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A DuPont Business

Petition for the Determination of Nonregulated Status for Insect-Resistant and Herbicide-Tolerant 4114 Maize

We submit this petition under 7 CFR §340.6 to request that the Administrator, Animal and Plant Health Inspection Service, make a determination that the article should no longer be regulated under 7 CFR §340.

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NO CBI

Table of Contents

List of Tables

List of Tables (continued)

List of Tables (continued)

List of Figures

List of Figures (continued)

List of Figures (continued)

Release of Information

Pioneer Hi-Bred International, Inc. (Pioneer) is submitting the information in this petition for review by USDA as part of the regulatory process. 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 (FOIA), 5 U.S.C., Section 552; USDA complies with the provisions of FOIA and USDA's implementation regulations (7 CFR Part 1.4); and this information is responsive to the specific request. Except in accordance with the Freedom of Information Act, Pioneer does not authorize the release, publication or other distribution of this information (including website posting) without Pioneer's prior notice or consent.

Certification

The undersigned certifies that, to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes all relevant data and information known to the petitioners, which are favorable and unfavorable to the petition.

_____

Date

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Summary

Pioneer Hi-Bred International, Inc., a DuPont Business (Pioneer) is submitting a Petition for Determination of Nonregulated Status for insect-resistant and herbicide-tolerant maize event DP-ØØ4114-3, hereafter referred to as 4114 maize. Maize line 4114 was developed by Pioneer. Pioneer requests a determination from USDA - Animal and Plant Health Inspection Service (APHIS) that 4114 maize and any crosses of this line with other nonregulated *Zea mays* no longer be considered regulated articles under 7 CFR §340.

4114 maize produces the Cry proteins Cry1F, Cry34Ab1, and Cry35Ab1, as well as the herbicide-tolerance protein PAT. The Cry1F protein confers resistance to certain lepidopteran pests, including European corn borer (*Ostrinia nubilalis*), a major maize pest. This protein and its associated genetic elements are identical to those in DAS-Ø15Ø7-1 maize (hereafter referred to as 1507 maize), which was deregulated by USDA, registered by EPA, and reviewed by FDA in 2001. The Cry34Ab1 and Cry35Ab1 proteins together comprise an active binary insecticidal crystal protein that confers resistance to 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 DAS-59122-7 maize (hereafter referred to as 59122 maize), which was deregulated by USDA in 2005, registered by EPA since 2005, and reviewed by FDA in 2004. Finally, 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; maize containing the PAT protein has been commercially grown in the U.S. since 1996. 1507 maize, 59122 maize, and the breeding stack of the two lines, 1507x59122 maize, were jointly developed by Pioneer and Dow AgroSciences and are now licensed broadly across the seed industry. In 2010, commercial products containing 1507x59122 maize were grown on approximately 14 million acres or approximately 16% of U.S. maize acres.

4114 maize is a new transformation event that, if deregulated, will provide an alternative to the breeding stack combination of two previously approved events: 1507 maize, which expresses the Cry1F and PAT proteins, and 59122 maize, which expresses the Cry34Ab1, Cry35Ab1, and PAT proteins. The 1507x59122 maize breeding stack combination was reviewed and registered by EPA in 2005. 4114 maize will provide similar insect resistance and herbicide tolerance to that of 1507x59122 maize. In 4114 maize, the genes for the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins are contained on a single transformation construct and have been integrated at a single locus in the genome; this is in contrast to the 1507x59122 maize where the insertions for the events are located at two unlinked loci. As a new event with all genes located at a single locus, 4114 maize will be bred more efficiently into new product offerings for growers that are customized to their local insect protection and agronomic needs. Efficient breeding of multiple traits in single commercial maize products is becoming more important as growers demand more complex products, including multiple modes of action for lepidopteran and corn rootworm insect resistance and tolerance to one or more classes of herbicides.

4114 maize was developed by *Agrobacterium*-mediated transformation using the DNA plasmid vector PHP27118. The T-DNA region of PHP27118 contains the *cry*1F, *cry*34Ab1, *cry*35Ab1, and *pat* genes. These gene cassettes are identical to those found in 1507 and 59122 maize, based on sequencing of the 4114 maize insertion, and the translated proteins were found to be identical. Furthermore, western blot analysis demonstrated similar molecular weight and immunoreactivity of the proteins. Therefore, identity of the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins in 4114 maize to those in 1507 and 59122 maize was confirmed.

Molecular characterization of the 4114 maize insertion by Southern blot analysis confirmed that a single, intact copy of the T-DNA of PHP27118 has been inserted into the maize genome and that the insertion is stable during the traditional breeding process. Southern blot analysis verified the absence of plasmid backbone DNA. Segregation analysis of five breeding generations of 4114 maize confirmed the stability and Mendelian inheritance of the insertion.

The concentrations of the introduced Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins were measured in a number of 4114 maize tissues and were compared to the concentrations in the respective 1507, 59122, and/or 1507x59122 maize tissues. In general, these comparisons revealed that 4114 maize tissues have similar or lower concentrations of the introduced proteins than those of 1507, 59122, and/or 1507x59122 maize. Exceptions were Cry1F and Cry34Ab1 concentrations in senescent tissue and also Cry1F concentrations in pollen, where concentrations in 4114 maize were higher. In general, senescent tissue can contain a variable degree of extractable protein and likely accounts for the variability in the comparison. Overall, these comparisons indicate that any previously conducted safety studies that used 1507, 59122, and 1507x59122 maize are applicable to 4114 maize. Safety studies that considered exposure to the Cry1F protein from 1507 or 1507x59122 pollen would require the reassessment of results for 4114 maize using the higher concentration.

The safety of the introduced proteins in 4114 maize has been previously evaluated by regulatory agencies in the U.S., as referenced above, and there is a history of safe use and exposure. The Cry1F, Cry34Ab1, and Cry35Ab1 proteins were derived from *Bacillus thuringiensis* and the PAT protein was derived from *Streptomyces viridochromogenes*. *B. thuringiensis and S. viridochromogenes* are naturally occurring soil bacteria and are not pathogenic; therefore, animals and humans are regularly exposed to these organisms and their components without adverse consequences.

The potential for allergenicity and toxicity of 4114 maize was evaluated by examining the allergenic potential of maize as a crop and by assessing the allergenic and toxic potential of the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins. Maize is not a common allergenic food and the modification in 4114 maize is not expected to alter the allergenic potential of maize. The Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins have been assessed previously for 1507 and 59122 maize and have been determined to be unlikely to be potential allergens or toxins to humans and animals. Previous assessments of these proteins included bioinformatic analyses, digestibility studies, and acute protein toxicity studies and are relevant for the assessment of

4114 maize. Updated bioinformatic analyses support the original conclusions that the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins are unlikely to be allergens or toxins. These data support the conclusion that the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins in 4114 maize are therefore safe for the food and feed supply. These data have been submitted to EPA to confirm the safety of these proteins and will be submitted to the FDA for their food and feed safety assessment of 4114 maize.

Comprehensive agronomic performance assessments for 4114 maize were conducted in replicated field studies at a total of 17 locations in the U.S. and Canada. These assessments were conducted independently of the intended effects of the introduced traits (*i.e.,* trait efficacy) so that appropriate comparisons would be made to conventional maize and any unintended effects due to the 4114 maize insertion would be evaluated. The following characteristics were measured: early population, final population, time to silking, time to pollen shed, pollen viability (measured at six locations), seedling vigor, stalk lodging, root lodging, stay green, disease incidence, insect damage, plant height, ear height, and yield (measured at 11 locations). Seed germination and dormancy data were also collected in laboratory experiments. Analysis of these data showed no statistically significant differences between 4114 maize and control maize lines, indicating the agronomic comparability of 4114 maize to conventional maize. In addition, 4114 maize has been field tested since 2006 in the U.S. and Puerto Rico. All releases in the U.S. have occurred under field permits and notifications granted by USDA - APHIS. All field trials of 4114 maize were observed for naturally occurring insects or diseases, and no unexpected differences between 4114 maize and control were observed. Together, these data support the conclusion that 4114 maize is unlikely to pose a greater plant pest risk than conventional maize.

Extensive nutrient composition analyses of grain (60 analytes) and forage (nine analytes) were conducted to compare the composition of 4114 maize to that of a control maize line and eight commercial maize varieties. These analyses were used to evaluate any changes in the levels of key nutrients, anti-nutrients and secondary metabolites. Based on the results of the compositional evaluation, the grain and forage of 4114 maize are comparable to commercially available maize and there would be no significant impact on raw or processed maize commodities. Along with the agronomic data included in this petition, compositional comparability is a general indicator that 4114 maize will not exhibit unexpected effects with respect to plant pest risk.

The potential environmental impact of the introduction of 4114 maize considered three primary areas: the potential for 4114 maize to become weedy or invasive; the potential for gene flow to sexually compatible wild relatives; and the potential impacts of the introduced Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins. Comparative analyses indicated that 4114 maize was comparable to conventional maize with respect to the nutrient composition and agronomic characteristics measured and independently of the introduced traits. In general, maize does not possess weediness characteristics and is not considered a weedy or invasive species. Therefore, 4114 maize does not exhibit any characteristics that would indicate it is any more likely than conventional maize to become a weed or plant pest. The potential for gene flow

examined maize pollination biology and the hybridization potential and geographic overlap of maize wild relatives. While maize does possess some pollination characteristics favorable to gene flow, the distribution of wild relative populations are limited in the U.S. and there is low fitness or sterility of hybrids; therefore, it is unlikely that the inserted DNA in 4114 maize would be introgressed significantly into these wild relative populations.

As referenced earlier, 4114 maize contains the same introduced proteins as those present in 1507, 59122, and/or 1507x59122 maize and has protein concentrations that are similar to or lower than these lines; therefore, previously conducted environmental safety studies for these lines were relevant for evaluating the environmental impact of 4114 maize cultivation. Based on the expected environmental exposure and the sufficient margin of safety of the proteins from 4114 maize, these environmental studies indicated that there is a low risk of the Cry1F and Cry34/35Ab1 proteins to non-target orders. A review of multiple field studies conducted for 1507, 59122, and/or 1507x59122 maize provide support that the abundance of non-target organisms would not be impacted by the cultivation of 4114 maize. In addition, certain threatened and endangered Lepidoptera and Coleoptera are unlikely to be adversely impacted from 4114 maize cultivation based on the lack of habitat overlap. Nutritional and toxicological studies on 1507, 59122, and/or 1507x59122 maize also support that 4114 maize will not adversely impact non-target vertebrates. Therefore, it is unlikely that 4114 maize will pose a risk to non-target organisms including beneficial, threatened and endangered species, as well as non-target vertebrates including birds, mammals, and humans. In support of this conclusion, 1507 maize has been commercially available in the U.S. since the 2003 growing season and 59122 and 1507x59122 maize have been available in the U.S. since 2006 with no negative safety or environmental effects. Taken together, it is unlikely that 4114 maize will pose a plant pest risk or impact non-target organisms in the environment.

In conclusion, based on the data contained herein, Pioneer requests that APHIS grant the request for a determination of nonregulated status for 4114 maize and any crosses of this line with other nonregulated *Zea mays*.

Abbreviations, Acronyms, and Definitions

~	approximately
%DB	percent dry basis
1507 maize	maize line containing the DAS-Ø15Ø7-1 event
1507x59122 maize	maize line containing the breeding stack DAS-Ø15Ø7-1xDAS-59122-7
32D78	Pioneer® commercial hybrid line
3394	Pioneer® commercial hybrid line
33G26	Pioneer® commercial hybrid line
33J24	Pioneer® commercial hybrid line
34A15	Pioneer® commercial hybrid line
34M94	Pioneer® commercial hybrid line
34P88	Pioneer® commercial hybrid line
35K02	Pioneer® commercial hybrid line
35T06	Pioneer® commercial hybrid line
35T36	Pioneer® commercial hybrid line
36M28	Pioneer® commercial hybrid line
37H24	Pioneer® commercial hybrid line
37Y12	Pioneer® commercial hybrid line
38B85	Pioneer® commercial hybrid line
4114 maize	maize line containing the DP-ØØ4114-3 event
59122 maize	maize line containing the DAS-59122-7 event
AACC	American Association of Cereal Chemists
ADF	acid detergent fiber
AOAC	Association of Official Analytical Chemists
AOSA	Association of Official Seed Analysis
APHIS	Animal and Plant Health Inspection Service of USDA
Bcl I	restriction enzyme from *Bacillus caldolyticus*
Bt	*Bacillus thuringiensis*
bp	base pair
CaMV	cauliflower mosaic virus
CCUR	Center for Crops Utilization Research
CFR	Code of Federal Regulations
CI	confidence interval
CMH	Cochran-Mantel-Haenszel test
*cry*1F	gene encoding the Cry1F protein from *Bacillus thuringiensis* var. *aizawai*
Cry1F	protein encoded by the *cry*1F gene from *Bacillus thuringiensis* var. *aizawai*
*cry*34Ab1	gene encoding the Cry34Ab1 protein from *Bacillus thuringiensis* strain PS149B1
Cry34Ab1	protein encoded by the *cry*34Ab1 gene from *Bacillus thuringiensis* strain PS149B1
*cry*35Ab1	gene encoding the Cry35Ab1 protein from *Bacillus thuringiensis* strain PS149B1
Cry35Ab1	protein encoded by the *cry*35Ab1 gene from *Bacillus thuringiensis* strain PS149B1
CS	compound symmetry
C_T	threshold cycle
df	degrees of freedom
DI	deionized
DIG	digoxygenin
DNA	deoxyribonucleic acid
DT_{50}	time for 50% dissipation

Abbreviations, Acronyms, and Definitions (continued)

E. coli	*Escherichia coli*
EEC	expected environmental concentration
E score	expectation score
ELISA	enzyme linked immunosorbent assay
EPA	Environmental Protection Agency
EUP	experimental use permit
FAME	fatty acid methyl ester
FARRP	Food Allergy Research and Resource Program
FDA	Food and Drug Administration
FDA CFSAN	Food and Drug Administration's Center for Food Safety and Applied Nutrition
FDR	false discovery rate
FOIA	Freedom of Information Act
GC/FID	gas chromatography with flame ionization detection
GLMM	Generalized Linear Mixed Model
GNAT	GCN 5-related *N*-acetyltransferases
Hae III	Restriction enzyme from *Haemophilus aegyptius*
HFCS	high-fructose corn syrup
*Hin*d III	Restriction enzyme from *Haemophilus influenzae*
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
ICP-OES	inductively coupled plasma optical emission spectroscopy
IgE	immunoglobulin E
kb	kilobase pair
kDa	kilodalton
KR	Kenward-Roger
LB	Left Border of the T-DNA
LC_{50}	lethal concentration at which 50% of a test population are affected
LLOQ	lower limit of quantification
LS-Mean	Least Squares Mean
LTP	lipid transfer protein
ML	maximum likelihood
MOE	margin of exposure
MS	mass spectrometry
NCBI	National Center for Biotechnology Information
NCGA	National Corn Growers Association
NDF	neutral detergent fiber
NOEC	no observed effect concentration
OD	optical density
OECD	Organisation for Economic Co-operation and Development
ORF	open reading frame
pat	gene encoding the PAT (phosphinothricin acetyltransferase) protein from *Streptomyces viridochromogenes*
PAT	phosphinothricin acetyltransferase protein from *Streptomyces viridochromogenes*
PBST	phosphate buffered saline plus Tween[a]-20

[a] Registered trademark of ICI Americas, Inc.

Abbreviations, Acronyms, and Definitions (continued)

PCR	polymerase chain reaction
PH09B	Pioneer proprietary maize inbred
PH1B5	Pioneer proprietary maize inbred
PHNAR	Pioneer proprietary maize inbred
PHR03	Pioneer proprietary maize inbred
PHTFE	Pioneer proprietary maize inbred
PHWWE	Pioneer proprietary maize inbred; used for initial transformation to produce 4114 maize
PIR	Protein Information Resource
PR	pathogenesis-related
PRF	Protein Research Foundation
*pin*II	proteinase inhibitor II
RB	Right Border of the T-DNA
REML	Residual Maximum Likelihood
SAS	Statistical Analysis Software
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
spc	spectinomycin resistance gene
ssp.	subspecies
T-DNA	portion of the *Agrobacterium* transformation plasmid between the Left and Right Borders that is expected to be transferred to the plant
tet	tetracycline resistance gene
TZ	tetrazolium chloride
*ubi*ZM1	*Zea mays* polyubiquitin gene
UPLC	ultra performance liquid chromatography
USDA	United States Department of Agriculture
USDA-APHIS BRS	United States Department of Agriculture-Animal and Plant Health Inspection Service, Biotechnology Regulatory Services
USDA-ERS	Economic Research Service of the U.S. Department of Agriculture
USDA-NASS	National Agricultural Statistics Service of the U.S. Department of Agriculture
USDA-NCRS	United States Department of Agriculture - Natural Resources Conservations Service
UTR	untranslated region
UV	ultraviolet
var.	variety
wwPDB	Worldwide Protein Data Bank

* Abbreviations of units of measurement and of physical and chemical quantities are used according to the standard format described in Instructions to Authors in the Journal of Biological Chemistry (http://www.jbc.org/)

1. Rationale for the Development of 4114 Maize

4114 maize (OECD Unique Identifier DP-ØØ4114-3) is a new event that has been transformed with a single genetic construct containing each of the proteins found in the previously approved maize events, DAS-Ø15Ø7-1 (1507 maize; Cry1F and PAT proteins) and DAS-59122-7 (59122 maize; Cry34Ab1, Cry35Ab1, and PAT proteins) and has been developed to provide an alternative to the 1507x59122 maize breeding stack for more complex stack combinations. 4114 maize 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 1507x59122 maize. 1507, 59122, and 1507x59122 maize contain familiar traits and are currently licensed broadly across the seed industry; in 2010, commercial products containing 1507x59122 maize were grown on approximately 14 million acres or approximately 16% of U.S. maize acres (GfK Kynetec, 2010).

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

1-A. Basis for the Request for a Determination of Nonregulated Status under 7 CFR §340.6

The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) has responsibility, under the Plant Protection Act (7 U.S.C. 7701-7772) and the Plant Quarantine Act (7 U.S.C. 151-167), to prevent the introduction or dissemination of plant pests into or within the U.S. Part 340 regulates introduction of organisms altered or produced through genetic engineering which are plant pests or for which there is a reason to believe are plant pests. The APHIS regulations at 7 CFR §340.6 provide that an applicant may petition APHIS to evaluate submitted data on the genetically engineered crop to determine that a regulated article does not present a plant pest risk and therefore should no longer be regulated.

Pioneer Hi-Bred International, Inc. is submitting data for genetically engineered insect-resistant and herbicide-tolerant 4114 maize and requests a determination from USDA - APHIS that event DP-ØØ4114-3 and any crosses with other *Zea mays* no longer be considered regulated articles under 7 CFR §340.

1-B. Similarity of 4114 Maize to Previously Approved 1507 and 59122 Maize

As mentioned earlier, 4114 maize is a new event with all genes at a single locus that will provide an alternative to 1507x59122 maize. 4114 maize contains each of the trait proteins found in

the previously approved 1507 maize (Cry1F and PAT proteins) and 59122 maize (Cry34Ab1, Cry35Ab1, and PAT proteins).

Both 1507 and 59122 maize and their combination 1507x59122 maize are fully approved by U.S. regulatory agencies (Table 1). 1507, 59122, and 1507x59122 maize products have been commercially available in the U.S. since 2003, 2006, and 2006, respectively. 4114 maize has the same introduced genetic material and proteins as 1507x59122 maize, and has similar agronomic properties, protein expression, and efficacy to the previously approved events; therefore, portions of previous regulatory data and analyses of 1507, 59122 and 1507x59122 are relevant to 4114 maize and will be cited in this petition.

Furthermore, 1507, 59122, and 1507x59122 maize were jointly developed by Pioneer and Dow AgroSciences; these commercial lines contain familiar traits and are currently licensed broadly across the seed industry. In 2010, commercial products containing 1507x59122 maize were grown on approximately 14 million acres, which represents approximately 16% of U.S. maize acres (GfK Kynetec, 2010).

Table 1. Regulatory History of 1507, 59122, and 1507x59122 Maize in the U.S.

Product	Agency	Approval Date[a]
1507 maize	USDA	August 14, 2001
	FDA	May 18, 2001[b]
	EPA	August 10, 2001; expires September 30, 2015
59122 maize	USDA	October 7, 2005
	FDA	October 4, 2004[b]
	EPA	August 31, 2005; expires September 30, 2015
1507x59122 maize	USDA	Not Applicable[c]
	FDA	Not Applicable[c]
	EPA	October 27, 2005; expires September 30, 2015

[a] Information accessed on U.S. regulatory agency websites (EPA, 2010a; EPA, 2010b; EPA, 2011a; FDA, 2001; FDA, 2004; USDA-APHIS, 2001; USDA-APHIS, 2005).

[b] Indicates completion date of biotechnology consultation.

[c] No review of 1507x59122 maize was required by USDA or FDA as the approved individual events were combined by conventional breeding.

1-C. Purpose and Need for 4114 Maize

Through the use of 4114 maize in new stacked product offerings, there will be similar insect resistance and herbicide tolerance benefits as those of 1507x59122 maize. Additionally, there are added breeding advantages of using a single transformation event in these products over the available 1507x59122 maize breeding stack, as described further below.

Benefits of Insect Resistance and Herbicide Tolerance of 4114 Maize

The use of 4114 maize in commercial products will provide similar insect and weed control benefits to those of 1507x59122 maize. 4114 maize contains the same Cry1F, Cry34Ab1, and Cry35Ab1 proteins as 1507x59122 maize and will provide growers with a simple, inexpensive, highly effective, and environmentally benign means of controlling lepidopteran and corn rootworm insects, including European corn borer (*Ostrinia nubilalis* (Hubner)) 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 U.S.; 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 4114 maize containing the Cry1F, Cry34Ab1, and Cry35Ab1 proteins has the potential to offer effective control of maize insect pests and a reduction in the use of highly toxic agricultural pesticide chemicals to control these insect pests (EPA, 2010a; EPA, 2010b). The Cry1F, Cry34Ab1, and Cry35Ab1 proteins also offer crop yield advantage under insect pressure (EPA, 2010a; EPA, 2010b). In general, for corn borer- and corn rootworm-protected maize varieties, a 5% yearly average yield advantage compared to conventional maize has been realized in the U.S. since these varieties were cultivated (Brookes and Barfoot, 2009; Brookes and Barfoot, 2010).

In contrast to the chemical pesticides used to control insect pests, the Cry1F, Cry34Ab1, and Cry35Ab1 proteins do not pose risks to humans or to the environment (EPA, 2010a; EPA, 2010b). As a result, 4114 maize would offer similar benefits as 1507 and 59122 maize -- increased worker safety and grower profits through reductions in the use of a number of highly toxic and expensive chemical pesticide control programs (EPA, 2010a; EPA, 2010b). In general, farm income has increased as a result of use of insect-protected maize (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 labeled rates. Glufosinate was registered as an herbicide in 1993 in the U.S. and is currently under re-review at EPA (CERA, 2002; EPA, 2011b). 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.

Benefits of 4114 Maize as a Replacement Option for 1507x59122 Maize

As discussed previously, 4114 maize will provide an alternative to 1507x59122 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 and by increasing product offerings 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 1507x59122 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. 4114 maize, 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.

4114 maize is expected to be more efficiently bred into a wide variety of maize genetic backgrounds and with additional traits, thus giving growers more choice in products that offer in-plant insect protection and that are customized to their local growing areas and agronomic needs. 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.

1-D. Prior Environmental Release and Submissions to Other Regulatory Agencies

As a new transformation event, 4114 maize is regulated by the United States Department of Agriculture-Animal and Plant Health and Inspection Service, Biotechnology Regulatory Services (USDA-APHIS BRS) and the United States Environmental Protection Agency (EPA). 4114 maize falls within the scope of the Food and Drug Administration's (FDA) policy statement concerning regulation of food products derived from new plant varieties, including those developed by recombinant DNA techniques.

4114 maize has been extensively field tested in the U.S. and Puerto Rico since 2006 in over 180 separate plantings as authorized by the USDA-APHIS permits and notifications listed in Appendix 1. No Experimental Use Permit (EUP) applications for 4114 maize were submitted to the EPA. 4114 maize has been grown in small-scale field tests under the 10-acre per pest EUP exemption 40 CFR §172.3(c)(1).

An application for a seed increase registration for 4114 maize was submitted to EPA on April 18, 2011. Seed increase registrations are used by EPA to authorize breeding and testing of events such as 4114 maize that will not be sold commercially without being stacked with other insect resistant traits. Upon EPA registration, 4114 maize will have county and national acreage limitations imposed by the EPA and will be prohibited from commercial use on its own. Future commercial breeding stacks of 4114 maize with other EPA-regulated Plant-Incorporated Protectant events are subject to EPA's regulatory authority and will require full registrations prior to commercialization.

A voluntary safety and nutritional assessment of 4114 maize will also be submitted to the FDA's Center for Food Safety and Applied Nutrition (FDA CFSAN) in the third quarter of 2011.

Regulatory submissions will be made in key U.S. maize export markets. These countries include Canada, Japan, Mexico, Taiwan, South Korea, and China. A full commercial launch of any products containing 4114 maize will only occur after obtaining all necessary authorizations in the U.S. and key import countries with functioning regulatory processes.

1-E. Maize Crop Cultivation in the U.S. and Usage

Maize is the largest crop grown in the U.S. in terms of acreage and net value. Maize has multiple downstream uses for feed, fuel, and food that are significant for U.S. and global supply. In 2010, over 12 billion bushels of maize were produced in the U.S. from approximately 81 million harvested acres with a crop value of $65.97 billion (NCGA, 2011; USDA-NASS, 2011). The U.S. is a major global exporter of maize at approximately 55% of the total trade market (NCGA, 2011). The largest maize U.S. export markets in 2009-2010 were Japan, Mexico, South Korea, Taiwan, Egypt, and Canada (NCGA, 2011). Exports accounted for 14.5% of the maize produced in 2010 (Figure 1; NCGA, 2011).

The 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 in the U.S. and globally. A significant portion of maize cultivated in the U.S. is genetically modified and contains similar insect resistant traits. In 2010, 86% of maize grown in the U.S. was genetically modified; insect resistant varieties accounted for approximately 63% of all maize acreage, which includes the percentage of insect resistant traits as well as stacked varieties (USDA-NASS, 2010). Over the past decade, maize yields and overall production have increased, in part due to improvements in seed varieties and agronomic production practices (NCGA, 2011; USDA-ERS, 2009).

Some background on maize processing and use for feed, fuel, and food industries is described below.

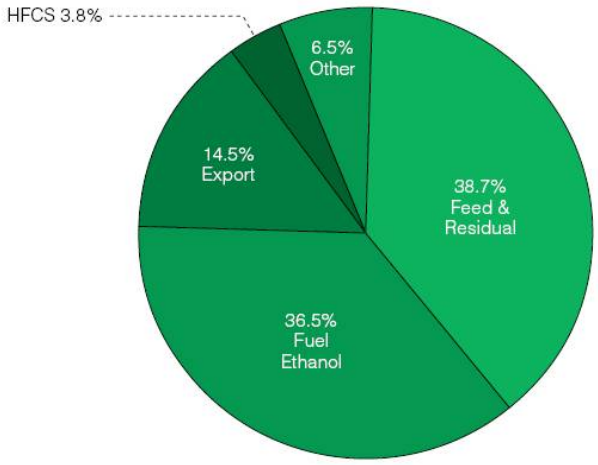


Figure 1. U.S. Maize Usage by Segment, 2010
HFCS: High-fructose corn syrup
Source: NCGA, 2011

Maize Processing for Feed, Fuel, and Food Uses

Maize grain requires processing for some downstream uses. Wet and dry milling processes are used to separate grain into components for food, feed, and fuel processing (OECD, 2002).

Wet milling starts with softening the kernel in hot water and sulfur dioxide prior to further fractionation and processing (OECD, 2002). Products produced in the wet milling process include germ meal, oil (further processed into margarine, cooking oil, baking and frying fats), corn gluten feed, corn gluten meal, and starch (further processed into ethanol and sweeteners) (OECD, 2002).

There are several means of dry milling maize grain, but by far the most widely used process begins with soaking the kernel in water to remove the pericarp and germ, followed by drying the remaining grain fraction before additional processing (OECD, 2002). Products produced in the dry milling process include flour, meal, germ meal, oil, beverage and fuel ethanol, distillers dried solubles, flaking grits, hominy feed, and grits (OECD, 2002). Maize grain may also be cooked in alkali and finely ground to produce what is known as *masa*, which is used for tortillas and snack chips (OECD, 2002).

The production of fuel ethanol typically begins with dry milling of maize grain, cooking, saccharification, and fermentation to produce ethanol and the by-product distiller dried grains or solubles (OECD, 2002).

Feed Use of Maize

The largest proportion, 38.7%, of maize produced in the U.S. is used for animal feed (Figure 1; NCGA, 2011).

Of the maize grain that is used for feed, the greatest percentage is consumed by beef cattle, followed by poultry, pork, and dairy cattle (Figure 2; NCGA, 2011). A number of different products from the maize plant and from grain processing may be used as feed.

The whole maize plant or its residue from harvesting are frequently used as animal feed. Silage, derived from the above-ground portions of the maize plant, is an important feed ingredient for feedlot and dairy cattle and preserves more than 90% of nutrients (OECD, 2002). In 2009, approximately 6% of the U.S. maize crop was used for silage (USDA-NASS, 2010). In addition, stalks from harvested maize plants can be grazed by ruminants in the field (OECD, 2002).

Maize ears, without shelling (*i.e.*, removing the grain from the cob), can be ground directly for ruminant feed (OECD, 2002). When ears are shelled to remove the grain, remnant cobs can also be used in animal feed (OECD, 2002). Maize grain can be fed to animals with minimal processing and can be fed whole, rolled, ground, or steam flaked (OECD, 2002). Rolled or

ground grain is fed to swine and poultry (OECD, 2002). Maize grain added to pet foods is ground, cooked, and pelleted or extruded (OECD, 2002).

Processed products from the milling and ethanol fermentation processes are also fed to livestock. A by-product of the wet milling process, corn gluten meal, is fed to ruminants, poultry, and swine (OECD, 2002). The ethanol fermentation process produces a co-product called distillers dried grains/solubles or corn gluten feed that is used as animal feed to dairy and beef cattle, poultry, and swine (USDA-ERS, 2009; USDA-ERS, 2010; USDA-NASS, 2007).

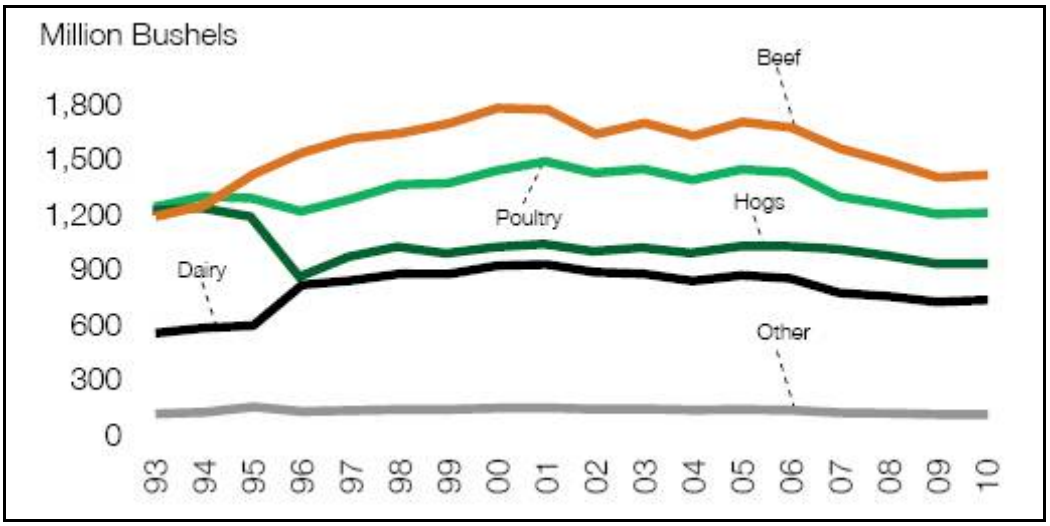


Figure 2. U.S. Maize Fed by Animal Group, 1993-2010
Source: NCGA, 2011

Fuel Use of Maize

Maize is the primary feedstock used to produce ethanol in the U.S.; 36.5% of maize grain produced is fermented into fuel ethanol (NCGA, 2011; USDA-ERS, 2010). In 2009, ethanol represented 8% of motor vehicle gasoline supplies and is expected to remain below 10% of annual gasoline use by 2015 (USDA-ERS, 2010).

Food Use of Maize

Starch, oil, grits, bran, meal, and flour from maize wet and dry milling are primarily used in foods (OECD, 2002). A majority of starch is converted to sweeteners, such as corn syrup, high fructose corn syrup, maltodextrins, and dextrose, and also fermented into ethanol (OECD, 2002). A significant portion of U.S. maize, 3.8%, goes to the production of high-fructose corn syrup as an end product (Figure 1; NCGA, 2011). Approximately 6.3% (classified in the "other" usage segment) comprises food purposes such as starch, sweeteners, cereal/other, and beverage alcohol (Figure 1; NCGA, 2011).

Starch is used for food such as bakery products/mixes, condiments, candies, and prepared (snack, dessert, meat) foods (CCUR, 2009). Sweeteners are used for soft drinks, candies, bakery products/mixes, condiments (jams, jellies, dressings), and prepared foods (CCUR, 2009). Whole maize is consumed as popcorn, sweet corn, and alkali processed grain for tortillas and snack chips (CCUR, 2009), though these uses comprise a very minor usage segment.

2. Introduced Trait, Development of the Transgenic Line, and Characterization of Insertion and Expressed Products

Characterization of the transgenic crop variety provides additional knowledge about the genetic modification and the trait as a starting point for the safety assessment. Knowledge about the transgenic crop in these areas provides background information for the food, feed, and environmental safety assessments and, if applicable, can also identify certain areas of greater potential risk or concern.

This section provides an overall characterization of 4114 maize and includes information about the maize crop, the source of the donor genetic elements, the identity and intended function of the expressed proteins, the development of the 4114 maize line, the structure and genetic stability of the DNA insertion, and the tissue-specific concentration of the expressed proteins. The information below provides background for safety assessments in later sections.

2-A. The Biology of Maize

Biology documents on the unmodified plant species have been published by the Organization for Economic Co-operation and Development (OECD) (OECD, 2003). The OECD document on maize provides background on the biology of *Zea mays* including:

- general description, including information on use of maize as a crop plant;
- taxonomic status of *Zea;*
- identification methods;
- center of origin / diversity and maize diversity;
- reproductive biology, including sexual and asexual reproduction;
- crosses, including intra- and inter-specific crosses and gene flow; and
- agro-ecology, including information about cultivation, volunteers and weediness, soil ecology, and maize-insect interactions.

2-B. Description of DNA Used for Transformation and Intended Phenotype of Introduced Proteins

Identity and Source of Genetic Material in the T-DNA Region of PHP27118

4114 maize was produced by *Agrobacterium tumefaciens*-mediated transformation with PHP27118 (Figure 3), which contains the *cry*1F*, cry*34Ab1*, cry*35Ab1*,* and *pat* expression cassettes (Figure 4). A summary of the genetic elements and their position in the T-DNA region is given in Table 2. As described earlier, 4114 maize is a new transformation event that contains the same gene cassettes as in previously registered 1507 and 59122 maize; these cassette comparisons are presented in Table 2.

The first cassette contains a truncated version of the *cry*1F gene from *Bacillus thuringiensis var. aizawai*. The expression of the *cry*1F gene is controlled by the maize polyubiquitin promoter

(Christensen *et al*., 1992), providing 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 *cry*1F gene is the polyadenylation signal from Open Reading Frame 25 (ORF 25) of the *Agrobacterium tumefaciens* Ti plasmid pTi15955 (Barker *et al*., 1983).

The second cassette contains the *cry*34Ab1 gene isolated from *Bacillus thuringiensis* strain PS149B1, which has been codon optimized for expression in plants (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 of *Solanum tuberosum* (*pin*II terminator) (An *et al*., 1989; Keil *et al*., 1986).

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

The fourth and final gene cassette contains the phosphinothricin acetyl transferase (*pat*) gene from *Streptomyces viridochromogenes,* which has been codon optimized for expression in plants. 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).

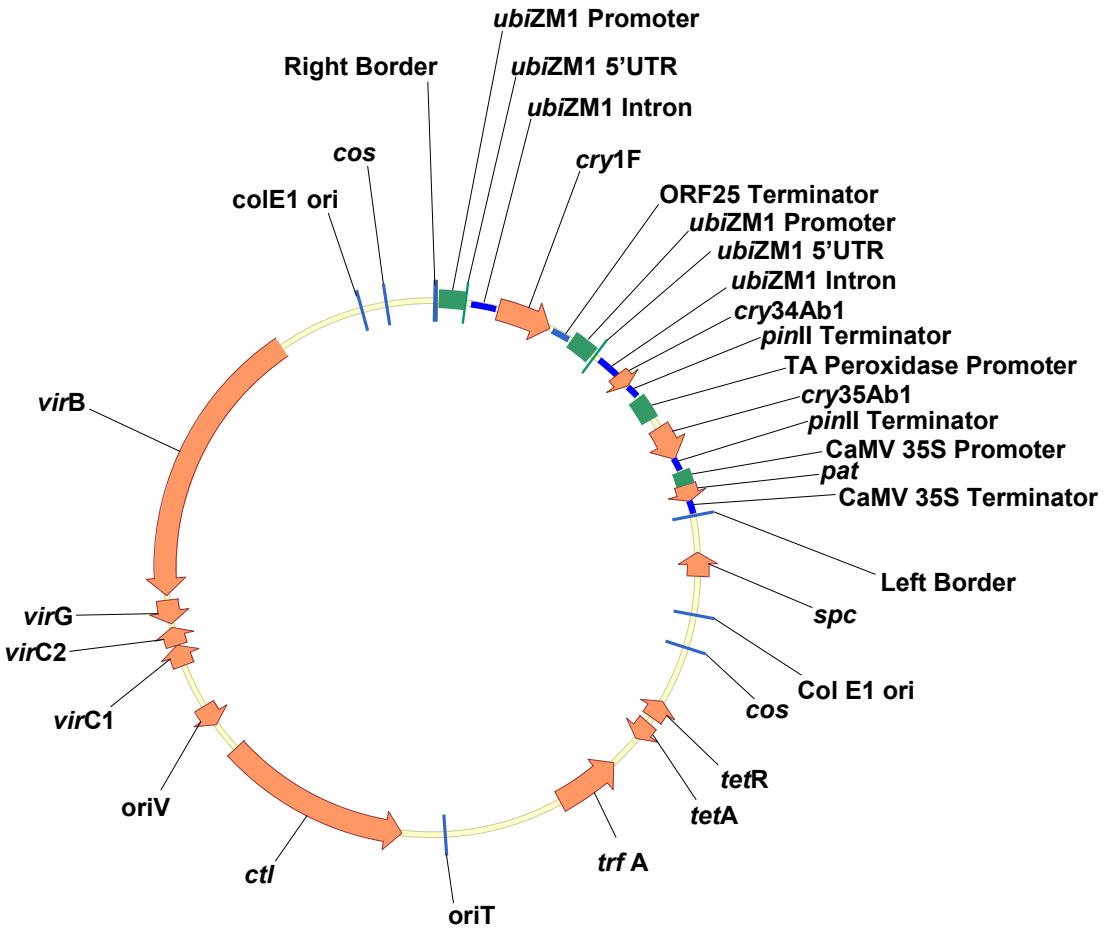


Figure 3. Schematic Diagram of Plasmid PHP27118

Schematic diagram of plasmid PHP27118 with genetic elements indicated. Plasmid size is 54910 bp.

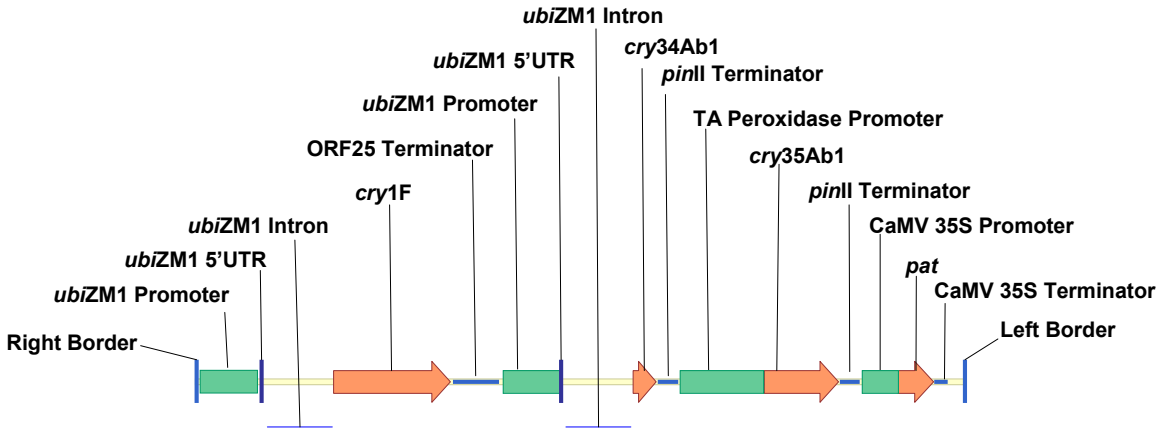


Figure 4. Schematic Diagram of the T-DNA Region from Plasmid PHP27118

Schematic diagram of the T-DNA indicating the *cry*1F, *cry*34Ab1, *cry*35Ab1, and *pat* genes along with their respective regulatory elements. The size of the T-DNA is 11978 bp.

Table 2. Description of Genetic Elements in the T-DNA Region of PHP27118

Comparison to Approved Event	Location on PHP27118 T-DNA (bp position)	Genetic Element	Size (bp)	Description
	1 to 25	Right Border	25	T-DNA Right Border region from Ti plasmid of *Agrobacterium tumefaciens*
	26 to 43	Ti Plasmid Region	18	Non-functional sequence from Ti plasmid of *Agrobacterium tumefaciens*
	44 to 114	Polylinker Region	71	Region required for cloning genetic elements
*cry*1F gene cassette in 4114 maize and 1507 maize	115 to 1014	*ubi*ZM1 Promoter	900	Promoter region from *Zea mays* polyubiquitin gene (Christensen *et al.*, 1992)
	1015 to 1097	*ubi*ZM1 5' UTR	83	5' untranslated region from *Zea mays* polyubiquitin gene (Christensen *et al.*, 1992)
	1098 to 2107	*ubi*ZM1 Intron	1010	Intron region from *Zea mays* polyubiquitin gene (Christensen *et al.*, 1992)
	2108 to 2129	Polylinker Region	22	Region required for cloning genetic elements
	2130 to 3947	*cry*1F Gene	1818	Truncated version of the *cry*1F gene from *Bacillus thuringiensis* var. *aizawai*
	3948 to 3992	Polylinker Region	45	Region required for cloning genetic elements
	3993 to 4706	ORF 25 Terminator	714	Terminator sequence from the *Agrobacterium tumefaciens* pTi15955 ORF 25 (Barker *et al.*, 1983)
	4707 to 4765	Polylinker Region	59	Region required for cloning genetic elements

Table 2. Description of Genetic Elements in the T-DNA Region of PHP27118 (continued)

Comparison to Approved Event	Location on PHP27118 T-DNA (bp position)	Genetic Element	Size (bp)	Description
*cry*34Ab1 gene cassette in 4114 maize and 59122 maize	4766 to 5665	*ubi*ZM1 Promoter	900	Promoter region from *Zea mays* polyubiquitin gene (Christensen *et al.*, 1992)
	5666 to 5748	*ubi*ZM1 5' UTR	83	5' untranslated region from *Zea mays* polyubiquitin gene (Christensen *et al.*, 1992)
	5749 to 6758	*ubi*ZM1 Intron	1010	Intron region from *Zea mays* polyubiquitin gene (Christensen *et al.*, 1992)
	6759 to 6786	Polylinker Region	28	Region required for cloning genetic elements
	6787 to 7158	*cry*34Ab1 Gene	372	Codon-optimized version of the *cry*34Ab1 gene encoding the 14 kDa delta-endotoxin parasporal crystal protein from the nonmotile strain PS149B1 of *Bacillus thuringiensis* (Ellis *et al*., 2002; Herman *et al*., 2002; Moellenbeck *et al*., 2001)
	7159 to 7182	Polylinker Region	24	Region required for cloning genetic elements
	7183 to 7492	*pin*II Terminator	310	Terminator region from *Solanum tuberosum* proteinase inhibitor II gene (An *et al.*, 1989; Keil *et al.,* 1986)
	7493 to 7522	Polylinker Region	30	Region required for cloning genetic elements

Table 2. Description of Genetic Elements in the T-DNA Region of PHP27118 (continued)

Comparison to Approved Event	Location on PHP27118 T-DNA (bp position)	Genetic Element	Size (bp)	Description
*cry*35Ab1 gene cassette in 4114 maize and 59122 maize	7523 to 8820	TA Peroxidase Promoter	1298	Promoter from *Triticum aestivum* (wheat) peroxidase including leader sequence (Hertig *et al.*, 1991)
	8821 to 8836	Polylinker Region	16	Region required for cloning genetic elements
	8837 to 9988	*cry*35Ab1	1152	Codon-optimized version of the *cry*35Ab1 gene encoding a 44 kDa delta-endotoxin parasporal crystal protein from the nonmotile strain PS149B1 of *Bacillus thuringiensis* (Ellis *et al*., 2002; Herman *et al*., 2002; Moellenbeck *et al*., 2001)
	9989 to 10012	Polylinker Region	24	Region required for cloning genetic elements
	10013 to 10322	*pin*II Terminator	310	Terminator region from *Solanum tuberosum* proteinase inhibitor II gene (An *et al.*, 1989; Keil *et al.,* 1986)
	10323 to 10367	Polylinker Region	45	Region required for cloning genetic elements
pat gene cassette in 4114 maize, 1507 maize and 59122 maize	10368 to 10897	CaMV 35S Promoter	530	35S promoter from Cauliflower Mosaic Virus (Franck *et al.*, 1980; Odell *et al.*, 1985; Pietrzak *et al.,* 1986)
	10898 to 10916	Polylinker Region	19	Region required for cloning genetic elements
	10917 to 11468	*pat* Gene	552	Codon-optimized phosphinothricin acetyltransferase gene from *Streptomyces viridochromogenes*.
	11469 to 11488	Polylinker Region	20	Region required for cloning genetic elements
	11489 to 11680	CaMV35S Terminator	192	35S terminator from Cauliflower Mosaic Virus (Franck *et al.*, 1980; Pietrzak *et al.,* 1986)
	11681 to 11874	Polylinker Region	194	Region required for cloning genetic elements
	11875 to 11953	Ti Plasmid Region	79	Non-functional sequence from Ti plasmid of *Agrobacterium tumefaciens*
	11954 to 11978	Left Border	25	T-DNA Left Border region from Ti plasmid of *Agrobacterium tumefaciens*

Activity and Function of Expressed Proteins in 4114 Maize

The insertion of the *cry*1F gene in 4114 maize confers resistance to plant damage by certain lepidopteran pests. The Cry1F protein is comprised of 605 amino acids and has a molecular weight of approximately 68 kDa (Figure 5). The Cry1F protein expressed in 4114 maize is identical to the one expressed in 1507 maize that has been deregulated by USDA, registered by EPA, and reviewed by FDA in 2001 (Table 1).

The *cry*34Ab1 and *cry*35Ab1 genes, isolated from the common soil bacterium *Bacillus thuringiensis* strain PS149B1, produce the proteins Cry34Ab1 and Cry35Ab1. The Cry34Ab1 protein is 123 amino acid residues in length and has a molecular weight of approximately 14 kDa (Figure 6). The full-length Cry35Ab1 protein has a length of 383 amino acids and a molecular weight of approximately 44 kDa (Figure 7). The Cry34Ab1 and Cry35Ab1 proteins together comprise an active insecticidal crystal protein (*i.e.,* the binary Cry34/35Ab1 protein), that confers resistance to certain corn rootworm pests. This binary protein is identical to the one expressed in 59122 maize deregulated by USDA in 2005, registered by EPA since 2005, and reviewed by FDA in 2004 (Table 1).

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 are lethal only when eaten by the larvae of 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* gene expresses the phosphinothricin acetyl transferase enzyme (PAT) that confers tolerance to glufosinate ammonium, the active ingredient in phosphinothricin herbicides. The PAT protein is 183 amino acids residues in length and has a molecular weight of approximately 21 kDa (Figure 8). 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; maize containing the PAT protein (*e.g.,* T25) has been commercially grown in the U.S. since 1996.

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 4114 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.

```
  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*
```

Figure 5. Deduced Amino Acid Sequence of the Cry1F Protein

Deduced amino acid sequence from translation of the *cry*1F gene from plasmid PHP27118. The Cry1F protein is 605 amino acids in length, has a molecular mass of approximately 68 kDa, and is identical to the one expressed in 1507 maize. The asterisk (*) indicates the translational stop codon.

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

Figure 6. Deduced Amino Acid Sequence of the Cry34Ab1 Protein

Deduced amino acid sequence from translation of the *cry*34Ab1 gene from plasmid PHP27118. The Cry34Ab1 protein is 123 amino acids in length, has a molecular mass of approximately 14 kDa, and is identical to the one expressed in 59122 maize. The asterisk (*) indicates the translational stop codon.

```
  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*
```

Figure 7. Deduced Amino Acid Sequence of the Cry35Ab1 Protein

Deduced amino acid sequence from translation of the *cry*35Ab1 gene from plasmid PHP27118. The Cry35Ab1 protein is 383 amino acids in length, has a molecular mass of approximately 44 kDa, and is identical to the one expressed in 59122 maize. The asterisk (*) indicates the translational stop codon.

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

Figure 8. Deduced Amino Acid Sequence of the PAT Protein

Deduced amino acid sequence from translation of the *pat* gene from plasmid PHP27118. The PAT protein is 183 amino acids in length and has a molecular mass of approximately 21 kDa. This protein is identical to the protein found in a number of approved events across several different crops that are currently in commercial use. The asterisk (*) indicates the translational stop codon.

Equivalency of Expressed Proteins in 4114 Maize and Previously Approved Events

The Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins expressed in 4114 maize are identical to the proteins expressed in previously approved 1507, 59122, and/or 1507x59122 maize. This equivalency was established using the following criteria:

- The same gene expression cassettes (promoters, protein coding sequences, and terminators) were used in 4114 maize as in 1507 and 59122 maize, so all genetic elements are identical (Table 2).
- The DNA insertion in 4114 maize was sequenced 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 amino acid sequences of those proteins in 1507 and 59122 maize (Figures 5 through 8). Therefore, the proteins in 4114 maize are also expected to be identical to the proteins in the breeding stack of 1507x59122 maize.
- Western blot analysis demonstrated the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins in 4114 maize migrate with equivalent molecular weight and similar relative immunoreactivity to the proteins expressed in 1507x59122 maize, indicating equivalency (Appendix 2).
- Bioequivalency of the expressed Cry1F and Cry34/35Ab1 proteins was established via side-by-side efficacy testing of 4114 maize with 1507x59122 maize in the field (data not shown; submitted to EPA).

Therefore, previously submitted safety data for Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins are also applicable for 4114 maize.

2-C. Description of Transformation, Selection, and Breeding

Pioneer proprietary inbred line PHWWE was transformed with plasmid PHP27118 to produce 4114 maize. Immature embryos of maize (*Zea mays* L.) line PHWWE were aseptically removed from the developing caryopsis nine to eleven days after pollination and inoculated with *Agrobacterium tumefaciens* strain LBA4404 containing plasmid PHP27118, essentially as described in Zhao *et al*., 2001. *Agrobacterium tumefaciens* strain LBA4404 is a disarmed strain that does not contain tumor-inducing factors, however with the inclusion of plasmid PHP27118 the strain will contain factors (*i.e.*, the *vir* genes) that enable the transfer of the T-DNA region to the inoculated host plant. After three to six days of embryo and *Agrobacterium* co-cultivation on solid culture medium with no selection, the embryos were then transferred to a medium without herbicide selection but containing carbenicillin for selection against *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 select against any remaining *Agrobacterium*. After six to eight weeks on the selective medium, healthy, growing calli that demonstrated resistance to bialaphos were identified.

Plants that were regenerated from transformation and tissue culture (designated T0 plants) were selected for further characterization by molecular analyses, herbicide and insect efficacy, and agronomic evaluations. Refer to Figure 9 for a schematic overview of the transformation and event development process for 4114 maize.

The subsequent breeding of 4114 maize proceeded as indicated in Figure 10 to produce specific generations for the characterization and assessments conducted, as well as for the development of commercial lines. Table 3 indicates the breeding generations used for each of the analyses described in this submission.

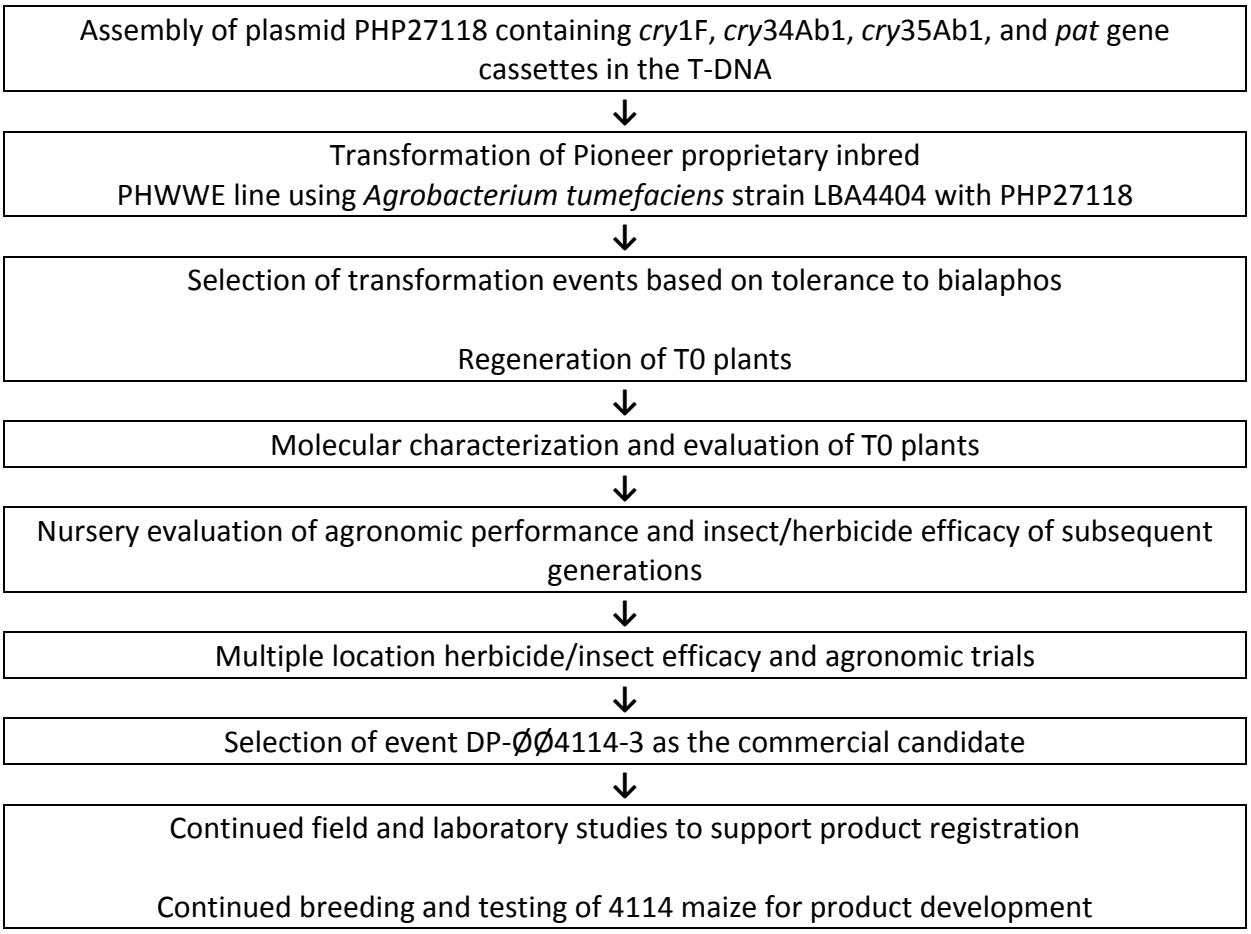


Figure 9. Schematic Diagram of the Development of 4114 Maize

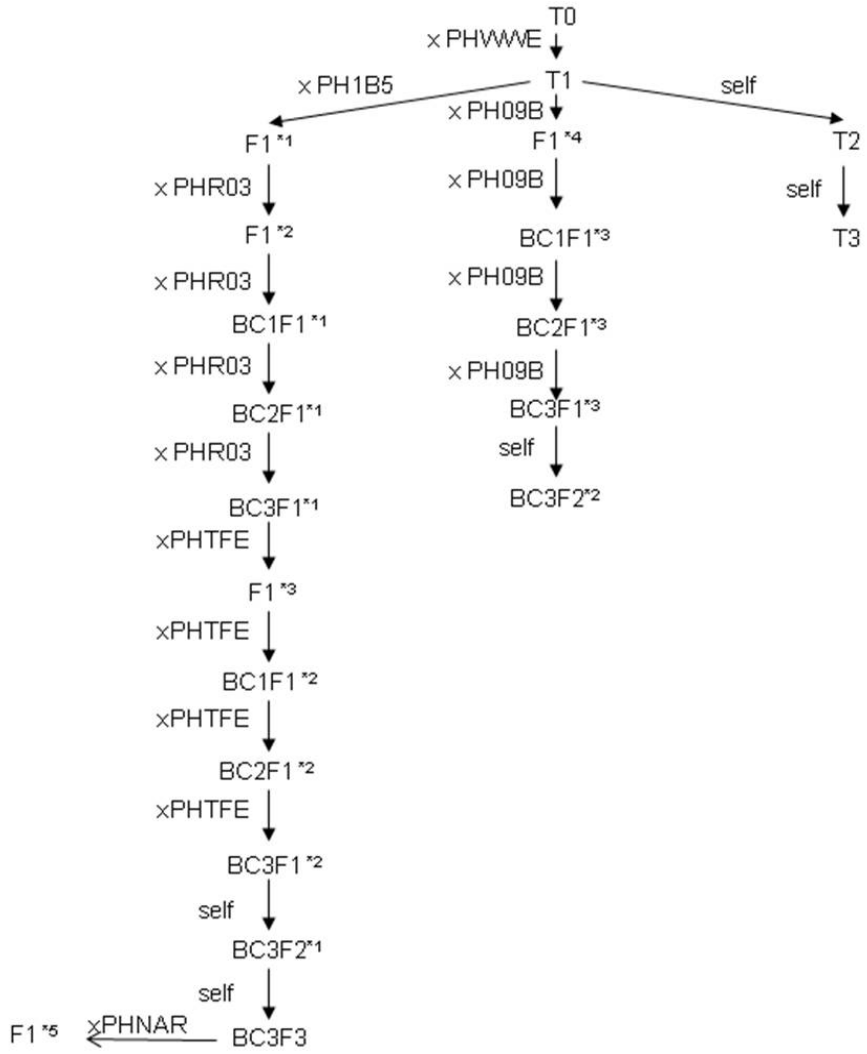


Figure 10. Breeding Diagram for 4114 Maize and Generations Used for Analyses

The breeding steps to produce the generations used for characterization, assessment, and the development of commercial lines are shown schematically. Pioneer proprietary inbred PHWWE was used for transformation to produce 4114 maize. Other Pioneer proprietary inbreds PH1B5, PHR03, PHTFE, PHNAR, and PH09B were used in crossing and backcrossing steps.

Table 3. Generations and Comparators Used for Analysis of 4114 Maize

Analysis	Generation	Comparators	Experimental Control and Reference Lines
Genetic Characterization and Absence of Backbone DNA (Section 2-D)	T2, T3, BC3F1[*3], BC3F2[*2]	Not applicable	Pioneer® proprietary maize inbreds PH09B and PHWWE
Stability and Inheritance of the DNA Insertion (Section 2-E)	F1[*1], BC2F1[*1], BC3F1[*1], BC2F1[*2], BC3F1[*2]	Not applicable	Not applicable
Concentrations of Cry1F, Cry34Ab1, Cry35Ab1, and PAT (Section 2-F)	F1[*5]	Not applicable[a]	1507, 59122, and 1507x59122 maize[a]
Compositional Assessment (Section 4-A)	Grain and forage from F1[*5]	PHNARxPHTFE	Pioneer® hybrids 34M94, 33G26, 33J24, 3394, 38B85, 37Y12, 34A15, and 34P88
Germination and Dormancy (Agronomic Performance) (Section 4-B)	F1[*5]	PHNARxPHTFE	Pioneer® hybrids 32D78 and 34P88
Field Trial Evaluations (Agronomic Performance) (Section 4-B)	F1[*5]	PHNARxPHTFE	Pioneer® hybrids 34M94, 33G26, 33J24, 3394, 38B85, 37Y12, 34A15, 34P88, 37H24, 36M28, 35T06, 35T36, and 35K02

[a] To determine actual protein concentrations in 4114 maize, no comparator lines were used. However, comparisons were made to the protein concentrations of 1507, 59122, and 1507x59122 maize lines in the same experiment to determine the applicability of previously conducted safety studies to 4114 maize.

Selection of Comparators, Experimental Controls, and Reference Lines for 4114 Maize

For the genetic characterization of the 4114 maize insertion, Pioneer proprietary maize inbreds were used as experimental controls (Table 3). These controls were selected because they represented the genetics of the maize lines that were crossed into the 4114 maize generations analyzed (Figure 10). Because certain probes were used that contained sequences in the endogenous maize genome, these controls containing the representative genetics eliminated background hybridization not related to the 4114 maize insertion.

For stability and inheritance of the DNA insertion, no comparators, controls, or reference lines were used or needed to conduct the analysis. For the measurement of concentrations of the expressed proteins in 4114 maize, no comparators were used, however 1507, 59122, and 1507x59122 maize reference lines were used to determine if 4114 maize protein concentrations were comparable to these lines. These comparisons were important to determine if previously conducted safety studies were applicable to the safety assessment of 4114 maize.

In the comparative assessments (*i.e.,* compositional analysis and agronomic performance), a control maize line was used (Table 3). For these analyses, the control comparator line had a genetic background approximately 99% similar to that of the 4114 maize generation used (*i.e.,* near-isoline), but did not go through the transformation process.

In addition, non-transgenic Pioneer commercial maize hybrid lines, as listed in Table 3, were used to obtain tolerance intervals for compositional and agronomic comparisons. These Pioneer commercial maize hybrids were chosen to represent a wide range of non-genetically modified varieties that would normally be planted commercially. These tolerance intervals represent the normal range of variation of the maize crop for compositional analytes and agronomic characteristics; they help to further determine the comparability of 4114 maize to conventional maize, if any statistical differences were observed.

2-D. Molecular Characterization of the Insertion in 4114 Maize

Molecular characterization of the inserted DNA evaluates the integrity of the introduced cassettes and provides a confirmation that the elements of the expression cassettes are intact. The inserted DNA is also evaluated over several generations to confirm its stability through traditional breeding methods.

The DNA insertion in 4114 maize was characterized by Southern blot analysis to evaluate the copy number, integrity, and stability of the inserted *cry*1F, *cry*34Ab1, *cry*35Ab1, and *pat* cassettes. As described earlier in Section 2-C, 4114 maize was produced by *Agrobacterium*-mediated transformation with plasmid PHP27118. The T-DNA region of PHP27118 contains four cassettes: *cry*1F, *cry*34Ab1, *cry*35Ab1, and *pat*. The *cry*1F cassette contains the *ubi*ZM1 promoter and intron, *cry*1F gene, and ORF25 terminator. The *cry*34Ab1 cassette contains a second copy of the *ubi*ZM1 promoter and intron, *cry*34Ab1 gene, and *pin*II terminator. The *cry*35Ab1 cassette contains the TA peroxidase promoter, *cry*35Ab1 gene, and *pin*II terminator, and the *pat* cassette contains the CaMV 35S promoter, *pat* gene, and CaMV 35S terminator. All probes used for the analysis are indicated on the schematic maps of PHP27118 and the PHP27118 T-DNA region (Figures 11 and 12, respectively) and outlined in Table 4. Plasmid PHP27118 was used as a positive control for probe hybridization and to verify fragment sizes internal to the T-DNA. The integration pattern of the insertion in 4114 maize was investigated using Southern blot analysis with *Bcl* I digested genomic DNA from individual plants of the T3 generation to determine copy number and *Hin*d III digested genomic DNA to determine insertion integrity. Copy number and integrity of each genetic element were determined using probes specific to each of the genetic elements present in the PHP27118 T-DNA region (Table 4, Figure 12). The stability of the insertion was analyzed using *Bcl* I digested genomic DNA from individual plants of the T2, T3, BC3F1[*3], and BC3F2[*2] generations (Section 2; Figure 10 and Table 3). Probes specific to each of the gene regions of PHP27118 T-DNA were used to confirm the stability of the insertion (Table 4, Figure 12). In addition, probes to the plasmid backbone region of PHP27118 located outside of the T-DNA region (Table 4, Figure 11) were used to show that these regions were not transferred to 4114 maize. Materials and methods for the molecular characterization of 4114 maize are described in Appendix 3.

Based on the Southern blot analyses described below, it was determined that a single, intact PHP27118 T-DNA was inserted into the genome of 4114 maize as diagramed in the insertion map (Figure 13) and that no region from the backbone of plasmid PHP27118 was inserted. In addition, these results confirmed the stability of the DNA insertion in 4114 maize across four breeding generations.

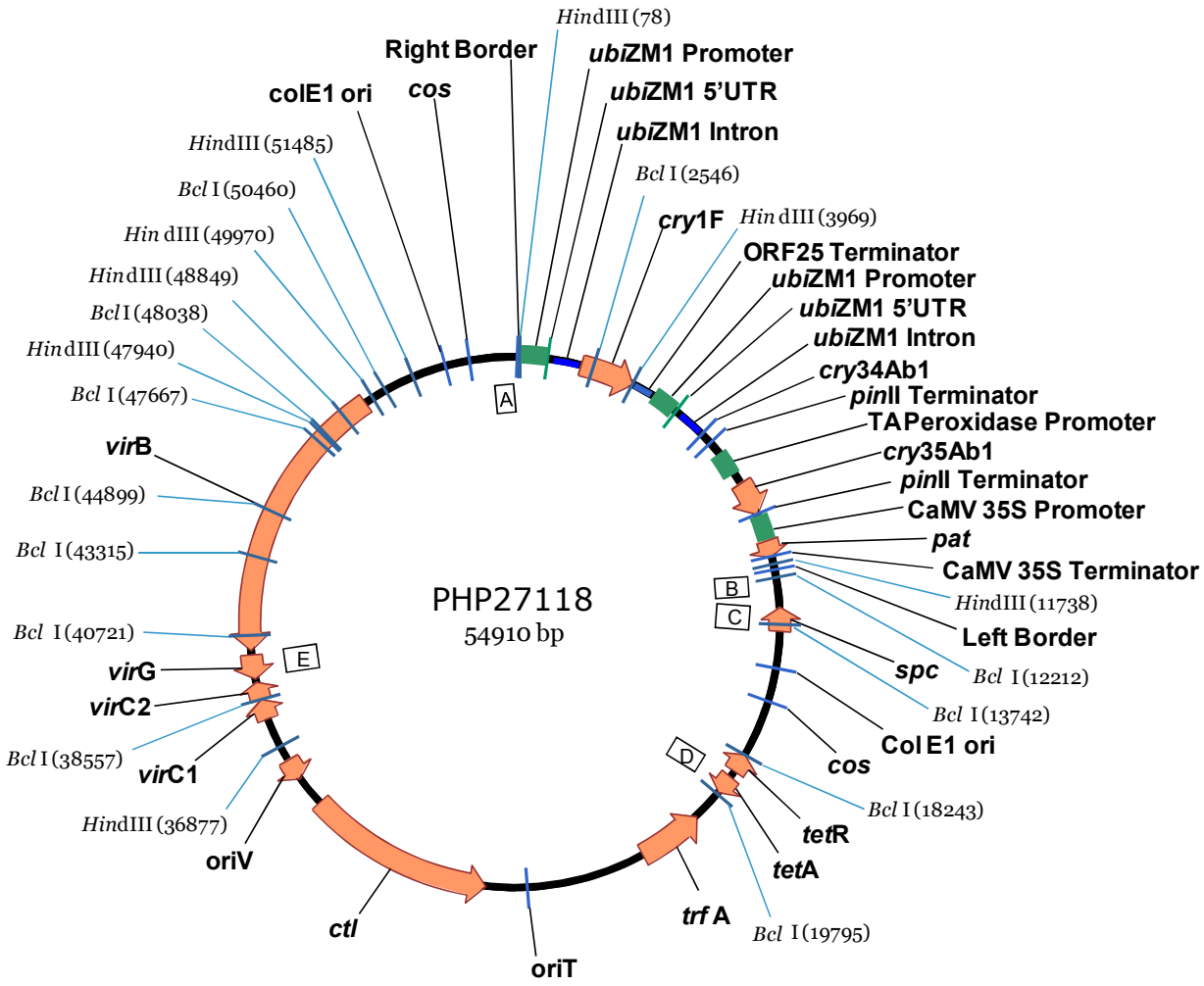


Figure 11. Plasmid Map of PHP27118

Schematic map of PHP27118 indicating *Bcl* I and *Hin*d III restriction enzyme sites with base pair positions. The Right Border and Left Border regions flank the T-DNA (Figure 12) that is expected to be transferred during *Agrobacterium*-mediated transformation. Plasmid backbone probe locations are indicated by boxes within the DNA map. **A**: RB probe; **B**: LB probe; **C**: *spc* probe; **D**: *tet* probe; **E**: *virG* probe.

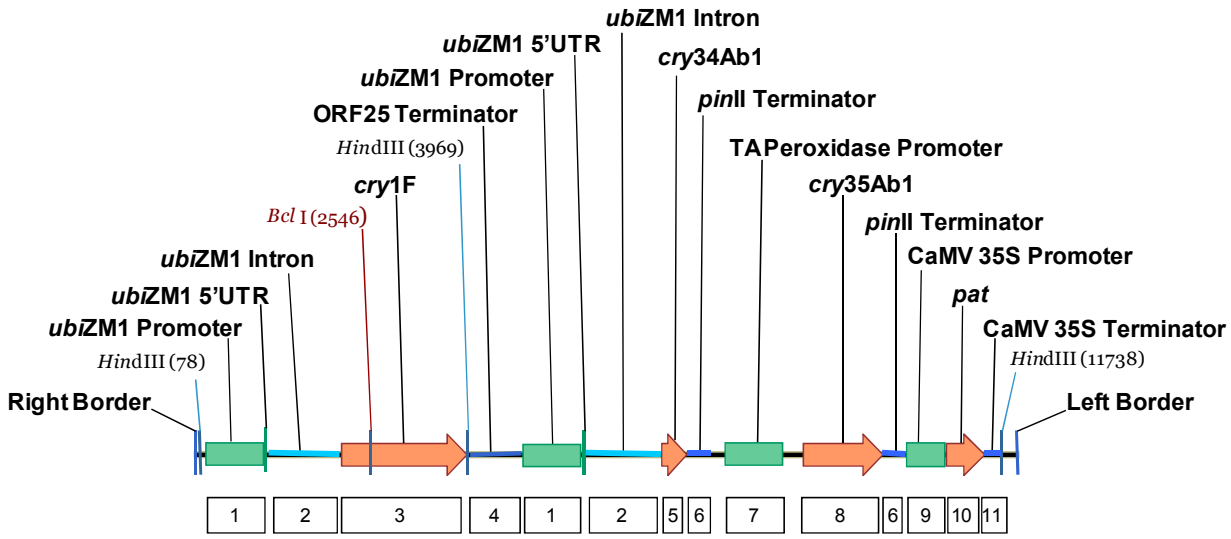


Figure 12. Map of PHP27118 T-DNA

Schematic map of T-DNA from PHP27118 indicating *Bcl* I and *Hin*d III restriction enzyme sites and the *cry*1F, *cry*34Ab1, *cry*35Ab1 and *pat* coding and regulatory regions. The T-DNA size is 11978 bp. The probe locations are indicated by boxes below the DNA map and are identified below:

Number	Probe Name
1	*ubi*ZM1 promoter
2	*ubi*ZM1 intron
3	*cry*1F
4	ORF25 terminator
5	*cry*34Ab1
6	*pin*II terminator
7	TA peroxidase promoter
8	*cry*35Ab1
9	35S promoter
10	*pat*
11	35S terminator

Table 4. Description of DNA Probes Used for Southern Blot Hybridization

Probe Name	Genetic Element	Figure *Probe*	Position on PHP27118 T-DNA (bp to bp) [a]	Position on PHP27118 (bp to bp) [b]	Length (bp)
*cry*1F[c]	*cry*1F gene	Figure 12 *Probe 3*	2133 to 3030 3038 to 3945	2133 to 3030 3038 to 3945	898 908
*cry*34Ab1	*cry*34Ab1 gene	Figure 12 *Probe 5*	6817 to 7133	6817 to 7133	317
*cry*35Ab1[c]	*cry*35Ab1 gene	Figure 12 *Probe 8*	8837 to 9303 9408 to 9979	8837 to 9303 9408 to 9979	467 572
pat	*pat* gene	Figure 12 *Probe 10*	10904 to 11451	10904 to 11451	548
*ubi*ZM1 promoter	*ubi*ZM1 promoter	Figure 12 *Probe 1*	150 to 1008 (copy 1) 4801 to 5659 (copy 2)	150 to 1008 (copy 1) 4801 to 5659 (copy 2)	859
*ubi*ZM1 intron	*ubi*ZM1 5' UTR and intron	Figure 12 *Probe 2*	1020 to 2100 (copy 1) 5671 to 6751 (copy 2)	1020 to 2100 (copy 1) 5671 to 6751 (copy 2)	1081
ORF25 terminator	ORF25 terminator	Figure 12 *Probe 4*	4003 to 4703	4003 to 4703	701
*pin*II terminator	*pin*II terminator	Figure 12 *Probe 6*	7235 to 7468 (copy 1) 10065 to 10298 (copy 2)	7235 to 7468 (copy 1) 10065 to 10298 (copy 2)	234
TA peroxidase promoter[c]	TA peroxidase promoter	Figure 12 *Probe 7*	7523 to 8415 8416 to 8813	7523 to 8415 8416 to 8813	893 398
35S promoter	CaMV 35S promoter	Figure 12 *Probe 9*	10383 to 10900	10383 to 10900	518
35S terminator	CaMV 35S terminator	Figure 12 *Probe 11*	11482 to 11692	11482 to 11692	211
LB	Plasmid backbone adjacent to T-DNA Left Border	Figure 11 *Probe B*	N/A[d]	12003 to 12348	346
spc	Spectinomycin resistance gene	Figure 11 *Probe C*	N/A[d]	13158 to 13932	775
tet[c]	Tetracycline resistance gene	Figure 11 *Probe D*	N/A[d]	19007 to 19545 19651 to 20108	539 458
*vir*G	*vir*G gene	Figure 11 *Probe E*	N/A[d]	39334 to 40077	744
RB	Plasmid backbone adjacent to T-DNA Right Border	Figure 11 *Probe A*	N/A[d]	54476 to 54865	390

[a] The probe position is based on the PHP27118 T-DNA map (Figure 12).

[b] The probe position is based on the PHP27118 plasmid map (Figure 11).

[c] The *cry*1F, *cry*35Ab1, TA peroxidase promoter, and *tet* probes each are comprised of two non-overlapping labeled fragments that are combined in the respective hybridization solutions.

[d] N/A = Not Applicable; these elements are not present in the PHP27118 T-DNA region.

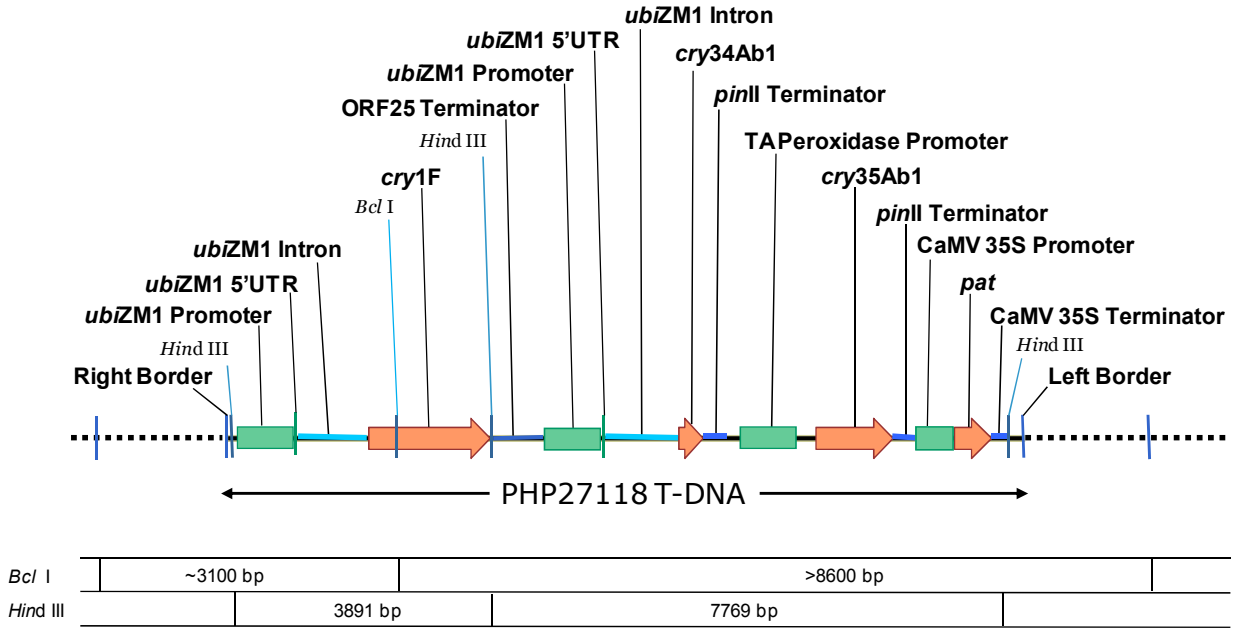


Figure 13. Map of the Insertion in 4114 Maize

Schematic map of the insertion in 4114 maize based on Southern blot analysis. The flanking maize genome is represented by the horizontal dotted line. A single, intact copy of the PHP27118 T-DNA integrated into the maize genome. *Bcl* I and *Hin*d 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 restriction enzyme sites outside the Right and Left Borders are not shown to scale.

Copy Number of the DNA Insertion in 4114 Maize

The restriction enzyme *Bcl* I has a single recognition site within the PHP27118 T-DNA (Figure 12) and provides information about the number of copies of the T-DNA that are integrated in the genome of 4114 maize. Hybridization with probes specific to the genetic elements of the PHP27118 T-DNA would indicate the number of copies of each element found in 4114 maize, as shown by the number of hybridizing bands. The site for *Bcl* I is located at bp 2546 of the T-DNA (Figure 12) and is predicted to yield fragments of greater than approximately 2500 bp and greater than approximately 9400 bp for a single inserted T-DNA (Table 5). The band of greater than 2500 bp will hybridize to the *ubi*ZM1 promoter and intron and the *cry*1F hybridization probes (Table 5, Figure 12). Probes specific to the following elements will hybridize to the fragment of greater than 9400 bp: the *ubi*ZM1 promoter and intron, the TA peroxidase promoter, CaMV 35S promoter, *cry*1F, *cry*34Ab1, *cry*35Ab1, *pat*, ORF25 terminator, *pin*II terminator, and CaMV 35S terminator (Figure 12). As the *Bcl* I restriction enzyme site is within the *cry*1F gene and between the duplicate copies of the *ubi*ZM1 promoter and intron regions, probes for the *cry*1F gene, the *ubi*ZM1 promoter and *ubi*ZM1 intron will hybridize to both fragments (Table 5, Figure 12). Therefore, the appearance of two bands for these probes indicates a single copy of the T-DNA. Although there are two copies of the *pin*II terminator in the T-DNA, both are located on the same *Bcl* I fragment and will thus yield a single band (Table 5, Figure 12). A single band for the remaining probes indicates a single copy of those genetic elements.

Hybridization of *Bcl* I-digested genomic DNA from 4114 maize with the *ubi*ZM1 promoter and intron probes resulted in a band of approximately 3100 bp and a band of greater than 8600 bp (Table 5, Figure 14). Based on the expected sizes for the genomic fragments, the band of approximately 3100 bp is derived from the Right Border or 5' end of the DNA insertion and the band of greater than 8600 bp is the band expected to be greater than 9400 bp and is derived from the Left Border or 3' end of the 4114 maize insertion. A band of approximately 4800 bp was observed in the 4114 maize plants and the PHWWE control maize with both probes (Table 5, Figure 14). This band was determined to be due to hybridization of the *ubi*ZM1 promoter and intron probes to the endogenous copy of these elements found in the native maize genome based upon its presence in the non-transformed PHWWE control maize line. A similar but slightly lower band is also seen in the non-transgenic PH09B control maize line. The presence of the two expected bands with the *ubi*ZM1 promoter and intron probes, and no additional insertion-derived bands, indicates that there is a single copy of the PHP27118 T-DNA in 4114 maize.

The *Bcl* I-digested PHP27118 plasmid lanes did not produce the expected size bands and produced two bands above the 8600 bp marker band; these bands were characteristic of an undigested plasmid and indicated that the plasmid DNA was likely methylated (Table 5, Figure 14). Sensitivity of the *Bcl* I enzyme to bacterial plasmid DNA methylation is well known and there is a bacterial Dam methylase recognition sequence in the site ($^{5'}$GATC$^{3'}$) (New England Biolabs, 2002). In all *Bcl* I sites (recognition sequence $^{5'}$TG<u>A</u>TCA$^{3'}$) 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-digested plasmid, only probe hybridization was confirmed.

Hybridization of the *Bcl* I-digested DNA with the *cry*1F probe also resulted in the same two bands of approximately 3100 bp and greater than 8600 bp (Table 5, Figure 15). Two bands are expected with the *cry*1F probe due to the location of the *Bcl* I restriction site within the *cry*1F gene (Figure 12), and the presence of these two bands indicates there is a single copy of the *cry*1F gene in 4114 maize.

When hybridized to the ORF25 terminator probe, the same blot resulted in a single band of greater than 8600 bp (Table 5, Figure 15). As the ORF25 terminator element is located on the 3' or Left Border side of the *Bcl* I site in the PHP27118 T-DNA (Figure 12), this demonstrates that the greater than 8600 bp band is indeed associated with the elements on this portion of the T-DNA.

The same band of greater than 8600 bp was observed when the *Bcl* I Southern blot was hybridized with the *cry*34Ab1 and *pin*II terminator probes (Table 5, Figure 16), the TA peroxidase promoter and *cry*35Ab1 probes (Table 5, Figure 17), and the 35S promoter, *pat*, and 35S terminator probes (Table 5, Figure 18). Although there are two copies of the *pin*II terminator in the PHP27118 T-DNA, both copies are located on the same *Bcl* I fragment so that they result in a single hybridizing band on the Southern blot. The presence of single bands with all of these elements confirms that there is a single copy of the PHP27118 T-DNA inserted in 4114 maize.

When taken together with the restriction map of the PHP27118 T-DNA (Figure 12), the presence of single bands with the ORF25 terminator, *cry*34Ab1, *pin*II terminator, TA peroxidase promoter, *cry*35Ab1, 35S promoter, *pat*, and 35S terminator probes, along with the expected two bands for the *ubi*ZM1 promoter, *ubi*ZM1 intron, and *cry*1F probes, demonstrate that there is a single inserted PHP27118 T-DNA in 4114 maize.

Additionally, *Bcl* I digestion of the 4114 maize insertion provides an event-specific hybridization pattern when certain probes are used. If the hybridization pattern is identical between different plants and generations, this demonstrates the 5' and 3' regions of the DNA insertion are stable during the breeding process and is discussed later in this section.

Table 5. Predicted and Observed Hybridizing Bands on Southern Blots; *Bcl* I Digest

Probe	Figure	Predicted Fragment Size from PHP27118 T-DNA (bp)[a]	Predicted Fragment Size from Plasmid PHP27118 (bp)[b]	Observed Fragment Size in 4114 Maize (bp)[c]
*ubi*ZM1 promoter	14	>9400 (border)[d, e] >2500 (border)[d, e]	9666 6996	>8600 ~3100 ~4800*
*ubi*ZM1 intron	14	>9400 (border)[d, e] >2500 (border)[d, e]	9666 6996	>8600 ~3100 ~4800*
*cry*1F	15, 14	>9400 (border)[d, f] >2500 (border)[d,f]	9666 6996	>8600 ~3100
ORF25 terminator	15	>9400 (border)[d]	9666	>8600
*cry*34Ab1	16, 23	>9400 (border)[d]	9666	>8600
*pin*II terminator	16	>9400 (border)[d, g]	9666	>8600
TA peroxidase promoter	17	>9400 (border)[d]	9666	>8600
*cry*35Ab1	17, 24	>9400 (border)[d]	9666	>8600
35S promoter	18	>9400 (border)[d]	9666	>8600
pat	18, 25	>9400 (border)[d]	9666	>8600
35S terminator	18	>9400 (border)[d]	9666	>8600
LB	28	N/A[h]	1530 (25139)[i]	No hybridization
spc	29	N/A[h]	1530 (25139)[i]	No hybridization
tet	30	N/A[h]	1552 (25139)[i]	No hybridization
*vir*G	31	N/A[h]	2164 (11063)[i]	No hybridization
RB	32	N/A[h]	6996 (3503)[i]	No hybridization

Note: An asterisk (*) and gray shading indicates the designated band is due to hybridization to endogenous sequences. These bands were identified in the maize control lines that were analyzed.

[a] Predicted size for hybridization in genomic DNA samples is based on the map of the T-DNA from PHP27118 (Figure 12). Border fragment sizes are rounded to the nearest 100 bp.

[b] Predicted size is based on the plasmid map of PHP27118 (Figure 11) and is the size expected for *Bcl* I digestion of an unmethylated plasmid. The methylated PHP27118 used in this study did not digest with *Bcl* I and therefore did not yield these fragments.

[c] Observed fragment sizes are approximated from the DIG-labeled DNA Molecular Weight Marker VII fragments on the Southern blots. Due to the inability to determine exact sizes on the blot, all approximated values are rounded to the nearest 100 bp.

[d] Border fragments are those in which one restriction site is in the inserted T-DNA and the other site is located in the flanking genomic DNA, providing a fragment of unique size for a given insertion. Border fragment sizes are rounded to the nearest 100 bp.

[e] There are two copies of the *ubi*ZM1 promoter and intron elements, located on either side of the *Bcl* I site, so both fragments will hybridize to these probes.

[f] The *Bcl* I site is located within the *cry*1F gene, so both fragments hybridize to the *cry*1F probe.

[g] Both copies of the *pin*II terminator in the PHP27118 T-DNA are located on the same *Bcl* I fragment.

[h] N/A=Not Applicable; these elements are not found on the PHP27118 T-DNA.

[i] The fragment size in parenthesis is for digestion of PHP27118 with *Hin*d III, which was included in Lanes 15 and 16 of certain *Bcl* I blots to show that the plasmid can be cut by a restriction enzyme that is not sensitive to methylation.

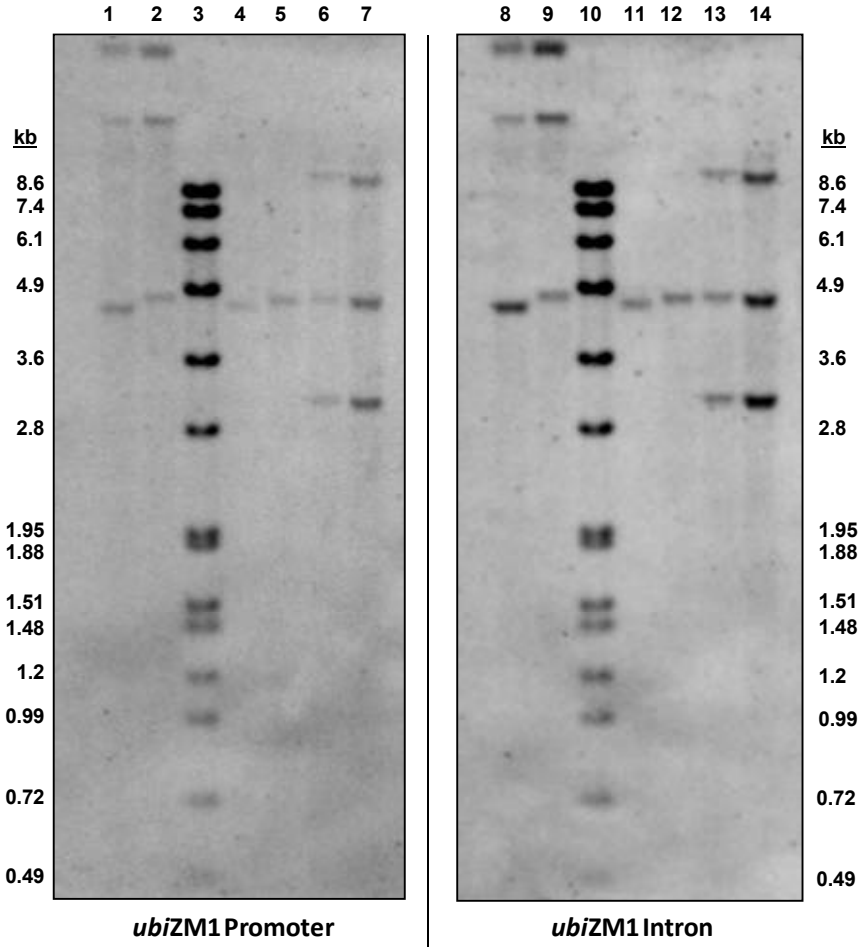


Figure 14. Southern Blot Analysis of the T3 Generation of 4114 Maize; *Bcl* I Digested DNA with *ubi*ZM1 Promoter and *ubi*ZM1 Intron Probes

DNA isolated from individual plants of 4114 maize was digested with *Bcl* I and hybridized to the *ubi*ZM1 promoter and *ubi*ZM1 intron probes. Approximately 3 - 4 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP27118 at the indicated approximate gene copy number and 4 µg of genomic DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	1 copy PHP27118 + PH09B control
2	3 copy PHP27118 + PHWWE control
3	DIGVII marker
4	PH09B control
5	PHWWE control
6	4114 maize plant 20 (T3 generation)
7	4114 maize plant 22 (T3 generation)

Lane	Sample
8	1 copy PHP27118 + PH09B control
9	3 copy PHP27118 + PHWWE control
10	DIGVII marker
11	PH09B control
12	PHWWE control
13	4114 maize plant 20 (T3 generation)
14	4114 maize plant 22 (T3 generation)

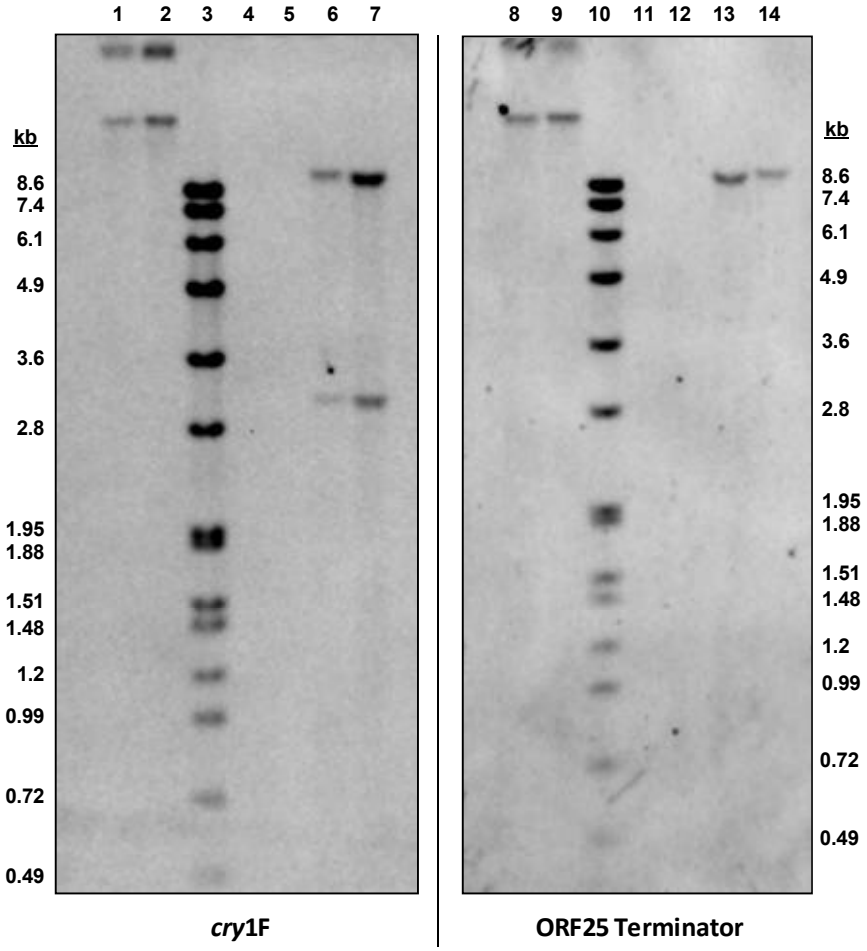


Figure 15. Southern Blot Analysis of the T3 Generation of 4114 Maize; *Bcl* I Digested DNA with *cry*1F and ORF25 Terminator Probes

DNA isolated from individual plants of 4114 maize was digested with *Bcl* I and hybridized to the *cry*1F and ORF25 terminator probes. Approximately 3 - 4 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP27118 at the indicated approximate gene copy number and 4 µg of genomic DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	1 copy PHP27118 + PH09B control
2	3 copy PHP27118 + PHWWE control
3	DIGVII marker
4	PH09B control
5	PHWWE control
6	4114 maize plant 20 (T3 generation)
7	4114 maize plant 22 (T3 generation)

Lane	Sample
8	1 copy PHP27118 + PH09B control
9	3 copy PHP27118 + PHWWE control
10	DIGVII marker
11	PH09B control
12	PHWWE control
13	4114 maize plant 20 (T3 generation)
14	4114 maize plant 22 (T3 generation)

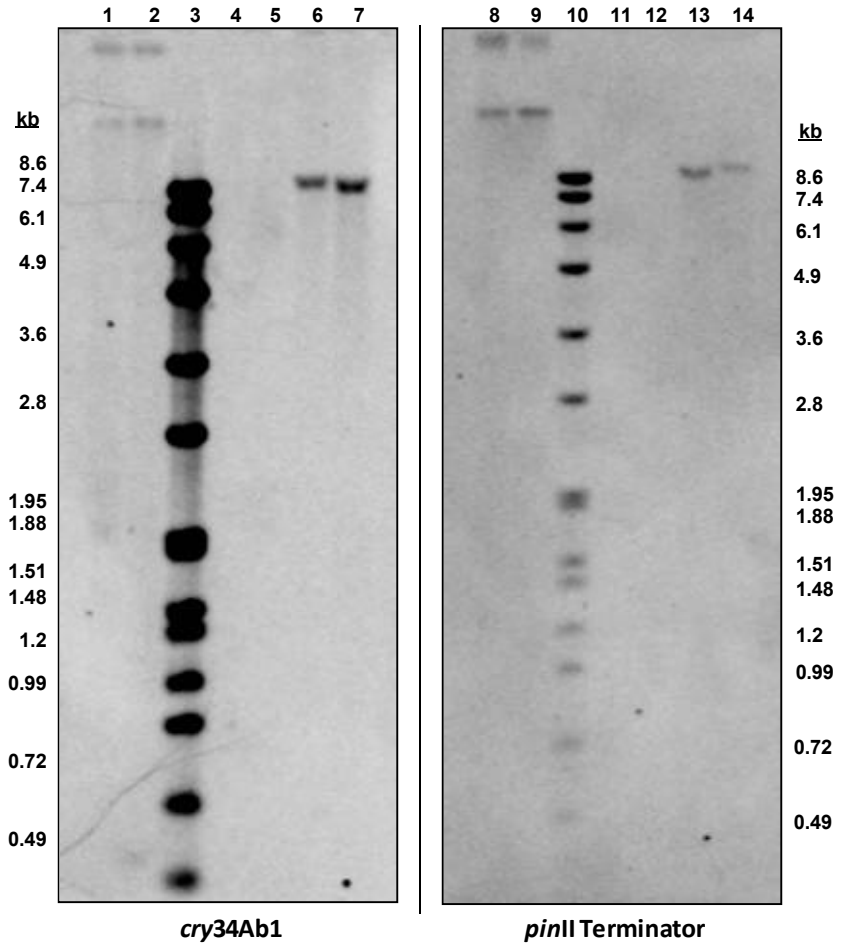


Figure 16. Southern Blot Analysis of the T3 Generation of 4114 Maize; *Bcl* I Digested DNA with *cry*34Ab1 and *pin*II Terminator Probes

DNA isolated from individual plants of 4114 maize was digested with *Bcl* I and hybridized to the *cry*34Ab1 and *pin*II terminator probes. Approximately 3 - 4 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP27118 at the indicated approximate gene copy number and 4 µg of genomic DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	1 copy PHP27118 + PH09B control
2	3 copy PHP27118 + PHWWE control
3	DIGVII marker
4	PH09B control
5	PHWWE control
6	4114 maize plant 20 (T3 generation)
7	4114 maize plant 22 (T3 generation)

Lane	Sample
8	1 copy PHP27118 + PH09B control
9	3 copy PHP27118 + PHWWE control
10	DIGVII marker
11	PH09B control
12	PHWWE control
13	4114 maize plant 20 (T3 generation)
14	4114 maize plant 22 (T3 generation)

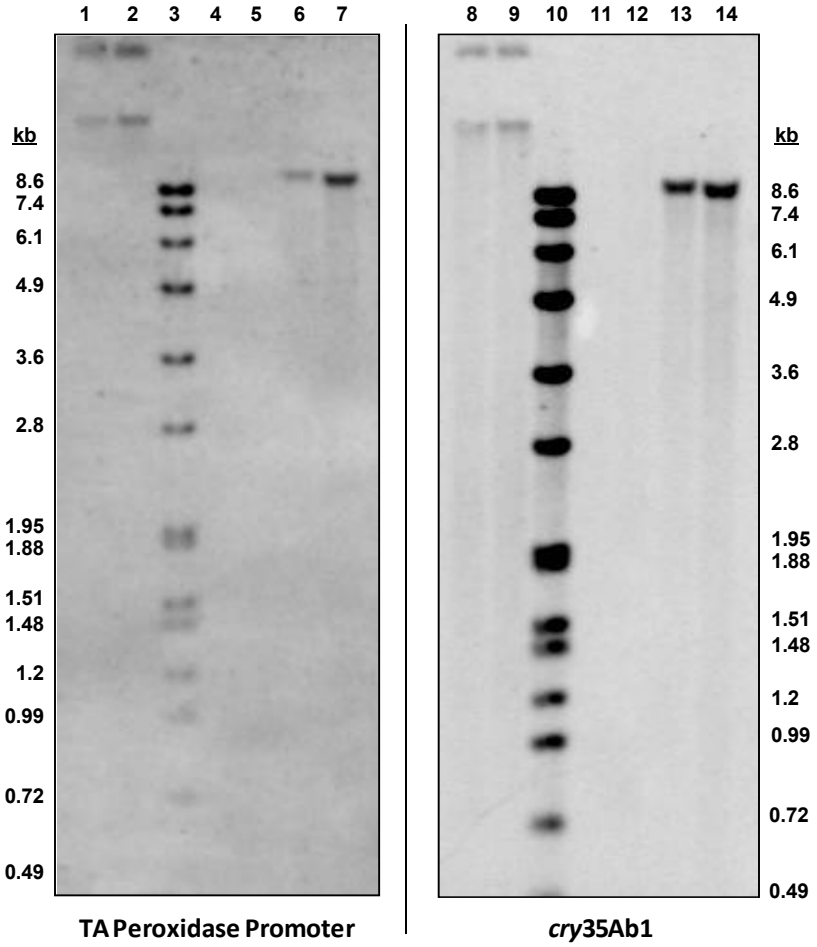


Figure 17. Southern Blot Analysis of the T3 Generation of 4114 Maize; *Bcl* I Digested DNA with TA Peroxidase Promoter and *cry*35Ab1 Probes

DNA isolated from individual plants of 4114 maize was digested with *Bcl* I and hybridized to the TA peroxidase promoter and *cry*35Ab1 probes. Approximately 3 - 4 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP27118 at the indicated approximate gene copy number and 4 µg of genomic DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	1 copy PHP27118 + PH09B control
2	3 copy PHP27118 + PHWWE control
3	DIGVII marker
4	PH09B control
5	PHWWE control
6	4114 maize plant 20 (T3 generation)
7	4114 maize plant 22 (T3 generation)

Lane	Sample
8	1 copy PHP27118 + PH09B control
9	3 copy PHP27118 + PHWWE control
10	DIGVII marker
11	PH09B control
12	PHWWE control
13	4114 maize plant 20 (T3 generation)
14	4114 maize plant 22 (T3 generation)

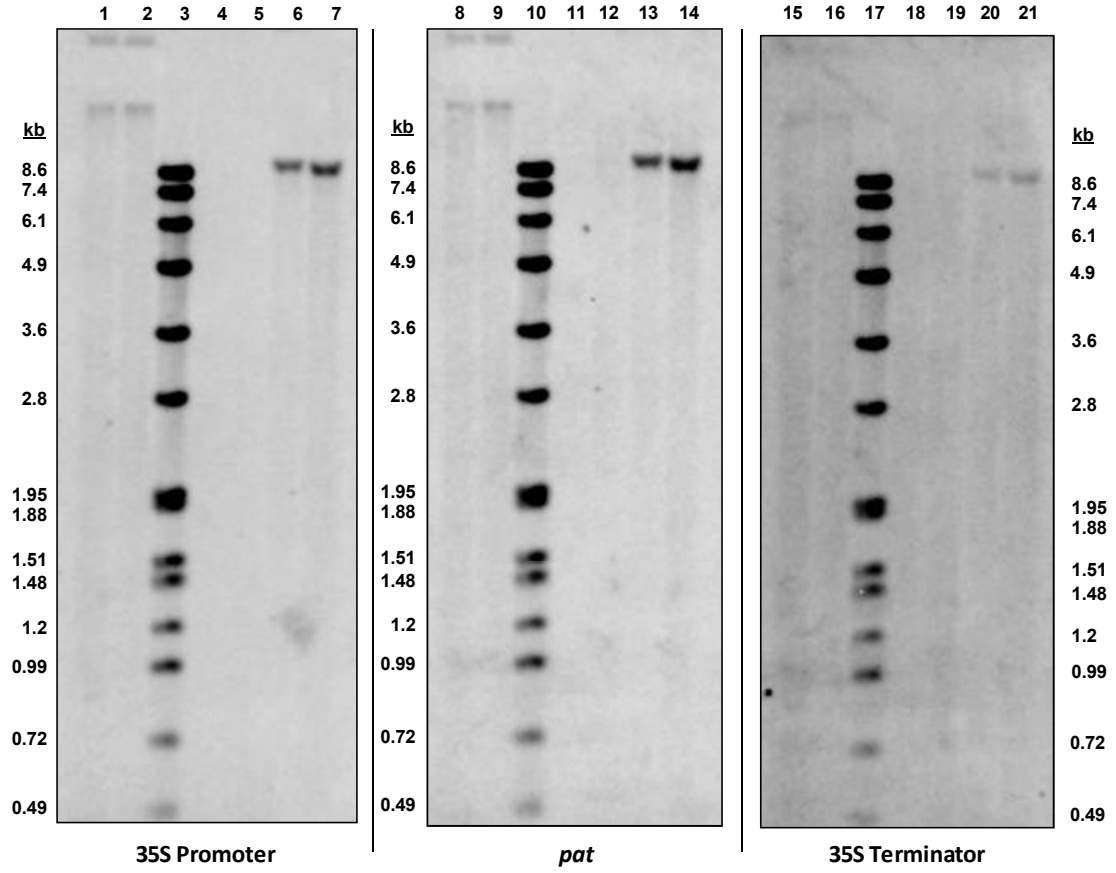


Figure 18. Southern Blot Analysis of the T3 Generation of 4114 Maize; *Bcl* I Digested DNA with 35S Promoter, *pat*, and 35S Terminator Probes

DNA isolated from individual plants of 4114 maize was digested with *Bcl* I and hybridized to the 35S promoter, *pat*, and 35S Terminator probes. Approximately 3 - 4 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP27118 at the indicated approximate gene copy number and 4 µg of genomic DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	1 copy PHP27118 + PH09B control
2	3 copy PHP27118 + PHWWE control
3	DIGVII marker
4	PH09B control
5	PHWWE control
6	4114 maize plant 20 (T3 generation)
7	4114 maize plant 22 (T3 generation)
8	1 copy PHP27118 + PH09B control
9	3 copy PHP27118 + PHWWE control
10	DIGVII marker
11	PH09B control

Lane	Sample
12	PHWWE control
13	4114 maize plant 20 (T3 generation)
14	4114 maize plant 22 (T3 generation)
15	1 copy PHP27118 + PH09B control
16	3 copy PHP27118 + PHWWE control
17	DIGVII marker
18	PH09B control
19	PHWWE control
20	4114 maize plant 20 (T3 generation)
21	4114 maize plant 22 (T3 generation)

Integrity of the DNA Insertion in 4114 Maize

The restriction enzyme *Hin*d III was used to confirm the integrity of the PHP27118 T-DNA insertion in 4114 maize. There are three *Hin*d III sites within the PHP27118 T-DNA: one site at bp 78, one after the *cry*1F gene at bp 3969, and one at bp 11738 just inside the Left Border of the T-DNA (Figure 12). An internal fragment of 3891 bp is expected to hybridize to the *cry*1F gene probe (Table 6, Figure 12), and another internal fragment of 7769 bp is expected to hybridize to the probes for the ORF25 terminator, *cry*34Ab1 gene, *pin*II terminator, *cry*35Ab1 gene, TA peroxidase promoter, *pat* gene and the CaMV 35S promoter and terminator regions (Table 6, Figure 12). Although there are two copies of the *pin*II terminator in the PHP27118 T-DNA, both copies are found on the same *Hin*d III fragment so the probe would hybridize to the single 7769 bp internal fragment. Because the central *Hin*d III restriction enzyme site is located between the duplicate copies of the *ubi*ZM1 promoter and intron regions, probes for these elements are expected to hybridize to both fragments (Table 6, Figure 12). The absence of any insert derived bands other than the expected bands provides a strong indication that the T-DNA is intact and was not truncated upon insertion. The *Hin*d III plasmid positive control run alongside the 4114 maize DNA samples was used to confirm that the sizes of the observed bands were identical to these internal fragments.

Hybridization of *Hin*d III-digested genomic DNA from 4114 maize with the *ubi*ZM1 promoter and intron probes resulted in the expected bands of 3891 bp and 7769 bp (Table 6, Figure 19). An additional band of approximately 6400 bp was observed in the 4114 plants and the PHWWE control maize with both probes (Table 6, Figure 19). This band resulted from the hybridization of the *ubi*ZM1 promoter and intron probes to the endogenous copy of these elements in the native maize genome based upon its presence in the PHWWE control maize line. Hybridization with the *cry*1F probe resulted in the expected band of 3891 bp in the 4114 maize plants (Table 6, Figure 20). As expected, the 7769 bp band was observed in the 4114 maize plants with the ORF25 terminator probe (Table 6, Figure 20), with the *cry*34Ab1 and *pin*II terminator probes (Table 6, Figure 21), the TA peroxidase promoter and *cry*35Ab1 probes (Table 6, Figure 22), and the 35S promoter, *pat*, and 35S terminator probes (Table 6, Figure 23). The presence of the expected size bands that were identical to the internal bands from the PHP27118 plasmid with all of these elements, and no other insertion-derived bands, indicate that the PHP27118 T-DNA inserted intact in 4114 maize and there were no truncated copies of the T-DNA.

Table 6. Predicted and Observed Hybridizing Bands on Southern Blots; *Hin*d III Digest

Probe	Figure	Predicted Fragment Size from PHP27118 T-DNA (bp)[a]	Predicted Fragment Size from Plasmid PHP27118 (bp)[b]	Observed Fragment Size in 4114 Maize (bp)[c]
*ubi*ZM1 promoter	19	7769[d] 3891[d]	7769 3891	7769[e] 3891[e]
				~6400*
*ubi*ZM1 intron	19	7769[d] 3891[d]	7769 3891	7769[e] 3891[e]
				~6400*
*cry*1F	20	3891	3891	3891[e]
ORF25 terminator	20	7769	7769	7769[e]
*cry*34Ab1	21	7769	7769	7769[e]
*pin*II terminator	21	7769[f]	7769	7769[e]
TA peroxidase promoter	22	7769	7769	7769[e]
*cry*35Ab1	22	7769	7769	7769[e]
35S promoter	23	7769	7769	7769[e]
pat	23	7769	7769	7769[e]
35S terminator	23	7769	7769	7769[e]

Note: An asterisk (*) and gray shading indicates the designated band is due to hybridization to endogenous sequences. These bands were identified in the maize control lines that were analyzed.

[a] Predicted size for hybridization in genomic DNA samples is based on the map of the T-DNA from PHP27118 (Figure 12).

[b] Predicted size is based on the plasmid map of PHP27118 (Figure 11).

[c] Observed fragment sizes are approximated from the DIG-labeled DNA Molecular Weight Marker VII fragments on the Southern blots. Due to the inability to determine exact sizes on the blot, all approximated values are rounded to the nearest 100 bp.

[d] There are two copies of the *ubi*ZM1 promoter and intron elements, located on either side of the central *Hin*d III site, so both fragments will hybridize to these probes.

[e] Size is same as predicted based on equal migration as the control plasmid fragment on the Southern blot.

[f] Both copies of the *pin*II terminator in the PHP27118 T-DNA are located on the same *Hin*d III fragment.

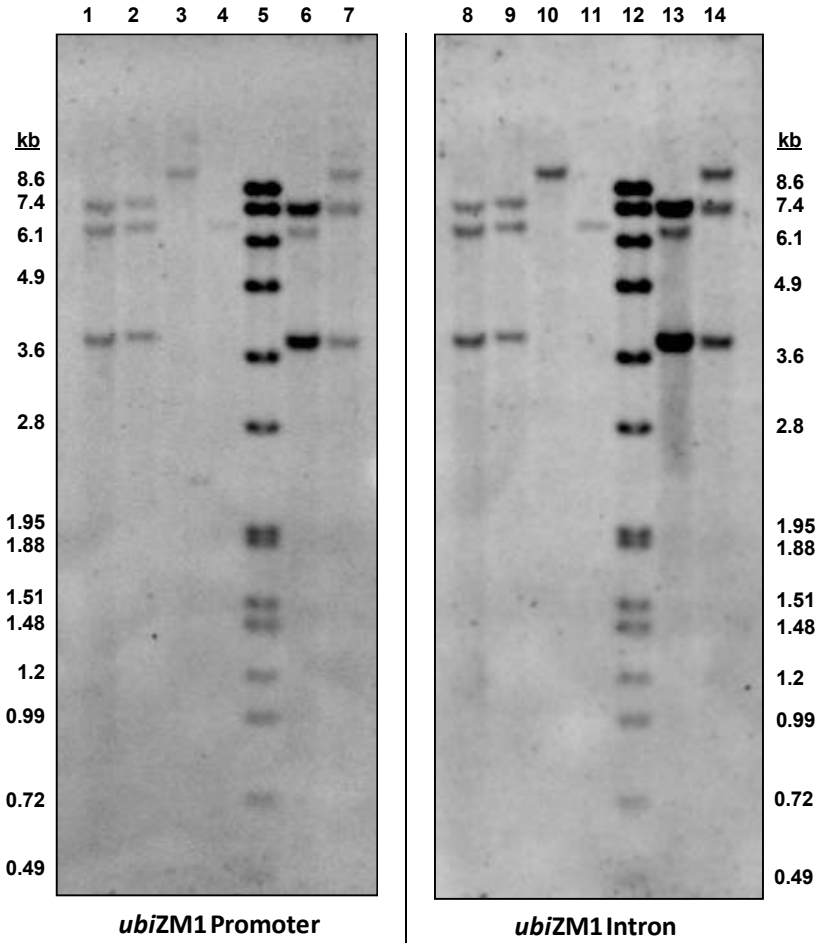


Figure 19. Southern Blot Analysis of the T3 Generation of 4114 Maize; *Hin*d III Digested DNA with *ubi*ZM1 Promoter and *ubi*ZM1 Intron Probes

DNA isolated from individual plants of 4114 maize was digested with *Hin*d III and hybridized to the *ubi*ZM1 promoter and *ubi*ZM1 intron probes. Approximately 3 - 4 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP27118 at the indicated approximate gene copy number and 4 µg of genomic DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	4114 maize plant 20 (T3 generation)
2	4114 maize plant 22 (T3 generation)
3	PH09B control
4	PHWWE control
5	DIGVII marker
6	3 copy PHP27118 + PHWWE control
7	1 copy PHP27118 + PH09B control

Lane	Sample
8	4114 maize plant 20 (T3 generation)
9	4114 maize plant 22 (T3 generation)
10	PH09B control
11	PHWWE control
12	DIGVII marker
13	3 copy PHP27118 + PHWWE control
14	1 copy PHP27118 + PH09B control

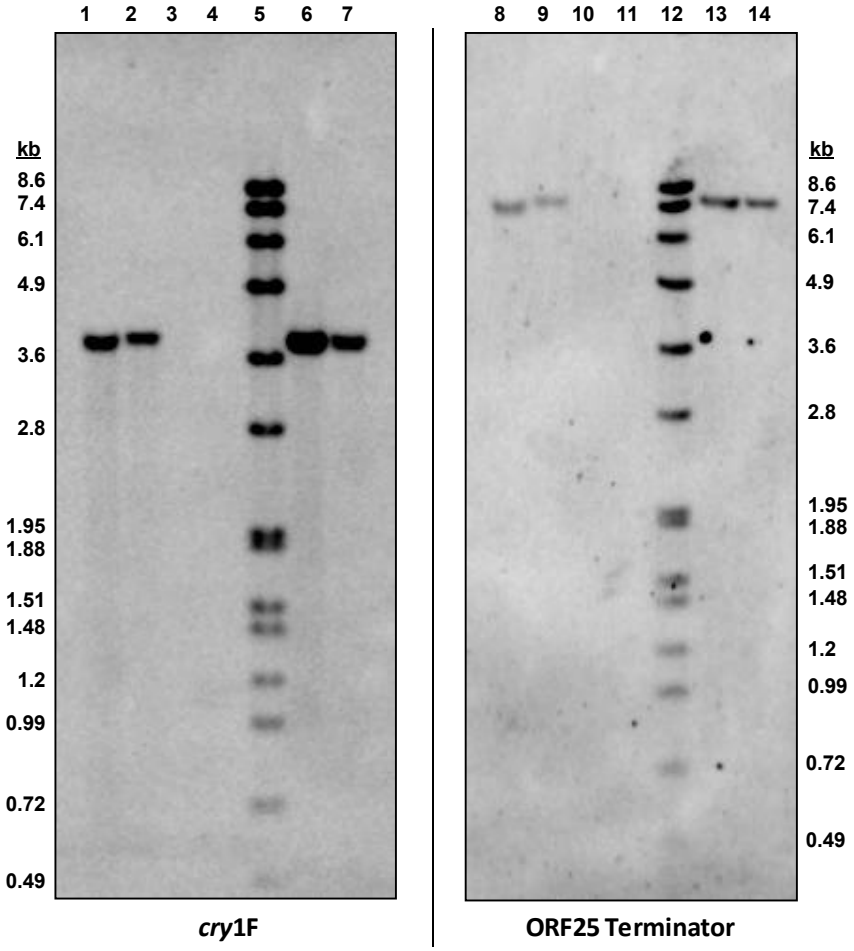


Figure 20. Southern Blot Analysis of the T3 Generation of 4114 Maize; *Hin*d III Digested DNA with *cry*1F and ORF25 Terminator Probes

DNA isolated from individual plants of 4114 maize was digested with *Hin*d III and hybridized to the *cry*1F and ORF25 terminator probes. Approximately 3 - 4 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP27118 at the indicated approximate gene copy number and 4 µg of genomic DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	4114 maize plant 20 (T3 generation)
2	4114 maize plant 22 (T3 generation)
3	PH09B control
4	PHWWE control
5	DIGVII marker
6	3 copy PHP27118 + PHWWE control
7	1 copy PHP27118 + PH09B control

Lane	Sample
8	4114 maize plant 20 (T3 generation)
9	4114 maize plant 22 (T3 generation)
10	PH09B control
11	PHWWE control
12	DIGVII marker
13	3 copy PHP27118 + PHWWE control
14	1 copy PHP27118 + PH09B control

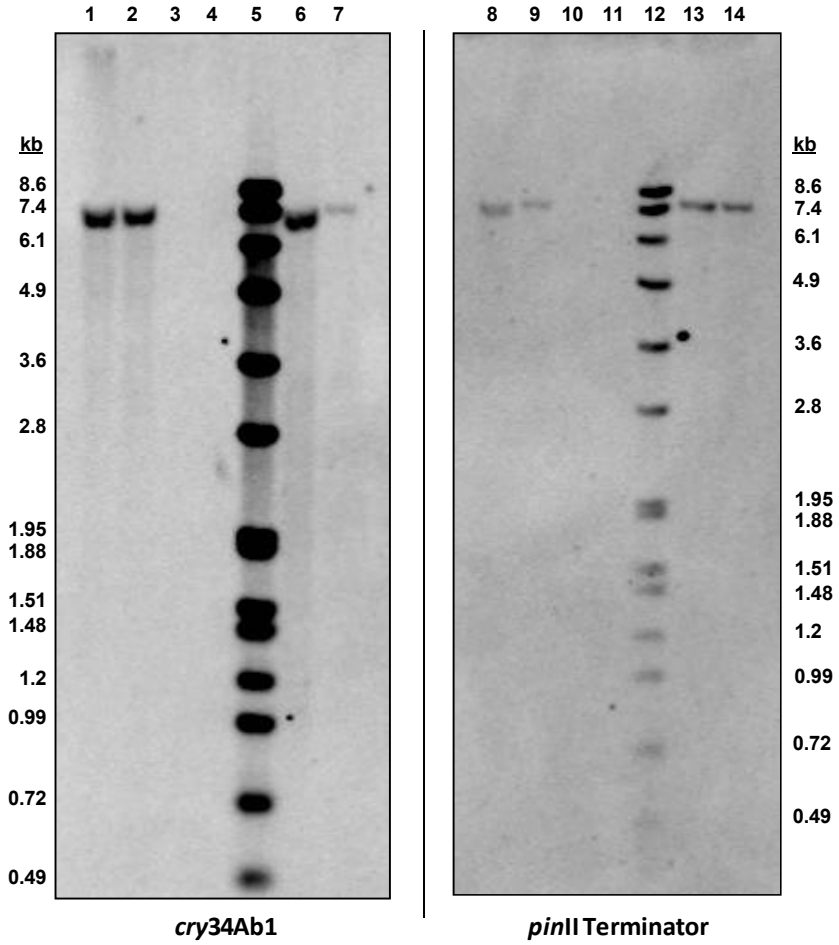


Figure 21. Southern Blot Analysis of the T3 Generation of 4114 Maize; *Hin*d III Digested DNA with *cry*34Ab1 and *pin*II Terminator Probes

DNA isolated from individual plants of 4114 maize was digested with *Hin*d III and hybridized to the *cry*34Ab1 and *pin*II terminator probes. Approximately 3 - 4 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP27118 at the indicated approximate gene copy number and 4 µg of genomic DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	4114 maize plant 20 (T3 generation)
2	4114 maize plant 22 (T3 generation)
3	PH09B control
4	PHWWE control
5	DIGVII marker
6	3 copy PHP27118 + PHWWE control
7	1 copy PHP27118 + PH09B control

Lane	Sample
8	4114 maize plant 20 (T3 generation)
9	4114 maize plant 22 (T3 generation)
10	PH09B control
11	PHWWE control
12	DIGVII marker
13	3 copy PHP27118 + PHWWE control
14	1 copy PHP27118 + PH09B control

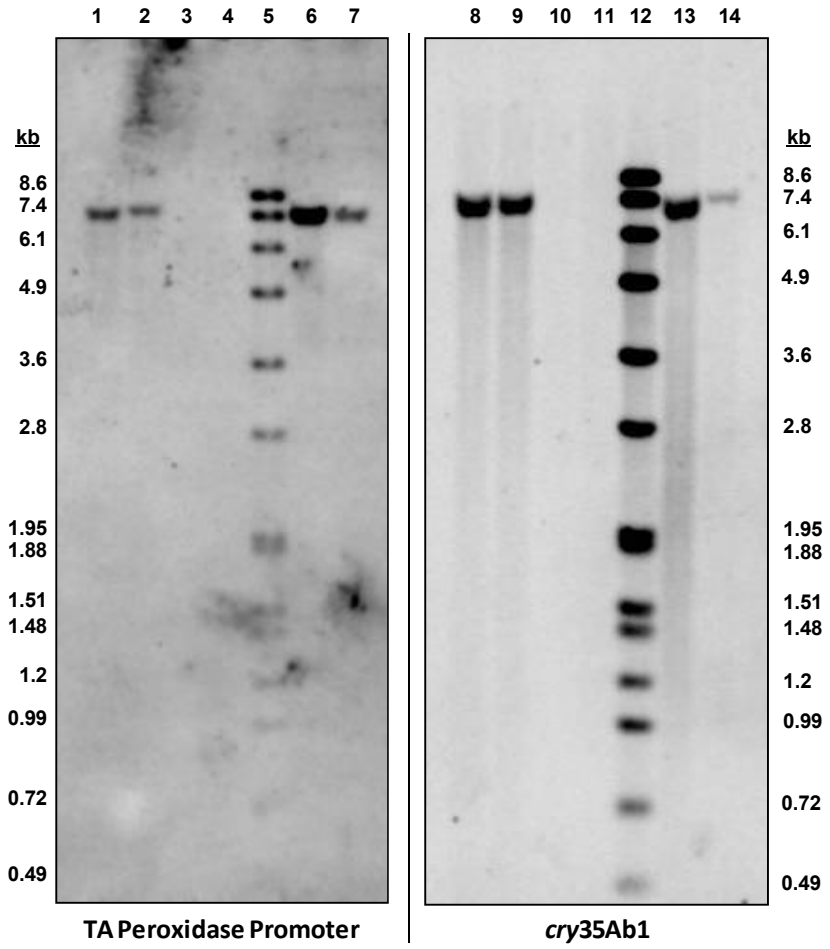


Figure 22. Southern Blot Analysis of the T3 Generation of 4114 Maize; *Hin*d III Digested DNA with TA Peroxidase Promoter and *cry*35Ab1 Probes

DNA isolated from individual plants of 4114 maize was digested with *Hin*d III and hybridized to the TA peroxidase promoter and *cry*35Ab1 probes. Approximately 3 - 4 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP27118 at the indicated approximate gene copy number and 4 µg of genomic DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb). **Note:** Blotches on the TA peroxidase panel are due to non-specific chemiluminescence from the detection process and are not due to hybridization of the probe to maize DNA.

Lane	Sample
1	4114 maize plant 20 (T3 generation)
2	4114 maize plant 22 (T3 generation)
3	PH09B control
4	PHWWE control
5	DIGVII marker
6	3 copy PHP27118 + PHWWE control
7	1 copy PHP27118 + PH09B control

Lane	Sample
8	4114 maize plant 20 (T3 generation)
9	4114 maize plant 22 (T3 generation)
10	PH09B control
11	PHWWE control
12	DIGVII marker
13	3 copy PHP27118 + PHWWE control
14	1 copy PHP27118 + PH09B control

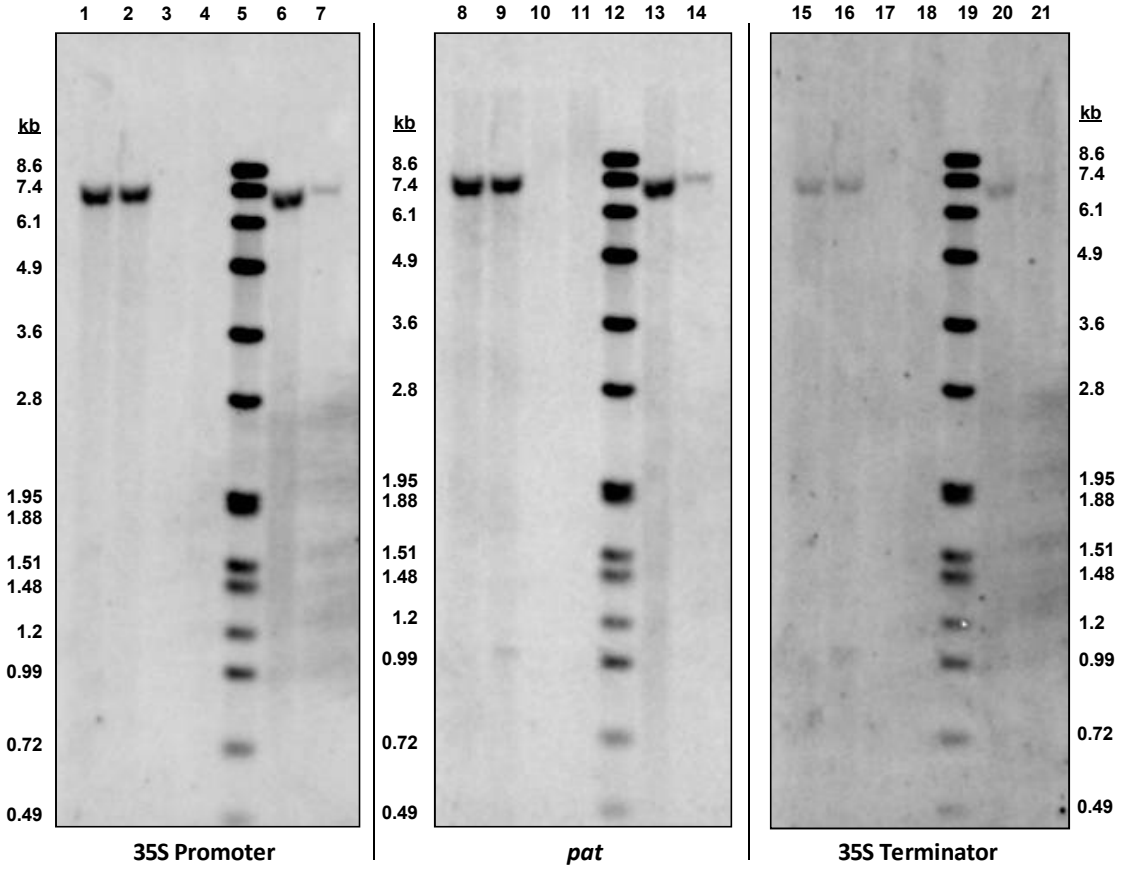


Figure 23. Southern Blot Analysis of the T3 Generation of 4114 Maize; *Hin*d III Digested DNA with 35S Promoter, *pat*, and 35S Terminator Probes

DNA isolated from individual plants of 4114 maize was digested with *Hin*d III and hybridized to the 35S promoter, *pat*, and 35S Terminator probes. Approximately 3 - 4 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP27118 at the indicated approximate gene copy number and 4 µg of genomic DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	4114 maize plant 20 (T3 generation)
2	4114 maize plant 22 (T3 generation)
3	PH09B control
4	PHWWE control
5	DIGVII marker
6	3 copy PHP27118 + PHWWE control
7	1 copy PHP27118 + PH09B control
8	4114 maize plant 20 (T3 generation)
9	4114 maize plant 22 (T3 generation)
10	PH09B control
11	PHWWE control

Lane	Sample
12	DIGVII marker
13	3 copy PHP27118 + PHWWE control
14	1 copy PHP27118 + PH09B control
15	4114 maize plant 20 (T3 generation)
16	4114 maize plant 22 (T3 generation)
17	PH09B control
18	PHWWE control
19	DIGVII marker
20	3 copy PHP27118 + PHWWE control
21	1 copy PHP27118 + PH09B control

Stability of the Insertion in 4114 Maize

Stability of the 4114 maize insertion was confirmed by performing Southern blot analysis on *Bcl* I-digested genomic DNA from individual plants of four generations of 4114 maize: T2, T3, BC3F1[*3], and BC3F2[*2]. As described earlier, hybridization of *Bcl* I-digested DNA to the probe for the *cry*1F gene resulted in an event-specific hybridization pattern consisting of a band of approximately 3100 bp and a band of greater than 8600 bp (Figure 13). This hybridization pattern can be used to demonstrate the stability of the 5' and 3' region of the 4114 maize insertion. Hybridizations with the *cry*34Ab1, *cry*35Ab1, and *pat* probes resulted in a single band of greater than 8600 bp (Figure 13), and can be used to demonstrate the stability of the 3' region of the insertion. The presence of these event-specific hybridization patterns in each of the four generations with these probes would confirm the stability of the insertion in 4114 maize during breeding.

Hybridization of the *Bcl* I-digested DNA with the *cry*1F probe resulted in two bands of approximately 3100 bp and greater than 8600 bp that were consistently observed in each of the four generations, indicating stability of the 5' and 3' regions of the 4114 maize insertion (Table 5, Figure 24). When hybridized to the *cry*34Ab1 (Table 5, Figure 25), *cry*35Ab1 (Table 5, Figure 26), and *pat* probes (Table 5, Figure 27), a band of greater than 8600 bp was consistently observed in all plants from the four generations. The observation of these expected event specific hybridization patterns with all probes indicated that the 4114 DNA insertion was stable during the breeding process.

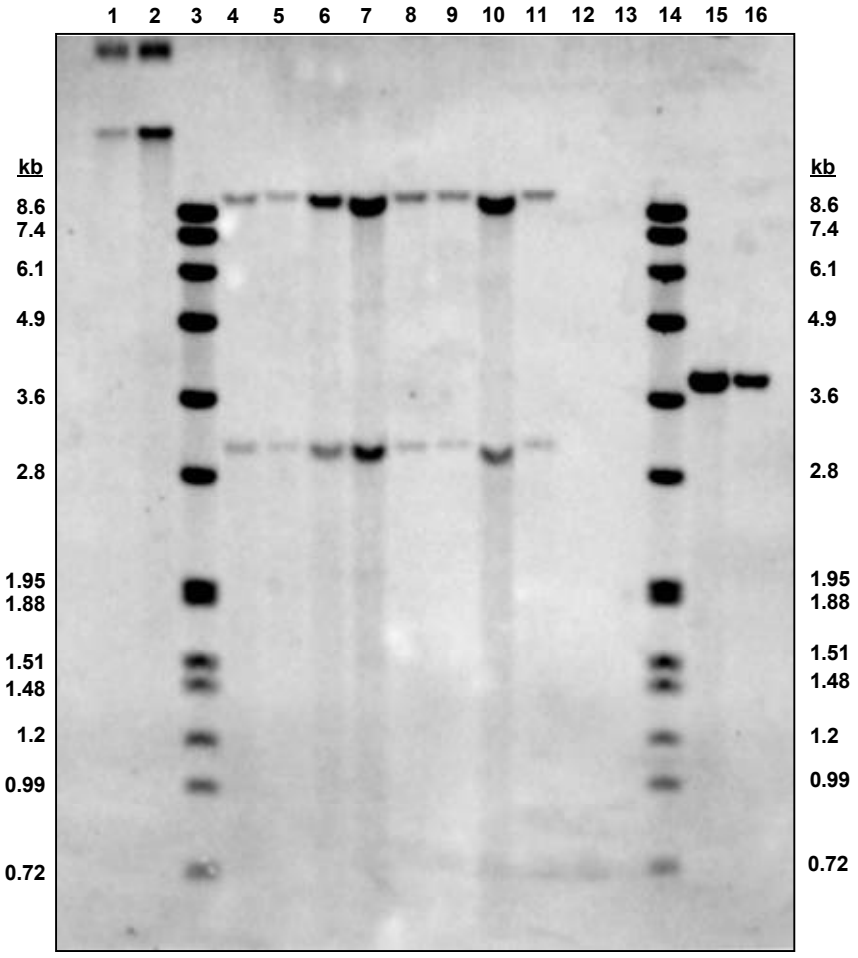


Figure 24. Southern Blot Analysis of the T2, T3, BC3F1[*3] and BC3F2[*2] Generations of 4114 Maize; *Bcl* I Digested DNA with *cry*1F Probe

DNA isolated from individual plants of 4114 maize was digested with *Bcl* I and hybridized to the *cry*1F probe. Approximately 3 - 6 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP27118 (digested with *Bcl* I in Lanes 1 and 2; digested with *Hin*d III in Lanes 15 and 16) at the indicated approximate gene copy number and 4 µg of genomic DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	1 copy PHP27118 + PH09B control
2	3 copy PHP27118 + PHWWE control
3	DIGVII marker
4	4114 maize plant 9 (T2 generation)
5	4114 maize plant 12 (T2 generation)
6	4114 maize plant 20 (T3 generation)
7	4114 maize plant 22 (T3 generation)
8	4114 maize plant 30 (BC3F1[*3] generation)

Lane	Sample
9	4114 maize plant 37 (BC3F1[*3] generation)
10	4114 maize plant 39 (BC3F2[*2] generation)
11	4114 maize plant 41 (BC3F2[*2] generation)
12	PH09B control
13	PHWWE control
14	DIGVII marker
15	3 copy PHP27118 + PH09B control (*Hin*d III)
16	1 copy PHP27118 + PHWWE control (*Hin*d III)

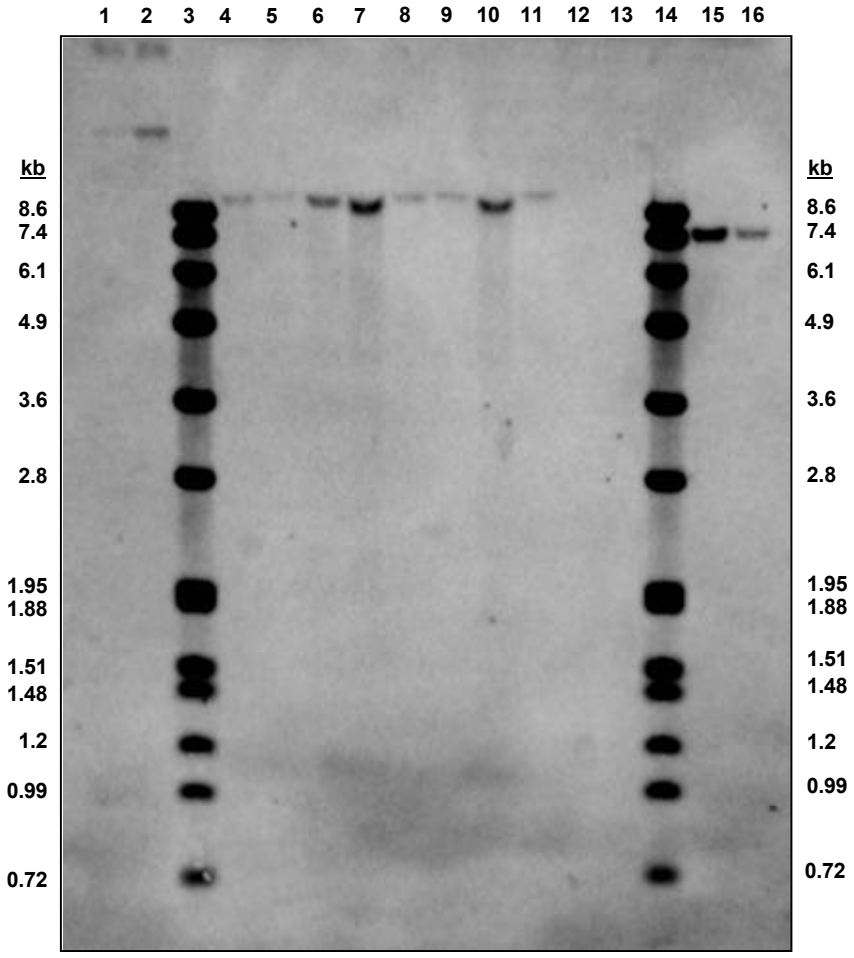


Figure 25. Southern Blot Analysis of the T2, T3, BC3F1*3 and BC3F2*2 Generations of 4114 Maize; *Bcl* I Digested DNA with *cry*34Ab1 Probe

DNA isolated from individual plants of 4114 maize was digested with *Bcl* I and hybridized to the *cry*34Ab1 probe. Approximately 3 - 6 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP27118 (digested with *Bcl* I in Lanes 1 and 2; digested with *Hin*d III in Lanes 15 and 16) at the indicated approximate gene copy number and 4 µg of genomic DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	1 copy PHP27118 + PH09B control
2	3 copy PHP27118 + PHWWE control
3	DIGVII marker
4	4114 maize plant 9 (T2 generation)
5	4114 maize plant 12 (T2 generation)
6	4114 maize plant 20 (T3 generation)
7	4114 maize plant 22 (T3 generation)
8	4114 maize plant 30 (BC3F1*3 generation)

Lane	Sample
9	4114 maize plant 37 (BC3F1*3 generation)
10	4114 maize plant 39 (BC3F2*2 generation)
11	4114 maize plant 41 (BC3F2*2 generation)
12	PH09B control
13	PHWWE control
14	DIGVII marker
15	3 copy PHP27118 + PH09B control (*Hin*d III)
16	1 copy PHP27118 + PHWWE control (*Hin*d III)

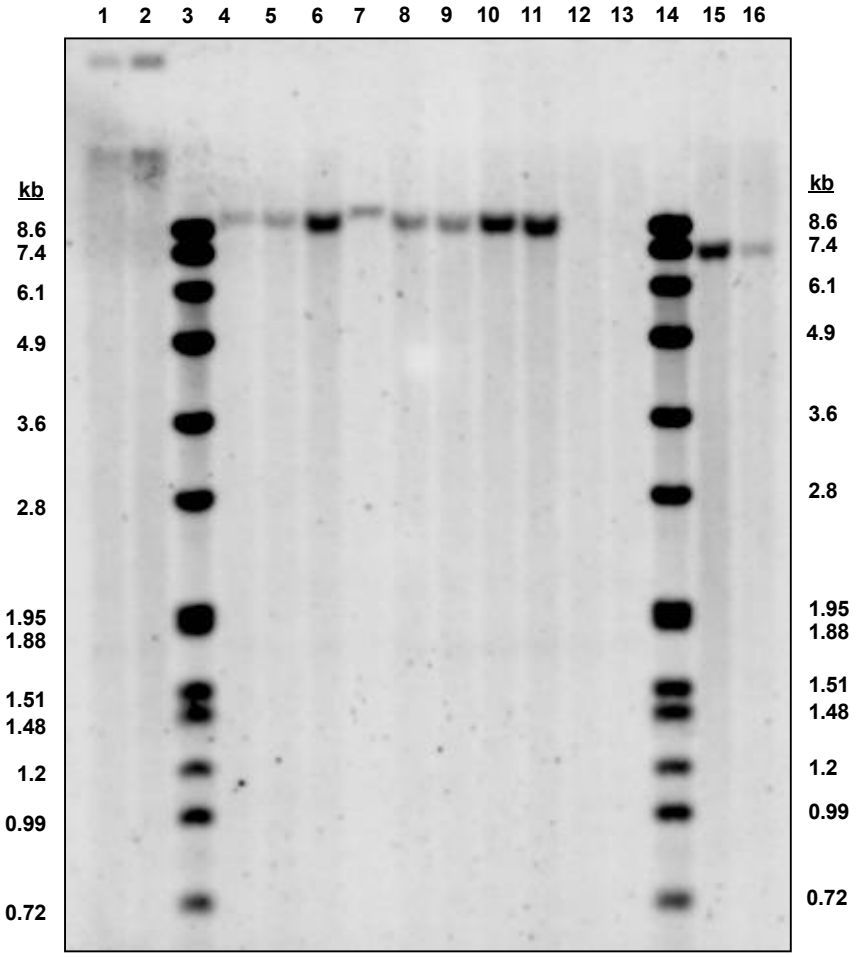


Figure 26. Southern Blot Analysis of the T2, T3, BC3F1[*3] and BC3F2[*2] Generations of 4114 Maize; *Bcl* I Digested DNA with *cry*35Ab1 Probe

DNA isolated from individual plants of 4114 maize was digested with *Bcl* I and hybridized to the *cry*35Ab1 probe. Approximately 3 - 6 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP27118 (digested with *Bcl* I in Lanes 1 and 2; digested with *Hin*d III in Lanes 15 and 16) at the indicated approximate gene copy number and 4 µg of genomic DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	1 copy PHP27118 + PH09B control
2	3 copy PHP27118 + PHWWE control
3	DIGVII marker
4	4114 maize plant 9 (T2 generation)
5	4114 maize plant 12 (T2 generation)
6	4114 maize plant 20 (T3 generation)
7	4114 maize plant 22 (T3 generation)
8	4114 maize plant 27 (BC3F1[*3] generation)

Lane	Sample
9	4114 maize plant 37 (BC3F1[*3] generation)
10	4114 maize plant 39 (BC3F2[*2] generation)
11	4114 maize plant 41 (BC3F2[*2] generation)
12	PH09B control
13	PHWWE control
14	DIGVII marker
15	3 copy PHP27118 + PH09B control (*Hin*d III)
16	1 copy PHP27118 + PHWWE control (*Hin*d III)

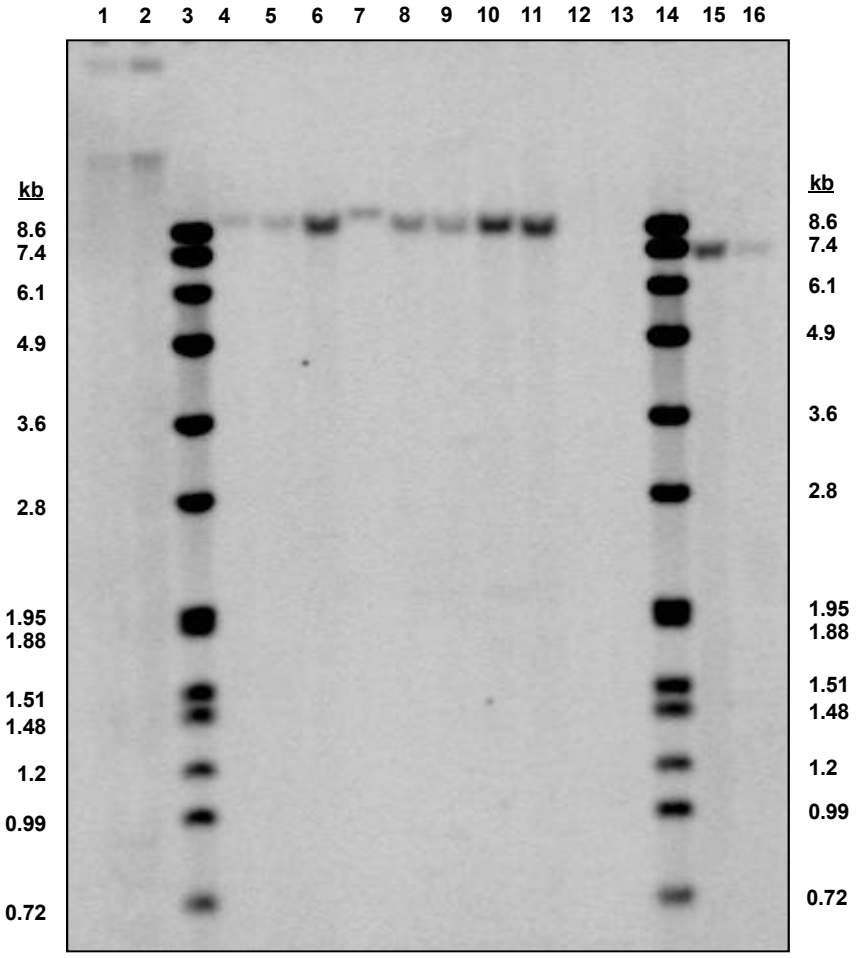


Figure 27. Southern Blot Analysis of the T2, T3, BC3F1[*3] and BC3F2[*2] Generations of 4114 Maize; *Bcl* I Digested DNA with *pat* Probe

DNA isolated from individual plants of 4114 maize was digested with *Bcl* I and hybridized to the *pat* probe. Approximately 3 - 6 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP27118 (digested with *Bcl* I in Lanes 1 and 2; digested with *Hin*d III in Lanes 15 and 16) at the indicated approximate gene copy number and 4 µg of genomic DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	1 copy PHP27118 + PH09B control
2	3 copy PHP27118 + PHWWE control
3	DIGVII marker
4	4114 maize plant 9 (T2 generation)
5	4114 maize plant 12 (T2 generation)
6	4114 maize plant 20 (T3 generation)
7	4114 maize plant 22 (T3 generation)
8	4114 maize plant 27 (BC3F1[*3] generation)

Lane	Sample
9	4114 maize plant 37 (BC3F1[*3] generation)
10	4114 maize plant 39 (BC3F2[*2] generation)
11	4114 maize plant 41 (BC3F2[*2] generation)
12	PH09B control
13	PHWWE control
14	DIGVII marker
15	3 copy PHP27118 + PH09B control (*Hin*d III)
16	1 copy PHP27118 + PHWWE control (*Hin*d III)

Absence of Plasmid Backbone DNA in 4114 Maize

Four generations of 4114 maize (T2, T3, BC3F1*3, and BC3F2*2) were analyzed by Southern blot analysis for plasmid sequences from the PHP27118 plasmid backbone. Probes for genes and regions on the PHP27118 plasmid backbone outside the T-DNA region were used to determine if any plasmid backbone was inserted in 4114 maize during transformation. The *spc*, *tet*, and *vir*G probes hybridize to the spectinomycin resistance, tetracycline resistance, and *vir*G genes respectively (Table 4, Figure 11). The LB probe hybridizes to the plasmid backbone region outside the Left T-DNA border, and the RB probe hybridizes to the region outside the Right Border of the T-DNA of PHP27118 (Table 4, Figure 11). These probes would show whether regions of the PHP27118 backbone outside the T-DNA was transferred into 4114 maize.

Genomic DNA from the T2, T3, BC3F1*3, and BC3F2*2 generations was digested with *Bcl* I and hybridized to the backbone probes described above. The LB (Figure 28), *spc* (Figure 29), *tet* (Figure 30), *vir*G (Figure 31), and RB (Figure 32) probes showed no bands from backbone hybridization in the 4114 maize or control maize plants. This confirmed that no sequence from the PHP27118 plasmid backbone was inserted during transformation. The plasmid lanes showed hybridization to the undigested plasmid in the *Bcl* I lanes (Figures 28 through 32, Lanes 1 and 2) and to the expected size bands for digestion with *Hin*d III (Figures 28 through 32, Lanes 15 and 16), thus demonstrating successful hybridization of the probes to plasmid backbone sequences.

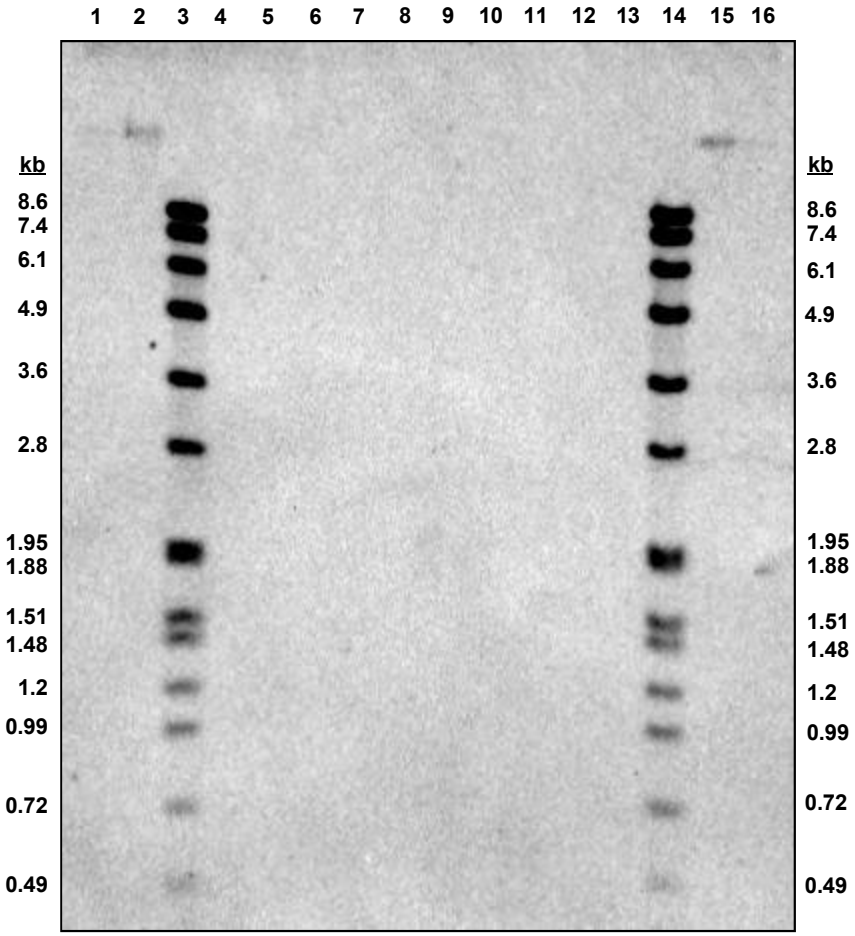


Figure 28. Southern Blot Analysis of the T2, T3, BC3F1*3 and BC3F2*2 Generations of 4114 Maize; *Bcl* I Digested DNA with LB Probe

DNA isolated from individual plants of 4114 maize was digested with *Bcl* I and hybridized to the LB probe. Approximately 3 - 6 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP27118 (digested with *Bcl* I in Lanes 1 and 2; digested with *Hin*d III in Lanes 15 and 16) at the indicated approximate gene copy number and 4 µg of genomic DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	1 copy PHP27118 + PH09B control
2	3 copy PHP27118 + PHWWE control
3	DIGVII marker
4	4114 maize plant 9 (T2 generation)
5	4114 maize plant 12 (T2 generation)
6	4114 maize plant 20 (T3 generation)
7	4114 maize plant 22 (T3 generation)
8	4114 maize plant 30 (BC3F1*3 generation)

Lane	Sample
9	4114 maize plant 37 (BC3F1*3 generation)
10	4114 maize plant 39 (BC3F2*2 generation)
11	4114 maize plant 41 (BC3F2*2 generation)
12	PH09B control
13	PHWWE control
14	DIGVII marker
15	3 copy PHP27118 + PH09B control (*Hin*d III)
16	1 copy PHP27118 + PHWWE control (*Hin*d III)

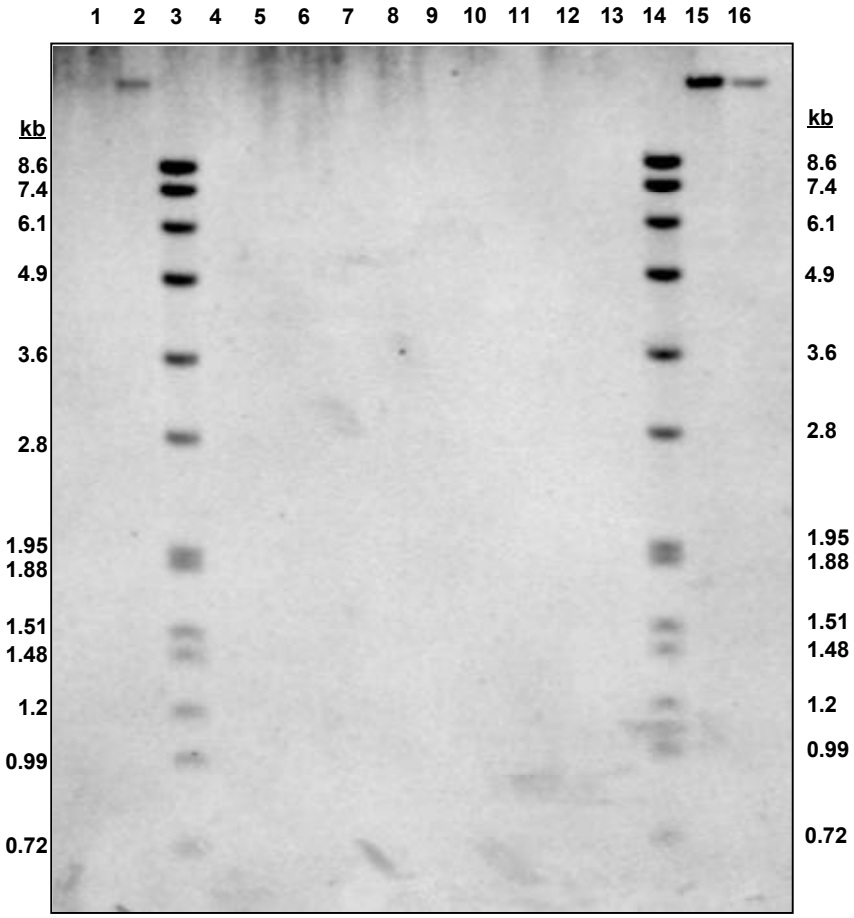


Figure 29. Southern Blot Analysis of the T2, T3, BC3F1*3 and BC3F2*2 Generations of 4114 Maize; *Bcl* I Digested DNA with *spc* Probe

DNA isolated from individual plants of 4114 maize was digested with *Bcl* I and hybridized to the *spc* probe. Approximately 3 - 6 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP27118 (digested with *Bcl* I in Lanes 1 and 2; digested with *Hin*d III in Lanes 15 and 16) at the indicated approximate gene copy number and 4 µg of genomic DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	1 copy PHP27118 + PH09B control
2	3 copy PHP27118 + PHWWE control
3	DIGVII marker
4	4114 maize plant 9 (T2 generation)
5	4114 maize plant 12 (T2 generation)
6	4114 maize plant 20 (T3 generation)
7	4114 maize plant 22 (T3 generation)
8	4114 maize plant 30 (BC3F1*3 generation)

Lane	Sample
9	4114 maize plant 37 (BC3F1*3 generation)
10	4114 maize plant 39 (BC3F2*2 generation)
11	4114 maize plant 41 (BC3F2*2 generation)
12	PH09B control
13	PHWWE control
14	DIGVII marker
15	3 copy PHP27118 + PH09B control (*Hin*d III)
16	1 copy PHP27118 + PHWWE control (*Hin*d III)

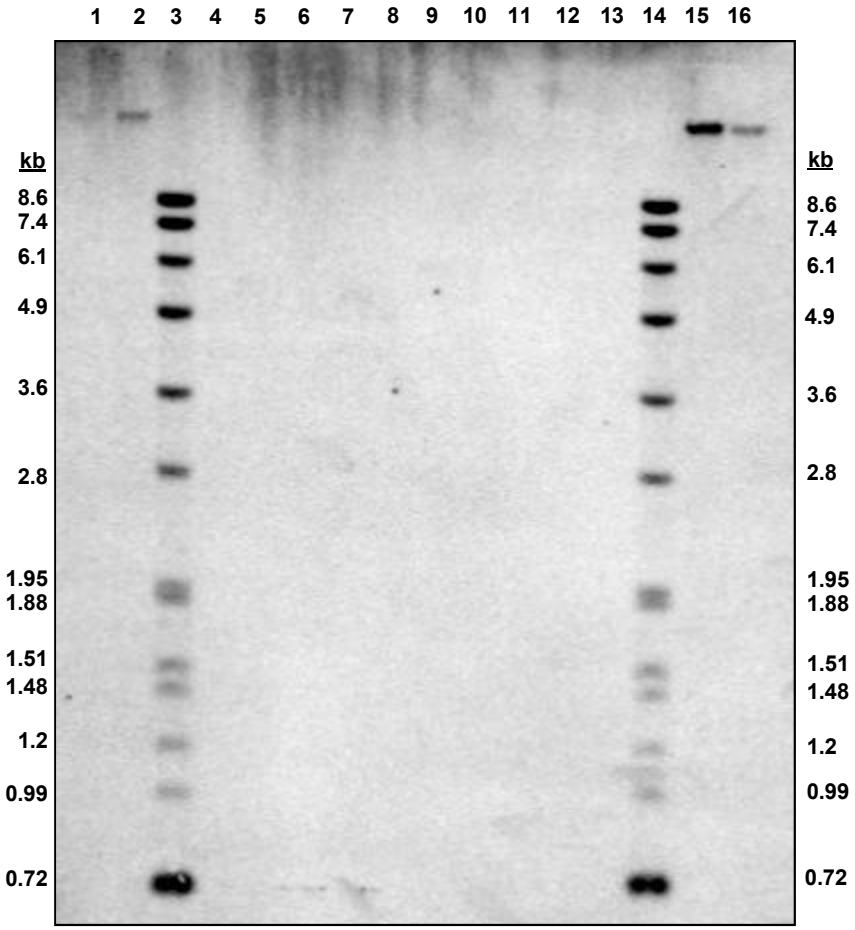


Figure 30. Southern Blot Analysis of the T2, T3, BC3F1[*3] and BC3F2[*2] Generations of 4114 Maize; *Bcl* I Digested DNA with *tet* Probe

DNA isolated from individual plants of 4114 maize was digested with *Bcl* I and hybridized to the *tet* probe. Approximately 3 - 6 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP27118 (digested with *Bcl* I in Lanes 1 and 2; digested with *Hin*d III in Lanes 15 and 16) at the indicated approximate gene copy number and 4 µg of genomic DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	1 copy PHP27118 + PH09B control
2	3 copy PHP27118 + PHWWE control
3	DIGVII marker
4	4114 maize plant 9 (T2 generation)
5	4114 maize plant 12 (T2 generation)
6	4114 maize plant 20 (T3 generation)
7	4114 maize plant 22 (T3 generation)
8	4114 maize plant 30 (BC3F1[*3] generation)

Lane	Sample
9	4114 maize plant 37 (BC3F1[*3] generation)
10	4114 maize plant 39 (BC3F2[*2] generation)
11	4114 maize plant 41 (BC3F2[*2] generation)
12	PH09B control
13	PHWWE control
14	DIGVII marker
15	3 copy PHP27118 + PH09B control (*Hin*d III)
16	1 copy PHP27118 + PHWWE control (*Hin*d III)

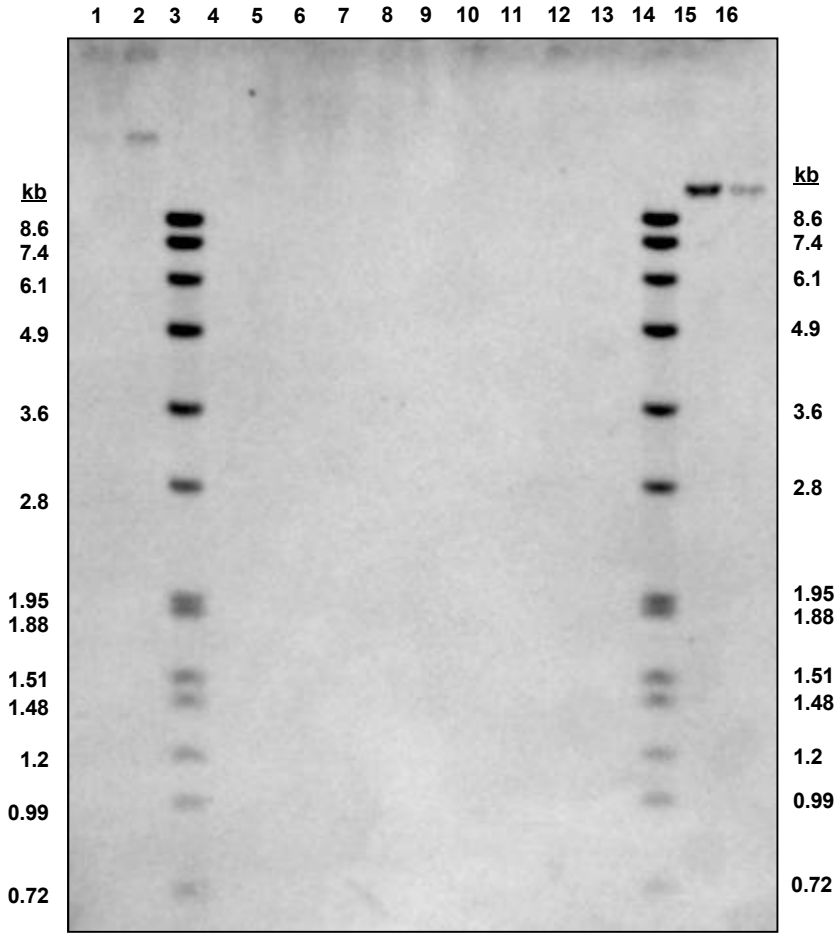


Figure 31. Southern Blot Analysis of the T2, T3, BC3F1[*3] and BC3F2[*2] Generations of 4114 Maize; *Bcl* I Digested DNA with *vir*G Probe

DNA isolated from individual plants of 4114 maize was digested with *Bcl* I and hybridized to the *virG* probe. Approximately 3 - 6 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP27118 (digested with *Bcl* I in Lanes 1 and 2; digested with *Hin*d III in Lanes 15 and 16) at the indicated approximate gene copy number and 4 µg of genomic DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	1 copy PHP27118 + PH09B control
2	3 copy PHP27118 + PHWWE control
3	DIGVII marker
4	4114 maize plant 9 (T2 generation)
5	4114 maize plant 12 (T2 generation)
6	4114 maize plant 20 (T3 generation)
7	4114 maize plant 22 (T3 generation)
8	4114 maize plant 30 (BC3F1[*3] generation)

Lane	Sample
9	4114 maize plant 37 (BC3F1[*3] generation)
10	4114 maize plant 39 (BC3F2[*2] generation)
11	4114 maize plant 41 (BC3F2[*2] generation)
12	PH09B control
13	PHWWE control
14	DIGVII marker
15	3 copy PHP27118 + PH09B control (*Hin*d III)
16	1 copy PHP27118 + PHWWE control (*Hin*d III)

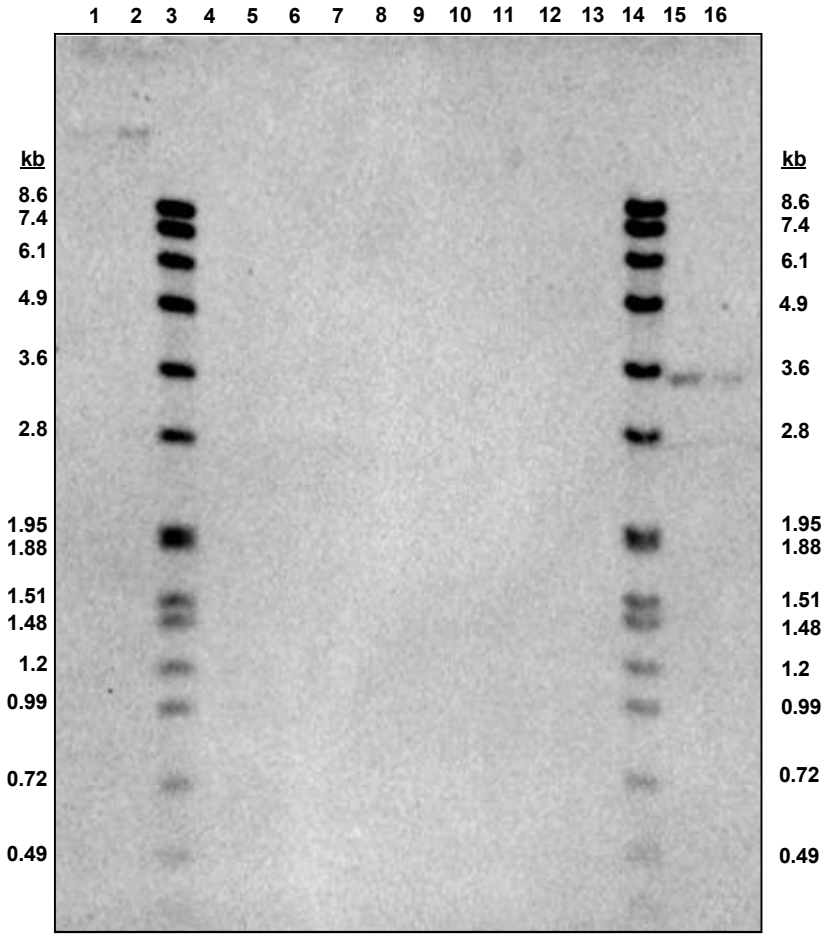


Figure 32. Southern Blot Analysis of the T2, T3, BC3F1*3 and BC3F2*2 Generations of 4114 Maize; *Bcl* I Digested DNA with RB Probe

DNA isolated from individual plants of 4114 maize was digested with *Bcl* I and hybridized to the RB probe. Approximately 3 - 6 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP27118 (digested with *Bcl* I in Lanes 1 and 2; digested with *Hin*d III in Lanes 15 and 16) at the indicated approximate gene copy number and 4 µg of genomic DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	1 copy PHP27118 + PH09B control
2	3 copy PHP27118 + PHWWE control
3	DIGVII marker
4	4114 maize plant 9 (T2 generation)
5	4114 maize plant 12 (T2 generation)
6	4114 maize plant 20 (T3 generation)
7	4114 maize plant 22 (T3 generation)
8	4114 maize plant 30 (BC3F1*3 generation)

Lane	Sample
9	4114 maize plant 37 (BC3F1*3 generation)
10	4114 maize plant 39 (BC3F2*2 generation)
11	4114 maize plant 41 (BC3F2*2 generation)
12	PH09B control
13	PHWWE control
14	DIGVII marker
15	3 copy PHP27118 + PH09B control (*Hin*d III)
16	1 copy PHP27118 + PHWWE control (*Hin*d III)

Physical Map of the DNA Insertion in 4114 Maize

Based on the Southern blot analysis, it was determined that a single, intact PHP27118 T-DNA was inserted into the genome of 4114 maize. A physical map of the DNA insertion in 4114 maize showing the restriction enzymes employed in the analysis was developed using these data and is presented in Figure 13.

Summary and Conclusions

Southern blot analysis was conducted on 4114 maize to confirm insertion copy number, integrity, and stability of the insertion. Analysis with *Bcl* I, examining the sequences flanking the DNA insertion, indicated a single copy of the PHP27118 T-DNA is present in 4114 maize. The *Hin*d III analysis indicated that the PHP27118 T-DNA had inserted intact in the genome. Analysis with *Bcl* I demonstrated that the 4114 DNA insertion is stable across the T2, T3, BC3F1[*3], and BC3F2[*2] generations and during the traditional breeding process, as identical hybridization patterns were observed in each of the four generations. In addition, analysis with probes for the regions of PHP27118 outside the T-DNA region demonstrated that no plasmid backbone sequences were incorporated into the 4114 maize genome.

2-E. Inheritance and Stability of the Inserted DNA

The stability of the inserted DNA during the breeding process is evaluated by examining the inheritance and segregation of the genes and/or traits in multiple generations. The segregation of these genes or traits as a single unit and as a single genetic locus will confirm that the inserted DNA will be predictably and stably inherited through the commercial breeding process.

For 4114 maize, the inheritance of both the DNA insertion and the herbicide-tolerance phenotype was evaluated to ensure stability of the traits during the plant breeding process and to confirm the traits were inserted at a single genetic locus. For the analyses, segregating generations of 4114 maize ($F1^{*1}$, $BC2F1^{*1}$, $BC3F1^{*1}$, $BC2F1^{*2}$, and $BC3F1^{*2}$ generations) were evaluated. The breeding history of these five generations is shown in the breeding diagram (Section 2, Figure 10 and Table 3). These generations were selected for analysis because they represent a range of different crossing and backcrossing points in a typical maize breeding program. Chi-square analysis was conducted on the five segregating generations to determine if the observed segregation ratios were consistent with the expected ratios. The assay methods and statistical analysis for the trait inheritance data are described in Appendix 4.

The presence of the 4114 event insertion was determined by event-specific and *cry*1F, *cry*34Ab1, *cry*35Ab1, and *pat* gene-specific endpoint PCR analyses performed on leaf punches from seedlings of each generation. 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.

Results from the segregation analysis are provided in Table 7. In every case, a positive plant tested positive for the presence of the 4114 event, the *cry*1F, *cry*34Ab1, *cry*35Ab1 and *pat* genes, and the herbicide-tolerance phenotype, indicating the 4114 insertion segregated as a unit. To confirm that the inserted DNA and the herbicide-tolerance phenotype segregate according to Mendel's laws of genetics for a single genetic locus, chi-square analysis was performed for the PCR and the herbicide-tolerance phenotype data.

No statistically significant differences were found between the observed and expected segregation ratios for the $F1^{*1}$, $BC2F1^{*1}$, $BC3F1^{*1}$, and $BC2F1^{*2}$ generations of 4114 maize (Table 7), indicating that these four generations all segregated as expected based on Mendel's laws. The 4114 insertion segregated as a unit and the observed ratios were indicative of an insertion at a single genetic locus.

The observed segregation ratio of the $BC3F1^{*2}$ generation in the original analysis of 99 seedlings was statistically significant (p-value <0.05) compared to the expected 1:1 segregation ratio, with a chi-square value of 5.34 and p-value of 0.0208. Typically 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 (ratio of 41:59 or 59:41). The $BC3F1^{*2}$ generation observed segregation ratio for the original sample was 38:61, only two plants outside of the acceptable

range. In order to determine if the statistical difference was a false positive result due to random sampling, additional sampling of 96 seedlings from the same seed source was performed. For the original sample, the chance of a false positive result due to random sampling was 5%; if a statistical difference was found when sampling was repeated, the chance of a false positive result would be greatly reduced to 5% x 5% = 0.25%. This additional sampling would not only confirm the prior result, but would also increase the statistical power of the BC3F1[*2] generation analysis.

Chi-square analysis of the combined data (n=195) for this single seed source (95% confidence) was performed and no statistically significant difference was found between the observed segregation ratio and the expected segregation ratio for the BC3F1[*2] generation (Table 7). The significant difference observed in the original BC3F1[*2] generation analysis was therefore likely due to a false positive result associated with random sampling.

These results indicate that the inserted DNA and the herbicide-tolerance phenotype in 4114 maize segregate predictably according to Mendel's laws of genetics and are consistent with the finding that the 4114 maize insertion is at a single genetic locus. Taken together with the stability results described earlier for the Southern analysis, these results confirm the stability of the insertion through the breeding process.

Table 7. Summary of Genotypic and Phenotypic Results for Segregating 4114 Maize

4114 Maize Generation[a]	Observed Values[b] (expected segregation = 1:1)			Statistical Analysis	
	Positive	Negative	Total	Chi-Square[c]	P-value
F1[*1]	52	46	98	0.367	0.545
BC2F1[*1]	48	52	100	0.160	0.689
BC3F1[*1]	47	53	100	0.360	0.549
BC2F1[*2]	53	47	100	0.360	0.549
BC3F1[*2]	87	108	195	1.62	0.1326

[a] 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. To increase the statistical power, 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.

[b] PCR analysis (consisting of event-specific PCR analysis to confirm the presence or absence of maize event 4114, 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 were conducted for each plant in each entry. All PCR results matched the corresponding herbicide tolerance result for each plant analyzed.

[c] Degrees of freedom = 1.

2-F. Concentrations of Cry1F, Cry34Ab1, Cry35Ab1, and PAT in 4114 Maize

Determining the concentrations of the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins in various tissues of 4114 maize is important background information for the conduct of exposure and safety assessments. To determine if previous safety studies conducted for 1507 (expresses Cry1F and PAT), 59122 (expresses Cry34Ab1, Cry35Ab1, and PAT), or 1507x59122 maize (expresses Cry1F, Cry34Ab1, Cry35Ab1, and PAT) are applicable to 4114 maize, it is necessary to determine if the concentrations of the expressed proteins in 4114 maize are comparable to these commercial lines.

In order to determine the concentrations of Cry1F, Cry34Ab1, Cry35Ab1, and PAT and to assess comparability, 4114 maize was grown side-by-side with commercial 1507, 59122, and 1507x59122 maize lines at five commercial maize-growing sites in the U.S. and Canada during the 2010 growing season. The sites were located in Iowa (two sites), Illinois, Nebraska, and Ontario, Canada. The F1*[5] generation of seed (Section 2; Figure 10 and Table 3) was used because F1 hybrid seed is representative of seed that growers would plant in commercial maize fields. Four replications (from different plants) per tissue were collected from each site, for a total of n=20 for each tissue and time point.

Plant tissue samples were collected throughout the growing season at various growth developmental stages (Table 8) and processed as described in Appendix 5. Time points for sampling were chosen to determine the range of protein concentrations throughout the growing season and for their relevance to commercial maize production practices. The protein concentrations in R6 tissues (*i.e.,* plant senescence and grain harvest) and R1 pollen values (*i.e.,* at pollen shed) have relevance for the evaluation of impact on non-target arthropods in the environment. The R4 stage of the whole plant sample (*i.e.,* forage) is the stage at which growers harvest plants for silage for animal feed. Grain is normally harvested at the R6 stage of development and is used for food and feed.

Concentrations of Cry1F, Cry34Ab1, Cry35Ab1, and PAT protein in plant tissue extracts were measured in the following tissues using specific quantitative enzyme-linked immunosorbent assay (ELISA) methods, as described in Appendix 5:

- Leaf—V6, V9, R1, R4, and R6
- Root—V6, V9, R1, R4, and R6
- Whole Plant (the above-ground portion of the plant, including the ear at R1 and R6)—V9, R1, and R6
- Pollen—R1
- Forage (the above-ground portion of the plant, including the ear)—R4
- Grain—R6

The ranges of transgenic protein mean concentrations in leaf, root, and whole-plant tissues over the course of the growing season, as well as the mean concentrations in pollen and root, are summarized in Table 9 for 4114 maize (data taken from Tables 10 through 13). The mean

PAT concentrations in 4114 maize pollen and grain were below the lower limit of quantification (LLOQ) of the ELISA assay.

Table 8. Maize Growth Stage Descriptions

Growth Stage[a]	Description
V6	The collar of the sixth leaf becomes visible.
V9	The collar of the ninth leaf becomes visible.
R1	Silks become visible.
R4	The material within the kernel produces a doughy consistency.
R6	Typical harvest maturity for grain (regarded as physiological maturity).

[a] Adapted from Ritchie *et al*., 2005

Table 9. Mean Concentrations of Transgenic Proteins in 4114 Maize

Tissue	Cry1F	Cry34Ab1	Cry35Ab1	PAT
	Range in ng/mg tissue dry weight[a]			
Leaf	2.0 - 34	19 - 110	27 - 90	0.52 - 14
Root	3.8 - 5.5	17 - 23	6.4 - 13	0.13 - 0.65
Whole Plant[b]	4.1 - 12	23 - 52	21 - 76	0.090 - 8.7
Pollen	35	9.2	0.34	<0.28[c]
Grain	3.3	24	1.1	<0.069[c]

[a] Ranges reflect the range of means at different growth stages.
[b] Whole plant ranges include forage.
[c] < Lower Limit of Quantification (LLOQ); indicates the values of the sample(s) were detected below the assay LLOQ

For each transgenic protein, the concentrations in each 4114, 1507, 59122, and 1507x59122 maize tissue were measured (Tables 10 through 13). The Cry1F, Cry34Ab1, and Cry35Ab1 protein concentrations in 4114 maize in each tissue were divided by the respective protein concentrations in 1507, 59122, and/or 1507x59122 maize to provide an "expression ratio" (Table 14). When the expression ratio was close to one, this indicated that 4114 maize had comparable expression to 1507, 59122, and/or 1507x59122 maize. If the expression ratio was less than one, this indicated that 4114 maize had lower expression. For values greater than one, expression was determined to be higher in 4114 maize.

The concentrations of Cry1F protein in 4114 maize were comparable to or lower than concentrations in 1507 maize and 1507x59122 maize in all tissues measured except for pollen (R1) and leaf (R6) tissues (Tables 10 and 14). Concentrations of Cry1F in pollen (R1) and leaf (R6) tissues were higher in 4114 maize.

The concentrations of Cry34Ab1 protein in 4114 maize were comparable to or lower than concentrations in 59122 maize and 1507x59122 maize in all tissues measured except for leaf (R6) tissue (Tables 11 and 14). The concentration of Cry34Ab1 in leaf (R6) tissue was higher in 4114 maize.

The concentrations of Cry35Ab1 protein in 4114 maize were comparable to or lower than concentrations in 59122 maize and 1507x59122 maize in all tissues measured (Tables 12 and 14).

The concentrations of PAT protein in 4114 maize were comparable to or greater than concentrations in 1507 maize and were comparable to or lower than concentrations in 59122 maize and 1507x59122 maize, with the exception of leaf (R6) which was higher (Tables 13 and 14). It was expected that PAT concentrations in 1507x59122 maize tissues would be greater than PAT concentrations in 4114, 1507, and/or 59122 maize, since 1507x59122 contains two copies of the *pat* gene (one each from 1507 and 59122 maize) and 4114, 1507, and 59122 maize each contain one copy of the *pat* gene. In general, PAT protein concentrations in pollen, R4 root tissue, and all R6 tissues (grain, leaf, root, and whole plant) were very low and, in some cases, below the lower limit of quantification.

It is noted that, in most tissues and time points, the concentrations of Cry1F, Cry34Ab1, Cry35Ab1 and PAT in 4114 maize differed from 1507, 59122, and/or 1507x59122 maize, even though the same promoter-gene combinations are present in 4114 maize as in 1507 and/or 59122 maize. Expression differences can likely be attributed to event-to-event variation and are generally considered minor for a biological system (*i.e.*, 5.4-fold or less in all cases and often less than 2-fold).

In addition, these expression differences are not necessarily biologically meaningful. For example, as described fully in insect efficacy data submitted to the EPA, despite the fact that concentrations of Cry1F, Cry34Ab1 and Cry35Ab1 are generally lower in 4114 maize than in 1507, 59122 and/or 1507x59122 maize, 4114 maize was equivalent to 1507 maize in efficacy

against target lepidopteran pests. 4114 maize may be slightly less efficacious and less consistent in efficacy against corn rootworm compared to 1507x59122 maize. At one location in 2010 where corn rootworm pressure was high, 4114 maize was less efficacious than 1507x59122 maize. However, for the remaining eight location-by-year comparisons, there were no significant differences in corn rootworm efficacy between 4114 and 1507x59122 maize. Therefore, although in many cases the concentrations of Cry1F, Cry34Ab1, and Cry35Ab1 proteins were generally lower in 4114 maize tissues than in 1507, 59122, and/or 1507x59122 maize, there was little to no biological impact in terms of efficacy.

As is summarized in Table 14, in most cases the concentrations of Cry1F, Cry34Ab1, Cry35Ab1, and PAT were generally comparable to or lower than 1507, 59122, and/or 1507x59122 maize. The notable exceptions were Cry1F and Cry34Ab1 in late-season R6 leaf tissue, where concentrations in 4114 maize were 2.3- to 5.4-fold higher than 1507, 59122, and 1507x59122 maize, and Cry1F in pollen, where concentrations in 4114 maize were 1.4- to 1.5-fold higher than 1507 and 1507x59122 maize. R6 leaf tissue is collected at harvest time, when tissue has begun to senesce to varying degrees, and can be variable in degree of senescence and total extractable protein levels. This may impact the concentrations of transgenic proteins reported; R6 leaf tissue ratios were not representative of comparisons between 4114 maize and 1507, 59122, and/or 1507x59122 maize in leaf tissues at other time points. In some cases, PAT concentrations were higher in 4114 maize as compared to 1507 maize and were also higher in R6 leaf for similar reasons described above; however, the concentrations of PAT overall were very low or below the lower limit of quantification which likely impacted ratios and the comparisons. The comparisons for all other tissues, with the exception of Cry1F pollen, demonstrate that 4114 maize tissues have similar or lower concentrations of Cry1F, Cry34Ab1, Cry35Ab1, and PAT than 1507, 59122, and/or 1507x59122 maize tissues.

In general, these comparisons indicate any previously conducted safety studies that used 1507, 59122, and 1507x59122 maize are applicable to 4114 maize. Safety studies that considered exposure to the Cry1F protein from 1507 or 1507x59122 pollen would require the reassessment of results for 4114 maize using the higher concentration.

Table 10. Cry1F Concentrations (ng/mg Tissue Dry Weight)

Tissue (Growth Stage)		4114 Maize	1507 Maize	1507x59122 Maize
Leaf (V6)	Mean[a]	11	18	18
	Range	7.8 - 17	11 - 26	13 - 23
	CI[b]	9.4 - 13	15 - 21	15 - 21
Root (V6)	Mean	4.2	5.7	6.3
	Range	0.69 - 6.0	1.1 - 7.8	3.3 - 8.7
	CI	2.8 - 5.5	4.4 - 7.1	5.0 - 7.6
Leaf (V9)	Mean	9.7	13	16
	Range	5.3 - 14	0.78 - 20	9.6 - 24
	CI	6.8 - 13	10 - 16	13 - 18
Root (V9)	Mean	5.0	7.2	6.9
	Range	1.3 - 7.5	4.2 - 12	4.2 - 11
	CI	3.1 - 6.9	5.3 - 9.1	5.0 - 8.7
Whole Plant (V9)	Mean	12	16	16
	Range	8.6 - 15	10 - 19	9.6 - 19
	CI	10 - 13	14 - 17	15 - 18
Leaf (R1)	Mean	13	16	17
	Range	7.2 - 28	10 - 23	11 - 26
	CI	9.6 - 18	12 - 21	12 - 23
Pollen (R1)	Mean	35	23	24
	Range	19 - 49	19 - 26	18 - 28
	CI	29 - 42	19 – 27	20 - 29
Root (R1)	Mean	5.5	7.0	6.1
	Range	3.9 - 7.8	5.1 - 9.9	3.9 - 7.5
	CI	4.8 - 6.3	6.1 - 7.9	5.3 - 7.0
Whole Plant (R1)	Mean	9.9	14	13
	Range	7.8 - 13	10 - 18	9.6 - 15
	CI	9.1 - 11	13 - 15	12 - 14
Forage (R4)	Mean	7.8	8.4	9.1
	Range	5.6 - 11	5.6 - 12	6.0 - 14
	CI	6.4 - 9.4	7.0 - 10	7.5 - 11
Leaf (R4)	Mean	34	37	37
	Range	19 - 56	25 - 49	23 - 58
	CI	27 - 42	30 - 47	29 - 46
Root (R4)	Mean	3.8	4.4	5.3
	Range	2.3 - 5.7	3.0 - 5.7	3.0 - 7.2
	CI	2.9 - 4.6	3.6 - 5.3	4.4 - 6.2
Grain (R6)	Mean	3.3	3.2	2.8
	Range	2.3 - 7.2	1.9 - 5.1	1.7 - 4.5
	CI	2.8 - 4.0	2.7 - 3.8	2.4 - 3.4
Leaf (R6)	Mean	2.0	0.79	0.37
	Range	0.32 - 21	<0.14[c]-21	<0.14[c]-16
	CI	0.38 - 10	0.15 - 4.1	0.072 - 1.9

Table 10. Cry1F Concentrations (ng/mg Tissue Dry Weight) (continued)

Tissue (Growth Stage)		4114 Maize	1507 Maize	1507x59122 Maize
Root (R6)	Mean	3.8	5.0	4.7
	Range	1.4 - 6.3	2.9 - 7.5	2.7 - 8.1
	CI	3.1 - 4.8	4.1 - 6.2	3.8 - 5.8
Whole Plant (R6)	Mean	4.1	4.9	3.8
	Range	2.4 - 9.4	2.2 - 8.4	2.2 - 7.4
	CI	3.0 - 5.5	3.6 - 6.6	2.8 - 5.1

[a] Least squares means (estimated from statistical models; see Appendix 5 for additional details).
[b] Statistical Confidence Interval
[c] < Lower Limit of Quantification (LLOQ); indicates the values of the sample(s) were detected below the assay LLOQ

Table 11. Cry34Ab1 Concentrations (ng/mg Tissue Dry Weight)

Tissue (Growth Stage)		4114 Maize	1507x59122 Maize	59122 Maize
Leaf (V6)	Mean[a]	21	32	33
	Range	17 - 25	25 - 43	24 - 46
	CI[b]	19 - 22	30 - 35	31 - 36
Root (V6)	Mean	17	29	26
	Range	9.0 - 23	19 - 42	16 - 36
	CI	13 - 22	24 - 34	22 - 31
Leaf (V9)	Mean	26	42	38
	Range	22 - 31	32 - 54	23 - 46
	CI	23 - 29	37 - 48	33-43
Root (V9)	Mean	21	36	37
	Range	13 - 28	30 - 42	23 - 48
	CI	18 - 24	31 - 41	31-41
Whole Plant (V9)	Mean	23	41	41
	Range	18 - 26	26 - 52	26 - 54
	CI	18 - 28	36 - 46	36 - 45
Leaf (R1)	Mean	50	70	75
	Range	36 - 84	52 - 110	54 - 110
	CI	33 - 67	53 - 87	58 - 92
Pollen (R1)	Mean	9.2	46	44
	Range	4.7 - 16	35 - 67	36 - 64
	CI	7.7 - 11	38 - 55	36 - 53
Root (R1)	Mean	19	29	29
	Range	8.7 - 30	14 - 45	16 - 39
	CI	10 - 28	20 - 38	19 - 38
Whole Plant (R1)	Mean	32	56	57
	Range	24 - 42	44 - 82	50 - 76
	CI	29 - 35	50 - 62	52 - 64
Forage (R4)	Mean	52	93	86
	Range	36 - 64	68 - 130	56 - 120
	CI	46 - 60	81 - 110	75 - 98
Leaf (R4)	Mean	110	200	190
	Range	66 - 140	150 - 250	84 - 270
	CI	84 - 130	180 - 220	160 - 210
Root (R4)	Mean	23	35	32
	Range	7.5 - 36	18 - 51	12 - 51
	CI	17 - 29	29 - 41	26 - 38
Grain (R6)	Mean	24	24	24
	Range	14 - 39	14 - 42	17 - 33
	CI	20 - 28	20 - 29	20 - 29
Leaf (R6)	Mean	19	6.0	7.7
	Range	4.2 - 66	0.30 - 130	0.90 - 120
	CI	4.4 - 82	1.4 - 26	1.8 - 34

Table 11. Cry34Ab1 Concentrations (ng/mg Tissue Dry Weight) (continued)

Tissue (Growth Stage)		4114 Maize	1507x59122 Maize	59122 Maize
Root (R6)	Mean	18	32	37
	Range	5.4 - 54	14 - 54	17 - 66
	CI	14 - 24	24 - 42	28 - 49
Whole Plant (R6)	Mean	36	48	48
	Range	20 - 62	26 - 88	28 - 70
	CI	29 - 44	39 - 58	40 - 59

[a] Least squares means (estimated from statistical models; see Appendix 5 for additional details).
[b] Statistical Confidence Interval

Table 12. Cry35Ab1 Concentrations (ng/mg Tissue Dry Weight)

Tissue (Growth Stage)		4114 Maize	1507x59122 Maize	59122 Maize
Leaf (V6)	Mean[a]	27	33	36
	Range	17 - 38	25 - 50	27 - 50
	CI[b]	22 - 33	26 - 41	29 - 44
Root (V6)	Mean	13	18	16
	Range	9.0 - 20	14 - 23	11 - 23
	CI	11 - 15	15 - 22	14 - 19
Leaf (V9)	Mean	33	46	40
	Range	28 - 39	35 - 58	35 - 47
	CI	30 - 37	42 - 50	37 - 44
Root (V9)	Mean	13	17	17
	Range	7.8 - 19	13 - 22	9.3 - 27
	CI	10 - 17	13 - 21	13 - 21
Whole Plant (V9)	Mean	76	79	85
	Range	58 - 100	52 - 100	66 - 100
	CI	67 - 86	70 - 89	76 - 96
Leaf (R1)	Mean	68	71	81
	Range	43 - 130	56 - 110	66 - 110
	CI	56 - 83	58 - 87	67 - 100
Pollen (R1)	Mean	0.34	<0.32[c]	0.33
	Range	<0.32[c] - 0.53	<0.32[c]	<0.32[c] - 0.44
	CI	NA[d]	NA	NA
Root (R1)	Mean	9.2	10	11
	Range	4.2 - 15	6.6 - 16	7.2 - 15
	CI	7.2 - 12	7.9 - 13	8.4 - 14
Whole Plant (R1)	Mean	66	75	69
	Range	44 - 100	58 - 110	54 - 98
	CI	57 - 75	66 - 86	60 - 79
Forage (R4)	Mean	29	28	30
	Range	18 - 52	17 - 42	20 - 52
	CI	23 - 35	23 - 34	24 - 36
Leaf (R4)	Mean	90	100	100
	Range	66 - 110	84 - 130	52 - 130
	CI	81 - 99	95 - 110	93 - 110
Root (R4)	Mean	6.4	8.0	8.0
	Range	2.1 - 12	3.9 - 14	1.1 - 13
	CI	3.6 - 9.3	5.1 - 11	5.1 - 11
Grain (R6)	Mean	1.1	1.4	1.4
	Range	0.54 - 2.3	0.69 - 2.0	0.75-2.3
	CI	0.84 - 1.4	1.1 - 1.8	1.1 - 1.8
Leaf (R6)	Mean	72	72	59
	Range	41 - 110	2.0 - 190	2.2 - 130
	CI	36 - 108	36 - 110	23 - 96

Table 12. Cry35Ab1 Concentrations (ng/mg Tissue Dry Weight) (continued)

Tissue (Growth Stage)		4114 Maize	1507x59122 Maize	59122 Maize
Root (R6)	Mean	6.9	8.9	10.0
	Range	2.2 - 14	3.3 - 18	3.3 - 17
	CI	3.5 - 10	5.5 - 12	6.6 - 13
Whole Plant (R6)	Mean	21	31	27
	Range	13 - 54	20 - 46	13 - 64
	CI	15 - 30	22 - 44	19 - 38

[a] Least squares means (estimated from statistical models; see Appendix 5 for additional details).
[b] Statistical Confidence Interval
[c] < Lower Limit of Quantification (LLOQ); indicates the values of the sample(s) were detected below the assay LLOQ
[d] Greater than 80% of samples were <LLOQ, statistical analysis not available (NA)

Table 13. PAT Concentrations (ng/mg Tissue Dry Weight)

Tissue (Growth Stage)		4114 Maize	1507 Maize	1507x59122 Maize	59122 Maize
Leaf (V6)	Mean[a]	9.0	5.6	16	12
	Range	4.2 - 14	3.1 - 10	7.2 - 24	8.4 - 18
	CI[b]	7.6 - 11	4.7 - 6.6	13 - 18	9.8 - 14
Root (V6)	Mean	0.44	0.29	1.7	1.1
	Range	0.14 - 0.78	0.075 - 0.45	0.93 - 2.9	0.42 - 2.1
	CI	0.32 - 0.62	0.21 - 0.40	1.2 - 2.3	0.78 - 1.5
Leaf (V9)	Mean	9.8	6.6	21	12
	Range	4.8 - 15	2.9 - 8.4	10 - 32	4.1 - 16
	CI	6.7 - 13	3.4 - 9.7	17 - 24	8.8 - 15
Root (V9)	Mean	0.65	0.36	2.2	1.5
	Range	0.39 - 0.90	0.22 - 0.57	1.1 - 3.0	0.99 - 2.6
	CI	0.30 - 1.00	0.013 - 0.71	1.8 - 2.5	1.1 - 1.8
Whole Plant (V9)	Mean	8.7	3.6	15	11
	Range	6.6 - 11	3.0 - 4.2	11 - 18	8.0 - 15
	CI	7.7 - 9.8	3.2 - 4.0	13 - 17	9.6 - 12
Leaf (R1)	Mean	14	6.5	27	19
	Range	5.0 - 24	2.0 - 9.6	17 - 31	12 - 26
	CI	9.9 - 17	2.8 - 10	23 - 31	15 - 22
Pollen (R1)	Mean	<0.28[c]	<0.28[c]	<0.28[c]	<0.28[c]
	Range	<0.28[c]	<0.28[c]	<0.28[c]	<0.28[c]
	CI	NA[d]	NA	NA	NA
Root (R1)	Mean	0.44	0.24	1.1	0.98
	Range	0.30 - 0.72	0.12 - 0.48	0.60 - 2.3	0.60 - 1.6
	CI	0.34 - 0.57	0.18 - 0.31	0.86 - 1.4	0.76 - 1.3
Whole Plant (R1)	Mean	4.9	2.4	8.4	6.7
	Range	3.2 - 7.4	1.6 - 3.8	5.4 - 14	4.6 - 9.4
	CI	4.0 - 5.9	1.9 - 2.9	6.9 - 10	5.5 - 8.1
Forage (R4)	Mean	1.9	0.75	3.5	2.5
	Range	1.1 - 2.8	0.54 - 1.2	1.8 - 4.8	1.0 - 4.0
	CI	1.4 - 2.4	0.23 - 1.3	3.0 - 4.0	2.0 - 3.0
Leaf (R4)	Mean	11	4.2	18	13
	Range	5.7 - 20	2.6 - 5.5	9.6 - 26	3.6 - 22
	CI	7.5 - 14	0.95 - 7.3	15 - 21	10 - 17
Root (R4)	Mean	0.16	0.13	0.62	0.33
	Range	<0.069[c] - 0.39	<0.069[c] - 0.33	0.30 - 1.9	<0.069[c] - 0.87
	CI	0.10 - 0.25	0.083 - 0.20	0.40 - 0.95	0.22 - 0.51
Grain (R6)	Mean	<0.069[c]	<0.069[c]	0.089	0.071
	Range	<0.069[c]	<0.069[c]	<0.069[c] - 0.45	<0.069[c] - 0.10
	CI	NA	NA	NA	NA
Leaf (R6)	Mean	0.52	0.25	0.33	0.25
	Range	<0.14[c] - 2.0	<0.14[c] - 1.0	<0.14[c] - 2.5	<0.14[c] - 1.1
	CI	NA	NA	NA	NA

Table 13. PAT Concentrations (ng/mg Tissue Dry Weight) (continued)

Tissue (Growth Stage)		4114 Maize	1507 Maize	1507x59122 Maize	59122 Maize
Root (R6)	Mean	0.13	0.13	0.41	0.41
	Range	<0.069[c] - 0.66	<0.069[c] - 0.39	0.078 - 1.8	0.11 - 1.6
	CI	0.084 - 0.21	0.084 - 0.21	0.26 - 0.64	0.26 - 0.64
Whole Plant (R6)	Mean	0.090	0.073	0.18	0.13
	Range	<0.046[c] - 0.76	<0.046[c] - 0.28	<0.046[c] - 1.1	<0.046[c] - 0.64
	CI	0.028 - 0.29	0.023 - 0.24	0.058 - 0.58	0.039 - 0.40

[a] Least squares means (estimated from statistical models; see Appendix 5 for additional details).
[b] Statistical Confidence Interval
[c] < Lower Limit of Quantification (LLOQ); indicates the values of the sample(s) were detected below the assay LLOQ
[d] Greater than 80% of samples were <LLOQ, statistical analysis not available (NA)

Table 14. Summary of 4114 Maize Protein Concentrations as a Ratio of 1507, 59122, and/or 1507x59122 Maize

Tissue (Growth Stage)	Protein Concentration Ratio		
	4114 Maize:1507 Maize	4114 Maize:59122 Maize	4114 Maize:1507x59122 Maize
Cry1F			
Leaf (V6)	0.611		0.611
Leaf (V9)	0.746		0.606
Leaf (R1)	0.813		0.765
Leaf (R4)	0.919		0.919
Leaf (R6)	2.53		5.41
Root (V6)	0.737		0.667
Root (V9)	0.694		0.725
Root (R1)	0.786		0.902
Root (R4)	0.864		0.717
Root (R6)	0.760		0.809
Whole Plant (V9)	0.750		0.750
Whole Plant (R1)	0.707		0.762
Whole Plant (R6)	0.837		1.08
Pollen (R1)	1.52		1.46
Forage (R4)	0.929		0.857
Grain (R6)	1.03		1.18
Cry34Ab1			
Leaf (V6)		0.636	0.656
Leaf (V9)		0.667	0.619
Leaf (R1)		0.667	0.714
Leaf (R4)		0.579	0.550
Leaf (R6)		2.32	3.17
Root (V6)		0.654	0.586
Root (V9)		0.568	0.583
Root (R1)		0.655	0.655
Root (R4)		0.719	0.657
Root (R6)		0.486	0.563
Whole Plant (V9)		0.561	0.561
Whole Plant (R1)		0.561	0.571
Whole Plant (R6)		0.750	0.750
Pollen (R1)		0.209	0.200
Forage (R4)		0.605	0.559
Grain (R6)		1.00	1.00

Table 14. Summary of 4114 Maize Protein Concentrations as a Ratio of 1507, 59122, and/or 1507x59122 Maize (continued)

Tissue (Growth Stage)	Protein Concentration Ratio		
	4114 Maize:1507 Maize	4114 Maize:59122 Maize	4114 Maize:1507x59122 Maize
Cry35Ab1			
Leaf (V6)		0.750	0.818
Leaf (V9)		0.825	0.717
Leaf (R1)		0.840	0.958
Leaf (R4)		0.900	0.900
Leaf (R6)		1.22	1.00
Root (V6)		0.813	0.722
Root (V9)		0.765	0.765
Root (R1)		0.836	0.920
Root (R4)		0.800	0.800
Root (R6)		0.690	0.775
Whole Plant (V9)		0.894	0.962
Whole Plant (R1)		0.957	0.880
Whole Plant (R6)		0.778	0.677
Pollen (R1)		1.03	1.06[a]
Forage (R4)		0.967	1.04
Grain (R6)		0.857	0.800
PAT			
Leaf (V6)	1.61	0.750	0.563
Leaf (V9)	1.48	0.817	0.467
Leaf (R1)	2.15	0.737	0.519
Leaf (R4)	2.62	0.846	0.611
Leaf (R6)	2.08	2.08	1.58
Root (V6)	1.52	0.400	0.259
Root (V9)	1.81	0.464	0.295
Root (R1)	1.83	0.449	0.400
Root (R4)	1.23	0.485	0.258
Root (R6)	1.00	0.317	0.317
Whole Plant (V9)	2.42	0.791	0.580
Whole Plant (R1)	2.04	0.731	0.583
Whole Plant (R6)	1.23	0.692	0.500
Pollen (R1)	1.00[b]	1.00[b]	1.00[b]
Forage (R4)	2.53	0.760	0.543
Grain (R6)	1.00[b]	0.972	0.775

[a] 1507x59122 maize mean Cry35Ab1 protein concentration value was < Lower Limit of Quantification (LLOQ); therefore, the LLOQ was used.

[b] Mean PAT concentration values were < LLOQ; therefore, the LLOQ was used.

3. Potential Allergenicity and Toxicity of 4114 Maize

The allergenicity and toxicity of 4114 maize was evaluated by examining the allergenic potential of maize as a crop and by assessing the allergenic and toxic potential of the introduced Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins. The proteins expressed in 4114 maize are identical to those in previously approved events 1507 and 59122 maize, as discussed in Section 1. As described in Section 2, the equivalency of the proteins expressed in 4114 maize to those expressed in 1507 and 59122 maize was established by sequencing of the protein coding regions of the insertion and by western blot. These analyses demonstrated that the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins introduced into 4114 maize are equivalent to those proteins expressed in 1507x59122 maize (Section 2 and Appendix 2). Therefore, previous safety studies conducted for 1507 and 59122 maize on the purified proteins are relevant for 4114 maize.

The Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins were derived from common soil bacteria and 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 and are planted on approximately 16% of U.S. maize acres (GfK Kynetec, 2010). 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.

The Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins were previously determined to have little potential for toxicity or allergenicity based on bioinformatics analyses as well as toxicity and allergenicity studies. In this section, published or previously submitted information on the allergenicity and toxicity potential of these proteins is summarized and updated bioinformatics analyses on the proteins are included.

3-A. Allergenicity of Maize

Although maize is widely grown worldwide with overall production at over 700 million tons per year and is the fourth most consumed food calorically, it is not considered a major allergenic food (Hefle *et al*., 1996; Moneret-Vautrin *et al.,* 1998). In a few case studies, allergenic reactions were reported and maize allergens identified. Specifically, the 9 kDa Zea m 14 protein (*i.e.,* the maize lipid transfer protein [LTP]) was identified as the major allergenic protein in maize (Fasoli *et al*., 2009; Pasini *et al.,* 2002; Pastorello *et al.,* 2000; Pastorello *et al.,* 2003). LTPs are small proteins that facilitate the transfer of phospholipids and other lipids across membranes. These proteins are widely distributed throughout the plant kingdom and belong to the pathogenesis-related (PR) protein family (Hoffmann-Sommergruber, 2002). The Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins are neither related to LTPs nor involved in lipid transfer across membranes.

Because maize is not a common allergenic food, it is not expected that the genetic modification in 4114 maize would alter the allergenic potential of maize.

3-B. Assessment of the Allergenicity of the Introduced Proteins in 4114 Maize

The allergenic potential of the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins was previously assessed for 1507 and 59122 maize through a "weight-of-evidence" approach and provided support that the proteins were unlikely to be allergens; this evaluation of allergenicity also has relevance for 4114 maize because it contains the same introduced proteins. A weight-of-evidence approach takes into account a variety of relevant factors and experimental observations used to derive an overall assessment of the allergenic potential of the introduced protein, because no single factor has been recognized as the primary indicator for allergenic potential, and no validated animal model that is predictive of allergenic potential is available (Codex, 2003). The allergenicity potential assessments are therefore based on what is known about food allergens, including the amino acid sequence identity to known human allergens; physicochemical properties such as thermal stability or stability to pepsin or pancreatin digestion *in vitro* (Thomas *et al.*, 2004); glycosylation status, and history of exposure and safety of the gene(s) source.

An important part of the weight-of-evidence approach for assessing the potential allergenicity of proteins introduced and expressed in genetically modified plants is an amino acid sequence comparison of the introduced protein against the sequences of known allergens. This is performed to identify the degree of potential for cross-reactivity between allergenic proteins and the protein of interest. These evaluations employ two techniques; a search for contiguous, identical stretches of eight amino acid residues or longer, and an identity search using the FASTA35 alignment algorithm (Pearson and Lipman, 1988) to search for alignments of 80 amino acid residues or longer possessing a 35% sequence identity or greater (Codex, 2003; FAO/WHO, 2001). If the introduced protein lacks both potential allergenic epitopes and identity to known allergens, the protein is "not a known allergen and is unlikely to be cross-reactive to known allergens" (Codex, 2003; Codex, 2009).

The allergenic protein sequences used for all comparisons were from the peer-reviewed database from the Food Allergy Research and Resource Program (FARRP) at the Department of Food Science and Technology at the University of Nebraska at Lincoln (FARRP Release 11 - January 2011; http://www.allergenonline.com), which contains 1491 entries representing both putative and proven food, environmental, and contact allergens as well as proteins implicated in celiac disease.

Source and History of Safe Exposure to the Cry1F, Cry34Ab1, Cry35Ab1, and PAT Proteins

The Cry1F, Cry34Ab1, and Cry35Ab1 proteins were derived from *Bacillus thuringiensis*, which is a naturally-occurring, common bacterium that is not a known mammalian pathogen and is present in soil, dust, insects, and leaves (EPA, 1998; McClintock *et al.*, 1995; Schnepf *et al.*, 1998). Some strains of *B. thuringiensis* have been shown to be opportunistic pathogens; however, this pathogenicity was not related to the Cry proteins (Hernandez *et al.*, 1999). Microbial preparations of *B. thuringiensis* containing Cry proteins have been used safely as

pesticide sprays for decades, and have been deemed to pose no toxic effects to mammals (EPA, 1998).

The PAT protein from *Streptomyces viridochromogenes* has a safe history of exposure to humans, animals, and the environment. The source organism *S. viridochromogenes* is widespread in soil and is not associated with human, animal, or plant pathogens (Hérouet *et al.*, 2005). Related PAT proteins are found in at least six other species of common soil bacteria, of which none have been reported as toxic or allergenic to humans or animals (Bartsch and Tebbe, 1989; Hérouet *et al.*, 2005; Kutzner, 1981). Furthermore, PAT has been a protein present in commercial maize events, as well as other crops, since 1996.

These proteins have also been present in commercial maize varieties such as 1507, 59122, and 1507x59122 maize since 2003, 2006, and 2006, respectively. These commercial lines are currently licensed broadly across the seed industry; in 2010, commercial products containing 1507x59122 maize were grown on approximately 14 million acres, which represents approximately 16% of U.S. maize acres (GfK Kynetec, 2010). Because of the broad use of 1507x59122 maize in commercial planting and lack of reported adverse effects, there is a history of safe use and exposure of these proteins.

The Potential Allergenicity of the Cry1F Protein

Previous work has established that the Cry1F protein does not have properties typically associated with allergens. The Cry1F protein was found to have no sequence homology with known allergens, to possess no N-glycosylation sites, to be rapidly degraded in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), and to be thermolabile (EPA, 2010a; Ladics *et al.*, 2006).

The bioinformatics analysis was repeated to include protein allergens that have been added to the updated FARRP allergen database since the original analysis was conducted. When the Cry1F protein sequence was used as a query in the search for contiguous, identical stretches of amino acids 8 residues or greater in length, no 8 residue or greater matches to known or putative allergens were returned. Similarly, there were no alignments greater than 35% identity over 80 or greater amino acids.

The updated bioinformatics analysis, the history of safe use of Cry1F, and studies demonstrating the lack of potential allergenicity, support the conclusion that the Cry1F protein is unlikely to be an allergen.

The Potential Allergenicity of the Cry34Ab1 and Cry35Ab1 Proteins

Previous work has established that the Cry34Ab1 and Cry35Ab1 proteins do not have properties typically associated with allergens. These proteins were found to have no sequence homology with known allergens, to possess no N-glycosylation sites, and to be degraded in SGF (EPA, 2010b).

The bioinformatics analysis was repeated to include protein allergens that have been added to the updated FARRP allergen database since the original analyses were conducted. When the Cry34Ab1 and Cry35Ab1 protein sequences were used as a query in the search for contiguous, identical stretches of eight amino acids residues or greater in length, no eight residue or greater matches to known or putative allergens were returned. Similarly, there were no alignments that were greater than 35% identity over 80 or greater amino acids.

The updated bioinformatics analysis, in addition to the history of safe use of the Cry34Ab1 and Cry35Ab1 proteins and studies demonstrating the lack of potential allergenicity, support the conclusion that these proteins are unlikely to be allergens.

The Potential Allergenicity of the PAT Protein

Previous work has established that the PAT protein does not have properties typically associated with allergens (Hérouet *et al.*, 2005). The PAT protein was found to have no sequence homology with known allergens, to possess no N-glycosylation sites, to be rapidly degraded in SGF and SIF, and to be thermolabile (Hérouet *et al.*, 2005).

The bioinformatics analysis of the PAT protein was repeated to take into account protein allergens that have been added to the updated FARRP allergen database since the original work took place. When the PAT protein sequence was used as a query in the search for contiguous, identical stretches of eight amino acids residues or greater in length, no eight contiguous amino acid residue or greater matches to known or putative allergens were returned. Similarly, there were no alignments that were greater than 35% identity over 80 or greater amino acids.

The updated bioinformatics analysis, in addition to the history of safe use of PAT protein and studies demonstrating the lack of potential allergenicity support the conclusion that the PAT protein is unlikely to be an allergen.

3-C. Assessment of the Toxicity of the Proteins Expressed in 4114 Maize

The potential toxicity of the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins was previously assessed for 1507 and 59122 maize by acute toxicity studies and by bioinformatic comparison of the protein sequences to publicly available protein sequences. These assessments provided evidence that the proteins are unlikely to be toxic and are also relevant for the assessment of the proteins in 4114 maize. As described earlier, the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins expressed in 4114 maize have been present in commercial maize lines such as 1507, 59122, and 1507x59122 maize for a number of years and represent a sizable portion of current maize acreage. To date, no known adverse reactions to these proteins have been reported.

Updated bioinformatics analyses were conducted on the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins to include proteins that have been added to public datasets since the original analyses

were conducted. The amino acid sequences of each of the proteins were compared with publicly available protein sequences. Proteins most similar to those produced in 4114 maize were manually inspected to identify any that could be potentially toxic to humans or animals. A close match could be an indicator of toxicological potential of these proteins.

To search for potential similarity to known toxins, the proteins were queried using the BLASTP 2.2.13 algorithm against Release 183.0 (4/15/11) of the NCBI Entrez Protein dataset, which incorporates non-redundant entries from all GenBank and RefSeq nucleotide translations along with protein sequences from SWISS-PROT (http://www.expasy.org/sprot/), PIR (Protein Information Resource; http://pir.georgetown.edu/), PRF (Protein Research Foundation; http://www.prf.or.jp/index-e.html), and wwPDB (Worldwide Protein Data Bank; http://www.wwpdb.org/). The scoring matrix used was the default (BLOSUM62), low complexity filtering was turned off, and the number of alignments returned was set to the maximum value of 2000.

One of the most important parameters to monitor when performing similarity searches is the expectation score, or *E* score. This score represents the probability that a particular alignment is due to random chance and can be used to evaluate the significance of an alignment. The calculated *E* score depends on the overall length of the aligned sequences (including inserted gaps), the number of identical and conserved residues within the alignment, and the size of the database (Baxevanis, 2005; Pearson and Lipman, 1988). When examining an alignment between two protein sequences, a very small *E* score is more likely to reflect a true similarity, while a large *E* score is more likely to be produced by chance and therefore less biologically relevant.

A cutoff expectation (*E*) value of 1.0 was used to generate biologically meaningful similarity between the protein of interest and proteins in the NCBI datasets. Although a statistically significant sequence similarity generally requires a match with an expectation value less than 0.01, a cutoff of $E < 1.0$ insures that proteins with even limited similarity will not be overlooked in the search (Pearson, 2000).

The Potential Toxicity of the Cry1F Protein

Previous work has established that the Cry1F protein does not have properties typically associated with toxins. Cry1F was found to lack sequence homology with known toxins and was not found to be acutely toxic in mice (EPA, 2010a).

In the updated bioinformatics analysis, the Cry1F protein sequence returned 544 protein accessions with an *E* score less than 1.0. Six accessions possessed virtually complete identity ($E = 0$); the top scoring alignment was to the originally isolated Cry1F protein (Chambers *et al.*, 1991) at 99% identity while the remaining 5 accessions were to closely related Cry1F-like proteins from *Bacillus thuringiensis*. Of the remaining 538 alignments returned, 465 are from the soil bacterium *Bacillus thuringiensis*, including 19 accessions classified as hypothetical or unnamed. These proteins are known by a variety of descriptions (parasporal crystal protein,

crystal protein, insecticidal protein, pesticidal crystal protein, insecticidal delta-endotoxin, crystalline entomocidal protoxin, crystal protein, insecticidal toxin, and delta-endotoxin, among others), and represent a class of highly specific insect toxins with a well characterized mode of action that are considered safe for humans and animals (Bravo *et al.,* 2007). Twenty-nine additional matches are to synthetic constructs, consisting primarily of transformation vectors containing intact, truncated, or chimeric versions of the Cry proteins mentioned above. The remaining 44 accessions represent Cry-related protein sequences from other *Bacillus* families, or translations of nucleotide sequences generated during genome sequencing projects. None of the protein sequences returned by the BLASTP search identified safety concerns that might arise from the expression of Cry1F in genetically modified plants.

The updated bioinformatics analysis, in addition to the history of safe use of Cry1F and studies demonstrating the lack of potential toxicity, support the conclusion that the Cry1F protein is unlikely to be a toxin to humans or animals.

The Potential Toxicity of the Cry34Ab1 and Cry35Ab1 Proteins

Previous work has established that the Cry34Ab1 and Cry35Ab1 proteins do not have properties typically associated with toxins. The Cry34Ab1 and Cry35Ab1 proteins were found to not be acutely toxic in mice (EPA, 2010b; Juberg *et al.*, 2009). Furthermore, earlier submitted bioinformatic analysis showed the proteins did not share sequence homology with any known protein toxins (EPA, 2010b).

In the updated bioinformatics analysis, the Cry34Ab1 protein sequence returned 41 protein accessions with an expectation (*E*) score less than 1.0. The top 16 accessions are derived from *Bacillus thuringiensis* (*Bt*). The two highest scoring accessions had identities of 100%; one is listed as a "13.6 kDa insecticidal crystal protein" while the other is annotated as a "Cry34Ab1-like" protein. The fourteen other accessions possess a variety of descriptions (crystal protein, insecticidal crystal protein, Cry34Ab1-like protein, among others), and represent a class of highly specific insect toxins with a well characterized mode of action that are considered safe for humans and animals (Bravo *et al.*, 2007).

The next highest scoring sequence (31% sequence identity) is to a recently added (March 2011) putative protein translation from the *Streptomyces* whole genome sequencing project (RefSeq accession ZP_08234223) that is described as an "aegerolysin"; this sequence has a significant match to the Pfam aegerolysin domain (PF06355) (NCBI, 2011). Sequences from these types of projects are not peer reviewed for annotation accuracy; while they may possess domain similarities to certain classes of proteins, they often have unconfirmed function (NCBI, 2009; NCBI, 2011). In addition, this match is not surprising given that three Cry34Ab1-like protein sequences were used as a scaffold to define the Pfam aegerolysin domain (NCBI, 2011).

Aegerolysins were originally isolated from edible mushrooms and are associated with primordial and fruiting body formation; these proteins have also been isolated from bacteria and plants (Berne *et al.*, 2002; Berne *et al.*, 2009). Only certain fungal aegerolysins have been

shown to have hemolytic properties (Berne *et al.*, 2002). While proteins may be described as aegerolysins and share the aegerolysin domain, many of these proteins possess diverse biological functions unrelated to hemolytic properties (Berne *et al.*, 2009). The few known hemolytic fungal aegerolysins include Aa-Pri1 from *Agrocybe aegerita* (*i.e.,* edible poplar or chestnut mushroom; Fernandez and Labarère, 1997), pleurotolysin A and ostreolysin from *Pleurotus ostreatus* (*i.e.,* edible oyster mushroom; Berne *et al.,* 2005; Sakurai *et al.,* 2004), and Asp-hemolysin from *Aspergillus fumigatus* (Ebina *et al.*, 1994). A CLUSTALW alignment was used to further compare the similarity of Cry34Ab1 and the ZP_08234223 accession with these sequences. Both Cry34Ab1 and the ZP_08234223 accession show very limited identity (13-20%) to any of these known fungal toxins, while the toxin sequences are more homologous to each other (41-80% identity). Furthermore, none of these known fungal toxins appeared in the Cry34Ab1 BLAST search. Therefore, it is unlikely that Cry34Ab1 possesses the toxic properties of the known fungal aegerolysins.

Of the remaining 24 sequences, 10 represent insecticidal crystal proteins from non-*Bt* species, while 12 others are annotated as hypothetical or predicted proteins derived from the translations of nucleotide sequences generated during genome sequencing projects. The two remaining sequences are low significance matches, one annotated as a beta-N-acetylglucosaminidase and the other a lysophospholipase Plb1. None of the protein sequences returned by the BLASTP search identified safety concerns that might arise from the expression of Cry34Ab1 protein in genetically modified plants.

The Cry35Ab1 protein sequence returned 45 protein accessions with an expectation (*E*) score less than 1.0. Nine accessions possessed an *E* score of 0; all 9 were derived from *Bacillus thuringiensis.* The top scoring alignment, a "43.8 kDa insecticidal crystal protein", displayed 100% identity over the entire length of the Cry35Ab1 sequence. There were an additional seven accessions from *Bacillus thuringiensis*, and 19 accessions displaying similarity to the mosquitocidal *Bin* toxins from *Lysinibacillus sphaericus* (synonym: *Bacillus sphaericus*) and *Bacillus cereus* (Baumann *et al.*, 1988; Baumann *et al.*, 1991; reviewed by Federici *et al.*, 2003). Of the remaining 10 sequences, six sequences of low significance (*E*=0.053-0.45) represent translations of nucleotide sequences generated during genome sequencing projects and four accessions are described as hypothetical proteins. None of the protein sequences returned by the BLASTP search identified safety concerns that might arise from the expression of the Cry35Ab1 protein in genetically modified plants.

The updated bioinformatics analysis, in addition to the history of safe use of the Cry34Ab1 and Cry35Ab1 proteins and studies demonstrating the lack of potential toxicity, support the conclusion that these proteins are unlikely to be toxins to humans or animals.

The Potential Toxicity of the PAT Protein

Previous work has established that the PAT protein does not have properties typically associated with toxins. PAT was found to lack sequence homology with known toxins and were found not to be acutely toxic in mice (Hérouet *et al.*, 2005).

In the updated bioinformatics analysis, the PAT protein sequence returned 1752 protein accessions with an expectation (*E*) score less than 1.0. The highest scoring alignments (*E* = 1 x 10^{-105} and 1 x 10^{-88}, respectively) were attributed to PAT proteins from *Streptomyces viridochromogene*s (100% identity over the entire 183 amino acid length of the query sequence) and *Streptomyces hygroscopicus* (84% identity to the query sequence). Both of these proteins have undergone extensive safety evaluations and have been deemed safe for expression in transgenic plants (Hérouet *et al.*, 2005). Seven other accessions represented sequences derived from these two bacterial strains.

Twelve of the alignments were to synthetic constructs, consisting primarily of transformation vectors containing portions of the PAT proteins mentioned above. Of the remaining 1731 sequences, 518 were annotated as phosphinothricin acetyltransferases. This bacterial enzyme deactivates the non-selective herbicide glufosinate, which is a potent inhibitor of glutamine synthesis (Dröge-Laser *et al.*, 1994) and is widely used for the generation of glufosinate-tolerant transgenic plants. Another 861 alignments are with sequences characterized as known or putative acetyltransferases, N-acetyltransferases, or GCN5 or GNAT acetyltransferases, based upon the possession of an acetyltransferase domain present in the PAT query sequence. One hundred and thirty-seven other accessions were classified as acetyltransferase related sequences including sortases, acyltransferases, antibiotic or toxin resistance proteins, ribosomal-protein-alanine acetyltransferases, or histone acetyltransferases. Ten other accessions were annotated as ArsR family transcriptional regulators, containing both the Arsenical Resistance Operon Repressor domain as well as the acetyltransferase domain. Fourteen accessions described as possessing a range of functionalities were returned; all of these sequences contained additional domains responsible for their annotations. Lastly, 191 of the accessions returned were classified as hypothetical, predicted, putative, unnamed, or unknown proteins. None of the protein sequences returned by the BLASTP search identified any safety concerns from the expression of PAT protein in genetically modified plants.

The updated bioinformatics analysis, in addition to the history of safe use of the PAT protein and studies demonstrating the lack of potential toxicity, support the conclusion that this protein is unlikely to be a toxin.

3-D. Overall Conclusions on the Potential Allergenicity and Toxicity of 4114 Maize

The potential for allergenicity and toxicity of 4114 maize was evaluated by examining the allergenic potential of maize as a crop and by assessing the allergenic and toxic potential of the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins. Maize is not a common allergenic food and the modification in 4114 maize is not expected to alter the allergenic potential of maize. The Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins have been assessed previously for 1507 and 59122 maize and have been determined to be unlikely to be potential allergens or toxins to humans and animals. Previous assessments of these proteins included bioinformatic analyses, digestibility, N-glycosylation studies, acute protein toxicity studies, and in some cases, heat stability studies; these studies are relevant for the assessment of the proteins in 4114 maize.

Updated bioinformatic analyses support the original conclusions that the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins are unlikely to be allergens or toxins. Additionally, 1507x59122 maize has been commercially available in the U.S. since the 2006 growing season with no reports of allergenicity or toxicity. These data support the conclusion that the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins in 4114 maize are unlikely to be potential allergens or toxins to humans and animals, and therefore support the food and feed safety of these proteins. These data have been provided to EPA in support of the safety of these proteins and also will be submitted to the FDA for the food and feed safety assessment of 4114 maize.

4. Comparative Assessment of 4114 Maize

In order to assess the safety of 4114 maize relative to conventional maize, a comparative assessment was conducted that was designed to be independent of the intended effects of the insect-resistant and herbicide-tolerant traits. The comparative assessment uses non-transformed (*i.e.,* conventional) comparators for compositional and agronomic comparisons and will establish the safety of 4114 maize relative to varieties that have a history of safe use in the environment and as food and feed. The objective is to determine if the modified organism presents any new or greater risks relative to its comparator, or whether it can be used interchangeably with its comparator without negatively affecting human and animal health and the environment in which it is grown. The compositional and agronomic analyses will highlight any differences between 4114 maize and its comparators in order to determine if 4114 maize is as safe as other lines that have a history of safe use. The studies were designed to eliminate the intended effects of the introduced traits (*i.e.,* trait efficacy) so that appropriate comparisons would be able to be made between 4114 maize and conventional maize and unintended effects due to the 4114 maize insertion would also be evaluated. Therefore, the receiving environment was managed to minimize insect and weed pressure in the evaluations.

To ensure an accurate comparative evaluation of the transgenic plant, a proper selection of comparator plants is important. As described earlier in Section 2, non-transgenic near-isoline maize lines were selected as comparators for 4114 maize in these analyses, since they had a similar genetic background (*i.e.*, approximately 99% similar). Statistical comparisons were made to these comparators to determine if there were any significant differences. In order to determine if any observed statistical differences were indicative of differences with conventional maize varieties, a statistical tolerance interval was established from non-transgenic commercial maize lines for the compositional and agronomic comparisons. These commercial lines are typical of those grown in maize-growing regions; represent a wide range of varieties that would normally be planted commercially; and represent the normal range of variation of the maize crop. In addition, for the compositional assessment, publicly available information was gathered on the range of natural variation of maize analyte concentrations (*i.e.,* literature range). If the measured values of 4114 maize fell within the statistical tolerance interval or the literature range, then these measured values would be considered comparable to conventional maize.

The compositional and agronomic comparative assessments of 4114 maize are discussed further below. These analyses indicated that 4114 maize was comparable to conventional maize with respect to the compositional analytes and agronomic characteristics measured and independently of the intended effects of the insect-resistant and herbicide-tolerant traits. Overall, these analyses indicate that 4114 maize is as safe as conventional maize varieties and does not pose a greater risk than conventional maize varieties in food, feed, and the environment.

4-A. Compositional Assessment

Compositional analysis of 4114 maize grain and forage was used to evaluate any changes in the levels of key nutrients, anti-nutrients, and secondary metabolites compared to the non-transformed, near-isoline control. For this analysis, grain was selected because of its use in both food and feed, and forage was selected for its use as feed. These analyses provided an indication whether 4114 maize is as nutritious as conventional maize. The U.S. FDA will review the details of the compositional analyses as a component of the food and feed safety assessment of 4114 maize.

Comprehensive compositional analyses were performed on grain and forage from 4114 maize and a near-isoline control grown in 2010 at six field locations in the U.S. and Canada (see Section 4-B; Table 28; Experiment B). These sites are representative of commercial maize-growing areas. The F1*[5] generation of seed (Section 2; Figure 10 and Table 3) was planted because F1 hybrid seed is representative of seed that growers would plant in commercial maize fields. The near-isoline control plants had a genetic background approximately 99% similar to that of the 4114 maize generation used, but did not go through the transformation process.

Although 4114 maize contains traits that are efficacious against certain lepidopteran and corn rootworm target pests and glufosinate-ammonium tolerance, each field trial site was managed to maintain a relatively insect-free and weed-free environment. Evaluation in this environment ensured that compositional characteristics measured would be independent of the intended effects of the insect-resistant and herbicide-tolerant traits; therefore, these evaluations would appropriately compare 4114 maize to conventional maize and also assess unintended effects of the transgene. Composition was not impacted by the intended effects of the traits and therefore could be used to assess comparability of 4114 maize to conventional maize.

Each location utilized a randomized complete block design containing four blocks. Each block contained 4114 maize and the control maize planted in two-row plots. One forage sample (sub-sample of 3 whole plants) was collected at the R4 growth stage from 4114 maize and the control maize from each of the four blocks. Forage samples were obtained by cutting the aerial portion of the plants from the root system approximately 1 inch (2.5 cm) above the soil surface after secondary or tertiary ears with exposed silks were removed. The plants (including the primary ear) were then cut into sections approximately 3 inches (7.5 cm) or less, and approximately 1/3 of the total sample was collected in a pre-labeled plastic-lined cloth bag. One grain sample (equal to five pooled ears) was collected at the R6 growth stage from 4114 maize and the control maize from each of the four blocks. All samples were collected from impartially selected, healthy individual plants that had previously been self-pollinated.

Forage and grain were also collected from non-modified commercial maize hybrids (reference hybrids) in two separate experiments. Pioneer® hybrids 34M94, 33G26, 33J24, and 3394 were analyzed in 2003 at six field locations in maize-growing areas of North America (Bagley, IA; York, NE; Chula, GA; New Holland, OH; Larned, KS and Hereford, PA). Pioneer® hybrids 38B85, 37Y12, 34A15, and 34P88 were analyzed in 2007 at six field locations in maize-growing areas of North

America (Tallahassee, FL; York, NE; Germansville, PA; Richland, IA; Larned, KS and Branchton, Ontario). These Pioneer commercial products were chosen to represent a wide range of non-genetically modified varieties that would normally be planted commercially.

For the reference hybrid trials, each location utilized a randomized complete block design containing three blocks. Each block contained two-row plots. Procedures for planting, harvesting, processing, and compositional analysis of the reference hybrid trials were similar to those employed for the trials containing near-isoline control and 4114 maize. Composition data collected from the reference hybrids was used to help determine the normal variation for the measured analytes in commercial maize.

The analytes for compositional assessment were selected considering the OECD consensus document on compositional considerations for new varieties of maize (OECD, 2002). Compositional analyses of grain samples included protein, fat, acid detergent fiber (ADF), neutral detergent fiber (NDF), ash, carbohydrates, fatty acids, amino acids, vitamins and minerals, key anti-nutrients, and key secondary metabolites. Compositional analyses of forage samples included protein, fat, ADF, NDF, ash, carbohydrates, calcium, and phosphorus.

Statistical analysis of nutrient composition data was conducted to test for differences in the analyte mean values between 4114 maize and the control. (For details of the statistical methodology, refer to Appendix 6). When numerous analytes are being evaluated on the same samples, controlling false positive outcomes is important. Since the introduction of the false discovery rate (FDR) approach in the mid-1990's, it has been widely employed across a number of scientific disciplines, including genomics, ecology, medicine, plant breeding, epidemiology, dairy science, and signal/image processing (*e.g.,* Pawitan *et al.,* 2005; Spelman and Bovenhuis, 1998). A false positive outcome occurs when an analyte mean of the transgenic line is deemed significantly different from the analyte mean of the control line, when in fact the two means are not different. If one uses a 5% type I error rate for each analyte, then the number of false positives increases as the number of analytes increase. In order to help manage the false positive rate, the false discovery rate (FDR) method of Benjamini and Hochberg was applied to account for making multiple comparisons (Benjamini and Hochberg, 1995; Westfall *et al*., 1999). P-values were adjusted accordingly, resulting in the false positive rate being held to 5%. Both adjusted and non-adjusted P-values are reported for the analyses that follow. A significant difference between the mean of 4114 maize and that of the control line was established with an FDR-adjusted P-value <0.05.

Using the data obtained from the reference hybrids, a statistical tolerance interval was calculated to contain, with 95% confidence, 99% of the values contained in the population of commercial maize. This statistical tolerance interval and the combined range of values for each analyte from the published literature, where available, provided further context for interpretation of the composition results for 4114 maize. 4114 maize analyte ranges that fell within the tolerance interval and/or combined literature range for that analyte were considered to be within the range of normal variability of commercial maize hybrids.

Key Nutrients in Maize Grain

As described in Section 1-E, maize grain is used in the U.S. for food, feed, and fuel. Approximately 38.7% of maize grown in the U.S. is used domestically for animal feed (primarily beef, poultry, pork and dairy) because of its high nutrient value and relative low cost (NCGA, 2011). Approximately 10.1% of maize grain is used for food products, with 3.8% going to high fructose corn syrup, and 6.3% comprising beverage alcohol, cereal, other sweeteners, and starch (NCGA, 2011). A significant portion of maize is used for fuel ethanol (36.5%) with the remainder being exported (14.5%) (NCGA, 2011).

Proximates in Maize Grain

Proximates were analyzed in 4114 maize and near-isoline control grain. Results are shown in Table 15. No statistically significant differences were observed between the 4114 maize and control mean values for crude protein, crude fat, crude fiber, acid detergent fiber (ADF), neutral detergent fiber (NDF), and carbohydrates (adjusted P-values > 0.05). A statistically significant difference was observed between the 4114 maize and control mean values for ash, however all of the individual values were within the tolerance interval.

In conclusion, analysis of proximates in maize grain demonstrates that 4114 maize is comparable to near-isoline and reference maize hybrids.

Table 15. Proximates in Maize Grain

Analyte (% Dry Weight)		Control (n=24)	4114 maize (n=24)	Tolerance Interval	Literature Range[a]
Crude Protein	Mean[b]	10.2	10.4	6.59 - 13.5	6.00 - 17.3
	Range[c]	8.92 - 11.4	7.32 - 11.7		
	CI[d]	9.54 - 10.7	9.78 - 10.9		
	Adjusted P-Value[e]		0.789		
	P-Value[f]		0.455		
Crude Fat	Mean	4.81	4.68	1.45 - 5.75	2.47 - 5.90
	Range	4.28 - 5.73	3.88 - 5.67		
	CI	4.47 - 5.17	4.35 - 5.04		
	Adjusted P-Value		0.679		
	P-Value		0.177		
Crude Fiber	Mean	2.57	2.48	0.941 - 3.73	0.490 - 5.50
	Range	1.36 - 3.38	1.34 - 3.16		
	CI	2.35 - 2.80	2.27 - 2.70		
	Adjusted P-Value		0.789		
	P-Value		0.520		
ADF	Mean	3.83	4.01	1.43 - 5.73	1.82 - 11.3
	Range	3.24 - 4.48	3.53 - 5.16		
	CI	3.56 - 4.12	3.72 - 4.32		
	Adjusted P-Value		0.679		
	P-Value		0.0965		
NDF	Mean	10.4	10.4	5.75 - 20.6	5.59 - 22.6
	Range	9.39 - 11.3	9.58 - 11.2		
	CI	9.97 - 10.8	9.93 - 10.8		
	Adjusted P-Value		0.908		
	P-Value		0.824		
Ash	Mean	1.33	1.44	0.531 - 2.16	0.616 - 6.28
	Range	1.12 - 1.52	1.30 - 1.60		
	CI	1.29 - 1.38	1.39 - 1.48		
	Adjusted P-Value		0.0498[g]		
	P-Value		0.00298		
Carbohydrates	Mean	83.7	83.6	80.3 - 89.7	77.4 - 89.5
	Range	82.5 - 84.9	82.2 - 85.7		
	CI	83.2 - 84.2	83.1 - 84.1		
	Adjusted P-Value		0.908		
	P-Value		0.758		

[a] Literature ranges are taken from published literature for maize (Codex, 1996; Codex, 2005; ILSI, 2006; OECD, 2002; Watson, 1982; Watson, 1987).
[b] Least squares mean
[c] Range denotes the lowest and highest individual value across locations.
[d] 95% Confidence Interval
[e] False Discovery Rate (FDR) adjusted P-value
[f] Non-adjusted P-value
[g] Statistically significant difference, adjusted P-value < 0.05

Vitamins and Minerals in Maize Grain

Maize is a nutritional source of vitamins and minerals for both humans and animals; therefore vitamins and minerals were measured in 4114 maize and near-isoline control grain. Based on OECD guidance, the following vitamins were analyzed: beta-carotene, vitamin B1 (thiamin), vitamin B2 (riboflavin), vitamin B3 (niacin), vitamin B6 (pyridoxine), vitamin B9 (folic acid), and vitamin E (α-tocopherol) (OECD, 2002). The following minerals were also analyzed, based on OECD guidance: calcium, copper, iron, magnesium, phosphorus, potassium, sodium and zinc (OECD, 2002).

Vitamin results are shown in Table 16 and mineral results are shown in Table 17. No statistically significant differences were observed between the 4114 maize and control mean values for any of the vitamins analyzed (adjusted P-values were > 0.05). For the minerals, there were no statistical differences for all of the analytes except phosphorus and potassium, however all individual data points were within the respective tolerance intervals.

In conclusion, vitamin and mineral analyses of maize grain demonstrate that 4114 maize is comparable to near-isoline and reference maize hybrids.

Table 16. Vitamins in Maize Grain

Analyte (mg/kg Dry Weight)		Control (n=24)	4114 maize (n=24)	Tolerance Interval	Literature Range[a]
Beta-Carotene	Mean[b]	16.4	18.1	0 - 68.3	0.190 - 46.8
	Range[c]	7.27 - 26.2	11.7 - 26.4		
	CI[d]	12.4 - 21.7	13.7 - 24.0		
	Adjusted P-Value[e]		0.679		
	P-Value[f]		0.267		
Vitamin B1 (Thiamine)	Mean	1.97	2.20	0.414 - 6.64	1.26 - 40.0
	Range	<1.80[g] - 2.92	<1.80[g] - 3.28		
	CI	1.44 - 2.70	1.62 - 2.99		
	Adjusted P-Value		0.679		
	P-Value		0.168		
Vitamin B2 (Riboflavin)	Mean	<0.900[g]	<0.900[g]	NC[h]	0.250 - 5.60
	Range	<0.900[g]	<0.900[g]		
	CI	NA[i]	NA[i]		
	Adjusted P-Value		NA[i]		
	P-Value		NA[i]		
Vitamin B3 (Niacin)	Mean	13.9	13.8	0 - 51.7	9.30 - 70.0
	Range	10.6 - 18.0	11.7 - 16.6		
	CI	12.8 - 15.1	12.7 - 15.0		
	Adjusted P-Value		0.908		
	P-Value		0.786		
Vitamin B6 (Pyridoxine)	Mean	4.68	4.29	1.83 - 11.1	3.68 - 11.3
	Range	2.35 - 6.88	2.50 - 6.36		
	CI	3.60 - 5.90	3.26 - 5.46		
	Adjusted P-Value		0.772		
	P-Value		0.404		
Vitamin B9 (Folic Acid)	Mean	0.872	0.792	0 - 2.30	0.147 – 683
	Range	0.181 - 1.91	0.0369 - 1.55		
	CI	0.656 - 1.09	0.577 - 1.01		
	Adjusted P-Value		0.789		
	P-Value		0.523		

Table 16. Vitamins in Maize Grain (continued)

Analyte (mg/kg Dry Weight)		Control (n=24)	4114 maize (n=24)	Tolerance Interval	Literature Range[a]
Vitamin E (α-Tocopherol)	Mean	5.47	5.68	2.18 - 28.2	1.50 - 68.7
	Range	2.09 - 13.3	2.14 - 14.8		
	CI	3.06 - 9.75	3.18 - 10.1		
	Adjusted P-Value		0.908		
	P-Value		0.672		

[a] Literature ranges are taken from published literature for maize (Codex, 1996; Codex, 2005; ILSI, 2006; OECD, 2002; Watson, 1982; Watson, 1987).
[b] Least squares mean
[c] Range denotes the lowest and highest individual value across locations
[d] 95% Confidence Interval
[e] False Discovery Rate (FDR) adjusted P-value
[f] Non-adjusted P-value
[g] <Lower limit of quantification (LLOQ); indicates that sample value(s) were detected below the assay LLOQ. Sample results which were below the LLOQ were assigned a value equal to the LLOQ for statistical analysis
[h] Tolerance interval not calculated (NC)
[i] Statistical analysis not available (NA)

Table 17. Minerals in Maize Grain

Analyte (% Dry Weight)		Control (n=24)	4114 maize (n=24)	Tolerance Interval	Literature Range[a]
Calcium	Mean[b]	0.00325	0.00335	0.00127 - 0.00902	0.00127 - 0.100
	Range[c]	0.00270 - 0.00399	0.00251 - 0.00448		
	CI[d]	0.00288 - 0.00367	0.00296 - 0.00378		
	Adjusted P-Value[e]		0.679		
	P-Value[f]		0.308		
Copper	Mean	0.0000645	0.0000653	0 - 0.000662	0.0000730 - 0.00185
	Range	<0.0000625[g] - 0.0000869	<0.0000625[g] - 0.0000876		
	CI	NA[h]	NA[h]		
	Adjusted P-Value		NA[h]		
	P-Value		NA[h]		
Iron	Mean	0.00168	0.00166	0.000857 - 0.00269	0.000100 - 0.0100
	Range	0.00146 - 0.00228	0.00147 - 0.00210		
	CI	0.00156 - 0.00181	0.00155 - 0.00179		
	Adjusted P-Value		0.908		
	P-Value		0.775		
Magnesium	Mean	0.133	0.135	0.0381 - 0.195	0.0594 - 1.00
	Range	0.103 - 0.155	0.112 - 0.157		
	CI	0.121 - 0.146	0.123 - 0.149		
	Adjusted P-Value		0.679		
	P-Value		0.251		
Phosphorus	Mean	0.289	0.311	0.127 - 0.472	0.147 - 0.750
	Range	0.258 - 0.334	0.296 - 0.330		
	CI	0.278 - 0.301	0.298 - 0.324		
	Adjusted P-Value		0.0115[i]		
	P-Value		0.000353		
Potassium	Mean	0.387	0.416	0.194 - 0.687	0.181 - 0.720
	Range	0.343 - 0.441	0.385 - 0.478		
	CI	0.373 - 0.401	0.401 - 0.431		
	Adjusted P-Value		0.0498[i]		
	P-Value		0.00306		
Sodium	Mean	0.0000582	0.0000628	0 - 0.00207	0 - 0.150
	Range	<0.0000625[g] - 0.000489	<0.0000625[g] - 0.000171		
	CI	0.0000409 - 0.0000828	0.0000447 - 0.0000882		
	Adjusted P-Value		0.908		
	P-Value		0.714		

Table 17. Minerals in Maize Grain (continued)

Analyte (% Dry Weight)		Control (n=24)	4114 maize (n=24)	Tolerance Interval	Literature Range[a]
Zinc	Mean	0.00174	0.00174	0.00104 - 0.00271	0.000650 - 0.00372
	Range	0.00150 - 0.00203	0.00149 - 0.00201		
	CI	0.00162 - 0.00188	0.00161 - 0.00188		
	Adjusted P-Value		0.946		
	P-Value		0.919		

[a] Literature ranges are taken from published literature for maize (Codex, 1996; Codex, 2005; ILSI, 2006; OECD, 2002; Watson, 1982; Watson, 1987).
[b] Least squares mean
[c] Range denotes the lowest and highest individual value across locations.
[d] 95% Confidence Interval
[e] False Discovery Rate (FDR) adjusted P-value
[f] Non-adjusted P-value
[g] <Lower limit of quantification (LLOQ); indicates that sample value(s) were detected below the assay LLOQ. Sample results which were below the LLOQ were assigned a value equal to the LLOQ for statistical analysis.
[h] Statistical analysis not available (NA)
[i] Statistically significant difference, adjusted P-value < 0.05

Fatty Acids in Maize Grain

The oil in maize consists mostly of five major fatty acids: palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) (OECD, 2002). Other fatty acids are present, but usually fall below 1% of total fatty acids (OECD, 2002). The complete fatty acid profile was measured in seed samples derived from 4114 maize, and compared with corresponding values from samples of control maize.

Results of the fatty acid analysis are shown in Table 18. The concentrations of the following fatty acids were below the lower limit of quantification for both 4114 maize and control and are not reported in Table 18: Caprylic Acid (C8:0); Capric Acid (C10:0); Lauric Acid (C12:0); Myristic Acid (C14:0); Myrisoleic Acid (C14:1); Pentadecanoic Acid (C15:0); Pentadecenoic Acid (C15:1); Heptadecenoic Acid (C17:1); γ- Linolenic Acid (C18:3); Nonadecanoic Acid (C19:0); Eicosatrienoic Acid (C20:3); Arachidonic Acid (C20:4); Heneicosanoic Acid (C21:0); Erucic Acid (C22:1); and Tricosanoic Acid (C23:0).

A small but statistically significant difference (adjusted P-value was < 0.05) was observed in the concentration of eicosenoic acid between 4114 maize and control samples, however all individual data points for 4114 maize were within the tolerance interval. No statistically significant differences were observed between the 4114 maize and near-isoline control for any of the other fatty acid mean values (adjusted P-values were > 0.05).

In conclusion, fatty acid analysis of maize grain demonstrates that 4114 maize is comparable to near-isoline and reference maize hybrids.

Table 18. Fatty Acids in Maize Grain

Analyte (% Total Fatty Acids)[a]		Control (n=24)	4114 maize (n=24)	Tolerance Interval	Literature Range[b]
Palmitic Acid (C16:0)	Mean[c]	14.1	14.2	5.51 - 18.4	7.00 - 20.7
	Range[d]	13.2 - 15.6	13.1 - 16.2		
	CI[e]	13.6 - 14.5	13.7 - 14.7		
	Adjusted P-Value[f]		0.789		
	P-Value[g]		0.518		
Palmitoleic Acid (C16:1)	Mean	0.124	0.139	0 - 0.337	0 - 1.00
	Range	0.0948 - 0.144	0.125 - 0.156		
	CI	NA[h]	NA		
	Adjusted P-Value		NA		
	P-Value		NA		
Heptadecanoic Acid (C17:0)	Mean	0.0940	0.100	0 - 0.189	0 - 0.111
	Range	0.0940 - 0.0940	0.100 - 0.100		
	CI	NA	NA		
	Adjusted P-Value		NA		
	P-Value		NA		
Heptadecadienoic Acid (C17:2)	Mean	0.307	0	NC[i]	NR[j]
	Range	0.307 - 0.307	0 – 0		
	CI	NA	NA		
	Adjusted P-Value		NA		
	P-Value		NA		
Stearic Acid (C18:0)	Mean	1.60	1.60	0.566 - 4.67	0 - 4.00
	Range	1.45 - 2.07	1.37 - 2.14		
	CI	1.46 - 1.75	1.47 - 1.75		
	Adjusted P-Value		0.908		
	P-Value		0.810		
Oleic Acid (C18:1)	Mean	22.6	22.1	10.4 - 65.6	17.4 - 50.0
	Range	20.7 - 25.8	20.3 - 23.8		
	CI	21.5 - 23.8	21.0 - 23.2		
	Adjusted P-Value		0.0817		
	P-Value		0.00629		
Linoleic Acid (C18:2)	Mean	60.1	60.5	30.4 - 81.7	34.0 - 70.0
	Range	54.9 - 62.9	56.3 - 63.5		
	CI	58.4 - 61.8	58.8 - 62.2		
	Adjusted P-Value		0.679		
	P-Value		0.203		
(9,15) Isomer of Linoleic Acid (C18:2)	Mean	0	0.249	0 - 6.92	NR
	Range	0 - 0	0.240 - 0.258		
	CI	NA	NA		
	Adjusted P-Value		NA		
	P-Value		NA		

Table 18. Fatty Acids in Maize Grain (continued)

Analyte (% Total Fatty Acids)[a]		Control (n=24)	4114 maize (n=24)	Tolerance Interval	Literature Range[b]
Linolenic Acid (C18:3)	Mean	0.787	0.770	0 - 3.34	0 - 2.25
	Range	0.410 - 1.22	0.305 - 1.12		
	CI	0.576 - 1.08	0.563 - 1.05		
	Adjusted P-Value		0.908		
	P-Value		0.697		
Arachidic Acid (C20:0)	Mean	0.392	0.394	0.159 - 0.849	0 - 2.00
	Range	0.343 - 0.505	0.347 - 0.481		
	CI	0.359 - 0.428	0.361 - 0.430		
	Adjusted P-Value		0.789		
	P-Value		0.518		
Eicosenoic Acid (C20:1)	Mean	0.235	0.265	0.213 - 0.370	0 - 1.92
	Range	0.204 - 0.255	0.229 - 0.336		
	CI	0.224 - 0.246	0.253 - 0.278		
	Adjusted P-Value		0.00472[k]		
	P-Value		<0.0001		
Eicosadienoic Acid (C20:2)	Mean	0.275	0	0 - 0.351[l]	0 - 0.533
	Range	0.275 - 0.275	0 – 0		
	CI	NA	NA		
	Adjusted P-Value		NA		
	P-Value		NA		
Behenic Acid (C22:0)	Mean	0	0.291	0 - 0.566	0 - 0.500
	Range	0 - 0	0.291 - 0.291		
	CI	NA	NA		
	Adjusted P-Value		NA		
	P-Value		NA		

Table 18. Fatty Acids in Maize Grain (continued)

Analyte (% Total Fatty Acids)[a]		Control (n=24)	4114 maize (n=24)	Tolerance Interval	Literature Range[b]
Lignoceric Acid (C24:0)	Mean	0.240	0.233	0 - 0.675	0 - 0.500
	Range	0.185 - 0.318	0.211 - 0.276		
	CI	0.205 - 0.280	0.200 - 0.273		
	Adjusted P-Value		0.908		
	P-Value		0.796		

[a] The concentrations of the following fatty acids were below the lower limit of quantification for both 4114 maize and control and are not reported here: Caprylic Acid (C8:0); Capric Acid (C10:0); Lauric Acid (C12:0); Myristic Acid (C14:0); Myrisoleic Acid (C14:1); Pentadecanoic Acid (C15:0); Pentadecenoic Acid (C15:1); Heptadecenoic Acid (C17:1); γ- Linolenic Acid (C18:3); Nonadecanoic Acid (C19:0); Eicosatrienoic Acid (C20:3); Arachidonic Acid (C20:4); Heneicosanoic Acid (C21:0); Erucic Acid (C22:1); and Tricosanoic Acid (C23:0).

[b] Literature ranges are taken from published literature for maize (Codex, 1996; Codex, 2005; ILSI, 2006; OECD, 2002; Watson, 1982; Watson, 1987).

[c] Least squares mean

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

[e] 95% Confidence Interval

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

[g] Non-adjusted P-value

[h] Statistical analysis not available (NA)

[i] A tolerance interval could not be calculated (NC)

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

[k] Statistically significant difference, adjusted P-value < 0.05

[l] No tolerance interval could be calculated for this analyte, and the listed interval is the min/max range.

Total Amino Acids in Maize Grain

Maize grain is generally a good source of essential and non-essential amino acids for most domestic animal species. Total levels of 18 amino acids were measured in 4114 maize and near-isoline control grain.

Results are shown in Table 19. No statistically significant differences were observed between the 4114 maize and near-isoline control maize for any of the amino acid mean values (adjusted P-values were > 0.05).

In conclusion, total amino acid analysis of maize grain demonstrates that 4114 maize is comparable to near-isoline and reference maize hybrids.

Table 19. Total Amino Acids in Maize Grain

Analyte (% Dry Weight)		Control (n=24)	4114 maize (n=24)	Tolerance Interval	Literature Range[a]
Alanine	Mean[b]	0.739	0.757	0.491 - 1.09	0.439 - 1.39
	Range[c]	0.604 - 0.840	0.460 - 0.886		
	CI[d]	0.676 - 0.797	0.695 - 0.813		
	Adjusted P-Value[e]		0.772		
	P-Value[f]		0.398		
Arginine	Mean	0.434	0.438	0.253 - 0.551	0.119 - 0.640
	Range	0.362 - 0.492	0.364 - 0.514		
	CI	0.408 - 0.463	0.411 - 0.466		
	Adjusted P-Value		0.908		
	P-Value		0.707		
Aspartic Acid	Mean	0.680	0.701	0.442 - 0.947	0.335 - 1.21
	Range	0.562 - 0.751	0.460 - 0.813		
	CI	0.634 - 0.724	0.657 - 0.743		
	Adjusted P-Value		0.679		
	P-Value		0.260		
Cystine	Mean	0.193	0.194	0.136 - 0.418	0.0800 - 0.514
	Range	0.157 - 0.253	0.144 - 0.252		
	CI	0.168 - 0.220	0.169 - 0.222		
	Adjusted P-Value		0.946		
	P-Value		0.931		
Glutamic Acid	Mean	1.93	1.96	1.17 - 2.88	0.965 - 3.54
	Range	1.59 - 2.24	1.21 - 2.31		
	CI	1.78 - 2.07	1.82 - 2.10		
	Adjusted P-Value		0.789		
	P-Value		0.546		
Glycine	Mean	0.372	0.377	0.249 - 0.485	0.184 - 0.539
	Range	0.318 - 0.442	0.305 - 0.445		
	CI	0.346 - 0.400	0.351 - 0.406		
	Adjusted P-Value		0.789		
	P-Value		0.461		
Histidine	Mean	0.293	0.303	0.180 - 0.362	0.137 - 0.434
	Range	0.257 - 0.342	0.230 - 0.426		
	CI	0.271 - 0.317	0.280 - 0.328		
	Adjusted P-Value		0.679		
	P-Value		0.274		
Isoleucine	Mean	0.339	0.349	0.143 - 0.587	0.179 - 0.710
	Range	0.288 - 0.388	0.229 - 0.408		
	CI	0.312 - 0.364	0.323 - 0.374		
	Adjusted P-Value		0.679		
	P-Value		0.251		

Table 19. Total Amino Acids in Maize Grain (continued)

Analyte (% Dry Weight)		Control (n=24)	4114 maize (n=24)	Tolerance Interval	Literature Range[a]
Leucine	Mean	1.26	1.31	0.659 - 1.95	0.642 - 2.49
	Range	1.02 - 1.49	0.775 - 1.56		
	CI	1.13 - 1.37	1.19 - 1.42		
	Adjusted P-Value		0.679		
	P-Value		0.206		
Lysine	Mean	0.293	0.288	0.112 - 0.551	0.0500 - 0.668
	Range	0.224 - 0.349	0.213 - 0.350		
	CI	0.256 - 0.335	0.252 - 0.330		
	Adjusted P-Value		0.789		
	P-Value		0.474		
Methionine	Mean	0.179	0.182	0.0724 - 0.490	0.100 - 0.468
	Range	0.144 - 0.231	0.136 - 0.230		
	CI	0.156 - 0.206	0.158 - 0.209		
	Adjusted P-Value		0.916		
	P-Value		0.846		
Phenylalanine	Mean	0.523	0.554	0.298 - 0.693	0.244 - 0.930
	Range	0.443 - 0.611	0.370 - 0.666		
	CI	0.474 - 0.568	0.508 - 0.596		
	Adjusted P-Value		0.679		
	P-Value		0.121		
Proline	Mean	0.899	0.926	0.454 - 1.64	0.462 - 1.63
	Range	0.763 - 1.01	0.624 - 1.11		
	CI	0.828 - 0.964	0.858 - 0.990		
	Adjusted P-Value		0.679		
	P-Value		0.235		
Serine	Mean	0.500	0.518	0.266 - 0.683	0.235 - 0.910
	Range	0.421 - 0.554	0.352 - 0.745		
	CI	0.459 - 0.545	0.475 - 0.564		
	Adjusted P-Value		0.679		
	P-Value		0.308		
Threonine	Mean	0.361	0.373	0.176 - 0.578	0.224 - 0.666
	Range	0.318 - 0.402	0.284 - 0.438		
	CI	0.341 - 0.381	0.354 - 0.392		
	Adjusted P-Value		0.679		
	P-Value		0.165		
Tryptophan	Mean	0.0634	0.0639	0.00876 - 0.127	0.0271 - 0.215
	Range	0.0367 - 0.0726	0.0377 - 0.0773		
	CI	0.0563 - 0.0692	0.0569 - 0.0696		
	Adjusted P-Value		0.908		
	P-Value		0.806		

Table 19. Total Amino Acids in Maize Grain (continued)

Analyte (% Dry Weight)		Control (n=24)	4114 maize (n=24)	Tolerance Interval	Literature Range[a]
Tyrosine	Mean	0.263	0.257	0.0707 - 0.505	0.103 - 0.790
	Range	0.199 - 0.333	0.163 - 0.367		
	CI	0.240 - 0.289	0.234 - 0.282		
	Adjusted P-Value		0.792		
	P-Value		0.561		
Valine	Mean	0.460	0.473	0.159 - 0.749	0.210 - 0.855
	Range	0.393 - 0.507	0.334 - 0.542		
	CI	0.428 - 0.488	0.443 - 0.500		
	Adjusted P-Value		0.679		
	P-Value		0.187		

[a] Literature ranges are taken from published literature for maize (Codex, 1996; Codex, 2005; ILSI, 2006; OECD, 2002; Watson, 1982; Watson, 1987).
[b] Least squares mean
[c] Range denotes the lowest and highest individual value across locations.
[d] 95% Confidence Interval
[e] False Discovery Rate (FDR) adjusted P-value
[f] Non-adjusted P-value

Key Anti-nutrients in Maize Grain

Maize grain contains several key anti-nutrients: raffinose, phytic acid, and trypsin inhibitor (OECD, 2002). Raffinose is a low molecular weight oligosaccharide that is non-digestible, creating flatulence from consumption, but typically can be removed through processing of maize (OECD, 2002). In addition, phytic acid chelates mineral nutrients including calcium, magnesium, potassium, iron and zinc, rendering them unavailable to monogastric animals. Trypsin inhibitor can interfere with the digestion of proteins, resulting in decreased animal growth.

Levels of key anti-nutrients were measured in 4114 maize and near-isoline control grain. Results are shown in Table 20. No statistically significant differences were observed between the mean values for 4114 maize and near-isoline control for any of the anti-nutrient measured (adjusted P-values were > 0.05).

In conclusion, anti-nutrient analysis of maize grain demonstrates that 4114 maize is comparable to near-isoline control maize and reference maize hybrids.

Table 20. Key Anti-Nutrients in Maize Grain

Analyte (% Dry Weight or as Indicated)		Control (n=24)	4114 maize (n=24)	Tolerance Interval	Literature Range[a]
Phytic Acid	Mean[b]	0.998	1.02	0.418 - 1.41	0.111 - 1.57
	Range[c]	0.842 - 1.17	0.827 - 1.52		
	CI[d]	0.917 - 1.09	0.941 - 1.11		
	Adjusted P-Value[e]		0.789		
	P-Value[f]		0.542		
Raffinose	Mean	0.0823	0.108	0 - 0.398	0.0200 - 0.320
	Range	<0.0800[g] - 0.210	<0.0800[g] - 0.236		
	CI	0.0503 - 0.134	0.0670 - 0.173		
	Adjusted P-Value		0.679		
	P-Value		0.0826		
Trypsin Inhibitor (TIU/mg)[h]	Mean	2.67	2.58	1.60 - 4.89	1.09 - 7.18
	Range	1.45 - 4.68	1.70 - 3.48		
	CI	2.24 - 3.18	2.16 - 3.07		
	Adjusted P-Value		0.908		
	P-Value		0.694		

[a] Literature ranges are taken from published literature for maize (Codex, 1996; Codex, 2005; ILSI, 2006; OECD, 2002; Watson, 1982; Watson, 1987).
[b] Least squares mean
[c] Range denotes the lowest and highest individual value across locations.
[d] 95% Confidence Interval
[e] False Discovery Rate (FDR) adjusted P-value
[f] Non-adjusted P-value
[g] <Lower limit of quantification (LLOQ); indicates that sample value(s) were detected below the assay LLOQ. Sample results which were below the LLOQ were assigned a value equal to the LLOQ for statistical analysis.
[h] Abbreviation: TIU = trypsin inhibitor units

Key Secondary Plant Metabolites in Maize Grain

Secondary plant metabolites are neither nutrients nor anti-nutrients, but can be analyzed as indicators of the absence of unintended effects of the genetic modification on metabolism (OECD, 2002). Characteristic plant metabolites in maize are the phenolic acids: ferulic acid, furfural, and *p*-coumaric acid.

Ferulic acid, furfural, and *p*-coumaric acid were measured in 4114 maize and near-isoline control grain. Results are shown in Table 21. No statistically significant differences were observed between the mean values for 4114 maize and near-isoline control for any of the secondary plant metabolites measured (adjusted P-values were > 0.05).

In conclusion, metabolite analysis of maize grain demonstrates that 4114 maize is comparable to near-isoline control maize and reference maize hybrids.

Table 21. Key Secondary Plant Metabolites in Maize Grain

Analyte (% Dry Weight)		Control (n=24)	4114 maize (n=24)	Tolerance Interval	Literature Range[a]
p-Coumaric Acid	Mean[b]	0.0183	0.0186	0.000341 - 0.0387	0.003 - 0.0576
	Range[c]	0.0120 - 0.0276	0.0151 - 0.0249		
	CI[d]	0.0159 - 0.0210	0.0162 - 0.0214		
	Adjusted P-Value[e]		0.789		
	P-Value[f]		0.546		
Furfural	Mean	<0.000100[g]	<0.000100[g]	NC[h]	0 - 0.000634
	Range	<0.000100[g]	<0.000100[g]		
	CI	NA	NA		
	Adjusted P-Value		NA		
	P-Value		NA		
Ferulic Acid	Mean	0.255	0.266	0.0553 - 0.309	0.0200 - 0.389
	Range	0.199 - 0.299	0.227 - 0.292		
	CI	0.243 - 0.268	0.253 - 0.280		
	Adjusted P-Value		0.679		
	P-Value		0.231		

[a] Literature ranges are taken from published literature for maize (Codex, 1996; Codex, 2005; ILSI, 2006; OECD, 2002; Watson, 1982; Watson, 1987).
[b] Least squares mean
[c] Range denotes the lowest and highest individual value across sites.
[d] 95% Confidence Interval
[e] False Discovery Rate (FDR) adjusted P-value
[f] Non-adjusted P-value
[g] <Lower Limit of Quantification (LLOQ); Indicates that the values of the sample or samples were detected below the assay's LLOQ. Sample results which were below the LLOQ were assigned a value equal to the LLOQ for statistical analysis.
[h] A tolerance interval could not be calculated (NC).

Key Nutrients in Maize Forage

Maize silage is an important feed ingredient for feedlot cattle and dairy cattle. In the U.S., approximately 10% of the maize crop is harvested as forage. The whole maize plant contains about one and one-half times the nutrients of the grain, and the ensiling process preserves more than 90% of the nutrients (OECD, 2002).

Proximates and Minerals in Maize Forage

Crude protein, crude fat, crude fiber, ADF, NDF, ash, carbohydrates, and the minerals calcium and phosphorus were measured in 4114 maize and near-isoline control forage.

Results are shown in Table 22. For each analyte measured, there was no statistical difference between 4114 maize and the near-isoline control maize (adjusted P-values were > 0.05).

In conclusion, proximate and mineral analysis of maize forage demonstrated that 4114 maize forage is comparable to near-isoline and reference maize forage.

Table 22. Proximates and Minerals in Maize Forage

Analyte (% Dry Weight)		Control (n=24)	4114 maize (n=24)	Tolerance Interval	Literature Range[a]
Crude Protein	Mean[b]	8.93	9.10	3.35 - 14.6	3.14 - 15.9
	Range[c]	5.82 - 11.0	5.72 - 11.0		
	CI[d]	7.52 - 10.0	7.75 - 10.1		
	Adjusted P-Value[e]		0.679		
	P-Value[f]		0.297		
Crude Fat	Mean	3.30	3.26	1.17 - 3.98	0.296 - 6.70
	Range	1.97 - 4.89	1.95 - 5.60		
	CI	2.63 - 4.15	2.60 - 4.10		
	Adjusted P-Value		0.938		
	P-Value		0.881		
Crude Fiber	Mean	21.8	21.2	13.4 - 32.6	19.0 - 62.8
	Range	15.6 - 27.6	17.6 - 25.1		
	CI	19.9 - 23.9	19.3 - 23.3		
	Adjusted P-Value		0.789		
	P-Value		0.524		
ADF	Mean	28.8	27.6	17.8 - 42.0	16.1 - 47.4
	Range	21.7 - 38.2	20.1 - 34.6		
	CI	25.3 - 32.9	24.2 - 31.4		
	Adjusted P-Value		0.679		
	P-Value		0.215		
NDF	Mean	47.4	47.5	30.8 - 71.4	20.3 - 63.7
	Range	39.5 - 57.4	41.2 - 52.8		
	CI	44.0 - 51.1	44.1 - 51.2		
	Adjusted P-Value		0.966		
	P-Value		0.966		
Ash	Mean	4.98	4.87	0.773 - 8.52	1.30 - 10.5
	Range	3.27 - 7.09	3.50 - 6.36		
	CI	4.19 - 5.92	4.10 - 5.79		
	Adjusted P-Value		0.760		
	P-Value		0.374		
Carbohydrates	Mean	82.8	82.7	76.7 - 90.9	76.4 - 92.1
	Range	78.7 - 87.6	80.1 - 86.6		
	CI	81.2 - 84.4	81.2 - 84.3		
	Adjusted P-Value		0.939		
	P-Value		0.896		
Calcium	Mean	0.259	0.216	0 - 0.563	0.0714 - 0.600
	Range	0.143 - 0.654	0.0878 - 0.375		
	CI	0.195 - 0.344	0.162 - 0.287		
	Adjusted P-Value		0.679		
	P-Value		0.156		

Table 22. Proximates and Minerals in Maize Forage (continued)

Analyte (% Dry Weight)		Control (n=24)	4114 maize (n=24)	Tolerance Interval	Literature Range[a]
Phosphorus	Mean	0.274	0.289	0.0198 - 0.465	0.0936 - 0.550
	Range	0.191 - 0.369	0.213 - 0.367		
	CI	0.243 - 0.308	0.257 - 0.326		
	Adjusted P-Value		0.679		
	P-Value		0.115		

[a] Literature ranges are taken from published literature for maize (ILSI, 2006; Watson, 1982).
[b] Least squares mean
[c] Range denotes the lowest and highest individual value across locations.
[d] 95% Confidence Interval
[e] False Discovery Rate (FDR) adjusted P-value
[f] Non-adjusted P-value

Conclusions on Compositional Assessment of 4114 maize

Extensive nutritional compositional analyses of grain and forage were conducted to evaluate the composition of 4114 maize as compared to a non-transgenic near-isoline control. Compositional analysis of 4114 maize was used to evaluate any changes in the levels of key nutrients, anti-nutrients or secondary metabolites.

Compositional analyses of grain included crude protein, crude fat, crude fiber, acid detergent fiber (ADF), neutral detergent fiber (NDF), ash, carbohydrates, fatty acids, total amino acids, key anti-nutrients, and key secondary metabolites. Compositional analyses of forage included crude protein, crude fat, crude fiber, ADF, NDF, ash, carbohydrates, calcium, and phosphorus. In total, data from 69 different analytical components (60 in grain, nine in forage) were analyzed. While statistical differences were observed in some of these analytes between 4114 maize and its near-isoline control, all values for 4114 maize were within the ranges for the commercial maize lines (*i.e.*, tolerance intervals) or within published literature ranges for maize. Therefore, based on these analyses, the grain and forage of 4114 maize are considered to be comparable to conventional maize with respect to nutrient composition. Furthermore, the presence of 4114 maize would not be expected to impact raw or processed maize commodities.

In conclusion, 4114 maize is comparable to conventional maize with respect to nutrient composition.

4-B. Agronomic Performance and Field Observations

Agronomic evaluations were conducted on 4114 maize and conventional and/or control maize. These evaluations form the basis to determine whether 4114 maize is agronomically comparable to conventional maize and they provide an appropriate scientific determination whether 4114 maize is no more likely to pose a plant pest risk when compared to conventional maize.

The agronomic evaluations were based on both laboratory experiments and replicated, multi-site field trials conducted by agronomists and scientists who are considered experts in the production and evaluation of maize. To evaluate the agronomic characteristics of 4114 maize, data were collected on representative characteristics that influence reproduction, crop survival, and potential weediness. In each of these assessments, 4114 maize was compared to a near-isoline control (*i.e.,* approximately 99% similar) that did not go through the transformation process. In each experiment, the agronomic characteristics of 4114 maize were comparable to the control or commercial comparators.

Field observations were made to assess responses of 4114 maize and control maize to naturally occurring insects and diseases in a wide variety of environmental conditions. In each case, 4114 maize demonstrated no unexpected responses to insects or diseases as compared to the control plants.

Based on the analyses described below, 4114 maize is comparable to conventional maize and will not pose a greater plant pest risk or increased weed potential when compared to conventional maize.

Germination and Dormancy Evaluations

In order to evaluate germination and dormancy, seeds from the $F1^{*5}$ generation (Section 2; Figure 10 and Table 3) of 4114 maize were tested for germination assays under warm, cold, and diurnal conditions (Table 23). The $F1*^{5}$ generation of seed was used because F1 hybrid seed is representative of seed that growers would plant in commercial maize fields. A near-isoline control was used for comparison. In addition, two commercial maize lines, Pioneer® hybrids 32D78 and 34P88, were evaluated in the study to establish a reference range for germination and dormancy evaluations but were not included in the statistical analysis. This reference range provided context for any statistical differences observed in the comparisons; if the values for 4114 maize fell within this reference range, it indicated that 4114 maize was comparable to conventional maize lines.

Each germination test contained eight replicates of 50 seeds each of 4114 maize, near-isoline control, and two commercial lines. The “Rules for Testing Seeds”, published by the Association of Official Seed Analysis, were used as guidelines for the germination methods and interpretation of results (AOSA, 2007). Each replicate was placed between sheets of moist germination toweling, rolled up, and placed in a growth chamber set to the appropriate test

conditions as specified in Table 23. At the end of each germination test, each seed was defined as either germinated or non-germinated. A given seed was classified as germinated if some or all of the essential structures necessary to produce a normal plant under favorable conditions had emerged, or non-germinated if none of these structures had emerged. Germination rates were reported as a percentage of germinating seed as follows: (number of germinated seeds/total seeds)*100. The results are presented in Tables 24, 25, and 26.

Non-germinated seed were further defined into three categories:

- hard (*i.e.,* did not absorb water);
- imbibed (*i.e.,* absorbed water but did not show signs of growth during the germination test); or
- dead (*i.e.,* absorbed water, did not show signs of growth during the germination test, and displayed distinct signs of decay such as an extremely soft interior that did not hold shape under gentle pressure).

Non-germinated seeds that were classified as hard or imbibed were further evaluated for potential dormancy using a standard Tetrazolium Chloride (TZ) test (AOSA, 2005). The TZ test was developed as a color test to evaluate seed viability. The color phase of the test occurs when the colorless testing solution consisting of water and 2,3,5-triphenyltetrazolium chloride is added to the seed. Once this solution penetrates into living cells, the TZ solution is reduced to a reddish (pink), water-insoluble compound. The absence or presence of variation in color characteristics within the tissues allows for recognition and location of living tissues within the embryo structure. Dead seed were excluded from the TZ test, due to the possibility of a false positive result from bacterial growth. The counts for each class of non-germinated seed and the corresponding TZ test result are presented in Table 27. In non-germinated hard seed, potential dormancy may be indicated if the seed is viable (*i.e.,* positive TZ test); however, seed dormancy is not commonly observed in maize.

Germination rates in 4114 maize under warm, cold, and diurnal growing conditions were comparable to those of control maize under corresponding growing conditions. No potentially dormant seed were identified using the TZ test.

The data provided here support the conclusion that 4114 maize is comparable to conventional maize with respect to germination and potential dormancy.

Table 23. Description of Seed Germination Conditions

Warm germination test	• Simulated optimal maize growth conditions • Continuous setting of 25°C and 90% relative humidity for 10 days • Germinated seed counted after 10 days
Cold germination test	• Simulated early spring planting conditions in Midwestern U.S. • Continuous setting of 10°C and 90% relative humidity for 10 days, followed by three days at a continuous setting of 25°C and 90% relative humidity • Germinated seed counted after 13 days
Diurnal germination test	• Simulated daily weather conditions in Midwestern U.S. • Cyclical setting of 10°C and 90% relative humidity for 16 hours and then 25°C and 90% relative humidity for 8 hours, repeated daily for 10 days • Germinated seed counted after 10 days

Table 24. Summary of Warm Germination Test Results

Statistic	4114 Maize	Control Maize	Reference Range
Frequency	396/400	395/399	96.0% - 100%
Mean	99.0%	99.0%	
Range	96.0% - 100%	96.0% - 100%	
P-value[a]	1.00		

[a] P-value was determined using Fisher's exact test for germination frequencies.

Table 25. Summary of Cold Germination Test Results

Statistic	4114 Maize	Control Maize	Reference Range
Frequency	371/398	383/399	86.0% - 98.0%
Mean	93.2%	96.0%	
Range	86.0% - 98.0%	91.8% - 100%	
LS-Mean	93.2%	96.0%	
SEM	1.26%	0.982%	
P-value[a]	0.0865		

[a] P-value was determined using GLMM-based statistical test for mean germination rates.

Table 26. Summary of Diurnal Germination Test Results

Statistic	4114 Maize	Control Maize	Reference Range
Frequency	395/400	395/399	96.0% - 100%
Mean	98.8%	99.0%	
Range	94.0% - 100%	96.0% - 100%	
P-value[a]	1.00		

[a] P-value was determined using Fisher's exact test for germination frequencies

Table 27. Tetrazolium Chloride (TZ) Testing of Non-Germinated Seed

Germination Test	Maize Line	Total Non-Germinated Seed			TZ Test of Non-Germinated Seed			Non-Germinated Seed Not Tested with TZ (*i.e.* Dead Seed)
					Result: Viable		Result: Non-Viable	
		Hard	Imbibed	Dead	Hard Seed	Imbibed Seed	Hard or Imbibed Seed	
Warm	4114 Maize	0	4	0	0	0	4	0
	Control Maize	0	3	1	0	0	3	1
	32D78 (Reference)	0	1	0	0	0	1	0
	34P88 (Reference)	0	6	0	0	0	6	0
Cold	4114 Maize	0	27	0	0	7	20	0
	Control Maize	0	16	0	0	1	15	0
	32D78 (Reference)	0	31	0	0	1	30	0
	34P88 (Reference)	0	15	0	0	2	13	0
Diurnal	4114 Maize	0	5	0	0	2	3	0
	Control Maize	0	4	0	0	4	0	0
	32D78 (Reference)	0	1	0	0	0	1	0
	34P88 (Reference)	0	5	0	0	1	4	0

Field Trial Evaluations

Agronomic data were collected from the F1*^{5} generation of 4114 maize and near-isoline control maize within two experiments (denoted A and B) that were conducted at 17 total field locations in 2010 (Table 28; Figure 33). For sites listed in the same location (*e.g.,* sites 3 and 12; Table 28), the field trials were conducted independently. The F1*^{5} generation of seed (Section 2; Figure 10 and Table 3) was used because F1 hybrid seed is representative of seed that growers would plant in commercial maize fields. The trial locations provided a range of environmental and agronomic conditions representative of the major maize-growing regions of the U.S. and Canada, where commercial production of 4114 maize is expected. Agronomic parameters observed for Experiments A and B are provided in Table 29. The data collected were identical for both experiments, except yield was only collected in Experiment A (11 locations) and pollen viability was only collected in Experiment B (6 locations) (Table 29). Details of the methods used are presented in Appendix 7.

Although 4114 maize contains traits that are efficacious against certain lepidopteran and corn rootworm target pests and glufosinate-ammonium tolerance, efficacy of the traits was not assessed in these agronomic evaluations. As with the compositional assessments, each field trial site was managed to maintain a relatively insect-free and weed-free environment in order to ensure that agronomic characteristics measured would be independent of the intended effects of the insect-resistant and herbicide-tolerant traits; therefore, these evaluations would appropriately compare 4114 maize to conventional maize and also assess unintended effects of the transgene. For example, yield or insect damage scores were not impacted by the intended effects of the traits and therefore could be used to assess comparability of 4114 maize to conventional maize.

Agronomic data were also collected from non-modified commercial maize hybrids (reference hybrids) in three separate experiments. Pioneer® hybrids 34M94, 33G26, 33J24, and 3394 were analyzed in 2003 at six field locations in maize-growing areas of North America (Bagley, IA; York, NE; Chula, GA; New Holland, OH; Larned, KS and Hereford, PA). Pioneer® hybrids 38B85, 37Y12, 34A15, and 34P88 were analyzed in 2007 at six field locations in maize-growing areas of North America (Tallahassee, FL; York, NE; Germansville, PA; Richland, IA; Larned, KS and Branchton, Ontario). Pioneer® hybrids 37H24, 36M28, 35T06, 35T36, 35K02, and 34P88 were analyzed in 2009 at four field locations in the maize-growing areas of Chile. These Pioneer commercial products were chosen to represent a wide range of non-genetically modified varieties that would normally be planted commercially.

For the reference hybrid trials, each location utilized a randomized complete block design containing three blocks. Each block contained two-row plots. Procedures for planting and collecting data from the reference hybrid trials were similar to those employed for the trials containing near-isoline control and 4114 maize. Agronomic data collected from the reference hybrids was used to help determine the normal range of variation for the agronomic characteristics in commercial maize.

A statistical analysis of agronomic data was conducted to test for differences in the mean values between the 4114 maize and the near-isoline control (see Appendix 7 for statistical model). When numerous comparisons are being made, it is important to control the rate of false-positive results. Since the introduction of the false discovery rate (FDR) approach in the mid-1990's, it has been widely employed across a number of scientific disciplines, including genomics, ecology, medicine, plant breeding, epidemiology, dairy science, and signal/image processing (*e.g.,* Pawitan *et al.,* 2005; Spelman and Bovenhuis, 1998). A false positive result occurs when two means are deemed significantly different when, in fact, they are not. If one uses a 5% type I error rate for each agronomic characteristic measured, then the number of false positives increases as the number of characteristics increase. In order to help manage the false positive rate, the FDR method of Benjamini and Hochberg was applied to account for making multiple comparisons (Benjamini and Hochberg, 1995; Westfall *et al.,* 1999). P-values were adjusted accordingly. This resulted in the false positive rate being held to 5%. Both adjusted and unadjusted P-values are provided for the agronomic data. In each experiment, a significant difference between the mean of 4114 maize and that of the control line was established with an FDA-adjusted P-value of less than 0.05.

Using the data obtained from the reference hybrids, a statistical tolerance interval was calculated to contain, with 95% confidence, 99% of the values contained in the population of commercial maize. This statistical tolerance interval provided further context for interpretation of the agronomic results for 4114 maize. The agronomic characteristic measurements for 4114 maize that fell within the tolerance interval for that characteristic were considered to be within the range of normal variability of commercial maize hybrids.

Table 28. Site Locations for 2010 Field Trial Experiments

Map Site Number	Country	Location
Experiment A: 2010 Field Trials		
1	U.S.	Sheridan, IN
2		Deerfield, MI
3		Richland, IA
4		Rochelle, IL
5		Bagley, IA
6		Seymour, IL
7		York, NE
8		Carlyle, IL
9		Wyoming, IL
10		Atlantic, IA
11		Geneva, MN
Experiment B: 2010 Field Trials		
12	U.S.	Richland, IA
13		Wyoming, IL
14		Geneva, MN
15		York, NE
16	Canada	Branchton, ON
17		Thorndale, ON

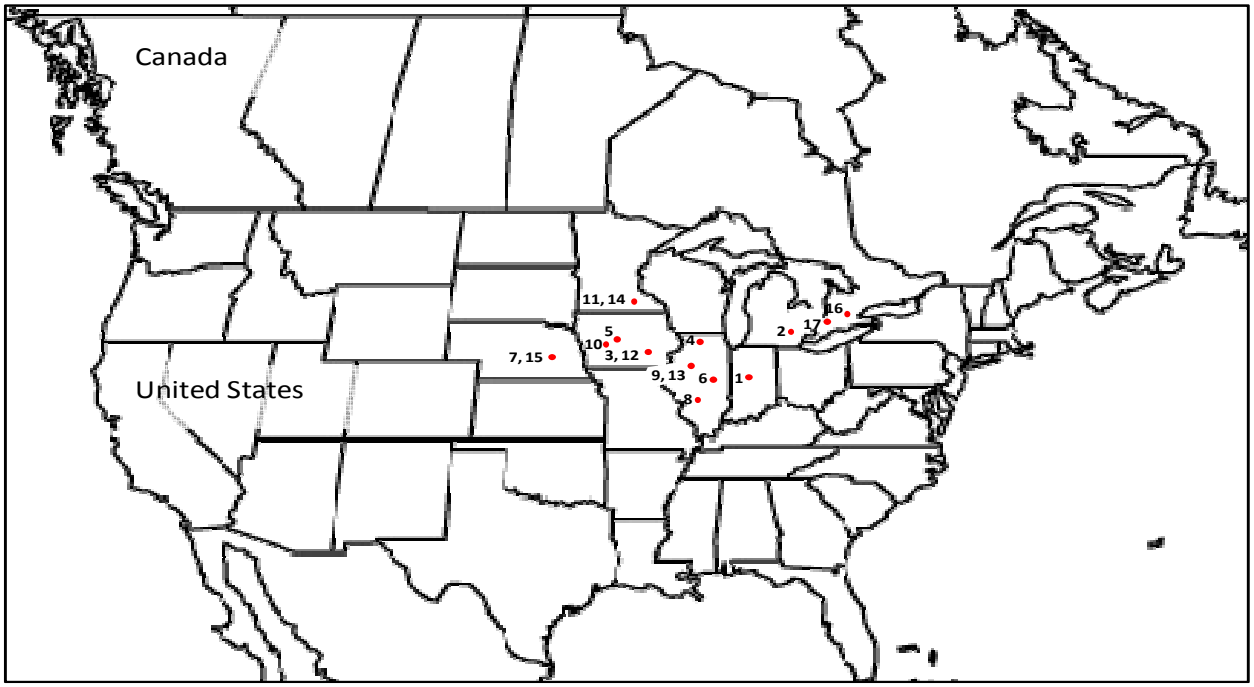


Figure 33. Map of Locations for Agronomic Data Collection for 4114 Maize

Experiment A contains data from 11 field site locations planted in the U.S. during the 2010 growing season (Table 28, Figure 33). The purpose of Experiment A was to evaluate and compare the agronomic characteristics and yield of 4114 maize with a near-isoline control.

Each site consisted of a randomized complete block design with four blocks, with each block containing 4114 maize and the control maize. Procedures employed to control the introduction of experimental bias in this study were as follows: non-systematic selection of trial areas and plot areas within each site, randomization of the maize entries within each block, and uniform maintenance treatments across each plot area.

The following agronomic characteristics were evaluated in all four blocks: early population, seedling vigor, time to silking, time to pollen shed, plant height, ear height, stalk lodging, root lodging, final population, stay green, disease incidence, insect damage, and yield. Descriptions of the characteristics and their measurement are found in Table 29.

The results of Experiment A are summarized in Table 30. No statistically significant differences were observed between 4114 maize and control maize values for all characteristics measured.

Based on the data obtained in this study, agronomic characteristics and yield were comparable between 4114 maize and the control maize.

Table 29. Agronomic Characteristics Measured

General Characteristic	Characteristic Measured	Evaluation Timing[a]	Data Description	Scale
Germination/ Emergence	Early Population	V2-V4	Number of plants emerged per plot	Actual count per plot
	Seedling Vigor	V2-V4	Visual estimate of average vigor of emerged plants per plot	From 1-9, where 1=short plants with small leaves, and 9=tall plants with large leaves
Vegetative Parameters	Plant Height	R4	Height from the soil surface to the tip of the tassel	Height in cm
	Ear Height	R4	Height from the soil surface to the base of the primary ear	Height in cm
	Stalk Lodging	R6	Visual estimate of percent of plants in the plot with stalks broken below the primary ear	0 to 100%
	Root Lodging	R6	Visual estimate of percent of plants in the plot leaning approximately 30° or more in the first 2 feet (0.6m) above the soil surface	0 to 100%
	Final Population	R6	The number of plants remaining per plot	Actual count per plot
	Stay Green	R6	Overall plant health	Ranging from 1-9 where 1=no visible green tissue; 5=approximately 50% green tissue remaining; 9=very green approximately 90% or greater green tissue remaining

Table 29. Agronomic Characteristics Measured (continued)

General Characteristic	Characteristic Measured	Evaluation Timing[a]	Data Description	Scale
Reproductive Parameters	Time to Silking	Approximately 50% silking	From the time of planting until approximately 50% of the plants have emerged silks	Number of Accumulated Heat Units (AHU)
	Time to Pollen Shed	Approximately 50% pollen shed	From the time of planting until approximately 50% of the plants have tassels shedding pollen	Number of Accumulated Heat Units (AHU)
	Yield	Approximately R6	Harvest weight per area adjusted to 13% moisture content	Bushels per acre
	Pollen Viability[b]	During pollen shed	Pollen Shape	Percentage of pollen grains with collapsed walls at 0, 30, 60, and 120 minutes
			Pollen Color	Percentage of pollen grains with intense yellow color at 0, 30, 60, and 120 minutes
Pest Response	Disease Incidence	Approximately R6	Visual estimate of foliar disease incidence	Ranging from 1-9 where 1=poor disease resistance or high infection; 9=best disease resistance or low infection
	Insect Damage	Approximately R6	Visual estimate of insect damage	Ranging from 1-9 where 1=poor insect resistance or high damage; 9=best insect resistance or low damage

[a] Refer to Ritchie *et al*., 2005 for a description of maize growth stages.
[b] Pollen viability has been correlated to pollen shape and color (Luna *et al.*, 2001)

Table 30. Experiment A: Summary of Agronomic Performance of 4114 Maize Across Eleven Locations

Agronomic Trait[a]		Control Maize	4114 Maize	Tolerance Interval[b]
Early Population (Count)	Mean[c]	56	56	41 - 60
	Range[d]	37 - 60	40 - 60	
	CI[e]	53 - 58	53 - 58	
	Adjusted P-Value[f]		0.823	
	P-Value[g]		0.690	
Final Population (Count)	Mean	54	55	34 - 60
	Range	39 - 60	38 - 63	
	CI	51 - 57	51 - 57	
	Adjusted P-Value		0.823	
	P-Value		0.823	
Time to Silking (Accumulated Heat Units)	Mean	1370	1370	841 - 1790
	Range	1020 - 1590	1020 - 1640	
	CI	1270 - 1480	1270 - 1480	
	Adjusted P-Value		0.823	
	P-Value		0.563	
Time to Pollen Shed (Accumulated Heat Units)	Mean	1410	1410	855 - 1810
	Range	1100 - 1620	1100 - 1670	
	CI	1310 - 1500	1310 - 1500	
	Adjusted P-Value		0.823	
	P-Value		0.759	
Seedling Vigor (1-9 Scale)	Mean	7	7	3 - 9
	Range	4 - 9	4 - 9	
	CI	6 - 8	6 - 8	
	Adjusted P-Value		0.823	
	P-Value		0.614	
Stalk Lodging (%)	Mean	2	2	0 - 20[i]
	Range	0 - 20	0 - 20	
	CI	NA[h]	NA	
	Adjusted P-Value		0.823	
	P-Value		0.386	
Root Lodging (%)	Mean	6	6	0 - 8[i]
	Range	0 - 45	0 - 45	
	CI	NA	NA	
	Adjusted P-Value		0.823	
	P-Value		0.485	
Stay Green (1-9 Scale)	Mean	5	5	1 - 9
	Range	1 - 9	1 - 9	
	CI	3 - 6	3 - 6	
	Adjusted P-Value		0.823	
	P-Value		0.121	

Table 30. Experiment A: Summary of Agronomic Performance of 4114 Maize Across Eleven Locations (continued)

Agronomic Trait		Control Maize	4114 Maize	Tolerance Interval
Disease Incidence (1-9 Scale)	Mean	7	7	1 – 9
	Range	5 - 9	5 - 9	
	CI	7 - 8	7 - 8	
	Adjusted P-Value		0.823	
	P-Value		0.749	
Insect Damage (1-9 Scale)	Mean	8	8	2 – 9
	Range	6 - 9	5 - 9	
	CI	NA	NA	
	Adjusted P-Value		0.823	
	P-Value		0.792	
Plant Height (cm)	Mean	292	294	112 – 389
	Range	232 - 323	234 - 325	
	CI	277 - 307	279 - 309	
	Adjusted P-Value		0.823	
	P-Value		0.196	
Ear Height (cm)	Mean	121	120	39 – 167
	Range	80 - 162	71 - 153	
	CI	106 - 136	105 - 135	
	Adjusted P-Value		0.823	
	P-Value		0.449	
Yield (bushels per acre)	Mean	174	176	NC[j]
	Range	68 - 257	105 - 240	
	CI	149 - 199	151 - 201	
	Adjusted P-Value		0.823	
	P-Value		0.632	

[a] Refer to Table 29 for descriptions of each agronomic characteristic measured.
[b] Tolerance Intervals were calculated from the non-modified commercial maize lines evaluated. These intervals contain 99% of the values with 95% confidence level unless otherwise noted.
[c] Least squares mean
[d] Range denotes the lowest and highest individual value across sites.
[e] 95% Confidence Interval
[f] False Discovery Rate (FDR) adjusted P-value
[g] Non-adjusted P-value
[h] Statistical analysis was not available (NA)
[i] No tolerance interval could be calculated; these values are minimum/maximum ranges
[j] No tolerance interval could be calculated (NC)

Experiment B contains data from six field site locations planted in the U.S. and Canada during the 2010 growing season (Table 28, Figure 33). The purpose of Experiment B was to evaluate and compare the agronomic and pollen characteristics of 4114 maize with a near-isoline control.

Each site included a randomized block design containing four blocks, with each block containing a plot of 4114 maize and one plot of control maize. Procedures employed to control the introduction of experimental bias in this study were as follows: non-systematic selection of trial areas and plot areas within each site, randomization of the maize entries within each block, and uniform maintenance treatments across each plot area.

The following agronomic characteristics were measured: early population, final population, time to silking, time to pollen shed, pollen viability, seedling vigor, stalk lodging, root lodging, stay green, disease incidence, insect damage, plant height, and ear height. Descriptions of the characteristics and their measurement are found in Table 29.

Results of Experiment B are summarized in Table 31. No statistically significant differences were observed between 4114 maize and control maize values for all characteristics measured.

Based on the data obtained in this study, agronomic characteristics and yield were comparable between 4114 maize and the control maize.

Table 31. Experiment B: Summary of Agronomic Characteristics of 4114 Maize Across Six Locations

Agronomic Characteristic[a]		Control Maize	4114 Maize	Tolerance Interval[b]
Early Population (count)	Mean[c]	57	58	41 – 60
	Range[d]	50 - 59	42 - 60	
	CI[e]	53 - 59	55 - 60	
	Adjusted P-Value[f]		0.354	
	P-Value[g]		0.0596	
Final Population (count)	Mean	55	57	34 – 60
	Range	38 - 59	46 - 60	
	CI	49 - 58	52 - 59	
	Adjusted P-Value		0.225	
	P-Value		0.0125	
Time to Silking (accumulated heat units)	Mean	1380	1390	841 – 1790
	Range	1300 - 1490	1300 - 1590	
	CI	1320 - 1450	1320 - 1450	
	Adjusted P-Value		0.494	
	P-Value		0.367	
Time to Pollen Shed (accumulated heat units)	Mean	1420	1430	855 – 1810
	Range	1350 - 1510	1350 - 1620	
	CI	1350 - 1480	1360 - 1490	
	Adjusted P-Value		0.494	
	P-Value		0.384	
Pollen Viability – Shape[h] (% of collapsed pollen) 0 minutes	Mean	3	2	0 – 65
	Range	0 - 80	0 - 70	
	CI	0 - 13	0 - 12	
	Adjusted P-Value		0.440	
	P-Value		0.244	
Pollen Viability - Shape (% of collapsed pollen) 30 minutes	Mean	67	55	0 – 100
	Range	15 - 100	20 - 90	
	CI	32 - 94	21 - 87	
	Adjusted P-Value		0.354	
	P-Value		0.0786	
Pollen Viability - Shape (% of collapsed pollen) 60 minutes	Mean	88	91	4 – 100
	Range	40 - 100	50 - 100	
	CI	63 - 100	67 - 100	
	Adjusted P-Value		0.494	
	P-Value		0.348	
Pollen Viability - Shape (% of collapsed pollen) 120 minutes	Mean	98	97	37 – 100
	Range	85 - 100	85 - 100	
	CI	NA[i]	NA	
	Adjusted P-Value		0.602	
	P-Value		0.502	

Table 31. Experiment B: Summary of Agronomic Characteristics of 4114 Maize Across Six Locations (continued)

Agronomic Characteristic		Control Maize	4114 Maize	Tolerance Interval
Pollen Viability - Color[h] (% of intense yellow pollen) 0 minutes	Mean	2	2	0 - 84
	Range	0 – 80	0 - 70	
	CI	0 – 12	0 - 10	
	Adjusted P-Value		0.376	
	P-Value		0.123	
Pollen Viability - Color (% of intense yellow pollen) 30 minutes	Mean	57	50	0 - 100
	Range	15 - 100	20 - 90	
	CI	22 - 89	16 - 84	
	Adjusted P-Value		0.440	
	P-Value		0.235	
Pollen Viability - Color (% of intense yellow pollen) 60 minutes	Mean	82	86	14 - 100
	Range	40 - 100	55 - 100	
	CI	50 - 99	56 - 100	
	Adjusted P-Value		0.376	
	P-Value		0.155	
Pollen Viability - Color (% of intense yellow pollen) 120 minutes	Mean	95	97	56 - 100
	Range	85 - 100	85 - 100	
	CI	NA	NA	
	Adjusted P-Value		0.376	
	P-Value		0.151	
Seedling Vigor (1-9 scale)	Mean	8	7	3 - 9
	Range	6 - 9	5 - 9	
	CI	NA	NA	
	Adjusted P-Value		0.354	
	P-Value		0.0522	
Stalk Lodging (%)	Mean	0	0	0 - 20[j]
	Range	0 - 4	0 - 5	
	CI	NA	NA	
	Adjusted P-Value		NA	
	P-Value		NA	
Root Lodging (%)	Mean	0	0	0 - 8[j]
	Range	0 - 2	0 - 1	
	CI	NA	NA	
	Adjusted P-Value		NA	
	P-Value		NA	
Stay Green (1-9 scale)	Mean	4	4	1 - 9
	Range	1 - 7	1 - 7	
	CI	2 - 6	3 - 6	
	Adjusted P-Value		0.735	
	P-Value		0.729	

Table 31. Experiment B: Summary of Agronomic Characteristics of 4114 Maize Across Six Locations (continued)

Agronomic Characteristic		Control Maize	4114 Maize	Tolerance Interval
Disease Incidence (1-9 scale)	Mean	8	8	1 – 9
	Range	5 - 9	5 - 9	
	CI	NA	NA	
	Adjusted P-Value		0.735	
	P-Value		0.735	
Insect Damage (1-9 scale)	Mean	8	8	2 – 9
	Range	7 - 9	7 - 9	
	CI	NA	NA	
	Adjusted P-Value		0.735	
	P-Value		0.731	
Plant Height (cm)	Mean	285	287	112 – 389
	Range	256 - 324	265 - 313	
	CI	268 - 302	270 - 304	
	Adjusted P-Value		0.494	
	P-Value		0.333	
Ear Height (cm)	Mean	113	115	39 – 167
	Range	82 - 150	79 - 145	
	CI	95 - 130	97 - 132	
	Adjusted P-Value		0.376	
	P-Value		0.167	

[a] Refer to Table 29 for descriptions of each agronomic characteristic measured.
[b] Tolerance Intervals were calculated from the non-modified commercial maize lines evaluated. These intervals contain 99% of the values with 95% confidence level unless otherwise noted.
[c] Least squares mean
[d] Range denotes the lowest and highest individual value across sites.
[e] 95% Confidence Interval
[f] False Discovery Rate (FDR) adjusted P-value
[g] Non-adjusted P-value
[h] Pollen viability has been correlated to pollen shape and color (Luna *et al.*, 2001)
[i] Statistical analysis was not available (NA)
[j] No tolerance interval could be calculated; these values are minimum/maximum ranges

Field Insect and Disease Observations

As discussed in Section 1, 4114 maize has been field tested in the U.S. and Puerto Rico since 2006, as authorized by USDA-APHIS permits and notifications. For each trial, a survey of the naturally occurring insects and diseases and any unexpected differences in the response of 4114 maize as compared to the control line (near-isoline and/or conventional maize lines) were recorded by experienced plant breeders and field staff at least every four weeks. A summary of these surveys for each trial and any differences seen between 4114 maize and control lines are presented in Appendix 8. These observations provide a means to determine if 4114 maize will respond differently from conventional maize lines to insects or diseases in the environment.

In every case, 4114 maize did not exhibit any unexpected responses to naturally occurring insects or diseases. These results support the conclusion that 4114 maize is comparable to control maize lines with similar genetics or to conventional maize lines with respect to insect or disease response.

Conclusions on Agronomic Performance and Field Observations

4114 maize was observed in laboratory experiments and at 17 field locations in the U.S. and Canada to measure agronomic parameters. These experiments and field studies evaluate the characteristics of maize over a broad range of environmental conditions that represent regions where 4114 maize will be grown. The agronomic parameters measured are characteristic traits for reproduction, survival, and potential weediness.

The agronomic data showed no significant differences between 4114 maize and control maize (near-isoline controls and/or commercial maize lines) with respect to early population, vegetative growth, reproductive parameters, yield, and pest responses. These data support the conclusion that 4114 maize is agronomically comparable to conventional maize.

Observations from U.S. and U.S. territory field trials showed no unexpected differences in the response of 4114 maize and control maize to naturally occurring insects and diseases. These results support the conclusion that 4114 maize is comparable to control maize lines with similar genetics and/or to conventional maize lines.

Based on these analyses, 4114 maize is comparable to conventional maize and will not pose a greater plant pest risk or increased weed potential than conventional maize.

4-C. Overall Conclusions to Comparative Assessment of 4114 Maize

A comparative assessment was conducted to determine if 4114 maize would present any new or greater risks relative to maize varieties that have a history of safe use in the environment and as food and feed. This comparative assessment included compositional and agronomic analyses with appropriate comparators to determine comparability to conventional maize. These comparative analyses were designed to eliminate the effects of the introduced insect-

resistant and herbicide-tolerant traits in 4114 maize, so that an appropriate comparison to conventional maize could be made and any unintended effects that may be due to the 4114 maize insertion could be evaluated.

Extensive nutrient composition analyses of grain and forage were conducted to compare the composition of 4114 maize to controls. These analyses were used to evaluate any changes in the levels of key nutrients, anti-nutrients and secondary metabolites. Based on the results of the compositional evaluation, the grain and forage of 4114 maize are as safe as commercially available maize and there would be no significant impact on raw or processed maize commodities.

A comprehensive agronomic evaluation for 4114 maize was conducted. The agronomic evaluations were based on both laboratory experiments and replicated, multi-site field trials. Data were collected on representative characteristics that influence reproduction, crop survival, and potential weediness. Field observations included observed responses of 4114 maize and control maize to naturally occurring insects and diseases in a wide variety of environmental conditions. These data support the conclusion that, independent of the introduced insect-resistant and herbicide-tolerant traits, 4114 maize is comparable to conventional maize and is unlikely to pose a greater plant pest risk than conventional maize.

In conclusion, these analyses indicated that 4114 maize was comparable to conventional maize with respect to the compositional analytes and agronomic characteristics measured. 4114 maize differs from conventional maize only in its herbicide tolerance and its efficacy against certain lepidopteran and corn rootworm pests. Overall, these comparative assessments indicate that 4114 maize is as safe as conventional maize varieties and does not pose a greater risk than conventional maize varieties in food, feed, and the environment.

5. Potential Environmental Impact of the Introduction of 4114 Maize

The potential environmental impact of a transgenic plant needs to be considered in the context of the characteristics of the recipient crop, the introduced trait, and the environment in which it will be introduced (OECD, 1993). Knowledge in each of these areas will provide background on which a risk or safety assessment can be made about the environmental release of the transgenic plant (OECD, 1993). In particular, weediness, gene transfer or flow, and trait effects are particular issues that may be relevant to evaluating the new transgenic line and its safety (OECD, 1993).

In order to evaluate the potential environmental impact of the introduction of 4114 maize, the potential for 4114 maize to become weedy or invasive, the potential for gene flow to sexually compatible wild relatives, and the potential impacts of the introduced proteins (Cry1F, Cry34Ab1, Cry35Ab1, and PAT) were considered. As described further below, in each case, it is not expected that 4114 maize will adversely impact the environment with respect to these considerations.

5-A. Potential for 4114 Maize to Have Altered Disease and Unintended Pest Susceptibilities or to Become Weedy or Invasive

In evaluating the potential for 4114 maize to become more weedy or invasive than conventional maize, general maize biology was considered. Maize is a cultivated annual plant that generally cannot survive temperatures below freezing and is typically grown in temperate regions (OECD, 2003). Maize is not classified as a weed, is not on the U.S. federal or state noxious weed lists, and possesses few characteristics of notably successful weeds (Baker, 1974; Keeler, 1989; USDA-NRCS, 2011). Therefore, the natural characteristics of maize do not indicate a high potential for weediness or invasiveness.

A comparative assessment of 4114 maize was conducted to determine if the DNA insertion altered the nutritive or agronomic characteristics of maize. Compositional and agronomic comparison data were collected on 4114 maize in multiple location field trials as described in Section 4; these analyses were designed to be independent of the introduced traits so that an appropriate comparison to conventional maize could be made and any unintended effects of the 4114 maize insertion could be assessed. These analyses showed that 4114 maize was comparable to conventional maize in composition, except for the introduced proteins, and was comparable in agronomics. In the agronomic analyses, a number of characteristics were measured, including certain ones that may be indicative of weediness: germination and emergence (germination rate, early population, seedling vigor); reproductive (time to silking, time to pollen shed, pollen viability, yield); vegetative (final population stalk lodging, root lodging, stay green, plant height, ear height); and pest response (disease incidence, insect damage). Characteristics related to seed germination, seed production, reproductive time and vegetative competitiveness have been identified with successful weeds (Baker, 1974); changes to these parameters relative to the conventional variety could indicate a change in the potential weediness of a crop. 4114 maize was comparable to conventional maize in each of

these characteristics, indicating that 4114 maize is unlikely to become more weedy or invasive than conventional maize.

In addition, 4114 maize has been field tested since 2006 in multiple locations that provide a range of environmental conditions and also include regions representative of maize cultivation in the U.S. These fields were frequently monitored by expert growers for the incidence of diseases and insects and the effect of these on 4114 maize and control plants. In all cases, no unexpected differences were observed between 4114 maize and the control comparators.

In summary, 4114 maize is unlikely to become more weedy or invasive than conventional maize when cultivated. Compositional and agronomic comparisons indicate no unexpected effects of the presence of the introduced proteins that alter the nutritional composition and weediness potential of maize. No unexpected differences were detected between 4114 maize and control maize in response to insects and diseases. Furthermore, the expression of the introduced proteins (Cry1F, Cry34Ab1, Cry35Ab1, PAT) is unlikely to increase the potential of 4114 maize to become weedy.

5-B. Potential for Gene Flow Between 4114 Maize and Sexually Compatible Wild Relatives

The potential for gene flow between a transgenic crop and its sexually compatible wild relatives is assessed through several factors. One factor includes the potential for pollen flow and outcrossing to occur significantly outside the cultivated field. Other factors include the overlap of the wild relative geographic distribution with the region of transgenic crop cultivation and the possibility of genetic compatibility between the crop and the relative. Finally, to determine the potential for widespread introgression of the trait into wild relative populations, whether the trait itself alters weediness characteristics and whether the wild relative is a noxious weed is considered.

4114 maize will be cultivated similarly to other commercial and conventional maize varieties; therefore, it is appropriate to examine maize pollination biology, regions of maize cultivation in the U.S. and the geographic distribution of sexually compatible wild relatives in order to determine the potential for gene flow. The regions of maize cultivation in the U.S. and the genetic compatibility and geographic distribution of sexually compatible wild relatives of maize, within the genera *Zea* and *Tripsacum*, are discussed further below. Based on this information, there is low potential for gene flow between 4114 maize and its wild relatives of the genera *Zea* and *Tripsacum* in the U.S.

The potential for the insertion in 4114 maize to become widespread in wild relative populations is also unlikely. The insertion, as discussed in Section 5-A, does not make 4114 maize more weedy than conventional cultivated maize; furthermore, none of the sexually compatible wild relatives are listed as noxious weeds.

Pollination Biology of Maize and Impact on Gene Flow

Maize is almost entirely cross-fertilizing and its pollen is typically wind dispersed (OECD, 2003); millions of pollen grains are produced per plant (Jarosz *et al.*, 2003). Despite pollination characteristics that are favorable for pollen flow, other factors make it highly unlikely that viable maize pollen will travel significantly outside of the cultivated field. Pollen viability is reduced in a matter of hours under high temperature and low humidity (Aylor, 2004). Studies also indicate that the majority of maize pollen is unlikely to be dispersed significant distances outside the originating field (Jarosz *et al.*, 2003). Numerous studies show the majority (84-92%) of pollen grains travel less than five meters (Pleasants *et al.*, 2001), with nearly all (>99.75%) pollen traveling less than 100 meters (Byrne and Fromherz, 2003; Matsuo *et al.*, 2004; Sears and Stanley-Horn, 2000). Therefore, the potential of cross-pollination between cultivated maize and its wild relatives will be highest where the wild relatives grow near or adjacent to areas of cultivation. Therefore, the geographic range of wild relatives and the regions of maize cultivation are one critical factor in determining the potential for gene flow.

Regions of Maize Cultivation in the U.S.

Field maize is a major crop worldwide, but represents the largest crop grown in the U.S. It is grown in most states, with production concentrated in the Heartland region (including Illinois, Iowa, Indiana, eastern portions of South Dakota and Nebraska, western Kentucky and Ohio, and the northern two-thirds of Missouri). Iowa and Illinois are the top maize-producing states and typically account for slightly more than one-third of the U.S. crop (USDA-ERS, 2009). Figure 34 indicates acres planted in the U.S. by county (USDA-NASS, 2011).

Additional maize varieties include popcorn and sweet corn, both of which are minor crops compared to field maize (OECD, 2002). While the range of cultivation of popcorn and sweet maize include the entire U.S., in total all acreage represents less than 1% of the acreage of field corn in 2007 (USDA-NASS, 2009).

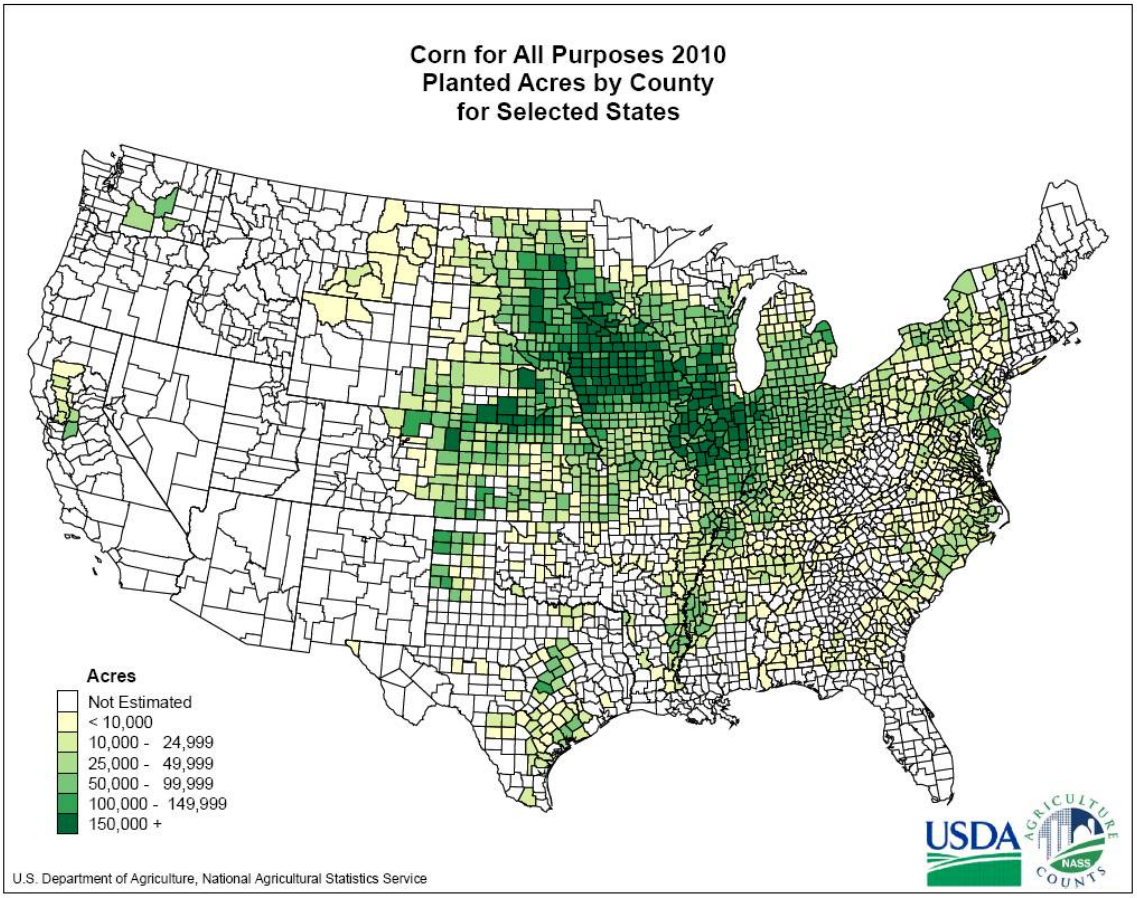


Figure 34. Maize Planted Acres by County in the U.S.
Source: USDA-NASS, 2011

Taxonomic Classification of Maize and Related Wild Relatives

Taxonomically, maize (*Zea mays* L.) is a member of the *Maydeae* tribe of the grass family, *Poaceae* (OECD, 2003). Teosinte, within the genus *Zea*, and the genus *Tripsacum* are the closest relatives to maize taxonomically. The genus *Tripsacum* is also included in the *Maydeae* tribe (OECD, 2003). Annual teosintes are grouped into the species *Zea mays*, although there is some dispute of this classification based on characteristics that prevent a high degree of introgression (OECD, 2003). Annual teosintes have been further classified into the subspecies *Zea mays* ssp. *mexicana* and *Zea mays* ssp. *parviglumis* (OECD, 2003). In contrast, perennial teosintes are classified as different species altogether: *Zea perennis* and *Zea diploperennis* (OECD, 2003). Both annual and perennial teosintes are considered the closest wild relatives of cultivated maize (OECD, 2003). Perennial plants of the genus *Tripsacum* are considered the next closest relatives of maize (OECD, 2003). Neither the *Zea* genus nor the *Tripsacum* genus are listed as noxious weeds on the federal or state noxious weed lists (USDA-NRCS, 2011).

Potential for Gene Flow with the Genus *Zea*

Both annual and perennial teosintes are normally confined to the tropical and subtropical regions of Mexico, Honduras, Guatemala, and Nicaragua (Iltis, 2011). In the U.S., sparsely dispersed introduced populations of annual teosintes *Zea mexicana* (synonym: *Zea mays* ssp. *mexicana*) and *Zea mays* ssp. *parviglumis* have been reported in Florida, Maryland, and Alabama (USDA, 2011). Also, an isolated population of *Zea perennis* (perennial teosinte) has been introduced in South Carolina (USDA, 2011). While maize can hybridize with these species under natural conditions, there is incompatibility between some maize populations and certain types of teosinte that results in low fitness of some hybrids and prevents a high rate of introgression (OECD, 2003). Together with the very limited geographic range of the teosinte population in the U.S., the probability of gene flow from cultivated maize fields to these wild relatives is very low.

Potential for Gene Flow with the Genus *Tripsacum*

Plants of the genus *Tripsacum* are mostly found in Mexico, Central, and South America (OECD, 2003). Three of these species (*T. dactyloides, T. floridanum*, and *T. lanceolatum*) exist as native species populations in the continental U.S.; and two species (*T. fasciculatum* and *T. latifolium*) were introduced in Puerto Rico (USDA, 2011). *T. dactyloides* occurs throughout the eastern half of the U.S. *T. lanceolatum* occurs in Arizona and New Mexico (USDA, 2011) and *T. floridanum* is native to southern Florida (USDA, 2011). Although it is extremely difficult, *Tripsacum* species (*T. dactyloides*, *T. floridanum*, and *T. lanceolatum*) can be crossed with maize; however, hybrids have a high degree of sterility and are genetically unstable (OECD, 2003). Successful crosses of maize with *Tripsacum* species have been made experimentally, however such crosses are not known to occur in the wild (OECD, 2003). Therefore, gene flow between cultivated maize and relatives of the genus *Tripsacum* is highly unlikely.

Conclusions on the Potential for Gene Flow between 4114 Maize and Wild Relatives

The potential for gene flow between maize and relatives of the genera *Zea* and *Tripsacum* is very low. While wild native or introduced populations of these genera occur where maize is cultivated, limited geographic range and low fitness or sterility of hybrids prevent successful gene flow. Furthermore, none of these wild relatives are considered to be noxious weeds and 4114 maize does not exhibit greater potential for weediness as determined from agronomic comparisons to conventional maize. Therefore, any incidental gene flow between 4114 maize and its wild relatives would not transform maize wild relatives into more weedy species, nor would the introduced trait be introgressed widely in wild relative populations.

5-C. Potential Environmental Impact of the Introduced Proteins in 4114 Maize

4114 maize is comparable in agronomic characteristics and compositional characteristics to conventional maize except for the presence of the introduced proteins, Cry1F, Cry34Ab1, Cry35Ab1, and PAT. Therefore, one aspect of assessing the potential for the environmental impact of 4114 maize cultivation is to consider the potential risk of the introduced proteins to non-target organisms; this risk is quantified by examining the environmental exposure and the potential hazard of the introduced proteins to non-target organisms. As described earlier, the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins in 4114 maize are identical to those in previously approved 1507 and/or 59122 maize, which USDA and EPA determined would not adversely impact the environment (EPA, 2010a; EPA, 2010b; Hunst and Rood, 2004; Shanahan and Stauffer, 2000; USDA-APHIS, 2001; USDA-APHIS, 2005).

The impact of the PAT protein from 4114 maize on the environment is expected to be negligible. The PAT protein is not toxic or allergenic and its food, feed, and environmental safety is well known and documented (CERA, 2011; Hérouet *et al.*, 2005; OECD, 1999). Furthermore, the PAT protein was evaluated for 1507 and 59122 maize and is not expected to have any significant impact on non-target organisms and threatened and endangered species (USDA-APHIS, 2001; USDA-APHIS, 2005).

The Cry1F and the binary Cry34Ab1 and Cry35Ab1 proteins (*i.e.,* the Cry34/35Ab1 protein) are known to have insecticidal activity against certain specific lepidopteran species and corn rootworm species, respectively. The specificity of this activity to target insects has been well established, and there is low risk of hazard to non-target orders, avian species, and mammals, as determined previously by USDA and EPA for 1507 and 59122 maize and by EPA for 1507x59122 maize (EPA, 2010a; EPA, 2010b; Hunst and Rood, 2004; Shanahan and Stauffer, 2000; USDA-APHIS, 2001; USDA-APHIS, 2005). With the exception of Cry1F protein concentrations in pollen, the concentrations of Cry1F and Cry34/35Ab1 in 4114 maize tissues are generally similar to or lower than 1507, 59122, and 1507x59122 maize, as shown in Section 2-F. Therefore, the previously conducted environmental safety studies for 1507, 59122 and 1507x59122 maize are relevant for evaluating the environmental impact of 4114 maize. The concentration of Cry1F protein in 4114 maize pollen is approximately 1.5 times that of 1507 or 1507x59122 maize. Despite this increase, the laboratory tests still indicate a sufficient margin

of safety for the expected environmental exposure to the Cry1F protein from 4114 maize pollen.

Similar to the previous conclusions for 1507 and 59122 maize, the cultivation of 4114 maize is unlikely to have adverse effects on non-target arthropods, including threatened and endangered arthropods, and non-target vertebrates, including birds, mammal, and humans. In support of this conclusion, 1507x59122 maize has been commercially available in the U.S. since the 2006 growing season with no negative safety or environmental effects.

Environmental Exposure and Environmental Persistence

One aspect of evaluating the potential environmental impact of the introduced proteins considers the probability and degree to which non-target organisms will be exposed to the proteins. Organisms can be exposed to the proteins in several ways. Animals and insects can feed on different parts of the plant and could be exposed to the proteins directly. Also, as the plant tissue residue degrades in the agricultural field, soil dwelling organisms may be exposed to these proteins if they persist in soil and aquatic organisms could be exposed from water runoff. Predatory insects may also be exposed to the Cry1F and Cry34/35Ab1 proteins from prey insects that feed on 4114 maize (*i.e.*, tri-trophic interactions), if these proteins accumulate in the prey insects.

For soil dwelling or aquatic organisms, protein exposure is highly dependent on protein degradation and dissipation in soils. Most Cry proteins do not persist or accumulate in soil over time (Clark *et al.*, 2005; Icoz and Stotzky, 2008). Similarly, the soil dissipation of Cry1F and Cry34/35Ab1 proteins is very rapid. In data previously provided for 1507 and 59122 maize, the time required for 50% of the Cry1F protein activity to dissipate in soil (DT_{50}) is 3.13 days and the DT_{50} for Cry34/35Ab1 proteins is approximately 3.2 days (Hunst and Rood, 2004; Shanahan and Stauffer, 2000; USDA-APHIS, 2001; USDA-APHIS, 2005). Later published work concluded that the Cry1F protein activity dissipated even more quickly in soil; it took less than one day for 50% of the activity to dissipate (Herman *et al.*, 2001). Additionally, no Cry1F protein was detected in soil sampled from fields following three years of successive cultivation of 1507 maize (Shan *et al.*, 2008). Lack of accumulation in soil would limit the amount of protein present in field water runoff. Because of the rapid dissipation of activity in soil and the low potential for the proteins to accumulate in soil, exposure to Cry1F and Cry34/35Ab1 from soil would not be significant, nor would these proteins accumulate in aquatic habitats.

For protein exposure through tri-trophic interactions, the degree of persistence and accumulation of these proteins in the prey insect (*i.e.*, bioaccumulate) will limit overall exposure to predatory insects. Proteins, in general, do not have the chemical characteristics needed to accumulate (Spacie *et al.,* 1995; USGS, 2010). Lack of accumulation and short half-life has been confirmed for certain Cry proteins in prey species (Li and Romeis, 2010; Lundgren and Wiedenmann, 2005; Meissle and Romeis, 2009; Obrist *et al.*, 2005; Romeis and Meissle, 2011), there have been no studies using validated and robust ELISA or western blot methods that have indicated bioaccumulation of Cry proteins in prey foods. Exposure to Cry proteins via

prey is a less significant route of exposure than direct feeding on plant material (*e.g.*, pollen). Therefore, predatory insects are unlikely to be significantly exposed to Cry1F or Cry34/Cry35Ab1 proteins through tri-tropic interactions.

Potential environmental exposure of the introduced proteins can be estimated to determine the significance of laboratory safety testing conducted. The mean concentrations of the Cry1F, Cry34Ab1, and Cry35Ab1 proteins were measured in 4114 maize plant tissues (Section 2-F) and were used to determine or estimate an expected environmental concentration (EEC). In certain instances, the protein concentration in a particular tissue was used directly to determine the EEC, depending on the feeding habits or the preferred habitat of the non-target organism. In other cases, estimations were made to determine the EEC. EEC can be estimated by considering the protein concentrations in the plant tissue consumed or contacted and the amount of plant matter present in the environment. Certain factors can limit environmental exposure and can be used to refine estimations. Degree of pollen dispersal and deposition were considered for pollen consumption by certain non-target organisms. For EEC in soil and aquatic environments, field planting density and residual field plant matter were used to guide numerical estimates. As general consideration, the lack of overlap between geographic range or preferred habitat of the wildlife species to regions of maize cultivation would further limit potential environmental exposure.

In summary, non-target organisms can be exposed to proteins from 4114 maize through direct consumption of plant tissue. In general, indirect exposure via soil and via tri-trophic interactions is not expected to be significant. In order to determine the significance of laboratory safety testing on non-target organisms, the potential environmental exposure for 4114 maize was estimated from the mean Cry1F and Cry34/35Ab1 protein concentrations. Potential environmental exposure was considered in determining the potential impact on non-target organisms.

Potential Hazard of the Introduced Proteins on Non-Target Organisms

In order to determine the potential hazard of the Cry1F and Cry34/35Ab1 proteins may have on non-target organisms, early-tier laboratory studies using purified protein were conducted on representative species of interest and surrogate species. These species were selected to provide an evaluation of the effects of Cry1F and Cry34/35Ab1 on different families and orders of organisms. Surrogate species are typically selected because they are amenable to the laboratory setting, are environmentally sensitive and representative of the agroecosystem, and can be used to predict potential impacts on related non-target organisms, including beneficial, threatened, or endangered species (Romeis *et al.*, 2011).

Early-tier testing is typically done to simulate higher than expected environmental exposure, typically 10-fold (Rose, 2007). The 10-fold margin of exposure also accounts for interspecies variability (Romeis *et al.*, 2011). The EPA has concluded that if there is no significant hazard (*i.e.*, greater than 50% adverse effect) observed at these unrealistically high laboratory exposures, then the risk of hazard at environmentally relevant concentrations is very low (Rose, 2007). Only when the early-tier hazard study detects an effect, should the next tier study be triggered (Romeis *et al.*, 2008).

Early-tier laboratory testing was conducted for previously approved 1507 and 59122 maize (EPA, 2010a; EPA, 2010b; Hunst and Rood, 2004; Shanahan and Stauffer, 2000; USDA-APHIS, 2001; USDA-APHIS, 2005). These studies include representative and surrogate species in order to predict the potential impacts of the Cry1F and Cry34/35Ab1 proteins on non-target organisms in the environment. These studies were considered in determining the potential hazard of the proteins and were used to determine the overall potential impact of 4114 maize on non-target organisms in the environment.

Potential Impact of the Cry1F and Cry34/35Ab1 Proteins on Non-Target Organisms

As determined for previously approved events 1507 and 59122 maize, the Cry1F and Cry34/35Ab1 proteins target certain specific lepidopteran species and corn rootworm species, respectively. In addition, the lack of adverse effects of these proteins on non-target organisms was confirmed through laboratory assays and field assessments. Based on these safety studies for 1507 and 59122 maize, the Cry1F and Cry34/35Ab1 proteins are not expected to adversely affect other invertebrates and all vertebrate organisms, including non-target birds, mammals, and humans, because of the high specificity of these insecticidal proteins to certain insect orders.

Many of the previously conducted safety studies for 1507 and 59122 maize are relevant for evaluating the environmental impact of 4114 maize, because the concentrations of Cry1F and Cry34/35Ab1 in 4114 maize tissues are generally similar to or lower than 1507, 59122, and 1507x59122 maize. One exception is the slight increase in Cry1F levels in pollen. Despite this increase, the laboratory tests still indicate a sufficient margin of safety for expected environmental concentration (EEC) to the Cry1F protein from 4114 maize pollen. Therefore, as

with 1507 and 59122 maize, the margin of safety of the Cry1F and Cry34/35Ab1 proteins indicated that 4114 maize would not adversely affect non-target organisms and the environment.

An overview of specificity of the proteins and an overview of the potential impact of the proteins on non-target organisms is discussed further below. The safety studies conducted on the purified Cry1F and Cry34/35Ab1 proteins were used to assess the potential hazard on a number of representative non-target species (Tables 32 and 33). The potential exposure to the proteins was calculated by estimating the EEC. Margins of exposure for 4114 maize were calculated by dividing the no observable effect concentration (NOEC) or the lethal concentration (LC_{50}) from the previously conducted safety studies for 1507 and 59122 maize by the EEC of each protein in 4114 maize. Both the potential hazard and the potential exposure were used to conclude the overall potential impact the proteins from 4114 maize would have on non-target organisms.

Specificity to Target Insect Pests

The insecticidal Cry1F and Cry34/35Ab1 proteins have been assessed by laboratory studies to evaluate the specificity of these proteins to target insects. In general, only insect species within a given order and closely related families are susceptible to a given insecticidal protein.

Extensive studies have determined that Cry1F has specificity for certain lepidopteran target pests. Cry1F is EPA labeled for protection against European corn borer (*Ostrinia nubilalis*), fall armyworm (*Spodoptera frugiperda*), corn earworm (*Helicoverpa zea*), western bean cut worm (*Striacosta albicosta* Smith), black cutworm (*Agrotis ipsilon*), lesser corn stalk borer (*Elasmopalpus lignosellus*), southwestern corn borer (*Diatraea grandiosella*), and sugarcane borer (*Diatraea saccharalis*) (EPA, 2011).

Similarly, studies have confirmed the specificity of Cry34Ab1 and Cry35Ab1 for certain corn rootworm target pests. Cry34/35Ab1 is EPA labeled for protection against northern corn rootworm (*Diabrotica barberi*), western corn rootworm (*Diabrotica virgifera virgifera*), and Mexican corn rootworm (*Diabrotica virgifera zeae*) (EPA, 2011). Cry34/35Ab1 also has activity for southern corn rootworm (*Diabrotica undecimpunctata howardi*) as determined by use in bioassays (EPA, 2010b).

Along with the non-target assessments described below, the specificity of the Cry1F and Cry34/35Ab1 proteins to the lepidopteran insects and corn rootworm species, respectively, provides support that these proteins are unlikely to pose a risk to unrelated non-target organisms in the environment.

Potential Impact on Soil Dwelling Non-Target Organisms

The potential hazard of the Cry1F and Cry34/35Ab1 proteins on two representative soil dwelling species, springtail and earthworm, was previously assessed for 1507 and 59122 maize though laboratory assays that used purified protein (EPA, 2010a; EPA, 2010b; Hunst and Rood, 2004; Shanahan and Stauffer, 2000; USDA-APHIS, 2001; USDA-APHIS, 2005). The results from the laboratory assays are given in Tables 32 and 33. Based on the calculation used by Hunst and Rood (2004) for 59122 maize, the EEC of the proteins in soil from 4114 maize was estimated based on factors such as planting density, dry weight of a maize plant, and mean protein concentrations in senescent (R6) maize tissue (Tables 32 and 33). None of the soil dwelling species tested showed significant adverse effects from the proteins and the margins of exposure were greater than 22-fold (Tables 32 and 33, Representative Soil Dwelling Species). Furthermore, as described earlier, soil dwelling non-target organisms are unlikely to be exposed to significant concentrations of the Cry1F and Cry34/35Ab1 proteins in soil, as realistic environmental exposures are expected to be significantly lower based on protein degradation and lack of accumulation in soil.

Taking together the potential protein hazard and potential exposure in soil, soil dwelling non-target invertebrates are unlikely to be impacted by the Cry1F and Cry34/35Ab1 proteins at the expected environmental concentrations for 4114 maize.

Table 32. 4114 Maize Estimated Margins of Exposure to Non-Target Organisms (Cry1F)

Organism: Common Name *Species* (Order)	Cry1F Test Material	Expected Environmental Concentration (EEC) from 4114 Maize	Results	Margin of Exposure (MOE)[a]
Representative Soil Dwelling Species				
Springtail *Folsomia candida* (Collembola)	Purified protein in yeast diet	0.041 mg/kg[b]	LC_{50} and NOEC >12.5 mg/kg diet	>300
Earthworm *Eisenia fetida* (Haplotaxida)	Purified protein in soil	0.041 mg/kg[b]	LC_{50} and NOEC >2.5 mg/kg dry soil	>60
Representative Coleopteran Species				
Ladybird beetle *Hippodamia convergens* (Coleoptera) Family: Coccinellidae	Purified protein in honey diet	35 µg/g Cry1F in pollen[c]	LC_{50} >480 ppm diet	>13
Representative Lepidopteran Species				
Monarch butterfly *Danaus plexippus* (Lepidoptera)	Purified protein in artificial diet	0.5 ng/mg Cry1F from pollen deposition on milkweed host plant[d]	LC_{50} > 10 ppm (10,000 ng/mL); NOEC < 10 ppm	20
Representative Arthropod Species				
Parasitic Hymenoptera *Nasonia vitripennis* (Hymenoptera) Family: Pteromalidae	Purified protein in honey diet	35 µg/g Cry1F in pollen[c]	LC_{50} >320 ppm diet	>9
Green lacewing *Chrysoperla carnea* (Neuroptera) Family: Chrysopidae	Purified protein in moth egg diet	35 µg/g Cry1F in pollen[c]	LC_{50} >480 ppm diet	>13
Honeybee *Apis mellifera* (Hymenoptera) Family: Apidae	Purified protein in sucrose solution	35 µg/g Cry1F in pollen[c]	NOEC >640 ng per larvae[e]	>9[d]

Table 32. 4114 Maize Estimated Margins of Exposure to Non-Target Organisms (Cry1F) (continued)

Organism: Common Name *Species* (Order)	Cry1F Test Material	Expected Environmental Concentration (EEC) from 4114 Maize	Results	Margin of Exposure (MOE)[a]
Representative Arthropod Species				
Water flea *Daphnia magna* (Cladocera) Family: Daphniidae	Purified protein dissolved in water	0.0046 µg/ml Cry1F[f]	LC_{50} and NOEC >100 mg/L	>21000

[a] The margin of exposure is calculated by dividing the NOEC (or LC_{50}) by the EEC

[b] For 4114 maize, the concentration of Cry1F protein in soil can be calculated based on estimating the amount of protein from senescent tissue in a field. Senescent tissue mean concentration is for R6 whole plant tissue as presented in Section 2-F. A modified version of the calculation used by Hunst and Rood (2004) was used to calculate the concentration of protein in soil. New assumptions were used for this calculation; plants per acre was increased to 30,000, based on current average maize plant population density (Farnham, 2001; Nielsen and Thomison, 2002) and the weight of the maize plant was now based on dry weight, since protein concentration is based on dry weight (Nguyen and Jehle, 2007). Protein in soil: (30,000 plants/acre x 300 g/plant (dry weight) x 4.1 µg/g Cry1F R6 whole plant tissue)/9.08 x 10^5 kg soil/acre (Hunst and Rood, 2004). This number is 0.041 mg/kg soil.

[c] Mean concentration as reported in Section 2-F.

[d] This number is calculated based on a number of metrics: the weight of an average pollen grain is 250 ng (Fonseca *et al.*, 2003); the high pollen deposition rate in a maize field is 1000 pollen grains/cm^2 (Pleasants *et al.*, 2001); the approximate weight of a milkweed leaf (17.7 mg/cm^2); and the amount of Cry1F per pollen grain based on the mean concentration as presented in Section 2-F and the weight of a pollen grain (0.0088 ng Cry1F/grain). The EEC = [0.0088 ng Cry1F/pollen grain X 1000 pollen grains/cm^2]/17.7 mg/cm^2.

[e] The MOE assumes that honey bee larvae will ingest 2 mg of pollen (dividing the final number by a factor of two). Taking this behavioral component into consideration, the tested concentration would be equivalent to an MOE of 9. However, if the pollen consumption is not factored in, the tested concentration would be equivalent to an MOE of 18.

[f] Estimate based on the protein from 4114 senescent maize tissue moving into a neighboring aquatic environment, using worst-case assumptions modified from the GENEEC model and Raybould and Vlachos (2011), as follows: 1) assume 10% of senescent maize tissue from a 10 hectare field will be deposited into a 1 hectare x 2 meter deep pond (20,000,000 L); 2) assume there are 30,000 plants per acre (average plant population density, as above), which is equivalent to 74,100 plants per hectare (1 hectare = 2.47 acres); 3) assume the dry weight of a maize plant is 300 g, since protein concentrations are expressed in dry weight, as above. Cry1F concentration in a pond = [4.1 µg/g (dry weight) X 74,100 plants/ha X 300 g/plant]/20,000,000L. Based on these assumptions, the concentration of Cry1F that would enter a pond under worst-case assumptions is 0.0046 µg/ml. The additional contribution of Cry1F protein via aerial deposition of maize pollen is negligible (low part per billion).

Table 33. 4114 Maize Estimated Margins of Exposure to Non-Target Organisms (Cry34/35Ab1)

Organism: Common Name *Species* (Order)	Cry34/35Ab1 Test Material	Expected Environmental Concentration (EEC) from 4114 Maize	Results	Margin of Exposure (MOE)[a]
Representative Soil Dwelling Species				
Springtail *Folsomia candida* (Collembola)	Purified protein in yeast diet	0.56 mg/kg in soil[b]	LC_{50} and NOEC >12.7 mg/kg diet	>22
Earthworm *Eisenia fetida* (Haplotaxida)	Purified protein in soil	0.56 mg/kg in soil[b]	LC_{50} and NOEC >25.3 mg/kg dry soil[c]	>45
Representative Coleopteran Species				
Ladybird beetle *Hippodamia convergens* (Coleoptera) Family: Coccinellidae	Purified protein in sugar water diet	9.54 µg/g Cry34/35Ab1 in pollen[d]	LC_{50} and NOEC >280 µg/mL diet	>29
Ladybird beetle *Coleomegilla maculata* (Coleoptera) Family: Coccinellidae	Purified protein in artificial diet	9.54 µg/g Cry34/35Ab1 in pollen[d]	LC_{50}> 901 ppm EC_{50} < 901ppm	94
Representative Arthropod Species				
Parasitic Hymenoptera *Nasonia vitripennis* (Hymenoptera) Family: Pteromalidae	Purified protein in sugar water diet	9.54 µg/g Cry34/35Ab1 in pollen[d]	LC_{50} >280 µg/mL diet	>29
Green lacewing *Chrysoperla carnea* (Neuroptera) Family: Chrysopidae	Solution of purified protein in moth egg diet	9.54 µg/g Cry34/35Ab1 in pollen[d]	LC_{50} and NOEC >280 ppm diet	>29
Honeybee *Apis mellifera* (Hymenoptera) Family: Apidae	Purified protein in sucrose solution	9.54 µg/g Cry34/35Ab1 in pollen[d]	NOEC >5600 ng per larvae[e]	>294[f]

Table 33. 4114 Maize Estimated Margins of Exposure to Non-Target Organisms (Cry34/35Ab1) (continued)

Organism: Common Name *Species* (Order)	Cry34/35Ab1 Test Material	Expected Environmental Concentration (EEC) from 4114 Maize	Results	Margin of Exposure (MOE)[a]
Representative Arthropod Species				
Water flea *Daphnia magna* (Cladocera) Family: Daphniidae	Purified protein dissolved in water	0.0634 µg/ml Cry34/35Ab1[f]	LC_{50} and NOEC >100 mg/L	>1500

[a] The MOE is calculated by dividing the NOEC (or LC50) by the EEC

[b] For 4114 maize, the concentration of Cry34/35Ab1 protein in soil can be calculated based on estimating the amount of protein from senescent tissue in a field. Senescent tissue concentration is the sum of the mean concentrations of Cry34Ab1 and Cry35Ab1 in R6 whole plant tissue as presented in Section 2-F. A modified version of the calculation used by Hunst and Rood (2004) was used to calculate the concentration of protein in soil. New assumptions were used for this calculation; plants per acre was increased to 30,000, based on current average maize plant population density (Farnham, 2001; Nielsen and Thomison, 2002) and the weight of the maize plant was now based on dry weight, since protein concentration is based on dry weight (Nguyen and Jehle, 2007). Protein in soil: [30,000 plants/acre x 300 g/plant (dry weight) x (36 µg/g Cry34Ab1 + 21 µg/g Cry35Ab1 R6 whole plant tissue)]/9.08 x 10^5 kg soil/acre. This number is 0.56 mg/kg soil.

[c] The final concentration of the experiment was previously reported as 76 mg/kg (EPA, 2010b); however, the original calculation was based on 76 mg in 3 kg of soil; therefore, the final concentration in the soil was 25.3 mg/kg.

[d] This concentration is the sum of the individual protein mean concentrations as reported in Section 2-F. Cry34Ab1 expression in pollen is 9.2 µg/g and Cry35Ab1 expression is 0.34 µg/g.

[e] The MOE assumes that honey bee larvae will ingest 2 mg of pollen (dividing the final number by a factor of two). Taking this behavioral component into consideration, the tested concentration would be equivalent to an MOE of 294. However, if the pollen consumption is not factored in, the tested concentration would be equivalent to an MOE of 587.

[f] Estimate based on the protein from 4114 senescent maize tissue moving into a neighboring aquatic environment, using worst-case assumptions modified from the GENEEC model and Raybould and Vlachos (2011), as follows: 1) assume 10% of senescent maize tissue from a 10 hectare field will be deposited into a 1 hectare x 2 meter deep pond; 2) assume there are 30,000 plants per acre (average plant population density, as above), which is equivalent to 74,100 plants per hectare (1 hectare = 2.47 acres); 3) assume the dry weight of a maize plant is 300 g, since protein concentrations are expressed in dry weight, as above. Cry34/35Ab1 concentration in a pond = [(36 µg/g Cry34Ab1 + 21 µg/g Cry35Ab1) (dry weight) X 74,100 plants/ha X 300 g/plant]/20,000,000L. Based on these assumptions, the concentration of Cry34/35Ab1 protein that would enter a pond under worst-case assumptions is 0.0634 µg/ml. The additional contribution of Cry34/35Ab1 protein via aerial deposition of maize pollen is negligible (low part per billion).

Potential Impact on Non-Target Coleoptera

Toxicity of the Cry1F and Cry34/35Ab1 protein on representative coleopteran species was evaluated for both 1507 and 59122 maize (EPA, 2010a; EPA, 2010b; Hunst and Rood, 2004; Shanahan and Stauffer, 2000; USDA-APHIS, 2001; USDA-APHIS, 2005). *Hippodamia convergens* was tested with the Cry1F and Cry34/35Ab1 proteins (Tables 32 and 33, respectively). In addition, *Coleomegilla maculata* was also tested for Cry34/35Ab1 (Table 33). The results from the laboratory assays are given in Tables 32 and 33. The EEC of the proteins is based on the mean concentrations of the proteins in 4114 maize pollen (Section 2-F). None of the coleopteran species tested showed significant adverse effects from the proteins and the margins of exposure were greater than 13-fold (Tables 32 and 33, Representative Coleopteran Species).

In addition, the EPA reviewed data from two studies that provided additional evidence for lack of impact of Cry34/35Ab1 on non-target Coleoptera. In one study, the toxicity of the purified Cry34/35Ab1 protein to carabid beetles (*Poecilus cupreus*) was assessed (EPA, 2010b). No statistically significant differences were observed in the populations fed Cry34/35Ab1 and the control. This study confirmed that, at field concentrations, there would be no impact of the proteins on carabid beetles. In the second study, the possibility of impact of the Cry34/35Ab1 protein in predator-prey feeding interactions was examined (EPA, 2010b). There was no effect on ladybird beetle (*Coleomegilla maculata*) larvae that were consuming aphids that had been feeding on maize plants expressing the Cry34/35Ab1 protein. Because levels of the Cry34/35Ab1 protein in 4114 maize pollen are comparable to or lower than those of 59122 maize, as described in Section 2-F, these studies and their conclusions are relevant to 4114 maize.

Based on these studies, non-target coleopteran species are unlikely to be impacted by the Cry1F and Cry34/35Ab1 proteins at the expected environmental concentrations for 4114 maize.

Potential Impact on Non-Target Lepidoptera

Due to the established specificity of the Cry1F protein for Lepidoptera, the potential impact of 1507 maize on a representative lepidopteran species of conservation interest, the monarch butterfly (*Danaus plexippus)*, was studied. The data indicated that 1507 maize would not pose a risk to monarch butterfly larvae (EPA, 2010a; Shanahan and Stauffer, 2000; USDA-APHIS, 2001).

The most likely route of exposure of non-target Lepidoptera to transgenic proteins from 4114 maize would be via pollen deposition on host plants (*i.e.,* milkweed) in maize fields and in field margins. Weed control practices make the likelihood of having host plants growing within the confines of the maize field remote; therefore, the most realistic exposure of monarch butterfly to maize pollen would be via incidental ingestion of maize pollen on milkweed plants located in field margins. The density of maize pollen deposition within maize fields and field margins has

been extensively studied (Pleasants *et al.*, 2001) and these values can be used to estimate the EEC for monarch butterfly to maize pollen. Pollen deposition rates were reported to be 1000 (high in-field), 171 (in-field average), and 63 (field edge) pollen grains/cm^2 (Pleasants *et al.*, 2001). In order to calculate the EEC and margin of exposure, the 1000 grains/cm^2 rate was used (Table 32); however, the more realistic case of pollen deposition would be the value at the field edge. The EEC was calculated based on a number of metrics: the weight of an average pollen grain; a high pollen deposition rate in a maize field; the approximate weight of a milkweed leaf per cm^2; and the amount of Cry1F per pollen grain based on the concentration as presented in Section 2-F (Table 32).

The results of the monarch butterfly study are provided in Table 32. Based on the concentration tested in the study and the EEC, the margin of exposure was calculated to be 20-fold (Table 32).

Furthermore, a laboratory feeding study was conducted that exposed monarch butterfly larvae to 1507x59122 maize pollen, containing both the Cry1F and Cry34/35Ab1 proteins, at up to 1600 pollen grains/cm^2 on milkweed leaves (EPA, 2010b). The results of the feeding study showed no reduction in the survival, weight gain, development, and leaf consumption of the larvae and no sub-chronic effects were seen (EPA, 2010b). The pollen deposition was higher than the maximum expected pollen deposition rate described above and the assay also contained the Cry1F and Cry34/35Ab1 proteins; therefore, the results of this study would be applicable to assessment of 4114 maize. Based on these studies, non-target lepidopteran species are unlikely to be impacted by incidental exposure to the Cry1F and Cry34/35Ab1 proteins from 4114 maize pollen at the expected pollen deposition rates.

Potential Impact on Other Non-Target Arthropods

Laboratory assays for four representative arthropods of the orders Hymenoptera (parasitic hymenoptera, *Nasonia vitripennis,* and honeybee, *Apis mellifera*), Neuroptera (green lacewing, *Chrysoperla carnea*), and Cladocera (*Daphnia magna*) were also conducted using purified Cry1F and Cry34/35Ab1 proteins at doses exceeding expected environmental exposures (Tables 32 and 33). These studies were submitted for 1507 and 59122 maize and supported the conclusion that Cry1F and Cry34/35Ab1 exposure does not have an adverse impact on non-target, beneficial arthropods (EPA, 2010a; EPA, 2010b; Hunst and Rood, 2004; Shanahan and Stauffer, 2000; USDA-APHIS, 2001; USDA-APHIS, 2005). The results from these assays are provided in Table 32 and 33. For three of the species tested (parasitic hymenoptera, green lacewing, and honeybee), the EEC of the proteins was based on the concentrations of the proteins in 4114 maize pollen (Section 2-F). For *Daphnia magna*, the potential EEC in an aquatic environment was estimated from a modified GENEEC model, which was used to make worst-case estimates for the amount of protein that could move into pond water from a neighboring field (Raybould and Vlachos, 2011). None of the other arthropod species tested showed significant adverse effects from the proteins and the margins of exposure were greater than 9-fold (Tables 32 and 33, Representative Arthropod Species).

In addition, to further test the impacts of Cry34/35Ab1 on non-target arthropods, EPA reviewed an additional study on the minute pirate bug (*Orius insidiosus*) in the order Hymenoptera (EPA, 2010b). EPA concluded that at field concentrations, it is unlikely that Cry34/35Ab1 would have any adverse effects on this insect.

These studies have relevance for 4114 maize, expressing the Cry1F and Cry34/Cry35Ab1 proteins. Based on the conclusions of these studies, it is unlikely that 4114 maize would have an adverse impact on non-target, beneficial arthropods.

Field Monitoring for Effects of Cry1F and Cry34/35Ab1 on Non-Target Arthropods

In addition to early tier laboratory studies, multiple field studies have been conducted to investigate the potential effects of cultivating transgenic maize hybrids containing an insect-resistant protein on non-target arthropod abundance. One field study reviewed by USDA showed that 1507 maize expressing the Cry1F protein had no effect on beneficial arthropod abundance (Shanahan and Stauffer, 2000; USDA-APHIS, 2001). A second three-year field study showed no impact on abundance of several different non-target organisms, including detritovores, herbivores, and predatory arthropods, by comparing fields of 1507 maize with near-isoline control fields (Higgins *et al.*, 2009).

Similar field studies were also conducted for 59122 maize and were reviewed by different U.S. regulatory agencies; results indicated that 59122 maize expressing the Cry34/35Ab1 proteins had no effect on beneficial arthropod abundance (EPA, 2010b; Hunst and Rood, 2004; USDA-APHIS, 2005). A three-year field study was evaluated by the EPA to determine the impact of 59122 maize, expressing the Cry34/Cry35Ab1 proteins, and 1507x59122 maize, expressing both Cry1F and Cry34/35Ab1 proteins, on non-target arthropods after continuous cropping at three locations in Iowa, Nebraska, and Indiana (EPA, 2010b). Similarly, the results showed no impact on non-target arthropod populations between fields of the control maize and 59122 and 1507x59122 maize hybrids (EPA, 2010b). The EPA concluded in their assessment that there is negligible potential for long-term adverse effects on non-target arthropods after continuous cultivation of both a maize line containing the Cry34/Cry35Ab1 proteins and a stacked line that includes the Cry34/35Ab1 and Cry1F proteins (EPA, 2010b).

Results from these field studies confirm laboratory testing and support the hypothesis that 1507 and 59122 maize are unlikely to adversely impact non-target arthropods occurring in maize fields. Because of comparable Cry1F and Cry34/35Ab1 protein expression, 4114 maize is not expected to adversely impact beneficial non-target arthropods in fields.

Potential Impact of 4114 Maize on Threatened and Endangered Arthropods

The impact of 4114 maize on threatened and endangered arthropods can also be evaluated by comparing to previous assessments of 1507 and 59122 maize. These assessments concluded that threatened and endangered species would unlikely be impacted by the cultivation of either maize line (EPA, 2010a; EPA, 2010b; USDA-APHIS, 2001; USDA-APHIS, 2005).

As of July 2011, 60 insect species are listed on the US Fish and Wildlife Service website as threatened or endangered (US FWS, 2011). Twenty-one of these insects are in the order Lepidoptera and 15 are in Coleoptera, with the remaining representing insects of other orders. Almost all of the species of Lepidoptera and Coleoptera currently listed were added prior to the deregulation of 1507 and 59122 maize, except for one coleopteran species, the Salt Creek Tiger Beetle (*Cicindela nevadica lincolniana*; Family: Carabidae) (US FWS, 2010; US FWS, 2011).

For 1507 maize, USDA considered the impact of the Cry1F protein on two lepidopteran species, the Karner blue butterfly (*Lycaeides melissa samuelis*) and Mitchell's satyr butterfly (US FWS, 2011; USDA-APHIS, 2001). USDA concluded that both species would not be expected to be present in or close to maize fields; therefore it is unlikely there would be any impact of 1507 maize cultivation (USDA-APHIS, 2001). For 59122 maize, USDA considered the impact on one primary threatened and endangered coleopteran species, the American burying beetle (US FWS, 2011; USDA-APHIS, 2005). From review of preferred habitats, this beetle was unlikely to be found in active maize fields and therefore would not be exposed significantly to the Cry34/35Ab1 protein from 4114 maize (USDA-APHIS, 2005).

For other lepidopteran and corn rootworm resistant maize events, EPA has made similar conclusions for the Karner blue butterfly and the American burying beetle and has not identified any new threatened or endangered species that would be impacted by cultivation (EPA, 2009; EPA, 2010b; EPA, 2010c). Furthermore, EPA examined the habitats of the other threatened and endangered insect species in the orders Diptera, Hemiptera, Odonata and Orthoptera and found that they primarily occupy dune, meadow or prairie, or open forest habitats and are not closely associated with row crop production, often times due to the specificity of the habitat of their host plants (EPA, 2009; EPA, 2010b; EPA, 2010c).

As mentioned earlier, only one coleopteran species, the Salt Creek Tiger Beetle (*Cicindela nevadica lincolniana*; Family: Carabidae), was added in 2005 to the threatened and endangered insect species list (US FWS, 2010; US FWS, 2011). This beetle is found in Nebraska (Lancaster and Saunders counties), the third largest state for maize cultivation (US FWS, 2010; USDA-NASS, 2010). However, the beetle's critical habitats have been characterized as non-vegetated stream banks or edges that are in saline or freshwater wetlands and the beetles prefer to be within a few meters of these habitats (US FWS, 2010). Wetland areas, stream edges, or saline wetlands are unlikely to be planted with maize. In addition, the beetle family is Carabidae, which is unrelated to those of corn rootworm species (Chrysomelidae). Therefore, it is highly unlikely that this beetle will be exposed to the Cry34/35Ab1 protein from 4114 maize or will be impacted due to the specificity of the Cry34/35Ab1 protein to corn rootworm species.

Similar to the conclusions for 1507 and 59122 maize, it is unlikely that threatened and endangered species such as the Karner blue and Mitchell's satyr butterflies and the American burying beetle would be impacted by 4114 maize cultivation. Based on critical habitat and the Cry34/35Ab1 target insect specificity, it is also unlikely that the Salt Creek Tiger Beetle would be adversely affected. Furthermore, the target specificity of the introduced proteins and the lack of habitat overlap with regions of maize cultivation also support the conclusion that 4114 maize would not adversely impact the other listed threatened and endangered insects.

Potential Impact on Non-Target Vertebrate Species

Wildlife in and around maize fields are of interest due to potential exposure to 4114 maize grain and plant residue. A wide variety of toxicology studies conducted over the past 40 years have established the safety of the microbial preparations of *B. thuringiensis*, including their expressed insecticidal Cry proteins (Betz *et al.*, 2000). As discussed in Section 3, the allergenicity and toxicity of the Cry1F, Cry34Ab1, and Cry35Ab1 proteins has been assessed and all three are unlikely to be potential allergens or toxins to humans or animals. Based on quail and mouse toxicological studies for Cry1F, both EPA and USDA concluded that the protein was unlikely to have an adverse effect on vertebrates, including non-target birds, mammals, and humans (EPA, 2010a; USDA-APHIS, 2001). EPA and USDA made similar conclusions for Cry34Ab1 and Cry35Ab1 on the basis of poultry, mouse, and rainbow trout toxicological studies (EPA, 2010b; USDA-APHIS, 2005). Providing additional evidence for the lack of toxicity of the Cry1F and Cry34/35Ab1 proteins, recently published rodent feeding studies showed no toxicological effects of diets containing 1507 and 1507x59122 maize grain (Appenzeller *et al.*, 2009; MacKenzie *et al.*, 2007). Additional feeding studies for laying hens, swine, beef cattle, and dairy cows with diets containing 1507 and 59122 maize have all shown no differences in nutritional equivalency from diets containing conventional maize (Faust *et al.*, 2007; Huls *et al.*, 2008; Jacobs *et al.*, 2008; Scheideler *et al.*, 2008; Sindt *et al.*, 2007; Stein *et al.*, 2009).

Results from these studies are relevant for 4114 maize, which expresses the Cry1F, Cry34Ab1, and Cry35Ab1 proteins, and provide evidence that 4114 maize is unlikely to have adverse effects on non-target vertebrate species, including birds, mammals, and humans.

Conclusions for the Environmental Impact of the Introduced Proteins in 4114 Maize

The environmental impact of 4114 maize focused on the introduced Cry1F and Cry34/35Ab1 proteins and considered the potential hazard and the level of environmental exposure to non-target organisms. Due to the presence of the same proteins and similar or lower protein expression, the previously conducted environmental safety studies for 1507, 59122, and 1507x59122 maize were relevant to the assessment of 4114 maize. As previously established for 1507 and 59122 maize, the Cry1F and Cry34/Cry35Ab1 proteins have insecticidal activity against lepidopteran and corn rootworm species, and there is low risk of hazard to non-target orders. The low hazard to several representative non-target organisms was confirmed in the laboratory at protein concentrations greater than those expected in the environment and the potential environmental exposure to the Cry1F and Cry34/35Ab1 proteins was determined. Based on this analysis, there is a sufficient margin of exposure for the proteins in the environment and, therefore, the cultivation of 4114 maize is unlikely to adversely impact non-target organisms. Likewise, multiple field studies have also confirmed that the abundance of non-target organisms under field conditions is not impacted by maize expressing the Cry1F and Cry34/35Ab1 proteins. Similar to the conclusions for 1507 and 59122 maize, the cultivation of 4114 maize is unlikely to have adverse effects on non-target arthropods, including threatened and endangered arthropods, and non-target vertebrates, including birds, mammal, and humans.

6. Overall Conclusions

In conclusion, based on the data contained herein, Pioneer requests that APHIS grant the request for a determination of nonregulated status for 4114 maize and any crosses of this line with other nonregulated *Zea mays*. 4114 maize was modified to produce the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins; these proteins and associated genetic elements in 4114 maize are identical to those in previously approved 1507, 59122, and 1507x59122 maize. In addition, the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins are unlikely to be allergens or toxins and are safe for the food and feed supply.

4114 maize contains all the gene cassettes that express the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins at a single genetic locus. Furthermore, molecular characterization confirmed that 4114 maize contains a single copy, intact insertion that is stable and segregates according to Mendel's laws of genetics.

Nutrient composition and agronomic comparative assessments were conducted to determine if 4114 maize would present any new or greater risks relative to maize varieties that have a history of safe use in the environment and as food and feed. These analyses indicated that 4114 maize is as safe as conventional maize varieties and does not pose a greater risk than conventional maize varieties in food, feed, and the environment.

It is unlikely that 4114 maize will pose a plant pest risk or impact non-target organisms in the environment. Maize is not considered a weed, and 4114 maize does not exhibit any characteristics that would indicate it is any more likely than conventional maize to become a weed or plant pest. Because sexually compatible wild relative populations are limited in the U.S. and there is low potential for gene flow, it is unlikely that the inserted DNA in 4114 maize would be introgressed significantly into these wild relative populations. Based on the evidence supporting the environmental safety of the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins, it is unlikely that 4114 maize will pose a risk to non-target organisms including beneficial, threatened and endangered species, as well as non-target vertebrates including birds, mammals, and humans.

In support for the safety of 4114 maize, 1507 maize has been commercially available in the U.S. since the 2003 growing season and 59122 and 1507x59122 maize have been available in the U.S. since 2006 with no adverse food, feed, or environmental effects. Maize products containing 1507x59122 maize were grown on more than 14 million acres in 2010.

Appendix 1. 4114 Maize USDA Release Permits and Notifications and Planted Acreage
Plantings through May 25, 2011 are listed

Year of Planting	Permit Name	Permit Valid Date	State	Number of Counties Where 4114 Maize[a] was Planted	Acreage
2006	06-019-03R	5/2/2006	PR	2	0.010
	06-019-04R	5/3/2006	HI	1	0.014
2007	06-019-03R	5/2/2006	CA	1	0.009
			PR	1	0.039
	06-019-04R	5/3/2006	HI	1	0.039
	07-040-101rm	5/3/2007	CA	1	0.009
			HI	1	0.146
			IA	2	0.072
			IL	4	0.092
			IN	1	0.030
			MN	1	0.030
			MO	1	0.020
			NE	1	0.020
			PR	2	0.110
			WI	1	0.020
2008	07-040-101rm	5/3/2007	HI	1	0.020
			PR	1	0.130
	08-014-111n	4/16/2008	CA	1	0.050
			IA	2	0.230
			IL	4	0.270
			IN	2	0.160
			MN	1	0.012
			MO	1	0.060
			NE	2	0.110
			PR	2	1.510
			WI	1	0.070
	08-014-131n	3/31/2008	HI	1	0.280
	08-095-105n	5/14/2008	IA	1	0.070
			IL	2	0.060
			MN	1	0.014
			NE	1	1.077
			OK	1	0.050
			TX	1	0.040
2009	08-014-111n	4/16/2008	PR	1	0.001
	09-013-108n	4/1/2009	IA	5	0.750
			IL	6	0.370
			IN	2	0.280
			MO	1	0.114
			NE	1	0.186
	09-016-103n	2/15/2009	IL	1	0.032
			NE	1	0.023

4114 Maize USDA Release Permits and Notifications and Planted Acreage (continued)

Year of Planting	Permit Name	Permit Valid Date	State	Number of Counties Where 4114 Maize[a] was Planted	Acreage
2009	09-035-109n	3/17/2009	AR	1	0.018
			CO	1	0.057
			DE	1	0.020
			GA	1	0.129
			HI	1	0.128
			KS	1	0.018
			MN	1	0.134
			PR	3	2.980
			TN	1	0.018
			WI	1	0.098
	09-264-102n	10/21/2009	HI	1	0.064
			PR	2	2.370
2010	09-264-102n	10/21/2009	HI	1	0.083
			PR	1	0.281
	10-015-106n	3/1/2010[b]	IA	3	0.090
			IL	3	0.080
			IN	1	0.011
			MI	1	0.011
			MN	1	0.034
			NE	1	1.057
	10-050-115n	3/9/2010[b]	NE	1	0.016
	10-052-101n	3/19/2010[b]	AR	1	0.017
			CA	1	0.200
			CO	2	0.060
			GA	1	1.006
			HI	1	0.630
			IA	9	2.500
			IL	6	2.210
			IN	4	0.920
			KS	1	0.224
			MI	1	0.286
			MN	2	0.310
			MO	3	0.540
			NE	1	0.619
			PA	1	0.186
			PR	4	1.020
			SD	1	0.039
			TN	1	0.068
			WI	1	0.372
	10-118-105n	5/6/2010[b]	IL	1	0.186
	10-281-101n	10/30/2010[b]	PR	1	0.069
	10-284-101n	11/16/2010[b]	PR	2	1.470

4114 Maize USDA Release Permits and Notifications and Planted Acreage (continued)

Year of Planting	Permit Name	Permit Valid Date	State	Number of Counties Where 4114 Maize[a] was Planted	Acreage
2011	11-040-123n	3/17/2011[b]	PR	2	0.404
	10-281-101n	10/30/2010[b]	TN	1	0.104
	10-284-101n	11/16/2010[b]	HI	1	0.030
			PR	1	0.684
	11-019-109n	3/1/2011[b]	IA	1	0.023
			IL	2	0.046
			NE	1	0.023
			OK	1	0.023
			TX	1	0.003
	11-040-123n	3/17/2011[b]	CO	1	0.049
			GA	1	0.260
			HI	1	0.698
			IA	7	2.880
			IL	6	4.019
			IN	5	2.114
			KS	1	0.654
			MI	1	0.554
			MN	2	2.137
			MO	3	1.182
			NE	1	0.847
			SD	1	0.498
			TN	1	0.041
			WI	2	0.518
	11-040-124n	3/17/2011[b]	HI	1	0.033
			IA	1	0.002

[a] 4114 maize is also referred to as DP-004114-3 and EA-2244.04.1.14 in USDA final reports.
[b] Final field permit test reports not yet due to USDA.

Appendix 2. Equivalency of the Proteins Produced in 4114 Maize to Those Produced in 1507 and 59122 Maize

2.1. Overview and Summary

As discussed earlier in Section 2, the introduced *cry*1F, *cry*34Ab1, *cry*35Ab1, and *pat* gene cassettes in 4114 maize are identical to those cassettes in 1507 and/or 59122 maize. Based on the DNA sequence of the genes, the translated protein sequences are also identical. Prior protein safety studies for 1507 and 59122 maize were conducted on the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins and are described in Section 3. These protein safety studies confirmed that none of the proteins are potential allergens or toxins. In order to verify the applicability and relevance of these protein safety studies for 4114 maize, the identity and equivalency of the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins should be established.

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.

As described in Section 2, the western blot analysis in conjunction with other analyses confirmed that the proteins in 4114 maize are the same as those of 1507 and/or 59122 maize; therefore, prior protein safety studies conducted for 1507 and/or 59122 maize on Cry1F, Cry34Ab1, Cry35Ab1, and PAT are relevant to the safety assessment of 4114 maize.

2.2. Western Blot Analysis of the Introduced Proteins

Cry1F

Western blot analysis using anti-Cry1F antibodies (Figure 2.1) 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.

Cry34Ab1

Western blot analysis using anti-Cry34Ab1 antibodies (Figure 2.2) 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.

Cry35Ab1

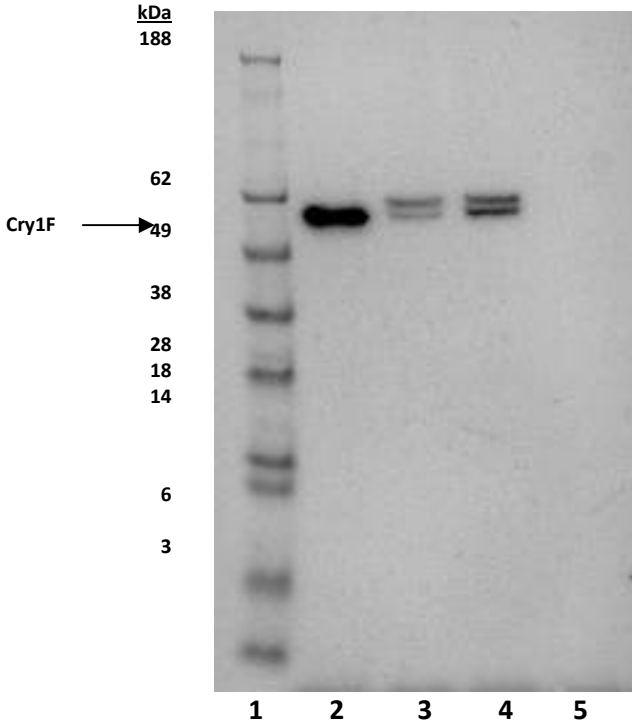
Western blot analysis using anti-Cry35Ab1 antibodies (Figure 2.3) demonstrated that the Cry35Ab1 protein derived from 4114 maize (Lane 3) had the same molecular weight and relative immunoreactivity as the Cry35Ab1 protein derived from 1507x59122 maize (Lane 4). Both protein samples migrated as two bands of approximately 40 kDa and 44 kDa in size. Like the Cry1F protein, the 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 (Lane 2) 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 (Figure 2.4) 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).

2.3. Conclusions

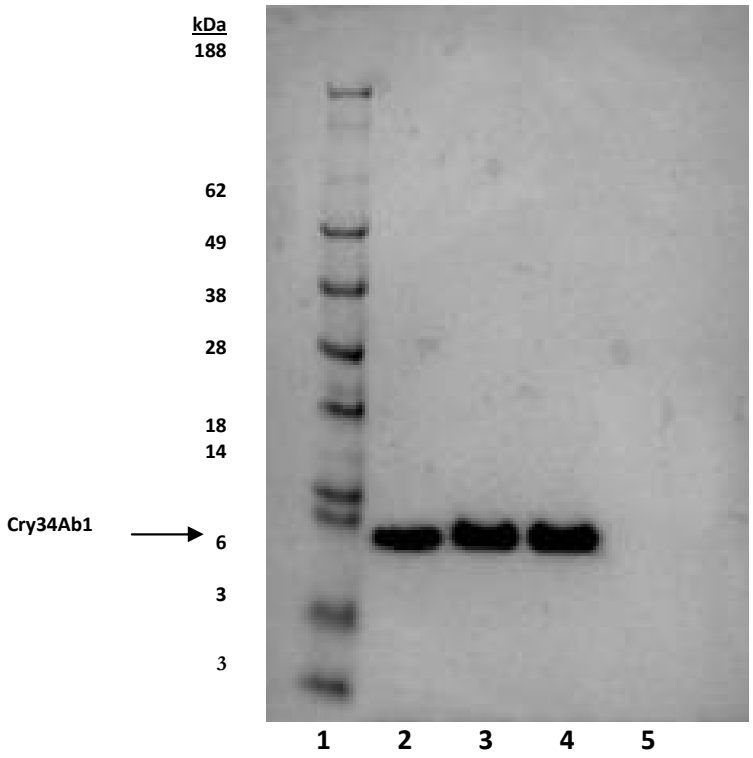
The western blot analyses demonstrated that the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins derived from 4114 maize have equivalent molecular weights and immunoreactivity to the proteins expressed in 1507x59122 maize. In all four western blots, the near-isoline control showed no immunoreactive proteins. 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. Therefore, prior safety studies conducted on Cry1F, Cry34Ab1, Cry35Ab1, and PAT for 1507 and/or 59122 maize are applicable to 4114 maize.



Lane	Sample ID
1	Molecular Weight Marker
2	Cry1F Standard (~4 ng)
3	Protein Extract of R1 Leaf from 4114 Maize (undiluted)
4	Protein Extract of R1 Leaf from 1507x59122 Maize (undiluted)
5	Protein Extract of R1 Leaf from Near-Isoline Control Maize (undiluted)

Figure 2.1. Western Blot Comparison of Cry1F Protein

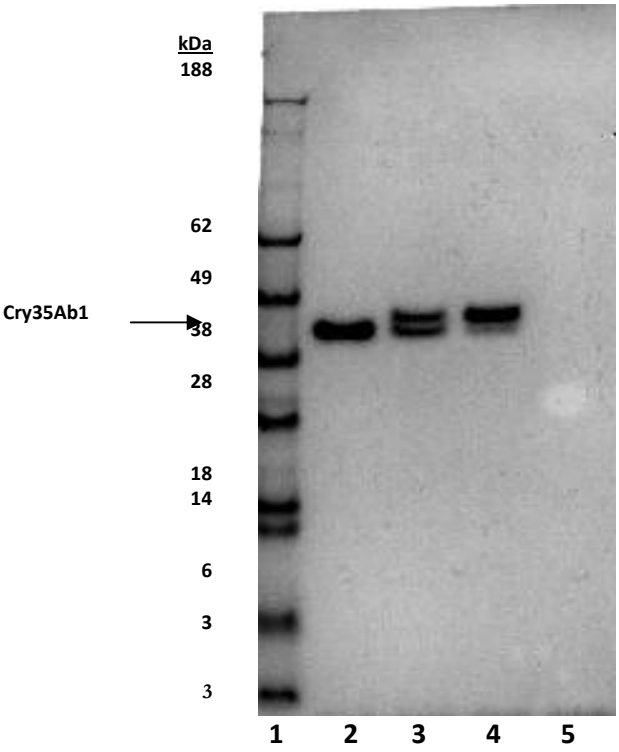
Plant-derived Cry1F protein can be partially degraded by plant proteases to a smaller, more stable truncated protein, therefore appearing as two bands (~60 kDa and ~62 kDa). The Cry1F standard protein consisted of the truncated protein only (~60 kDa).



Lane	Sample ID
1	Molecular Weight Marker
2	Cry34Ab1 Standard (~10 ng)
3	Protein Extract of R1 Leaf from 4114 Maize (1:2 dilution)
4	Protein Extract of R1 Leaf from 1507x59122 Maize (1:3 dilution)
5	Protein Extract of R1 Leaf from Near-Isoline Control Maize (undiluted)

Figure 2.2. Western Blot Comparison of Cry34Ab1 Protein

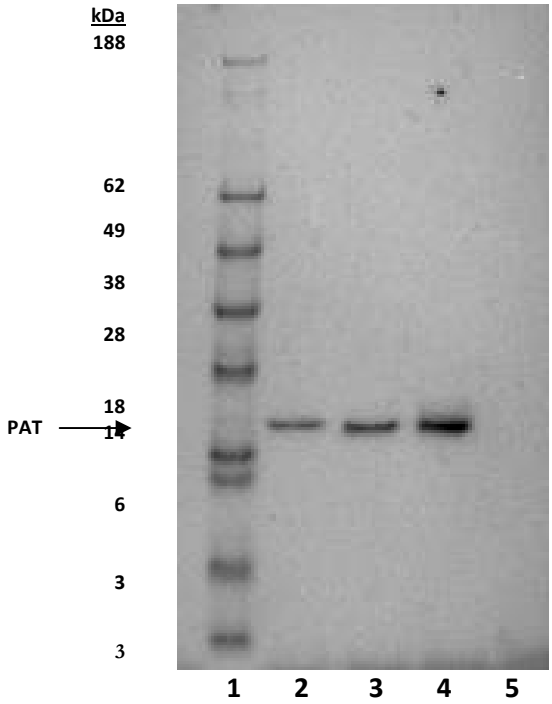
Plant-derived and standard Cry34Ab1 proteins migrated at the expected mass of ~14 kDa.



Lane	Sample ID
1	Molecular Weight Marker
2	Cry35Ab1 Standard (~10 ng)
3	Protein Extract of R1 Leaf from 4114 Maize (1:2 dilution)
4	Protein Extract of R1 Leaf from 1507x59122 Maize (1:2 dilution)
5	Protein Extract of R1 Leaf R1 from Near-Isoline Control Maize (undiluted)

Figure 2.3. 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).



Lane	Sample ID
1	Molecular Weight Marker
2	PAT Standard (~4 ng)
3	Protein Extract of R1 Leaf from 4114 Maize (undiluted)
4	Protein Extract of R1 Leaf from 1507x59122 Maize (1:2 dilution)
5	Protein Extract of R1 Leaf from Near-Isoline Control Maize (undiluted)

Figure 2.4. Western Blot Comparison of PAT Protein

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

2.4. Experimental Methods

2.4.1. 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.

2.4.2. 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, 1507x59122 maize, and the control maize were collected and processed as described in Appendix 5. A representative leaf sub-sample from each maize line was weighed at a target weight of 10 mg $\pm$ 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.

2.4.3. 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[b]-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[c] (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 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.

2.4.4. 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.

[b] Registered trademark of ICI Americas
[c] Registered trademark of SPEX CertiPrep, Inc.

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

All sample extracts and protein standard solutions were prepared for electrophoresis by adding 25% NuPAGE[d] 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[d] Pre-Stained Standard (Invitrogen) was loaded at 10 µl/well.

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

2.4.6. 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[d] module (Invitrogen). 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 specific monoclonal primary antibody (Table 2.1), 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 2.1; 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[e] 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 1507x59122 maize.

[d] Registered trademark of Invitrogen Corporation
[e] Registered trademark of Pierce Biotechnology, Inc.

Table 2.1. Primary and Secondary Antibody Descriptions Used for Qualitative Western Blot 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)

Appendix 3. Materials and Methods for Genetic Characterization of 4114 Maize

3.1. 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 were analyzed by Southern blot 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*d III restriction enzymes. Southern blot analysis was conducted on individual plants of the four generations to confirm stability of the insertion across generations and to verify the absence of backbone sequences from plasmid PHP27118.

3.2. 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.

3.3. Control Material

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

3.4. 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 methylase recognition sequence ($^{5'}$GATC$^{3'}$) (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 $^{5'}$TGATCA$^{3'}$) was methylated and did not digest as expected (Pioneer data not shown). Therefore, the *Bcl* I-digested plasmid served only as a positive control to demonstrate probe hybridization and not to provide any band size data. As Dam methylase 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*d III 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) labeled (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.

3.5. 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 plants for event-specific PCR analysis. DNA was extracted from each leaf sample using the Extract-N-Amp[f] 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[g] 7500 Fast Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA). TaqMan[g] 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[g] 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.

[f] Registered trademark of Sigma-Aldrich
[g] Registered Trademarks of Applied Biosystems, Inc.

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.

3.6. 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[h] (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 then washed with 70% ethanol. After drying, the DNA was re-dissolved with 0.5 ml distilled water and stored at 4°C.

3.7. Quantification of Genomic DNA

Following extraction, the DNA samples were quantified on a spectrofluorometer using PicoGreen[i] 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.

3.8. 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 to 6 μg of genomic DNA was digested using 50 units of enzyme according to manufacturer's 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 (10mM Tris, 1 mM EDTA, pH 7.5). The reference plasmid, PHP27118, was spiked into a control 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.

[h] Registered trademark of SPEX CertiPrep, Inc.
[i] Registered trademark of Molecular Probes, Inc.

3.9. 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 labeled 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 (3M NaCl, 0.3 M sodium citrate) using the method as described for the TURBOBLOTTER[j] Rapid Downward Transfer System (Whatman, Inc., Piscataway, NJ). The DNA was then bound to the membrane by UV crosslinking (Stratalinker, Stratagene, La Jolla, CA).

3.10. DNA Probe Labeling for Southern Blot Hybridization

Probes for the *cry*1F, *cry*34Ab1, *cry*35Ab1 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, *pin*II 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 labeled nucleotide, [DIG-11]-dUTP, into the fragment. PCR labeling of isolated fragments was carried out according to the procedures supplied in the PCR DIG Probe Synthesis Kit (Roche).

3.11. Probe Hybridization and Visualization

Labeled probes 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-labeled probes and DIG-labeled 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

[j] Registered trademark of Whatman, Inc.

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.

3.12. Stripping of Probes and Subsequent Hybridizations

Following hybridization and detection, membranes were stripped of DIG-labeled 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 labeled with the alkali-labile DIG.

Appendix 4. Materials and Methods for Segregation Analysis of Five Generations 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.

4.1. Greenhouse Experimental Design

Five separate generations (F1[*1], BC2F1[*1], BC3F1[*1], BC2F1[*2], and BC3F1[*2]) of 4114 maize were planted and grown in a greenhouse under standard maize production environmental conditions. Leaf punch samples were collected from each generation and analyzed using PCR analyses specific for the event DP-ØØ4114-3, *cry*1F gene, *cry*34Ab1 gene, *cry*35Ab1 gene, and *pat* gene. After sample collection, all plants were treated with a broadcast application of glufosinate and then visually evaluated for herbicide resistance.

4.2. Planting and Leaf Sample Collection

Maize seeds, approximately 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 (≤ -10°C) for storage. Individual plants and corresponding leaf punch samples were uniquely labeled 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.

4.3. 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 *cry*1F, *cry*34Ab1, *cry*35Ab1, and *pat* genes.

4.4. Phenotypic Analysis

After sample collection, all plants were treated with a tank mixture containing Ignite[k] 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 liter). The tank mix was applied at an approximate rate of 22 fluid ounces per acre (1.6 liters per hectare) and the spray volume was 21.6 gallons per acre (202 liters 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).

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.

4.5. Statistical Analysis

A chi-square analysis (95%) was performed on the segregation results of each 4114 maize generation to compare the observed segregation ratio to the expected segregation ratio (1:1).

[k] Registered trademark of Bayer CropScience

Appendix 5. Materials and Methods for Determination of Cry1F, Cry34Ab1, Cry35Ab1, and PAT Protein Concentrations

Concentrations of the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins were determined in various tissues of 4114 maize using enzyme-linked immunosorbent assays (ELISA). Plant tissues were collected from 4114 maize plants from a 2010 field trial and analyzed as described further below.

5.1. Field Trial Experimental Design

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

Each field site utilized a randomized complete block design with 4114 maize, 1507 maize, 59122 maize, 1507x59122 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.

5.2. Plant Material Collection, Shipping, Processing, and Storage

5.2.1. Tissue Collection

Leaf, root, whole plant, pollen, forage, and grain samples for protein concentration analysis were collected 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.

The following tissues were collected with the plant growth stages indicated in parentheses: leaf (V6, V9, R1, R4, and R6), root (V6, V9, R1, R4, and R6), whole plant (V9, R1, and R6), pollen (R1), forage (R4), and grain (R6). Plant growth stages are explained in Table 5.1 (Ritchie *et al*., 2005).

Table 5.1. Maize Growth Stage Descriptions

Growth Stage[a]	Description[a]
V6	The collar of the sixth leaf becomes visible
V9	The collar of the ninth leaf becomes visible
R1	The silks become visible
R4	The material within the kernel produces a doughy consistency
R6	The typical harvest maturity for grain; regarded as physiological maturity

[a] Growth stages are as described in Ritchie *et al.*, 2005

Leaf

Four leaf samples (one sample per plant) were each collected from 4114 maize, 1507 maize, 59122 maize, and 1507x59122 maize and one sample was collected from the control maize at each site. Samples were obtained from the youngest healthy leaf that had emerged at least 8 inches (20 cm) from the whorl.

Root

Four root samples (one sample per plant) were each collected from 4114 maize, 1507 maize, 59122 maize, and 1507x59122 maize and one sample was collected from the control maize at each site. Samples were obtained by cutting a circle 10-15 inches (25-38 cm) in diameter around the base of the plant to a depth of 7-9 inches (18-23 cm). The root ball was removed from the soil, shaken to remove excess soil, and thoroughly cleaned with water. A representative sub-sample of root tissue was collected.

Whole Plant

Following the collection of all other samples for a respective growth stage, whole plant samples were collected from the remaining aerial portion of the plants. Four whole plant samples (one sample equals one plant) were each collected from 4114 maize, 1507 maize, 59122 maize, and 1507x59122 maize and one sample was collected from the control maize at each site. Samples were obtained by cutting the plants from the root system approximately 1 inch (2.5 cm) above the soil surface line. The plants were chopped into sections less than 3 inches (7.5 cm) in length.

Pollen

Four pollen samples (one sample per plant) were each collected from 4114 maize, 1507 maize, 59122 maize, and 1507x59122 maize and one sample was collected from the control maize at each site. The tassel selected for sampling had half to three-quarters of the main spikes shedding and was bagged no more than one day prior to collection. Samples were obtained by shaking or tapping the bagged tassel to dislodge the pollen.

Forage

Following the collection of R4 leaf and root samples, forage samples were collected from the remaining aerial portion of the plants. Four forage samples (one sample equals one plant) were each collected from 4114 maize, 1507 maize, 59122 maize, and 1507x59122 maize and one sample was collected from the control maize at each site. All plants used for sampling contained self-pollinated primary ears. Any secondary or tertiary ears with exposed silks were removed prior to sampling. Samples were obtained by cutting the plants from the root system approximately 1 inch (2.5 cm) above the soil surface line. The plants were chopped into sections less than 3 inches (7.5 cm) in length.

Grain

Four grain samples (one sample equals the grain from one self pollinated primary ear per plant) were each collected from 4114 maize, 1507 maize, 59122 maize, and 1507x59122 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. Husks and cobs were retained for inclusion with the R6 whole plant samples.

5.2.2. Sample Shipping, Processing, and Storage

All tissue samples were shipped frozen and stored at ≤ -5 °C. Whole plant and forage samples were coarsely homogenized. Leaf, root, whole plant, pollen, forage, and grain samples were lyophilized under vacuum until dry. Following lyophilization, leaf, root, whole plant, forage, and grain samples were finely homogenized and stored frozen until analysis.

5.3. Protein Concentration Determination

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

5.3.1. Protein Extraction

Analytical samples were weighed into 1.2-ml tubes at target weights (±5%) of 10 mg for leaf; 20 mg for root and grain; 30 mg for forage, and whole plant; and 5 mg for pollen. 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[l]-20). Following centrifugation, supernatants were removed, diluted, and analyzed.

5.3.2. Cry1F ELISA Procedure

The Cry1F ELISA method utilized a sequential ELISA format to determine the concentration of the Cry1F protein in sample extracts. The Cry1F ELISA kit employed was obtained from Envirologix, Inc. (Portland, Maine). 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 colored 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.

5.3.3. 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. The Cry34Ab1 ELISA kit employed was obtained from Envirologix, Inc.

5.3.4. 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. The Cry35Ab1 ELISA kit employed was obtained from Acadia BioScience, LLC (Portland, Maine).

5.3.5. 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. The PAT ELISA kit employed was obtained from Envirologix, Inc.

5.3.6. Calculations for Determining Protein Concentrations

SoftMax[m] 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.

[l] Registered trademark of ICI Americas, Inc.

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.

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

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:

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

5.4. Statistical Analysis

Statistical analyses were conducted using SAS[n] software, Version 9.2 (SAS Institute, Inc., Cary, NC) to estimate mean concentrations of Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins derived from 4114 maize, 1507 maize, 59122 maize, and 1507x59122 maize.

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[m] Registered trademark of Molecular Devices Corporation
[n] Registered trademark of SAS Institute, Inc.

5.4.1. Processing of Data

Transformation

Protein concentrations were first subjected to a natural logarithm transformation "ln(y)" before statistical analyses. Residuals were examined for validation of the normality and homogeneous variance assumptions. When these assumptions were questionable, other types of transforms or none were considered. The following proteins and tissue types did not undergo "ln(y)" transformation because the original data achieved model validation assumptions.

- Cry1F in leaf (V9), root (R4, V6, and V9), and whole plant (V9)
- Cry34Ab1 in leaf (R1 and R4), root (R1, R4, and V6), and whole plant (V9)
- Cry35Ab1 in leaf (R4 and R6), root (R4 and R6), and grain (R6)
- PAT in leaf (R1, R4 and V9), forage (R4), and root (V9)

If the protein concentration underwent "ln(y)" transformation, the statistical analyses were conducted based on the transformed data. The estimated mean values and the confidence limits were then back-transformed to the original data scale for reporting purposes.

Partial LLOQ Sample Values

For a given protein in a given tissue, the number of samples below the LLOQ determined whether statistical analysis was conducted. The following rules were implemented:

If < 80% of samples for each entry were below the LLOQ, then the statistical model considering LLOQ was utilized to conduct statistical analysis.

If ≥ 80% of samples for a single entry within the study were below the LLOQ, then statistical analysis was not conducted.

5.4.2. Statistical Models

Default Model

For a given protein concentration in a given tissue, data were analyzed using the following linear mixed model:

$y_{ijk} = \mu_i + \ell_j + r_{k(j)} + (\mu\ell)_{ij} + \varepsilon_{ijk}$ 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. The estimated means are known as least squares means (LS-Means).

SAS PROC MIXED was utilized to fit Model 1 and to generate LS-Means, 95% confidence intervals.

Model Considering LLOQ

For Cry1F in leaf (R6), PAT in root (R4 and R6), and whole plant (R6), <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:

$y_{ijk} = \mu_i + \ell_j + r_{k(j)} + (\mu\ell)_{ij} + \varepsilon_{ijk}$ 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 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 residual for the observation obtained from the plot assigned to the ith entry in the kth block of the jth site.

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 (i.e. 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 θ denotes the vector of all random effects, ϕ denotes the standard normal density function, Φ 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 (ML) procedure was then used to generate estimates of variance components and entry means (i.e. LS-Means).

SAS PROC NLMIXED was utilized to fit Model 2 and generate LS-Means and 95% confidence intervals.

5.4.3. Reported Statistics

For each expressed trait protein in each tissue that was statistically analyzed, entry LS-Mean (back-transformed, if needed), range, and 95% confidence interval (back-transformed, if needed) (labeled as Mean, Range, and CI, respectively) are provided in Section 2; Tables 10-13. In these tables, descriptive statistics (arithmetic means and ranges) are reported for proteins and tissues that were not statistically analyzed using mixed model analyses due to insufficient above-LLOQ samples.

The Cry1F, Cry34Ab1, and Cry35Ab1 protein mean concentrations in 4114 maize in each tissue were divided by the respective protein mean concentrations in 1507, 59122, and/or 1507x59122 maize to provide an “expression ratio” (Section 2; Table 14). When the expression ratio was close to one, this indicated that 4114 maize had comparable expression to 1507, 59122, and/or 1507x59122 maize. If the expression ratio was less than one, this indicated that 4114 maize had lower expression. For values greater than one, expression was determined to be higher in 4114 maize.

Appendix 6. Materials and Methods for Nutrient Composition

Nutrient composition was determined in 4114 maize using plants from a 2010 field trial and analyzed as described further below.

6.1. Field Trial and Experimental Design

4114 maize plants of the F1[*5] generation were grown at six sites in 2010 in commercial maize growing regions of North America, with four sites in the U.S. and two sites in Canada [Richland, IA; Wyoming, IL; Geneva, MN; York, NE; Branchton, ON Canada; and Thorndale, ON Canada].

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. Maintenance fertilizer, herbicides, and pesticides were applied uniformly to 4114 maize and the control maize at each site, as needed. In order to ensure grain purity for compositional analyses, 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.

6.2. Plant Material Collection, Shipping, Processing, and Storage

6.2.1. 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 forage and 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.

<u>Forage</u>

One forage sample (composite of 3 whole plants) was collected from 4114 maize and the control maize from each of the four blocks. Any secondary or tertiary ears with exposed silks were removed prior to sampling. Forage samples were obtained by cutting the aerial portion of the plants from the root system approximately 1 inch (2.5 cm) above the soil surface line. The plants (including ears) were then cut into sections approximately 3 inches (7.5 cm) or less, and approximately 1/3 of the total sample was collected in a pre-labeled plastic-lined cloth bag.

Grain

One grain sample (five pooled ears equaled 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-labeled plastic-lined cloth bag.

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

6.2.2. Sample Shipping, Processing, and Storage

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

6.3. Nutrient Composition Analyses

Nutrient composition analyses of maize forage and grain were conducted and included the analysis methods described in Table 6.1.

Table 6.1. Methods for Compositional Analysis of 4114 Maize

Nutritional Analyte	Method
Moisture in forage and 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 forced air oven (forage) or vacuum oven (grain).
Ash in forage and 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 forage and 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.

Table 6.1. Methods for Compositional Analysis of 4114 Maize (continued)

Nutritional Analyte	Method
Crude fat in forage and 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 3N 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 forage and 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 fiber in forage and grain	The analytical procedure for crude fiber determination was based on methods provided by the manufacturer of the extraction apparatus (Ankom Technology). Samples were analyzed to determine the percentage of crude fiber by digestion and solubilization of other materials present.
Neutral detergent fiber in forage and grain	The analytical procedure for neutral detergent fiber (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 fiber in forage and grain	The analytical procedure for acid detergent fiber (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 dried and weighed to determine the ADF content.
Minerals in forage and grain	The analytical procedure for the determination of minerals is based on methods published by the AOAC and CEM Corporation. The maize forage minerals determined were calcium and phosphorus. 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*.

Table 6.1. Methods for Compositional Analysis of 4114 Maize (continued)

Nutritional Analyte	Method
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.
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.

Table 6.1. Methods for Compositional Analysis of 4114 Maize (continued)

Nutritional Analyte	Method
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 hemicalcium 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.

Table 6.1. Methods for Compositional Analysis of 4114 Maize (continued)

Nutritional Analyte	Method
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.
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.
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.
Phytic acid in grain	The analytical procedure for the determination of phytic acid was based on a method published by the AOAC. The samples were analyzed to determine the amount of phytic acid by extracting the phytic acid with dilute hydrochloric acid (HCl) and isolating it using an aminopropyl silica solid phase extraction column. Once isolated and eluted, the phytic acid was analyzed for elemental phosphorus by ICP-OES.

6.4. Statistical Analyses

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

6.4.1. Processing of Data

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 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 both forage and 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.

[o] Registered trademark of SAS Institute, Inc.

6.4.2. 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 analysis was conducted.
- If, for a single entry, ≥80% of samples were below the LLOQ, then the 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.

Default Model

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

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

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

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. 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:

$y_{ijk} = \mu_i + \ell_j + r_{k(j)} + (\mu\ell)_{ij} + \varepsilon_{ijk}$ 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 i[th] entry (fixed effect), ℓ_j denotes the effect of the j[th] site (random effect), $r_{k(j)}$ denotes the effect of the k[th] block within the j[th] 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 i[th] entry in the k[th] block of the j[th] 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 θ denotes the vector of all random effects, ϕ denotes the standard normal density function, Φ 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). 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).

FDR Adjustment

The FDR method of Benjamini and Hochberg (Benjamini and Hochberg, 1995; Westfall *et al*., 1999) was applied as a post-hoc procedure to account for multiple comparisons due to multiple compositional analytes, and P-values were adjusted accordingly. The FDR adjustment was conducted for each set of comparisons. A significant difference was identified if an adjusted P-value <0.05.

SAS PROC MULTTEST was utilized to provide adjusted P-values.

6.4.3. Interpretations of Statistical Results

Where a statistically significant difference (adjusted P-value <0.05) was identified for a given analyte in the 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 (*i.e.,* 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 (Codex, 1996; Codex, 2005; ILSI, 2006; OECD, 2002; Watson, 1982; Watson, 1987).

6.4.4. Reported Statistics

For each analyte that was statistically analyzed using mixed model analysis, entry LS-Mean (back-transformed, if needed), range, and 95% confidence interval (back-transformed, if needed) (labeled as Mean, Range, and CI, respectively) are provided in Section 4; Tables 15-22. Both the non-adjusted P-values and FDR-adjusted P-values (labeled as P-Value and Adjusted P-Value, respectively) are provided for comparisons between 4114 maize and the control maize. For each analyte, a tolerance interval (Section 4; Tables 15-22) and a literature range (Section 4; Tables 15-22), if available, are provided.

In Section 4, Tables 15-22, descriptive statistics (arithmetic means and ranges) are reported for analytes that were not statistically analyzed using mixed model analysis. Note: for fatty acid analytes, means and ranges were calculated based on assay values above the LLOQ. When all fatty acid samples values were below the LLOQ for a given entry, means and ranges were 0 and 0-0, respectively, and were not reported.

Appendix 7. Material and Methods for Analysis of Agronomic Characteristics and Germination Evaluation

7.1. Germination and Dormancy Evaluation

4114 maize and two commercial Pioneer® hybrid maize reference lines (32D78 and 34P88) were evaluated for germination rates under warm, cold, and diurnal growing conditions.

7.1.2. Experimental Design

Three separate germination tests (warm, cold, and diurnal) were conducted. For a given germination test, approximately 400 seed from each of 4114 maize, the control maize, 32D78 maize, and 34P88 maize were evaluated. The seed from each maize line were arranged into eight individual replicates, with approximately 50 seed per replicate. No broken or damaged seed were included in any of the germination tests. To control experimental bias in this study, the following procedures were utilized: randomization of each sample replication within the growth chamber and uniform maintenance of environmental conditions across all entries and replicates within the growth chamber.

7.1.3. Germination Tests

The warm germination test simulated optimal maize growth conditions. Each replicate was placed between sheets of moist germination toweling and rolled up. The rolls were then placed in a dark growth chamber at a continuous setting of 25 °C and 90% relative humidity for 10 days. After 10 days, the number of germinated seed in each replicate was counted.

The cold germination test simulated early spring planting conditions in the midwestern United States. Each replicate was placed between sheets of moist germination toweling and rolled up. The rolls were then placed in a dark growth chamber at a continuous setting of 10 °C and 90% relative humidity for 10 days, followed by three days at a continuous setting of 25 °C and 90% relative humidity. After 13 days, the number of germinated seed in each replicate was counted.

The diurnal germination test simulated daily weather conditions in the midwestern United States. Each replicate was placed between sheets of moist germination toweling and rolled up. The rolls were then placed in a growth chamber at a cyclical setting of 10 °C and 90% relative humidity for 16 hours and then 25 °C and 90% relative humidity for 8 hours, repeated daily for 10 days. After 10 days, the number of germinated seed in each replicate was counted.

7.1.4. Seed Evaluation

Classification of Germinated and Non-Germinated Seed

At the end of each germination test, each seed was defined as either germinated or non-germinated. A given seed was classified as germinated if any of the essential structures

necessary to produce a normal plant under favorable conditions had emerged, or non-germinated if these structures had not emerged.
Non-germinated seed were further defined into three categories: hard (*i.e.,* did not absorb water), imbibed (*i.e.,* absorbed water but did not show signs of growth during the germination test), or dead (*i.e.,* absorbed water, did not show signs of growth during the germination test, and displayed distinct signs of decay such as an extremely soft interior that did not hold shape under gentle pressure).

Descriptions of all germination classifications are summarized in Table 7.1.

Table 7.1. Description of Germination Test Classifications

Germination Classification		Description
Germinated Seed		Visible emergence of any structures necessary to produce a healthy plant.
Non-Germinated Seed	Hard	No emergence of structures necessary to produce a healthy plant. Seed did not absorb water during the germination test.
	Imbibed	No emergence of structures necessary to produce a healthy plant. Seed did absorb water during the germination test but showed no signs of growth.
	Dead	No emergence of structures necessary to produce a healthy plant. Seed did absorb water during the germination test but showed no signs of growth. Seed displayed distinct signs of decay (*i.e.*, extremely soft interior that did not hold shape under gentle pressure).

Evaluation of Non-Germinated Seed Viability

Germinated seed were considered viable. Non-germinated seed classified as dead were considered non-viable and no further assessments of viability were conducted. Non-germinated seed classified as hard or imbibed were further evaluated for viability using a tetrazolium chloride (TZ) test (AOSA, 2005). In non-germinated hard seed, potential dormancy can be indicated by seed viability; however, seed dormancy is not commonly observed in maize.

The TZ test was conducted as follows: seeds were bisected longitudinally and placed in a separate 50 ml vial for each entry, then stained with 1% TZ solution (prepared by dissolving 10 g of 2,3,5-triphenyltetrazolium chloride into one liter of water) for approximately two hours at 25 °C. Any living cells were stained a reddish-pink color by the TZ solution, allowing identification of viable tissues. Seed with staining patterns indicative of 100% viable tissue in the essential seed structures (*i.e.,* radical, embryo axis, plumule, and coleoptiles), were considered viable. Seed with staining patterns (or non-staining) indicative of less than 100% viable tissue in the essential seed structures were considered to be non-viable.

7.1.5. Statistical Analysis

Statistical analyses of germination data were conducted to evaluate the germination rate of seed derived from 4114 maize compared to the germination rate of seed derived from the control maize. Statistical analyses were conducted separately for each of the three germination tests (warm, cold, and diurnal) using SAS[p] software, Version 9.2 (SAS Institute, Inc., Cary, NC).

For a given germination test, when the minimum value of the expected count of total non-germinated seed derived from either 4114 maize or the control maize was less than five, a Fisher's exact test was conducted to compare total germination frequencies. A significant difference was identified if the P-value < 0.05. SAS PROC FREQ was utilized to conduct Fisher's exact tests.

For a given germination test, if the expected count of total non-germinated seed derived from both 4114 maize and the control maize was five or more, then a Generalized Linear Mixed Model (GLMM) assuming binomial distribution with the "logit" link function was utilized to analyze the data. Maximum likelihood method with Laplace approximation (SAS Institute Inc., 2008; Vonesh, 1996) was utilized to estimate and compare mean germination rates between 4114 maize and the control maize. A significant difference was identified if the P-value < 0.05. SAS PROC GLIMMIX was utilized to implement this type of analysis.

Let y_{ij} represent the number of germinated seed in the j^{th} replicate (each replicate contained a total of 50 seed) of the i^{th} entry, j = 1, 2, …, 8. y_{ij} ~ Binomal (n_{ij}, π_{ij}), where n_{ij} denotes the total

[p] Registered trademark of SAS Institute, Inc.

number of seed in the j[th] replicate of the i[th] entry, and π_{ij} denotes the probability of a seed being germinated in the j[th] replicate of the i[th] entry. The "logit" link function was expressed as:

$$\eta_{ij} = \text{logit}(\pi_{ij}) = \log\left(\frac{\pi_{ij}}{1-\pi_{ij}}\right)$$

Which transformed the parameter π_{ij} on the unit scale into a parameter on the linear predictor scale, denoted as η_{ij}. Logit link is default for binomial data.

The GLMM on the linear predictor scale was:

$$\eta_{ij} = \mu_i + r_{j(i)}$$

Where μ_i denotes the mean response for the i[th] entry (fixed effect) and $r_{j(i)}$ denotes the effect of the j[th] replicate within the i[th] entry (random effect nested within fixed effect). For this model, it was assumed that random effects $r_{j(i)} \sim N(0, \sigma^2_R)$ were independently and identically distributed of each other.

Reported statistics for each germination test included descriptive statistics (total germination frequency, mean, and range of individual replicate germination rates) for 4114 maize and the control maize, and P-values for statistical comparisons in Section 4; Tables 24-26. When GLMM was utilized, estimated mean germination rate and standard error of the estimate were also provided (labeled as LS-Mean and SEM, respectively) for 4114 maize and the control maize Section 4; Tables 24-26. For each germination test, the range of germination rates for individual replicates across reference maize lines (labeled as Reference Range) is provided Section 4; Tables 24-26.

7.2. Agronomic Characteristics Evaluation

Agronomic characteristics were evaluated in 4114 maize using plants from two experiments in 2010 and analyzed as described below.

7.2.1. Field Trial and Experimental Design

4114 maize plants of the F1[*5] generation were grown in two experiments at a total of 17 sites in 2010, with 15 sites in the U.S. and two sites in Canada. For those sites in the same location (*e.g.*, 3 and 12), the field trials were conducted independently of each other.

Site	Country	Location
Experiment A: 2010 Field Trials		
1	U.S.	Sheridan, IN
2		Deerfield, MI
3		Richland, IA
4		Rochelle, IL
5		Bagley, IA
6		Seymour, IL
7		York, NE
8		Carlyle, IL
9		Wyoming, IL
10		Atlantic, IA
11		Geneva, MN
Experiment B: 2010 Field Trials		
12	U.S.	Richland, IA
13		Wyoming, IL
14		Geneva, MN
15		York, NE
16	Canada	Branchton, ON
17		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. 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. Maintenance fertilizer, herbicides, and pesticides were applied uniformly to 4114 maize and the control maize at each site, as needed. In plots where grain yield was collected, plants were allowed to open-pollinate. 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.

7.2.2. Agronomic Data Collection

Agronomic characteristics were recorded for each maize line within all blocks at each site. Descriptions of maize growth stages are provided in Table 7.2.

Table 7.2. Maize Growth Stage Descriptions

Growth Stage[a]	Description[a]
V2	The stage when the collar of the second leaf becomes visible.
V4	The stage when the collar of the fourth leaf becomes visible.
V6	The stage when the collar of the sixth leaf becomes visible.
V7	The stage when the collar of the seventh leaf becomes visible.
V9	The stage when the collar of the ninth leaf becomes visible.
R1	The stage when silks become visible.
R4	The stage when the material within the kernel produces a doughy consistency.
R6	Typical grain harvest would occur. This stage is regarded as physiological maturity.

[a] Growth stages as described in Ritchie *et al.*, 2005

The following characteristics were recorded:

Early Population

The total number of plants emerged per plot was determined between V2 and V4 growth stages.

Seedling Vigor

Seedling vigor was visually estimated per plot when plants were between V2 and V4 growth stages using a 1-9 scale, with 1 corresponding to poor vigor (short plants with small, thin leaves) and 9 corresponding to good vigor (tall plants with large, robust leaves).

Time to Silking

The number of accumulated heat units[q] was calculated per plot from the planting date to the date when approximately 50% of plants had produced silks, except for two sites, which did not record 50% silking date for 4114 maize in Blocks 2, 3, and 4; and the control maize in Block 4.

Time to Pollen Shed

The number of accumulated heat units was calculated per plot from the planting date to the date when approximately 50% of plants had shed pollen, except for one site which did not record the dates to 50% pollen shed.

Pollen Viability

Pollen viability was only assessed at six of the 17 sites, with four sites in the U.S. and two sites in Canada. Pollen viability has been correlated to pollen shape and color (Luna *et al.*, 2001). Pollen viability per plot was assessed indirectly by evaluating pollen shape and color when approximately 50% of the plants had shed pollen. The percentage of pollen grains with collapsed walls[r] and intense yellow color[s] were recorded at four time points (0 minutes, 30 minutes, 60 minutes, and 120 minutes).

Plant Height

Plant height was measured in centimeters from the soil surface to the tip of the tassel for ten plants per plot at the R4 growth stage, except for two field sites which measured plant height at the R4 or R5 growth stages.

Ear Height

Ear height was measured in centimeters from the soil surface to the base of the primary ear for ten plants per plot at the R4 growth stage, except for two field sites which measured ear height at the R4 or R5 growth stages.

Stalk Lodging

Stalk lodging severity was visually estimated per plot as the percentage of plants broken below the primary ear at the R6 growth stage.

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[q] Heat units = ((maximum temperature + minimum temperature)/2) – 50 °F
If the maximum temperature was greater than 86 °F then 86 °F was used. If the minimum temperature was less than 50 °F, 50 °F was used. Heat units were calculated for each growing day and summed to give a total accumulated heat unit value. If a daily heat unit was negative, 0 (zero) was assigned.

[r] Percentage of collapsed pollen compared to spherical pollen.

[s] Percentage of pollen with intense yellow color compared to pollen white in color.

Root Lodging

Root lodging severity was visually estimated per plot as the percentage of plants leaning approximately 30 degrees or more in the first 2 feet (0.6 m) above the soil surface at the R6 growth stage.

Final Population

The total number of remaining plants per plot was recorded at the R6 growth stage. Previously sampled plants were included in the final population total.

Stay Green

Overall plant health was visually estimated per plot at the R6 growth stage using a 1 to 9 scale, with 1 corresponding to no visible green tissue remaining, 5 corresponding to approximately 50% green tissue remaining, and 9 corresponding to approximately ≥90% green tissue remaining.

Disease Incidence

Foliar disease incidence was visually estimated per plot at the R6 growth stage using a 1-9 scale, with 1 corresponding to poor disease resistance (high infection) and 9 corresponding to excellent disease resistance (low infection).

Insect Damage

Insect damage was visually estimated per plot at the R6 growth stage using a 1-9 scale, with 1 corresponding to poor insect resistance (high damage) and 9 corresponding to excellent insect resistance (low damage).

Yield

Yield was only assessed at 11 of the 17 sites in the U.S. during the 2010 growing season. Maize grain was collected from each plot at typical harvest maturity (R6 growth stage). Yield was determined based on the weight (kg) and moisture content (%) of the grain. The following formulas were used to determine yield:

Grain weights from each plot were adjusted to 0% moisture content.

$$Grain_{(dry\ weight)}\ (kg) = Grain_{(fresh\ weight)}\ (kg) - (Grain_{(fresh\ weight)}\ (kg) * \%\ actual\ moisture)$$

Example: $Grain_{(fresh\ weight)} = 9.66$ kg, Actual Moisture = 21.5%

$$Grain_{(dry\ weight)} = 9.66\ kg - (9.66\ kg * 21.5\%) = 7.58\ kg$$

The grain dry weights were then adjusted to 15.5% moisture content.

$$Grain_{(@\ 15.5\%\ moisture)}\ (kg) = Grain_{(dry\ weight)}\ (kg) / (1 - 15.5\%\ moisture)$$

Example: $Grain_{(dry\ weight)} = 7.58$ kg

$Grain_{(@\ 15.5\%\ moisture)} = 7.58\ kg / (1 - 15.5\%) = 8.98$ kg

The grain weights at 15.5% moisture were then converted to bu/A.

$$Yield\ (bu/A) = \frac{Grain_{(@\ 15.5\%\ moisture)}\ (kg) * 43560\ ft^2/A}{Plot\ Area\ (ft^2) * 25.4\ kg/bu}$$

Example: $Grain_{(@\ 15.5\%\ moisture)} = 8.98$ kg, Plot Area = 125 ft^2

$$Yield\ (bu/A) = \frac{8.98\ kg * 43560\ ft^2/A}{125\ ft^2 * 25.4\ kg/bu} = 123\ bu/A$$

7.2.3. Statistical Methods

Statistical analyses were conducted separately for two studies to evaluate and compare agronomic characteristics of 4114 maize and the control maize using SAS software, Version 9.2 (SAS Institute, Inc. Cary, NC).

7.2.4. Processing of Data

Plant and Ear Height

Plant height and ear height were measured on ten plants per plot. Plot average (average of ten plants) was treated as the response.

Transformation

Pollen viability (color and shape) at 0, 30, and 60 minutes, were percentage data. They were converted to proportion data. Early population and final population were binomial count data. They were also converted to proportion data according to:

y= number of stand count / max (target seedling rate, which is 60. Maximum stand count across plots which could be more or less than 60)

Proportion data was subjected to the arcsine square-root transformation,

$$z = \arcsin(\sqrt{y})$$

where *y* denotes proportion value, and z denotes transformed value.

The transformed data met statistical assumptions of mixed model analyses, namely normality and constant variance assumptions. The statistical results were then transformed back to the original scale.

7.2.5. Statistical Analyses

In one of the two experiments, greater than 80% of data values for root lodging and stalk lodging were at a uniform value (*i.e.,* 0% lodging); therefore, the analysis was not conducted for these two characteristics.

Mixed Model Analysis

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

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

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

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. The estimated means are known as least squares means (LS-means). The statistical comparison was conducted by testing for difference in LS-means for contrasts of interest. The approximated degrees of freedom for statistical test were derived by Kenward-Roger (KR) method (Kenward and Roger, 1997).

SAS PROC MIXED was utilized to fit Model 1 and generate LS-means and 95% confidence intervals. The same procedure also provided 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 drastically larger than what was expected under the original experimental design.

In order to make the degrees of freedom remain stable across all agronomic characteristics, 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). However, expanding the parameter space sometimes can cause the model to fail to converge. In one of the two studies, for time to silking, the model with CS structure failed to converge and parameters were estimated using the default setting.

Generalized Cochran-Mantel-Haenszel (CMH) Test

In one agronomic study the characteristics insect damage, stalk lodging, and root lodging had very discrete values. In the other agronomic study, the characteristics disease incidence, insect damage, seedling vigor, and pollen viability (color and shape) at 120 minutes, had very discrete values. This violated the normality assumption in Model 1; hence, it was not feasible to apply the mixed model approach on these characteristics. Instead, comparisons of entries were based on the generalized CMH test.

The generalized CMH test was developed specifically for stratified nominal-by-ordinal contingency tables (Agresti, 2002; Koch *et al.*, 1990). It compares entries (a nominal variable) based on their values (recorded on an ordinal scale) while controlling for location (the stratifying variable). The test's P-values can be directly interpreted as testing for the difference between the arithmetic means for each entry, because the data values were used as the scores in the generalized CMH test.

Note that when applying the generalized CMH test, the block-nested-within-location effect and the location-by-entry effect in the design were ignored. This does not present any issues because for these agronomic characteristics, the block-nested-within-location effect and the location-by-entry effect are often negligible compared with the location effect.

SAS PROC FREQ was used to perform the generalized CMH test.

7.2.6. FDR (False Discovery Rate) Adjustment

The FDR method of Benjamini and Hochberg (Benjamini and Hochberg, 1995; Westfall *et al.*, 1999) was applied as a post-hoc procedure to account for multiple comparisons due to multiple agronomic characteristics and P-values were adjusted accordingly. The FDR adjustment was conducted for results. A significant difference was identified if an adjusted P-value <0.05.

SAS PROC MULTTEST was utilized to provide adjusted P-values.

7.2.7. Interpretation of Statistical Results

Where a statistically significant difference (adjusted P-value <0.05) was identified for a given agronomic characteristic, the respective range of individual values was compared to a tolerance

interval. Tolerance intervals containing 99% of the values for corresponding characteristics of the conventional maize population with 95% confidence level (Graybill, 1976) were derived from data collected under multiple historical studies. In those studies, a total of eight non-modified commercial maize lines were grown in a total of twelve sites in North America and a total of six non-modified commercial maize lines were grown in a total of four sites in Chile under normal agronomic practices. The selected maize lines represent a non-modified maize population with a history of safe use, and the selected environments (site and year combinations) represent maize growth under a wide range of environmental conditions (*i.e.,* soil type, temperature, precipitation, and irrigation) and maize maturity group zones similar to the sites used in these 4114 maize agronomic studies.

7.2.8. Reported Statistics

For each agronomic characteristic that was statistically analyzed using mixed model analysis, entry LS-mean (back-transformed, if needed), range, and 95% confidence interval of the mean (labeled as Mean, Range, and CI, respectively) are provided in Section 4; Tables 30 and 31. Both the non-adjusted P-values and the FDR-adjusted P-values (labeled as P-Value and Adjusted P-Value, respectively) are provided for statistical comparisons. For each agronomic characteristic, a tolerance interval, if available, is provided for the results in Section 4; Tables 30 and 31. In the tables (Section 4; Tables 30 and 31), descriptive statistics (arithmetic means and ranges) are reported for characteristics that were not statistically analyzed using mixed model analysis.

Appendix 8. Field Insect and Disease Observations

4114 maize has been field tested in the U.S. and Puerto Rico since 2006, as authorized by USDA-APHIS permits and notifications (Appendix 1). For each trial, a survey of the naturally occurring insects and diseases and any unexpected differences in the response of 4114 maize as compared to the control line (near-isoline and/or conventional maize lines) were recorded by experienced plant breeders and field staff at least every four weeks. The plant breeders and field staff were familiar with plant pathology and entomology and also recorded the severity of any insect or disease in the field. These observations provide a means to determine if 4114 maize will respond differently from conventional maize lines to insects or diseases in the environment.

A summary of the naturally-occurring insects present in the fields and any unexpected differences seen between 4114 maize and control lines is presented in Table 8.1. A summary of diseases present in the fields is presented in Table 8.2.

The following scale was used to evaluate 4114 maize and control lines (Tables 8.1 and 8.2; "Range of Severity in 4114 Maize"):

- Mild – very little disease or insect injury (<10%) visible
- Moderate – noticeable plant tissue damage (10% to 30%)
- Severe – significant plant tissue damage (>30%)

In every case, the 4114 maize did not exhibit any unexpected responses to naturally-occurring insects or diseases as compared to control line.

Table 8.1. Observations of Insects Present and Comparison Between 4114 Maize and Control

Year of Planting	Permit Number	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2006	06-019-03R	PR	Guayama	Fall armyworm (*Spodoptera frugiperda*)	mild to moderate	no
				Corn earworm (*Helicoverpa zea*)	mild	no
	06-019-04R	HI	Kauai	Corn earworm (*Helicoverpa zea*)	mild to severe	no
				Rose beetle (*Adoretus sinicus*)	mild	no
				Leafhopper (Cicadellidae)	mild to moderate	no
				Thrips (*Frankliniella* spp.)	mild	no
				Spider mites (Tetranychidae)	mild	no
	06-019-03R	PR	Salinas	Corn earworm (*Helicoverpa zea*)	mild	no
				Fall armyworm (*Spodoptera frugiperda*)	mild, moderate	no
				Aphids (Aphididae)	mild	no
2007	07-040-101rm	CA	Yolo	Painted ladies (*Vanessa* spp.)	mild	no
		HI	Kauai	Corn earworm (*Helicoverpa zea*)	mild	no
				Aphids (Aphididae)	mild	no
				Leafhopper (Cicadellidae)	mild	no
				Rose beetle (*Adoretus sinicus*)	mild	no
				Fall armyworm (*Spodoptera frugiperda*)	mild	no
		IA	Linn	Corn rootworm (*Diabrotica* spp.)	mild to moderate	no
				Japanese beetle (*Popillia japonica*)	mild	no
				European corn borer (*Ostrinia nubilalis*)	moderate	no
			Polk	Corn rootworm (*Diabrotica* spp.)	mild	no
				European corn borer (*Ostrinia nubilalis*)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
				Bean leaf beetles (*Cerotoma trifurcata*)	mild	no
		IL	Bureau	European corn borer *(Ostrinia nubilalis)*	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
			Champaign	Corn rootworm (*Diabrotica* spp.)	mild	no
				Grasshoppers (Orthoptera)	mild	no
			McDonough	Corn rootworm (*Diabrotica* spp.)	mild to moderate	no

Table 8.1. Observations of Insects Present and Comparison Between 4114 Maize and Control (continued)

Year of Planting	Permit Number	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2007	07-040-101rm	IL	McDonough	European corn borer (*Ostrinia nubilalis*)	mild to moderate	no
			Ogle	Corn rootworm (*Diabrotica* spp.)	mild	no
				Japanese beetle (*Popillia japonica*)	mild	no
		IN	Tipton	Corn rootworm (*Diabrotica* spp.)	mild	no
		MN	Blue Earth	Aphids (Aphididae)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
		PR	Juana Diaz	Corn earworm (*Helicoverpa zea*)	mild	no
				Fall armyworm (*Spodoptera frugiperda*)	mild to moderate	no
			Salinas	Corn earworm (*Helicoverpa zea*)	mild	no
				Fall armyworm (*Spodoptera frugiperda*)	mild to moderate	no
		WI	Rock	Corn rootworm (*Diabrotica* spp.)	moderate	no
				Aphids (Aphididae)	mild	no
		NE	York	European corn borer (*Ostrinia nubilalis*)	mild	no
				Lady beetles (Coccinellidae)	mild	no
				Aphids (Aphididae)	mild to moderate	no
				Corn rootworm (*Diabrotica* spp.)	mild to moderate	no
				Spider mites (Tetranychidae)	mild	no
				Lacewings (Neuroptera)	mild	no
2008	07-040-101rm	PR	Salinas	Corn earworm (*Helicoverpa zea*)	mild	no
				Fall armyworm (*Spodoptera frugiperda*)	mild to moderate	no
				Corn planthoppers (Delphacidae)	mild	no
		HI	Kauai	Leafhopper (Cicadellidae)	mild	no
				Spider mites (Tetranychidae)	mild to moderate	no
				Corn earworm (*Helicoverpa zea*)	moderate	no
	08-014-111n	CA	Yolo	Painted ladies (*Vanessa* spp.)	mild	no
		IN	Gibson	Japanese beetle (*Popillia japonica*)	mild	no
			Tipton	Corn rootworm (*Diabrotica* spp.)	mild	no
				European corn borer (*Ostrinia nubilalis*)	mild	no

Table 8.1. Observations of Insects Present and Comparison Between 4114 Maize and Control (continued)

Year of Planting	Permit Number	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2008	08-014-111n	IA	Polk	European corn borer *(Ostrinia nubilalis)*	mild	no
				Japanese beetle (*Popillia japonica*)	mild	no
			Linn	Japanese beetle (*Popillia japonica*)	mild to moderate	no
				European corn borer (*Ostrinia nubilalis*)	mild	no
		NE	Nance	European corn borer (*Ostrinia nubilalis*)	mild	no
				Lady beetles (Coccinellidae)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
				Aphids (Aphididae)	mild	no
				Grasshoppers (Orthoptera)	mild	no
				Stink bug (Pentatomidae)	mild	no
			York	European corn borer (*Ostrinia nubilalis*)	mild to moderate	no
				Lady beetles (Coccinellidae)	mild to moderate	no
				Stink bug (Pentatomidae)	mild	no
				Beetles (Coleoptera)	mild to moderate	no
				Grasshoppers (Orthoptera)	mild to moderate	no
				Aphids (Aphididae)	mild to moderate	no
				Corn rootworm (*Diabrotica* spp.)	mild to moderate	no
				Lacewings (Neuroptera)	mild	no
		MN	Blue Earth	European corn borer *(Ostrinia nubilalis)*	mild to moderate	no
				Corn rootworm (*Diabrotica* spp.)	moderate	no
		PR	Juana Diaz	Fall armyworm (*Spodoptera frugiperda*)	mild to moderate	no
				Corn earworm (*Helicoverpa zea*)	mild to moderate	no
				Aphids (Aphididae)	severe	no
			Salinas	Fall armyworm (*Spodoptera frugiperda*)	mild to severe	no
				Corn earworm (*Helicoverpa zea*)	mild to severe	no
				Cornsilk fly (*Euxesta stigmatias*)	mild	no
				Aphids (Aphididae)	mild	no
				Grasshoppers (Orthoptera)	mild to moderate	no

Table 8.1. Observations of Insects Present and Comparison Between 4114 Maize and Control (continued)

Year of Planting	Permit Number	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2008	08-014-111n	IL	Bureau	Corn rootworm (*Diabrotica* spp.)	mild to moderate	no
			Champaign	Corn rootworm (*Diabrotica* spp.)	mild	no
			McDonough	Japanese beetle (*Popillia japonica*)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
			Ogle	Japanese beetle (*Popillia japonica*)	severe	no
				Corn rootworm (*Diabrotica* spp.)	severe	no
		WI	Rock	Japanese beetle (*Popillia japonica*)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
				Aphids (Aphididae)	mild	no
				Grasshoppers (Orthoptera)	mild	no
	08-014-131n	HI	Kauai	Leafhopper (Cicadellidae)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild to moderate	no
				Spider mites (Tetranychidae)	mild to moderate	no
				Rose beetle (*Adoretus sinicus*)	mild	no
				Thrips (*Frankliniella* spp.)	mild	no
				Leafhopper (Cicadellidae)	mild	no
	08-095-105n	OK	Caddo	Fall armyworm (*Spodoptera frugiperda*)	mild to moderate	no
				Green June beetles (*Cotinus nitida*)	mild	no
				Corn earworm (*Helicoverpa zea*)	moderate	no
		IL	Clinton	Corn earworm (*Helicoverpa zea*)	mild	no
				Fall armyworm (*Spodoptera frugiperda*)	mild	no
				Japanese beetle (*Popillia japonica*)	mild	no
			Stark	Corn rootworm (*Diabrotica* spp.)	mild	no
				Aphids (Aphididae)	mild	no
				European corn borer (*Ostrinia nubilalis*)	mild	no
		IA	Jefferson	European corn borer (*Ostrinia nubilalis*)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no

Table 8.1. Observations of Insects Present and Comparison Between 4114 Maize and Control (continued)

Year of Planting	Permit Number	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2008	08-095-105n	MN	Freeborn	Aphids (Aphididae)	mild	no
				European corn borer (*Ostrinia nubilalis*)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
		NE	York	Corn rootworm (*Diabrotica* spp.)	mild	no
				Aphids (Aphididae)	mild	no
				Grasshoppers (Orthoptera)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild to moderate	no
		TX	Wharton	Corn earworm (*Helicoverpa zea*)	mild	no
				Corn stalk borers (Pyralidae)	mild	no
				Fall armyworm (*Spodoptera frugiperda*)	mild	no
2009	08-014-111n	PR	Salinas	Fleahoppers (Miridae)	moderate to severe	no
				Corn earworm (*Helicoverpa zea*)	mild to severe	no
				Corn sap beetle (*Carpophilus* spp.)	severe	no
				Fall armyworm (*Spodoptera frugiperda*)	moderate	no
	09-013-108n	IL	Bureau	Corn rootworm (*Diabrotica* spp.)	mild	no
				Japanese beetle (*Popillia japonica*)	mild	no
			Fulton	Corn rootworm (*Diabrotica* spp.)	mild	no
				Aphids (Aphididae)	mild	no
				Grasshoppers (Orthoptera)	mild	no
			McDonough	European corn borer (*Ostrinia nubilalis*)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
		IN	Gibson	Japanese beetle (*Popillia japonica*)	mild	no
			Tipton	Japanese beetle (*Popillia japonica*)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
		IA	Dallas	Bean leaf beetles (*Cerotoma trifurcata*)	mild	no
				European corn borer (*Ostrinia nubilalis*)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no

Table 8.1. Observations of Insects Present and Comparison Between 4114 Maize and Control (continued)

Year of Planting	Permit Number	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2009	09-013-108n	IA	Dallas	Aphids (Aphididae)	mild	no
				Lady beetles (Coccinellidae)	mild	no
				Stink bug (Pentatomidae)	mild	no
			Kossuth	Corn rootworm (*Diabrotica* spp.)	mild	no
				Aphids (Aphididae)	mild	no
			Linn	Corn earworm (*Helicoverpa zea*)	mild	no
				Japanese beetle (*Popillia japonica*)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
			Polk	Beetles (Coleoptera)	mild	no
				Aphids (Aphididae)	mild	no
		NE	York	European corn borer (*Ostrinia nubilalis*)	mild	no
				Corn blotch leafminers (*Agromyza parvicornis*)	mild	no
				Western bean cutworm (*Richia albicosta*)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
				Aphids (Aphididae)	mild	no
	09-035-109n	AR	Crittenden	Corn earworm (*Helicoverpa zea*)	mild to severe	no
				European corn borer (*Ostrinia nubilalis*)	severe	no
				Fall armyworm (*Spodoptera frugiperda*)	severe	no
		CO	Weld	Spider mites (Tetranychidae)	mild	no
		GA	Grady	Fall armyworm (*Spodoptera frugiperda*)	mild to moderate	no
				Aphids (Aphididae)	moderate	no
				Spider mites (Tetranychidae)	moderate	no
		HI	Kauai	Leafhopper (Cicadellidae)	mild	no
				Spider mites (Tetranychidae)	mild	no
				Rose beetle (*Adoretus sinicus*)	mild	no
				Aphids (Aphididae)	mild	no
				Cornsilk fly (*Euxesta stigmatias*)	mild	no

Table 8.1. Observations of Insects Present and Comparison Between 4114 Maize and Control (continued)

Year of Planting	Permit Number	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2009	09-035-109n	HI	Kauai	Corn sap beetle (*Carpophilus* spp.)	mild	no
				Beet armyworm (*Spodoptera exigua*)	mild	no
				Rose beetle (*Adoretus sinicus*)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
		MN	Blue Earth	European corn borer *(Ostrinia nubilalis)*	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
				Aphids (Aphididae)	mild	no
		PR	Guayama	Fall armyworm (*Spodoptera frugiperda*)	mild to severe	no
				Corn earworm (*Helicoverpa zea*)	mild to moderate	no
				Aphids (Aphididae)	moderate to severe	no
				Corn sap beetle (*Carpophilus* spp.)	mild	no
			Juana Diaz	Fall armyworm (*Spodoptera frugiperda*)	mild to severe	no
				Corn earworm (*Helicoverpa zea*)	mild to moderate	no
				Corn sap beetle (*Carpophilus* spp.)	mild to severe	no
				Cornsilk fly (*Euxesta stigmatias*)	mild to severe	no
				Aphids (Aphididae)	mild to severe	no
				Spider mites (Tetranychidae)	moderate	no
				Corn planthoppers (Delphacidae)	mild	no
				Leafhopper (Cicadellidae)	mild	no
				Fleahoppers (Miridae)	moderate	no
				Whiteflies (Aleyrodidae)	moderate	no
			Salinas	Corn earworm (*Helicoverpa zea*)	mild to severe	no
				Fall armyworm (*Spodoptera frugiperda*)	mild to severe	no
				Fleahoppers (Miridae)	mild to severe	no
				Cornsilk fly (*Euxesta stigmatias*)	moderate	no
		TN	Obion	European corn borer *(Ostrinia nubilalis)*	mild	no
				Southwestern corn borer (*Diatraea grandiosella)*	mild	no
				Grasshoppers (Orthoptera)	mild	no

Table 8.1. Observations of Insects Present and Comparison Between 4114 Maize and Control (continued)

Year of Planting	Permit Number	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2009	09-035-109n	TN	Obion	Fall armyworm (*Spodoptera frugiperda*)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
		WI	Rock	Aphids (Aphididae)	mild	no
				Japanese beetle (*Popillia japonica*)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild to moderate	no
	09-264-102n	HI	Kauai	Corn earworm (*Helicoverpa zea*)	mild	no
				Beetles (Coleoptera)	mild	no
				Corn flea beetle (*Chaetocnema pulicaria*)	mild	no
				Whiteflies (Aleyrodidae)	mild	no
				Leafhopper (Cicadellidae)	mild	no
				Lacewings (Neuroptera)	mild	no
				Wasps (Hymenoptera)	mild	no
				Spider mites (Tetranychidae)	mild	no
				Aphids (Aphididae)	mild	no
				Lady beetles (Coccinellidae)	mild	no
		PR	Juana Diaz	Fall armyworm (*Spodoptera frugiperda*)	mild to moderate	no
				Aphids (Aphididae)	mild to moderate	no
				Spider mites (Tetranychidae)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
				Corn sap beetle (*Carpophilus* spp.)	mild	no
				Cornsilk fly (*Euxesta stigmatias*)	mild	no
			Salinas	Fall armyworm (*Spodoptera frugiperda*)	mild to severe	no
				Grasshoppers (Orthoptera)	mild	no
				Corn planthoppers (Delphacidae)	mild to moderate	no
				Corn earworm (*Helicoverpa zea*)	mild to moderate	no
				Corn sap beetle (*Carpophilus* spp.)	mild to severe	no
				Cornsilk fly (*Euxesta stigmatias*)	mild to moderate	no
				Spider mites (Tetranychidae)	mild to severe	no

Table 8.1. Observations of Insects Present and Comparison Between 4114 Maize and Control (continued)

Year of Planting	Permit Number	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2009	09-264-102n	PR	Salinas	Aphids (Aphididae)	mild to severe	no
	09-016-103n	IL	Stark	Aphids (Aphididae)	mild	no
				European corn borer (*Ostrinia nubilalis*)	mild	no
		NE	York	Black cutworms (*Agrotis ipsilon*)	mild	no
				European corn borer (*Ostrinia nubilalis*)	mild	no
2010	09-264-102n	HI	Kauai	Thrips (*Frankliniella* spp.)	mild	no
				Leafhopper (Cicadellidae)	mild	no
				Fall armyworm (*Spodoptera frugiperda*)	mild	no
				Lacewings (Neuroptera)	mild	no
				Lady beetles (Coccinellidae)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
				Spider mites (Tetranychidae)	mild	no
				Aphids (Aphididae)	mild	no
				Wasps (Hymenoptera)	mild	no
		PR	Salinas	Fall armyworm (*Spodoptera frugiperda*)	mild to moderate	no
				Spider mites (Tetranychidae)	mild to moderate	no
				Leafhopper (Cicadellidae)	mild to moderate	no
				Corn sap beetle (*Carpophilus* spp.)	mild to moderate	no
				Cornsilk fly (*Euxesta stigmatias*)	moderate	no
				Corn earworm (*Helicoverpa zea*)	mild to moderate	no
				Aphids (Aphididae)	mild to moderate	no
				Corn planthoppers (Delphacidae)	moderate	no
	10-050-101n	NE	Keith	Corn rootworm (*Diabrotica* spp.)	mild	no
				Grasshoppers (Orthoptera)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
	10-052-101n	AR	Crittenden	Fall armyworm (*Spodoptera frugiperda*)	moderate to severe	no
				Corn earworm (*Helicoverpa zea*)	moderate to severe	no
				Southwestern corn borer (*Diatraea grandiosella*)	moderate to severe	no

Table 8.1. Observations of Insects Present and Comparison Between 4114 Maize and Control (continued)

Year of Planting	Permit Number	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2010	10-052-101n	AR	Crittenden	European corn borer (*Ostrinia nubilalis*)	moderate to severe	no
		CA	Yolo	Spider mites (Tetranychidae)	mild to moderate	no
		CO	Philips	Grasshoppers (Orthoptera)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
			Weld	Spider mites (Tetranychidae)	mild	no
		GA	Grady	Aphids (Aphididae)	mild	no
				Fall armyworm (*Spodoptera frugiperda*)	mild	no
				Spider mites (Tetranychidae)	mild	no
				Aphids (Aphididae)	mild	no
		HI	Kauai	Aphids (Aphididae)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
				Spider mites (Tetranychidae)	mild	no
				Fall armyworm (*Spodoptera frugiperda*)	mild	no
				Beet armyworm (*Spodoptera exigua*)	mild	no
				Lacewings (Neuroptera)	mild	no
				Thrips (*Frankliniella* spp.)	mild	no
				Leafhopper (Cicadellidae)	mild	no
				Lady beetles (Coccinellidae)	mild	no
		IL	Bureau	Japanese beetle (*Popillia japonica*)	mild	no
			Champaign	Corn rootworm (*Diabrotica* spp.)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
			Fulton	Grasshoppers (Orthoptera)	mild	no
				Lady beetles (Coccinellidae)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
				Stink bugs (Pentatomidae)	mild	no
				Aphids (Aphididae)	mild	no

Table 8.1. Observations of Insects Present and Comparison Between 4114 Maize and Control (continued)

Year of Planting	Permit Number	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2010	10-052-101n	IL	La Salle	Japanese beetle (*Popillia japonica*)	mild	no
			McDonough	Lady beetles (Coccinellidae)	mild	no
				Grasshoppers (Orthoptera)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
			Ogle	Corn rootworm (*Diabrotica* spp.)	mild to severe	no
				Japanese beetle (*Popillia japonica*)	severe	no
		IN	Gibson	Japanese beetle (*Popillia japonica*)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
			Tipton	Corn rootworm (*Diabrotica* spp.)	mild	no
		IA	Bremer	Corn earworm (*Helicoverpa zea*)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
			Dallas	Bean leaf beetles (*Cerotoma trifurcata*)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
				Lady beetles (Coccinellidae)	mild	no
				Fall armyworm (*Spodoptera frugiperda*)	mild	no
			Delaware	Corn earworm (*Helicoverpa zea*)	mild	no
				Japanese beetle (*Popillia japonica*)	mild	no
			Kossuth	Lady beetles (Coccinellidae)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
				Lady beetles (Coccinellidae)	mild	no
			Linn	Corn earworm (*Helicoverpa zea*)	mild	no
				Japanese beetle (*Popillia japonica*)	mild	no
			Madison	Bean leaf beetles (*Cerotoma trifurcata*)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no

Table 8.1. Observations of Insects Present and Comparison Between 4114 Maize and Control (continued)

Year of Planting	Permit Number	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2010	10-052-101n	IA	Madison	Lady beetles (Coccinellidae)	mild	no
				Fall armyworm (*Spodoptera frugiperda*)	mild	no
			Polk	Corn earworm (*Helicoverpa zea*)	mild	no
				Japanese beetle (*Popillia japonica*)	mild	no
				Beetles (Coleoptera)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
			Van Buren	Corn rootworm (*Diabrotica* spp.)	mild	no
		KS	Finney	Flea beetle (Galerucinae)	mild	no
				Cucumber beetle (Chrysomelidae)	mild	no
		MI	Gratiot	Western bean cutworm (*Richia albicosta*)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	No
				Lady beetles (Coccinellidae)	mild	No
		MN	Blue Earth	European corn borer *(Ostrinia nubilalis)*	mild	No
				Corn rootworm (*Diabrotica* spp.)	mild	No
		MO	Scott	European corn borer *(Ostrinia nubilalis)*	mild	No
				Fall armyworm (*Spodoptera frugiperda*)	mild	No
				Grasshoppers (Orthoptera)	mild	No
		NE	York	Corn rootworm (*Diabrotica* spp.)	mild	No
				European corn borer *(Ostrinia nubilalis)*	mild	No
				Western bean cutworm (*Loxagrotis albicosta*)	mild	No
		PA	Lebanon	European corn borer *(Ostrinia nubilalis)*	mild	No
				Fall armyworm (*Spodoptera frugiperda*)	mild	No
		PR	Guayama	Corn sap beetle (*Carpophilus* spp.)	mild to severe	No
				Cornsilk fly (*Euxesta stigmatias*)	mild to severe	No
				Fall armyworm (*Spodoptera frugiperda*)	mild to severe	No
				Grasshoppers (Orthoptera)	mild	No
				Corn planthoppers (Delphacidae)	mild to moderate	No
				Corn earworm (*Helicoverpa zea*)	mild to severe	No

Table 8.1. Observations of Insects Present and Comparison Between 4114 Maize and Control (continued)

Year of Planting	Permit Number	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2010	10-052-101n	PR	Guayama	Aphids (Aphididae)	mild to severe	no
				Fall armyworm (*Spodoptera frugiperda*)	mild to severe	no
				Corn sap beetle (*Carpophilus* spp.)	mild to severe	no
				Spider mites (Tetranychidae)	mild to severe	no
				Corn flea beetle (*Chaetocnema pulicaria*)	mild	no
			Juana Diaz	Cornsilk fly (*Euxesta stigmatias*)	mild to severe	no
				Aphids (Aphididae)	mild to severe	no
				Spider mites (*Tetranychidae*)	mild to moderate	no
				Corn planthoppers (Delphacidae)	mild to moderate	no
				Spider mites (*Tetranychidae*)	moderate to severe	no
				Fall armyworm (*Spodoptera frugiperda*)	mild to moderate	no
				Grasshoppers (Orthoptera)	mild to moderate	no
				Corn earworm (*Helicoverpa zea*)	mild to moderate	no
				Corn sap beetle (*Carpophilus* spp.)	mild to severe	no
				Thrips (*Frankliniella* spp.)	mild to moderate	no
			Salinas	Fall armyworm (*Spodoptera frugiperda*)	moderate	no
				Aphids (Aphididae)	mild to moderate	no
				Corn planthoppers (Delphacidae)	moderate	no
				Cornsilk fly (*Euxesta stigmatias*)	mild to moderate	no
				Corn sap beetle (*Carpophilus* spp.)	mild to moderate	no
			Santa Isabel	Fall armyworm (*Spodoptera frugiperda*)	mild	no
				Corn planthoppers (Delphacidae)	mild	no
				Corn sap beetle (*Carpophilus* spp.)	moderate	no
				Spider mites (Tetranychidae)	moderate	no
		TN	Obion	Fall armyworm (*Spodoptera frugiperda*)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
				Southwestern corn borer (*Diatraea grandiosella)*	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no

Table 8.1. Observations of Insects Present and Comparison Between 4114 Maize and Control (continued)

Year of Planting	Permit Number	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2010	10-052-101n	TN	Obion	Grasshoppers (Orthoptera)	mild	no
		WI	Rock	Corn rootworm (*Diabrotica* spp.)	mild	no
				Grasshoppers (Orthoptera)	mild	no
				Japanese beetle (*Popillia japonica*)	mild	no
	10-118-105n	IL	Gallatin	Japanese beetle (*Popillia japonica*)	mild	no
	10-281-101n	HI	Kauai	Leafhopper (Cicadellidae)	mild	no
				Spider mites (Tetranychidae)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
		PR	Salinas	Fall armyworm (*Spodoptera frugiperda*)	mild to moderate	no
				Corn planthoppers (Delphacidae)	mild to moderate	no
				Grasshoppers (Orthoptera)	mild	no
				Cornsilk fly (*Euxesta stigmatias*)	mild to moderate	no
				Corn sap beetle (*Carpophilus* spp.)	mild to moderate	no
				Aphids (Aphididae)	mild to moderate	no
				Corn earworm (*Helicoverpa zea*)	mild to moderate	no
				Spider mites (Tetranychidae)	mild to moderate	no
	10-284-101n	HI	Kauai	Corn earworm (*Helicoverpa zea*)	mild	no
				Spider mites (Tetranychidae)	mild	no
				Leafhopper (Cicadellidae)	mild	no
				Lacewings (Neuroptera)	mild	no
		PR	Salinas	Corn sap beetle (*Carpophilus* spp.)	mild to severe	no
				Corn earworm (*Helicoverpa zea*)	mild to severe	no
				Cornsilk fly (*Euxesta stigmatias*)	mild to severe	no
				Spider mites (*Tetranychidae*)	mild to severe	no
				Corn planthoppers (Delphacidae)	mild to severe	no
				Chinch bugs (Lygaeidae)	moderate	no
				Thrips (*Frankliniella* spp.)	mild to severe	no
				Lady beetles (Coccinellidae)	mild	no

Table 8.1. Observations of Insects Present and Comparison Between 4114 Maize and Control (continued)

Year of Planting	Permit Number	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2010	10-284-101n	PR	Salinas	Leafhopper (Cicadellidae)	mild	no
				Lacewings (Neuroptera)	mild	no
				Fall armyworm (*Spodoptera frugiperda*)	mild to severe	no
				Aphids (Aphididae)	mild to severe	no
				Grasshoppers (Orthoptera)	mild to moderate	no
			Guayama	Corn planthoppers (Delphacidae)	mild to moderate	no
				Spider mites (Tetranychidae)	moderate	no
				Corn earworm (*Helicoverpa zea*)	moderate	no
				Fall armyworm (*Spodoptera frugiperda*)	moderate to severe	no
				Corn sap beetle (*Carpophilus* spp.)	moderate	no
				Thrips (*Frankliniella* spp.)	moderate	no
				Cornsilk fly (*Euxesta stigmatias*)	moderate	no
				Fall armyworm (*Spodoptera frugiperda*)	mild to moderate	no
				Aphids (Aphididae)	mild to moderate	no
				Spider mites (Tetranychidae)	moderate	no
				Grasshoppers (Orthoptera)	moderate	no
	10-015-106n	IL	Champaign	Fall armyworm (*Spodoptera frugiperda*)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
			Clinton	Corn earworm (*Helicoverpa zea*)	mild	no
				Western bean cutworm (*Richia albicosta*)	mild	no
				Grasshoppers (Orthoptera)	mild	no
				Japanese beetle (*Popillia japonica*)	mild	no
			Stark	Corn rootworm (*Diabrotica* spp.)	mild	no
				Aphids (Aphididae)	mild	no
				European corn borer (*Ostrinia nubilalis*)	mild	no
		IA	Guthrie	Grasshoppers (Orthoptera)	mild	no
				European corn borer (*Ostrinia nubilalis*)	mild	no
				Western bean cutworm (*Richia albicosta*)	mild	no

Table 8.1. Observations of Insects Present and Comparison Between 4114 Maize and Control (continued)

Year of Planting	Permit Number	State	County	Insect	Overall Severity in Field	Unexpected Difference in Comparison?
2010	10-015-106n	IA	Jefferson	Corn rootworm (*Diabrotica* spp.)	mild	no
				European corn borer (*Ostrinia nubilalis*)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
			Shelby	European corn borer (*Ostrinia nubilalis*)	mild	no
				Corn earworm (*Helicoverpa zea*)	moderate	no
				Aphids (Aphididae)	mild to moderate	no
				Grasshoppers (Orthoptera)	mild	no
		MI	Lenawee	Corn earworm (*Helicoverpa zea*)	mild	no
				Aphids (Aphididae)	mild	no
		MN	Freeborn	Aphids (Aphididae)	mild	no
				European corn borer (*Ostrinia nubilalis*)	mild	no
		NE	York	Corn earworm (*Helicoverpa zea*)	mild	no
				European corn borer (*Ostrinia nubilalis*)	mild	no
				Grasshoppers (Orthoptera)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
				Beetles (Coleoptera)	mild	no
				Picnic beetles (Nitidulidae)	mild	no
				Aphids (Aphididae)	mild	no
2011	10-284-101n	PR	Guayama	Fall armyworm (*Spodoptera frugiperda*)	mild to severe	no
				Aphids (Aphididae)	mild to severe	no
				Spider mites (Tetranychidae)	mild to severe	no
				Corn planthoppers (Delphacidae)	mild to moderate	no
				Cornsilk fly (*Euxesta stigmatias*)	mild to severe	no
				Thrips (*Frankliniella* spp.)	mild to severe	no
				Corn earworm (*Helicoverpa zea*)	mild to severe	no
				Corn sap beetle (*Carpophilus* spp.)	moderate	no
	11-040-123n	PR	Guayama	Corn planthoppers (Delphacidae)	mild to moderate	no
				Thrips (*Frankliniella* spp.)	mild to moderate	no

Table 8.2. Observations of Diseases Present and Comparison Between 4114 Maize and Control

Year of Planting	Permit Number	State	County	Disease	Overall Severity in Field	Unexpected Difference in Comparison?
2006	06-019-04R	HI	Kauai	Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
2007	07-040-101rm	CA	Yolo	Common smut (*Ustilago zeae*)	mild	no
		IA	Linn	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
			Polk	Stewart's wilt (*Pantoea stewartii*)	mild to moderate	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild to moderate	no
				Common corn rust (*Puccinia sorghi*)	mild to moderate	no
				Common smut (*Ustilago zeae*)	mild to moderate	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild to moderate	no
				Southern corn rust (*Puccinia polysora*)	mild to moderate	no
				Anthracnose (*Colletotrichum graminicola*)	mild	no
		IL	Bureau	Common corn rust (*Puccinia sorghi*)	mild	no
			Champaign	Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
			McDonough	Gray leaf spot (*Cercospora zeae-maydis*)	mild to moderate	no
				Northern corn leaf blight (*Exserohilum turcicum*)	moderate	no
			Ogle	Common corn rust (*Puccinia sorghi*)	mild	no
				Common smut (*Ustilago zeae*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
		IN	Tipton	Common corn rust (*Puccinia sorghi*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild to severe	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild to moderate	no
		MO	Saline	Gray leaf spot (*Cercospora zeae-maydis*)	mild to moderate	no
				Southern corn rust (*Puccinia polysora*)	mild	no
		WI	Rock	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
		NE	York	Common corn rust (*Puccinia sorghi*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild to moderate	no

Table 8.2. Observations of Diseases Present and Comparison Between 4114 Maize and Control (continued)

Year of Planting	Permit Number	State	County	Stressor	Overall Severity in Field	Unexpected Difference in Comparison?
2008	08-014-111n	IN	Gibson	Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
			Tipton	Common corn rust (*Puccinia sorghi*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
		IA	Polk	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Southern corn rust (*Puccinia polysora*)	mild	no
				Crazy top (*Sclerophthora macrospora*)	mild	no
			Linn	Common corn rust (*Puccinia sorghi*)	mild	no
				Northern corn leaf blight *(Exserohilum turcicum*)	mild	no
		NE	Nance	Common corn rust (*Puccinia sorghi*)	mild to moderate	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild to moderate	no
				Common smut (*Ustilago zeae*)	mild to moderate	no
				Crazy top (*Sclerophthora macrospora*)	moderate	no
			York	Common corn rust (*Puccinia sorghi*)	mild to moderate	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild to moderate	no
		MO	Saline	Gray leaf spot (*Cercospora zeae-maydis*)	mild to moderate	no
		IL	Bureau	Common corn rust (*Puccinia sorghi*)	mild	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Anthracnose (*Colletotrichum graminicola*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
			Champaign	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
			McDonough	Common corn rust (*Puccinia sorghi*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no

Table 8.2. Observations of Diseases Present and Comparison Between 4114 Maize and Control (continued)

Year of Planting	Permit Number	State	County	Disease	Overall Severity in Field	Unexpected Difference in Comparison?
2008	08-014-111n	WI	Rock	Common corn rust (*Puccinia sorghi*)	mild to moderate	no
				Gray leaf spot (*Cercospora zeae-maydis*)	moderate	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Gibberella stalk rot (*Gibberella zeae*)	mild	no
	08-095-105n	OK	Caddo	Common smut (*Ustilago zeae*)	mild	no
		IL	Clinton	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
			Stark	Anthracnose (*Colletotrichum graminicola*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
		IA	Jefferson	Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
		NE	York	Common corn rust (*Puccinia sorghi*)	mild to moderate	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Corn eyespot (*Aureobasidium zeae*)	mild	no
2009	09-013-108n	IL	Bureau	Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
			Douglas	Gray leaf spot (*Cercospora zeae-maydis*)	mild to severe	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild to severe	no
				Common corn rust (*Puccinia sorghi*)	mild	no
				Southern corn rust (*Puccinia polysora*)	mild	no
				Anthracnose (*Colletotrichum graminicola*)	mild	no
			Fulton	Gray leaf spot (*Cercospora zeae-maydis*)	mild to moderate	no
				Common corn rust (*Puccinia sorghi*)	mild	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild to moderate	no
			McDonough	Gray leaf spot (*Cercospora zeae-maydis*)	mild to moderate	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild to moderate	no
		IN	Gibson	Gray leaf spot (*Cercospora zeae-maydis*)	mild to moderate	no

Table 8.2. Observations of Diseases Present and Comparison Between 4114 Maize and Control (continued)

Year of Planting	Permit Number	State	County	Disease	Overall Severity in Field	Unexpected Difference in Comparison?
2009	09-013-108n	IN	Gibson	Northern corn leaf blight (*Exserohilum turcicum*)	mild to moderate	no
				Southern corn rust (*Puccinia polysora*)	mild to moderate	no
			Tipton	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
		IA	Dallas	Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
				Southern corn rust (*Puccinia polysora*)	mild	no
			Kossuth	Gray leaf spot (*Cercospora zeae-maydis*)	mild to moderate	no
				Corn eyespot (*Aureobasidium zeae*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild to moderate	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild to moderate	no
				Common smut (*Ustilago zeae*)	mild to moderate	no
			Linn	Common corn rust (*Puccinia sorghi*)	mild	no
				Common smut (*Ustilago zeae*)	mild	no
			Polk	Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Common smut (*Ustilago zeae*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
				Corn eyespot (*Aureobasidium zeae*)	mild	no
		NE	York	Gray leaf spot (*Cercospora zeae-maydis*)	mild to moderate	no
				Common corn rust (*Puccinia sorghi*)	mild to moderate	no
				Common smut (*Ustilago zeae*)	mild to moderate	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild to moderate	no
		MO	Saline	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
	09-035-109n	AR	Crittenden	Common corn rust (*Puccinia sorghi*)	mild to moderate	no

Table 8.2. Observations of Diseases Present and Comparison Between 4114 Maize and Control (continued)

Year of Planting	Permit Number	State	County	Disease	Overall Severity in Field	Unexpected Difference in Comparison?
2009	09-035-109n	AR	Crittenden	Southern corn rust (*Puccinia polysora*)	mild to moderate	no
				Northern corn leaf blight (*Exserohilum turcicum*)	moderate	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild to severe	no
		GA	Grady	Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Southern corn leaf blight (*Bipolaris maydis*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
		HI	Kauai	Maize chlorotic mottle machlomovirus	moderate	no
				Aspergillus ear and kernel rot (Aspergillus spp.)	mild	no
				Fusarium (Fusarium spp.)	mild	no
		MN	Blue Earth	Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
		TN	Obion	Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
				Southern corn rust (*Puccinia polysora*)	mild	no
				Brown Spot (*Physoderma maydis*)	mild	no
		WI	Rock	Anthracnose (*Colletotrichum graminicola*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
				Common smut (*Ustilago zeae*)	mild	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Anthracnose (*Colletotrichum graminicola*)	mild	no
	09-264-102n	HI	Kauai	Common corn rust (*Puccinia sorghi*)	mild	no
				Common smut (*Ustilago zeae*)	mild	no
				Maize chlorotic mottle machlomovirus	mild	no
	09-016-103n	IL	Stark	Common corn rust (*Puccinia sorghi*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
2010	09-264-102n	HI	Kauai	Maize chlorotic mottle machlomovirus	mild	no
				Common smut (*Ustilago zeae*)	mild	no
				Maize mosaic rhabdovirus	mild	no

Table 8.2. Observations of Diseases Present and Comparison Between 4114 Maize and Control (continued)

Year of Planting	Permit Number	State	County	Disease	Overall Severity in Field	Unexpected Difference in Comparison?
2010	09-264-102n	HI	Kauai	Maize dwarf mosaic potyvirus	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
	10-050-101n	NE	Keith	Common corn rust (*Puccinia sorghi*)	mild	no
		AR	Crittenden	Diplodia ear rot (*Stenocarpella maydis*)	mild to moderate	no
				Fusarium ear mold (Fusarium spp.)	mild to moderate	no
				Gray leaf spot (*Cercospora zeae-maydis*)	moderate	no
				Southern corn rust (*Puccinia polysora*)	moderate	no
				Southern corn leaf blight (*Bipolaris maydis*)	moderate	no
		GA	Grady	Northern corn leaf blight (*Exserohilum turcicum*)	mild to moderate	no
				Common corn rust (*Puccinia sorghi*)	mild	no
				Southern corn leaf blight (*Bipolaris maydis*)	mild to moderate	no
				Southern corn rust (*Puccinia polysora*)	mild to moderate	no
				Common smut (*Ustilago zeae*)	moderate	no
		HI	Kauai	Common corn rust (*Puccinia sorghi*)	mild	no
				Common smut (*Ustilago zeae*)	mild	no
				Northern corn leaf spot (*Bipolaris zeicola*)	mild	no
		IL	Bureau	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
			Champaign	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Southern corn rust (*Puccinia polysora*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
			Fulton	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Anthracnose (*Colletotrichum graminicola*)	mild	no
				Northern corn leaf spot (*Bipolaris zeicola*)	mild	no
			La Salle	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no

Table 8.2. Observations of Diseases Present and Comparison Between 4114 Maize and Control (continued)

Year of Planting	Permit Number	State	County	Disease	Overall Severity in Field	Unexpected Difference in Comparison?
2010	10-052-101n	IL	La Salle	Common corn rust (*Puccinia sorghi*)	mild	no
			McDonough	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Common smut (*Ustilago zeae*)	mild	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
			Ogle	Common corn rust (*Puccinia sorghi*)	mild to moderate	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild to moderate	no
		IN	Gibson	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
			Shelby	Common corn rust (*Puccinia sorghi*)	mild	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
			Tipton	Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
			White	Common corn rust (*Puccinia sorghi*)	mild	no
		IA	Bremer	Common corn rust (*Puccinia sorghi*)	mild	no
			Dallas	Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
			Delaware	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
			Kossuth	Common corn rust (*Puccinia sorghi*)	mild	no
				Common smut (*Ustilago zeae*)	mild	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
			Linn	Common smut (*Ustilago zeae*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
			Madison	Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	moderate	no
			Polk	Northern corn leaf blight (*Exserohilum turcicum*)	mild to moderate	no

Table 8.2. Observations of Diseases Present and Comparison Between 4114 Maize and Control (continued)

Year of Planting	Permit Number	State	County	Disease	Overall Severity in Field	Unexpected Difference in Comparison?
2010	10-052-101n	IA	Polk	Common corn rust (*Puccinia sorghi*)	mild to moderate	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild to moderate	no
				Common smut (*Ustilago zeae*)	moderate	no
			Van Buren	Common smut (*Ustilago zeae*)	mild	no
		KS	Finney	Common corn rust (*Puccinia sorghi*)	mild	no
		MI	Gratiot	Common smut (*Ustilago zeae*)	mild	no
				Stewart's wilt (*Pantoea stewartii*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
		MN	Blue Earth	Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Common smut (*Ustilago zeae*)	mild	no
			Kandiyohi	Common corn rust (*Puccinia sorghi*)	mild	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
		MO	Scott	Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
		NE	York	Common smut (*Ustilago zeae*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
				Goss's wilt (*Clavibacter michiganensis*)	mild	no
				Common smut (*Ustilago zeae*)	mild	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
		PA	Lebanon	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
			Juana Diaz	Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Common smut (*Ustilago zeae*)	mild	no
		TN	Obion	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
				Southern corn rust (*Puccinia polysora*)	mild	no
				Brown spot (*Physoderma maydis*)	mild	no

Table 8.2. Observations of Diseases Present and Comparison Between 4114 Maize and Control (continued)

Year of Planting	Permit Number	State	County	Disease	Overall Severity in Field	Unexpected Difference in Comparison?
2010	10-052-101n	TN	Obion	Common corn rust (*Puccinia sorghi*)	mild	no
		WI	Rock	Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Common smut (*Ustilago zeae*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Corn eyespot (*Aureobasidium zeae*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
	10-118-105n	IL	Gallatin	Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild to moderate	no
	10-281-101n	HI	Kauai	Common smut (*Ustilago zeae*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
		PR	Salinas	Maize stripe tenuivirus	mild	no
				Corn leaf blight (Pleosporaceae)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
	10-284-101n	HI	Kauai	Common smut (*Ustilago zeae*)	mild	no
				Maize stripe tenuivirus	mild	no
		PR	Salinas	Common corn rust (*Puccinia sorghi*)	mild	no
				Maize stripe tenuivirus	mild	no
				Corn leaf blight (Pleosporaceae)	mild	no
			Guayama	Maize stripe tenuivirus	mild	no
				Southern corn leaf blight (*Bipolaris maydis*)	mild	no
	10-015-106n	IN	Clinton	Anthracnose (*Colletotrichum graminicola*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
		IL	Champaign	Diplodia ear rot (*Stenocarpella maydis*)	mild	no
			Clinton	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
			Stark	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no

Table 8.2. Observations of Diseases Present and Comparison Between 4114 Maize and Control (continued)

Year of Planting	Permit Number	State	County	Disease	Overall Severity in Field	Unexpected Difference in Comparison?
2010	10-015-106n	IL	Stark	Anthracnose (*Colletotrichum graminicola*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
		IA	Guthrie	Eye spot of corn (*Aureobasidium zeae*)	mild	no
				Gibberella stalk rot (*Gibberella zeae*)	mild	no
				Common smut (*Ustilago zeae*)	mild	no
			Jefferson	Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
				Southern corn leaf blight (*Bipolaris maydis*)	mild	no
				Eye spot of corn (*Aureobasidium zeae*)	mild	no
			Shelby	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
				Common smut (*Ustilago zeae*)	mild	no
		MI	Lenawee	Common corn rust (*Puccinia sorghi*)	mild	no
		MN	Freeborn	Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Common corn rust (*Puccinia sorghi*)	mild	no
		NE	York	Common corn rust (*Puccinia sorghi*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no

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