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

Supporting Document 1

SAFETY ASSESSMENT

SUMMARY AND CONCLUSIONS

Dual herbicide-tolerant maize DP-098140-6 has been genetically modified for tolerance to the broad-spectrum herbicide glyphosate and to acetolactate synthase (ALS)-inhibiting herbicides. Tolerance is conferred by expression in the plant of two novel proteins: GAT4621 and ZM-HRA. The GAT4621 enzyme is an optimised acetyltransferase with activity that results in the inactivation of the glyphosate-containing herbicides, rendering them non-phytotoxic. The ZM-HRA enzyme is a modified version of a maize ALS that can function in the presence of the ALS-inhibiting class of herbicides, thereby conferring tolerance to those herbicides.

In conducting a safety assessment of food derived from dual herbicide-tolerant maize DP-098140-6, a number of criteria have been addressed including: a characterisation of the transferred genes, their origin, function and stability in the maize genome; the changes at the level of DNA, protein and in the whole food; compositional analyses; evaluation of intended and unintended changes; and the potential for the newly expressed proteins to be either allergenic or toxic in humans.

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

History of Use

Maize is one of the major cereal crops with global production levels similar to those of wheat and rice. Maize-derived products are routinely used in a large number and diverse range of foods and have a long history of safe use. Products derived from DP-098140-6 maize (hereafter referred to as maize 98140) may include flour and other starch products, breakfast cereals, corn syrup, and corn oil.

Novel Gene Characterisation

Maize 98140 contains two novel genes, *gat4621* and *zm-hra*. The *gat4621* gene is based on the sequence of three genes from the common soil bacterium *Bacillus licheniformis*. The *zm-hra* gene is a modified maize gene. Detailed molecular analyses indicate that one copy of each novel gene has been inserted at a single site in the plant genome and the genes are stably inherited from one generation to the next. No antibiotic resistance marker genes are present in maize 98140.

Characterisation of Novel Proteins

Maize 98140 expresses two novel proteins: GAT4621 and ZM-HRA. The GAT4621 sequence is based on the GAT enzyme sequences from three strains of *B. licheniformis* that have been optimised for enhanced glyphosate acetylation activity.

The amino acid sequence of the GAT4621 protein is 75-78% identical and 90-91% similar to the translated protein sequences of the three original *gat* alleles from *B. licheniformis* from which *gat4621* was derived. GAT4621 is 147 amino acids in length and has an approximate molecular weight of 17 kDa. The GAT4621 protein is expressed at low levels in maize 98140 grain, with a mean concentration of 7.7 ng/mg of grain (dry weight).

The ZM-HRA protein is a modified version of the native ALS from maize. The herbicide tolerant ZM-HRA protein contains two specific amino acid changes in the mature ALS protein that are known to confer tolerance to sulfonylurea herbicides. The ZM-HRA protein is 638 amino acids in length with an approximate molecular weight of 69 kDa. Following transport into the chloroplast and cleavage of the transit peptide, the mature protein is 598 amino acids with a predicted molecular weight of 65 kDa. The ZM-HRA protein is also expressed at low levels in maize 98140 grain, with a mean concentration of 0.34 ng/mg of grain (dry weight).

A large number of studies have been conducted to confirm the identity and physicochemical and functional properties of the expressed GAT4621 and ZM-HRA proteins, as well as to determine their potential toxicity and allergenicity. Both proteins conform in size and amino acid sequence to that expected, do not exhibit glycosylation, and demonstrate the expected enzymatic activity.

Bioinformatic studies with the GAT4621 and ZM-HRA proteins confirmed the absence of any biologically significant amino acid sequence similarity to known protein toxins or allergens and digestibility studies demonstrated that both proteins would be rapidly degraded following ingestion, similar to other dietary proteins. Acute oral toxicity studies in mice with both proteins also confirmed the absence of toxicity. Taken together, the evidence indicates that neither protein is toxic nor likely to be allergenic in humans.

Compositional Analyses

Compositional analyses were conducted to establish the nutritional adequacy of maize 98140, and to compare it to a non-transgenic conventional maize under typical cultivation conditions. The components analysed were protein, fat, carbohydrate, amino acids, fatty acids, vitamins, minerals, and the anti-nutrients raffinose, phytic acid and trypsin inhibitor.

The compositional analyses of key components in maize 98140 indicate that, for the majority of components, there are no compositional differences of biological significance in forage or grain from transgenic maize 98140, compared to the non-GM control. Several minor differences in key nutrients and other constituents were noted, however, the mean levels observed were within the range of values observed for the non-transgenic comparator and within the range of natural variation.

As the GAT4621 enzyme also acetylates amino acids, the levels of N-acetylglutamate (NAGlu), N-acetylaspartate (NAAsp), N-acetylthreonine (NAThr), N-acetylserine (NASer) and N-acetylglycine (NAGly) in maize 98140 are elevated compared with conventional maize. Together, these acetylated amino acids account for only 0.5% of the total amino acid content in maize 98140 grain.

Both NAGlu and NAAsp were found to be present in a number of common foods, indicating that they are normal components of human diets. The other acetylated amino acids, NAThr, NASer and NAGly are also present in conventional maize and these compounds can therefore also not be considered novel. Although commercialisation of maize 98140 could potentially increase dietary exposure to acetylated amino acids slightly above current levels of exposure, acetylated amino acids are readily metabolised in humans and raise no safety issues.

Nutritional Impact

The introduction of dual herbicide-tolerant maize 98140 into the food supply would be expected to have negligible nutritional impact. This was supported by the results of two feeding studies, one in broiler chickens and another in rats. The results showed no differences in health and growth performance of broiler chickens and rats fed diets containing maize 98140 and those fed conventional maize.

Conclusion

No potential public health and safety concerns have been identified in the assessment of dual herbicide-tolerant maize 98140. On the basis of the data provided in the present Application, and other available information, food derived from maize 98140 is considered as safe for human consumption as food derived from conventional maize varieties.

1. INTRODUCTION

Dual herbicide tolerant corn line 98140 has been genetically modified for tolerance to the broad-spectrum herbicide glyphosate and acetolactate synthase (ALS)-inhibiting herbicides. The intended brand name for this product is Optimum®GAT® maize.

Maize 98140 plants express two novel proteins, GAT4621 (glyphosate acetyltransferase) and ZM-HRA (modified version of a maize acetolactate synthase). The GAT4621 protein, encoded by the *gat4621* gene, confers tolerance to glyphosate-containing herbicides by acetylating glyphosate which renders it non-phytotoxic. The ZM-HRA protein, encoded by the *zm-hra* gene, contains two specific amino acid changes to the maize GM-ALS enzyme, an essential enzyme in the biosynthesis of branched chain amino acids in plants. Expression of the ZM-HRA enzyme confers tolerance to the ALS-inhibiting class of herbicides such as the sulfonylureas.

2. HISTORY OF USE

2.1 Donor organisms

2.1.1 The gat4621 gene

The *gat4621* gene is based on the sequence of three *gat* genes from the common soil bacterium *Bacillus licheniformis*. *B. licheniformis* is an approved bacterial source for the production of a number of enzymes used as food processing aids, such as α-amylase, pullulanase (a glucanase) and serine protease. The U.S. Environmental Protection Agency has determined that this organism presents a low risk to human health and the environment when used under specific conditions for general commercial use (EPA, 1996). However, while *B. licheniformis* is widespread in the environment and people are regularly exposed to it without any associated adverse effects, non-proteinaceous toxins produced by isolates of *B. licheniformis* have been associated with food involving food poisoning incidents (see Salkinoja-Salonen *et al.*, 1999 and references therein).

2.1.2 The zm-hra gene

The *zm-hra* gene is derived from the crop plant maize, which has a long history of use as food (see following section).

2.2 Host organism

Corn (*Zea mays L)*, otherwise known as maize, is one of the leading cereal crops in the world, along with wheat and rice, and is grown in over 25 countries (OECD, 2002). In 2007, worldwide production of corn was over 700 million tonnes, with the United States and China being the major producers (FAOSTAT, 2008).

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

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, could produce lower yields (CFIA, 1994). The commercial production of corn now utilises controlled cross-pollination of two inbred lines (using conventional techniques) to combine desired genetic traits and produce hybrid varieties known to be superior to open-pollinated varieties in terms of their agronomic characteristics. This inbred-hybrid concept and resulting yield response is the basis of the modern corn seed industry and hybrid corn varieties are used in most developed countries for consistency of performance and production.

3. DESCRIPTION OF THE GENETIC MODIFICATION

3.1 Method used for genetic modification

Maize 98140 was produced by transformation of an inbred maize variety (PHWVZ) with a plasmid containing the *gat4621* and *zm-hra* expression cassettes. The genetic elements of the plasmid (PHP24279) are described in Section 3.2. Transformation was achieved by infection of maize PHWVZ with the bacterium *Agrobacterium tumefaciens* containing the PHP24279 plasmid.

A schematic diagram of the development process for maize 98140 is shown in Figure 1. A breeding diagram for maize 98140 is shown in Figure 2.

Figure 1: Development of maize 98140

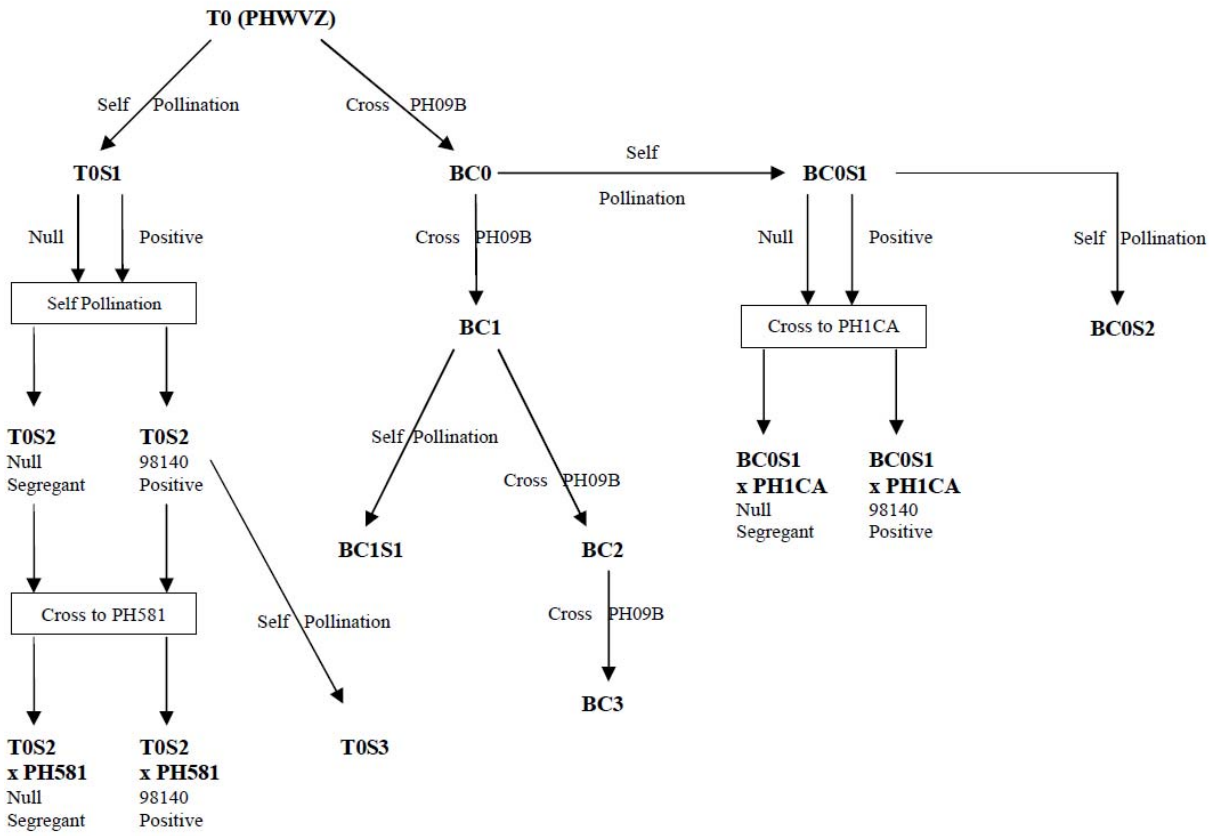

Figure 2: Breeding Diagram for maize 98140

The generations of maize 98140 and control maize used for the various analyses are shown in Table 1 below.

Table 1: Control and maize 98140 generations used for analysis

Analysis	Maize 98140 generation	Control maize generation
Molecular (DNA)	T0S3, BC1, BC0S2, BC1S1	PH09B, PHWVZ & null segregants of BC1 and BC1S1
Inheritance	BC0S1, BC1S1, BC2, BC3	Not applicable
Concentrations of GAT4621 and ZM-HRA	BC0S1 x PH1CA	Null segregants of BC0S1 x PH1CA
Compositional assessment	Grain from BC0S1 x PH1CA	Grain from null segregants of BC0S1 x PH1CA and four commercial reference hybrids
Poultry feeding study	Grain from BC0S1 x PH1CA	Grain from null segregants of BC0S1 x PH1CA and three commercial reference hybrids

3.2 Genetic elements in the plasmid DNA

A schematic map of plasmid PHP24279 is shown in Figure 3. The region from the left border to the right border is known as the transfer DNA (T-DNA) region and is shown in detail in Figure 4. The T-DNA region is the DNA fragment found in the Ti (tumour inducing) plasmid harboured by the soil bacterium *Agrobacterium tumefaciens*, which is commonly used to transfer genes to plants. A summary of the genes and regulatory elements and their position on the plasmid is provided in Table 2.

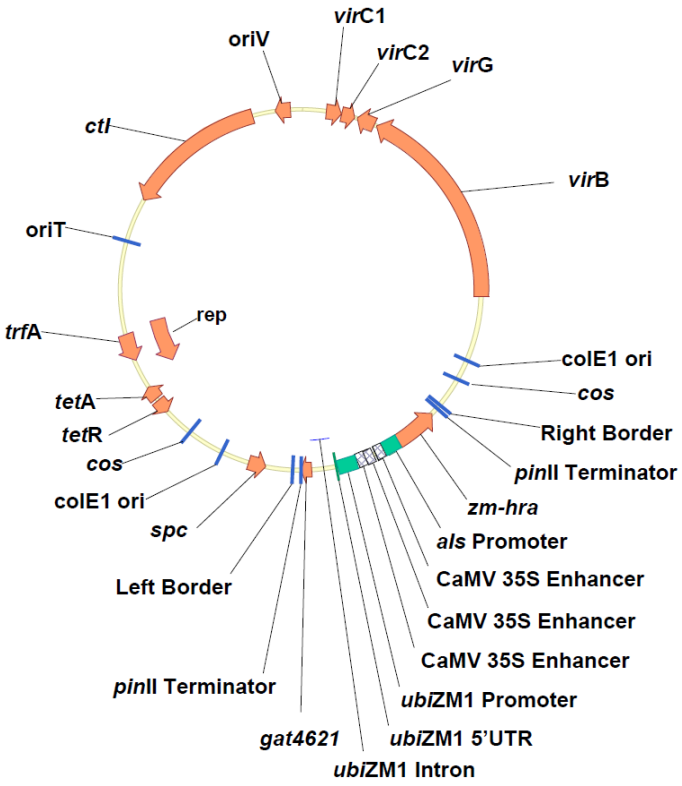

Figure 3: Map of plasmid PHP24279 with the location of genes and regulatory elements indicated. Plasmid size is 50371 base pairs.

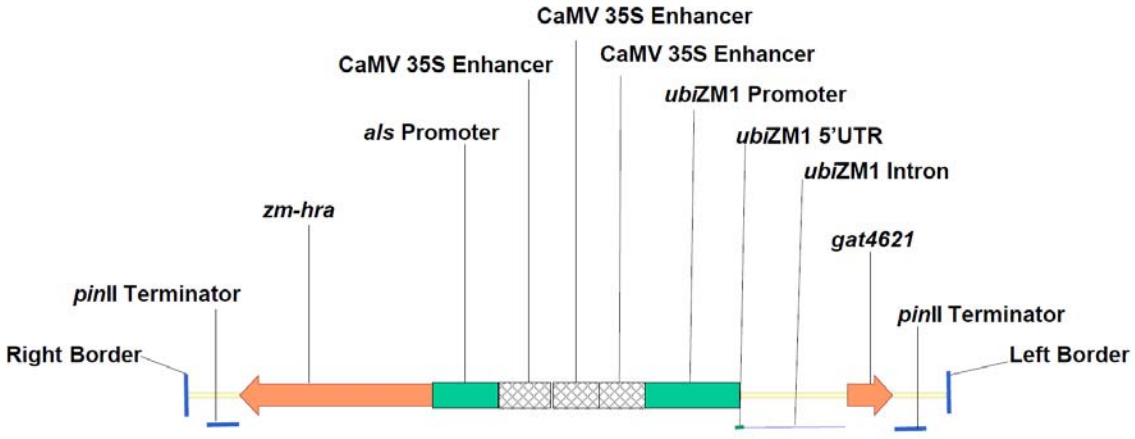

Figure 4: Map of the T-DNA region of the PHP24279 plasmid indicating the gat4621 gene and the zm-hra gene, along with their respective regulatory elements. The size of the T-DNA is 7440 base pairs.

Table 2: Genetic elements in the T-DNA of PHP24279

Location on T-DNA (base pair position)	Genetic Element	Size (base pairs)	Description
1 to 25	Right border	25	T-DNA Right Border region, from Ti plasmid of *Agrobacterium tumefaciens*
26 to 177	Ti plasmid region	152	Non-functional sequence from Ti plasmid of *A. tumefaciens*
178 to 210	Polylinker region	33	Region required for cloning genetic elements
211 to 521	*pin*II terminator	311	Terminator region from *Solanum tuberosum* proteinase inhibitor II gene (Keil *et al.,* 1986; An *et al.*, 1989). *(reverse orientation)*
522 to 537	Polylinker region	33	Region required for cloning genetic elements
538 to 2454	*zm-hra* gene	1917	Modified endogenous *Zea mays* acetolactate synthase gene (Fang *et al.*, 1992). *(reverse orientation)*
2455 to 3115	*zm-als* promoter	661	Promoter region from *Zea mays* acetolactate synthase gene (Fang *et al.*, 1992). *(reverse orientation)*
3116 to 3189	Polylinker region	74	Region required for cloning genetic elements
3190 to 3625	CaMV 35S enhancer	436	Enhancer region from the Cauliflower Mosaic Virus genome (Franck *et al.*, 1980; Odell *et al.*, 1985).
3626 to 3648	Polylinker region	23	Region required for cloning genetic elements
3649 to 4086	CaMV 35S enhancer	438	Enhancer region from the Cauliflower Mosaic Virus genome (Franck *et al.*, 1980; Odell *et al.*, 1985).
4087 to 4093	Polylinker region	7	Region required for cloning genetic elements
4094 to 4531	CaMV 35S enhancer	438	Enhancer region from the Cauliflower Mosaic Virus genome (Franck *et al.*, 1980; Odell *et al.*, 1985).
4532 to 4566	Polylinker region	35	Region required for cloning genetic elements
4567 to 5466	ubiZM1 promoter	900	Promoter region from *Zea mays* ubiquitin gene (Christensen *et al*., 1992).
5467 to 5549	ubiZM1 5' untranslated region	83	UTR 5' untranslated region from *Zea mays* ubiquitin gene (Christensen *et al*., 1992).
5550 to 6558	ubiZM1 intron	1009	Intron region from *Zea mays* ubiquitin gene (Christensen *et al*., 1992).
6559 to 6586	Polylinker region	28	Region required for cloning genetic elements
6587 to 7030	gat4621 gene	444	Synthetic glyphosate N-acetyltransferase gene (Castle *et al*., 2004; Siehl *et al*., 2005).
7031 to 7046	Polylinker region	16	Region required for cloning genetic elements
7047 to 7362	pinII terminator	316	Terminator region from *Solanum tuberosum* proteinase inhibitor II gene (Keil *et al*., 1986; An *et al*., 1989).
7363 to 7415	Ti plasmid region	53	Non-functional sequence from Ti plasmid of *A. tumefaciens*
7416 to 7440	Left border T-DNA	25	Left Border region, from Ti plasmid of *A. tumefaciens*

The T-DNA region of plasmid PHP24279 contains two gene expression cassettes. The first cassette contains the synthetic glyphosate N-acetyltransferase gene (*gat4621*) that encodes

the GAT4621 protein. The *gat* genes were isolated from three strains of *Bacillus licheniformis*, and the *gat4621* sequence was generated by functional optimisation of these genes using DNA shuffling to enhance the acetylation activity of the GAT enzyme. This process of fragmentation and recombination followed by selection can be repeated using those progeny with improved properties as parents for the next round of shuffling. In the case of the *gat4621* gene, this process was repeated eleven times using a combination of multi-gene shuffling and the introduction of genetic diversity via the polymerase chain reaction (PCR). The promoter for the *gat4621* coding region is the promoter from the maize ubiquitin gene, including a 5' untranslated region and an intron (Christensen *et al*., 1992). The terminator for the *gat4621* gene is the 3' terminator sequence from the proteinase inhibitor II gene of *Solanum tuberosum* (Keil *et al*., 1986; An *et al.*, 1989).

The second cassette of the T-DNA region of PHP24279 contains *zm-hra*, a modified version of the endogenous maize acetolactate synthase gene (*als*). The *zm-hra* gene encodes the ZM-HRA protein, which has two amino acid residues modified in comparison to the native maize ALS protein. The full-length ZM-HRA protein (including the chloroplast transit peptide sequence) is 638 amino acids in length and has an approximate molecular weight of 69 kDa. The expression of the *zm-hra* gene is controlled by the maize *als* promoter (Fang *et al*., 1992). The terminator for the *zm-hra* gene is the 3' terminator sequence from the proteinase inhibitor II gene of *Solanum tuberosum* (pinII terminator) (Keil *et al*., 1986; An *et al*., 1989).

In addition to the regulatory elements mentioned above for the gat4621 and zm-hra cassettes, three copies of the CaMV 35S enhancer region from cauliflower mosaic virus serve to enhance expression of both the *gat4621* and *zm-hra* genes (Franck *et al*., 1980; Odell *et al*., 1985).

3.3 Function and regulation of the novel genes

The *gat4621* gene is a synthetic glyphosate N-acetyltransferase gene constructed from sequences isolated from three strains of *B. licheniformis* to produce a novel gene encoding a GAT enzyme with enhanced glyphosate acetylation activity. The relevant gene in these *B. licheniformis* strains was identified using a mass spectrometry method to detect the desired product, N-acetylglyphosate (Castle *et al.*, 2004). The three *gat* genes were used as parents for fragmentation-based multigene shuffling to create enzymes with higher efficiency and increased specificity for glyphosate.

The native *gm-als* gene, which encodes for the herbicide sensitive ALS enzyme, was modified to encode two specific amino acid changes that are known to confer herbicide tolerance to the ALS enzyme, resulting in the ZM-HRA enzyme.

3.4 Characterisation of the novel genes in maize 98140

3.4.1 Insert and copy number

Studies submitted:

Brink, K. and Dietrich, N. (2007a) Characterization of Maize Event DP-Ø9814Ø-6: Insertion Stability, Copy Number, and Backbone Analysis in Two Generations. Unpublished Pioneer Report PHI-2006-100.

Brink, K. and Dietrich, N. (2007b) Characterization of DP-Ø9814Ø-6 Maize: Detailed Physical Map of Insert Region. Unpublished Pioneer Report PHI-2006-194.

Weber, N. and Igo, E. (2007) Characterization of DP-Ø9814Ø-6 Maize: Genetic Equivalence of the Inserted DNA and Mendelian Segregation within a Single Generation. Unpublished Pioneer Report PHI-2006-204.

Southern blot analysis was conducted on DNA from maize 98140 to confirm insertion copy number, genetic stability of the inserted DNA across two generations, and absence of plasmid backbone DNA. Genomic DNA samples from individual maize 98140 plants were analysed by digestion with the restriction enzymes *Eco*R V and *Spe* I followed by hybridisation with *gat4621* and *zm-hra* probes (see Figure 5 below for a schematic map of the T-DNA region and the probes used for Southern blot analysis). Analysis with *Eco*R V, examining sites in the bordering genome, indicated single copies of both the *gat4621* and *zm-hra* genes. From analysis with *Spe* I, it was determined that the PHP24279 T-DNA had likely inserted intact in the genome, as the internal restriction sites are present. In addition, all hybridisations showed consistency of the hybridisation pattern across the two generations analysed, BC0S2 and BC1, indicating genetic stability of the inserted DNA. Backbone regions not intended to be transferred from plasmid PHP24279 were not present in maize 98140.

In another study, Southern blot analysis was conducted on DNA from maize 98140 to confirm insertion copy number and integrity and to generate a physical restriction enzyme map of the insertion. Genomic DNA samples from individual maize 98140 plants (TS03 generation) were analysed by digestion with the restriction enzymes *Bam*H I, *Bgl* II, *Eco*R I, *Eco*R V, *Hind* III, and *Spe* I followed by hybridisation with probes to the *gat4621* and *zm-hra* genes and the regulatory regions, including the *als* promoter, *ubi* promoter, *ubi* intron, *pin*II terminator, and 35S enhancer. Analysis with *Bgl* II and *Eco*R V, examining sites in the bordering genome, indicated a single copy of the PHP24279 T-DNA is present in maize 98140. The *Spe* I analysis demonstrated that the PHP24279 T-DNA had likely inserted intact in the genome, as the internal restriction sites are present. Analysis with the remaining three enzymes was used in conjunction with these data to create a physical restriction map of the T-DNA insertion in maize 98140.

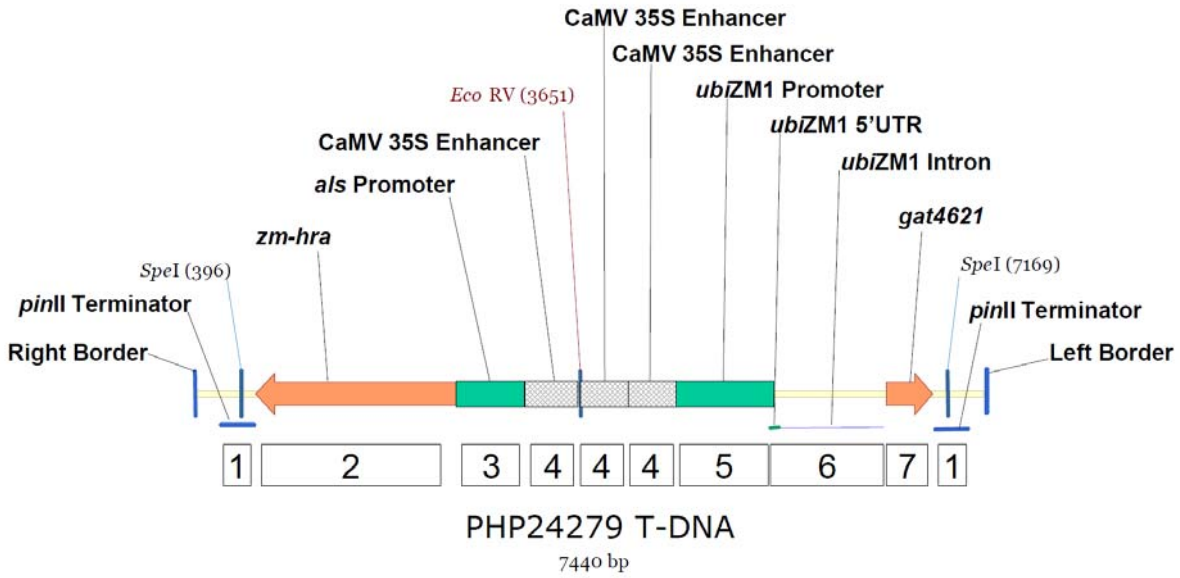

Figure 5. Map of PHP24279 T-DNA with genetic element probes indicated. Base pair positions for EcoR V and Spe I restriction enzyme sites are also shown. The total T-DNA size is 7440 base pairs. Probes used for Southern blotting are indicated schematically as numbered boxes below the map. Probe 1: pinII terminator; 2: zm-hra; 3: als promoter; 4: 35S enhancer; 5 ubiZM1 promoter; 6: ubiZM1 intron; 7: gat4621.

3.4.2 PCR and sequence analysis

Studies submitted:

Henderson, N., Zhong, C. and Cressman, M.S. (2008) Sequence characterization of inserts and genomic border regions of maize event DP-Ø9814Ø-6. Unpublished Pioneer Report PHI-2006-092/041.

The sequence of the insert and genomic border regions was determined to confirm the integrity of the inserted DNA and to characterise the genomic sequence flanking the insertion site present in maize 98140. In total, 7986 bp of maize 98140 genomic sequence was confirmed, comprising 300 bp of the 5' genomic border sequence, 300 bp of the 3' genomic border sequence, and 7386 bp of inserted DNA. The inserted T-DNA in maize 98140 was found to be intact and identical to plasmid PHP24279 except for small deletions on the T-DNA termini, a 30 base pair deletion on the Right Border terminus and a 24 base pair deletion on the Left Border terminus. The 5' and 3' genomic border regions of maize 98140 were verified to be of maize origin by PCR amplification and sequencing of the genomic border regions from both maize 98140 and control samples.

3.4.3 Bioinformatic analysis of the 5' and 3' junction regions

Studies submitted:

Henderson, N., Zhong, C. and Cressman, M.S. (2008) Sequence characterization of inserts and genomic border regions of maize event DP-Ø9814Ø-6. Unpublished Pioneer Report PHI-2006-092/041.

Both the 5' and 3' flanking genomic border sequences of maize 98140 were subjected to BLASTn analysis in an effort to characterize the location and nature of the T-DNA insertion in

the maize genome (http://blast.ncbi.nlm.nih.gov). The searches were performed against a combined dataset ("all_maize_nt") consisting of proprietary maize genomic and EST nucleotide sequences and maize-specific sequences from several public sources, including the NCBI Genbank Nucleotide ("nt") dataset (http://www.ncbi.nlm.nih.gov, Release 162), maize expressed sequence tags (ESTs) from the EST subset of Genbank, maize genomic sequences from the Genome Survey Sequence (GSS), sequence tagged sites (STS), high throughput genomic (HTG) subsets of Genbank, maize genomic assemblies and maize repeated sequences from The Institute for Genomic Research (TIGR; http://maize.tigr.org), as well as complete maize mitochondrial (AY506529), and chloroplast (X86563) DNA sequences. Default parameters were used in all cases. The insert with attached 5' and 3' genomic border sequences was also screened for the presence of novel open reading frames (ORFs) containing both a start and stop codon that spanned either the 5' or 3' insert/genomic junction and were longer than or equal to 100 amino acids (300 bp) in length using Vector NTI 9.1 sequence analysis software (Invitrogen, Carlsbad, California, USA).

When the 300 bp sequence from the 5' genomic border region of maize 98140 was used as a query in a BLASTn search against the all_maize_nt dataset, numerous highly significant overlapping alignments with public maize genomic sequences were returned encompassing the entire 300 nucleotide region. The highest scoring alignment was to nucleotides 1266 - 1565 of a 2527 bp TIGR maize assembly (AZM5_16010_TIGR), possessing 97% identity to nucleotides 1 – 300 of the maize 98140 5' genomic border. Six additional maize genomic sequences matched the same region at the same level of identity. This was followed by a single alignment between a maize genomic sequence and nucleotides 1-295 of the 5' flanking region. The next highest scoring alignment was to the same region (nt 1-299) at 97% identity to one of the 34 unordered pieces from a 205 Kb public maize chromosome 2 genomic clone (AC210003.1). Following three additional matches to maize genomic sequences, there was a significant match between nucleotides 53 – 300 of the 5' flanking region and nucleotides 311 – 559 of a 7142 bp chromosome 2 genomic clone encompassing the LIGULELESS 1 (lg1) locus. The lg1 mRNA, which encodes a transcription factor responsible for maize ligule development, begins at nucleotides 3441, 2782 nucleotides downstream from the aligned region and terminates at nucleotide 6758 of the published sequence. The remaining alignments were all to genomic sequences, and there was no evidence of expressed sequences occurring within the 5' flanking genomic region.

The 300 bp sequence from the 3' genomic border region of maize 98140 also produced numerous high scoring alignments to multiple maize genomic sequences present in the all_maize_nt dataset. The highest scoring alignment was to the same TIGR maize assembly returned from the 5' genomic border sequence. Displaying 97% identity, this alignment occurred between nucleotides 7687 - 7986 of the 3' genomic border region and nucleotides 1599 – 1898 of the assembled maize genomic sequence. There were 6 additional alignments common to both the 5' and 3' flanking regions, including a 95% identity alignment between nucleotides 7687 – 7986 of the 3' flanking region and nucleotides 593 – 894 of the lg1 genomic clone (AF451895.1). The remaining alignments were all to maize genomic sequences, and there was no evidence of expressed sequences occurring within the 3' flanking genomic region. The 5' and 3' junction regions between the maize genomic border sequence and the insertion in maize 98140 were analysed for the presence of putative novel ORFs containing both start and stop codons. No ORFs longer than or equal to 100 amino acids were identified spanning the 5' or 3' insert/genomic border regions, indicating that no novel ORFs were generated as a result of the insertion.

3.5 Stability of the genetic changes

3.5.1 Segregation data

Study submitted:

Linderblood, C. (2007) Segregation Analysis of GAT/HRA Maize Event DP-Ø9814Ø-6. Unpublished Pioneer Report PHI-2007-002.

Gene specific and event specific PCR analyses were used to confirm the presence of the *gat4621* and *zm-hra* genes, and event DP-098140-6, respectively, in four generations of maize 98140 (BC0S1, BC1S1, BC2 and BC3). The plants from the BC0S1 and BC1S1 generations were expected to segregate 3:1, and the plants from the BC2 and BC3 generations were expected to segregate 1:1 for the presence of the *gat4621* and *zm-hra* genes. In order to confirm the expected segregation ratios, polymerase chain reaction (PCR) analysis was performed on leaf punches from seedlings. Results from the segregation analysis are summarised in Table 3. As the *gat4621* and *zm-hra* gene cassettes are physically linked on the maize 98140 insert, they are expected to co-segregate. In every case, plants that were positive for the *gat4621* gene were also positive for the *zm-hra* gene, confirming co-segregation of the two genes as expected. Segregation of the *gat4621* and *zm-hra* genes, and event DP-098140-6 was evaluated by chi-square analysis which tested the goodness-of-fit of the observed segregation ratios to the expected segregation ratios based on Mendelian inheritance patterns of a single genetic locus. All P-values were greater than 0.05, indicating no statistically significant differences between the observed and expected frequencies of the *gat4621* and/or *zm-hra* genes in the four generations of maize 98140. Based on this analysis, the observed segregation ratios of the *gat4621* and *zm-hra* genes, and event DP-098140-6 were consistent with expected ratios, and indicated Mendelian inheritance patterns of a single genetic locus.

Table 3: Comparison of Observed and Expected Segregation Ratios for maize 98140

Generation	Observed		Expected			Chi-Square Test
	Positive for *gat4621* and *zm-hra* genes	Negative for *gat4621* and *zm-hra* genes	Expected Ratio	Positive for *gat4621* and *zm-hra* genes	Negative for *gat4621* and *zm-hra* genes	P-value
BC0S1	55	22	3:1	57.75	19.25	0.5537
BC1S1	45	20	3:1	48.75	16.25	0.3519
BC2	51	48	1:1	49.5	49.5	0.8407
BC3	52	45	1:1	48.5	48.5	0.5424

3.5.2 Stability of the inserted DNA

Studies submitted:

Brink, K. and Dietrich, N. (2007a) Characterization of Maize Event DP-Ø9814Ø-6: Insertion Stability, Copy Number, and Backbone Analysis in Two Generations. Unpublished Pioneer Report PHI-2006-100.
Weber, N. and Igo, E. (2007) Characterization of DP-Ø9814Ø-6 Maize: Genetic Equivalence of the Inserted DNA and Mendelian Segregation Within a Single Generation. Unpublished Pioneer Report PHI-2006-204.

The stability of the genetic change in maize 98140 over two generations was demonstrated by Southern blot analyses as described in Section 3.4. Genomic DNA from two generations (BC0S2 and BC1) of maize 98140 was analysed by digestion with the restriction enzymes *Eco*R V and *Spe* I followed by hybridisation with *gat4621* and *zm-hra* probes. All hybridisations showed consistency of the hybridisation pattern across the two generations analysed indicating genetic stability of the inserted DNA.

Southern blot analysis was conducted on the BC1S1 generation of maize 98140 to verify the stability of the insertion amongst a large number of individuals from this generation. Genomic DNA samples from a subset of individual positive and negative plants of this generation were analysed by Southern blot analysis using *Eco*R V digestion and hybridisation with the *gat4621* and *zm-hra* probes. The hybridisation pattern was consistent in all of the BC1S1 positive plants analysed and was consistent with previous Southern blot analysis on earlier generations thus confirming the stability of the insertion.

3.6 Antibiotic resistance genes

The BC0S2 and BC1 generations were analysed to confirm the absence of plasmid sequence from PHP24279 outside of the T-DNA region. The analysis confirmed the absence of backbone sequences, including any genes that encode resistance to antibiotics, in maize 98140. The *vir*G, *tet*, *spc*, LB, and RB probes were designed to detect key areas of plasmid PHP24279 outside of the T-DNA that may have been inserted during *Agrobacterium*-mediated transformation. None of the backbone probes hybridised to maize 98140 samples confirming the absence of these sequences. The observed hybridisation of the probes to the plasmid control samples indicate the probes were able to detect the target sequences and would have identified backbone sequences if they had been present in maize 98140. The Southern blots demonstrated there was no hybridisation of the probes to genomic DNA from maize 98140, indicating that plasmid regions outside of the PHP24279 T-DNA were not inserted in maize 98140. As expected, neither the *tet* gene that encodes for resistance to tetracycline in bacteria nor the *spc* gene that encodes for resistance to spectinomycin in bacteria was inserted in maize 98140.

4. CHARACTERISATION OF NOVEL PROTEINS

Maize 98140 expresses two novel enzymes: GAT4621 (a glyphosate acetyltransferase) and ZM-HRA (an acetolactate synthase), which are described below.

4.1 Function and phenotypic effects

4.1.1 GAT4621

GAT4621 is a glyphosate acetyltransferase enzyme based on N-acetyltransferase protein sequences from *Bacillus licheniformis*, a gram positive saprophytic bacterium that is widespread in nature. The GAT4621 protein is 147 amino acids in length and has an approximate molecular weight of 17 kDa. The sequence is 75-78% identical and 90-91% similar to the translated protein sequences of the three original *B. licheniformis* gat alleles from which GAT4621 was derived.

Expression of the GAT4621 protein in maize 98140 plants confers tolerance to the broad spectrum herbicide glyphosate. The GAT4621 protein detoxifies glyphosate to the non-phytotoxic N-acetylglyphosate, by acetylating the secondary amine of glyphosate using acetyl coenzyme A as an acetyl donor, as shown in Figure 6. Aminomethylphosphonic acid (AMPA) and N-acetyl AMPA are also formed as minor metabolites during metabolism of glyphosate in maize 98140 plants.

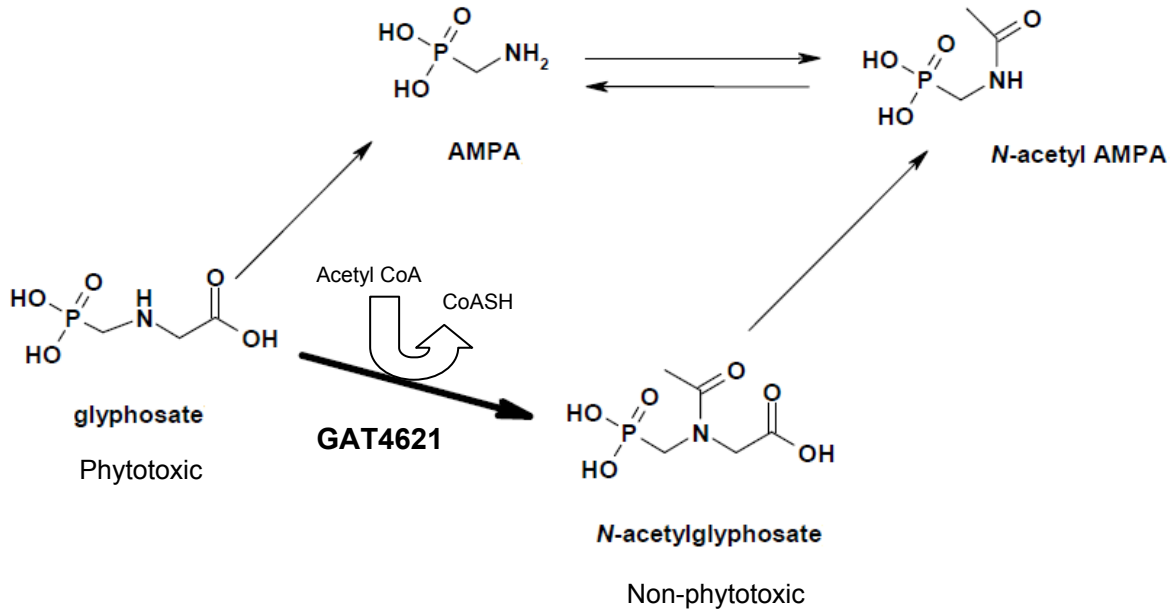

Figure 6: Enzymatic activity of GAT4621

This mechanism is an alternative mode of tolerance to that widely used in other glyphosate tolerant crops; these crops express a variant of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) which is insensitive to glyphosate inhibition, allowing continued biosynthesis of aromatic amino acids even in the presence of glyphosate.

GAT proteins are members of the GCN5-related family of N-acetyl transferases, also known as the GNAT superfamily (Dyda *et al*., 2000). This large enzyme superfamily contains over 10,000 representatives and is found in plants, animals and microbes. Members of the GNAT superfamily contain a conserved GNAT motif, but are otherwise highly sequence divergent.

Derivation of GAT4621

The GAT4621 sequence was obtained by a process of selecting optimal GAT enzyme sequences from three strains of *B. licheniformis* followed by further enhancement for increased glyphosate acetylation activity. This process is described below.

An enzyme with glyphosate acetyltransferase activity was first identified by screening a collection of several hundred *B. licheniformis* isolates using a mass spectrometry method to detect N-acetylglyphosate. Several strains of *B. licheniformis* exhibited GAT activity reproducibly (Castle *et al*., 2004). Genomic DNA fragments from two of these strains were screened in recombinant *E. coli* to identify the gene encoding GAT activity. Another gene variant was isolated from a third *B. licheniformis* strain.

The *gat* genes from these three *B. licheniformis* strains were then used as parents for fragmentation-based multigene shuffling to create enzymes with improved activity on the substrate glyphosate. In the case of the *gat4621* gene, this process was repeated eleven times using a combination of multi-gene shuffling and the introduction of genetic diversity via PCR. The initial diversity represented among the three native GAT protein sequences occurred at 12 of the 146 total amino acid positions.

To select for improved GAT activity, libraries of shuffled gene variants were created, expressed in *E. coli*, and screened for glyphosate acetylation. Variants that showed increased accumulation of N-acetylglyphosate were selected for additional rounds of shuffling. In each round of DNA shuffling, approximately 5,000 gene variants were screened and 24-48 purified enzymes were analysed to determine their kinetic properties. Typically, three to twelve variants exhibiting enhanced enzymatic properties were chosen to be the parents for the next round.

At the fifth round of shuffling, two advances were made: 1) the additional introduction of diversity by PCR incorporation of oligonucleotides based on related DNA sequences from *Bacillus cereus* and *Bacillus subtilis* during the fragment reassembly step that allowed for substitutions at 27 amino acid residues; and 2) a functional pre-screen based on resistance to glyphosate of *E. coli* strains expressing GAT (Castle *et al.*, 2004).

At the end of the 11th round of gene shuffling, the GAT activity was approximately 7000-fold improved over the native enzymes (Castle *et al.*, 2004; Siehl *et al.*, 2005).

4.1.2 ZM-HRA

The ZM-HRA protein is a modified version of the native acetolactate synthase (ALS) protein from maize. The native ALS, also known as acetohydroxyacid synthase (AHAS), is an enzyme that catalyses the first common step in the biosynthesis of the essential branched-chain amino acids isoleucine, leucine, and valine (LaRossa and Schloss, 1984; LaRossa and Falco, 1984; Duggleby and Pang, 2000; Coruzzi and Last, 2000). Two reactions are catalysed by ALS enzymes: the conversion of two molecules of pyruvate to form acetolactate leading to the synthesis of leucine and valine, and the condensation of pyruvate with 2-ketobutyrate to form 2-acetohydroxybutyrate in the pathway to isoleucine.

The ZM-HRA protein confers tolerance to the class of herbicides which act by inhibiting ALS. The herbicide tolerant *zm-hra* gene was derived by isolating an endogenous maize *als* gene, which codes for a herbicide-sensitive protein, and introducing two specific amino acid changes in the mature protein. The full-length ZM-HRA protein (including the chloroplast transit peptide sequence) is 638 amino acids in length and has an approximate molecular weight of 69 kDa.

ALS proteins contain N-terminal transit peptides, and the mature protein is formed following transport into the chloroplast and subsequent cleavage of the transit peptide. The mature protein is 598 amino acids in length with a predicted molecular weight of 65 kDa. The amino acid sequence of the ZM-HRA enzyme differs from the endogenous maize ALS sequence at only two positions: Proline165 → Alanine and Tryptophan542 → Leucine.

The ALS enzyme was first identified as the target site for sulfonylurea herbicides in the late 1970's (Levitt, 1978), and the first enzymes with herbicide tolerant activity were identified in bacteria (LaRossa and Schloss, 1984), yeast (Falco and Dumas, 1985), and plants (Chaleff and Mauvais, 1984). The respective genes were then isolated from various species and amino acid sequence changes accountable for the tolerant phenotype were identified. Several reviews are available on amino acid substitutions that result in tolerance to ALS inhibitors (Hartnett *et al*.,1990, 1991; Falco *et al*., 1989; Duggleby and Pang, 2000). For example, a resistant tobacco line was isolated through two successive rounds of tissue culture selection in the presence of sulfonylurea herbicides (Creason and Chaleff, 1988). The gene responsible for the altered ALS was identified, and the sequence of the gene identified two amino acid substitutions that contributed to the herbicide tolerant activity: mutations P196A and W573L. The locations of these two mutations are equivalent to the locations of the commonly found natural tolerance mutations, P197 and W574 (Duggleby and Pang, 2000). Both individual mutations conferred tolerance to herbicides, but the two mutations combined together typically resulted in a higher level of tolerance (Mazur and Falco, 1989; Hartnett *et al*., 1990; Creason and Chaleff, 1988).

In the case of ZM-HRA, mutations analogous to those described above in the double mutant tobacco enzyme were introduced into the sensitive version of the maize *als* gene (P165A and W542L) by site-directed mutagenesis in order to produce the *zm-hra* gene encoding the ZM-HRA herbicide tolerant enzyme.

The involvement of ALS in branched chain amino acid synthesis in plants is shown in Figure 7.

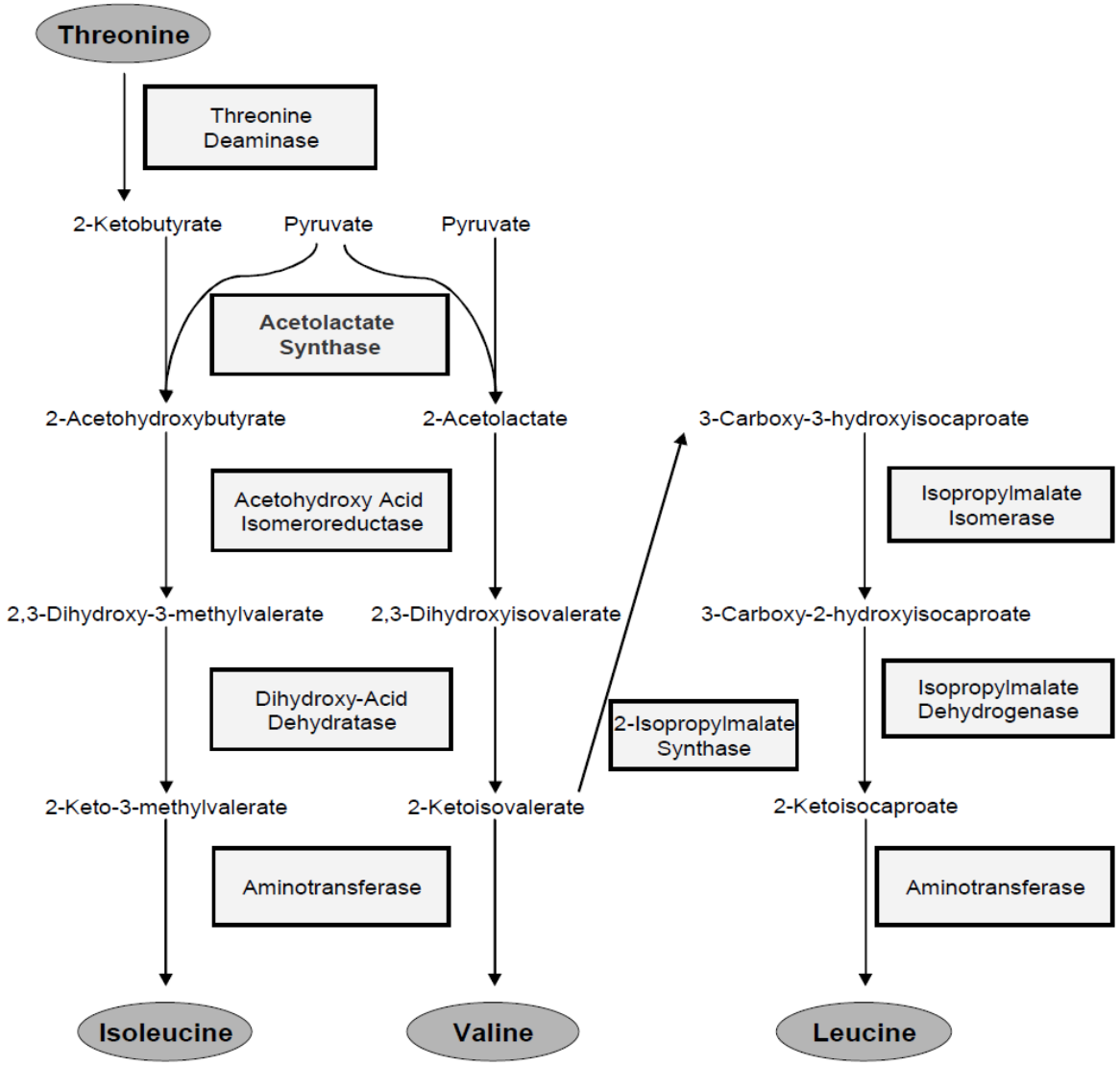

Figure 7: Branched chain amino acid biosynthesis in plants (adapted from Coruzzi and Last, 2000)

4.2 Protein Expression Analysis

Studies submitted:

Linderblood C. (2007) Expressed Trait Protein Concentration of a Maize GAT Event DP-Ø9814Ø-6: US and Canada Locations. Unpublished Pioneer Report PHI-2006-038/010.

Linderblood C. (2007) Expressed Trait Protein Concentration of a Maize GAT Event DP-Ø9814Ø-6 Treated with Herbicides: US and Canada Locations. Unpublished Pioneer Report PHI-2006-038/011.

Concentrations of the GAT4621 and ZM-HRA proteins were determined in leaf, root, whole plant, pollen, stalk, forage, and grain samples for near isoline control maize, unsprayed maize 98140, maize 98140 treated with glyphosate, maize 98140 treated with the ALS inhibiting herbicides nicosulfuron and rimsulfuron, and maize 98140 treated with glyphosate plus nicosulfuron and rimsulfuron. GAT4621 and ZM-HRA protein levels were determined using specific quantitative enzyme linked immunosorbent assay (ELISA) methods.

Site design, crop maintenance practices, and collection of leaf, root, whole plant, pollen, stalk, forage, and grain samples were described in study reports PHI-2006-038/001 and PHI-2006-038/002 (see Section 5.1). Three replicate samples per tissue per location were collected for maize 98140, and one sample per tissue per location for the control maize. Sets of samples were collected at various growth stages (see footnote to Table 4).

For the determination of GAT4621 levels, lower limits of quantification (LLOQs) were 0.072 ng/mg dry weight for stalk, forage and whole plant; 0.11 ng/mg dry weight for root and grain; 0.22 ng/mg dry weight for leaf; and 0.43 ng/mg dry weight for pollen.

For ZM-HRA, LLOQs were 0.14 ng/mg dry weight for grain; 0.18 ng/mg dry weight for stalk, and whole plant (R6); 0.27 ng/mg dry weight for root; 0.36 ng/mg dry weight for whole plant (V9 and R1) and forage; and 0.54 ng/mg dry weight for leaf and pollen.

Levels of GAT4621 and ZM-HRA were similar for non-herbicide treated and herbicide treated maize 98140 regardless of herbicide treatment. For example, the mean level of GAT4621 in grain from non-herbicide treated maize 98140 was 7.9 ng/mg (dry weight) while levels in herbicide treated maize 98140 ranged from 7.4 to 7.7 ng/mg (dry weight). The mean level of ZM-HRA in grain from herbicide-treated and non-herbicide treated maize 98140 was 0.34 ng/mg (dry weight). GAT4621 and ZM-HRA were not detected in control maize tissues. For unsprayed and sprayed maize 98140, the levels of GAT4621 and ZM-HRA protein at various growth stages are shown in Tables 4 to 7.

Table 4: Levels of GAT4621 and ZM-HRA protein in unsprayed maize 98140 at various growth stages

Growth Stage[1]	Tissue	GAT4621 (ng/mg tissue dry weight)		ZM-HRA (ng/mg tissue dry weight)	
		Mean ± SD	*Range*	*Mean ± SD*	*Range*
V9	Leaf	44 ± 14	18 – 67	6.7 ± 3.5	0.66 – 11
	Root	13 ± 7.4	1.8 – 23	0.55 ± 0.48	0 – 1.4
	Whole plant	40 ± 5.9	28 – 51	8.6 ± 2.3	4.0 – 14
R1	Pollen	13 ± 1.6	11 – 18	0 ± 0 [2]	0
	Leaf	51 ± 5.3	38 – 59	5.9 ± 3.4	2.2 – 13
	Stalk	28 ± 4.6	18 – 36	1.4 ± 0.47	0.46 – 2.2
	Root	11 ± 7.6	0.63 – 22	0.36 ± 0.39	0 – 1.3
	Whole plant	28 ± 4.9	19 – 36	4.0 ± 0.86	2.4 – 5.2
R4	Leaf	34 ± 14	3.3 – 51	5.5 ± 4.2	0 – 13
	Root	6.9 ± 4.6	0.28 – 14	0.17 ± 0.24	0 – 0.67
	Forage	16 ± 7.0	5.6 – 28	2.7 ± 1.4	0.73 – 4.8
R6	Leaf	4.3 ± 11	0 – 41	0.048 ± 0.21	0 – 0.87
	Grain	7.9 ± 3.5	3.6 – 20	0.34 ± 0.27	0 – 0.92
	Root	2.6 ± 2.8	0 – 11	0.026 ± 0.11	0 – 0.46
	Whole plant	3.5 ± 2.6	0 – 8.0	0.23 ± 0.32	0 – 0.90

[1] Growth stages are defined as follows:

V9 - The stage when the collar of the ninth leaf becomes visible;

R1 - The stage when silks become visible;

R4 - The stage when the material within the kernel produces a doughy consistency;

R6 - Typical harvest maturity for grain. This stage is regarded as physiological maturity.

[2] If all replicate concentrations were less than the LLOQ then a value of zero was reported for the mean, standard deviation and range.

Table 5: Levels of GAT4621 and ZM-HRA protein in maize 98140 treated with glyphosate

Growth Stage	Tissue	GAT4621 (ng/mg tissue dry weight)		ZM-HRA (ng/mg tissue dry weight)	
		Mean ± SD	*Range*	*Mean ± SD*	*Range*
V9	Leaf	40 ± 12	17 - 62	5.1 ± 2.2	0.94 - 8.7
	Root	14 ± 7.2	2.4 - 28	0.50 ± 0.45	0 - 1.2
	Whole plant	40 ± 4.1	30 - 46	8.7 ± 1.6	5.8 - 12
R1	Pollen	14 ± 1.5	11 - 16	0 ± 0	0
	Leaf	49 ± 12	26 - 71	5.3 ± 3.5	1.5 - 12
	Stalk	30 ± 8.6	16 - 50	1.2 ± 0.61	0 - 2.2
	Root	15 ± 5.9	4.5 - 25	0.31 ± 0.32	0 - 0.81
	Whole plant	27 ± 6.1	16 - 34	3.9 ± 0.97	2.2 - 5.7
R4	Leaf	34 ± 14	5.5 - 58	4.5 ± 4.0	0 - 11
	Root	6.3 ± 4.1	0.45 - 13	0.16 ± 0.18	0 - 0.48
	Forage	15 ± 6.2	5.6 - 28	2.2 ± 1.1	0.77 - 3.8
R6	Leaf	2.2 ± 5.5	0 - 22	0 ± 0	0
	Grain	7.7 ± 2.0	3.6 - 12	0.34 ± 0.27	0.14 - 0.89
	Root	2.0 ± 1.7	0 - 5.1	0.037 ± 0.11	0 - 0.33
	Whole plant	3.0 ± 2.2	0.38 - 7.6	0.18 ± 0.26	0 - 0.74

Table 6: Levels of GAT4621 and ZM-HRA protein in maize 98140 treated with nicosulfuron and rimsulfuron

Growth Stage	Tissue	GAT4621 (ng/mg tissue dry weight)		ZM-HRA (ng/mg tissue dry weight)	
		Mean ± SD	*Range*	*Mean ± SD*	*Range*
V9	Leaf	41 ± 8.5	24 - 57	7.0 ± 2.2	3.1 - 11
	Root	14 ± 7.0	3.9 - 25	0.65 ± 0.47	0 - 1.7
	Whole plant	39 ± 5.5	28 - 50	8.9 ± 2.5	5.7 - 15
R1	Pollen	14 ± 1.4	9.4 - 16	0.033 ± 0.14	0 - 0.60
	Leaf	52 ± 12	21 - 67	6.6 ± 4.5	0.55 - 15
	Stalk	28 ± 5.6	18 - 38	1.3 ± 0.65	0.51 - 2.8
	Root	12 ± 5.8	2.8 - 21	0.37 ± 0.38	0 - 1.0
	Whole plant	27 ± 5.6	14 - 36	4.3 ± 1.2	2.2 - 7.0
R4	Leaf	36 ± 14	8.0 - 57	5.5 ± 4.5	0 - 14
	Root	6.8 ± 5.3	0.48 - 21	0.13 ± 0.23	0 - 0.72
	Forage	16 ± 6.8	5.7 - 30	2.5 ± 1.2	0.61 - 4.2
R6	Leaf	2.6 ± 6.0	0 - 19	0 ± 0	0
	Grain	7.4 ± 1.7	5.6 - 10	0.34 ± 0.26	0 - 0.85
	Root	2.5 ± 2.0	0 - 6.1	0.064 ± 0.15	0 - 0.42
	Whole plant	3.5 ± 2.3	0.50 - 8.6	0.26 ± 0.38	0 - 1.3

Table 7: Levels of GAT4621 and ZM-HRA protein in maize 98140 treated with glyphosate, nicosulfuron and rimsulfuron

Growth Stage	Tissue	GAT4621 (ng/mg tissue dry weight)		ZM-HRA (ng/mg tissue dry weight)	
		Mean ± SD	*Range*	*Mean ± SD*	*Range*
V9	Leaf	37 ± 4.4	28 - 43	7.6 ± 2.4	3.3 - 12
	Root	15 ± 8.6	4.1 - 30	0.60 ± 0.42	0 - 1.5
	Whole plant	38 ± 4.9	26 - 46	8.7 ± 2.6	5.3 - 14
R1	Pollen	14 ± 1.6	12 - 17	0 ± 0	0
	Leaf	54 ± 7.4	39 - 68	5.0 ± 3.1	1.6 - 12
	Stalk	28 ± 5.9	18 - 38	1.2 ± 0.41	0.59 - 2.2
	Root	10 ± 7.2	0.84 - 23	0.26 ± 0.38	0 - 1.4
	Whole plant	28 ± 4.3	20 - 40	3.9 ± 0.75	2.6 - 5.2
R4	Leaf	35 ± 13	9.6 - 59	4.6 ± 3.9	0 - 13
	Root	5.8 ± 4.2	0.77 - 14	0.13 ± 0.19	0 - 0.60
	Forage	16 ± 5.6	9.6 - 28	2.4 ± 0.98	0.64 - 4.0
R6	Leaf	2.4 ± 4.6	0 - 15	0 ± 0	0
	Grain	7.4 ± 2.1	3.9 - 12	0.34 ± 0.27	0 - 0.96
	Root	2.9 ± 2.1	0 - 8.3	0.043 ± 0.13	0 - 0.47
	Whole plant	4.2 ± 2.9	0 - 9.2	0.34 ± 0.38	0 - 0.88

4.3 Characterisation of the novel proteins in maize 98140

Studies submitted:

Buffington, J. (2007) Equivalency Assessment of the Glyphosate N-Acetyltransferase 4621 Protein (GAT4621) Derived from a Microbial Expression System (Lot# PCF-0005) with the GAT4621 Protein Derived from Maize Containing Event DP-Ø9814Ø-6. Unpublished Pioneer Report, PHI-2006-205.

Comstock, B. (2007) Equivalency Assessment of the Modified Form of the Acetolactate Synthase Enzyme (ZM-HRA Protein) Derived from a Microbial Expression System (Lot # PCF-0009) with the ZM-HRA Protein Derived from Maize Containing Event DP-Ø9814Ø-6. Unpublished Pioneer Report, PHI-2006-206.

Studies were conducted to fully characterise the GAT4621 and ZM-HRA proteins produced in maize 98140. A range of analytical techniques was used to determine the identity as well as the physicochemical and functional properties of the plant-produced GAT4621 and ZM-HRA proteins isolated from maize 98140 and to compare them to the reference proteins produced in *E. coli*. These techniques included sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot analysis, mass spectrometry (MS), N-terminal sequencing, and glycosylation analysis. Enzyme activity was also determined for the purified GAT4621 and ZM-HRA proteins expressed in *E. coli*.

Due to the difficulties in extracting and purifying sufficient quantities of the GAT4621 and ZM-HRA proteins from maize tissues where the protein is expressed at low levels, the proteins were also produced in a bacterial (*E. coli*) expression system thus facilitating high expression levels and ease of purification.

GAT4621

The GAT4621 protein extracted from maize 98140 leaf tissue was partially purified by immunoaffinity chromatography using a GAT-specific mouse monoclonal antibody. The GAT4621 protein was expressed in *E. coli* strain BL21 (DE3) and was purified in a series of chromatography steps followed by diafiltration. The molecular identity and biochemical characteristics of the GAT4621 proteins expressed in maize and in *E. coli* were examined using a variety of biochemical techniques.

The GAT4621 protein derived from maize 98140 and the *E. coli* expressed GAT4621 protein each migrated as a band with a molecular weight of approximately 16 kDa by SDS-PAGE. The maize 98140 derived GAT4621 protein and the microbially expressed GAT4621 protein each demonstrated the expected immunoreactivity in Western blot analysis using a GAT-specific antibody, which bound to the single protein band migrating at approximately 16 kDa. The primary N-terminal amino acid sequences obtained for both the plant derived and the microbially expressed GAT4621 proteins matched the theoretical sequence of the GAT4621 protein with the exception that both proteins lacked the N-terminal methionine. Electrospray mass spectrometry of purified GAT4621 expressed in *E. coli* identified a major peak at 16500 Da. The theoretical molecular weight of the *E. coli* expressed GAT4621, as determined by *in silico* translation of the *gat4621* gene is 16630 Da. The observed difference (130 Da) is consistent with the absence of the N-terminal methionine. Matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) analysis demonstrated that a tryptic digest of the microbially expressed and maize GAT4621 proteins each produced eight peptides which matched theoretical GAT4621 peptide masses, resulting in coverage of 76% and 83%, respectively, of the GAT4621 amino acid sequence. Glycosylation of the GAT4621 proteins expressed in maize and *E. coli* was not detected as demonstrated through the use of a glycoprotein staining kit. For the *E. coli* expressed GAT4621 protein, the mean K_M, k_{cat}, and k_{cat}/K_M values for the N-acetylation of glyphosate were reported to be 0.076 mM, 837 min^{-1} and 11300 $min^{-1}mM^{-1}$, respectively.

The results of this study indicate that the GAT4621 protein derived from an *E. coli* expression system is equivalent to the GAT4621 protein derived from the maize 98140 leaf tissue. The GAT4621 protein derived from the *E. coli* expression system is therefore appropriate for use in safety assessment studies as a proxy for the GAT4621 protein expressed in maize plants.

ZM-HRA

The ZM-HRA protein extracted from maize 98140 leaf tissue was partially purified by immunoaffinity chromatography using HRA-specific mouse monoclonal antibodies. The ZM-HRA protein was expressed in *E. coli* strain BL21 (DE3) RIPL as an N-terminal His-T7 fusion protein which was purified by metal-affinity chromatography followed by cleavage with thrombin. Diafiltration was used to remove the cleaved tag and thrombin. The molecular identity and biochemical characteristics of the ZM-HRA proteins expressed in maize and in *E. coli* were examined using a variety of biochemical techniques.

The ZM-HRA protein from both sources migrated as the major electrophoretic band on SDS-PAGE with a molecular weight of approximately 65 kDa and showed expected immunoreactivity with a ZM-HRA-specific antibody in Western blot analysis. The N-terminal amino acid sequences of the *E. coli* expressed and maize 98140 derived ZM-HRA proteins differed by the presence of an additional sequence of glycine-serine-cysteine at the N-terminus of the *E. coli* expressed ZM-HRA as shown in Table 8. The N-terminal glycine, serine, and cysteine residues are not part of the mature form of the maize derived ZM-HRA protein sequence but remain following thrombin cleavage of the His-T7 fusion tag used for purification of the *E. coli* expressed ZM-HRA protein.

Table 8. N-terminal amino acid sequences of ZM-HRA from maize 98140 and *E. coli*

ZM-HRA from maize 98140:				S	A	A	S	P	A	M	P	M	A
ZM-HRA expressed in *E. coli:*	G	S	C	S	A	A	S	P	A	M	P	M	A

Analysis of the *E. coli* expressed ZM-HRA protein by electrospray mass spectroscopy identified a major peak at 65086 Da. This was consistent with the calculated molecular mass of 65074 Da. MALDI-MS analysis demonstrated that trypsin digestion of the *E. coli* expressed and maize plant derived ZM-HRA proteins yielded 26 and 28 peptides matching the theoretical ZM-HRA peptide masses, respectively. The matching peptides of the *E. coli* expressed and the maize derived ZM-HRA proteins covered 73% and 77%, respectively, of the expected ZM-HRA amino acid sequence. For both the *E. coli* expressed and the maize 98140 derived ZM-HRA proteins, glycosylation was not detected using a glycoprotein staining kit. The ALS enzymatic activity of the *E. coli* expressed ZM-HRA protein was demonstrated by the formation of product of the ALS enzymatic reaction. Additionally, in the presence of chlorsulfuron, an inhibitor of ALS activity, the enzymatic activity did not decrease confirming that the protein in the assay was the herbicide-resistant ZM-HRA.

The results of this study indicate that the ZM-HRA protein derived from an *E. coli* expression system differs from the ZM-HRA protein derived from maize 98140 leaf tissue only by the presence of three additional amino acids at the N-terminus of the *E. coli* expressed protein. The ZM-HRA protein derived from the *E. coli* expression system is considered appropriate for use in safety assessment studies as a proxy for the ZM-HRA protein contained in maize plants.

Conclusion

The GAT4621 and ZM-HRA proteins expressed in maize 98140 have been analysed to confirm their identity and physicochemical and properties as well as to characterise their similarity to *E. coli*-produced GAT4621 and ZM-HRA proteins. These studies have demonstrated that the two novel proteins expressed in maize 98140 both conform in size and amino acid sequence to that expected.

The *E. coli*-produced proteins were shown to be closely similar to the plant produced proteins in terms of their size and amino acid sequence. In addition, the enzymatic activities of the *E. coli* expressed GAT4621 and ZM-HRA were demonstrated. The herbicide insensitivity of the *E. coli* expressed ZM-HRA was also demonstrated. The *E. coli*-produced proteins are considered suitable to use as substitutes for the plant-produced proteins in further safety studies.

4.4 Potential toxicity of novel proteins

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

4.4.1 History of use

GAT4621

The GAT4621 protein sequence was synthesised from the sequence of three GAT enzymes from the common soil bacterium *Bacillus licheniformis*. The GAT4621 amino acid sequence is 75-78% identical and 90-91% similar to the three GAT proteins from which it is derived. *B. licheniformis* is widespread in the environment and is an approved bacterial source for the production of a number of enzymes used as food processing aids, such as α-amylase, pullulanase (a glucanase) and serine protease. The synthesised GAT4621 enzyme is a member of the GNAT superfamily of N-acetyltransferases, which is present in all organisms, including plants, mammals, fungi, algae and bacteria (Vetting *et al*., 2005).

ZM-HRA

The ZM-HRA protein is derived from the native maize GM-ALS protein. The herbicide tolerant ZM-HRA differs from the native maize GM-ALS protein at two specific amino acids. ALS proteins are present in many species, including bacteria, fungi, algae and higher plants. Herbicide-tolerant ALS proteins are components of some existing commercial crop varieties, including maize and canola.

4.4.2 Similarities with known protein toxins

Studies submitted:
Cressman, R. (2007) Evaluation of the Amino Acid Sequence Similarity of the GAT4621 Protein to the NCBI Protein Sequence Datasets. Unpublished Pioneer Report PHI-2007-009.

Cressman, R.F. (2007) Evaluation of the Amino Acid Sequence Similarity of the ZM-HRA Protein to the NCBI Protein Sequence Datasets. Unpublished Pioneer Report PHI-2007-011.

Bioinformatic analyses are useful for assessing whether the GAT4621 and ZM-HRA proteins share any amino acid sequence similarity with known protein toxins.

The GAT4621 (147 amino acids) and ZM-HRA (656 amino acids) sequences were compared with the non-redundant ('nr') protein sequence database available from the US National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov/). The 'nr' database incorporates non-redundant entries from all Genbank nucleotide translations along with protein sequences from the SWISS-PROT, PIR, PRF and PDB databases. The NCBI database is a public database containing over 3 million protein sequences, and thus provides a robust source from which to identify any potential protein toxin homologies.

The similarity search used the BLASTP algorithm (version 2.2.13), now frequently used for searching for similarities between protein sequences (Altschul *et al*., 1997). All protein sequence alignments with an EXPECT value (*E* value) of less than 1 were identified by the BLASTP program. The *E* value reflects the degree of similarity between a pair of aligned protein sequences and can be used to evaluate the significance of the alignment (see Section 3.4.3). Although a statistically significant sequence similarity generally requires a match with an *E* value of less than 0.01, setting a threshold *E* value of 1.0 ensures that proteins with even limited similarity will not be excluded.

GAT4621

The BLASTP analysis with the GAT4621 protein sequence returned 231 entries with *E* values less than 1. The highest *E* value returned was 0.98, confirming that sequences with limited similarity were not overlooked in the search. Four of the entries returned by the

search are identical or closely similar (identity 78-100%) to acetyltransferase proteins from *Bacillus licheniformis*. Thirty-nine other accessions represent acetyltransferases from related Bacillus species, such as *B. subtilis*, *B. cereus*, and *B. thuringiensis*. The majority of the remaining 188 matching accessions represented both known and putative acetyltransferase proteins from various bacterial, archaebacterial, and eukaryotic species. The protein sequences returned by the BLASTP search do not give rise to any safety concerns for the expression of GAT4621 in genetically modified plants.

ZM-HRA

The ZM-HRA similarity search identified 2636 protein sequence entries with an *E* value of less than 1, confirming the widespread presence of similar proteins in nature. The highest *E* value returned was 0.81, confirming that sequences with limited similarity were not overlooked in the search. One hundred and five of the entries returned by the search gave a rounded *E* value = 0 and represented very closely related acetohydroxyacid synthase (AHAS) or acetolactate synthase (ALS) proteins from various plant species. A total of 1199 proteins were identified as either AHAS or ALS proteins from various bacterial, archaebacterial, and eukaryotic species. The remaining 1437 hits represented a variety of proteins, the great majority of which were functionally related by the possession of one or more well characterised conserved thiamine pyrophosphate (TPP) binding domains. The protein sequences returned by the BLASTP search do not give rise to any safety concerns for the expression of ZM-HRA in genetically modified plants.

4.4.3 Digestibility

See Section 4.5.3.

4.4.4 Thermolability

See Section 4.5.4.

4.4.5 Acute oral toxicity studies

Acute oral toxicity studies using mice were conducted to examine the potential toxicity of the GAT4621 and ZM-HRA proteins. As it is difficult to extract and purify sufficient quantities of the subject protein from transgenic plants for the acute oral toxicity studies, it has become standard practice to instead use equivalent proteins that have been produced using bacterial expression systems. For these studies, *E. coli*-produced GAT4621 and ZM-HRA proteins were used as the test substances. The equivalence of the *E. coli*- and maize 98140-produced GAT4621 and ZM-HRA proteins was established using a range of methods including SDS-PAGE, Western blot analysis, N-terminal sequencing, MALDI-MS, enzyme activity assays and glycosylation analysis (see Section 4.3).

Studies submitted:

Finlay, C. (2006) GAT4621: Acute Oral Toxicity Study in Mice.
Unpublished Pioneer Report PHI-2005-110.

Finlay, C. (2007) ZM-HRA: Acute Oral Toxicity Study in Mice.
Unpublished Pioneer Report PHI-2006-009.

GAT4621

Test material	GAT4621 preparation from *E. coli* (82% GAT4621 protein)
Vehicle	Deionised water
Test Species	Crl:CD®-1(ICR)BR mice
Dose	2000 mg/kg bw by oral gavage (GAT4621 dose 1640 mg/kg)
Control	Bovine serum albumin (2000 mg/kg) or vehicle alone

Ten fasted mice (5/sex) received a single dose of GAT4621 protein administered by oral gavage at a target dose of 2000 mg/kg corresponding to an actual dose of 1640 mg/kg. Control groups of ten fasted mice (5/sex) were administered bovine serum albumin at a dose of 2000 mg/kg, or water, once by oral gavage. Mice were approximately 9 weeks old on the day of dosing.

Mice were observed for mortality, body weight effects and clinical signs for 14 days after dosing. At the end of the study all animals were killed and examined post mortem for organ or tissue damage or dysfunction.

All mice survived through the duration of the study. No clinical signs of systemic toxicity were observed. No gross lesions were present in the mice at necropsy on day 14.

Under the conditions of this study, administration of GAT4621 to male and female mice at a dose of 1640 mg/kg bw produced no test substance-related clinical signs of toxicity, body weight loss, gross lesions, or mortality. These results support the conclusion that the GAT4621 protein is not acutely toxic.

ZM-HRA

Test material	ZM-HRA preparation from *E. coli* (61.8% ZM-HRA)
Vehicle	0.5% aqueous methylcellulose
Test Species	Crl:CD®-1(ICR)BR mice
Dose	2000 mg/kg bw by oral gavage (ZM-HRA dose 1236 mg/kg)
Control	Bovine serum albumin (2000 mg/kg) or vehicle alone

Ten fasted mice (5/sex) received a single dose of ZM-HRA protein administered by oral gavage at a dose of 1236 mg/kg. Control groups of ten fasted mice (5/sex) were administered bovine serum albumin at a dose of 2000 mg/kg, or vehicle, once by oral gavage. Mice were approximately 9 weeks old on the day of dosing.

Mice were observed for mortality, body weight gain and clinical signs for 14 days. At the end of the study all animals were killed and examined post mortem for organ or tissue damage or dysfunction. All mice survived through the duration of the study. No clinical signs of systemic toxicity were observed. No gross lesions were present in the mice at necropsy on day 14.

Under the conditions of this study, administration of ZM-HRA to male and female mice at a dose of 1236 mg /kg bw produced no test substance-related clinical signs of toxicity, body weight loss, gross lesions, or mortality. These results support the conclusion that the ZM-HRA protein is not acutely toxic.

4.5 Potential allergenicity of novel proteins

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

4.5.1 Source of protein

GAT4621

The GAT4621 protein in maize 98140 is similar to the glyphosate acetyltransferase from *Bacillus licheniformis*, a ubiquitous gram-positive soil bacteria. *B. licheniformis* has not been associated with any allergic effects in humans.

ZM-HRA

The ZM-HRA protein is similar to the native maize GM-ALS protein, differing only at two specific amino acids. Maize is not considered an allergenic food, although in a few case studies allergenic reactions were reported and maize allergens identified (Pasterello *et al*., 2000, 2003; Pasini *et al*., 2002). The maize ALS protein from which the ZM-HRA protein is derived, however, has not been characterised as an allergen (Weichel *et al*., 2006).

4.5.2 Similarity to known allergens

Studies submitted:

Cressman, R. (2007) Comparison of the Amino Acid Sequence Identity between the GAT4621 Protein and Known Protein Allergens. Unpublished Pioneer Report PHI-2007-008.

Cressman, R. (2007) Comparison of the Amino Acid Sequence Identity between the ZM-HRA Protein and Known Protein Allergens. Unpublished Pioneer Report PHI-2007-010.

To determine whether the GAT4621 or ZM-HRA proteins have significant sequence identity to proteins known or suspected to be allergens, the amino acid sequences of GAT4621 and ZM-HRA were compared to the Food Allergy Research and Resource Program (FARRP, University of Nebraska) Protein AllergenOnline Database (Version 7.0, January 2007) which contains 1251 amino acid sequences of known and putative allergenic proteins (www.allergenonline.com/about.asp) using established criteria (Codex, 2004). Potential similarities between the novel proteins in maize 98140 and proteins in the allergen database were evaluated using the FASTA sequence alignment algorithm (Pearson and Lipman, 1988). Alignments were inspected for identities greater than or equal to 35% over 80 or greater residues. The GAT4621 and ZM-HRA proteins were also evaluated for any 8 or greater contiguous identical amino acid matches to entries in the FARRP allergen database. These two approaches aim to identify both short contiguous regions of identity that could potentially correspond to shared IgE binding epitopes, as well as longer stretches of sequence similarity that may indicate a potentially cross-reactive protein structure.

GAT4621

None of the FASTA alignments between the GAT4621 protein sequence and the sequences in the FARRP Allergen Database exceeded the 35% threshold over 80 or greater amino acids. There were no eight or greater contiguous identical amino acid stretches in common between the GAT4621 protein sequence and any of the protein sequences in the allergen dataset. The results indicate that the GAT4621 protein does not show significant sequence identity with known allergens.

ZM-HRA

None of the FASTA alignments between the ZM-HRA protein sequence and the sequences in the FARRP Allergen Database exceeded the 35% threshold over 80 or greater amino acids. There were no eight or greater contiguous identical amino acid stretches in common between the ZM-HRA protein sequence and any of the protein sequences in the allergen dataset. The results indicate that the ZM-HRA protein does not show significant sequence identity with known allergens.

4.5.3 In vitro digestibility

Typically, food proteins that are allergenic tend to be resistant to the acidic pH of the stomach and stable to degradation from digestive enzymes such as pepsin and trypsin. The resulting intact proteins are consequently available for interaction with IgE antibodies potentially leading to an allergic response (Astwood and Fuchs, 1996; Metcalfe *et al*., 1996; Kimber *et al*., 1999). Therefore a correlation exists between the resistance of a protein to degradation by gastrointestinal enzymes and allergenic potential. As a consequence, one of the criteria for assessing potential allergenicity is to examine the stability of novel proteins in conditions mimicking human digestion. Proteins that are rapidly degraded in such conditions are considered less likely to elicit an allergic response.

Pepsin digestibility assays using simulated gastric fluid (SGF) were conducted to determine the stability of the GAT4621 and ZM-HRA proteins. In addition to the pepsin protocol, a second digestibility study was conducted using simulated intestinal fluid (SIF) containing pancreatin, which is a mixture of many enzymes including amylase, trypsin, lipase and ribonuclease.

Simulated gastric fluid studies

Studies submitted:

Comstock, B. (2007) Characterization of the *In Vitro* Pepsin Resistance of Glyphosate N-acetyltransferase 4621 Protein (GAT4621). Unpublished Pioneer Report PHI-2006-120.

Comstock, B. (2007) Characterization of the *In Vitro* Pepsin Resistance of ZM-HRA. Unpublished Pioneer Report PHI-2006-121.

The *in vitro* digestibility of the *E. coli*-derived GAT4621 and ZM-HRA proteins in SGF containing pepsin at pH 1.2 was evaluated by SDS-PAGE. Digestibility of the proteins in SGF was measured by incubating samples at 37° C for selected times (0.5, 1, 2, 5, 10, 20, 30 and 60 minutes) and subjecting these to SDS-PAGE. Two control proteins were treated in parallel with incubation times of 0, 1 and 60 minutes: bovine serum albumin (BSA) is known to hydrolyse readily in pepsin and served as a positive control; β-lactoglobulin is known to persist in pepsin and was used as a negative control.

Both the GAT4621 and ZM-HRA proteins were rapidly hydrolysed in SGF, with no GAT4621 or ZM-HRA protein detectable after exposure to SGF for 30 seconds. The BSA positive control was also rapidly hydrolysed (< 1 minute) while the β-lactoglobulin negative control showed no degradation.

Simulated intestinal fluid studies

Studies submitted:

Comstock, B. (2007) Characterization of the *In Vitro* Pancreatin Resistance of Glyphosate N-acetyltransferase 4621 Protein (GAT4621). Unpublished Pioneer Report PHI-2006-122.

Comstock, B. (2007) Characterization of the *In Vitro* Pancreatin Resistance of ZM-HRA. Unpublished Pioneer Report PHI-2006-123.

The digestibility of *E. coli*-derived GAT4621 and ZM-HRA proteins in SIF containing pancreatin was assessed using SDS-PAGE. Digestibility of the proteins in SIF was measured by incubating samples with SIF at pH 7.5 and 37° C, for specified time intervals (0, 0.5, 1, 2, 5, 10, 20, 30 and 60 minutes), and analysing by SDS-PAGE with protein staining, and also Western blot analysis.

Two control proteins were treated in parallel: bovine serum albumin (BSA) as a negative control (pancreatin resistant) and β-lactoglobulin as a positive control (pancreatin sensitive). The controls were incubated in SIF for 0, 1 and 60 minutes. Control proteins were detected by protein staining.

No visible GAT4621 protein was present following Western blot analysis at five minutes, indicating that the GAT4621 protein was hydrolysed in less than five minutes in SIF. No visible ZM-HRA band was observed following Western blot analysis at one minute.

The β-lactoglobulin positive control was also hydrolysed, with a faint band visible on a protein stained gel after one minute incubation, but no band visible after 60 minutes. The BSA negative control was only partially hydrolysed after 60 minutes.

4.5.4 Thermolability

Studies submitted:

Siehl, D. and Locke, M. (2007) Characterization of the Thermal Stability of Glyphosate Acetyltransferase Enzyme Activity: GAT4621. Unpublished Pioneer Report PHI-2006-184/018.

Comstock, B. (2007a) Characterization of the Thermal Stability of ZM-HRA Enzyme Activity. Unpublished Pioneer Report PHI-2007-089.

Comstock, B. (2007b) Characterization of the Effect of Heat Treatment on the Immunoreactivity of the ZM-HRA Protein using Western Blot Analysis. Unpublished Pioneer Report PHI-2007-130.

The heat stabilities of the *E. coli*-produced GAT4621 and ZM-HRA proteins were evaluated by examining loss of enzyme activity after heating to temperatures ranging from 36 to 60° C for 15 minutes. Following heat treatment, the enzyme activities of GAT4621 and ZM-HRA were evaluated using a continuous absorbance spectrophotometric assay with glyphosate and pyruvate as respective substrates. The onset of the decline in GAT4621 enzyme activity occurred at approximately 46° C while a 90-93% reduction in activity was observed at temperatures ≥ 53° C. The onset of the decline in ZM-HRA enzyme activity occurred at

36° C while a 93% reduction in activity was observed at 46° C. No residual ZM-HRA enzyme activity was observed at ≥ 50° C.

The heat stability of the *E. coli*-produced ZM-HRA protein was also evaluated by examining loss of immunoreactivity after heating at 100° C for 30 minutes. Immunoreactivity, as estimated by the intensity of bands on a Western blot, decreased by at least 70% relative to unheated ZM-HRA.

4.6 Conclusion from studies on the novel proteins

Maize 98140 expresses two novel proteins, GAT4621 and ZM-HRA, at relatively low levels in the grain. For non-herbicide and herbicide treated maize 98140, the mean concentrations of GAT4621 ranged from 7.4 to 7.9 ng/mg (dry weight) while the mean concentration of ZM-HRA was 0.34 ng/mg (dry weight) irrespective of herbicide treatment.

A large number of studies on the GAT4621 and ZM-HRA proteins have been conducted to confirm their identity and physicochemical and functional properties as well as to examine their potential toxicity and allergenicity. These studies demonstrate that both proteins conform in size and amino acid sequence to that expected, do not exhibit glycosylation, and also demonstrate the expected enzyme activity.

Bioinformatic studies with the GAT4621 and ZM-HRA proteins confirmed the absence of any significant amino acid sequence similarity to known protein toxins or allergens. Digestibility studies demonstrated that both proteins would be rapidly degraded in the stomach following ingestion, similar to other dietary proteins. Furthermore, both the GAT4621 and ZM-HRA proteins are heat labile. Acute oral toxicity studies in mice with both proteins also confirmed the absence of toxicity in animals. Taken together, these results provide strong evidence that both proteins are unlikely to be toxic or allergenic in humans.

5. COMPOSITIONAL ANALYSES

A comparison of similarities and differences in composition between a GM plant and its conventional counterpart aids in the identification of potential safety and nutritional issues and is considered the most appropriate strategy for the safety and nutritional assessment of GM foods (WHO, 2000). Ideally, the comparator should be the near isogenic parental line grown under identical conditions. The composition of both herbicide-treated and untreated maize 98140 was compared to that of the non-transgenic control which was the parent maize line used for the initial transformation. In addition, compositional analyses of four different conventional maize varieties provide additional comparators to establish reference ranges for compositional constituents. Any statistically significant differences between herbicide-tolerant maize 98140 and the control maize can be compared to the reference ranges to assess whether the differences are likely to be biologically relevant.

5.1 Key components

When determining similarities and differences in composition between a GM plant and its conventional counterpart, the critical components measured are determined by identifying key nutrients, key toxicants and anti-nutrients for the food source in question (FAO, 1996). The key nutrients and anti-nutrients are those components in a particular food that can have a substantial impact on the overall diet. These can be major constituents (*e.g.*, fats, proteins, carbohydrates) or minor components (*e.g.*, minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in the plant, such as those compounds whose potency and level may be significant to health (*e.g.*, increased levels of solanine in potatoes).

Studies submitted:

Buffington J. (2005) Nutrient Composition and Agronomic Characteristics of Commercial Maize Hybrids: U.S. 2003. Unpublished Pioneer Report PHI-2003-031.
Linderblood C. (2007a) Agronomic Characteristics of a Maize GAT Event DP-Ø9814Ø-6: U.S. and Canada Locations. Unpublished Pioneer Report PHI-2006-038/001.

Linderblood C. (2007a) Agronomic Characteristics of a Maize GAT Event DP-Ø9814Ø-6 Treated with Herbicides: U.S. and Canada Locations. Unpublished Pioneer Report PHI-2006-038/002.

Buffington J. (2007a) Nutrient Composition of Maize Event DP-Ø9814Ø-6: US and Canada Locations. Unpublished Pioneer Report PHI-2006-038/020.

Buffington J. (2007b) Nutrient Composition of Maize Event DP-Ø9814Ø-6 Treated with Herbicides: US and Canada Locations. Unpublished Pioneer Report PHI-2006-038/021.

Buffington J. (2007c) Concentration of Free Amino Acids in GAT/HRA Maize Forage and Grain (Event DP-Ø9814Ø-6). Unpublished Pioneer Report PHI-2007-019.

As a minimum, the key nutrients of maize grain appropriate for a comparative study include the proximates (crude protein, fat, ash, acid detergent fibre and neutral detergent fibre), amino acids and fatty acids. In addition, international guidance suggests levels of the key anti-nutrients phytic acid and raffinose and the secondary plant metabolites furfural, ferulic acid and *p*-coumaric acid should be determined for new varieties of maize (OECD, 2002). Phytic acid is present in maize and binds about 60-75% of the phosphorus in the form of phytate. Because of phytate binding, the bioavailability of phosphorus in maize is less than 15% for non-ruminant animals. Raffinose is a non-digestible oligosaccharide considered to be an anti-nutrient due to gas production and resulting flatulence caused by its consumption.

Secondary plant metabolites are neither nutrients nor anti-nutrients. They are important though for a comparative approach to compositional analysis. Characteristic plant metabolites in maize are furfural and the phenolic acids ferulic acid and *p*-coumaric acid. Their biological function is not completely characterised, but furfural might play a role in toxicity and the phenolic acids might influence digestion, while other data suggest beneficial effects. Furfural is a heterocyclic aldehyde. It occurs in several vegetables, fruits and cereals. The phenolic acids, ferulic acid and *p*-coumaric acid are structural and functional components of plant cells. Their function is, amongst others, to act as a natural pesticide against insects and fungi.

5.2 Design and conduct of studies

Compositional analyses were conducted on maize 98140 grain to evaluate any changes in the levels of key nutrients, anti-nutrients, and secondary metabolites compared to the near-isogenic control. Analyses were conducted on non-herbicide treated maize 98140, maize 98140 treated with glyphosate, maize 98140 treated with nicosulfuron and rimsulfuron, and maize 98140 treated with glyphosate, nicosulfuron and rimsulfuron.

In a separate study, grain was also collected from four conventional (*i.e.*, non-modified) commercial maize hybrids ("reference hybrids") grown in 2003 at six field locations in maize growing areas of North America (Bagley, Iowa; York, Nebraska; Chula, Georgia; New Holland, Ohio; Larned, Kansas and Hereford, Pennsylvania). The reference hybrids were planted, harvested, processed, and analysed using similar methods to those employed for the near-isogenic control and maize 98140. Composition analysis of the reference varieties was used to help determine the normal variation for the measured analytes.

In each study, seed was planted in rows approximately 7.5 m long with spacing between rows of approximately 0.75 m. Each experimental block was planted in a randomised design of two-row plots. Each two-row plot was bordered on each side by one row of non-transgenic, commercial maize of a similar relative maturity. Three replicate samples (1 sample per block) per location per treatment were collected for a total of 18 replicates across 6 locations. Each sample replicate contained approximately 300 g of grain.

The compositional assessment was conducted in accordance with the OECD consensus document on compositional considerations for new varieties of maize (OECD, 2002). Compositional analyses of grain samples included protein, fat, acid detergent fibre (ADF), neutral detergent fibre (NDF), ash, carbohydrates, fatty acids, amino acids, vitamins and minerals, key anti-nutrients (raffinose and phytic acid) and key secondary metabolites (furfural, ferulic acid and *p*-coumaric acid). Analyses were also conducted for trypsin inhibitor, although in maize the levels are typically low and not considered nutritionally significant (OECD, 2002).

Most crops exhibit considerable variability in their nutrient composition. Environmental factors and the genotype of the plant have a significant impact on composition. Variation in nutrient parameters is a natural phenomenon and is considered normal. Therefore, in addition to a comparison of the composition of the GM food to a closely related non-GM control, it is appropriate to include a further comparison to the range of natural variation found in the conventional (non-GM) food crop.

5.2.1 Statistical analysis

Statistical analysis of composition data was conducted to test for differences in the analyte mean values between maize 98140 under spray and non-spray conditions and the near-isogenic control. Statistical evaluation of the compositional data compared the seed from the GM maize population to the non-transgenic control population and tested for statistically significant differences.

In assessing the significance of any difference between the mean analyte value for maize 98140 and the non-transgenic control, a *p*-value of less than 0.05 was defined as a statistically significant difference. In studies comprising multiple comparisons, such as numerous analytes, statistical methods exist to manage the false discovery rate (FDR) by reducing the probability of errors. The Applicant has presented data adjusted according to the method of Benjamini and Hochberg (1995) to account for making multiple comparisons. The method aims to maintain a false positive rate of 5%. The trade-off in using multiple testing correction is that the rate of false negatives (comparisons that are called non-significant when they are) is increased. Data are presented with both FDR-adjusted and non-adjusted *p*-values. In considering the compositional data provided, this assessment focussed on the non-adjusted *p*-values.

For those comparisons in which the maize 98140 test result was statistically different from the control, the test mean was compared to the tolerance interval derived from the commercial varieties. Using the data obtained from the reference hybrids, a statistical tolerance interval was calculated to contain, with 95% confidence, 99% of the values contained in the population of commercial maize. This statistical tolerance interval and the combined range of values for each analyte from the published literature, where available, provided further context for interpretation of the composition results for maize 98140. Maize 98140 analyte ranges that fell within the tolerance interval and/or combined literature range for that analyte were considered to be within the range of normal variability of commercial maize hybrids.

The results of the comparisons of grain from maize 98140, sprayed and unsprayed, and the conventional counterpart are presented in Tables 9 to 14.

Although the Applicant provided results for the compositional analyses of forage, the focus of this assessment is on the food uses of maize and therefore the forage data are not presented in this report.

5.3 Key nutrients

Levels of proximates, fatty acids, total amino acids, vitamins and minerals were measured in near-isogenic control maize grain, unsprayed maize 98140, maize 98140 treated with glyphosate, maize 98140 treated with nicosulfuron and rimsulfuron, and maize 98140 treated with glyphosate, nicosulfuron and rimsulfuron.

Proximates

Protein, fat, ADF, NDF, ash, and carbohydrates were measured in sprayed and unsprayed maize 98140 and near-isogenic control maize grain. Results are shown in Table 9. For each analyte measured, there was no statistically significant difference between maize 98140 and the near-isogenic control maize (all *p*-values were > 0.05). Hence, proximate analysis of maize grain demonstrated that maize 98140 grain is comparable to near-isogenic control and reference maize lines.

Table 9: Proximates in Grain from Unsprayed and Sprayed Maize 98140

Analyte		Control	98140 Non-spray	98140 Treated with Glyphosate, Nicosulfuron and Rimsulfuron	Tolerance Intervals[a]	Literature Ranges[b]
Proximates, Fiber, and Minerals Composition (%Dry Weight)						
Crude Protein	Mean[c]	10.8	10.6	10.9	4.11 - 15.6	6 - 17.3
	Range[d]	8.95 - 14.0	9.14 - 11.9	9.75 - 12.2		
	Standard Error	0.421	0.492	0.421		
	FDR[e]		0.8513	0.9085		
	P-value[f]		0.6939	0.5787		
Crude Fat	Mean	4.16	4.13	4.16	1.04 - 6.48	2.47 - 5.90
	Range	3.18 - 4.89	3.02 - 4.91	3.06 - 4.81		
	Standard Error	0.184	0.176	0.184		
	FDR		0.8854	0.9907		
	P-value		0.7555	0.9773		
ADF	Mean	3.12	3.08	3.14	0.958 - 6.49	1.82 - 11.3
	Range	1.81 - 4.23	1.82 - 4.02	1.78 - 4.30		
	Standard Error	0.317	0.327	0.317		
	FDR		0.7978	0.9705		
	P-value		0.6276	0.8112		
NDF	Mean	9.94	9.29	9.79	2.34 - 20.6	5.59 - 22.6
	Range	6.53 - 13.6	6.24 - 11.6	5.89 - 13.1		
	Standard Error	0.898	0.925	0.898		
	FDR		0.7324	0.9253		
	P-value		0.166	0.6865		
Ash	Mean	1.54	1.47	1.48	0.338 - 2.54	0.616 - 6.28
	Range	1.14 - 2.07	1.05 - 1.76	1.23 - 1.87		
	Standard Error	0.0860	0.0858	0.0860		
	FDR		0.7434	0.6438		
	P-value		0.2226	0.2483		
Carbohydrates (calculated)	Mean	83.5	83.8	83.4	78.2 - 91.6	77.4 - 89.5
	Range	79.3 - 85.8	81.5 - 86.7	81.6 - 85.2		
	Standard Error	0.573	0.651	0.573		
	FDR		0.7681	0.9705		
	P-value		0.5467	0.7987		

The following footnotes apply to Tables 9 to 14:

[a] Negative tolerance limits have been set to zero.

[b] Literature ranges are taken from published literature for maize (Watson, 1982, 1987; Codex, 1996; Codex, 2001; OECD, 2002 and ILSI, 2006).

[c] Least Square Means.

[d] Range denotes the lowest and highest individual value across sites.

[e] False Discovery Rate (FDR) adjusted P-value.

[f] Non-adjusted P-value.

[g] Statistical analysis was not available (NA).

[h] Analyte ranges were not reported (NR) in the published literature references.

[i] < Lower Limit of Quantification (LLOQ); Indicates that the values of the sample or samples were detected below the LLOQ of the assay.

LLOQ, Sample results which were below the LLOQ were assigned a value equal to the LLOQ for statistical analysis.

[j] TIU = trypsin inhibitor units.

[k] Adjusted P-value < 0.05.

[†] Ammonia and ethanolamine are not amino acids but are typically measured as part of a free amino acid analysis. Ornithine and γ-amino-n-butyric acid are amino acids but are not incorporated into proteins. All four compounds are included in the total free amino acid calculation.

[‡] The maize 98140 unsprayed and sprayed parameters were statistically analysed separately using two analysis of variance (ANOVA) comparisons: i) maize 98140 (unsprayed) with non GM control, and ii) maize 98140 (sprayed) with non GM control.

Fatty Acids

Levels of 28 fatty acids were measured in 98140 and near-isogenic control maize grain under both non-spray and spray conditions. Levels of 17 fatty acids were below the limit of quantification for the assay: caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), myristoleic acid (C14:1), pentadecanoic acid (C15:0), pentadecenoic acid (C15:1), heptadecenoic acid (C17:1), heptadecadienoic acid (C17:2), γ-linolenic acid (C18:3), nonadecanoic acid (C19:0), eicosadienoic acid (C20:2), eicosatrienoic acid (C20:3), arachidonic acid (C20:4), heneicosanoic acid (C21:0), erucic acid (C22:1) and tricosanoic acid (C23:0). Therefore, no statistical analyses were conducted on these fatty acids and data are not shown. Levels of the remaining 11 fatty acids are shown in Table 10. Statistically significant differences ($p < 0.05$) were observed for heptadecanoic acid (C17:0), linolenic acid (C18:3), arachidic acid (C20:0) and lignoceric acid (C24:0). However, the mean values for these fatty acids were within the ranges observed for the non-transgenic control line and were also within the range of natural variation reported in the literature. In conclusion, the fatty acid composition of maize 98140 is comparable to near-isogenic control and reference maize hybrids, irrespective of the spray or non-spray conditions.

Table 10: Major Fatty Acids in Grain from Unsprayed and Sprayed Maize 98140

Analyte		Control	98140 Non-spray	98140 Treated with Glyphosate, Nicosulfuron and Rimsulfuron	Tolerance Interval[a]	Literature Ranges[b]
Fatty Acids Composition (% Total Fatty Acids)						
Palmitic acid (C16:0)	Mean	14.1	14.2	14.3	4.85 - 19.3	7 - 20.7
	Range	13.2 - 15.6	13.4 - 15.4	13.2 - 15.6		
	Standard Error	0.291	0.290	0.291		
	FDR		0.5365	0.5759		
	P-value		0.076	0.159		
Palmitoleic acid (C16:1)	Mean	0.0989	0.0974	0.0993	NA	0 - 1
	Range	0 - 0.187	0 - 0.164	0 - 0.191		
	Standard Error	0.0321	0.0314	0.0321		
	FDR		0.9134	0.9907		
	P-value		0.7916	0.931		
Heptadecanoic acid (C17:0)	Mean	0.0645	0.0846	0.0908	NA	0 - 0.111
	Range	0 - 0.125	0 - 0.150	0 - 0.168		
	Standard Error	0.0261	0.0244	0.0261		
	FDR		0.4575	0.1221		
	P-value		0.0367[k]	0.0033[k]		
Stearic acid (C18:0)	Mean	1.39	1.36	1.37	0.635 - 2.04	0 - 4.0
	Range	1.12 - 1.75	1.06 - 1.68	1.11 - 1.64		
	Standard Error	0.0756	0.0789	0.0756		
	FDR		0.4575	0.5913		
	P-value		0.0549	0.2051		
Oleic acid (C18:1)	Mean	20.8	21.2	21.2	0 - 73.4	17.4 - 50
	Range	19.5 - 21.4	20.0 - 22.9	19.9 - 22.6		
	Standard Error	0.264	0.251	0.264		
	FDR		0.7434	0.5201		
	P-value		0.2897	0.1148		
Linoleic acid (C18:2)	Mean	61.4	61.0	60.8	21.4 - 97.3	34.0 - 70
	Range	60.7 - 63.4	59.6 - 62.0	58.6 - 62.0		
	Standard Error	0.253	0.244	0.253		
	FDR		0.7434	0.4858		
	P-value		0.307	0.0863		
Linolenic acid (C18:3)	Mean	1.21	1.16	1.22	0 - 2.91	0 - 2.25
	Range	1.03 - 1.44	1.02 - 1.39	0.975 - 1.37		
	Standard Error	0.0474	0.0446	0.0474		
	FDR		0.4575	0.939		
	P-value		0.0323[k]	0.7108		
Arachidic acid (C20:0)	Mean	0.380	0.360	0.367	NA	0 - 2
	Range	0.339 - 0.477	0.292 - 0.444	0.311 - 0.447		
	Standard Error	0.0161	0.0173	0.0161		
	FDR		0.3725	0.2664		
	P-value		0.0149[k]	0.0144[k]		
Eicosenoic acid (C20:1)	Mean	0.328	0.317	0.357	NA	0 - 1.92
	Range	0.241 - 0.456	0.240 - 0.501	0.252 - 0.536		
	Standard Error	0.0318	0.0282	0.0318		
	FDR		0.7681	0.6358		
	P-value		0.5218	0.2377		
Behenic acid (C22:0)	Mean	0.0421	0.0307	0.0416	NA	0 - 0.5
	Range	0 - 0.196	0 - 0.189	0 - 0.195		
	Standard Error	0.0301	0.0310	0.0301		
	FDR		0.7434	0.9907		
	P-value		0.2878	0.9752		
Lignoceric acid (C24:0)	Mean	0.203	0.151	0.160	NA	0 - 0.5
	Range	0 - 0.302	0 - 0.268	0 - 0.287		
	Standard Error	0.0461	0.0410	0.0461		
	FDR		0.5365	0.4129		
	P-value		0.0884	0.0372[k]		

Total amino acids

Total levels of 18 amino acids were measured in maize 98140 and near-isogenic control maize grain under both spray and non-spray conditions. As asparagine and glutamine are converted to aspartate and glutamate respectively during the analysis, listed aspartate levels include both aspartate and asparagine, while glutamate levels include both glutamate and glutamine. Results are shown in Table 11.

For 17 of the measured amino acids, there were no statistically significant differences in total levels between maize 98140, sprayed or unsprayed, and the control line. For tryptophan, the levels in unsprayed and sprayed maize 98140 were statistically significantly higher than those of the control line ($p < 0.05$). The mean values for tryptophan were however within the ranges observed for the non-transgenic control line and were also within the statistical tolerance intervals for commercial maize varieties, and within the range of natural variation reported in the literature (OECD, 2002; ILSI 2006). Therefore, maize 98140 is comparable to near-isogenic and reference maize hybrids with respect to total amino acid analysis.

Table 11: Total Amino Acids in Grain from Unsprayed and Sprayed Maize 98140

Analyte		Control	98140 Non-spray	98140 Treated with Glyphosate, Nicosulfuron and Rimsulfuron	Tolerance Intervals[a]	Literature Ranges[b]
Amino Acids Composition (% Dry Weight)						
Methionine	Mean	0.255	0.254	0.239	0.0923 - 0.535	0.10 - 0.468
	Range	0.153 - 0.369	0.142 - 0.345	0.101 - 0.374		
	Standard Error	0.0234	0.0205	0.0234		
	FDR		0.9272	0.5759		
	P-value		0.8655	0.1759		
Cystine	Mean	0.245	0.249	0.243	0.0831 - 0.360	0.08 - 0.514
	Range	0.166 - 0.346	0.186 - 0.344	0.154 - 0.352		
	Standard Error	0.0156	0.0154	0.0156		
	FDR		0.8421	0.9705		
	P-value		0.6737	0.8415		
Lysine	Mean	0.348	0.352	0.348	0.214 - 0.537	0.05 - 0.668
	Range	0.248 - 0.462	0.265 - 0.478	0.282 - 0.416		
	Standard Error	0.0154	0.0168	0.0154		
	FDR		0.867	0.9957		
	P-value		0.7283	0.9912		

Table 11 (continued):

Analyte		Control	98140 Non-spray	98140 Treated with Glyphosate, Nicosulfuron and Rimsulfuron	Tolerance Interval[a]	Literature Ranges[b]
Tryptophan	Mean	0.0653	0.0681	0.0707	0 - 0.134	0.0271 - 0.215
	Range	0.0566 - 0.0803	0.0592 - 0.0908	0.0467 - 0.0878		
	Standard Error	0.00378	0.00299	0.00378		
	FDR		0.4575	0.2192		
	P-value		0.0391[k]	0.0079[k]		
Threonine	Mean	0.455	0.455	0.450	0.158 - 0.660	0.224 - 0.666
	Range	0.353 - 0.540	0.350 - 0.556	0.379 - 0.535		
	Standard Error	0.0246	0.0214	0.0246		
	FDR		0.9967	0.9705		
	P-value		0.9834	0.816		
Isoleucine	Mean	0.359	0.351	0.371	0.121 - 0.532	0.179 - 0.71
	Range	0.305 - 0.485	0.313 - 0.392	0.325 - 0.415		
	Standard Error	0.0127	0.0135	0.0127		
	FDR			0.7319		
	P-value		0.79780.625	0.333		
Histidine	Mean	0.292	0.286	0.301	0.142 - 0.389	0.137 - 0.434
	Range	0.217 - 0.343	0.220 - 0.358	0.253 - 0.341		
	Standard Error	0.0103	0.00967	0.0103		
	FDR		0.7593	0.7749		
	P-value		0.4309	0.363		
Valine	Mean	0.483	0.472	0.494	0.179 - 0.616	0.21 - 0.855
	Range	0.424 - 0.620	0.437 - 0.549	0.447 - 0.550		
	Standard Error	0.0158	0.0160	0.0158		
	FDR		0.7681	0.7948		
	P-value		0.553	0.3938		
Analyte		**Control**	**98140 Non-spray**	**98140 Treated with Glyphosate, Nicosulfuron and Rimsulfuron**	**Tolerance Interval[a]**	**Literature Ranges[b]**
Leucine	Mean	1.36	1.30	1.38	0.333 - 2.10	0.642 - 2.49
	Range	1.11 - 1.96	1.15 - 1.52	1.18 - 1.67		
	Standard Error	0.0620	0.0663	0.0620		
	FDR		0.7681	0.9253		
	P-value		0.4896	0.6833		
Arginine	Mean	0.496	0.509	0.513	0.162 - 0.620	0.119 - 0.64
	Range	0.418 - 0.560	0.426 - 0.572	0.437 - 0.631		
	Standard Error	0.0181	0.0128	0.0181		
	FDR		0.7434	0.5913		
	P-value		0.3093	0.2014		

Table 11 (continued):

Analyte		Control	98140 Non-spray	98140 Treated with Glyphosate, Nicosulfuron and Rimsulfuron	Tolerance Interval[a]	Literature Ranges[b]
Phenylalanine	Mean	0.556	0.548	0.582	0.180 - 0.774	0.244 - 0.930
	Range	0.461 - 0.804	0.493 - 0.635	0.501 - 0.693		
	Standard Error	0.0197	0.0179	0.0197		
	FDR		0.8513	0.5759		
	P-value		0.7037	0.1439		
Glycine	Mean	0.381	0.371	0.386	0.205 - 0.528	0.184 - 0.539
	Range	0.323 - 0.445	0.333 - 0.438	0.330 - 0.422		
	Standard Error	0.0126	0.0125	0.0126		
	FDR		0.7373	0.9085		
	P-value		0.1966	0.6014		
Alanine	Mean	0.852	0.816	0.864	0.298 - 1.27	0.439 - 1.39
	Range	0.636 - 1.16	0.716 - 0.918	0.724 - 1.00		
	Standard Error	0.0407	0.0436	0.0407		
	FDR		0.7681	0.9253		
	P-value		0.4978	0.6877		
Aspartic Acid	Mean	0.819	0.797	0.820	0.332 - 1.02	0.335 - 1.21
	Range	0.689 - 1.08	0.661 - 0.921	0.694 - 0.916		
	Standard Error	0.0301	0.0308	0.0301		
	FDR		0.7593	0.9907		
	P-value		0.448	0.9609		
Glutamic Acid	Mean	2.19	2.10	2.21	0.742 - 3.26	0.965 - 3.54
	Range	1.64 - 2.89	1.66 - 2.52	1.75 - 2.90		
	Standard Error	0.141	0.122	0.141		
	FDR		0.7533	0.9675		
	P-value		0.3917	0.7445		
Analyte		**Control**	**98140 Non-spray**	**98140 Treated with Glyphosate, Nicosulfuron and Rimsulfuron**	**Tolerance Interval[a]**	**Literature Ranges[b]**
Proline	Mean	1.02	0.992	1.01	0.501 - 1.84	0.462 - 1.63
	Range	0.873 - 1.26	0.854 - 1.19	0.811 - 1.16		
	Standard Error	0.0396	0.0403	0.0396		
	FDR		0.7978	0.9907		
	P-value		0.6071	0.9426		
Serine	Mean	0.543	0.522	0.550	0.209 - 0.780	0.235 - 0.91
	Range	0.464 - 0.734	0.434 - 0.599	0.456 - 0.631		
	Standard Error	0.0220	0.0204	0.0220		
	FDR		0.7533	0.9085		
	P-value		0.3613	0.6462		
Tyrosine	Mean	0.296	0.284	0.305	0.138 - 0.435	0.103 - 0.79
	Range	0.214 - 0.359	0.225 - 0.340	0.195 - 0.367		
	Standard Error	0.0135	0.0111	0.0135		
	FDR		0.7434	0.8021		
	P-value		0.3278	0.4052		

Free amino acids

Because GAT4621 has been shown to acetylate some amino acids with low efficiency under certain *in vitro* conditions, the levels of the free amino acids were measured in 98140 and control maize grain.

Results of the analyses of free amino acid levels are presented in Table 12. There were statistically significantly differences ($p < 0.05$) compared to the control line for L-asparagine, L-lysine, L-histidine and L-arginine. In all cases the mean values in maize 98140 fell within the range of values measured for the control line. The observed differences would not be expected to have any nutritional impact. Literature values and statistical tolerance intervals for free amino acids in maize grain are not available.

Free amino acid analysis of maize grain demonstrates that maize 98140 is comparable to near-isogenic control maize, irrespective of the spray or non-spray conditions.

Table 12: Free Amino Acids in Grain from Unsprayed and Sprayed Maize 98140

Analyte		Control	98140 Non-Spray	98140 Treated with Glyphosate plus Nicosulfuron and Rimsulfuron
Free Amino Acids Composition (mg/g Dry Weight)				
L-Aspartic Acid	Mean[a]	0.235	0.238	0.222
	Range[b]	0.00832 - 0.453	0.0117 - 0.524	0.0107 - 0.446
	Std Error	0.0710	0.0710	0.0679
	FDR[c]		0.9513	0.8007
	P-Value[d]		0.933	0.5295
L-Threonine	Mean	0.0350	0.0386	0.0360
	Range	0.00129 - 0.124	0.00238 - 0.0996	0.00307 - 0.121
	Std Error	0.0134	0.0134	0.0151
	FDR		0.7125	0.9623
	P-Value		0.5083	0.859
L-Serine	Mean	0.0509	0.0603	0.0586
	Range	0.00434 - 0.163	0.00691 - 0.146	0.00415 - 0.171
	Std Error	0.0194	0.0193	0.0222
	FDR		0.4647	0.6167
	P-Value		0.0537	0.2293
L-Asparagine	Mean	0.333	0.312	0.294
	Range	0.000246 - 0.600	0 - 0.555	0 - 0.507
	Std Error	0.102	0.102	0.0968
	FDR		0.6672	0.2184
	P-Value		0.227	0.027[e]
L-Glutamic Acid	Mean	0.117	0.127	0.126
	Range	0.0118 - 0.253	0.00853 - 0.279	0.0188 - 0.262
	Std Error	0.0287	0.0285	0.0259
	FDR		0.7125	0.825
	P-Value		0.5344	0.6091

Table 12 (continued)

L-Glutamine	Mean	0.0342	0.0381	0.0376
	Range	0.00188 - 0.117	0 - 0.101	0.000358 - 0.102
	Std Error	0.0133	0.0133	0.0149
	FDR		0.7125	0.8007
	P-Value		0.4146	0.5434
L-Cysteine	Mean	0.00750	0.0186	0.0142
	Range	0 - 0.0278	0 - 0.0359	0 - 0.0387
	Std Error	0.00390	0.00386	0.00401
	FDR		0.4647	0.3891
	P-Value		0.0877	0.0878
L-Proline	Mean	0.488	0.469	0.436
	Range	0.00309 - 1.13	0.00336 - 0.907	0.00512 - 0.929
	Std Error	0.151	0.151	0.144
	FDR		0.8422	0.5524
	P-Value		0.7841	0.1894
L-Glycine	Mean	0.0432	0.0409	0.0384
	Range	0.00194 - 0.101	0.00150 - 0.0883	0.00212 - 0.0868
	Std Error	0.0130	0.0130	0.0132
	FDR		0.7125	0.5524
	P-Value		0.4955	0.1728
L-Alanine	Mean	0.105	0.109	0.0985
	Range	0.00199 - 0.300	0.00205 - 0.243	0 - 0.310
	Std Error	0.0302	0.0301	0.0372
	FDR		0.8422	0.8424
	P-Value		0.7035	0.6588
L-Valine	Mean	0.0387	0.0519	0.0468
	Range	0 - 0.125	0.00272 - 0.106	0 - 0.133
	Std Error	0.0126	0.0125	0.0143
	FDR		0.4647	0.7173
	P-Value		0.0656	0.4232

Table 12 (continued)

Analyte		Control	98140 Non-Spray	98140 Treated with Glyphosate plus Nicosulfuron and Rimsulfuron
Free Amino Acids Composition (mg/g Dry Weight)				
L-Cystine	Mean	0.000225	0.00214	0
	Range	0 - 0.00406	0 - 0.0386	0 - 0
	Std Error	0.00165	0.00161	0
	FDR		0.7125	0.9623
	P-Value		0.3865	0.8718
L-Methionine	Mean	0.0122	0.0155	0.0125
	Range	0 - 0.0368	0 - 0.0642	0.000489 - 0.0316
	Std Error	0.00463	0.00458	0.00440
	FDR		0.7125	0.9793
	P-Value		0.3856	0.9429
L-Isoleucine	Mean	0.0218	0.0289	0.0256
	Range	0.00125 - 0.0701	0.00291 - 0.0634	0.00152 - 0.0774
	Std Error	0.00781	0.00777	0.00904
	FDR		0.4923	0.7173
	P-Value		0.1136	0.4254
Leucine	Mean	0.0378	0.0472	0.0381
	Range	0.00236 - 0.132	0.00707 - 0.200	0.00378 - 0.118
	Std Error	0.0138	0.0136	0.0139
	FDR		0.7125	0.9856
	P-Value		0.4351	0.9856
L-Tyrosine	Mean	0.0490	0.0599	0.0501
	Range	0.000725 - 0.113	0.00547 - 0.0910	0.00206 - 0.104
	Std Error	0.0108	0.0108	0.0138
	FDR		0.4647	0.9623
	P-Value		0.0966	0.8801
L-Phenylalanine	Mean	0.0312	0.0372	0.0296
	Range	0.00349 - 0.0803	0.00135 - 0.120	0.00340 - 0.0710
	Std Error	0.00762	0.00751	0.00891
	FDR		0.7125	0.9527
	P-Value		0.434	0.7756
γ-Amino-n-Butyric Acid	Mean	0.192	0.124	0.152
	Range	0.00128 - 0.497	0 - 0.286	0.00220 - 0.436
	Std Error	0.0520	0.0516	0.0572
	FDR		0.6772	0.7173
	P-Value		0.3172	0.4053
Ethanolamine†	Mean	0.0198	0.0218	0.0209
	Range	0 - 0.0420	0 - 0.0363	0 - 0.0447
	Std Error	0.00504	0.00502	0.00574
	FDR		0.7125	0.9623
	P-Value		0.4529	0.81
Ammonia†	Mean	0.0342	0.0480	0.0531
	Range	0.00964 - 0.0855	0.0209 - 0.165	0.0191 - 0.141
	Std Error	0.00858	0.00854	0.0283
	FDR		0.6068	0.825
	P-Value		0.1867	0.6288

Table 12 (continued)

Analyte		Control	98140 Non-Spray	98140 Treated with Glyphosate plus Nico-sulfuron and Rimsulfuron
Free Amino Acids Composition (mg/g Dry Weight)				
L-Ornithine	Mean	0.00788	0.00891	0.00929
	Range	0 - 0.0201	0 - 0.0661	0 - 0.0366
	Std Error	0.00273	0.00265	0.00239
	FDR		0.8422	0.872
	P-Value		0.7872	0.6905
L-Tryptophan	Mean	0.00615	0.00570	0.00610
	Range	0 - 0.0225	0 - 0.0225	0 - 0.0182
	Std Error	0.00191	0.00188	0.00199
	FDR		0.8994	0.9793
	P-Value		0.8648	0.973
L-Lysine	Mean	0.104	0.155	0.135
	Range	0.00505 - 0.381	0.00783 - 0.512	0.00830 - 0.381
	Std Error	0.0418	0.0415	0.0480
	FDR		0.4647	0.2184
	P-Value		0.0983	0.035[e]
L-Histidine	Mean	0.0379	0.0468	0.0449
	Range	0 - 0.0951	0 - 0.0795	0.00282 - 0.0920
	Std Error	0.0114	0.0113	0.0125
	FDR		0.3757	0.249
	P-Value		0.0289[e]	0.0431[e]
L-Arginine	Mean	0.116	0.247	0.238
	Range	0 - 0.326	0 - 0.469	0 - 0.513
	Std Error	0.0514	0.0513	0.0698
	FDR		0.3137	0.1159
	P-Value		0.0122[e]	0.0046[e]

Minerals

Based on OECD guidance, the following minerals were analysed: calcium, copper, iron, magnesium, phosphorus, potassium, sodium and zinc (OECD, 2002). The level of phosphorus in maize 98140 treated with glyphosate, nicosulfuron and rimsulfuron was 0.344% (dry weight) which was statistically significantly lower (p = 0.033) than the level in control maize (0.379% dry weight). However, all mean values were within the ranges observed for the non-transgenic control line and were also within the range of natural variation reported in the literature (Table 13). There were no other statistically significant differences observed between maize 98140 (sprayed or unsprayed) and control maize. Therefore, maize 98140 grain is comparable to near isogenic and reference maize hybrids with regard to these mineral levels.

Table 13: Minerals in Grain from Unsprayed and Sprayed Maize 98140

Analyte		Control	98140 Non-spray	98140 Treated with Glyphosate, Nicosulfuron and Rimsulfuron	Tolerance Interval[a]	Literature Ranges[b]
Minerals Composition (% Dry Weight)						
Calcium	Mean	0.00471	0.00475	0.00431	0 - 0.00961	0.00127 - 0.100
	Range	0.00117 - 0.0103	0.00257 - 0.0105	0.00231 - 0.00673		
	Standard Error	0.000691	0.00106	0.000690		
	FDR		0.9272	0.7898		
	P-value		0.8952	0.3822		
Copper	Mean	0.000218	0.000190	0.00018	0 - 0.00114	0.000073 - 0.00185
	Range	0.000145 - 0.000490	0.000138 - 0.000249	0.000119 - 0.000285		
	Standard Error	0.0000216	0.0000238	0.0000215		
	FDR		0.7533	0.4858		
	P-value		0.3802	0.0941		
Iron	Mean	0.00216	0.00222	0.00204	0.000898 - 0.00274	0.0001 - 0.010
	Range	0.00142 - 0.00362	0.00138 - 0.00352	0.00121 - 0.00332		
	Standard Error	0.000239	0.000262	0.000238		
	FDR		0.7681	0.5742		
	P-value		0.5442	0.1319		
Magnesium	Mean	0.149	0.140	0.136	0.0613 - 0.193	0.0594 - 1.00
	Range	0.103 - 0.195	0.0607 - 0.185	0.0902 - 0.169		
	Standard Error	0.0105	0.0116	0.0105		
	FDR		0.7533	0.4858		
	P-value		0.3854	0.0724		
Phosphorus	Mean	0.379	0.363	0.344	0.103 - 0.533	0.147 - 0.750
	Range	0.261 - 0.463	0.181 - 0.501	0.256 - 0.423		
	Standard Error	0.0255	0.0301	0.0255		
	FDR		0.7593	0.3832		
	P-value		0.4391	0.0328[k]		
Potassium	Mean	0.460	0.467	0.437	0 - 0.835	0.181 - 0.720
	Range	0.270 - 0.699	0.251 - 0.766	0.315 - 0.645		
	Standard Error	0.0520	0.0451	0.0519		
	FDR		0.9272	0.9085		
	P-value		0.8535	0.6371		
Sodium	Mean	0.000931	0.00140	0.000646	0 - 0.000999	0.0 - 0.150
	Range	0.0000417 - 0.00334	0.0000417 - 0.00509	0.0000417 - 0.00218		
	Standard Error	0.000401	0.000458	0.000400		
	FDR		0.7324	0.5759		
	P-value		0.1489	0.1786		
Zinc	Mean	0.00189	0.00181	0.00177	0.00113 - 0.00254	0.00065 - 0.00372
	Range	0.00133 - 0.00293	0.00101 - 0.00291	0.00119 - 0.00251		
	Standard Error	0.000180	0.000183	0.000179		
	FDR		0.7593	0.5086		
	P-value		0.4155	0.1008		

Vitamins

Based on OECD guidance, the following vitamins were analysed: beta-carotene, vitamin B1 (thiamin), vitamin B2 (riboflavin), vitamin B6 (pyridoxine), vitamin B3 (niacin), folic acid and α-tocopherol (OECD, 2002). The level of vitamin B1 in unsprayed maize 98140 was 3.28 mg/kg (dry weight) which was statistically significantly lower (p = 0.002) than the level in control maize (3.85 mg/kg dry weight) but was within the ranges observed for the non-transgenic control line (2.18 – 4.93 mg/kg dry weight) and was also within the range of natural variation reported in the literature (Table 14). This small difference is therefore not expected to have any nutritional impact. There were no statistically significant differences in the levels of the remaining vitamins. Therefore, maize 98140 grain is comparable to near isogenic and reference maize hybrids with regard to these vitamin levels.

Table 14: Vitamins in Grain from Unsprayed and Sprayed Maize 98140

Analyte		Control	98140 Non-spray	98140 Treated with Glyphosate, Nicosulfuron and Rimsulfuron	Tolerance Interval[a]	Literature Ranges[b]
Vitamins Composition (mg/kg Dry Weight)						
Beta-carotene	Mean	14.9	13.9	14.6	0 - 30.9	0.19 - 46.8
	Range	3.86 - 28.8	4.17 - 26.6	6.01 - 25.4		
	Standard Error	3.09	3.29	3.09		
	FDR		0.7434	0.9705		
	P-value		0.2744	0.8259		
Vitamin B1 (Thiamin)	Mean	3.85	3.28	3.79	0 - 33.4	1.26 - 40.0
	Range	2.18 - 4.93	1.85 - 4.58	2.19 - 6.72		
	Standard Error	0.328	0.285	0.328		
	FDR		0.075	0.9705		
	P-value		0.002[k]	0.8252		
Vitamin B2 (Riboflavin)	Mean	1.05	<1.00[i]	<1.00[i]	NA	0.25 - 5.6
	Range	<1.00[i] - 1.85	<1.00[i]	<1.00[i]		
	Standard Error	0.0236	0.0334	0.0236		
	FDR		0.7434	0.5759		
	P-value		0.337	0.174		
Vitamin B6 (Pyridoxine)	Mean	3.80	3.80	4.58	NA	3.68 - 11.3
	Range	2.23 - 5.88	2.22 - 5.85	2.27 - 8.44		
	Standard Error	0.573	0.417	0.573		
	FDR		1	0.5189		
	P-value		1	0.108		
Vitamin B3 (Niacin)	Mean	18.2	19.8	20.2	NA	9.3 - 70
	Range	6.90 - 22.9	14.8 - 25.7	17.0 - 23.3		
	Standard Error	0.916	1.00	0.916		
	FDR		0.7434	0.5759		
	P-value		0.2421	0.1413		
Folic acid	Mean	1.15	0.800	1.00	0.114 - 1.49	0.147 - 683
	Range	0.484 - 4.23	0.470 - 1.24	0.389 - 3.44		
	Standard Error	0.241	0.211	0.241		
	FDR		0.7366	0.9017		
	P-value		0.1866	0.5646		
α-tocopherol	Mean	4.17	4.42	3.74	0 - 53.6	1.5 - 68.7
	Range	<0.500[i] - 9.52	<0.500[i] - 13.6	<0.500[i] - 9.66		
	Standard Error	1.52	1.61	1.52		
	FDR		0.7681	0.6814		
	P-value		0.5389	0.2885		
δ-tocopherol	Mean	0.663	0.815	0.697	0 - 2.39	0.38 - 16.1
	Range	<0.500[i] - 1.09	<0.500[i] - 1.72	<0.500[i] - 1.02		
	Standard Error	0.0823	0.105	0.0823		
	FDR		0.4575	0.9017		
	P-value		0.0545	0.5485		

5.4 Anti-nutrients and secondary plant metabolites

The anti-nutrients raffinose, phytic acid and trypsin inhibitor were measured in 98140 and near-isogenic control maize grain. Results are shown in Table 15. No statistically significant differences were observed between the mean values for 98140 and near-isogenic control maize for these anti-nutrients (all *p*-values were > 0.05).

Secondary plant metabolites are neither nutrients nor anti-nutrients, but it has been suggested they can be analysed as indicators of the absence of unintended effects of the genetic modification on metabolism (OECD, 2002). The characteristic plant metabolites in maize (furfural, ferulic acid and *p*-coumaric acid) were measured in 98140 and near-isogenic control maize grain. Results are shown in Table 15. No statistically significant differences were observed between the mean values for 98140 and near-isogenic control maize for any of the secondary plant metabolites measured (all *p*-values were > 0.05).

Based on these results, the levels of anti-nutrients and secondary plant metabolites in maize 98140 are comparable to those found in conventional maize.

Table 15: Anti-nutrients and Secondary Metabolites in Grain from Unsprayed and Sprayed Maize 98140

Analyte		Control	98140 Non-spray	98140 Treated with Glyphosate, Nicosulfuron and Rimsulfuron	Tolerance Interval[a]	Literature Ranges[b]
Secondary Metabolites and Anti-Nutrients Composition (% Dry Weight or as Indicated)						
Furfural	Mean	0.000119	0.000127	<0.000100[i]	NA	0 - 0.000634
	Range	<0.000100[i] - 0.000367	<0.000100[i] - 0.000412	<0.000100[i]		
	Standard Error	0.0000125	0.0000240	0.0000122		
	FDR		0.7978	0.5913		
	P-value		0.609	0.1978		
p-Courmaric acid	Mean	0.0109	0.00977	0.0122	0 - 0.0415	0.003 - 0.0576
	Range	0.00450 - 0.0182	0.00547 – 0.0137	0.00818 - 0.0165		
	Standard Error	0.000840	0.00104	0.000840		
	FDR		0.7593	0.6814		
	P-value		0.4657	0.2846		
Ferulic acid	Mean	0.126	0.117	0.141	0.0585 - 0.300	0.02 - 0.389
	Range	0.0400 - 0.239	<0.0400[i] - 0.193	0.0650 - 0.216		
	Standard Error	0.0162	0.0192	0.0162		
	FDR		0.7978	0.7077		
	P-value		0.5942	0.3149		
Raffinose	Mean	0.168	0.163	<0.160[i]	0 - 0.495	0.020 - 0.320
	Range	<0.160[i] - 0.226	<0.160[i] - 0.205	<0.160[i]		
	Standard Error	0.00310	0.00413	0.00304		
	FDR		0.7533	0.4858		
	P-value		0.3629	0.0784		
Phytic acid	Mean	0.826	0.825	0.794	0.188 - 1.29	0.111 - 1.57
	Range	0.651 - 1.22	0.629 - 1.09	0.495 - 1.10		
	Standard Error	0.0601	0.0507	0.0597		
	FDR		0.9272	0.9017		
	P-value		0.9025	0.5617		
Trypsin Inhibitor (TIU[j]/mg)	Mean	3.69	3.61	3.68	1.26 - 5.05	1.09 - 7.18
	Range	2.47 - 5.80	2.61 - 5.41	2.52 - 6.34		
	Standard Error	0.489	0.486	0.489		
	FDR		0.7593	0.9907		
	P-value		0.4655	0.9426		

5.5 Compositional differences

Comparative compositional analyses of grain were conducted for maize 98140, non-transgenic near isogenic and four conventional maize hybrids. Compositional analysis of maize 98140 grain was used to evaluate any changes in the levels of key nutrients, antinutrients or secondary metabolites. Compositional analyses of grain included protein, fat, acid detergent fibre (ADF), neutral detergent fibre (NDF), ash, carbohydrates, fatty acids, total amino acids, free amino acids, key anti-nutrients (raffinose, phytic acid and trypsin inhibitor) and key secondary metabolites (furfural, ferulic acid and *p*-coumaric acid).

None of the statistically significant differences ($p < 0.05$) between levels of key nutrients in control maize grain and maize 98140 grain were considered to represent nutritionally significant differences. All key nutrient, anti-nutrient and secondary metabolite levels fell within the ranges taken from the published literature on maize. In conclusion, the composition of maize 98140 grain is considered to be comparable to conventional maize grain.

5.6 Characterisation of metabolites

Studies submitted:

Siehl, D. & Locke M. (2007) Characterization of Substrate Specificity of a Microbial Acetyltransferase Optimized for Activity with Glyphosate: GAT4621. Unpublished Pioneer Report PHI-2006-184/017.

Buffington, J.S. (2007) Concentration of N-Acetyl-L-Aspartate and N-Acetyl-L-Glutamate in GAT/HRA Maize Forage and Grain (Event DP-Ø9814Ø-6). Unpublished Pioneer Report PHI-2007-016.

Buffington, J.S. (2008) Concentration of N-Acetylglycine, N-Acetylserine, and N-Acetylthreonine in Grain from Maize GAT Event DP-Ø9814Ø-6. Unpublished Pioneer Report PHI-2008-052.

Maize 98140 expresses the enzyme GAT4621, with optimised activity for the acetylation of glyphosate. A closely related GAT enzyme, differing from GAT4621 by only one amino acid, has been shown to catalyse the acetylation of substrates other than glyphosate such as the amino acids L-aspartate and L-glutamate and glyphosate analogues such as D-2-amino-3-phosphonopropionate (Siehl *et al.*, 2005). Therefore, consideration has been given to the potential for the production of acetylated metabolites in maize 98140, and any subsequent potential impact on food safety.

To determine the substrate specificity of the GAT4621 enzyme, a survey of substrates was completed using a preliminary assay to detect any activity with the GAT4621 enzyme. Substrates that showed activity with GAT4621 were further evaluated using a continuous spectrophotometric assay to determine the kinetic properties for these substrates with the GAT4621 enzyme. A broad sampling of agrochemicals, antibiotics, and amino acids were evaluated for activity with the GAT4621 enzyme using the preliminary assay. Of these compounds, only five indicated measurable enzyme activity (above the limit of quantification of the assay). These five compounds were the amino acids L-aspartate, L-glutamate, L-serine, L-threonine, and glycine. GAT4621 activity on glyphosate, L-aspartate, L-glutamate, L-serine, L-threonine, and glycine was further characterised using the continuous spectrophotometric assay. The catalytic efficiency (k_{cat}/K_M) of GAT4621 on L-aspartate, L-glutamate, L-serine, and L-threonine was about 1%, 0.8%, 0.05 and 0.06%, respectively, of the activity on glyphosate. The affinity of the GAT4621 enzyme for glycine was too low for estimation of K_M, and the catalytic efficiency could therefore not be calculated.

Following the substrate specificity study, the levels of the acetylated amino acids N-acetylglutamate (NAGlu), N-acetylaspartate (NAAsp), N-acetylthreonine (NAThr), N-acetylserine (NASer) and N-acetylglycine (NAGly) were measured in unsprayed control maize grain, grain from unsprayed maize 98140 (Table 16) and grain from maize 98140 sprayed with glyphosate, rimsulfuron + nicosulfuron, and glyphosate + rimsulfuron + nicosulfuron (Table 17). NAAsp, NAGlu, NAThr, NASer, and NAGly were found in control maize grain, indicating that these substances are not novel. Mean values for NAAsp, NAGlu, NAThr, NASer, and NAGly in maize 98140 grain were significantly higher ($p < 0.05$) than those of control maize grain. This observation is not unexpected, given the ability of GAT enzymes to acetylate amino acids like glutamate and aspartate, threonine, serine and glycine albeit with low catalytic efficiency (Siehl *et al*., 2005). The total level of these acetylated amino acids is 0.05% on a dry weight basis. Compared to NAAsp and NAGlu, the concentrations of NAThr, NASer, and NAGly were more than 100 fold lower at less than 0.0003% individually on a mean dry weight basis. This observation is consistent with the much lower level of catalytic efficiency of GAT4621 on L-serine, glycine and L-threonine as determined in the substrate specificity study.

Table 16: Levels of Acetylated Amino Acids in Grain from Unsprayed Maize 98140

		Analyte level (µg/g dry weight)	
Analyte		**Control Maize**	**Unsprayed Maize 98140**
NAAsp	Mean [a]	0.918	403
	Range [b]	0.0983 – 7.32	103 – 926
	p-value [c]	-	0.0072
NAGlu	Mean	0.472	78.6
	Range	<0.075 [d] – 4.04	0.622 – 195
	p-value	-	0.033
NAThr	Mean	0.177	2.99
	Range	0.125 – 0.300	1.77 – 4.42
	p-value	-	< 0.0001
NASer	Mean	0.900	1.97
	Range	0.301 – 1.88	0.732 – 2.87
	p-value	-	< 0.0001
NAGly	Mean	0.0719	0.233
	Range	0.0345 – 0.129	0.108 – 0.373
	p-value	-	< 0.0001

[a] Least squares mean
[b] Range denotes the lowest and highest individual value across sites.
[c] Non-adjusted *p*-value
[d] < Lower Limit of Quantification (LLOQ); indicates that the values of the sample or samples were detected below the LLOQ of the assay. Sample results which were below the LLOQ were assigned a value equal to the LLOQ for statistical analysis.

Table 17: Levels of Acetylated Amino Acids in Grain from Sprayed Maize 98140

		Analyte level (µg/g dry weight)			
Analyte		**Control Maize**	**Glyphosate Maize 98140**	**Nic + Rim [e] Maize 98140**	**Glyphosate + Nic + Rim Maize 98140**
NAAsp	Mean [a]	0.789	393	414	406
	Range [b]	0.0983 – 7.32	95.4 – 962	108 – 909	96.3 – 992
	Adjusted *p*-value [c]	-	0.001	0.0009	0.0009
	p-value [d]	-	0.0005	0.0003	0.0003
NAGlu	Mean	0.958	80.6	94.3	91.0
	Range		0.627 – 175	0.663 – 217	0.645 – 221
	Adjusted *p*-value	-	0.0026	0.0011	0.0012
	p-value	-	0.0026	0.0007	0.001
NAThr	Mean	0.177	2.81	3.44	2.84
	Range	0.125 – 0.300	1.93 – 3.84	2.38 – 5.38	1.34 – 4.19
	Adjusted *p*-value	-	<0.0001	<0.0001	<0.0001
	p-value	-	<0.0001	<0.0001	<0.0001
NASer	Mean	0.900	1.94	2.14	1.95
	Range	0.301 – 1.88	0.690 – 4.17	0.493 – 4.57	0.798 – 3.71
	Adjusted *p*-value	-	0.0001	<0.0001	0.0001
	p-value	-	0.0001	<0.0001	0.0001
NAGly	Mean	0.0719	0.216	0.230	0.195
	Range	0.0345 – 0.129	0.105 – 0.323	0.0862 – 0.445	0.0727 – 0.430
	Adjusted *p*-value	-	<0.0001	<0.0001	0.0002
	p-value	-	<0.0001	<0.0001	0.0002

[a] Least squares mean
[b] Range denotes the lowest and highest individual value across sites.
[c] False Discovery Rate (FDR) adjusted *p*-value
[d] Non-adjusted *p*-value
[e] Nicosulfuron + Rimsulfuron

In order to determine whether acetylation of amino acids affects their incorporation into proteins, the distribution of total amino acids into three categories (amino acids that are incorporated into proteins, free amino acids, and acetylated amino acids) was calculated for maize 98140 and near-isogenic control maize grain (Table 18). As expected, the majority of amino acids are incorporated into proteins, and a small proportion of amino acids are in the free amino acid pool for both maize 98140 grain and control maize grain. Together, acetylated amino acids make up less than 0.5% of the total amino acids in maize 98140 grain and 99.9% of this is attributed to NAAsp and NAGlu. In addition, the levels of protein in maize 98140 grain are comparable to near-isogenic control maize.

Therefore, the acetylation of L-aspartate, L-glutamate, L-threonine, L-serine, and glycine in maize 98140 did not affect the incorporation of amino acid into proteins or the composition of the free amino acid pool.

Table 18: Distribution of Amino Acids in maize 98140 grain and control maize grain

Amino acid category	Control maize		Maize 98140	
	Mean (mg/g dry weight)	% Total amino acids	Mean (mg/g dry weight)	% Total amino acids
Total amino acids [a]	110.15	100%	107.26	100%
Free amino acids [b]	2.16	1.96%	2.35	2.19%
Acetylated amino acids [c]	0.0026	0.0023%	0.49	0.45%
Amino acids incorporated into proteins [d]	107.99	98.04%	104.42	97.35%

[a] Concentrations of individual amino acids from Table 7 (% dry weight of tissue) were totalled and converted to mg/g dry weight.
[b] Concentrations of free amino acids from Table 8 (% dry weight of tissue) were totalled and converted to mg/g dry weight.
[c] Concentrations of acetylated amino acids from Table 12 (% dry weight of tissue) were totalled and converted to mg/g dry weight.
[d] The amount of incorporated amino acids was calculated by subtracting total free amino acids and acetylated amino acids from the total amino acid amount.

5.6.1 Safety of acetylated amino acids

The N-acetylated amino acids NAAsp, NAGlu, NAThr, NASer, and NAGly were found in control maize grain, indicating that these substances are not novel. Mean levels for these compounds in maize 98140 were statistically significantly higher than those of control maize grain; however, the increases were largest for NAAsp and NAGlu. These acetylated amino acids make up less than 0.5% of the total amino acids in maize 98140 grain and 99.9% of this is attributable to NAAsp and NAGlu. As the levels of NAAsp and NAGlu were increased most markedly in maize 98140, consideration has been given to the potential impacts on the safety of the food.

The biochemistry, metabolism and toxicity of NAAsp and NAGlu, as well as their concentrations in various foods, have been reviewed during the assessment of Application 1006 - Food derived from herbicide-tolerant soybean line DP-356043-5). (http://www.foodstandards.gov.au/standardsdevelopment/applications/applicationa1006food3900.cfm). NAAsp and NAGlu are typical constituents of the human diet, being present in a wide range of foods. Both compounds are also produced endogenously, with high levels of NAAsp in the central nervous system in humans and other species. Both acetylated amino acids are expected to be readily metabolisable by acylase enzymes present in the gastrointestinal tract and in numerous organs including the liver and kidney. In normal individuals, NAAsp and NAGlu would be expected to be metabolised to the constituent amino acid and acetate. Toxicity studies in rats have shown that NAAsp is not acutely toxic and causes no adverse effects when present in the diet at high doses for 28 days. For the soybean 356043 assessment, dietary modelling was used to estimate intakes of NAAsp and NAGlu for the Australian and New Zealand populations. For these calculations, it was assumed that 45% of soybean products would be replaced with soybean 356043 (considered to be a conservative over-estimate). For all population groups/sub-groups, the estimated mean dietary intake of NAAsp was predicted to increase by no more than 3 fold (1.8 - 2.9x), while intake of NAGlu was estimated to increase only marginally (1.1x). Based on the available information, the increased levels of NAAsp and NAGlu in soybean 356043 were considered to be nutritionally insignificant and of no food safety concern.

The applicant has provided a dietary exposure assessment using levels of NAAsp and NAGlu in control maize and maize 98140. The dietary exposures to NAAsp and NAGlu were estimated using DEEM/FCID (Dietary Exposure Evaluation Model – Food Commodity Intake Database, Version 2.14, Exponent Inc., Washington, DC). This model is commonly used by

the EPA Office of Prevention, Pesticides and Toxic Substances to estimate human dietary exposure. Mean and 90th percentile exposures were calculated for the U.S. population and several subpopulations. The applicant stated that the exposure analysis presented will also be valid for the Australian population since the food consumption patterns in Australia are similar to food consumption patterns in the United States (http://www.who.int/foodsafety/chem/gems/en/index1.html).

NAAsp and NAGlu concentrations in unprocessed grain were taken from Table 16 (NAAsp: 0.9 mg/kg for non-maize 98140, and 403 mg/kg for maize 98140; NAGlu: 0.5 mg/kg for non-maize 98140 and 79 mg/kg for maize 98140) and adjusted using relevant processing factors. For example, NAAsp levels in flour were 62% of those in unprocessed grain (processing factor = 0.62). NAAsp and NAGlu were not found in maize oil. Analyses were not conducted to determine NAAsp and NAGlu levels in high fructose maize syrup (HFCS) so starch processing factors were used as a worst-case surrogate since maize starch is the feedstock for producing HFCS.

The results of two dietary exposure assessments were provided. The first used NAAsp or NAGlu concentrations in control maize ("Baseline" columns in Tables 19 and 20), and the second assessment used NAAsp or NAGlu concentrations in maize 98140 combined with control maize ("98140 maize" columns in Tables 19 and 20). In the second assessment, maize 98140 made up 40% of the consumed maize, and non-maize 98140 made up the remaining 60%. The results of the assessments for the U.S. population and selected population subgroups are summarised in Table 19 for NAAsp and Table 20 for NAGlu. Assuming that maize 98140 made up 40% of the consumed maize, the mean intakes of NAAsp and NAGlu were estimated to be 24.6 and 6.7 µg/kg body weight/day, respectively, for the US population. These intakes represent increases of 8.8-fold and 3.4-fold, respectively over the estimated baseline intakes. For children 1-6 years of age, the estimated intakes of NAAsp and NAGlu were increased by 11.2-fold and 4.4-fold, respectively.

Table 19: Dietary intake estimates for NAAsp

Population Subgroup	NAAsp Exposure mg/kg body weight/day			
	Baseline[a] Mean	Baseline 90th Percentile	98140 maize[b] Mean	98140 maize 90th Percentile
U.S. Population	0.0028	0.0060	0.0246	0.0661
Hispanics	0.0034	0.0073	0.0452	0.1266
Non-hispanic whites	0.0026	0.0055	0.0211	0.0568
Non-hispanic blacks	0.0033	0.0075	0.0290	0.0778
Non-hispanic/non-white/non-black	0.0031	0.0068	0.0218	0.0611
All infants	0.0022	0.0079	0.0169	0.0440
Children 1-6 yrs	0.0057	0.0124	0.0639	0.1700
Children 7-12 yrs	0.0037	0.0080	0.0497	0.1294
Youth 13-19 yrs	0.0027	0.0058	0.0302	0.0811
Adults 20-49 yrs	0.0024	0.0051	0.0180	0.0485
Adults 50+ yrs	0.0020	0.0042	0.0116	0.0351

[a] Baseline intake calculations used NAAsp concentrations in control maize.
[b] Maize 98140 intake calculations used NAAsp concentrations as would be measured in a mixture of 40% control maize and 60% maize 98140.

Table 20. Dietary intake estimates for NAGlu

Population Subgroup	NAGlu Exposure mg/kg body weight/day			
	Baseline[a] Mean	Baseline 90th Percentile	98140 maize[b] Mean	98140 maize 90th Percentile
U.S. Population	0.0020	0.0044	0.0067	0.0169
Hispanics	0.0022	0.0048	0.0124	0.0330
Non-hispanic whites	0.0019	0.0041	0.0057	0.0143
Non-hispanic blacks	0.0022	0.0052	0.0076	0.0194
Non-hispanic/non-white/non-black	0.0025	0.0058	0.0066	0.0173
All infants	0.0021	0.0051	0.0049	0.0109
Children 1-6 yrs	0.0038	0.0080	0.0166	0.0421
Children 7-12 yrs	0.0028	0.0059	0.0128	0.0325
Youth 13-19 yrs	0.0020	0.0042	0.0077	0.0201
Adults 20-49 yrs	0.0018	0.0039	0.0051	0.0126
Adults 50+ yrs	0.0013	0.0029	0.0034	0.0088

[a] Baseline intake calculations used NAGlu concentrations in control maize.
[b] Maize 98140 intake calculations used NAGlu concentrations as would be measured in a mixture of 40% control maize and 60% maize 98140.

The biochemical and toxicological evidence indicates that the mammalian system can adequately cope with an increase in the levels of NAAsp and NAGlu of the magnitude expected if maize 98140 were to be introduced into the Australian and New Zealand food supplies. Overall, the weight of evidence indicates that the increased levels of NAAsp and NAGlu in food derived from maize 98140 are unlikely to result in any adverse effects.

5.6.2 Conclusion

In summary, acetylated amino acids such as NAGlu and NAAsp are typical constituents of the human diet, being present in a large range of foods. Because acetylated amino acids are metabolisable and are normally consumed by humans and animals, their higher levels in maize 98140 grain compared to conventional maize grain does not raise safety concerns.

5.8 Conclusion from compositional studies

The levels of key nutrients and anti-nutrients in maize 98140 were compared to levels in the non-transgenic parental line and to a range of conventional maize varieties. The compositional analyses indicate that, for the majority of components, there are no compositional differences of biological significance in forage or grain from transgenic maize 98140, compared to the non-GM control. Several minor differences in key nutrients and other constituents were noted, however, the mean levels observed were within the range of values observed for the non-transgenic comparator and within the range of natural variation.

The compositional data are consistent with the conclusion that there are no biologically significant differences in the levels of key components in grain from maize 98140 when compared with the nontransgenic counterpart and to conventional maize varieties currently on the market.

The GAT4621 enzyme was shown to acetylate several amino acids with greatest efficiency for L-glutamate and L-aspartate. Consequently, levels of NAGlu, NAAsp, NAThr, NASer and NAGly in maize 98140 are significantly higher than those of the non-GM parent and are also outside the established tolerance interval for maize. Acetylated amino acids occur naturally, and the deacetylation and bioavailability of N-acetylated amino acids appears to be a general phenomenon. Both NAGlu and NAAsp were found to be present in common foods, indicating that they are normal components of human diets. NAThr, NASer and NAGly were also present in conventional maize and these compounds can therefore also not be considered novel. These analyses demonstrate a safe history of exposure to these metabolites, and therefore no food safety concerns were identified.

6. NUTRITONAL IMPACT

Establishing that a GM food is safe for human consumption is generally achieved through an understanding of the genetic modification and its direct consequences in the plant, together with an extensive compositional analysis of the food components derived from the GM plant and the non-GM counterpart.

To date, all approved GM plants with modified agronomic production traits (*e.g.* herbicide tolerance) have been shown to be compositionally equivalent to their conventional counterparts. In the case of maize 98140, there are unintended changes in the levels of several acetylated amino acids that are outside the tolerance interval for maize. As discussed in section 5, all of these components are typical constituents of the human diet, and are likely to be readily metabolised, and thus raise no safety issues resulting from their presence in food derived from maize 98140.

The extent of the compositional and other available data is considered sufficient to establish the nutritional adequacy of maize 98140. However, the Applicant submitted the results of a feeding study with maize 98140 using chickens. This has been evaluated by FSANZ as additional supporting information.

6.1 Feeding study in chickens

Study submitted:
Delaney, B. and Smith, B. (2007) Nutritional Equivalency Study of Transgenic Maize Grain Containing Event DP-098140-6: Poultry Feeding Study. Unpublished Pioneer Study PHI-2006-185.

Study aim

To assess the nutritional performance in chickens of diets containing grain produced from maize 98140 in comparison to a diet containing conventional maize grain.

Study conduct

Two lots of maize 98140 were used: the first lot was produced from plants that received no herbicide treatment (98140 unsprayed) and the second lot was from plants treated with a mixture of glyphosate, nicosulfuron, and rimsulfuron (98140 sprayed). Diets produced with grain from non-transgenic near-isoline (control), 98140 sprayed and unsprayed, and non-transgenic commercial reference hybrids (33J56, 33P66, and 33R77) were fed to Ross x Cobb broilers (n = 60/sex/group) for a period of 42 days. Diets were fed in three phases in accordance with standard commercial poultry production practice: Starter (Days 0-21), Grower (Days 22-35), and Finisher (Days 36-42). Starter diets contained 58.5% maize, Grower diets 64% maize, and Finisher diets 71.5% maize.

Analysis

Body weights were measured on Day 0 and every 7 days and feed intakes calculated every 7 days during the growing period; weight gain, feed intake, and mortality-adjusted feed intake:weight gain ratio (feed efficiency) were calculated for Days 0 to 42. Standard carcass and organ yield data were collected at the end of the feeding trial.

Results

No statistically significant ($p < 0.05$) differences were observed in weight gain, mortality, mortality-adjusted feed efficiency, and carcass yields between broilers consuming diets produced with 98140 sprayed or unsprayed maize grain and those consuming a diet containing near isoline control maize grains. Pre-chill liver weights were slightly higher in females receiving the 98140 unsprayed (mean liver weight was 3.57% of live bird weight; $p = 0.035$) or 98140 sprayed (3.71%; $p = 0.0008$) maize grain diets compared to those fed the control diet (3.34%). There were no statistically significant liver weight changes in males. These small liver weight differences observed in females are consistent with normal variation and are not considered to be attributable to any differences in the diets.

Conclusion

No biologically significant differences were detected between the test diets used in this study in terms of bird health, growth performance and carcass measurements. The maize 98140 sprayed and unsprayed diets were comparable to a conventional maize diet with regard to nutritional qualities.

7. OTHER STUDIES

In the case of herbicide-tolerant maize 98140, the extent of the molecular, compositional and other available data is considered sufficient to establish the safety of the food. However, the Applicant also published the results of a 90-day toxicity study in rats fed a diet containing maize 98140 grain (Appenzeller *et al*., 2009). While FSANZ does not routinely require animal toxicity studies to be undertaken, where such studies already exist, FSANZ evaluates them as additional supporting information.

This approach is consistent with the recommendations of an expert panel FSANZ convened to consider the role of animal feeding studies in the safety assessment of genetically modified foods[1]. The panel noted that whole-food animal feeding studies may be informative in some limited circumstances, but that any potential adverse health effects can generally be identified by a scientifically informed comparative assessment of the GM food against its

[1] The workshop report is available at http://www.foodstandards.gov.au/foodmatters/gmfoods/roleofanimalfeedings3717.cfm

conventional counterpart. The panel also recommended that, where the results of relevant animal feeding studies are available, FSANZ evaluate them with critical attention to the methodology and potential limitations in interpretation of the results.

Study evaluated:

Munley, S.M. (2009) Thirteen-week Rat Feeding Study with Maize Grain Containing Event DP-Ø9814Ø-6. Unpublished Pioneer Report PHI-2006-176.

Appenzeller, L.M., Munley, S.M., Hoban, D., Sykes, G.P., Malley, L.A. and Delaney, B. (2009) Subchronic feeding study of grain from herbicide-tolerant maize DP-Ø9814Ø-6 in Sprague-Dawley rats. *Food Chem. Toxicol*. **47**(9):2269-2280.

Study aim

To evaluate the potential nutritional and health effects of maize 98140 when fed to rats for at least 90 days.

Study conduct

The study design was based on guidelines for rodent subchronic toxicology studies, the OECD Guidelines for Testing of Chemicals, Test No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents (OECD, 1998)[2].

Six groups of 7 week old Sprague-Dawley rats, each consisting of 12 animals/sex/group, were used in a 93 day feeding study with a standard feed for rats formulated to contain approximately 35% (w/w) of milled maize grain. The diets were formulated to conform to the specifications for PMI Certified Rodent LabDiet #5002, for protein and calorie content. The control group received a diet formulated to contain milled maize grain from the near-isogenic control line 091. One test group was administered a diet containing maize 98140 from unsprayed plants. The second test group received a diet formulated to contain maize 98140 from plants treated with a herbicide mix containing glyphosate, nicosulfuron and rimsulfuron. The three remaining groups were fed diets containing three non-transgenic commercially-available reference maize varieties, 33J56, 33P66 and 33R77.

Parameters Evaluated

All animals were observed at least twice daily for mortality, moribundity or abnormal behaviour or appearance. Detailed clinical examinations were performed weekly and included evaluation of coat condition, skin, eyes, mucous membranes, occurrence of secretions and excretions, autonomic nervous system activity (lacrimation, piloerection, unusual respiratory pattern), changes in gait, posture, response to handling, and presence of clonic, tonic, stereotypical or abnormal behaviour.

Individual body weights and food consumption were determined daily for the first week and weekly thereafter. Food conversion efficiency was calculated from the food consumption and body weight data.

An ophthalmological examination was conducted on all rats prior to grouping and on all surviving rats on day 86.

[2] OECD Guidelines for the Testing of Chemicals are described and available at http://www.oecd.org/document/40/0,3343,en_2649_34377_37051368_1_1_1_1,00.html

A neurobehavioural evaluation was conducted prior to grouping and during the final week of the study. The evaluation included assessment for potential neurobehavioural effects, functional observational battery evaluations and measurement of sensory function, grip strength and motor activity.

Clinical pathology was assessed after 13 weeks. Evaluations included haematology, coagulation, serum chemistry and urinalysis.

At completion of the study, a complete gross pathology examination was conducted on all animals. The following organs were weighed (paired organs weighed together): liver, kidneys, adrenal glands, thymus, brain, spleen, heart, ovaries and uterus (females) and testes and epididymides (males). Tissues and organs were collected and fixed.

The following tissues from the control (091) and two test (98140 sprayed and un-sprayed) diet groups were examined microscopically: digestive system (liver, oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, salivary glands, and pancreas), urinary system (kidneys and urinary bladder), respiratory system (lungs, trachea, nose, larynx/pharynx), cardiovascular system (heart and aorta), hematopoietic system (spleen, thymus, mandibular lymph node, mesenteric lymph node, bone marrow, and Peyer's patches), endocrine system (pituitary gland, thyroid gland, parathyroid glands, and adrenal glands), nervous system (brain [including cerebrum, cerebellum, and medulla/pons], spinal cord [cervical, mid-thoracic, and lumbar], sciatic nerve, optic nerves, and eyes), skin, musculoskeletal system (skeletal muscle, femur/knee joint, and sternum), and reproductive system of males (testes, epididymides, prostate, and seminal vesicles) and females (ovaries, uterus, mammary glands, and vagina).

Statistical calculations were performed using the SAS/STAT™ (Version 8, SAS™, 1999) software package. Response variable values from animals in the two test groups (98140 unsprayed and sprayed) were compared separately with values from animals in the control group (091). Quantitative and categorical data from male and female rats were analysed within gender. For all comparisons, differences between values were considered statistically significant at a p-value < 0.05. Data from animals in the three reference groups (33J56, 33P66, and 33R77) were used to construct a within-study range of natural variation for each response variable, but were not included in comparative statistical calculations.

Results

One male rat in the reference group 33R77 died on day 60 with pyelonephritis as the probable cause of death. This death was not considered to be related to administration of the reference diet. All other animals survived to the scheduled necropsy.

No differences in body weights, body weight gains, food consumption or food efficiency between rats in the control group and those in either test group were observed. There were no clinical signs of toxicity of ophthalmological lesions attributable to dietary exposure. Nor were there any statistically significant differences in the results of the neurobehavioural evaluation for rats in either test group compared with rats in the control group.

No biologically meaningful differences in clinical chemistry parameters were observed between male or female rats in either test group compared with animals in the control group. Although mean alkaline phosphatase (ALKP) concentrations were higher (p < 0.05) at test days 92–96 in male rats in the 98140 test group and at test days 97–99 in female rats in the 98140 sprayed group compared with rats in the 091 control group, these statistical differences were considered to be unrelated to consumption of the test diets. The ALKP group mean for male rats in the 98140 unsprayed group (92 U/L) was within the range of mean values from male rats in the three reference groups (90–93 U/L). The group mean

from female rats in the 98140 sprayed group (72 U/L) was within the 97.5% confidence interval (26–88 U/L) of the testing facility's historical control data for female rats of this age and strain. Also, a higher serum ALKP concentration was not consistently observed for both test diets containing maize 98140 grain.

There were no statistically significant differences in mean haematology and coagulation parameters for male or female rats in either test group compared with animals in the control group.

No statistically significant differences were observed in urinalysis parameters for male or female rats in the 98140 unsprayed or sprayed groups compared with rats in the 091 control group.

No statistically significant differences were observed in mean relative organ weights for male or female rats in the 98140 unsprayed or sprayed test groups compared with rats in the 091 control group.

Rats killed during the terminal sacrifice presented no atypical or diet-related gross observations. Additionally, there was no evidence of increased incidence or severity of microscopic findings in the tissues of male and female rats in the 98140 unsprayed or sprayed groups compared with rats in the 091 control group. All gross and microscopic observations reported were unremarkable and consistent with spontaneous background lesions in adult Sprague-Dawley rats.

Conclusion

The lack of diet related differences in the findings from this study support the conclusion that administration of milled grain from maize 98140 at concentrations of approximately 35% (w/w) in the diet for at least 90 days had no adverse effects on the growth or health of Sprague-Dawley rats.

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