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October 20, 2021

Bernadette Juarez
APHIS Deputy Administrator
Biotechnology Regulatory Services

RE: Amendment of Confirmation Request 21-271-01cr

Dear Ms. Juarez,

Recently Agrivida, Inc. submitted a request for confirmation that a variety of maize (*Zea mays*) genetically engineered to produce the animal feed enzyme phytase (Event PY1203) is exempt from regulation under 7 CFR part 340, §340.1(c). Agrivida, Inc. is submitting an amended version of this request in order to address several comments provided by USDA reviewers (Michael Stulberg, email of October 12, 2021). Included with this letter is the amended request that addresses and incorporates the comments from the reviewers.

Agrivida, Inc. does not consider any of the information in this document to be "Confidential Business Information" (CBI). If you have any questions concerning this request, please direct them to me.

Sincerely,

James Ligon, Ph.D.
V.P., Regulatory Affairs
Agrivida, Inc.

Agrivida

Request for Confirmation of Exemption of Maize Event PY1203 from Regulation Under 7 CFR Part 340

Agrivida, Inc. is submitting this information to support a request for confirmation that the Administrator, Animal and Plant Health Inspection Service, make a determination that the article is not regulated under 7 CFR Part 340 due to exemptions described in 7 CFR Part 340.1(c).

No CBI

Agrivida, Inc. does not consider any information contained in this petition to be confidential business information or to be a trade secret.

October 19, 2021

Summary

Using recombinant DNA technology, Agrivida, Inc. has developed a variety of maize that produces the animal feed enzyme phytase in the grain. The corn grain from this maize variety, maize Event PY203, is harvested and ground into a coarse cornmeal that is sold to animal feed producers for inclusion in the feed of poultry and swine in order to improve the nutritional availability of phytate-bound phosphorous in the diet. Agrivida, Inc. submitted a petition to USDA seeking the deregulation of maize Event PY203 and after reviewing this petition, USDA has determined that Event PY203 is not regulated (19-0176-01p). Subsequent to the development of maize Event PY203, Agrivida, Inc. has developed a second phytase producing maize event, Event PY1203, with increased levels of phytase expression. The phytase gene *phy02* that was used in maize Event PY203 was optimized to better reflect the codon preference of maize to create the *phy02opt* gene that was introduced into the genome of maize Event PY1203. In addition, research by Agrivida, Inc. scientists indicated that the addition of a single alanine residue in the coding sequence of the γ-zein signal sequence of the *phy02* gene increased expression levels of the phytase gene in maize and this modification was engineered into the *phy02opt* gene present in maize Event PY1203. These modifications in the *phy02opt* gene resulted in a threefold increase in the production of phytase in the grain of maize Event PY1203 compared to that of maize Event PY203. The phytase enzyme produced in maize Event PY1203 is identical to that produced by maize Event PY203 since the codon optimization of the *phy02* gene did not result in changes to the amino acid sequence of the encoded phytase enzyme. In addition, the additional alanine residue present in the *phy02opt* gene is in the γ-zein signal sequence that is cleaved from the phytase upon transit of the protein into the endoplasmic reticulum and therefore is not present in the final phytase enzyme.

The phytase produced by maize Event PY1203 was compared to the phytase produced in the antecedent Event PY203 and it was demonstrated that the two phytases are identical based upon comparison of N-terminal amino acid sequence, enzyme kinetic properties, thermal and pH optima, molecular weight and immunoreactivity. Agrivida, Inc. has concluded that based on this information that demonstrates the identity of the phytase enzymes in maize Events PY203 and PY1203 and on the fact that the phytase from maize Event PY203 is "Generally Regarded as Safe" (GRAS) for inclusion in the feed of poultry and swine (Agrivida, 2017 and 2018)), that the phytase produced by maize Event PY1203 is also GRAS for this use (Agrivida, 2019). In the course of a biotechnology consultation with the US FDA, Agrivida, Inc. conducted a food and feed safety evaluation of maize Event PY203 and concluded that this maize event is as safe and nutritious as conventional maize (Agrivida, 2020). Accordingly, Agrivida, Inc. has concluded that under 7 CFR §340.1(c), maize Event PY1203 is exempt from regulation. Contained in this document is information to support a request for confirmation of exemption, including a molecular description of the inserted genetic material, methods used to

produce maize Event PY1203, function of the modified gene, molecular characterization, and DNA sequence data.

Description of the Genetic Material Introduced into the Genome of Maize Event PY1203

The Phy02 phytase protein that is expressed in maize Event PY1203 is identical to the Phy02 phytase protein that is expressed in maize Event PY203 that has been determined by USDA/APHIS not to be regulated (19-176-01p). The expression constructs containing the phytase genes in Event PY1203 are different from those used to create Event PY203. A transformation gene cassette (pAG4916) was constructed containing two copies of the *phy02opt* phytase gene, each with a different monocot derived promoter that provide endosperm-specific gene expression, including the *Oryza sativa* glutelin-1 gene promoter and the promoter from the *Zea mays Zc2* gene that encodes the 27-kDa zein storage protein. Like the transformation construct used to create Event PY203, plasmid pAG4916 contains the *manA* gene encoding phosphomannose isomerase (PMI) 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. As is the case of the *phy02* genes inserted into the genome of Event PY203, the coding sequence of *phy02opt* genes engineered into Event PY1203 contain the γ–zein signal peptide at the N-terminus that directs the phytase enzyme to the endoplasmic reticulum. They also include the six amino acid (SEKDEL) coding sequence of the maize endoplasmic reticulum retention signal that functions to sequester the phytase enzyme in the endoplasmic reticulum. The genetic elements of plasmid pAG4916 are shown in Figure 1, and the individual genetic elements in plasmid pAG4916 are described in Table 1. An alignment of the deduced amino acid sequences of the Phy02 phytase protein produced by the *phy02* gene of Event PY203 and the *phy02opt* gene of Event PY1203 is presented in Figure 2.

Plasmid pAG4916 was introduced into maize by *Agrobacterium*-mediated transformation as described by Negrotto *et al*. (2000) and transformants were selected based on the presence of the plant selectable marker *manA* gene that was also used in the selection of the Phy02 expressing Event PY203. Data from Southern analyses and DNA sequencing demonstrated the presence of a single insertion of the *phy02opt* gene containing T-DNA in maize chromosome 10. The insertion was shown to contain two copies of the T-DNA organized as an inverse tandem repeat.

Figure 1. Diagram of transformation plasmid pAG4916. See Table 1 for descriptions of individual genetic elements. Cleavage sites for the restriction enzyme *Bam*HI are indicated, as are nucleotide (nt) positions that are referenced elsewhere in this document.

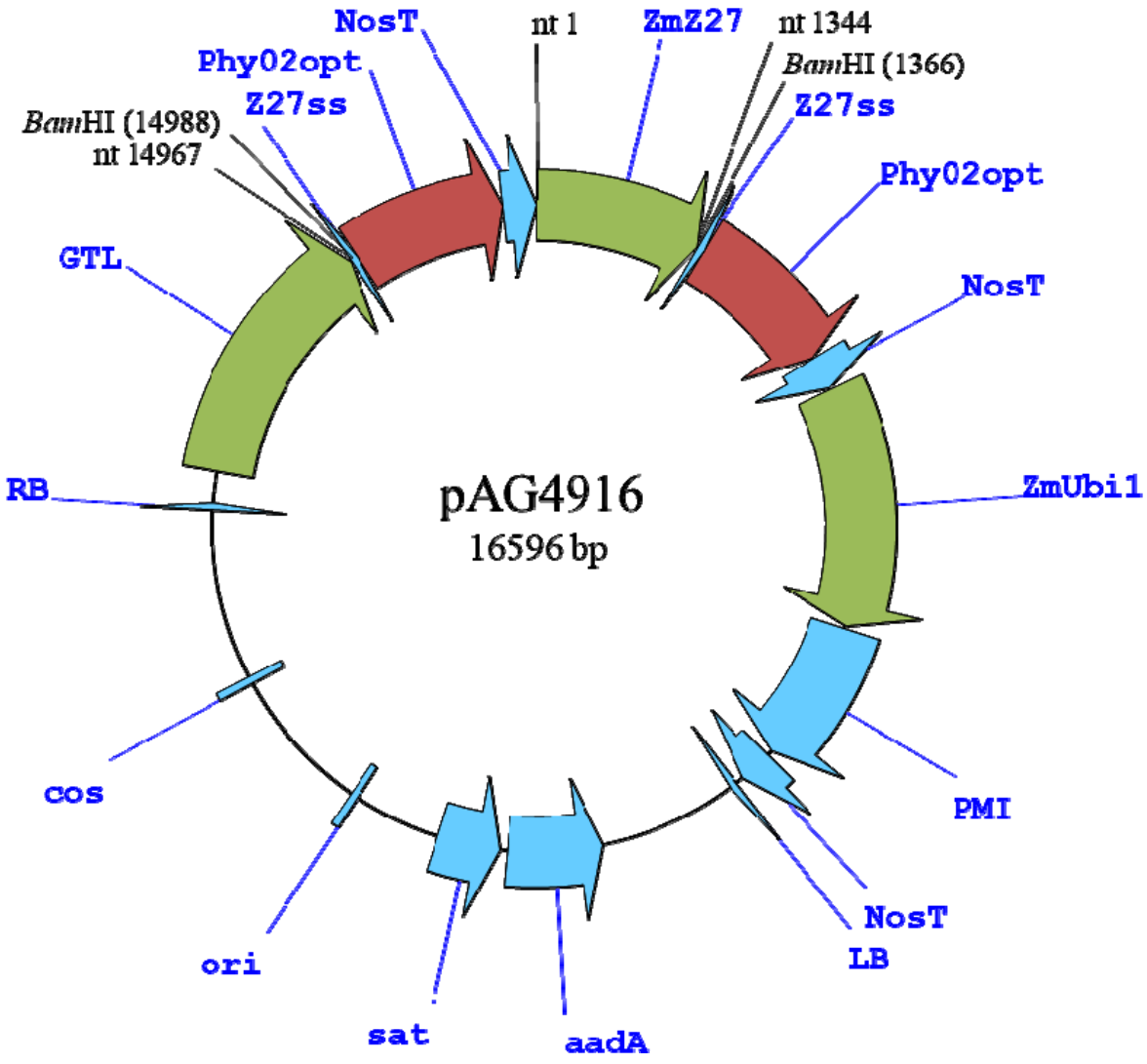

Table 1. Description of the genetic elements in the T-DNA element of plasmid pAG4916 that was inserted in the genome of Event PY1203.

Genetic Element	Description	Position*	Reference
RB	Right border region of T-DNA from *A, tumefaciens* nopaline Ti plasmid (GenBank accession number J01826).	12596-12620	Wang *et al*., 1984
GTL	Promoter derived from the *Oryza sativa* Glutelin-1 gene (similar to Genbank accession number EU264103.1); provides endosperm-specific gene expression	12916-14986	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	14996-15052	Torrent *et al*., 2009
Phy02opt	Coding sequence of the modified Phy02 phytase gene that was originally derived from the *E. coli appA* phytase gene (Genbank accession number EFE63517.1). In pAG4916, each copy of the Phy02opt coding sequence includes an additional C-terminal SEKDEL sequence (see below)	15053-16288	This document
SEKDEL (not shown)	Sequence encoding an endoplasmic reticulum retention peptide	16289-16309	Semenza and Pelham, 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	16316-16591	Depicker *et al*., 1982
ZmZ27	Promoter sequence derived from the Z. mays Zc2 gene (Genbank accession number X53514.1); provides endosperm-specific gene expression.	2-1364	Reina *et al*., 1990; Russell & Fromm, 1997
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 (identical to Z27ss, above)	1374-1430	Torrent *et al*., 2009
Phy02opt	Coding sequence of the modified Phy02 phytase gene that was originally derived from the *E. coli appA* phytase gene (Genbank accession number EFE63517.1). In pAG4916, each copy of the Phy02opt coding sequence includes an additional C-terminal SEKDEL sequence (see below); (identical to Phy02opt, above)	1431-2666	This document
SEKDEL (not shown)	Sequence encoding an endoplasmic reticulum retention peptide; (identical to SEKDEL, above)	2667-2687	Semenza and Pelham, 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	2694-2969	Depicker *et al*., 1982
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*.	2976-4967	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	4983-6158	Negrotto *et al*., 2000
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	6207-6482	Depicker *et al*., 1982
LB	Left border region of T-DNA from *A. tumefaciens* nopaline Ti plasmid (GenBank accession number J01825).	6553-6577	Zambryski *et al*., 1982

*nucleotide coordinates relative to numbering used in Figure 1.

Figure 2. Alignment of the deduced amino acid sequences of the Phy02 phytases encoded by the *phy02* and *phy02opt* genes. Dots in the lower line of each pair indicate identical amino acids. Letters indicate amino acids that differ. Dashes indicate gaps in one sequence relative to the other. The two phytase proteins differ only by the addition of an alanine residue at the end of the γ-zein signal sequence (Z27ss, underlined). The endoplasmic retention signal at the C-terminus (SEKDEL) is underlined.

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4758 Phy02 1    MRVLLVALALLALAASATSA-QSEPELKLESVVIVSRHGVRAPTKFTQLMQDVTPDAFYT  59
4916 Phy02 1    ....................A.....................................  60

4758 Phy02 60   WPVKLGELTPRGGELIAYLGHYWRQRLVADGLLPKKGCPQSGQVAIIADVDERTRKTGEA 119
4916 Phy02 61   ............................................................ 120

4758 Phy02 120  FAAGLAPDCAITVHTQADTSSPDPLFNPLKTGVCQLDVAQVTDAILERAGGSIADFTGHY 179
4916 Phy02 121  ............................................................ 180

4758 Phy02 180  QTAFRELERVLNFPQSNLALKREKQDESASLTQALPSELKVSADNVSLTGAWSLASMLTE 239
4916 Phy02 181  ............................................................ 240

4758 Phy02 240  IFLLQQAQGMPEPGWGRITDSHQWNTLLSLHNAQFDLLQRTPEVARSRATPLLDLIKTAL 299
4916 Phy02 241  ............................................................ 300

4758 Phy02 300  TPHPPQKQAYGVTLPTSVLFIAGHDTNLANLGGALELQWTLPGQPDNTPPGGELVFERWR 359
4916 Phy02 301  ............................................................ 360

4758 Phy02 360  RLSDNSQWIQVSLVFQTLQQMRDKTPLFLNTPPGEVKLTLAGCEERNAQGMCSLAGFTQI 419
4916 Phy02 361  ............................................................ 420

4758 Phy02 420  VNEARIPACSLSEKDEL  436
4916 Phy02 421  ................  437
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Absence of Vector Plasmid Backbone Fragments in the Genome of Event PY1203

The absence in the genome of Event PY1203 of DNA fragments derived from outside of the T-DNA of vector pAG4916 was demonstrated by Southern blot analysis. Overlapping probes were generated from pAG4916 via PCR, which collectively represented the entire region of the plasmid vector from the LB to the RB, including the antibiotic resistance markers, *sat* and *aadA* genes, as well as the bacterial origin of replication and the *cos* site. The primers that were used to generate these PCR products that were used as probes are listed in Table 2, and the relative positions of these PCR products are depicted in Figure 3.

Genomic DNA samples from wild type (untransformed) *Z. mays* and Event PY1203 as well as the purified transformation vector pAG4916 were each digested with either *Bam*HI or *Eco*RI, separated via agarose gel electrophoresis, and subjected to Southern analysis using the PCR-generated DIG-labeled probes (Table 2). As depicted in Figure 4, the probes readily detected the backbone fragments in lanes loaded with vector DNA (lanes 5 and 6). The same probes, however, did not detect any fragments in either the

wild type or Event PY1203 genomic DNA (lanes 1-4). These results indicate that no vector backbone-derived sequences are present in the genome of Event PY1203.

Table 2. PCR primers used to generate hybridization probes to detect presence of plasmid backbone sequences in Event PY1203.

Primer #	Sequence	From*	To*	sense	probe ID
a1	ACC AGC CAG CCA ACA GCT	6577	6594	-->	a
a2	CAT CCA ACT ACG ACA TTT CTC C	7538	7559	<--	a
b1	GAG TTG TCG TAG TTG CTT GGA	7520	7540	-->	b
b2	CGA TGT ACT GGT ACT GGT T	8624	8642	<--	b
c1	GAG AAC CAG TAC CAG TAC A	8621	8639	-->	c
c2	AGC CAG TAT ACA CTC CGC T	9550	9568	<--	c
d1	AGC GGA GTG TAT ACT GGC T	9550	9568	-->	d
d2	CCT CAC TGA TTA AGC ATT GG	10610	10629	<--	d
e1	GCT TAA TCA GTG AGG CAC CT	10615	10634	-->	e
e2	GGA TGG CAT GAC AGT AAG AG	11550	11569	<--	e
f1	CTC TTA CTG TCA TGC CAT CC	11550	11569	-->	f
f2	CCT AAG AGA AAA GAG CGT TTA T	12575	12596	<--	f

*Positions "from" and "to" are relative to the diagram of pAG4916 as shown in Figure 3. For reference, the LB corresponds to positions 6553-6577, and the RB corresponds to positions 12596-12620.

Figure 3. Positions of probes used for Southern blotting of vector backbone sequences. Probes a-f (red) were prepared by PCR, labeled with DIG, pooled, and used to probe genomic DNA derived from Event PY1203. Positions of *Eco*RI and *Bam*HI sites as well as the +1 position are indicated for reference.

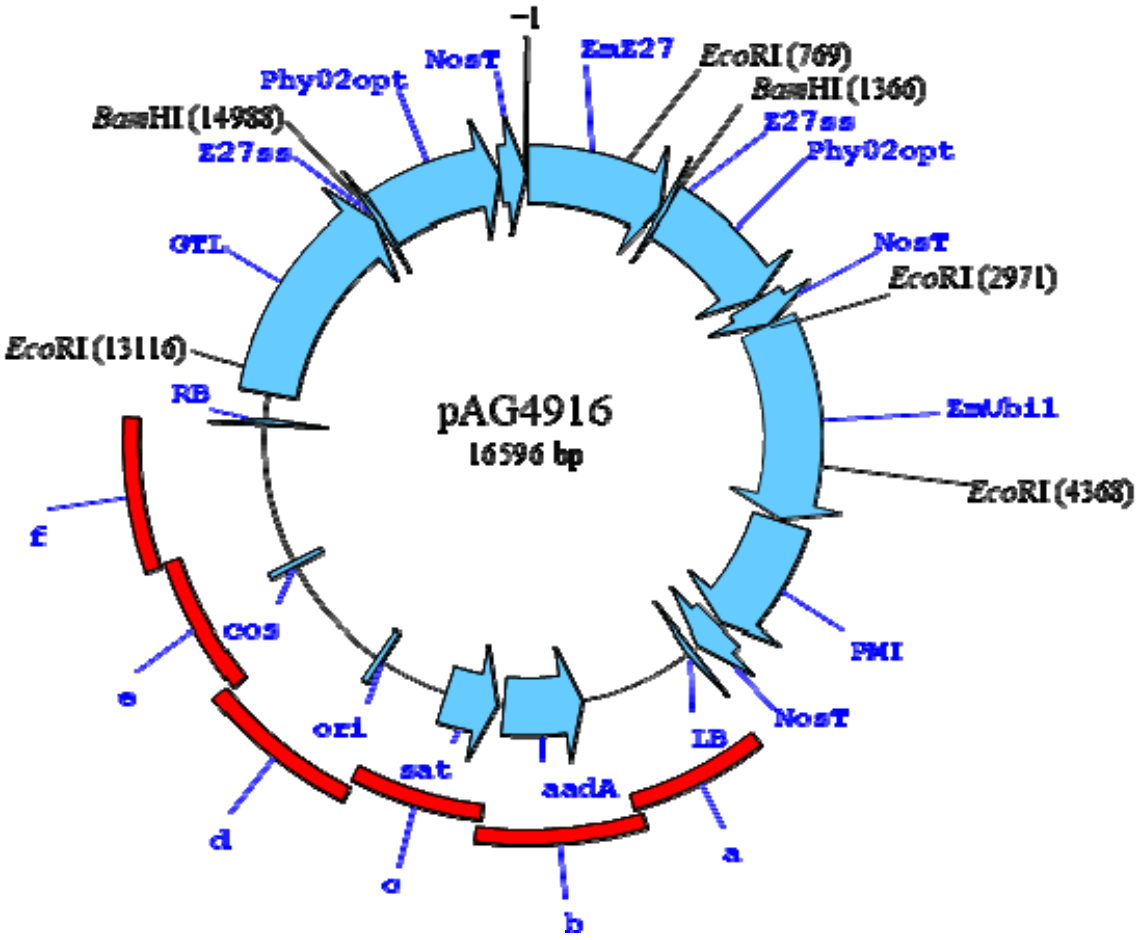
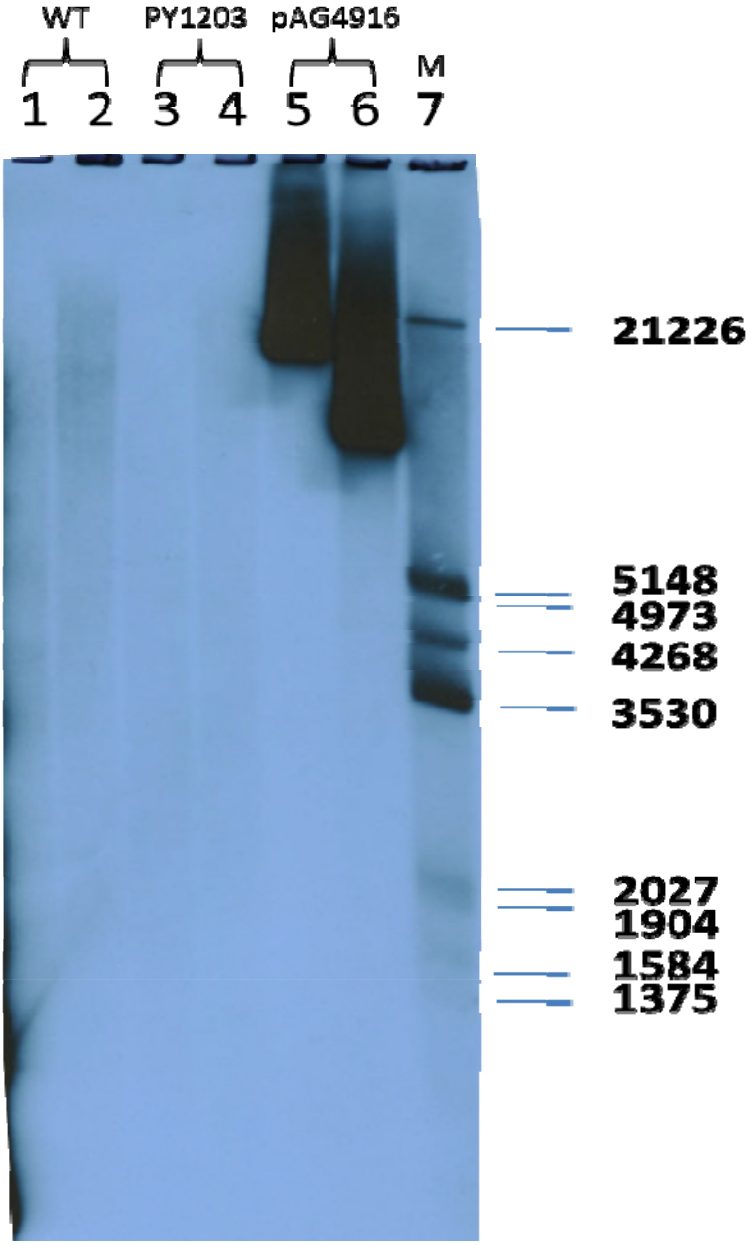


Figure 4. Southern blot showing the lack of vector backbone-derived sequences in the genomic DNA of Event PY1203. Lane 1: wild type (WT) *Z. mays* DNA digested with *Bam*HI; lane 2: WT *Z. mays* DNA digested with *Eco*RI; lane 3: PY1203 DNA digested with *Bam*HI; lane 4: PY1203 DNA digested with *Eco*RI; lane 5: pAG4916 DNA, equivalent to one genome-copy equivalent, digested with *Bam*HI; lane 6: pAG4916 DNA, equivalent to one gene-copy equivalent, digested with *Eco*RI; lane 7: DIG-labeled molecular weight marker. The relative sizes (in base pairs) of the labeled fragments in lane 7 are indicated to the right.



Characterization of the T-DNA insertion in maize Event PY1203

DNA Sequencing of the Insert and Maize Flanking Regions

Combining several sequencing strategies such as target enrichment followed by PacBio sequencing, inverse PCR, long range PCR, and high-fidelity PCR for gap closure, the DNA sequence of the entire insert in Event PY1203 was elucidated and is presented in Figure 5. The overall length of the PY1203 locus is 31,814 nucleotides, including two complete T-DNA copies from the pAG4916 construct and maize genomic DNA flanks. The two T-DNAs in Event PY1203 are arranged as tandem inverted repeats and flanked by the maize genomic DNA sequences of 5819 nt (nucleotides 131245876-131251694 on chromosome 10) at the left side of the insertion, as depicted in Figure 6 and Table 2, and 4981 nt (nucleotides 131245833-131240851 on chromosome 10) at the right side of the insertion (Figure 6; Table 2). The left-most T-DNA ("T-DNA 1" in Figure 6) is composed of 10,480 nt, while the right most T-DNA ("T-DNA 2" in Figure 6) has a length of 10,527 nt. The 47 nt sequence length difference between two T-DNAs is due to differences in the amount of non-functional (spacer) sequence from the extreme ends of the T-DNA (up to and including the RB or LB) that was integrated at the locus. For example, the entire RB sequence was deleted from both copies of the inserted T-DNAs. The entire LB sequence as well as about 45 nucleotides of spacer sequence was deleted from the extreme left end of T-DNA 1 (as defined in Figure 6), and all but 3 nucleotides were deleted from the LB at the extreme right end of T-DNA 2.

The genomic organization of the PY1203 locus was confirmed by PCR and Southern Blot analyses. Analysis of nucleotide sequence identity between flanking sequences of the PY1203 locus and the reference maize genome B73 (version RefGen_v4, Woodhouse *et al*., 2021) revealed that the T-DNA sequences were integrated into the maize genome between nucleotides 131245833-131245876 on maize chromosome 10 and displaced 43 nucleotides of the wild type maize genomic DNA sequence. No additional vector sequences have been observed at this genetic locus or any other genomic locations as demonstrated by PCR, locus sequencing, and Southern Blot analyses. Furthermore, the PY1203 locus appeared to have no internal deletions or rearrangements of the genetic elements within the two integrated T-DNAs. The inserted T-DNA sequences are 100% identical to the T-DNA sequence of pAG4916, with the exception of two nucleotide substitutions 5346G>T and 5347C>T, which occurred in a single codon of the phosphomannose isomerase (PMI) selectable marker gene (*manA*) that is positioned on the T-DNA adjacent to the right maize genomic DNA flank. The identified mutations lead to a single amino acid substitution (122Ala>Phe) in the PMI protein sequence. No annotated or predicted gene sequences were determined for the host genomic DNA sequence at the site of the T-DNA integration in the PY1203 locus.

A complete description of the genetic characterization of Event PY1203 is presented in the GRAS notice submitted by Agrivida, Inc. to the FDA Center for Veterinary Medicine (Agrivida, 2020).

Figure 5. Nucleotide sequence of the locus of T-DNA insertion and the flanking maize genomic DNA in Event PY1203. The flanking maize genomic sequence is designated in lower case letters while the sequence of the T-DNA insertion is designated in capital letters. The positions of key genetic elements within the locus sequence are indicated in Table 3.

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>PY1203 full locus sequence
cttttatgtaaactagttcaattatgcacttctatacttgctttggtttgtgttagcatc
aatcaccaaaaaggggagattgaaagggaattaggcttacacctatttcctaaatgatt
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Figure 6. Diagram of the T-DNA insertion locus within maize chromosome 10 in Event PY1203. Descriptions and positions of individual genetic elements can be found in Table 3. For simplicity, NosT, Z27ss, and SEKDEL elements have been omitted from the figure. Cleavage positions for restriction enzymes *Hin*dIII, *Pme*I and *Pvu*II are indicated.

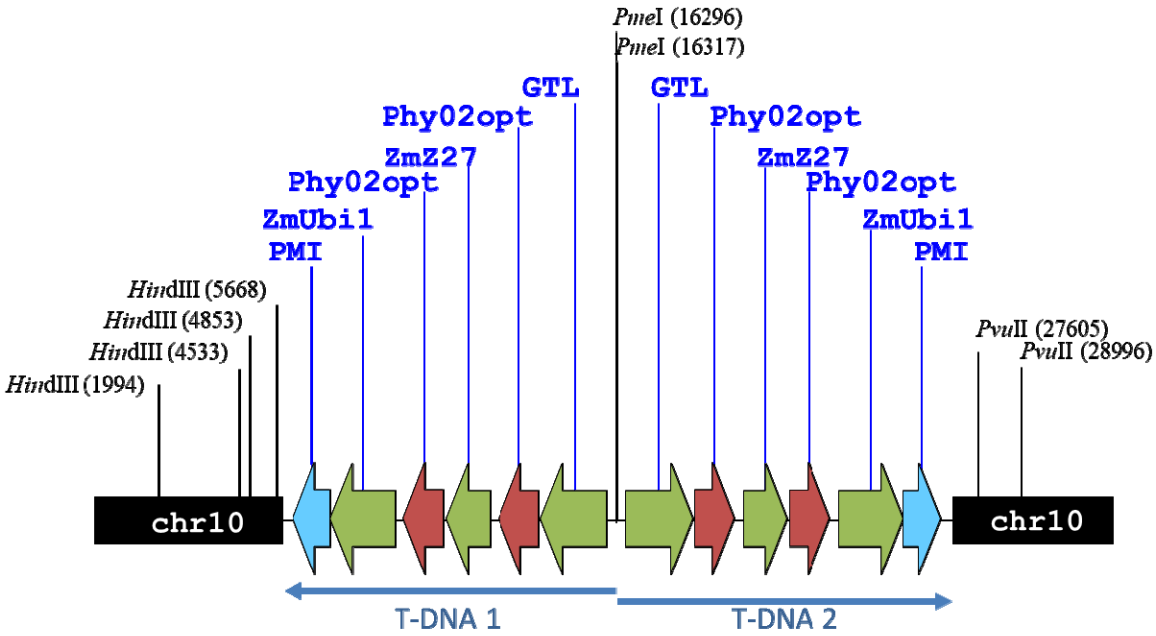

Table 3. **Positions of key genetic elements within the PY1203 T-DNA locus.** The genetic elements are described in Table 1. The complete DNA sequence of this locus is presented in Figure 5.

Start	End	Genetic Element
1	5819	Maize chromosomal DNA ("chr10")
5827	**16306**	**T-DNA 1**
5852	6127	NosT
6176	7351	*manA* gene encoding PMI
7367	9358	Promoter from the *Z. mays* ubiquitin 1 gene including the first intron (ZmUbi1)
9365	9640	NosT (not shown in Figure 6)
9647	10903	*phy02opt* gene with SEKDEL at C-terminus
10904	10960	γ-zein signal sequence Z27ss
10970	12332	Promoter from the *Z. mays* Zc2 gene (ZmZ27)
12339	12614	NosT
12621	13877	*phy02opt* gene with SEKDEL at C-terminus
13878	13934	γ-zein signal sequence Z27ss
13944	16014	GTL promoter from the *Oryza sativa* Glutelin-1 gene
16307	**26833**	**T-DNA 2**
16598	18668	GTL promoter from the *Oryza sativa* Glutelin-1 gene
18678	18734	γ-zein signal sequence Z27ss
18735	19991	*phy02opt* gene with SEKDEL at C-terminus
19998	20273	NosT
20280	21642	Promoter from the *Z. mays* Zc2 gene (ZmZ27)
21652	21708	Z27ss (not shown)
21709	22965	*phy02opt* gene with SEKDEL at C-terminus
22972	23247	NosT
23254	25245	Promoter from the *Z. mays* ubiquitin 1 gene including the first intron (ZmUbi1)
25261	26436	*manA* gene encoding PMI
26485	26760	NosT
26831	26833	Partial LB (3 nucleotides only)
26834	31814	Maize chromosomal DNA ("chr10")

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