

Supporting document 1

Safety assessment report – Application A1106 (at Approval)

Food derived from Herbicide-tolerant & Insect-protected Corn Line 4114

Summary and conclusions

Background

A genetically modified (GM) corn line with OECD Unique Identifier DP-004114-3, hereafter referred to as line 4114, has been developed by Pioneer Hi-Bred International Inc. The corn has been modified to be tolerant to the herbicide glufosinate ammonium and protected against lepidopteran insect pests, particularly European corn borer (*Ostrinia nubilalis*) and coleopteran insect pests, particularly western corn rootworm (*Diabrotica virgifera virgifera*).

Tolerance to glufosinate ammonium is achieved through expression of the enzyme phosphinothricin acetyltransferase (PAT) encoded by the *pat* gene derived from the common soil bacterium *Streptomyces viridochromogenes*. Protection against lepidopteran insect pests is conferred by the *cry1F* gene, which is a synthetic and truncated version of a gene from *Bacillus thuringiensis* var. *aizawai*. Protection against coleopteran insect pests is conferred by two genes, *cry34Ab1* and *cry35Ab1* both from *B. thuringiensis* strain PS149B1.

In conducting a safety assessment of food derived from line 4114, a number of criteria have been addressed including: a characterisation of the transferred gene sequences, their origin, function and stability in the corn genome; the changes at the level of DNA, and protein in the whole food; compositional analyses; and evaluation of intended and unintended changes.

This safety assessment report addresses only food safety and nutritional issues. It therefore does not address:

- environmental risks related to the environmental release of GM plants used in food production
- the safety of animal feed, or animals fed with feed, derived from GM plants
- the safety of food derived from the non-GM (conventional) plant.

History of Use

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

Molecular characterisation

Line 4114 was generated through *Agrobacterium*-mediated transformation and contains four expression cassettes: Comprehensive molecular analyses of line 4114 indicate there is a single insertion site comprising a single, complete copy of each of the *cry1F*, *cry34Ab1*, *cry35Ab1*, and *pat* genes. The introduced genetic elements are stably inherited from one generation to the next. There are no antibiotic resistance marker genes present in the line and no plasmid backbone has been incorporated into the transgenic locus.

Characterisation and safety assessment of new substances

Newly expressed proteins

Corn line 4114 is a molecular stack¹ expressing four novel proteins, Cry1F, Cry34Ab1, Cry35Ab1 and PAT. These proteins have previously been assessed in two corn lines DAS-01507-1 (line 1507) and DAS-59122-7 (line 59122) where the coding regions and regulatory elements of the various gene cassettes are the same as in line 4114. A breeding stack created from these two lines is also commercially available

For Cry34Ab1, Cry35Ab1 and PAT, mean levels were lowest in the pollen (9.2, 0.34 µg/g dry weight and <LOQ respectively). The highest levels of these three proteins were in the leaf samples at either the R1 or R4 stages. In contrast, the level of Cry1F was lowest in leaf tissue at R6 (the stage at which grain is harvested) and highest in pollen (35 µg/g dry weight). In the grain, levels of Cry1F, Cry34Ab1, Cry35Ab1 and PAT were 3.3, 24, 1.1 and <LOQ µg/g dry weight respectively.

Western blot analyses confirmed that the four novel proteins in line 4114 have the expected molecular weights and immunoreactivity and are equivalent to the corresponding proteins produced in the breeding stack.

Herbicide Metabolites

The herbicide residues resulting from the application of glufosinate to lines carrying the *pat* or *bar* genes have been assessed in previous applications. There are no concerns that the spraying of line 4114 with glufosinate would result in the production of any novel metabolites that have not been previously considered.

Compositional analyses

Detailed compositional analyses were done to establish the nutritional adequacy of grain from line 4114 and to characterise any unintended compositional changes. Analyses were done of proximates, fibre, minerals, amino acids, fatty acids, vitamins, secondary metabolites and anti-nutrients. The levels were compared to levels in a) an appropriate non-GM hybrid line, PHNAR x PHTFE, b) a tolerance interval compiled from results taken for eight non-GM hybrid lines grown in similar field trials in different seasons, and c) levels recorded in the literature. Only five of the 56 analytes that were considered in a statistical analysis deviated in level from the control in a statistically significant manner. However, the mean levels of all five of these analytes fell within both the tolerance interval and the historical range from the literature.

¹ Gene stacking refers to the process of combining two or more genes of interest into a single plant and can be achieved essentially in one of two ways. A molecular stack is made when those genes are introduced simultaneously or sequentially into the target plant by standard transformation systems. A breeding stack is made when the genes are added via traditional crossing between GM parent plants containing the genes.

It is further noted that the differences between the line 4114 and control means for each of the five analytes were less than the variation found within the control. It can therefore be concluded that grain from line 4114 is compositionally equivalent to grain from conventional corn varieties.

Conclusion

No potential public health and safety concerns have been identified in the assessment of herbicide-tolerant and insect-protected corn line 4114. On the basis of the data provided in the present Application, and other available information, food derived from line 4114 is considered to be as safe for human consumption as food derived from conventional corn varieties.

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List of Abbreviations

ADF	acid detergent fibre
BLASTP	Basic Local Alignment Search Tool: Protein
bp	base pairs
<i>Bt</i>	<i>Bacillus thuringiensis</i>
Cry	crystal
DIG	digoxigenin
DNA	deoxyribonucleic acid
T-DNA	transferred DNA
dw	dry weight
ELISA	enzyme linked immunosorbent assay
FAO	Food and Agriculture Organization of the United Nations
FARRP	Food Allergy Research and Resource Program
FASTA	Fast Alignment Search Tool - All
FDR	False discovery rate
FSANZ	Food Standards Australia New Zealand
GM	genetically modified
kDa	kilo Dalton
LB	Left Border of T-DNA
LOQ	Limit of quantitation
MRL	maximum residue limit
NDF	neutral detergent fibre
OECD	Organisation for Economic Co-operation and Development
OGTR	Office of the Gene Technology Regulator
ORF	open reading frame
PAT	phosphinothricin acetyltransferase
PCR	polymerase chain reaction
P-value	probability value
RB	Right Border of T-DNA
SAS	Statistical Analysis Software
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
U.S.	United States of America
WHO	World Health Organization

1 Introduction

Pioneer Hi-Bred Australia Ltd on behalf of Pioneer Hi-Bred International Inc has submitted, an application to FSANZ to vary Standard 1.5.2 – Food produced using Gene Technology – in the *Australia New Zealand Food Standards Code* (the Code) to include food from a new genetically modified (GM) corn line with OECD Unique Identifier DP-004114-3 (also referred to as line 4114). The corn has been modified to be tolerant to the herbicide glufosinate ammonium (glufosinate) and protected against lepidopteran insect pests, particularly European corn borer (*Ostrinia nubilalis*) and coleopteran insect pests, particularly western corn rootworm (*Diabrotica virgifera virgifera*) (Diehn et al. 2011).

Tolerance to glufosinate is achieved through expression of the enzyme phosphinothricin acetyltransferase (PAT) encoded by the *pat* gene derived from the common soil bacterium *Streptomyces viridochromogenes*. This protein has been considered in 19 previous FSANZ approvals and globally is represented in six major crop species and over 30 approved GM single plant events².

Protection against lepidopteran insect pests is conferred by the *cry1F* gene, which is a synthetic version of a gene from *Bacillus thuringiensis* var. *aizawai*, and encodes a truncated version of an insecticidal protein, Cry1F. Protection against coleopteran insect pests is conferred by two genes, *cry34Ab1* and *cry35Ab1* both from *B. thuringiensis* strain PS149B1 and encoding the insecticidal proteins Cry34Ab1 and Cry35Ab1. These proteins have both been considered previously by FSANZ

Line 4114 is a molecular stack³ that, in terms of traits, is the equivalent of a breeding stack (known commercially as Herculex® XTRA⁴) obtained by crossing two corn lines, DAS-01507-1 (line 1507) and DAS-59122-7 (line 59122). Food from both of these lines has been approved by FSANZ in applications A446 (FSANZ 2003) and A543 (FSANZ 2005) respectively and hence, food from the breeding stack is also approved to enter the Australian and New Zealand food supplies. The breeding stack was grown on approximately 10,000,000 ha in the U.S. in 2013 (Pioneer Hi-Bred proprietary data).

Food from line 4114 requires a separate approval since it represents a unique molecular event although the expressed traits are the same as those already assessed by FSANZ. The purpose in developing the line 4114 molecular stack was to have all of the inserted genes on a single transformation construct integrated at a single genetic locus in the corn genome. Line 4114 therefore has an advantage over the breeding stack because having three linked traits at a single locus will simplify future breeding efforts. The Applicant has stated that line 4114 is not intended to be a stand-alone product and will be crossed by conventional breeding with other approved GM corn lines as well as conventional lines.

The Applicant indicates that any lines containing the DP-004114-3 event will be grown primarily in North America, and approval for cultivation in Australia or New Zealand is not currently being sought. Therefore, if approved, food derived from this line may enter the Australian and New Zealand food supply as imported food products.

² FAO GM Foods Platform - <http://www.fao.org/food/food-safety-quality/gm-foods-platform/browse-information-by/commodity/en/>

³ Gene stacking refers to the process of combining two or more genes of interest into a single plant and can be achieved essentially in one of two ways. A molecular stack is made when those genes are introduced simultaneously or sequentially into the target plant by standard transformation systems. A breeding stack is made when the genes are added via traditional crossing between GM parent plants containing the genes.

⁴ <http://www.dowagro.com/herculex/about/herculextra/>

2 History of Use

2.1 Host organism

The host organism is a conventional Pioneer proprietary inbred corn (*Zea mays*) line PHWWE (Wang et al. 2008). It is a homozygous line with an elite genotype that also has high transformability and good response in tissue culture. The line was produced by initially crossing a Hi-II hybrid corn line with pollen from the elite line PHO9B and then going through a series of backcrossing to PHO9B and self-pollination while selecting for the desired characteristics.

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

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

In terms of production, corn is the world's dominant cereal crop, ahead of wheat and rice and is grown in over 160 countries (FAOSTAT 2014). In 2012, worldwide production of corn was over 872 million tonnes, with the United States and China being the major producers (~273 and 208 million tonnes, respectively) (FAOSTAT 2014). Corn is not a major crop in Australia or New Zealand and in 2012, production was approximately 450,000 and 211,00 tonnes respectively (FAOSTAT 2014).

Domestic production is supplemented by the import of corn grain and corn-based products, the latter of which are used, for example, in breakfast cereals, baking products, extruded confectionery and food coatings. In 2011, Australia and New Zealand imported, respectively, 856 and 5,800 tonnes of corn grain, 10,600 and 306 tonnes of frozen sweet corn and 8,427 and 900 tonnes of otherwise-processed sweet corn (FAOSTAT 2014). Corn product imports to Australia and New Zealand included 4,734 and 2,100 tonnes of corn flour and 1,520 and 13 tonnes of corn oil respectively (FAOSTAT 2014). Corn is a major source of crystalline fructose and high fructose corn syrup, both of which are processed from cornstarch. Approximately 3,000 tonnes of crystalline fructose, but negligible high fructose corn syrup, were imported into Australia in 2011 (Green Pool 2012); neither Australia nor New Zealand currently produce fructose (either crystalline or as high fructose corn syrup).

The majority of grain and forage derived from corn is used as animal feed, however corn also has a long history of safe use as food for human consumption. There are five main types of corn grown for food:

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

Dent corn is the most commonly grown for grain and silage and is the predominant type grown in the U.S. (OGTR 2008). Line 4114 is a yellow dent corn but could be crossed with other types. Two main grain processing routes are followed for dent corn (White and Pollak 1995):

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

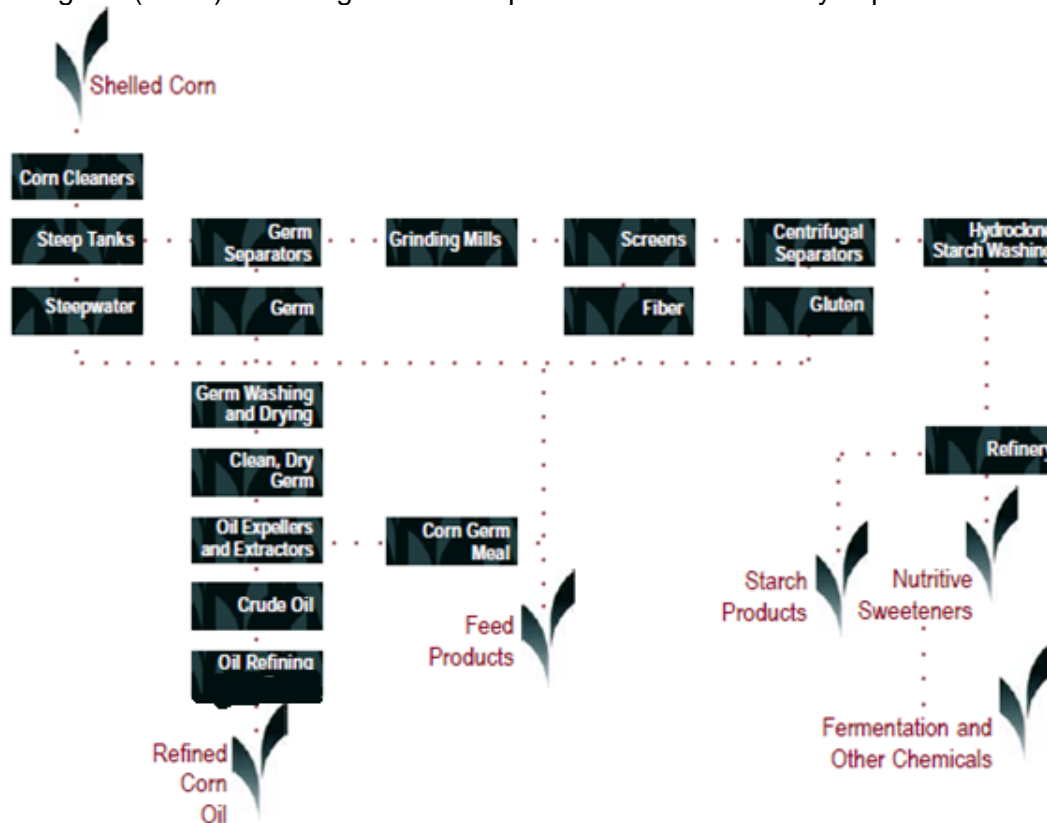


Figure 1: The corn wet milling process (diagram taken from CRA (2006))

2.2 Donor organisms

2.2.1 *Bacillus thuringiensis*

Many different subspecies of *Bacillus thuringiensis* (*Bt*) have been isolated from dead or dying insects, mostly from the orders Coleoptera, Diptera and Lepidoptera, but many subspecies have also been found in the soil, aquatic environments and other habitats (WHO 1999). The source of the *cry3Bb1* gene used in line 4114 is the *Bt* variety *aizawai*.

Studies on mammals, particularly laboratory animals, demonstrate that *B. thuringiensis* is mostly non-pathogenic and non-toxic. *B. thuringiensis* has been demonstrated to be highly specific in its insecticidal activity and has demonstrated little, if any, direct toxicity to non-target insects (see NPTN 2000; OECD 2007 and references therein). Infection in humans is unusual although there have been at least two clinical reports, one in the wounds of a soldier (Hernandez et al. 1998) and one in burn wounds (Damgaard et al. 1997), and in both cases impaired immunosuppression was implicated in the cause of the infection.

B. thuringiensis has also been rarely associated with gastroenteritis (see eg Jackson et al. 1995) but generally, *B. thuringiensis* present in drinking water or food has not been reported to cause adverse effects on human health (WHO 1999; NPTN 2000; OECD 2007).

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

Commercial *Bt* products are powders containing a mixture of dried spores and toxin crystals. Such products are approved for use on crops in Australia⁵ and New Zealand⁶ and in both countries there is an exemption from maximum residue limits (MRLs) when *Bt* is used as an insecticide⁷.

2.2.2 *Streptomyces viridochromogenes*

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

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

2.2.3 Other organisms

Genetic elements from several other organisms have been used in the genetic modification of line 4114 (refer to Table 1). These non-coding sequences are used to drive, enhance, target or terminate expression of the novel genetic material. None of the sources of these genetic elements is associated with toxic or allergenic responses in humans. The genetic elements derived from plant pathogens are not pathogenic in themselves and do not cause pathogenic symptoms in line 4114.

3 Molecular characterisation

Molecular characterisation is necessary to provide an understanding of the genetic material introduced into the host genome and helps to frame the subsequent parts of the safety assessment. The molecular characterisation addresses three main aspects:

- the transformation method together with a detailed description of the DNA sequences introduced to the host genome

⁵ <https://portal.apvma.gov.au/pubcris>

⁶ <http://www.biosecurity.govt.nz/pests-diseases/forests/white-spotted-tussock-moth/about-btk.htm>

⁷ (<http://www.foodsafety.govt.nz/elibrary/industry/nz-mrl-agricultural-compounds-food-standards-07-2014.pdf> ; <http://www.comlaw.gov.au/Details/F2014C00821>).

- a characterisation of the inserted DNA including any rearrangements that may have occurred as a consequence of the transformation
- the genetic stability of the inserted DNA and any accompanying expressed traits.

Studies submitted:

2011. Characterization of DP-ØØ4114-3 Maize: Insertion Integrity, Stability, Copy Number, and Backbone Analysis. Study ID **PHI-2009-015**. Pioneer Hi-Bred International, Inc (unpublished).
 2011. Maize Event DP-004114-3 and Methods for Detection Thereof. Patent No. US2011/0154523 A1. <http://www.google.com/patents/about?id=XwjoAQAAEBAJ&dq=SSA&ie=ISO-8859-1>
 2011. Segregation Analysis of Five Generations of a Maize Line Containing Event DP-004114-3. Study ID **PHI-2010-197/010**. Pioneer Hi-Bred International, Inc (unpublished)
 2015. Junction Reading Frame Analysis at the Insertion Site of maize Event DP-004114-3. Study ID **PHI-R036-Y15**. Pioneer Hi-Bred International, Inc (unpublished)

3.1 Method used in the genetic modification

Immature embryos from line PHWWE were aseptically removed from 9 – 11 day post-pollination ears and transformed, using a disarmed strain of *Agrobacterium tumefaciens* (LBA4404), with the T-DNA from plasmid vector PHP27118 (see Figure 2) following the method of Zhao et al (2001)

After co-culturing with the *Agrobacterium* carrying the vector, the embryos were placed on two sequential selection media - firstly containing carbenicillin (to inhibit the growth of untransformed plant cells and excess *Agrobacterium*) and secondly containing plant hormones (to stimulate somatic embryogenesis) and bialaphos (glufosinate - to select for expression of the *pat* gene). Rooted plantlets (generation T₀) with normal phenotypic characteristics, polymerase chain reaction (PCR)-verified presence of the four novel genes, confirmed presence of a single copy of the T-DNA, and negative for *Agrobacterium* backbone sequences were selected and transferred to soil for growth and further assessment following crossing to inbred lines. Ultimately, line 4114 was selected as the lead event.

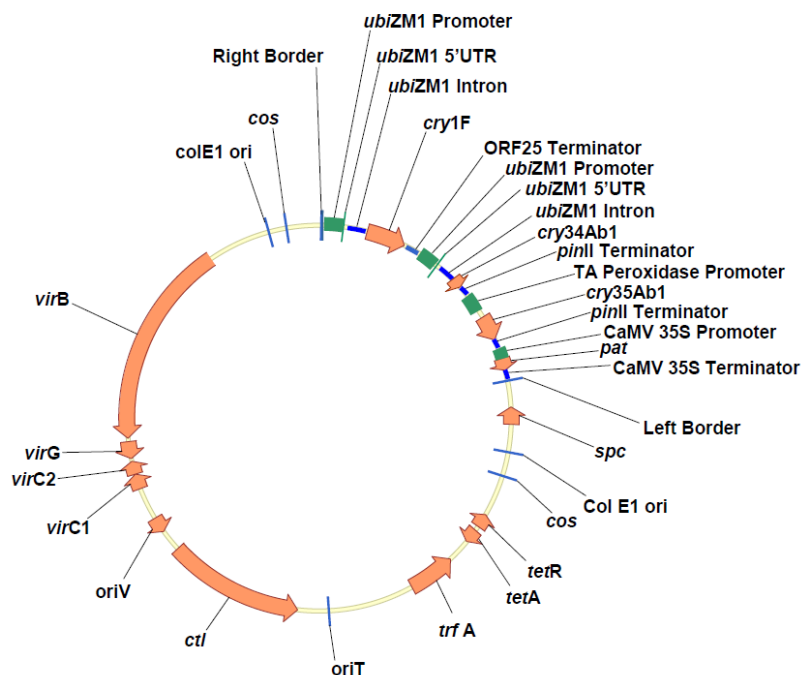


Figure 2: Genes and regulatory elements contained in plasmid PHP27118

3.2 Function and regulation of introduced genes

A diagram of the T-DNA insert in plasmid PHP27118 is given in Figure 3.

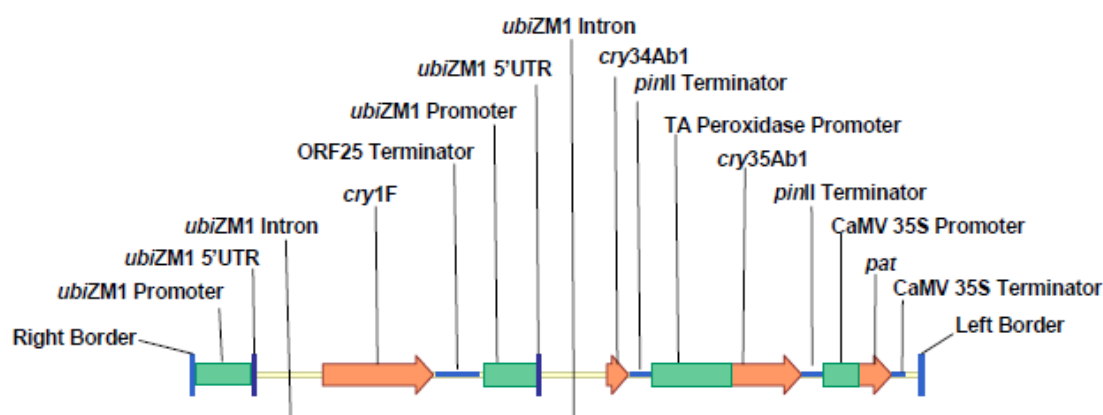


Figure 3: Representation of the genetic elements in the T-DNA insert of plasmid PHP27118

Information on the genetic elements in the T-DNA insert present in line 4114 is summarised in Table 1. There are four cassettes plus non-functional Ti sequences comprising a total of 11,928 base pairs (bp) located between a 25 bp Left Border (LB) and a 25 bp Right Border (RB). The complete plasmid is 54,910 bp in size (i.e. the vector backbone comprises 42,932 bp). The coding regions and regulatory elements are the same as those found in the breeding stack obtained by crossing lines 1507 and 59122 (1507 x 59122).

Table 1: Description of the genetic elements contained in the T-DNA of PHP27118

Genetic element	bp location on plasmid	Size (bp)	Source	Orient.	Description & Function	References
RIGHT BORDER	1 - 25	25				
Ti plasmid region	26 - 43	18			Non-functional sequence	
cry1F cassette						
Intervening sequence	44 - 114	71				
<i>ubiZM1</i>	115 - 2107	1993	<i>Zea mays</i> (corn)	Anti-clockwise	<ul style="list-style-type: none"> Promoter region from the polyubiquitin gene (including 5'UTR and intron) Directs transcription of the <i>cry1F</i> gene 	Christensen <i>et al.</i> (1992)
Intervening sequence	2108 - 2129	22				
<i>cry1F</i>	2130 - 3947	1818	<i>Bacillus thuringiensis</i> var <i>aizawai</i>	Anti-clockwise	<ul style="list-style-type: none"> Codon optimised synthetic and truncated version of the coding region of the <i>cry1F</i> gene 	Chambers (1991)
Intervening sequence	3948 - 3992	45				
<i>ORF25</i>	3993 - 4706	714	<i>Agrobacterium tumefaciens</i> pTi15955	Anti-clockwise	<ul style="list-style-type: none"> Transcriptional terminator and polyadenylation site of open reading frame 25 	Barker <i>et al.</i> (1983)
cry34Ab1 cassette						
Intervening sequence	4707 - 4765	59				
<i>ubiZM1</i>	4766 - 6758	1993	<i>Zea mays</i> (corn)	Anti-clockwise	<ul style="list-style-type: none"> Promoter region from the polyubiquitin gene (including 5'UTR and intron) Directs transcription of the <i>cry34Ab1</i> gene 	Christensen <i>et al.</i> (1992)

Genetic element	bp location on plasmid	Size (bp)	Source	Orient.	Description & Function	References
Intervening sequence	6759 - 6786	28				
<i>cry34Ab1</i>	6787 - 7158	372	<i>Bacillus thuringiensis</i> non-motile strain PS149E1	Anti-clockwise	<ul style="list-style-type: none"> Synthesised, codon-optimized version of the <i>cry34Ab1</i> gene encoding the 14 kDa delta-endotoxin parasporal crystal protein 	Ellis <i>et al.</i> (2002); Herman <i>et al.</i> (2002); Moellenbeck <i>et al.</i> (2001)
Intervening sequence	7159 - 7182	24				
<i>pinII</i>	7183 - 7492	310	<i>Solanum tuberosum</i> (potato)	Anti-clockwise	<ul style="list-style-type: none"> Terminator region from the proteinase inhibitor II gene Directs polyadenylation of the <i>cry34Ab1</i> gene 	An <i>et al.</i> (1989); Keil <i>et al.</i> (1986)
<i>cry35Ab1</i> cassette						
Intervening sequence	7493 - 7522	30				
<i>TA peroxidase</i>	7523 - 8820	1298	<i>Triticum aestivum</i> (wheat)	Anti-clockwise	<ul style="list-style-type: none"> Promoter and leader sequence from the <i>peroxidase</i> gene Directs transcription of the <i>cry35Ab1</i> gene 	Hertig <i>et al.</i> (Hertig <i>et al.</i> 1991)
Intervening sequence	8821 - 8836	16				
<i>cry35Ab1</i>	8837 - 9988	1152	<i>Bacillus thuringiensis</i> non-motile strain PS149E1	Anti-clockwise	<ul style="list-style-type: none"> Synthesises, codon-optimized version of the <i>cry35Ab1</i> gene encoding the 44 kDa delta-endotoxin parasporal crystal protein 	Ellis <i>et al.</i> (2002); Herman <i>et al.</i> (2002); Moellenbeck <i>et al.</i> (2001)
Intervening sequence	9989 - 10012	24				
<i>pinII</i>	10013 - 10322	310	<i>Solanum tuberosum</i> (potato)	Anti-clockwise	<ul style="list-style-type: none"> Terminator region from the proteinase inhibitor II gene Directs polyadenylation of the <i>cry34Ab1</i> gene 	An <i>et al.</i> (1989); Keil <i>et al.</i> (1986)
<i>pat</i> cassette						
Intervening sequence	10323 - 10367	45				
35S	10368 - 10897	530	Cauliflower mosaic virus	Anti-clockwise	<ul style="list-style-type: none"> Promoter from the 35S gene Drives transcription of the <i>pat</i> gene 	Franck <i>et al.</i> (1980); Odell <i>et al.</i> (Odell <i>et al.</i> 1985); Pietrzak <i>et al.</i> (1986)
Intervening sequence	10898 - 10916	19				
<i>pat</i>	10917 - 11468	552	<i>Streptomyces viridochromogenes</i>	Anti-clockwise	Codon-optimized phosphinothricin acetyltransferase coding sequence	Wohlleben <i>et al.</i> (1988)
Intervening sequence	11469 - 11488	20				
35S	11489 - 11680	192			<ul style="list-style-type: none"> Terminator region from the 35S gene Directs polyadenylation of the <i>pat</i> gene 	Franck <i>et al.</i> (1980); Pietrzak <i>et al.</i> (1986)
Intervening sequence	11681 - 11874	194				
Ti plasmid region	11875 - 11953	79			Non-functional sequence	
LEFT BORDER	11954 - 11978	25				

3.2.1 *cry1F* expression cassette

The bacterial *cry1F* gene sequence has been shown to provide high levels of protection against certain insect pests when it is expressed in plants.

The gene encodes one of the family of Bt insecticidal proteins, Cry1F, that specifically inhibits European and southwestern corn borer insects, black cutworm and armyworms.

The introduced *cry1F* gene has been re-synthesised in the laboratory prior to transformation to optimise expression levels in the plant; naturally occurring *Bt* genes tend to be A:T rich, while plant genes have higher G:C content

The corresponding amino acid sequence of the Cry1F protein is unchanged by the modified DNA sequence, except for one change at the carboxy terminus of the protein. A leucine residue occurs in place of a phenylalanine residue at position 604 of the 605 amino acids of the plant expressed protein. This single amino acid change results from an intended nucleotide change required to facilitate processing steps in the laboratory. The leucine substitution represents a conservative change in terms of the naturally occurring amino acid at the corresponding position in other Bt proteins.

Under the regulation of the constitutive Ubi-1 promoter from corn, including the 5' untranslated region (UTR) and intron associated with the native polyubiquitin promoter, expression of the *cry1F* gene would be expected in all parts of the plant, conferring insect protection at the whole plant level. Transcription is terminated by the polyadenylation signal from ORF25 of *Agrobacterium tumefaciens*.

The promoter, coding region and terminator in this cassette are identical to the corresponding elements in line 1507 (FSANZ 2003). The UbiZM1 promoter in line 4114 is described as being 7 bp longer than the same promoter in line 1507 but a more recent sequence analysis showed that the 7 bp previously identified as a polylinker in line 1507 is in fact a part of the UbiZM1 promoter.

3.2.2 cry34Ab1 and cry35Ab1 expression cassettes

The genes encode the proteins Cry34Ab1 (123 amino acids) and Cry35Ab1 (383 amino acids). Both proteins are required for maximal activity against western corn rootworm (Ellis et al. 2002; Herman et al. 2002). The transgenes that encode these proteins were optimised for expression in corn plants. The proteins encoded by the synthetic transgenes are identical in sequence to the native Bt crystal proteins. The *cry34Ab1* gene is regulated by the ubiquitin promoter from *Zea mays* and the *Solanum tuberosum* proteinase inhibitor terminator. The *cry35Ab1* gene is regulated by the wheat peroxidase gene promoter and the *Solanum tuberosum* proteinase inhibitor terminator. The promoter, coding region and terminator in each cassette are identical to the corresponding elements in line 59122 (FSANZ 2005). Again, apparent small discrepancies between the sizes of elements in line 4114 and in line 59122 are due to more sophisticated recent sequence analysis

3.2.3 pat expression cassette

As with the genes in the other expression cassettes, the codon usage pattern of the native *Streptomyces pat* gene has been modified in the laboratory prior to introduction into the plant. The amino acid sequence of the resulting PAT protein however is not changed. The gene is under the regulation of the constitutive promoter and terminator from Cauliflower mosaic virus (CaMV) and therefore the new protein is expected to be expressed in all parts of the plant, including the grain. The promoter, coding region and terminator are identical to the corresponding elements in both lines 1507 and 59122 with, again, apparent small discrepancies being due to more sophisticated recent sequence analysis.

3.3 Breeding of corn line DP-004114-3

The breeding pedigree for the various generations is given in Figure 4.

From a single T₀ plant, breeding then proceeded in order to produce specific generations that were used in characterisation and analysis (as indicated in Table 2). Pioneer proprietary inbreds PH1B5, PHR03, PHTFE, PHNAR, and PH09B were used in crossing and backcrossing steps and, because of the complexity of the crossing, a number of different non-GM lines were used as controls, depending on the particular generation(s) used for analysis. In addition, since line 4114 contains the same traits as the approved GM lines 1507 and 59122, these two lines and the breeding stack produced from the two lines were used as comparators for the protein expression analysis.

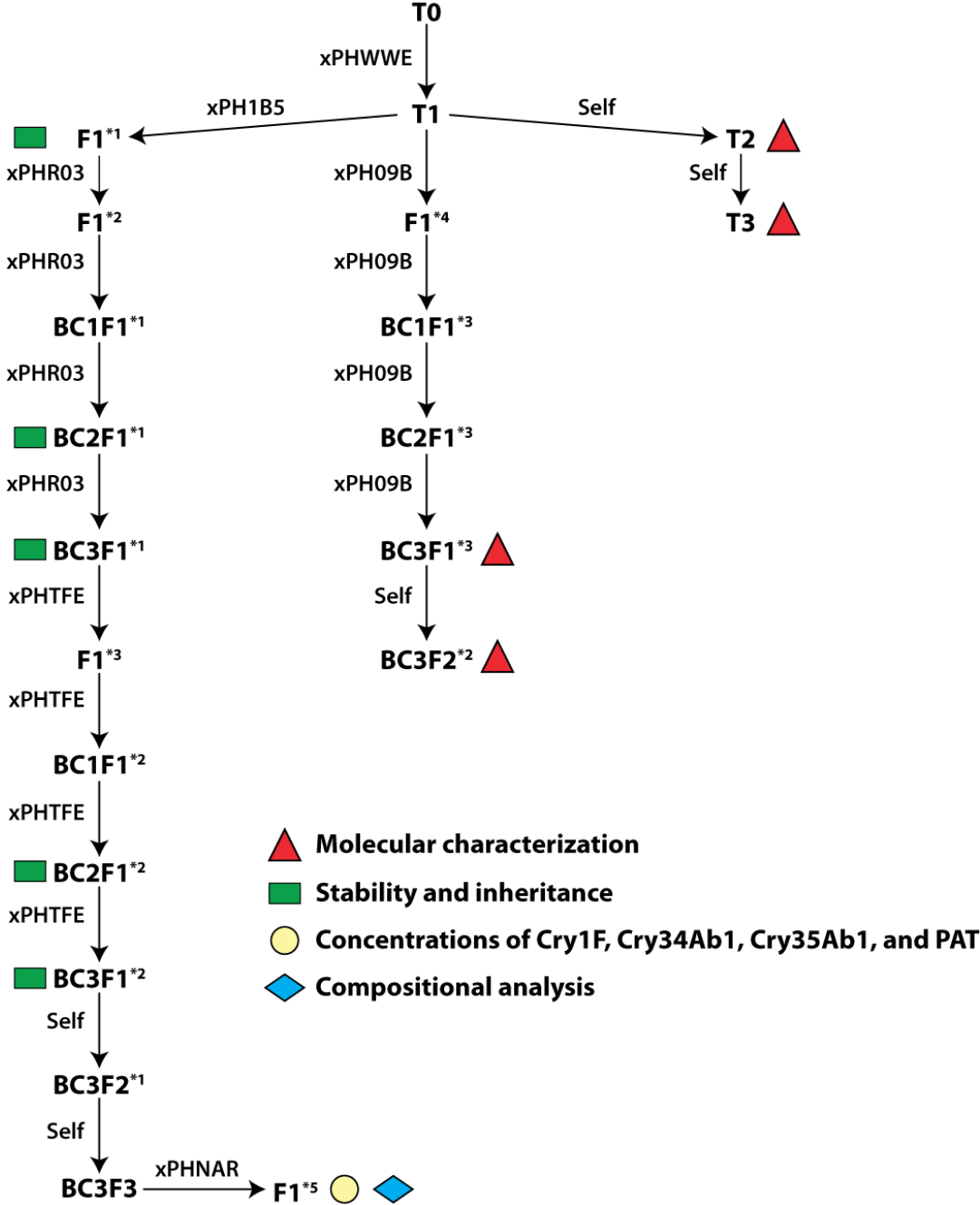


Figure 4: Breeding diagram for corn line DP-004114-3

Table 2: Line 4114 generations used for various analyses

Analysis	Line 4114 generation used	Control(s) used	Reference comparators
Molecular characterisation (Section 3.4)	T ₂ , T ₃ , BC ₃ F ₁ ² , BC ₃ F ₂ ²	PH09B; PHWWE	
Genetic stability (Section 3.5)	T ₂ , T ₃ , BC ₃ F ₁ ² , BC ₃ F ₂ ²	PH09B, PHWWE	
Mendelian inheritance (Section 3.5)	F ₁ ¹ , BC ₂ F ₁ ¹ , BC ₃ F ₁ ¹ , BC ₂ F ₁ ² , BC ₃ F ₁ ²	N/A	
Protein expression levels in plant parts (Section 4.1.3)	F ₁ ^{*5}		line 1507; line 59122; 1507 x 59122
Protein characterisation (Section 4.1.4)	F ₁ ^{*5}	Non-GM, near-isoline	1507 x 59122
Compositional analysis (Section 5)	F ₁ ^{*5}	PHNAR x PHTFE	Pioneer® hybrids 34M94, 33G26, 33J24, 3394, 38B85, 37Y12, 34A15, and 34P88

3.4 Characterisation of the genetic modification in the plant

A range of analyses were undertaken to characterise the genetic modification in line 4114. These analyses focussed on the nature of the insertion of the introduced genetic elements and whether any unintended genetic re-arrangements may have occurred as a consequence of the transformation procedure.

3.4.1 Transgene copy number, insertion integrity and plasmid backbone analysis

Total genomic DNA from pooled leaves of plants of the T3 generation from verified line 4114 (using lateral flow immunoassay⁸ to confirm expression of the proteins, and event-specific PCR analysis) and from the negative controls (PHO9B and PHWWE) was used for Southern blots to determine the number of T-DNA insertions and the sequence integrity of the introduced DNA in line 4114, and test for the presence or absence of plasmid vector backbone sequences. Positive controls (DNA from PHO9B and PHWWE spiked with digested plasmid PHP27118) were also included in the Southern blot analysis.

Southern analysis of *Bcl I*-digested DNA was used to determine insert copy number in line 4114. The *Bcl I* restriction enzyme has one recognition site within the T-DNA of PHP27118 thereby cutting it into two fragments. Digested DNA fragments were separated and transferred to a membrane for hybridisation with 11 different digoxigenin (DIG)-labelled probes specific to each of the genetic elements within the PHP27118 T-DNA. Two anomalous results were noted. Firstly, the detection of an extra fragment in digested DNA from line 4114, PHWWE and PHO9B when a *ubiZM1* probe was used, was consistent with the presence of native copies of the *ubiZM1* promoter and intron. Secondly, the occurrence of an unusual banding pattern in *Bcl I*-digested PHP27118 plasmid DNA was explained by the methylation of the DNA adenine methylase (Dam) recognition sequence in all *Bcl I* sites on the plasmid⁹.

⁸ http://www.envirologix.com/artman/publish/article_34.shtml

⁹ The PHP27118 plasmid was prepared in a *dam+* strain of *E. coli* and hence the Dam recognition sequence was present. Dam is specific to bacteria and therefore not found in corn, so maize genomic DNA is digested normally by *Bcl I*.

The effect of this would be to block digestion by *Bcl I*. For all other results, the expected sizes of two fragments were obtained thereby confirming that there is integration of a single copy of the T-DNA within the genome of line 4114.

Southern blot analysis of *Hind III*-digested DNA (three sites located within the T-DNA) from line 4114 was used to confirm the integrity of the T-DNA insert. The same 11 probes as used for the copy number analysis were used. As for the *Bcl I* digestion, an extra fragment associated with the *ubiZM1* probes reflected the presence of native sequences. The results indicated the predicted size fragments were detected and hence that the single insert contained the intact T-DNA from plasmid PHP27118 without truncation, rearrangement or deletion of nucleotide sequences.

To test whether plasmid backbone DNA is present in line 4114, Southern analysis of *Bcl I*-digested DNA obtained from 4 generations (T2, T3, BC3F1^{*3}, and BC3F2^{*2}) from verified plants was undertaken. Five DIG-labelled probes spanning the backbone were used. No hybridisation fragments were detected, thus confirming the lack of integration of any plasmid backbone sequences.

3.4.2 Insert organisation and sequence

The experimental work to determine the organisation and sequence of the insert in line 4114 is available in a patent (Diehn et al. 2011). Genomic DNA was obtained from verified leaf tissue and the samples were used to characterise the DNA sequence in the transgene insertion and its flanking border regions.

Six overlapping polymerase chain reaction (PCR) fragments spanning the inserted sequences and border regions in event DP-004114-3 were amplified, purified and then cloned into a bacterial vector. For each fragment, the DNA from vector colonies was sequenced individually and the sequences were aligned to obtain a consensus sequence. Commercially available software (Sequencher®) was then used to assemble the consensus sequences to obtain a final sequence for the insert. Sequence annotation was performed using Vector NTI® 9.1.0 software by comparing the T-DNA insert sequences from line 4114 with sequences in the T-DNA of plasmid PHP27118.

In order to confirm that the 5' and 3' border regions are of corn origin, PCR amplification and sequencing of the border regions from both line 4114 and the PHWEE control was carried out and the sequences were compared.

The sequence of the insert and border regions was determined and 16,752 bp of line 4114 genomic sequence was confirmed (Diehn et al. 2011). This comprised 2,398 bp of the 5' genomic border sequence, 2,405 bp of the 3' genomic border sequence, and 11,949 bp of inserted T-DNA from PHP27118. The inserted T-DNA in line 4114 was found to have a 29 bp deletion on the RB) end and a 24 bp deletion on the LB end; this truncation of the border sequences is not uncommon for *Agrobacterium*-mediated transformation events (Kim et al. 2007). The comparison of border region sequences from line 4114 and the control confirmed they are of corn genomic origin.

3.4.3 Open reading frame (ORF) analysis

Sequences spanning the 5' and 3' junctions of the insert were translated from start codon to stop codon (TGA, TAG, TAA) in all six reading frames, and encoding sequences of 30 or more amino acids. ORFs shorter than 29 amino acids were not evaluated since a minimum 35% identity requires at least a match of 29 amino acids over 80 amino acids. The 35% identity is a recommended criterion for indicating potential allergenicity (Codex 2009).

A single ORF (putatively encoding a sequence of 99 amino acids) was identified spanning the 5' genomic border. No analysis was done to determine whether any potential regulatory elements were associated with it.

The putative polypeptide was analysed using a bioinformatic strategy to determine similarity to known protein toxins or allergens (refer to Section 4.1.6).

3.5 Stability of the genetic changes in line 4114

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

The genetic stability of event DP-004114-3 was evaluated by Southern blot analysis of *Bcl I*-digested genomic DNA from plants from four generations (T_2 , T_3 , $BC_3F_1^2$, BC_3F_2). Four DIG-labelled probes corresponding to the four coding regions in the T-DNA insert were used for hybridisation. The expected hybridisation patterns were obtained thereby indicating the stable inheritance of the insert.

Phenotypic stability was assessed by determining the segregation of the *cry1F*, *cry34Ab1*, *cry35Ab1* and *pat* genes within five generations (F_1^{*1} , $BC_2F_1^1$, $BC_3F_1^1$, $BC_2F_1^2$, $BC_3F_1^{*2}$). Since it was demonstrated that the insert resides at a single locus within the genome of line 4114, the expectation would be that the genetic material within it would be inherited according to Mendelian principles.

Leaf punches from individual plants from each generation were analysed by event-specific PCR for the presence of the T-DNA insert and by gene-specific PCR for the presence of each of the four genes. In addition, plants were sprayed with glufosinate to evaluate whether they possessed the herbicide-tolerance phenotype.

In all cases, a plant that tested positive for the T-DNA insert also tested positive for the presence of the *cry1F*, *cry34Ab1*, *cry35Ab1* and *pat* genes and for a positive herbicide tolerance phenotype. Chi-squared (χ^2) analysis was used to compare the observed segregation data to the hypothesised ratio of 1:1 (positive:negative). The results (Table 3) indicated that there were no significant differences between the observed and expected segregation ratios in any of the generations. This both supported the conclusion that the T-DNA resides at a single locus and showed that the T-DNA is inherited according to Mendelian principles.

Table 3: Segregation of the DP-004114-3 T-DNA sequences over five generations

Generation	Total plants	Ratio positive:negative		χ^2	Probability (P) ¹
		Observed	Expected		
F_1^{*1}	98	1:0.88	1:1	0.367	0.545
$BC_2F_1^1$	100	1:1.08	1:1	0.160	0.689
$BC_3F_1^1$	100	1:1.12	1:1	0.360	0.549
$BC_2F_1^2$	100	1:0.88	1:1	0.360	0.549
$BC_3F_1^{*2}$	195	1:1.24	1:1	1.62	0.132

¹Statistical significance is when $P \leq 0.05$

3.6 Antibiotic resistance marker genes

No antibiotic marker genes are present in line 4114. The insert sequence analysis (Section 3.4.1) showed that no plasmid backbone has been integrated into the 4114 genome during transformation, i.e. the *spc* gene, which was used as a bacterial selectable marker gene, is not present in line 4114.

3.7 Conclusion

Corn line 4114 contains four expression cassettes: Comprehensive molecular analyses of line 4114 indicate there is a single insertion site comprising a single, complete copy of each of the *cry1F*, *cry34Ab1*, *cry35Ab1*, and *pat* genes. The introduced genetic elements are stably inherited from one generation to the next. There are no antibiotic resistance marker genes present in the line and no plasmid backbone has been incorporated into the transgenic locus.

4 Characterisation and safety assessment of new substances

4.1 Newly expressed proteins

In considering the safety of novel proteins it is important to note that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects, although a small number have the potential to impair health, e.g., because they are allergens or anti-nutrients (Delaney et al. 2008). As proteins perform a wide variety of functions, different possible effects have to be considered during the safety assessment including potential toxic, anti-nutritional and allergenic effects. To effectively identify any potential hazards requires knowledge of the characteristics, concentration and localisation of all novel proteins expressed in the organism as well as a detailed understanding of their biochemical function and phenotypic effects. It is also important to determine if the novel protein is expressed as expected, including whether any post-translational modifications have occurred.

Two types of novel proteins were considered:

- Those that were expected to be directly produced as a result of the translation of the introduced genes. A number of different analyses were done to characterise these proteins and determine *in planta* expression.
- Those that may be potentially translated as a result of the creation of ORFs during the transformation process (see Section 3.4.3).

4.1.1 Cry proteins

Crystal (Cry) proteins produced by *Bacillus thuringiensis* (*Bt*) are classified by their primary amino acid sequence and more than 500 different *cry* gene sequences have been classified into 73 groups (Cry1–Cry73)¹⁰ The largest family is the 3D-Cry group and contains proteins subdivided into further groups based on their specificity for target insects; Cry 3 proteins (such as Cry34Ab1 and Cry35Ab1) act specifically on Coleopterans while Cry 1 proteins (such as Cry1F) act specifically on Lepidopterans (Höfte and Whiteley 1989).

¹⁰ Cry protein classification can be accessed at http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/toxins2.html

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

4.1.1.1 *Cry1F*

In its native form, Cry 1F is produced in *B. thuringiensis* as a large protoxin of 1174 amino acids. Following solubilisation and proteolytic processing in the gut of susceptible insect larvae, the active toxin moiety corresponds to approximately 600 amino acids at the N-terminal end of the full-length protein. Although precise cleavage has not been shown, the activated toxin is estimated to correspond to amino acids 28-612, based on laboratory data and computer simulations. Therefore, to confer insect resistance, a truncated form of the *cry1F* gene, encoding only the active toxin moiety, was inserted into the corn plants.

The truncated Cry1F protein present in line 4114 should be identical to amino acids 1-605 of the N-terminal domain of the native Cry1F protoxin with the exception of a single amino acid substitution, leucine in place of phenylalanine, at position 604 (F₆₀₄L – see Figure 5). The amino acid change was made to facilitate production in the laboratory of large quantities of a microbially-produced Cry1F/Cry1A(b) chimeric protein that was used as a source of the Cry1F moiety required for safety studies. The chimeric protein is a fusion of the gene sequence coding for the Cry1A(b) C-terminal domain with the gene sequence coding for the Cry1F core toxin. The decision to use F₆₀₄L substitution was based on the occurrence of leucine in the homologous position of other Cry1 proteins, and is therefore a conservative substitution. The predicted molecular weight is 68 kDa.

Cry1F provides control of not only European corn borer and southwestern corn borer, but also provides protection against damaging infestations of other lepidopteran pests including sugarcane borer, fall armyworm, black cutworm, and western bean cutworm (Siebert et al. 2014)

```

1  MENNIQNQCV PYNCLNNPEV EILNEERSTG RLPLDISLSL TRFLLSEFVP GVGVAFGLFD
61  LIWGFITPSD WSLFLLQIEQ LIEQRIETLE RNRAITTLRG LADSYEIIYIE ALREWEANPN
121 NAQLREDVRI RFANTDDALI TAINNFTLTS FEIPLLSVYV QAANLHLSLL RDAVSFGQGW
181 GLDIATVNNH YNRLINLIHR YTKHCLDTYN QGLENLRGTN TRQWARFNQF RRDLTTLTVLD
241 IVALFPNYDV RTYPIQTSSQ LTREIYTSSV IEDSPVSANI PNGFNRAEFG VRPPHLMDFM
301 NSLFVTAETV RSQTVWGGHL VSSRNTAGNR INFPSYGVFN PGGAIWIAD E DPRPFYRTLS
361 DPVFVRGGFG NPHYVLGLRG VAFQQTGTNH TRFRNSGTI DSLDEIPPQD NSGAPWNDYS
421 HVLNHVTFVR WPGEISGSDS WRAPMFSWTH RSATPTNTID PERITQIPLV KAHTLQSGTT
481 VVRGPGFTGG DILRRTSGGP FAYTIVNING QLPQRYRARI RYASTTNLRI YVTVAGERIF
541 AGQFNKTMDDT GDPLTFQSFS YATINTAFTF PMSQSSFTVG ADTFSSGNEV YIDRFELIPV
601 TATLE

```

Figure 5: Amino acid sequence of the *Cry1F* protein

4.1.1.2 Cry34Ab1 and Cry35Ab1

Both proteins are required together for mortality of the corn rootworm larvae. Although the Cry34Ab1 protein is active alone in corn rootworm larvae when applied at high concentrations in bioassays, transgenic plants which expressed only the Cry34Ab1 protein do not control western corn rootworm. The activity of the Cry34Ab1 protein in bioassays is greatly potentiated by Cry35Ab1. The Cry35Ab1 protein alone is not active against corn rootworm. *In vivo*, only a small quantity of Cry35Ab1 is needed in the Cry34/35Ab1 insecticidal crystal protein (ICP). Therefore, the majority of the activity seen with mixtures of Cry34Ab1 and Cry35Ab1 may be explained by the concentration of the Cry34Ab1 protein.

It is not known exactly how the Cry34/35Ab1 ICP exerts its toxicity. Histological studies have shown that the ICP causes disruption of the western corn rootworm larval mid-gut membranes. In experiments using artificial membranes, the ICP produces ion channels or pores which is at least partially responsible for the disruption of the synthetic membranes (Masson et al. 2004). The formation of ion channels in artificial membranes has also been reported for Cry34Ab1 (Baum et al. 2004). Meaningful *in vivo* activity with the ICP has only been observed in a subset of coleopteran larvae (corn rootworm). *In vivo* activity has not been found in adult corn rootworms, a corn aphid species or certain lepidopteran pests, indicating selective activity for corn rootworm larvae. Cry34Ab1 and Cry35Ab1 have not been observed to associate to form a hetero-dimer.

The amino acid sequences of the Cry34Ab1 (mw = 14 kDa) and Cry35Ab1 (mw = 44 kDa) proteins are given in Figure 6.

Cry34Ab1

```
1 MSAREVHIDV NTKTGH TLQL EDKTKLDGGR WRTSPTNVAN DQIKTFVAES NGFMTGTEGT
61 IYYSINGEAE ISLYFDNPPA GSNKYDGHSN KSQYEIITQG GSGNQSHVTY TIQTSSRYG
121 HKS
```

Cry35Ab1

```
1 MLDTNKVYEI SNHANGLYAA TYLSLDDSGV SLMNKND DDI DDYNLKWFLF PIDDDQYIIT
61 SYAANNCKVW NVNNDKINVS TYSSTNSIQK WQIKANGSSY VIQSDNGKVL TAGTGQALGL
121 IRLTDESSNN PNQQWNLT SV QTIQLPQKPI IDTKLKDY PK YSPTGNIDNG TSPQLMGWTL
181 VPCIMVNDPN IDKNTQIKTT PYYILKKYQY WQRAVGSNVA LRPHEKKS YT YEWGTEIDQK
241 TTIINTLGFQ INIDSGMKFD IPEVGGGTDE IKTQLNEELK IEYSHETKIM EK YQE QSEID
301 NPTDQSMNSI GFLTITSLEL YRYNGSEIRI MQIQTSDNDT YNVTSYPNHQ QALLLLTNHS
361 YEEVEEITNI PKSTLKKLKK YYF
```

Figure 6: Amino acid sequences of the Cry34Ab1 and Cry35Ab1 proteins

4.1.2 PAT

The PAT protein consists of 183 amino acids (Figure 7), has a molecular weight of 21 kDa, and exhibits a high degree of enzyme specificity; recognising only one substrate. PAT functions by detoxifying phosphinothricin (PPT), the active constituent of glufosinate ammonium herbicides. PPT acts by inhibiting the endogenous enzyme glutamine synthetase, an enzyme involved in amino acid biosynthesis in plant cells. By inhibiting this enzyme, PPT causes rapid accumulation of ammonia in the plant cell, leading to plant death. In transformed corn plants, the introduced PAT enzyme chemically inactivates the PPT by acetylation of the free ammonia group, giving rise to herbicide tolerance in the whole plant.

1 MSPERRPVEI RPATAADMAA VCDIVNHYIE TSTVNFRTPEP QTPQEWIDDL ERLQDRYPWL
61 VAEVEGVVAG IAYAGPWKAR NAYDWTVEST VYVSHRHQRL GLGSTLYTHL LKSMEAQGFK
121 SVVAVIGLPN DPSVRLHEAL GYTARGTLRA AGYKHGGWHD VGFWRQDFEL PAPP RPVRPV
181 TQI

Figure 7: Amino acid sequence of the PAT protein

4.1.3 Protein expression in the tissues of line 4114

Study submitted:

2011. Expressed Trait Protein Concentration of a Maize Line Containing Events DP-004114-3, DAS-01507-1, DAS-59122-7, and Combined Trait Product DAS-01507 x DAS-59122-7: US and Canada Test Sites. Study ID **PHI-2010-059**. Pioneer Hi-Bred International, Inc (unpublished).

Plants of line 4114 (generation F₁^{*5} – since F₁ hybrid seed is representative of seed that would be commercially planted) were grown from verified seed lots at five field sites in the USA and Canada¹¹ during the 2010 growing season. Since the genetic material in line 4114 is equivalent to that contributed by lines 1507 and 59122 in the commercial breeding stack, plants of both parents and the stack (all grown from verified seed) were also analysed to provide comparative data.

Three of the planting sites overlapped with those used for the compositional analysis described in Section 5.2. There were four replicated plots at each site planted in a randomised complete-block design. Maintenance fertiliser and pesticide were applied to all plots as appropriate to the site conditions.

Four replicated samples from different plants were taken at various stages of growth (Table 4) to give a total of 20 samples for each tissue and time point. Levels of Cry1F, Cry34Ab1, Cry35Ab1 and PAT were determined for each sample type using a validated enzyme linked immunosorbent assay (ELISA). For each protein, plates were pre-incubated with an appropriate-specific antibody to bind the protein to the plate wells. The protein was then detected by incubation with a different specific antibody conjugated to horseradish peroxidase followed by addition of substrate specific to the enzyme. Plates were analysed on a microplate spectrophotometer, and commercial software (SoftMax Pro GxP, Molecular Devices) was used to convert optical density values to protein concentration

The results, averaged over all sites, are given in Table 4.

Table 4: Cry1F, Cry34Ab1, Cry35Ab1 and PAT protein content in line 4114 parts at different growth stages (averaged across 5 sites)

Tissue/Growth stage ¹	Cry1F µg/g dw		Cry34Ab1 ug/g dw		Cry35Ab1 µg/g dw		PAT µg/g dw	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Leaf V6	11	7.8 - 17	21	17 - 25	27	17 - 38	9	4.2 - 14
Leaf V9	9.7	5.3 - 14	26	22 - 31	33	28 - 39	9.8	4.8 - 15
Leaf R1	13	7.2 - 28	50	36 - 84	68	43 - 130	14	5 - 24
Leaf R4	34	19 - 56	110	66 - 140	90	66 - 110	11	5.7 - 20
Leaf R6	2	0.32 - 21	19	4.2 - 66	72	41 - 110	0.52	<LOQ - 2
Root V6	4.2	0.69 - 6	17	9 - 23	13	9 - 20	0.44	0.14 - 0.78

¹¹ Bagley, Iowa; Atlantic, Iowa; Wyoming, Illinois; York, Nebraska, Branchton, Ontario

Tissue/Growth stage ¹	Cry1F µg/g dw		Cry34Ab1 ug/g dw		Cry35Ab1 µg/g dw		PAT µg/g dw	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Root V9	5	1.3 – 7.5	21	13 - 28	13	7.8 - 19	0.65	0.39 – 0.9
Root R1	5.5	3.9 – 7.8	19	8.7 - 30	9.2	4.2 - 15	0.44	0.3 – 0.72
Root R4	3.8	2.3 – 5.7	23	7.5 - 36	6.4	2.1 - 12	0.16	<LOQ – 0.39
Root R6	3.8	1.4 – 6.3	18	5.4 - 54	6.9	2.2 - 14	0.13	<LOQ – 0.66
Whole plant V9	12	8.6 - 15	23	18 - 26	76	58 - 100	8.7	6.6 - 11
Whole Plant R1	9.9	7.8 - 13	32	24 - 42	66	44 - 100	4.9	3.2 – 7.4
Whole Plant R6	4.1	2.4 – 9.4	36	20 - 62	21	13 - 54	0.09	<LOQ – 0.76
Pollen R1	35	19 - 49	9.2	4.7 - 16	0.34	<LOQ – 0.53	< LOQ ²	<LOQ
Forage ³ R4	7.8	5.6 - 11	52	36 - 64	29	18 - 52	1.9	1.1 – 2.8
Grain R6	3.3	2.3 – 7.2	24	14 - 39	1.1	0.54 – 2.3	<LOQ	<LOQ

¹For information on corn growth stages see e.g. Ransom & Endres (2014)

²LOQ = limit of quantitation

³Forage is the above ground plant parts used for animal feed.

For Cry34Ab1, Cry35Ab1 and PAT, mean levels were lowest in the pollen (9.2, 0.34 µg/g dry weight and <LOQ respectively). The highest levels of these three proteins were in the leaf samples at either the R1 or R4 stages. In contrast, the level of Cry1F was lowest in leaf tissue at R6 (the stage at which grain is harvested) and highest in pollen (35 µg/g dry weight).

The levels of the four proteins in line 4114 were compared to levels in line 1507, line 59122 and the breeding stack of these parental lines, by dividing the levels in line 4114 by the respective protein levels in line 1507, line 59122 and 1507 x 59122 to provide an 'expression ratio' (see Table 5) i.e. a ratio of close to 1 indicated comparable levels. For all proteins, the levels in line 4114 were comparable to, or lower than, the levels in the other lines with the following exceptions (see yellow highlight in Table 5):

- Cry1F was higher in pollen and R6 leaf of line 4114 than in the same tissues from either comparator line.
- Cry34Ab1 was higher in R6 leaf of line 4114 than in R6 leaf of either comparator line.
- Cry35Ab1 was higher in R6 leaf of line 4114 than in R6 leaf of line 59122
- PAT was higher in R6 leaf of line 4114 than in all three comparator lines.
- PAT was higher in all tissue types (except R6 root, pollen and grain.) of line 4114 than in the same tissue types of line 1507.

These differences are likely to be explained by event-to-event variation and do not impact on food safety where the grain is the major source of food products.

Table 5: Summary of line 4114 protein concentrations as a ratio of line 1507, line 59122 and 1507 x 59122

Tissue (Growth Stage)	Protein Concentration Ratio			
	4114 Maize:1507 Maize	4114 Maize:59122 Maize	4114 Maize:1507x59122 Maize	
Cry1F				
Leaf (V6)	0.611	Cry1F not present in DAS-59122-7	0.611	
Leaf (V9)	0.746		0.606	
Leaf (R1)	0.813		0.765	
Leaf (R4)	0.919		0.919	
Leaf (R6)	2.53		5.41	
Root (V6)	0.737		0.667	
Root (V9)	0.694		0.725	
Root (R1)	0.786		0.902	
Root (R4)	0.864		0.717	
Root (R6)	0.760		0.809	
Whole Plant (V9)	0.750		0.750	
Whole Plant (R1)	0.707		0.762	
Whole Plant (R6)	0.837		1.08	
Pollen (R1)	1.52		1.46	
Forage (R4)	0.929		0.857	
Grain (R6)	1.03		1.18	
Cry34Ab1				
Leaf (V6)	Cry34Ab1 not present in DAS- 1507-1		0.636	0.656
Leaf (V9)		0.667	0.619	
Leaf (R1)		0.667	0.714	
Leaf (R4)		0.579	0.550	
Leaf (R6)		2.32	3.17	
Root (V6)		0.654	0.586	
Root (V9)		0.568	0.583	
Root (R1)		0.655	0.655	
Root (R4)		0.719	0.657	
Root (R6)		0.486	0.563	
Whole Plant (V9)		0.561	0.561	
Whole Plant (R1)		0.561	0.571	
Whole Plant (R6)		0.750	0.750	
Pollen (R1)		0.209	0.200	
Forage (R4)		0.605	0.559	
Grain (R6)		1.00	1.00	
Cry35Ab1				
Leaf (V6)		Cry35Ab1 not present in DAS-01507-1	0.750	0.818
Leaf (V9)	0.825		0.717	
Leaf (R1)	0.840		0.958	
Leaf (R4)	0.900		0.900	
Leaf (R6)	1.22		1.00	
Root (V6)	0.813		0.722	
Root (V9)	0.765		0.765	
Root (R1)	0.836		0.920	
Root (R4)	0.800		0.800	
Root (R6)	0.690		0.775	
Whole Plant (V9)	0.894		0.962	
Whole Plant (R1)	0.957		0.880	
Whole Plant (R6)	0.778		0.677	
Pollen (R1)	1.03		1.06	
Forage (R4)	0.967		1.04	
Grain (R6)	0.857		0.800	
PAT				
Leaf (V6)	1.61		0.750	0.563
Leaf (V9)	1.48	0.817	0.467	
Leaf (R1)	2.15	0.737	0.519	
Leaf (R4)	2.62	0.846	0.611	
Leaf (R6)	2.08	2.08	1.58	
Root (V6)	1.52	0.400	0.259	
Root (V9)	1.81	0.464	0.295	
Root (R1)	1.83	0.449	0.400	
Root (R4)	1.23	0.485	0.258	
Root (R6)	1.00	0.317	0.317	
Whole Plant (V9)	2.42	0.791	0.580	
Whole Plant (R1)	2.04	0.731	0.583	
Whole Plant (R6)	1.23	0.692	0.500	
Pollen (R1)	1.00	1.00 ^b	1.00	
Forage (R4)	2.53	0.760	0.543	
Grain (R6)	1.00	0.972	0.775	

4.1.4 Characterisation of the plant-produced proteins

The expected characteristics of the four expressed novel proteins are discussed in Sections 4.1.1. and 4.1.2. A translation of the DNA insert in line 4114 indicated that the amino acid sequences of the encoded Cry1F, Cry34Ab1, Cry35Ab1 and PAT proteins are as provided in Figures 5-7 and are identical to the sequences found in lines 1507 and 59122. A full characterisation of the proteins *in planta* had been done for lines 1507 and 59122 (FSANZ 2003; FSANZ 2005) and therefore for line 4114 it was only necessary to confirm whether the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins expressed in line 4114 are equivalent to those expressed in the breeding stack 1507 x 59122. Accordingly, the Applicant used a Western blot analysis to do this.

Study submitted:

2011. Expressed trait protein concentration of a maize line containing events DP-004114-3, DAS-01507-1, DAS-59122-7, and a combined trait product DAS-01507-1 x DAS-59122-7: US and Canada test sites. Study ID **PHI-2010-059**. Pioneer Hi-Bred International, Inc (unpublished).
2011. Western Blot Analysis of Maize Lines Containing Event DP-004114-3 and Combined Trait Product DAS-01507-1xDAS-59122-7. Study ID **PHI-2011-067**. Pioneer Hi-Bred International, Inc (unpublished).

Leaf samples were collected at the R1 growth stage from plants of each of line 4114 (generation F₁*5), the breeding stack 1507 x 59122 and a verified non-GM control line. Crude extracts of proteins were prepared by extraction in buffer, before separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to polyvinylidene fluoride membranes. Each novel protein was then visualised following incubation in an appropriate monoclonal mouse primary antibody and a horseradish peroxidase-linked anti-mouse secondary antibody. There was no detection of any of the novel proteins in the samples from the control.

For Cry1F, the samples from both line 4114 and the breeding stack showed a double banding pattern with sizes of approximately 60 kDa and 62 kDa. This doublet was not unexpected since it had also been observed for the Cry1F protein in line 1507 where the following explanation was given in the safety assessment (FSANZ 2003) - "The results of the analyses of Cry1F protein expression in plant tissues demonstrated that under denaturing conditions the Cry1F protein was detected as two bands with almost identical mobility (a doublet) of approximately 65 to 68 kDa in leaf, pollen, grain and whole plant tissue". No other bands indicative of a partial Cry1F protein or a fusion protein of greater molecular size were observed. Due to the presence of the known enzyme cleavage sites near the amino-terminus of the protein, the doublet most likely resulted from limited N-terminal processing by a plant protease with trypsin-like specificity. It is noted that the protein obtained from a bacterial source, and used as a standard, appears as a single immunoreactive band. Importantly, the Western blot confirms the equivalent immunoreactivity of the Cry1F protein from line 4114 and the breeding stack.

Similarly, for the Cry35Ab1 protein, a doublet (approximately 40 kDa and 44 kDa) was also observed in samples from both line 4114 and the breeding stack. Again, this doublet was also noted for the Cry35Ab1 protein in line 59122 (FSANZ 2005) – "the full length upper band protein of the 44-kDa is originally expressed. However, the protein is susceptible to the proteases in plant cells, resulting in the lower band form". As for the Cry1F protein, there was only a single immunoreactive band for the bacterially-produced protein standard but, importantly the Western blot confirms the equivalent immunoreactivity of the Cry35Ab1 protein from line 4114 and the breeding stack.

The Western blots for both the Cry34Ab1 and PAT proteins showed single bands at approximately 14 kDa and 21 kDa respectively.

Overall, the results confirm that the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins expressed in line 4114 have the approximate molecular weights expected of the proteins (within the limitations of a Western blot for estimating molecular weight) and that each protein has the same immunoreactivity as the equivalent protein expressed in the 1507 x 59122 breeding stack.

4.1.5 The safety of the introduced proteins

All of the introduced novel proteins have been assessed by FSANZ in a number of previous applications and all have been considered safe.

- Cry1F occurs in line 1507 which was assessed in Application A446 (FSANZ 2003).
- Cry34Ab1 and Cry35Ab1 occur in line 59122 which was assessed in Application A543 (FSANZ 2005).
- The PAT protein, encoded by either the *pat* or *bar* genes (Wehrmann et al. 1996), has now been considered in 19 FSANZ approvals (A372, A375, A380, A385, A386, A446, A481, A518, A533, A543, A589, A1028, A1040, A1046, A1073 A1080, A1081, A1087 and A1094) as well as being accepted in the literature as having neither toxicity nor allergenicity concerns (Hérouet et al. 2005; see e.g. Delaney et al. 2008).

A summary of these previous characterisations is provided in Table 6 which shows the application in which the most recent detailed study or information was considered by FSANZ and is available on the FSANZ website.

For the bioinformatic studies, which analyse sequence similarity to known protein toxins and allergens, where the Applicant provided searches (see updated studies listed below) using a more recent (and hence larger) database, the results did not alter the conclusions reached previously that Cry1F, Cry34Ab1, Cry35Ab1 and PAT are not allergenic or toxic.

Table 6: Summary of consideration of Cry1F, Cry34Ab1, Cry35Ab1 and PAT in previous FSANZ safety assessments

Consideration	Sub-section	Cry1F (link)	Cry34Ab1 & Cry35Ab1 (link)	PAT (link)
Potential toxicity	History of human consumption	A446 (FSANZ, 2003)	A543 (FSANZ, 2005)	A1087 (FSANZ 2013a)
	Amino acid sequence similarity to protein toxins	This application – using search updated in June 2014	This application – using search updated in July 2014	This application – using search updated in June 2014
	Stability to heat	A446 (FSANZ, 2003)	A543 (FSANZ, 2005)	A1080 (FSANZ 2013b)
	Acute oral toxicity	A446 (FSANZ, 2003)	A543 (FSANZ, 2005)	A1080 (FSANZ 2013b)
Potential allergenicity	<i>In vitro</i> digestibility	A446 (FSANZ, 2003)	A543 (FSANZ, 2005)	A1080 (FSANZ 2013b)
	Amino acid sequence similarity to allergens	This application – using search updated in April 2014	This application – using search updated in April 2014	This application – using search updated in April 2014

Updated bioinformatics studies submitted

2014. Evaluation of the Amino Acid Sequence Similarity of the Cry1F Protein to the NCBI Protein Sequence Datasets. Study ID **PHI-2008-240/074**. Pioneer Hi-Bred International, Inc (unpublished).
2014. Evaluation of the Amino Acid Sequence Similarity of the Cry34Ab1 and Cry35Ab1 Protein to the NCBI Protein Sequence Datasets. Study ID **PHI-2008-243/074**. Pioneer Hi-Bred International, Inc (unpublished).
2014. Evaluation of the Amino Acid Sequence Similarity of the PAT Protein to the NCBI Protein Sequence Datasets. Study ID **PHI-2008-242/074**. Pioneer Hi-Bred International, Inc (unpublished).
2014. Comparison of the Amino Acid Sequence Identity Between the Cry1F Protein and Known Protein Allergens ($\geq 35\%$ Identity over ≥ 80 Amino Acids and 8 Amino Acid Exact Match as Search Criteria). Study ID **PHI-2008-236/074**. Pioneer Hi-Bred International, Inc (unpublished).
2014. Comparison of the Amino Acid Sequence Identity Between the Cry34Ab1 and Cry35Ab1 Proteins and Known and Putative Protein Allergens ($\geq 35\%$ Identity over ≥ 80 Amino Acids and 8 Amino Acid Exact Match as Search Criteria). Study ID **PHI-2008-237/074**. Pioneer Hi-Bred International, Inc (unpublished).
2014. Comparison of the Amino Acid Sequence Identity Between the PAT Protein and Known and Putative Protein Allergens ($\geq 35\%$ Identity over ≥ 80 Amino Acids and 8 Amino Acid Exact Match as Search Criteria). Study ID **PHI-2008-238/074**. Pioneer Hi-Bred International, Inc (unpublished).

4.1.6 Bioinformatic analysis of additional ORFs created by the transformation procedure

Study submitted:

2015. Junction Reading Frame Analysis at the Insertion Site of maize Event DP-004114-3. Study ID **PHI-R036-Y15**. Pioneer Hi-Bred International, Inc (unpublished)

A bioinformatics analysis was performed to assess the similarity to known allergens and toxins of the putative 99 amino acid polypeptide encoded by the sequence obtained from the ORF analysis (refer to Section 3.4.3).

To evaluate the similarity to known allergens of proteins that might potentially be produced by translation of the ORF, an epitope search was carried out to identify any short sequences of amino acids that might represent an isolated shared allergenic epitope. This search compared the sequences with known allergens residing in the FARRP (Food Allergy Research and Resource Program) dataset within version 15 of AllergenOnline (University of Nebraska)¹². The Fast Alignment Search Tool - All (FASTA) algorithm (Pearson and Lipman 1988), version 35.4.4, was used to search the database. No alignments generated an E-score¹³ of $\leq 1e^{-4}$, no alignment met or exceeded the Codex Alimentarius (Codex 2009) FASTA alignment threshold for potential allergenicity (35% identity over 80 amino acids) and no alignments of eight or more consecutive identical amino acids (Metcalf et al. 1996) were found. It was concluded that the ORF does not contain any cross-reactive Immunoglobulin E (IgE) binding epitopes with known allergens.

The ORF amino acid sequence was also compared with non-redundant sequences present in the National Center for Biotechnology (NCBI) database¹⁴ which incorporated entries from several databases including GenBank, RefSeq, Swiss-Prot, Protein Information Resource, Protein Research Foundation and Worldwide Protein Data Bank database.

¹² <http://www.allergenonline.org/>

¹³ Comparisons between highly homologous proteins yield E-values approaching zero, indicating the very low probability that such matches would occur by chance. A larger E-value indicates a lower degree of similarity. Typically, alignments between two sequences will need to have an E-score of $1e^{-5}$ (1×10^{-5}) or smaller to be considered to have significant homology.

¹⁴ <ftp://ftp.ncbi.nlm.nih.gov/blast/db/>

The search used the BLASTP (Basic Local Alignment Search Tool Protein – version 2.2.26) (Altschul et al. 1997) algorithm. No significant similarities of any sequence to any sequences in the databases (including those of known toxins) were found.

It is concluded that, in the unlikely event the 99-amino acid potential ORF was expressed in line 4114, there is no significant similarity between the encoded sequence and any known protein toxins or allergens.

4.1.7 Conclusion

Corn line 4114 is a molecular stack expressing four novel proteins, Cry1F, Cry34Ab1, Cry35Ab1 and PAT. These proteins have previously been assessed in two corn lines 1507 and 59122 where the coding regions and regulatory elements of the various gene cassettes are the same as in line 4114. Food from the breeding stack created from these two lines is also commercially available.

For Cry34Ab1, Cry35Ab1 and PAT, mean levels were lowest in the pollen (9.2, 0.34 µg/g dry weight and <LOQ respectively). The highest levels of these three proteins were in the leaf samples at either the R1 or R4 stages. In contrast, the level of Cry1F was lowest in leaf tissue at R6 (the stage at which grain is harvested) and highest in pollen (35 µg/g dry weight). In the grain, levels of Cry1F, Cry34Ab1, Cry35Ab1 and PAT were 3.3, 24, 1.1 and <LOQ µg/g dry weight respectively.

Western blot analyses confirmed that the four novel proteins in line 4114 have the expected molecular weights and immunoreactivity and are equivalent to the corresponding proteins produced in the breeding stack 1507 x 59122.

4.2 Herbicide metabolites

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

The glufosinate-tolerance trait is present in lines from 19 previous applications to FSANZ (see Section 4.1.5). The enzyme activity of PAT results in the acetylation of the free amino group of glufosinate to produce the non-herbicidal N-acetyl glufosinate. This is a well-known metabolite in glufosinate-tolerant plants and was previously considered in detail by FSANZ in cotton line LL25 (FSANZ 2006). There are no concerns that the spraying of line 4114 with glufosinate would result in the production of any novel metabolites that have not been previously assessed.

5 Compositional analysis

The main purpose of compositional analysis is to determine if, as a result of the genetic modification, any unexpected changes have occurred to the food. These changes could take the form of alterations in the composition of the plant and its tissues and thus its nutritional adequacy. Compositional analysis can also be important for evaluating the intended effect where there has been a deliberate change to the composition of the food.

The classic approach to the compositional analysis of GM food is a targeted one. Rather than analysing every single constituent, which would be impractical, the aim is to analyse only those constituents most relevant to the safety of the food or that may have an impact on the whole diet.

Important analytes therefore include the key nutrients, toxicants and anti-nutrients for the food in question. The key nutrients and anti-nutrients are those components in a particular food that may have a substantial impact in the overall diet. They may be major constituents (fats, proteins, carbohydrates or enzyme inhibitors such as anti-nutrients) or minor constituents (minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in an organism, such as compounds whose toxic potency and level may be significant to health (e.g. solanine in potatoes).

5.1 Key components

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

Corn contains a number of anti-nutrients: phytic acid, raffinose, 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA) and trypsin and chymotrypsin inhibitor. Only phytic acid and raffinose are considered to be biologically relevant (OECD 2002). DIMBOA is present at highly variable levels in corn hybrids and little evidence is available on either its toxicity or anti-nutritional effects, and corn contains only low levels of trypsin and chymotrypsin inhibitor, neither of which is considered nutritionally significant.

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

Secondary metabolites are defined as those natural products which do not function directly in the primary biochemical activities which support the growth, development and reproduction of the organism in which they occur. Secondary plant metabolites are neither nutrients nor anti-nutrients but are sometimes analysed as further indicators of the absence of unintended effects of the genetic modification on metabolism (OECD 2002). Characteristic metabolites in corn are furfural and the phenolic acids, ferulic acid and p-coumaric acid.

There are no generally recognised anti-nutrients in corn at levels considered to be harmful, but for the purposes of comparative assessment, the OECD has recommended considering analytical data for the content of the anti-nutrients phytic acid and raffinose, and the secondary metabolites furfural, ferulic acid, p-coumaric acid.

5.2 Study design and conduct for key components

Study submitted:

2011. Agronomic Characteristics and Nutrient Composition of a Maize Line Containing Event DP-ØØ4114-3: US and Canada Test Sites. Study ID **PHI-2010-058/021**. Pioneer Hi-Bred International, Inc (unpublished).

The line 4114 hybrid generation F_1^{*5} (refer to Figure 4) was used for compositional analysis because it represents a commercial hybrid form of line 4114 that would be most applicable to food and feed use. Ideally, the comparator in compositional analyses should be the near isogenic parental line grown under identical conditions (OECD 2002).

In the case of line 4114, the control was the hybrid produced by crossing PHNARxPHTFE since this represents the closest non-GM genetic line to F₁⁵ for the purposes of comparison.

The test and control lines were grown from verified seed lots at six field sites in the US and Canada¹⁵ during the 2010 growing season. Three of these sites were the same as those used for the protein expression analysis (Section 4.1.3). There were four replicated blocks at each site planted in a randomised complete-block design. Maintenance fertilizer and pesticides were applied to all lines as needed in order to maintain a relatively weed-free and insect-free environment. In addition, a sub-set of line 4114 plants was sprayed at the V4 and V7 growth stages with glufosinate herbicide (Ignite™ 268 SL) at application rates of 0.44 – 0.47 and 0.43 – 0.46 kg ai/ha¹⁶ respectively. No symptoms of plant injury were observed in these glufosinate-treated plants. Compositional data from a total of four proprietary non-GM lines grown at six locations in 2003¹⁷ and four lines grown at six locations in 2007¹⁸ was collected. Procedures for planting, harvesting and processing, of these reference lines were similar to those employed for the trials containing line 4114 and the PHNARxPHTFE control. These data were used to aid in the determination of the normal variation found in corn analyte levels.

Grain was harvested at physiological maturity (R6) and samples (each sample comprised grain from five pooled ears) were analysed for proximates, fibre (acid detergent fibre – ADF; neutral detergent fibre – NDF; total dietary fibre), fatty acids, amino acids, minerals, vitamins, anti-nutrients and secondary metabolites.

Key analyte levels (proximates, fibre, calcium and phosphorus) for forage (harvested at R4) were also obtained but are not reported here; it is noted, however, that in the combined site analysis none of the analyte levels in line 4114 differed significantly from those of the control.

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

5.3 Analyses of key components in grain

The analysis of line 4114 is only for glufosinate-treated plants. In total, 81 analyte levels were measured and carbohydrate was calculated rather than being measured i.e. there was a total of 82 analytes considered. Moisture values were measured for conversion of components to dry weight, but were not statistically analysed. A total of 26 analytes had more than 80% of the observations below the assay limit of quantitation (LOQ) and were excluded from the statistical analysis. The data for 56 analytes were therefore analysed. Data were transformed into Statistical Analysis Software¹⁹ (SAS) data sets and analysed using SAS version 9.2. A least squares mean (LSM) value was generated (combined across all sites) and used for each analyte comparison, and minimum and maximum values were also noted for each analyte (summarised in Tables 7 – 13). A mixed model Analysis of Variance was used for over-all analysis. The significance of an overall treatment effect was estimated using an F-test, while paired contrasts were made using t-tests. Probability values were adjusted using False Discovery Rate (FDR) procedures to improve discrimination of true differences (Benjamini and Hochberg 1995).

¹⁵ Richland, Iowa; Wyoming, Illinois; Geneva Minnesota; York, Nebraska, Branchton, Ontario, Thorndale Ontario

¹⁶ ai = active ingredient

¹⁷ 2003 plantings contained lines 34M94, 33G26, 33J24, 3394 grown in Bagley, Iowa, York, Nebraska, Chula, Georgia; New Holland, Ohio; Larned, Kansas; Hereford, Pennsylvania.

¹⁸ 2007 plantings contained lines 38B85, 37Y12, 34A15, 34P88 grown in Tallahassee, Florida; York, Nebraska; Germansville, Pennsylvania; Richland, Iowa; Larned, Kansas; Branchton, Ontario.

¹⁹ SAS website - <http://www.sas.com/technologies/analytics/statistics/stat/index.html>

In assessing the significance of any difference between the mean analyte value for line 4114 and the control, an FDR-adjusted P-value of 0.05 was used. This means that approximately 5% of statistically significant differences are expected to occur due to chance alone.

Any statistically significant differences between line 4114 and the hybrid control have been compared to the 99% tolerance interval compiled from the results for each analyte of the eight non-GM reference lines combined across all sites/times, to assess whether the differences are likely to be biologically meaningful. These tolerance intervals contain, with 95% confidence, 99% of the values contained in the population of commercial lines. Additionally, the results for line 4114 and the hybrid control have been compared to a combined literature range for each analyte, compiled from published literature for commercially available corn²⁰. It is noted, however, that information in the published literature is limited and is unlikely to provide a broad reflection of the natural diversity that occurs within corn (Harrigan et al. 2010; Ridley et al. 2011; Zhou et al. 2011). Therefore, even if means fall outside the published range, this does not necessarily mean the value falls outside the natural variation.

5.3.1 Proximates and fibre

Results of the proximate and fibre analysis are shown in Table 7. There are no significant differences between the means of line 4114 and the control for any analyte and all means are also within both the tolerance interval and the literature range.

Table 7: Mean (range) percentage dry weight (%dw) of proximates and fibre in grain from glufosinate-treated line 4114 and the hybrid (PHNAR x PHTFE) control.

Analyte	Line 4114 (%dw)	PHNARxPHTFE (%dw)	adj P-value	Tolerance interval (%dw)	Combined literature range (%dw)
Ash	1.43 (1.31 – 1.58)	1.33 (1.12 – 1.52) ²	0.0539	0.531, 2.16	0.62 – 6.28
Crude Protein	10.2 (7.64 – 11.6)	10.2 (8.92 – 11.4)	0.943	6.59, 13.5	6.15 – 17.26
Crude Fat	4.65 (4.32 – 5.86)	4.81 (4.28 – 5.73)	0.332	1.45, 5.75	1.74 – 5.82
Carbohydrate ¹	83.8 (82.3 – 86.4)	83.7 (82.5 – 84.9)	0.874	80.3, 89.7	77.4 – 89.5
ADF	4.05 (3.27 – 4.76)	3.83 (3.24 – 4.48)	0.219	1.43, 5.73	1.82 – 11.34
NDF	10.7 (9.42 – 12.1)	10.4 (9.39 – 11.3)	0.363	5.75, 20.6	5.59 – 22.64
Crude Fibre	2.70 (2.39 – 3.09)	2.57 (1.36 – 3.38)	0.672	0.941, 3.73	0.49 – 5.50

¹ Carbohydrate calculated as 100% - (protein %dw+ fat %dw + ash %dw)

5.3.2 Fatty Acids

The levels of 29 fatty acids were measured. Of these, the following had ≥80% of observations below the LOQ and were therefore excluded from analysis - C8:0 caprylic, C10:0 capric, C12:0 lauric, C14:0 myristic, C14:1 myristoleic, C15:0 pentadecanoic, C15:1 pentadecenoic, C16:1 palmitoleic, C17:0 heptadecanoic, C17:1 heptadecenoic, C17:2 heptadecadienoic, C18:2 (9,15) isomer of linoleic, C18:3 gamma linolenic, C19:0 nonadecanoic, C20:2 eicosadienoic, C20:3 eicosatrienoic, C20:4 arachidonic, C21:0 heneicosanoic, C22:0 behenic, C22:1 erucic and C23:0 tricosanoic. Results for the remaining eight fatty acids are given in Table 8 and can be summarised as follows:

²⁰ Published literature for corn incorporates references used to compile listings in the ILSI Crop Composition Database Version 4 (ILSI 2014).

- There was no significant difference between the means of line 4114 and the control for palmitic, stearic, oleic, linoleic, linolenic, arachidic, and lignoceric acids.
- The mean level of eicosenoic acid was significantly higher in grain of line 4114 compared with grain from the control but fell within both the tolerance interval and the combined literature range.
- The mean level of oleic acid was significantly lower in grain of line 4114 compared with grain from the control but fell within both the tolerance interval and the combined literature range.

Table 8: Mean (range) percentage composition, relative to total fat, of major fatty acids in grain from glufosinate-treated line 4114 and the hybrid (PHNAR x PHTFE) control

Analyte	Line 4114 ¹ (%total)	PHNARxPHTFE (%total)	Adj P- value	Tolerance interval (%total)	Combined literature range (%total)
Palmitic acid (C16:0)	13.9 (13.2 – 14.7)	14.1 (13.2 – 15.6)	0.672	5.51, 18.4	7.94 – 20.71
Stearic acid (C18:0)	1.55 (1.40 – 1.93)	1.6 (1.45 – 2.07)	0.351	0.566, 4.67	1.02 – 3.40
Oleic acid (C18:1)	22.0 (20.0 – 23.9)	22.6 (20.7 – 25.8)	0.0354	10.4, 65.6	17.4 – 40.2
Linoleic acid (C18:2)	60.9 (58.0 – 64.2)	60.1 (54.9 – 62.9)	0.157	30.4, 81.7	36.2 – 66.5
Linolenic acid (C18:3)	0.844 (0.432 – 1.23)	0.787 (0.410 – 1.22)	0.530	0, 3.34	0.57 – 2.25
Arachidic acid (C20:0)	0.384 (0.330 – 0.462)	0.392 (0.343 – 0.505)	0.219	0.159, 0.849	0.28 – 0.965
Eicosenoic acid (C20:1)	0.258 (0.231 – 0.282)	0.235 (0.204 – 0.255)	0.0206	0.213, 0.37	0.17 – 1.917
Lignoceric (C24:0)	0.264 (0.222 - 0.346)	0.240 (0.185 – 0.318)	0.609	0, 0.675	0.14 – 0.23

¹ orange shading represents line 4114 mean that is significantly higher than the control mean; mauve shading represents line 4114 mean that is significantly lower than the control mean

5.3.3 Amino acids

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

The results in Table 9 show there was no significant difference between the control and line 4114 for any of the analyte means. All means also fell within both the tolerance interval and the literature range.

Table 9: Mean (range) %dw of amino acids in grain from glufosinate-treated line 4114 and the hybrid (PHNAR x PHTFE) control

Analyte	Line 4114 ¹ (%dw)	PHNARxPHTFE (%dw)	Adj P-value	Tolerance interval (%dw)	Combined literature range (%dw)
Alanine	0.738 (0.521 - 0.890)	0.739 (0.604 – 0.84)	0.980	0.491, 1.09	0.439 – 1.393
Arginine	0.430 (0.363 – 0.516)	0.434 (0.362 – 0.492)	0.874	0.253, 0.551	0.119 – 0.639
Aspartate	0.691 (0.524 – 0.791)	0.680 (0.562 – 0.751)	0.817	0.442, 0.947	0.335 – 1.208
Cystine	0.188 (0.132 – 0.241)	0.193 (0.157 – 0.253)	0.874	0.136, 0.418	0.125 – 0.514
Glutamate	1.92 (1.36 – 2.31)	1.93 (1.59 – 2.24)	0.943	1.17, 2.88	0.965 – 3.536
Glycine	0.371 (0.303 – 0.439)	0.372 (0.318 – 0.442)	0.932	0.249, 0.485	0.184 – 0.539
Histidine	0.295 (0.222 – 0.365)	0.293 (0.257 – 0.342)	0.874	0.18, 0.362	0.137 – 0.434
Isoleucine	0.341 (0.237 – 0.417)	0.339 (0.288 – 0.388)	0.874	0.143, 0.587	0.179 – 0.692
Leucine	1.27 (0.813 – 1.61)	1.26 (1.02 – 1.49)	0.874	0.659, 1.95	0.642 – 2.492
Lysine	0.286 (0.223 – 0.334)	0.293 (0.224 – 0.349)	0.556	0.112, 0.551	0.172 – 0.668
Methionine	0.179 (0.127 – 0.225)	0.179 (0.144 – 0.231)	0.982	0.072, 0.49	0.124 – 0.468
Phenylalanine	0.535 (0.353 – 0.679)	0.523 (0.443 – 0.611)	0.805	0.298, 0.693	0.244 – 0.930
Proline	0.905 (0.642 – 1.10)	0.899 (0.763 – 1.01)	0.874	0.454, 1.64	0.462 – 1.632
Serine	0.506 (0.364 – 0.620)	0.5 (0.421 – 0.554)	0.874	0.266, 0.683	0.235 – 0.769
Threonine	0.364 (0.272 – 0.426)	0.361 (0.318 – 0.402)	0.874	0.176, 0.578	0.224 – 0.666
Tryptophan	0.0656 (0.0530 – 0.0740)	0.0634 (0.0367 – 0.0726)	0.551	0.0087, 0.127	0.0271 – 0.215
Tyrosine	0.248 (0.175 – 0.332)	0.263 (0.199 – 0.333)	0.480	0.0707, 0.505	0.103 – 0.642
Valine	0.462 (0.346 – 0.550)	0.460 (0.393 – 0.507)	0.897	0.159, 0.749	0.266 – 0.855

5.3.4 Minerals

The levels of nine minerals in grain from line 4114 and the hybrid control were measured. For copper, ≥ 80% of the data points were below the LOQ and no statistical analysis was done. Results for the remaining analytes are given in Table 10 and can be summarised as follows:

- There was no significant difference between the means of line 4114 and the control for calcium, iron, magnesium, manganese, sodium and zinc.
- The mean levels of phosphorus and potassium were significantly higher in grain of line 4114 compared with grain from the control but fell within both the tolerance interval and the combined literature range.

Table 10: Mean (range) levels of minerals in the grain of glufosinate-treated line 4114 and the hybrid (PHNAR x PHTFE) control

Analyte	Unit	Line 4114 ¹	PHNARxPHTFE	Adj P-value)	Tolerance interval	Combined literature range
Calcium	mg/kg dw	33.5 (26.1 – 52.7)	32.5 (27 – 39.9)	0.575	12.7, 90.2	12.7 – 208.4
Iron	mg/kg dw	17.5 (15.0 – 33.4)	16.8 (14.6 – 22.8)	0.599	8.57 – 26.9	10.4 – 49.1
Magnesium	%dw	0.134 (0.109 – 0.168)	0.133 (0.103 – 0.155)	0.874	0.038 – 0.19	0.0594 – 0.194
Manganese	mg/kg dw	6.27 (4.94 – 9.70)	6.21 (4.92 – 9.58)	0.874	2.5, 12.2	1.69 – 14.30
Phosphorus	%dw	0.311 (0.283 – 0.362)	0.289 (0.258 – 0.334)	0.0206	0.127 – 0.472	0.147 – 0.533
Potassium	%dw	0.421 (0.381 – 0.514)	0.387 (0.343 – 0.441)	0.0208	0.194, 0.687	0.181 – 0.603

Analyte	Unit	Line 4114 ¹	PHNARxPHTFE	Adj P-value)	Tolerance interval	Combined literature range
Sodium	mg/kg dw	0.732 (<LOQ – 2.84)	0.582 (<LOQ – 4.89)	0.575	0 – 20.7	0.2 - 732
Zinc	mg/kg dw	17.5 (15.0 – 20.1)	17.4 (15.0 – 20.3)	0.874	10.4, 27.1	6.5 – 37.2

¹ orange shading represents line 4114 means that are significantly higher than the control means.

5.3.5 Vitamins

Levels of 12 vitamins were measured. For riboflavin, β -tocopherol and δ -tocopherol because more than 80% of the readings were below the LOQ, no analysis was done.

Results for the remaining nine vitamins are given in Table 11 and show the mean level of Vitamin B₁ in line 4114 was significantly higher than the mean in the control. However, both means fell within both the tolerance interval and the literature range. There were no significant differences between the means of line 4114 and the control for any other analytes.

Table 11: Mean (range) weight (mg/k g dw) of vitamins in grain from glufosinate-treated line 4114 and the hybrid (PHNAR x PHTFE) control

Analyte	Line 4114 (mg/kg dw) ¹	PHNARxPHTFE (mg/kg dw)	Adj P-value)	Tolerance interval (mg/kg dw)	Combined literature range (mg/kg dw)
Vitamin A (β -carotene)	18.9 (12.0 – 33.6)	16.4 (7.27 – 26.2)	0.351	0, 68.3	0.19 – 46.81
Vitamin B ₁ (Thiamine HCl)	2.59 (<LOQ – 3.61)	1.97 (<LOQ – 2.92)	0.0479	0.414, 6.64	1.26 – 40.00
Vitamin B ₃ (Niacin)	13.7 (10.5 – 16.7)	13.9 (10.6 – 18)	0.836	0, 51.7	10.37 – 46.94
Vitamin B ₅ (Pantothenic acid)	6.06 (3.94 – 27.0)	4.92 (<LOQ – 10.9)	0.322	3.02, 8.20	3.50 – 14.0
Vitamin B ₆ (Pyridoxine HCl)	4.83 (1.81 – 8.14)	4.68 (2.35 – 6.88)	0.874	1.83, 11.1	3.68 – 11.32
Vitamin B ₉ (Folic acid)	0.943 (0.138 – 1.48)	0.872 (0.181 – 1.91)	0.824	0, 2.3	0.147 – 1.464
Vitamin E (α -tocopherol)	6.50 (3.02 – 13.0)	5.47 (2.09 – 13.3)	0.303	2.18, 28.2	1.5 – 68.7
γ -tocopheroal	4.05 (2.88 – 5.54)	3.38 (2.30 – 5.04)	0.0662	0, 39.9	6.46 – 61.0
Total tocopherols	11.0 (7.09 – 17.9)	9.23 (4.77 – 17.0)	0.195	0, 53.6	8.69 - 133

¹ orange shading represents line 4114 mean that is significantly higher than the control mean.

5.3.6 Anti-nutrients

Levels of three key anti-nutrients were measured. Results in Table 12 show that none of the means differed significantly between line 4114 and the control. All means fell within both the tolerance interval and the literature range.

Table 12: Mean (range) of anti-nutrients in grain from glufosinate-treated line 4114 and the hybrid (PHNAR x PHTFE) control

Analyte	Line 4114 ¹	PHNARxPHTFE	Adj P-value	Tolerance interval	Combined literature range
Phytic acid (%dw)	1.04 (0.796 – 1.36)	0.998 (0.842 – 1.17)	0.575	0.418, 1.41	0.111 – 1.570
Raffinose %dw)	0.0928 (<LOQ – 0.227)	0.0823 (<LOQ – 0.210)	0.672	0, 0.398	0.020 – 0.320
Trypsin inhibitor (TIU/mg)	2.39 (1.60 – 3.05)	2.67 (1.45 – 4.68)	0.530	1.60, 4.89	1.09 – 7.18

5.3.7 Secondary metabolites

The levels of four secondary metabolites were measured but furfural was below the level of quantitation in line 4114, the control and all of the reference hybrids. For the three remaining metabolites p-coumaric acid, ferulic acid and inositol (see Table 13) there was no significant difference between the control and line 4114 for either of the means.

Table 13: Mean %dwt (range) of three secondary metabolites in grain from glufosinate-treated line 4114 and the hybrid (PHNAR x PHTFE) control

Analyte	Line 4114 (%dw)	PHNARxPHTFE (%dw)	Adj P-value	Tolerance interval (%dw)	Combined literature range (%dw)
p-coumaric acid	0.0190 (0.0152 – 0.0255)	0.0183 (0.0120 – 0.0276)	0.530	0.000341, 0.0387	0.003 – 0.0576
Ferulic acid	0.275 (0.237 – 0.329)	0.255 (0.199 – 0.299)	0.219	0.0553, 0.0309	0.0200 – 0.0389
Inositol	0.0224 (0.0151 – 0.0325)	0.0256 (0.0197 – 0.0341)	0.132	0, 0.0461	0.0089 – 0.377

5.3.8 Summary of analysis of key components

Statistically significant differences in the analyte levels found between grain of line 4114 and the PHNAR x PHTFE control are summarised in Table 14.

Table 14: Summary of analyte levels found in grain of glufosinate-treated line 4114 that are significantly (P < 0.05) different from those found in grain of the hybrid (PHNAR x PHTFE) control

Analyte	Unit	Line 4114 mean ¹	Hybrid control mean	diff between line411 & hybrid control means	diff between max and min in hybrid control	Line 4114 within tolerance interval?	line 4114 within literature range?
Ash	% dw	1.44	1.33	0.11	0.40	yes	yes
Oleic acid	% total fatty acid	22.0	22.6	0.6	3.9	yes	yes
Eicosenoic acid	% total fatty acid	0.265	0.235	0.030	0.051	yes	yes
Phosphorus	% dw	0.311	0.289	0.022	0.076	yes	yes

Analyte	Unit	Line 4114 mean ¹	Hybrid control mean	diff between line411 & hybrid control means	diff between max and min in hybrid control	Line 4114 within tolerance interval?	line 4114 within literature range?
Potassium	% dw	0.416	0.387	0.029	0.098	yes	yes
Vitamin B ₁	mg/kg dw	2.59	1.97	0.62	> 1.12	yes	yes

¹ orange shading represents line 4114 means that are significantly higher than the hybrid control means; mauve shading represents line 4114 mean that is significantly lower than the control mean

5.4 Conclusion from compositional analysis

Detailed compositional analyses were done to establish the nutritional adequacy of grain from line 4114 and to characterise any unintended compositional changes. Analyses were done of proximates, fibre, minerals, amino acids, fatty acids, vitamins, secondary metabolites and anti-nutrients. The levels were compared to levels in a) an appropriate non-GM hybrid line, PHNAR x PHTFE, b) a tolerance interval compiled from results taken for eight non-GM hybrid lines grown in similar field trials in different seasons, and c) levels recorded in the literature. Only five of the 56 analytes reported in Tables 7 - 13 deviated in level from the control in a statistically significant manner. However, the mean levels of all of these analytes fell within both the tolerance interval and the historical range from the literature. It is further noted that the differences between the line 4114 and control means for each analyte in Table 14 were less than the variation found within the control. It can therefore be concluded that grain from line 4114 is compositionally equivalent to grain from conventional corn varieties.

6 Nutritional impact

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

If the compositional analysis indicates biologically significant changes to the levels of certain nutrients in the GM food, additional nutritional assessment should be undertaken to assess the consequences of the changes and determine whether nutrient intakes are likely to be altered by the introduction of such foods into the food supply.

Where a GM food has been shown to be compositionally equivalent to conventional varieties, the evidence to date indicates that feeding studies using target livestock species will add little to the safety assessment and generally are not warranted (see e.g. OECD 2003; Bartholomaeus et al. 2013; Herman and Ekmay 2014). Line 4114 is the result of a genetic modification designed to confer protection against corn rootworm and tolerance to the herbicide glufosinate with no intention to significantly alter nutritional parameters in the food. In addition, the extensive compositional analyses of grain that have been undertaken to demonstrate the nutritional adequacy of line 4114 indicate it is equivalent in composition to conventional corn cultivars. The introduction of food from line 4114 into the food supply is therefore expected to have little nutritional impact and as such no additional studies, including animal feeding studies, are required.

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