Application to Food Standards Australia New Zealand for the Inclusion of Insect-Resistant and Herbicide-Tolerant DP-ØØ4114-3 Maize in Standard 1.5.2 – Food Produced Using Gene Technology

Submitting Company:

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OECD Unique Identifier: DP-ØØ4114-3

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STATUTORY DECLARATION – AUSTRALIA

Statutory Declarations Act 1959

I, [redacted] Managing Director for Pioneer Hi-Bred Australia, LMB 9001 204 Wyreema Road, Toowoomba, QLD 4350 AU, make the following declaration under the *Statutory Declarations Act 1959*:

1. the information provided in this application fully sets out the matters required
2. the information provided in this application is true to the best of my knowledge and belief
3. no information has been withheld that might prejudice this application, to the best of my knowledge and belief

I understand that a person who intentionally makes a false statement in a statutory declaration is guilty of an offence under section 11 of the *Statutory Declarations Act 1959*, and I believe that the statements in this declaration are true in every particular.

....

Signature [redacted]

Declared at Toowoomba on 3rd of December 2014

[place] [day] [month] [year]

Before me,

Signature of person before whom this declaration is made

[redacted] ....

[Full name, qualification and address of person before whom this declaration is made]

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APPLICANT DETAILS

(a)	Applicant's name/s	
(b)	Company/organisation name	Pioneer Hi-Bred Australia
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(d)	Telephone number	+ 61 – 7- 4637 3600
(e)	Email address	
(f)	Nature of applicant's business	Pioneer Hi-Bred International, Inc. (Pioneer) is the world's leading developer and supplier of advanced plant genetics and biotechnology products to farmers worldwide. DuPont is a science company, delivering science-based solutions that make a difference in people's lives in food and nutrition, health care, apparel, home and construction, electronics and transportation.
(g)	Details of other individuals, companies or organisations associated with the application	Not applicable.

PURPOSE OF THE APPLICATION

The purpose of this submission is to make an application to amend the *Australia New Zealand Food Standards Code* to allow for the inclusion of insect-resistant and herbicide-tolerant 4114 maize in Standard 1.5.2–Food Produced Using Gene Technology. This variation would permit the import and use of food derived from insect-resistant and herbicide-tolerant 4114 maize developed by Pioneer.

RELEVANT OVERSEAS APPROVALS

Maize event 4114 has been approved for all uses (e.g., cultivation, food and feed use) in the United States and Canada, and approved for food and feed use in Mexico, Korea, and Taiwan (Table 1).

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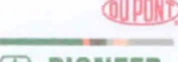

Table 1: Current approvals for 4114 maize

Country	Competent National Authority	Type of Authorisation	Approval Date
United States	United States Department of Agriculture (USDA)	Determination of nonregulated status	20 June 2013
	Food and Drug Administration (FDA)	Consultation completed; no further questions regarding use in food or feed	25 March 2013
	Environmental Protection Agency (EPA)	Biopesticide registration	7 June 2012
Canada	Canadian Food Inspection Agency (CFIA)	Unconfined environmental release	21 June 2013
		Use in livestock feed	21 June 2013
	Health Canada	No objection to use in food	19 June 2013
Mexico	Department of Health (COFEPRIS)	Authorisation for food and feed use	7 January 2014
Korea	Ministry of Food and Drug Safety (MFDS)	Approval for food use	10 October 2014
	Rural Development Administration (RDA)	Approval for feed use	4 September 2014
Taiwan	Ministry of Health and Welfare (MOHW)	Registration for food and feed use	27 June 2014

In addition, submissions have been made to the following regulatory authorities/agencies, where they are currently in process:

1. Ministry of Health, Labour and Welfare (MHLW) for internal review of food safety assessment in Japan on November 8, 2012; Ministry of Agriculture, Forestry and Fisheries (MAFF) for environmental assessment on March 27, 2013 and MAFF for feed safety assessment on February 1, 2013;

2. Ministry of Agriculture in China on June 25, 2012.

3. European Food Safety Authority on 19 November, 2014

Responsible environmental stewardship and deployment of biotechnology-derived products are very important to Pioneer, its parent company E.I. du Pont de Nemours and Company (DuPont), and its affiliate companies. Pioneer and DuPont are founding members of Excellence Through Stewardship® (ETS), an industry-coordinated initiative that promotes the global adoption of stewardship programs and quality management systems for the full life cycle of biotechnology-derived plant products. The ETS "*Guide Product Launch Stewardship of Biotechnology-Derived Products*" (ETS, 2010) also references and is consistent with the product launch policies of the Biotechnology Industry Organization and Crop Life International.

JUSTIFICATION FOR THE APPLICATION

Maize event 4114 has been developed to provide an alternative to the 1507 × 59122 maize breeding stack for more complex stack combinations. Maize 4114 is not intended to be a stand-alone commercial product and will be combined with other approved events using conventional breeding to create stacked products with multiple modes of action for control of pest insects and with tolerance to one or more classes of herbicides. As part of a complex breeding stack, 4114 maize will have similar insect resistance and herbicide tolerance benefits as those containing the combination of 1507 and 59122 maize, and will have added breeding advantages over the available 1507 × 59122 maize, which was grown on approximately 26 million acres or approximately 27% of U.S. maize acres in 2013 (Data source: Pioneer Hi-Bred International, Inc. Proprietary Data).

Maize is the largest crop grown in the United States in terms of acreage and net value, and has multiple downstream uses for feed, fuel, and food that are significant for global supply. The

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introduction of new maize trait offerings that meet grower needs, such as stacked products containing 4114 maize, is critical to help keep pace with increasing maize demand globally.

Australia imports a range of processed food products from various countries, which may in future contain ingredients derived from 4114 maize. This variation to the Standard would permit the import and use of food derived from insect-resistant and herbicide-tolerant 4114 maize developed by Pioneer.

ASSESSMENT PROCEDURE

Pioneer Hi-Bred Australia is anticipating that this application will be considered under the **General Procedure** for Administrative Assessment process by Food Standards Australia New Zealand.

EXCLUSIVE CAPTURABLE COMMERCIAL BENEFIT

Pioneer Hi-Bred Australia acknowledges that the proposed amendment to the Standard will likely result in an exclusive capturable commercial benefit being accrued to the parent company (Pioneer International) as defined in Section 8 of the *FSANZ Act*.

INTERNATIONAL AND OTHER NATIONAL STANDARDS

The Codex Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants (CAC/GL 45-2003) is applicable to the assessment of this application to amend the *Australia New Zealand Food Standards Code* to allow for the inclusion of insect-resistant and herbicide-tolerant 4114 maize in Standard 1.5.2–Food Produced Using Gene Technology.

COST-BENEFIT EFFECT

In countries where they are cultivated, commercial maize hybrids containing event 4114 will provide similar insect and weed control benefits to those of current 1507 × 59122 hybrids. 4114 maize contains the same Cry1F, Cry34Ab1, and Cry35Ab1 proteins as 1507x59122 maize and will provide growers with a simplified means of controlling lepidopteran and corn rootworm insects, including European corn borer (*Ostrinia nubilalis*) and western corn rootworm (*Diabrotica virgifera virgifera)*. Both European corn borer and the corn rootworm species complex (*Diabrotica* ssp.) are major maize insect pests throughout the United States and monetary losses resulting from feeding damage and insect control for each pest exceed $1 billion each year (Gray *et al.*, 2009; Ostlie *et al.*, 2002).

Similar to the benefits of 1507 and 59122 maize, the use of hybrids containing event 4114 has the potential to offer effective control of maize insect pests (US-EPA, 2010a; US-EPA, 2010b). The Cry1F, Cry34Ab1, and Cry35Ab1 proteins also offer crop yield advantage under insect pressure (US-EPA, 2010a; US-EPA, 2010b). In general, for corn borer- and corn rootworm-protected maize varieties, a 5 percent yearly average yield advantage compared to conventional maize has

been realized in the United States since these varieties were cultivated (Brookes and Barfoot, 2009; Brookes and Barfoot, 2010)

In addition, 4114 maize also contains the PAT protein that confers tolerance to the herbicidal active ingredient glufosinate-ammonium at current labelled rates. Glufosinate was registered as an herbicide in 1993 in the United States and is approved for use in Australia. Glufosinate-ammonium tolerance will allow growers to proactively manage weed populations and, in a proper herbicide rotation program, delay the development of adverse populations of weeds.

As discussed previously, 4114 maize will provide an alternative to 1507 × 59122 maize in new complex stacked product offerings. Many maize products will be stacked through traditional breeding with multiple conventional and genetically modified traits to meet evolving grower needs of insect, weed, disease, and abiotic stress management. As a single event replacing a breeding stack with separate unlinked segregating events, 4114 maize will potentially benefit growers by reducing the development time for new products that meet grower and trait durability needs.

The option to use 4114 maize, which contains all genes at a single breeding locus, will reduce the number of breeding loci over 1507 × 59122 maize, thus increasing the speed at which new products will be available to growers. This is because the complexity and expense of breeding multiple traits or events into one maize product increases with each breeding locus added. For example, the effort required to breed two transgenic loci into an inbred line is double the effort required for one transgenic locus. Furthermore, each trait locus must be homozygous in an inbred line and as more loci are combined, the proportion of plants that are homozygous for each locus becomes smaller, resulting in more seed discard during the breeding process.

With the ability to efficiently create stacked products containing additional traits, both transgenic and native, there is an opportunity to enhance product durability and to provide custom offerings that better address grower regional needs. Growers are requiring sophisticated stacked products that include insect resistance (i.e., *Bacillus thuringiensis* or *Bt* traits) and herbicide tolerance, as well as native traits such as disease tolerance, drought tolerance, and higher yields. For *Bt* traits, the maize seed industry is transitioning from products with a single mode of action to products with multiple modes of action, in order to extend the durability of the traits that many growers rely on to manage pests. Therefore, it is important that multiple native and transgenic traits -- including *Bt* traits with different modes of action -- are bred into a single maize product to efficiently meet the needs of growers. Maize event 4114, as one component of future *Bt* breeding stack products with multiple modes of action, will provide benefits to growers in the form of multi-trait products with enhanced durability and more diverse germplasm suited for diverse growing regions. Ultimately, these offerings will allow growers to continue to increase their overall farm productivity to meet the food security needs of a growing global population.

Most of the sweet corn consumed in Australia is grown domestically. Domestic production of corn in Australia (1994–2013 *ca.* 3,840,000 metric tonnes) and New Zealand is supplemented by import of a small amount of corn-based products usually frozen or canned, largely as high-fructose corn syrup, which is not currently manufactured in either Australia or New Zealand.

About 80 percent of sweet corn production in Australia goes to the industrial food processing sector rather than the fresh food market. The sweet corn is processed into breakfast cereals, baking products, extruded confectionery and corn chips. Sweet corn is also processed into the following products:

- Frozen
  - cobetts (portions of whole cobs)
  - whole cobs
  - whole kernels
- Canned
  - whole kernels
  - cream style
- Other
  - soups
  - mixed vegetable packs
  - Cornjacks®
  - long-life vacuum-packed cobs (using a special laminated plastic bag to withstand heat during processing).

Kernel products are the most popular processed lines, with frozen whole cob and cobett products expected to improve their market share. Other corn products such as maize starch are also imported, which is used by the food industry for the manufacture of dessert mixes and canned food. Although not requiring a FSANZ approval for livestock feed, from time to time, mainly during periods of drought where local supply of feed grain is limited, maize is imported from the United States for use as stock feed, predominantly in the pig and poultry markets.

This variation to the Standard would permit the import and use of food derived from insect-resistant and herbicide-tolerant 4114 maize developed by Pioneer.

RELEASE OF INFORMATION

Pioneer Hi-Bred International, Inc. (Pioneer) is submitting the information in this assessment for review by the Food Standards Australia New Zealand (FSANZ) as part of the regulatory process. Pioneer holds proprietary rights to the extent allowable by law to all such information and by submitting this information, Pioneer does not authorize its release to any third party except to the extent it is requested under the *Freedom of Information Act 1982* (*FOIA*) or in compliance with the responsibility of FSANZ to publish all documents required under sections 8, 8(A), 8(C) and 8(D) of the *FOIA*; and this information is responsive to the specific request. Accordingly, except as specifically stated above, Pioneer does not authorize the release, publication or other distribution of this information (including website posting or otherwise), nor does Pioneer authorize any third party to use, obtain, or rely upon any such information, directly or indirectly, as part of any other application or for any other use, without Pioneer's prior notice and written consent. Submission of this information does not in any way waive Pioneer's rights (including rights to exclusivity and compensation) to such information in accordance with the *FOIA*, Pioneer does not authorize the release, publication or other distribution of this information (including website posting) without Pioneer's prior notice and consent.

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Confidential Commercial Information (CCI)

The following report (in the List of Supporting Studies, page 104) is marked as Confidential Commercial information (CCI) and attached as a separate CCI report:

Annex III: Event-Specific Detection Method for 4114 Maize (CCI)

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PART 2 SPECIFIC DATA REQUIREMENTS FOR SAFETY ASSESSMENT

A. Technical Information on the GM Food

A.1. NATURE AND IDENTITY OF THE GENETICALLY MODIFIED FOOD

Description of the new GM organism

Pioneer Hi-Bred has developed maize event 4114 which contains the combined traits from maize lines 1507 (DAS-Ø15Ø7-1) and 59122 (DAS-59122-7). Maize line 4114 expresses the same insect-resistance and herbicide-tolerance traits as present in currently commercialized hybrids containing 1507 × 59122, which are marketed under the trade name Herculex®[2] XTRA. Event 4114 maize expresses the Cry1F protein, conferring resistance to certain lepidopteran insect pests (e.g., European corn borer), the Cry34Ab1 and Cry35Ab1 proteins, which together form an active binary insecticidal protein conferring resistance to corn rootworm, and the phosphinothricin acetyltransferase (PAT) enzyme, which inactivates glufosinate herbicide via acetylation.

Name, number or other identifier of each new line or strain

In accordance with OECD's "Guidance for the Designation of a Unique Identifier for Transgenic Plants", this event has an OECD identifier of DP-ØØ4114-3.

The Name the Food will be Marketed Under

Maize event 4114 is at pre-commercialization stage and has not yet been assigned a commercial product name. In the event that maize event 4114 is commercialized as a stand-alone product, Pioneer Hi-Bred will provide Food Standards Australia New Zealand the commercial name of once it is available.

The Types of Products Likely to Include the Food or Food Ingredient

The introduced traits of insect-resistance and herbicide-tolerance in 4114 maize are not intended to change any of the end-use characteristics of maize grain and the commercial introduction of maize hybrids containing event 4114 is not anticipated to change the usage and consumption patterns of maize grain. It is anticipated that following commercialization, any food containing maize products may contain material derived from 4114 maize.

A.2. HISTORY OF USE OF THE HOST AND DONOR ORGANISMS

Donor Organisms

Bacillus thuringiensis

Strains of *Bacillus thuringiensis* (order: Bacillales; family: Bacillaceae) were sources of the *cry1*F, *cry34*Ab1, and *cry35*Ab1 genes used in the development of event 4114 maize. *Bacillus thuringiensis* (*Bt*) is a naturally occurring soil bacterium (Lang and Otto, 2010) whose ability to form spores containing insecticidal proteins is one of its principal features (Sanahuja *et al.*, 2011). The first commercial *Bt* materials were available for field-testing in the United States in 1958 (Faust, 1974) and formulations containing combinations of several groups of Cry proteins have

[2] Herculex is a registered trademark of Dow AgroSciences LLC.

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been registered in the United States since 1961 (Betz *et al.*, 2000; Schnepf *et al.*, 1998). In their history of widespread and continuous use over 50 years, *Bt* microbial pesticides have caused no known adverse human or environmental effects (Betz *et al.*, 2000; US-EPA, 1998). In a comparative review of the toxicity studies submitted to the United States Environmental Protection Agency to support the registration of *Bt* products, McClintock *et al.* (1995) found no evidence of any significant adverse effects in body weight gain, clinical observations, or upon necropsy, and that the existing data supported the lack of mammalian toxicity or pathogenicity. Similarly, the World Health Organization's (WHO) International Program on Chemical Safety report on environmental health criteria for *Bt* concluded that: *"Owing to their specific mode of action, Bt products are unlikely to pose any hazard to humans or other vertebrates..."* and *"Bt has not been documented to cause any adverse effects on human health when present in drinking water or food"* (IPCS, 1999).

<u>Streptomyces viridochromogenes</u>

Streptomyces viridochromogenes (order: Actinomycetales; family: Streptomycetaceae) is a ubiquitous gram-positive sporulating soil bacterium and was the source of the PAT encoding gene that is present in event 4114 maize. Few *Streptomyces* have been isolated from animal or human sources and pathogenicity is not a typical property of these organisms. *Streptomyces viridochromogenes* is itself not known to be a human pathogen and nor has it been associated with other properties (e.g., production of toxins) known to affect human health.

<u>Other Donor Organisms</u>

Other donor organisms, including cauliflower mosaic virus (CaMV), *Agrobacterium tumefaciens*, wheat (*Triticum aestivum*), potato (*Solanum tuberosum*), and maize (*Zea mays*) were used as sources of various regulatory sequences that are not expressed in the transformed plant. These sequences include: the CaMV 35S promoter and terminator sequences; the promoter, 5′ untranslated region, and intron sequences from the maize polyubiquitin gene; the wheat peroxidase promoter; the terminator region from the potato proteinase inhibitor II gene; and the terminator sequences from the pTi59555 ORF 25 of *A. tumefaciens*. Since none of these sequences encode expressed products in 4114 maize, their donor organisms are of little relevance to assessing potential toxicity or potential allergenicity.

Host Organism

Maize (*Zea mays* L.) is a grass that was first domesticated in Mexico more than 8000 years ago and is now cultivated worldwide as the third most planted crop after wheat and rice. Maize has a long history of safe use and represents a staple food for a significant proportion of the world's population. No significant endogenous toxins are reported to be associated with the genus *Zea* (IFBC, 1990).

The biology of maize, with particular reference to the Australian environment, cultivation, and use, has been described by the Office of the Gene Technology Regulator (OGTR, 2008), and is not further elaborated in this application.

With respect to food safety assessment, it is relevant to note that all maize food products are derived from maize kernels (grain). Maize grain is processed by wet or dry milling to yield

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products such as starch, oil, grits, bran, meal, and flour, which are used extensively in the food industry (OECD, 2002). A majority of starch is converted to sweeteners (e.g., corn syrup, high fructose corn syrup, maltodextrins, and dextrose), and also fermented into ethanol (OECD, 2002).

A.3. NATURE OF THE GENETIC MODIFICATION

Transformation Method

Maize event 4114 was produced by *Agrobacterium tumefaciens*-mediated transformation of immature maize (*Zea mays* L.) embryos inoculated with *Agrobacterium tumefaciens* strain LBA4404 containing plasmid PHP27118 (Figure 2), essentially as described by Zhao *et al.* (2001). After three to six days of embryo and *Agrobacterium* co-cultivation on solid culture medium without selection, the embryos were transferred to a medium without herbicide selection but containing the antibiotic carbenicillin to kill residual *Agrobacterium*. After three to five days on this medium, embryos were then transferred to selective medium that was stimulatory to maize somatic embryogenesis and contained bialaphos for selection of cells expressing the *pat* transgene. The medium also contained carbenicillin to kill any remaining *Agrobacterium*. Following six to eight weeks on the selective medium, healthy, growing calli that demonstrated resistance to bialaphos were identified.

Regenerated plants from transformation and tissue culture (designated T0 plants) were selected for further characterization by molecular analyses, herbicide and insect efficacy, and agronomic evaluations (Figure 1).

Intermediate hosts (eg. bacteria)

Pioneer proprietary inbred line PHWWE was transformed with plasmid PHP27118 by *Agrobacterium tumefaciens*-mediated transformation to produce DP4114 maize without the use of bacteria as intermediate host organism.

Description of the Potentially Introduced Genetic Material

The gene expression cassettes contained within the T-DNA region of plasmid PHP27118 are exactly the same as used in the production of 1507 maize (*cry1*F, *pat*) and 59122 maize (*cry34*Ab*1*, *cry35*Ab1, *pat*), and are briefly described below, and summarized in Table 2.

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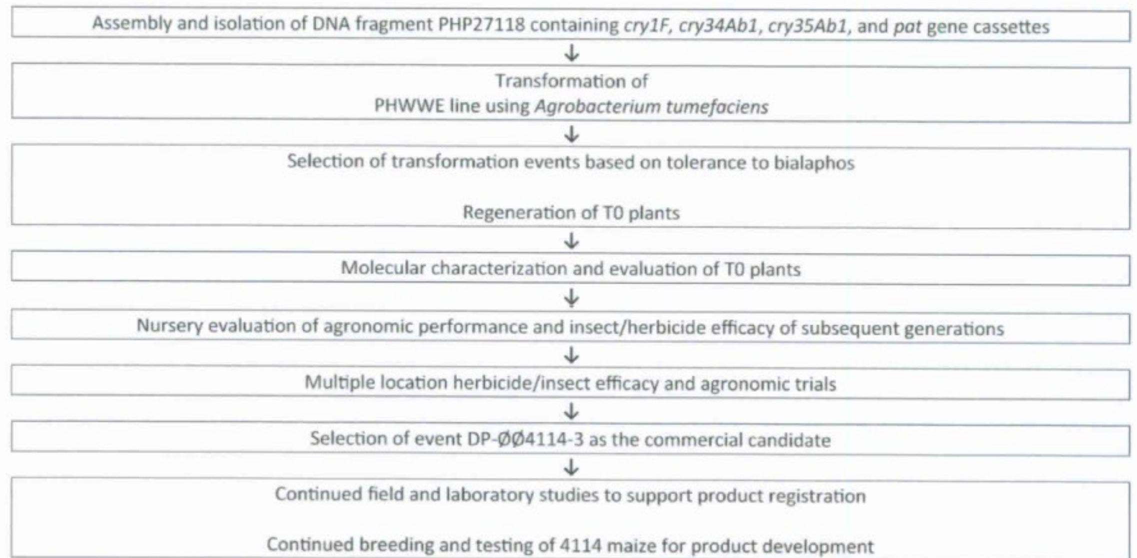

Figure 1. Schematic diagram of the development of 4114 maize

The first cassette contains a truncated version of the *cry1*F gene from *Bacillus thuringiensis* var. *aizawai*. Transcriptional control is provided by the maize polyubiquitin promoter (*ubiZM1*; Christensen *et al.*, 1992), resulting in constitutive expression of the Cry1F protein in maize. This region also includes the 5′ untranslated region (UTR) and intron associated with the native polyubiquitin promoter. The terminator for the *cry1*F gene is the polyadenylation signal from open reading frame 25 (ORF25) of the *A. tumefaciens* Ti plasmid pTi15955 (Barker *et al.*, 1983).

The second cassette contains the *cry34Ab1* gene isolated from *B. thuringiensis* strain PS149B1 (Ellis *et al.*, 2002; Herman *et al.*, 2002; Moellenbeck *et al.*, 2001). The expression of the *cry*34Ab1 gene is controlled by a second copy of the maize polyubiquitin promoter with 5′ UTR and intron (Christensen *et al.*, 1992). The terminator for the *cry*34Ab1 gene is the 3′ terminator sequence from the proteinase inhibitor II gene (*pinII* terminator) of *Solanum tuberosum* (An *et al.*, 1989; Keil *et al.*, 1986).

The third gene cassette contains the *cry*35Ab1 gene, also isolated from *B. thuringiensis* strain PS149B1 (Ellis *et al.*, 2002; Herman *et al.*, 2002; Moellenbeck *et al.*, 2001). The expression of the *cry35Ab1* gene is controlled by the *Triticum aestivum* (wheat) peroxidase promoter and leader sequence (Hertig *et al.*, 1991). The terminator for the *cry35Ab1* gene is a second copy of the *pinII* terminator (An *et al.*, 1989; Keil *et al.*, 1986).

The fourth and final gene cassette contains a version of the phosphinothricin *N*-acetyltransferase (*pat*) gene from *Streptomyces viridochromogenes* that has been optimized for expression in maize. Expression of the *pat* gene is controlled by the promoter and terminator regions from the cauliflower mosaic virus (CaMV) 35S transcript (Franck *et al.*, 1980; Odell *et al.*, 1985; Pietrzak *et al.*, 1986).

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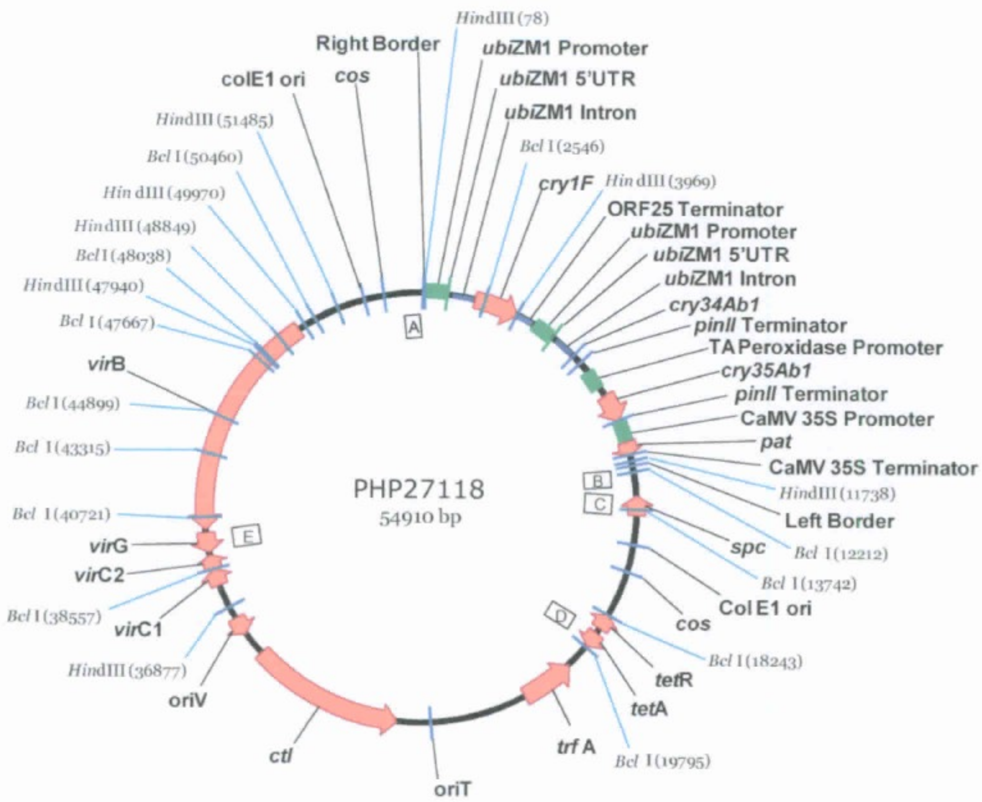

Figure 2. Schematic diagram of plasmid PHP27118

Schematic map of PHP27118 indicating *Bcl*I and *Hin*dIII restriction enzyme sites with base pair positions. The Right Border and Left Border regions flank the T-DNA (Figure 3) that is intended to be integrated within the host plant genome during *Agrobacterium*-mediated transformation. For reference in the discussion on Southern hybridization analysis performed to demonstrate the lack of incorporation of plasmid backbone sequences (section 0, page 30), the locations of relevant plasmid backbone probes are indicated by boxes within the plasmid map. A: RB probe; B: LB probe; C: *spc* (spectinomycin resistance gene) probe; D: *tet* (tetracycline resistance) probe; E: *vir*G probe.

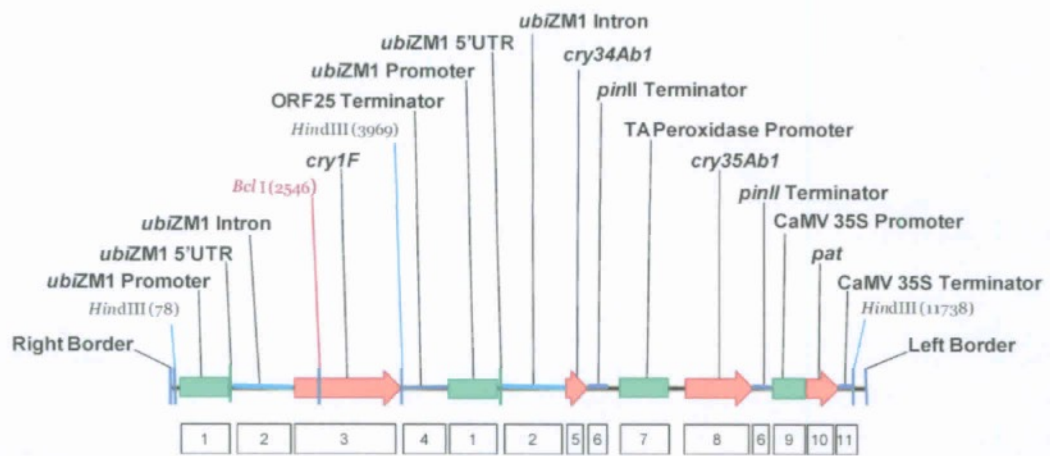

Figure 3. Schematic diagram of the T-DNA region from plasmid PHP27118

Schematic diagram of the PHP27118 T-DNA indicating the *cry1*F, *cry34Ab1*, *cry35Ab1*, and *pat* genes along with their respective regulatory elements. The locations of restriction endonucleases (*Bcl*I: position 2546 bp; *Hin*dIII: positions 78, 3969, and 11738 bp) and hybridization probes used for Southern blot analyses are also shown, with the numbers 1 through 11 corresponding to probes specific for: 1–*ubi*ZM1 promoter; 2–*ubi*ZM1 intron; 3–*cry1*F; 4–ORF25 terminator; 5–*cry*34Ab1; 6–*pinII* terminator; 7–TA peroxidase promoter; 8–*cry*35Ab1; 9–CaMV 35S promoter; 10–*pat*; and 11–CaMV 35S terminator. The size of the T-DNA is 11978 bp.

Table 2: Description of the genetic elements within the T-DNA of PHP27118

Comparison to Approved Event	Location on PHP27118 T-DNA (bp position)	Genetic Element	Size (bp)	Description
	1–25	Right border	25	T-DNA right border region from Ti plasmid of *A. tumefaciens*
	26–43	Ti Plasmid region	18	Non-functional sequence from Ti plasmid of *A. tumefaciens*
	44–114	Polylinker region	71	Region required for cloning genetic elements
*cry1*F gene cassette in 4114 and 1507 maize	115–1014	*ubi*ZM1 Promoter	900	Promoter region from *Zea mays* polyubiquitin gene (Christensen *et al.*, 1992)
	1015–1097	*ubi*ZM1 5' UTR	83	5' Untranslated region from *Z. mays* polyubiquitin gene (Christensen *et al.*, 1992)
	1098–2107	*ubi*ZM1 Intron	1010	Intron region from *Z. mays* polyubiquitin gene (Christensen *et al.*, 1992)
	2108–2129	Polylinker region	22	Region required for cloning genetic elements
	2130–3947	*cry1*F Gene	1818	Truncated version of the *cry1F* gene from *B. thuringiensis* var. *aizawai*
	3948–3992	Polylinker region	45	Region required for cloning genetic elements
	3993–4706	ORF25 Terminator	714	Terminator sequence from *A. tumefaciens* pTi15955 ORF 25 (Barker *et al.*, 1983)
	4707–4765	Polylinker region	59	Region required for cloning genetic elements
*cry34*Ab1 gene cassette in 4114 and 59122 maize	4766–5665	*ubi*ZM1 Promoter	900	Promoter region from *Z. mays* polyubiquitin gene (Christensen *et al.*, 1992)
	5666–5748	*ubi*ZM1 5' UTR	83	5' Untranslated region from *Z. mays* polyubiquitin gene (Christensen *et al.*, 1992)
	5749–6758	*ubi*ZM1 Intron	1010	Intron region from *Z. mays* polyubiquitin gene (Christensen *et al.*, 1992)
	6759–6786	Polylinker region	28	Region required for cloning genetic elements
	6787–7158	*cry34*Ab1 Gene	372	Synthesized version of the *cry34*Ab1 gene encoding the 14 kDa delta-endotoxin parasporal crystal protein from the nonmotile strain PS149B1 of *Bacillus thuringiensis* (Moellenbeck *et al.*, 2001; Ellis *et al.*, 2002; Herman *et al.*, 2002)
	7159–7182	Polylinker region	24	Region required for cloning genetic elements
	7183–7492	*pinII* Terminator	310	Terminator region from *S. tuberosum* proteinase inhibitor II gene (An *et al.*, 1989; Keil *et al.*, 1986)
	7493–7522	Polylinker region	30	Region required for cloning genetic elements
*cry35*Ab1 gene cassette in 4114 and 59122 maize	7523–8820	TA Peroxidase promoter	1298	Promoter from *T. aestivum* peroxidase including leader sequence (Hertig *et al.*, 1991)
	8821–8836	Polylinker region	16	Region required for cloning genetic elements
	8837–9988	*cry35Ab1* Gene	1152	Synthesized version of the *cry35*Ab1 gene encoding a 44 kDa delta-endotoxin parasporal crystal protein from the nonmotile strain PS149B1 of *Bacillus thuringiensis* (Moellenbeck *et al.*, 2001; Ellis *et al.*, 2002; Herman *et al.*, 2002)
	9989–10012	Polylinker region	24	Region required for cloning genetic elements
	10013–10322	*pinII* Terminator	310	Terminator region from *S. tuberosum* proteinase inhibitor II gene (An *et al.*, 1989; Keil *et al.*, 1986)
	10323–10367	Polylinker region	45	Region required for cloning genetic elements
pat gene cassette in 4114, 1507, and 59122 maize	10368–10897	CaMV 35S Promoter	530	35S Promoter from cauliflower mosaic virus (Franck *et al.*, 1980; Odell *et al.*, 1985; Pietrzak *et al.*, 1986)
	10898–10916	Polylinker region	19	Region required for cloning genetic elements
	10917–11468	*pat* Gene	552	Synthesized, plant-optimized phosphinothricin acetyltransferase coding sequence from *S. viridochromogenes*
	11469–11488	Polylinker region	20	Region required for cloning genetic elements
	11489–11680	CaMV 35S Terminator	192	35S Terminator from cauliflower mosaic virus (Franck *et al.*, 1980; Pietrzak *et al.*, 1986)
	11681–11874	Polylinker region	194	Region required for cloning genetic elements
	11875–11953	Ti Plasmid region	79	Non-functional sequence from Ti plasmid of *A. tumefaciens*
	11954–11978	Left border	25	T-DNA Left border region from Ti plasmid of *A. tumefaciens*

Breeding Process to Obtain Maize Line 4114

Beginning with a single T0 plant, the subsequent breeding of 4114 maize proceeded as indicated in Figure 4 to produce specific generations that were used in various characterizations and analyses, as described in Table 3.

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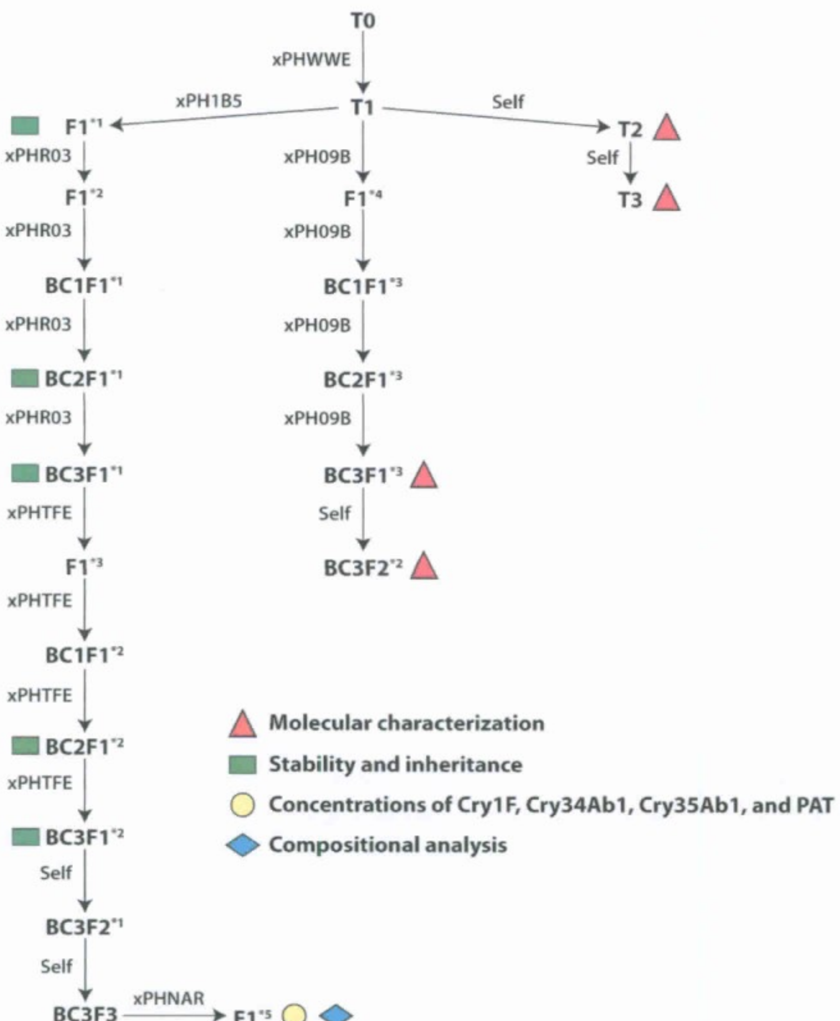

Figure 4. Breeding diagram for 4114 maize and generations used for analyses

Table 3: Generations and comparators used for analysis of 4114 maize

Analysis	Generation(s)	Comparators	Experimental Control and Reference Lines
Molecular-genetic characterization and absence of plasmid backbone DNA	T2, T3, BC3F1*3, BC3F2*2	Not applicable	PH09B, PHWWE
Stability and inheritance of the DNA insertion	F1*1, BC2F1*1, BC3F1*1, BC2F1*2, BC3F1*2	Not applicable	Not applicable
Concentrations of Cry1F, Cry34Ab1, Cry35Ab1, PAT	F1*5	Not applicable†	1507, 59122, and 1507 × 59122 maize†
Compositional analyses	F1*5	PHNAR x PHTFE	Pioneer® hybrids 34M94, 33G26, 33J24, 3394, 38B85, 37Y12, 34A15, and 34P88

† To determine actual protein concentrations in 4114 maize, no comparator lines were used. However, statistical comparisons were made to the Cry1F, Cry34Ab1, Cry35Ab1, and PAT protein concentrations in grain samples from 1507, 59122, and 1507 × 59122 maize lines that were grown alongside 4114 maize plants in the same field trials.

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Molecular Genetic Characterization of 4114 Maize including Identification of Genetic Elements

Southern blot analyses (Southern, 1975) were performed to investigate the number of sites of insertion of the PHP27118 T-DNA, the integrity of introduced genetic elements, the absence of plasmid backbone sequences, and the multigenerational stability of the inserted DNA. A complete description of the methods used, including locations and identities of various hybridization probes, is presented in Annex I, page 61.

Insert Copy Number and Organization within the 4114 Maize Genome

The restriction enzyme *Bcl*I has a single recognition site at position 2546 within the PHP27118 T-DNA and was used to provide information about the number of copies of the T-DNA integrated within the 4114 maize genome. The predicted fragment sizes following *Bcl*I digestion are >2500 bp, corresponding to the 5′ (Right Border) proximal region spanning the junction of the PHP27118 T-DNA insert and the maize genome, and >9400 bp, corresponding to the 3′ (Left Border) proximal region spanning the junction of the PHP27118 T-DNA insert and the maize genome. Thus, the insertion of a single copy of the PHP27118 T-DNA in 4114 maize should result in two detectable hybridization fragments of >2400 and >9400 bp following *Bcl*I digestion and hybridization with either the *ubi*ZM1 promoter, *ubi*ZM1 intron, or *cry*1F probes (Table 4). This is due to the presence of the *Bcl*I restriction enzyme site within the *cry*1F gene and between duplicate copies of the *ubi*ZM1 promoter and intron in the PHP27118 T-DNA (Figure 3). For each of the other probes (ORF25 terminator, *cry*34Ab1, *pinII* terminator, TA peroxidase promoter, *cry*35Ab1, CaMV 35S promoter, *pat*, and CaMV 35S terminator), a single hybridizing fragment of >9400 bp following *Bcl*I digestion is predicted based on integration of a single copy of the PHP27118 T-DNA in 4114 maize (Table 4). Although there are two copies of the *pinII* terminator in the PHP27118 T-DNA, both are located within the same *Bcl*I fragment and will thus yield a single band.

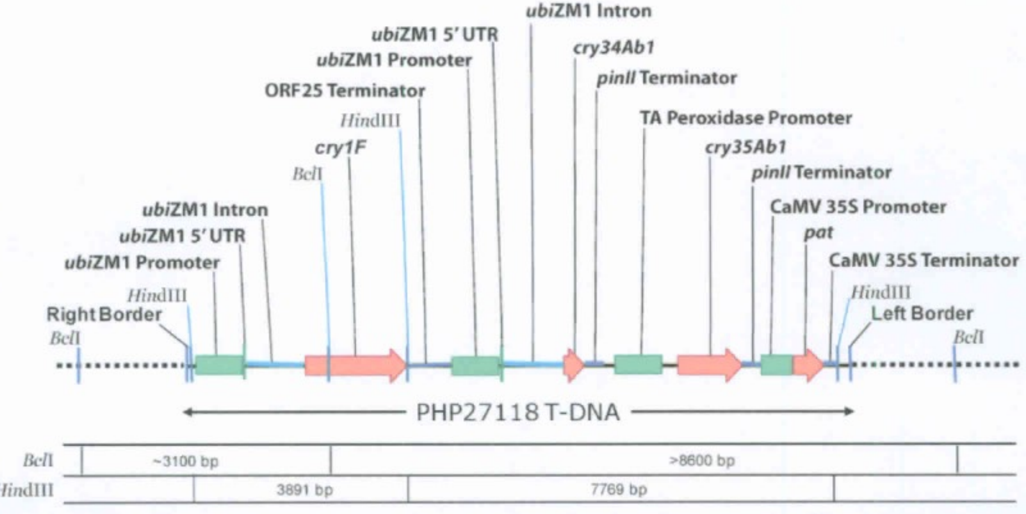

Figure 5. Schematic map of the PHP27118 T-DNA insert within the 4114 maize genome
Schematic map of the insertion in 4114 maize based on Southern blot analyses. The flanking maize genome is represented by the horizontal dotted line. A single, intact copy of the PHP27118 T-DNA was integrated into the maize genome. The *Bcl*I and *Hind*III restriction sites are indicated with the sizes of observed fragments on Southern blots shown below the map in base pairs (bp). The locations of *Bcl*I restriction enzyme sites outside the Right and Left Borders are not shown to scale.

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Table 4: Correlation of predicted and observed fragment sizes based on a single site of insertion of the PHP27118 T-DNA within maize line 4114

Probe	Enzymes	Figure	Event 4114 Genomic DNA Fragment Size (bp)			Plasmid PHP27118 DNA Fragment Size (bp)		
			Lanes	Predicted	Observed[a]	Lanes	Predicted	Observed[a]
*ubi*ZM1 Promoter	*Bcl*I	6	6, 7	>9400[b], >2500	>8600, ~3100 ~4800[c]	1, 2	9666, 6996	NA[d] ~4600[c], ~4800[c]
	*Hin*dIII	9	1, 2	7769, 3891	7769, 3891 ~6400[c]	6, 7	7769, 3891	7769, 3891 ~6400[c], >8600[c]
*ubi*ZM1 Intron	*Bcl*I	6	13, 14	>9400[b], >2500	>8600, ~3100 ~4800[c]	8, 9	9666, 6996	NA ~4600[c], ~4800[c]
	*Hin*dIII	9	8, 9	7769, 3891	7769, 3891 ~6400[c]	13, 14	7769, 3891	7769, 3891 ~6400[c], >8600[c]
*cry*1F	*Bcl*I	6	20, 21	>9400[b], >2500	>8600, ~3100	15, 16	9666, 6996	NA
	*Hin*dIII	9	15, 16	3891	3891	20, 21	3891	3891
ORF25 Terminator	*Bcl*I	6	27, 28	>9400	>8600	22, 23	9666	NA
	*Hin*dIII	9	22, 23	7769	7769	27, 28	7769	7769
*cry*34Ab1	*Bcl*I	7	6, 7	>9400	>8600	1, 2	9666	NA
	*Hin*dIII	10	1, 2	7769	7769	6, 7	7769	7769
pinII Terminator	*Bcl*I	7	13, 14	>9400	>8600	8, 9	9666	NA
	*Hin*dIII	10	8, 9	7769	7769	13, 14	7769	7769
TA Peroxidase promoter	*Bcl*I	7	20, 21	>9400	>8600	15, 16	9666	NA
	*Hin*dIII	10	15, 16	7769	7769	20, 21	7769	7769
*cry*35Ab1	*Bcl*I	7	27, 28	>9400	>8600	22, 23	9666	NA
	*Hin*dIII	10	22, 23	7769	7769	27, 28	7769	7769
CaMV 35S Promoter	*Bcl*I	8	6, 7	>9400	>8600	1, 2	9666	NA
	*Hin*dIII	11	1, 2	7769	7769	6, 7	7769	7769
pat	*Bcl*I	8	13, 14	>9400	>8600	8, 9	9666	NA
	*Hin*dIII	11	8, 9	7769	7769	13, 14	7769	7769
CaMV 35S Terminator	*Bcl*I	8	20, 21	>9400	>8600	15, 16	9666	NA
	*Hin*dIII	11	15, 16	7769	7769	20, 21	7769	7769

a. Observed fragment sizes were approximated from the DIG-labelled DNA molecular size marker VII fragments on the Southern blots. Due to the inability to determine exact sizes on the blot, all values are approximations to the nearest 100 bp. The exception to this was when the observed fragment had equal migration as the control plasmid hybridization fragment, in which case the predicted size was assigned (e.g., 7769 bp or 3891 bp, in the case of *Hin*dIII digestions).

b. There are two copies each of the *ubi*ZM1 promoter and intron sequences, one on each side of the *Bcl*I site yielding two predicted hybridization fragments. The location of the *Bcl*I restriction site within the *cry*1F gene results in hybridization of the *cry*1F probe to both fragments.

c. Hybridization fragment due to the presence of native *ubi*ZM1 promoter and intron sequences in maize genome. These fragments were also present in the PHWWE and PH09B negative control, as well as the positive controls consisting of PHWWE or PH09B DNA spiked with PHP27118 plasmid DNA. For PHWWE control DNA, the sizes of these native hybridization fragments were *ca.* 4800 or *ca.* 6400 bp in the case of *Bcl*I or *Hin*dIII digestion, respectively. For PH09B control DNA, the sizes of these native hybridization fragments were *ca.* 4600 or >8600 in the case of *Bcl*I or *Hin*dIII digestion, respectively.

d. NA = Not applicable. The PHP27118 plasmid used was prepared in a *dam*+ strain of *E. coli* resulting in methylation of all *Bcl*I sites and lack of digestion. Therefore on all Southern blots with *Bcl*I-treated plasmid, only probe hybridization was confirmed. Note, that due to the presence of native *ubi*ZM1 promoter and intron sequences in PHWWE and PH09B carrier DNA, hybridization with these sequences was detected following *Bcl*I digestion of positive control samples.

Hybridization of *Bcl*I-digested genomic DNA from 4114 maize with the *ubi*ZM1 promoter and intron probes detected fragments of approximately 3100 bp and greater than 8600 bp, which were derived from the Right Border and Left Border regions, respectively (Table 4; Figure 6, lanes 6, 7, 13, 14). The presence of native copies of the *ubi*ZM1 promoter and intron sequences within the maize genome resulted in detection of a *ca.* 4800 bp fragment in samples of *Bcl*I-digested 4114 maize genomic DNA, and in samples of similarly digested PHWWE control DNA, using both probes. A similar but slightly smaller (*ca.* 4600 bp) fragment was detected in samples of *Bcl*I-digested PH09B control DNA using the *ubi*ZM1 promoter and intron probes (Table 4; Figure 6, lanes 4, 11).

Hybridization of the *Bcl*I-digested event 4114 DNA with the *cry*1F probe also detected two fragments of *ca.* 3100 bp and >8600 bp (Table 4; Figure 6, lanes 20, 21), which was expected

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considering the location of the *Bcl*I restriction site within the *cry*1F gene (Figure 3). For each of the other probes (ORF25 terminator, *cry*34Ab1, *pinII* terminator, TA peroxidase promoter, *cry*35Ab1, CaMV 35S promoter, *pat*, and CaMV 35S terminator), a single hybridizing fragment of >8600 bp was detected following *Bcl*I digestion of 4114 maize genomic DNA (Table 4; Figures 6, 7, and 8).

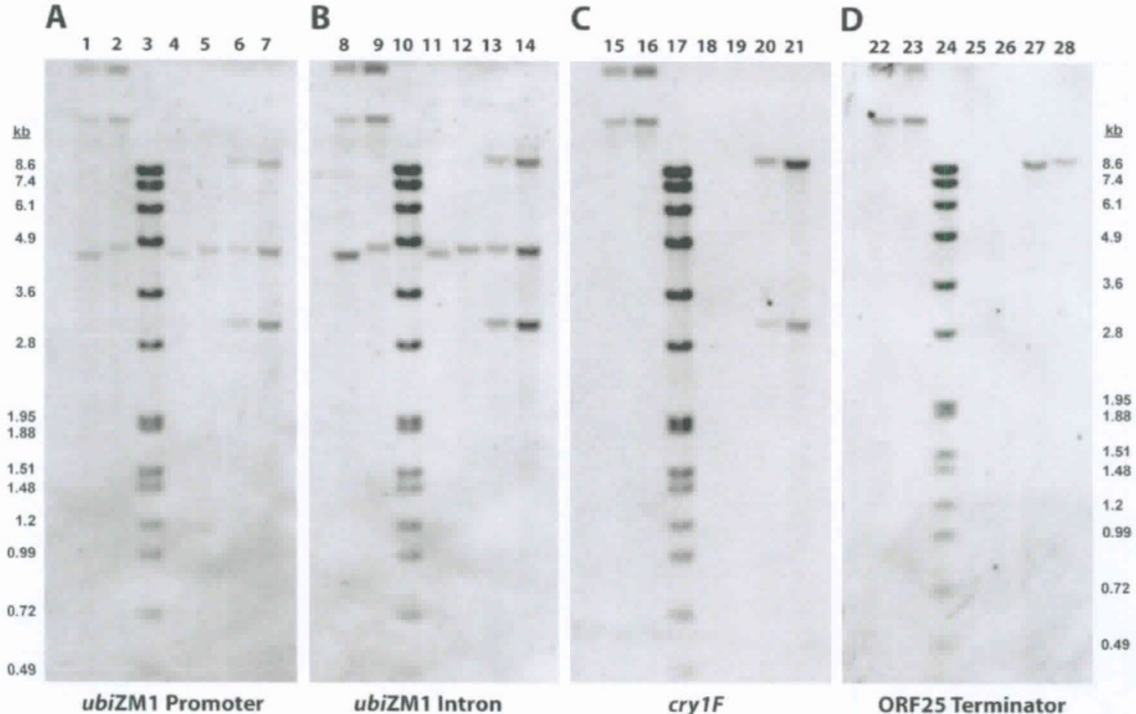

Figure 6. Southern blot hybridizations of *Bcl*I-digested 4114 maize genomic DNA with *ubi*ZM1 promoter, *ubi*ZM1 intron, *cry1F*, and ORF25 terminator probes

Samples of genomic DNA (*ca.* 3–4 µg) from individual plants of the T3 generation of 4114 maize (lanes 6, 7, 13, 14, 20, 21, 27, 28), negative control DNA from maize lines PH09B (lanes 4, 11, 18, 25) and PHWWE (lanes 5, 12, 19, 26), negative control PH09B DNA (4 µg) containing one copy equivalent of plasmid PHP27118 (lanes 1, 8, 15, 22), and negative control PHWWE DNA (4 µg) containing three copy equivalents of plasmid PHP27118 (lanes 2, 9, 16, 23), were subjected to restriction endonuclease digestion with *Bcl*I followed by agarose gel electrophoresis and transfer onto nylon membrane. Blots were hybridized with DIG-labelled probes specific for the *ubi*ZM1 promoter (panel A), *ubi*ZM1 intron (panel B), *cry*1F gene (panel C) or the ORF25 terminator (panel D). Following washing, hybridized probes and DIG-labelled molecular weight markers VII (lanes 3, 10, 17, 24) were visualized using a chemiluminescent detection system followed by electronic image capture.

It was observed that *Bcl*I-treated PHP27118 plasmid DNA did not result in the production of the expected size fragments, but instead two fragments >8600 bp were detected with all probes. This pattern was characteristic of undigested plasmid DNA, likely due to DNA methylation. The sensitivity of the *Bcl*I enzyme to bacterial plasmid DNA methylation is well known and there is a bacterial Dam (DNA adenine methylase) recognition sequence (5'GATC3') within the restriction enzyme site (New England Biolabs, 2002). In all *Bcl*I sites (recognition sequence 5'TG**A**TCA3') on the plasmid, the central adenine will be methylated, blocking digestion by *Bcl*I. The PHP27118 plasmid used was prepared in a *dam*+ strain of *E. coli* and thus all *Bcl*I sites would be methylated and would not digest as expected. Therefore on all Southern blots with *Bcl*I-treated plasmid, only probe hybridization was confirmed.

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In summary, Southern blot analyses of *Bcl*I-digested 4114 maize genomic DNA using probes specific for each of the genetic elements within the PHP27118 T-DNA were consistent with the integration of a single copy of the T-DNA within the 4114 maize genome, as illustrated in Figure 5.

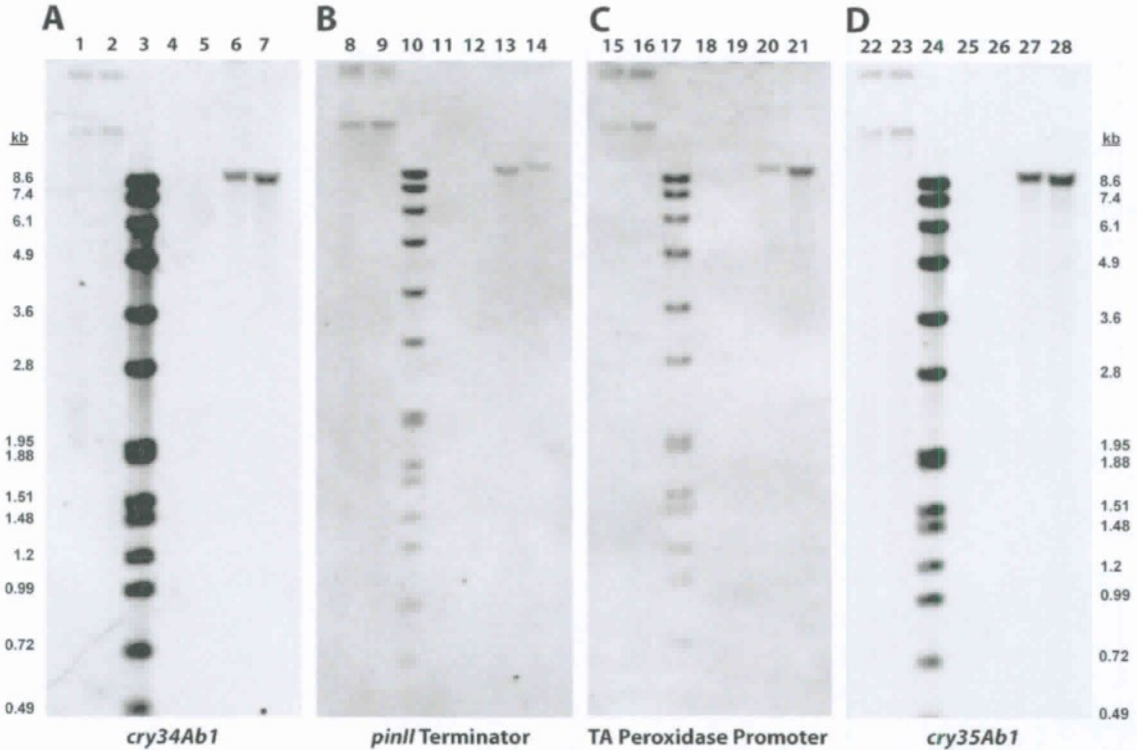

Figure 7. Southern blot hybridizations of *Bcl*I-digested 4114 maize genomic DNA with *cry34*Ab1, *pinII* terminator, TA peroxidase promoter, and *cry35*Ab1 probes

Samples of genomic DNA (*ca.* 3–4 µg) from individual plants of the T3 generation of 4114 maize (lanes 6, 7, 13, 14, 20, 21, 27, 28), negative control DNA from maize lines PH09B (lanes 4, 11, 18, 25) and PHWWE (lanes 5, 12, 19, 26), negative control PH09B DNA (4 µg) containing one copy equivalent of plasmid PHP27118 (lanes 1, 8, 15, 22), and negative control PHWWE DNA (4 µg) containing three copy equivalents of plasmid PHP27118 (lanes 2, 9, 16, 23), were subjected to restriction endonuclease digestion with *Bcl*I followed by agarose gel electrophoresis and transfer onto nylon membrane. Blots were hybridized with DIG-labelled probes specific for the *cry34*Ab1 gene (panel A), *pinII* terminator (panel B), TA peroxidase promoter (panel C) or the *cry35*Ab1 gene (panel D). Following washing, hybridized probes and DIG-labelled molecular weight markers VII (lanes 3, 10, 17, 24) were visualized using a chemiluminescent detection system followed by electronic image capture.

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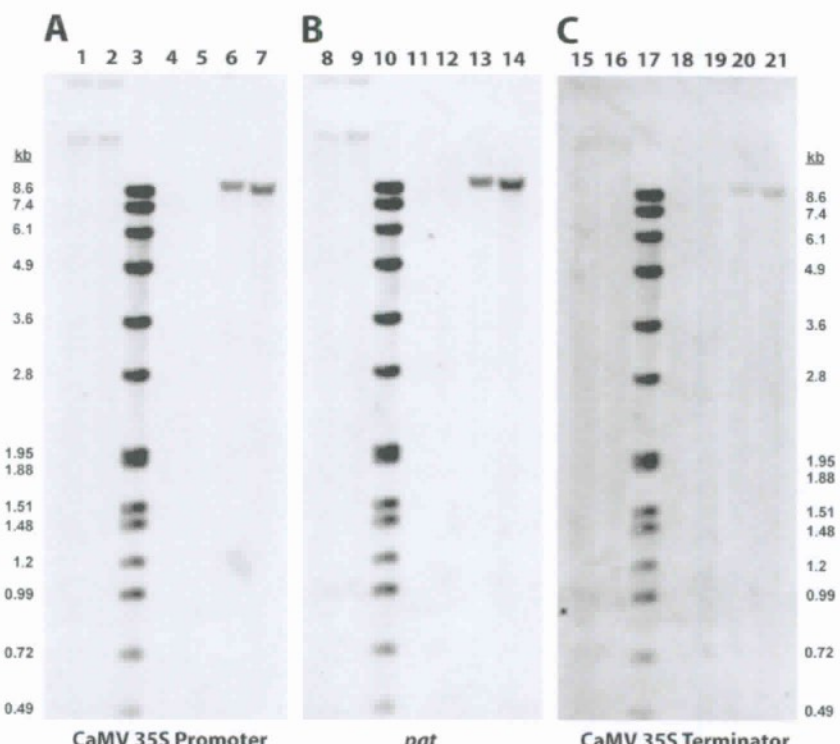

Figure 8. Southern blot hybridizations of *BclI*-digested 4114 maize genomic DNA with CaMV 35S promoter, *pat*, and CaMV 35S terminator probes

Samples of genomic DNA (*ca.* 3–4 μg) from individual plants of the T3 generation of 4114 maize (lanes 6, 7, 13, 14, 20, 21), negative control DNA from maize lines PH09B (lanes 4, 11, 18) and PHWWE (lanes 5, 12, 19), negative control PH09B DNA (4 μg) containing one copy equivalent of plasmid PHP27118 (lanes 1, 8, 15), and negative control PHWWE DNA (4 μg) containing three copy equivalents of plasmid PHP27118 (lanes 2, 9, 16), were subjected to restriction endonuclease digestion with *BclI* followed by agarose gel electrophoresis and transfer onto nylon membrane. Blots were hybridized with DIG-labelled probes specific for the CaMV 35S promoter (panel A), *pat* gene (panel B), or the CaMV 35S terminator (panel C). Following washing, hybridized probes and DIG-labelled molecular weight markers VII (lanes 3, 10, 17) were visualized using a chemiluminescent detection system followed by electronic image capture.

Integrity of the Inserted DNA in 4114 Maize

Southern blot analyses of *Hin*dIII-digested 4114 maize DNA were used to confirm the integrity of the PHP27118 T-DNA insert. The PHP27118 T-DNA contains three *Hin*dIII restriction sites, located at positions 78, 3969 (immediately 3′ of the *cry1F* gene), and 11738 bp (Figure 3). Insertion of an intact copy of the PHP27118 T-DNA was predicted to result in the detection of two hybridizing fragments of 3891 bp and 7769 bp following *Hin*dIII digestion of 4114 maize DNA and hybridization with probes specific to either the *ubi*ZM1 promoter or intron sequences (Table 4). This is because the *Hin*dIII site located at position 3969 bp lies between duplicate copies of the *ubi*ZM1 promoter and intron sequences in the PHP27118 T-DNA. Similar hybridization with the *cry1F* probe should detect a single 3891 bp fragment, and hybridizations using each of the remaining probes (ORF25 terminator, *cry34Ab1*, *pinII* terminator, TA peroxidase promoter, *cry35Ab1*, CaMV 35S promoter, *pat*, and CaMV 35S terminator) should each detect a single 7769 bp fragment (Table 4). Although there are two copies of the *pin*II terminator in the PHP27118 T-DNA, both copies are found on the same 7769 bp *Hin*dIII restriction fragment (Figure 3).

The results of the Southern analyses (Figures 9, 10, and 11) demonstrated that the predicted size fragments were detected for each of the hybridization probes tested (Table 4). In addition, and as

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expected, hybridization with *Hin*dIII restriction fragments containing copies of the native *ubi*ZM1 promoter and intron sequences was also detected using probes specific to these sequences (Figure 9, panel A and B). These fragments had apparent sizes of *ca.* 6400 bp or >8600 bp for PHWWE or PH09B control maize lines, respectively.

The complete correlation between predicted and observed *Hin*dIII restriction fragments using all eleven hybridization probes, plus the lack of any other insertion-derived fragments, indicated that the PHP27118 T-DNA integrated within the 4114 maize genome was intact, without truncation, rearrangement, or deletion of nucleotide sequences within any of the gene expression cassettes.

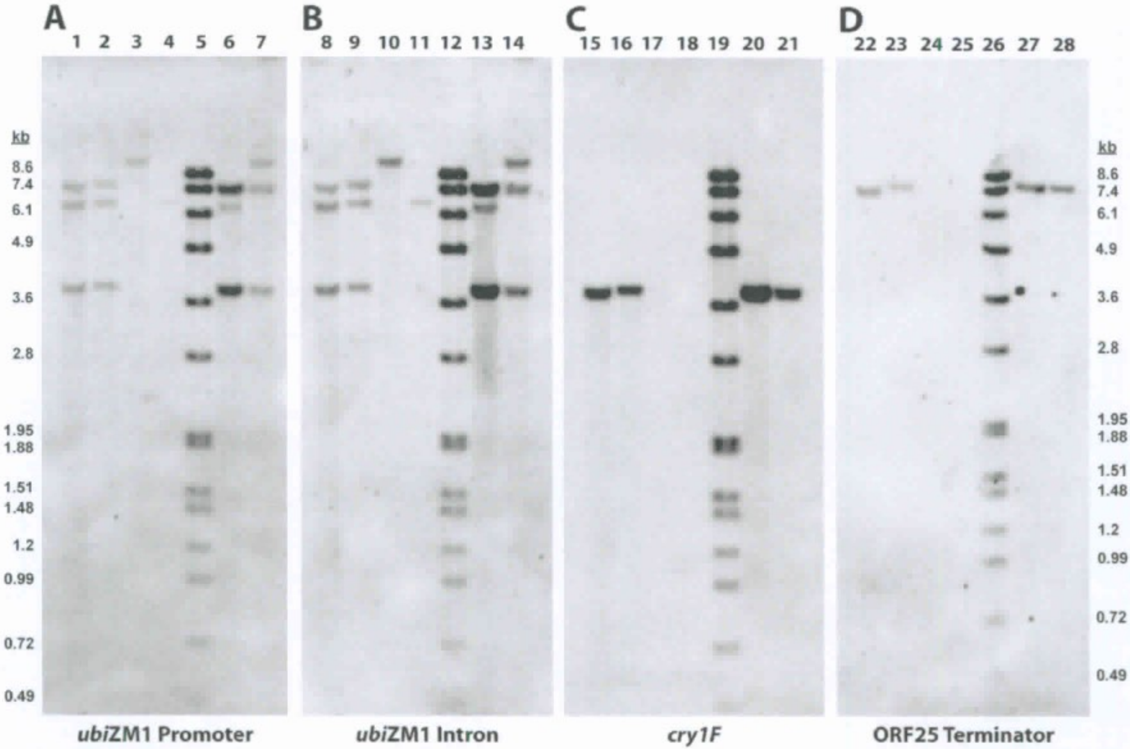

Figure 9. Southern blot hybridizations of *Hin*dIII-digested 4114 maize genomic DNA with *ubi*ZM1 promoter, *ubi*ZM1 intron, *cry*1F, and ORF25 terminator probes

Samples of genomic DNA (*ca.* 3–4 µg) from individual plants of the T3 generation of 4114 maize (lanes 1, 2, 8, 9, 15, 16, 22, 23), negative control DNA from maize lines PH09B (lanes 3, 10, 17, 24) and PHWWE (lanes 4, 11, 18, 25), negative control PHWWE DNA (4 µg) containing three copy equivalents of plasmid PHP27118 (lanes 6, 13, 20, 27), and negative control PH09B DNA (4 µg) containing one copy equivalent of plasmid PHP27118 (lanes 7, 14, 21, 28), were subjected to restriction endonuclease digestion with *Hin*dIII followed by agarose gel electrophoresis and transfer onto nylon membrane. Blots were hybridized with DIG-labelled probes specific for the *ubi*ZM1 promoter (panel A), *ubi*ZM1 intron (panel B), *cry1F* gene (panel C) or the ORF25 terminator (panel D). Following washing, hybridized probes and DIG-labelled molecular weight markers VII (lanes 5, 12, 19, 26) were visualized using a chemiluminescent detection system followed by electronic image capture.

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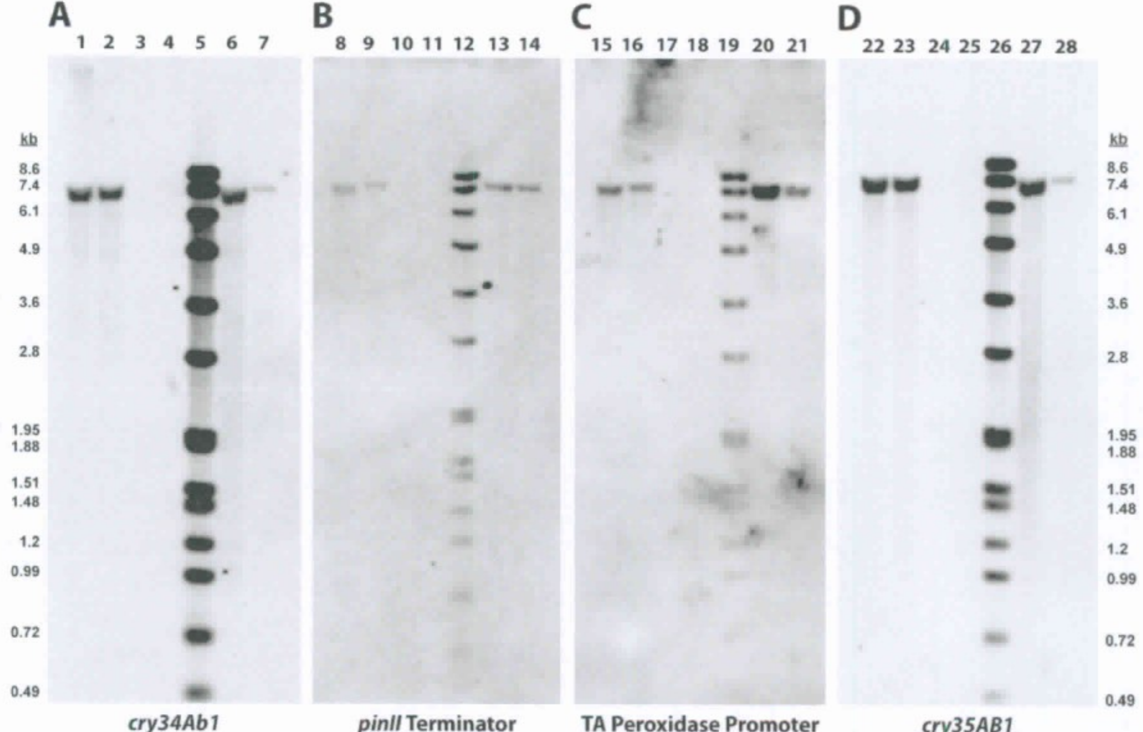

Figure 10. Southern blot hybridizations of *Hin*dIII-digested 4114 maize genomic DNA with *cry34*Ab1, *pinII* terminator, TA peroxidase promoter, and *cry35*Ab1 probes

Samples of genomic DNA (*ca.* 3–4 µg) from individual plants of the T3 generation of 4114 maize (lanes 1, 2, 8, 9, 15, 16, 22, 23), negative control DNA from maize lines PH09B (lanes 3, 10, 17, 24) and PHWWE (lanes 4, 11, 18, 25), negative control PHWWE DNA (4 µg) containing three copy equivalents of plasmid PHP27118 (lanes 6, 13, 20, 27), and negative control PH09B DNA (4 µg) containing one copy equivalent of plasmid PHP27118 (lanes 7, 14, 21, 28), were subjected to restriction endonuclease digestion with *Hin*dIII followed by agarose gel electrophoresis and transfer onto nylon membrane. Blots were hybridized with DIG-labelled probes specific for the *cry34Ab1* gene (panel A), *pin*II terminator (panel B), TA peroxidase promoter (panel C) or the *cry*35Ab1 gene (panel D). Following washing, hybridized probes and DIG-labelled molecular weight markers VII (lanes 5, 12, 19, 26) were visualized using a chemiluminescent detection system followed by electronic image capture. Blotches on panel C were due to non-specific chemiluminescence from the detection process and were not due to probe hybridization.

Table 5: Predicted fragment sizes following restriction endonuclease digestion of PHP27118 plasmid DNA and hybridization with plasmid backbone probes

		Predicted Fragment Size from Plasmid PHP27118 (bp)		Event 4114 Genomic DNA Fragment Size Observed (bp)
Probe	**Figure**	***Bcl*I**	***Hin*dIII†**	***Bcl*I**
LB	12, panel A	1530	25139	No hybridization
spc	12, panel B	1530	25139	No hybridization
tet	12, panel C	1552	25139	No hybridization
*vir*G	12, panel D	2164	11063	No hybridization
RB	13, panel A	6996	3503	No hybridization

† Digestion with *Hin*dIII was included only for samples of PHP27118 plasmid DNA to demonstrate that the plasmid could be digested by a restriction enzyme that is not sensitive to Dam methylation. As described in Table 4, plasmid PHP27118 was produced in a *dam*+ strain of *E. coli* resulting in methylation of all *Bcl*I sites and lack of digestion.

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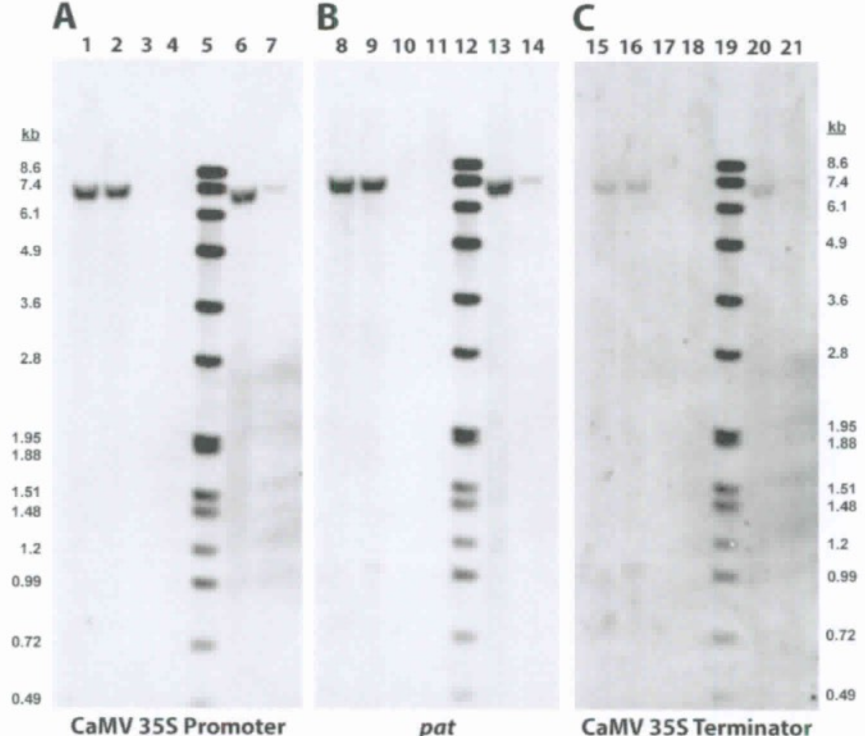

Figure 11. Southern blot hybridizations of *Hind*III-digested 4114 maize genomic DNA with CaMV 35S promoter, *pat*, and CaMV 35S terminator probes

Samples of genomic DNA (*ca.* 3–4 µg) from individual plants of the T3 generation of 4114 maize (lanes 1, 2, 8, 9, 15, 16), negative control DNA from maize lines PH09B (lanes 3, 10, 17) and PHWWE (lanes 4, 11, 18), negative control PHWWE DNA (4 µg) containing three copy equivalents of plasmid PHP27118 (lanes 6, 13, 20), and negative control PH09B DNA (4 µg) containing one copy equivalent of plasmid PHP27118 (lanes 7, 14, 21), were subjected to restriction endonuclease digestion with *Hind*III followed by agarose gel electrophoresis and transfer onto nylon membrane. Blots were hybridized with DIG-labelled probes specific for the CaMV 35S promoter (panel A), the *pat* gene (panel B), or the CaMV 35S terminator (panel C). Following washing, hybridized probes and DIG-labelled molecular weight markers VII (lanes 5, 12, 19) were visualized using a chemiluminescent detection system followed by electronic image capture.

Absence of Plasmid Backbone DNA in 4114 Maize

Southern blot analyses of *Bcl*I-digested genomic DNA obtained from four generations of 4114 maize (T2, T3, BC3F1*3, and BC3F2*2; Figure 4) were performed to demonstrate the lack of integration of any sequences derived from the PHP27118 plasmid backbone. Five different probes were utilized for these analyses, including probes specific for the spectinomycin (*spc*) and tetracycline (*tet*) resistance genes, the *vir*G gene, and probes specific for the plasmid backbone sequences adjacent to either the Left Border (LB) or Right Border (RB) of the PHP27118 T-DNA (Figure 2). Hybridizing fragments were not detected for any of these probes when tested against samples of *Bcl*I-digested 4114 maize genomic DNA (Table 5; Figures 12 and 13), confirming the lack of integration of any plasmid backbone sequences.

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Figure 12. Southern blot hybridizations of *Bcl*I-digested 4114 maize genomic DNA with LB, *spc*, *tet*, and *vir*G plasmid backbone probes

Samples of genomic DNA (*ca.* 3–6 µg per lane) from individual plants of the T2 (lanes 4, 5), T3 (lanes 6, 7), $BC3F1^{*3}$ (lanes 8, 9), or $BC3F2^{*2}$ (lanes 10, 11) generations of 4114 maize, negative control DNA from maize lines PH09B (lane 12) and PHWWE (lane 13), control PH09B DNA (4 µg) containing one copy equivalent of plasmid PHP27118 (lane 1), and control PHWWE DNA (4 µg) containing three copy equivalents of plasmid PHP27118 (lane 2), were subjected to digestion with *Bcl*I. In addition, samples containing either three or one copy equivalents, respectively, of PHP27118 plasmid DNA in PH09B (lane 15) or PHWWE (lane 16) carrier DNA were digested with *Hin*dIII. All samples were subjected to agarose gel electrophoresis and transfer onto nylon membrane. Blots were hybridized with DIG-labelled probes specific for plasmid backbone sequences adjacent to the Left Border (LB; panel A), the *spc* gene (panel B), the *tet* gene (panel C), or the *vir*G gene (panel D). Following washing, hybridized probes and DIG-labelled molecular weight markers VII (lanes 3, 14) were visualized using a chemiluminescent detection system followed by electronic image capture.

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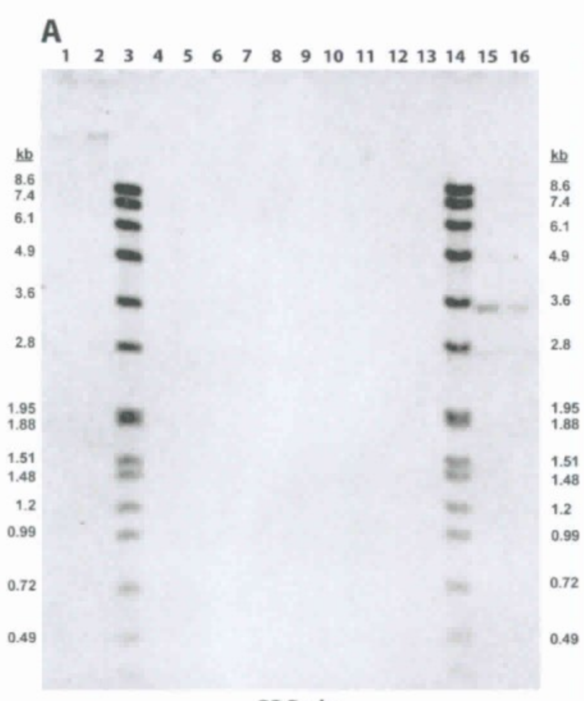

Figure 13. Southern blot hybridizations of *BclI*-digested 4114 maize genomic DNA with RB plasmid backbone probe

Samples of genomic DNA (*ca.* 3–6 µg per lane) from individual plants of the T2 (lanes 4, 5), T3 (lanes 6, 7), BC3F1*3 (lanes 8, 9), or BC3F2*2 (lanes 10, 11) generations of 4114 maize, negative control DNA from maize lines PH09B (lane 12) and PHWWE (lane 13), control PH09B DNA (4 µg) containing one copy equivalent of plasmid PHP27118 (lane 1), and control PHWWE DNA (4 µg) containing three copy equivalents of plasmid PHP27118 (lane 2), were subjected to digestion with *BclI*. In addition, samples containing either three or one copy equivalents, respectively, of PHP27118 plasmid DNA in PH09B (lane 15) or PHWWE (lane 16) carrier DNA were digested with *Hin*dIII. All samples were subjected to agarose gel electrophoresis and transfer onto nylon membrane. The blot was hybridized with a DIG-labelled probe specific for plasmid backbone sequences adjacent to the Right Border (RB; panel A). Following washing, hybridized probes and DIG-labelled molecular weight markers VII (lanes 3, 14) were visualized using a chemiluminescent detection system followed by electronic image capture.

Nucleotide Sequencing of the Introduced DNA and Genomic Flanking Regions

As part of the molecular characterization of 4114 maize, the nucleotide sequence of the T-DNA insert and genomic border regions was determined to confirm the integrity of the inserted DNA and to characterize the genomic sequence flanking the insertion site in 4114 maize. In total, 16,752 base pairs (bp) of 4114 maize genomic sequence was confirmed, comprising 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 (Diehn *et al.*, 2011). The inserted T-DNA in 4114 maize was found to have a 29 bp deletion on the Right Border (RB) end and a 24 bp deletion on the Left Border (LB) end, which is not uncommon for *Agrobacterium*-mediated transformation events (Kim *et al.*, 2007). Furthermore, at the 5' end of the insert, position 1-24 bp of the insert consists of 15bp of T-DNA vector sequence (identical to bp 2,462-2,476) and 9bp of cry1F sequence (identical to bp 4,922-4,930). All remaining sequence is intact and identical to that in plasmid PHP27118. The 5′ and 3′ genomic border regions of the 4114 maize insert were verified to be of maize origin by polymerase chain reaction amplification and sequencing of the genomic border regions from both 4114 maze and control maize plants.

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Novel Open Reading Frame (ORF) Analysis

To investigate the possibility of creating novel new ORFs as a consequence of the T-DNA insertion in event 4114 maize, an open reading frame analysis was conducted to look for potential start-to-stop ORFs that spanned either the 5′ or 3′ junctional regions. This analysis examined each of three possible reading frames in both orientations (i.e., six possible reading frames in total) for potential ORFs encoding sequences of 30 or more amino acids (i.e., equal or greater than *ca.* 3,300 Da). A single putative ORF was identified in the reverse (complementary) orientation that spanned the 5′ T-DNA insert–genomic DNA border, extending from nucleotide 2557 upstream to nucleotide 2261 within the 5′ genomic border, encoding a sequence of 99 amino acids (Figure 14).

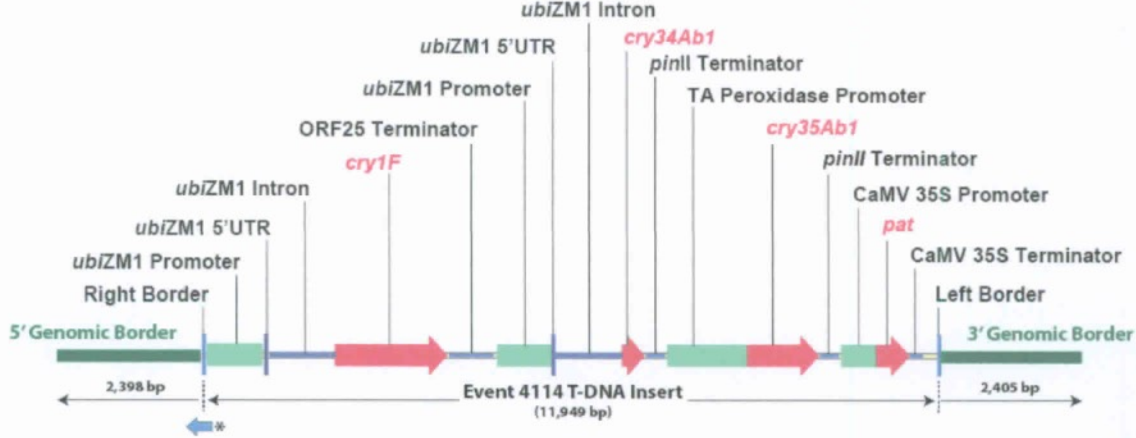

Figure 14. Schematic diagram of the T-DNA insert within 4114 maize
Schematic diagram of the 4114 maize T-DNA insert indicating the *cry*1F, *cry*34Ab1, *cry*35Ab1, and *pat* genes along with their respective regulatory elements. In total, 16,752 bp of 4114 maize genomic sequence was confirmed, comprising 2,398 bp of the 5′ genomic border sequence, 2,405 bp of the 3′ genomic border sequence, and 11,949 bp of the inserted T-DNA from PHP27118. The location of a putative ORF spanning the 5′ junction of the T-DNA insert and the maize genomic DNA is indicated by the asterisk (*). This potential ORF extends from position 2557 upstream to position 2261 within the 5′ genomic border and encodes a sequence of 99 amino acids (Table 6).

To assess the potential for allergenicity, the deduced amino acid sequence of the 5′ junctional potential ORF was compared to a peer-reviewed database of 1630 known and putative allergen and celiac protein sequences residing in the FARRP13 dataset at the University of Nebraska.[3] Potential identities between the 99-amino acid peptide sequence and proteins in the allergen database were evaluated with the FASTA35 sequence alignment tool using the default parameters. The standard 35 percent or greater identity threshold over any 80 or greater amino acid length sequence alignment between the query sequence and an allergen was used to indicate the potential for cross-reactivity. No identity matches of ≥ 35 percent over ≥ 80 residues were observed. The putative peptide sequence was also evaluated for any eight or greater contiguous identical amino acid matches to the allergens contained in the FARRP database. This was done using an algorithm that generates all possible eight-word peptides from both the query and dataset proteins and evaluates each query "word" against all dataset "words" for perfect matches. There were no eight or greater contiguous identical amino acid matches observed with the 99-amino acid sequence encoded by the putative 5′ junctional ORF.

[3] The FARRP allergen protein database resides at AllergenOnline (http://www.allergenonline.org). Version 13 was released on February 12, 2013, and contains 1630 peer-reviewed sequences representing 612 taxonomic-protein groups.

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To search for potential similarity to known toxins, the 99-amino acid sequence was queried using the BLASTP 2.2.25 algorithm against Release 194.0 (2/15/13) of the non-redundant ("nr") protein dataset, which incorporates non-redundant entries from all GenBank and RefSeq nucleotide translations along with protein sequences from SWISS-PROT, PIR, PRF, and PDB.[4] A cut-off expectation (E) value[5] of 1.0 was used to identify biological meaningful similarity between the target sequence and proteins in the National Center for Biotechnology Information (NCBI) datasets. Although a statistically significant sequence similarity generally requires a match with an expectation value less than 0.01, a cut-off of $E < 1.0$ ensures that proteins with even limited similarity will not be overlooked in the search (Pearson, 2000). Other than the E-value cut-off and the removal of low complexity filtering, the default BLASTP parameters were used and the number of alignments returned was set to the maximum value of 2000. The BLASTP search resulted in no significant hits returned (Table 6).

Table 6: Location and identity of the putative ORF spanning the 5′ T-DNA–genomic DNA junction in 4114 maize

Nucleotide Location	Strand	Length (amino acids)	Deduced Amino Acid Sequence	No. of Allergen Hits	No. of BLASTP Hits
2557–2261	–	99	MLIISREGHDRVTLHCRHARGYRWAPAEASVRVTALPVEERLNYQICVE ASVRVTTCKCYKREAPKPEPCVVNRVLESRAAEMRTGKAARKGGNKSMI V*	None	None

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

Stability and Inheritance of the Inserted DNA

The stability and inheritance of the inserted DNA in 4114 maize was investigated using a combination of methods, including Southern blot analyses of *Bcl*I-digested genomic DNA isolated from four different generations, and polymerase chain reaction (PCR) analyses for the *cry1*F, *cry34*Ab1, *cry35*Ab1, and *pat* genes, correlated with a phenotypic assessment of the herbicide-tolerance trait in five different generations (see Annex II, page 64, for materials and methods).

[4] The Basic Local Alignment Search Tool for Proteins (BLASTP) is located at: http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins.

[5] The Expect value (E) is a parameter that describes the number of hits one can "expect" to see by chance when searching a database of a particular size. It decreases exponentially as the Score (S) of the match increases. The lower the E-value, or the closer it is to zero, the more "significant" the match.

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A

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

kb 8.6 7.4 6.1 4.9 3.6 2.8 1.95 1.88 1.51 1.48 1.2 0.99 0.72

cry1F Probe

B

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

kb 8.6 7.4 6.1 4.9 3.6 2.8 1.95 1.88 1.51 1.48 1.2 0.99 0.72

cry34Ab1 Probe

C

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

kb 8.6 7.4 6.1 4.9 3.6 2.8 1.95 1.88 1.51 1.48 1.2 0.99 0.72

cry35Ab1 Probe

D

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

kb 8.6 7.4 6.1 4.9 3.6 2.8 1.95 1.88 1.51 1.48 1.2 0.99 0.72

pat Probe

Figure 15. Southern blot analysis of *cry1*F, *cry34*Ab1, *cry35*Ab1, and *pat* gene stability across multiple generations of 4114 maize

Samples of genomic DNA (*ca.* 3–6 µg per lane) from individual plants of the T2 (lanes 4, 5), T3 (lanes 6, 7), BC3F1*3 (lanes 8, 9), or BC3F2*2 (lanes 10, 11) generations of 4114 maize, negative control DNA from maize lines PH09B (lane 12) and PHWWE (lane 13), control PH09B DNA (4 µg) containing one copy equivalent of plasmid PHP27118 (lane 1), and control PHWWE DNA (4 µg) containing three copy equivalents of plasmid PHP27118 (lane 2), were subjected to digestion with *Bcl*I. In addition, samples containing either three or one copy equivalents, respectively, of PHP27118 plasmid DNA in PH09B (lane 15) or PHWWE (lane 16) carrier DNA were digested with *Hind*III. All samples were subjected to agarose gel electrophoresis and transfer onto nylon membrane. Blots were hybridized with DIG-labelled probes specific for the *cry1*F gene (panel A), the *cry34*Ab1 gene (panel B), the *cry35*Ab1 gene (panel C), or the *pat* gene (panel D). Following washing, hybridized probes and DIG-labelled molecular weight markers VII (lanes 3, 14) were visualized using a chemiluminescent detection system followed by electronic image capture.

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Southern Blot Analyses Confirming Stability of the Inserted DNA

For the Southern blot analyses, samples of *Bcl*I-digested genomic DNA corresponding to individual plants from the T2, T3, BC3F1*3, and BC3F2*2 generations (Figure 4) were tested using probes specific for the *cry*1F, *cry*34Ab1, *cry*35Ab1, and *pat* gene sequences. The detection of two hybridization fragments of *ca.* 3100 bp and >8600 bp using the *cry*1F probe, and the detection of a single >8600 bp hybridizing fragment using each of the remaining probes, in each sample from each generation, confirmed stable integration and inheritance of the inserted DNA in 4114 maize (Figure 15).

Mendelian Inheritance of the T-DNA Insert

The inheritance pattern of the T-DNA insert within 4114 maize was investigated by determining segregation of the *cry*1F, *cry*34Ab1, *cry*35Ab1, and *pat* genes within five generations (F1*1, BC2F1*1, BC3F1*1, BC2F1*2, and BC3F1*2; Figure 4) that represented a range of different crossing and backcrossing points in a typical maize breeding program. Leaf punches from individual plants from each generation were analyzed for the presence of the T-DNA insert by event-specific PCR and for the presence of each of the introduced genes using gene-specific PCR. The herbicide-tolerance phenotype was determined by treating the plants with glufosinate herbicide and visually evaluating each plant for herbicide injury. A positive plant exhibited no herbicidal injury and a negative plant exhibited severe herbicide injury. The expected Mendelian inheritance ratio of positive and negative plants for a hemizygous trait in these populations is 1:1.

Table 7: Summary of genotypic and phenotypic results for segregating generations of 4114 maize

		Expected		Observed†		
Generation	Total	Positive	Negative	Positive	Negative	Chi-Square[a]
F1*1 (1:1)	98	49	49	52	46	0.367
BC2F1*1 (1:1)	100	50	50	48	52	0.160
BC3F1*1 (1:1)	100	50	50	47	53	0.360
BC2F1*2 (1:1)	100	50	50	53	47	0.360
BC3F1*2 (1:1)[b]	195	97.5	97.5	87	108	1.62

† PCR analysis (consisting of event-specific PCR analysis to confirm the presence or absence of the T-DNA insert, and gene-specific PCR analysis to confirm the presence or absence of the *cry*1F, *cry*34Ab1, *cry*35Ab1, and *pat* genes) and herbicide (i.e., glufosinate) tolerance analysis was conducted for each plant from each generation. All PCR results matched the corresponding herbicide tolerance result for each plant analyzed.

a. This analysis tested the hypothesis that the introduced traits were segregating in a Mendelian fashion. The critical value to reject the hypothesis at the 5 percent level is 3.84. Since the Chi-square value was less than 3.84 within each generation, the observed differences were not statistically significant.

b. The BC3F1*2 generation analysis was conducted during two time points. The first analysis was conducted with 99 seedlings and found to be statistically significant by Chi-square analysis (Chi-square = 5.34). An observed segregation ratio would not be statistically different compared to a 1:1 expected ratio (50:50 based on 100 plants) if it fell in the range of 50:50 ± 9 (i.e., ratio of 41:59 or 59:41). The BC3F1*2 generation observed segregation ratio for the original sample was 38:61, only two or three plants outside of the acceptable range. In order to determine if the statistical difference was not a false positive result due to random sampling, an additional 96 seedlings from the same seed lot were tested. The combined results of the two time points within the single seed lot are shown and were not statistically different from the expected segregation ratio.

In every case, a positive plant tested positive for the presence of the 4114 T-DNA insert, the *cry*1F, *cry*34Ab1, *cry*35Ab1 and *pat* genes, and the herbicide-tolerance phenotype, indicating that the inserted T-DNA and its included genetic elements within 4114 maize segregated together. A Chi-square (χ^2) analysis was performed on the data and no statistically significant differences were found between the observed and expected segregation ratios for the F1*1, BC2F1*1, BC3F1*1, BC2F1*2, and BC3F1*2 generations of 4114 maize (Table 7), indicating that within these five generations, each of the introduced genes segregated according to Mendelian rules of inheritance for a single genetic locus. These results were consistent with Southern blot data indicating the stable integration of the insert at a single site in the genome.

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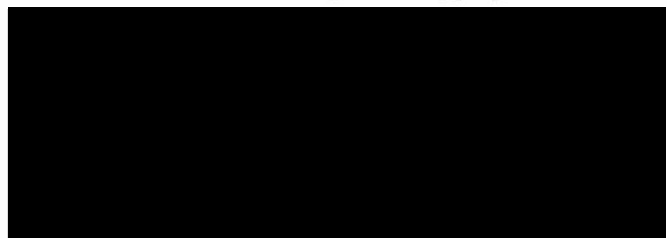
A.4. Analytical Method for Detection

Pioneer has developed a quantitative event-specific polymerase chain reaction (PCR) amplification method for maize event 4114 using a FAM/MGB[6] labelled detection system. The detection method specifically amplifies a 90-bp DNA fragment spanning the junction between the maize genome and the 5′ region of the T-DNA insert within event 4114. Since the site of T-DNA insertion, and thus the host genomic flanking sequence, is unique to each event, PCR amplification using these specific primers can be used to unambiguously detect event 4114. The limit of quantification of the assay is 0.08 percent event 4114 DNA in event 4114-free maize DNA, with an average relative standard deviation of 14.29 percent and an average trueness[7] of 12.50 percent.

A complete description of the event-specific detection method for 4114 maize is provided in Annex III (page 66). Pioneer will provide appropriate reference materials upon request:



B. Information Related to the Safety of the GM Food

B.1. Equivalence Studies

Identity and Equivalence of Newly Expressed Proteins in 4114 Maize

The *cry1F*, *cry34Ab1*, *cry35Ab1*, and *pat* gene cassettes introduced into 4114 maize are identical to those cassettes introduced into 1507 maize (*cry1F*, *pat*) or 59122 maize (*cry34Ab1*, *cry35Ab1*, *pat*), and are also present in commercial breeding stacks of 1507 × 59122 maize. The identity of these introduced genes is known, not just based on the method of production of 4114 maize, but also from nucleotide sequencing of the DNA insertion in 4114 maize (Diehn *et al.*, 2011), from which the deduced amino acid sequences of the Cry1F, Cry34Ab1, Cry35Ab1, PAT proteins were determined and found to be identical to the corresponding deduced amino acid sequences for these proteins in previously authorized 1507 and 59122 maize (Figure 16).

[6] FAM = 6-carboxyfluorescein; MGB = dihydrocyclopyrroloindole tripeptide minor groove binder.
[7] Trueness is the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value.

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A

Cry1F (605 amino acids; *ca.* 68 kDa)

```
  1  MENNIQNQCV PYNCLNNPEV EILNEERSTG RLPLDISLSL TRFLLSEFVP
 51  GVGVAFGLFD LIWGFITPSD WSLFLLQIEQ LIEQRIETLE RNRAITTLRG
101  LADSYEIYIE ALREWEANPN NAQLREDVRI RFANTDDALI TAINNFTLTS
151  FEIPLLSVYV QAANLHLSLL RDAVSFGQGW GLDIATVNNH YNRLINLIHR
201  YTKHCLDTYN QGLENLRGTN TRQWARFNQF RRDLTLTVLD IVALFPNYDV
251  RTYPIQTSSQ LTREIYTSSV IEDSPVSANI PNGFNRAEFG VRPPHLMDFM
301  NSLFVTAETV RSQTVWGGHL VSSRNTAGNR INFPSYGVFN PGGAIWIADE
351  DPRPFYRTLS DPVFVRGGFG NPHYVLGLRG VAFQQTGTNH TRTFRNSGTI
401  DSLDEIPPQD NSGAPWNDYS HVLNHVTFVR WPGEISGSDS WRAPMFSWTH
451  RSATPTNTID PERITQIPLV KAHTLQSGTT VVRGPGFTGG DILRRTSGGP
501  FAYTIVNING QLPQRYRARI RYASTTNLRI YVTVAGERIF AGQFNKTMDT
551  GDPLTFQSFS YATINTAFTF PMSQSSFTVG ADTFSSGNEV YIDRFELIPV
601  TATLE*
```

B

Cry34Ab1 (123 amino acids; *ca.* 14 kDa)

```
  1  MSAREVHIDV NNKTGHTLQL EDKTKLDGGR WRTSPTNVAN DQIKTFVAES
 51  NGFMTGTEGT IYYSINGEAE ISLYFDNPFA GSNKYDGHSN KSQYEIITQG
101  GSGNQSHVTY TIQTTSSRYG HKS*
```

C

Cry35Ab1 (383 amino acids; *ca.* 44 kDa)

```
  1  MLDTNKVYEI SNHANGLYAA TYLSLDDSGV SLMNKNDDDI DDYNLKWFLF
 51  PIDDDQYIIT SYAANNCKVW NVNNDKINVS TYSSTNSIQK WQIKANGSSY
101  VIQSDNGKVL TAGTGQALGL IRLTDESSNN PNQQWNLTSV QTIQLPQKPI
151  IDTKLKDYPK YSPTGNIDNG TSPQLMGWTL VPCIMVNDPN IDKNTQIKTT
201  PYYILKKYQY WQRAVGSNVA LRPHEKKSYT YEWGTEIDQK TTIINTLGFQ
251  INIDSGMKFD IPEVGGGTDE IKTQLNEELK IEYSHETKIM EKYQEQSEID
301  NPTDQSMNSI GFLTITSLEL YRYNGSEIRI MQIQTSDNDT YNVTSYPNHQ
351  QALLLLTNHS YEEVEEITNI PKSTLKKLKK YYF*
```

D

PAT (183 amino acids; *ca.* 21 kDa)

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

Figure 16. Deduced amino acid sequences of the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins
The amino acid sequences of the Cry1F (panel A), Cry34Ab1 (panel B), Cry35Ab1 (panel C), and PAT (panelD) proteins were determined from the nucleotide sequences of the corresponding *cry1F*, *cry34Ab1*, *cry35Ab1*, and *pat* genes present in the event 4114 maize genome. In each case, these amino acid sequences were identical to the corresponding amino acid sequences determined based on the nucleotide sequence of T-DNA region of plasmid PHP27118, and they were identical to the deduced amino acid sequences of the respective proteins expressed in either 1507 or 59122 maize.

Additional western immunoblot analysis comparing the molecular weight and immunoreactivity of each of these proteins isolated from either 4114 maize or the breeding stack of 1507 × 59122 maize is presented below.

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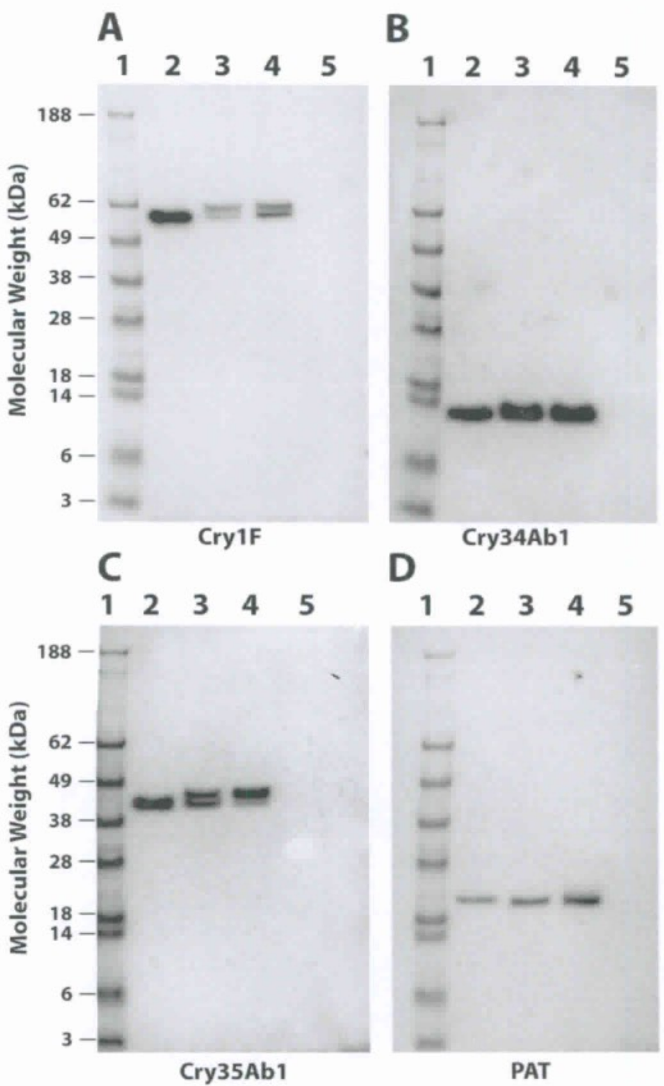

Figure 17. Western immunoblot analysis of Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins produced in 4114 and 1507 × 59122 maize

Samples containing protein standards for Cry1F (*ca.* 4 ng; lane 2, panel A), Cry34Ab1 (*ca.* 10 ng; lane 2, panel B), Cry35Ab1 (*ca.* 10 ng; lane 2, panel C), and PAT (*ca.* 4 ng; lane 2, panel D), and leaf tissue protein extracts from 4114 maize (lane 3, each panel), 1507 × 59122 maize (lane 4, each panel), and near isogenic-control maize (lane 5, each panel), were analyzed by SDS-PAGE followed by electroblotting onto PVDF membrane. Blots were probed with monoclonal antibodies specific for Cry1F (panel A), Cry34Ab1 (panel B), Cry35Ab1 (panel C), or PAT (panel D), followed by horseradish peroxidase (HRP)-conjugated anti-mouse IgG. Bound HRP-conjugated second antibody was visualized by developing the blots with a chemiluminescent substrate followed by electronic image capture. Molecular weight markers (SeeBlue Plus2®, Invitrogen) were included in lane 1 of each panel.

Western Blot Analysis of the Introduced Proteins

Samples of leaf tissue extracts prepared from 4114, 1507 × 59122, and near-isogenic control maize plants were evaluated by western immuoblot analysis using monoclonal antibodies specific for each of the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins (see Annex IV, page 67, for method details).

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In each case, comparisons of molecular weight and immunoreactivity for Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins derived from either 4114 or 1507 × 59122 maize demonstrated that the respective proteins were equivalent (Figure 17).

In the case of Cry1F, two immunoreactive species of *ca.* 60 kDa and *ca.* 62 kDa, respectively, were detected in samples prepared from both 4114 and 1507 × 59122 maize. This was expected as it is known that plant-derived Cry1F protein is susceptible to partial degradation by plant proteases, yielding a more stable *ca.* 60 kDa form of the same relative mobility as the Cry1F standard included in the analysis (Figure 17, panel A). A similar phenomenon was observed for the Cry35Ab1 protein, where partial degradation by plant proteases resulted in two immunoreactive species of *ca.* 44 kDa and *ca.* 40 kDa, respectively, with the smaller of these migrating at the same relative mobility as the Cry35Ab1 standard (Figure 17, panel C). There was no proteolytic degradation observed for either the Cry34Ab1 (*ca.* 14 kDa) or PAT (*ca.* 21 kDa) proteins.

B.2. Information on Antibiotic Resistance Marker Genes

No antibiotic resistance marker genes are present in maize event 4114. Analysis for the presence of plasmid backbone sequences (section 0 demonstrated that no plasmid backbone was incorporated into the maize genome during transformation. Thus the spectinomycin (*spc*) resistance gene, which was used as a bacterial selectable marker gene, is not present in 4114 maize.

B.3. Characterisation of Novel Proteins or Other Novel Substances

Biochemical function and phenotypic effects of novel protein(s)

DP4114 maize produces the Cry proteins Cry1F, Cry34Ab1, and Cry35Ab1, as well as the herbicide-tolerance protein PAT. The Cry1F protein provides protection against certain lepidopteran pests, including European corn borer (*Ostrinia nubilalis*), a major maize pest. This protein and its associated genetic elements are identical to those in1507 maize which was assessed and approved by FSANZ in 2003. The Cry34Ab1 and Cry35Ab1 proteins together comprise an active binary insecticidal crystal protein that provides protection against corn rootworm pests, including western corn rootworm (*Diabrotica virgifera virgifera)*, also a major maize pest. This binary protein and the associated genetic elements are identical to those in 59122 maize which was approved by FSANZ in 2005. .

Cry proteins (*i.e.,* delta-endotoxins), including the Cry1F and Cry34/35Ab1 proteins expressed in 4114 maize, act by selectively binding to specific sites localized on the lining of the midgut of susceptible insect species (de Maagd *et al.*, 2003; Schnepf *et al.*, 1998). Following binding, pores are formed that disrupt midgut ion flow, causing gut paralysis and eventual death due to bacterial sepsis (Bravo *et al.*, 2007). The Cry1F and Cry34/35Ab1 proteins provide protection against certain lepidopteran or coleopteran insects, respectively, and their specificity of action can be attributed to the presence of their respective binding sites in the unique environment of the target insect midgut that is required for their activity (Chambers *et al.*, 1991; de Maagd *et al.*, 2003; Ellis *et al.*, 2002; Hua *et al.*, 2001; Moellenbeck *et al.*, 2001). There are no binding sites for the delta-endotoxins of *B. thuringiensis* on the surface of mammalian intestinal cells and the mammalian digestive system environment does not support the steps required for toxicity of these proteins, therefore, livestock animals and humans are not susceptible to these proteins (Siegel, 2001).

The PAT protein confers tolerance to the herbicidal active ingredient glufosinate-ammonium at current labeled rates. This protein is identical to the protein found in a number of approved events across several different crops that are currently in commercial use, including 1507 and 59122 maize.

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Glufosinate chemically resembles the amino acid glutamate and acts to inhibit an enzyme, called glutamine synthetase, which is involved in the synthesis of glutamine. Glutamine synthetase is also involved in ammonia detoxification. Due to its similarity to glutamate, glufosinate blocks the activity of glutamine synthetase, resulting in reduced glutamine levels and a corresponding increase in concentrations of ammonia in plant tissues, leading to cell membrane disruption and cessation of photosynthesis resulting in plant death. The PAT protein expressed in DP4114 maize acetylates glufosinate to N-acetylglufosinate. This action prevents the inhibition of glutamine synthetase and therefore the plant is able to survive applications of herbicides containing glufosinate at current labeled rates.

Identification of novel substances (e.g. metabolites), levels and site

There are no novel substances produced as an effect of the insertion of the 4114 event.

Concentrations of Cry1F, Cry34Ab1, Cry35Ab1, and PAT in 4114 Maize Grain

All maize-derived food products are produced from the grain (kernels), and this represents the only realistic route of dietary exposure to the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins present in 4114 maize. In order to demonstrate that the potential human and animal dietary exposure to these proteins derived from 4114 maize is equivalent to the existing exposure potential from commercial maize hybrids containing events 1507 and/or 59122, their concentrations were determined in samples of grain harvested from multi-location field trials.

The concentrations of Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins were measured by specific quantitative enzyme linked immunosorbent assay (ELISA) of samples of maize kernels (grain) harvested from hybrid plants of lines 4114 (derived from F1*5 generation seed; Figure 4), 1507, 59122, and the breeding stack of 1507 × 59122, which were grown in side-by-side trials across five locations in the United States and Canada in 2010 (see Annex V, page 69, for materials and methods). For each protein, the concentration in grain samples from 4114 maize was compared to the concentration in grain samples from 1507, 59122, and 1507 × 59122 maize, as applicable.

The mean values and ranges of Cry1F, Cry34Ab1, and Cry35Ab1 protein concentrations in grain samples derived from 4114 maize were similar to corresponding values measured in samples of 1507, 59122, and 1507 × 59122 maize (Table 8). For all grain samples, the concentrations of PAT protein were consistently near, or below, the lower limit of quantification.

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Table 8: Concentrations of Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins in samples of maize grain derived from maize lines 4114, 1507, 59122, and the breeding stack of 1507 × 59122

Protein	Protein Concentration (ng/mg tissue dry weight)†			
	Event 4114	Event 1507	Event 59122	Stack 1507 x 59122
Cry1F	3.3 (2.3–7.2)	3.2 (1.9–5.1)	– –	2.8 (1.7–4.5)
Cry34Ab1	24 (14–39)	– –	24 (17–33)	24 (14–42)
Cry35Ab1	1.1 (0.54–2.3)	– –	1.4 (0.75–2.3)	1.4 (0.69–2.0)
PAT	<LOQ[a] (<LOQ–<LOQ)	<LOQ (<LOQ–<LOQ)	0.071 (<LOQ–0.10)	0.089 (<LOQ–0.45)

† Protein concentrations were measured by specific quantitative ELISA and values are expressed as the least squares means of four replicate maize grain (R6) samples collected from hybrid plants of each genotype across five locations in the United States and Canada during the 2010 growing season (n=20). For each protein, the lowest and highest individual values across locations are shown in parentheses.

a. LOQ = Lower limit of quantification, which for the PAT protein was 0.069 ng/mg tissue dry weight for samples of maize grain.

The similarity in concentrations of Cry1F, Cry34Ab1, Cry35Ab1, PAT proteins in grain derived from 4114 maize when compared with the corresponding concentrations of these proteins in 1507 and 59122 maize, and in breeding stacks of 1507 × 59122 maize, means that evaluations of potential dietary exposure conducted during the previous assessments of 1507 and 59122 maize are directly applicable to 4114 maize. There is no anticipated change in potential dietary exposure to the Cry1F, Cry34Ab1, Cry35Ab1, or PAT proteins as a consequence of commercial introduction of maize hybrids containing event 4114.

Post-translational modifications to the novel protein(s)

Equivalency of the proteins produced in 4114 maize to those produced in 1507 and 59122 maize

In order to verify the identity of the introduced proteins in 4114 maize and the equivalency to the proteins in 1507 and/or 59122 maize, western blot analysis of the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins was conducted in sample extracts of 4114 maize and 1507x59122 maize. The western blot analysis demonstrated that the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins derived from **4114 maize** have equivalent molecular weights and immunoreactivity to the proteins expressed in 1507x59122 maize. In all four western blots, the near-isoline control showed no immunoreactive proteins.

Western Blot Analysis of the Introduced Proteins

Cry1F

Western blot analysis using anti-Cry1F antibodies (Annex VII) demonstrated that the Cry1F protein derived from 4114 maize had the same molecular weight and relative immunoreactivity as the Cry1F protein derived from 1507x59122 maize. Both protein samples migrated as two bands of approximately 60 kDa and 62 kDa in size. The double banding pattern was expected because plant-derived Cry1F protein can be partially degraded by plant proteases to a smaller, more stable truncated protein, therefore appearing as two bands (the larger intact protein and the smaller truncated protein). Relative amounts of the two bands can vary from sample to sample. The Cry1F standard protein included on the blot consisted of the truncated form of the protein and migrated at the expected approximately 60 kDa.

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Cry34Ab1

Western blot analysis using anti-Cry34Ab1 antibodies demonstrated that the Cry34Ab1 protein derived from 4114 maize had the same molecular weight and relative immunoreactivity as the Cry34Ab1 protein derived from 1507x59122 maize, with each sample migrating at approximately 14 kDa. Similarly, the Cry34Ab1 standard protein migrated at approximately 14 kDa and appeared to be equivalent to the plant-derived proteins.

Cry35Ab1

Western blot analysis using anti-Cry35Ab1 antibodies demonstrated that the Cry35Ab1 protein derived from 4114 maize had the same molecular weight and relative immunoreactivity as the Cry35Ab1 protein derived from 1507x59122. Both protein samples migrated as two bands of approximately 40 kDa and 44 kDa in size. Like the Cry1F protein, the Cry35Ab1 protein can be partially degraded to a more stable truncated protein by plant proteases, and the relative amounts of each protein can vary from sample to sample. The Cry35Ab1 standard protein consisted of the truncated form of the protein and migrated at the expected approximately 40 kDa.

PAT Protein

Western blot analysis using anti-PAT antibodies (Annex VII, Figure 2.4) demonstrated that the PAT protein derived from 4114 maize had the same molecular weight and relative immunoreactivity as the PAT protein derived from 1507x59122 maize, with each sample migrating at approximately 21 kDa. The PAT standard protein migrated at the same molecular weight as the plant-derived PAT proteins (approximately 21 kDa).

2.3. Conclusions

The western blot analyses demonstrated that the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins derived from 4114 maize have equivalent molecular weights and immunoreactivity to the proteins expressed in 1507x59122 maize. In all four western blots, the near-isoline control showed no immunoreactive proteins. These data, in addition to the identical DNA sequences encoding the proteins, demonstrates equivalency of the transgenic proteins in 4114 maize to those in 1507 and/or 59122 maize.

History of human consumption of novel substances or similarity to substances previously consumed in food

The safety of the introduced proteins in DP4114 maize has been previously evaluated by FSANZ, as referenced above. The Cry1F, Cry34Ab1, and Cry35Ab1 proteins were derived from *Bacillus thuringiensis* and the PAT protein was derived from *Streptomyces viridochromogenes* and all four proteins have a history of safe use in agricultural crop commodities. These proteins have been present in commercial maize varieties such as 1507, 59122, and 1507x59122 maize since 2003, 2006, and 2006, respectively. These commercial lines contain familiar traits and are currently licensed broadly across the seed industry; commercial products containing 1507x59122 maize were grown on approximately 26 million acres or approximately 27% of U.S. maize acres in 2013 (Data source: Pioneer Hi-Bred International, Inc. Proprietary Data). In addition to these varieties the PAT protein has also been present in a number of other commercial crops and commercially planted in the U.S. since 1996. Further, *B. thuringiensis and S. viridochromogenes*

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are naturally occurring soil bacteria and are not pathogenic. Animals and humans are regularly exposed to these organisms and their components without any reported adverse consequences.

B.4-B.5. Prior Safety Assessments of Cry1F, Cry34Ab1, Cry35Ab1, and PAT Proteins

The information and data provided herein have established that the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins present in 4114 maize are equivalent to the corresponding proteins produced in 1507 (Cry1F, PAT) and 59122 (Cry34Ab1, Cry35Ab1, PAT) maize. This equivalency was established based on the following criteria:

1. The same gene expression cassettes (promoters, protein coding sequences, and terminators) were used in the production of 4114 maize as in previously authorized 1507 and 59122 maize lines, so all genetic elements are identical.
2. The DNA insertion in 4114 maize was sequenced (Diehn *et al.*, 2011) and the translated amino acid sequences of the encoded Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins were determined. The translated amino acid sequences were compared and found to be identical to the deduced amino acid sequences of those proteins in previously authorized 1507 and 59122 maize lines, which are also present in breeding stacks of 1507 × 59122 maize.
3. Western immunoblot analysis demonstrated the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins in 4114 maize have the same molecular weight and immunoreactivity as the corresponding proteins expressed in 1507 × 59122 maize.

Each of these proteins has been previously assessed for potential toxicity and potential allergenicty by Food Standards Australia New Zealand (FSANZ, 2003; FSANZ, 2005) and numerous other regulatory agencies worldwide, and determined to pose no significant risks to the environment, human or animal health. In addition, there is a considerable body of public information supporting the safety of *B. thuringiensis*-derived insect control proteins, including Cry1F, Cry34Ab1, and Cry35Ab1 (OECD, 2007), as well as the PAT protein (Hérouet *et al.*, 2005).

Therefore, the previous safety assessments of the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins conducted during the evaluations of 1507 and 59122 maize are also applicable for 4114 maize.

B.6 Potential Toxicity of Glufosinate Ammonium Metabolites

Glufosinate ammonium herbicide contains both the L-isomer and the D-isomer of glufosinate. Unlike the L-isomer, the D-isomer does not competitively inhibit the glutamine synthase enzyme in plants and is not herbicidally active. In plants expressing the *pat* gene, the herbicidally active component of glufosinate ammonium, the L-isomer, is rapidly metabolized by the action of the PAT enzyme into the non-phytotoxic stable metabolite *N*-acetyl-L-glufosinate (2-acetamido-4-methylphosphinicobutanoic acid; NAG). This metabolite does not inhibit glutamine synthetase, therefore the plants will survive applications of this herbicide.

In season applications of glufosinate ammonium herbicide on hybrids containing event 4114 will be according to the same good agricultural practice standards as applicable for other glufosinate ammonium-tolerant maize hybrids, including hybrids containing events 1507, 59122, and 1507 × 59122. The potential toxicity of metabolites derived from glufosinate ammonium has previously been reviewed by Food Standards Australia New Zealand during the safety assessments of 1507

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(FSANZ, 2003) and 59122 (FSANZ, 2005) maize, and those conclusions apply equally for 4114 maize.

B.7 Compositional Analyses of the GM Food

Since all maize-derived food products are produced from kernels (grain), analysis of the composition of grain is the most appropriate test for food use and is the approach that was followed for 4114 maize. As stated in the OECD consensus document on compositional considerations for new maize varieties, *"if only agronomical traits are influenced by the genetic modification, derived products need not be analysed separately"* (OECD, 2002). For new varieties without purposefully altered nutritional properties, such as 4114 maize, the nutritional evaluation is part of the weight-of-evidence approach for evaluating whether there were any unanticipated consequences of the genetic modification.

Compositional analyses were performed on samples of maize kernels (grain) harvested from hybrid plants of 4114 (derived from F1*5 generation seed; Figure 4) and near-isogenic control maize grown in side-by-side trials across six locations in the United States and Canada in 2010. The near-isogenic control plants had a genetic background that was greater than 97% percent similar to that of the 4114 maize generation used, but did not go through the transformation process. Each location utilized a randomized complete block design containing four blocks of 4114 and control maize planted together in two-row plots. One grain sample (equal to five pooled ears), from each of the four blocks, was collected from 4114 and control maize at the R6 growth stage. All samples were collected from impartially selected, healthy individual plants that had previously been self-pollinated. Further details on sampling, processing, testing, and statistical analyses are presented in Annex VI, page 72.

In two separate experiments, grain samples were also harvested from trials of non-modified commercial reference hybrids grown at six locations in 2003 (Pioneer® hybrids 34M94, 33G26, 33J24, and 3394) and at six locations in 2007 (Pioneer® hybrids 38B85, 37Y12, 34A15, and 34P88). Compositional analyses of these samples were used to determine a statistical tolerance interval that was calculated to contain, with 95 percent confidence, 99 percent of the values comprising the population of commercial maize. This statistical tolerance interval, together with the range of values for each analyte reported in the published literature (e.g., OECD, 2002) or available from the ILSI Crop Composition Database (ILSI, 2010), provided further context for interpretation of the composition results for 4114 maize. Analyte ranges for 4114 maize that fell within the tolerance interval and/or combined literature range for that analyte were considered to be within the range of normal variability of commercial maize hybrids.

The analytes for compositional assessment were selected taking into account the OECD consensus document on compositional considerations for new varieties of maize (OECD, 2002). Compositional analyses of grain samples included protein, fat, fibre, ash, carbohydrates, minerals, vitamins, amino acids, fatty acids, key antinutrients and secondary metabolites.

Compositional Data

Proximates

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The major constituents, or proximates, of maize grain are carbohydrates, protein, fat and ash. Fibre also makes up an important component of the indigestible carbohydrate in maize grain. Fibre is measured by the neutral detergent fibre method (NDF), which measures the insoluble fibre: lignin, cellulose and hemicellulose. Total dietary fibre (TDF) consists of the insoluble and soluble fibre (pectin). The soluble fibre fraction in maize is low, so the NDF value in maize grain is comparable to that of TDF. The acid detergent fibre (ADF) method solubilizes hemicellulose, measuring only cellulose and lignin (Watson, 1987).

Comparisons of proximates in grain samples derived from 4114 and near-isogenic control maize hybrids resulted in no statistically significant differences in crude fat, crude protein, carbohydrates, ADF, NDF, or crude fibre (Table 9). The only statistically significant difference measured was a slightly higher (*ca.* 8.3 percent) mean ash content in event 4114 maize grain samples. The mean values for all proximates in 4114 maize grain, including ash, were within the tolerance interval and ranges reported in the literature.

Table 9: Proximate and fibre composition of maize grain harvested from event 4114 and control hybrids grown in six locations across the U.S. and Canada in 2010

	Proximates (% dry weight)†				Fibre (% dry weight)†		
Samples	**Ash**	**Crude Fat**	**Crude Protein**	**CHO[a]**	**ADF**	**NDF**	**Crude Fibre**
Event 4114	1.44 (1.30–1.60)	4.68 (3.88–5.67)	10.4 (7.32–11.7)	83.6 (82.2–85.7)	4.01 (3.53–5.16)	10.4 (9.58–11.2)	2.48 (1.34–3.16)
Control	1.33 (1.12–1.52)	4.81 (4.28–5.73)	10.2 (8.92–11.4)	83.7 (82.5–84.9)	3.83 (3.24–4.48)	10.4 (9.39–11.3)	2.57 (1.36–3.38)
p-Value	***0.003***	0.177	0.455	0.758	0.0965	0.824	0.520
TI (99%)[b]	0.531–2.16	1.45–5.75	6.59–13.5	80.3–89.7	1.43–5.73	5.75–20.6	0.941–3.73
Literature Values							
OECD (2002)	1.1–3.9	3.1–5.8	6–12.7	82.2–82.9	3.0–4.3	8.3–11.9	NR[c]
ILSI (2010)	1.44 (0.62–6.28)	3.62 (2.74–5.90)	10.29 (6.15–17.26)	84.7 (77.4–89.5)	4.07 (1.82–11.34)	11.25 (5.59–22.64)	2.35 (0.49–5.50)

† Values represent the least squares mean of four replicate samples collected from each of six trial site locations across the U.S. and Canada where event 4114 and control maize hybrids were grown in 2010 (n=24). For each analyte, the lowest and highest individual values across locations are shown in parentheses.

a. CHO = carbohydrate, by calculation; ADF = acid detergent fibre; NDF = neutral detergent fibre.

b. TI = Tolerance Interval, negative limits set to zero. Using data from a range of non-modified commercial maize hybrids grown at twelve field locations across the U.S. and Canada in 2003 (six sites) and 2007 (six sites), a statistical tolerance interval was calculated to contain, with 95% confidence, 99% of the values comprising the population of commercial maize.

c. NR = Values for this analyte not reported.

Minerals

Several mineral ions are recognized as essential plant nutrients and are required by the plant in significant quantities. These macronutrients include calcium, phosphorous, and potassium. The micronutrient minerals, iron, copper, sodium, and zinc are incorporated in plant tissues in only trace amounts. Comparison of the mineral composition in samples of 4114 and near-isogenic control maize grain is shown in Table 10. No statistically significant differences were observed for concentrations of calcium, copper, iron, magnesium, manganese, sodium or zinc. Although statistically significant differences were noted for phosphorus and potassium, which were both slightly elevated in 4114 grain samples by *ca.* 7.6 and 7.5 percent, respectively, the mean values for these two minerals were both within the tolerance interval and range reported in the literature.

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Table 10: Mineral composition of maize grain harvested from event 4114 and control hybrids grown in six locations across the U.S. and Canada in 2010

Samples	Minerals (mg/100g dry weight)†								
	Ca[a]	Cu	Fe	Mg	Mn	P	K	Na	Zn
Event 4114	3.35 (2.51–4.48)	0.0653 (<LOQ[c]–0.09)	1.66 (1.47–2.10)	135 (112–157)	0.650 (0.44–1.04)	311 (296–330)	416 (385–478)	0.063 (<LOQ-0.17)	1.74 (1.49–2.01)
Control	3.25 (2.70–3.99)	0.0645 (<LOQ-0.09)	1.68 (1.46–2.28)	133 (103–155)	0.627 (0.49–0.97)	289 (258–334)	387 (343–441)	0.058 (<LOQ-0.49)	1.74 (1.50–2.03)
p-Value	0.308	NA[d]	0.775	0.251	0.314	***0.0004***	***0.0031***	0.714	0.919
TI (99%)[b]	1.27–9.02	0–0.662	0.857–2.69	38.1–195	0.25–1.22	127–472	194–687	0–2.07	1.04–2.71
Literature Values									
OECD (2002)	3–100	0.09–1.0	0.1–10	82–1000	NR[e]	234–750	320–720	0–150	1.2–3.0
ILSI (2010)	4.65 (1.3–20.8)	0.174 (0.07–1.85)	2.18 (1.04–4.91)	119 (59–194)	0.619 (0.17–1.43)	327 (147–533)	385 (181–603)	3.24 (0.02–73.2)	2.16 (0.65–3.72)

† Values represent the least squares mean of four replicate samples collected from each of six trial site locations across the U.S. and Canada where event 4114 and control maize hybrids were grown in 2010 (n=24). For each analyte, the lowest and highest individual values across locations are shown in parentheses.

a. Ca = calcium; Cu = copper; Fe = iron; Mg = magnesium; Mn = manganese; P = phosphorus; K = potassium; Na = sodium; and Zn = zinc.

b. TI = Tolerance Interval, negative limits set to zero. Using data from a range of non-modified commercial maize hybrids grown at twelve field locations across the U.S. and Canada in 2003 (six sites) and 2007 (six sites), a statistical tolerance interval was calculated to contain, with 95% confidence, 99% of the values comprising the population of commercial maize.

c. LOQ = Lower limit of quantification, which for copper and sodium was 0.0625 mg/100g dry weight of tissue.

d. NA = Statistical analysis not applicable.

e. NR = Values for this analyte not reported.

Vitamins

Maize contains two fat-soluble vitamins, vitamin-A (β-carotene) and vitamin E, and most of the water-soluble vitamins. Vitamin A occurs in two forms in nature. Its true form, retinol, is present in foods of animal origin such as fish oils and liver. Provitamin A, in the form of the carotenoids β-carotene and cryptoxanthin, is found in plants and converted in the body to vitamin A. Vitamin E (tocopherol) occurs in a variety of vegetable, nut, and oilseed crops, and of the various structural isomers (alpha-, beta-, delta- and gamma-tocopherol), α-tocopherol is the most biologically important as a natural antioxidant. Alpha-tocopherol is the only form of vitamin E that is actively maintained in the human body, and has the greatest nutritional significance (Linus Pauling Institute, 2004).

There were no statistically significant differences in concentrations of β-carotene, thiamine, niacin, pantothenic acid, pyridoxine, folic acid, α-tocopherol, γ-tocopherol, or total tocopherols between grain samples from 4114 or near-isogenic control maize (Table 11). Concentrations of riboflavin, β-tocopherol, and δ-tocopherol in both 4114 and control maize grain samples were near, or below, the lower limit of quantification such that statistical analysis was not possible.

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Table 11: Vitamin composition of maize grain harvested from event 4114 and control hybrids grown in six locations across the U.S. and Canada in 2010

Samples	β-Carotene and B Vitamins (mg/kg dry weight)†						
	β-carotene	B1 Thiamine	B2 Riboflavin	B3 Niacin	B5 Pantothenic Acid	B6 Pyridoxine	B9 Folic Acid
Event 4114	18.1 (11.7–26.4)	2.20 (<LOQ–3.28)	<LOQ[b] (<LOQ-<LOQ)	13.8 (11.7–16.6)	5.11 (<LOQ–7.49)	4.29 (2.50–6.36)	0.792 (0.037–1.55)
Control	16.4 (7.27–26.2)	1.97 (<LOQ–2.92)	<LOQ (<LOQ-<LOQ)	13.9 (10.6–18.0)	4.92 (<LOQ–10.9)	4.68 (2.35–6.88)	0.872 (0.181–1.91)
p-Value	0.267	0.168	NA[c]	0.786	0.737	0.404	0.523
TI (99%)[a]	0–68.3	0.414–6.64	NC[d]	0–51.7	3.02–8.20	1.83–11.1	0–2.30
Literature Values							
OECD (2002)	0.49–2.18	2.3–8.6	0.25–5.6	9.3–70	NR[e]	4.6–9.6	NR
ILSI (2010)	6.63 (0.19–46.8)	5.33 (1.26–40.0)	1.25 (0.50–2.36)	23.84 (10.4–46.9)	NR	6.46 (3.68–11.32)	0.645 (0.147–1.46)

Samples	Tocopherols (mg/kg dry weight)†				
	α-Tocopherol	β-Tocopherol	γ-Tocopherol	δ-Tocopherol	Total Tocopherols
Event 4114	5.68 (2.14–14.8)	0.511 (<LOQ–0.756)	3.64 (2.52–5.64)	<LOQ (<LOQ–<LOQ)	9.90 (4.95–18.2)
Control	5.47 (2.09–13.3)	<LOQ (<LOQ–<LOQ)	3.38 (2.30–5.04)	<LOQ (<LOQ–<LOQ)	9.23 (4.77–17.0)
p-Value	0.672	NA	0.199	NA	0.342
TI (99%)	2.18–28.2	0–1.50	0–39.9	0–2.36	0–53.6
Literature Values					
ILSI (2010)	10.38 (1.54–68.7)	12.65 (0.58–22.8)	29.6 (6.46–61.0)	1.98 (0.38–16.1)	40.53 (8.69–133)

† Values represent the least squares mean of four replicate samples collected from each of six trial site locations across the U.S. and Canada where event 4114 and control maize hybrids were grown in 2010 (n=24). For each analyte, the lowest and highest individual values across locations are shown in parentheses.

a. TI = Tolerance Interval, negative limits set to zero. Using data from a range of non-modified commercial maize hybrids grown at twelve field locations across the U.S. and Canada in 2003 (six sites) and 2007 (six sites), a statistical tolerance interval was calculated to contain, with 95% confidence, 99% of the values comprising the population of commercial maize.

b. LOQ = Lower limit of quantification, which for thiamine, riboflavin, pantothenic acid, β-tocopherol, and δ-tocopherol was 1.80, 0.900, 1.33, 0.500, and 0.500 mg/kg dry weight of tissue, respectively.

c. NA = Statistical analysis not applicable.

d. NC = Not calculated.

e. NR = Values for this analyte not reported.

Amino Acids

The quality of protein produced by different maize hybrids can be determined by measuring the content of different amino acids. Eighteen amino acids commonly found in maize are considered to be important for compositional analysis (EuropaBio, 2003). Levels of methionine and cysteine are important for formulation of animal feed, as are lysine and tryptophan, which cannot be produced by non-ruminant animals such as swine and poultry, and are present at low concentrations in maize.

Comparison of the amino acid composition of 4114 grain and the near-isogenic control grain is shown in Table 12. No significant differences were noted for any of the 18 amino acids measured. Mean concentrations of all amino acids in 4114 maize grain were within the tolerance interval and ranges reported in the literature.

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Table 12: Amino acid composition of maize grain harvested from event 4114 and control hybrids grown in six locations across the U.S. and Canada in 2010

Samples	Concentration (mg/g dry weight)†								
	Asp[a]	Thr	Ser	Glu	Pro	Gly	Ala	Cys	Val
Event 4114	7.01 (4.60–8.13)	3.73 (2.84–4.38)	5.18 (3.52–7.45)	19.6 (12.1–23.1)	9.26 (6.24–11.1)	3.77 (3.05–4.45)	7.57 (4.6–8.86)	1.94 (1.44–2.52)	4.73 (3.34–5.42)
Control	6.80 (5.62–7.51)	3.61 (3.18–4.02)	5.00 (4.21–5.54)	19.3 (15.9–22.4)	8.99 (7.63–10.1)	3.72 (3.18–4.42)	7.39 (6.04–8.4)	1.93 (1.57–2.53)	4.60 (3.93–5.07)
p-Value	0.260	0.165	0.308	0.546	0.235	0.461	0.398	0.931	0.187
TI (99%)[b]	4.42–9.47	1.76–5.78	2.66–6.83	11.7–28.8	4.54–16.4	2.49–4.85	4.91–10.9	1.36–4.18	1.59–7.49
Literature Values									
OECD (2002)	4.8–8.5	2.7–5.8	3.5–9.1	12.5–25.8	6.3–13.6	2.6–4.9	5.6–10.4	0.8–3.2	2.1–8.5
ILSI (2010)	6.88 (3.35–12.1)	3.73 (2.24–6.66)	5.13 (2.35–7.69)	20.1 (9.65–35.4)	9.50 (4.62–16.3)	3.86 (1.84–5.39)	7.90 (4.39–13.9)	2.21 (1.25–5.14)	4.90 (2.66–8.55)
Samples	**Met**	**Ile**	**Leu**	**Tyr**	**Phe**	**His**	**Lys**	**Arg**	**Trp**
Event 4114	1.82 (1.36–2.30)	3.49 (2.29–4.08)	13.1 (7.75–15.6)	2.57 (1.63–3.67)	5.54 (3.70–6.66)	3.03 (2.30–4.26)	2.88 (2.13–3.50)	4.38 (3.64–5.14)	0.639 (0.38–0.77)
Control	1.79 (1.44–2.31)	3.39 (2.88–3.88)	12.6 (10.2–14.9)	2.63 (1.99–3.33)	5.23 (4.43–6.11)	2.93 (2.57–3.42)	2.93 (2.24–3.49)	4.34 (3.62–4.92)	0.634 (0.37–0.73)
p-Value	0.846	0.251	0.206	0.561	0.121	0.274	0.474	0.707	0.806
TI (99%)	0.72–4.90	1.43–5.87	6.59–19.5	0.71–5.05	2.98–6.93	1.80–3.62	1.12–5.51	2.53–5.51	0.09–1.27
Literature Values									
OECD (2002)	1.0–4.6	2.2–7.1	7.9–24.1	1.2–7.9	2.9–6.4	1.5–3.8	0.5–5.5	2.2–6.4	0.4–1.3
ILSI (2010)	2.10 (1.24–4.68)	3.68 (1.79–6.92)	13.4 (6.42–24.9)	3.37 (1.03–6.42)	5.25 (2.44–9.30)	2.96 (1.37–4.34)	3.16 (1.72–6.68)	4.35 (1.19–6.39)	0.627 (0.27–2.15)

† Values represent the least squares mean of four replicate samples collected from each of six trial site locations across the U.S. and Canada where event 4114 and control maize hybrids were grown in 2010 (n=24). For each analyte, the lowest and highest individual values across locations are shown in parentheses.

a. Asp=aspartic acid; Thr=threonine; Ser=serine; Glu=glutamic acid; Pro=proline; Gly=glycine; Ala=alanine; Cys=cystine; Val=valine; Met=methionine; Ile=isoleucine; Leu=leucine; Tyr=tyrosine; Phe=phenylalanine; His=histidine; Lys=lysine; Arg=arginine; Trp=tryptophan.

b. TI = Tolerance Interval, negative limits set to zero. Using data from a range of non-modified commercial maize hybrids grown at twelve field locations across the U.S. and Canada in 2003 (six sites) and 2007 (six sites), a statistical tolerance interval was calculated to contain, with 95% confidence, 99% of the values comprising the population of commercial maize.

Fatty Acids

Five fatty acids account for *ca.* 98 percent of the total fatty acids in maize grain (ILSI, 2010), with the most abundant being linoleic (C18:2 Δ9,12; 57.7%) and oleic (C18:1 Δ9; 25.7%) acids. Less abundant, but occurring at measurable levels are palmitic (C16:0; 11.5%), stearic (C18:0; 1.8%) and α-linolenic (C18:3 Δ9,12,15; 1.2%) acids.

The desaturation of oleic acid to form linoleic acid, and its subsequent desaturation to form α-linolenic acid, occurs only in plants, hence both linoleic and α-linolenic acids are essential fatty acids for mammals. For this reason, it was desirable to measure for any unintended changes in the levels of linoleic and α-linolenic acids, and their key precursors, palmitic, stearic and oleic acids, in grain from 4114 maize.

Other polyunsaturated and longer chain polyunsaturated fatty acids, such as γ-linolenic (C:18 Δ6,9,12), eicosatrienoic (C20:3 Δ8,11,14) and arachidonic (C20:4 Δ5,8,11,14) acids can all be synthesized by mammals from dietary sources of α-linolenic and linoleic acid. And the synthesis of palmitoleic (C16:1 Δ9) and saturated fatty acids with chain lengths greater than 18 (e.g., C20:0, C22:0, C24:0) can be accomplished in mammals through *de novo* fatty acid synthesis without dietary requirements for palmitic and stearic acids, respectively. Hence, small changes in the concentrations of any of these trace fatty acids in 4114-derived grain would have little or no biological significance to either humans or animals consuming 4114 maize grain products.

Table 13: Fatty acid composition of maize grain harvested from event 4114 and control hybrids grown in six locations across the U.S. and Canada in 2010

	Amount (% of total fatty acids)†							
Samples	**Palmitic (C16:0)**	**Stearic (C18:0)**	**Oleic (C18:1)**	**Linoleic (C18:2)**	**α-Linolenic (C18:3)**	**Arachidic (C20:0)**	**Eicosenoic (C20:1)**	**Lignoceric (C24:0)**
Event 4114	14.2 (13.1–16.2)	1.60 (1.37–2.14)	22.1 (20.3–23.8)	60.5 (56.3–63.5)	0.770 (0.305–1.12)	0.394 (0.347–0.481)	0.265 (0.229–0.336)	0.233 (0.211–0.276)
Control	14.1 (13.2–15.6)	1.60 (1.45–2.07)	22.6 (20.7–25.8)	60.1 (54.9–62.9)	0.787 (0.410–1.22)	0.392 (0.343–0.505)	0.235 (0.204–0.255)	0.240 (0.185–0.318)
p-Value	0.518	0.810	***0.0063***	0.203	0.697	0.518	***<0.0001***	0.796
TI (99%)[a]	5.51–18.4	0.566–4.67	10.4–65.6	30.4–81.7	0–3.34	0.159–0.849	0.213–0.370	0–0.675
Literature Values								
ILSI (2010)	11.50 (7.94–20.71)	1.82 (1.02–3.40)	25.7 (17.4–40.2)	57.7 (36.2–66.5)	1.20 (0.57–2.25)	0.412 (0.28–0.97)	0.297 (0.170–1.92)	0.168 (0.140–0.230)

† Values represent the least squares mean of four replicate samples collected from each of six trial site locations across the U.S. and Canada where event 4114 and control maize hybrids were grown in 2010 (n=24). For each analyte, the lowest and highest individual values across locations are shown in parentheses. The concentrations of the following fatty acids were below the lower limit of quantification for more than 80% of the samples from 4114 and control maize such that statistical analysis was not possible, and are not reported: caprylic (C8:0); capric (C10:0); lauric (C12:0); myristic (C14:0); myristoleic (C14:1); pentadecanoic (C15:0); pentadecenoic (C15:1); palmitoleic (C16:1); heptadecanoic (C17:0); heptadecenoic (C17:1); heptadecadienoic (C17:2); (9,15) isomer of linoleic (C18:2); γ-linolenic (C18:3); nonadecanoic (C19:0); eicosadienoic (C20:2); eicosatrienoic (C20:3); arachidonic (C20:4); heneicosanoic (C21:0); behenic (C22:0); erucic (C22:1); and tricosanoic (C23:0).

a. TI = Tolerance Interval, negative limits set to zero. Using data from a range of non-modified commercial maize hybrids grown at twelve field locations across the U.S. and Canada in 2003 (six sites) and 2007 (six sites), a statistical tolerance interval was calculated to contain, with 95% confidence, 99% of the values comprising the population of commercial maize.

The complete fatty acid profile of maize grain from 4114 and control hybrids was determined and the results are summarized in Table 13. The concentrations of a number of fatty acids occurring in trace amounts in both 4114 and control maize grain samples were below the lower limit of quantification for more than 80 percent of the samples such that statistical analysis was not possible (these are itemized in the footnotes to Table 13). The only statistically significant differences observed were for oleic (C18:1) acid, which was slightly lower (*ca.* 2.2 percent) in samples of 4114 maize grain, and for eicosenoic (C20:1) acid, where the mean concentration was slightly elevated by *ca.* 12.8 percent in samples of 4114 maize grain. Mean concentrations of all quantifiable fatty acids in 4114 grain samples, including oleic acid and eicosenoic acid, were within the tolerance interval and ranges reported in the literature.

Secondary Metabolites and Antinutrients

Secondary metabolites are defined as those natural products that do not function directly in the primary biochemical activities that support growth, development and reproduction of the organism in which they occur (EuropaBio, 2003). One class of secondary metabolites, antinutrients, is responsible for deleterious effects related to the absorption of nutrients and micronutrients from foods (Shahidi, 1997). There are generally no recognized antinutrients in maize at levels considered to be harmful, but for the purposes of safety assessment OECD recommends testing for the following secondary metabolites in maize: ferulic acid, ρ-coumaric acid, furfural, phytic acid, inositol, raffinose, and trypsin inhibitor. These secondary metabolites and antinutrients were analyzed in grain samples from 4114 and control hybrids (Table 14).

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Table 14: Composition of secondary metabolites and antinutrients in maize grain harvested from event 4114 and control hybrids grown in six locations across the U.S. and Canada in 2010

	Concentration (mg/100g dry weight)†						
Samples	**ρ-Coumaric Acid**	**Furfural**	**Ferulic Acid**	**Phytic Acid**	**Inositol**	**Raffinose**	**Trypsin Inhibitor (TIU/mg)**
Event 4114	18.6 (15.1–24.9)	<LOQ[b] (<LOQ–<LOQ)	266 (227–292)	1020 (827–1520)	22.9 (16.2–30.3)	108 (<LOQ–236)	2.58 (1.70–3.48)
Control	18.3 (12.0–27.6)	<LOQ (<LOQ–<LOQ)	255 (199–299)	998 (842–1170)	25.6 (19.7–34.1)	82.3 (<LOQ–210)	2.67 (1.45–4.68)
p-Value	0.546	NA[c]	0.231	0.542	***0.0339***	0.0826	0.694
TI (99%)[a]	0.341–38.7	NC[d]	55.3–309	418–1410	0–46.1	0–398	1.60–4.89
Literature Values							
OECD (2002)	3–30	<0.001	20–300	450–1000	NR[e]	210–310	NR
ILSI (2010)	22.0 (5.34–57.6)	0.370 (0.300–0.634)	220 (29.2–389)	748 (111.5–1570)	136.9 8.90–376.5	133 (20.4–320)	2.73 (1.09–7.18)

† Values represent the least squares mean of four replicate samples collected from each of six trial site locations across the U.S. and Canada where event 4114 and control maize hybrids were grown in 2010 (n=24). For each analyte, the lowest and highest individual values across locations are shown in parentheses. Values are expressed as mg/100g dry weight tissue, except for trypsin inhibitor, which is expressed in trypsin inhibitor units (TIU)/mg dry weight tissue.

a. TI = Tolerance Interval, negative limits set to zero. Using data from a range of non-modified commercial maize hybrids grown at twelve field locations across the U.S. and Canada in 2003 (six sites) and 2007 (six sites), a statistical tolerance interval was calculated to contain, with 95% confidence, 99% of the values comprising the population of commercial maize.

b. LOQ = Lower limit of quantification, which for furfural and raffinose was 0.100 and 80.0 mg/100g dry weight of tissue, respectively.

c. NA = Statistical analysis not applicable.

d. NC = Not calculated.

e. NR = Values for this analyte not reported.

Phenolic acids —may have beneficial health effects because of their anti-oxidant properties. Ferulic acid and ρ-coumaric acid are weak anti-oxidants, and are found in vegetables, fruit and cereals. They are also used as flavouring in foods, as supplements and in traditional Chinese herbal medicine. Daily intake of phenolic acids by humans is estimated to be 0.2–5.2 mg/day (Clifford, 1999; Radtke *et al.*, 1998).

There were no statistically significant differences in mean concentrations of ferulic and ρ-coumaric acid between 4114 and control grain samples, and the concentrations of both these phenolic acids were within the tolerance interval and ranges published in the literature.

Furfural – is a heterocyclic aldehyde that occurs in several vegetables, fruits and cereals. It is generally recognized as safe (GRAS) under conditions of intended use as a flavour ingredient, i.e., at levels 100 times lower than the occurrence of furfural as a natural ingredient in traditional foods. Field maize generally contains < 0.001 mg/100g furfural (Adams *et al.*, 1997). Furfural was below the lower limit of quantification (0.100 mg/100g) in all samples of 4114 and control grain.

Phytic acid and inositol – (myo-inositol 1,2,3,4,5,6-hexakis[dihydrogen phosphate]) is considered to be an antinutrient due to its ability to bind minerals, proteins and starch at physiological pH (Rickard and Thompson, 1997). Phytic acid is present in maize germ and binds 60–75 percent of phosphorus in the form of phytate (NRC, 1998), decreasing the bioavailability of phosphorus in maize for non-ruminant animals. Phytic acid levels in maize grain vary from 0.45–1.0 percent of dry matter (Watson, 1982).

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There were no statistically significant differences between mean concentrations phytic acid in grain samples from 4114 and control hybrids (Table 14). There was a statistically significant difference in mean inositol levels, which were slightly reduced (*ca.* 10.5 percent) in samples of 4114 maize grain compared to control samples.

Protease inhibitors – are found in abundance in raw cereals and legumes, especially soybeans. Trypsin inhibitors in soybean give rise to inactivation and loss of trypsin in the small intestine, triggering the induction of excess trypsin in the pancreas at the expense of sulfur-containing amino acids (Shahidi, 1997). Maize contains low levels of trypsin and chymotrypsin inhibitors, neither of which is considered nutritionally significant (White and Pollak, 1995). No statistically significant difference was observed between mean trypsin inhibitor concentrations in grain samples from 4114 and control hybrids, and all values were within the tolerance interval and range reported in the literature (Table 14).

Alpha-galactosides – of sucrose, including raffinose, are widely distributed in higher plants (Naczk *et al.*, 1997). Due to the absence of α-galactosidase activity in human and non-ruminant animal mucosa, raffinose cannot be broken down by enzymes in the gastrointestinal tract and is considered an antinutrient, although it is not toxic.

Raffinose concentrations were generally low for both 4114 and control maize grain samples, with some values below the lower limit of quantification. There was no statistically significant difference in mean concentrations of raffinose between 4114 and control grain samples.

C. Nutritional Impact

Maize hybrids derived from the 1507 x 59122 breeding stack, expressing the same insect-resistance and herbicide-tolerance traits as present in 4114 maize, have been commercially cultivated on significant acreages in both the United States and Canada. Hence, there has been a history of safe use and exposure to the introduced Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins present in 4114 maize.

Detailed molecular characterization confirmed that the genes encoding the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins were incorporated within a single site in the 4114 maize genome, that they were stably inherited across multiple generations, and that they segregated as a single genetic locus according to Mendelian rules of inheritance.

As confirmed through measurements of protein expression and by extensive compositional analysis, the genetic modification resulting in 4114 maize did not introduce any new characteristics relative to existing 1507 x 59122 maize, nor did it result in the loss of any existing characteristic or in one or more characteristics falling outside the normal range of variation for maize. The concentrations of the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins in 4114 maize grain were similar to their respective concentrations in 1507 x 59122 grain. Relative to 1507 x 59122, maize line 4114 does not produce any other new novel proteins, nor does it produce any new secondary metabolites. And, based on the compositional comparison between 4114 and

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conventional maize, it can be concluded that 4114 maize is substantially equivalent to conventional maize.

C.1. Data on Nutritional Impact of Compositional Changes

The conclusion based on these data was that grain from 4114 maize was compositionally equivalent to grain from unmodified near-isogenic control maize, and to other commercial maize hybrids. Processing is unlikely to alter the compositional components of maize grain, thus, products derived from 4114 grain are anticipated to be compositionally equivalent to their conventional counterparts. Food products derived from 4114 maize grain are anticipated to be equivalent to food products derived from other commercially available maize.

C.2. Data From Animal Feeding Study, If Available

Compositional analysis is the cornerstone of the nutritional assessment of a food derived from a new plant variety. When compositional equivalence between the new food and its conventional counterpart has been established, the results of numerous published livestock feeding trials with modified varieties of maize, soybean, canola, cotton, and sugar beet, have confirmed that there are no significant differences in digestibility of nutrients, animal health, or animal performance (Flachowsky *et al.*, 2005; Van Eenennaam and Young, 2014). Therefore, once compositional equivalence has been established, nutritional equivalence may be assumed (EFSA, 2006; OECD, 2003).

Considering the compositional equivalence between 4114 maize and conventional maize, and the lack of any observed phenotypic characteristics indicative of unexpected unintended effects arising from the genetic modification process, there were no plausible risk hypotheses that would indicate the need for animal feeding studies.

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Annex I Methods for Southern Blot Analysis of 4114 Maize

I-1 Southern Blot Characterization of 4114 Maize

Southern blot analysis was conducted to characterize the DNA insertion in 4114 maize. Individual plants of the T2, T3, BC3F1*3, and BC3F2*2 generations (Figure 4) were analyzed by Southern hybridization experiments to determine the number of each of the genetic elements of the expression cassette that were inserted and to verify that the integrity of the PHP27118 T-DNA was maintained upon integration. The integration patterns of the insertion in 4114 maize were investigated with *Bcl*I and *Hin*dIII restriction enzymes. Southern blot analysis was conducted on individual plants from each of the four generations to confirm stability of the insertion across generations and to verify the absence of backbone sequences from plasmid PHP27118.

I-2 Test Material

Seeds from the T2, T3, BC3F1*3, and BC3F2*2 generations of 4114 maize were planted and leaf tissue harvested from individual plants was used for genomic DNA extraction.

I-3 Control Material

Seeds from the unmodified maize varieties PHWWE and PH09B were planted and leaf tissue harvested from individual plants was used for genomic DNA extraction.

I-4 Reference Material

Plasmid DNA from PHP27118 was prepared from *E. coli* (Invitrogen, Carlsbad, CA) and was used as a positive control for Southern blot analysis to verify probe hybridization and to verify sizes of fragments internal to the plasmid. The plasmid stock was a copy of the plasmid used for transformation to produce 4114 maize and was digested with restriction enzymes to confirm the plasmid map. The probes used in this study were derived from plasmid PHP27118 or from a plasmid containing equivalent genetic elements.

The *Bcl*I recognition sequence contains a Dam (DNA adenine methylase) recognition sequence (5'GATC3') (New England Biolabs, 2002). The PHP27118 plasmid used in this analysis was prepared in a *dam*+ strain of *E. coli* and thus the central adenine residue in all *Bcl*I sites (recognition sequence 5'TG**A**TCA3') was methylated and did not digest as expected. Therefore, the *Bcl*I-treated plasmid served only as a positive control to demonstrate probe hybridization and not to provide any fragment size data. As Dam is specific to bacteria and not found in maize plants, maize genomic DNA will be digested normally by *Bcl*I. Plasmid PHP27118 digested with *Hin*dIII was included on some of the *Bcl*I Southern blots to show that the plasmid used in this analysis was of sufficient quality and cut properly when digested with an enzyme that is not sensitive to methylation.

DNA molecular weight markers for gel electrophoresis and Southern blot analysis were used to determine approximate molecular weights. For Southern analysis, DNA Molecular Weight Marker VII, digoxigenin (DIG) labelled (Roche, Indianapolis, IN), was used as a size standard for hybridizing fragments. ΦX174 RF DNA/*Hae*III Fragments (Invitrogen, Carlsbad, CA) was used as a molecular weight standard to determine sufficient migration and separation of the fragments on the gel.

I-5 Identification of the 4114 Maize Plants Used for Southern Blot Analysis

Phenotypic analysis of 4114 maize plants and control plants was carried out by the use of lateral flow devices able to detect the Cry1F, Cry34Ab1 and PAT proteins to confirm the absence or presence of these proteins in material used for Southern blot analysis.

Leaf extracts were prepared by grinding leaf punches to homogeneity in 400 µl of EB2 extraction buffer (Envirologix, Inc., Portland, ME). Lateral flow devices (Envirologix) were placed in the

homogenate and allowed to develop. After incubation, the results were read from the lateral flow devices. A single stripe indicated a negative result and a double stripe indicated the sample was positive for the Cry1F, Cry34Ab1, or PAT proteins.

Genotypic analysis of the 4114 maize and control maize plants was carried out by real-time polymerase chain reaction (PCR) using assays specific for the DNA insertion. A leaf sample was taken from each test and control plant for event-specific PCR analysis. DNA was extracted from each leaf sample using the Extract N Amp™ Plant PCR kit using the described procedure (Sigma Aldrich, St. Louis, MO).

Real-time PCR was performed on each DNA sample utilizing an ABI PRISM® 7500 Fast Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA). TaqMan® probe (Applied Biosystems, Inc.) and primer sets (Integrated DNA Technologies, Coralville, IA) were designed to detect target sequences from the insertion in 4114 maize. In addition, a second TaqMan® probe and primer set for a reference maize endogenous gene was used to confirm the presence of amplifiable DNA in each reaction. The assay analysis consisted of real-time PCR determination of qualitative positive/negative calls. The extracted DNA was assayed using TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems, Inc.). Initial incubation was at 95°C for 10 minutes followed by 40 cycles as follows: 95°C for 15 seconds, 60°C for one minute.

Positive or negative determination for each plant was based on comparison of the threshold cycle (C_T) of the insertion target PCR to that of the maize endogenous reference target. If the event-specific and endogenous PCR targets amplified above C_T, then the plant was scored as positive for the 4114 event. If the endogenous target amplified and the event target did not, then the plant was scored as negative.

A subset of 4114 maize plants that were identified as containing the inserted DNA and expressing the Cry1F, Cry34Ab1, and PAT proteins from the four generations described previously were selected for Southern blot analysis.

I-6 GENOMIC DNA EXTRACTION

Genomic DNA was extracted from leaf tissue harvested from individual test and control plants. The tissue was pulverized in tubes containing grinding beads using a Geno/Grinder™ (SPEX CertiPrep, Inc., Metuchen, NJ) instrument and the genomic DNA was isolated using a urea-based procedure (modification from Chen and Dellaporta, 1994). Approximately 1 gram of ground tissue per sample was extracted with 5 ml urea extraction buffer (7 M urea, 0.34 M NaCl, 0.05 M Tris-HCl, pH 8.0, 0.02 M EDTA, 1% *N*-lauroylsarcosine) for 15-18 minutes at 37°C, followed by two extractions with phenol/chloroform/isoamyl alcohol (25:24:1) and one extraction with water saturated chloroform. The DNA was precipitated from the aqueous phase by the addition of 1/10 volume of 3 M NaOAc (pH 5.2) and 1 volume of isopropyl alcohol, followed by centrifugation to pellet the DNA. After washing the pellet twice with 70% ethanol, the DNA was dissolved in 0.5 ml distilled water and treated with 10 µg ribonuclease A for 15 minutes at 37°C. The sample was precipitated a second time and the pellet was washed with 70% ethanol. After drying, the DNA was re-dissolved with 0.5 ml distilled water and stored at 4°C.

I-7 QUANTIFICATION OF GENOMIC DNA

Following extraction, the DNA samples were quantified on a spectrofluorometer using PicoGreen® reagent (Molecular Probes, Inc., Eugene, OR) following a standard procedure. The DNA was also visualized on an agarose gel to confirm quantification values from the PicoGreen® analysis and to determine DNA quality.

I-8 DIGESTION OF DNA FOR SOUTHERN BLOT ANALYSES

Genomic DNA samples extracted from selected 4114 maize and control maize plants were digested with restriction enzymes following a standard procedure. Approximately 3–6 µg of genomic DNA was digested using 50 units of enzyme according to manufacturer's

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recommendations. The digestions were carried out at 37°C for approximately three hours, followed by ethanol precipitation with 1/10 volume of 3 M NaOAc (pH 5.2) and 2 volumes of 100% ethanol. After incubation at ≤-5°C and centrifugation, the DNA was allowed to dry and then re-dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). The reference plasmid, PHP27118, was spiked into a control (carrier) plant DNA sample in an amount equivalent to approximately one or three gene copies per maize genome and digested with the same enzyme to serve as a positive control for probe hybridization and to verify sizes of fragments internal to the plasmid on the Southern blot.

I-9 Electrophoretic Separation and Southern Transfer

Following restriction enzyme digestion, the resultant DNA fragments were electrophoretically separated by size through an agarose gel. A molecular weight standard [ΦX174 RF DNA/*Hae*III Fragments (Invitrogen)] was used to determine sufficient migration and separation of the fragments on the gel. DIG-labelled DNA Molecular Weight Marker VII (Roche), which is visible after DIG detection, as described below, was used to determine hybridizing fragment size on the Southern blots.

Agarose gels containing the separated DNA fragments were depurinated, denatured, and neutralized *in situ*, and transferred to a nylon membrane in 20x SSC buffer (3 M NaCl, 0.3 M sodium citrate) using the method as described for the TURBOBLOTTER™ Rapid Downward Transfer System (Whatman, Inc., Piscataway, NJ). The DNA was then bound to the membrane by UV cross-linking (Stratalinker, Stratagene, and La Jolla, CA).

I-10 DNA Probe Labelling for Southern Blot Hybridization

Probes for the *cry1F*, *cry34Ab1*, *cry35Ab1* and *pat* genes were used to detect genes within the T-DNA insertion. Probes for the *ubi*ZM1 promoter, *ubi*ZM1 5' UTR and intron region, ORF25 terminator, *pinII* terminator, TA peroxidase promoter, and CaMV 35S promoter and terminator regions were used to detect regulatory regions within the T-DNA insertion. To determine whether PHP27118 backbone was incorporated during T-DNA insertion, the Right Border (RB) and Left Border (LB) backbone probes were used to analyze the backbone regions directly outside the T-DNA borders, and probes for the spectinomycin resistance (*spc*), tetracycline resistance (*tet*), and *vir*G genes were used to confirm absence of these genes and other portions of the backbone. DNA fragments of the probe elements were generated by PCR from plasmid PHP27118 or a plasmid with equivalent elements using specific primers. PCR fragments were electrophoretically separated on an agarose gel, excised and purified using a gel purification kit (Qiagen, Valencia, CA). DNA probes were generated from these fragments by PCR that incorporated a DIG labelled nucleotide, [DIG-11]-dUTP, into the fragment. PCR labelling of isolated fragments was carried out according to the procedures supplied in the PCR DIG Probe Synthesis Kit (Roche).

I-11 Probe Hybridization and Visualization

Labelled probes (Table 15) were hybridized to the target DNA on the nylon membranes for detection of the specific fragments using the procedures essentially as described for DIG Easy Hyb solution (Roche). After stringent washes, the hybridized DIG-labelled probes and DIG-labelled DNA standards were visualized using CDP-Star Chemiluminescent Nucleic Acid Detection System with DIG Wash and Block Buffer Set (Roche). Blots were exposed to X-ray film for one or more time points to detect hybridizing fragments and to visualize molecular weight standards bound to the nylon membrane. Images were digitally captured by detection with the Luminescent Image Analyzer LAS-3000 (Fujifilm Medical Systems, Stamford, CT). Digital images were compared to original X-ray film exposures as verification for use in this submission. The sizes of detected bands were documented for each digest and each probe.

I-12 Stripping of Probes and Subsequent Hybridizations

Following hybridization and detection, membranes were stripped of DIG-labelled probe to prepare the blot for subsequent re-hybridization to additional probes. Membranes were rinsed

briefly in distilled, de-ionized water and then stripped in a solution of 0.2 M NaOH and 0.1% SDS at 37°C with constant shaking. The membranes were then rinsed in 2x SSC and used directly for subsequent hybridizations. The alkali-based stripping procedure effectively removes probes labelled with the alkali-labile DIG.

Table 15: Locations and sizes of DNA probes used for Southern blot analyses

Probe	Genetic Element	Position on PHP27118 (bp)†	Length
cry1F[a]	*cry1F* gene	2133 to 3030 3038 to 3945	898 908
cry34Ab1	*cry34Ab1* gene	6817 to 7133	317
cry35Ab1[a]	*cry35Ab1* gene	8837 to 9303 9408 to 9979	467 572
pat	*pat* gene	10904 to 11451	548
*ubi*ZM1 promoter	*ubi*ZM1 promoter	150 to 1008 (copy 1) 4801 to 5659 (copy 2)	859
*ubi*ZM1 intron	*ubi*ZM1 5' untranslated region and intron	1020 to 2100 (copy 1) 5671 to 6751 (copy 2)	1081
ORF25 terminator	ORF25 terminator	4003 to 4703	701
pinII terminator	*pinII* terminator	7235 to 7468 (copy 1) 10065 to 10298 (copy 2)	234
TA peroxidase promoter[a]	TA peroxidase promoter	7523 to 8415 8416 to 8813	893 398
CaMV 35S promoter	CaMV 35S promoter	10383 to 10900	518
CaMV 35S terminator	CaMV 35S terminator	11482 to 11692	211
LB	Plasmid backbone adjacent to T-DNA Left Border	12003 to 12348	346
spc	Spectinomycin resistance gene	13158 to 13932	775
tet[a]	Tetracycline resistance gene	19007 to 19545 19651 to 20108	539 458
*vir*G	*vir*G gene	39334 to 40077	744
RB	Plasmid backbone adjacent to T-DNA Right Border	54476 to 54865	390

† Position numbering is relative to the PHP27118 plasmid map (Figure 2, page 20), which for probes within the T-DNA region also matches the numbering relative to the PHP27118 T-DNA (Figure 3, page 20). In other words, position numbering on plasmid PHP27118 begins with the first nucleotide residue of the T-DNA Right Border.

a. The *cry1F*, *cry35Ab1*, TA peroxidase promoter, and *tet* probes were each comprised of two non-overlapping labelled fragments that were combined in the respective hybridization solutions.

Annex II Materials and Methods for Multi-Generation Segregation Analysis of 4114 Maize

Five generations of 4114 maize were evaluated using polymerase chain reaction (PCR) analyses and herbicide-tolerance testing to confirm Mendelian inheritance of the genotype and phenotype.

II-1 Greenhouse Experimental Design

Five separate generations (F1*1, BC2F1*1, BC3F1*1, BC2F1*2, and BC3F1*2; Figure 4) of 4114 maize were planted and grown in a greenhouse under standard environmental conditions for maize production. Leaf punch samples were collected from each generation and analyzed using PCR amplification methods specific for the event DP-ØØ4114-3, *cry1F* gene, *cry34Ab1* gene, *cry35Ab1* gene, and *pat* gene. After sample collection, all plants were treated with a broadcast application of glufosinate and then visually evaluated for herbicide-tolerance.

II-2 Planting and Leaf Sample Collection

Maize seeds, *ca.* 126 for each generation, were planted in separate cell-divided flats and grown in a greenhouse using typical greenhouse procedures. Ten days after planting, each generation was thinned to a final population of approximately 100 plants.

When plants were at approximately the V2 growth stage (the growth stage when the collar of the second leaf is visible) and prior to herbicide application, leaf samples were collected from each plant. The samples each consisted of one leaf punch distributed into an individual bullet tube and placed on dry ice until they were transferred to a freezer (≤-0°C) for storage. Individual plants and corresponding leaf punch samples were uniquely labelled to allow a given sample to be traced back to the originating plant.

After the data from the original entries were analyzed, it was determined that additional plants for the BC3F1*2 generation were needed to verify the original result and increase the statistical power. An additional 126 seeds from same BC3F1*2 seed source and 91 seeds from a second BC3F1*2 generation seed source were planted and sampled following the previously outlined procedures. No thinning was performed on the plants from the second seed source before leaf punch samples were collected.

II-3 Genotypic Analysis

Leaf punch samples were analyzed using event-specific PCR analysis to confirm the presence or absence of event DP-ØØ4114-3 as well as gene-specific PCR analysis to confirm the presence or absence of the *cry1F*, *cry34Ab1*, *cry35Ab1*, and *pat* genes.

II-4 Phenotypic Analysis

After sample collection, all plants were treated with a tank mixture containing Ignite[8] 280 SL herbicide (Bayer CropScience AG, Monheim am Rhein, Germany) and ammonium sulfate. The Ignite 280 SL herbicide contained 24.5% glufosinate (by weight) in the form of glufosinate ammonium, equivalent to 2.34 pounds glufosinate active ingredient per gallon (0.28 kilograms glufosinate active ingredient per litre). The tank mix was applied at an approximate rate of 22 fluid ounces per acre (1.6 litres per hectare) and the spray volume was 21.6 gallons per acre (202 litres per hectare).

Each plant was visually evaluated 7 days after herbicide application for the presence or absence of herbicide injury, and was identified as presenting either an herbicide-tolerant phenotype (plant exhibited no herbicidal injury) or an herbicide susceptible phenotype (plant exhibited severe herbicide injury).

[8] Registered trademark of Bayer CropScience.

For the additional BC3F1*2 generation plants Ignite 280 SL herbicide was applied with a spray volume of 19.7 gallons per acre (184 l/ha) without the addition of ammonium sulfate and plants were visually evaluated for herbicide injury 4 days after herbicide application.

II-5 Statistical Analysis

A Chi-square analysis was performed on the segregation results of each 4114 maize generation to compare the observed segregation ratio to the expected segregation ratio (1:1). This analysis tested the hypothesis that the introduced traits were segregating in a Mendelian fashion. The critical value to reject the hypothesis at the 5 percent level is 3.84. The summary of segregation data is presented in Table 7, on page 367.

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Annex III Event-Specific Detection Method for 4114 Maize

This annex describes the development and preliminary validation of a quantitative, event-specific polymerase chain reaction (PCR) amplification detection method in a simplex format for 4114 maize (DP-ØØ4114-3) using a FAM/MGB[9] labelled detection system. Preliminary validation of the detection method was done in accordance (where applicable) with Annex I to Regulation (EC) 641/2004 and with the technical guidance documents of the European Network of GMO Laboratories (ENGL) on minimum performance requirements for analytical methods of GMO testing from version 13-10-2008.[10] Validation acceptance criteria set prior to experimental verification are cited in each section, together with the respective evaluation of the results of the experiments.

Refer to Confidential Commercial Information Report for information and details.

[9] FAM = 6-carboxyfluorescein; MGB = dihydrocyclopyrroloindole tripeptide minor groove binder.

[10] Available on the Internet at: http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf

Annex IV Materials and Methods for Western Immunoblot Analysis

IV-1 EXPERIMENTAL DESIGN

One maize leaf tissue sample from each of 4114 maize, 1507 × 59122 maize, and the control maize was extracted and evaluated using western blot analysis for expression of the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins.

IV-2 SAMPLE COLLECTION, PROCESSING, AND STORAGE

Maize leaf tissue samples were collected at the R1 growth stage (Ritchie *et al.*, 2005). One sample from each of 4114 maize, 1507 × 59122 maize, and the control maize were collected and processed as described in Annex V. A representative leaf sub-sample from each maize line was weighed at a target weight of 10 mg ± 5% into an individual 1.2 ml tube, assigned a unique identification number, and stored in a temperature-monitored freezer at ≤-10 °C until prepared for extraction.

IV-3 SAMPLE EXTRACTION AND PREPARATION FOR SDS-PAGE

Two chilled (2–8°C) 5/32" chrome balls and 0.6 ml of chilled (2–8°C) extraction buffer (PBST: phosphate buffered saline, pH 7.4, with 0.05% Tween[11]-20) were added to each tube, and the tubes were covered securely with strip caps. The samples were extracted for 30 seconds at a setting of 1500 strokes per minute on a Geno/Grinder[12] (SPEX CertiPrep, Inc., Metuchen, NJ). The extracts were then centrifuged for 10 minutes at a setting of 4000 rpm at 4 °C and the supernatants were transferred to new tubes.

The samples were diluted as needed in PBST to adjust for relative band intensity in the western blots; the 4114 maize extract was not diluted for the PAT and Cry1F western blots but was diluted 1:2 for the Cry34Ab1 and Cry35Ab1 western blots, and the 1507 × 59122 maize extract was not diluted for the Cry1F western blot but was diluted 1:3 for the Cry34Ab1 western blot and 1:2 for the Cry35Ab1 and PAT western blots.

IV-4 PREPARATION OF PROTEIN STANDARD SOLUTIONS

The Cry1F and PAT protein standards were each diluted to a concentration of 200 ng/ml in PBST, and the Cry34Ab1 and Cry35Ab1 protein standards were each diluted to a concentration of 500 ng/ml in PBST.

IV-5 SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

All sample extracts and protein standard solutions were prepared for electrophoresis by adding 25% NuPAGE[13] 4X LDS (Invitrogen Corporation, Carlsbad, CA) and 10% 10X reducing buffer containing dithiothreitol (DTT) (Invitrogen), heated for approximately seven minutes at 95°C, and then loaded onto two NuPAGE Novex 4-12% Bis-Tris 12-well gels (Invitrogen). Samples were loaded at 20 µl/well and SeeBlue[5] Pre-Stained Standard (Invitrogen) was loaded at 10 µl/well.

Electrophoresis was conducted with an XCell SureLock[c] Mini-Cell electrophoresis unit (Invitrogen) with NuPAGE[c] MES SDS running buffer (Invitrogen) at a constant voltage of 200 V for approximately 40–44 minutes.

IV-6 WESTERN BLOT ANALYSIS

After electrophoresis, gels were removed from the gel cassette and proteins were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Invitrogen) for approximately seven minutes using an iBlot[5] module (Invitrogen). Following transfer, the PVDF

[11] Registered trademark of ICI Americas.

[12] Registered trademark of SPEX CertiPrep, Inc.

[13] Registered trademark of Invitrogen Corporation.

membrane blots were blocked with a 5% non-fat dry milk (NFDM)/PBST solution for approximately 30 minutes at 20–25°C, followed by an incubation of approximately 1 hour at 20–25°C in a specific monoclonal primary antibody (Table 16), which was diluted 1:2000 in 1% NFDM/PBST. The blots were then washed with PBST three times, for at least 10 minutes per wash. Following the third wash, the blots were incubated in a secondary antibody (Table 16; Promega U.S., Madison, WI), which was conjugated to the enzyme horseradish peroxidase (HRP) and diluted 1:10,000 in 1% NFDM/PBST, for approximately 1 hour at 20–25°C, after which the blots were washed with PBST three times for at least 10 minutes per wash.

The blots were developed using SuperSignal[14] West Dura Extended Duration Substrate detection kit (Pierce Biotechnology, Inc., Rockford, IL) followed by image capture using the Kodak Image Station 4000R Pro imaging system (Carestream Health, Inc., Rochester, NY). The resulting images were evaluated for similarities between 4114 maize and 1507 × 59122 maize (see Figure 17, page 39).

Table 16: Descriptions of primary and secondary antibodies used for western immunoblot analysis

Target Protein	Primary Antibody	Secondary Antibody
Cry1F	Cry1F Monoclonal Antibody 205A62.1	Anti-Mouse IgG HRP Conjugate (Promega)
Cry34Ab1	Cry34Ab1 Monoclonal Antibody 1E1.G6	Anti-Mouse IgG HRP Conjugate (Promega)
Cry35Ab1	Cry35Ab1 Monoclonal Antibody 8B5.1A10	Anti-Mouse IgG HRP Conjugate (Promega)
PAT	PAT Monoclonal Antibody 22G6	Anti-Mouse IgG HRP Conjugate (Promega)

[14] Registered trademark of Pierce Biotechnology, Inc.

Annex V Materials and Methods for Determination of Cry1F, Cry34Ab1, Cry35Ab1, and PAT Protein Concentrations in Grain Samples

Concentrations of the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins were determined by specific enzyme-linked immunosorbent assay (ELISA) of grain samples obtained from 4114, 1507, 59122, and 1507 × 59122 maize plants grown across five field trial locations in the United States and Canada in 2010.

V-1 Field Trial Experimental Design

Event 4114 maize plants of the F1*5 generation (Figure 4) were grown in 2010 at five locations within the commercial maize growing regions of the United States and Canada, with four sites in the United States [Bagley, IA; Atlantic, IA; Wyoming, IL; and York, NE] and one site in Canada [Branchton, ON].

Each field site utilized a randomized complete block design with 4114, 1507, 59122, 1507 x 59122 maize, and the control maize planted in two-row plots within four replicate blocks. Approximately 30 seeds were planted in each 25 ft (7.6 m) row resulting in a seed spacing of approximately 10 inches (25 cm). Row spacing was approximately 30 inches (76 cm) and every two-row plot was bordered on either side by one row of commercial maize of similar maturity. In addition, blocks were separated by an alley distance of at least 36 inches (0.9 m) and each site was surrounded by a minimum of four external border rows. In order to ensure grain purity, ear shoots of test and control plants were covered prior to silk emergence and the primary ear was self-pollinated by hand. To control experimental bias in this study, the following procedures were utilized: non-systematic selection of trial and plot areas within each site, randomization of maize entries within each block, and uniform maintenance across blocks in each field site.

V-2 Plant Material Collection, Shipping, Processing, and Storage

V-2.a) Tissue Collection

Grain samples for protein concentration analysis were collected at the R6 growth stage (the typical harvest maturity for grain, regarded as physiological maturity) from impartially selected, healthy, representative plants from each entry. Each sample was placed on dry ice after collection before being transferred to a freezer (≤-10°C) for storage until shipment.

Grain

Four grain samples (one sample equals the grain from one self-pollinated primary ear per plant) were each collected from 4114, 1507, 59122, and 1507 × 59122 maize and one sample was collected from the control maize at each site. The grain samples were obtained by husking and shelling each ear. A representative sub-sample of 15 kernels from each ear was collected.

V-2.b) Sample Shipping, Processing, and Storage

All tissue samples were shipped frozen and stored at ≤-5°C. Grain samples were lyophilized under vacuum until dry. Following lyophilization, grain samples were finely homogenized and stored frozen until analysis.

V-3 Protein Concentration Determination

Concentrations of the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins were determined using specific quantitative ELISA methods.

V-3.a) Protein Extraction

Analytical samples were weighed into 1.2-ml tubes at target weights (±5%) of 20 mg for grain. Each sample analyzed for protein concentrations was extracted with 0.6 ml of chilled PBST buffer

(phosphate buffered saline, pH 7.45 and 0.05% Tween[15]-20). Following centrifugation, supernatants were removed, diluted, and analyzed.

V-3.b) Cry1F ELISA Procedure

The Cry1F ELISA method utilized a sequential ELISA format to determine the concentration of the Cry1F protein in sample extracts. Standards (analyzed in triplicate wells) and diluted sample extracts (analyzed in duplicate wells) were incubated in a plate pre-coated with a Cry1F-specific antibody. Following incubation, unbound substances were washed from the plate. A different Cry1F-specific antibody conjugated to the enzyme horseradish peroxidase (HRP) was added to the plate and incubated. The unbound substances were washed from the plate. Detection of the bound Cry1F-antibody complex was accomplished by the addition of substrate, which generated a coloured product in the presence of HRP. The reaction was stopped with an acid solution and the optical density (OD) of each well was determined using a plate reader. An average of the results from duplicate wells was used to determine the concentration of the Cry1F protein in ng/mg sample weight.

V-3.c) Cry34Ab1 ELISA Procedure

A similar procedure was used as for the Cry1F ELISA, except a Cry34Ab1-specific antibody was used on the pre-coated plate and a second Cry34Ab1-specific antibody conjugated to HRP was used.

V-3.d) Cry35Ab1 ELISA Procedure

A similar procedure was used as for the Cry1F ELISA, except a Cry35Ab1-specific antibody was used on the pre-coated plate and a second Cry35Ab1-specific antibody conjugated to HRP was used.

V-3.e) PAT ELISA Procedure

A similar procedure was used as for the Cry1F ELISA, except a PAT-specific antibody was used on the pre-coated plate and a second PAT-specific antibody conjugated to HRP was used.

V-3.f) Calculations for Determining Protein Concentrations

SoftMax[16] Pro GxP (Molecular Devices, Sunnyvale, CA) software was used to perform the calculations required to convert OD values obtained by the microtiter plate reader to protein concentration values.

Standard Curve

A standard curve was included on each ELISA plate. The equation for the standard curve was generated by the software, which used a quadratic fit to relate the mean OD values obtained for the standards to the respective standard concentration (ng/ml).

The quadratic regression equation was applied as follows:

$$y = Cx^2 + Bx + A$$

Where x = known standard concentration and y = respective mean absorbance value (OD)

Sample Concentration

Interpolation of the sample concentration (ng/ml) was accomplished by solving for x in the above equation using values for A, B, and C determined by the standard curve.

[15] Registered trademark of ICI Americas, Inc.
[16] Registered trademark of Molecular Devices Corporation.

$$\text{Sample concentration (ng/ml)} = \frac{-B + \sqrt{B^2 - 4C(A - \text{sample OD})}}{2C}$$

The sample concentration values were adjusted for a dilution factor expressed as 1:N by multiplying the interpolated concentration by N.

Adjusted sample concentration values obtained from SoftMax Pro GxP software were converted from ng/ml to ng/mg sample weight as follows:

ng/mg Sample Weight = ng/ml x Extraction Volume (ml)/Sample Weight (mg)

Lower Limit of Quantification (LLOQ)

The LLOQ, in ng/mg sample weight, was calculated as follows:

$$\text{LLOQ} = \frac{\text{Reportable Assay LLOQ x Extraction Volume}}{\text{Sample Target Weight}}$$

V-4 STATISTICAL ANALYSIS AND REPORTING

Statistical analyses were conducted using SAS[17] software, Version 9.2 (SAS Institute, Inc., Cary, NC) to estimate mean concentrations of Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins in grain samples derived from 4114, 1507, 59122, and 1507 × 59122 maize. For each expressed trait protein in grain, the least squares (LS) mean concentration (ng/mg tissue dry weight) and range are provided in Table 8 (page 42).

[17] Registered trademark of SAS Institute, Inc.

Annex VI Materials and Methods for Compositional Analyses of Grain Samples

Compositional analyses were performed on samples of maize kernels (grain) harvested from 4114 and near-isogenic control maize hybrids grown in side-by-side trials across six locations in the United States and Canada in 2010.

VI-1 Field Trial and Experimental Design

Event 4114 maize plants of the F1*5 generation (Figure 4) and near-isogenic control maize plants were grown in 2010 at six locations within the commercial maize growing regions of the United States and Canada, with four sites in the United States [Richland, IA; Wyoming, IL; Geneva, MN; and York, NE] and two sites in Canada [Branchton, ON; and Thorndale, ON].

Each field site utilized a randomized complete block design with 4114 maize and the control maize planted in two-row plots within four replicate blocks. Approximately 30 seeds were planted in each 25 ft (7.6 m) row resulting in a seed spacing of approximately 10 inches (25 cm). Row spacing was approximately 30 inches (76 cm) and every two-row plot was bordered on either side by one row of commercial maize of similar maturity. In addition, blocks were separated by an alley distance of at least 36 inches (0.9 m) and each site was surrounded by a minimum of four external border rows. In order to ensure grain purity, ear shoots of test and control plants were covered prior to silk emergence and the primary ear was self-pollinated by hand. To control experimental bias in this study, the following procedures were utilized: non-systematic selection of trial and plot areas within each site, randomization of maize entries within each block, and uniform maintenance across blocks in each field site.

VI-2 Tissue Collection, Shipping, Processing, and Storage

VI-2.a) Tissue Collection

All samples were collected from impartially selected, healthy individual plants. All control maize samples were collected prior to collection of 4114 maize samples. All grain samples were assigned unique sample identification numbers that described the sample by site, entry, block, sample number, and tissue type. Samples were placed on dry ice within thirty minutes of collection and were maintained in coolers on wet or dry ice and/or in the freezers until shipment.

Grain

One grain sample (five pooled ears equalled one sample) was collected from 4114 maize and the control maize from each of the four blocks at typical harvest maturity. Each ear was husked and shelled, and the grain was collected into a pre-labelled plastic-lined cloth bag.

After collection, grain samples were shipped from each field site to EPL Bio-Analytical Services (EPL-BAS) for processing and nutrient composition analysis. Each sample was labelled by site, entry, block, sample number, tissue type, and growth stage. A unique sample identification number was also included on each label.

VI-2.b) Sample Shipping, Processing, and Storage

After collection, grain samples were shipped and stored frozen at -20°C. Samples were lyophilized, ground, and homogenized before nutrient composition analysis.

VI-3 Nutrient Composition Analyses

Nutrient composition analyses of maize grain were conducted using the analytical methods described in Table 17.

Table 17: Methods for compositional analysis of grain samples from 4114 and control maize

Compositional Component	Method
Moisture in grain	The analytical procedure for moisture determination was based on a method published by the Association of Official Analytical Chemists (AOAC). Samples were assayed to determine the percentage of moisture by gravimetric measurement of weight loss after drying in a vacuum oven (grain).
Ash in grain	The analytical procedure for ash determination was based on a method published by the AOAC. Samples were analyzed to determine the percentage of ash by gravimetric measurement of the weight loss after ignition in a muffle furnace.
Crude protein in grain	The analytical procedure for crude protein determination utilized an automated Kjeldahl technique based on a method provided by the manufacturer of the titrator unit (Foss-Tecator). Ground samples were digested in the presence of a catalyst. The digestate was then distilled and titrated with a Foss-Tecator Kjeltec titrator unit.
Crude fat in grain	The analytical procedure for crude fat determination was based on methods provided by the manufacturer of the hydrolysis and extraction apparatus (Ankom Technology). Samples were hydrolyzed with 3 N hydrochloric acid at 90°C for 60 minutes. The hydrolysates were extracted with a petroleum ether/ethyl ether/ethyl alcohol solution at 90°C for 60 minutes. The ether extracts were evaporated and the fat residue remaining determined gravimetrically.
Carbohydrate in grain	The carbohydrate content in maize grain on a dry weight basis was calculated using a formula obtained from the United States Department of Agriculture "*Energy Value of Foods,*" in which the percent dry weight of crude protein, crude fat, and ash was subtracted from 100%.
Crude fibre in grain	The analytical procedure for crude fibre determination was based on methods provided by the manufacturer of the extraction apparatus (Ankom Technology). Samples were analyzed to determine the percentage of crude fibre by digestion and solubilization of other materials present.
Neutral detergent fibre (NDF) in grain	The analytical procedure for neutral detergent fibre (NDF) determination was based on a method provided by the manufacturer of the extraction apparatus (Ankom Technology). Samples were analyzed to determine the percentage of NDF by digesting with a neutral detergent solution, sodium sulfite, and alpha amylase. The remaining residue was dried and weighed to determine the NDF content.
Acid detergent fibre (ADF) in grain	The analytical procedure for acid detergent fibre (ADF) determination was based on a method provided by the manufacturer of the extraction apparatus (Ankom Technology). Samples were analyzed to determine the percentage of ADF by digesting with an acid detergent solution and washing with reverse osmosis water. The remaining was weighed to determine the ADF content.
Minerals in grain	The analytical procedure for the determination of minerals is based on methods published by the AOAC and CEM Corporation. The maize grain minerals determined were calcium, copper, iron, magnesium, manganese, phosphorus, potassium sodium, and zinc. The samples were digested in a microwave based digestion system and the digestate was diluted using deionized water. Samples were analyzed by inductively coupled plasma optical emission spectroscopy (ICP-OES).
Tryptophan in grain	The analytical procedure for tryptophan determination was based on an established lithium hydroxide hydrolysis procedure with reverse phase ultra performance liquid chromatography (UPLC) with ultraviolet (UV) detection published by the *Journal of Micronutrient Analysis.*
Cystine and methionine in grain	The analytical procedure for cystine and methionine determination was based on methods obtained from Waters Corporation, AOAC, and *Journal of Chromatography A*. The procedure converts cystine to cysteic acid and methionine to methionine sulfone, after acid oxidation and hydrolysis, to the 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatives which are then analyzed by reverse phase UPLC with UV detection.
Additional amino acids in grain	Along with tryptophan, cystine, and methionine, 15 additional amino acids were determined. The analytical procedure for analysis of these amino acids was based on methods obtained from Waters Corporation and the *Journal of Chromatography A*. The procedure converts the free acids, after acid hydrolysis, to the 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatives, which are analyzed by reverse phase UPLC with UV detection.
Fatty acids in grain	The analytical procedure for determination of fatty acids was based on methods published by the AOAC and American Oil Chemist Society (AOCS). The procedure converts the free acids, after ether extraction and base hydrolysis, to the fatty acid methyl ester (FAME) derivatives, which are analyzed by gas chromatography with flame ionization detection (GC/FID). Results are reported as percent total fatty acids but presented in the raw data as %DB.

Compositional Component	Method
Thiamine (vitamin B1) and Riboflavin (vitamin B2) in grain	The analytical procedure for the determination of Thiamine (Vitamin B1) and Riboflavin (Vitamin B2) was based on a method published by the American Association of Cereal Chemists (AACC). The samples were extracted with 10% acetic acid/4.3% trichloroacetic acid solution. A 50-fold dilution was performed then the samples were analyzed by reverse phase HPLC tandem mass spectrometry (MS/MS).
Niacin (vitamin B3) in grain	The analytical procedure for the determination of Niacin (vitamin B3) was based on a method published by the AACC. Niacin (vitamin B3) was extracted from the sample by adding deionized (DI) water and autoclaving. A tube array was prepared using three different dilutions of the samples. This tube array was inoculated with *Lactobacillus plantarum* and allowed to incubate for approximately 18 to 22 hours. After incubation, the bacterial growth was determined using a spectrophotometer at an absorbance of 660 nm. The absorbance readings were compared to a standard curve generated using known concentrations of nicotinic acid.
Pantothenic acid (vitamin B5) in grain	The analytical procedure for the determination of pantothenic acid (Vitamin B5) was based on a method from the AOAC. Pantothenic acid (Vitamin B5) content was determined using a microbiological assay. Pantothenic acid (Vitamin B5) was extracted from the sample by an acetic acid buffer solution, consisting of acetic acid adjusted to a pH of 5.65 with sodium hydroxide, and autoclaving the samples. A tube array was prepared using three different dilutions of the samples. This tube array was inoculated with *Lactobacillus plantarum* and allowed to incubate for approximately 18–22 hours. After incubation, the bacterial growth was determined using a spectrophotometer at an absorbance of 660 nm. The absorbance readings were compared to a standard curve generated using known concentrations of D-pantothenic acid hemi-calcium salt.
Pyridoxine (vitamin B6) in grain	The analytical procedure for the determination of pyridoxine (Vitamin B6) was based on a method from the AACC. Pyridoxine (Vitamin B6) was determined using a microbiological assay. Pyridoxine (Vitamin B6) was extracted from the sample by adding sulfuric acid and autoclaving. The pH was adjusted and a tube array was prepared using four different dilutions of the samples. This tube array was inoculated with *Saccharomyces cerevisiae* and allowed to incubate for approximately 18–22 hours. After incubation, the microbial growth was determined using a spectrophotometer at an absorbance of 600 nm. The absorbance readings were compared to a standard curve generated using known concentrations of pyridoxine hydrochloride.
Total folate as folic acid (vitamin B9) in grain	The analytical procedure for determination of total folate as folic acid was based on a microbiological assay published by the AACC. Samples were hydrolyzed and digested by protease and amylase enzymes to release the folate from the grain. A conjugase enzyme was used to convert the naturally occurring folypolyglutamates. An aliquot of the extracted folates was mixed with a folate and folic acid free microbiological growth medium. The mixture was inoculated with *Lactobacillus casei*. The total folate content was determined by measuring the turbidity of the *Lactobacillus casei* growth response in the sample and comparing it to the turbidity of the growth response with folic acid standards.
Tocopherols (vitamin E) in grain	The analytical procedure for determination of tocopherols (Vitamin E) was based on methods from the *Journal of the American Oil Chemists' Society* and *Analytical Sciences*. Alpha, beta, delta, and gamma-tocopherols were extracted with hot hexane and the extracts were analyzed by normal phase HPLC with fluorescence detection.
Beta-carotene in grain	The analytical procedure for determination of beta-carotene was based on a method published by the AOAC. Fat-soluble pigments from the ground maize grain were extracted and determined spectrophotometrically and expressed as carotene.
Trypsin inhibitor in grain	The analytical procedure for the determination of trypsin inhibitor was based on a method published by the AOCS. Trypsin inhibitor was extracted with sodium hydroxide. Trypsin was added and reacted with trypsin inhibitor. The amount of trypsin present in the sample was measured using a spectrophotometer, and the amount of inhibitor was calculated based on how much trypsin remained.
Inositol and raffinose in grain	The analytical procedure for the determination of inositol and raffinose was based on a gas chromatography (GC) method published in the *Handbook of Analytical Derivatization Reactions*, an AACC method, and a method from the *Journal of Agricultural and Food Chemistry*. Extracted inositol is derivatized to the butylboronic acid derivative and analyzed by GC/FID. Extracted raffinose is analyzed by reverse phase HPLC with refractive index detection.
Furfural in grain	The analytical procedure for the determination of furfural was based on methods published in the *Journal of Agricultural and Food Chemistry*. Ground maize grain was analyzed for furfural content by reverse phase HPLC with UV detection.

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Compositional Component	Method
p-Coumaric and ferulic acid in grain	The analytical procedure for the determination of *p*-coumaric and ferulic acids was developed based on methods published in *Journal of Agricultural and Food Chemistry* and *The Journal of Chemical Ecology*. Ground maize grain was analyzed to determine the amounts of *p*-coumaric acid and ferulic acid by separating the total content of phenolic acids using reverse phase HPLC and UV detection.

VI-4 STATISTICAL ANALYSES

Statistical analyses were conducted using SAS[18] software, Version 9.2 (SAS Institute, Inc., Cary, NC) to evaluate and compare the nutrient composition of grain derived from 4114 maize and the control maize.

VI-4.a) Data Processing

Fatty Acids
For some fatty acid analytes, absolute sample values were detected below the assay lower limit of quantification (LLOQ). When sample values for each fatty acid analyte were converted from an absolute value to a relative proportion (percentage of total fatty acids), sample results below the LLOQ were reported as zero to reflect a negligible proportion. However, these zeros were not "true" zeros, but were some unknown small positive values. Therefore, these "zero" sample values were treated as missing values during subsequent statistical analysis.

Data Transformation
A natural logarithmic "ln(y)" transformation was performed for the raw data of all analytes before statistical analyses. For each analyte, residuals from the across sites analysis were examined for validation of the normality and homogeneous variance assumptions.

Residual distributions skewed to the left for the following analytes after "ln(y)" transformation and therefore, either no transformation, a cubic "$(y)^3$" transformation, a square "$(y)^2$" transformation, or a square root "$\sqrt{y}$" transformation was performed to the raw data instead:

- Vitamin B9 – No transformation was performed.
- Crude protein in grain, and tryptophan and valine in grain – A cubic "$(y)^3$" transformation was performed.
- NDF, alanine, aspartic acid, glutamic acid, isoleucine, leucine, phenylalanine, proline, and threonine – A square "$(y)^2$" transformation was performed.
- Vitamin B6 – A square root "$\sqrt{y}$" transformation was performed.

The model assumptions were reasonably satisfied for these analytes after the more-appropriate transformation or non-transformation.

For a given analyte, the same type of transformation or non-transformation was used for all statistical analyses and comparisons performed. The statistical results were then back-transformed to the original data scale for reporting purposes.

VI-4.b) Statistical Analyses
For a given analyte, the number of samples below the assay LLOQ value determined whether a statistical analysis was conducted. The following rules were implemented:

- If, for each entry, <80% of samples were below the LLOQ, then the across sites analysis was conducted.

[18] Registered trademark of SAS Institute, Inc.

- If, for a single entry, ≥80% of samples were below the LLOQ, then across sites analysis was not conducted.

If, for a given analyte, a statistical analysis was not conducted due to insufficient data, the logistic regression likelihood-ratio test was used to test if the proportion of assay values below the LLOQ was different between 4114 maize and the control maize.

Across Sites Analysis

Default Model

For a given analyte, data were analyzed using the following linear mixed model:

$$y_{ijk} = \mu_i + \ell_j + r_{k(j)} + (\mu\ell)_{ij} + \varepsilon_{ijk} \qquad \textbf{Model 1}$$

$\ell_j \sim iid\ N(0, \sigma^2_{Site})$, $r_{k(j)} \sim iid\ N(0,\sigma^2_{Rep})$, $(\mu\ell)_{ij} \sim iid\ N(0, \sigma^2_{Ent\times Site})$, and $\varepsilon_{ijk} \sim iid\ N(0, \sigma^2_{Error})$

Where μ_i denotes the mean of the ith entry (fixed effect), ℓ_j denotes the effect of the jth site (random effect), $r_{k(j)}$ denotes the effect of the kth block within the jth site (random effect), $(\mu\ell)_{ij}$ denotes the interaction between the entries and sites (random effect) and ε_{ijk} denotes the effect of the plot assigned the ith entry in the kth block of the jth site (random effect or residual). Notation $\sim iid\ N(0,\ \sigma^2_a)$ indicates random variables that are identically independently distributed (*iid*) as normal with zero mean and variance σ^2_a.

The residual maximum likelihood (REML) estimation procedure was utilized to generate estimates of variance components and entry means across sites. The estimated means are known as least squares means (LS-Means). The statistical comparison was conducted by testing for differences in LS-Means between 4114 maize and the control maize. The approximated degrees of freedom for the statistical test were derived by the Kenward-Roger (KR) method (Kenward and Roger, 1997).

SAS PROC MIXED was utilized to fit Model 1 and to generate LS-Means, 95% confidence intervals, and statistical comparisons (p-values). By default, the variance components in Model 1 are all constrained to be non-negative. When the estimated value of $\sigma^2_{Ent\times Site}$ is zero, the KR method pools degrees of freedom for the interaction term with the degrees of freedom for residuals. Consequently, the degrees of freedom for the statistical test could be larger than what was expected under the original experimental design. In order to stabilize the degrees of freedom across all analytes, effect $(\mu\ell)_{ij}$ in Model 1 was combined with ε_{ijk} and the compound symmetry (CS) structure was used to model the corresponding residual variance structure. This approach allows $\sigma^2_{Ent\times Site}$ to take negative values without affecting the degrees of freedom (Littell *et al.*, 2006).

Model Considering LLOQ

For the analytes sodium, vitamin B1, vitamin B5, and raffinose, <80% of sample values were detected below the assay LLOQ. Sample results below the LLOQ were treated as left-censored observations at the respective assay LLOQ value. Data were analyzed using the following linear mixed model:

$$y_{ijk} = \mu_i + \ell_j + r_{k(j)} + (\mu\ell)_{ij} + \varepsilon_{ijk} \qquad \textbf{Model 2}$$

$\ell_j \sim iid\ N(0, \sigma^2_{Site})$, $r_{k(j)} \sim iid\ N(0,\sigma^2_{Rep})$, $(\mu\ell)_{ij} \sim iid\ N(0, \sigma^2_{Entry\times Site})$, $\varepsilon_{ijk} \sim iid\ N(0, \sigma^2_{Error})$

Where μi denotes the mean of the ith entry (fixed effect), ℓ_j denotes the effect of the jth site (random effect), $r_{k(j)}$ denotes the effect of the kth block within the jth site (random effect), $(\mu\ell)_{ij}$ denotes the interaction between the entries and sites (random effect) and ε_{ijk} denotes the residual for the observation obtained from the plot assigned to the ith entry in the kth

block of the jth site. Notation $\sim iid\ N(0,\ \sigma^2_a)$ indicates random variables that are identically independently distributed (*iid*) as normal with zero mean and variance σ^2_a.

Model 2 can also be written as:

$$y_{ijk} = \mu_{ijk} + \varepsilon_{ijk} = \mu_i + \ell_j + r_{k(j)} + (\mu\ell)_{ij} + \varepsilon_{ijk}$$

Where μ_{ijk} denotes the mean of y_{ijk}. The conditional likelihood for each observation, given the random effects, was formulated according to the status of the observation (observed or left-censored) (Thiébaut and Jacqmin-Gadda, 2004):

$$f(y_{ijk} \mid \theta) = \begin{cases} P(y_{ijk} = y^*_{ijk}) = \phi(\dfrac{y^*_{ijk} - \mu_{ijk}}{\sigma}) & observed \\ P(y_{ijk} \le c) = \Phi(\dfrac{c - \mu_{ijk}}{\sigma}) & left-censored \end{cases}$$

Where □ denotes the vector of all random effects, □□denotes the standard normal density function, □□denotes the standard normal cumulative distribution function, y^*_{ijk} denotes the observed sample value of y_{ijk}, and *c* denotes the assay LLOQ value.

The conditional likelihood function was a product of all individual conditional likelihoods, and the marginal likelihood function was formed when the conditional likelihood function was integrated over all random effects.

The maximum likelihood procedure was then used to generate estimates of variance components and entry means (LS-Means) across sites. The statistical comparison was conducted by testing for difference in LS-Means between 4114 maize and the control maize. The approximated degrees of freedom for the statistical test were derived by the KR method.

SAS PROC NLMIXED was utilized to fit Model 2 and generate LS-Means, 95% confidence intervals, and statistical comparisons (p-values).

VI-4.c)Interpretations of Statistical Results

Where a statistically significant difference (p-value <0.05) was identified for a given analyte in the across sites analysis between 4114 maize and the control maize, the respective range of individual values from 4114 maize was compared to a tolerance interval. Tolerance intervals containing 99% of the values for corresponding analytes of the conventional maize population with 95% confidence level (Graybill, 1976) were derived from data collected under previous studies. In each of these studies, four non-modified commercially available maize lines were grown at six sites in North America, and were harvested, processed, and analyzed using methods similar to those employed in this nutrient composition study for 4114 maize. The selected maize varieties represent the non-modified maize population with a history of safe use, and the selected environments (site and year combinations) represent maize growth under a range of environmental conditions (e.g., soil type, temperature, precipitation, and irrigation) and maize maturity group zones similar to the sites used in the 4114 maize agronomic study. Ranges containing individual values outside the tolerance interval for a given analyte were then compared to the respective literature range obtained from published literature (ILSI, 2010; OECD, 2002; Watson, 1982; Watson, 1987).

VI-5 REPORTED STATISTICS

For each analyte that was statistically analyzed using mixed model analysis, entry LS-Mean (back-transformed, if needed), and range are provided in Tables 9–14 (pages 46–51). The p-values are provided for comparisons between 4114 maize and the control maize. For each analyte, a tolerance interval [TI (99%)], and a literature range, if available, are provided.

Annex VII – Materials and Methods for Analysis of Protein Expression via Western Blot

METHODS

D.1.

A. EXPERIMENTAL DESIGN

One maize leaf tissue sample from each of 4114 maize, 1507x59122 maize, and the control maize was extracted and evaluated using western blot analysis for expression of the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins.

1. **Sample Collection, Processing, and Storage**
   Maize leaf tissue samples were collected at the R1 growth stage (when silks became visible (*1*)). One sample from each of 4114 maize, 1507x59122 maize, and the control maize were collected and processed under study PHI-2010-059 (*2*). A representative leaf sub-sample from each maize line was weighed at a target weight of 10 mg ± 5% into an individual 1.2 ml tube, assigned a unique identification number, and stored in a temperature-monitored freezer at ≤ -10 °C until prepared for extraction.

2. **Sample Extraction and Preparation for SDS-PAGE**
   Two chilled (2-8 °C) 5/32" chrome balls and 0.6 ml of chilled (2-8 °C) extraction buffer (PBST: phosphate buffered saline, pH 7.4, with 0.05% Tween[19]-20) were added to each tube, and the tubes were covered securely with strip caps. The samples were extracted for 30 seconds at a setting of 1500 strokes per minute on a Geno/Grinder[20] (SPEX CertiPrep, Inc., Metuchen, NJ, USA). The extracts were then centrifuged for 10 minutes at a setting of 4000 rpm at 4 °C and the supernatants were transferred to new tubes.

   The samples were diluted as needed in PBST to adjust for relative band intensity in the western blots; the 4114 maize extract was not diluted for the PAT and Cry1F western blots but was diluted 1:2 for the Cry34Ab1 and Cry35Ab1 western blots, and the 1507x59122 maize extract was not diluted for the Cry1F western blot but was diluted 1:3 for the Cry34Ab1 western blot and 1:2 for the Cry35Ab1 and PAT western blots.

3. **Preparation of Protein Standard Solutions**
   The Cry1F and PAT protein standards were each diluted to a concentration of 200 ng/ml in PBST, and the Cry34Ab1 and Cry35Ab1 protein standards were each diluted to a concentration of 500 ng/ml in PBST.

4. **Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

[19] Registered trademark of ICI Americas

[20] Registered trademark of SPEX CertiPrep, Inc.

All sample extracts and protein standard solutions were prepared for electrophoresis by adding 25% NuPAGE[21] 4X LDS (Invitrogen Corporation, Carlsbad, CA, USA; #NP0007) and 10% 10X reducing buffer containing dithiothreitol (DTT) (Invitrogen, #NP0009), heated for approximately seven minutes at 95 °C, and then loaded onto two NuPAGE Novex 4-12% Bis-Tris 12-well gels (Invitrogen, #NP0322). Samples were loaded at 20 µl/well and SeeBlue© Pre-Stained Standard (Invitrogen, #LC5625) was loaded at 10 µl/well.

Electrophoresis was conducted with a XCell SureLock© Mini-Cell electrophoresis unit (Invitrogen, #EI0001) with NuPAGE© MES SDS running buffer (Invitrogen, #NP0002) at a constant voltage of 200V for approximately 40-44 minutes.

5. **Western Blot Analysis**

After electrophoresis, gels were removed from the gel cassette and proteins were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Invitrogen, #IB4010-01) for approximately seven minutes using an iBlot© module (Invitrogen, #IB1001). Following transfer, the PVDF membrane blots were blocked with a 5% non-fat dry milk (NFDM)/PBST solution for approximately 30 minutes at 20-25 °C, followed by an incubation of approximately 1 hour at 20-25 °C in a monoclonal primary antibody, which was diluted 1:2000 in 1% NFDM/PBST. The blots were then washed with PBST three times, for at least 10 minutes per wash. Following the third wash, the blots were incubated in a secondary antibody (Promega U.S., Madison, WI, USA), which was conjugated to the enzyme horseradish peroxidase (HRP) and diluted 1:10,000 in 1% NFDM/PBST, for approximately 1 hour at 20-25 °C, after which the blots were washed with PBST three times for at least 10 minutes per wash. Additional details pertaining to individual antibodies and dilutions used for western blot analysis are provided in Table 1.

The blots were developed using SuperSignal[22] West Dura Extended Duration Substrate detection kit (Pierce Biotechnology, Inc., Rockford, IL, USA; #34076) followed by image capture using the Kodak Image Station 4000R Pro imaging system (Carestream Health, Inc., Rochester, NY, USA). The resulting images were evaluated for similarities between 4114 maize and 1507x59122 maize.

[21] Registered trademark of Invitrogen Corporation

[22] Registered trademark of Pierce Biotechnology, Inc.

RESULTS AND DISCUSSION

Overall, the western blot analysis demonstrated that the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins derived from 4114 maize have equivalent molecular weights and immunoreactivity to the proteins expressed in 1507x59122 maize. In all four western blots, the near-isoline control showed no immunoreactive proteins. Images of the western blots are provided in Figures 1-4.

A. Cry1F Protein

Western blot analysis using anti-Cry1F antibodies (Figure 1) demonstrated that the Cry1F protein derived from 4114 maize (Lane 3) had the same molecular weight and relative immunoreactivity as the Cry1F protein derived from 1507x59122 maize (Lane 4). Both protein samples migrated as two bands of approximately 60 kDa and 62 kDa in size. The double banding pattern was expected because plant-derived Cry1F protein can be partially degraded by plant proteases to a smaller, more-stable truncated protein, therefore appearing as two bands (the larger intact protein and the smaller truncated protein). Relative amounts of the two bands can vary from sample to sample. The Cry1F standard protein included on the blot (Lane 2) consisted of the truncated form of the protein and migrated at the expected approximately 60 kDa.

B. Cry34Ab1 Protein

Western blot analysis using anti-Cry34Ab1 antibodies (Figure 2) demonstrated that the Cry34Ab1 protein derived from 4114 maize (Lane 3) had the same molecular weight and relative immunoreactivity as the Cry34Ab1 protein derived from 1507x59122 maize (Lane 4), with each sample migrating at approximately 14 kDa. Similarly, the Cry34Ab1 standard protein (Lane 2) migrated at approximately 14 kDa and appeared to be equivalent to the plant-derived proteins.

C. Cry35Ab1 Protein

Western blot analysis using anti-Cry35Ab1 antibodies (Figure 3) demonstrated that the Cry35Ab1 protein derived from 4114 maize (Lane 3) had the same molecular weight and relative immunoreactivity as the Cry35Ab1 protein derived from 1507x59122 maize (Lane 4). Both protein samples migrated as two bands of approximately 40 kDa and 44 kDa in size. Like the Cry1F protein, the larger Cry35Ab1 band can be partially degraded to a more stable truncated protein by plant proteases, and the relative amounts of each band can vary from sample to sample. The Cry35Ab1 standard protein (Lane 2) consisted of the truncated form of the protein and migrated at the expected approximately 40 kDa.

D. PAT Protein

Western blot analysis using anti-PAT antibodies (Figure 4) demonstrated that the PAT protein derived from 4114 maize (Lane 3) had the same molecular weight and relative immunoreactivity as the PAT protein derived from 1507x59122 maize (Lane 4), with each sample migrating at approximately 21 kDa. The PAT standard protein (Lane 2) migrated at the same molecular weight as the plant-derived PAT proteins (approximately 21 kDa).

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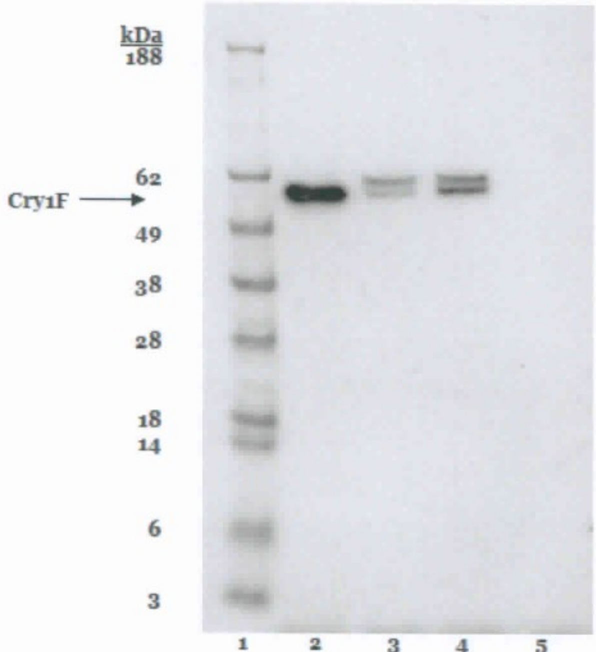
Table 1. Primary and Secondary Antibody Descriptions Used for Qualitative Western Blot Analysis

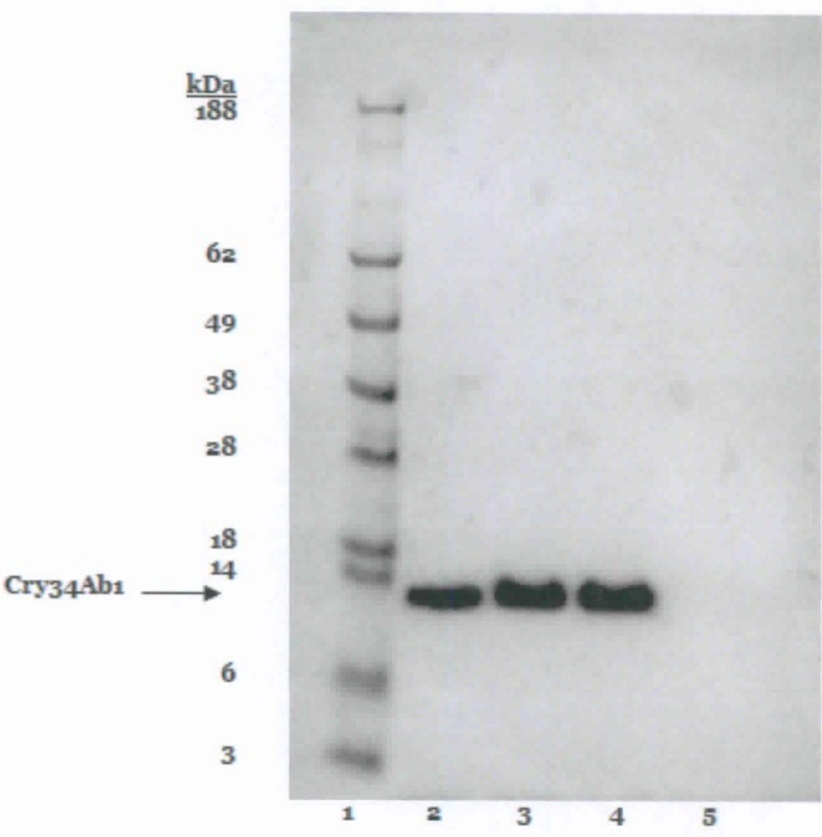
Target Protein	Primary Antibody	Primary Antibody Dilution	Secondary Antibody	Secondary Antibody Dilution
Cry1F	Cry1F Monoclonal Antibody 205A62.1 (Lot #031308-3)	1:2,000	Anti-Mouse IgG HRP Conjugate (Promega, #W4021)	1:10,000
Cry34Ab1	Cry34 Monoclonal Antibody 1E1.G6 (Lot #12282010-1)	1:2,000	Anti-Mouse IgG HRP Conjugate (Promega, #W4021)	1:10,000
Cry35Ab1	Cry35 Monoclonal Antibody 8B5.1A10 (Lot #062607-2)	1:2,000	Anti-Mouse IgG HRP Conjugate (Promega, #W4021)	1:10,000
PAT	PAT Monoclonal Antibody 22G6 (Lot #07152010-5)	1:2,000	Anti-Mouse IgG HRP Conjugate (Promega, #W4021)	1:10,000



Lane #	Sample ID
1	Molecular Weight Marker
2	Cry1F Standard Protein (200 ng/mL)
3	Leaf R1 from 4114 Maize
4	Leaf R1 from 1507x59122 Maize
5	Leaf R1 from Near-Isoline Control Maize

Figure 1. Western Blot Comparison of Cry1F Protein

Plant-derived Cry1F protein can be partially degraded by plant proteases to a smaller, more stable truncated protein, therefore appearing as two bands (~60 kDa and ~62 kDa). The Cry1F standard protein consisted of the truncated protein only (~60 kDa).



Lane #	Sample ID
1	Molecular Weight Marker
2	Cry34 Standard (500 ng/mL)
3	Leaf R1 from 4114 Maize
4	Leaf R1 from 1507x59122 Maize
5	Leaf R1 from Near-Isoline Control Maize

Figure 2. Western Blot Comparison of Cry34Ab1 Protein

Plant-derived and standard Cry34Ab1 proteins migrated at the expected mass of ~14 kDa.

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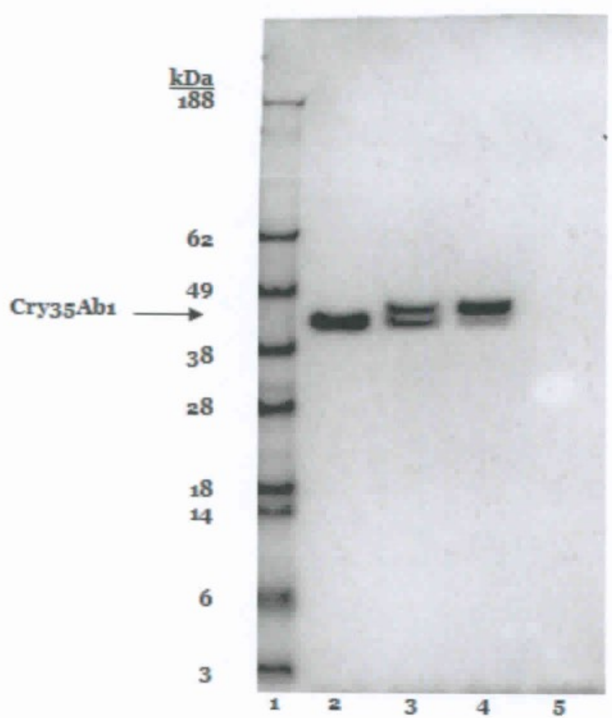

Lane #	Sample ID
1	Molecular Weight Marker
2	Cry35 Standard (500 ng/mL)
3	Leaf R1 from 4114 Maize
4	Leaf R1 from 1507x59122 Maize
5	Leaf R1 from Near-Isoline Control Maize

Figure 3. Western Blot Comparison of Cry35Ab1 Protein

Plant-derived Cry35Ab1 protein can be partially degraded by plant proteases to a smaller, more stable truncated protein, therefore appearing as two bands (~40 kDa and ~44 kDa). The Cry35Ab1 standard protein consisted of the truncated protein only (~40 kDa).

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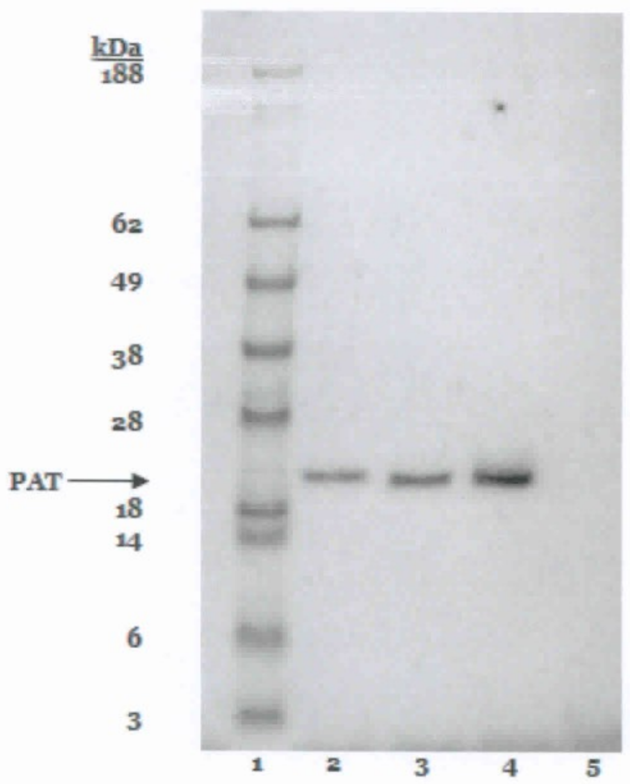

Lane #	Sample ID
1	Molecular Weight Marker
2	PAT Standard (200 ng/mL)
3	Leaf R1 from 4114 Maize
4	Leaf R1 from 1507x59122 Maize
5	Leaf R1 from Near-Isoline Control Maize

Figure 4. Western Blot Comparison of PAT Protein

Plant-derived and standard PAT proteins showed the expected mass of ~21 kDa.

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