RECEIVED
By BRS Document Control Officer at 4:20 pm, Jun 25, 2019

Petition for the Determination of Nonregulated Status of Maize Event PY203

Zea mays expressing a phytase gene derived from *Escherichia coli* strain K12

Agrivida, Inc. is submitting 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 not be regulated under 7 CFR Part 340.

Submitted by:

James M. Ligon, Ph.D.
VP, Regulatory Affairs and Stewardship
Agrivida, Inc.
1023 Christopher Drive
Chapel Hill, NC 27517

This document was prepared by James Ligon, Phillip Lessard, and Michael Raab

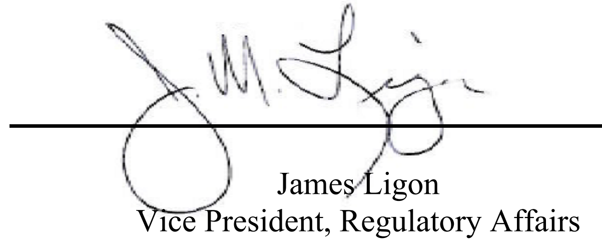
OECD Unique Identifier: AGV-PY203-4

No CBI
Agrivida, Inc. does not consider any information contained in this petition to be confidential business information or to be a trade secret.

June 20, 2019

Statement of Grounds Unfavorable

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



James Ligon
Vice President, Regulatory Affairs

Agrivida, Inc.
78E Olympia Avenue
Woburn, MA 01801

Tel: (919) 675-6666
Fax: (781) 391-4262

Table of Contents

Section	Title	Page
	Statement of Grounds Unfavorable	2
	Table of Contents	3
	List of Figures	6
	List of Tables	7
	List of Abbreviations Used in this Petition	9
I.	Petition from Agrivida, Inc. for the Determination of Non-Regulated Status of Maize Event PY203	11
A.	Summary	11
B.	Rationale for the Development of Maize Event PY203	12
C.	Status of Maize Event PY203 with Other Federal Agencies	14
D.	Status of USDA APHIS Regulatory Permits	15
II.	The Maize Family	16
A.	General Description of *Zea mays* L. (Maize)	16
B.	Origin of the Species *Zea mays* L.	16
C.	Cultivation and Use of Maize	17
D.	Pollination of Maize	18
E.	Inter-Species/Genus Hybridization	19
F.	Potential for Introgression from *Z. mays* into Relatives	19
G.	Volunteers and Weediness in Maize	19
III.	Characterization of the Genetic Material Introduced into the Genome of Maize Event PY203	21

Section	Title	Page
III.A.	Summary	21
B.	Introduction	21
C.	Origin of the Gene Encoding Phytase Phy02	21
D.	Characteristics of the Phy02 Expression Construct	23
E.	Characterization of the Maize Event PY203	26
1.	Determination of the Number of DNA Insertions	27
2.	Absence of Vector Backbone Fragments	35
3.	Sequence of the *phy02* Gene Insertions and the Flanking Maize Genome	40
4.	Genetic Stability of the Inserts Over Multiple Generations	41
5.	Mendelian Inheritance of the *phy02* Gene Insertions	44
IV.	Expression of Phy02 Protein in Event PY203	47
V.	The Phy02 Phytase Protein	51
A.	Enzyme Name	51
B.	Enzyme Identity	51
C.	Source and Amino Acid Sequence of the Phy02 Phytase	51
D.	Phytases in Other Organisms and Prior Exposure	51
E.	Summary of the Food and Feed Safety of the Phy02 Protein	53
VI.	The Phosphomannose Isomerase Protein	56
VII.	Phenotypic Evaluation of Event PY203	57
A.	Agronomic Trial Methodology	57
B.	Seed Dormancy and Germination Characteristics	69

Section	Title	Page
VIII.	Assessment of the Nutrient Composition of Grain and Forage	72
A.	Nutrient Analysis of Grain	73
B.	Nutrient Analysis of Forage	80
C.	Conclusions from Nutrient Analysis of Grain and Forage Derived from Maize Event PY203	81
IX.	Cultivation Practices	83
A.	Intended Cultivation Area	83
B.	Standard Cultivation Practices for Maize	83
C.	Cultivation Practices for Maize Event PY203	84
X.	Environmental Consequences of Event PY203	86
A.	The Safety of Maize Event PY203 to Wildlife	86
B.	Estimates of Phy02 Phytase Levels for Wildlife Exposure Assessment	87
C.	The Composition of Maize Event PY203	89
D.	The Environmental Fate of the Phy02 Phytase Protein	89
1.	Phy02 Protein in Cultivated Maize Event PY203	89
2.	Fate of Phy02 in the Soil	90
E.	Weediness Potential of Event PY203	90
XI.	References	92
	Appendix 1: Complete nucleotide sequence of Event PY203 locus 3293 including flanking maize DNA	104
	Appendix 2: Complete nucleotide sequence of Event PY203 locus 3507 including flanking maize DNA	110

List of Figures

Figure	Description	Page
1	Comparison of the amino acid sequences of the Phy02 and *E. coli* AppA phytases.	22
2	Plasmid map of pAG4758 that was used in the transformation of maize to create the phytase producing Event PY203	24
3	Breeding diagram for maize Event PY203	26
4	Diagram of the T-DNA from pAG4758 and location of annealing PCR primers in genome walking to determine the number of T-DNA inserts	28
5	Gene maps of Event PY203 loci 3293 and 3507	31
6	Southern hybridization blots of restriction enzyme-digested genomic DNA of Event PY203	32
7	Maps of Locus 3293 and and Locus 3507 from PY203, indicating the locations of PCR products that were used to detect T-DNA-derived insertions within the genome of PY203	34
8	Plasmid map of pAG4758 with location of probes used for Southern analysis and location of overlapping backbone (BB) PCR fragments	36
9	Southern blot hybridization of digested genomic DNA from Event PY203 with DNA fragments from the pAG4758 TDNA	37
10	PCR results with primers corresponding to pAG4758 backbone (BB) DNA sequences	39
11	Alignment of genomic DNA sequence from locus 3293 of Event PY203 to sequences from PCR amplicons from 4 successive BC generations	42
12	Alignment of genomic DNA sequence from locus 3507 of Event PY203 to sequences from PCR amplicons from 4 successive BC generations	42
13	Southern blot of four BC generations of Event PY203 hybridized with a T-DNA RB probe	43
14	Map of PY203 locus 3293 and sequence of a 163 bp fragment amplified from the T-DNA into the flanking genomic region	45
15	Map of PY203 3507 locus and sequence of a 113 bp fragment amplified from the T-DNA into the flanking genomic region	45

List of Tables

Table	Description	Page
1	Status of USDA APHIS notifications/permits and field trial reports for Event PY203	15
2	Description of the genetic elements in the 21,195 bp plasmid pAG4758 used to transform maize	25
3	PCR Primers used for genomic walking to determine the number of T-DNA insertions	29
4	List of primers used for PCR segregation analysis	33
5	Segregation of T-DNA-derived sequences among of progeny from PY203	35
6	List of primers used amplify pAG4758 plasmid backbone Southern probes containing the ColE1, *aadA*, and *sat* fragments	36
7	Description of primer pairs and expected size of DNA products from PCR tiling analysis of Event PY203 genomic DNA	38
8	Size of predicted and observed DNA restriction fragments from the genome of Event PY203 that hybridize to the RB probe	44
9	Segregation of the PY203 genetic loci in four backcross generations	46
10	Levels of Phy02 protein measured in tissues derived from maize Event PY203	50
11	Description of the agronomic characteristics that were assessed for Event PY203	59
12	Assessment of vegetative characteristics of Event PY2023	60
13	Assessment of reproductive characteristics of Event PY2023	64
14	Statistical comparison of agronomic characteristics for Event PY203 and the non-transgenic control line	67
15	Seed germination after incubation at 10°C and 25°C at 4, 7, and 10 days post-planting	70

Table	Description	Page
16	Proximate nutrient analysis of grain from Event PY203	73
17	Amino acid analysis of grain from Event PY203	75
18	Fatty acid analysis of grain from Event PY203	76
19	Mineral analysis of grain from Event PY203	77
20	Vitamin analysis of grain from Event PY203	79
21	Anti-nutrient analysis of grain from Event PY203	80
22	Proximate nutrient analysis of forage from Event PY203	81
23	Acres planted to corn in the U.S. by state in 2018	84
24	Levels of Phy02 phytase for wildlife exposure assessment	88

List of Abbreviations

aadA	Aminoglycoside adenylyltransferase gene
APHIS	Animal and Plant Health Inspection Service
appA/AppA	phytase gene/protein from *Escherichia coli* strain K12
bp	base pair
BC	backcross
BLASTN	Basic Local Alignment Search Tool for nucleotide sequence
°C	degree Celsius
CFIA	Canadian Food Inspection Agency
CFR	Code of Federal Regulations
Cos	Cohesive end (DNA fragment)
CVM	Center for Veterinary Medicine (of the U.S. FDA)
DIG	digoxigenin
DNA	deoxyribonucleic acid
DW	dry weight
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
FTU	phytase activity units
FW	fresh weight
GRAS	generally recognized as safe
ILSI-CCDB	International Life Science Institute-Crop Composition Database
IU	international unit
kb	kilobase pair
kDa	kilodalton
kg	kilogram
lb	pound
LB	left border
LOD	limit of detection
LOQ	limit of quantitation
M	molar
manA	gene from *E. coli* encoding the phosphomannose isomerase enzyme; also called *pmi*
min	minute
mm	millimeter
NCBI	National Center for Biotechnology Information
nos	nopaline synthase terminator
nM	nanomole
nt	nucleotide
OECD	Organization for Economic Cooperation and Development
ori	origin of replication
PCR	Polymerase chain reaction
phy02/Phy02	Modified version of the *E. coli* phytase gene/protein engineered into Event PY203

pmi/PMI	phosphomannose isomerase gene/protein; *pmi* is a synonym for *manA*
ppm	parts per million
PVDF	polyvinylidene difluoride
RB	right border
rpm	revolutions per minute
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gene electrophoresis
sec	second
SEKDEL	6 amino acid endoplasmic reticulum retention signal from maize
ss	signal sequence
ssp	subspecies
T0	initial transformed plant
T-DNA	transformed DNA
TIU	trypsin international units
™	trade mark
USDA	United States Department of Agriculture
USDA-NASS	U.S.D.A. National Agricultural Statistics Service
UTR	untranslated region
var	variety

I. Petition from Agrivida, Inc. for the Determination of Non-Regulated Status of Maize Event PY203

A. Summary

Agrivida, Inc. is submitting this petition to USDA APHIS to determine that maize Event PY203 that has been genetically engineered to produce the enzyme 6-phytase poses neither a plant pest risk nor any risks to the environment and therefore should no longer be considered to be a regulated plant under 7 CFR 340.

The phytase producing maize Event PY203 developed by Agrivida, Inc. contains transgenes that produce two proteins novel to maize, phytase and phosphomannose isomerase. Phytases are ubiquitous in nature and are produced by many microbes and plants. Phytases catalyze the step-wise removal of phosphate from the plant produced phosphate storage molecule phytin or phytic acid and they are commonly added to the diets of monogastric animals to increase the nutritional availability of phosphate. The gene encoding the phytase enzyme (referred to as phytase Phy02) in maize Event PY203 was derived from the native *appA* gene of *Escherichia coli* strain K12. The native *E. coli appA* gene was optimized to increase the thermotolerance of the encoded Phy02 phytase and its susceptibility to digestion in the gastric environment. Maize Event PY203 was generated by *Agrobacterium*-mediated transformation into immature maize embryo tissue as described by Negrotto *et al*. (2000) using a gene cassette containing three copies of the optimized *appA* phytase gene, herein referred to as the *phy02* gene, and the *manA* gene encoding the phosphomannose isomerase (PMI) enzyme. The three copies of the *phy02* gene are each expressed from three different monocot derived promoters, including the *Oryza sativa* glutelin-1 gene promoter that provides endosperm-specific gene expression, the promoter from the *Zea mays Zc2* gene that encodes the 27-kDa zein storage protein, that provides endosperm-specific gene expression, and the *Z. mays* globulin Glb1 gene promoter that provides embryo-specific gene expression. The *manA* gene encoding phosphomannose isomerase (PMI) was included in the transformation cassette as a plant selectable marker. The *manA* gene is expressed by the promoter from the *Z. mays* ubiquitin 1 gene that provides expression in all maize tissues.

Multiple lines of evidence, including Southern analyses of genomic DNA from Event PY203 indicate the presence of two independent insertions of T-DNA from the transformation plasmid pAG4758. The nucleotide sequence of both insertions, including the flanking genomic maize DNA, was determined and demonstrates that one of the insertions consists of the complete T-DNA and is located on maize chromosome 8, while the other insertion is truncated consisting of two complete phytase genes and lacking the third phytase gene and the *manA* selectable marker gene. The location of this insertion was determined to be maize chromosome 2. Both phytase gene insertions were demonstrated to be stable and inherited in a Mendelian manner over multiple generations. Southern analyses were also used to demonstrate the absence of DNA fragments derived from the vector backbone of plasmid pAG4758 in the genome of Event PY203.

Maize Event PY203 was grown at six locations across the Midwestern United States including sites in Ohio, Indiana, Iowa, and Nebraska and at two locations in Argentina. Agronomic

characteristics of Event PY203 and near isogenic non-transgenic control plants grown at these locations were assessed throughout the life cycle of the plants. The agronomic performance and phenotypic data generated demonstrate that the genetic modifications introduced into Event PY203 did not have any unintended effects on seed germination, agronomic characteristics, or yield. These data support the conclusion that Event PY203 maize is unlikely to develop into feral persistent populations or to be more weedy or invasive in the environment compared to conventional maize varieties.

The nutrient composition of grain and forage samples derived from Event PY203 and a near-isogenic non-transgenic control was determined and compared. Grain samples were analyzed for proximate nutrients, amino acids, fatty acids, minerals, vitamins, and other bioactive metabolites (phytic acid, trypsin inhibitor, p-coumaric acid, raffinose, and ferulic acid). Forage samples were analyzed for proximate nutrients only. The data from this study demonstrate that there were minor differences in the nutrient levels of samples from Event PY203 and the control plants, however all nutrient values were within the historical ranges determined for maize as reported in the ILSI Crop Composition Database (ILSI-CCDB, 2018). Based on these results it is concluded that the nutrient composition of grain and forage derived from Event PY203 is substantially equivalent to that of other conventional maize varieties.

Agrivida has conducted a food and feed safety assessment of the Phy02 phytase and concluded that this protein does not present any safety concerns related to consumption of maize from Event PY203 by humans or animals. Agrivida, Inc. has completed an Early Food Safety Evaluation of the Phy02 phytase with the U.S. Food and Drug Administration (FDA), and a submission under FDA's Policy for Foods Derived from New Plant Varieties has been submitted (BNF No. 000167). The safety and efficacy of Phy02 as a feed additive in poultry has been reviewed by the FDA Center for Veterinary Medicine who had no questions concerning Agrivida Inc.'s conclusion that the Phy02 phytase is GRAS for this purpose (CVM, 2017b). A detailed assessment of human and animal safety of the Phy02 phytase protein has been provided to the FDA as part of a food and feed safety and nutritional assessment for maize Event PY203. The safety of the PMI protein has been extensively characterized (Privalle *et al*, 2006) and this selectable marker has been widely used in maize and other crop species that have been approved for food use by regulatory authorities in the United States and other countries.

Based on the information and data contained in this petition, Agrivida, Inc. requests that USDA APHIS make a determination of nonregulated status for maize Event PY203, any progeny derived from crosses between Event PY203 and other maize varieties, and progeny derived from crosses of Event PY203 with other transgenic maize varieties that have received nonregulated status under 7 CFR Part 340.

B. Rationale for the Development of Maize Event PY203

Phytases are a class of acid phosphatase enzymes that hydrolyze phosphates from phytic acid (also referred to as phytate) to produce free phosphate and inositol. Phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate) accounts for up to 80% of the phosphorus

in the seeds of cereals and legumes and is the primary storage form of phosphate in these materials (Reddy *et al*., 1982). Phytate phosphorus is nutritionally unavailable to monogastric animals such as poultry and swine and therefore, inorganic forms of phosphorus are commonly added to animal feed to supply the nutritional needs for this important nutrient. The addition of inorganic phosphorus to animal feeds results in the generation of high-phosphorus manure that can contaminate rivers and streams resulting in algal blooms, oxygen depletion and the death of fish and aquatic animals due to eutrophication (Jongbloed and Lenis, 1998; Correll, 1999; Mallin, 2000; Poulsen, 2000). In addition, phytate also forms a complex salt with several mineral ions such as Fe^{+2}, K^{+}, Mg^{+2}, Ca^{+2} and Zn^{+2} that is called phytin. In this complexed state, the minerals in phytin are nutritionally unavailable to monogastric animals (Lin *et al*., 2005; Harland and Morris, 1995; Minihane, 2002). For these reasons, phytate is considered an anti-nutrient (Coulibaly et al., 2011).

One strategy for making phosphorus from phytate nutritionally available to monogastric animals is the addition of phytase to animal feeds (Jongbloed and Lenis, 1998; Onyango *et al*., 2005). The use of phytase in the diets of poultry and swine has been shown to improve feed and phosphorus utilization (Augspurger *et al*., 2003; Nyannor *et al*., 2007 and 2009). A number of phytase products are currently marketed for this use and include Natuphos™ (BASF) a phytase derived from *Aspergillus niger*, Ronozyme™ (DSM) a phytase derived from *Peniophora lycii*, and Quantum and Quantum Blue (AB Vista), two phytases that are also derived from the AppA phytase of *Escherichia coli*. The use of phytase in animal feeds allows a reduction in the amount of inorganic phosphorus added to animal feeds and has been reported to result in reductions in fecal phosphorus as high as 60% (Nahm, 2002; Sharpley *et al*., 1994; Wodzinski and Ullah 1996). In December, 2002, a regulation issued from the US EPA was implemented that regulates the application of manure from concentrated animal farming operations onto land based on the amount of phosphorus being applied (EPA, 2002). The use of phytase in poultry and swine feed results in a more efficient utilization of phosphorus and reduces phosphorus in animal wastes. Therefore, its use may assist concentrated animal farming operations in meeting the EPA guidelines without reducing the size of their operations or having to utilize other more expensive waste handling technologies.

All of the current phytase animal feed products are produced by genetically modified microorganisms through fermentation and purification of the phytase from the fermentation medium. The Phy02 phytase that is being developed by Agrivida, Inc. is produced in the grain of maize (*Zea mays*). Genes encoding the Phy02 phytase under the expression of embryo and endosperm specific promoters were introduced into maize to achieve the production of Phy02 phytase specifically in the grain of maize. The Phy02 phytase is derived from the native *E. coli* phytase AppA and differs from the AppA phytase by only 16 of the 411 total amino acid residues in the mature protein. Grain produced from Event PY203 will be ground into a meal and is intended to be added to the feed of poultry and swine (non-ruminants) in order to improve the nutritional availability of phosphorus in the feed. For this purpose, one to four pounds of the product are sufficient to treat one ton of feed. In addition, examination of manure from cattle (a ruminant) has demonstrated the presence of a significant amount of phytic acid (Toor *et al*., 2005), indicating that the microbial

populations in the rumen do not produce sufficient amounts of phytase to completely digest all of the phytic acid in the diet. However, a series of studies published in peer-reviewed journals support the conclusion that ruminant animals may derive the same dietary benefits from the addition of exogenous phytase to their diet as do monogastric animals. Also, the passage rate of digesta, Ca-complexes formed with phytate, and processing of dietary ingredients have been shown to reduce phytase activity in the rumen (Konishi *et a*l., 1999; Park *et al*., 1999; Kincaid *et al*., 2005). The addition of exogenous phytase to the diet of both dairy and beef cattle has been shown to result in a significant improvement of phytic acid digestion (Brask-Pedersen *et al*., 2013; Hurley *et al*., 2002; Kincaid *et al*., 2005; Knowlton *et al*., 2007). Several recent studies have demonstrated that the addition of exogenous phytase to the diets of ruminant animals results in a reduction of phosphorus in the manure (Bravo *et al*., 2003; Buendia *et al*., 2014; Kincaid *et al*., 2005; Winter *et al*., 2015). However, the practice of phytase inclusion in cattle feed has not been widely adopted in the commercial setting as the relatively large quantities of phytase that are necessary are prohibitively expensive if the phytase was sourced from microbial fermentation. The cultivation of Phy02 phytase producing maize for grain or silage for cattle would be a cost efficient means of delivering phytase to cattle for the improvement of phytic acid digestion and leading to reduced phosphorous content of the manure.

C. Status of Maize Event PY203 with Other Federal Agencies

Agrivida, Inc. intends to produce Phy02 phytase containing grain through the cultivation of Event PY203 with a limited number of select growers who will cultivate Event PY203 under contract and deliver the grain to Agrivida, Inc. for processing into a cornmeal that will be packaged and sold as a feed additive for poultry and swine. Agrivida, Inc. may also introgress the Phy02 phytase trait by traditional breeding techniques into forage varieties of maize that will be sold as seed to cattle producers to be cultivated as forage. As Event PY203 is within the scope of the U.S. Food and Drug Administration's 1992 Statement of Policy: Foods Derived from New Plant Varieties, including genetically engineered varieties pursuant to 21 CFR Section 192.25 of the Federal Food, Drug, and Cosmetic Act (FFDCA), Agrivida, Inc. will complete the voluntary biotechnology consultation process with FDA before offering Event PY203 varieties for commercial sale in the United States as a forage crop. Agrivida, Inc. has completed an Early Food Safety Evaluation of New Non-Pesticidal Proteins Produced by New Plant Varieties Intended for Food Use: Early Food Safety Assessment for the Phy02 Phytase Protein (NPC 000015) on August 7, 2015. A Pre-market Biotechnology Notification (PBN) consultation for Event PY203 was submitted to the U.S. FDA in June 2018 (BNF 000167). In addition, Agrivida, Inc. has determined that ground cornmeal derived from Event PY203 grain that contains the Phy02 phytase enzyme is safe and effective when added to the feed of poultry and therefore it is Generally Recognized as Safe (GRAS) for this use. Data and information supporting the GRAS nature of the Phy02 phytase was submitted to, and reviewed by, the FDA Center for Veterinary Medicine (CVM, 2017a). In a letter dated May 23, 2017, CVM informed Agrivida, Inc. that it had no further questions related to Agrivida's conclusion that the maize produced Phy02 phytase enzyme is GRAS for use in poultry feed (CVM, 2017b). Agrivida, Inc. has also determined that a similar use of the Phy02 phytase in swine feed is safe and effective and that the Phy02 phytase is GRAS for this use. Likewise, data and information supporting Agrivida, Inc.'s conclusion of the GRAS nature of the Phy02

phytase for use in swine feed has been submitted to CVM and CVM notified Agrivida, Inc. in a letter dated September 6, 2018 that this notice is complete and they have initiated a review of the document.

D. Status of USDA APHIS Regulatory Permits

Agrivida, Inc. has conducted field trials of Event PY203 in the United States for the purposes of research, developing data for regulatory submissions, seed production, and Phy02 phytase product production (Table 1). Phy02 producing Event PY203 has been planted in several states across the U.S. corn belt since 2015.

Table 1. Status of USDA APHIS notifications/permits and field trial reports for Event PY203.

Year	USDA Notification or Permit No.	Trial Site Locations by State	Status of field trial reports
2015	15-065-101rm-a1	IN (2 sites), IA (1 site)	Submitted
2016	16-032-101rm-a1	NE (2 sites), OH	Submitted
	16-063-102rm	IA (2 sites), IL (2 sites), IN (3 sites), NE (2 sites)	Submitted
2017	17-044-101n	NE (4 sites), OH	Submitted
	17-052-102n	IA (3 sites), IL (1 site), IN (1 site), OH (1 site), NE (2 sites)	Submitted
	17-102-102n	IN (1 site)	Submitted

II. The Maize Family

The following was excerpted, with minor edits, from USDA APHIS Environmental Assessment 92-042-01 (authored by Dr. James Lackey), the Canadian Food and Inspection Agency (CFIA) Regulatory Directive Dir94-11 (CFIA, 1994) and the Organization for Economic Cooperation and Development (OECD) Consensus Document on the Biology of *Zea mays* subsp. mays (Maize) (OECD, 2003). Full descriptions and complete references can be obtained from these documents.

A. General Description of *Zea mays* L. (Maize)

Zea is a genus of the family Graminae (Poaceae), commonly known as the grass family. Maize (*Zea mays* L.) is a tall, monecious annual grass with overlapping sheaths and broad conspicuously distichous blades. Plants have staminate spikelets in long spike-like racemes that form large spreading terminal panicles (tassels) and pistillate inflorescences in the leaf axils, in which the spikelets occur in 8 to 16 rows, approximately 30 cm long, on a thickened, almost woody axis (cob). The whole structure (ear) is enclosed in numerous large foliaceous bracts and long styles (silks) protrude from the tip of the ear as a mass of silky threads (Hitchcock and Chase, 1971). Pollen is produced entirely in the staminate inflorescence and eggs, entirely in the pistillate inflorescence. Maize is wind-pollinated and both self and cross-pollination are usually possible. Shed pollen usually remains viable for 10 to 30 minutes, but can remain viable for longer durations under favorable conditions (Coe et al., 1988). Cultivated maize is presumed to have been derived from teosinte (*Z. mexicana*) and is thought to have been introduced into the old world in the sixteenth century. Maize is cultivated worldwide and represents a staple food for a significant proportion of the world's population. No significant native toxins are reported to be associated with the genus *Zea* (International Food Biotechnology Council, 1990).

B. Origin of the Species *Zea mays* L.

It is generally agreed that teosinte (*Z. mexicana*) is an ancestor of maize, although opinions vary as to whether maize is a domesticated version of teosinte (Galinat, 1988; for reviews see OECD, 2003; CFIA, 1994). Teosinte is an ancient wild grass found in Mexico and Guatemala. Because it has differentiated into various races, species and plant habits, taxonomic classification is still a matter of controversy. Doebley and Iltis (1980) and Iltis and Doebley (1980) classified the annual teosintes into two subspecies of *Z. mays*: ssp. *mexicana* (including races Chalco, Central Plateau and Nobogame) and ssp. *parviglumis*-var. *parviglumis* (race Balsas) and var. *huehuetenangensis* (race Huehuetenango) and the species *Z. luxurians* (race Guatemala). The perennial teosintes from Jalisco, Mexico are separated into two more species according to ploidy, *Z. perennis* and *Z. diploperennis*. The Meso-American region located within middle South Mexico and Central America is recognized as one of the main centers of origin and development of agriculture as well as center of origin and diversification of more than one hundred crops (Vavilov, 1951; Smith, 1995; Harlan, 1992). At the present time, there is no agreement about where exactly maize was domesticated and there are several proposals in this regard. Based on the findings of archaeological materials from the maize plant (pollen, cobs, husks, and other remnants) in

the United States and Mexico, which are older than those found in South America, Randolph (1959) proposed that maize was domesticated, independently, in the southwestern United States, Mexico, and Central America. Mangelsdorf (1974) proposed that "corn had not one origin but several in both Mexico and South America", because the archaeological evidences are found in Mexico and several morphological characteristics of extant populations are found in the maize races of South America (Andes region) in comparison to those races of Meso-America.

C. Cultivation and Use of Maize

As discussed above, the indigenous peoples of North America have cultivated maize for thousands of years. The modern era of maize hybrid production began in the United States where research conducted in the early part of the last century proved that hybrid maize could produce a yield superior to open-pollinated varieties (Sprague and Eberhart, 1977). Gradually, hybrid-derived varieties replaced the open-pollinated types in the 1930's and 1940's. Almost all maize grown in the United States now comes from hybrid seed that is obtained every planting season from private enterprises; the older open-pollinated varieties are virtually unknown in commerce (Hallauer *et al*., 1988).

The production of hybrid seed requires the development and maintenance of inbred lines and subsequent controlled crosses to produce commercial seed. Self-pollination is essential for inbred development while controlled cross-pollination is mandatory for hybrid seed production. Mechanisms have been developed to ensure the correct form of pollination for each process and to prevent genetic contamination of seed stocks (Wych, 1988). Breeder or foundation seed is produced from self-pollinated seed after the eighth or ninth generation of inbreeding. A high degree of self-pollination is assured by planting in blocks that are isolated by a distance of at least 200 meters (~660 ft.) from any other contaminating source of pollen. Hybrid seed production is accomplished by inter-planting rows of the male and female inbred parents (e.g., one row of male to four female rows). Hybrid seed production requires isolation similar to that for foundation seed. Self-pollination of the female parent is prevented through detasseling prior to pollen shed or by the use of male sterile females. Genetic conformity of inbreds and hybrids is monitored and assured through grow-outs of representative seed lots and laboratory screening using such criteria as isozyme profiles.

Maize is planted when soil temperatures are warm (greater than or equal to 10°C) usually mid to late April through to mid-May in the U.S. Corn Belt. Optimum yields occur when the appropriate hybrid maturity and population density are chosen. In addition, exogenous sources of nitrogen fertilizer are generally applied and weed and insect control measures are generally recommended. Choice of the appropriate hybrid for the intended growing area helps to ensure that the crop will mature before frost halts the growth of the plant at the end of the season; hybrids are categorized according to the amount of "heat units" that will be required for maturity. Therefore, a hybrid developed for a specific heat unit zone, will not mature in (cooler) areas that receive fewer "heat units". Traditional cultivation practices in maize often result in bare soil that is susceptible to erosion by wind or water; increasingly, "no till" maize is being grown in an effort to reduce this soil loss.

In 2017, there were more than 82 million acres planted to maize in the United States producing over 14.6 billion bushels of grain (USDA, 2018). Maize grown in the United States is predominantly of the yellow dent type, a commodity crop largely used to feed domestic animals, either as grain or silage. The remainder of the crop is exported or processed by wet or dry milling to yield products such as high fructose corn syrup and starch or oil, grits and flour. These processed products are used extensively in the food industry. For example, maize starch serves as a raw material for an array of processed foods, and in industrial manufacturing processes. Since the early 1980's, a significant amount of grain has also been used for fuel ethanol production. The by-products from these processes are often used in animal feeds. For a full discussion of the uses of maize see Watson (1988).

D. Pollination of Maize

Maize is primarily wind-pollinated; insects are responsible for insignificant amounts of pollen dispersal (Russell and Hallauer, 1980). Pollination, fertilization, and caryopsis development of maize follows the same pattern for chasmogamous wind-pollinated grasses, with the following exceptions:

1. Pollen is produced entirely in the staminate inflorescences. Eggs are produced entirely in the pistillate inflorescences.

2. Self-pollination and fertilization and cross-pollination and fertilization are usually possible, and frequencies of each are usually determined by physical proximity and other environmental influences on pollen transfer. A number of complicating factors, such as genetic sterility factors and differential growth rates of pollen tubes may also influence the frequencies of self-fertilization versus cross-fertilization.

3. Maize styles and maize pollen tubes are the longest known in the plant kingdom.

4. Shed pollen typically remains viable for 10 to 30 minutes, but may remain viable for much longer under refrigerated conditions (Coe *et al*., 1988).

5. Pollen dispersal is limited due to its large size (0.1 mm diameter) and spherical nature. Numerous studies have shown that greater than 98% of pollen settles to the ground within 60 meters of its source (Raynor *et al*., 1972; Luna *et al*., 2001; Paterniani and Stort 1974).

6. The staminate and pistillate inflorescences do not develop at the same time. The pistillate inflorescence is precocious. However, there is the appearance of slight protandry because the elongating styles (silks) are delayed for about seven days in emergence from the bracts of the pistillate inflorescence, while the later-developing staminate inflorescence is fully visible. The silks are receptive to pollen up to 10 days after emergence, but receptivity decreases rapidly thereafter (Walden and Everett, 1961).

E. Inter-Species/Genus Hybridization

Maize and other species and subspecies of teosinte are sexually compatible and can produce fertile hybrids (Wilkes, 1977). Related *Zea* species are geographically restricted and occur only in Mexico and Guatemala. The closest known relative of *Zea* is *Tripsacum*, a genus of eleven species, widely distributed between 42°N and 24°S latitude (de Wet *et al*., 1981). Three species occur in the United States, two of which, *Tripsacum floridanum* (Florida Gamagrass) and *Tripsacum lanceolatum* (Mexican Gamagrass), are confined to the southernmost states of the United States. Only one, *Tripsacum dactyloides* (Eastern Gamagrass), has a distribution that includes the northern (U.S.) maize belt (Gould, 1968).

F. Potential for Introgression from *Z. mays* into Relatives

An examination of the literature prior to 1980 would lead to the conclusion that there is constant gene flow between maize and teosinte, and that the weedy teosinte (*Z. mays* ssp. *mexicana*) is a hybrid of maize and *Tripsacum*, and functions as a genetic bridge between the two (de Wet and Harlan, 1972; de Wet, 1975; Galinat, 1973). However, this premise has been re-evaluated using techniques of gene mapping, which failed to show any evidence of recent introgression between maize and teosinte (Smith *et al*., 1985). Moreover, *Z. mays* ssp. *mexicana* seems not to be a hybrid of the wild and cultivated forms of *Zea* and therefore probably does not serve as a genetic bridge as the physical similarities appear to be due to parallel adaptation to the same habitat (Doebley, 1984). There is evidence of highly restricted gene flow between *Zea* spp. that apparently occurs predominantly from teosinte into maize (Doebley *et al*., 1987). *Tripsacum* and *Zea* have different chromosome numbers (n = 9 versus n = 10). Crosses between *Z. mays* and *T. dactyloides* can be made, but only through human intervention and, even then, only with extreme difficulty. Moreover, the progeny are frequently sterile or genetically unstable (Mangelsdorf, 1974). The process of transferring *Tripsacum* germplasm into maize is technically difficult. The transmission rate of the single extra *Tripsacum* chromosome added to the genome is so low and the rate of maize *Tripsacum* crossing over so reduced, as to practically exclude the general use of experimentally introduced *Tripsacum* germplasm in maize improvement (Galinat, 1988).

G. Volunteers and Weediness in Maize

Maize has lost the ability to survive in the wild due to its long process of domestication and needs human intervention to disseminate its seed. Although maize from the previous crop year can overwinter and germinate the following year, it does not persist as a weed (OECD, 2003). The presence of maize in soybean fields following the maize crop from the previous year is a common occurrence. Measures are often taken to either eliminate the plants with cultivation or use of herbicides to kill the plants in soybean fields, but the plants that remain and produce seed usually do not persist during the following years. Volunteers are common in many agronomic systems, but they are easily controlled; however, maize is incapable of sustained reproduction outside of domestic cultivation. Maize plants are non-invasive in natural habitats (Gould, 1968). Some *Zea* species are successful wild plants in Central America, but they have no pronounced weedy tendencies (Galinat, 1988). In contrast to weedy plants, maize has a pistillate inflorescence (ear) with a cob enclosed with husks.

Consequently seed dispersal of individual kernels does not occur naturally. However, individual kernels of maize may be distributed in fields and along avenues of travel from the field operations while transporting the grain from the harvested fields to storage facilities (Hallauer, 2000).

III. Characterization of the Genetic Material Introduced into the Genome of Maize Event PY203

A. Summary

Data from Southern analyses and DNA sequencing demonstrated that two independent copies of the T-DNA from the transformation construct pAG4758 are inserted into the genome of Event PY203. The nucleotide sequence of the T-DNA and the flanking maize genomic DNA of both insertions was determined and revealed that one insertion (locus 3293) contains a complete copy of the T-DNA with three copies of the *phy02* phytase gene and a single copy of the phosphomannose isomerase (*manA*) gene. The second insertion (locus 3507) is a truncated version of the T-DNA that includes two of the three *phy02* genes from the T-DNA fragment. This insertion lacks the third, downstream copy of the *phy02* gene and much of the ZmGlb1 promoter from which the gene is expressed as well as the entire *manA* gene. Event PY203 does not contain any vector backbone sequences of the transformation plasmid pAG4758. Both insertions in Event PY203 were demonstrated to be stable over several generations and to be inherited in the expected Mendelian pattern typical of native plant genes.

B. Introduction

The following is a summary of data and information relevant to the genetic characterization of the two T-DNA insertions of Event PY203 and this information was previously submitted to the FDA/CVM in Agrivida, Inc.'s GRAS notice (AGRN 21) for the use of phytase produced by Event PY203 in poultry feed.

C. Origin of the Gene Encoding Phytase Phy02

The native *E. coli appA* phytase gene was optimized using a combination of modeling and site-directed mutagenesis to generate a gene encoding the Phy02 phytase with increased thermotolerance and susceptibility to digestion in a gastric environment. Thermotolerance is a desirable trait for commercial feed enzymes since many animal feeds are produced by a pelleting process that involves a heat treatment that inactivates thermolabile enzymes. Examination of the sensitivity of the native AppA protein to digestion in a simulated gastric environment revealed that it is resistant to digestion in this environment. Although resistance to gastric digestion has been hypothesized to correlate with a higher potential for a protein to be allergenic, experimentally this correlation has not been validated (Fu *et al*., 2002). Nevertheless, the stability of a new protein in the simulated gastric environment is considered in the evaluation of the safety of new proteins in food. The nucleotide coding sequence and deduced amino acid sequence of the *phy02* phytase gene are shown in Figure 1. Since the Phy02 phytase exhibits the enzymatic properties common to phytases (CVM, 2017a), it is clear that none of the amino acid changes have significantly changed its structure or biological function.

Figure 1. Comparison of the amino acid sequences of the Phy02 and *E. coli* AppA phytases. The amino acid sequence of the *E. coli* AppA phytase is presented. Amino acid substitutions in the Phy02 phytase relative to the AppA phytase are shown below the AppA sequence and these are shaded in gray. The amino acid sequence at the amino terminus varies between the two phytases since in the Phy02 phytase 31 of the amino terminal residues of AppA, which contain the bacterial periplasmic signal peptide, were replaced with the maize Z27 γ-zein signal peptide (underlined) that directs the protein to the endoplasmic reticulum. In addition, six amino acids representing the endoplasmic retention peptide (underlined) were added to the carboxy terminus of the Phy02 protein. The mature Phy02 phytase protein consists of 417 amino acids with a predicted molecular weight of 45,684 kDa. The consensus phytase active site (RHGxRxP) is shown in yellow shading. Other residues that are involved in substrate binding that are conserved in other phytases are shaded in blue. Amino acid residues that have no corresponding residue in the other protein are indicated by a hyphen. Excluding the signal peptides at the termini of the proteins, the AppA and Phy02 phytases are homologous across 411 amino acids with 16 differences in Phy02 relative to the AppA phytase.

```
1      MSDMKSGNISMKAILIPFLSLLIPLTPQSAFAQSEPELKLESVVIVSRHGVRAPTKATQL   60
       ------------MRVLLVALALLALAASATS                         F

61     MQDVTPDAWPTWPVKLGWLTPRGGELIAYLGHYQRQRLVADGLLAKKGCPQSGQVAIIAD   120
                FY       E               W          P

121    VDERTRKTGEAFAAGLAPDCAITVHTQADTSSPDPLFNPLKTGVCQLDNANVTDAILSRA   180
                                                        V Q      E

181    GGSIADFTGHRQTAFRELERVLNFPQSNLCLKREKQDESCSLTQALPSELKVSADNVSLT   240
                  Y                  A         A

241    GAVSLASMLTEIFLLQQAQGMPEPGWGRITDSHQWNTLLSLHNAQFYLLQRTPEVARSRA   300
         W                                           D

301    TPLLDLIKTALTPHPPQKQAYGVTLPTSVLFIAGHDTNLANLGGALELNWTLPGQPDNTP   360
                                                        Q

361    PGGELVFERWRRLSDNSQWIQVSLVFQTLQQMRDKTPLSLNTPPGEVKLTLAGCEERNAQ   420
                                              F

421    GMCSLAGFTQIVNEARIPACSL------
                             SEKDEL
```

D. Characteristics of the Phy02 Expression Construct

A transformation gene cassette containing three copies of the Phy02 phytase gene, each with a different monocot derived promoter and NosT terminator was constructed in plasmid pAG4758. The genetic elements of plasmid pAG4758 that was used to transform maize are shown in Figure 2. The individual genetic elements within plasmid pAG4758 are described in Table 2. This plasmid was transformed by *Agrobacterium*-mediated transformation into immature maize embryo tissue as described by Negrotto *et al*. (2000) and transformants were selected based on the presence of the plant selectable marker *manA* gene on the transformed DNA fragment that encodes the enzyme phosphomannose isomerase (PMI). The PMI enzyme enables maize tissue to grow on mannose as a sole source of carbon (Negrotto *et al*., 2000). The *manA* gene has been used as a selectable gene in several genetically modified maize varieties that have completed review by the USDA, FDA, and EPA for food and feed safety, including maize Events 5307 and Mir604 maize with resistance to corn rootworm, lepidoptera resistant Mir162, and α-amylase expressing Event 3272, all products of Syngenta Seeds. The US EPA has reviewed the safety and environmental impact of expressing PMI in maize and issued an exemption from the requirement for a tolerance (EPA, 2004). Maize plants containing the Phy02 phytase gene were cultivated and were demonstrated to produce approximately 3500 units of phytase activity (FTU) per gram of grain. Based upon the specific activity determined for the Phy02 phytase of 426 FTU/mg protein there is approximately 8.2 mg of the Phy02 phytase protein per gram of grain from Event PY203. The transformation event chosen as a development candidate was designated PY203.

Figure 2. Plasmid map of pAG4758 that was used in the transformation of maize to create the phytase producing Event PY203. The genetic elements shown are described in Table 2.

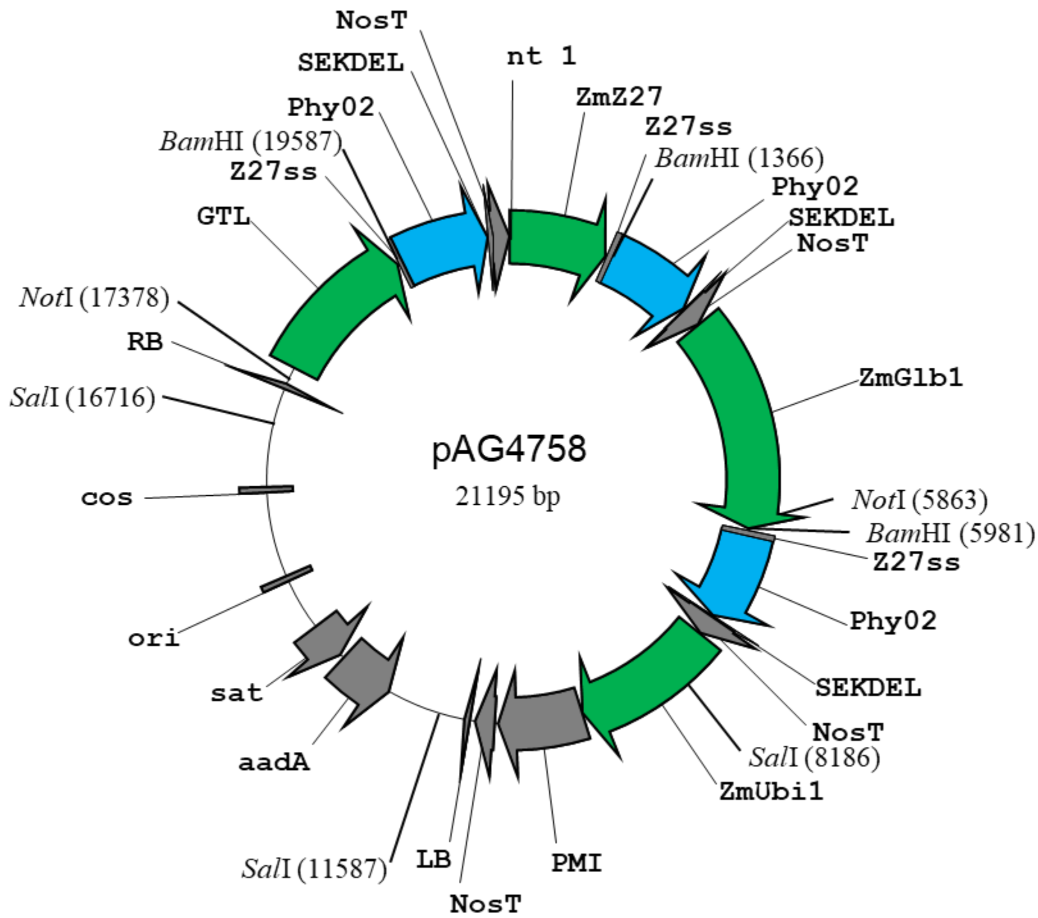


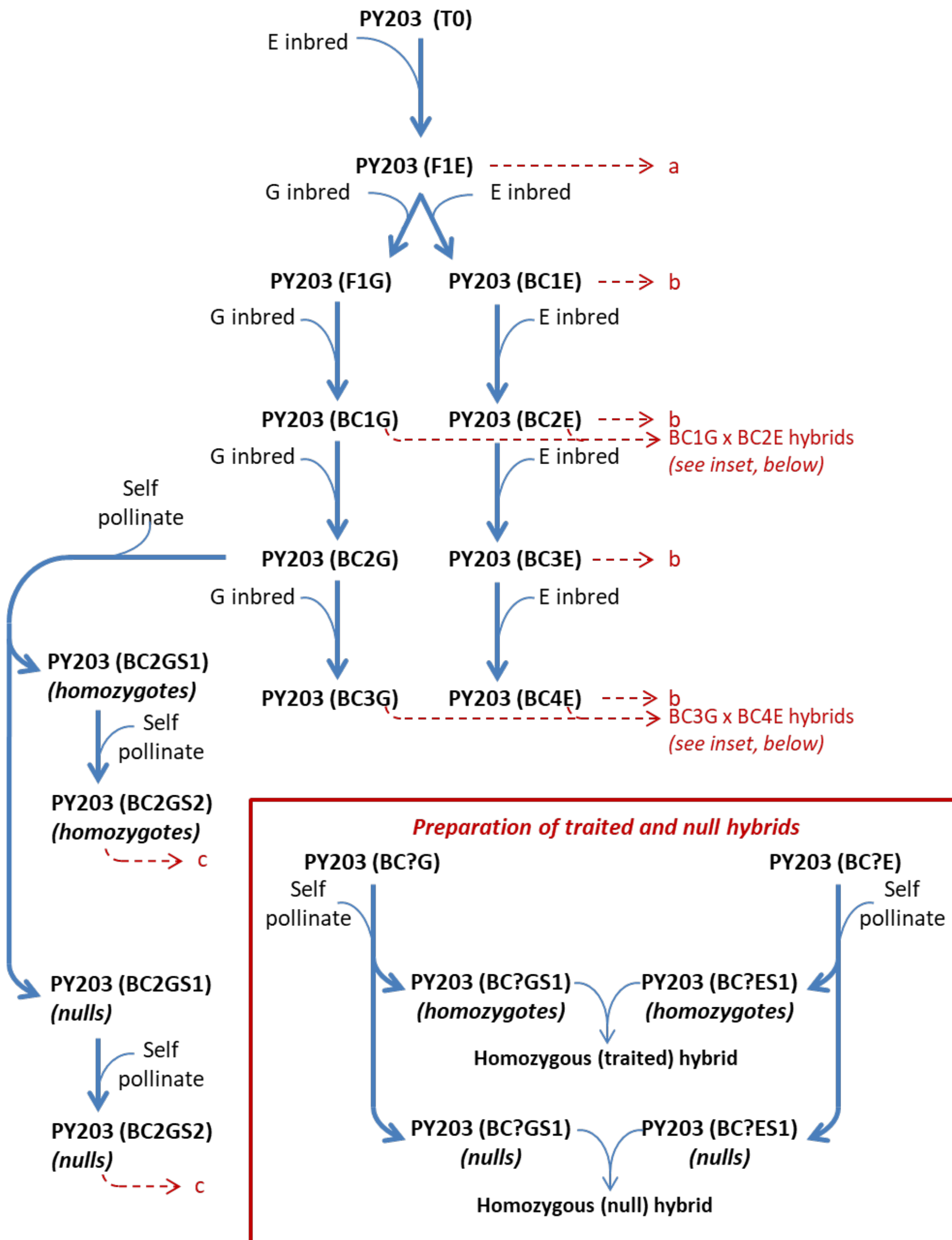
Table 2. Description of the genetic elements in the 21,195 bp plasmid pAG4758 used to transform maize. Genetic elements highlighted in bold font are within the T-DNA borders.

Genetic Element	Description	Size (bp)	Reference
GTL	Promoter derived from the *Oryza sativa* Glutelin-1 gene (similar to Genbank accession number EU264103.1); provides endosperm-specific gene expression	2071	Qu *et al*., 2008
Z27ss	Signal peptide sequence derived from the 27 kDa γ-zein seed storage protein of *Zea mays* (similar to Genbank accession number AB086264.1); directs proteins to the endoplasmic reticulum	57	Torrent *et al*., 2009
Phy02	Coding sequence of the modified Phy02 phytase gene that was originally derived from the *E. coli appA* phytase gene (Genbank accession number EFE63517.1)	1257	This document
SEKDEL	Sequence encoding an endoplasmic reticulum retention peptide	18	Semenza *et al*., 1992
NosT	Terminal sequence of the nopaline synthase (*nos*) gene from *Agrobacterium tumefaciens* (similar to Genbank accession number AJ237588.1). Terminates gene transcription by providing polyadenylation signals	276	Depicker *et al*., 1982
ZmZ27	Promoter sequence derived from the *Z. mays Zc2* gene (Genbank accession number X53514.1); provides endosperm-specific gene expression.	1363	Reina *et al*., 1990 Russell & Fromm, 1997
ZmGlb1	Promoter derived from the *Z. mays Glb1* gene (similar to Genbank accession AH001354.2); provides embryo-specific gene expression.	3004	Streatfield *et al*., 2010
ZmUbi1	Promoter derived from the *Z. mays* ubiquitin 1 gene including the first intron (ZmUbi1 intron) (Genbank accession number S94464.1); directs expression in all tissues of *Z. mays*.	1992	Christensen and Quail, 1996
PMI	Gene sequence of the *manA* gene encoding phosphomannose isomerase (PMI) derived from *E. coli* (similar to Genbank accession number EGI11220.1); selectable marker gene	1176	Negrotto *et al*., 2000
LB	Left border region of T-DNA from *A. tumefaciens* nopaline Ti plasmid (GenBank accession number J01825).	25	Zambryski *et al*., 1982
RB	Right border region of T-DNA from *A, tumefaciens* nopaline Ti plasmid (GenBank accession number J01826).	25	Wang *et al*., 1984
sat	Streptothricin acetyltransferase (*sat*) gene from *Streptomyces lavendulae* (similar to Genbank accession number LT898487.1), confers resistance to streptothricin in bacteria.	567	Horinouchi *et al*., 1987
ori	ColE1 origin of replication; permits replication of plasmid in *E. coli*. (similar to GenBank accession number V00268)	807	Itoh and Tomizawa, 1978
cos	Cohesive ends of the bacteriophage lambda genome (Genbank accession number KT232076.1); promote circularization of plasmids	12	Collins and Hohn, 1978
aadA	Streptomycin adenyltransferase, *aadA* gene from *E. coli* Tn7 (GenBank accession number X03043). Confers resistance to erythromycin, streptomycin, and spectinomycin; used as a bacterial selectable marker.	786	Fling *et al*., 1985

E. Characterization of Maize Event PY203

In the following sections various analyses and characterizations of maize Event PY203 are described. The grain and maize tissues used in these analyses were derived from different stages of a breeding program for Event PY203. A diagram that presents the different stages of breeding beginning with the initial transformed line (T0) and the sources of the plant material used in each of the analyses described below is shown in Figure 3.

Figure 3. Breeding diagram for maize Event PY203.



The original maize line used in the initial transformation was a variety known as High II B and was obtained from the Maize Genetics and Genomics Database (stock# T940B; http://www.maizegdb.org/data_center/stock?id=96986). The maize germplasms E and G are proprietary inbred lines. The initial transformant (T0) plant was crossed with the wild type inbred E, creating F1E seed (see Figure 3). Progeny from this cross were grown, and then either crossed with a second inbred ("G"), creating "F1G" seed, or backcrossed with inbred E, creating BC1E seed. Multiple generations of continued backcrossing and self-pollination were accomplished as indicated in Figure 3. At various points in this process, progeny were taken for characterization (red in Figure 3), as follows: a) T-DNA genomic flank characterization and complete sequencing of the loci carrying the T-DNA insertions; b) determination of segregation ratio among progeny from four consecutive generations; c) evaluation of Phy02 protein expression in various tissues as well as nutritional composition of forage and grain from field-grown plants. At two different generations, hybrid plants were prepared, as indicated in the inset of Figure 3, for use in agronomic trials and seed germination tests. In each case, progeny from backcrosses into the G and E lines were self-pollinated to produce populations of segregating seed. Seedlings were grown and genetically characterized to identify progeny that were homozygous or null for both of the T-DNA loci. Seedlings that were hemizygous for either T-DNA locus were discarded. Upon maturing, homozygous plants from the G and E backgrounds were crossed, creating traited hybrids, while null plants from the G and E backgrounds were crossed, creating null hybrids.

E1. Determination of the Number of DNA Insertions

Three lines of evidence support the conclusion that Event PY203 contains two independently-segregating T-DNA insertions:

- Genome walking involving generation by PCR, and sequencing of, DNA fragments containing T-DNA/maize genome junction regions
- Southern blotting of genomic DNA from Event PY203 using probes derived from three distinct regions of the T-DNA
- Segregation analysis using PCR primers that independently detect each of the four expression cassettes in the T-DNA

Each of these is described in detail below.

A genome walking method as described by Mishra *et al*. (2002) was used to identify the junctions between T-DNA fragments and maize genomic DNA (the "flanking sequences") in event PY203 (see also § III.E.3 below). This PCR-based approach involves pairing a primer that anneals to a known, T-DNA-derived sequence (see Table 3), with a second, partially degenerate primer that will anneal in the uncharacterized maize genomic sequence further downstream. The amplified DNA fragments are then cloned and sequenced to reveal the identity of the adjacent sequence. More than 80 unique primers were employed in this strategy to test for insertions that may have originated from virtually any part of the T-DNA, with a primer laying down on average every 125 basepairs (bp) along the length of the 15,177 bp T-DNA (Figure 4, Table 3). Sequencing hundreds of clones derived in this manner revealed that all of the identified flanking sequences were derived from two locations in the

maize genome, one of which is on chromosome 2 and the other on chromosome 8. These two maize genomic regions were the only sequences that were found to be associated with T-DNA-derived sequences, and are consistent with complementary data (see below) that indicate that there were two T-DNA insertion sites. These two T-DNA insertions were designated locus 3293 and locus 3507. The complete DNA sequence of the insertions and their flanking sequences were determined as described in §E.3 below.

Figure 4. Diagram of the T-DNA from pAG4758 and location of annealing PCR primers in genome walking to determine the number of T-DNA inserts. The genetic elements shown are described in Table 2. ZmZ27ss and SEKDEL elements are not shown for clarity. Red and orange arrowheads indicate the approximate annealing positions for each of the reverse and forward primers, respectively, listed in Table 3. At the bottom of the diagram is a scale in kilobase pairs (kb) for reference to Table 3.

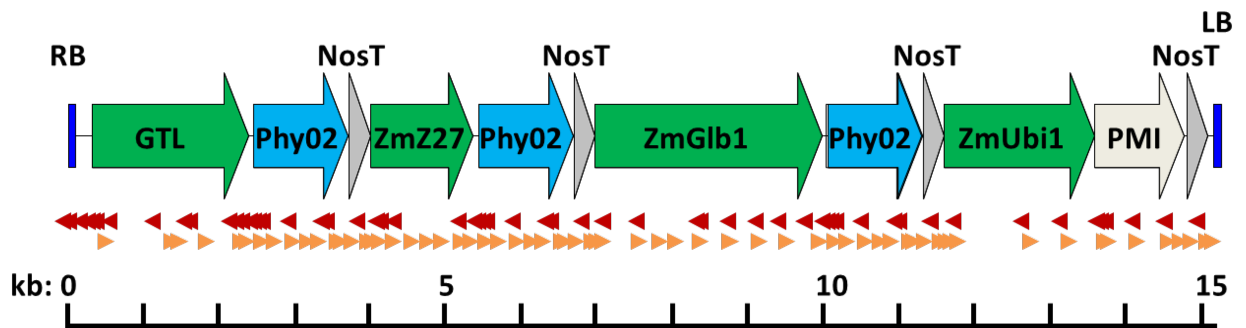


Table 3: PCR Primers used for genomic walking to determine the number of T-DNA insertions. The genetic elements to which the primers anneal in the T-DNA, the specific nucleotides within the T-DNA to which they anneal (relative to the scale shown in Figure 4), and the orientation of each primer (F, forward; R, reverse) are presented. The location where each primer anneals within the T-DNA is also depicted graphically in Figure 4.

Primer #	Region of T-DNA	Anneals to (nt):	Orientation
1759	RB and spacer	71-90	R
1758		121-140	R
1756		297-316	R
2418	GTL	378-397	R
2419		401-420	R
2695		402-421	F
2420		463-482	R
2421		668-687	R
3093		1148-1167	R
2990		1164-1184	F
3087		1330-1349	F
3094		1696-1715	R
3088		1742-1761	F
2993		1759-1778	R
2702		2174-2193	F
3089		2224-2243	F
3095		2224-2243	R
2699		2330-2350	R
2697		2379-2401	R
2723	Phy02	2483-2501	F
2718		2534-2553	R
2705		2589-2606	R
2719		2620-2638	R
3090		2632-2651	F
3096		2632-2651	R
3147		2886-2905	F
3097		2998-3017	R
3091		3106-3125	F
3148		3188-3207	F
2715		3496-3515	F
3149		3497-3516	R
2713		3504-3523	F
3098		3504-3523	R
1488	NosT	3738-3757	F
2731		3924-3944	F
2734		3927-3946	R
1490		3935-3954	F
507	ZmZ27	4082-4101	F
383		4188-4207	R
2701		4230-4249	R
508	ZmZ27	4381-4400	F
510		4381-4400	R
506		4696-4715	F
509		4863-4882	F
384		5105-5124	F
2709		5136-5155	F
512		5334-5353	R
2723	Phy02	5457-5475	F
2718		5508-5527	R
2705		5563-5580	R
2719		5594-5612	R
3090		5606-5625	F
3096		5606-5625	R
3147		5860-5879	F
3097		5972-5991	R
3091		6080-6099	F
3148		6162-6181	F
2715		6470-6489	F
3149		6471-6490	R
2713		6478-6497	F
3098		6478-6497	R
1488	NosT	6712-6731	F
2731		6898-6918	F
2734		6901-6920	R
1490		6909-6928	F
2888		6959-6980	F
2710	ZmGlb1	7168-7187	R
3152		7459-7480	F
3155		7641-7664	R
3153		7751-7772	F
3154		7942-7962	F
2720		8243-8264	F
2714		8416-8435	R
2716		8452-8470	R
3156		8617-8636	F
3162		8832-8851	R
3157		9047-9066	F
3161		9186-9206	R
3158		9437-9460	F
3160		9539-9558	R
1762	ZmGlb1	9885-9902	F
3159		9890-9909	R
2723	Phy02	10072-10090	F
2718		10123-10142	R
2705		10178-10195	R
2719		10209-10227	R
3090		10221-10240	F
3096		10221-10240	R
3147		10475-10494	F
3097		10587-10606	R
3091		10695-10714	F
3148		10777-10796	F
2715		11085-11104	F
3149		11086-11105	R
2713		11093-11112	F
3098		11093-11112	R
1488	NosT	11327-11346	F
2731		11512-11532	F
2734		11515-11534	R
1490		11523-11542	F
2728	ZmUbi1	11682-11702	F
2722		11809-11831	R
397		12641-12660	F
396		12722-12741	R
399		13135-13155	F
402		13137-13156	R
2732	PMI	13628-13647	F
2730		13630-13649	F
2727		13745-13764	R
2726		13778-13797	R
2729		13813-13831	R
2829		14036-14056	F
2830		14201-14219	R
1482		14471-14490	F
1484		14630-14649	F
3163		14643-14662	R
1488	NosT	14824-14843	F
2731		15010-15030	F
2734		15013-15032	R
1490		15021-15040	F

Three different Southern hybridization strategies were also used to confirm the presence of two independent insertions of the T-DNA from transformation plasmid pAG4758 in Event PY203.

The first test used a 268 bp probe derived from the unique T-DNA sequences that are adjacent to the RB (the "RB probe") within the T-DNA. Genomic DNA from Event PY203 was digested independently with the restriction endonucleases *Eco*RI or *Hin*dIII. *Eco*RI and *Hin*dIII restriction sites are present in the T-DNA and/or the genomic maize flanking DNA (Figure 5). As shown in Figure 6A, in the *Eco*RI digest, a 587 bp fragment is generated from Locus 3293 and a 5,160 bp fragment is generated from Locus 3507 (cf. Figure 5). In the *Hin*dIII digest, a 10,192 bp fragment is generated from Locus 3293 and an 11,900 bp fragment is generated from Locus 3507 (cf. Figure 5).

In the second test, genomic DNA from Event PY203 was digested with *Bam*HI and subjected to Southern blot analysis using a 461 bp probe that was derived from the PMI region of the T-DNA (Figure 6B). Since Locus 3507 lacks this portion of the T-DNA, only the 7,751 bp fragment derived from Locus 3293 can be detected (cf. Figure 5).

In a third test, the presence of two independent T-DNA insertions in Event PY203 was also demonstrated by examination of independently segregating progeny from an outcross between a hemizygous Event PY203 plant and a non-transformed inbred. The progeny of this outcross were first tested for the presence of the Phy02 coding sequence by PCR using primers that detected a 306 bp region from the middle of any one of the three Phy02 coding sequences that are present in the T-DNA. This made it possible to identify transgenic and null plants among the progeny. Genomic DNA was isolated from each plant, digested with *Bam*HI, and subjected to Southern blot analysis using a 400 bp probe derived from the rice glutelin promoter (GTL) region of the T-DNA (Figure 6C). Whereas no hybridizing DNA fragments could be detected in DNA from null plants (Null2, Null23), the expected fragments of 2,519 bp (derived from Locus 3293) and 10,462 bp (derived from Locus 3507) were found to segregate independently among the Phy02-positive progeny. Whereas many plants carried both loci (e.g. #48), others carried only Locus 3293 (e.g. #50) or Locus 3507 (e.g. #52). The fact that all of the progeny that carried Phy02 sequences (as detected by PCR) harbored at least one of the two previously characterized loci and no other GTL-hybridizing fragments supports the conclusion that these are the only two T-DNA insertion sites in Event PY203.

Figure 5. Gene maps of PY203 3293 and 3507 insertion loci. The regions from which the RB (right border), GTL and PMI probes are derived are indicated. Only those *Eco*RI and *Hin*dIII, and *Bam*HI sites closest to the regions where the probes hybridize are indicated. The locations of NosT elements have been omitted for clarity. ZmZ27ss:Phy02:SEKDEL coding sequences are labeled simply as "Phy02". Locus 3293 lacks 27 bp from the RB end of the T-DNA, including the entire 25 bp RB element. The truncated portion of the ZmGlb1 promoter in locus 3507 (see below) is labeled as ZmGlb1*. Flanking regions derived from maize chromosome 8 (chr8) and chromosome 2 (chr2) that were cloned and sequenced directly are indicated as heavy black boxes. Dotted lines: examination of the publicly-available reference sequence for the maize B73 genome (NCBI Reference Sequence: NC_024460.2) predicts the presence of a *Bam*HI site within the chromosomal sequence (relative nucleotide position: 19,518), downstream of the portion of the flanking sequence that was directly cloned and sequenced from Locus 3293 (as described in Appendix 1) Similarly, *Bam*HI, *Eco*RI and *Hin*dIII sites were predicted to be 5,993 bp, 2,563 bp and 1,076 bp, respectively, upstream of the region of Locus 3507 that was sequenced directly. See Table 2 for descriptions of other elements.

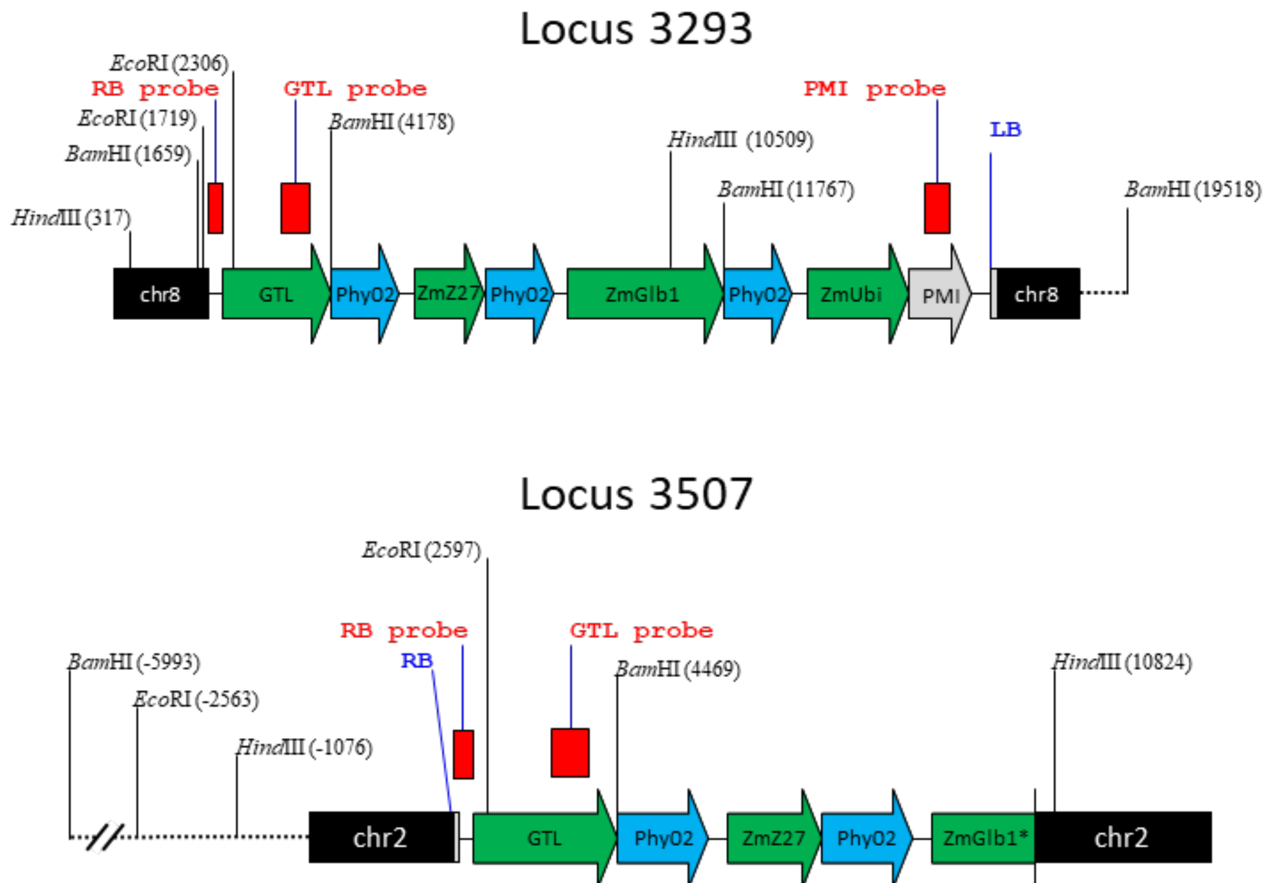
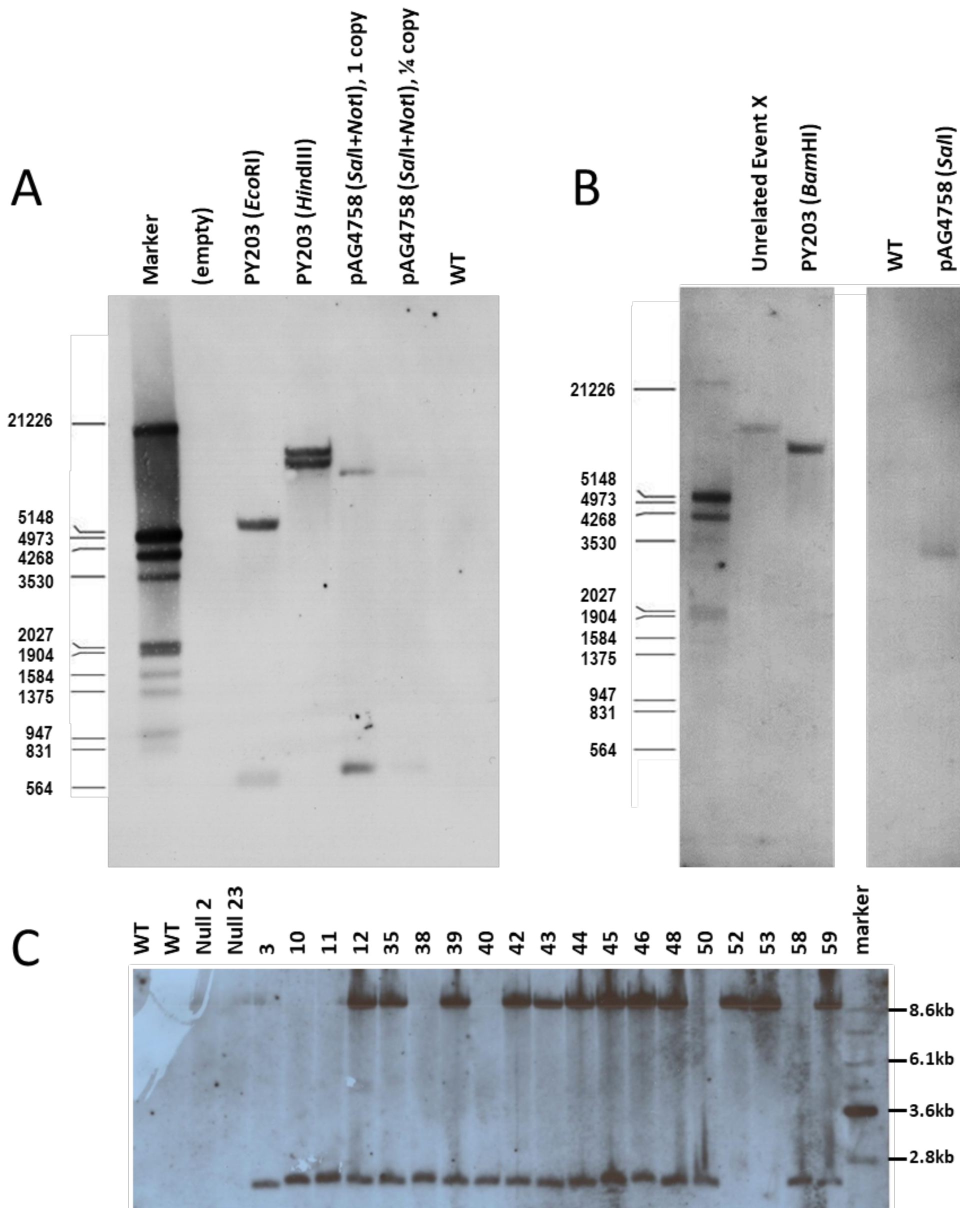


Figure 6. Southern hybridization blots of restriction enzyme-digested genomic DNA of Event PY203. **A.** PY203 genomic DNA digested with either *Eco*RI or *Hind*III hybridized with a probe derived from the T-DNA RB region. DIG-labeled DNA marker fragments are shown (left lane) with their corresponding sizes in bp indicated to the left of the blot. Samples of *Sal*I- and *Not*I-digested pAG4758 (cf. Figure 2), included to estimate copy numbers of 1 and ¼ copies/genome, reveal the expected 662 bp 9680 bp fragments. A separate lane of digested genomic DNA from untransformed maize (WT) probed with the T-DNA RB probe is shown on the right to demonstrate that the probe does not hybridize to WT genomic maize DNA. **B.** *Bam*HI-digested genomic DNA from Event PY203 (and an unrelated "Event X" that had been generated in a separate transformation experiment) hybridized with a probe derived from the PMI region of the T-DNA. *Sal*I-digested pAG4758 produced the expected 3,401 bp fragment (cf. Figure 2). **C.** *Bam*HI-digested genomic DNA from independently-segregating progeny of Event PY203 hybridized with a probe derived from the GTL region of the T-DNA. Null2 and Null23, samples from plants that scored negative in a PCR screen for the Phy02 coding sequence. 3-59, samples from plants that scored positive in a PCR screen for the Phy02 coding sequence. A molecular weight marker and the corresponding fragment sizes (in kb) are shown at the right.



The number of T-DNA insertions in Event PY203 was further investigated by examining segregation ratios of specific pAG4758 T-DNA elements in progeny from outcrosses between PY203 and a non-transformed inbred. Two replicate experiments (Group 1 and Group 2) produced a total of 61 progeny for analysis. PCR primers were designed that would detect each of the 4 expression cassettes (3 distinct Phy02 expression cassettes and the PMI expression cassette) that are present in the T-DNA. To distinguish the 3 Phy02 expression cassettes, one primer was designed that would anneal to the 3' end of the promoter for that cassette, while a second primer was designed that would anneal within the 5' end of the Phy02 coding sequence. The resulting ~200 bp PCR products would span the end of the promoter, the entire ZmZ27ss signal sequence and the beginning of the Phy02 gene. A fourth set of primers would detect a 461 bp region from the middle of the PMI coding sequence (see Figure 7 for relative positions of these PCR products; Table 4 provides the sequences of the primers that were used for each test). PCR amplification of the three unique promoter-Phy02 junctions in the T-DNA from these plants resulted in 72% segregation for two of the three junctions closest to the RB (GTL-Phy02 and Z27-Phy02; Table 5), which is not significantly different from the expected 75% segregation for an event carrying two independently segregating loci (Chi Square *p*-value = 0.605). Segregation of the Glb1 promoter-Phy02 (Glb-Phy02) PCR fragment was 49%, which was not significantly different from 50% as expected for a single locus (Chi Square *p*-value = 0.898). For 36 of the plants from this population (T1 Progeny Group 1), a PMI PCR fragment (PMI11) co-segregated with the ZmGlb1-Phy02 PCR fragment at 44%, which was also not significantly different from 50% as expected for a single locus (Chi Square *p*-value = 0.505). (Plants from Group 2 were not tested for presence of the PMI fragment.) These results support the conclusions that (1) PY203 contains two T-DNA insertions as follows: one insertion (locus 3293) that contains all four of the expression cassettes that are present in the pAG4758 T-DNA, and one insertion (locus 3507) that lacks elements (Glb-Phy02 junction and PMI) close to the LB, and (2) no other T-DNA insertions can be detected in Event PY203 that carry either PMI or any of the promoter-Phy02 elements.

Table 4. **List of primers used for PCR segregation analysis.**

Primer Name	Sequence	Target Region to which Primer Hybridizes
420	GATCGACACCGGATCCTAAAC	ZmZ27 promoter
421	ATCAGCTGGGTGAACTTGG	Phy02
422	GCCACCTCCGCTTTAGTT	ZmGlb1 promoter
436	CACAAAAAGCATTCAGTTCA	GTL promoter
11	ACAGCCACTCTCCATTCAGGTTCA	PMI
12	CGCCAGGGTTCAATTTCACCACAT	PMI

Figure 7. Maps of Locus 3293 and Locus 3507 from PY203, indicating the locations of the three promoter-Phy02 PCR products (GTL-Phy02, ZmZ27-Phy02, and ZmGlb1-Phy02) and the PMI11 PCR product that were used to detect T-DNA-derived insertions within the genome of PY203. Primers used for amplifications are shown in parentheses. Labels are as in Figure 5. See also Table 4.

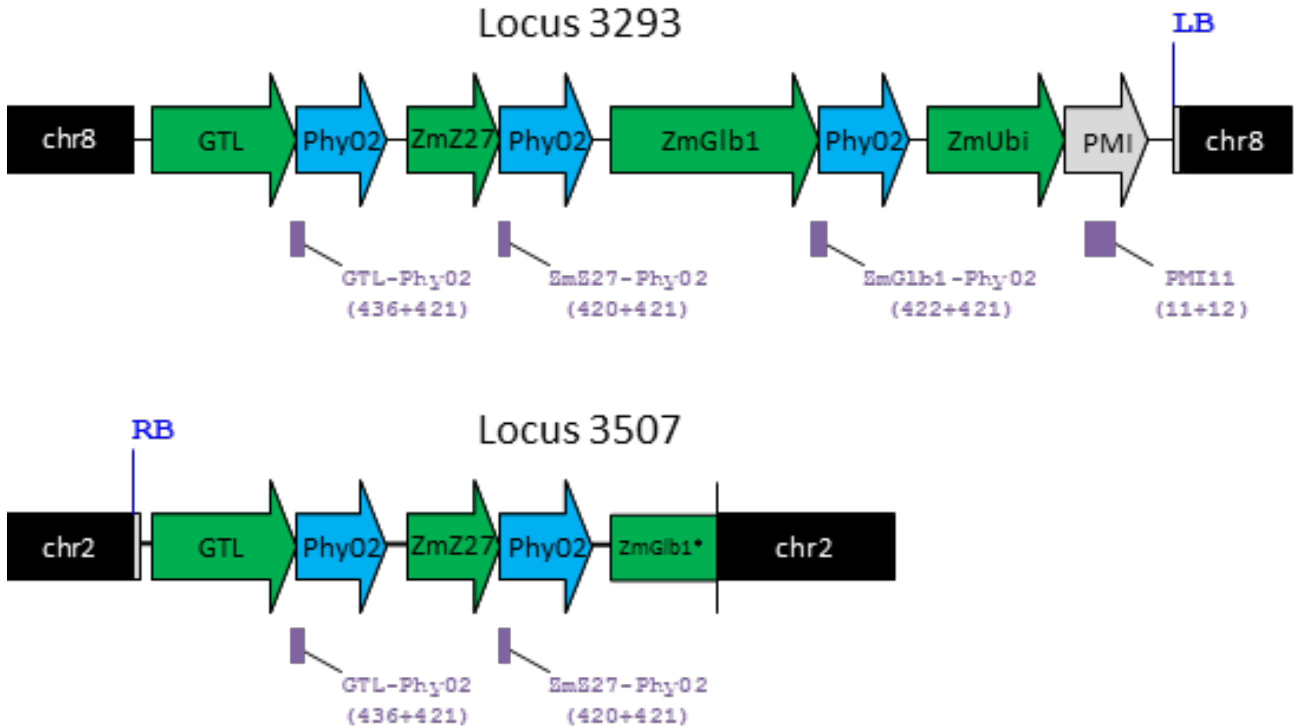


Table 5. Segregation of T-DNA-derived sequences among of progeny from PY203. Two groups of progeny were tested by PCR for the presence of each of several distinct T-DNA regions. Seg. = segregation; Est. = estimated; 1 loc, 2 loc = percentage of progeny expected to score as PCR-positive depending on whether the plant carries 1 or 2 T-DNA loci .

Generation (Event cross)	# of Plants	PCR Product (size)	# PCR-positive	Expected % Seg. (1 loc, 2 loc)	Observed % Seg.	Est. # of Loci
T1 progeny Group 1	36	GTL-Phy02 (194 bp)	25	50%, 75%	69%	2
		Z27-Phy02 (168 bp)	25	50%, 75%	69%	2
		Glb-Phy02 (233 bp)	16	50%, 75%	44%	1
		PMI11 (461 bp)	16	50%, 75%	44%	1
T1 progeny Group 2	25	GTL-Phy02 (194 bp)	19	50%, 75%	76%	2
		Z27-Phy02 (168 bp)	19	50%, 75%	76%	2
		Glb-Phy02 (233 bp)	14	50%, 75%	56%	1
T1 progeny Total	61	GTL-Phy02 (194 bp)	44	50%, 75%	72%	2
		Z27-Phy02 (168 bp)	44	50%, 75%	72%	2
		Glb-Phy02 (233 bp)	30	50%, 75%	49%	1

E2. Absence of Vector Backbone Fragments

The absence of DNA fragments outside of the T-DNA that are derived from the vector portion of plasmid pAG4758 in the genome of the Phy02 expressing maize Event PY203 was demonstrated by two different approaches.

First, DNA fragments derived from the genetic elements within the vector portion of plasmid pAG4758, including the ColE1 origin of plasmid replication and the streptothricin acetyltransferase and *aadA* genes (Figure 8), were used as hybridization probes in Southern blots containing restricted genomic DNA of maize Event PY203. The PCR primers used to generate the DNA probes containing these genetic elements are described in Table 6. None of the DNA fragments containing the genetic elements from the vector portion of plasmid pAG4758 demonstrated hybridization to genomic DNA from the Phy02 expressing maize Event PY203 (Figure 9). This result demonstrates the absence of DNA fragments containing the key genetic elements of the vector portion of plasmid pAG4758 in the genome of maize Event PY203.

Figure 8. Plasmid map of pAG4758 with location of probes used for Southern analysis and location of overlapping backbone (BB) PCR fragments (a thru i) that were used for PCR tiling.

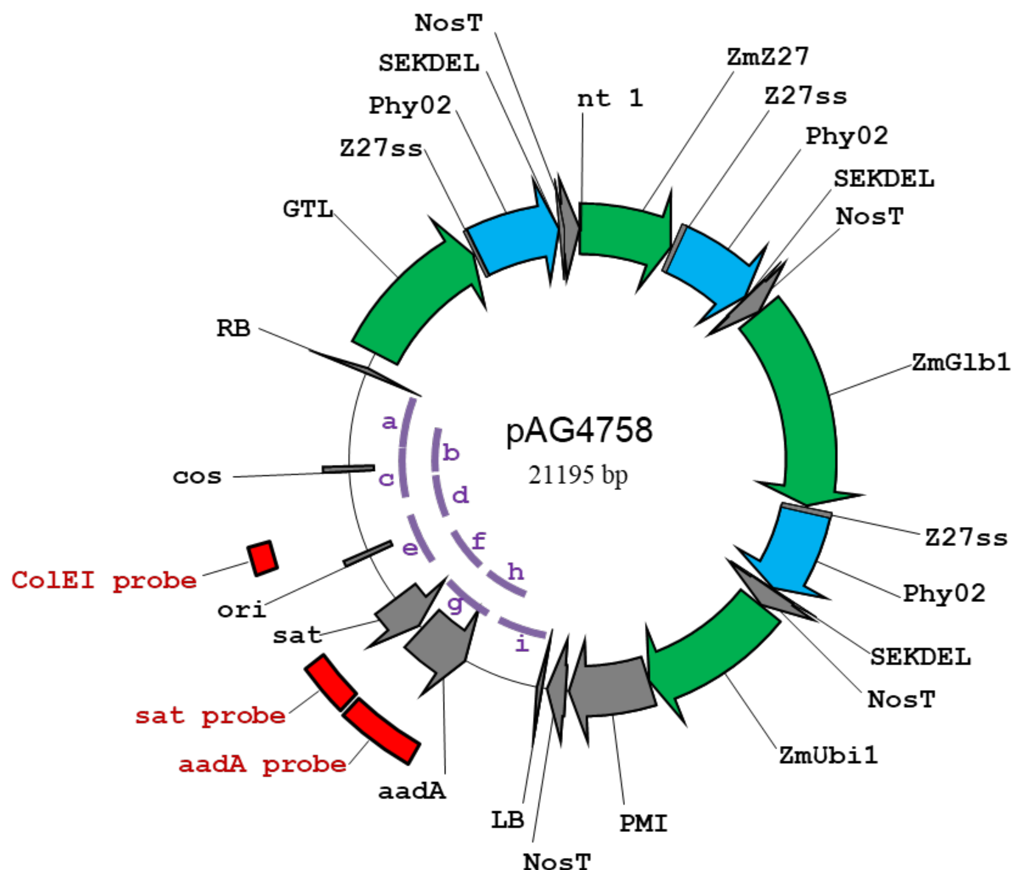
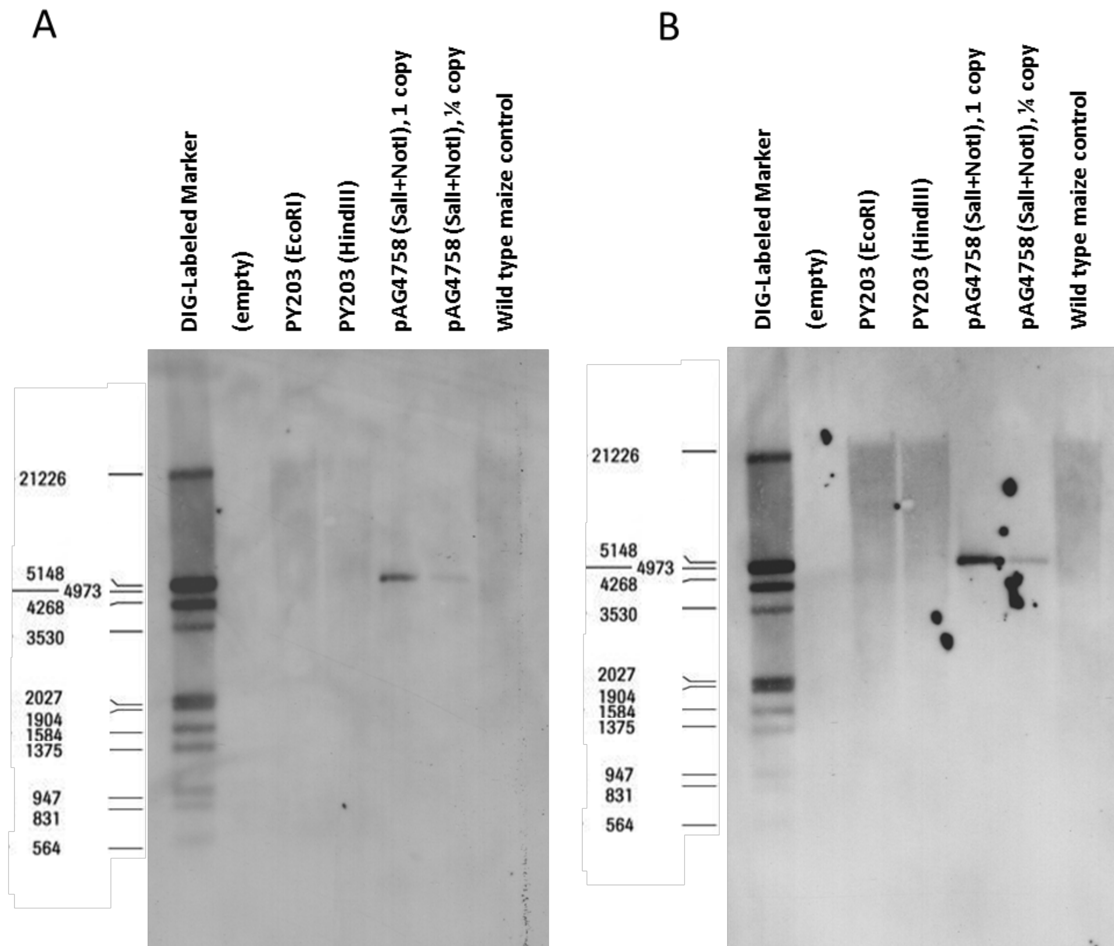


Table 6. List of primers used to amplify the pAG4758 plasmid backbone Southern probes ColE1, *aadA*, and *sat*.

Primer Name	Sequence	Target	Primer pair (Probe size)
722	AACTATCGTCTTGAGTCCAACC	ColE1	722+731 (278 bp)
731	TTTCTGCGCGTAATCTGCTG	ColE1	
735	TTATTTGCCGACTACCTTGGTG	*aadA*	735+736 (789 bp)
736	ATGAGGGAAGCGGTGATCG	*aadA*	
737	TTAGGCGTCATCCTGTGCT	*sat*	737+738 (525 bp)
738	ATGAAGATTTCGGTGATCCCTGA	*sat*	

Figure 9. Southern blot hybridization of digested genomic DNA from Event PY203 with DNA fragments from the ColE1 (A) and a combination of the two antibiotic resistance genes, streptomycin adenylyltransferase (*aadA*) and streptothricin acetyltransferase (*sat*) (B). DIG-labeled DNA marker fragments are shown (left lanes of each respective blot) with their corresponding sizes in bp indicated to the left of each blot. Separate lanes of restricted genomic DNA from untransformed maize probed with the ColE1 or *sat* + *aadA* probes are shown on the right of each blot to demonstrate that the probe does not hybridize to genomic maize DNA. A band ≈5,129 bp in size was detected in the *Sal*I+*Not*I-digested pAG4758 plasmid control (1 & ¼ copy) as expected (see Figure 2 for relative positions of *Sal*I and *Not*I sites in pAG4758).



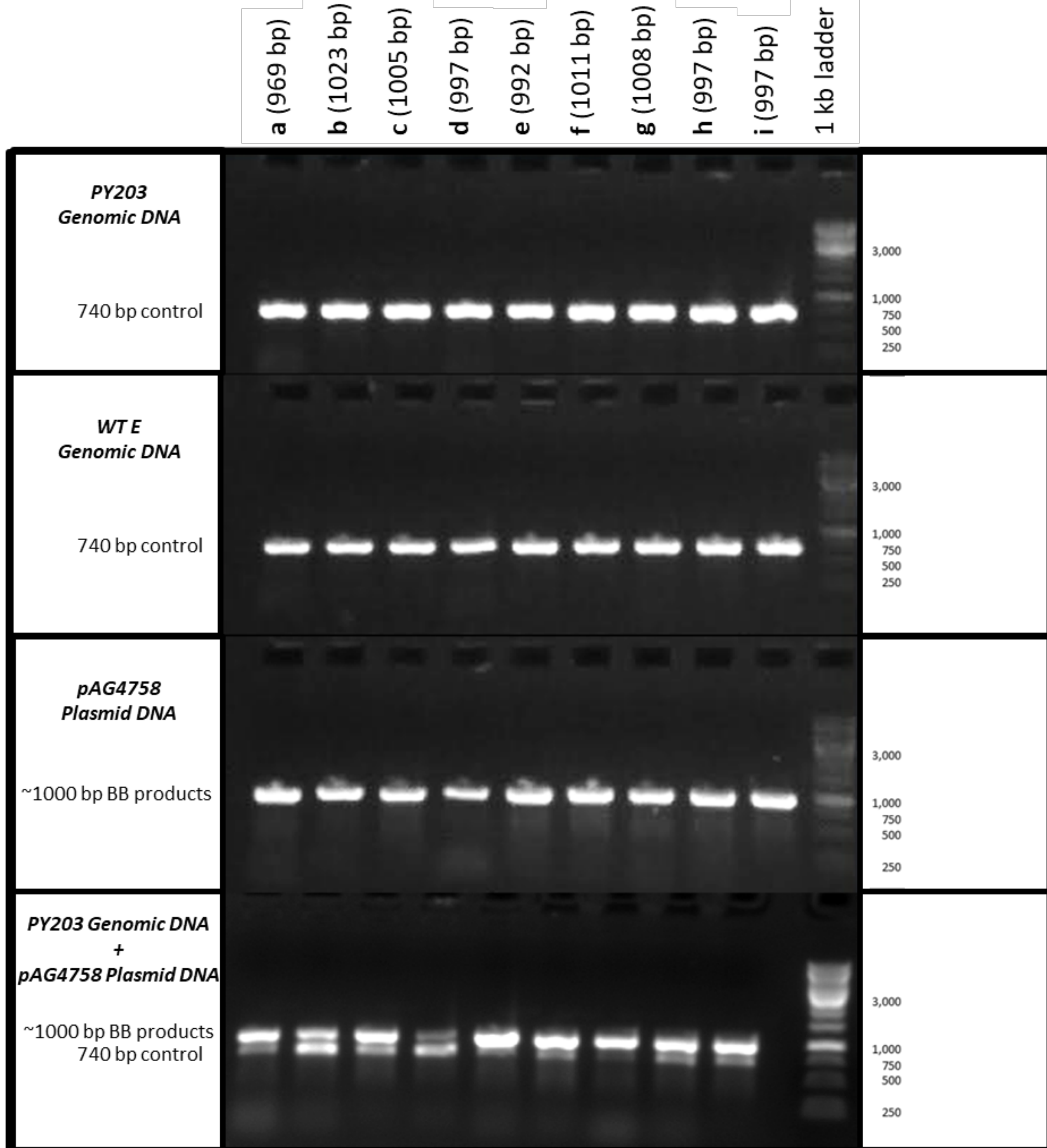
Second, PCR tiling using overlapping primers across the entire pAG4758 plasmid backbone was used to screen PY203 for any detectable backbone DNA (Figure 8 and Table 7). Maize genomic control primers that amplify the endogenous maize α-glucan water dikinase gene (GWD) were included with each PCR reaction as an internal control to confirm the ability to amplify DNA sequences from these samples. As shown in Figure 10, when PY203 genomic DNA was used as the template (top panel), only the GWD gene (the maize control) could be detected, while none of the BB-specific products could be detected. Similarly, when WT genomic DNA from non-transformed maize inbred "E" was used (second panel), only the

GWD gene could be detected. This set of reactions served as the negative control for each of the BB PCRs and as the positive control for the GWD PCR. When pAG4758 plasmid DNA was used as the template with no maize DNA present (third panel), each primer set (a-i) produced the expected ~1 kb product. However, the GWD primer set failed to generate the 740 bp GWD product. This set of reactions served as the positive control for each BB primer set (a-i) and as the negative control for the GWD primer set. Finally, when plasmid DNA was mixed with genomic DNA from Event PY203 (bottom panel), both the BB-specific primer set and the GWD primer set produced the expected products, which demonstrated that both the BB and GWD PCR products could be produced simultaneously if both templates had been present. Therefore, the failure to detect BB products in PY203 genomic DNA (top panel) indicates that the corresponding regions of the vector backbone are not present in Event PY203. Taken together with the data from Southern blots (Figure 9), these results support the conclusion that vector backbone-derived sequences and antibiotic resistance markers are not present in the genome of Event PY203.

Table 7. Description of primer pairs and expected size of DNA products from PCR tiling analysis of Event PY203 genomic DNA. PCR products a-i refer to the regions of the vector backbone that are depicted in Figure 8, along with the nucleotide positions (relative to the map of pAG4758; see also Figure 2 for reference) to which each primer anneals. na, not applicable.

PCR product/ region amplified	Number	Sequence	From	To	length of product
a	603	CGGCGTCAACACGGGATAATA	16251	16271	968
	482	TGACAGGATATATTGGCGGGTAAAC	17195	17219	
b	591	GAAGAACGGAAACGCCTTAAAC	15672	15693	1022
	592	GCCTCGTGATACGCCTATTT	16675	16694	
c	593	CCTATCTCAGCGATCTGTCTATTT	15231	15254	1004
	594	GTCGCCGCATACACTATTCT	16216	16235	
d	595	GATACCTGTCCGCCTTTCTC	14596	14615	996
	596	GCCTCTGTCGTTTCCTTTCT	15573	15592	
e	599	CTTCCGGCTCGATGTCTATTG	13921	13941	991
	600	CAGAGCGCAGATACCAAATACT	14891	14912	
f	597	GGTGTCGGCTTGAATGAATTG	13177	13197	1010
	598	GCTCTGATGCCGCATAGTTA	14168	14187	
g	589	TTGGTGATCTCGCCTTTCAC	12368	12387	1007
	590	GCTCCTTGGCATACGATTAGAG	13354	13375	
h	601	CGCAGAAGCTCCCATCTTT	11859	11877	996
	602	ATCATTCCGTGGCGTTATCC	12836	12855	
i	479	GTTTACACCACAATATATCCTGCCA	11152	11176	996
	588	CGACATTTCTCCAAGCAACTAC	12127	12148	
GWD	531	GACCACACCACTCTATCTGAAC	na		740
	532	ACTGCATGGCCAACTTCT	na		

Figure 10. **PCR results with primers corresponding to pAG4758 backbone (BB) DNA sequences.** BB primer sets, which each amplify ~1kb products (see Table 7), were multiplexed with a maize control primer set (which amplifies a 740 bp region of the endogenous single-copy GWD gene) to test for presence of vector backbone within the genomic DNA of Event PY203. Individual reactions included the GWD-specific primer set as well as one BB-specific primer pair (a-i, as indicated at the top of each lane along with the predicted product sizes of the BB fragments). The templates used in each set of reractions were as follows: Top panel, genomic DNA from Event PY203; second panel, genomic DNA from untransformed (WT inbred "E") genomic DNA; Third panel, pAG4758 plasmid DNA alone; bottom panel, a mixture of PY203 genomic DNA and pAG4758 plasmid DNA.



E3. Sequence of the *phy02* Gene Insertions and the Flanking Maize Genome

As described in § III.E.1 above, the Phy02 expressing maize Event PY203 contains two independent loci in its genome that contain the *phy02* gene that are derived from the T-DNA fragment of pAG4758. These independent loci have been designated locus 3293 and locus 3507. Using genome walking and PCR cloning and sequencing strategies, the nucleotide sequence of each locus, including the inserted DNA and the flanking maize genomic DNA, has been determined. The insertion at locus 3293 includes the complete T-DNA fragment of pAG4758 with three copies of the *phy02* phytase gene. The complete nucleotide sequence and a genetic map of the insertion at locus 3293 and flanking maize genomic DNA is presented in Appendix 1 and Figure 5, respectively. The other insertion at locus 3507 contains a truncated version of the T-DNA fragment that includes two of the three *phy02* genes from the T-DNA fragment. This insertion lacks the third, downstream copy of the *phy02* gene and most of the ZmGlb1 promoter from which the gene is expressed. The complete nucleotide sequence and a genetic map of the insertion at locus 3507 and flanking maize genomic DNA is presented in Appendix 2 and Figure 5, respectively.

The nucleotide sequence of 1812 bp of maize genomic DNA at the right border of the T-DNA of locus 3293 was determined. A BLASTN comparison of this sequence against the publicly available B73 maize genome sequence database (http://www.maizegdb.org) demonstrated that it has 100% identity to sequence on maize chromosome 8 (nucleotide position: 89933570-89935378). This genomic region does not contain annotated genes or defined genetic elements. At the left border of the T-DNA of locus 3293, the nucleotide sequence of 1662 bp of the maize genome was determined. A BLASTN comparison of this sequence against the publicly available B73 maize genome sequence database revealed 99.94% sequence identity to a region of maize chromosome 8 (nucleotide position: 89935403-89937064). Analysis of the complete nucleotide sequence at locus 3293 and comparison to the genomic sequence of the B73 maize chromosome 8 revealed that the T-DNA insertion into the maize genome at locus 3293 resulted in the deletion of 24 bp of maize chromosome 8. It also revealed that the insertion is located 308 bp downstream of the stop codon of the annotated gene model GRMZM2G159344 whose cDNA expression has been confirmed in the inbred line B73 (Sekhon *et al*., 2011). The predicted gene GRMZM2G159344 and its corresponding protein have not been characterized and its function is not known.

The nucleotide sequence of 2098 bp of maize genomic DNA at the right border of the T-DNA of locus 3507 was determined. A BLASTN comparison of this sequence against the publicly available B73 maize genome sequence database demonstrated that it has 100% identity to a sequence on maize chromosome 2 (nucleotide position: 141216135-141214035). This genomic region contains a 99 nt unannotated open reading frame, that has been disrupted by the T-DNA insertion. There is no gene model associated with this ORF in the annotated B73 maize genome, suggesting that it is unlikely to correspond to a functional gene. Furthermore, a BLASTp analysis of the inferred amino acid sequence against the NCBI non-redundant protein sequences database identified no proteins with significant similarity. At the left border of the T-DNA at locus 3507, the nucleotide sequence of 2569 bp of the maize genome was determined. A BLASTN comparison of this sequence against the publicly available B73

maize genome sequence database revealed 99.96% sequence identity to a region of maize chromosome 2 (nucleotide position: 141213994-141211426). This genomic region does not contain annotated genes or defined genetic elements. Comparison of the maize genomic sequences flanking the T-DNA insertion of locus 3507 with the B73 maize genome sequence revealed that the insertion resulted in the deletion of 40 nt of the maize genomic DNA at the insertion site in maize chromosome 2.

The discovery of a deletion of a portion of the T-DNA insert at locus 3507 resulted in a loss of 1517 nucleotides of the proximal portion of the 3004 bp ZmGlb1 promoter and the entire *phy02* gene associated with it, as well as the entire *manA* gene, nos terminator, and left T-DNA border. Agrivida evaluated whether the remaining distal portion of the promoter (now fused to maize genomic DNA) would be sufficient to drive transcription, potentially leading to expression of a novel protein. Liu *et al*. (1998) used a series of 5' terminal deletions to identify the portions of the ZmGlb1 promoter that control transcription and regulate its responsiveness to the plant hormone abscisic acid. They found that deletions of the proximal region of the promoter between 86 and 295 bp upstream of the transcription initiation site completely abolished expression and the plant's ability to respond to abscisic acid. Furthermore, these researchers examined the role of the Em1a element, a 9-bp consensus sequence located approximately 124 bp upstream of the transcription initiation site, and found that changing as few as 3 nucleotides within this element abolished both expression and abscisic acid responsiveness of the promoter. Since the truncated ZmGlb1 promoter found in T-DNA at locus 3507 carries only the most distal portion of the 3 kb promoter and completely lacks the proximal 1.4 kb of the promoter, including the above-mentioned regulatory sequences, Agrivida concludes that the ZmGlb1 fragment within locus 3507 is not transcriptionally active.

E4. Genetic Stability of the Inserts Over Multiple Generations

The genetic stability of the two insertion loci in maize Event PY203 were evaluated by two different methods in four different backcross (BC) generations in an inbred genetic background designated "E". Genomic DNA was isolated from fresh leaf tissue of the successive BC generations BC1E, BC2E, BC3E and BC4E (Figure 3). A PCR primer set consisting of one primer specific to the T-DNA Right Border (RB) element at the edge of the inserted T-DNA and a second primer specific to maize genomic DNA sequence in the flanking region of locus 3923 or locus 3507 was developed and used in PCR reactions with genomic DNA from Event PY203 as the template. Two separate PCR reactions were conducted with each primer set and the resulting amplified DNA fragments were sequenced. Alignments of the sequence of the corresponding region from the genome of Event PY203 with the sequences of the PCR amplified fragments from each of the PY203 backcrossing generations and primer set are shown for loci 3293 and 3507 in Figures 11 and 12, respectively. All four generations, BC1E-BC4E, had identical insertion site sequences for both loci indicating that the sequence of the maize flanking DNA adjacent to the RB of each insertion did not change and was stable across 4 generations. This result demonstrates that both loci that contain the *phy02* gene in Event PY203 are stable and have not moved from their original genomic locations over the four BC generations studied.

Figure 11. Alignment of genomic DNA sequence from locus 3293 of Event PY203 to sequences from PCR amplicons from 4 successive BC generations. PCR products (163 bp) that straddled the leftmost (RB) T-DNA/genomic DNA junction of Locus 3293 were amplified from PY203 plants representing 4 generations of backcrossing (BC1E-BC4E) and sequenced. Maize genomic DNA-derived sequences are highlighted in yellow. T-DNA-derived sequences are highlighted in blue. Nucleotides that were identical over 4 generations are shown at the bottom of the alignment, marked "Consensus." This analysis revealed no changes at the T-DNA/genomic DNA junction over four generations.

```
                   1                                                                              80
       Event PY203 CACCAACCTTCTGGCATTTGAGAATTCATCTCATCCAAAAACTTCTATAATATATCATTCAGTGTCAGGCCTCTCCCTTG
PY203-3293_BC1E-RB CACCAACCTTCTGGCATTTGAGAATTCATCTCATCCAAAAACTTCTATAATATATCATTCAGTGTCAGGCCTCTCCCTTG
PY203-3293_BC2E-RB CACCAACCTTCTGGCATTTGAGAATTCATCTCATCCAAAAACTTCTATAATATATCATTCAGTGTCAGGCCTCTCCCTTG
PY203-3293_BC3E-RB CACCAACCTTCTGGCATTTGAGAATTCATCTCATCCAAAAACTTCTATAATATATCATTCAGTGTCAGGCCTCTCCCTTG
PY203-3293_BC4E-RB CACCAACCTTCTGGCATTTGAGAATTCATCTCATCCAAAAACTTCTATAATATATCATTCAGTGTCAGGCCTCTCCCTTG
         Consensus CACCAACCTTCTGGCATTTGAGAATTCATCTCATCCAAAAACTTCTATAATATATCATTCAGTGTCAGGCCTCTCCCTTG

                   81                                                                                 163
       Event PY203 GCTAGGGCTAGGAGAGGTTCTAGTAACTTGGGGAAGCACTGATAGTTTAAACTGAAGGCGGGAAACGACAACCTGATCATGAG
PY203-3293_BC1E-RB GCTAGGGCTAGGAGAGGTTCTAGTAACTTGGGGAAGCACTGATAGTTTAAACTGAAGGCGGGAAACGACAACCTGATCATGAG
PY203-3293_BC2E-RB GCTAGGGCTAGGAGAGGTTCTAGTAACTTGGGGAAGCACTGATAGTTTAAACTGAAGGCGGGAAACGACAACCTGATCATGAG
PY203-3293_BC3E-RB GCTAGGGCTAGGAGAGGTTCTAGTAACTTGGGGAAGCACTGATAGTTTAAACTGAAGGCGGGAAACGACAACCTGATCATGAG
PY203-3293_BC4E-RB GCTAGGGCTAGGAGAGGTTCTAGTAACTTGGGGAAGCACTGATAGTTTAAACTGAAGGCGGGAAACGACAACCTGATCATGAG
         Consensus GCTAGGGCTAGGAGAGGTTCTAGTAACTTGGGGAAGCACTGATAGTTTAAACTGAAGGCGGGAAACGACAACCTGATCATGAG
```

Figure 12. Alignment of genomic DNA sequence from locus 3507 of Event PY203 to sequences from PCR amplicons from 4 successive BC generations. PCR products (113 bp) that straddled the leftmost (RB) T-DNA/genomic DNA junction of Locus 3507 were amplified from PY203 plants representing 4 generations of backcrossing (BC1E-BC4E) and sequenced. Maize genomic DNA-derived sequences are highlighted in yellow. T-DNA-derived sequences are highlighted in blue. Nucleotides that were identical over 4 generations are shown at the bottom of the alignment, marked "Consensus." This analysis revealed no changes at the T-DNA/genomic DNA junction over four generations.

```
                   1                                                                              80
       Event PY203 AATCTGGGCCGCTATTTCCCTGACCTCACAACCAGCCGCAAAGGCGCGGTGGACATCCTCCTCAAACACTGATAGTTTAA
PY203-3507_BC1E-RB AATCTGGGCCGCTATTTCCCTGACCTCACAACCAGCCGCAAAGGCGCGGTGGACATCCTCCTCAAACACTGATAGTTTAA
PY203-3507_BC2E-RB AATCTGGGCCGCTATTTCCCTGACCTCACAACCAGCCGCAAAGGCGCGGTGGACATCCTCCTCAAACACTGATAGTTTAA
PY203-3507_BC3E-RB AATCTGGGCCGCTATTTCCCTGACCTCACAACCAGCCGCAAAGGCGCGGTGGACATCCTCCTCAAACACTGATAGTTTAA
PY203-3507_BC4E-RB AATCTGGGCCGCTATTTCCCTGACCTCACAACCAGCCGCAAAGGCGCGGTGGACATCCTCCTCAAACACTGATAGTTTAA
         Consensus AATCTGGGCCGCTATTTCCCTGACCTCACAACCAGCCGCAAAGGCGCGGTGGACATCCTCCTCAAACACTGATAGTTTAA

                   81                            113
       Event PY203 ACTGAAGGCGGGAAACGACAACCTGATCATGAG
PY203-3507_BC1E-RB ACTGAAGGCGGGAAACGACAACCTGATCATGAG
PY203-3507_BC2E-RB ACTGAAGGCGGGAAACGACAACCTGATCATGAG
PY203-3507_BC3E-RB ACTGAAGGCGGGAAACGACAACCTGATCATGAG
PY203-3507_BC4E-RB ACTGAAGGCGGGAAACGACAACCTGATCATGAG
         Consensus ACTGAAGGCGGGAAACGACAACCTGATCATGAG
```

The genomic stability of the two insertion loci was also demonstrated using Southern hybridization. Genomic DNA was isolated from plants from each of the above-described PY203 BC generations (BC1E, BC2E, BC3E, and BC4E) and subjected to digestion with the restriction endonuclease *Hin*dIII. From the sequence of the maize genome flanking regions for loci 3293 and 3507 and of the inserted DNA at these loci it is predicted that digestion of genomic DNA from PY203 with *Hin*dIII will produce one unique *Hin*dIII restriction fragment from each of the insertion loci that each contain DNA from the corresponding maize genome flanking region and the T-DNA insertion. The predicted sizes of the *Hin*dIII restriction digest fragments from locus 3923 and locus 3507 of PY203 are 10,192 bp and 11,900 bp, respectively. *Hin*dIII digested genomic DNA from each of the four PY203 BC generations was

subjected to agarose gel electrophoresis, blotted to a membrane and hybridized with a DNA fragment corresponding to the right border region of the T-DNA in both insertion loci. Two hybridizing fragments of approximately 10,000 and 12,000 bp were observed in the DNA from each of the four PY203 BC generations (Figure 13, Table 8). These results confirm the results of analysis of DNA sequence of maize genomic DNA flanking the insertion loci and demonstrate that the maize genomic DNA adjacent to both loci in PY203 transformed maize from four successive backcross generations is unchanged and stable.

Figure 13. Southern blot of four BC generations of Event PY203 hybridized with a T-DNA RB probe. Genomic DNA was isolated from wild type maize and from PY203 plants representing four consecutive generations of backcrossing (BC1E-BC4E), digested with *Hin*dIII, separated by agarose gel electrophoresis, and subjected to Southern blot analysis using the RB probe (see also Figure 5 and Figure 6). *Sal*I and *Not*I-digested pAG4578 was also loaded onto the gel to estimate copy numbers of approximately 1 copy and ¼ copy per genome. From this digest, the RB probe will hybridize to two plasmid fragments (662 bp and 9680 bp). A PCR product that is identical in sequence to the RB probe was also loaded onto the gel as a positive control for hybridization. Sizes of the molecular weight markers are shown to the left of the figure, in bp. The sizes of other observed fragments are listed in Table 8.

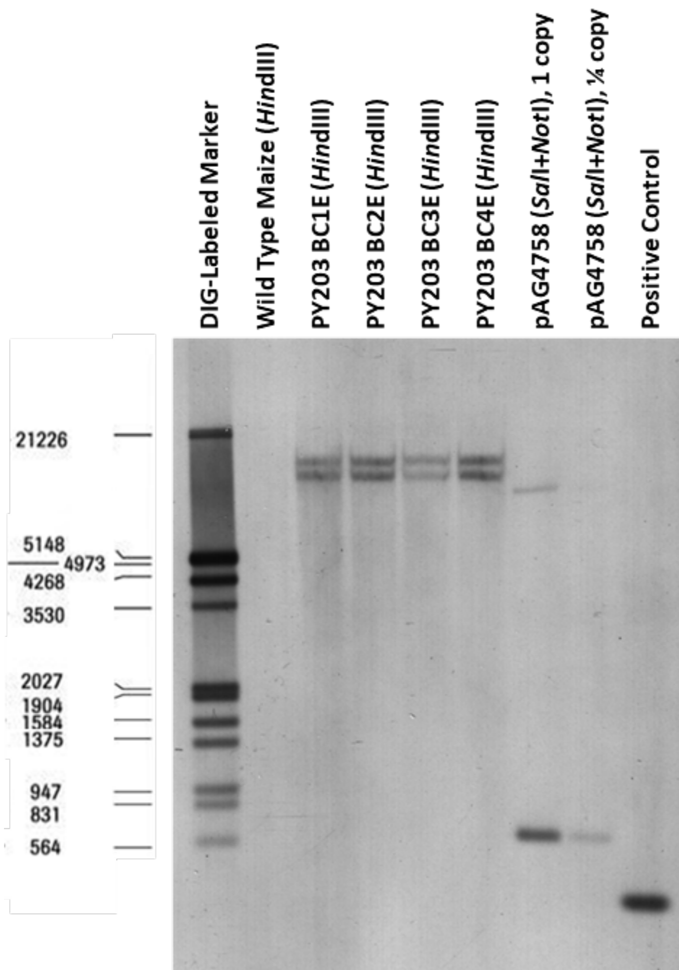


Table 8. The size of predicted and observed DNA restriction fragments from the genome of Event PY203 that hybridize to the T-DNA RB probe.

Sample	Locus	Predicted Fragment Size	Observed Fragment Size
PY203 (*HindIII*)	3293	10,192 bp	≈10,000 bp
PY203 (*HindIII*)	3507	11,900 bp	≈12,000 bp
pAG4758 (*SalI+NotI*), 1 & ¼ copy	--	9,680 bp & 662 bp	≈9,500 bp & ≈700 bp
Wild type maize control	--	--	--
Positive Control	--	274 bp	≈200-300 bp

E.5. Mendelian Inheritance of the *phy02* Gene Insertions

Inheritance of the two *phy02* gene loci of Event PY203 was investigated by PCR using seed from each of four BC generations (BC1E, BC2E, BC3E, and BC4E; Figure 3). DNA from seed of four breeding generations of PY203 was isolated and subjected to PCR using locus-specific primers for each locus such that presence of Locus 3293 can be detected by PCR as a 163 bp PCR product, and presence of Locus 3507 can be detected as a 113 bp PCR product (Figures 14 and 15). This makes it possible to separately identify the two PY203 loci in a population of segregating seed. The number of seed that were observed to carry each locus was scored. The resulting data were statistically analyzed, and the Chi square p-value was calculated using the standard formula, $\Sigma(\text{Observed}_i - \text{Expected}_i)/(\text{Expected}_i)$. Statistical significance was set at $p \leq 0.05$.

Figure 14. Map of PY203 locus 3293 and sequence of a 163 bp fragment amplified from the T-DNA into the flanking genomic region showing relative positions of the locus-specific primers that were used to identify this locus by PCR.

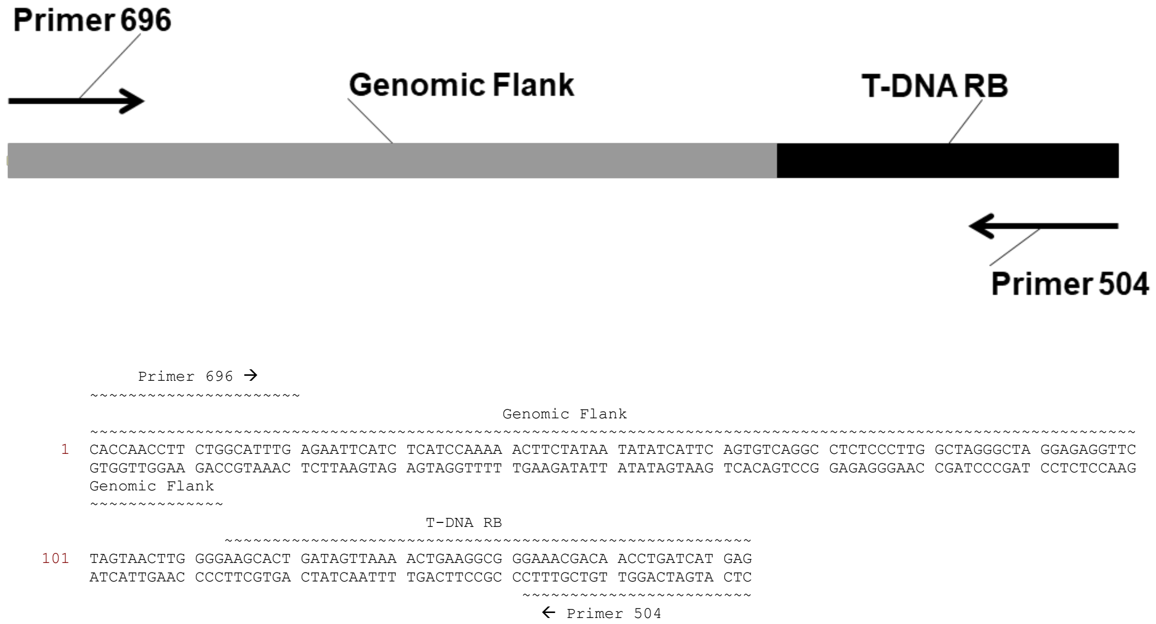
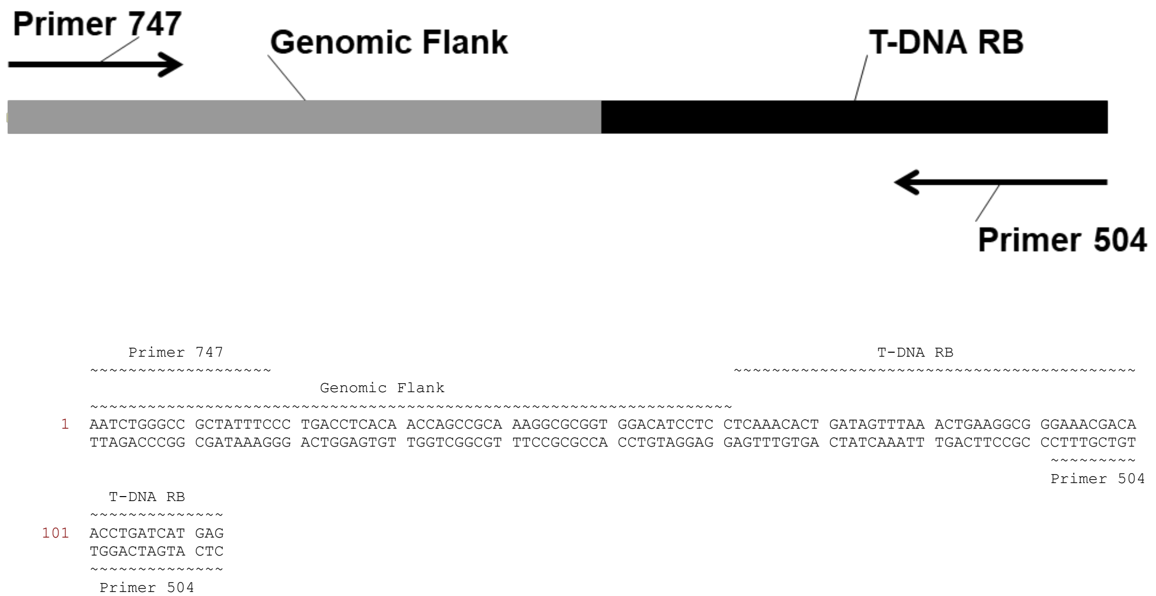


Figure 15. Map of PY203 3507 locus and sequence of a 113 bp fragment amplified from the T-DNA into the flanking genomic region.



The results of the analysis of seed from four BC generations of Event PY203 for the presence of locus 3293 and locus 3507 are presented in Table 9. These results demonstrate that neither of the *phy02* gene loci deviated significantly from expected segregation of a single locus (50%), thereby confirming the expected Mendelian inheritance of both PY203 loci over four generations.

Table 9. Segregation of the PY203 genetic loci in four backcross generations.

Generation	Seed (total)	Seed with 3293	Seed with 3507	Seed Expected per Locus	% 3293 positives	% 3507 positives	Chi Square *p* value (3293)	Chi Square *p* value (3507)
BC1E	129	61	62	64.5	47%	48%	0.538	0.660
BC2E	69	39	32	34.5	57%	46%	0.279	0.547
BC3E	137	72	69	68.5	53%	50%	0.550	0.932
BC4E	115	52	49	57.5	45%	43%	0.305	0.113

IV. Expression of Phy02 Protein in Event PY203

As noted above, the three *phy02* genes present in the T-DNA of plasmid pAG4758 are expressed from three different monocot derived promoters that direct production of the Phy02 phytase protein to the developing seed. In addition, each of the Phy02 gene coding sequences includes at the N-terminus the γ-zein seed storage protein signal sequence (Z27ss) of *Zea mays* that directs proteins to the endoplasmic reticulum (Geli *et al*., 1994). The coding sequences also include the six amino acid maize endoplasmic reticulum retention signal (SEKDEL) that has no impact on the enzymatic activity of the Phy02 phytase. The 19 amino acid signal sequence of the γ-zein protein is typically cleaved from peptides during transport into the endoplasmic reticulum to generate the mature protein (Esen *et al*., 1982). Prat *et al*. (1985) noted that the 19 amino acid signal sequence of γ-zein has structural features commonly found among eukaryotic signal peptides (Walter *et al*., 1984). By comparing the N-terminal amino acid sequence of γ-zein with the coding sequence that includes the signal peptide, Esen *et al*. (1982) determined that the signal peptide is cleaved upon transport into the endoplasmic reticulum immediately following the sequence Ser-Ala-Thr-Ser. The γ-zein signal peptide has been successfully used to target numerous heterologous proteins to the endoplasmic reticulum (de Virgilio *et al*., 2008; Harrison *et al*., 2011; Torrent *et al*., 2009).

Protein from extracts derived from the grain of maize Event PY203 was subject to SDS-PAGE and transferred to a PVDF membrane that was stained with Coomassie Blue without heating to visualize the protein bands. The band corresponding to the correct molecular weight of the Phy02 phytase was excised and the N-terminal amino acid sequence of the protein was determined by Edman degradation by Alphalyse, Inc. (Palo Alto, CA). The predicted cleavage site of the γ-zein signal peptide is immediately after the serine residue at position 19 of the Phy02 phytase protein (Figure 1) (Esen *et al*., 1982). The results demonstrated that the γ-zein signal peptide is cleaved at two different locations within the Phy02 pre-protein since there were two different amino acid residues at the N-terminus of the mature Phy02 phytase protein. The N-terminal amino acid sequence of the mature Phy02 phytase protein was shown to be either AQSEPEL or SEPEL (see Figure 1). In the case of the Phy02 phytase, it appears that the site of cleavage of the γ-zein signal peptide is not precise and cleavage may also occur between residues 21 (Q, glutamine) and 22 (S, serine) to produce a mature protein that begins with the sequence SEPEL. These results confirm that the mature Phy02 phytase protein that is produced in the grain of maize has the N-terminal amino acid sequence that is expected from the coding sequence of the *phy02* gene with the exception of the slight variability due to variable cleavage of the γ-zein signal peptide at the C-terminus of either residue 19 or 21 of the Phy02 phytase pre-protein.

Since the *phy02* genes present in the T-DNA from transformation plasmid pAG4758 are expressed from three different monocot derived seed specific promoters (Table 2), it is expected that nearly all expression of the *phy02* genes in Event PY203 will be in the grain with minimal expression in other tissues. In order to confirm this expectation, the levels of Phy02 protein in Event PY203 were determined in tissues

derived from field grown plants cultivated at two locations (Nash County, IN; York County, NE) in 2016. Four separate field plots were planted with both Event PY203 and a negative-segregant control (nulls) at each location. Plots were organized in a complete randomized block design.

Tissue samples were collected from 2 plants per replicate plot for Event PY203 and from 1 plant per replicate plot for the negative control plants at the following developmental stages (as defined by Mueller and Pope, 2009):

> V8 (8 leaf collars present): roots, leaves, stalks
> R1 (any silk is visible): roots, leaves, pollen
> R6 (physiological maturity; the milk line is no longer visible and a black layer forms at the kernel's attachment): roots, leaves, grain

Developing ears and silks at stages R1 were bagged prior to pollen maturation and were self-pollinated to ensure that the grain produced would be homozygous or null, respectively, for the Phy02 phytase gene.

Tissue samples were collected as follows:

- Roots – excavate root ball
- Leaves – one whole leaf, corresponding to the leaf directly above the uppermost collared leaf. At V8 this corresponds to leaf 9.
- Stalks – a 6-inch stalk section, including the longest internode, cut into smaller sections.
- Pollen
- Grain

Following tissue processing, Phy02 protein was quantified in duplicate aliquots from each individual tissue sample by ELISA (Envirologix "QuantiPlateTM Kit for GraINzyme® Phytase" protocol; Catalog Number AP 071 NW V10). The data presented in Table 10 represent the mean of 16 replicate leaf and root tissue samples or 8 replicate stem, pollen and seed tissue samples from each location at each specific developmental stage. Phytase protein levels were determined on a µg/g dry weight (DW) basis. Purified Phy02 protein from Event PY203 was used as a protein standard and was diluted to 1, 2, 4, 8, 10, 12 and 15 ppb for the ELISA test.

As the *phy02* genes in Event PY203 are under the control of monocot derived endosperm and embryo specific promoters, gene expression is anticipated to be primarily in the seed. The results of this study confirm that production of the Phy02 protein in Event PY203 does occur primarily in the seed with little to no expression in other tissues (Table 10). The average amount of Phy02 protein in seeds of Event PY203 was 6670 µg/g DW at the IN field site and 6258 µg/g DW at the NE location. Overall the amount of Phy02 protein in grain ranged from 4548 to 9079 µg/g DW. The level of Phy02 protein in PY203 leaf, stem, and pollen was either below the limit of detection (LOD) or close to the limit of quantitation (LOQ) (Table 10). The LOD and

LOQ were determined based on the mean plus 3-fold, or 10-fold, respectively, of the standard deviation of the measurement from null samples in each specific tissue at each development stage, respectively. The monoclonal antibody used in the ELISA does not recognize the two endogenous phytases of maize that are expressed primarily during seed germination and at low levels in roots (Maugenest *et al*., 1999). Therefore the low ELISA responses from the null tissues were close to zero and were considered to be background noise.

Table 10. Levels of Phy02 protein measured in tissues derived from maize Event PY203 grown in Nash County, IN and York County, NE.

Tissue	Location	V8 Stage		R1 Stage		R6 Stage	
		Mean ± SD (range)	LOQ/LOD (µg/g)	Mean ± SD (range)	LOQ/LOD (µg/g)	Mean ± SD (range)	LOQ/LOD (µg/g)
Leaves	IN	ND[1]	0.062/0.023	0.011±0.011 (ND - 0.024)	0.000/0.000	0.016±0.013 (ND - 0.039)	0.019/0.008
	NE	0.020±0.009 (ND - 0.034)	0.016/0.005	0.007±0.008 (ND - 0.016)	0.000/0.000	0.03±0.017 (ND - 0.054)	0.009/0.004
Stems	IN	0.106±0.032 (ND -< LOQ)	0.212/0.077				
	NE	0.128±0.035 (0.087 - 0.220)	0.034/0.015				
Roots	IN	0.497±1.349 (ND - 5.004)*	0.045/0.023	0.044±0.014 (0.022 - 0.063)	0.028/0.016	0.178±0.322 (ND - 1.028)	0.052/0.027
	NE	0.058±0.054 (0.023 - 0.188)	0.037/0.015	0.017±0.003 (0.015 - 0.023)	0.021/0.008	0.031±0.017 (ND - 0.068)**	0.039/0.017
Pollen	IN			ND	0.048/0.030		
	NE			0.047±0.069 (ND - 0.237)	0.068/0.035		
Kernels	IN					6670.3±1138.6 (5240.2 - 9079.5)	0.000/0.000
	NE					6258.3±1072.8 (4548.0 - 7926.8)	0.000/0.000

[1]Not detected.

*2 of the 8 root samples collected at the V8 stage demonstrated high phytase levels compared to the other samples (5.004 and 1.433 µg/g); all other samples had less than 0.087 µg/g Phy02 phytase.

**1 of the 8 root samples collected at the V8 stage demonstrated high phytase levels compared to the other samples (1.028 µg/g); all other samples had less than 0.175 µg/g Phy02 phytase.

V. The Phy02 Phytase Protein

A. Enzyme Name

The phytase protein produced in Event PY203 maize is designated Phy02.

B. Enzyme Identity

The enzyme properties of the Phy02 phytase are described below:

Classification:	6-Phytase, a histidine acid phosphatase
IUBMB classification:	EC 3.1.3.26
CAS No.:	9001-89-2

Phytase enzymes catalyze the step-wise hydrolysis of *myo*-inositol hexakisphosphate to orthophosphate and lower *myo*-inositol phosphates. The lower *myo*-inositol phosphates released from the reaction may become substrates of the enzyme and further cycles of activity result in the release of more orthophosphate groups and ever lower *myo*-inositol phosphates. The Phy02 phytase is derived from the native AppA phytase of *Escherichia coli* strain K12. This phytase is classified as a 6-phytase since it preferentially initiates the removal of the phosphate at the L6 position of the *myo*-inositol ring (Konietzny and Greiner, 2002).

C. Source and Amino Acid Sequence of the Phy02 Phytase

The *E. coli appA* phytase gene (GenBank accession No. JF274478; https://www.ncbi.nlm.nih.gov/nuccore/JF274478.1) was optimized using a combination of modeling and site-directed mutagenesis (Short, 2001) to generate a version of the phytase protein with (1) increased thermo-tolerance and (2) increased lability in a simulated gastric environment. Thermo-tolerance is a desirable trait for commercial feed enzymes since many animal feeds are produced by a pelleting process that involves a heat treatment that inactivates thermolabile enzymes. Further, examination of the sensitivity of the AppA protein to digestion in a simulated gastric environment revealed that it is resistant to digestion in this environment. Although resistance to gastric digestion has been hypothesized to correlate with a higher potential for a protein to be allergenic, experimentally this correlation has not been validated (Fu *et al*., 2002). Nevertheless, the stability of a new protein in the simulated gastric environment is considered in the evaluation of the safety of new proteins in food. The Phy02 phytase is 45,684 kDa in size and its amino acid sequence is presented in Figure 1 (§ III.C).

D. Phytases in Other Organisms and Prior Exposure

Phytase enzymes are ubiquitous in nature and are produced by many microbes and plants. Phytases in bacteria have been identified in *Pseudomonas* sp. (Richardson and Hadobas, 1997), *Bacillus subtilis* (Powar and Jagannathan, 1982; Shimizu, 1992;

Kerovuo *et al*., 1998), *B. amyloliquefaciens* (Kim *et al*., 1998a), *Klebsiella* sp. (Tambe *et al*., 1994; Greiner *et al*., 1997), *E. coli* (Greiner *et al*., 1993) and *Enterobacter* (Yoon *et al*., 1996). Pure culture studies of anaerobic ruminal bacteria have clearly demonstrated phytate-degrading activity in numerous strains, particularly in *Selenomonas ruminantium*, *Megasphaera elsdenii*, *Prevotella* sp., and *Mitsuokella multiacidus* (Yanke *et al*., 1998). In addition, microorganisms used in food fermentation have been shown to degrade phytate during the fermentation process and so are assumed to produce phytase. Some yeasts that are used in food production, such as *S. cerevisiae* (Nakamura *et al*., 2000) and *Schwanniomyces castellii* (Lambrechts *et al*., 1992), produce phytase enzymes. Phytase enzymes are also widespread in filamentous fungi, having been identified in *Aspergillus niger, Aspergillus terreus, Aspergillus oryzae, ,* and *Penicillium simplicissimum* among others (Konietzny and Greiner, 2002).

In plants, phytase enzymes occur mostly in grains, seeds and pollen of higher plants, such as cereals, legumes, oilseeds and nuts, but low levels of phytase activity is also found in the roots of plants. Phytic acid accounts for up to 80% of the phosphorus in the seeds of cereals and legumes and is the primary storage form of phosphate in these materials (Reddy *et al*., 1982). Phytase enzymes in grains, seeds and pollen have been shown to be responsible for phytate degradation during germination to make phosphate, minerals and myoinositol available for the purpose of plant growth and development (Reddy *et al*., 1989). It has been suggested that phytase in plant roots increases the availability of soil phosphorus (Hayes *et al*., 2000). Plant grains, seeds and pollen contain both constitutive and germination-inducible phytases (Lin *et al*., 1987; Greiner *et al*., 2000) and large increases in phytase activities have been extensively reported in germinating seeds as well as in germinating pollen. Significant levels of endogenous phytase activity (>1000 FTU/kg) have been reported in rye, wheat, rye bran and wheat bran (Viveros, 2000) and multiple forms of phytase have been reported in barley, maize, rice, wheat, spelt, soybean, rape seed, pumpkin, lily (Konietzny and Greiner, 2002).

Bacteria that inhabit the intestinal tracts of animals are known to produce phytase and phytase activity has been measured in the gastrointestinal tracts of animals, including humans (Iqbal, 1994). In ruminants, production of phytase by anaerobic ruminate bacteria is most likely responsible for the increased rate of phytate degradation that has been noted in these animals (Yanke, 1998).

Phytases are included in human dietary supplements currently marketed in the U.S. and are suggested to improve the digestion of foods and the absorption of minerals. The absorption of iron in humans has been shown to be dramatically improved when at least 2 of the 6 phosphate groups of phytic acid are removed by phytase (Sandberg, 1996), thereby demonstrating the positive nutritional effects of phytase in alleviating the anti-nutritive properties of phytic acid. General Nutrition Centers (Pittsburgh, PA) markets a dietary supplement (Natural Brand™ Super Digestive Enzymes; GNC, 2018) consisting of a mixture of different enzymes including phytase. Nutriteck Inc. (Montreal, Canada) markets a dietary phytase supplement

that contains 200 FTU/g of a phytase derived from *Aspergillus niger* (Nutriteck, 2018). Global Healing Center (Houston, TX) markets a phytase-containing enzyme mixture named VeganZyme® (GHC, 2018). CereCalase (NEC, 2018) is another phytase-containing human dietary supplement. It is produced by the National Enzyme Company (Forsyth, MO) and contains a phytase from *A. niger*. Most of the phytase enzymes included in the abovementioned dietary supplement products are derived from *Aspergillus niger*. The AppA phytase that is nearly identical to the Phy02 phytase has been shown to be structurally similar to the phytase from *Aspergillus niger* (Lim *et al*., 2000).

Phytase enzymes have been added to the feed of monogastric animals for approximately three decades in order to increase the digestibility of phytic acid derived phosphorus in the feed. Phytase is the most widely used feed enzyme and globally it is included in approximately 90% of poultry and 70% of swine diets. Based on the above, it is well established that humans and animals have had a long and extensive exposure to phytase enzymes in their diet.

E. Summary of the Food and Feed Safety of the Phy02 Protein

A detailed evaluation of the food and feed safety of the Phy02 phytase protein has been submitted to the FDA in a food and feed safety and nutritional assessment for maize Event PY203. A summary of that information is provided below:

1. There is a long history of safe exposure of phytases in human food from natural sources and in dietary supplements. Likewise, phytase enzymes have been added safely to the feed of monogastric animals for decades to improve phosphorus digestibility.

2. Differing in only 16 out of the total of 411 amino acids in the mature protein, the Phy02 phytase is nearly identical and substantially equivalent to a commercial phytase (Quantum®, AB Vista) (CVM, 2017a) for which extensive safety studies have demonstrated no mammalian toxicity (EFSA, 2008).

3. The application of a decision tree developed by Pariza and Johnson (2001) for the evaluation of the safety of enzymes used in food processing indicates that the Phy02 phytase is safe in human food. The authors recognized that protein engineering techniques that are applied to food and feed enzymes to improve the performance of the enzymes and which result in a small number of amino acid changes will not result in any increased toxicity of the new enzyme relative to its progenitor.

4. A global sequence similarity search of the Phy02 amino acid sequence was conducted using the NCBI Protein dataset and no similarities to known toxic proteins was discovered.

5. Bioinformatic analyses of the Phy02 amino acid sequence and comparison

with a database of known or suspected allergenic proteins revealed no similarities to known or putative protein allergens.

6. The Phy02 phytase protein in simulated gastric fluid containing pepsin was rapidly digested, thereby indicating that it is unlikely to be an allergenic protein.

7. The donor organism for the Phy02 protein is not known to be a source of allergenic proteins and maize, the Phy02 production host, has a long and safe history of use as human food and animal feed.

8. Analysis of the Phy02 phytase protein expressed in Event PY203 revealed no evidence of post-translational glycosylation.

The safety of the Phy02 phytase is further supported by two tolerance studies, one in poultry and another in swine, that were conducted by Agrivida, Inc. In the poultry study a feed containing approximately 60,000 units of Phy02 phytase activity (FTU) per kilogram was fed to broiler chickens from 1 to 42 days of age. The chickens showed improved performance as expected from the addition of a phytase enzyme to the feed and demonstrated no signs of toxicity or adverse health (CVM, 2017a). Blood chemistry and hematology analyses as well as examination of organs after euthanasia demonstrated no differences or signs of toxicity between the Phy02-treated and untreated groups. Similarly, a tolerance study in swine in which the feed was amended with the Phy02 phytase to contain 45,000 FTU phytase per kg showed that the Phy02 treated animals had good performance and no adverse health symptoms (GRAS Notice AGRN #27 currently under review by FDA/CVM). As in the poultry tolerance study there were no hematological or tissue differences between the Phy02-treated and untreated animals.

A commercial phytase feed product named Quantum® (AB Vista) has been used safely and effectively in poultry and swine diets for the past decade. The phytase in Quantum® is also derived from the native *E. coli* AppA phytase and it is very similar to the Phy02 phytase, differing in only 12 amino acids (CVM, 2017a). Grain from transgenic maize expressing a gene encoding the Quantum® phytase was used to replace all of the corn in the feed of two independent poultry feeding trials. The feed contained up to approximately 363,000 FTU of phytase activity per kilogram of diet, and after 14 days the chickens demonstrated good performance and no adverse health effects (Nyannor and Adeola, 2008; Nyannor *et al*., 2009). Nyannor *et al*. (2007) also tested the effects of high doses of Quantum® phytase in swine demonstrating that feeding up to 49,500 FTU/kg feed was safe and effective. The safety of the Quantum® phytase was also demonstrated in a subchronic 90-day rat feeding trial in which 400 mg of purified phytase enzyme representing 462,000 FTU/kg body weight/day was administered orally to rats without any indications of toxicity (EFSA, 2008). These studies with the Quantum® phytase demonstrate that it is safe for animals at very high doses. Due to the high level of identity between the Quantum® and Phy02 phytases it is reasonable to conclude that the Phy02 phytase

is equally as safe.

VI. The Phosphomannose Isomerase Protein

The PMI protein produced in Event PY203 plants is encoded by the native *manA* gene from *E. coli* strain K12 (Miles and Guest, 1984). PMI catalyzes the reversible inter-conversion of mannose-6-phospate and fructose-6-phosphate and requires zinc for activity. The PMI enzyme is specific for mannose-6-phospate and fructose-6-phosphate and no other natural substrates for PMI are known (Freeze, 2002). Plant cells expressing the *manA* gene are capable of survival and growth in the presence of mannose as the only or primary carbon source. Under the same conditions, plant cells lacking PMI accumulate mannose-6-phosphate and fail to grow. The *manA* gene was used as a selectable marker for the transformation of plasmid construct pAG4758 containing the *phy02* gene. The *manA* gene and associated regulatory sequences introduced into Event PY203 are identical to the genetic sequences for which Syngenta Seeds was granted an exemption for the requirement of a tolerance in all plants (https://www.federalregister.gov/documents/2004/05/14/04-10877/phosphomannose-isomerase-and-the-genetic-material-necessary-for-its-production-in-all-plants). The nucleotide sequence of the single copy of this marker gene in Event PY203 was confirmed to be identical as well. Further, the PMI protein expressed in Event PY203 was readily detectable using commercially available PMI test strips and ELISA plates. The food safety of this protein has been extensively characterized (Privalle *et al*, 2006). This selectable marker has been widely used in maize and other crop species that have been approved for food use by regulatory authorities in the United States. This weight of evidence and history of safe use supports Agrivida's conclusion that the PMI protein expressed in maize Event PY203 is safe for food use and the environment.

VII. Phenotypic Evaluation of Event PY203

An assessment of the phenotypic properties of maize Event PY203 was conducted in order to demonstrate that Event PY203 does not have any unintended or unanticipated traits and will not present a plant pest risk when released into the environment.

The introduction of the *phy02* gene into Event PY203 was intended to result only in the production of phytase in the grain of the maize. It was not intended to alter other natural phenotypes of the maize plant or the methods used in its cultivation. In order to confirm that the typical agronomic characteristics of maize Event PY203 were not altered by the genetic modifications used to create it, the agronomic characteristics of Event PY203 and a near-isogenic null segregant used as a control were compared in field studies conducted at six locations in the corn belt of the United States in 2017 (under USDA notifications 17-044-101n and 17-052-102n) and at two locations in Argentina in the 2016/17 growing season.

A. Agronomic Trial Methodology

The agronomic assessment trials were conducted at three separate locations in IA (Dallas, Scott, and Jasper counties), two locations in NE (York and Antelope counties), and one location in OH (Miami county) in the growing season of 2017. Similar trials using the same trial protocol as was used in the trials conducted in the United States were conducted at two sites in Argentina located near the towns of Rojas and Salto, which are separated by 52 km (32 miles), during the 2016/17 growing season. At each trial location Event PY203 and a near-isogenic null segregant hybrid without the *phy02* gene insertion were planted in a randomized complete block design with 4 replicate plots per entry per location. Each plot consisted of 4 rows 17.5 feet in length planted with 30 inch spacing and a plant population equivalent to 34,000 plants per acre. Plants in the center two rows were assessed for agronomic characteristics. The only exception to this protocol was that at two of the locations in the United States, six rows were planted in order to provide plants for use in a nutritional composition study (§ IV). The agronomic practices for the cultivation of the trials reflected common agronomic practices used for maize cultivation. All trials were cultivated as conventional non-transgenic maize with respect to herbicides. Weed control was achieved by hand weeding as needed or by the application of selective herbicides approved for use on maize. Insect control was achieved by the application of maize-approved insecticides as needed.

A list of the agronomic assessments that were conducted in all trials is presented in Table 11. Abiotic stressors (e.g. flood, frost, hail, high temperature, nutrient deficiency, sunscald, and wind), as well as differences in the incidence of insect predation (e.g. aphids, *Colaspis* spp., cutworms, grasshoppers, sap beetles, woolybear caterpillars, stink bugs, and thrips) and plant disease (e.g. anthracnose, *Cercospora zeae-maydis*, *Colletotrichum graminicola*, dumping off, ear rot,

Helminthosporium maydis, *Helminthosporium turcicum*, Mal de Río IV virus, *Pseudomonas alboprecipitans*, rust, stalk rot, and *Ustilago maydis*) between the PY203 and null control plants were evaluated at all growth stages up to maturity. Differences in the populations of other biota such as bees, butterflies, ladybeetles, grasshoppers and other insects, birds and mammals in the PY203 and null control fields were also observed.

Table 11. Description of the agronomic characteristics that were assessed for maize Event PY203.

General Characteristic	Trait	Growth Stage *	Description	Scale
Emergence	Early Stand count (EmStCo)	V2 - V4	Plant emergence (14 days after planting)	Percentage of plants to emerge after germination per plot
Vegetative characteristics	Ear height (Ear Ht)	Maturity R5	5 plants per plot; Measure from ground to point of attachment of primary ear	Avg measurement from 10 plants in inches
	Plant height (Plant Ht)	Maturity R5	5 plants per plot; Measure from ground to base of flag leaf	Avg measurement from 10 plants in inches
	Stay green	R6 (black layer)	% plant tissue per plot that is green	Scale where 0% = entire plant is brown; 100% = entire plant is green
	Final stand count (FiStCo)	R6 Pre- harvest	Total number of plants in two rows per plot	Number of plants per plot
	Stalk lodging	R6 Pre- harvest	Plants broken below ear	Percentage of total (% per plot)
	Root lodging	R6 Pre- harvest	Plants leaning greater than 30° from vertical	Percentage of total (% per plot)
Reproductive characteristics	Days to 50% pollen shed	Pollen- shed	Days from planting to when 50% of plants are shedding pollen.	Number of days
	Days to 50% silking	Silking	Days from planting t when 50% of plants have silks emerged from the primary ea	Number of days
	Dropped ears	Pre- harvest	Ears on the ground, no longer attached to the stalk.	Ears per plot (# per plot)
	Barrenness	Pre- harvest	Plants with no ears.	Plants per plot
	Grain Weight	Harvest	Grain weight corrected to 15% moisture.	Pounds per plot
	Grain moisture	R6 Harvest	Moisture content of harvested, shelled grain.	Percent moisture
	Grain test weight	R6 Harvest	Harvested, shelled grain density	Pounds

*Abbreviations for Maize Growth Stages (Abendroth et al., 2011):

Vegetative:
V2 first two leaves collared
V3 first three leaves collared
V4 first four leaves collared
V5 first five leaves collared
V6 first six leaves collared
V7 first seven leaves collared
V8 first eight leaves collared
V9 first nine leaves collared
V10 first ten leaves collared
V11 first eleven leaves collared
V12 first twelve leaves collared
V13 first thirteen leaves collared
VT tassel

Reproductive:
R1 silking
R2 blister
R3 milk
R4 dough
R5 dent
R6 physiological maturity

In order to compare the growth habits and vegetative vigor of Event PY203 and the near isogenic control line, assessments of seedling emergence (Early Stand Count), ear height, plant height, green plants (Stay Green), final stand count, stalk lodging and root lodging were conducted. Reproductive parameters of the two entries were assessed through measurements of days to 50% pollen shed and silking, number of dropped ears, barrenness, grain weight, grain moisture, and grain test weight.

The two trial locations in Argentina near the cities of Rojas and Salto are located west of Buenos Aires in the Humid Pampas region, a region of flat, fertile grassland of loessic origin. It has an average precipitation of 900 mm (~35 inches) per year. Humid, temperate weather characterizes spring and fall and summer days can be hot.(Government of Argentina, 2010). Maize and soybeans are the prominent crops cultivated in this region of Argentina. The climate and environmental conditions in this region are similar to that of the Midwestern U.S. and other regions in the U.S. where maize is commonly cultivated and therefore the agronomic data collected at these locations is representative of data collected in the U.S. corn belt.

The experimental design was a randomized complete block design and location was used as the blocking criteria. Each of the 8 blocks had 4 replicate plots to which the treatments (n = 4 per treatment) were randomly distributed. Plots were used as experimental unit (n=32 per treatment) for each analyzed variable. Data was analyzed using Fit Least Squares procedure of the JMP software (version 12, SAS Institute Inc., Cary, NC). The ANOVA model included treatment and block. Least Square Mean values were separated using Tukey's honesty significant difference procedure and P-values < 0.05 were considered significant in all comparisons.

The results of the assessments of vegetative and reproductive characteristics conducted for PY203 and the null control plants are presented in Tables 12 and 13, respectively.

Table 12. Assessment of vegetative characteristics of maize Event PY203. Abbreviations for the measured characteristics are presented in Table 11. ND = Not Determined.

Location	Trt	Rep	EmStCo	Ear Ht	Plant Ht	Stay Green	FiStCo	Stalk Lodge	Root Lodge
Antelope	PY203	1	65	52	96	40	64	0	0
		2	59	44	84	50	59	0	0
		3	51	45	81	50	51	0	0
		4	58	40	82	60	58	0	0
	PY203Null	1	59	52	94	40	59	0	0
		2	59	49	86	50	59	0	0
		3	58	40	81	40	58	0	0
		4	61	42	82	50	61	0	0
Dallas	PY203	1	68	44.3	91.7	ND*	64	10.9	0
		2	67	45.7	93.6	ND	65	6.2	0
		3	65	47.2	93.4	ND	61	9.8	0
		4	70	47.1	96.8	ND	64	10.9	0
	PY203Null	1	66	48.0	93.0	ND	63	20.6	0
		2	69	49.3	95.8	ND	65	21.5	0
		3	69	46.9	96.7	ND	65	16.9	0
		4	58	44.6	95.5	ND	56	10.7	0
Jasper	PY203	1	63	42.5	87.4	70	63	11.1	0
		2	66	45.3	91.7	75	64	17.2	0
		3	71	46.6	92.0	70	64	0.0	0
		4	70	45.8	92.4	70	64	1.6	0
	PY203Null	1	70	47.1	91.6	75	64	0.0	0
		2	64	44.5	90.4	80	64	18.8	0
		3	69	45.1	90.2	75	64	0.0	0
		4	65	50.2	93.4	80	64	1.6	0

Table 12. Assessment of vegetative characteristics of maize Event PY203 (continued). ND = Not Determined.

Location	Trt	Rep	EmStCo	Ear Ht	Plant Ht	Stay Green	FiStCo	Stalk Lodge	Root Lodge
Miami	PY203	1	62	52.3	103.2	35	63	0.0	0
		2	62	51.6	102.3	40	66	0.0	0
		3	68	52.1	101.0	50	64	5.0	0
		4	69	48.8	99.6	50	66	0.0	0
	PY203Null	1	70	52.3	101.1	50	62	0.0	0
		2	61	52.8	103.4	45	63	5.0	0
		3	66	54.6	104.5	40	68	5.0	0
		4	65	52.5	104.0	45	63	0.0	0
Rojas	PY203	1	60	38.5	78.0	ND	60	3	1
		2	48	38.3	78.7	ND	48	3	0
		3	61	38.0	81.1	ND	61	1	1
		4	54	39.8	79.8	ND	54	0	2
	PY203Null	1	79	42.6	89.4	ND	79	27	0
		2	70	40.8	84.1	ND	70	11	0
		3	70	38.1	74.6	ND	70	6	3
		4	65	39.7	81.2	ND	65	4	0
Salto	PY203	1	55	32.9	75.1	ND	55	8	4
		2	60	41.3	80.0	ND	60	2	0
		3	59	37.6	81.6	ND	59	1	2
		4	49	36.3	79.1	ND	49	7	10
	PY203Null	1	68	37.8	75.7	ND	68	2	0
		2	68	38.6	76.5	ND	68	3	5
		3	63	39.7	79.0	ND	63	4	7
		4	67	40.3	77.6	ND	67	6	22

Table 12. Assessment of vegetative characteristics of maize Event PY203 (continued).

Location	Trt	Rep	EmStCo	Ear Ht	Plant Ht	Stay Green	FiStCo	Stalk Lodge	Root Lodge
Scott	PY203	1	67	48	91	5	62	1.6	0.0
		2	69	44	87	5	64	0.0	0.0
		3	69	48	88	5	64	3.1	0.0
		4	68	45	88	5	62	1.6	4.8
	PY203Null	1	71	48	93	5	66	6.1	0.0
		2	64	46	86	5	64	3.1	1.6
		3	68	46	88	5	63	0.0	0.0
		4	71	44	86	5	65	6.2	1.5
York	PY203	1	64	56	98	60	61	5.0	0.0
		2	63	56	98	70	64	0.0	0.0
		3	62	59	103	60	66	5.0	0.0
		4	64	57	102	60	61	5.0	0.0
	PY203Null	1	67	55	100	60	63	5.0	0.0
		2	63	58	100	60	61	5.0	0.0
		3	63	54	97	60	61	5.0	0.0
		4	62	50	94	60	65	5.0	0.0

Table 13. Assessment of reproductive characteristics of maize Event PY203.

Location	Trt	Rep	Days 50 PS	Days 50 Silk	Drop Ears	Barren	Grain Wt	Grain Moist	Test Wt
Antelope	PY203	1	61	66	0	0	27.62	22.2	58.7
		2	61	67	0	0	27.14	22.3	58.7
		3	62	67	0	0	27.20	22.9	58.7
		4	60	67	0	0	27.39	22.9	58.9
	PY203Null	1	61	66	0	0	31.25	22.3	60.8
		2	61	65	0	0	29.61	21.8	60.4
		3	62	67	0	0	27.64	22.0	60.0
		4	61	67	0	0	27.36	22.0	58.3
Dallas	PY203	1	56	56	1	0	21.11	20.6	51.3
		2	56	56	0	0	20.95	19.5	52.0
		3	56	56	0	0	17.83	19.5	50.5
		4	56	56	0	0	23.62	19.2	51.7
	PY203Null	1	56	56	1	0	24.59	18.5	55.6
		2	56	56	1	0	25.51	18.2	55.0
		3	56	56	0	0	25.56	17.3	54.8
		4	56	56	0	0	23.03	18.2	55.2
Jasper	PY203	1	58	58	0	0	24.22	19.3	51.9
		2	58	58	0	0	22.40	19.9	49.7
		3	58	58	0	0	25.01	19.7	51.6
		4	58	58	0	0	25.69	19.8	52.1
	PY203Null	1	58	58	0	0	28.01	18.6	54.8
		2	58	58	0	0	28.15	17.8	54.0
		3	58	58	0	0	27.05	17.9	55.3
		4	58	58	0	0	28.10	18.5	54.1

Table 13. Assessment of reproductive characteristics of maize Event PY203 (continued).

Location	Trt	Rep	Days 50 PS	Days 50 Silk	Drop Ears	Barren	Grain Wt	Grain Moist	Test Wt
Miami	PY203	1	61	62	0	0	22.25	26.1	50.8
		2	60	62	0	0	22.51	25.7	50.8
		3	60	62	0	1	20.99	26.0	50.8
		4	61	62	0	1	20.38	25.2	51.8
	PY203Null	1	60	62	0	1	22.14	22.4	54.0
		2	61	61	0	1	25.55	22.7	54.0
		3	61	62	0	0	21.14	21.3	54.7
		4	61	62	0	1	21.71	22.0	54.2
Rojas	PY203	1	62	61	1	0	16.0	19.5	68.2
		2	62	62	0	2	11.3	20.8	65.6
		3	61	60	2	0	21.1	20.4	66
		4	62	62	2	0	17.0	19	68
	PY203Null	1	61	61	2	0	15.1	18.8	69.6
		2	62	61	1	0	18.2	18.4	70.8
		3	62	62	4	0	14.5	19.8	69.5
		4	61	61	5	0	16.2	17.1	67.4
Salto	PY203	1	60	59	4	0	9.0	23.1	59.9
		2	59	60	5	0	10.2	23	61.2
		3	60	60	6	0	10.8	21.9	60.8
		4	60	60	2	0	8.5	21.3	62.8
	PY203Null	1	61	60	3	0	8.9	21.5	60.6
		2	59	59	6	0	11.9	22.1	61
		3	60	58	4	0	13.3	22.1	61.7
		4	61	60	9	0	9.4	20.9	60.4

Table 13. Assessment of reproductive characteristics of maize Event PY203 (continued).

Location	Trt	Rep	Days 50 PS	Days 50 Silk	Drop Ears	Barren	Grain Wt	Grain Moist	Test Wt
Scott	PY203	1	65	67	0	0	22.72	18.7	53.9
		2	65	67	0	0	20.28	18.8	54.3
		3	67	69	0	0	22.75	18.7	55.0
		4	65	67	0	0	20.41	19.2	54.4
	PY203Null	1	67	69	0	0	28.35	17.4	57.6
		2	65	67	0	0	25.45	18.2	56.4
		3	65	67	1	0	24.41	18.0	56.6
		4	65	67	0	1	25.89	17.6	57.3
York	PY203	1	56	61	1	0	17.68	18.0	57.1
		2	56	59	0	0	19.04	17.8	57.9
		3	56	59	0	0	18.49	18.1	57.0
		4	56	60	1	0	17.75	17.9	57.1
	PY203Null	1	56	60	2	0	17.87	17.3	59.1
		2	55	57	1	0	21.26	17.7	59.0
		3	57	58	1	0	19.64	17.8	58.1
		4	56	60	2	0	18.77	17.8	58.7

Table 14. Statistical comparison of the vegetative and reproductive characteristics for maize Event PY203 and the non-transgenic null control line across all locations. Abbreviations for the measured characteristics are presented in Table 11. Data that are significantly different (P<0.05) from one another are indicated by different letter designations (A, B).

	EmStCo	Ear Ht	Plant Ht	Stay Green	FiStCo	Stalk Lodge	Root Lodge
PY203	62.69 B	45.81	89.89	46.50	60.94 B	3.719 B	0.775
PY203null	65.88 A	46.55	90.15	46.50	64.25 A	6.203 A	1.253
Std Error	0.84	0.46	0.58	1.07	0.78	0.852	0.471
P Value	0.0098	0.27	0.76	1.00	0.0038	0.044	0.48

	Days 50 PS	Days 50 Silk	Drop Ears	Barren	Grain Wt	Grain Moist	Test Wt
PY203	59.81	61.38	0.781 B	0.125	19.98 B	20.84 A	56.54 B
PY203null	59.91	61.09	1.344 A	0.125	22.05 A	19.50 B	58.72 A
Std Error	0.10	0.13	0.174	0.060	0.33	0.13	0.18
P Value	0.52	0.12	0.026	1.000	<0.0001	<0.0001	<0.0001

The statistical comparison of the agronomic characteristics presented in Table 14 demonstrate that there were no statistical differences in ear height, plant height, stay green, root lodging, days to 50% pollen shed, days to 50% silking, or barren plants between Event PY203 and the near-isogenic, non-transgenic control line (Table 14). Although there were statistically significant differences in stalk lodging and dropped ears between Event PY203 and the control line, the values for each of these characteristics were lower for Event PY203 compared to the control. The emergent stand count was slightly lower for Event PY203 compared to the control line and this difference was statistically significant (62.69 vs. 65.88; Table 14). This also resulted in a lower final stand count for Event PY203. The lower stand count for Event PY203 resulted in statistically lower grain weight for Event PY203. However, when the grain weight data is normalized on a per plant basis the adjusted grain weight data for Event PY203 and the control line is much closer and not significantly different (21.07 vs. 22.05 lbs per plot for Event PY203 and the control, respectively).

The grain test weights for Event PY203 were also significantly lower than those measured for the control line. Lower grain test weights are often seen in kernels that have a greater abundance of non-vitreous, floury or "opaque" endosperm. In maize, the floury or opaque phenotypes are associated with changes in the formation of protein storage bodies (Gerde, *et al.*, 2016). In vitreous endosperm, the physicochemical properties of individual storage proteins, including the α-, β-, γ- and δ-zeins, allow them to assemble in precise ratios and positions. Disrupting this organization, either by altering the abundance of one or more of these proteins or by introducing a new or modified protein into the protein body, will change the size, shape and abundance of protein bodies in the endosperm, leading to an opaque or floury phenotype (Wang *et al.*, 2014; Wu and Messing, 2010; Wu *et al.*, 2010). The abundance of the 27 kDa γ-zeins, in particular, correlates with test weight (Gerde *et al.*, 2016). In Event PY203, the heterologous Phy02 phytase, is targeted to the protein body by virtue of the signal peptide derived from the 27 kDa γ-zein protein, and it accumulates among the endogenous storage proteins. This likely leads to changes in the organization of the native proteins, which causes a floury phenotype. Although opaque or floury phenotypes are not preferred by dry-grind maize processors, this phenotype does have some associated benefits. Sorghum varieties with the floury phenotype, which are similarly caused by alterations in the assembly of storage proteins within the protein body, are valued for their increased digestibility in ruminant diets (Wang *et al.*, 2014). Also, Quality Protein Maize varieties take advantage of the altered protein body formation seen in floury and opaque kernels that lead to improvements in the amino acid profile of protein in the grain (Wu *et al.*, 2010). Therefore, the lower grain test weights that were seen for Event PY203 compared to the control line are not surprising.

During the course of the growing season the plants of Event PY203 and the maize control line were assessed for diseases and insect predation as described above (see § VII.A). Although disease issues and insects were controlled by the application of

fungicides and insecticides, there were no observed differences in the incidence of diseases or insect predation between the Phy02 phytase producing Event PY203 and the near isogenic null comparator line. No differences between the field plots of Event PY203 and the null controls in the populations of other animals, including insects, birds and mammals were noted during the growing season at any of the field locations nor were any adverse environmental effects from the cultivation of Event PY203 relative to the null controls observed.

Conventional maize plants have been highly bred and require human intervention in order to grow well in the environment (Gould, 1968). Although maize plants may occasionally grow in uncultivated fields and by roadsides or occur as volunteers in cultivated crops in the year following cultivation, these plants are generally not competitive and rarely produce viable seed (CFIA, 1994). Although statistical differences between Event PY203 and the non-transgenic control line were seen in some agronomic characteristics these would not be expected to significantly impact the ability of maize Event PY203 to survive or to be more persistent in the environment compared to conventional maize varieties. In the case of stalk lodging, Event PY203 demonstrated 3.7 percent lodging compared to 6.2 percent for the non-transgenic control line (Table 12). Maize Event PY203 demonstrated slightly less dropped ears compared to the non-transgenic control, 0.78 vs. 1.34 ears per plot (Table 13). These values and their differences are relatively low and are not expected to significantly impact the environmental competitiveness of maize Event PY203. In both emergent and final stand counts Event PY203 was slightly lower compared to the non-transgenic control line (62.7 vs. 65.9 plants/plot for emergent stand count and 60.9 vs. 64.2 plants/plot for final stand count; Table 12). These differences in stand counts are also not great and would not be expected to significantly alter the environmental competitiveness of Event PY203.

B. Seed Dormancy and Germination Characteristics

Many plants use complex mechanisms to release and disperse seeds in order to survive over multiple seasons (Anderson, 1996). Hard seed is a dormancy characteristic that allows seeds to survive the cold temperatures of winter or long periods of drought. Some plant seeds are capable of germination at low temperatures in order to establish themselves in the environment before competitor species thereby providing a competitive advantage to the young seedlings. Primary seed dormancy is exceedingly rare in most field crops, including maize (Gould, 1968; Galinat, 1988).

Standardized germination assays are routinely conducted to measure the seed viability and germination potential of maize seeds (AOSA, 1998). In order to assess the seed germination and dormancy potential of seed of maize Event PY203, Agrivida, Inc. conducted laboratory experiments using seed from Event PY203 and a near isogenic null control line. Event PY203 was backcrossed for three generations into inbred variety G and for four generations into inbred variety E. The resulting

backcrosses were then self–pollinated to create a segregating population of progeny from which homozygous (with respect to the PY203 loci) and null progeny were selected. Pollen from homozygous E plants was used to pollinate silks from homozygous G plants to create hybrid PY203 seed. Similarly, pollen from null E segregants was used to pollinate silks from null G segregants to create null hybrid seed.

The seed produced as described above was planted in small pots containing potting soil and the pots were moved into two growth chambers, one that was maintained at 10°C and the other at 25°C. In each growth chamber there were three replicate groups of 50 pots for each maize line. The moisture of the soil was checked daily and maintained by watering as necessary. The germination state of the seed was assessed and recorded at 4, 7, and 10 days after planting.

Table 15. Seed germination after incubation at 10°C and 25°C at 4, 7, and 10 days post-planting.

Incubation Temp.			**Number Germinated Seed**		
	Days Post-Planting		**4**	**7**	**10**
25°C	PY203	rep1	12	47	47
		rep2	14	45	45
		rep3	20	44	46
	PY203null	rep1	24	48	50
		rep2	22	50	50
		rep3	24	49	47
10°C	PY203	rep1	0	0	0
		rep2	0	0	0
		rep3	0	0	0
	PY203null	rep1	0	0	0
		rep2	0	0	0
		rep3	0	0	0
Statistical analysis of germination data at 25°C; Average (Avg), Standard Deviation (Std).		PY203 Avg	15.3	45.3	46.0
		PY203 Std	4.2	1.5	1.0
		null Avg	23.3	49.0	49.0
		null Std	1.2	1.0	1.7
		P value	0.074	0.111	0.122

The results of the seed germination study demonstrate that seed of Event PY203 is not able to germinate at the lower temperature of 10°C. At the conclusion of the experiment there was no germination of any seed from either Event PY203 or the null control seed at this temperature (Table 15). This demonstrates that, similar to other cultivated maize varieties, seed of Event PY203 is not capable of germination under cool conditions. Therefore, Event PY203 is unlikely to demonstrate early

germination in the environment that can result in a competitive advantage compared to other plant species. At 25°C the germination rate of Event PY203 and the null control was not statistically different ($P \leq 0.05$) after 4, 7, or 10 days. After 10 days the average seed germination was 92 (±2.0%) and 98% (±3.4%) for Event PY203 and the null control, respectively (Table 15). A T-test revealed that these differences were not significant (p = 0.12). The null seed appeared to germinate slightly more quickly than the PY203 seed, as seen after 4 days. However, even these differences did not achieve statistical significance (p = 0.07). These results demonstrate that the seed germination characteristics of Event PY203 are equivalent to those of the null segregant line and of cultivated maize varieties and that the potential for Event PY203 to become a weed pest is no different than it is for other cultivated maize varieties.

VIII. Assessment of the Nutrient Composition of Grain and Forage

The nutrient composition of grain and forage derived from Event PY203 was compared to similar materials derived from a near isogenic non-transformed control hybrid as well as nutrient values from the published literature. The production of the Phy02 phytase in Event PY203 is neither intended nor expected to substantially alter the nutritional composition of the grain or forage produced by it but establishing this is typically a component of a weight of evidence approach for evaluating whether there are any unintended or unforeseen consequences of the genetic modification. In addition, establishing that the nutrient composition of grain and forage from PY203 is substantially equivalent to that of typical maize contributes to a conclusion that Event PY203 poses no increased plant pest risk or weediness potential. A description of the nutrient composition study of Event PY203 and the results derived that are presented herein have been previously submitted to the US-FDA as part of a food and feed safety evaluation of grain and forage derived from Event PY203 (BNF 000167).

Event PY203 grain and forage was harvested from plants cultivated at 5 field locations in 2016: Hamilton and Tipton counties, IN; Dallas county, IA; and Brunswick and York counties, NE. These field trials were conducted under USDA Permit# 16-063-102rm and each trial included 4 replicate plots of Event PY203 and a non-transgenic null segregant (negative control) organized in a randomized complete block design. For forage tissue collection, the entire above ground portion of one plant per replicate plot was collected at the R4 stage (Mueller & Pope, 2009). At physical maturity a total of 2 kg of grain from several plants in each replicate plot was collected. The forage and grain samples from each replicate plot at each trial site were sent to Eurofins (www.eurofins.com) for analysis. Grain samples were analyzed for proximates, amino acids, fatty acids, minerals, vitamins, and other bioactive metabolites (phytic acid, trypsin inhibitor, p-coumaric acid, raffinose, and ferulic acid). Analysis of the forage samples was limited to proximates. The experimental design was a randomized complete block design, with location used as the blocking criteria for grain composition and forage proximates. Each of the 5 blocks had 4 replicate plots to which the treatments (n = 4 per treatment) were randomly distributed. Due to incomplete sample collection within and across locations (n=6 for PY203 null and n=9 for PY203) location wasn't included as blocking criteria for grain proximate analysis. Plots were used as the experimental unit for each analyzed variable. Data was analyzed using Fit Least Squares procedure of the JMP software (version 14, SAS Institute Inc., Cary, NC). The ANOVA model included treatment and block (grain composition and forage proximates) or treatment only (grain proximates). Least Square Mean values were separated using Tukey's honesty significant difference procedure and P-values < 0.05 were considered significant in all comparisons.

A. Nutrient Analysis of Grain

Proximates: The values for moisture and crude fat from grain of PY203 were significantly greater than the corresponding values from grain of the negative control, whereas those for carbohydrates were significantly lower. The values determined for protein, crude fiber and ash were not statistically different (Table 16). The means for each nutrient for Event PY203 and the negative control grain were compared to the historic means and ranges for maize grain for all years and all countries in the ILSI Crop Composition Database (ILSI-CCDB, 2018). The mean values for protein, crude fat, ash and carbohydrates of grain from Event PY203 and the null segregant were similar to each other, and within the ranges for these nutrients as reported in the ILSI-CCDB (Table 16). For all proximate nutrients except crude fat, the means for PY203 grain were within the ranges for the same nutrients from the negative control grain. In the case of crude fat, Event PY203 grain had higher values compared to the negative control, but similar amounts compared to the ILSI-CCDB.

Table 16. Proximate nutrient analysis of grain from Event PY203. Differences in values reported for PY203 and the non-transgenic null control that are presented in bold font are statistically significant ($p<0.05$).

Analyte (%DW)		PY203 Transgenic	PY203 Non-transgenic control	ILSI Data[1]
Protein	Mean ± SD	11.48 ± 1.21	10.69 ± 1.14	10.31
	Range	10.71 - 14.65	8.74 - 12.23	5.72 - 17.26
Moisture (%FW)	Mean ± SD	**12.79 ± 1.84**	**8.95 ± 2.00**	14.6
	Range	8.43 - 14.47	7.93 - 13.03	5.1 - 40.5
Crude Fat	Mean ± SD	**4.29 ± 0.48**	**3.14 ± 0.26**	3.82
	Range	3.57 - 4.93	2.85 - 3.51	1.363 - 7.83
Crude Fiber	Mean ± SD	2.02 ± 0.25	1.80 ± 0.32	NA[2]
	Range	1.84 - 2.62	1.30 - 2.18	-
Ash	Mean ± SD	1.68 ± 0.25	1.50 ± 0.19	1.41
	Range	1.52 - 2.35	1.20 - 1.71	0.616 - 6.28
Carbo-hydrates	Mean ± SD	**82.54 ± 1.46**	**84.67 ± 1.34**	84.5
	Range	78.95 - 83.64	82.60 - 86.69	77.4 - 89.7

[1]ILSI Crop Composition Database Version 6.0; Generated 02/15/2017
[2]Not Available

Amino acids: The abundance of amino acids in Event PY203 and negative control grain was measured, including alanine, arginine, aspartic acid, cystine/cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. While the values for 14 of the amino acids from grain of PY203 were significantly greater than the corresponding values from grain of the negative control, the values for glutamic acid, leucine, proline, and tyrosine were not statistically different (Table 17). For all amino acids the mean values for Event PY203 and the negative control were similar to each other and to the means for maize grain reported by the ILSI-CCDB (Table 17). In all cases, the means for all amino acids in Event PY203 grain were within the historical ranges for maize grain. These results demonstrate that expression of Phy02 protein in Event PY203 does not significantly affect the amino acid composition in the grain.

Fatty Acids: Fatty acid levels were evaluated in the grain from Event PY203 and the negative control maize (Table 18). While the values for palmitic, oleic, linoleic, linolenic and total fatty acids from grain of PY203 were significantly greater than the corresponding values from grain of the negative control, the values for stearic, arachidic, gondoic, and lignoceric acids were not statistically different (Table 18). For all fatty acids the mean values for Event PY203 and the negative control were similar to each other and to the means from the ILSI-CCDB. The mean values for all fatty acids in PY203 grain were within the historical ranges for the same fatty acids in the ILSI-CCDB. These results demonstrate that expression of Phy02 protein in Event PY203 does not significantly affect the fatty acid composition in the grain.

Minerals: The levels of numerous minerals were evaluated in the grain from Event PY203 and the negative control maize, including calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, selenium, and zinc. While the values for calcium, magnesium, manganese, phosphorus, potassium, and zinc from grain of PY203 were significantly greater than the corresponding values from grain of the negative control, the values for copper, iron, sodium, and selenium were not statistically different (Table 19). For all minerals, the mean values for Event PY203 and the negative control were similar to each other and to the means for maize grain from the ILSI-CCDB (Table 19). The means for all minerals in Event PY203 grain were within the historical ranges for the same minerals in grain from the ILSI-CCDB. These results demonstrate that expression of the Phy02 protein in Event PY203 does not significantly affect the mineral composition of the grain.

Table 17. Amino acid analysis of grain from Event PY203. Differences in values reported for PY203 and the non-transgenic null control that are presented in bold font are statistically significant ($p<0.05$).

Analyte (%FW)		PY203 Transgenic	Non-Transgenic	ILSI Data[1]
Alanine	Mean ± SD	**0.76 ± 0.11**	**0.71 ± 0.09**	0.67
	Range	0.62 - 0.95	0.56 - 0.87	0.33 - 1.28
Arginine	Mean ± SD	**0.54 ± 0.06**	**0.42 ± 0.03**	0.4
	Range	0.46 - 0.67	0.35 - 0.48	0.11 - 0.61
Aspartic Acid	Mean ± SD	**0.76 ± 0.09**	**0.62 ±0.05**	0.58
	Range	0.63 - 0.89	0.54 - 0.70	0.31 - 1.11
Cystine/Cysteine	Mean ± SD	**0.22 ± 0.03**	**0.20 ± 0.03**	0.18
	Range	0.16 - 0.28	0.15 - 0.23	0.09 - 0.46
Glutamic Acid	Mean ± SD	1.87 ± 0.29	1.82 ± 0.24	1.66
	Range	1.54 - 2.44	1.46 - 2.26	0.78 - 3.25
Glycine	Mean ± SD	**0.46 ± 0.05**	**0.37 ± 0.05**	0.33
	Range	0.4 - 0.55	0.32 - 0.54	0.17 - 0.52
Histidine	Mean ± SD	**0.29 ± 0.04**	**0.26 ± 0.03**	0.24
	Range	0.24 - 0.36	0.21 - 0.31	0.12 - 0.4
Isoleucine	Mean ± SD	**0.37 ± 0.05**	**0.33 ± 0.03**	0.31
	Range	0.30 - 0.46	0.27 - 0.40	0.16 - 0.64
Leucine	Mean ± SD	1.22 ± 0.20	1.21 ± 0.17	1.1
	Range	0.97 - 1.63	0.94 - 1.54	0.51 - 2.29
Lysine	Mean ± SD	**0.38 ± 0.08**	**0.27 ± 0.05**	0.25
	Range	0.26 - 0.51	0.19 - 0.35	0.09 - 0.59
Methionine	Mean ± SD	**0.28 ± 0.04**	**0.24 ± 0.02**	0.18
	Range	0.21 - 0.36	0.19 - 0.27	0.09 - 0.42
Phenylalanine	Mean ± SD	**0.50 ± 0.07**	**0.47 ± 0.05**	0.45
	Range	0.40 - 0.63	0.37 - 0.57	0.21 - 0.86
Proline	Mean ± SD	0.86 ± 0.14	0.85 ± 0.12	0.78
	Range	0.69 - 1.10	0.66 - 1.07	0.36 - 1.5
Serine	Mean ± SD	**0.51 ± 0.07**	**0.46 ± 0.05**	0.42
	Range	0.42 - 0.64	0.37 - 0.55	0.15 - 0.71
Threonine	Mean ± SD	**0.43 ± 0.05**	**0.35 ± 0.03**	0.31
	Range	0.36 - 0.51	0.30 - 0.40	0.18 - 0.6
Tryptophan	Mean ± SD	**0.12 ± 0.01**	**0.08 ± 0.01**	0.061
	Range	0.11 - 0.15	0.07 - 0.09	0.024 - 0.186
Tyrosine	Mean ± SD	0.35 ± 0.05	0.31 ± 0.04	0.3
	Range	0.27 - 0.45	0.24 - 0.38	0.09 - 0.59
Valine	Mean ± SD	**0.55 ± 0.06**	**0.46 ± 0.04**	0.41
	Range	0.44 - 0.65	0.38 - 0.55	0.22 - 0.79

[1]ILSI-CCDB Version 6.0; Generated 06/23/2017

Table 18. Fatty acid analysis of grain from Event PY203. Differences in values reported for PY203 and the non-transgenic null control that are presented in bold font are statistically significant (p<0.05).

Analyte (%FW)		PY203 Transgenic	Non-transgenic	ILSI Data[1]
Palmitic (C16:0)	Mean ± SD	**0.46 ± 0.06**	**0.41 ± 0.04**	0.37
	Range	0.26 - 0.54	0.32 - 0.51	0.14 ± 0.90
Stearic (18:0)	Mean ± SD	0.05 ± 0.01	0.05 ± 0.01	0.06
	Range	0.04 - 0.06	0.04 - 0.06	0.02 - 0.15
Oleic (C18:1)	Mean ± SD	**0.64 ± 0.09**	**0.56 ± 0.08**	0.77
	Range	0.40 - 0.78	0.45 - 0.74	0.32 - 2.02
Linoleic (C18:2)	Mean ± SD	**2.03 ± 0.33**	**1.81 ± 0.24**	1.63
	Range	1.02 - 2.36	1.32 - 2.24	0.47 - 2.73
Linolenic (C18:3)	Mean ± SD	**0.05 ± 0.01**	**0.04 ± 0.01**	0.04
	Range	0.02 - 0.06	0.02 - 0.05	0.01 - 0.07
Arachidic (C20:0)	Mean ± SD	0.01 ± 0.00	0.01 ± 0.00	0.01
	Range	0.01 - 0.02	0.01 - 0.02	0.01 - 0.04
Gondoic (C20:1)	Mean ± SD	0.01 ± 0.00	0.01 ± 0.00	0.01
	Range	0.01 - 0.02	0.01 - 0.02	0.002 - 0.04
Lignoceric (C24:0)	Mean ± SD	0.01 ± 0.00	0.01 ± 0.00*	NA[2]
	Range	0.01 - 0.01	<0.01 - 0.01	-
Total Fatty Acids	Mean ± SD	**3.27 ± 0.50**	**2.91 ± 0.36**	NA
	Range	1.80 - 3.82	2.21 - 3.56	-

*One sample measured 0.01 while all others were <0.01.
[1]ILSI-CCDB Version 6.0; Generated 06/23/2017
[2]Not Available

Other fatty acid species were below the limit of detection (<0.01 %FW)

Table 19. Mineral analysis of grain from Event PY203. Differences in values reported for PY203 and the non-transgenic null control that are presented in bold font are statistically significant ($p<0.05$).

Analyte (ppm FW)		PY203 Transgenic	Non-transgenic	ILSI Data[1]
Calcium	Mean ± SD	**60 ± 40**[2]	**40 ± 10**	37.2
	Range	40 - 200	30 - 60	8.5 - 687.3
Copper	Mean ± SD	1.49 ± 0.38	1.50 ± 0.20	1.47
	Range	1.0 - 2.0	1.20 - 2.00	0.51 - 17.17
Iron	Mean ± SD	18 ± 11	17 ± 6	17.48
	Range	11 - 58	13 - 34	6.19 - 155.09
Magnesium	Mean ± SD	**1150 ± 120**	**990 ± 100**	1030.3
	Range	990 - 1320	880 - 1200	424.4 - 1726.6
Manganese	Mean ± SD	**6.18 ± 1.30**	**4.85 ± 0.70**	5.46
	Range	3.90 - 9.00	3.10 - 5.90	1.43 - 13
Phosphorus	Mean ± SD	**3130 ± 260**	**2830 ± 220**	2676.3
	Range	2700 - 3500	2500 - 3400	841.1 - 4598.2
Potassium	Mean ± SD	**4060 ± 210**	**3310 ± 210**	3148.4
	Range	3700 - 4500	3000 - 3800	1268.1 - 5366.7
Sodium	Mean ± SD	20 ± 0.0[3]	<20[4]	20.15
	Range	20 - 20	-	0.15 - 654
Selenium	Mean ± SD	0.275 ± 0.133	0.256 ± 0.136	0.22
	Range	0.110 - 0.540	0.100 - 0.510	0.03 - 0.99
Zinc	Mean ± SD	**20.53 ± 1.90**	**17.80 ± 1.76**	19.4
	Range	16.0 - 25.0	14.0 - 21.0	5.6 - 35.2

[1]ILSI-CCDB Version 6.0; Generated 06/23/2017
[2]Only 3 of the samples had >100 ppm Calcium; the remainder had an average calcium content of 46±6 ppm, with a range of 40-60 ppm
[3]All samples were less than the LOQ (20 ppm) except two that were equal to the LOQ
[4]All samples were less than the LOQ (20 ppm)

Vitamins: Vitamin levels were evaluated in Event PY203 and the negative control grain, including β-carotene (Vitamin A precursor), niacin (Vitamin B_3), thiamine (Vitamin B_1), riboflavin (Vitamin B_2), pantothenic acid (Vitamin B_5), ascorbic acid (Vitamin C), pyridoxine (Vitamin B_6), and total tocopherols (Vitamin E). The values for niacin, riboflavin, and pyridoxine from grain of PY203 were significantly greater than the corresponding values from grain of the negative control, whereas those for β-carotene and thiamine were significantly lower. The values determined for

pantothenic acid, vitamin C and total tocopherols were not statistically different (Table 20). For all vitamins tested, the means for Event PY203 and the negative control samples were similar to each other and, except for β-carotene, were similar to the means reported by ILSI-CCDB (Table 20). The values for β-carotene in the ILSI-CCDB are reported in mg/100g FW, whereas those reported by Eurofins were in International Units (IU)/100g FW. The ILSI values for β-carotene were converted to IU/100g FW using the standard ratio of 1 IU β-carotene equals 0.6 μg β-carotene (NIH, 2018). Mean values for β-carotene in Event PY203 and the negative control grain were 66.21 and 113.39 IU/100g FW, respectively, compared to a historical mean of 633.3 IU/100g FW in the ILSI-CCDB after conversion to IU. However, it should be noted that the historical range for β-carotene in the ILSI-CCDB is broad (28.3 – 5966 IU/100g FW). The mean values for β-carotene for both Event PY203 and negative control grains are within this historical range as reported in the ILSI-CCDB. In all cases, the means for all vitamins in PY203 grain were within the historical ranges for the same vitamins as reported for maize grain in the ILSI-CCDB. These results demonstrate that expression of the Phy02 protein in Event PY203 does not significantly affect the vitamin composition of the grain.

Anti-nutritional factors: The levels of anti-nutritional metabolites known to be present in maize were evaluated in Event PY203 and negative control grain, including phytic acid, trypsin inhibitor, inositol, *p*-coumaric acid, raffinose, and ferulic acid. The value for *p*-coumaric acid from grain of PY203 was significantly greater than the corresponding value from grain of the negative control, whereas those for phytic acid, trypsin inhibitor, and raffinose were significantly lower. The values determined for ferulic acid and inositol were not statistically different (Table 21). For all metabolites tested except phytic acid, the mean values for Event PY203 and the negative control were similar to each other and to the published ILSI-CCDB values (Table 21). In the case of phytic acid, the mean for Event PY203 grain was approximately half the value of the means for both the negative control and the ILSI-CCDB. This result is to be expected given that the biological activity of the Phy02 phytase is the sequential removal of phosphate from phytic acid with the eventual production of phosphates and inositol. In all cases, the means for all metabolites (including phytic acid) in Event PY203 grain were within published historical ranges. These results demonstrate that with the exception of phytic acid, expression of the Phy02 protein in Event PY203 grain does not significantly affect the composition these metabolites.

Table 20. Vitamin analysis of grain from Event PY203. Differences in values reported for PY203 and the non-transgenic null control that are presented in bold font are statistically significant ($p<0.05$).

Analyte (Units in FW)		PY203 Transgenic	Non-transgenic	ILSI Data[1]
β-carotene (IU/100g)	Mean ± SD	**66.21 ± 15.86**	**113.39 ± 24.91**	633.3*
	Range	35.9 - 91	63.6 - 140	28.3 - 5966.7
Niacin, Vit. B_3 (mg/100g)	Mean ± SD	**1.92 ± 0.52**	**1.50 ± 0.33**	1.77
	Range	0.73 - 2.82	0.80 - 1.97	0.47 - 4.29
Thiamine, Vit. B_1 (mg/100g)	Mean ± SD	**0.33 ± 0.04**	**0.36 ± 0.04**	0.32
	Range	0.26 - 0.40	0.28 - 0.43	0.096 - 3.56
Riboflavin, Vit. B_2 (mg/100g)	Mean ± SD	**0.25 ± 0.11**	**0.16 ± 0.04**	0.17
	Range	0.15 - 0.56	0.12 - 0.27	0.045 - 0.66
Pantothenic Acid, Vit. B_5 (mg/100g)	Mean ± SD	0.30 ± 0.17	0.23 ± 0.06	0.39
	Range	0.18 - 0.76	0.15 - 0.37	0.22 - 0.75
Vit. C, Ascorbic Acid (mg/100g)	Mean ± SD	0.66 ± 0.08	0.51 ± 0.05	NA[2]
	Range	0.60 - 0.71	0.48 - 0.54	-
Vit. B_6, Pyridoxine (mg/100g)	Mean ± SD	**0.51 ± 0.06**	**0.44 ± 0.04**	0.51
	Range	0.42 - 0.64	0.36 - 0.52	0.087 - 1.08
Total Vit. E, Tocopherols (IU/100g)	Mean ± SD	3.09 ± 2.47	2.08 ± 0.65	3.28
	Range	0.24 - 12.3	0.69 - 3.17	0.17 - 11.40

* The values for β-carotene in the ILSI-CCDB are reported as mg/100g FW whereas those reported by Eurofins for PY203 and the negative control were reported as IU/100g FW. The values reported in the ILSI-CCDB for β-carotene are:

Min	Max	Mean	Units
0.017	3.578	0.381	mg/100g FW

The ILSI-CCDB values were converted to IU using the following formula: 1 IU B-carotene = 0.6 μg β-carotene.

[1]ILSI-CCDB Version 6.0; Generated 06/26/2017

[2]Not Available

Table 21. Anti-nutrient analysis of grain from Event PY203. Differences in values reported for PY203 and the non-transgenic null control that are presented in bold font are statistically significant (p<0.05).

Analyte (Units)		PY203 Transgenic	Non- transgenic	ILSI Data[1]
Phytic Acid (%FW)	Mean ± SD	**0.38 ± 0.08**	**0.76 ± 0.09**	0.73
	Range	0.15 - 0.45	0.63 - 0.94	0.1 - 1.44
Trypsin Inhibitor (TIU/g)	Mean ± SD	**1578.9 ± 342.51**	**1755 ± 160.51**	2949.59
	Range	1200 - 2300	1500 - 2000	516.45 - 7262.50
Inositol (μg/g FW)	Mean ± SD	250.63 ± 70.1.9	242.3 ± 38.22	147.13
	Range	128 - 344	171 - 308	46.9 - 416.1
p-Coumaric Acid (μg/g FW)	Mean ± SD	**186.22 ± 39.93**	**159.26 ± 20.92**	192.3
	Range	116.27 - 255	127 - 204	46.9 - 734.7
Raffinose (%FW)	Mean ± SD	**0.127 ± 0.04**	**0.172 ± 0.069**	0.149
	Range	0.06 - 0.2	0.06 - 0.34	0.015 - 0.398
Ferulic Acid (ppm FW)	Mean ± SD	1727.69 ± 256.39	1422.09 ± 126.22	1933.5
	Range	1335.63 - 2230	1191 - 1620.75	173.7 - 3940

[1]ILSI-CCDB Version 6.0; Generated 06/26/2017

B. Nutrient Analysis of Forage

Proximate nutrient values of forage samples from Event PY203 and the negative control maize, including protein, crude fat, crude fiber, ash, carbohydrates, acid detergent and neutral detergent fiber, total dietary fiber, calcium and phosphorus, were determined (Table 22). The values for total dietary fiber from forage samples of PY203 were significantly greater than the corresponding values from forage of the non-transgenic control, whereas those for all other nutrients were not statistically different (Table 22). The mean values for Event PY203 and the negative control samples were similar to each other and within the historical ranges for the same nutrients in the ILSI-CCDB. These results demonstrate that expression of Phy02 protein in Event PY203 does not significantly affect the composition these nutrients in forage.

Table 22. Proximate nutrient analysis of forage from Event PY203. Differences in values reported for PY203 and the non-transgenic null control that are presented in bold font are statistically significant ($p<0.05$).

Analyte (% DW)		PY203 Transgenic	Non- transgenic	ILSI Data[1]
Protein	Mean ± SD	8.72 ± 1.11	8.3 ± 1.25	7.68
	Range	6.88 - 11.64	5.88 - 11.09	3.14 - 16.32
Crude Fat	Mean ± SD	2.33 ± 0.41	1.94 ± 0.52	2.09
	Range	1.47 - 3.46	1.14 - 3.15	0.30 - 6.75
Crude Fiber	Mean ± SD	27.99 ± 3.82	26.74 ± 4.18	23
	Range	22.02 - 33.65	20.43 - 31.56	15.1 - 30.1
Ash	Mean ± SD	5.19 ± 0.60	5.25 ± 0.90	4.28
	Range	4.03 - 6.28	3.96 - 6.82	0.66 - 13.2
Carbohydrate	Mean ± SD	83.78 ± 1.83	84.51 ± 2.15	86
	Range	78.72 - 86.37	80.36 - 87.77	73.3 - 92.9
Fiber, Acid Detergent	Mean ± SD	33.44 ± 4.61	32.56 ± 5.13	25.85
	Range	26.66 - 40.00	23.12 - 39.20	9.9 - 47.39
Fiber, Neutral Detergent	Mean ± SD	54.96 ± 7.15	55.08 ± 6.32	42.16
	Range	45.25 - 63.73	41.47 - 63.25	20.29 - 67.8
Fiber, Total Dietary	Mean ± SD	**61.23 ± 6.44**	**59.57 ± 6.55**	49.49
	Range	51.68 - 70.56	47.34 - 68.60	35.88 - 62.83
Calcium	Mean ± SD	0.29 ± 0.03	0.24 ± 0.03	0.19
	Range	0.21 - 0.35	0.19 - 0.30	0.06 - 0.58
Phosphorus	Mean ± SD	0.25 ± 0.03	0.24 ± 0.02	0.2
	Range	0.20 - 0.30	0.19 - 0.28	0.07 - 0.44

[1]ILSI-CCDB Version 6.0; Generated 06/26/2017

C. Conclusions from Nutrient Analysis of Grain and Forage Derived from Maize Event PY203

The results of the nutrient composition study indicate that both grain and vegetative tissue derived from field grown Event PY203 plants is substantially equivalent to its near-isogenic control and within published historical ranges for maize. For proximate nutrients, amino acids, fatty acids, minerals, vitamins and other metabolites (except phytic acid), the mean values for grain from Event PY203 and the negative control maize were similar to each other and to means for grain as reported in the ILSI-CCDB. Similarly, the proximate nutrient composition of forage samples collected at the R4 developmental stage from Event PY203 and the negative

control maize were determined and compared to means for these nutrients in maize forage samples in the ILSI-CCDB. The results demonstrate that the forage proximate nutrient composition of PY203 is very similar to that of the forage samples collected from the negative control plants and to means for forage samples reported in the ILSI-CCDB. Overall, these results demonstrate that the expression of Phy02 protein in Event PY203 does not significantly affect the nutrient composition in the grain or forage of maize Event PY203.

IX. Cultivation Practices

A. Intended Cultivation Area

Agrivida, Inc. intends to cultivate maize Event PY203 in the corn belt of the United States, primarily in the Midwestern region. No cultivation of Event PY203 is anticipated in any regions where maize is not currently planted.

B. Standard Cultivation Practices for Maize

In 2018 the U.S. was the world's leading producer of corn having produced 380 million metric tons (MMT) of corn grain (USDA-FAS, 2019) from 89,129,000 planted acres (USDA-NASS, 2019). The next five highest corn grain producing countries in 2018 included China (265 MMT), the European Union (147 MMT), Brazil (103 MMT), Argentina (57 MMT) and Ukraine (45 MMT). The total world production of corn grain in 2018 equaled 1,393 MMT (USDA-FAS, 2019). Much of the production of corn in the U.S. occurs in the Midwestern states with Iowa, Illinois, Nebraska, Minnesota, and Kansas as the top five states by acres planted in 2018 (USDA-NASS, 2019; Table 23).

In 2016 USDA-NASS conducted a survey of maize producers to collect data about fertilizer and pesticide use as well as pest management practices in growing maize (USDA-NASS, 2017). USDA-NASS conducted the survey in 19 states that together accounted for 92 percent of the 94 million acres planted to maize in the United States in 2016: Colorado, Georgia, Illinois, Indiana, Iowa, Kansas, Kentucky, Michigan, Minnesota, Missouri, Nebraska, New York, North Carolina, North Dakota, Ohio, Pennsylvania, South Dakota, Texas, and Wisconsin. Maize cultivation in the United States typically includes the application of fertilizers to the soil. A fertilizer is defined as a soil-enriching input that contains one or more plant nutrients. In the 2016 crop year, farmers applied nitrogen to 97 percent of planted maize acres, at an average rate of 145 pounds per acre, for a total of 12 billion pounds. They applied phosphate to 79 percent of maize planted acres, potash to 65 percent, and sulfur to 33 percent.

Pesticides, including herbicides (targeting weeds), insecticides (targeting insects), fungicides (targeting fungal pathogens), or other chemicals (targeting all other pests) are commonly applied to maize. Herbicides were applied to 97 percent of planted acres in 2016 and insecticides and fungicides were each applied to 12 percent of planted acres (USDA-NASS, 2017). Among herbicides, atrazine was the most widely used active ingredient (applied to 60 percent of planted acres). It was also the most widely applied ingredient in the 2014 survey of maize growers. The most widely used monitoring practice in growing maize was scouting for weeds, used on 90 percent of maize planted acres. The top avoidance practice was crop rotation (79 percent) and the top prevention practice was no-till or minimum till prior to planting (64 percent). In each category, the most widely used practice in 2016 was also the top practice in the 2014 survey.

In the process of domesticating maize, it lost its ability to disperse seeds and compete in the environment and it requires much human intervention in order to grow well in the environment (Gould, 1968). Maize plants occasionally grow in uncultivated fields and by roadsides or occur as volunteers in cultivated crops in the year following cultivation of a maize crop (CFIA, 1994), but these plants are generally not competitive and rarely produce viable seed for the next growing season. Control of volunteer maize is commonly achieved by mechanical means and herbicides.

No changes to agronomic practices typically applied in management of conventional maize are required for the cultivation of maize Event PY203. Specifically, no increases in pesticides and fertilizers are required as well as no changes in cultivation, planting, harvesting, or volunteer control.

Table 23. Acres planted to corn in the U.S. by state in 2018 (USDA-NASS, 2019).

State	Acres	State	Acres	State	Acres
Iowa	13,200,000	Virginia	485,000	Utah	70,000
Illinois	11,000,000	Mississippi	480,000	W. Virginia	46,000
Nebraska	9,600,000	Louisiana	460,000	Maine	31,000
Minnesota	7,900,000	Maryland	450,000	Connecticut	23,000
Kansas	5,450,000	California	430,000	Massachusetts	14,000
Indiana	5,350,000	Idaho	360,000	Nevada	13,000
S. Dakota	5,300,000	S. Carolina	340,000	N. Hampshire	13,000
Wisconsin	3,900,000	Georgia	325,000	Rhode Island	2,000
Missouri	3,500,000	Oklahoma	320,000		
Ohio	3,500,000	Alabama	260,000		
N. Dakota	3,150,000	Delaware	170,000		
Michigan	2,300,000	Washington	165,000		
Texas	2,200,000	N. Mexico	135,000		
Colorado	1,470,000	Montana	115,000		
Pennsylvania	1,350,000	Florida	100,000		
Kentucky	1,340,000	Wyoming	95,000		
N. York	1,100,000	Vermont	85,000		
N. Carolina	910,000	Oregon	80,000		
Tennessee	740,000	N. Jersey	72,000		
Arkansas	660,000	Arizona	70,000		

C. Cultivation Practices for Maize Event PY203

Maize Event PY203 will be cultivated in order to produce grain containing the Phy02 phytase enzyme that will in turn be ground into a coarse meal that will be

packaged into labeled containers and sold for inclusion in relatively small amounts (less than 2 kg/ton) in the feed of poultry and swine. Growers under contract to Agrivida, Inc. will conduct the cultivation of maize Event PY203 maize for this purpose. Agrivida, Inc. will provide the seed of Event PY203 to the growers and the growers will plant and cultivate it using agronomic practices that are typical for maize. During cultivation, maize Event PY203 will remain the property of Agrivida, Inc. and after harvest the grower will deliver all of the harvested grain to Agrivida, Inc. for processing into the phytase feed additive product. As the source of a valuable feed additive enzyme, grain of Event PY203 has a much higher value than normal maize grain and therefore, Agrivida, Inc. will not sell or deliver grain from Event PY203 for other uses, including food uses, dry milling, etc. The primary purpose for the development of maize Event PY203 is for the production of phytase enzyme to be used as a feed additive in the feed of monogastric animals in order to increase the digestibility of phytate phosphorus present in corn and other grains that are major components of animal feed. Given the relatively low inclusion rate of the Phy02 phytase in animal feed, the area required to produce sufficient product to meet the demands of the poultry and swine production markets is only about 10,000 acres.

In the event that Agrivida, Inc. markets GraINzyme® Phytase for use in dairy or beef cattle, it may sell a limited amount of seed of maize Event PY203 to dairy or beef producers to enable them to plant and cultivate this maize event for the sole purpose of producing Phy02 phytase-containing maize silage. The cultivation of maize Event PY203 for this purpose will not be different than the cultivation of other maize varieties for the production of silage. In this case, dairy or beef producers will be required to agree that plant material derived from maize Event PY203 will only be used to produce feed for cattle and that seed or grain will not be saved or used for any other purpose.

X. Environmental Consequences of Event PY203

The purpose of this chapter is to review and summarize data relevant to the environmental safety of maize Event PY203 and to present conclusions on the likely environmental impact of the cultivation of Event PY203 in the environment. There are two important aspects of the consideration of the environmental safety of Event PY203: the potential for Event PY203 to cause harm to wildlife, including species beneficial to agriculture and endangered and threatened species; and the potential for Event PY203 to become a serious weed of agricultural or non-agricultural habitats.

A fundamental assumption is that conventional, non-transgenic maize has been cultivated for millennia without adverse impacts on the environment and therefore poses no unacceptable environmental risks. The data presented in this submission demonstrates that maize Event PY203 is substantially equivalent in its agricultural and growth characteristics and in nutrient composition to that of conventional maize. Based upon these findings, we conclude that the cultivation of Event PY203 in the environment is as safe as is the cultivation of conventional maize.

As maize Event PY203 does not differ substantially from conventional maize varieties in key agronomic and compositional qualities, the establishment of two endpoints is achieved. The first is the protection and maintenance of the abundance and variety of wildlife within and outside of fields where Event PY203 is cultivated. Included in this consideration are animals that consume maize or pests of maize, insects that benefit agriculture, animals that ingest or are otherwise exposed to maize or its derivatives, and plants that grow in habitats that could be invaded by maize. Secondly, is the consideration of the impact of the yield of crops for which maize may be considered a potential weed. In both cases, if Event PY203 is reasonably demonstrated to pose no greater threat to these two factors compared to conventional maize then the requirements of several environmental protection statutes, including the Federal Plant Protection Act, the Endangered Species Act and the National Environmental Policy Act are satisfied.

A. The Safety of Maize Event PY203 to Wildlife

The potential for wildlife to be exposed to harmful amounts of toxic substances derived from Event PY203 in the environment is evaluated in this section. The approach toward a safety assessment for wildlife first compares the nutrient composition of Event PY203 maize with that of near isogenic, non-transgenic maize and with the historical compositional database for maize in general. The goal of this comparison is to identify substances produced in Event PY203 that are different in concentration relative to non-transgenic maize. The potential routes of exposure of these substances in the environment to wildlife are considered in an assessment of the potential hazard or toxicity to wildlife. Together, the level of hazard and the potential for exposure in the environment are considered to assess the risk of harm that is defined as the likelihood that Event PY203 maize may cause reduced

abundance or diversity of wildlife. The biological activity of phytase enzymes is well understood (Oh et al., 2004) and it is not similar to that of any known toxic proteins. Second, the amino acid sequence of the Phy02 protein has been shown not to have homology to toxic proteins (FDA, 2015). Phytase enzymes have been routinely added to the diets of monogastric animals in order to increase the digestibility of phosphorus and to improve the availability of minerals in the diet. The safety and functionality of the Phy02 phytase enzyme in animal diets has been intensively investigated and these studies have been reviewed by the FDA-CVM. After reviewing the safety and functionality studies of the Phy02 phytase, FDA-CVM informed Agrivida, Inc. that that it had no further questions regarding Agrivida, Inc.'s conclusion that the Phy02 phytase is safe and effective when added to the diets of poultry and that it is therefore GRAS for this use (CVM, 2017b). Agrivida, Inc. has conducted similar safety and functionality studies with swine and based upon the results of these studies has concluded that the Phy02 phytase is also GRAS for use in swine feed. The data and information that supports this conclusion are currently under review by FDA-CVM. Based upon these studies, the long history of the use of phytase enzymes in animal feed, and the safety of the Phy02 phytase as outlined in § V.E, it is reasonable to assume that, in the case that wildlife or domesticated animals were to consume grain of Event PY203 that was dispersed in fields where this crop had been cultivated, the animals would not suffer any adverse consequences. Indeed, it is anticipated that in the event of the unintended consumption of Phy02 maize grain by wildlife or domesticated animals that they would derive similar benefits related to increased phosphorus and mineral availability as do poultry and swine that consume it routinely in their diets. Since the level of the hazard posed to wildlife that may inadvertently consume grain from Event PY203 is expected to be extremely low or nonexistent, even though it is possible that some wildlife may consume small amounts of grain of Event PY203 that is dispersed in fields where this maize was cultivated, the likelihood that this consumption will result in reduced abundance or diversity of wildlife is extremely low.

B. Estimates of Phy02 Phytase Levels for Wildlife Exposure Assessment

Wildlife may be exposed to the Phy02 phytase through consumption of leaves and kernels of Event PY203 in the field. A highly conservative estimate of potential wildlife exposure to the Phy02 phytase was calculated based on the highest level detected in the tissues of Event PY203 that would be consumed. The highest level of Phy02 phytase detected in kernels was 9079.5 μg/g and in leaves it was 0.054 μg/g (Table 10). In the event of the consumption by wildlife of tissues of Event PY203 that contain the Phy02 phytase, no adverse effects are expected based on the rationale presented in § V.E. The Phy02 phytase would be expected to increase the digestibility of phosphorus from phytate in the diets of the wildlife that consumes tissues of Event PY203 as it does in the diets of poultry and swine fed diets treated with the Phy02 phytase. The values presented in Table 24 are used in the environmental risk assessment described in § X.

Table 24. Levels of Phy02 phytase for wildlife exposure assessment

Tissue	Development Stage	Highest Level (μg/g)
kernels	R6 (physiological maturity)	9079.5
leaves	R6 (physiological maturity)	0.054

The results of studies in which animals were dosed with high levels of the Phy02 phytase or the nearly identical Quantum® phytase that were described in § V.E indicate that high doses of phytase are well tolerated by animals and that in the event of the consumption of grain from Event PY203 by wildlife in the environment there would be no adverse effects to the animals that consumed the grain. In a subchronic exposure study, rats were dosed orally each day for 90 days with 400 mg of purified Quantum® phytase representing 462,000 FTU/kg body weight. The rats in this study showed no adverse health effects or any signs of toxicity (EFSA, 2008). The USDA has estimated that it takes 70.7 ears of dry corn to produce a bushel of corn weighing approximately 56 pounds. Therefore, a typical ear of dry corn contains about 0.8 lbs of corn or 363 grams. The corn from a single ear from Event PY203 that contains approximately 4,000 FTU of phytase activity per gram would contain a total of approximately 1.5 million FTUs from the Phy02 phytase (363 g corn grain/cobb x 4,000 FTU/g grain from Event PY203). In the case of a small animal weighing 6 to 8 lbs, the consumption of an ear of corn from Event PY203 containing a total of 1.5 million FTU of Phy02 phytase would represent a one-time dose of phytase in the same range as the dose administered to rats daily in the 90-day subchronic study described in § V.E. Larger animals consuming corn from Event PY203 would be exposed to smaller doses of Phy02 on a per body weight basis. Collectively, this information indicates that the consumption of grain from Event PY203 by wildlife in the environment is unlikely to have any adverse effects or negative impacts on the consuming animals. Conversely, as indicated by many studies on the effects of delivering high doses of phytase to animals (see § V.E.), consumption of corn grain from Event PY203 in the environment is likely to improve the dietary availability of phosphorus and minerals in the animals consuming it.

There are many insects that feed on maize in the environment. Based on the demonstration that there is little to no production of the Phy02 phytase protein in leaf and/or stalk tissue at any developmental stage (§ IV) it is concluded that consumption of these tissues by insects would not have adverse affects or otherwise impact their normal life cycle. Other insects may feed on roots or pollen which also were demonstrated in Event PY203 to produce little to no Phy02 phytase protein (§ IV) and so it is similarly concluded that consumption of roots or pollen by insects would have no adverse impacts on the individuals consuming these tissues. Insects that may consume developing or mature grain from Event PY203 would be exposed to higher dietary levels of Phy02 phytase compared to insects that consumed other

tissues. Depending on the pH of the digestive system of the consuming insect, the Phy02 phytase may or may not demonstrate phytase enzymatic activity. The Phy02 phytase in maize grain has optimal activity in the range of pH 2 to 7 (CVM, 2017) so it would not be expected to have significant activity in the digestive tracts of insects that have digestive systems with a more basic pH. In this case, the Phy02 phytase protein that is consumed would be digested into its constituent amino acids and no adverse impact to the insect is expected. Even if the Phy02 phytase maintains some activity in the digestive process of consuming insects, this activity is known not to be toxic or otherwise harmful. Seed and grain of many plants have been demonstrated to contain phytase enzymes and consumption of these by insects has not been demonstrated to be harmful. Based on this it is concluded that consumption by insects of tissues of Event PY203, including grain or developing kernels, would have no adverse environmental or health impacts on the consuming individuals or their populations.

C. The Composition of Maize Event PY203

The results of a comparison of the nutritional composition of grain and forage derived from maize Event PY203 are described in § VIII. The conclusion of this study is that, except for phytic acid in grain, there are no significant differences in the composition of grain or forage between Event PY203 and the near isogenic non-transgenic control maize, or with the historical maize composition databases. In the case of phytic acid, the level detected in grain of Event PY203 was approximately half the value of that for both the near isogenic control maize and the historical average for maize, as recorded in the ILSI database. This result is not surprising given that the Phy02 phytase is produced primarily in grain and its biological activity is the degradation of phytic acid. However, phytic acid is considered to be an anti-nutrient in grains due to its ability to chelate important dietary minerals that renders them nutritionally unavailable. Therefore it is reasonable to conclude that the reduction of phytic acid in maize poses no increased risk to wildlife exposed to the grain of Event PY203.

D. The Environmental Fate of the Phy02 Phytase Protein

The environmental fate of the Phy02 phytase produced in the grain of Event PY203 is considered in order to determine potential routes of exposure of wildlife to this protein.

1. Phy02 Protein in Cultivated Maize Event PY203

The pattern of the expression of the *phy02* gene and the production of the Phy02 phytase in maize is described in § IV. The Phy02 protein is present primarily in the grain during all stages of grain development. There is no appreciable production of the Phy02 protein in leaves, shoots, roots or pollen.

2. Fate of Phy02 in the Soil

The most likely route for the Phy02 protein to enter soil is by the spillage of grain on the field during harvest or from ears dropping from the plants during growth. Although it is recognized that plants exude proteins from their roots into the soil, Agrivida, Inc. has demonstrated that there is very little production of the Phy02 protein in the roots of maize Event PY203 (§ IV) and so this route of entry into the soil is not expected to be significant. Most proteins do not persist or accumulate in the soil because they are readily degraded by proteases in soils that are the product of microbial activity (Burns, 1982; Marx *et al*., 2005, and references therein). Phytase enzymes are commonly produced by soil organisms (§ V.D) and these proteins are not known to persist or accumulate in soil. Although no studies have been conducted to specifically investigate the stability of the Phy02 protein in soil, the Phy02 protein has been shown to be susceptible to digestion by proteases in simulated gastric and intestinal environments. Therefore, it is expected that the Phy02 protein will be similarly degraded in the soil as other microbial phytases that are naturally present in the soil.

A third theoretical route for Phy02 protein to enter the soil is by horizontal transfer of the *phy02* gene from Event PY203 into soil microorganisms. Many studies have been conducted to investigate the potential for gene transfer from plants into other organisms in the environment and they all conclude that there is very little potential for horizontal gene transfer between transgenic plants and soil microorganisms or other organisms in the environment (EPA, 2001; Conner *et al*., 2003). In the unlikely event that a *phy02* gene were transferred from Event PY203 into a soil microorganism, it is unlikely that the gene would be expressed or that any Phy02 protein would be produced since the promoters used to express the *phy02* gene in Event PY203 are plant specific and would not function in a microbial host. Therefore, it is concluded that it is extremely unlikely that Phy02 protein will be produced in the soil by microbes as a result of potential horizontal gene transfer from maize Event PY203. In the unlikely event that the *phy02* gene was transferred horizontally from maize Event PY203 to a soil microbe and that the *phy02* gene was expressed in this organism thereby resulting in the production of the Phy02 phytase in the soil, it is anticipated that this would have no impact on the soil environment since many soil microbes already produce similar, if not nearly identical, phytase enzymes.

In summary, the consequences of cultivating Event PY203 in the environment would be no different from those resulting from the cultivation of conventional maize. This conclusion is supported by the information presented in this document that demonstrates that the agronomic characteristics, grain and forage nutrient composition, seed germination, and other characteristics of Event PY203 are substantially equivalent to those of conventional maize varieties. The well-established safety of the Phy02 phytase for humans and animals, the long and safe history of oral exposure by humans and animals to phytase enzymes, and the ubiquitous nature of phytase enzymes in the environment provide further support

for the conclusion that the cultivation of Event PY203 would not result in any additional adverse environmental impacts relative to the cultivation of conventional maize.

E. Weediness Potential of Event PY203

Maize has been cultivated as a crop for centuries and it has never shown any tendency to become a weed. Due to this experience maize is not on the list of noxious weed species prepared by the federal government (7 CFR § 360). Currently cultivated varieties of maize are highly domesticated as a result of many years of selection by humans for desirable traits. In the process of this domestication, traits that are associated with weediness such as seed dispersal mechanisms, seed dormancy, or the ability to develop vibrant populations in the environment without human cultivation, have not been selected or maintained. Although maize from the previous crop year can overwinter and germinate the following year, it does not persist as a weed (OECD, 2003). The presence of maize in soybean fields following the maize crop from the previous year is a common occurrence. Measures are often taken to either eliminate the plants with cultivation or use of herbicides to kill the plants in soybean fields, but the plants that remain and produce seed usually do not persist during the following years. Maize plants are non-invasive in natural habitats (Gould, 1968) and are not capable of weediness (Keeler, 1989). Corn does not persist in habitats outside agriculture because, in addition to the features listed above, it requires disturbed ground to germinate and it is very uncompetitive against perennial vegetation (Raybould *et al*. 2012). In contrast to weedy plants, maize has a pistillate inflorescence (ear) with a cob enclosed with husks. Consequently seed dispersal of individual kernels does not occur naturally.

Studies were conducted in order to compare the characteristics of maize Event PY203 and a conventional, near isogenic , nontransgenic maize variety. The results of these studies demonstrated that Event PY203 is equivalent to conventional maize varieties in properties including vegetative and reproductive characteristics, interactions with insects and diseases, nutrient composition of grain and forage, and seed germination. Two of the agronomic parameters that were assessed in the agronomic comparison study that would be expected to affect weediness potential were emergent stand count (the number of plants in a row 14 days after planting) and final stand count (the number of plants in a row at maturity). In this study Event PY203 had similar, but statistically lower, emergent and final stand counts compared to the conventional control (Table 14). This result confirms that Event PY203 does not have an increased potential to become a weed. Collectively, these results support a conclusion that maize Event PY203 has no increased weed potential compared to conventional maize and it is no more likely to become a weed than conventional maize.

XI. References

Abendroth L.J., R.W. Elmore, M.J. Boyer, and S.K. Marlay (2011). Corn Growth and Development. PMR 1009. Ames, IA: Iowa State University Extension. 49 pp.

Anderson, W.P. (1996). Weed ecology. In: Weed science principles and applications. St. Paul, MN: West Publishing Company. pp 27-38.

AOSA. (1998). Rules for testing seeds. Lincoln, NE: Association of Official Seed Analysis.

Augspurger, N.R., D. M. Webel, X. G. Lei, and D. H. Baker (2003). Efficacy of an *E. coli* phytase expressed in yeast for releasing phytate-bound phosphorus in young chicks and pigs. J. Anim. Sci. **81**:474-483.

Brask-Pedersen, D.N., L.V. Glitsø, L.K. Skov, P. Lund, and J. Sehested (2013). Effect of exogenous phytase on degradation of inositol phosphate in dairy cows. J. Dairy Sci. **96**: 1691–1700.

Bravo, D., F. Meschy, C. Bogaert, and D. Sauvant (2003). Phosphorus availability of oilseed meals determined by the apparent faecal digestibility technique. Anim. Feed Sci. Technol. **108**:43–60.

Buendia, G., M.S. González, S.M. Pérez, N.I. Ortega, H.C. Aceves, F.M.D. Montoya, B.I. Almaraz, P.J.E. Partida, and A.Z.M. Salem (2014). Effect of an exogenous phytase on growth performance in growing Holstein calves. Anim. Nutr. Feed Technol. **14**:169–174.

Burns, R.G. (1982). Enzyme activity in soil: location and possible role in microbial ecology. Soil Biol. Biochem. **14**: 423-427.

CFIA (1994). The biology of Zea mays L. (corn/maize). Plant Health and Production Division, Plant Biosafety Office. Regulatory Directive Dir94-11. http://www.inspection.gc.ca/english/plaveg/bio/dir/dir9411e.shtml.

Christensen, A. H., and P.H. Quail (1996). Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Research, **5**(3), 213–218.

Coe, E.H., M.G. Nueffer, and D.A. Hoisington. (1988). The genetics of maize. In: G.F Sprague and J.W. Dudley (Eds.), Corn and corn improvement (Agronomy Monographs No. 18; pp. 81-236). Madison, WI: American Society of Agronomy.

Collins, J. and B. Hohn (1978). Cosmids: a type of plasmid gene-cloning vector that is packageable in vitro in bacteriophage lambda heads. PNAS USA **75**:4242–4246.

Conner, A.J., T.R. Glare and J.-P. Nap (2003). The release of genetically modified crops into the environment. Part II. Overview of ecological risk assessment. The Plant Journal 33, 19–46.

Correll, D. L. (1999). Phosphorus: a rate limiting nutrient in surface waters. Poultry Science **78**: 674-682.

Coulibaly, A., B. Kouakou, and J. Chen (2011). Phytic acid in cereal grains: Structure, healthy or harmful ways to reduce phytic acid in cereal grains and their effects on nutritional quality. Amer. J. Plant Nutr. Fertilization Tech. 1 (1): 1-22.

CVM (2017a). GraINzyme® Phytase: A phytase feed enzyme produced by Zea mays expressing a phytase gene derived from Escherichia coli. Current Animal Food GRAS Notices Inventory, available at: https://www.fda.gov/downloads/AnimalVeterinary/Products/AnimalFoodFeeds/GenerallyRecognizedasSafeGRASNotifications/UCM581398.pdf

CVM (2017b). Letter from CVM stating that it has no further questions related to Agrivida's conclusion that its maize produced phytase enzyme is GRAS for use in poultry feed. https://www.fda.gov/downloads/AnimalVeterinary/Products/AnimalFoodFeeds/GenerallyRecognizedasSafeGRASNotifications/UCM581397.pdf

Depicker, A., Stachel, S., Dhaese, P., Zambryski, P., Goodman, H.M. (1982). Nopaline synthase: transcript mapping and DNA sequence. J. Mol. Appl. Genet. **1**, 561-573.

de Virgilio, M., F. De Marchis, M. Bellucci, D. Mainieri, M. Rossi, E. Benvenuto, S.Arcioni and A. Vitale (2008). The human immunodeficiency virus antigen Nef forms protein bodies in leaves of transgenic tobacco when fused to zeolin. J. Exper. Botany **59:**2815-2829.

de Wet, J.M.J. (1975). Evolutionary dynamics of cereal domestication. Bull. Torr. Bot. Club **102**: 307-312.

de Wet, J.M.J. and J.R. Harlan (1972). The origin of maize: the tripartite hypothesis. Euphytica **21**: 271-279.

de Wet, J.M.J., Timothy, D.H., Hilu, K.W., and G.B. Fletch. (1981). Systematics of South American Tripsicum (Graminae) Am. J. Bot. **68**: 269-276.

Doebley, J.F. and Iltis, H.H. (1980). Taxonomy of Zea (Graminae). I. Subspecific classification with key to taxa. Am. J. Bot. 67, 986-983.

Doebley, J.F. (1984). Maize introgression into teosinte- a reappraisal. Ann. Miss. Bot. Gard. 71, 1100-1112.

Doebley, J., Goodman, M.M., Stuber, C.W. (1987). Patterns of isozyme variation between maize and Mexican annual teosinte. Eco. Bot. 41, 234-246.

EFSA (European Food Safety Authority) (2008). Safety and efficacy of the product Quantum™ Phytase 5000 L and Quantum™ Phytase 2500 D (6-phytase) as a feed additive for chickens for fattening, laying hens, turkeys for fattening, ducks for fattening and piglets (weaned). The EFSA Journal **627**:1-27.

EPA (2001). Biopesticides Registration Action Document – Bacillus thuringiensis Plant Incorporated Protectants.
https://archive.epa.gov/pesticides/biopesticides/web/html/bt_brad.html

EPA (2002). Notice of Data Availability; National Pollutant Discharge Elimination System Permit Regulation and Effluent Limitations Guidelines and Standards for Concentrated Animal Feeding Operations. Federal Register **67**(141): 48099-48110.

EPA (2004). Phosphomannose isomerase and the genetic material necessary for its production in all plants; Exemption from the requirement of a tolerance. 40 CFR Part 180. Fed. Reg. 69(94), 26770-26775, May 14, 2004.

Esen, A. (1982). Tandem repeats in the N-terminal sequence of a proline-rich protein from corn endosperm. Nature **296**:678-679.

FDA (2015). Early food safety assessment for the Phy02 phytase. New Protein Consultation (NPC) 000015;
https://www.accessdata.fda.gov/scripts/fdcc/?set=NPC

Fling, M.E., Kopf, J., Richards, C. (1985). Nucleotide sequence of the transposon Tn7 gene encoding an aminoglycoside-modifying enzyme, 3'(9)-Onucleotidyltransferase. Nucleic Acids Res. 13, 7095-7106.

Freeze, H.H. (2002). Phosphomannose isomerase. In: N. Taniguchi, K. Honke & M. Fukuda (Eds.), Handbook of glycosyltransferases and related genes (1st Edition, pp. 595-599). Tokyo & New York: Springer-Verlag.

Fu T.-J., U.R. Abbott, and C. Hatzos (2002). Digestibility of allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid—a comparative study. J Agric Food Chem. 50:7154–7160.

Galinat, W.C. (1973). Preserve Guatemalan teosinte, a recent link in corn's evolution. Science 180, 323.

Galinat, W.C. (1988). The origin of corn. In: G.F Sprague & J.W. Dudley (Eds.), Corn and corn improvement (Agronomy Monographs No. 18; pp. 1-31). Madison, WI; American Society of Agronomy.

Geli, M. I., M. Torrent, and D. Ludevid (1994). Two structural domains mediate two sequential events in γ-zein targeting: Protein endoplasmic reticulum retention and protein body formation. Plant Cell **6:**1911-1922.

Gerde, J. A., S. Tamagno, J.C. Di Paola, and L. Borrás (2016). Genotype and nitrogen effects over maize kernel hardness and endosperm zein profiles. Crop Science, **56**(3): 1225–1233. http://doi.org/10.2135/cropsci2015.08.0526.

GHC (2018). VeganZyme®; http://www.globalhealingcenter.com/supplements/digestive-enzymes-supplement.html?gclid=CjwKEAiAxNilBRD88r2azcqB2zsSJABy2B96KcLMTx6IVQsDjU; accessed 6 July 2018.

GNC (2018). Natural BrandTM Super Digestive Enzymes; http://www.gnc.com/enzymes/180313.html?mrkgcl=1098&mrkgadid=3200889660&product_id=180313&flagtype=brand&vendor=none&channel=ppc&adpos=1o1&creative=189757865731&device=c&matchtype=&network=g&gclid=EAIaIQobChMIi_-4hY-L3AIVQeDICh34wwSCEAQYASABEgI7X_D_BwE;
Accessed 6 July 2018.

Gould, F.W. (1968). Grass systematics. New York: McGraw Hill, pp.1-382.

Government of Argentina (2010). About Argentina. https://web.archive.org/web/20101220215355/http://www.argentina.gov.ar/argentina/portal/paginas.dhtml?pagina=1483

Greiner, R., E. Haller, U. Konietzny, and K.-D. Jany (1997). Purification and characterization of a phytase from *Klebsiella terrigena*. Archives of Biochemistry and Biophysics **341**:201–206.

Greiner, R., K.-D. Jany, and M. Larsson Alminger (2000). Identification and properties of myo-inositol hexakisphosphate phosphohydrolases (phytases) from barley (*Hordeum vulgare*). Journal of Cereal Science, **31**:127–139.

Greiner, R., U. Konietzny, andK.-D. Jany (1993). Purification and characterization of two phytases from *Escherichia coli*. Archives of Biochemistry and Biophysics **303**:107–113.

Hallauer, A.R., Russell, W.A., Lamkey, K.R. (1988). Corn Breeding. In: G.F. Sprague & J.W. Dudley (Eds.), Corn and corn improvement. (Agronomy Monographs No 18; pp. 463-564). Madison, WI; American Society of Agronomy.

Hallauer, A.R. (2000). Potential for outcrossing and weediness of genetically modified insect protected corn. APHIS-USDA.

Harlan, J.R. (1992). Crops and man. Crop Science Society of America, Inc. (2nd Edition, 284 pp.), Madison, WI: American Society of Agronomy.

Harland, B. F. and E. R. Morris (1995). Phytate: a good or a bad food component? Nutrition Research **15**:733-754.

Harrison, M.D., J. Geijskes, H.D. Coleman, K. Shand, M. Kinkema , A. Palupe, R. Hassall, M. Sainz, R. Lloyd, S. Miles and J.L. Dale (2011). Accumulation of recombinant cellobiohydrolase and endoglucanase in the leaves of mature transgenic sugar cane. Plant Biotechnol. J. **9**:884-896.

Hayes, J.E., R.J. Simpson, and A.E. Richardson (2000). The growth and phosphorus utilisation of plants in sterile media when supplied with inositol hexaphosphate, glucose 1-phosphate or inorganic phosphate. Plant and Soil, **220:**165–174.

Hitchcock, A.S. and Chase, A. (1971). Manual of the grasses of the United States (Vol. 2. pp. 790-796), New York: Dover Publications.

Horinouchi, S., K. Furuya, M. Nishiyama, H. Suzuki, and T. Beppu (1987). Nucleotide sequence of the streptothricin acetyltransferase gene from *Streptomyces lavendulae* and its expression in heterologous hosts. J. Bacteriol. 169:129-137.

Hurley, L.A., T.L. Stanton, M.J. Jarosz, and D. Shutz (2002). Case Study: Effects of dairy phosphorus and microbial phytase level on beef finishing performance. Professional Animal Scientist **18**:286-292.

ILSI-CCDB (2018). Crop Composition Database, International Life Science Institute. Available at: https://www.cropcomposition.org/query/index.html.

Iltis, H. H. and J. F. Doebley (1980). Taxonomy of *Zea* (*Gramineae*). II. Subspecific categories in the *Zea mays* complex and a generic synopsis. Amer. J. Bot. **67**:994-1004.

International Food Biotechnology Council. (1990). Biotechnologies and food: assuring the safety of foods produced by genetic modification. Reg. Tox. Pharma. 12, S1-S196.

Iqbal, T. H., K.O. Lewis, and B.T. Cooper (1994). Phytase activity in the human and rat small intestine. Gut **35**:1233-1236.

Itoh, T. and J. Tomizawa (1978). Initiation of replication of plasmid ColE1 DNA by RNA polymerase, ribonuclease H and DNA polymerase I. Cold Spring Harbor Symposium on Quantitative Biology. 43, 409-418.

Jongbloed, A. W. and N. P. Lenis (1998). Environmental concerns of animal manure. Journal of Animal Science **76**:2641-2648.

Keeler, K.H. (1989). Can genetically engineered crops become weeds? Bio/Technology **7**:1134-1139.

Kerovuo, J., M. Lauraeus, P. Nurminen, N. Kalkinnen, and J. Apajalahti (1998). Isolation, characterization, molecular gene cloning and sequencing of a novel phytase from *Bacillus subtilis*. Applied and Environmental Microbiology,**64:**2079–2085.

Kim, Y.-O., H.-K. Kim, K.-S. Bae, J.H. Yu, and T.K. Oh (1998a). Purification and properties of a thermostable phytase from *Bacillus* sp. DS11. Enzyme and Microbial Technology, **22**:2–7.

Kincaid, R.L., D.K. Garikipati, T.D. Nennich, and J.H. Harrison (2005). Effect of grain source and exogenous phytase on phosphorus digestibility in dairy cows. J. Dairy Sci. **88**:2893–2902.

Knowlton, K.F., M.S. Taylor, S.R. Hill, C. Cobb, and K.F. Wilson (2007). Manure nutrient excretion by lactating cows fed exogenous phytase and cellulase. J. Dairy Science **90**:4356-4360.

Konietzny, U. and R. Greiner (2002). Molecular and catalytic properties of phytate-degrading enzymes (phytases). International Journal of Food Science and Technology **37**:791–812.

Konishi, C., Matsui, T., Park, W.Y., Yano, H., Yano, F. (1999). Heat treatment of soybean meal and rapeseed meal suppresses rumen degradation of phytate phosphorus in sheep. Anim. Feed Sci. Technol. 80, 112–115.

Lambrechts, C., H. Boze, G. Moulin, P. Galzy (1992). Utilization of phytate by some yeasts. Biotechnology Letters, **14**:61–66.

Lim, D., S. Golovan, C.W. Forsberg, and Z. Jia (2000). Crystal structures of Escherichia coli phytase and its complex with phytate. Nat. Struct. Biol. **7**:108-113.

Lin, J.-J., D.B.Dickinson, and T.-H.D. Ho (1987). Phytic acid metabolism in Lily (*Lilium longiflorum* Thunb.) pollen. Plant Physiology, **83**:408–413.

Lin, L., I. Ockenden, and J.N.A. Lott (2005). The concentrations and distributions of phytic acid-phosphorus and other mineral nutrients in wild-type and low phytic acid1-1 (*lpa1-1*) corn (*Zea mays* L.) grains and grain parts. Can. J. Botany **83**(1):131-141.

Liu, S., Kriz, A., Duncan, D., & Widholm, J. (1998). Abscisic acid-regulated Glb1 transient expression in cultured maize P3377 cells. *Plant Cell Reports*, *17*, 650–655.

Luna, S., V.J. Figueroa, M.B. Baltazar, M.R. Gomez, L.R. Townsend, J.B. Schoper. (2001). Maize pollen longevity and distance isolation requirements for effective pollen control. Crop Sci. 41, 1551-1557.

Mallin, M. A. (2000). Impact of industrial animal production on rivers and estuaries. American Scientist **88**:26-37.

Mangelsdorf, P.C. (1974). Corn: its origin, evolution and improvement. (pp. 113-120). Cambridge: Harvard University Press.

Marx, M.C., Kandeler, E., Wood, M., Wermbter, N., Jarvis, S.C. (2005). Exploring the enzymatic landscape: distribution and kinetics of hydrolytic enzymes in soil particle-size fractions. Soil Biol. Biochem. 37, 35-48.

Maugenest, S., I. Martinez, B. Godin, P. Perez and A.-M. Lescure (1999). Structure of two maize phytase genes and their spatio-temporal expression during seedling development. Plant Molecular Biology **39**:502–514.

Miles, J.S. and J.R. Guest (1984). Nucleotide sequence and transcriptional start point of the phosphomannose isomerase gene (*manA*) of *Escherichia coli.* Gene **32**:41-48.

Minihane, A. M., and G. Rimbach (2002). Iron absorption and the iron binding and anti-oxidant properties of phytic acid. International Journal of Food Science and Technology **37**:741-748.

Mishra, R.N., S.L. Singla-Pareek, S. Nair, S.K. Sopory, and M.K. Reddy (2002). Directional genome walking using PCR. BioTechniques **33**:830-834.

Mueller, D., & Pope, R. (Eds.). (2009). Corn Field Guide: A reference for identifying diseases, insect pests, and disorders of corn*.* Ames: Iowa State University of Science and Technology.

Nahm, K.H. (2002). Efficient feed nutrient utilization to reduce pollutants in poultry and swine manure. Critical Revs. Environ. Sci. & Technol. **32:**1-16.

Nakamura, Y., H. Fukuhara, and K. Sano (2000). Secreted phytase activities of yeasts. Bioscience, Biotechnology, and Biochemistry, **64**:841–844.

NEC (2018). CereCalase; https://www.ulprospector.com/en/na/Food/Detail/6662/223773/CereCalase; accessed 27 March 2019.

Negrotto, D., Jolley, M., Beer, S., Wenck, A.R., Hansen, G. (2000). The use of phosphomannose-isomerase as a selectable marker to recover transgenic maize plants (Zea mays L.) via Agrobacterium transformation. Plant Cell Rep. 19, 798-803.

NIH (2018). Vitamin A: A fact sheet for health professionals. National Institute of Health; Office of Dietary Supplements: https://ods.od.nih.gov/factsheets/VitaminA-HealthProfessional/.

Nutriteck (2018). Phytase Supplement; http://www.nutriteck.com/phytase.html; Accessed 6 July 2018.

Nyannor, E. and O. Adeola (2008). Corn expressing an *Escherichia Coli*-derived phytase gene: comparative evaluation study in broiler chicks. Poultry Sci. **87**:2015–2022.

Nyannor, E.K.D., M.R. Bedford, and O. Adeola (2009). Corn expressing an *Escherichia coli*-derived phytase gene: Residual phytase activity and microstructure of digesta in broiler chicks. Poultry Sci. **88:**1413-1420.

Nyannor, E., P. Williams, M. Bedford, and O. Adeola (2007). Corn expressing an *Escherichia coli*-derived phytase gene: A proof-of-concept nutritional study in pigs. J. Anim. Sci. **85**:1946-1952.

OECD (2003). Consensus document on the biology of Zea mays subsp. mays. Series on Harmonisation of Regulatory Oversight in Biotechnology, No. 27. http://www.olis.oecd.org/olis/2003doc.nsf/43bb6130e5e86e5fc12569fa005d004c/f70b80eb7cd25728c1256d57003e5f0c/$FILE/JT00147699.PDF.

Oh, B.-C., W.-C. Choi, S. Park, Y.-O. Kim, and T.-K. Oh (2004). Biochemical properties and substrate specificities of alkaline and histidine acid phytases. Appl. Microbiol. Biotechnol. **63**(4):362-372.

Onyango, E. M., M. R. Bedford, and O. Adeola (2005). Efficacy of an evolved *Escherichia coli* phytase in diets of broiler chicks. Poult. Sci. **84**:248–255.

Pariza, M.W. and Johnson, E.A. (2001). Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. Regul. Toxicol. Pharmacol. 33(2), 173-186.

Park, W.Y., Matsui, T., Konishi, C., Kim, S.W., Yano, F., Yano, H., 1999. Formaldehyde treatment suppresses ruminal degradation of phytate in soybean meal and rapeseed meal. Br. J. Nutr. 81, 467–471.

Paterniani, E. and A.C. Stort (1974). Effective maize pollen dispersal in the field. Euphytica **23**:129-134.

Poulsen, H. (2000). Phosphorus utilization and exdretion in pig production. Journal of Environmental Quality **29**:24-27.

Powar, V.K. and V. Jagannathan (1982). Purification and properties of phytate-specific phosphatase from *Bacillus subtilis*. Journal of Bacteriology, **151**:1102–1108.

Prat, S., J. Cortadas, P. Puigdomenech and J. Palau (1985). Nucleic acid (cDNA) and amino acid sequences of the maize endosperm protein glutelin-2. Nucleic Acids Res. **13** (5): 1493-1504.

Privalle, L., *et al*. (2006). Phosphomannose Isomerase, A Novel Selectable Plant Selection System: Mode of Action and Safety Assessment. Proceedings of the 6th International Symposium on The Biosafety of Genetically Modified Organisms, Saskatoon, Canada.

Qu, L.Q., Y.P. Xing, W.X. Liu, X.P. Xu and Y.R. Song (2008). Expression pattern and activity of six glutelin gene promoters in transgenic rice. J. Exper. Botany **59:**2417–2424.

Quail, P.H., A.H. Christensen, H.P. Hershey, R.A. Sharrock and T.D. Sullivan (1996). Plant ubiquitin promoters system. U.S. Patent No. 5,510,474. USPTO.

Randolph, L.F. (1959). The origin of maize. Indian J. Genet. Plant Breed. 19, 1-12.

Raybould A., L.S. Higgins, M.J. Horak, R.J. Layton, N.P. Storer, J.M. De La Fuente, and R.A. Herman (2012). Assessing the ecological risks from the persistence and spread of feral populations of insect-resistant transgenic maize. Transgenic Research **21**:655-664.

Raynor, G.S., Eugene, C.O., Janet, V.H. (1972). Dispersion and deposition of corn pollen from experimental sources. Agron. J. 64, 420-427.

Reddy, N.R., M.D. Pierson, S.K. Sathe, and D.K. Salunkhe (1989). Phytates in Cereals and Legumes. Boca Raton, FL: CRC Press Inc.

Reddy, N. R., S. K. Sathe, et al. (1982). Phytates in legumes and cereals. Advances in Food Chemistry. C. O. Chichester, E. M. Mrak and G. F. Stewart. New York, Academic Press, pp 1-92.

Reina, M., P. Guillen, I. Ponte, A. Boronat and J. Palau (1990). DNA sequence of the gene encoding the Zc1 protein from *Zea mays* W64 A. Nucleic Acids Res. **18:**6425.

Richardson, A.E. and P.A. Hadobas (1997). Soil isolates of *Pseudomonas* spp. that utilize inositol phophates. Canadian Journal of Microbiology, **43**:509–516.

Russell, D.A. and Fromm, M.E. (1997). Tissue-specific expression in transgenic maize of four endosperm promoters from maize and rice. Transgenic Res. 6, 157-168.

Russell, W.A., Hallauer, A.R. (1980). Corn. In: W.R Fehr & H.H. Hadley (Eds.),

Hybridization of crop plants (pp. 299-312). Madison, WI: American Society of Agronomy and Crop Science Society of America.

Sandberg, A. S., L.R. Hulthen, and M. Turk (1996). Dietary Aspergillus niger phytase increases iron absorption in humans. Journal of Nutrition **126**:476-480.

Sekhon, R.S., H. Lin, K.L. Childs, C.N. Hansey, C. R. Buell, N. de Leon, and S.M. Kaeppler (2011). Genome-wide atlas of transcription during maize development. The Plant Journal **66**:553-563.

Semenza, J.C. and H.R.B. Pelham (1992). Changing the specificity of the sorting receptor for luminal endoplasmic reticulum proteins. J. Mol. Biol. **224:**l-5.

Sharpley, A., S.C. Chapra, R. Wedepohl, J.T. Sims, T.C. Daniel, and K.R. Reddy (1994). Managing agricultural phosphorus for protection of surface waters: Issues and options. J. Environmental Quality **23**:437-451.

Shimizu, M. (1992). Purification and characterization of a phytase from *Bacillus subtilis* (natto) N-77. Bioscience, Biotechnology, and Biochemistry, **56**:1266–1269.

Short, J. (2001). Saturation mutagenesis in directed evolution, US Patent Number 6,171,820 B1. US Patent Office.

Smith, B.D. (1995). The emergence of Agriculture (231 pp.), New York: Scientific American Library.

Smith, J.S.C., Goodman, M.M., C.W., Stuber. (1985). Relationships between maize and teosinte of Mexico and Guatemala: numerical data analysis of allozyme data. Eco. Bot. 39, 12-24.

Sprague, G.F. and Eberhart, S.A.. (1976). Maize breeding. In: Corn and corn improvement. (Agronomy Monographs No. 18, pp. 312-313), Madison, WI: American Society of Agronomy.

Streatfield, S.J., J. Bray, R.T. Love, M.E. Horn, J.R. Lane, C.F. Drees, E.M. Egelkrout and J.A. Howard (2010). Identification of maize embryo-preferred promoters suitable for high-level heterologous protein production. GM Crops, **1:**162-172.

Tambe, S.M., G.S. Kaklij, S.M. Keklar, and L.J. Parekh (1994). Two distinct molecular forms of phytase from *Klebsiella aerogenes*: evidence for unusually small active enzyme peptide. Journal of Fermentation and Bioengineering, **77**:23–27.

Toor, G.S., B.J. Cade-Menun, and J. T. Sims (2005). Establishing a linkage between phosphorus forms in dairy diets, feces, and manures. J. Environ. Qual. **34**:1380–1391.

Torrent, M., B. Llompart, S. Lasserre-Ramassamy, I. Llop-Tous, M. Bastida, P. Marzabal, A. Westerholm-Parvinen, M. Saloheimo, P.B. Heifetz and M.D. Ludevid (2009). Eukaryotic protein production in designed storage organelles. BMC Biology **7**:5.

USDA (2018). Corn Yield and Soybean Production Up in 2017, USDA Reports Winter Wheat Seedings and Grain Stocks also reported. USDA National Agricultural Statistics Service. News Release, Jan. 12, 2018. https://www.nass.usda.gov/Newsroom/2018/01_12_2018.php.

USDA-FAS (2019). United States Department of Agriculture-Foreign Agricultural Service; Grain: World Markets and Trade, May 2019.

USDA-NASS (2019). Quick Stats. Accessed 30 May 2019. https://quickstats.nass.usda.gov/

USDA-NASS (2017). 2016 Agricultural Chemical Use Survey. USDA National Agricultural Statistics Service; No. 2017-2, May 2017. https://www.nass.usda.gov/Surveys/Guide_to_NASS_Surveys/Chemical_Use/2016_Corn_Potatoes/ChemUseHighlights_Corn_2016.pdf.

Vavilov, N.I. (1951). The origin, variation, immunity and breeding of cultivated plants (94 pp.), Translated from Russian by K. Starr Chester. New York: The Ronald Press Co.

Viveros, A., C. Centeno, A. Brenes, R. Canales, and A. Lozano (2000). Phytase and acid phosphatase activities in plant feedstuffs. Journal of Agricultural Food Chemistry **48**:4009-4013.

Walden, D.B. and Everett, H.L. (1961). A quantitative method for the in vivo measurement of the viability of corn pollen. Crop Sci. 1, 21-25.

Walter, P., R. Gilmore, and G. Blobel (1984). Protein translocation across the endoplasmic reticulum. Cell **38**:5-8.

Wang, K., Herrera-Estrella L., Van Montagu, M., Zambryski, P. (1984). Right 25 bp terminus sequence of the nopaline T-DNA is essential for and determines direction of DNA transfer from Agrobacterium to the plant genome. Cell 38, 455-462.

Wang, G., W. Qi, Q. Wu, D. Yao, J. Zhang, J. Zhu, J., and R. Song (2014). Identification and Characterization of Maize floury4 as a Novel Semidominant Opaque Mutant That Disrupts Protein Body Assembly. Plant Physiology, **165**(2): 582–594. http://doi.org/10.1104/pp.114.238030.

Watson, S.A. (1988). Corn marketing, processing and utilization. In: G.F. Sprague &

J.W. Dudley (Eds.), Corn and corn improvement (Agronomy Monographs No. 18; pp. 881-934). Madison, WI: American Society of Agronomy.

Wilkes, H.G. (1977). Hybridization of maize and teosinte, in Mexico and Guatemala and the improvement of maize. Eco. Bot. 31, 254-293.

Winter, L., U. Meyer, D. von Soosten, M. Gorniak, P. Lebzien, and S. Dänicke (2015). Effect of phytase supplementation on rumen fermentation characteristics and phosphorous balance in lactating dairy cows. Ital. J. Anim. Sci. **14**:53–60.

Wodzinski, R. J., and A.H.J. Ullah (1996). Phytase. Advances in Applied Microbiology **42**:263-302.

Wu, Y., D.R. Holding, and J. Messing (2010). γ -Zeins are essential for endosperm modification in quality protein maize. Proceedings of the National Academy of Sciences of the United States of America, **107**(29): 825–838. http://doi.org/10.1073/pnas.1004721107/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1004721107.

Wu, Y., and J. Messing (2010). RNA Interference-Mediated Change in Protein Body Morphology and Seed Opacity through Loss of Different Zein Proteins. Plant Physiology, **153**(1): 337–347. . http://doi.org/10.1104/pp.110.154690.

Wych, R.D. (1988). Production of hybrid seed corn. In: G.F Sprague & J.W. Dudley (Eds.), Maize and maize improvement (Agronomy Monographs No. 18; pp. 565-605). Madison, WI: American Society of Agronomy.

Yanke, L.J., H.D. Bae, L.B. Selinger, and K.-J. Cheng (1998). Phytase activity of anaerobic ruminal bacteria. Microbiology **144**:1565–1573.

Yoon, S.J., Y.J. Choi, H.K. Min, et al. (1996). Isolation and identification of phytase-producing bacterium, *Enterobacter* sp.4, and enzymatic properties of phytase enzyme. Enzyme and Microbial Technology, **18**:449–454.

Zambryski, P., Depicker, A., Kruger, K., Goodman, H.M. (1982). Tumor induction by Agrobacterium tumefaciens: analysis of the boundaries of T-DNA. J. Mol. Appl. Genet. 1, 361-370.

APPENDIX 1

Nucleotide sequence of Locus 3293 and maize genomic flanking DNA in Event PY203. Maize genomic DNA sequence is presented in lower case letters while the sequence of the T-DNA insert is presented in upper case. The table below presents the positions of key genetic elements relative to the nucleotide numbering.

Nucleotide Position		
Start	**End**	**Genetic Element**
1	1812	Maize chromosomal DNA (“chr8”)
2106	4176	GTL
4189	4245	Z27ss
4246	5478	Phy02
5479	5496	SEKDEL
5506	5781	NosT
5788	7150	ZmZ27
7163	7219	Z27ss
7220	8452	Phy02
8453	8470	SEKDEL
8480	8755	NosT
8762	11765	ZmGlb1
11778	11834	Z27ss
11835	13067	Phy02
13068	13085	SEKDEL
13095	13370	NosT
13377	15368	ZmUbi1
15369	16544	PMI
16593	16868	NosT
16939	16959	LB
16960	18621	Maize chromosomal DNA (“chr8”)

```
1      tgggtcgactgattcactgttttccctttttccatttttataattttaatttaagtttga  60
61     tttccatgttcaaataaattgtgatccaaatcacttcaaaaaatcataaataattagaag  120
121    agcactttagtaaagtttgatctcatttcttggtgaggatttttatacaaaaataatgtt  180
181    ttccctttctgtatttgaggaaaacatatgtaaactcatgtttaaagggaaaagaaatca  240
241    aattcctctctaaaacttgtgacatgtgtttgtgtattcatagtggttttgtgtcataca  300
301    aaggtagagtgttacaagcttacatgtatgttctacttatggaccctacctatcatcagg  360
361    cctactgcttctatctcattgtgatctcatatctaaacaaacacaaactctatacctcat  420
421    agccatgcaatgcacatggagcgcgcatcagtgttaatcatttctattgaggtgaccttc  480
481    caagctaggcctcgctgagtgaggacatgtgccttaaggggactagtacaccataataga  540
541    atctcagtatggttccttgtacttttactacatgtaagtatattcatactacctagtcat  600
601    cgctatagtatttttttccattccttggtaaagggcatgattgcctgacctggtatggcat  660
661    taaatgtgatggtagacctacacaatggcaacgagtacatctgtagccctaagagcatgt  720
721    caggttgaaggtataaccataactcatataataatgtagtttgaagcattacttatctcc  780
781    ccatttgtagttgttcctgtctagattttatgttacgtttgaggactttctcgaggtgat  840
841    tttcataccagttggtgggacatgtatgataagttgtgtctatgggtgcccaggcctgtg  900
901    tcgcttggtatgatgtaattggtacctaatgtagccttttgtacttgattgtgttttatg  960
```

```
961    gtacactttgtgtgtgcacgctcaactatagggttagtgcacacaccatattgtggtcat  1020
1021   gagtaccctatagcctagtcgtggcaacaacatgtgtgtcagggcttgcaatatcgccca  1080
1081   tcacacatttcatgctttgtacctaagacttatctactagactatcgctaagaaatagtc  1140
1141   tataaacccttagatgtgggatcgatttcctagggatataacatccatggtggcccctaa  1200
1201   tcacatccctctatctttgcaaaaaagattacacttcgagcccatgccccaaagtgccca  1260
1261   catctcgattgcccacaaagtacgagatcagagactcttcagggaacctagcatcaacgg  1320
1321   atcctaagcagctaagtggtgtagtgtatagggtttagactgtcctaagcatccacagtc  1380
1381   ttcttatcatcatagaacatgaggggctccgagaacccatatttactagcgtttagggac  1440
1441   aatgaggggctccaaggacttaggcttataagtgtctcaggacaggtgaggcctgaggac  1500
1501   ttataagagacccccctgatgagtgatgccatgcgggtatagtctcatggatgttctagga  1560
1561   catgggggttgtggacttattctttggtcaatgcattgtggcacaaggtgccttagagac  1620
1621   ttagaccattttacctttgaaggtcttggggagctatggatcccgagcctcatggcgtgt  1680
1681   tggtgactcaaaaagccaccaaccttctggcatttgagaattcatctcatccaaaaactt  1740
1741   ctataatatatcattcagtgtcaggcctctcccttggctagggctaggagaggttctagt  1800
1801   aacttggggaagCACTGATAGTTTAAACTGAAGGCGGGAAACGACAACCTGATCATGAGC  1860
1861   GGAGAATTAAGGGAGTCACGTTATGACCCCCGCCGATGACGCGGGACAAGCCGTTTTACG  1920
1921   TTTGGAACTGACAGAACCGCAACGTTGAAGGAGCCACTCAGCCTAAGCGGCCGCATTGGA  1980
1981   CTTAATTAAGTGAGGCCGGCCAAGCGTCGATTTAAATGTACCACATGGCGCGCCAACTAT  2040
2041   CATGCGATCGCTTCATGTCTAACTCGAGTTACTGGTACGTACCAAATCCATGGAATCAAG  2100
2101   GTACCTCCATGCTGTCCTACTACTTGCTTCATCCCCTTCTACATTTTGTTCTGGTTTTTG  2160
2161   GCCTGCATTTCGGATCATGATGTATGTGATTTCCAATCTGCTGCAATATGAATGGAGACT  2220
2221   CTGTGCTAACCATCAACAACATGAAATGCTTATGAGGCCTTTGCTGAGCAGCCAATCTTG  2280
2281   CCTGTGTTTATGTCTTCACAGGCCGAATTCCTCTGTTTTGTTTTTCACCCTCAATATTTG  2340
2341   GAAACATTTATCTAGGTTGTTTGTGTCCAGGCCTATAAATCATACATGATGTTGTCGTAT  2400
2401   TGGATGTGAATGTGGTGGCGTGTTCAGTGCCTTGGATTTGAGTTTGATGAGAGTTGCTTC  2460
2461   TGGGTCACCACTCACCATTATCGATGCTCCTCTTCAGCATAAGGTAAAAGTCTTCCCTGT  2520
2521   TTACGTTATTTTACCCACTATGGTTGCTTGGGTTGGTTTTTTCCTGATTGCTTATGCCAT  2580
2581   GGAAAGTCATTTGATATGTTGAACTTGAATTAACTGTAGAATTGTATACATGTTCCATTT  2640
2641   GTGTTGTACTTCCTTCTTTTCTATTAGTAGCCTCAGATGAGTGTGAAAAAAACAGATTAT  2700
2701   ATAACTTGCCCTATAAATCATTTGAAAAAAATATTGTACAGTGAGAAATTGATATATAGT  2760
2761   GAATTTTTAAGAGCATGTTTTCCTAAAGAAGTATATATTTTCTATGTACAAAGGCCATTG  2820
2821   AAGTAATTGTAGATACAGGATAATGTAGACTTTTTGGACTTACACTGCTACCTTTAAGTA  2880
2881   ACAATCATGAGCAATAGTGTTGCAATGATATTTAGGCTGCATTCGTTTACTCTCTTGATT  2940
2941   TCCATGAGCACGCTTCCCAAACTGTTAAACTCTGTGTTTTTTGCCAAAAAAAAAATGCATA  3000
3001   GGAAAGTTGCTTTTAAAAAATCATATCAATCCATTTTTTAAGTTATAGCTAATACTTAAT  3060
3061   TAATCATGCGCTAATAAGTCACTCTGTTTTTCGTACTAGAGAGATTGTTTTGAACCAGCA  3120
3121   CTCAAGAACACAGCCTTAACCCAGCCAAATAATGCTACAACCTACCAGTCCACACCTCTT  3180
3181   GTAAAGCATTTGTTGCATGGAAAAGCTAAGATGACAGCAACCTGTTCAGGAAAACAACTG  3240
3241   ACAAGGTCATAGGGAGAGGGAGCTTTTGGAAAGGTGCCGTGCAGTTCAAACAATTAGTTA  3300
3301   GCAGTAGGGTGTTGGTTTTTGCTCACAGCAATAAGAAGTTAATCATGGTGTAGGCAACCC  3360
3361   AAATAAAACACCAAAATATGCACAAGGCAGTTTGTTGTATTCTGTAGTACAGACAAAACT  3420
3421   AAAAGTAATGAAAGAAGATGTGGTGTTAGAAAAGGAAACAATATCATGAGTAATGTGTGG  3480
3481   GCATTATGGGACCACGAAATAAAAAGAACATTTTGATGAGTCGTGTATCCTCGATGAGCC  3540
3541   TCAAAAGTTCTCTCACCCCGGATAAGAAACCCTTAAGCAATGTGCAAAGTTTGCATTCTC  3600
3601   CACTGACATAATGCAAAATAAGATATCATCGATGACATAGCAACTCATGCATCATATCAT  3660
3661   GCCTCTCTCAACCTATTCATTCCTACTCATCTACATAAGTATCTTCAGCTAAATGTTAGA  3720
3721   ACATAAACCCATAAGTCACGTTTGATGAGTATTAGGCGTGACACATGACAAATCACAGAC  3780
3781   TCAAGCAAGATAAAGCAAAATGATGTGTACATAAAACTCCAGAGCTATATGTCATATTGC  3840
3841   AAAAAGAGGAGAGCTTATAAGACAAGGCATGACTCACAAAAATTCATTTGCCTTTCGTGT  3900
3901   CAAAAAGAGGAGGGCTTTACATTATCCATGTCATATTGCAAAAGAAAGAGAGAAAGAACA  3960
3961   ACACAATGCTGCGTCAATTATACATATCTGTATGTCCATCATTATTCATCCACCTTTCGT  4020
4021   GTACCACACTTCATATATCATGAGTCACTTCATGTCTGGACATTAACAAACTCTATCTTA  4080
4081   ACATTTAGATGCAAGAGCCTTTATCTCACTATAAATGCACGATGATTTCTCATTGTTTCT  4140
4141   CACAAAAAGCATTCAGTTCATTAGTCCTACAACAACGGATCCTAAACCATGCGCGTGCTG  4200
4201   CTCGTGGCCCTGGCCCTGCTGGCTCTTGCTGCCAGCGCCACCTCTGCCCAGAGCGAGCCG  4260
4261   GAGCTGAAGCTGGAGAGCGTGGTGATCGTGAGCAGGCACGGCGTGAGGGCCCCGACCAAG  4320
4321   TTCACCCAGCTGATGCAGGACGTGACCCCGGACGCGTTCTACACCTGGCCGGTGAAGCTG  4380
4381   GGCGAGCTGACCCCGAGGGGCGGCGAGCTGATCGCCTACCTGGGCCACTACTGGAGGCAG  4440
4441   AGGCTGGTGGCCGACGGCCTGCTGCCGAAGAAGGGCTGCCCGCAGAGCGGCCAGGTGGCC  4500
4501   ATCATCGCCGACGTGGACGAGAGGACCAGGAAGACCGGTGAGGCCTTCGCCGCCGGCCTG  4560
4561   GCCCCGGACTGCGCCATCACCGTGCACACCCAGGCCGACACCAGCAGCCCGGACCCGCTG  4620
4621   TTCAACCCGCTGAAGACCGGCGTGTGCCAGCTGGACGTGGCCCAGGTGACCGACGCCATC  4680
4681   CTGGAGAGGGCCGGCGGCAGCATCGCCGACTTCACCGGCCACTACCAGACCGCCTTCAGG  4740
```

```
4741   GAGCTGGAGAGGGTGCTGAACTTCCCGCAGAGCAACCTGGCCCTGAAGAGGGAGAAGCAG  4800
4801   GACGAGAGCGCCAGCCTGACCCAGGCCCTGCCGAGCGAGCTGAAGGTGAGCGCCGACAAC  4860
4861   GTGAGCCTGACCGGCGCCTGGAGCCTGGCCAGCATGCTGACCGAGATTTTCCTGCTGCAG  4920
4921   CAGGCCCAGGGCATGCCGGAGCCGGGCTGGGGCAGGATCACCGACAGCCACCAGTGGAAC  4980
4981   ACCCTGCTGAGCCTGCACAACGCCCAGTTCGACCTGCTGCAGAGGACCCCGGAGGTGGCC  5040
5041   AGGAGCAGGGCCACCCCGCTGCTGGACCTGATCAAGACCGCCCTGACCCCGCACCCGCCG  5100
5101   CAGAAGCAGGCCTACGGCGTGACCCTGCCGACCAGCGTGCTGTTCATCGCCGGCCACGAC  5160
5161   ACCAACCTGGCCAACCTGGGCGGCGCCCTCGAGCTGCAGTGGACCCTGCCGGGCCAGCCG  5220
5221   GACAACACCCCGCCGGGCGGCGAGCTGGTGTTCGAGAGGTGGAGGAGGCTGAGCGACAAC  5280
5281   AGCCAGTGGATTCAGGTGAGCCTGGTGTTCCAGACCCTGCAGCAGATGAGGGACAAGACC  5340
5341   CCGCTGTTCCTGAACACCCCGCCGGGCGAGGTGAAGCTGACCCTGGCCGGCTGCGAGGAG  5400
5401   AGGAACGCCCAGGGCATGTGCAGCCTGGCCGGCTTCACCCAGATCGTGAACGAGGCCAGG  5460
5461   ATTCCGGCCTGCAGCCTGAGCGAGAAGGACGAGCTGTGACCTAGGTCCCCGAATTTCCCC  5520
5521   GATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCG  5580
5581   ATGATTATCATATAATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGC  5640
5641   ATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATAC  5700
5701   GCGATAGAAAACAAAATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCT  5760
5761   ATGTTACTAGATCGGGAATTGGGTACCAAAGTAATCATATTATTTTATGTGTGAATCTTC  5820
5821   TTTACTTTTTCATTTGATTATGATTATGAAGGTATGACCTTCATAACCTTCGTCCGAAAT  5880
5881   CCATTATATCCAAAGGAAAATAATGCTTCGAAGGACGAAGGATTTTGATATTTAACATTT  5940
5941   TATGTTGCCTTGTTCTTAATTCATAGCATTTGAGAACAAGTCCCCAACACCAATCTTTAT  6000
6001   CTTTACTATATTAAAGCACCAGTTCAACGATCGTCTCGTGTCAATATTATTAAAAAACTC  6060
6061   CTACATTTCTTTATAATCAACCCGCACTCTTATAATCTCTTCTCTTACTACTATAATAAG  6120
6121   AGAGTTTATGTACAAAATAAGGTGAAATTATGTATAAGTGTTCTGGACCTTGGTTGTTGG  6180
6181   CTCATATTCACACAACCTAATCAATAGAAAACATATGTTTTATTAAAACAAAATTTATCA  6240
6241   TATATATATATATATATATATATATATATATATATATATATATAATATAAACCGTAGCAATG  6300
6301   CACAGGCATATGACTAGTGGCAACTTAATACCATGTGTGTATTAAGATGAATAAGAGGTA  6360
6361   TCCAAATAAATAACTTGTTCGCTTACGTCTGGATCGAAAGGGGTTGGAAACGATTAAATC  6420
6421   TCTTCCTAGTCAAAATTAAATAGAAGGAGATTTAATCGATTTCTCCCAATCCCCTTCGAT  6480
6481   CCAGGTGCAACCGAATAAGTCCTTAAATGTTGAGGAACACGAAACAACCATGCATTGGCA  6540
6541   TGTAAAGCTCCAAGAATTCGTTGTATCCTTAACAACTCACAGAACATCAACCAAAATTGC  6600
6601   ACGTCAAGGGTATTGGGTAAGAAACAATCAAACAAATCCTCTCTGTGTGCAAAGAAACAC  6660
6661   GGTGAGTCATGCCGAGATCATACTCATCTGATATACATGCTTACAGCTCACAAGACATTA  6720
6721   CAAACAACTCATATTGCATTACAAAGATCGTTTCATGAAAAATAAAATAGGCCGGAACAG  6780
6781   GACAAAAATCCTTGACGTGTAAAGTAAATTTACAACAAAAAAAAAGCCATATGTCAAGCT  6840
6841   AAATCTAATTCGTTTTACGTAGATCAACAACCTGTAGAAGGCAACAAAACTGAGCCACGC  6900
6901   AGAAGTACAGAATGATTCCAGATGAACCATCGACGTGCTACGTAAAGAGAGTGACGAGTC  6960
6961   ATATACATTTGGCAAGAAACCATGAAGCTGCCTACAGCCGTCTCGGTGGCATAAGAACAC  7020
7021   AAGAAATTGTGTTAATTAATCAAAGCTATAAATAACGCTCGCATGCCTGTGCACTTCTCC  7080
7081   ATCACCACCACTGGGTCTTCAGACCATTAGCTTTATCTACTCCAGAGCGCAGAAGAACCC  7140
7141   GATCGACACCGGATCCTAAACCATGCGCGTGCTGCTCGTGGCCCTGGCCCTGCTGGCTCT  7200
7201   TGCTGCCAGCGCCACCTCTGCCCAGAGCGAGCCGGAGCTGAAGCTGGAGAGCGTGGTGAT  7260
7261   CGTGAGCAGGCACGGCGTGAGGGCCCCGACCAAGTTCACCCAGCTGATGCAGGACGTGAC  7320
7321   CCCGGACGCGTTCTACACCTGGCCGGTGAAGCTGGGCGAGCTGACCCCGAGGGGCGGCGA  7380
7381   GCTGATCGCCTACCTGGGCCACTACTGGAGGCAGAGGCTGGTGGCCGACGGCCTGCTGCC  7440
7441   GAAGAAGGGCTGCCCGCAGAGCGGCCAGGTGGCCATCATCGCCGACGTGGACGAGAGGAC  7500
7501   CAGGAAGACCGGTGAGGCCTTCGCCGCCGGCCTGGCCCCGGACTGCGCCATCACCGTGCA  7560
7561   CACCCAGGCCGACACCAGCAGCCCGGACCCGCTGTTCAACCCGCTGAAGACCGGCGTGTG  7620
7621   CCAGCTGGACGTGGCCCAGGTGACCGACGCCATCCTGGAGAGGGCCGGCGGCAGCATCGC  7680
7681   CGACTTCACCGGCCACTACCAGACCGCCTTCAGGGAGCTGGAGAGGGTGCTGAACTTCCC  7740
7741   GCAGAGCAACCTGGCCCTGAAGAGGGAGAAGCAGGACGAGAGCGCCAGCCTGACCCAGGC  7800
7801   CCTGCCGAGCGAGCTGAAGGTGAGCGCCGACAACGTGAGCCTGACCGGCGCCTGGAGCCT  7860
7861   GGCCAGCATGCTGACCGAGATTTTCCTGCTGCAGCAGGCCCAGGGCATGCCGGAGCCGGG  7920
7921   CTGGGGCAGGATCACCGACAGCCACCAGTGGAACACCCTGCTGAGCCTGCACAACGCCCA  7980
7981   GTTCGACCTGCTGCAGAGGACCCCGGAGGTGGCCAGGAGCAGGGCCACCCCGCTGCTGGA  8040
8041   CCTGATCAAGACCGCCCTGACCCCGCACCCGCCGCAGAAGCAGGCCTACGGCGTGACCCT  8100
8101   GCCGACCAGCGTGCTGTTCATCGCCGGCCACGACACCAACCTGGCCAACCTGGGCGGCGC  8160
8161   CCTCGAGCTGCAGTGGACCCTGCCGGGCCAGCCGGACAACACCCCGCCGGGCGGCGAGCT  8220
8221   GGTGTTCGAGAGGTGGAGGAGGCTGAGCGACAACAGCCAGTGGATTCAGGTGAGCCTGGT  8280
8281   GTTCCAGACCCTGCAGCAGATGAGGGACAAGACCCCGCTGTTCCTGAACACCCCGCCGGG  8340
8341   CGAGGTGAAGCTGACCCTGGCCGGCTGCGAGGAGAGGAACGCCCAGGGCATGTGCAGCCT  8400
8401   GGCCGGCTTCACCCAGATCGTGAACGAGGCCAGGATTCCGGCCTGCAGCCTGAGCGAGAA  8460
8461   GGACGAGCTGTGACCTAGGTCCCCGAATTTCCCCGATCGTTCAAACATTTGGCAATAAAG  8520
```

```
8521   TTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAA  8580
8581   TTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTT  8640
8641   TATGATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATATAGCGCGC  8700
8701   AAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCGGGAATTGGGTAC  8760
8761   CCGGTATGAATTTGGAAACAAATTCAGTACTTTTAAAAAAATTTGTTGTAGGGAGCAAAT  8820
8821   AATACATAAAATAATTTATGCATTATTTTATTTTTTATTTGTAATAATATGCTTGAAACG  8880
8881   ATAATTCAGTATGCATGTTGTGCCAGTGTACTACACGGGCGGGGGGAGGGGATTGAGTGG  8940
8941   GCCAGCGCGGTGCGTAGGGTAGATGGGCTGAAATTGATAACTCAAGTCCGACTAGGTTCT  9000
9001   CTTTTTATTTCCCTTCCTTTTCTATTTTCCTTTCTTTTAATTTTCATGCTTTCAAACTAA  9060
9061   ATTCAAATTCGAGTTTTGAATTTCAGCTTCTAAATTGTACACTAAAATTATATGATAAGG  9120
9121   TAACCCCTACTATTACTTTTAATTTTTTTATTCTACCCCATATTGTTTACTTAGGGGAGA  9180
9181   ATAATTGACTTAATCACATTCTTCCTTAGGTTTCAATTCTCAATCTTTCAAATCCACATT  9240
9241   TTTAGATTTCTATTTTGAATTTAAATACCAGTTTGGATTTAGAGTTCAATTTCAAAATAC  9300
9301   ACAACCAAAATACCAGCATGAATGCAAATATATTTTATGTTTATGTATTTACTTTTCTTT  9360
9361   TATACTTTGCTCAAAATAGTTATTTTCATGTATGAAACTCAATAAGCAAGGAACTCACGT  9420
9421   TATTATATAACCTAATAGGAATAATTTAGGTAACATAATTTATCATCCTCTTGATTTAAA  9480
9481   AGAGATATGCCTCCAGAATAAGACACATACTAAAAATAACTCTAATATTGAATAACTAAA  9540
9541   GTCGTACAAATCTCTACTATTATTCCTATAAAATAATAAAGAACTAGCTACAACTTCTTT  9600
9601   AAGGCATTATTCAGGGTTTACAGCTTGAGAGGCATGAACCCATCCTGTATACTCCTGGAC  9660
9661   TTGGAAGACAAAATGTCAACCAAAGTGAAAGGTTTTCTTATGGTTGCTGCTAAGAGATAG  9720
9721   ATTGAACACTAGATCTCTCCTAAGACGTCAGGGCATGCGTTTAGACTCCTACACATGCGA  9780
9781   AAACTGCATCTTACAGTTGGAAGAAACTATATCTCACCACTTCCTGCGGTGTAACTTTGC  9840
9841   CCAAAGATGTTGGCTCACTGTTGGAATCACTCCGCCCCGAACTTTGGATCTAACGCTTGC  9900
9901   AGTGCTACATATTAGAGCAAGACTAACAATGCCGTGGAGAATGGAAGGTATTATAACCAT  9960
9961   GTCATGGTGCATATGGAAATGTCGAAATAACTGGATATTCGAAAACATACCGCCAACGGT 10020
10021  GGCGGCCTGCAAGGAAATGTTCAAGACTGAAATGAACTACATCTGCTACCAAGTTAAGCT 10080
10081  CGAGACAGGAGCTAAAAGTAGAAACTGGATACAACACTTTGTAACATAGTGACACTCCCC 10140
10141  TTTTCCTTTCTTTTACCTTAGAACTATACATACAATCCACATTCAATAAAAATTTGTAGG 10200
10201  TACGCCATACACACTACCGGAATCCGGCTCTTTGCCGAGTGTGAGGCGCTTTGTCGAGTG 10260
10261  CTTTTTGTCCAGCACTCGGCAAAAAAGTCTTTGCCATGTGCCGCACTCGGCAAAGTCCTG 10320
10321  CTCTCGGTAACGACCGCGTTTACCGAGAGCAGGACTCTCGACACAGAAATACACTCGACA 10380
10381  AAGAAATCTTTGCCGAGAGCCAAACACTCGGCGAACGGCAGCGCTCGGCAAAGGGTCGTC 10440
10441  AGCCGCCGTCTAAAGCTGACGGTCGTTATCTTTGTCGAGTGCCCCCTCGTCCGACACTCA 10500
10501  GTAGAGCAAGCTTGCCGAGTGCCATCCTTGGACACTCGATAAAGTATATTTTATTTTTTT 10560
10561  TTATTTTGCCAACCAAACTTTTTGTGGTATGTTCCTACACTATGTAGATCTACATGTACC 10620
10621  ATTTTGGCACAATTACAAAAATGTTTTCTATAACTATTAGATTTAGTTCGTTTATTTGAA 10680
10681  TTTCTTCGGAAAATTCACATATGAACTGCAAGTCACTCGAAACATGAAAAACCGTGCATG 10740
10741  CAAAATAAATGATATGCATGTTATCTAGCACAAGTTACGACCGAATTCAGAAGCAGACCA 10800
10801  GAATCTTCAAGCACCATGCTCACTAAACATGACCGTGAACTTGTTATCCAGTTGTTTAAA 10860
10861  AATTGTATAAAACACAAATAAAGTCAGAAATTAATGAAACTTGTCCACATGTCATGATAT 10920
10921  CATATATAGAGGTTGTGATAAAAATTTGATAATGTTTCGGTAAAGTTGTGACGTACTATG 10980
10981  TGTAGAAACCTAAGTGACCTACACATAAAATCATAGAGTTTCAATGTAGTTCACTCGACA 11040
11041  AAGACTTTGTCAAGTGTCCGATAAAAAGTATTCAGCAAAGAAGCCGTTGTCGATTTACTG 11100
11101  TTCGTCGAGATCTCTTTGCCGAGTGTCACACTAGGCAAAGTCTTTACGGAGTGTTTTTCA 11160
11161  GGCTTTGACACTCGGCAAAGCGCTCGATTCCAGTAGTGACAGTAATTTGCATCAAAAATA 11220
11221  GCCGAGAGATTTAAAATGAGTCAACTAATAGACCAACTAATTATTAGCTATTAGTCGTTA 11280
11281  GCTTCTTTAATCTAAGCTAAAACCAACTAATAGCTTATTTGTTGAATTACAATTAGCTCA 11340
11341  ACGGAATTCTCTGTTTTTTCTATAAAAAAAAGGGAAACTGCCCCTCATTTACAGCAAACT 11400
11401  GTCCGCTGCCTGTCGTCCAGATACAATGAACGTACCTAGTAGGAACTCTTTTACACGCTC 11460
11461  GGTCGCTCGCCGCGGATCGGAGTCCCAGGAACACGACACCACTGTGGAACACGACAAAGT 11520
11521  CTGCTCAGAGGCGGCCACACCCTGGCGTGCACCGAGCCGGAGCCCGGATAAGCACGGTAA 11580
11581  GGAGAGTACGGCGGGACGTGGCGACCCGTGTGTCTGCTGCCACGCAGCCTTCCTCCACGT 11640
11641  AGCCGCGCGGCCGCGCCACGTACCAGGGCCCGGCGCTGGTATAAATGCGCGCCACCTCCG 11700
11701  CTTTAGTTCTGCATACAGCCAACCCAACACACACCCGAGCATATCACAGTGACAGACACT 11760
11761  ACACGGGATCCTAAACCATGCGCGTGCTGCTCGTGGCCCTGGCCCTGCTGGCTCTTGCTG 11820
11821  CCAGCGCCACCTCTGCCCAGAGCGAGCCGGAGCTGAAGCTGGAGAGCGTGGTGATCGTGA 11880
11881  GCAGGCACGGCGTGAGGGCCCCGACCAAGTTCACCCAGCTGATGCAGGACGTGACCCCGG 11940
11941  ACGCGTTCTACACCTGGCCGGTGAAGCTGGGCGAGCTGACCCCGAGGGGCGGCGAGCTGA 12000
12001  TCGCCTACCTGGGCCACTACTGGAGGCAGAGGCTGGTGGCCGACGGCCTGCTGCCGAAGA 12060
12061  AGGGCTGCCCGCAGAGCGGCCAGGTGGCCATCATCGCCGACGTGGACGAGAGGACCAGGA 12120
12121  AGACCGGTGAGGCCTTCGCCGCCGGCCTGGCCCCGGACTGCGCCATCACCGTGCACACCC 12180
12181  AGGCCGACACCAGCAGCCCGGACCCGCTGTTCAACCCGCTGAAGACCGGCGTGTGCCAGC 12240
12241  TGGACGTGGCCCAGGTGACCGACGCCATCCTGGAGAGGGCCGGCGGCAGCATCGCCGACT 12300
```

12301 TCACCGGCCACTACCAGACCGCCTTCAGGGAGCTGGAGAGGGTGCTGAACTTCCCGCAGA 12360
12361 GCAACCTGGCCCTGAAGAGGGAGAAGCAGGACGAGAGCGCCAGCCTGACCCAGGCCCTGC 12420
12421 CGAGCGAGCTGAAGGTGAGCGCCGACAACGTGAGCCTGACCGGCGCCTGGAGCCTGGCCA 12480
12481 GCATGCTGACCGAGATTTTCCTGCTGCAGCAGGCCCAGGGCATGCCGGAGCCGGGCTGGG 12540
12541 GCAGGATCACCGACAGCCACCAGTGGAACACCCTGCTGAGCCTGCACAACGCCCAGTTCG 12600
12601 ACCTGCTGCAGAGGACCCCGGAGGTGGCCAGGAGCAGGGCCACCCCGCTGCTGGACCTGA 12660
12661 TCAAGACCGCCCTGACCCCGCACCCGCCGCAGAAGCAGGCCTACGGCGTGACCCTGCCGA 12720
12721 CCAGCGTGCTGTTCATCGCCGGCCACGACACCAACCTGGCCAACCTGGGCGGCGCCCTCG 12780
12781 AGCTGCAGTGGACCCTGCCGGGCCAGCCGGACAACACCCCGCCGGGCGGCGAGCTGGTGT 12840
12841 TCGAGAGGTGGAGGAGGCTGAGCGACAACAGCCAGTGGATTCAGGTGAGCCTGGTGTTCC 12900
12901 AGACCCTGCAGCAGATGAGGGACAAGACCCCGCTGTTCCTGAACACCCCGCCGGGCGAGG 12960
12961 TGAAGCTGACCCTGGCCGGCTGCGAGGAGAGGAACGCCCAGGGCATGTGCAGCCTGGCCG 13020
13021 GCTTCACCCAGATCGTGAACGAGGCCAGGATTCCGGCCTGCAGCCTGAGCGAGAAGGACG 13080
13081 AGCTGTGACCTAGGTCCCCGAATTTCCCCGATCGTTCAAACATTTGGCAATAAAGTTTCT 13140
13141 TAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTACG 13200
13201 TTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGA 13260
13261 TTAGAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATATAGCGCGCAAACT 13320
13321 AGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCGGGAATTGGAATTCCTGC 13380
13381 AGTGCAGCGTGACCCGGTCGTGCCCCTCTCTAGAGATAATGAGCATTGCATGTCTAAGTT 13440
13441 ATAAAAAATTACCACATATTTTTTTTGTCACACTTGTTTGAAGTGCAGTTTATCTATCTT 13500
13501 TATACATATATTTAAACTTTACTCTACGAATAATATAATCTATAGTACTACAATAATATC 13560
13561 AGTGTTTTAGAGAATCATATAAATGAACAGTTAGACATGGTCTAAAGGACAATTGAGTAT 13620
13621 TTTGACAACAGGACTCTACAGTTTTATCTTTTTAGTGTGCATGTGTTCTCCTTTTTTTTT 13680
13681 GCAAATAGCTTCACCTATATAATACTTCATCCATTTTATTAGTACATCCATTTAGGGTTT 13740
13741 AGGGTTAATGGTTTTTATAGACTAATTTTTTTAGTACATCTATTTTATTCTATTTTAGCC 13800
13801 TCTAAATTAAGAAAACTAAAACTCTATTTTAGTTTTTTTATTTAATAATTTAGATATAAA 13860
13861 ATAGAATAAAATAAAGTGACTAAAAATTAAACAAATACCCTTTAAGAAATTAAAAAAACT 13920
13921 AAGGAAACATTTTTCTTGTTTCGAGTAGATAATGCCAGCCTGTTAAACGCCGTCGACGAG 13980
13981 TCTAACGGACACCAACCAGCGAACCAGCAGCGTCGCGTCGGGCCAAGCGAAGCAGACGGC 14040
14041 ACGGCATCTCTGTCGCTGCCTCTGGACCCCTCTCGAGAGTTCCGCTCCACCGTTGGACTT 14100
14101 GCTCCGCTGTCGGCATCCAGAAATTGCGTGGCGGAGCGGCAGACGTGAGCCGGCACGGCA 14160
14161 GGCGGCCTCCTCCTCCTCTCACGGCACGGCAGCTACGGGGGATTCCTTTCCCACCGCTCC 14220
14221 TTCGCTTTCCCTTCCTCGCCCGCCGTAATAAATAGACACCCCCTCCACACCCTCTTTCCC 14280
14281 CAACCTCGTGTTGTTCGGAGCGCACACACACACACAACCAGATCTCCCCCAAATCCACCCGT 14340
14341 CGGCACCTCCGCTTCAAGGTACGCCGCTCGTCCTCCCCCCCCCCCCCTCTCTACCTTCTC 14400
14401 TAGATCGGCGTTCCGGTCCATGGTTAGGGCCCGGTAGTTCTACTTCTGTTCATGTTTGTG 14460
14461 TTAGATCCGTGTTTGTGTTAGATCCGTGCTGCTAGCGTTCGTACACGGATGCGACCTGTA 14520
14521 CGTCAGACACGTTCTGATTGCTAACTTGCCAGTGTTTCTCTTTGGGGAATCCTGGGATGG 14580
14581 CTCTAGCCGTTCCGCAGACGGGATCGATTTCATGATTTTTTTTGTTTCGTTGCATAGGGT 14640
14641 TTGGTTTGCCCTTTTCCTTTATTTCAATATATGCCGTGCACTTGTTTGTCGGGTCATCTT 14700
14701 TTCATGCTTTTTTTTGTCTTGGTTGTGATGATGTGGTCTGGTTGGGCGGTCGTTCTAGAT 14760
14761 CGGAGTAGAATTCTGTTTCAAACTACCTGGTGGATTTATTAATTTTGGATCTGTATGTGT 14820
14821 GTGCCATACATATTCATAGTTACGAATTGAAGATGATGGATGGAAATATCGATCTAGGAT 14880
14881 AGGTATACATGTTGATGCGGGTTTTACTGATGCATATACAGAGATGCTTTTTGTTCGCTT 14940
14941 GGTTGTGATGATGTGGTGTGGTTGGGCGGTCGTTCATTCGTTCTAGATCGGAGTAGAATA 15000
15001 CTGTTTCAAACTACCTGGTGTATTTATTAATTTTGGAACTGTATGTGTGTGTCATACATC 15060
15061 TTCATAGTTACGAGTTTAAGATGGATGGAAATATCGATCTAGGATAGGTATACATGTTGA 15120
15121 TGTGGGTTTTACTGATGCATATACATGATGGCATATGCAGCATCTATTCATATGCTCTAA 15180
15181 CCTTGAGTACCTATCTATTATAATAAACAAGTATGTTTTATAATTATTTTGATCTTGATA 15240
15241 TACTTGGATGATGGCATATGCAGCAGCTATATGTGGATTTTTTTAGCCCTGCCTTCATAC 15300
15301 GCTATTTATTTGCTTGGTACTGTTTCTTTTGTCGATGCTCACCCTGTTGTTTGGTGTTAC 15360
15361 TTCTGCAGATGCAGAAACTCATTAACTCAGTGCAAAACTATGCCTGGGGCAGCAAAACGG 15420
15421 CGTTGACTGAACTTTATGGTATGGAAAATCCGTCCAGCCAGCCGATGGCCGAGCTGTGGA 15480
15481 TGGGCGCACATCCGAAAAGCAGTTCACGAGTGCAGAATGCCGCCGGAGATATCGTTTCAC 15540
15541 TGCGTGATGTGATTGAGAGTGATAAATCGACTCTGCTCGGAGAGGCCGTTGCCAAACGCT 15600
15601 TTGGCGAACTGCCTTTCCTGTTCAAAGTATTATGCGCAGCACAGCCACTCTCCATTCAGG 15660
15661 TTCATCCAAACAAACACAATTCTGAAATCGGTTTTGCCAAAGAAAATGCCGCAGGTATCC 15720
15721 CGATGGATGCCGCCGAGCGTAACTATAAAGATCCTAACCACAAGCCGGAGCTGGTTTTTG 15780
15781 CGCTGACGCCTTTCCTTGCGATGAACGCGTTTCGTGAATTTTCCGAGATTGTCTCCCTAC 15840
15841 TCCAGCCGGTCGCAGGTGCACATCCGGCGATTGCTCACTTTTTACAACAGCCTGATGCCG 15900
15901 AACGTTTAAGCGAACTGTTCGCCAGCCTGTTGAATATGCAGGGTGAAGAAAAATCCCGCG 15960
15961 CGCTGGCGATTTTAAAATCGGCCCTCGATAGCCAGCAGGGTGAACCGTGGCAAACGATTC 16020
16021 GTTTAATTTCTGAATTTTACCCGGAAGACAGCGGTCTGTTCTCCCCGCTATTGCTGAATG 16080

```
16081  TGGTGAAATTGAACCCTGGCGAAGCGATGTTCCTGTTCGCTGAAACACCGCACGCTTACC 16140
16141  TGCAAGGCGTGGCGCTGGAAGTGATGGCAAACTCCGATAACGTGCTGCGTGCGGGTCTGA 16200
16201  CGCCTAAATACATTGATATTCCGGAACTGGTTGCCAATGTGAAATTCGAAGCCAAACCGG 16260
16261  CTAACCAGTTGTTGACCCAGCCGGTGAAACAAGGTGCAGAACTGGACTTCCCGATTCCAG 16320
16321  TGGATGATTTTGCCTTCTCGCTGCATGACCTTAGTGATAAAGAAACCACCATTAGCCAGC 16380
16381  AGAGTGCCGCCATTTTGTTCTGCGTCGAAGGCGATGCAACGTTGTGGAAAGGTTCTCAGC 16440
16441  AGTTACAGCTTAAACCGGGTGAATCAGCGTTTATTGCCGCCAACGAATCACCGGTGACTG 16500
16501  TCAAAGGCCACGGCCGTTTAGCGCGTGTTTACAACAAGCTGTAAGAGCTTACTGAAAAAA 16560
16561  TTAACATCTCTTGCTAAGCTGGGAGCTCTAGATCCCCGAATTTCCCCGATCGTTCAAACA 16620
16621  TTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATAT 16680
16681  AATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTA 16740
16741  TGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAAAACA 16800
16801  AAATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATGTATGTTACTAGATC 16860
16861  GGGAATTGGCGAGCTCGAATTAATTCAGTACATTAAAAACGTCCGCAATGTGTTATTAAG 16920
16921  TTGTCTAAGCGTCAATTTGTTTACACCACAATATATCCTagtggacctcgaagtctatct 16980
16981  ttcgagcagaaatttttgaatgcgtattagtggcagatggatgaatgtctggcctttccc17040
17041  aaacgttaacaagcatatgcattttcaaatattactttcaaatgtttcagcttggcaatc 17100
17101  acttgcaaaactctccaacttaaaaagacttgcttttgaatttcttattataaacccaga 17160
17161  caacccaagctcatatatcttagagcctttattcttttgtgaccgaactcatcaatatta 17220
17221  ttcatcaccaatcaccattggttatataaatcaagatagatacgtagctaatcgtcttcc 17280
17281  atgcatattgtccgagatcgcatcgcatctcgcatcaaacgctcagcatccatgttcttt 17340
17341  cttgacgcaggtgctgttgcagttgctttggcaggtggggaagcatgactggaacatacc 17400
17401  ttgcttgcatgcttggcaatctgtatagtcgtcatggatagaaaggcaggcgttctgggc 17460
17461  ttccaagttgcagtcccggctgcaggtgccgtagctgcagtaggccgcctggcacccctg 17520
17521  gaggacctgcctttcgcagtcctcccgggcagccgtggtgtcgttgtcgcagtagcttct 17580
17581  gcaggttgtggagttggcagcctctgcagtgcagttcgtattgcacagcgacccacactc 17640
17641  gctgcataatggccctttgtttggatgccccatggaagatacgaccagaatggcgagaac 17700
17701  ggcggcggtggcgccggcgccggcggcatcagaagccattgctgcagagatggacacggc 17760
17761  aaggcaatacctagatatagagaggagataggatgtcaatgtcaacgagattaattggtg 17820
17821  ctcagatgattgagcggctgatgctatttataggctccaaactaattaagggaacatcta 17880
17881  gcctaaattattatatagacggggtatctaatgtataattaagggttttagtgttgtata 17940
17941  tacaagttgacttgaagtcgtcgaagaggagaatatcatattcacctttcactgggttcc 18000
18001  atttgatttacaatccgaggtgaagctaaaagttgactggtcacacttaactcagacag 18060
18061  gtccaaatgtgaaacaattgtaggatcaaaaaatgcacattcattaattctcattgcaga 18120
18121  gaacgtaaccacgcgtgcgtcatttttatttattatgtgctaagaatatatttgagaaa 18180
18181  gatgcaaagggtttcgtttattagataaaacaacatgtaaattttggtattaagcttta 18240
18241  ggttgaaccggctgatgcgagaggaaattaagtggtgaaactgaggagctcacaatgtta 18300
18301  ctgacaatcaagtgtctagctaatggagcattaatttttaacaaagtcagaggcagtaga 18360
18361  gataactcttacaagtagatcgaagattcacgtggagtaacatgcagtgagtacgacact 18420
18421  cagtttacttttgatggacaacttgcttttagtcaactgcgtgcatgtccaatcctgtgg 18480
18481  cgttttccccatcttggttgatacttgctaactatgtacggcacaaagttgacgagcaag 18540
18541  agatggcactcggcatggtcgccgcttccgtgcttgctatcttcgagcgagctcgcgcgt 18600
18601  tgttctcatgtggagcagatt  18621
```

APPENDIX 2

Nucleotide sequence of Locus 3507 and maize genomic flanking DNA in Event PY203. Maize genomic DNA sequence is presented in lower case letters while the sequence of the T-DNA insert is presented in upper case. The table below presents the positions of key genetic elements relative to the nucleotide numbering.

Nucleotide Position		
Start	**End**	**Genetic Element**
1	2098	Maize chromosomal DNA ("chr2")
2099	2101	RB (partial)
2397	4467	GTL
4480	4536	Z27ss
4537	5769	Phy02
5770	5787	SEKDEL
5797	6072	NosT
6079	7441	ZmZ27
7454	7510	Z27ss
7511	8743	Phy02
8744	8761	SEKDEL
8771	9046	NosT
9053	10540	ZmGlb1 (truncated)
10541	13109	Maize chromosomal DNA ("chr2")

```
1       aagctgccagaagactgagctgataggcgagagctcctacgggtaggtagaacatgtttc  60
61      tctttgcacatgtgctttttggggtgtttggtttgcccctgctaaaatttagcccctat  120
121     cacatcgaatgtttgaacctccgttcgaggtattaaatgtagtcggattataaaactaat  180
181     ttctcagccgaacattaaaaagtgagacgaatctagtacagttggttgggtctatatttc  240
241     atactcctatttaaaagtcaaacgcttgatgtgacccgggctaaattttagtccacagaa  300
301     ccaaacacccccttggctcggggtggcgcagacagagggtatgcaatagcgggggagcag  360
361     tggcgaagcatcgcgacgagggtggggttcttgctgtcagacaataagtcaagagtggtg  420
421     ggtgtagcggcacggcggggcgagcaatagtgcgccagagatggtgagcgaggtacatgt  480
481     ggtgggcttctagggtcaactgcaggggaggtggttaggattttgtaggcatggcatggt  540
541     gagcaaggagggcaaacatcacggataagacaaaaagttcgatgagggtgcttgtgtggg  600
601     accggtttgtgggggttcgctgtccacagaattggcggcagctagagatgtccaaacggg  660
661     ccgcccggcccggtccggcccgggcccggtgaagcccggtccgttttgggcccggcccgc  720
721     caggcacgattaaaaaccgggccgggccggctaagcacgcgggcttaattccttgtccg  780
781     agcccggcccgcaatgggcctaaacgggccgggccggcccgtttagcacgaaaaaagcgg  840
841     gccgaaaagcgggctatgcgggccgaaaagcacgttttagtgtaaaaaaagcgggcttaa  900
901     cgggcttagagctaaacgggtcgtgccgggctagcccaccgtgcctaatttcctgtccaa  960
961     gcccggcccgcttattcgtgccgggccggcccgggcccgcatataaccgggccgtgccgg  1020
1021    gctcggaccgggcccaaaaaacgggcttcgtgccgggctcgcgggcctcgtgcttattgg  1080
1081    acatctatagcggcagcaggggccgggggacatttggtgggttgtcacgaggttgggccc  1140
1141    tggcctggggattgacggaagatgggcctaaagtagccatgcatgtgtagctactctcgc  1200
1201    ctgttgatcacttgttctcaattgctatacacaattgaaaataaggcaacacagtgttaa  1260
1261    aaagataattaaccttcgtcctccaacaattatcctttcggaataacttttcgaggatga  1320
1321    aggttaggattttaatcgaatgaatcatacatcagtatataaatgagtatacatgaaatg  1380
1381    taaagaacttaaacatgttaactcatctcagatatctcaatatctcatttatctcctttc  1440
1441    atataataaatttacaatcgtaccttcggttctacaaagtgagttagagcgaaggtgctc  1500
1501    tagggaagagattacaagaggcaattatctagcccaccaagaggtactttatgcaggaca  1560
```

1561 ttgtttatctatttataggggaacatatagcttcgtgcaaaattacaaatatatctgtaa 1620
1621 aaccagtacgtatgatttacaaatgatgtgagggcaatattgttttgtgcctttagcatt 1680
1681 atggatgacgccttctccctggtacgttgatattctgcctttgtctccttgtgttgttat 1740
1741 gacgaagttaacatcttcgtctttctccgtgccttgcttcaaaggtatttcgtcgccgaa 1800
1801 gctcctgcgacacttaataacactgcgacacttaataacacgttggcaaccaacagctag 1860
1861 atgtgtttttaggaccttcaaaagatgaaggcccctaacatcgccttcgccaaatctgg 1920
1921 accggcagcaccttggccacacacccttctcggacgaaaccaaacctcttagaaatccag 1980
1981 agctcatgggggatgtcaaggtggtggttgacctaaatgaccaccctgattaggccaaat 2040
2041 ctgggccgctatttccctgacctcacaaccagccgcaaaggcgcggtggacatcctccTC 2100
2101 AAACACTGATAGTTTAAACTGAAGGCGGGAAACGACAACCTGATCATGAGCGGAGAATTA 2160
2161 AGGGAGTCACGTTATGACCCCCGCCGATGACGCGGGACAAGCCGTTTTACGTTTGGAACT 2220
2221 GACAGAACCGCAACGTTGAAGGAGCCACTCAGCCTAAGCGGCCGCATTGGACTTAATTAA 2280
2281 GTGAGGCCGGCCAAGCGTCGATTTAAATGTACCACATGGCGCGCCAACTATCATGCGATC 2340
2341 GCTTCATGTCTAACTCGAGTTACTGGTACGTACCAAATCCATGGAATCAAGGTACCTCCA 2400
2401 TGCTGTCCTACTACTTGCTTCATCCCCTTCTACATTTTGTTCTGGTTTTTGGCCTGCATT 2460
2461 TCGGATCATGATGTATGTGATTTCCAATCTGCTGCAATATGAATGGAGACTCTGTGCTAA 2520
2521 CCATCAACAACATGAAATGCTTATGAGGCCTTTGCTGAGCAGCCAATCTTGCCTGTGTTT 2580
2581 ATGTCTTCACAGGCCGAATTCCTCTGTTTTGTTTTTCACCCTCAATATTTGGAAACATTT 2640
2641 ATCTAGGTTGTTTGTGTCCAGGCCTATAAATCATACATGATGTTGTCGTATTGGATGTGA 2700
2701 ATGTGGTGGCGTGTTCAGTGCCTTGGATTTGAGTTTGATGAGAGTTGCTTCTGGGTCACC 2760
2761 ACTCACCATTATCGATGCTCCTCTTCAGCATAAGGTAAAAGTCTTCCCTGTTTACGTTAT 2820
2821 TTTACCCACTATGGTTGCTTGGGTTGGTTTTTTCCTGATTGCTTATGCCATGGAAAGTCA 2880
2881 TTTGATATGTTGAACTTGAATTAACTGTAGAATTGTATACATGTTCCATTTGTGTTGTAC 2940
2941 TTCCTTCTTTTCTATTAGTAGCCTCAGATGAGTGTGAAAAAAACAGATTATATAACTTGC 3000
3001 CCTATAAATCATTTGAAAAAAATATTGTACAGTGAGAAATTGATATATAGTGAATTTTTA 3060
3061 AGAGCATGTTTTCCTAAAGAAGTATATATTTTCTATGTACAAAGGCCATTGAAGTAATTG 3120
3121 TAGATACAGGATAATGTAGACTTTTTGGACTTACACTGCTACCTTTAAGTAACAATCATG 3180
3181 AGCAATAGTGTTGCAATGATATTTAGGCTGCATTCGTTTACTCTCTTGATTTCCATGAGC 3240
3241 ACGCTTCCCAAACTGTTAAACTCTGTGTTTTTTGCCAAAAAAAAATGCATAGGAAAGTTG 3300
3301 CTTTTAAAAAATCATATCAATCCATTTTTTAAGTTATAGCTAATACTTAATTAATCATGC 3360
3361 GCTAATAAGTCACTCTGTTTTTCGTACTAGAGAGATTGTTTTGAACCAGCACTCAAGAAC 3420
3421 ACAGCCTTAACCCAGCCAAATAATGCTACAACCTACCAGTCCACACCTCTTGTAAAGCAT 3480
3481 TTGTTGCATGGAAAAGCTAAGATGACAGCAACCTGTTCAGGAAAACAACTGACAAGGTCA 3540
3541 TAGGGAGAGGGAGCTTTTGGAAAGGTGCCGTGCAGTTCAAACAATTAGTTAGCAGTAGGG 3600
3601 TGTTGGTTTTTGCTCACAGCAATAAGAAGTTAATCATGGTGTAGGCAACCCAAATAAAAC 3660
3661 ACCAAAATATGCACAAGGCAGTTTGTTGTATTCTGTAGTACAGACAAAACTAAAAGTAAT 3720
3721 GAAAGAAGATGTGGTGTTAGAAAAGGAAACAATATCATGAGTAATGTGTGGGCATTATGG 3780
3781 GACCACGAAATAAAAAGAACATTTTGATGAGTCGTGTATCCTCGATGAGCCTCAAAAGTT 3840
3841 CTCTCACCCCGGATAAGAAACCCTTAAGCAATGTGCAAAGTTTGCATTCTCCACTGACAT 3900
3901 AATGCAAAATAAGATATCATCGATGACATAGCAACTCATGCATCATATCATGCCTCTCTC 3960
3961 AACCTATTCATTCCTACTCATCTACATAAGTATCTTCAGCTAAATGTTAGAACATAAACC 4020
4021 CATAAGTCACGTTTGATGAGTATTAGGCGTGACACATGACAAATCACAGACTCAAGCAAG 4080
4081 ATAAAGCAAAATGATGTGTACATAAAACTCCAGAGCTATATGTCATATTGCAAAAAGAGG 4140
4141 AGAGCTTATAAGACAAGGCATGACTCACAAAAATTCATTTGCCTTTCGTGTCAAAAAGAG 4200
4201 GAGGGCTTTACATTATCCATGTCATATTGCAAAAGAAAGAGAGAAAGAACAACACAATGC 4260
4261 TGCGTCAATTATACATATCTGTATGTCCATCATTATTCATCCACCTTTCGTGTACCACAC 4320
4321 TTCATATATCATGAGTCACTTCATGTCTGGACATTAACAAACTCTATCTTAACATTTAGA 4380
4381 TGCAAGAGCCTTTATCTCACTATAAATGCACGATGATTTCTCATTGTTTCTCACAAAAAG 4440
4441 CATTCAGTTCATTAGTCCTACAACAACGGATCCTAAACCATGCGCGTGCTGCTCGTGGCC 4500
4501 CTGGCCCTGCTGGCTCTTGCTGCCAGCGCCACCTCTGCCCAGAGCGAGCCGGAGCTGAAG 4560
4561 CTGGAGAGCGTGGTGATCGTGAGCAGGCACGGCGTGAGGGCCCCGACCAAGTTCACCCAG 4620
4621 CTGATGCAGGACGTGACCCCGGACGCGTTCTACACCTGGCCGGTGAAGCTGGGCGAGCTG 4680
4681 ACCCCGAGGGGCGGCGAGCTGATCGCCTACCTGGGCCACTACTGGAGGCAGAGGCTGGTG 4740
4741 GCCGACGGCCTGCTGCCGAAGAAGGGCTGCCCGCAGAGCGGCCAGGTGGCCATCATCGCC 4800
4801 GACGTGGACGAGAGGACCAGGAAGACCGGTGAGGCCTTCGCCGCCGGCCTGGCCCCGGAC 4860
4861 TGCGCCATCACCGTGCACACCCAGGCCGACACCAGCAGCCCGGACCCGCTGTTCAACCCG 4920
4921 CTGAAGACCGGCGTGTGCCAGCTGGACGTGGCCCAGGTGACCGACGCCATCCTGGAGAGG 4980
4981 GCCGGCGGCAGCATCGCCGACTTCACCGGCCACTACCAGACCGCCTTCAGGGAGCTGGAG 5040
5041 AGGGTGCTGAACTTCCCGCAGAGCAACCTGGCCCTGAAGAGGGAGAAGCAGGACGAGAGC 5100
5101 GCCAGCCTGACCCAGGCCCTGCCGAGCGAGCTGAAGGTGAGCGCCGACAACGTGAGCCTG 5160
5161 ACCGGCGCCTGGAGCCTGGCCAGCATGCTGACCGAGATTTTCCTGCTGCAGCAGGCCCAG 5220
5221 GGCATGCCGGAGCCGGGCTGGGGCAGGATCACCGACAGCCACCAGTGGAACACCCTGCTG 5280
5281 AGCCTGCACAACGCCCAGTTCGACCTGCTGCAGAGGACCCCGGAGGTGGCCAGGAGCAGG 5340

```
5341  GCCACCCCGCTGCTGGACCTGATCAAGACCGCCCTGACCCCGCACCCGCCGCAGAAGCAG  5400
5401  GCCTACGGCGTGACCCTGCCGACCAGCGTGCTGTTCATCGCCGGCCACGACACCAACCTG  5460
5461  GCCAACCTGGGCGGCGCCCTCGAGCTGCAGTGGACCCTGCCGGGCCAGCCGGACAACACC  5520
5521  CCGCCGGGCGGCGAGCTGGTGTTCGAGAGGTGGAGGAGGCTGAGCGACAACAGCCAGTGG  5580
5581  ATTCAGGTGAGCCTGGTGTTCCAGACCCTGCAGCAGATGAGGGACAAGACCCCGCTGTTC  5640
5641  CTGAACACCCCGCCGGGCGAGGTGAAGCTGACCCTGGCCGGCTGCGAGGAGAGGAACGCC  5700
5701  CAGGGCATGTGCAGCCTGGCCGGCTTCACCCAGATCGTGAACGAGGCCAGGATTCCGGCC  5760
5761  TGCAGCCTGAGCGAGAAGGACGAGCTGTGACCTAGGTCCCCGAATTTCCCCGATCGTTCA  5820
5821  AACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATC  5880
5881  ATATAATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTA  5940
5941  TTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAA  6000
6001  AACAAAATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTA  6060
6061  GATCGGGAATTGGGTACCAAAGTAATCATATTATTTTATGTGTGAATCTTCTTTACTTTT  6120
6121  TCATTTGATTATGATTATGAAGGTATGACCTTCATAACCTTCGTCCGAAATCCATTATAT  6180
6181  CCAAAGGAAAATAATGCTTCGAAGGACGAAGGATTTTGATATTTAACATTTTATGTTGCC  6240
6241  TTGTTCTTAATTCATAGCATTTGAGAACAAGTCCCCAACACCAATCTTTATCTTTACTAT  6300
6301  ATTAAAGCACCAGTTCAACGATCGTCTCGTGTCAATATTATTAAAAAACTCCTACATTTC  6360
6361  TTTATAATCAACCCGCACTCTTATAATCTCTTCTCTTACTACTATAATAAGAGAGTTTAT  6420
6421  GTACAAAATAAGGTGAAATTATGTATAAGTGTTCTGGACCTTGGTTGTTGGCTCATATTC  6480
6481  ACACAACCTAATCAATAGAAAACATATGTTTTATTAAAACAAAATTTATCATATATATAT  6540
6541  ATATATATATATATATATATATATATATATATAATATAAACCGTAGCAATGCACAGGCAT  6600
6601  ATGACTAGTGGCAACTTAATACCATGTGTGTATTAAGATGAATAAGAGGTATCCAAATAA  6660
6661  ATAACTTGTTCGCTTACGTCTGGATCGAAAGGGGTTGGAAACGATTAAATCTCTTCCTAG  6720
6721  TCAAAATTAAATAGAAGGAGATTTAATCGATTTCTCCCAATCCCCTTCGATCCAGGTGCA  6780
6781  ACCGAATAAGTCCTTAAATGTTGAGGAACACGAAACAACCATGCATTGGCATGTAAAGCT  6840
6841  CCAAGAATTCGTTGTATCCTTAACAACTCACAGAACATCAACCAAAATTGCACGTCAAGG  6900
6901  GTATTGGGTAAGAAACAATCAAACAAATCCTCTCTGTGTGCAAAGAAACACGGTGAGTCA  6960
6961  TGCCGAGATCATACTCATCTGATATACATGCTTACAGCTCACAAGACATTACAAACAACT  7020
7021  CATATTGCATTACAAAGATCGTTTCATGAAAAATAAAATAGGCCGGAACAGGACAAAAAT  7080
7081  CCTTGACGTGTAAAGTAAATTTACAACAAAAAAAAAAGCCATATGTCAAGCTAAATCTAAT  7140
7141  TCGTTTTACGTAGATCAACAACCTGTAGAAGGCAACAAAACTGAGCCACGCAGAAGTACA  7200
7201  GAATGATTCCAGATGAACCATCGACGTGCTACGTAAAGAGAGTGACGAGTCATATACATT  7260
7261  TGGCAAGAAACCATGAAGCTGCCTACAGCCGTCTCGGTGGCATAAGAACACAAGAAATTG  7320
7321  TGTTAATTAATCAAAGCTATAAATAACGCTCGCATGCCTGTGCACTTCTCCATCACCACC  7380
7381  ACTGGGTCTTCAGACCATTAGCTTTATCTACTCCAGAGCGCAGAAGAACCCGATCGACAC  7440
7441  CGGATCCTAAACCATGCGCGTGCTGCTCGTGGCCCTGGCCCTGCTGGCTCTTGCTGCCAG  7500
7501  CGCCACCTCTGCCCAGAGCGAGCCGGAGCTGAAGCTGGAGAGCGTGGTGATCGTGAGCAG  7560
7561  GCACGGCGTGAGGGCCCCGACCAAGTTCACCCAGCTGATGCAGGACGTGACCCCGGACGC  7620
7621  GTTCTACACCTGGCCGGTGAAGCTGGGCGAGCTGACCCCGAGGGGCGGCGAGCTGATCGC  7680
7681  CTACCTGGGCCACTACTGGAGGCAGAGGCTGGTGGCCGACGGCCTGCTGCCGAAGAAGGG  7740
7741  CTGCCCGCAGAGCGGCCAGGTGGCCATCATCGCCGACGTGGACGAGAGGACCAGGAAGAC  7800
7801  CGGTGAGGCCTTCGCCGCCGGCCTGGCCCCGGACTGCGCCATCACCGTGCACACCCAGGC  7860
7861  CGACACCAGCAGCCCGGACCCGCTGTTCAACCCGCTGAAGACCGGCGTGTGCCAGCTGGA  7920
7921  CGTGGCCCAGGTGACCGACGCCATCCTGGAGAGGGCCGGCGGCAGCATCGCCGACTTCAC  7980
7981  CGGCCACTACCAGACCGCCTTCAGGGAGCTGGAGAGGGTGCTGAACTTCCCGCAGAGCAA  8040
8041  CCTGGCCCTGAAGAGGGAGAAGCAGGACGAGAGCGCCAGCCTGACCCAGGCCCTGCCGAG  8100
8101  CGAGCTGAAGGTGAGCGCCGACAACGTGAGCCTGACCGGCGCCTGGAGCCTGGCCAGCAT  8160
8161  GCTGACCGAGATTTTCCTGCTGCAGCAGGCCCAGGGCATGCCGGAGCCGGGCTGGGGCAG  8220
8221  GATCACCGACAGCCACCAGTGGAACACCCTGCTGAGCCTGCACAACGCCCAGTTCGACCT  8280
8281  GCTGCAGAGGACCCCGGAGGTGGCCAGGAGCAGGGCCACCCCGCTGCTGGACCTGATCAA  8340
8341  GACCGCCCTGACCCCGCACCCGCCGCAGAAGCAGGCCTACGGCGTGACCCTGCCGACCAG  8400
8401  CGTGCTGTTCATCGCCGGCCACGACACCAACCTGGCCAACCTGGGCGGCGCCCTCGAGCT  8460
8461  GCAGTGGACCCTGCCGGGCCAGCCGGACAACACCCCGCCGGGCGGCGAGCTGGTGTTCGA  8520
8521  GAGGTGGAGGAGGCTGAGCGACAACAGCCAGTGGATTCAGGTGAGCCTGGTGTTCCAGAC  8580
8581  CCTGCAGCAGATGAGGGACAAGACCCCGCTGTTCCTGAACACCCCGCCGGGCGAGGTGAA  8640
8641  GCTGACCCTGGCCGGCTGCGAGGAGAGGAACGCCCAGGGCATGTGCAGCCTGGCCGGCTT  8700
8701  CACCCAGATCGTGAACGAGGCCAGGATTCCGGCCTGCAGCCTGAGCGAGAAGGACGAGCT  8760
8761  GTGACCTAGGTCCCCGAATTTCCCCGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAG  8820
8821  ATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTACGTTAA  8880
8881  GCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAG  8940
8941  AGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGA  9000
9001  TAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCGGGAATTGGGTACCCGGTATGA  9060
9061  ATTTGGAAACAAATTCAGTACTTTTAAAAAAATTTGTTGTAGGGAGCAAATAATACATAA  9120
```

```
9121   AATAATTTATGCATTATTTTATTTTTTATTTGTAATAATATGCTTGAAACGATAATTCAG  9180
9181   TATGCATGTTGTGCCAGTGTACTACACGGGCGGGGGGAGGGGATTGAGTGGGCCAGCGCG  9240
9241   GTGCGTAGGGTAGATGGGCTGAAATTGATAACTCAAGTCCGACTAGGTTCTCTTTTTATT  9300
9301   TCCCTTCCTTTTCTATTTTCCTTTCTTTTAATTTTCATGCTTTCAAACTAAATTCAAATT  9360
9361   CGAGTTTTGAATTTCAGCTTCTAAATTGTACACTAAAATTATATGATAAGGTAACCCCTA  9420
9421   CTATTACTTTTAATTTTTTTATTCTACCCCATATTGTTTACTTAGGGGAGAATAATTGAC  9480
9481   TTAATCACATTCTTCCTTAGGTTTCAATTCTCAATCTTTCAAATCCACATTTTTAGATTT  9540
9541   CTATTTTGAATTTAAATACCAGTTTGGATTTAGAGTTCAATTTCAAAATACACAACCAAA  9600
9601   ATACCAGCATGAATGCAAATATATTTTATGTTTATGTATTTACTTTTCTTTTATACTTTG  9660
9661   CTCAAAATAGTTATTTTCATGTATGAAACTCAATAAGCAAGGAACTCACGTTATTATATA  9720
9721   ACCTAATAGGAATAATTTAGGTAACATAATTTATCATCCTCTTGATTTAAAAGAGATATG  9780
9781   CCTCCAGAATAAGACACATACTAAAAATAACTCTAATATTGAATAACTAAAGTCGTACAA  9840
9841   ATCTCTACTATTATTCCTATAAAATAATAAAGAACTAGCTACAACTTCTTTAAGGCATTA  9900
9901   TTCAGGGTTTACAGCTTGAGAGGCATGAACCCATCCTGTATACTCCTGGACTTGGAAGAC  9960
9961   AAAATGTCAACCAAAGTGAAAGGTTTTCTTATGGTTGCTGCTAAGAGATAGATTGAACAC 10020
10021  TAGATCTCTACTAAGACGTCAGGGCATGCGTTTAGACTCCTACACATGCGAAAACTGCAT 10080
10081  CTTACAGTTGGAAGAAACTATATCTCACCACTTCCTGCGGTGTAACTTTGCCCAAAGATG 10140
10141  TTGGCTCACTGTTGGAATCACTCCGCCCCGAACTTTGGATCTAACGCTTGCAGTGCTACA 10200
10201  TATTAGAGCAAGACTAACAATGCCGTGGAGAATGGAAGGTATTATAACCATGTCATGGTG 10260
10261  CATATGGAAATGTCGAAATAACTGGATATTCGAAAACATACCGCCAACGGTGGCGGCCTG 10320
10321  CAAGGAAATGTTCAAGACTGAAATGAACTACATCTGCTACCAAGTTAAGCTCGAGACAGG 10380
10381  AGCTAAAAGTAGAAACTGGATACAACACTTTGTAACATAGTGACACTCCCCTTTTCCTTT 10440
10441  CTTTTACCTTAGAACTATACATACAATCCACATTCAATAAAAATTTGTAGGTACGCCATA 10500
10501  CACACTACCGGAATCCGGCTCTTTGCCGAGTGTGAGGCGCgagccactaggcgtgtgaaa 10560
10561  aaaacgtttcgaggtttttcaagacgctcgaaagaaaaggacgtaccttcgaaggccag 10620
10621  gggaatgaagtgatgaacgtaggaggtgaatcgaaggagtgccacgccatatcagagggc 10680
10681  agcaggtccatcgggatctcgggagcaatggtttggaggcaggcgcgtaactggtctatg 10740
10741  gactctagcataaccacatcaccctcggatgtgtaggcatgcaggagccagcacttgaga 10800
10801  gcgtggttgctgggaggcaactaagcttctagatactatgtgtgactcccagcattttct 10860
10861  ttattgcccgagctttcctccgaagctgaggactcctccccaccctgagagtggccggcg 10920
10921  tccgacaccgaggaggcgaagcaggatgtagtagcactgacacggttatggcttggaaag 10980
10981  ataggggcgaccggtgtccactagagggggagggggtagcgacatgaatgcctacctccca 11040
11041  ctagggaagaagatggacgttaaccgtgcacacatagaaagacgaggcaacaacttcatc 11100
11101  ggtaggggaggccccatgatcaccacagtgcaagcatccggggtgggtggagatatgcag 11160
11161  aggggcgtgcaggggacgagggtggcgtgtgaggccgcgcggcctcccccctccgctcgca 11220
11221  tgctggctacagacgcgccccttgccgtgcagcgcttcccctggaagcccctctacgca 11280
11281  ggggaaaggaacaggagcacgctgcagatagcgtgagcccgtgacgccccctgccactgt 11340
11341  ttcttcgttgggtgagtttttaatatgtgaaaaaaaaatggtaataaatgatgagtaggt 11400
11401  ttggatagtgatattttatagttgtagtggggtataggggggagaatatagagggaaccgc 11460
11461  tgcgggagatgaaaaagtaggggatgaaatgttgatgatgtgacgcaaaaaagtgatata 11520
11521  gggggacaaatttaggggtaaccgttgcagatagtctgaggcagcgtcgcagcggttcca 11580
11581  agctggaggagaggtcattgatgggaacaaccacaacagagaaggggtggagcggacatg 11640
11641  gatcaaagggtggcggcggtggaatgaggcgaggacgtggttgatcggcgtggcgtggga 11700
11701  tgggctctaatgaccaaagtccctatagtttctacaacaaacggggtcgcaacagtcttt 11760
11761  gacttggtgatcggaggcgagacaatggaagcagcggtcggggaggtgataggcttgggt 11820
11821  tttggagagcgaggtggttgatggctggagagaggagtacttcctggaacaagggggcta 11880
11881  cattgagcatggagagagtgtgagggtttaagacgacgctagggtttcttgggggaagac 11940
11941  gagggtaagtgtggacaagacgtccaattgaacacgaagagatagctgagtcaggtttgt 12000
12001  gagagagggagtaaatactttgaccggagtcaatcttagggacgaaggcataggggcga 12060
12061  gaagatctttgatagtcaggctcgccgacttgctgcacgccagctgcgtacggggagggc 12120
12121  aggtggcgtaagaggccattgaagtcttgcacaggcccgagcagcaaagcaaattttgaa 12180
12181  ttcgaaacattcccgccgtggctgcgtgtggcgcaaaggctatgaggcactatggccatg 12240
12241  gcagagagctccaagggatatgggcattacgtaacaccatacatgcaccctcctacccac 12300
12301  acctatatatccaacatgtgtgttgggggaaagagggaacattacgtaacagcttgatg 12360
12361  cgctggtcgatgggacagagaaaggttctcggctgtcgacgaactggtagatgggcaaga 12420
12421  ccacgatgccacagatgatcctgagcacctgcttggggtctggtagcgacccaccgccta 12480
12481  ccacgatgtgcgagatccactccacccaccttgtcacggtcgacaagccaagcacaaatc 12540
12541  caataagatttaccacaaaggcgtcgagttgtagcctgattgttttgtgattacatacgc 12600
12601  tatcctcggggtggaagcaagggtgtaggatgtcattcatgtcgaggtagaggttgacga 12660
12661  actcgaggttggggttgggcttggaggtgtcgatgggaaccgggactccctcgatctcgc 12720
12721  tgggctcctcctccaccatgtccaccaccaccatcggtgacttctcaactagccaccagt 12780
12781  aaaacgctggcgcgcccctatcaccactgactccgccggaatgctaaccctagaagtggt 12840
12841  gtttgtgggcgcggatccatgtagaagaggcgcgcaggaagtatagcgggagctcacatg 12900
```

```
12901  gagtgatgggcgagagggctaggaggataaaaacctatatgcagcgtactcgttcgtgca 12960
12961  actggacaggagagaggtaggaccggggttggttaagagggaggttaaaattaataaaga 13020
13021  atttgtggtagttggtatagtgtagagatttaaggtgaatatgattgtggaaaattgtga 13080
13081  gatagggtagaaaatctcgctgaccagga  13109
```