Event 5307 Maize:

Genomic Insertion Site Analysis

AMENDED REPORT NO.1

Data Requirement:	Not applicable
Author:	Stephen New
Study Completion Date:	November 11, 2010
Performing Laboratory:	Syngenta Biotechnology, Inc. Product Safety 3054 East Cornwallis Road PO Box 12257 Research Triangle Park, NC 27709-2257, USA
Syngenta Study No.:	Not applicable
Report No.:	SSB-202-10 A1

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Company: *Syngenta Seeds, Inc.*

Company Representative:

Demetra Vlachos — November 11, 2010

Demetra Vlachos
Regulatory Affairs Manager

Date

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STATEMENT CONCERNING GOOD LABORATORY PRACTICES STANDARDS

This study was not conducted in compliance with the relevant provisions of Good Laboratory Practices Standards (GLPS) (40 CFR Part 160, US EPA 1989) pursuant to the Federal Insecticide, Fungicide, and Rodenticide Act. However, all components of the study were performed according to accepted scientific practices, and relevant study records (including raw data) have been retained.

Study Director:

November 11, 2010

Stephen New
Technical Expert I
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REPORT AMENDMENTS

Amendment No. 1: November 11, 2010

This amended report has the following corrections:

On page 2, the confidentiatliy statement has been updated and the Regulatory Affairs Manager has been updated.

On page 3, the Regulatory Affairs Manager has been updated, and the positions for the Study Director and the Sponsor have been updated.

On page 4, the Table of Contents has been updated.

On page 6, a new section has been added listing the Report Amendments.

On pages 8, 12, and 13, the number of base pairs that were deleted during the integration of the 5307 insert has been corrected.

On page 14, the positions for the Study Director and the Sponsor have been updated.

The corrected pages in this amended report SSB-202-10 A1 are indicated as "*REVISED*".

LIST OF ACRONYMS AND ABBREVIATIONS

3′	three prime
5′	five prime
bp	base pair
CMP	cestrum yellow leaf curling virus promoter
Cry1Ab	Cry1Ab protein
Cry3A	Cry3A protein
CTAB	cetyltrimethyl ammonium bromide
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
ecry3.1Ab	eCry3.1Ab gene
eCry3.1Ab	eCry3.1Ab protein
EDTA	ethylenediaminetetraacetic acid
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
g	gram
GLPS	Good Laboratory Practices Standards
LB	left border
M	molar
manA	phosomannose isomerase gene
mCry3A	mCry3A protein
ml	milliliter
mM	millimolar
NaCl	sodium chloride
NOS	nopaline synthase
NTI	New Technologies Informax
PCR	polymerase chain reaction
pmi	phosomannose isomerase gene
PMI	phosomannose isomerase protein
TE	Tris-EDTA
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
US EPA	United States Environmental Protection Agency
v/v	volume to volume
w/v	weight to volume
ZmUbiInt	*Zea mays* ubiquitin promoter with intron
µg	microgram
°C	degrees Celsius
× *g*	times gravity
®	registered trademark
™	trademark

SUMMARY

Using the techniques of modern molecular biology, Syngenta has transformed maize (*Zea mays*) to produce Event 5307 maize, a new cultivar that has insecticidal activity against certain corn rootworm (*Diabrotica*) species. Maize plants derived from transformation Event 5307 ("5307 maize") contain the gene *ecry3.1Ab* encoding an eCry3.1Ab protein and the gene *pmi* (also known as *manA*) encoding the enzyme phosphomannose isomerase (PMI).

The purpose of this study is to determine the genomic insertion site of the 5307 maize insert and to assess the changes in the maize genome that may have occurred at the genomic insertion site during integration of the 5307 maize insert. The maize genomic sequence at the point of integration of the 5307 maize insert was determined using polymerase chain reaction and sequencing. The deoxyribonucleic acid (DNA) sequence of the nontransgenic maize genome at this location was then sequenced and compared to the genomic sequences flanking the 5307 maize insert. This analysis was used to assess the changes in the maize genome that may have occurred at the genomic insertion site during integration of the 5307 maize insert

Analysis of sequence data demonstrated that 33 base pairs from the maize genomic sequence were deleted during the integration of the 5307 maize insert.

INTRODUCTION

Using the techniques of modern molecular biology, Syngenta has transformed maize (*Zea mays*) to produce Event 5307 maize, a new cultivar that has insecticidal activity against certain corn rootworm (*Diabrotica*) species. Maize plants derived from transformation Event 5307 ("5307 maize") contain the gene *ecry3.1Ab* encoding an eCry3.1Ab protein and the gene *pmi* (also known as *manA*) encoding the enzyme phosphomannose isomerase (PMI). The eCry3.1Ab protein is an engineered chimera of modified Cry3A (mCry3A) and Cry1Ab proteins. The gene *pmi* was obtained from *Escherichia coli* strain K-12 and the protein it encodes was utilized as a plant selectable marker during development of 5307 maize.

The purpose of this study is to determine the genomic insertion site of 5307 maize and to assess the changes in the maize genome that may have occurred at the genomic insertion site during integration of the 5307 maize insert. The maize genomic sequence at the point of integration of the 5307 maize insert was determined using polymerase chain reaction (PCR) and sequencing. Analysis comparing the sequence of the genomic insertion site from nontransgenic maize to the genomic sequences flanking the 5307 maize insert was used to assess the changes in the maize genome that may have occurred at the genomic insertion site during integration of the 5307 maize insert.

MATERIALS AND METHODS

Control Substance

The control substance for this study was nontransgenic maize seed (NP2222 maize). This material was selected because it was the transformation recipient used in the creation of 5307 maize. Table 1 shows the description and pedigree code for the control substance.

Table 1. Control substance

Seed identification	Pedigree
NP2222 maize	NP2222

The control substance was characterized by real-time PCR (Ingham *et al.* 2001) analysis to confirm the identity and purity. Results from the real-time PCR analysis confirmed that the control substance was nontransgenic (*i.e.*, the control substance tested negative for any transgenic events).

Plant Tissue for Genomic Deoxyribonucleic Acid (DNA) Extraction

Control substance seed was grown in a Syngenta Biotechnology, Inc. greenhouse in Research Triangle Park, North Carolina, USA. Following verification of the plants' identity by real-time PCR analysis, leaf tissue from plants grown from the control substance was pooled into one sampling bag and stored at -80°C ± 10°C.

Genomic DNA Extraction

Genomic DNA was isolated from the pooled leaf tissue using a modification of the method described in Saghai-Maroof *et al*. (1984).

Pooled leaf tissue was ground into a fine powder using a pre-chilled mortar and pestle, with liquid nitrogen, and then placed into a bottle for storage. For each DNA extraction, approximately 40 g of this tissue and 200 ml of prewarmed CTAB buffer (100 mM Tris pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 2% CTAB [w/v], 0.2% [v/v] β-mercaptoethanol) were combined in a bottle; the sample was then mixed gently and incubated for 90 minutes at 65°C ± 5°C. An equal volume of chloroform:isoamyl alcohol (24:1) was then added, followed by gentle mixing and centrifugation for 10 minutes at 7277 × *g* at room temperature.

The resulting aqueous phase was transferred to a clean container, and 10 µg of ribonuclease per ml of aqueous phase was added. The sample was mixed and incubated for 30 minutes at 37°C ± 2°C. An equal volume of chloroform:isoamyl alcohol (24:1) was then added, followed by gentle mixing and centrifugation for 10 minutes at 7277 × *g* at room temperature. The aqueous phase was collected in a clean bottle, and the DNA was precipitated with a 0.8 volume of isopropanol. The DNA was then pelleted by centrifugation at 291 × *g* and washed once with 70% ethanol. The DNA pellet was air-dried and dissolved in 2.5 ml of prewarmed 0.1X TE.

DNA Quantitation

The concentration of DNA was measured using a Quant-iT™ PicoGreen® dsDNA kit. A two-point standard curve was generated using a Lambda DNA standard. Genomic DNA was quantified by interpolation from the two point standard curve and was assayed in triplicate using the TBS-380 Mini-Fluorometer.

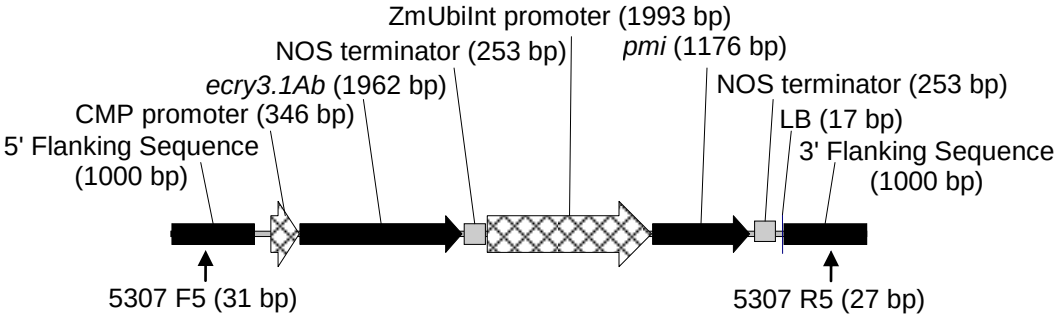
PCR Amplification

The genomic insertion site was amplified from genomic DNA extracted from the nontransgenic maize line NP2222 maize using PCR analysis. Primers for PCR amplification were designed based on the genomic sequence flanking the 5′ and 3′ regions of the 5307 maize insert previously reported (New 2010). Table 2 lists the primers used to amplify the genomic insertion site, and Figure 1 is a map showing the location of the primers. PCR amplification was carried out using the Expand™ High-Fidelity PCR System. Table 3 contains the thermal cycling parameters.

Table 2. Primers used for amplification of the genomic insertion site of 5307 maize

Forward primer name	Forward primer sequence (5' to 3')	Reverse primer name	Reverse primer sequence (5' to 3')
5307_F5	CTGCGTAAGGGTATCGCT CTCCCTTGGTCAT	5307_R5	GAGAGCCAAACGTGTACCTT ACAACCT

Figure 1. Map of 5307 maize insert including genomic sequences flanking the insert and locations of primers used for amplification of the genomic insertion site



bp = base pair

Table 3. Cycling parameters

Cycle	Step	Temperature (°C)	Time	Number of cycles
A	1	4	5 min	1
B	1	95	5 min	1
C	1	95	15 sec	35
C	2	55	15 sec	35
C	3	72	2 min	35
D	1	72	10 min	1
E	1	4	Hold	1

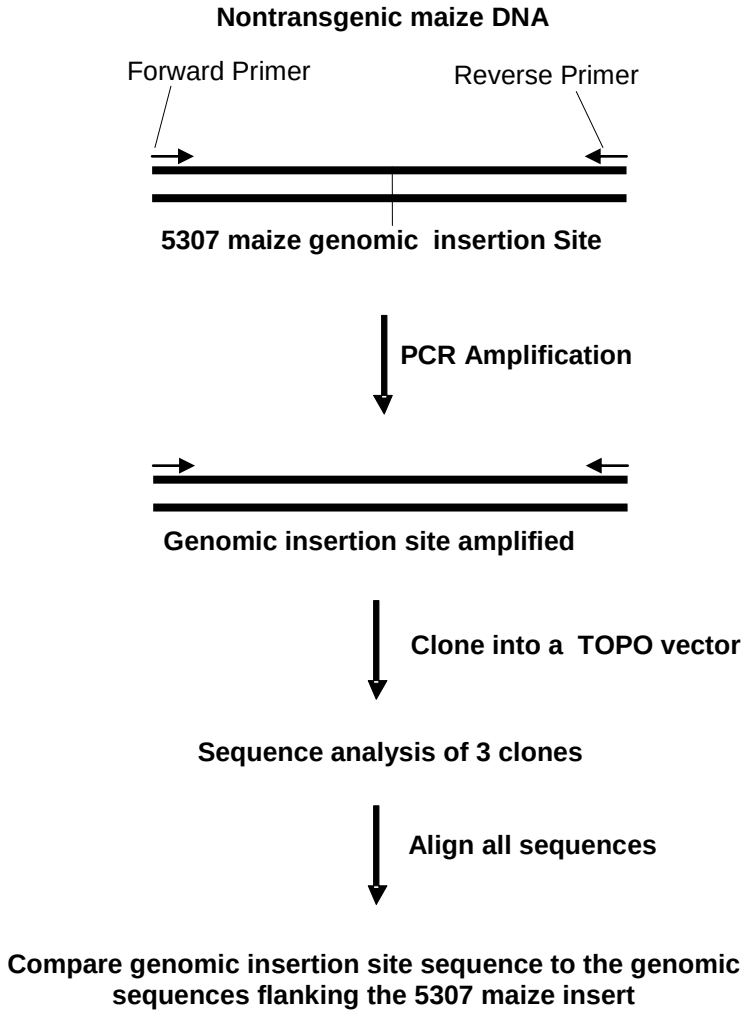
The PCR fragment was cloned into pCR®-XL-TOPO® vector, and 3 colonies were randomly selected and grown. The plasmid DNA was then independently extracted, and the resulting plasmid preparations, which contained the PCR amplification product, were subsequently sequenced.

Sequencing

Dye-terminator sequencing, a modification of the dideoxynucleotide chain-terminator sequencing method, was carried out using the ABI3730XL analyzer with ABI BigDye® 3.1 terminator chemistry. The sequence analysis was done using the Phred, Phrap, and Consed package (from the University of Washington), and was carried out to an error rate of less than 1 in 10,000 bases (Ewing and Green 1998).

Three individual clones were sequenced individually, and a consensus sequence was generated for each clone. These sequences were aligned using AlignX™, a component of Vector NTI Advance™, version 10.3.0, to obtain the final consensus sequence for the genomic insertion site sequence. Figure 2 shows a diagram of the process used to determine the genomic insertion site of the 5307 maize insert.

Figure 2. Process used to determine the genomic insertion site of the 5307 maize insert



Statistical Analysis

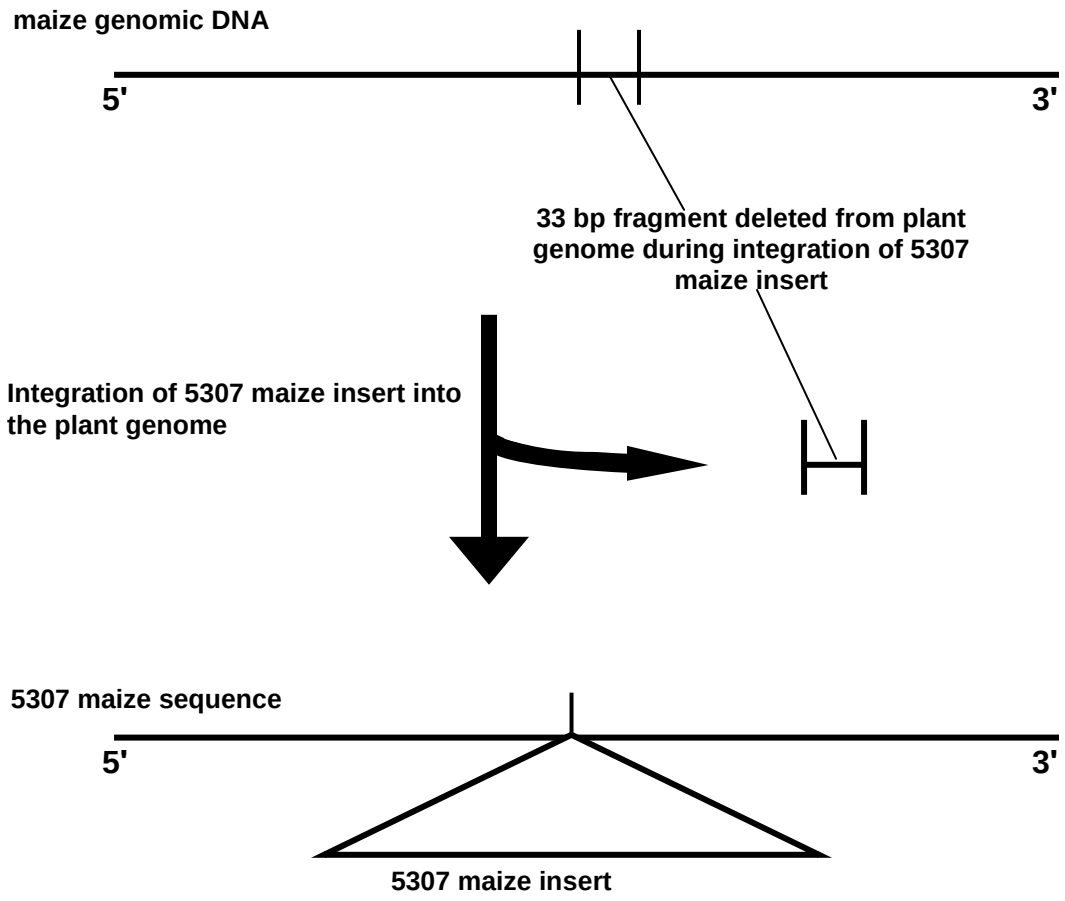
No statistical analysis was used during this study.

RESULTS

Genomic Insertion Site Sequencing

The Appendix shows the alignment of the genomic insertion site sequence from nontransgenic maize to the genomic sequence flanking the 5307 maize insert. This comparison demonstrated that a 33 base pair deletion occurred during integration of the 5307 maize insert into the maize genome. Figure 3 shows a diagram of the integration of the 5307 maize insert into the maize genome.

Figure 3. Integration of the 5307 maize insert into the maize genome



bp = base pair

Data Quality and Integrity

No circumstances occurred during the conduct of this study that would have adversely affected the quality or integrity of the data generated.

CONCLUSIONS

Sequence analysis of the 5307 maize genomic insertion site demonstrated that 33 base pairs of maize genomic sequence were deleted when the 5307 maize insert integrated into the maize genome.

RECORDS RETENTION

Raw data, the original copy of this report, and other relevant records are archived at Syngenta Biotechnology, Inc., 3054 East Cornwallis Road, Research Triangle Park, NC 27709-2257, USA.

CONTRIBUTING SCIENTISTS

The analytical work reported herein was conducted by Stephen New, B.S. This work was conducted at Syngenta Biotechnology, Inc.

Reported by: _____ November 11, 2010
Date

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Approved by: Hope Hart _____ November 11 2010
Date

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Ingham DJ, Beer S, Money S, Hansen G. 2001. Quantitative real-time PCR assay for determining transgene copy number in transformed plants. *BioTechniques* 31:132–140.

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW. 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *P Natl Acad Sci USA* 81:8014–8018.

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Unpublished

New S. 2010. *Event 5307 Maize: Flanking Sequence Determination*. Report No. SSB-160-10 (unpublished). Research Triangle Park, NC: Syngenta Biotechnology.

APPENDIX

Alignment of the genomic insertion site sequence from nontransgenic maize to the genomic sequences flanking the 5307 maize insert

{CBI Cross Reference Number 1}

Event 5307 maize:

Genomic Insertion Site Analysis

CONTAINS CONFIDENTIAL BUSINESS INFORMATION

Data Requirement:	Not applicable
Author:	Stephen New
Study Completion Date:	November 11, 2010
Performing Laboratory:	Syngenta Biotechnology, Inc. Product Safety 3054 East Cornwallis Road PO Box 12257 Research Triangle Park, NC 27709-2257, USA
Syngenta Study No.:	Not applicable
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CBI Cross-Reference Number 1	This cross-reference number noted on a place holder page is used in place of the following whole page at the indicated volume and page references.

Deleted Pages are attached immediately behind this page.

Pages	Reason for Deletion	FIFRA Reference
15	Discloses information concerning the composition of the product	§10(d)(1)(A)

APPENDIX

Alignment of the genomic insertion site sequence from nontransgenic maize to the genomic sequences flanking the 5307 maize insert

```
                                  5′ Flanking Sequence
         5307 maize      (1) CTGCGTAAGGGTATCGCTCTCCCTTGGTCATCGCAAGAAC
nontransgenic maize      (1) CTGCGTAAGGGTATCGCTCTCCCTTGGTCATCGCAAGAAC

                                  5′ Flanking Sequence
         5307 maize     (41) CAAGTGCTCACAACGAGATGATCCTTTGCCACTCCGGCGC
nontransgenic maize     (41) CAAGTGCTCACAACGAGATGATCCTTTGCCACTCCGGCGC

                                  5′ Flanking Sequence
         5307 maize     (81) GGTGGATCCCTCACGACCGCTTACAAACTTGAGTCGGGTC
nontransgenic maize     (81) GGTGGATCCCTCACGACCGCTTACAAACTTGAGTCGGGTC

                                  5′ Flanking Sequence
         5307 maize    (121) ACCAACAAGATCTCCACGGTGATCACCGAGCTCCCAACGC
nontransgenic maize    (121) ACCAACAAGATCTCCACGGTGATCACCGAGCTCCCAACGC

                                  5′ Flanking Sequence
         5307 maize    (161) CACCAAGCCGTCTAGGTGATGCCGATCACCAAGAGTAATA
nontransgenic maize    (161) CACCAAGCCGTCTAGGTGATGCCGATCACCAAGAGTAATA

                                  5′ Flanking Sequence
         5307 maize    (201) AGCCATAGACTTTCACTTGACCAAGAGAAGCCTAATGCAT
nontransgenic maize    (201) AGCCATAGACTTTCACTTGACCAAGAGAAGCCTAATGCAT

                                  5′ Flanking Sequence
         5307 maize    (241) GCGGTGTGTGCTCTAGGTGGCTCTCGCTAGCGTTAATGAG
nontransgenic maize    (241) GCGGTGTGTGCTCTAGGTGGCTCTCGCTAGCGTTAATGAG

                                  5′ Flanking Sequence
         5307 maize    (281) GTCCAAATGCGGGATTAAGATTCTCAAGTCACCTCACTAG
nontransgenic maize    (281) GTCCAAATGCGGGATTAAGATTCTCAAGTCACCTCACTAG

                                  5′ Flanking Sequence
         5307 maize    (321) GCTTTGTGGTGCTTGCAATGCTCTACCAATGTGTAGGAGT
nontransgenic maize    (321) GCTTTGTGGTGCTTGCAATGCTCTACCAATGTGTAGGAGT

                                  5′ Flanking Sequence
         5307 maize    (361) AAATGTGGGCAGCAAGACCATCAATATGGTAGGTGGATGG
nontransgenic maize    (361) AAATGTGGGCAGCAAGACCATCAATATGGTAGGTGGATGG

                                  5′ Flanking Sequence
         5307 maize    (401) GGTATAAATAGCCCTCACCCACCAACTAGCCATTACCAGG
nontransgenic maize    (401) GGTATAAATAGCCCTCACCCACCAACTAGCCATTACCAGG

                                  5′ Flanking Sequence
         5307 maize    (441) AATCTGCTGCGCATGGGCGCACCGGACAGTCCGGTGTGCC
nontransgenic maize    (441) AATCTGCTGCGCATGGGCGCACCGGACAGTCCGGTGTGCC
```

Alignment of the genomic insertion site sequence from nontransgenic maize to the genomic sequences flanking the 5307 maize insert *(Continued)*

```
                                  5′ Flanking Sequence
         5307 maize   (481) ACCGGTGCGCCAACGGTCGACTCAAACGGCTAGTTCTGAC
nontransgenic maize   (481) ACCGGTGCGCCAACGGTCGACTCAAACGGCTAGTTCTGAC

                                  5′ Flanking Sequence
         5307 maize   (521) AGCTAGCCGTTGGACAGATGGCATACCGGACAGTCCGATA
nontransgenic maize   (521) AGCTAGCCGTTGGACAGATGGCATACCGGACAGTCCGATA

                               5′ Flanking Sequence
         5307 maize   (561) CGCTGTCCGGTGTGCCTCTAAAATTCAACTCACGA-----
nontransgenic maize   (561) CGCTGTCCGGTGTGCCTCTAAAATTCAACTCACGAACAGC

                                                  3′ Flanking Sequence
         5307 maize   (596) -----(5307 maize insert)----CCCTCTTCCCTG
nontransgenic maize   (601) GCGCTCTCGGGTTTCTGCGCGCAGGGAACCCTCTTCCCTG

                                  3′ Flanking Sequence
         5307 maize   (606) GGCCAGGCTGGGCCCACTGGCAAAGGGTGCACCGGACAGT
nontransgenic maize   (641) GGCCAGGCTGGGCCCACTGGCAAAGGGTGCACCGGACAGT

                                  3′ Flanking Sequence
         5307 maize   (646) CCGGTGCCCCAAAGCCAGAAACCCTAGCTTCTGTTTTGTG
nontransgenic maize   (681) CCGGTGCCCCAAAGCCAGAAACCCTAGCTTCTGTTTTGTG

                                  3′ Flanking Sequence
         5307 maize   (686) CTGTTTTTTCAATTTGGTTTTTGTTCTAACTTGTGAGTAT
nontransgenic maize   (721) CTGTTTTTTCAATTTGGTTTTTGTTCTAACTTGTGAGTAT

                                  3′ Flanking Sequence
         5307 maize   (726) GTTCTAGAGTTACACCTAGCACTATATGTGAGTGTGAATA
nontransgenic maize   (761) GTTCTAGAGTTACACCTAGCACTATATGTGAGTGTGAATA

                                  3′ Flanking Sequence
         5307 maize   (766) TGCACCAACACTACACTAGAACTCTTTTGGTCAAACTACT
nontransgenic maize   (801) TGCACCAACACTACACTAGAACTCTTTTGGTCAAACTACT

                                  3′ Flanking Sequence
         5307 maize   (806) TATCGACAACCCCTCTTTATAGTACGGCTAAAACAAAATA
nontransgenic maize   (841) TATCGACAACCCCTCTTTATAGTACGGCTAAAACAAAATA

                                  3′ Flanking Sequence
         5307 maize   (846) AAAGACCTAACTATATCACGAGTGTCCGCAACTCCTTGAC
nontransgenic maize   (881) AAAGACCTAACTATATCACGAGTGTCCGCAACTCCTTGAC

                                  3′ Flanking Sequence
         5307 maize   (886) ACTCGGAATACGAAGACCTTCACTTTTTGTTTCGTCGCTT
nontransgenic maize   (921) ACTCGGAATACGAAGACCTTCACTTTTTGTTTCGTCGCTT

                                  3′ Flanking Sequence
         5307 maize   (926) TAGCCGTTGCTTCAAGTTTTTATCTCCGGGATTGTTTTCA
nontransgenic maize   (961) TAGCCGTTGCTTCAAGTTTTTATCTCCGGGATTGTTTTCA
```

Alignment of the genomic insertion site sequence from nontransgenic maize to the genomic sequences flanking the 5307 maize insert *(Continued)*

```
                                  3′ Flanking Sequence
         5307 maize   (966) CCATTGTAGTACATCTACCTGTAATGCGACCTAACTTACC
nontransgenic maize  (1001) CCATTGTAGTACATCTACCTGTAATGCGACCTAACTTACC

                                  3′ Flanking Sequence
         5307 maize  (1006) ATTTGCCTCTGCAAAACACATGTTAGTCACATATAAAATT
nontransgenic maize  (1041) ATTTGCCTCTGCAAAACACATGTTAGTCACATATAAAATT

                                  3′ Flanking Sequence
         5307 maize  (1046) ACGTTGTCATTAATCACTAAAACCAACCAGGGGCCTAGAT
nontransgenic maize  (1081) ACGTTGTCATTAATCACTAAAACCAACCAGGGGCCTAGAT

                                  3′ Flanking Sequence
         5307 maize  (1086) GCTTTCTAGTTTAAATCCCCAACAAGTCAAAATTCTTTCT
nontransgenic maize  (1121) GCTTTCTAGTTTAAATCCCCAACAAGTCAAAATTCTTTCT

                                  3′ Flanking Sequence
         5307 maize  (1126) ATTTTTTTTTTGCAAGTTCCAATTGACATCTGAAAGGTTGT
nontransgenic maize  (1161) ATTTTTTTTTTGCAAGTTCCAATTGACATCTGAAAGGTTGT


                           3′ Flanking Sequence
         5307 maize  (1166) AAGGTACACGTTTGGCTCTC
nontransgenic maize  (1201) AAGGTACACGTTTGGCTCTC
```