert in 5307 corn. However, this nucleotide change had no effect on the functionality of the insert. Additionally, the analysis indicates that some truncation of the nucleotide sequence occurred at the 5' and 3' ends of the T-DNA during the transformation process that resulted in 5307 corn; such truncation occurs commonly in transformation via *Agrobacterium*. The entire right border and three bp of non-coding sequence at the 5' end of the insert, and eight bp of the left border were truncated; however, these deletions had no effect on the functionality of the insert.

Integration Site

Sequence analysis of the 5307 insertion site demonstrates that 33 bp of corn genomic sequence were deleted when the 5307 corn insert integrated into the corn genome. Basic Local Alignment Search Tool (BLAST) analyses comparing the corn genomic sequence flanking the 5307 insert to sequences in public databases indicate that the insert does not disrupt any known endogenous corn gene.

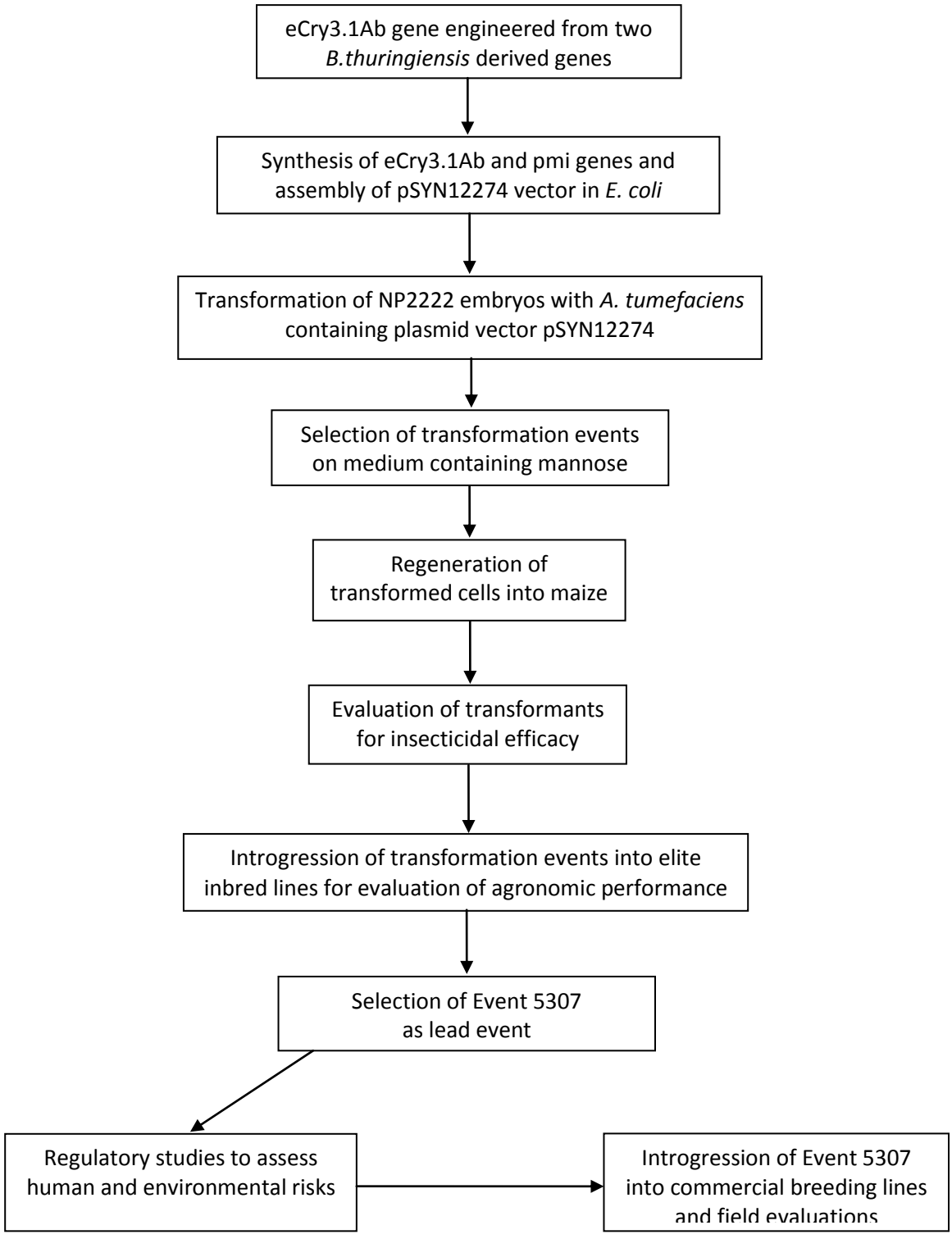
Translations of any putative open reading frame (ORF) spanning either the junction between corn genomic sequence and the 3' region of the 5307 corn insert or the junction between corn genomic sequence and the 5' region of the 5307 corn insert were screened for amino acid sequence similarity to known or putative allergens or toxins. Comparisons to the FARRP AllergenOnline database suggest that the amino acid translation of the identified putative ORF(s) shows no biologically relevant amino acid sequence similarity to any known or putative protein allergens. Additionally, the results of a comprehensive amino acid similarity search of the NCBI Entrez® Protein Database suggest that the amino acid translation of the identified putative ORF(s) shows no biologically relevant amino acid sequence similarity to any known or putative toxins. These data demonstrate that there are no deleterious changes in the 5307 corn genome as a result of the T-DNA insertion.

Genetic Stability

The *ecry3.1Ab* and *pmi* segregation ratios over several generations of 5307 corn plants are consistent with linkage of these transgenes at a single locus in the corn nuclear genome. These data and the results from Southern blot analyses of several generations of 5307 corn indicate that the transgenic locus is stably inherited during conventional breeding.

e) A description of how the line or strain from which food is derived was obtained from the original transformation (i.e. provide a family tree or describe the breeding process).

Progeny of the original transformants (T_0 plants) were field tested for resistance to insect feeding damage and for agronomic performance after introgression of the transgenes into multiple elite lines of corn. 5307 corn was selected as the lead commercial candidate and placed into regulatory trials. A schematic showing the steps in development of 5307 corn is shown in the diagram below:



Steps in the development of 5307 corn.

f) Evidence of the stability of the genetic changes, including:

i. The pattern of inheritance of the transferred gene(s) and the number of generations over which this has been monitored; and
ii. The pattern of inheritance and expression of the phenotype over several generations and where appropriate, across different environments.

Mendelian Inheritance Analysis

Event 5307 Maize: Mendelian Inheritance Analysis. SSB-203-10 A1 - Appendix 8.

Molecular analysis was performed to determine whether the 5307 corn insert integrated into a chromosome within the corn nuclear genome.

Individual plants from four generations were tested for the presence of *ecry3.1Ab* and *pmi* by real-time polymerase chain reaction (PCR) analysis. The results from the PCR analysis were used to determine the segregation ratios of *ecry3.1Ab* and *pmi.* Chi-squared analysis of the segregation data for three of the generations was performed to test the hypothesis that 5307 corn insert is inherited in a predictable manner, according to Mendelian principles and consistent with insertion into a chromosome within the corn nuclear genome.

Plants from one additional generation were grown from seed that was produced by crossing between plants from a nontransgenic corn line and plants that were hemizygous for *ecry3.1Ab* and *pmi.* The plants were all expected to be positive for *ecry3.1Ab* and *pmi*. Because the expected ratio of positive to negative plants is 1:0, Chi-square analysis could not be applied to the data for this generation. Still, the observed ratio and the expected ratio for this generation were identical. This suggested that the 5307 insert is inherited according to Mendelian principles.

Segregation data from four 5307 corn generations confirmed the expected segregation ratios for *ecry3.1Ab* and *pmi*, indicating that the 5307 corn insert is inherited according to Mendelian principles. This supports the conclusion that the 5307 corn insert integrated into a chromosome within the corn nuclear genome.

Multi-Generational Equivalence of eCry3.1Ab and PMI Protein Expression

Evaluation of Transgenic Protein Levels in Multiple Generations of Plants Derived from Transformation Event 5307 Maize - SSB-011-08 – Appendix 9.

The equivalence of eCry3.1Ab and PMI protein expression in 5307 corn from four generations of backcrosses (F1, NP2171 x BC5F3, BC6, and BC7) was determined. Plants derived from these generations were grown under standard greenhouse conditions. Plants were screened using TaqMan® PCR analysis to distinguish hemizygous plants containing the *ecry3.1Ab* and *pmi* (*manA*) transgenes from the negative segregant plants from each backcross generation.

Leaf, root, and pollen samples were collected at vegetative growth stage VT-R1 from five plants for each generation that were hemizygous for the transgenes, and the concentrations of eCry3.1Ab and PMI in these samples were measured by enzyme-linked immunosorbent assay (ELISA). Samples from a corresponding nontransgenic, near-isogenic corn plant were analysed to identify background effects of the plant extract on the ELISA.

The mean concentrations of eCry3.1Ab, measured in leaves from the four generations, without correcting for extraction efficiency, ranged from 83.40 to 93.67 µg/g dry weight (20.39 to 23.71 µg/g fresh weight.) After correcting for extraction efficiency, the mean concentrations of eCry3.1Ab in leaves ranged from 108.18 to 119.89 µg/g dry weight (26.45 to 30.76 µg/g fresh weight). The mean eCry3.1Ab concentrations (without correcting for extraction efficiency) measured in roots from the four generations ranged from 23.88 to 35.39 µg/g dry weight (2.58 to 3.79 µg/g fresh weight). After correcting for extraction efficiency, the mean concentrations of eCry3.1Ab in roots ranged from 31.99 to 47.40 µg/g dry weight (3.45 to 5.08 µg/g fresh weight). The eCry3.1Ab concentrations (without correcting for extraction efficiency) measured in pollen from the F1, NP2171 x BC5F3, and BC7 generations were below the limit of detection (0.08 µg/g dry weight). The eCry3.1Ab concentrations measured in pollen from the BC6 generation ranged from below the limit of detection (0.08 ug/g dry weight) to 0.15 µg/g dry weight (without correcting for extraction efficiency). Tables 1 and 2 below provide the uncorrected values, the extraction efficiencies and the values corrected for extraction efficiency for leaf and root tissue, respectively.

The mean concentrations of PMI measured in leaves from the F1, NP2171 x BC5F3, BC6, and BC7 generations, without correcting for extraction efficiency, ranged from 1.77 to 1.95 µg/g dry weight (0.44 to 0.48 µg/g fresh weight). The mean PMI concentrations measured in roots from the F1, NP2171 x BC5F3, BC6, and BC7 generations, without correcting for extraction efficiency, ranged from 1.05 to 1.19 µg/g dry weight (0.12 to 0.13 µg/g fresh weight). The mean PMI concentrations measured in pollen from the F1, NP2171 x BC5F3, BC6, and BC7 generations ranged from 18.96 to 25.58 µg/g dry weight (18.46 to 25.18 µg/g fresh weight).

The concentrations of eCry3.1Ab and PMI measured across four generations of 5307 corn at VT-R1 were comparable, indicating consistency of expression of these proteins across multiple generations.

Table 1. Expression Levels of eCry3.1Ab in four generations of 5307 corn: Leaf tissue (DW: dry weight; FW: fresh weight; %EE: extraction efficiency).

Generation	µg/g DW	%EE	Corrected µg/g DW	Average µg/g DW	µg/g FW	%EE	Corrected µg/g FW	Average µg/g FW
F1	101.72	77.09%	131.95		25.33	77.09%	32.86	
	82.1	77.09%	106.50		20.21	77.09%	26.22	
	91.29	77.09%	118.42		22.42	77.09%	29.08	
	95.06	77.09%	123.31		22.59	77.09%	29.30	
	75.17	77.09%	97.51	115.54	18.04	77.09%	23.40	**28.17**
BC5F3 x NP2171	79.27	77.09%	102.83		19.56	77.09%	25.37	
	82.81	77.09%	107.42		20.20	77.09%	26.20	
	81.35	77.09%	105.53		18.81	77.09%	24.40	
	96.54	77.09%	125.23		24.28	77.09%	31.50	
	77.02	77.09%	99.91	108.18	19.09	77.09%	24.76	**26.45**
BC6	77.45	77.09%	100.47		19.46	77.09%	25.24	
	105.52	77.09%	136.88		26.83	77.09%	34.80	
	105.8	77.09%	137.24		25.36	77.09%	32.90	
	81.91	77.09%	106.25		20.35	77.09%	26.40	
	97.69	77.09%	126.72	121.51	23.79	77.09%	30.86	**30.04**
BC7	78.24	77.09%	101.49		19.69	77.09%	25.54	
	79.01	77.09%	102.49		20.51	77.09%	26.61	
	113.06	77.09%	146.66		29.41	77.09%	38.15	
	100.32	77.09%	130.13		26.37	77.09%	34.21	
	91.48	77.09%	118.67	119.89	22.58	77.09%	29.29	**30.76**

Table 2. Expression Levels of eCry3.1Ab in four generations of 5307 corn: Root tissue (DW: dry weight; FW: fresh weight; %EE: extraction efficiency).

Generation	µg/g DW	%EE	Corrected µg/g DW	Average µg/g DW	µg/g FW	%EE	Corrected µg/g FW	Average µg/g FW
F1	25.13	74.66%	33.66		3.17	74.66%	4.25	
	26.16	74.66%	35.04		2.68	74.66%	3.59	
	24.63	74.66%	32.99		2.36	74.66%	3.16	
	29.12	74.66%	39.00		2.97	74.66%	3.98	
	24.42	74.66%	32.71	34.68	2.92	74.66%	3.91	**3.78**
BC5F3 x NP2171	33.29	74.66%	44.59		3.35	74.66%	4.49	
	36.72	74.66%	49.18		4.10	74.66%	5.49	
	41.21	74.66%	55.20		3.60	74.66%	4.82	
	31.38	74.66%	42.03		4.12	74.66%	5.52	
	34.33	74.66%	45.98	47.40	3.78	74.66%	5.06	**5.08**
BC6	25.43	74.66%	34.06		2.87	74.66%	3.84	
	23.12	74.66%	30.97		2.93	74.66%	3.92	
	27.71	74.66%	37.11		3.47	74.66%	4.65	
	21.92	74.66%	29.36		2.28	74.66%	3.05	
	23.96	74.66%	32.09	32.72	2.62	74.66%	3.51	**3.80**
BC7	22.04	74.66%	29.52		2.30	74.66%	3.08	
	21.5	74.66%	28.80		1.81	74.66%	2.42	
	25.16	74.66%	33.70		2.99	74.66%	4.00	
	26.51	74.66%	35.51		3.12	74.66%	4.18	
	24.2	74.66%	32.41	31.99	2.66	74.66%	3.56	**3.45**

4. Information on the labelling of the GM food

This part includes both of the following:

a) Information on whether novel DNA or protein is likely to be present in final food.

Quantitation of eCry3.1Ab and Phosphomannose Isomerase in Key Processed Fractions Prepared from Event 5307 Maize Grain – SSB-004-10 – Appendix 10.

The concentrations of eCry3.1Ab and phosphomannose isomerase (PMI) proteins in various wet- and dry-milled fractions produced from corn grain derived from transformation 5307 corn was determined. Using laboratory scale milling methodology, corn grain from 5307 corn and from nontransgenic, near-isogenic corn grain were processed following wet-mill and dry-mill procedures. Both the 5307 test grain and the control, nontransgenic grain were processed into food and feed fractions at the Food Protein Research and Development Center, Texas A&M University (Bryan, Texas) using standard wet- and dry-milling processes. Figures 2 and 3 represent the processing flowchart.

Figure 2. Wet-milling flowchart

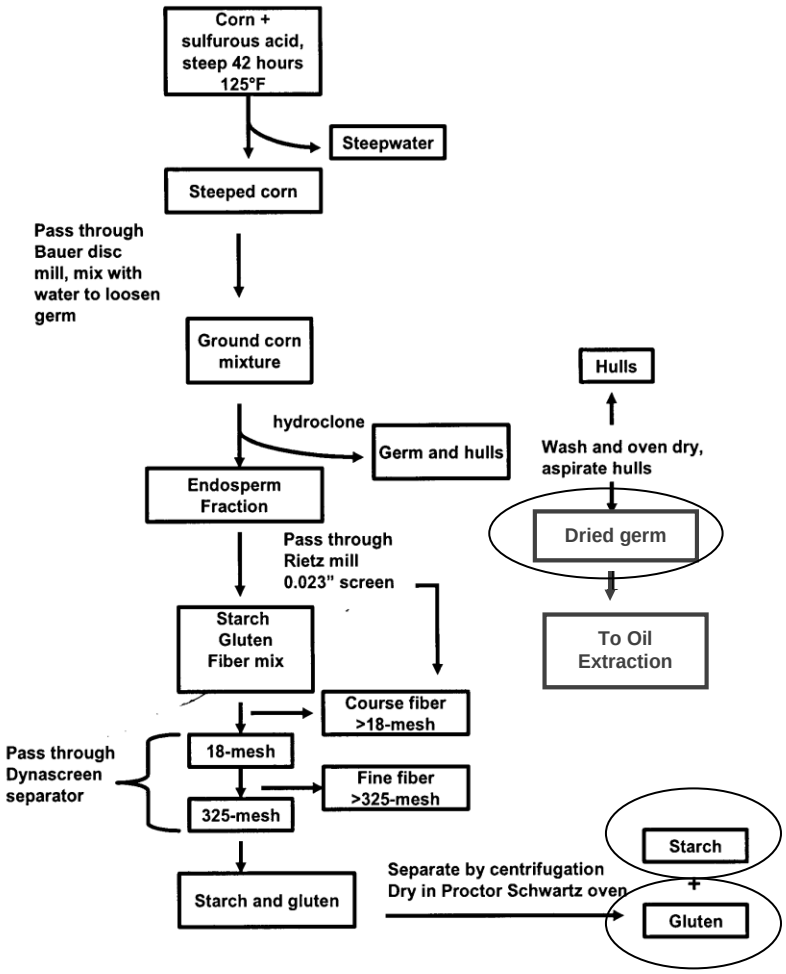
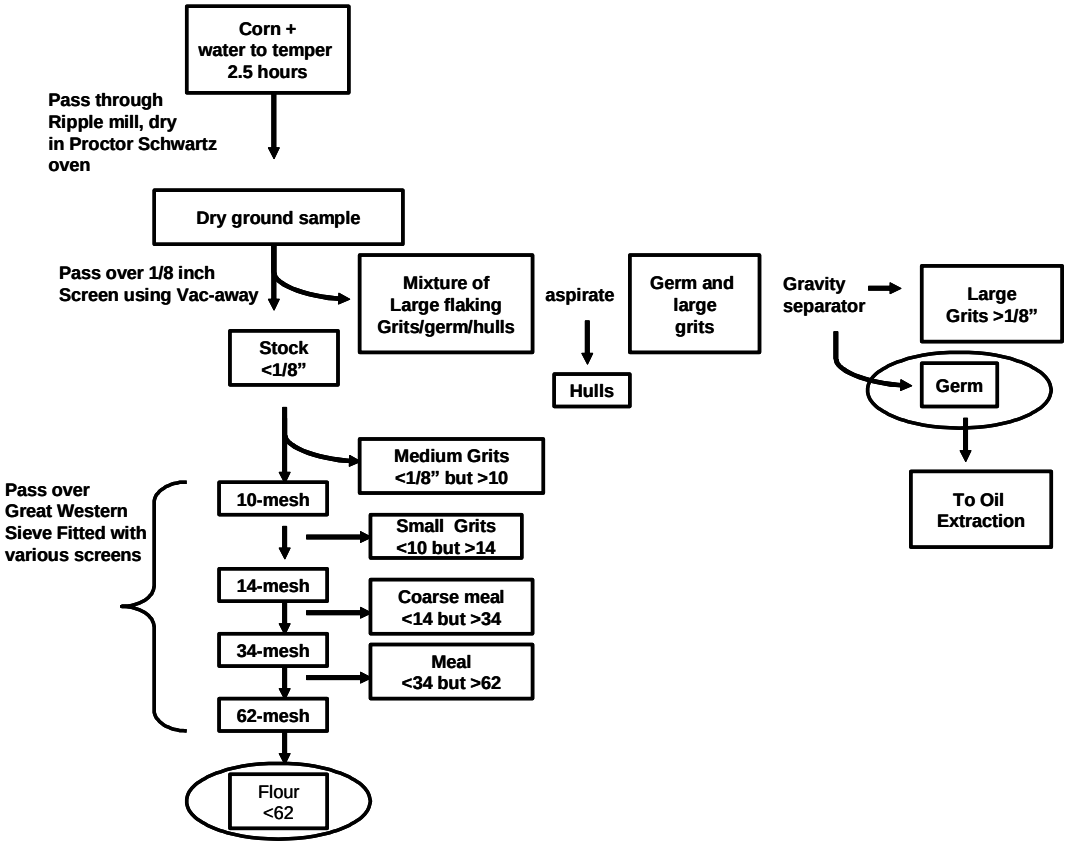


Figure 3. Dry-milling flowchart



The fractions most likely to enter the food and feed chain are circled in Figures 2 and 3. These end products of the wet- and dry-milling processes were selected for quantitative analysis; Table 1 below lists the fractions.

Table 1. Key processed fractions produced

Wet-mill processing	Dry-mill processing
Dried germ Starch Gluten	Flour Germ

Protein extractions were performed on three representative aliquots of each fraction. For each sample, the insoluble material was subjected to iterative extractions. The extracts were analysed by ELISA to quantify the amount of eCry3.1Ab and PMI in each sample. The amount of eCry3.1Ab and PMI protein in each sample was reported as the sum of the recovery from all quantifiable extractions. Table 2 presents the concentrations of eCry3.1Ab and PMI in 5307 grain, and key processed fractions derived from 5307 grain.

Table 2. Concentrations of eCry3.1Ab and PMI in 5307 grain, and key processed fractions derived from 5307 grain

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

n = 3 replicate analyses for each sample
[a] LOD = 0.048 µg/g sample
[b] LOD = 0.048 µg/g sample
[c] LOD = 0.024 µg/g sample
[d] LOD = 0.003 µg/g sample
[e] LOD = 0.002 µg/g sample
[f] LOD = 0.003 µg/g sample

Analysis of nontransgenic sample extracts confirmed the absence of matrix effects for extracts of each sample type.

The concentrations of eCry3.1Ab and PMI are higher in the dry-mill corn germ than in the grain; this is consistent with the distribution of total protein within the kernel. Corn germ, which only accounts for 10% of the total dry weight of mature kernel, is 18% protein whereas the intact kernel is 8% to 10% protein. More protein is extracted during dry-milling than wet-milling, and is likely to account for the differences in concentrations of eCry3.1Ab and PMI proteins in the dry-mill germ compared to the wet-mill germ.

The results of this study indicate that eCry3.1Ab and PMI proteins were present at quantifiable levels in flour and germ when produced by standard dry-mill processing of 5307 grain. In contrast, the eCry3.1Ab and PMI proteins were below the limit of detection in the dried germ, starch, and gluten when produced by standard wet-mill processing of 5307 grain.

b) Detection methodology for the GM food suitable for analytical purposes.

An event specific polymerase chain reaction (PCR) method was developed to detect 5307 deoxribonucleic acid (DNA) extracted from grain and seed samples. The method and validation are provided as Appendix 11.

C. Information related to the safety of the genetically-modified food

1. Information on antibiotic resistance marker genes (if used)

Not relevant. No antibiotic resistance marker genes were used in the development of 5307 corn. The *pmi* gene obtained from *Escherichia coli* strain K-12 and the protein it encodes were utilised as the plant selectable marker in 5307 corn.

2. The characterisation of novel proteins or other novel substances

This part includes all of the following;

a) A full description of the biochemical function and phenotypic effects of all novel substances (e.g. a protein or an untranslated RNA) that could potentially be expressed in the new GM organism, including those resulting from the transfer of marker genes.

5307 corn produces the eCry3.1Ab and PMI proteins. The eCry3.1Ab protein is a chimeric protein representing portions of modified Cry3A (mCry3A) protein and Cry1Ab protein, which are derived from *Bacillus thuringiensis*. The 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. Vigifera zeae* Krysan and Smith). These important coleopteran pests cause significant damage to U.S. corn crops annually. The *pmi* gene, also known as *manA*, is derived from *Eschericha coli* strain K-12 and encodes the PMI protein, which was utilised as a selectable marker during the development of 5307 corn. GM corn 5307 in breeding stack combinations with other insect-resistant corn products will allow growers to have optimal broad-spectrum insect control. No intended compositional changes are expected as a result of the genetic modification in 5307 corn. 5307 corn is as safe and nutritious as food and feed derived from conventional corn varieties.

b) The identification of any other novel substances, (e.g. metabolites) that might accumulate on or in the GM organism as a result of the genetic modification, and their levels and site of accumulation.

No other novel substances were identified as a result of the genetic modification.

c) Data on the site of expression of all novel substance, particularly whether they are likely to be present in the edible portions of the organism, and levels of expression.

Data on the site expression of protein was described under in section 3(f) of Part B above. And is presented in the following report, *Quantification of eCry3.1Ab and phosphomannose*

isomerase in corn tissues derived from transformation Event 5307. SSB-016-09 A2 – Appendix 12.

This study measured the concentrations of the proteins eCry3.1Ab and PMI in several tissues of corn plants derived from transformation Event 5307, grown in four different different locations in 2008. The concentrations of eCry3.1Ab and PMI were measured by ELISA in various plant tissues from a 5307 corn hybrid sampled at four developmental stages: whorl, anthesis, maturity and senescence. As controls, plants of a nontrangenic, near-isogenic hybrid were also grown, and samples were collected and analysed concurrently with the samples of the 5307 corn hybrid.

The corn plants used in the study were grown according to local agronomic practices. At each location, ten plants of 5307 hybrid were collected at each of the four growth stages. Five of the ten plants were retained as whole-plant samples; leaves and roots were sampled from the remaining five plants. At maturity and senescenes stages, kernel samples were also collected along with the leaf and root samples. Pollen samples were analysed by ELISA to measure the concentrations of eCry3.1Ab and PMI in the 5307 corn tissues. The tissues of the nontransgenic corn hybrid were also analysed concurrently with those of the 5307 corn hybrid to identify matrix effects on the ELISAs.

The concentrations of eCry3.1Ab across all locations and plant stages on a fresh-weight basis ranged from <LOQ to 71.21 µg/g in leaves and from 0.40 µg/g to 9.29 µg/g in roots. Across all locations and plant stages, the concentrations of eCry3.1Ab ranged from 1.70 µg/g to 28.64 µg/g in whole plants and 1.60 µg/g to 7.29 µg/g in kernals on a fresh-weight basis. Concentrations of eCry3.1Ab in pollen samples across locations ranged from <LOQ to 0.09 µg/g on a fresh weight basis.

The concentrations of PMI across all locations and plant stages on a fresh-weight basis ranged from <LOD to 1.66 µg/g in leaves, and from <LOQ to 1.07 µg/g in roots. Across locations, the concentrations of PMI ranged from 0.15 µg/g to 2.13 µg/g in whole plants, and from 0.50 µg/g to 2.38 µg/g in kernals on a fresh weight basis. Concentrations of PMI across locations in pollen samples ranged from 5.16 µg/g to 6.06 µg/g on a fresh-weight basis.

The concentrations of eCry3.1Ab and PMI measured in this study represent the levels of these proteins in 5307 corn in various tissue types at four developmental stages across four different locations.

Concentrations of eCry3.1Ab were either detectable or quantifiable in all tissues analysed. Concentrations of PMI were either detectable or quantifiable in all tissues analysed except for some leaf tissue samples at senescence stage.

d) Information on whether any newly expressed protein has undergone any unexpected post-translational modification in the new host.

Comparison of eCry3.1Ab Protein Produced in Event 5307-Derived Maize Plants and eCry3.1Ab Protein Produced in Recombinant Escherchia coli, SSB-002-09 – Appendix 13.

Characterization of Test Substance ECRY3.1AB-028 and certificate of Analysis, SSB-010-08 – Appendix 14.

There was no evidence of post-translational glycosylation of eCry 3.1Ab purified from an extract of leaf material from 5307 corn or from the microbially-produced test substance.

e) Evidence of non-expression of a gene, in the case where a transferred gene is not expected to express any novel substances (e.g. because it has a 'silencing' role or is in a non-functional form).

There is no evidence of transfer of a gene in a non-functional form.

f) Information about prior history of human consumption of the novel substances, if any, or their similarity to substances previously consumed in food.

History of Safe Use of Cry3A, mCry3A, Cry1Ab

There has been no prior human consumption of the eCry3.1Ab protein. Syngenta engineered eCry3.1Ab; it is a chimera of mCry3A and Cry1Ab. There has been dietary exposure to these related proteins.

The native Cry3A protein has had a long history of safe use as a component of spore preparations of *B.t.* subsp*. tenebrionis*-based insecticides, as an encapsulated component of a microbial insecticide derived from *B.t*. subsp. *san diego*, and as an insect control protein in NewLeaf Bt potatoes. An extensive assessment of the human and environmental safety of Cry3A as expressed in *B.t.* potato was completed by the U.S. EPA in 2001 and no safety concerns were identified.

The safety of mCry3A in Syngenta's MIR604 corn has also been evaluated previously. An extensive reassessment of the human and environmental safety of mCry3A as expressed in MIR604 plants was completed by the U.S. EPA in 2010 and FSANZ in 2006 (A564) and no safety concerns were identified. Studies supporting the safety of mCry3A in MIR604 corn showed that this protein is nontoxic to vertebrate species. A permanent exemption from U.S. food and feed tolerances for the mCry3A protein was granted in 2006.

The full-length c*ry1Ab* gene can be derived from the bacterium *B.t.* subsp. *kurstaki* HD-1. The corresponding full-length protein is 1155 amino acids in length. The Cry1Ab proteins as introduced in Syngenta's Bt11 corn and Syngenta's 176 corn are truncated proteins of 615

and 648 amino acids in length, respectively. An extensive reassessment of the human safety of Cry1Ab as expressed in Bt11 corn was completed by the U.S. EPA in 2010 and no safety concerns were identified. The first permanent exemption for Cry1Ab food and feed tolerances was granted by the US EPA in 1995, in connection with the safety assessment and approval of Bt176 corn (now a discontinued product). Studies supporting the safety of Cry1Ab in transgenic corn have repeatedly demonstrated that the protein is nontoxic to vertebrates. Bt11 and Bt176 corn were evaluated and approved by FSANZ in 2001.

There are many reports available showing that *B.t.* δ-endotoxins do not have adverse toxic effects on mammalian or avian species, or freshwater fish. Both the long history of safe use of *B.t.*-based products and the acute oral toxicity data that exists on Cry3A, mCry3A and Cry1Ab verify that these and other δ-endotoxins pose negligible toxicity risk to humans.

History of Safe Use and Exposure to PMI

Data and information are available to support a history of safe use of PMI proteins. This protein can be currently found in commercially available products including Syngenta's MIR162 corn approved by FSANZ in 2009 as application A1001 and MIR604 corn approved by FSANZ in 2006 as application A564. It is also conceivable, and indeed likely, that small amounts of PMI proteins from various sources have always been present in the food and feed supply due to the ubiquitous occurrence of PMI proteins in nature, including food plants and animals. PMI proteins have been found in such diverse plant species as tobacco, walnut, and *Brassica* species, as well as in seeds of soybeans and other legumes. Genes encoding putative PMI proteins have been purified and characterised from many other organisms, including bacteria, yeast, rats, pigs, and humans and have been demonstrated to be essential for many organisms, including humans.

3. The potential toxicity of novel proteins or other novel substances

This part includes all of the following:

a) A bioinformatic comparison of the amino acid sequence of each of the novel proteins to known protein toxins and anti-nutrients (e.g. protease inhibitors, lectins).

eCry3.1Ab

eCry3.1Ab (Entrez® Database Accession Number ADC30135): Assessment of Amino Acid Sequence Similarity to Known or Putative Toxins, SSB-165-11 – Appendix 15

To determine whether the eCry3.1Ab protein had any significant amino acid sequence homology with proteins identified as toxins, the protein sequence (Entrez Accession No. ADC30135) was systematically compared to the latest posting of the National Center for Biotechnology Information (NCBI, 2011) Entrez Protein Database. The Basic Local Alignment Search Tool for Proteins program (BLASTP) was used to search the NCBI database. This process

identified (1) whether any proteins in the database showed significant similarity to the eCry3.1Ab amino acid sequence (*i.e*., alignments with BLASTP Expectation values [*E* -values] below an established threshold), indicating that the amino acid sequence might be closely related to the eCry3.1Ab amino acid sequence, and (2) whether any proteins showing sequence similarity to the eCry3.1Ab amino acid sequence were known or putative toxins.

The NCBI Entrez® Protein Database search identified 550 sequences with significant similarity to the eCry3.1Ab amino acid sequence (i.e., E-values less than 0.1). None of the 550 alignments described in the analysis are known toxins to humans or mammals.

Of the 550 alignments, there were 505 proteins from 15 species identified as known or putative delta-endotoxin proteins (also known as Cry proteins or insecticidal crystal proteins); included in this count were alignments to synthetic Cry protein constructs. The *E*-values for alignments between these sequences and the eCry3.1Ab amino acid sequence ranged from 0 to 0.0355.

Additional alignments to 26 proteins from 6 species were identified as hypothetical or unknown proteins of unspecified function. The E-values for alignments between these sequences and the eCry3.1Ab amino acid sequence ranged from 2.8×10^{-15} to 0.0605. None of these proteins are known or putative toxins.

Additinal alignments to 16 parasporin proteins from *Bacillus thuringiensis* were identified. The term parasporin is defined as Bacillus *thuringiensis* and related bacterial parasporal proteins. There are no published reports of *in vivo* parasporin toxicity to humans or mammals. The *E*-values for alignments between these sequences and the eCry3.1Ab amino acid sequence ranged from 1.1×10^{-54} to 5.1×10^{-9}.

Additional alignments to 2 proteins from 2 species were identified as twin arginine translocation pathway signal proteins. The *E*-values for alignments between these sequences and the eCry3.1Ab amino acid sequence ranged from 0.002 to 0.0464. Neither of these proteins are known or putative toxins.

An additional single alignment to a protein from *Bacillus thuringiensis* was identified as fructose-6- phosphate amidotransferase; the amidotransferase protein aligns with the eCry3.1Ab sequence in a region that is described as a delta endotoxin domain. The *E*-value for the alignment between this sequence and the eCry3.1Ab sequence was 1.12×10^{-72}.

The results of a comprehensive amino acid similarity search of the NCBI Entrez® Protein Database support the conclusion that the eCry3.1Ab amino acid sequence shows no significant similarity with any known or putative toxins other than known or putative delta-endotoxin proteins (also known as Cry proteins or insecticidal crystal proteins).

PMI

PMI (Entrez® Database Accession Number AAA24109): Assessment of Amino Acid Sequence Similarity to Known or Putative Toxins, SSB-145-11 – Appendix 16.

Amino acid sequence comparisons between novel proteins and known protein toxins and allergens are part of the weight-of-evidence approach to assessing potential mammalian toxicity and allergenicity. For example, in combination with other supporting data, the presence of significant homologies with known allergens could indicate that the novel protein might elicit an allergic cross-reaction in sensitized individuals.

To determine whether the PMI protein had any significant amino acid sequence homology with proteins identified as toxins, the protein sequence (Entrez Accession No. AAA24109) was systematically compared to the latest posting of the National Center for Biotechnology Information (NCBI, 2011) Entrez Protein Database. The Basic Local Alignment Search Tool for Proteins program (BLASTP) was used to search the NCBI database. This process identified (1) whether any proteins in the database showed significant similarity to the PMI amino acid sequence (*i.e*., alignments with BLASTP Expectation values [*E* -values] below an established threshold), indicating that the amino acid sequence might be closely related to the PMI amino acid sequence, and (2) whether any proteins showing sequence similarity to the PMI amino acid sequence were known or putative toxins.

The NCBI Entrez Protein Database search identified 1384 sequences with significant similarity to the PMI amino acid sequence (*i.e*., *E*-values less than 1.3).

Of the 1384 sequences, 1208 alignments to proteins from 587 species were identified as known or putative phosphomannose isomerase enzymes or proteins involved in carbohydrate/sugar metabolism or transport. The *E*-values for alignments between these sequences and the PMI amino acid sequence ranged from 0 to 0.99.

An additional alignment was to ADP-L-glycero-D-manno-heptose-6-epimerase (AGME) from *Asperigillus terreus*. AGME is responsible for the synthesis of heptose, which is not present in mammalian cells. AGME is not a known toxin and the product of AGME bioactivity, heptose, is not a known toxin. The *E*-value for this alignment was 4.8×10^{-11}.

An additional 175 alignments to proteins from 128 species were identified as hypothetical or unknown proteins of unspecified function. The *E*-values for alignments between these sequences and the PMI amino acid sequence ranged from $2.6 \times ^{10\text{-}73}$ to 0.99. None of the proteins were identified as known or putative toxins.

The results of a comprehensive amino acid similarity search of the NCBI Entrez® Protein Database support the conclusion that the PMI amino acid sequence shows no significant similarity with any known or putative toxins.

b) Information on the stability to heat or processing and/or to degradation in appropriate gastric and intestinal model systems.

eCry3.1Ab

Digestibility of eCry3.1Ab in SGF

In vitro Digestibility of eCry3.1Ab Protein under Simulated Mammalian Gastric Conditions (Syngenta Study No. TK0028111) – Appendix 17.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot and densitometry analyses were used to evaluate the *in vitro* digestibility of eCry3.1Ab in simulated mammalian gastric fluid (SGF) over a 15 minute time course at 37°C.

The eCry3.1Ab protein degraded rapidly upon exposure to the pepsin enzyme in SGF. Intact eCry3.1Ab (molecular weight 74.8 kDa) was readily digested in less than 30 seconds, as assessed by SDS-PAGE analysis. Further analysis of the eCry3.1Ab protein bands in the SDS-PAGE gel by densitometry revealed that 3% and 0% of the eCry3.1Ab remained after incubation in SGF for 15 and 30 seconds, respectively. Following incubation in SGF for 30 seconds, no intact eCry3.1Ab or eCry3.1Ab derived fragments were observed by Western blot analysis; supporting the conclusion that eCry3.1Ab is readily digested by the mammalian gastric enzyme, pepsin.

Digestibility of eCry3.1Ab in SIF

In vitro Digestibility of eCry3.1Ab Protein as Contained in Test Substance ECRY3.1AB-0208 Under Simulated Mammalian Intestinal Conditions (SSB-015-09 A1) – Appendix 18

The susceptibility of eCry3.1Ab to proteolytic degradation in simulated mammalian intestinal fluid (SIF) containing pancreatin was evaluated using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. The intact eCry3.1Ab protein (molecular weight 74.8 kDa) from recombinant *Escherichia coli* was rapidly degraded in SIF. Following exposure of eCry3.1Ab protein to SIF for 1 minute, no intact eCry3.1Ab (74.8 kDa) was evident, as assessed by SDS-PAGE and Western blot analysis (Figures 1 and 2, Lane 9). However, a protein band corresponding to a molecular weight of approximately 56 kDa appeared in the 1 minute sample; it remained stable over the 48 hour sample time course as shown by the Western blot analysis (Figure 2, Lanes 9 - 19). A band corresponding to a molecular weight of approximately 62 kDa also appeared in the 1 minute sample (Figure 2, Lane 9); it diminished in intensity over the time course and disappeared after 30 minutes. Because these bands cross-reacted with the antibodies capable of detecting eCry3.1Ab, they were identified to be degradation products of eCry3.1Ab protein. An additional immunoreactive band corresponding to a molecular weight of approximately 40 kDa was observed in the 1 hour sample and increased in intensity over the remaining time course (Figure 2, Lanes 14 - 19). This band most

likely corresponded to a breakdown product of the eCry3.1Ab protein. The SIF with pancreatin contains proteins that co-migrate with eCry3.1Ab degradation products so it is difficult to follow the breakdown of eCry3.1Ab on the SDS-PAGE gel. The Western blot more clearly shows the degradation of eCry3.1Ab.

An immunoreactive protein band with a molecular weight of approximately 6 kDa was visible starting in the 1 minute sample (Figure 2, Lane 9). This band diminished in intensity over the time course and disappeared after 1 hour (Figure 2, Lanes 9 – 14). Another immunoreactive band with a molecular weight of approximately 5 kDa appeared after 5 minutes (Figure 2, Lane 11) and remained visible over the remaining time course (Figure 2, Lanes 12 – 19). These two bands most likely represent breakdown fragments of the eCry3.1Ab protein.

Faint protein bands were noted on the Western blot with molecular weights higher than the band representing intact eCry3.1ab (i.e., higher than 74.8 kDa). These bands likely represented dimer and trimer forms of eCry3.1Ab, and were consistent with the corresponding molecular weights of two or three eCry3.1Ab polypeptides, respectively, at approximately 150 kDa (Figures 1 and 2, Lanes 4, 5, 6 and 8) and 224 kDa (Figure 2, Lanes 4 - 6). The limit of detection (LOD) for SDS-PAGE was determined to be 8 ng eCry3.1Ab. The LOD for Western blot analysis was determined to be 0.25 ng eCry3.1Ab.

The results of this study indicate that intact eCry3.1Ab produced in recombinant *E. coli* is readily degraded under simulated mammalian intestinal conditions. The eCry3.1Ab protein was degraded so rapidly that no intact eCry3.1Ab was detected upon sampling of the reaction mixture at 1 minute. However, immunoreactive fragments of eCry3.1Ab protein (molecular weights ca. 56, 40 and 5 kDa) were detected over the 48-hour time course of the study. Based on these data, it is evident that the eCry3.1Ab protein is sensitive to proteolysis by pancreatin and is rapidly degraded to constituent peptides.

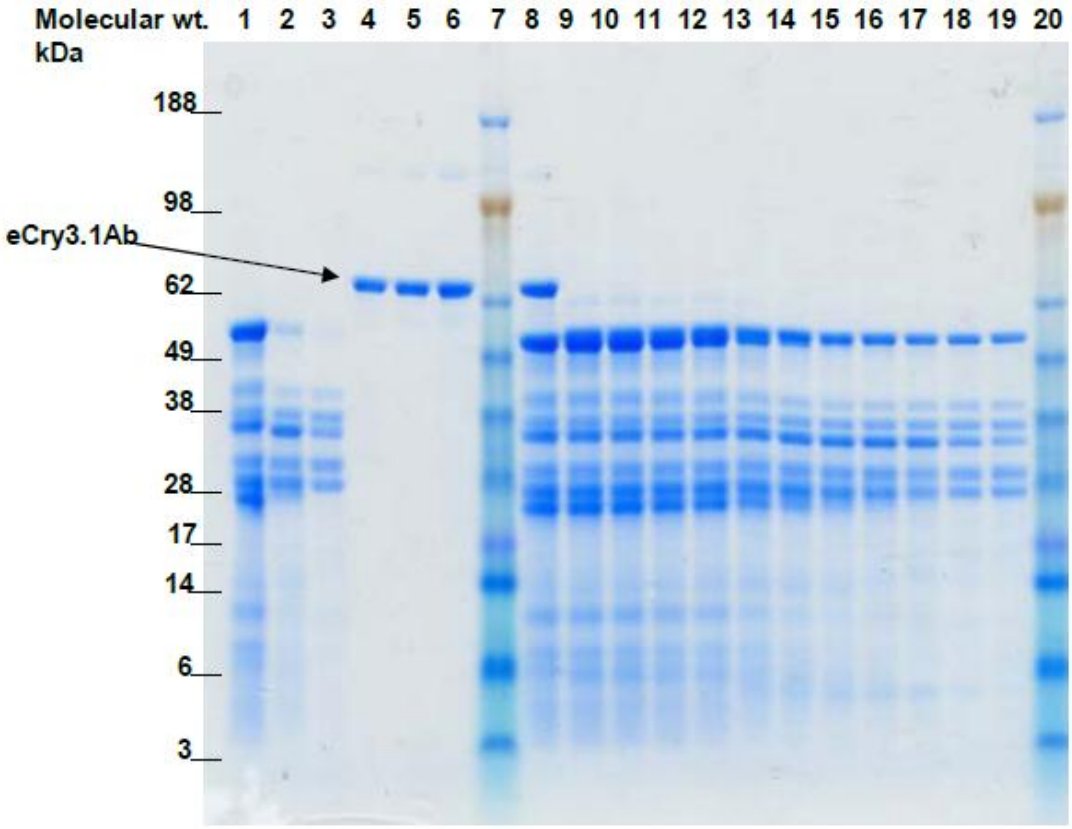


Figure 1. SDS-PAGE analysis of eCry3.1Ab following digestion in SIF.

Lane 1: SIF only control – time point 0 hours
Lane 2: SIF only control – time point 2 hours
Lane 3: SIF only control – time point 48 hours
Lane 4: eCry3.1Ab incubated in SIF without pancreatin – time point 0 hours
Lane 5: eCry3.1Ab incubated in SIF without pancreatin – time point 2 hours
Lane 6: eCry3.1Ab incubated in SIF without pancreatin – time point 48 hours
Lane 7: molecular weight standard
Lane 8: eCry3.1Ab incubated in SIF (with pancreatin) for 0 minutes
Lane 9: eCry3.1Ab incubated in SIF (with pancreatin) for 1 minutes
Lane 10: eCry3.1Ab incubated in SIF (with pancreatin) for 2 minutes
Lane 11: eCry3.1Ab incubated in SIF (with pancreatin) for 5 minutes
Lane 12: eCry3.1Ab incubated in SIF (with pancreatin) for 10 minutes
Lane 13: eCry3.1Ab incubated in SIF (with pancreatin) for 30 minutes
Lane 14: eCry3.1Ab incubated in SIF (with pancreatin) for 60 minutes
Lane 15: eCry3.1Ab incubated in SIF (with pancreatin) for 2 hours
Lane 16: eCry3.1Ab incubated in SIF (with pancreatin) for 3 hours
Lane 17: eCry3.1Ab incubated in SIF (with pancreatin) for 6 hours
Lane 18: eCry3.1Ab incubated in SIF (with pancreatin) for 24 hours
Lane 19: eCry3.1Ab incubated in SIF (with pancreatin) for 48 hours
Lane 20: molecular weight standard

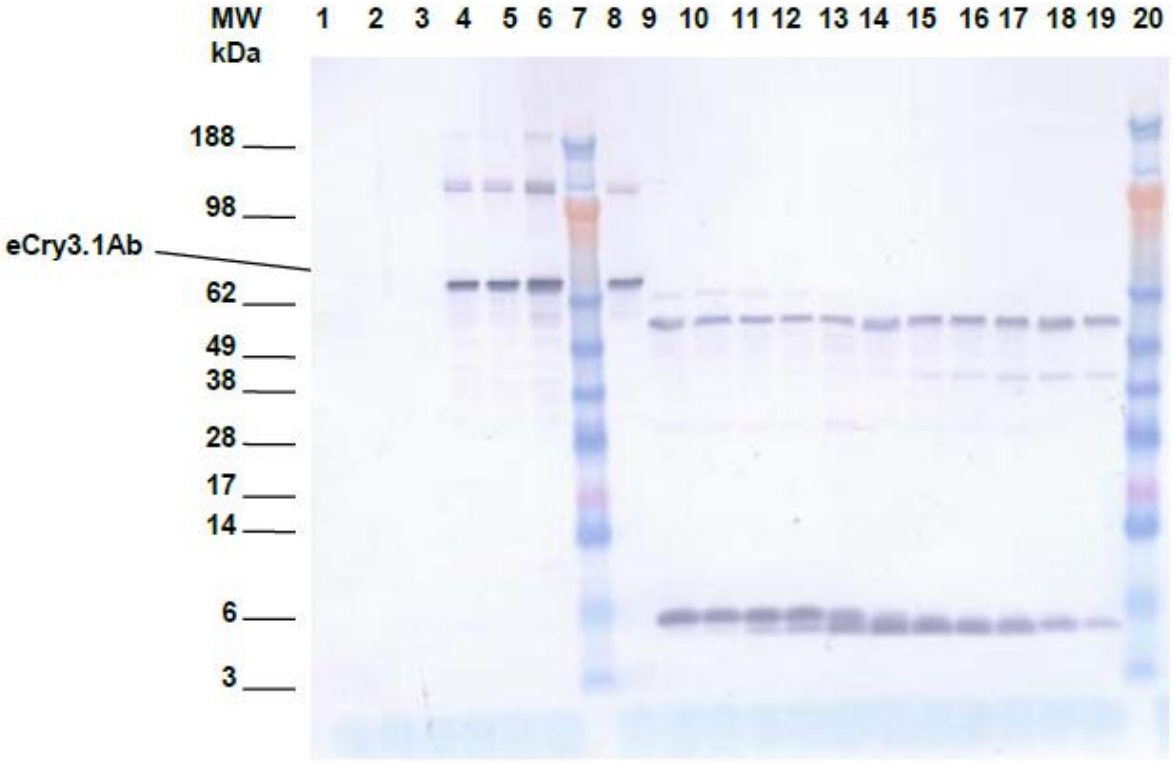


Figure 2. Western blot analysis of eCry3.1Ab following digestion in SIF.

Lane 1: SIF only control – time point 0 hours
Lane 2: SIF only control – time point 2 hours
Lane 3: SIF only control – time point 48 hours
Lane 4: eCry3.1Ab incubated in SIF without pancreatin – time point 0 hours
Lane 5: eCry3.1Ab incubated in SIF without pancreatin – time point 2 hours
Lane 6: eCry3.1Ab incubated in SIF without pancreatin – time point 48 hours
Lane 7: molecular weight standard
Lane 8: eCry3.1Ab incubated in SIF (with pancreatin) for 0 minutes
Lane 9: eCry3.1Ab incubated in SIF (with pancreatin) for 1 minutes
Lane 10: eCry3.1Ab incubated in SIF (with pancreatin) for 2 minutes
Lane 11: eCry3.1Ab incubated in SIF (with pancreatin) for 5 minutes
Lane 12: eCry3.1Ab incubated in SIF (with pancreatin) for 10 minutes
Lane 13: eCry3.1Ab incubated in SIF (with pancreatin) for 30 minutes
Lane 14: eCry3.1Ab incubated in SIF (with pancreatin) for 60 minutes
Lane 15: eCry3.1Ab incubated in SIF (with pancreatin) for 2 hours
Lane 16: eCry3.1Ab incubated in SIF (with pancreatin) for 3 hours
Lane 17: eCry3.1Ab incubated in SIF (with pancreatin) for 6 hours
Lane 18: eCry3.1Ab incubated in SIF (with pancreatin) for 24 hours
Lane 19: eCry3.1Ab incubated in SIF (with pancreatin) for 48 hours
Lane 20: molecular weight standard

PMI

Digestibility of PMI in SGF

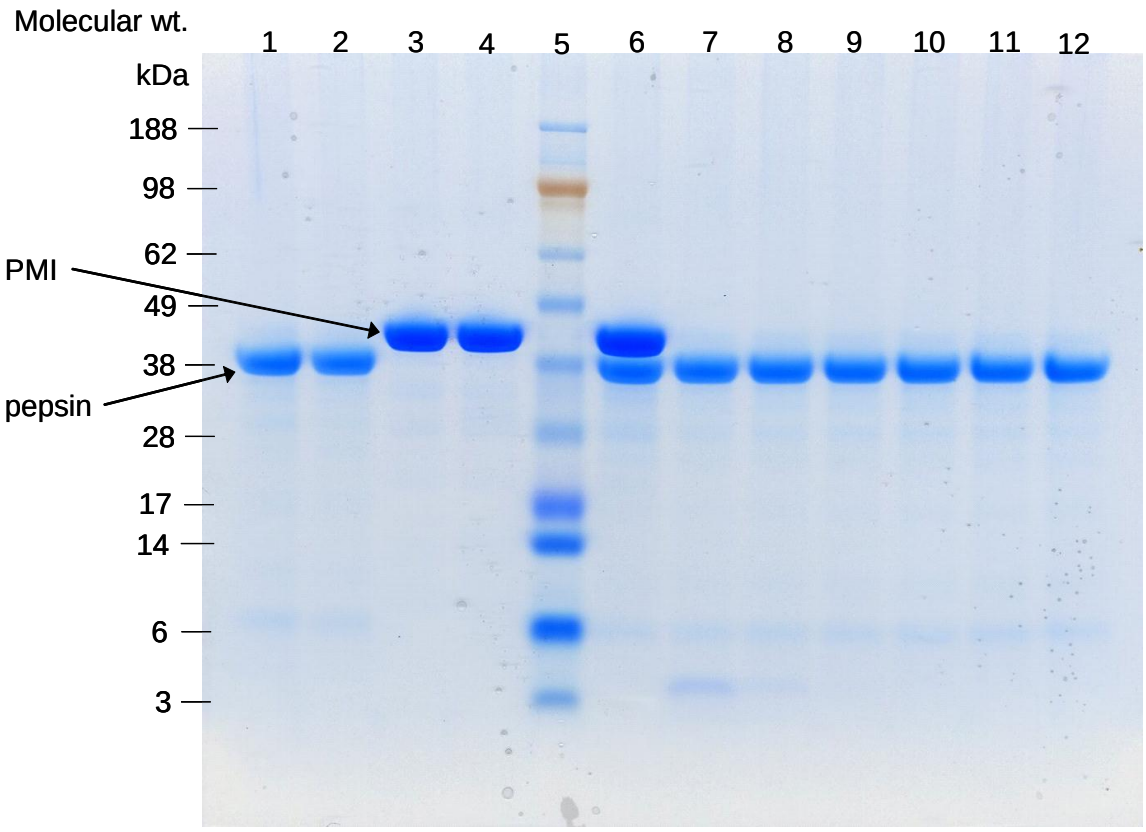
In vitro Digestibility of Phosphomannose Isomerase (PMI) as Contained in Test Substance PMI-0105 Under Simulated Mammalian Gastric Conditions SSB -034-07A1 - Appendix 19.

The susceptibility of phosphomannose isomerase (PMI) to proteolytic degradation in simulated mammalian gastric fluid containing pepsin (SGF) was evaluated. Test substance PMI-0105, a purified preparation of microbially produced PMI, was used as the source of test protein in this study. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis were used to assess the digestibility of PMI in SGF over a 60 minute time course at 37°C.

Following exposure to SGF for 1 min, no intact PMI (approximately 42.8 kDa molecular weight) was evident, as assessed by SDS-PAGE and Western blot analysis (Figures 1 and 2, Lane 7). Faint, diffuse bands of lower molecular weight (approximately 4 kDa) were visible on SDS-PAGE starting at 1 min, diminished in intensity in the 2 min sample and were not detectable in the 5 min sample and later time points (Figure 1, Lanes 7 through 12). These bands did not cross-react with the anti-PMI antibody, as shown on the corresponding Western blot (Figure 2, Lanes 7 through 12).

SGF controls without pepsin showed no significant degradation of PMI over the 60-min time course (Figures 1 and 2, Lanes 3 and 4). Incubation of the SGF solution alone for 60 min showed no significant degradation of pepsin (Figure 1, Lanes 1 and 2).

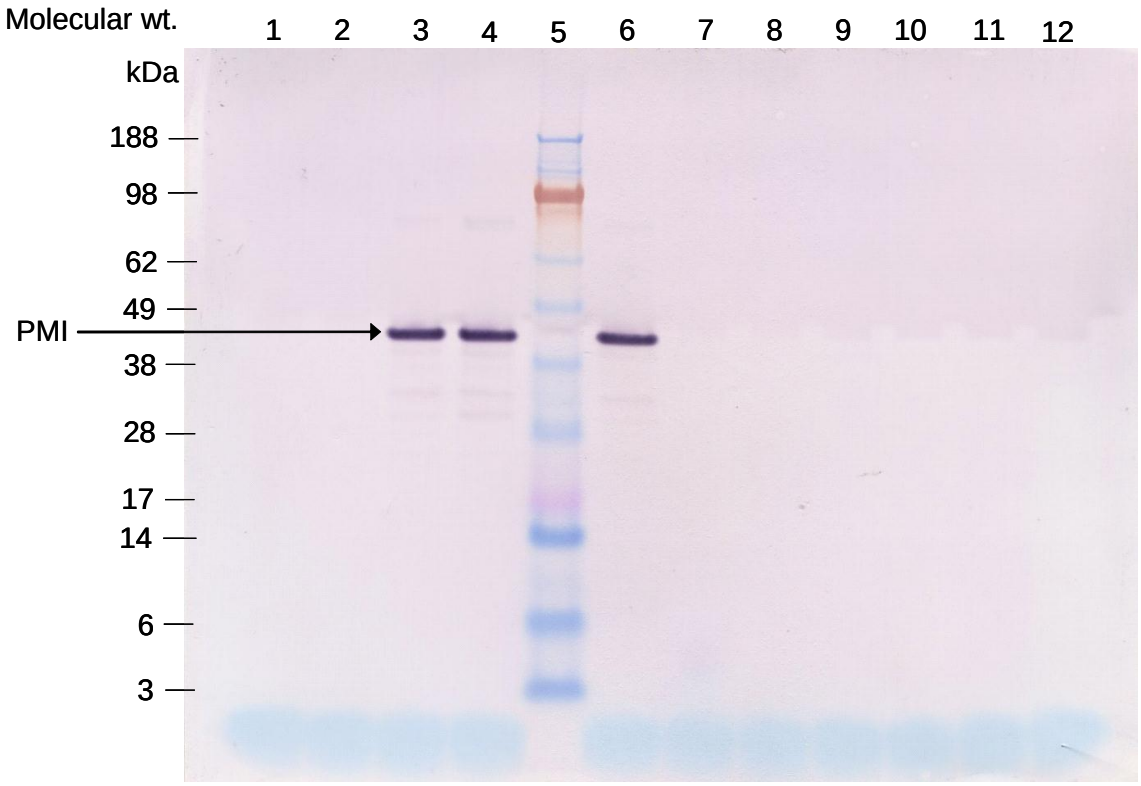
Figure 1. SDS-PAGE analysis of PMI (test substance PMI-0105) following digestion in SGF



Lane 1: SGF only incubated for 0 min
Lane 2: SGF only incubated for 60 min
Lane 3: PMI incubated in SGF without pepsin for 0 min
Lane 4: PMI incubated in SGF without pepsin for 60 min
Lane 5: molecular weight standard.
Lane 6: PMI incubated in SGF for time 0 min
Lane 7: PMI incubated in SGF for time 1 min
Lane 8: PMI incubated in SGF for time 2 min
Lane 9: PMI incubated in SGF for time 5 min
Lane 10: PMI incubated in SGF for time 10 min
Lane 11: PMI incubated in SGF for time 30 min
Lane 12: PMI incubated in SGF for time 60 min

The molecular weight of pepsin corresponds to approximately 35 kDa.
The molecular weight of PMI corresponds to approximately 42.8 kDa.

Figure 2. Western blot analysis of PMI (test substance PMI-0105) following digestion in SGF



Lane 1: SGF only incubated for 0 min
Lane 2: SGF only incubated for 60 min
Lane 3: PMI incubated in SGF without pepsin for 0 min
Lane 4: PMI incubated in SGF without pepsin for 60 min
Lane 5: molecular weight standard.
Lane 6: PMI incubated in SGF for time 0 min
Lane 7: PMI incubated in SGF for time 1 min
Lane 8: PMI incubated in SGF for time 2 min
Lane 9: PMI incubated in SGF for time 5 min
Lane 10: PMI incubated in SGF for time 10 min
Lane 11: PMI incubated in SGF for time 30 min
Lane 12: PMI incubated in SGF for time 60 min

The molecular weight of pepsin corresponds to approximately 35 kDa.
The molecular weight of PMI corresponds to approximately 42.8 kDa.

Digestibility of PMI in SIF

In vitro Digestibility of Phosphomannose Isomerase (PMI) as Contained in Test Substance PMI-0105 Under Simulated Mammalian Intestinal Conditions – SSB-036-07 – Appendix 20

The susceptibility of phosphomannose isomerase (PMI) to proteolytic degradation in simulated mammalian intestinal fluid containing pancreatin (SIF) was evaluated. Test substance PMI-0105, a purified preparation of microbially produced PMI, was used as the source of test protein in this study. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis were used to assess the digestibility of PMI in SIF over a 60 minute time course at 37°C.

Simulated mammalian intestinal fluid (USP 1990) was freshly prepared and contained 50 mM potassium phosphate monobasic, 38 mM sodium hydroxide, 10 mg/ml pancreatin (Sigma-Aldrich Cat. No. P7545) and the pH was adjusted to 7.5 ± 0.05 using 0.2 N sodium hydroxide. Immediately before use, the proteolytic activity of pancreatin was confirmed using azoalbumin as the substrate. SIF without pancreatin was prepared for use as a negative control.

Following exposure to SIF for 15 minutes, no intact PMI (approximately 42.8 kDa molecular weight) or degradation products were evident, as assessed by Western blot analysis (Figure 2, Lane 12). The analysis of the SDS polyacrylamide gel showed that the SIF contained a protein that co-migrated with PMI which made it difficult to distinguish between the two proteins. This made it difficult to clearly follow the degradation of PMI on the gel. However, the intensity of the co-migrating bands appeared to diminish significantly after 15 minutes of incubation (Figure 1, Lanes 8-12). The diminished intensity was most likely the result of the digestion of PMI in the SIF. This result was confirmed by Western blot analysis which showed that an immunoreactive PMI band was visible at 1, 2, and 5 minutes (Figure 2, Lanes 9, 10, and 11) and completely disappeared after 15 minutes (Figure 2, Lane 12). Incubation of the SIF control solution for 48 hours resulted in significant degradation of the pancreatin itself, as shown by the decreasing intensity of the protein bands (Figure 1, Lanes 5, 6 and 7). The 48-hour SIF control showed faint bands of higher molecular weights when compared to the zero time point of the SIF control (Figure 1, Lanes 5 and 7). These unexpected higher molecular weight bands could be explained by dimerization of some proteins found in the pancreatin over the time course of the experiment (Figure 1, Lanes 7 and 19). SIF controls without pancreatin showed no significant degradation of PMI over the 48-hour time course (Figures 1 and 2, Lanes 2, 3 and 4).

These results indicate that PMI is readily degraded under simulated mammalian intestinal conditions. The majority of intact PMI was digested after 5 minutes of incubation in simulated mammalian intestinal fluid and no intact PMI or degradation products were detected by Western blot analysis upon sampling of the reaction mixture after 15 minutes of incubation. The results of this study support the conclusion that PMI is readily digested under typical mammalian intestinal conditions.

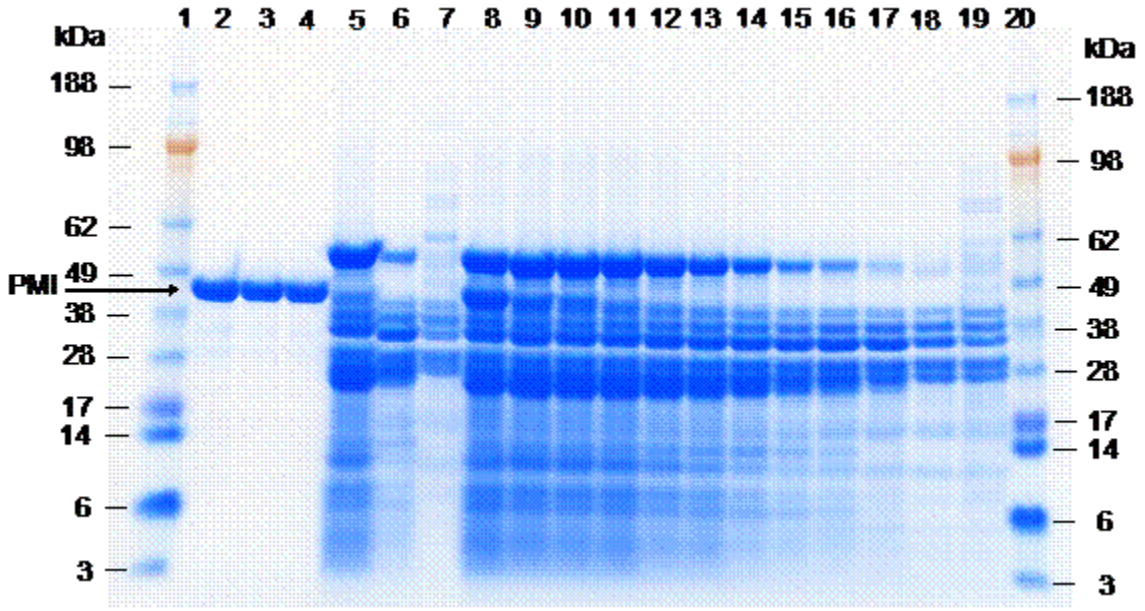


Figure 1. SDS-PAGE analysis of PMI (test substance PMI-0105) following digestion in SIF.

Lane 1: Molecular weight standard.
Lane 2: PMI incubated in SIF without pancreatin for 0 hours.
Lane 3: PMI incubated in SIF without pancreatin for 2 hours
Lane 4: PMI incubated in SIF without pancreatin for 48 hours
Lane 5: SIF incubated for 0 hours
Lane 6: SIF incubated for 2 hours
Lane 7: SIF incubated for 48 hours
Lane 8: PMI incubated in SIF for 0 minutes
Lane 9: PMI incubated in SIF for 1 minute
Lane 10: PMI incubated in SIF for 2 minutes
Lane 11: PMI incubated in SIF for 5 minutes
Lane 12: PMI incubated in SIF for 15 minutes
Lane 13: PMI incubated in SIF for 30 minutes
Lane 14: PMI incubated in SIF for 60 minutes
Lane 15: PMI incubated in SIF for 2 hours
Lane 16: PMI incubated in SIF for 3 hours
Lane 17: PMI incubated in SIF for 6 hours
Lane 18: PMI incubated in SIF for 24 hours
Lane 19: PMI incubated in SIF for 48 hours
Lane 20: Molecular weight standard.

The molecular weight of PMI corresponds to approximately 42.8 kDa.

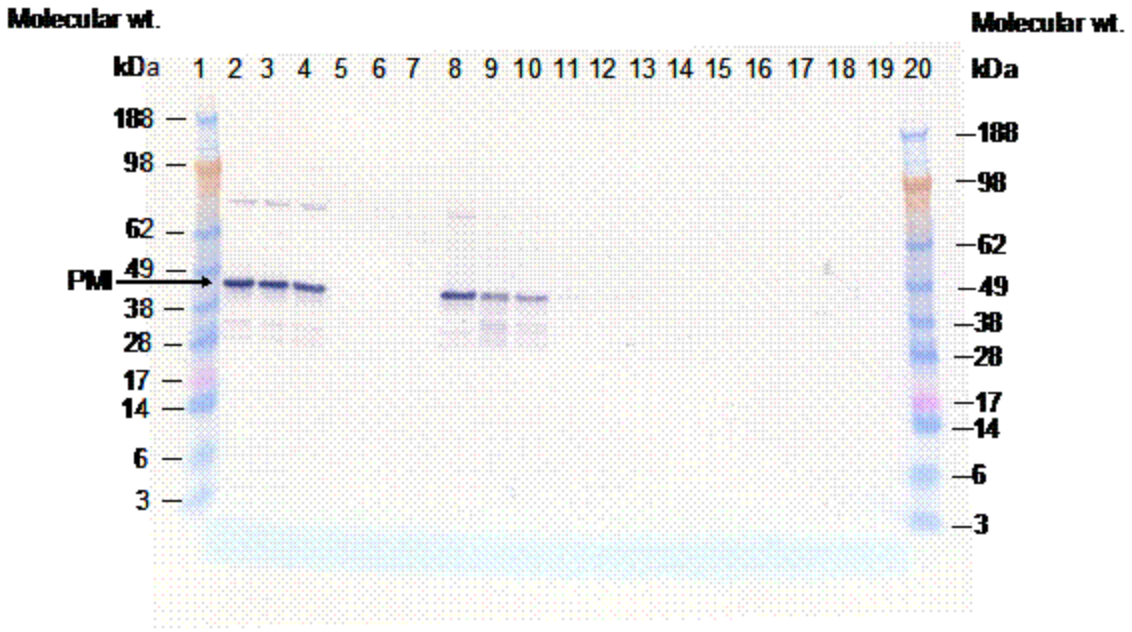


Figure 2. Western blot analysis of PMI (test substance PMI-0105) following digestion in SIF.

Lane 1: Molecular weight standard.
Lane 2: PMI incubated in SIF without pancreatin for 0 hours.
Lane 3: PMI incubated in SIF without pancreatin for 2 hours
Lane 4: PMI incubated in SIF without pancreatin for 48 hours
Lane 5: SIF incubated for 0 hours
Lane 6: SIF incubated for 2 hours
Lane 7: SIF incubated for 48 hours
Lane 8: PMI incubated in SIF for 0 minutes
Lane 9: PMI incubated in SIF for 1 minute
Lane 10: PMI incubated in SIF for 2 minutes
Lane 11: PMI incubated in SIF for 5 minutes
Lane 12: PMI incubated in SIF for 15 minutes
Lane 13: PMI incubated in SIF for 30 minutes
Lane 14: PMI incubated in SIF for 60 minutes
Lane 15: PMI incubated in SIF for 2 hours
Lane 16: PMI incubated in SIF for 3 hours
Lane 17: PMI incubated in SIF for 6 hours
Lane 18: PMI incubated in SIF for 24 hours
Lane 19: PMI incubated in SIF for 48 hours
Lane 20: Molecular weight standard.

The molecular weight of PMI corresponds to approximately 42.8 kDa.

eCry3.1Ab

Effect of temperature on the bioactivity of eCry3.1Ab protein as contained in test substance ECRY3.1AB-0208. - SSB-014-09 A1 – Appendix 21

The temperature stability of microbially produced eCry3.1Ab was evaluated by incubating the test substance at various temperatures [4°C (control), 25°C, 37°C, 65°C and 95°C] for 30 minutes and determining the loss of insecticidal activity in an insect bioassay with *Leptinotarsa decemlineata* (Colorado potato beetle) larvae.

At 25 °C, 37 °C, and 65°C eCry3.1Ab retained bioactivity when compared with that observed at 4°C. The estimated LC_{50} values and 95% confidence intervals at 4°C, 25°C, 37°C, and 65°C were 0.918 (0.419 – 1.617), 1.802 µg eCry3.1Ab/ml (1.066 – 2.714), 1.814 µg eCry3.1Ab/ml (0.411 – 4.714) and 4.682 µg eCry3.1Ab/ml (1.321 – 10.092), respectively. However, after treatment at 95ºC for 30 minutes, LC_{50} values and 95% confidence intervals could not be estimated due to the low mortality in these samples at all concentrations tested. These data demonstrate that eCry3.1Ab is inactivated, and therefore, denatured upon heating at temperatures of 95°C and above.

PMI

Effect of Temperature on Phosphomannose Isomerase (PMI) as Contained in Test Substance PMI-0105 as Assessed by Enzymatic Activity – SSB-023-09 – Appendix 22

The temperature stability of microbially produced PMI was evaluated by incubating the test substance at various temperatures [4° (control), 25, 37, 65 and 95°C] for 30 minutes and determining the specific PMI enzymatic activity. Loss or decrease of specific PMI enzymatic activity was used to determine the effect elevated temperatures have on the protein. At 25 and 37°C there was no significant effect on PMI enzymatic activity compared to that observed at 4°C. Incubation at 65°C resulted in a loss of enzymatic activity below the assay limit of quantitation (i.e., less than 52.2 milli-OD or 6.25 µg NADPH/ml), whereas incubation at 95°C resulted in no detectable enzymatic activity (i.e., below 15.0 milli-OD or 1.90 µg NADPH/ml). These data demonstrate that PMI is inactivated and therefore denatured upon heating at temperatures of 65°C and greater.

c) Detailed reports of all available acute or short term oral toxicity studies in animals on the novel proteins or other novel substances.

eCry3.1Ab

Single-Dose Oral (Gavage) Toxicity Study of ECRY3.1AB-0208 in Mice with a 14-Day Observation Period –WIL 639031– Appendix 23

The potential toxicity of the eCry3.1Ab protein was evaluated in an acute oral toxicity

study in the mouse conducted by WIL Research Laboratories, LLC (Ashland, OH, USA). The microbially produced, lyophilized test substance containing eCry3.1Ab protein (89.6% purity w/w), was administered as a single oral dose via gavage to groups of five male and five female Crl:CD-1 (ICR) mice at 0 or 2000 mg eCry3.1Ab/ kg body weight. The dosing vehicle, 0.5% (w/v) aqueous carboxymethylcellulose was administered to the control group. The dosing formulations were administered at a dose volume of 10 mL/kg for all groups. All animals were euthanized after a 14-day observation period following dosing.

All animals were observed twice daily for mortality and moribundity. Clinical examinations were performed at the time of dosing, approximately 1-2 hours post-dosing and approximately 4-5 hours post-dosing on the day of dose administration (study day 0) and once daily on nondosing days (study days 1-13). Detailed physical examinations were performed weekly. Individual body weights and food consumption were recorded daily during the study. Complete necropsies were conducted on all animals, and selected tissues were examined microscopically from all animals.

All animals survived the 14-day observation period to the scheduled necropsy. There were no observed clinical signs of distress or impairment. There were no test substance-related clinical observations. All clinical findings in the test substance-treated groups were limited to single animals and/or were common findings for laboratory mice of this age and strain.

At the end of the 14-day observation period, a complete necropsy was conducted on all animals. Animals were euthanized by carbon dioxide anesthesia and exsanguinated. The necropsies included, but were not limited to, examination of the external surface, all orifices, and the cranial, thoracic, abdominal and pelvic cavities, including viscera. The following tissues and organs were collected:

Adrenals (2)
Aorta
Bone with marrow
  Femur with joint
  Sternum
Bone marrow smear
Brain
  Cerebrum (2 levels)
  Cerebellum with medulla/pons
Cervix
Epididymides (2)
Eyes with optic nerve (2)
Gallbladder
Gastrointestinal tract
  Esophagus *
  Stomach *
  Duodenum *
  Jejunum *
  Peyer's patches *
  Ileum *
  Cecum *
  Colon *
  Rectum *
Heart
Kidneys (2)
Liver
Lungs (fixed by inflation with fixative)
Lymph nodes
  Mandibular *
  Mesenteric *
Ovaries (2) with oviducts
Pancreas
Peripheral nerve (sciatic)
Pituitary
Prostate
Salivary glands [mandibular (2)]
Seminal vesicles (2)
Skeletal muscle (rectus femoris)
Skin with mammary gland
Spinal cord (cervical, thoracic, lumbar)
Spleen *
Testes (2)
Thymus *
Thyroid [with parathyroids, if
  present (2)]
Trachea
Urinary bladder
Uterus
Vagina
Gross lesions (when possible) *

* Tissues processed for histopathological examination from all animals at the scheduled necropsy.

Histopathology evaluations were made on all the gastro-intestinal tissues of all the animals.

Body weights and food consumption were unaffected by test substance administration. Statistically significantly higher mean body weight gain was noted for the 2000 mg/kg group males on study days 3-4 and 13-14 compared to the control group. Statistically significantly lower mean body weight gain was noted for the 2000 mg/kg females on study days 2-3 and 9-10. These differences in body weight gain were considered incidental and not related to test substance administration because the magnitude of the change was very small.

Statistically significantly higher mean food consumption was noted for the 2000 mg/kg group males on study days 8-9 and 12-13. This difference in food consumption was considered incidental and not related to test substance administration because the magnitude of the change was small and no other changes in food consumption were noted for the remaining study intervals.

Review of the gross necropsy observations revealed no observations that were considered to be associated with administration of the test substance. Occasional histological changes were noted in both treated and control animals; these were all considered to be incidental findings

or related to some aspect of experimental manipulation other than administration of the test substance. There was no test substance related alteration in the prevalence, severity or histological character of those incidental tissue alterations.

In summary, test substance ECRY3.1AB-0208, containing the active ingredient eCry3.1Ab protein (89.6% purity w/w), administered as a single oral dose at 2000 mg eCry3.1Ab/ kg body weight followed by a 14-day nondosing observation period was well tolerated in male and female CD-1 mice. No toxicity was observed in mice given an acute oral gavage dose of 2000 mg eCry3.1Ab/kg body weight. Based on the results of this study, the estimated no observed adverse effect level (NOAEL) for eCry3.1Ab protein in male and female mice was > 2000 mg/kg body weight.

PMI

Single-Dose Oral (Gavage) Toxicity Study of PMI-0105 in Mice with a 14-Day Observation Period- WIL-639011 – Appendix 24

The potential toxicity of the PMI protein was evaluated in an acute oral toxicity study in the mouse conducted by WIL Research Laboratories, LLC (Ashland, OH, USA).

Test substance PMI-0105, containing the active ingredient phosphomannose isomerase protein (89.5% purity w/w), was administered as a single oral gavage dose to groups of five male and five female Crl:CD-1(ICR) mice at 0 or 2000 mg active ingredient/ kg body weight. Deionised water was used as the vehicle and administered to the control group. The dosing formulations were administered at a dose volume of 10 mL/kg. All animals were euthanized after a 14-day observation period following dosing.

All animals were observed twice daily for mortality and moribundity. Clinical examinations were performed at the time of dosing, approximately 1-2 hours post-dosing and approximately 4-5 hours post-dosing on the day of dose administration (study day 0) and once daily on non-dosing days (study days 1-13). Detailed physical examinations were performed weekly. Individual body weights and food consumption were recorded daily during the study. Clinical pathology evaluations (haematology and serum chemistry) were performed on all animals prior to the scheduled necropsy. Complete necropsies were conducted on all animals, and selected organs were weighed at the scheduled necropsy. Selected tissues were examined microscopically from all animals. The following tissues and organs were collected:

Adrenals (2)
Aorta
Bone with marrow
  Femur with joint
  Sternum
Bone marrow smear [a]
Brain
  Cerebrum (2 levels)
  Cerebellum with medulla/pons
Cervix (females)
Epididymides (2) [b] (males)
Eyes with optic nerve (2) [c]
Gallbladder
Gastrointestinal tract
  Esophagus
  Stomach
  Duodenum
  Jejunum
  Ileum
  Cecum
  Colon
  Rectum
  Heart
  Kidneys (2)
  Liver
  Lungs (fixed by inflation with fixative)
Lymph nodes
  Mandibular
  Mesenteric
Ovaries (2) with oviducts [d] (females)
Pancreas
Peripheral nerve (sciatic)
Peyer's patches
Pituitary
Prostate (males)
Salivary glands [mandibular (2)]
Seminal vesicles (2) (males)
Skeletal muscle (rectus femoris)
Skin with mammary gland [e]
Spinal cord (cervical, thoracic, lumbar)
Spleen
Testes (2) [b] (males)
Thymus
Thyroid [with parathyroids, if present (2)] [d]
Trachea
Urinary bladder
Uterus (females)
Vagina (females)
Gross lesions (when possible)

[a] - Bone marrow smears were obtained at scheduled necropsy, but not placed in formalin; slides were examined only if scientifically warranted.
[b] - Fixed in Bouin's solution
[c] - Fixed in Davidson's solution
[d] - Parathyroids and oviducts were examined histologically if in the plane of section and in all cases when a gross lesion was present
[e] - For females; a corresponding section of skin was collected from the same anatomic area for males

All animals survived to the scheduled necropsy. There were no test substance-related clinical observations. There were no test substance-related effects on body weight or weight gain, food consumption or haematology parameters. There were no macroscopic or microscopic findings that were attributable to the test substance.

Higher urea nitrogen levels (males only), slightly higher alkaline phosphatase levels (males only) and slightly higher alanine aminotransferase levels (females only) were noted in the 2000 mg/kg group compared with the control group, and were considered potentially test substance-

related. However, these changes in serum chemistry parameters were considered non-adverse as there were no histopathological correlates and the group mean values were within WIL Historical control ranges (Version 2.5), except for one male mouse which had urea nitrogen levels exceeding the historical control range.

Statistically significantly lower testicular and epididymal weights in males and slightly higher adrenal weights in females were noted in the 2000 mg/kg group compared with the control group, and were considered test substance-related. However, there were no distinct microscopic changes in these organs, and the organ weights were within the WIL Historical control ranges (Version 2.6), suggesting that organ weight alterations probably represented physiologic responses of a non-adverse nature.

4. The potential allergenicity of novel proteins

This part includes all of the following:

a) Source of the introduced protein.

The eCry3.1Ab protein is an engineered chimera of modified Cry3A (mCry3A) and Cry1Ab proteins, which are derived from *Bacillus thuringiensis*. The *pmi* gene was obtained from *Escherichia coli* strain K-12 and the PMI protein it encodes was utilised as a plant selectable marker during development of 5307 corn.

b) Any significant similarity between the amino acid sequence of the protein and that of known allergens.

eCry3.1Ab

eCry3.1Ab (Entrez® Database Accession Number ADC30135): Assessment of Amino Acid Sequence Similarity to Known or Putative Allergens – SSB-112-11 - Appendix 25

Amino acid sequence comparisons between novel proteins and known protein toxins and allergens are part of the weight-of-evidence approach to assessing potential mammalian toxicity and allergenicity. For example, in combination with other supporting data, the presence of significant homologies with known allergens could indicate that the novel protein might elicit an allergic cross-reaction in sensitised individuals.

To determine whether or not the eCry3.1Ab amino acid sequence showed biologically relevant amino acid sequence similarity to known or putative allergens, two different searches were performed against the Food Allergy Research and Resource Program Protein (FARRP) Allergen Online database, version 11.0, which contained 1,491 amino acid sequences of known or putative allergens. A full-length sequence search using FASTA and a search for exact matches of eight or more contiguous amino acid when compared to each of the known or putative

allergens sequences were used in the assessment. In the first search, no sequence similarity greater than 35% shared identity over 80 amino acids or more was observed between the eCry3.1Ab amino acid sequence and any entry in the FARRP Allergen Online database. In the second search, no matches of eight or more contiguous amino acids were observed between eCry3.1Ab amino acid sequence and sequences in the FARRP Allergen Online database. These results support the conclusion that eCry3.1Ab shares no biologically relevant amino acid sequence similarity to known or putative protein allergens.

PMI

PMI (Entrez® Database Accession Number AAA24109): Assessment of Amino Acid Sequence Similarity to Known or Putative Allergens, SSB-102-11 - Appendix 26

To determine whether or not the PMI amino acid sequence showed biologically relevant amino acid sequence similarity to known or putative allergens, two different searches were performed against the Food Allergy Research and Resource Program Protein (FARRP) Allergen Online database, version 11.0, which contained 1,491 amino acid sequences of known or putative allergens. A full-length sequence search using FASTA and a search for exact matches of eight or more contiguous amino acid when compared to each of the known or putative allergens sequences were used in the assessment. In the first search, no sequence similarity greater than 35% shared identity over 80 amino acids or more was observed between the PMI amino acid sequence and any entry in the FARRP Allergen Online database. In the second search, a single match of eight identical amino acids between PMI and α–parvalbumin from *Rana* species CH2001 was observed. This match has been previously reported in assessments of the sequence similarity of PMI to known and putative allergens. Further investigation using sensitive serum IgE antibody screening demonstrated no cross-reactivity between PMI and the α–parvalbumin protein using serum from the single individual known to have demonstrated IgE-mediated allergy to this specific α–parvalbumin from *Rana* species 2001. The results indicated that the allergic patient's serum IgE does not recognise any portion of PMI as an allergenic epitope. The present study was undertaken to reassess the similarity of PMI to known allergens based on an updated FARRP Online database. This study found no new significant amino acid sequence similarity to known or putative allergens other than what has previously been reported. These results support the conclusion that eCry3.1Ab shares no biologically relevant amino acid sequence similarity to known or putative protein allergens.

c) Its structural properties, including but not limited to, its susceptibility to enzymatic degradation (e.g. digestion by pepsin), heat stability and/or, acid and enzymatic treatment.

eCry3.1Ab

Effect of temperature on the bioactivity of eCry3.1Ab protein as contained in test substance ECRY3.1AB-0208. - SSB-014-09 A1 – Appendix 21

The temperature stability of microbially produced eCry3.1Ab was evaluated by incubating the test substance at various temperatures [4°C (control), 25°C, 37°C, 65°C and 95°C] for 30 minutes and determining the loss of insecticidal activity in an insect bioassay with *Leptinotarsa decemlineata* (Colorado potato beetle) larvae.

At 25°C, 37°C, and 65°C eCry3.1Ab retained bioactivity when compared with that observed at 4°C. The estimated LC_{50} values and 95% confidence intervals at 4°C, 25°C, 37°C, and 65°C were 0.918 (0.419 – 1.617), 1.802 µg eCry3.1Ab/ml (1.066 – 2.714), 1.814 µg eCry3.1Ab/ml (0.411 – 4.714) and 4.682 µg eCry3.1Ab/ml (1.321 – 10.092), respectively. However, after treatment at 95°C for 30 minutes, LC_{50} values and 95% confidence intervals could not be estimated due to the low mortality in these samples at all concentrations tested. These data demonstrate that eCry3.1Ab is inactivated, and therefore, denatured upon heating at temperatures of 95°C and above.

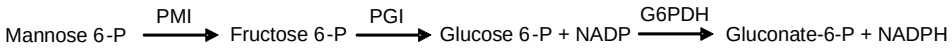
PMI

Effect of Temperature on Phosphomannose Isomerase (PMI) as Contained in Test Substance PMI-0105 as Assessed by Enzymatic Activity – SSB-023-09 – Appendix 22

The stability of the PMI protein was evaluated at a range of temperatures, with loss of enzyme activity used to determine the instability of the protein after exposure to the various treatments. A solution containing PMI-0105, was exposed to a range of temperatures (25ºC, 37ºC, 65ºC and 95ºC) for 30 min, including incubation at 4ºC for 30 min as a control to determine the baseline PMI concentration. Loss, or decrease, of specific PMI enzymatic activity was used to determine the effect of elevated temperature on the protein.

For the temperature analysis, test substance PMI-0105 was dissolved in 50 mM Tris (pH 7.0) buffer to a final concentration of 5 µg PMI/ml. Three 1 ml aliquots of the test substance solution were then incubated at each temperature (25ºC ± 2ºC, 37ºC ± 2ºC, 65ºC ± 2ºC and 95ºC ± 2ºC) for 30 min. For a baseline control, three additional aliquots were incubated at 4ºC ± 2ºC for 30 min. After incubation each 1ml (5 µg PMI/ml) solution was further diluted in 1 ml of 50 mM Tris (pH7.0) with 0.1% Tween 20 to a concentration of 0.1 ng PMI/µl.

The enzymatic activity of PMI was monitored for each treatment in triplicate (i.e. three 25ºC samples each were aliquoted three times into the assay for a total of nine reactions). The enzymatic reactions were conducted in Corning 96-well plates containing 10 mM mannose 6-phosphate , 1 mM β-nicotinamide adenine dinucleotide phosphate sodium salt hydrate, 2U/ml phosphoglucose isomerase and 2U/ml glucose 6-phosphate dehydrogenase in 50 mM Tris buffer (pH 7.0). The reaction was initiated by the addition of 5 ng PMI (50µl of 0.1 ng/ul dilution) per well to the assay mixture (total reaction volume 200 µl per well). PMI activity was measured by the production of NADPH in a coupled reaction containing phosphoglucose isomerase (PGI) and glucose 6-phosphate dehydrogenase (G6PDH) and validated for use in this study.



PMI = Phosphomannose isomerase, PGI = Phosphoglucose isomerase, G6PDH = Glucose 6 -phosphate dehydrogenase

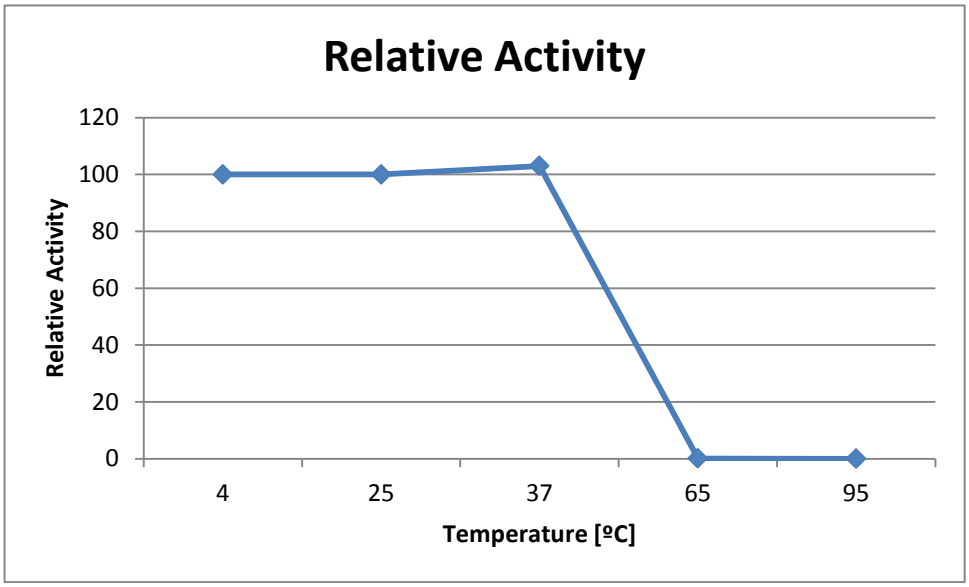
The production of NADPH was monitored by measuring absorbance increase at 340 nm using a SpectraMax Plus 384 spectrophotometer (Molecular Devices) and SoftMax Pro software. One unit of PMI is defined as the amount of enzyme required to catalyse the isomerisation of mannose 6-phosphate to fructose 6-phosphate per min under the described reaction conditions.

The results shown in Table 1 and Figure 1 indicate that incubation of PMI at temperatures up to 37ºC for 30 minutes did not decrease the enzymatic activity of PMI. Incubation of PMI at 65ºC for 30 minutes resulted in loss of enzymatic activity below the enzymatic assays limit of quantitation, and the incubation at 95ºC resulted in no detectable enzymatic activity.

Table 1. Effect of Temperature on Immunoreactivity of PMI. For each temperature three aliquots were incubated and analysed in triplicate by ELISA for immunoreactivity.

Temperature [ºC]	Mean Specific Activity ± SD [U/mg PMI]	Relative Activity
4 (control)	420 ± 23	100
25	439 ± 11	100
37	422 ± 11	103
65	<LOQ	<LOQ
95	Not detectable	Not detectable

Figure 1. Effect of Temperature on Enzymatic Activity.



d) Specific serum screening where a newly expressed protein is derived from a source known to be allergenic or has sequence homology with a known allergen.

Not relevant, the protein is not derived from a source known to be allergenic or have any sequence homology with a known allergen.

5. Compositional analyses of the GM food

This part includes all of the following:

a) The levels of key nutrients, toxicants and anti-nutrients in the GM food compared with the levels in an appropriate comparator (usually the GM non-counterpart, near isogenic parental line). The statistical significance of any observed differences must be assessed in the context of the range of natural variations for that parameter to determine its biological significance.

b) The levels of any other constituents that may potentially be influenced by the genetic modification, as a result, for example, of downstream metabolic effects, compared with the levels in an appropriate comparator.

c) The levels of any naturally occurring allergenic proteins in the GM food compared with the levels in an appropriate comparator.

The nutritional composition of corn grain and forage derived from 5307 corn was investigated in the study titled, *"Compositional Analysis of Forage and Grain from Event 5307 Hybrid Corn Grown During 2008 in the USA [2008 Composition Study (SSB-170-09 A2)]". – Appendix 27.*

Transgenic 5307 corn was not developed to have any intentionally altered nutritional value, but rather to exhibit the agronomic trait of resistance to certain coleopteran insect pests. 5307 corn produces the eCry3.1Ab and phosphomannose isomerase (PMI) proteins.

A comparative assessment was conducted where the transformed plant was compared to its conventional counterpart in order to establish whether there was any unintended biologically relevant changes in the transformed plant. Since all corn-derived food products are produced from kernels (grain), analysis of the composition of kernels is the most appropriate test for food use and is the approach that was followed for 5307 corn. Additionally, forage was also evaluated.

Method

Key nutritional components in corn grain and forage derived from 5307 corn and near isogenic nontransgenic control plants were compared. Replicate trials of transgenic and corresponding

near-isogenic nontransgenic control hybrids were planted in multiple locations in the United States in 2008. The locations of the trial sites were selected to be representative of the range of environmental conditions under which the hybrid varieties would typically be grown. Six locations that produced sufficient grain and forage were selected for this study as follows:

Location Code	City and State	Location Identifier
L1	Stanton, Minnesota	Stanton 4536
L2	Janesville, Wisconsin	Janesville 5629
L4	New Haven, Indiana	New Haven 6742
L6	Shirley, Illinois	Shirley 7630
L7	Marshall, Missouri	Marshall 8409
L8	Bloomington, IL	Blm Nursery 761N

Data for each component were subjected to analysis of variance across all locations. Average values for each analyte in the hybrid pair were compared to data for forage and grain composition published in both the International Life Sciences Institute (ILSI) crop composition database (ILSI, 2008) and the Organization for Economic Co-operation and Development consensus document on new corn varieties (OECD, 2002) to assess whether any observed variation was within the natural range for cultivated corn forage and grain.

At each field trial location, three replicate plots of each genotype were planted in randomized complete blocks and plants were grown according to local agronomic practices. Both the 5307 hybrid and the non-transgenic control hybrid were treated with conventional pesticides as needed. Plants were self-pollinated by hand and the developing ears were bagged to avoid cross-pollination.

The hybrid pair used for this study was identified as follows:

Description	Entry	Genotype
Event 5307	1	NP2171/NP2460(5307)
Nontransgenic, near-isogenic hybrid	2	NP2171/NP2460

Figure 5.1 on the following page gives the pedigree chart of 5307 corn used in compositional study.

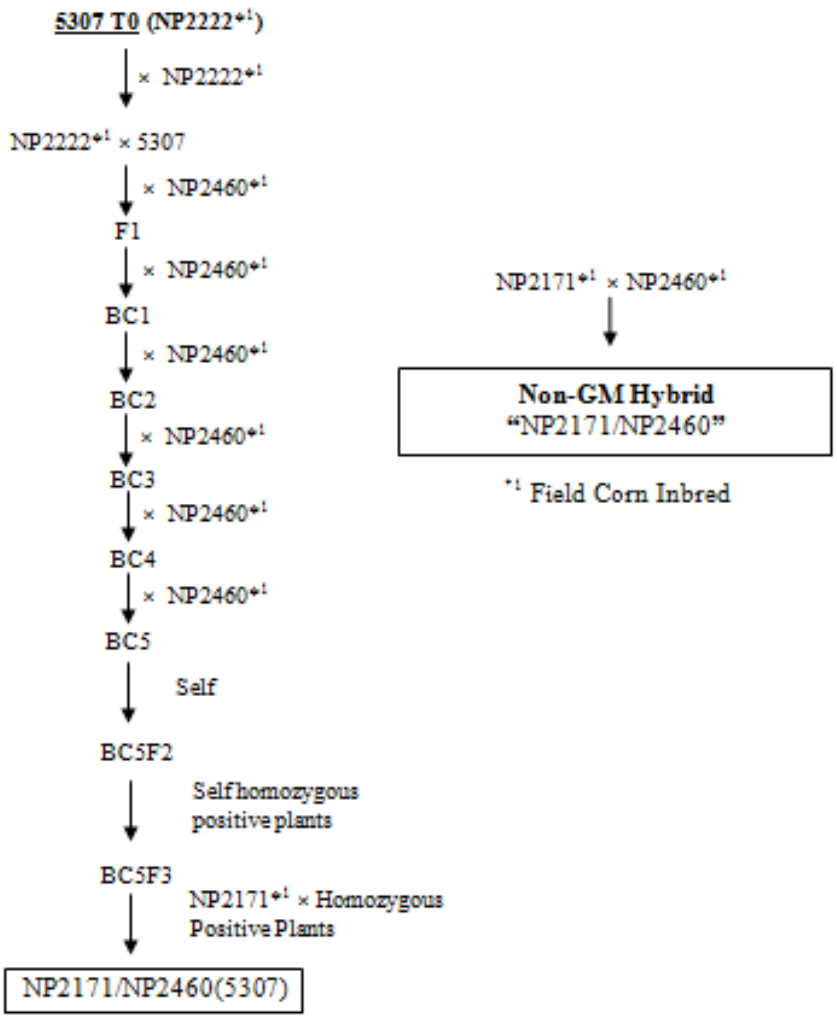


Figure 5.1 Pedigree chart of 5307 corn used in compositional study.

Forage sampling and processing

For each hybrid described above, from each of the three replicated plots at each location, the entire above ground portions of five plants were harvested at the dough stage (R4, approximately the stage at which silage would be prepared), pooled to create a composite sample, and ground with a chipper-shredder. A sub-sample from each composite sample was shipped overnight on wet ice to Syngenta Crop Protection, Inc. (Greensboro, NC, USA). Samples were stored frozen at -20±10°C, then finely ground and shipped on dry ice to Covance Laboratories, Inc. (Madison, WI, USA) where they were stored frozen at -20±10°C until analysed.

Grain sampling and processing

Ears were harvested after physiological maturity (R6) at approximately 18–24 percent moisture and dried post-harvest to approximately 10–13 percent moisture. Each grain sample was pooled from ears harvested from fifteen plants from each of the 3 replicated plots at each location. A subsample of approximately 500 g of grain from each replicate plot was shipped at ambient temperature to Syngenta Crop Protection, Inc. where it was stored frozen at -20±10°C, then finely ground and shipped on dry ice to Covance Laboratories, Inc. Samples were stored frozen at -20±10°C until analysed.

Analyses performed

All compositional analyses were conducted by Covance Laboratories, Inc., using methods published and approved by AOAC International, or other industry-standard analytical methods. Based on the moisture content of each sample, analyte levels were converted to equivalent units of dry weight.

Statistical Analysis

Across-location comparisons

The data for each component were subjected to analysis of variance using the following mixed model:

$$Y_{ijk} = U + T_i + L_j + B(L)_{jk} + LT_{ij} + e_{ijk}$$

In this model, Y_{ijk} is the observed response for genotype i at location j block k, U is the overall mean, T_i is the genotype effect, L_j is the location effect, $B(L)_{jk}$ is the effect of block within a location, LT_{ij} is the location-by-genotype interaction effect, and e_{ijk} is the residual error. Genotype was regarded as a fixed effect, while the effects of location, block within location, and location-by-genotype were regarded as random.

For each quantifiable component, an F test was used to assess the statistical significance of the genotype effect with an alpha level of 0.05 and with the denominator degrees of freedom determined using the Kenward-Roger method. Moisture content of grain was not statistically analysed because the samples had been mechanically dried.

Individual-location comparisons

The data for each component at each location were subjected to an analysis of variance with genotype and block included in the statistical model. Significance was based on an alpha level of 0.05. Statistical analysis was performed using SAS v. 9.2 (SAS Institute, Inc., Cary NC).

Moisture levels in grain were not subjected to analysis of variance since the moisture analysis was performed on grain that had been mechanically dried, thus altering the original moisture

content of the harvested grain. Mechanical drying after harvest is a standard agronomic practice for improving storage characteristics of grain.

Compositional Data

Proximates

Analysis of the major constituents of corn, or proximates, was used to determine the nutritional properties of corn grain from different hybrids. The major constituents of corn grain and forage are carbohydrates, protein, fat and ash. Fibre is the predominant form of carbohydrate present in forage and starch is the major carbohydrate in corn grain. Fibre is measured by the neutral detergent fibre method (NDF), which measures the insoluble fibre: lignin, cellulose and hemicellulose. Total dietary fibre (TDF) consists of the insoluble and soluble fibre (pectin). The soluble fibre fraction in corn is negligible, so the NDF value in corn grain is comparable to that of TDF. The acid detergent fibre (ADF) method solubilises hemicellulose, measuring only cellulose and lignin.

As shown in Table 5.1, in the across-location comparisons, levels of protein, fat, ash carbohydrates, ADF, NDF, TDF, and starch did not differ significantly between the 5307 corn grain and the nontransgenic corn grain. Moisture levels were not compared statistically because grain samples were mechanically dried. In the comparisons at each location, levels of fat, carbohydrates, ADF, NDF, and TDF did not differ significantly between the two genotypes at any of the six locations. Levels of protein differed at only one location; levels of ash differed at another location; and levels of starch differed significantly at a third location. The mean levels across locations and for each location were within the ranges reported in the ILSI database, except for starch which was slightly higher than the ILSI range in the nontransgenic hybrid at one location.

As shown in Table 5.2, in the across-location comparisons, levels of moisture, protein, fat, ash, carbohydrates, ADF, and NDF did not differ significantly between the 5307 corn forage and the nontransgenic corn forage. In the comparisons at each location, levels of fat, ash, carbohydrates, ADF, and NDF did not differ significantly between the two genotypes at any of the six locations. Levels of moisture and protein each differed significantly at one (different) location. The mean levels of all proximates, across locations and for each location were within the ranges reported in literature.

Minerals

Several mineral ions are recognised as essential plant nutrients and are required by the plant in significant quantities. These macronutrients include calcium, phosphorus, potassium and sodium. The micronutrient minerals, iron, copper and zinc are incorporated in plant tissues in only trace amounts. Corn is an important source of selenium in animal feed, and this analyte was also included in the analyses of grain.

As shown in Table 5.3, in the across-location comparisons, levels of calcium, copper, iron, magnesium, manganese, phosphorus, potassium, and zinc did not differ significantly between the 5307 corn grain and the nontransgenic corn grain. In the comparisons at each location, levels of calcium, copper, iron, and potassium did not differ between the two genotypes at any of the six locations. Levels of magnesium, manganese, phosphorus, and zinc each differed significantly at one location. For selenium and sodium, levels below the limit of quantitation (LOQ) precluded statistical comparison across-locations at all six locations for sodium, and at four of the six locations for selenium. At the two locations where statistical analysis was performed for selenium, the levels did not differ significantly. For all quantifiable components, mean levels across locations and for each location were within the ranges reported in literature.

As shown in Table 5.4, in the across-location comparisons, neither calcium nor phosphorus levels differed significantly between the 5307 corn forage and the nontransgenic corn forage. In the comparisons at each location, levels of calcium and phosphorus each differed significantly at one location. The mean levels across locations and for each location were within the ranges reported in literature.

Vitamins

Although animal feed formulations are usually supplemented with additional vitamins to achieve nutritional balance, corn contains two fat-soluble vitamins, vitamin-A (β-carotene) and vitamin E, and most of the water-soluble vitamins. Vitamin A occurs in two forms in nature. Its true form, retinol, is present in foods of animal origin such as fish oils and liver. Provitamin A, in the form of the carotenoids β-carotene and cryptoxanthin are found in plants and converted in the body to vitamin A. Vitamin E (tocopherol) occurs in a variety of vegetable, nut, and oilseed crops, and of the various structural isomers (alpha-, beta-, delta- and gamma-tocopherol), α-tocopherol is the most biologically important as a natural antioxidant. Alpha-tocopherol is the only form of vitamin E that is actively maintained in the human body, and has the greatest nutritional significance. The water-soluble vitamins B_1 and B_6 (pyridoxine) are present in corn grain at quantities sufficient to be important in animal rations.

As shown in Table 5.5, in the across-location comparisons, levels of vitamins B_1, B_2, B_3, and E did not differ significantly between the 5307 corn grain and the nontransgenic corn grain. Statistically significant differences were observed in levels of vitamins A, B_6, and B_9. In the comparisons at each location, levels of vitamins B_1, B_2, B_3, B_9, and E did not differ between the two genotypes at any of the six locations. Levels of vitamin B_6 differed significantly at one location and levels of vitamin A differed significantly at two locations. All mean levels across locations and for each location were within the ranges reported in the ILSI database, except for vitamin B_2 which was slightly higher than the ILSI range for a nontransgenic hybrid at one location.

Amino Acids

The quality of protein produced by different corn hybrids can be determined by measuring the content of different amino acids. Eighteen amino acids commonly found in corn are considered to be important for compositional analysis. Levels of methionine and cysteine are important for formulation of animal feed, as are lysine and tryptophan, which cannot be produced by non-ruminant animals such as swine and poultry and are present at low concentrations in corn.

As shown in Table 5.6, in the across-location comparisons, none of the amino acid levels differed significantly between the 5307 corn and the nontransgenic corn. In the comparisons at each location, levels of eleven amino acids did not differ between the genotypes at any of the six locations. Levels of asparagine, threonine, glutamic acid, glycine, alanine, valine, leucine, and histidine all differed significantly at one single location (L2). All mean levels across locations and for each location were within the ranges reported in literature.

Fatty Acids

Five fatty acids account for nearly 98 percent of the total fatty acids in corn grain, with the most abundant being linoleic (C18:2 Δ9,12; 57.6%) and oleic (C18:1 Δ9; 26.0%) acids. Less abundant, but occurring at measurable levels are palmitic (C16:0; 11.03%), stearic (C18:0; 1.8%) and α-linolenic (C18:3 Δ9,12,15; 1.13%) acids. The desaturation of oleic acid to form linoleic acid, and its subsequent desaturation to form α- linolenic acid, occurs only in plants, hence both linoleic and α-linolenic acids are essential fatty acids for mammals. For this reason, it was desirable to measure for any unintended changes in the levels of linoleic and α-linolenic acids, and their key precursors, palmitic, stearic and oleic acids, in grain from 5307.

Other polyunsaturated and longer chain polyunsaturated fatty acids, such as γ-linolenic (C:18 Δ6,9,12), eicosatrienoic (C20:3 Δ8,11,14) and arachidonic (C20:4 Δ5,8,11,14) acids can all be synthesized by mammals from dietary sources of α-linolenic and linoleic acid. Hence, small changes in the levels of these trace fatty acids in 5307-derived grain would have little or no biological significance to either humans or animals consuming 5307 grain products. And the synthesis of palmitoleic (C16:1 Δ9) and saturated fatty acids with chain lengths greater than 18 (e.g., C20:0, C22:0, C24:0), can be accomplished in mammals through *de novo* fatty acid synthesis without dietary requirements for palmitic and stearic acids, respectively.

The complete fatty acid profile of corn grain from 5307 and non-transgenic control hybrids was determined and the results are summarized in Table 5.7. As shown in the across-location comparisons of Table 5.7, the proportions of 18:1 oleic acid, 18:2 linoleic acid, 20:0 arachidic acid, and 22:0 behenic acid as fractions of total fatty acids did not differ significantly between the 5307 corn and the nontransgenic corn. Statistically significant differences were observed in proportions of four other fatty acids (16:0 palmitic acid, 18:0 stearic acid, 18:3 linolenic acid, and 20:1 eicosenoic acid). In the comparisons at each location, the proportions of 18:1 oleic acid, 18:2 linoleic acid, 20:0 arachidic acid, and 22:0 behenic acid as fractions of total fatty acids did not differ between the two genotypes at any of the six locations. Statistically

significant differences were observed in proportions of 20:1 eicosenoic acid at one location; and 16:0 palmitic acid, 18:0 stearic acid, and 18:3 linolenic acid at two locations. All four fatty acids with statistically significant differences in the individual-location comparisons were different at Location 4 (L4). For 16:1 palmitoleic acid, levels below the LOQ precluded statistical comparison across-locations, and at five of the six locations. At the one location where statistical analysis was performed for 16:1 palmitoleic acid, the proportions did not differ significantly. For all quantifiable components, mean levels across locations and for each location were within the ranges reported in literature.

Secondary Metabolites and Antinutrients

Secondary metabolites are defined as those natural products which do not function directly in the primary biochemical activities that support growth, development and reproduction of the organism in which they occur. One class of secondary metabolites, antinutrients, is responsible for deleterious effects related to the absorption of nutrients and micronutrients from foods. There are generally no recognised antinutrients in corn at levels considered to be harmful, but for the purposes of safety assessment OECD recommends testing for the following secondary metabolites in corn: ferulic acid, ρ-coumaric acid, furfural, inositol, phytic acid, raffinose and trypsin inhibitor. These secondary metabolites and antinutrients were analysed in grain samples from 5307 and non-transgenic control hybrids (Table 5.8).

As shown in Table 5.8, in the across-location comparisons, levels of ferulic acid, *p*-coumaric acid, inositol, phytic acid, trypsin inhibitor and raffinose did not differ significantly between the 5307 corn grain and the nontransgenic corn grain; nor did these components differ at any of the six locations. For furfural, levels below the LOQ precluded statistical comparison across-locations, and at all six locations. For all quantifiable components, mean levels across locations and for each location were within the ranges reported in literature.

Nutritional Impact/Conclusions

Among the numerous compositional analyses that were carried out, most showed no statistically significant differences. Of the seven grain components with statistically significant across-location comparisons, all were significantly different at two or fewer locations. For all components compared statistically, including those for which statistically significant differences were observed, mean levels were within literature ranges for conventional corn hybrids, except starch and vitamin B_2 in grain. Levels of starch and vitamin B_2 were each slightly higher than the ILSI range in the nontransgenic grain, each from a single location (ILSI, 2008). These findings support the conclusion that no biologically significant changes in composition occurred as an unintended result of the transformation process or expression of the transgenes in 5307 corn. The conclusion based on these data was that grain and forage from 5307 corn were substantially equivalent in composition to both the non-transgenic control hybrid included in this study, and to other commercial corn with an established history of safe use.

Table 5.1. Proximate and starch composition of grain from 5307 corn and nontransgenic corn

Proximate and starch levels shown in % DW, except moisture (% FW). Results significantly different at $P < 0.05$ are shown in bold italic type.
For across location analyses $N = 18$.

Location	Data source	Statistic	Moisture[a]	Protein	Fat	Ash	Carbo-hydrates	ADF	NDF	TDF	Starch
Across all	Event 5307	mean	10.13	10.86	4.54	1.46	83.1	2.74	8.85	11.8	69.4
		range	9.54–11.4	9.12–12.6	3.85–4.93	1.22–1.60	81.0–85.3	2.23–3.34	7.68–9.52	10.8–13.4	62.0–73.7
	Nontransgenic	mean	10.18	10.92	4.72	1.40	83.0	2.85	8.83	11.7	70.3
		range	9.21–12.2	9.20–13.0	4.43–5.09	1.09–1.67	80.7–84.7	2.47–3.48	7.79–10.2	10.6–13.5	63.1–77.3
	ANOVA (*F* test)										
	Genotype effect	*P*	–	0.737	0.053	0.138	0.515	0.281	0.930	0.700	0.589
		SEM	–	0.375	0.067	0.044	0.44	0.069	0.128	0.19	1.21
	ILSI (2008)	mean	11.3	10.30	3.555	1.439	84.6	4.05	11.23	16.43	57.7
		range	6.1–40.5	6.15–17.26	1.742–5.823	0.616–6.282	77.4–89.5	1.82–11.34	5.59–22.64	8.85–35.31	26.5–73.8
		N	1434	1434	1174	1410	1410	1350	1349	397	168
	OECD (2002)	range	7.0–23	6–12.7	3.1–5.8	1.1–3.9	82.2–82.9	3.0–4.3	8.3–11.9	11.1	NA

– = not applicable
NA = not available
[a]Grain was mechanically dried after harvest so moisture levels were not subject to ANOVA

Table 5.2. Proximate composition of forage from 5307 corn and nontransgenic corn

Proximate levels are shown in % DW, except for moisture (% FW). Results significantly different at *P* <0.05 are shown in bold italic type.

For across location analyses *N* = 18.

Location	Data source	Statistic	Moisture	Protein	Fat	Ash	Carbo-hydrates	ADF	NDF
Across all	Event 5307	mean	73.0	7.72	1.90	4.12	86.3	29.1	44.9
		range	66.5–79.5	5.91–10.3	0.893–2.81	2.89–5.35	82.9–88.9	22.3–40.1	35.5–56.1
	Nontransgenic	mean	72.3	7.57	1.89	4.34	86.2	28.6	45.4
		range	66.7–78.0	6.27–10.0	0.843–2.63	3.43–6.18	82.3–89.0	19.0–41.5	32.3–57.4
	ANOVA (*F* test)								
	Genotype effect	*P*	0.126	0.525	0.893	0.076	0.895	0.696	0.785
		SEM	1.49	0.449	0.118	0.295	0.64	1.33	1.91
	ILSI (2008)	mean	70.2	7.78	2.039	4.628	85.6	27.00	41.51
		range	49.1–81.3	3.14–11.57	<LOQ–4.570	1.527–9.638	76.4–92.1	16.13–47.39	20.29–63.71
		[a]*N*	945	945	921	945	945	945	945
	OECD (2002)	range	62–78	4.7–9.2	1.5–3.2	2.9–5.7	NA	25.6–34	40–48.2

NA = not available

[a]*N* is the number of ILSI values used to calculate the mean and excludes values <LOQ

Table 5.3. Mineral composition of grain from 5307 corn and nontransgenic corn

Mineral levels shown in mg/kg DW except OECD values in mg/100g. Results significant at $P < 0.05$ are shown in bold italic type. For across location analyses $N = 18$.

Location	Data source	Statistic	Ca	Cu	Fe	Mg	Mn	P	K	Se[a,c]	Na[b,c]	Zn
Across all	Event 5307	mean	43.9	1.52	23.7	1323	5.65	3228	3758	–	–	23.0
		range	38.6–49.3	0.89–4.20	21.2–28.0	1150–1430	4.69–6.61	2620–3520	3400–4010	<LOQ–0.363	< LOQ	19.5–26.9
	Nontransgenic	mean	44.0	1.89	23.3	1336	5.43	3307	3776	–	–	23.4
		range	40.3–50.1	1.02–4.36	20.3–28.1	1220–1450	4.43–6.38	2650–3600	3240–4150	<LOQ–0.400	< LOQ	20.5–27.9
	ANOVA (*F* test)											
	Genotype effect	*P*	0.891	0.058	0.308	0.401	0.131	0.110	0.707	–	–	0.355
		SEM	1.28	0.253	0.85	21.2	0.249	94.7	81.0	–	–	0.78
	ILSI (2008)	mean	46.4	1.75	21.81	1193.8	6.18	3273.5	3842	0.20	31.75	21.6
		range	12.7–208.4	<LOQ–18.50	10.42–49.07	594.0–1940.0	1.69–14.30	1470.0–5330.0	1810.0–6030.0	<LOQ–0.75	<LOQ–731.54	6.5–37.2
		[d]*N*	1344	1249	1255	1257	1256	1349	1257	89	223	1257
	OECD (2002)	range	3–100	0.09–1.0	0.1–10	82–1000	NA	234–750	320–720	0.001–0.1	0–150	1.2–3.0

– = not applicable
NA = not available
[a]The LOQ for selenium was 0.055–0.056 mg/kg DW
[b]The LOQ for sodium was 110–114 mg/kg DW
[c]Where some or all values were <LOQ, calculation of the mean and statistical comparison was not possible so only the range is shown
[d]*N* is the number of ILSI values used to calculate the mean and excludes value <LOQ

Table 5.4. Calcium and phosphorus composition of forage from 5307 corn and nontransgenic corn

Calcium and phosphorus levels shown in mg/kg DW. Results significantly different at $P < 0.05$ are shown in bold italic type. For across location analyses $N = 18$.

Location	Data source	Statistic	Ca	P
Across all	Event 5307	mean	2346	1906
		range	1450–3470	1420–2870
	Nontransgenic	mean	2354	1953
		range	1660–3350	1390–2890
	ANOVA (*F* test)			
	Genotype effect	*P*	0.886	0.491
		SEM	209.3	163.9
	ILSI (2008)	mean	2028.6	2066.1
		range	713.9–5767.9	936.2–3704.1
		N	481	481
	OECD (2002)	range	0.15–0.31	0.20–0.27

Table 5.5. Vitamin composition of grain from 5307 corn and nontransgenic corn

Vitamin levels shown in mg/100 g DW except as indicated for vitamin E (mg/g) and OECD values (mg/kg). Results significant at $P < 0.05$ are shown in bold italic type.

For across location analyses $N = 18$.

Location	Data source	Statistic	Vitamin A β-carotene	Vitamin B_1 Thiamine	Vitamin B_2 Riboflavin	Vitamin B_3 Niacin	Vitamin B_6 Pyridoxine	Vitamin B_9 Folic Acid	Vitamin E[a] α-tocopherol
Across all	Event 5307	mean	0.155	0.449	0.198	3.13	0.692	0.0397	0.0093
		range	0.133–0.185	0.399–0.511	0.156–0.264	2.53–4.11	0.587–0.769	0.0305–0.0460	0.00719–0.0111
	Nontransgenic	mean	0.176	0.458	0.198	3.18	0.737	0.0382	0.0090
		range	0.155–0.216	0.408–0.518	0.152–0.318	2.51–3.70	0.621–0.815	0.0289–0.0463	0.00607–0.0110
	ANOVA (*F* test)								
	Genotype effect	*P*	***<0.001***	0.146	0.941	0.674	***0.005***	***0.031***	0.074
		SEM	0.0049	0.0126	0.0096	0.104	0.0167	0.00199	0.00055
	ILSI (2008)	mean	0.684	0.530	0.125	2.376	0.644	0.0651	0.0103
		range	0.019–4.681	0.126–4.000	0.050–0.236	1.037–4.694	0.368–1.132	0.0147–0.1464	0.0015–0.0687
		N	276	894	704	415	415	895	863
	OECD (2002)	range	NA	2.3–8.6	0.25–5.6	9.3–70	4.6–9.6	NA	NA

NA = not available

[a]Original units of mg/100 g reported by Covance Laboratories were converted to mg/g

Table 5.6. Amino acid composition of grain from 5307 corn and nontransgenic corn

Amino acid levels shown in mg/g DW except OECD values in % DW. Results significant at $P < 0.05$ are shown in bold italic type. For across location analyses $N = 18$.

Location	Data source	Statistic	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Cys	Val
Across all	Event 5307	mean	6.93	3.80	5.14	20.4	9.23	3.95	8.21	2.33	5.12
		range	5.82–8.15	3.19–4.39	4.10–6.10	16.4–24.9	7.55–11.0	3.52–4.39	6.64–9.97	2.07–2.50	4.33–6.08
	Nontransgenic	mean	6.88	3.79	5.17	20.6	9.24	3.97	8.24	2.36	5.13
		range	6.00–8.20	3.36–4.47	4.44–6.28	17.4–25.3	7.84–10.9	3.61–4.35	7.06–10.0	2.14–2.59	4.28–6.01
	ANOVA (*F* test)										
	Genotype effect	*P*	0.625	0.908	0.736	0.715	0.973	0.761	0.846	0.284	0.877
		SEM	0.236	0.123	0.203	0.90	0.375	0.087	0.345	0.043	0.179
	ILSI (2008)	mean	6.88	3.75	5.12	20.09	9.51	3.85	7.90	2.21	4.90
		range	3.35–12.08	2.24–6.66	2.35–7.69	9.65–35.36	4.62–16.32	1.84–5.39	4.39–13.93	1.25–5.14	2.66–8.55
		N	1350	1350	1350	1350	1350	1350	1350	1350	1350
	OECD (2002)	range	0.48–0.85	0.27–0.58	0.35–0.91	1.25–2.58	0.63–1.36	0.26–0.49	0.56–1.04	0.08–0.32	0.21–0.85

(continued)

Table 5.6 (*continued*). Amino acid composition of grain from 5307 corn and nontransgenic corn

Amino acid levels shown in mg/g DW except OECD values in % DW. Results significant at $P < 0.05$ are shown in bold italic type. For across location analyses $N = 18$.

Location	Data source	Statistic	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Trp
Across all	Event 5307	mean	2.29	3.92	13.8	3.18	5.50	3.10	2.99	4.81	0.570
		range	1.97–2.51	3.19–4.77	10.8–17.1	1.57–4.18	4.34–6.68	2.76–3.36	2.57–3.44	3.72–5.56	0.381–0.704
	Nontransgenic	mean	2.36	3.91	13.8	3.26	5.52	3.09	3.01	4.82	0.557
		range	2.08–2.56	3.23–4.71	11.5–17.3	1.67–3.98	4.73–6.70	2.74–3.38	2.57–3.43	4.20–5.32	0.380–0.700
	ANOVA (*F* test)										
	Genotype effect	*P*	0.102	0.947	0.789	0.711	0.883	0.902	0.684	0.892	0.722
		SEM	0.049	0.163	0.66	0.153	0.239	0.059	0.088	0.144	0.0298
	ILSI (2008)	mean	2.09	3.68	13.41	3.36	5.25	3.15	2.96	4.33	0.627
		range	1.24–4.68	1.79–6.92	6.42–24.92	1.03–6.42	2.44–9.30	1.72–6.68	1.37–4.34	1.19–6.39	0.271–2.150
		N	1350	1350	1350	1350	1350	1350	1350	1350	1350
	OECD (2002)	range	0.10– 0.46	0.22–0.71	0.79–2.41	0.12–0.79	0.29–0.64	0.05–0.55	0.15–0.38	0.22–0.64	0.04–0.13

Table 5.7. Fatty acid[a] composition of grain from 5307 corn and nontransgenic corn

Fatty acids shown as % of total fatty acids except OECD values in % DW. Results significant at *P* <0.05 are shown in bold italic type. For across location analyses *N* = 18.

Location	Data source	Statistic	16:0 Palmitic	16:1 Palmitoleic[b]	18:0 Stearic	18:1 Oleic	18:2 Linoleic	18:3 Linolenic	20:0 Arachidic	20:1 Eicosenoic	22:0 Behenic
Across all	Event 5307	mean	15.7	–	1.74	24.5	55.6	1.60	0.392	0.250	0.220
		range	15.1–16.1	<LOQ–0.137	1.50–2.04	22.0–27.0	53.2–58.1	1.48–1.71	0.353–0.453	0.238–0.265	0.186–0.252
	Nontransgenic	mean	15.2	–	1.81	24.9	55.7	1.50	0.387	0.242	0.213
		range	14.6–15.9	<LOQ–0.450	1.54–2.17	22.6–26.4	53.8–58.4	1.40–1.57	0.361–0.437	0.232–0.261	0.194–0.247
	ANOVA (*F* test)										
	Genotype effect	*P*	***<0.001***	–	***0.038***	0.108	0.599	***<0.001***	0.186	***<0.001***	0.243
		SEM	0.07	–	0.059	0.54	0.60	0.017	0.0098	0.0029	0.0056
	ILSI (2008)	mean	11.50	0.154	1.82	25.8	57.6	1.20	0.412	0.297	0.176
		range	7.94–20.71	<LOQ–0.447	1.02–3.40	17.4–40.2	36.2–66.5	0.57–2.25	0.279–0.965	0.170–1.917	<LOQ–0.349
		N	1344	596	1344	1344	1344	1344	988	987	924
	OECD (2002)	range	0.29–0.79	NA	0.04–0.17	0.70–1.39	0.67–2.81	0.03–0.10	NA	NA	NA

– = not applicable

NA = not available

[a] Where some or all values were <LOQ, % of total fatty acids could not be calculated and statistical analysis could not be performed. Levels <LOQ were observed for all replicates at all locations for 8:0 caprylic, 10:0 capric, 12:0 lauric, 14:0 myristic, 14:1 myristoleic, 15:0 pentadecanoic, 15:1 pentadecenoic, 17:0 heptadecanoic, 17:1 heptadecenoic, 20:2 eicosadienoic, 20:3 eicosatrienoic, and 20:4 arachidonic fatty acids

[b] Some values were <LOQ so only the range is shown

Table 5.8. Secondary metabolite and antinutrient composition of grain from 5307 corn and nontransgenic corn

Analyte units as in column headings except OECD values for ferulic acid and ρ-coumaric acid in % DW. Results significant at $P < 0.05$ are shown in bold italic type. For across location analyses $N = 18$.

Location	Data source	Statistic	Ferulic acid (mg/kg DW)	ρ-Coumaric acid (mg/kg DW)	Inositol (ppm DW)	Phytic acid (% DW)	Trypsin inhibitor (TIU/mg DW)	Furfural[a,b] (mg/kg DW)	Raffinose (% DW)
Across all	Event 5307	mean	1906	186	2510	0.910	3.34	–	0.156
		range	1670–2190	153–229	2120–3160	0.671–1.03	2.39–4.42	< LOQ	0.115–0.199
	Nontransgenic	mean	1889	186	2504	0.942	3.46	–	0.163
		range	1620–2090	148–226	1980–3060	0.729–1.06	2.22–3.94	< LOQ	0.119–0.188
	ANOVA (*F* test) Genotype effect	*P*	0.691	0.926	0.951	0.216	0.393	–	0.066
		SEM	52.4	9.1	86.1	0.0261	0.118	–	0.0087
	ILSI (2008)	mean	2201.1	218.4	1331.5	0.745	2.73	3.697	0.132
		range	291.9–3885.8	53.4–576.2	89.0–3765.4	0.111–1.570	<LOQ–7.18	<LOQ–6.340	<LOQ–0.320
		[c] *N*	817	817	504	1196	696	14	701
	OECD (2002)	range	0.02–0.3	0.003–0.03	NA	0.45–1.0	NA	< 0.01	0.21–0.31

– = not applicable

NA = not available

[a] The LOQ for furfural was 0.55–0.57 mg/kg DW

[b] All values were <LOQ and therefore statistical comparison was not possible

[c] *N* is the number of ILSI values used to calculate the mean and excludes value <LOQ

D. Information related to the nutritional impact of the genetically-modified food

The application must contain the following information if the compositional analysis indicates biologically significant changes to the levels of certain nutrients in the GM food compared to the non-GM counterpart food:

1. Data to allow the nutritional impact of compositional changes in the food to be assessed

This part includes data on the anticipated dietary intake of the GM food in relation to the overall diet, together with any information which may indicate a change to the bioavailability of the nutrients from the GM food.

Quantification of eCry3.1Ab and phosphomannose isomerase in maize tissues derived from transformation Event 5307. SSB-016-09 A1 – Appendix 12.

Protein Expression Levels

The concentrations of the eCry3.1Ab and PMI proteins in various 5307 plant tissues were quantified using ELISA. The protein concentrations were measured in leaves and kernels as well as whole plants at four growth stages (whorl, anthesis, maturity and senescence) from a 5307 corn hybrid grown at four locations in the Midwest of the USA in 2008. Additionally, eCry3.1Ab and PMI concentrations were measured in kernel at plant maturity and senescence. The means across all locations for eCry3.1Ab and PMI proteins were determined on a dry- and fresh-weight basis (Tables 1 and 2).

The concentrations of eCry3.1Ab and PMI measured in this study represent the levels of these proteins in 5307 corn in various tissue types across four different locations throughout the life of the plant.

Mammalian Exposure Potential

Kernels from 5307 corn are the most likely tissue to enter the food supply, either as grain or grain by-products. The average eCry3.1Ab concentration measured in kernels from 5307 corn (4.45 µg eCry3.1Ab/g dry weight [gdw] at senescence, Table 1) represents approximately 0.004% of the total protein. Humans would potentially consume corn at the senescence stage of development, whereas livestock would be more likely to consume the kernels at maturity. The average eCry3.1Ab concentration measured in kernels at maturity was 6.19 µg eCry3.1Ab/gdw (Table 1), representing approximately 0.006% of the total protein.

The average PMI concentration measured in kernels from 5307 corn (1.11 µg PMI/gdw at senescence, Table 2) represents approximately 0.001% of the total protein. The average PMI concentration measured in kernels at maturity was 2.08 µg PMI/gdw (Table 2), representing approximately 0.002% of the total protein (these calculations are based on corn grain or kernels containing 10% total protein by weight).

Given the low levels of eCry3.1Ab and PMI in 5307 kernels, dietary exposure potential can be considered minimal. Since no health hazards have been identified for the eCry3.1Ab or PMI proteins (see safety assessment sections above), specific dietary exposure estimates are not necessary to support a conclusion regarding the safety of 5307 corn.

Table 1. Concentrations of eCry3.1Ab in 5307 corn tissues at four stages on a dry-weight (DW) and a fresh-weight (FW) basis

		µg/g DW		µg/g FW	
Stage	**Location**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**
Whorl	Leaves	142.96 ± 53.44	88.65–279.79	23.75 ± 3.16	16.81–33.80
	Whole plants	111.08 ± 38.36	75.16–178.22	15.78 ± 2.47	11.41–28.64
Anthesis	Leaves	84.34 ± 9.85	61.37–112.62	20.23 ± 2.59	13.83–27.59
	Whole plants	38.14 ± 8.72	14.18–55.67	8.11 ± 2.11	3.10–13.12
Maturity	Leaves	49.04 ± 31.79	1.46–105.60	25.33 ± 17.99	0.89–71.21
	Whole plants	16.03 ± 5.45	6.37–38.94	8.86 ± 3.93	3.36–21.96
	Kernels	6.19 ±1.87	2.37–9.64	4.56 ± 1.40	1.60–7.29
Senescence	Leaves	–	<LOQ–26.50	–	<LOQ–20.29
	Whole plants	8.27 ± 2.90	3.41–25.46	3.60 ± 0.94	1.70–10.65
	Kernels	4.45 ± 0.82	2.92–6.76	3.24 ± 0.41	2.38–4.66

Means represent data from 4 locations. At each location, 5 plants were analysed at each sampling stage. All data are corrected for extraction efficiency.
– = Not Applicable. It was not possible to calculate the mean as some values were below the limit of quantification (LOQ). LOQ = 0.10 µg/g DW and 0.02 µg/g FW.

Table 2. Concentrations of PMI in 5307 corn tissues at four stages on a dry-weight (DW) and a fresh-weight (FW) basis

		µg/g DW		µg/g FW	
Stage	**Location**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**
Whorl	Leaves	4.83 ± 1.47	2.97–8.33	0.81 ± 0.10	0.59–1.13
	Whole plants	4.23 ± 1.38	2.59–7.69	0.62 ± 0.22	0.34–1.13
Anthesis	Leaves	2.91 ± 0.33	1.74–5.20	0.70 ± 0.08	0.43–1.28
	Whole plants	4.38 ± 2.43	2.00–8.83	0.93 ± 0.47	0.44–2.13
Maturity	Leaves	–	<LOQ–3.50	–	<LOQ–1.66
	Whole plants	1.83 ± 0.51	0.68–2.56	0.96 ± 0.31	0.41–1.57
	Kernels	2.08 ± 0.49	1.04–3.82	1.36 ± 0.29	0.74–2.38
Senescence	Leaves	–	<LOD–0.54	–	<LOD–0.42
	Whole plants	0.97 ± 0.37	0.39–2.02	0.43 ± 0.14	0.15–0.71
	Kernels	1.11 ± 0.05	0.70–1.62	0.82 ± 0.06	0.50–1.28

Means represent data from 4 locations. At each location, 5 plants were analysed at each sampling stage. All data are corrected for extraction efficiency.
– = Not Applicable. It was not possible to calculate the mean as some values were below the LOQ or Limit of Detection (LOD); LOQ = 0.06 µg/g DW and 0.03 µg/g FW for leaves at maturity; LOQ = 0.05 µg/g DW and 0.03 µg/g FW for leaves at senescence; LOD = 0.01 µg/g DW and FW for leaves at senescence.

2. Data from an animal feeding study, if available

This part includes an animal feeding study with the GM food using a species that consumes the non-GM counterpart food. Such studies are typically conducted over a period of rapid growth of the animal. Other studies in animals may be conducted to enable specific effects to be measured.

Evaluation of Event 5307 transgenic maize grain in a broiler chicken feeding study. SSB-211-10 – Appendix 28.

Chickens (*Gallus domesticus*) consume large quantities of corn grain in commercial feeds. Broiler chickens, in particular, have relatively high corn consumption because conventional feeding regimens have been designed to provide maximal body weight gain in the shortest amount of time. In addition, broiler chickens are highly sensitive to small nutrient changes within their diets because of their extremely rapid growth rates. A broiler chicken study model has previously been used to assess whether consumption of transgenic corn grain can result in

adverse effects. A 49-day feeding study was performed to evaluate whether standard poultry diets prepared with 5307 corn grain had any adverse effect on male or female broiler chicken survival, growth, feed conversion (an indicator of how efficiently a bird converts feed to live body weight), or carcass yield when compared with control corn grains.

Three lots of corn grain were used to prepare poultry diets as follows: diets prepared with grain from 5307 corn grain, diets prepared with grain from a nontransgenic, near-isogenic control corn grain, and diets prepared with a commercially available lot of North Carolina (US) corn grain. The diets were formulated based on the individual nutrient analyses for each of the grains to meet standard nutritional recommendations for growing chickens (diets prepared with 55% to 63% corn) and were fed to groups of 90 male and 90 female birds for 49 consecutive days.

Parameters evaluated in the study included survival, body weight, feed conversion and carcass yield. Broiler chickens fed diets prepared with 5307 corn grain did not exhibit any adverse effects compared with chickens fed diets prepared with either the nontransgenic, near-isogenic grain or the commercially available grain. There were no statistically significant differences between groups for body weight, feed consumption, survival, overall feed conversion, and carcass yield (when expressed on an absolute weight basis). A few statistically significant differences were noted in carcass yield (when expressed as a percentage of total body weight). Male broilers fed 5307 corn grain had decreased thigh weights compared with male broilers fed nontransgenic diets, but they were not different from the males consuming the commercially available control diets. Female broilers fed 5307 diets had decreased thigh and *pectoralis minor* weights compared with female broilers fed the nontransgenic and NCSU 2007 diets. There were no differences noted in the other carcass parts including: fat pads, drums, wings, and *pectoralis* major muscle.

Overall, diets containing 5307 corn grain supported rapid broiler chicken growth at low mortality rates and excellent feed conversion ratios and there were no adverse effects on carcass yield. There were no adverse dietary effects on broiler chickens consuming diets prepared with 5307 corn grain when compared with those consuming diets prepared with nontransgenic corn grain, either as a direct effect of the transgenic proteins in the diet or as a result of any unintended compositional changes in the grain that may have altered its nutritional value. The results of this study support the conclusion that 5307 corn is nutritionally comparable to and as safe as conventional corn for consumers.

Diet samples prepared with either the 5307 corn grain or the nontransgenic, near-isogenic corn grain were evaluated for the concentrations of the eCry3.1Ab and PMI transgenic proteins. Neither eCry3.1Ab nor PMI was detected in diet samples prepared with the nontransgenic, near-isogenic corn grain. Diets prepared with the 5307 transgenic corn grain had concentrations of eCry3.1Ab ranging from 0.34 to 2.13 µg/g and concentrations of PMI ranging from less than the limit of detection (LOD = 0.012 µg/g) to 0.67 µg/g.

E. References

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F. Appendices

1. Event 5307: Copy Number Functional Element Southern Blot Analysis, SSB-189-10 A1

2. Event 5307 Maize: Insert Sequence Analysis, SSB-159-10 A1

3. Event 5307 Maize: Flanking Sequence Determination, SSB-160-10

4. Event 5307 Maize: Genetic Stability Analysis, SSb-184-10 A1

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, SSB-187-10

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, SSB-188-10

7. Event 5307 Maize: Genomic Insertion Site Analysis, SSB-202-10 A1

8. Event 5307 Maize: Mendelain Inheritance Analysis, SSB-203-10 A1

9. Evaluation of Transgenic Protein Levels in Multiple Generations of Plants Derived from Transformation Event 5307 Maize, SSB-011-08

10. Quantitation of eCry3.1Ab and Phosphomannose Isomerase in Key Processed Fractions Prepared from Event 5307 Maize Grain, SSB-004-10

11. Event 5307 Maize: Real time, Event-specific Polymerase Chain Reaction Method. SSB-237-10 & Validation of Real-time, Event-specific Polymerase Chain Reaction Method. SSB-238 -10

12. Quantification of eCry3.1Ab and phosphomannose isomerase in maize tissues derived from transformation Event 5307, SSB-016-09 A1

13. Comparison of eCry3.1Ab Protein Produced in Event 5307-Derived Maize Plants and eCry 3.1Ab Protein Produced in Recombinant *Escherchia coli*, SSB-002-09

14. Characterization of Test Substance ECRY3.1AB-028 and certificate of analysis, SSB-010-08

15. eCry3.1Ab (Entrez® Database Accession Number ADC30135): Assessment of Amino Acid Sequence Similarity to Known or Putative Toxins, SSB-165-11

16. PMI (Entrez® Database Accession Number AAA24109): Assessment of Amino Acid Sequence Similarity to Known or Putative Toxins, SSB-145-11

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

18. *In vitro* Digestibility of eCry3.1Ab Protein as Contained in Test Substance ECRY3.1AB-0208 Under Simulated Mammalian Intestinal Conditions, SSB-015-09 A1

19. *In vitro* Digestibility of Phosphomannose Isomerase (PMI) as Contained in Test Substance PMI-0105 Under Simulated Mammalian Gastric Conditions, SSB-034-07 A1

20. *In vitro* Digestibility of Phosphomannose Isomerase (PMI) as Contained in Test Substance PMI-0105 Under Simulated Mammalian Intestinal Conditions, SSB-036-07

21. Effect of temperature on the bioactivity of eCry3.1Ab protein as contained in test substance ECRY3.1AB-0208, SSB-014-09 A1

22. Effect of Temperature on Phosphomannose Isomerase (PMI) as Contained in Test Substance PMI-0105 as Assessed by Enzymatic Activity, SSB-023-09

23. Single-Dose Oral (Gavage) Toxicity Study of ECRY3.1AB-0208 in Mice with a 14-Day Observation Period WIL 639031

24. Single-Dose Oral (Gavage) Toxicity Study of PMI-0105 in Mice with a 14-Day Observation Period- WIL-639011.

25. eCry3.1Ab (Entrez® Database Accession Number ADC30135): Assessment of Amino Acid Sequence Similarity to Known or Putative Allergens, SSB-112-11

26. PMI (Entrez® Database Accession Number AAA24109): Assessment of Amino Acid Sequence Similarity to Known or Putative Allergens, SSB-102-11

27. Compositional Analysis of Forage and Grain from Event 5307 Hybrid Corn Grown During 2008 in the USA [2008 Composition Study], SSB-170-09 A1

28. Evaluation of Event 5307 transgenic corn grain in a broiler chicken feeding study, SSB-211-10