Event 5307 Maize:

Genetic Stability Analysis

AMENDED REPORT NO.1

Data Requirement:	Not applicable
Author:	Stephen New
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Performing Laboratory:	Syngenta Biotechnology, Inc. Product Safety 3054 East Cornwallis Road PO Box 12257 Research Triangle Park, NC 27709-2257, USA
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Report No.:	SSB-184-10 A1

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Company: *Syngenta Seeds, Inc.*

Company Representative:

Demetra Vlachos — November 2, 2010

Demetra Vlachos
Regulatory Affairs Manager

Date

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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 3, 2010

Stephen New
Technical Expert I
Product Safety
Syngenta Biotechnology, Inc.

Date

Submitted by:

November 2, 2010

Demetra Vlachos
Regulatory Affairs Manager
Syngenta Seeds, Inc.
3054 East Cornwallis Road
PO Box 12257
Research Triangle Park, NC 27709-2257, USA

Date

Sponsor:

November 3, 2010

Linda Meyer
Technical Leader III
Product Safety
Syngenta Biotechnology, Inc.

Date

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LIST OF ACRONYMS AND ABBREVIATIONS

aadA	streptomycin adenylyltransferase gene from *Escherichia coli* that confers resistance to streptomycin and spectinomycin
BC	backcross
bp	base pair
CMP	cestrum yellow leaf curling virus promoter
ColE1 ori	*Escherichia coli* origin of replication 1
Cry	crystal protein
cry1Ab	Cry1Ab gene
Cry1Ab	Cry1Ab protein
cry3A	Cry3A gene
Cry3A	Cry3A protein
CTAB	cetyltrimethyl ammonium bromide
dCTP	deoxycytidine triphosphate
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
HCl	hydrochloric acid
kb	kilobase
LB	left border
M	molar
manA	phosphomannose isomerase gene
mcry3A	modified Cry3A gene
mCry3A	modified Cry3A protein
mg	milligram
ml	milliliter
mM	millimolar
N	normal
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram
NOS	nopaline synthase
PCR	polymerase chain reaction
pg	picogram
pmi	phosphomannose isomerase gene
PMI	phosphomannose isomerase protein
RB	right border

LIST OF ACRONYMS AND ABBREVIATIONS *(Continued)*

repA	pVS1 replication gene from *Pseudomonas aeruginosa*
spec	streptomycin adenylyltransferase gene from *Escherichia coli*
RB	right border
SDS	sodium dodecyl sulfate
SSC	sodium chloride–sodium citrate
T_0	original transformant
T-DNA	transferred DNA
TAE	Tris-Acetate-EDTA
TE	Tris-EDTA
Tris	2-amino-2(hydroxymethyl)-1,3-propanediol
US EPA	United States Environmental Protection Agency
v/v	volume to volume
vir	virulence regulon in *Agrobacterium tumefaciens*
virG	part of the two-component regulatory system for the virulence regulon in *Agrobacterium tumefaciens*
VirGN54D	VirG protein with a N54D substitution
VS1 ori	plasmid pVS1 origin of replication and partitioning region
w/v	weight to volume
ZmUbiInt	*Zea mays* ubiquitin promoter with intron
× *g*	times gravity
×	cross
⊗	self-pollination
α-^{32}P	phosphorus-32 radioisotope
°C	degrees Celsius
®	registered trademark
™	trademark
µg	microgram
µl	microliter

REPORT AMENDMENTS

Amendment No. 1: November 3, 2010

This amended report has the following corrections:

On page 1, the department title has been changed to Product Safety.

On page 2, the Regulatory Affairs Manager name has been updated.

On page 3, the Regulatory Affairs Manager and Sponsor names have been updated, and position and department titles for the Study Director and the Sponsor have been updated.

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

On page 8, a new section has been added listing the Report Amendments.

On page 11, a reference citation was added.

On page 13, the Accession number for the gene *ecry3.1Ab* has been updated, and typographical errors in the description of *ecry3.1Ab* have been corrected.

On page 14, typographical errors in the description of *ecry3.1Ab* have been corrected.

On page 37, the Sponsor name has been updated, and position and department titles for the Study Director and the Sponsor have been updated.

On page 39, an additional reference was added.

On page 40, references were shifted as a result of the addition of a reference on the previous page.

The corrected pages in this amended report SSB-184-10 A1 are indicated as "*REVISED*".

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).

Molecular analyses were performed to characterize the 5307 maize insert and to demonstrate its genetic stability over four generations. Southern blot analyses were performed using standard molecular biology techniques. Each Southern blot contained a positive control and a negative control. The positive control, representing one copy of a fragment of known size in the maize genome, was included to demonstrate the sensitivity of each experiment; the negative control, deoxyribonucleic acid (DNA) extracted from plants grown from nontransgenic maize seed, was included in order to identify possible endogenous DNA sequences that hybridize with the probe. Two restriction enzyme digestion strategies were used in these Southern blot analyses. In the first strategy, maize genomic DNA was digested with an enzyme that cut once within the 5307 maize insert; the other recognition sites for this enzyme were located in the maize genome flanking the 5307 maize insert. This first strategy was used twice with two different enzymes to determine the copy number of the 5307 maize insert and the presence or absence of extraneous DNA fragments of plasmid pSYN12274 in other regions of the 5307 maize genome. In the second strategy, maize genomic DNA was digested with two enzymes that cut within the 5307 maize insert such that a DNA fragment of predictable size was generated. This strategy was used to determine the presence or absence of any closely linked extraneous DNA fragments of plasmid pSYN12274. Two probes were used: a full length transferred DNA (T-DNA)-specific probe containing every base of the plasmid pSYN12274 T-DNA and a plasmid pSYN12274 backbone-specific probe covering every base of plasmid pSYN12274 present outside of the T-DNA region.

With the first digestion strategy, Southern blots probed with a full length T-DNA-specific probe resulted in two hybridization bands specific to the 5307 maize insert. This was as expected as the restriction enzyme cut once within the insert yielding two fragments, each containing insert sequence detected by the probe. Detection of only two hybridization bands indicated that the T-DNA integrated into a single location in the genome and that there were no extraneous DNA fragments of the plasmid pSYN12274 T-DNA in other regions of the 5307 maize genome.

With the second digestion strategy, Southern blots probed with a full length T-DNA-specific probe resulted in one hybridization band of the expected size associated with the insert. Detection of only one hybridization band of the expected size confirmed that the T-DNA integrated into a single location in the genome and that there were no closely linked extraneous DNA fragments of the plasmid pSYN12274 T-DNA present in the 5307 maize genome. With each of these digestion strategies, Southern blots probed with the plasmid pSYN12274 backbone-specific probe resulted in no hybridization bands, indicating that there was no backbone sequence in the 5307 maize genome.

Southern blot analyses demonstrated that 5307 maize contains a single, complete copy of the insert and that there are no extraneous DNA fragments of plasmid pSYN12274 T-DNA inserted elsewhere in the 5307 maize genome. Identical hybridization patterns across all generations of 5307 maize analyzed in this study indicates that the insert is stably inherited from one generation to the next. Additionally, every generation of 5307 maize examined is free of backbone sequence from the transformation plasmid pSYN12274.

INTRODUCTION

Description of the Event 5307 Maize

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 (Walters *et al.* 2010). 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.

Description of the Transformation System and Method

Transformation of *Z. mays* to produce 5307 maize was accomplished using immature embryos of a proprietary maize line *via Agrobacterium tumefaciens*-mediated transformation, as described in Negrotto *et al*. 2000. Using this method, genetic elements within the left and right border regions of the transformation plasmid were efficiently transferred and integrated into the genome of the plant cell, while genetic elements outside these border regions were not transferred. Immature embryos were excised from 8 to 12 day old maize ears and rinsed with fresh medium in preparation for transformation. Embryos were mixed with a suspension of *A. tumefaciens* strain LBA4404 harboring plasmid pSB1 (Komari *et al.* 1996). The transformation plasmid pSYN12274 was vortexed for thirty seconds, and allowed to incubate for an additional five minutes. Excess *A. tumefaciens* suspension was aspirated and embryos were moved to plates containing a non-selective culture medium. Embryos were co-cultured with the remaining *A. tumefaciens* at 22°C for 2 to 3 days in the dark. Embryos were then transferred to culture medium supplemented with ticarcillin (200 mg/l) and silver nitrate (1.6 mg/l) and incubated in the dark for ten days. The gene *pmi* was used as a selectable marker during the transformation process (Negrotto *et al*. 2000). The embryos producing embryogenic callus were transferred to a cell culture medium containing mannose. After initial incubation with *A. tumefaciens*, the transformed tissue was transferred to selective media containing 500 mg/l of the broad-spectrum antibiotic cefotaxime and grown for four months, ensuring that the *A. tumefaciens* was cleared from the transformed tissue. Cefotaxime has been shown to kill *A. tumefaciens* at this concentration (Xing *et al*. 2008). Regenerated plantlets were tested for the presence of *pmi* and *ecry3.1Ab* and for the absence of the spectinomycin resistance gene (*spec*) present on the vector backbone, by real-time polymerase chain reaction (PCR) analysis (Ingham *et al*. 2001). This screen allows for the selection of transgenic events that carry the transferred deoxyribonucleic acid (T-DNA) and are free of vector backbone DNA. Plants positive for *pmi* and *ecry3.1Ab* and negative for *spec* were transferred to the greenhouse for further propagation.

Genetic Stability Analysis

Southern blot analyses were performed to characterize the 5307 maize insert. Included in this report are data and information describing the genetic elements of plasmid

pSYN12274 (the transformation plasmid used to generate 5307 maize) and the molecular characterization of multiple generations of 5307 maize.

The purpose of this study is to determine the copy number of the insert, the presence or absence of extraneous fragments of plasmid pSYN12274 T-DNA, the stability of the insert over four generations, and the presence or absence of plasmid backbone sequence in the 5307 maize genome.

MATERIALS AND METHODS

Genetic Elements for 5307 Maize in Plasmid pSYN12274

The genetic elements in plasmid pSYN12274, the 5307 maize transformation plasmid, are listed in Table 1 and mapped in Figure 1. Table 1 also contains a description of each constituent of plasmid pSYN12274, including the size in base pairs (bp) and the position within the plasmid. Figure 1 also shows the positions of the restriction sites used in the Southern blot analyses described in this report.

Table 1. Genetic elements in plasmid pSYN12274

Active ingredient cassette			
Genetic element	**Size (bp)**	**Position**	**Description**
Intervening sequence	203	26 to 228	Intervening sequence with restriction sites used for cloning
CMP promoter	346	229 to 574	Cestrum Yellow Leaf Curling Virus promoter region (Hohn *et al*. 2007). Provides constitutive expression in maize.
Intervening sequence	9	575 to 583	Intervening sequence with restriction sites used for cloning
ecry3.1Ab	1962	584 to 2545	An engineered Cry gene active against certain corn rootworm (*Diabrotica*) species (Entrez® Accession No. GU327680 [NCBI 2010]). As an engineered chimeric protein, eCry3.1Ab has similarities to other well characterized Cry proteins. Because Cry proteins share structural similarities, chimeric Cry genes can be engineered *via* the exchange of domains that are homologous between different Cry genes. The gene *ecry3.1Ab* consists of a fusion between the 5′ end (Domain I, Domain II and 15 AA of Domain III) of a modified Cry3A gene (*mcry3A*) and the 3′ end (Domain III and Variable Region 6 [Hofte and Whiteley 1989]) of a synthetic Cry1Ab gene (see descriptions of *mcry3A* and *cry1Ab* below). Upstream of the *mcry3A* domain, the gene *ecry3.1Ab* carries a 67 bp long oligomer extension at its 5′ end, which was introduced during the engineering of the variable regions and is translated into the following 22 amino acid residues: MTSNGRQCAGIRPYDGRQQHRG. The next 459 amino acid residues are identical to those of mCry3A, followed by 172 residues of Cry1Ab. Description of *mcry3A*: a maize-optimized *cry3A* was synthesized to accommodate the preferred codon usage for maize (Murray *et al*. 1989). The synthetic sequence was based on the native Cry3A protein sequence from *Bacillus thuringiensis* subsp. *tenebrionis* (Sekar *et al*. 1987). The maize-optimized gene was then modified to incorporate a consensus cathepsin-G protease recognition site within the expressed protein. The amino acid sequence of the encoded mCry3A corresponds to that of the native Cry3A, except that (1) its N-terminus

Table 1. Genetic elements in plasmid pSYN12274 (*Continued*)

Genetic element	Size (bp)	Position	Description
			corresponds to methionine-48 of the native protein and (2) a cathepsin G protease recognition site has been introduced, beginning at amino acid residue 155 of the native protein. This cathepsin-G recognition site has the sequence alanine-alanine-proline-phenylalanine, and has replaced the amino acids valine-155, serine-156, and serine-157 in the native protein (Chen and Stacy 2007). Description of *cry1Ab*: the gene *cry1Ab* was originally cloned from *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1 (Geiser *et al*. 1986). Its amino acid sequence has been codon-optimized (Koziel *et al*. 1997) to accommodate the preferred codon usage for maize (Murray *et al*. 1989)
Intervening sequence	30	2546 to 2575	Intervening sequence with restriction sites used for cloning
NOS terminator	253	2576 to 2828	Terminator sequence from the nopaline synthase gene of *Agrobacterium tumefaciens* (Entrez® Accession No. V00087 [NCBI 2010]). Provides a polyadenylation site (Depicker et al. 1982)
Selectable marker cassette			
Genetic element	**Size (bp)**	**Position**	**Description**
Intervening sequence	25	2829 to 2853	Intervening sequence with restriction sites used for cloning
ZmUbiInt promoter	1993	2854 to 4846	Promoter region from the maize polyubiquitin gene which contains the first intron (Entrez® Accession Number S94464 [NCBI 2010]). Provides constitutive expression in monocots (Christensen *et al*. 1992)
Intervening sequence	12	4847 to 4858	Intervening sequence with restriction sites used for cloning
pmi	1176	4859 to 6034	*Escherichia coli* gene *pmi* encoding the enzyme phosphomannose isomerase (PMI) (Entrez® Accession Number M15380 [NCBI 2010]); this gene is also known as *manA*. Catalyzes the isomerization of mannose-6-phosphate to fructose-6-phosphate (Negrotto *et al*. 2000)
Intervening sequence	60	6035 to 6094	Intervening sequence with restriction sites used for cloning
NOS terminator	253	6095 to 6347	Terminator sequence from the nopaline synthase gene of *Agrobacterium tumefaciens* (Entrez® Accession No. V00087 [NCBI 2010]). Provides a polyadenylation site (Depicker *et al*. 1982)

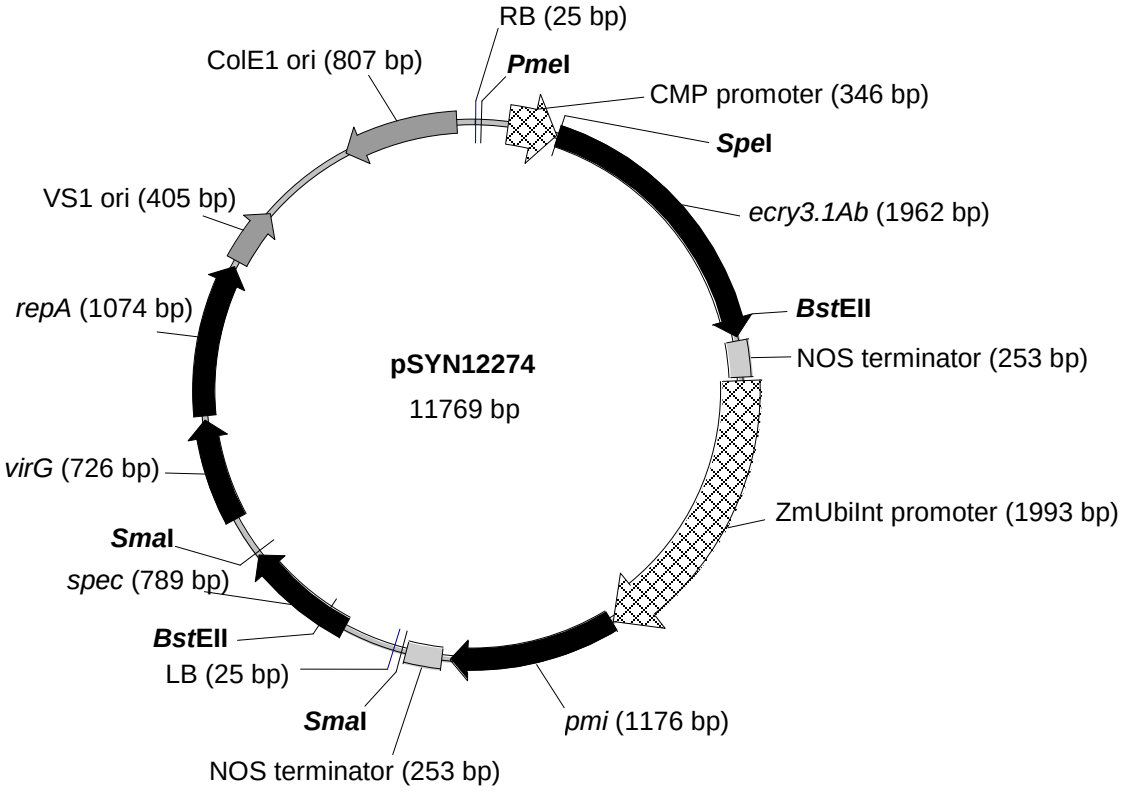
Table 1. Genetic elements in plasmid pSYN12274 (*Continued*)

Genetic element	**Size (bp)**	**Position**	**Description**
Intervening sequence	88	6348 to 6435	Intervening sequence with restriction sites used for cloning
Plasmid backbone			
Genetic element	**Size (bp)**	**Position**	**Description**
Left border (LB)	25	6436 to 6460	Left border region of T-DNA from *Agrobacterium tumefaciens* nopaline Ti-plasmid (Entrez® Accession Number J01825 [NCBI 2010]). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell (Zambryski *et al*. 1982)
Intervening sequence	349	6461 to 6809	Intervening sequence with restriction sites used for cloning
spec	789	6810 to 7598	Streptomycin adenylyltransferase, *aadA* gene from *Escherichia coli transposon* Tn7 (similar to Entrez® Accession Number X03043 [NCBI 2010]). Confers resistance to streptomycin and spectinomycin and is used as a bacterial selectable marker (Fling *et al*. 1985)
Intervening sequence	299	7599 to 7897	Intervening sequence with restriction sites used for cloning
virG	726	7898 to 8623	The VirGN54D gene (*virG*) from pAD1289 (similar to Entrez® Accession Number AF242881 [NCBI 2010]). The N54D substitution results in a constitutive *virG* phenotype. VirG is part of the two-component regulatory system for the virulence *(vir)* regulon in *Agrobacterium tumefaciens* (Hansen *et al*. 1994)
Intervening sequence	29	8624 to 8652	Intervening sequence with restriction sites used for cloning
repA	1074	8653 to 9726	Gene encoding the pVS1 replication protein from *Pseudomonas aeruginosa* (similar to Entrez® Accession Number AF133831 [NCBI 2010]), which is a part of the minimal pVS1 replicon that is functional in Gram-negative, plant-associated bacteria (Heeb *et al*. 2000)
Intervening sequence	42	9727 to 9768	Intervening sequence with restriction sites used for cloning
VS1 ori	405	9769 to 10173	Consensus sequence for the origin of replication and partitioning region from plasmid pVS1 of *Pseudomonas aeruginosa* (Entrez® Accession Number U10487 [NCBI 2010]). Serves as origin of replication in *Agrobacterium tumefaciens* host (Itoh *et al*. 1984)
Intervening sequence	677	10174 to 10850	Intervening sequence with restriction sites used for cloning

Table 1. Genetic elements in plasmid pSYN12274 (*Continued*)

Genetic element	Size (bp)	Position	Description
ColE1 ori	807	10851 to 11657	Origin of replication (similar to Entrez® Accession Number V00268 [NCBI 2010]) that permits replication of plasmids in *Escherichia coli* (Itoh and Tomizawa 1979)
Intervening sequence	112	11658 to 11769	Intervening sequence with restriction sites used for cloning
Right border (RB)	25	1 to 25	Right border region of T-DNA from *Agrobacterium tumefaciens* nopaline Ti-plasmid (Entrez® Accession Number J01826 [NCBI 2010]). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell (Wang *et al*. 1984)

Figure 1. Map of plasmid pSYN12274 indicating the restriction sites used in the Southern blot analyses (shown in bold type)



Test, Control, and Reference Substances

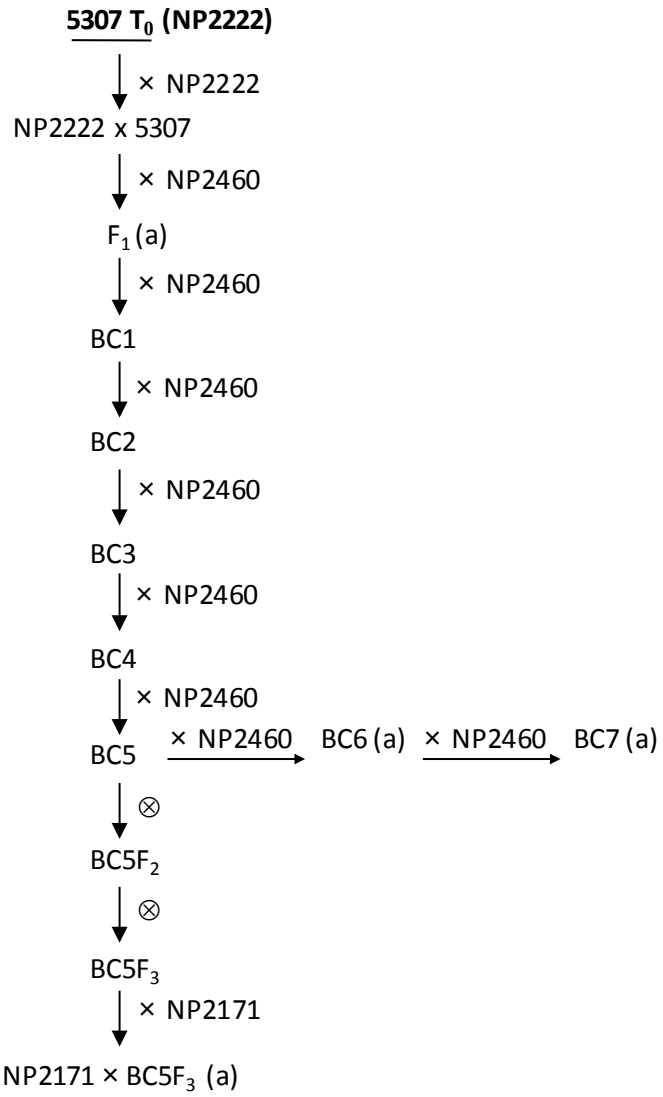
The test substances for this study were 5307 maize seed from generations F_1, BC6, BC7, and NP2171 × $BC5F_3$. The control substances were nontransgenic maize seed (NP2171/NP2460) near-isogenic to 5307 NP2171 × $BC5F_3$ and seed of nontransgenic maize lines NP2222, NP2460, and NP2171. Table 2 shows the descriptions and pedigree codes for the test and control substances. Figure 2 illustrates a pedigree chart demonstrating the production of the test substances. The reference substance for Southern blot analyses was the Analytical Marker DNA wide range molecular weight marker.

Table 2. Test and control substances

Seed identification	Pedigree
5307 F_1 (test)	NP2460//NP2222/(5307)1
5307 BC6 (test)	(NP2460*//NP2222//(5307)1)B>B>B>B<2>4>
5307 BC7 (test)	(NP2460*//NP2222/(5307)1)B>B>B>B<2>1>2>
5307 NP2171 × $BC5F_3$ (test)	NP2171 /(NP2460*//NP2222/(5307)1) B>B>B> B<2>B-B(T++)-
NP2171/NP2460 (control)	NP2171/NP2460:B(#1)-
NP2222 (control)	NP2222
NP2460 (control)	NP2460
NP2171 (control)	NP2171

The test and control substances were characterized by real-time PCR analysis (Ingham *et al.* 2001) to confirm the identity and purity.

Figure 2. Pedigree history for 5307 maize indicating the generations used in the study presented in this report



(a) = Southern blot analyses
T_0 = original transformant
× = cross
BC = backcross
⊗ = self-pollination

Plant Tissue for Genomic DNA Extraction

Seed of each test and control substance 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 5307 F_1 was pooled into a sampling bag and stored at -80°C ± 10°C. This process was repeated for 5307 BC6, 5307 BC7, 5307 NP2171 × $BC5F_3$, NP2171/NP2460, NP2222, NP2460, and NP2171 plants.

Genomic DNA Extraction

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

For each test and control substance, 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 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 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 using the TBS-380 Mini-Fluorometer.

Southern Blot Analyses

Southern blot analyses were performed using standard molecular biology techniques (Chomczynski 1992). Each lane contained 7.5 µg of genomic DNA that was digested with the appropriate restriction enzyme(s) for 8 to 16 hours.

A positive control, representing one copy of a fragment of known size in the maize genome, was included on each Southern blot. The positive control for these Southern blot analyses was digested DNA from plasmid pSYN12274. This positive control was loaded in a well together with 7.5 µg of digested DNA from NP2171/NP2460 plants, so

that the migration of this positive control DNA reflected, more accurately, the migration of the restriction fragment in the maize genome.

The amount of positive control (picograms for one copy) was calculated by the following formula with a maize genome size of 2.67×10^9 bp (Arumuganathan and Earle, 1991).

$$\left\{\left(\frac{Positive\,control\,size(bp)}{Genome\,size(bp) * Ploidy}\right) * \mu g\,loaded\right\} * 1 \times 10^6 = pg\ \ for\,1\ copy$$

The following factors were used to calculate the amounts of positive control:

maize genome size (bp)	2.67×10^9
maize ploidy	2
DNA loaded in each lane (µg)	7.5
Positive control size (bp)	11,769

The following amount of positive control was calculated:

Plasmid pSYN12274 (pg)	16.53

The molecular weight marker (serving as the reference substance), the digested genomic DNA, and the positive control were loaded onto 1% SeaKem® Gold agarose gels, and the DNA fragments were then separated by electrophoresis in 1X TAE buffer.

Following a 10 minute depurination in 0.25 N HCl, the DNA in the gel was denatured in 0.5 M NaOH and 1.5 M NaCl for 30 minutes. The DNA was then transferred to a Zeta-Probe GT membrane, by downward alkaline transfer, for 90 minutes using a Bio-Rad Appligene Vacuum Blotter. After rinsing the membrane briefly in 2X SSC, the DNA was cross-linked to the membrane using ultraviolet light.

All PCR-generated probes (the full length T-DNA-specific probe and the plasmid pSYN12274 backbone-specific probe) and the molecular weight marker-specific probe were labeled with phosphorus-32-deoxycytidine triphosphate ([α-^{32}P]-dCTP) by random priming using the Megaprime™ DNA labeling system. Unincorporated label ([α-^{32}P]-dCTP) was removed using the Micro Bio-Spin® Chromatography Columns.

Membranes were incubated in 30 ml of PerfectHyb™ Plus Hybridization Buffer (which contained 100 µg/ml denatured Calf Thymus DNA) for at least 30 minutes at 65°C ± 5°C. Both the molecular weight marker-specific probe and either the full length T-DNA-specific probe or backbone-specific probe were added to the hybridization solution, and the membranes were incubated for 16 hours at 65°C ± 5°C. Incubation was followed by a combination of washes at 65°C ± 5°C in 2X SSC with 0.1% SDS and washes at 65°C ± 5°C in 0.1X SSC with 0.1% SDS. Finally, the membranes were subjected to imaging using a Molecular Dynamics Storm 860® phosphorimager.

Genetic Stability of 5307 Maize Using a Full Length T-DNA-specific Probe

Genetic stability of the insert during conventional breeding of 5307 maize was determined by Southern blot analyses using a full length T-DNA-specific probe. The Southern blot analyses included genomic DNA extracted from plants grown from the test and control substances (5307 F_1, 5307 BC6, 5307 BC7, 5307 NP2171 × $BC5F_3$, NP2171/NP2460, NP2222, NP2460, and NP2171) (Figure 2 breeding pedigree). The full length T-DNA-specific probe, which contains sequence of the maize polyubiquitin promoter (ZmUbiInt), cross-hybridizes to genomic DNA fragments of different sizes in the different maize lines due to restriction fragment length polymorphism of the genomic DNA that carries the endogenous maize polyubiquitin promoter. Control substances NP2222, NP2460, and NP2171 were needed because the 5307 maize generations analyzed were created by crossing with these maize lines.

For these experiments, genomic DNA was analyzed using two restriction enzyme digestion strategies. In the first strategy, the maize genomic DNA was digested with an enzyme that cut once within the 5307 maize insert. The other recognition sites for this enzyme were located in the maize genome flanking the 5307 maize insert. This first strategy was used twice with two different enzymes (*Bst*EII and *Spe*I) to determine the copy number of the 5307 maize insert and the presence or absence of extraneous DNA fragments of the plasmid pSYN12274 T-DNA in other regions of the 5307 maize genome. These digests were expected to result in only two hybridization bands corresponding to the 5307 maize insert when a full length T-DNA-specific probe was used. More than two bands with either digest would have indicated that there were multiple copies of the insert in the plant genome.

In the second strategy, the maize genomic DNA was digested with *Sma*I + *Pme*I, which cut within the 5307 maize insert such that a DNA fragment of predictable size was released. This strategy was used to determine the presence of any closely linked extraneous DNA fragments of the plasmid pSYN12274 T-DNA.

Genetic Stability of 5307 Maize Using a Plasmid Backbone-specific Probe

The absence of plasmid backbone sequence in 5307 maize was assessed by Southern blot analyses using plasmid pSYN12274 backbone sequence as a probe on Southern blots of DNA subjected to the two restriction enzyme digestion strategies described above. This plasmid backbone-specific probe contained every base of the plasmid pSYN12274 backbone present outside of the T-DNA region. With both restriction enzyme digestion strategies, no hybridization bands were expected.

Statistical Analysis

No statistical analysis was used during this study.

RESULTS

Genetic Stability of 5307 Maize Using a Full Length T-DNA-specific Probe

Genetic stability of the insert during conventional breeding of 5307 maize was determined by Southern blot analyses using a full length T-DNA-specific probe. The Southern blot analyses included genomic DNA extracted from plants grown from the test and control substances (5307 F_1, 5307 BC6, 5307 BC7, 5307 NP2171 × $BC5F_3$, NP2171/NP2460, NP2222, NP2460, and NP2171).

Figure 3 shows a map of the T-DNA of the 5307 maize transformation plasmid pSYN12274, indicating the location of the full length T-DNA-specific probe and restriction sites for *Bst*EII, *Spe*I, *Sma*I, and *Pme*I. Figure 4 depicts the results of the corresponding Southern blot analyses, and Table 3 outlines the expected and observed sizes of the hybridization bands.

For Southern blot analysis with genomic DNA digested with *Bst*EII and probed with the full length T-DNA-specific probe, two hybridization bands of approximately 2.9 kb and 7.2 kb were observed in lanes containing DNA extracted from 5307 F_1, 5307 BC6, 5307 BC7, and 5307 NP2171 × $BC5F_3$ plants as expected (Figure 4A, Lanes 3, 4, 5, and 6) (Table 3). These hybridization bands were absent in lanes containing DNA extracted from the control substances plants (Figure 4A, Lanes 7, 8, 9, and 10) and were therefore, specific to the 5307 maize insert. Three hybridization bands of approximately 11.8 kb, 14 kb, and 20 kb were observed in the lane containing the positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Spe*I and loaded with DNA extracted from NP2171/NP2460 plants) (Figure 4A, Lane 19).

Additional bands resulting from cross-hybridization of the ZmUbiInt promoter sequence present on the full length T-DNA-specific probe with the endogenous maize polyubiquitin promoter sequence were also detected in all material analyzed. Two hybridization bands of approximately 8.4 kb and 12 kb, corresponding to the hybridization bands observed in lanes containing DNA extracted from NP2460 plants (Figure 4A, Lane 9) and NP2222 plants (Figure 4A, Lane 8), respectively, were observed in lanes containing DNA extracted from 5307 F_1, 5307 BC6, and 5307 BC7 plants (Figure 4A, Lanes 3, 4, and 5). Two hybridization bands of approximately 12 kb and 18 kb, corresponding to the hybridization bands observed in lanes containing DNA extracted from NP2222 plants (Figure 4A, Lane 8) and NP2171 plants (Figure 4A, Lane 10), respectively, were observed in the lane containing DNA extracted from 5307 NP2171 × $BC5F_3$ plants (Figure 4A, Lane 6). Finally, two hybridization bands of approximately 8.4 kb and 18 kb, corresponding to the hybridization bands observed in lanes containing DNA extracted from NP2460 plants (Figure 4A, Lane 9) and NP2171 plants (Figure 4A, Lane 10), respectively, were observed in lanes containing DNA extracted from NP2171/NP2460 plants (Figure 4A, Lane 7).

For Southern blot analysis with genomic DNA digested with *Spe*I and probed with the full length T-DNA-specific probe, two hybridization bands of approximately 2.6 kb and 7.0 kb were observed in lanes containing DNA extracted from 5307 F_1, 5307 BC6, 5307 BC7, and 5307 NP2171 × $BC5F_3$ plants as expected (Figure 4A, Lanes 11, 12, 13, and

14) (Table 3). These bands were absent in lanes containing DNA extracted from the control substances plants (Figure 4A, Lanes 15, 16, 17, and 18) and were therefore specific to the 5307 maize insert. Three hybridization bands of approximately 11.8 kb, 14 kb, and 20 kb were observed in the lane containing the positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Spe*I and loaded with DNA extracted from NP2171/NP2460 plants) (Figure 4A, Lane 19).

Additional bands resulting from cross-hybridization of the ZmUbiInt promoter sequence present on the full length T-DNA-specific probe with the endogenous maize polyubiquitin promoter sequence were also detected in all material analyzed. Two hybridization bands of approximately 20 kb and 25 kb, corresponding to the hybridization bands observed in lanes containing DNA extracted from NP2460 plants (Figure 4A, Lane 17) and NP2222 plants (Figure 4A, Lane 16), respectively, were observed in lanes containing DNA extracted from 5307 F_1, 5307 BC6, and 5307 BC7 plants (Figure 4A, Lanes 11, 12, and 13). Two hybridization bands of approximately 14 kb and 25 kb, corresponding to the hybridization bands observed in lanes containing DNA extracted from NP2171 plants (Figure 4A, Lane 18) and NP2222 plants (Figure 4A, Lane 16), respectively, were observed in the lane containing DNA extracted from 5307 NP2171 × $BC5F_3$ plants (Figure 4A, Lane 14). Finally, two hybridization bands of approximately 14 kb and 20 kb, corresponding to the hybridization bands observed in lanes containing DNA extracted from NP2171 plants (Figure 4A, Lane 18) and NP2460 plants (Figure 4A, Lane 17), respectively, were observed in lanes containing DNA extracted from NP2171/NP2460 plants (Figure 4A, Lane 15).

For Southern blot analysis with genomic DNA digested with *Sma*I + *Pme*I and probed with the full length T-DNA-specific probe, one hybridization band of approximately 6.3 kb was observed in lanes containing DNA extracted from 5307 F_1, 5307 BC6, 5307 BC7, and 5307 NP2171 × $BC5F_3$ plants as expected (Figure 4B, Lanes 3, 4, 5, and 6) (Table 3). This hybridization band was absent in lanes containing DNA extracted from the control substances plants (Figure 4B, Lanes 7, 8, 9, and 10) and was therefore, specific to the 5307 maize insert. One hybridization band of approximately 6.3 kb and a high molecular weight band (greater than 30 kb) was observed in the lane containing the positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Sma*I + *Pme*I and loaded with DNA extracted from NP2171/NP2460 plants) (Figure 4B, Lane 11).

At least one additional band resulting from cross-hybridization of the ZmUbiInt promoter sequence present on the full length T-DNA-specific probe with the endogenous maize polyubiquitin promoter sequence was also detected in all material analyzed. One hybridization band of approximately 18 kb, corresponding to the hybridization band observed in the lane containing DNA extracted from NP2222 plants (Figure 4B, Lane 8), was observed in lanes containing DNA extracted from 5307 F_1, 5307 BC6, 5307 BC7 and 5307 NP2171 × $BC5F_3$ plants (Figure 4B, Lanes 3, 4, 5, and 6). An additional high molecular weight band (greater than 30 kb) was observed in lanes containing DNA extracted from NP2460 plants (Figure 4B, Lane 9) and NP2171 plants (Figure 4B, Lane 10). This faint high molecular weight band (greater than 30 kb) was observed in all lanes containing DNA extracted from plants carrying either the NP2460 polyubiquitin promoter allele (5307 F_1, 5307 BC6, 5307 BC7, and NP2171/NP2460) (Figure 4B, Lanes

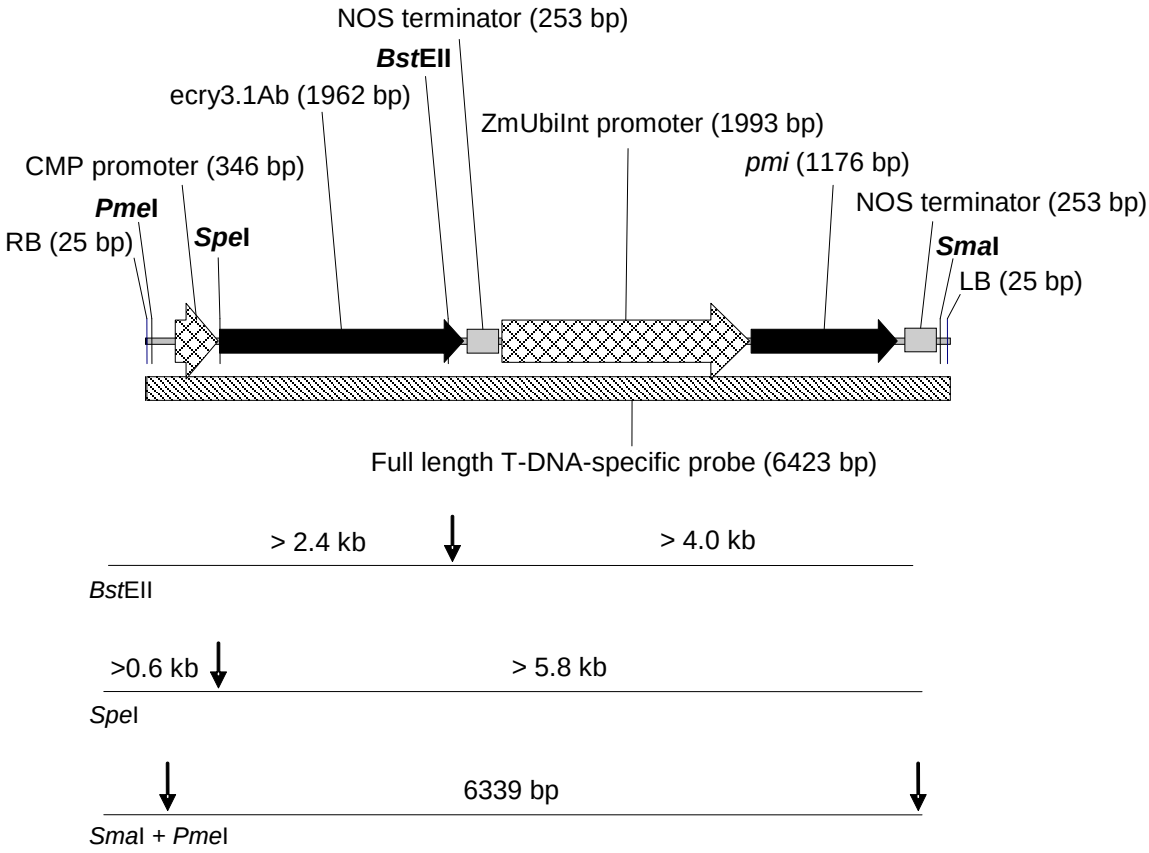
3, 4, 5, and 7) and/or the NP2171 polyubiquitin promoter allele (5307 NP2171 × $BC5F_3$ and NP2171/NP2460) (Figure 4B, Lanes 6 and 7).

Data from these Southern blot analyses demonstrated that the 5307 maize insert integrated into a single locus of the maize genome as only two hybridization bands specific to the 5307 maize insert were observed when genomic DNA was digested with *Bst*EII and probed with a full length T-DNA-specific probe and only two hybridization bands specific to the 5307 maize insert were observed when the genomic DNA was digested with *Spe*I and probed with a full length T-DNA-specific probe. These hybridization bands were specific to the 5307 maize insert and corresponded to each side of the restriction site of the enzyme used for Southern blot analysis. Additional hybridization bands observed resulted from cross-hybridization between the ZmUbiInt promoter sequence present on the full length T-DNA-specific probe with the maize endogenous polyubiquitin promoter sequence; these bands were consistent with the genetic make-up of the various generations analyzed. As expected, the 5307 F_1, 5307 BC6, and 5307 BC7 generations carry the maize polyubiquitin promoter allelic forms present in NP2222 and NP2460, the 5307 NP2171 × $BC5F_3$ generation carries the maize polyubiquitin promoter allelic forms present in NP2171 and NP2222, and the control substance NP2171 × NP2460 carries the maize polyubiquitin promoter allelic forms present in NP2171 and NP2460.

Data from these Southern blot analyses also demonstrated that a complete copy of the 5307 maize insert integrated into the maize genome as the hybridization band specific to the 5307 maize insert observed when the genomic DNA was digested with *Sma*I + *Pme*I was the predicted size. The approximately 18 kb band observed on this Southern blot was present in lanes containing DNA extracted from NP2222 plants and all generations carrying the NP2222 polyubiquitin promoter allelic form (5307 F_1, 5307 BC6, 5307 BC7, and 5307 NP2171 × $BC5F_3$). The faint and high molecular weight band (greater than 30 kb) observed on this Southern blot was present in lanes containing DNA extracted from NP2460 plants and NP2171 plants and all generations carrying either the NP2460 polyubiquitin promoter allelic form (5307 F_1, 5307 BC6, 5307 BC7, and NP2171/NP2460) and/or the NP2171 polyubiquitin promoter allelic form (5307 NP2171 × $BC5F_3$ and NP2171/NP2460).

Because no additional bands were observed (other than those associated with the 5307 maize insert and the maize endogenous sequence), Southern blot analyses indicated that there were no extraneous DNA fragments of plasmid pSYN12274 T-DNA in other regions of the 5307 maize genome. The data depicted in the Southern blot analyses showed that the hybridization bands specific to the insert were identical in lanes containing DNA extracted from plants grown from all generations (5307 F_1, 5307 BC6, 5307 BC7, and 5307 NP2171 × $BC5F_3$); these results indicated that the 5307 maize insert is stably inherited from one generation to the next.

Figure 3. Location of the *Bst*EII, *Spe*I, *Sma*I, and *Pme*I restriction sites and position of the 6423 bp full length T-DNA-specific probe in the T-DNA region of the transformation plasmid pSYN12274



The vertical arrows indicate the site of restriction digestion
Sizes of the expected restriction fragments are indicated

Table 3. Expected and observed hybridization band sizes in Southern blot analysis of 5307 maize, using a full length T-DNA-specific probe and restriction enzymes *Bst*EII, *Spe*I, and *Sma*I + *Pme*I

Lane	Source of DNA	Restriction enzyme	Expected number of bands	Expected band size (kb)	Observed band size (kb)
Figure 4A, 3	5307 F_1	*Bst*EII	2 5307 insert x endogenous	>2.4 >4 unknown	~2.9 ~7.2 ~8.4 (endogenous) ~12 (endogenous)
Figure 4A, 4	5307 BC6	*Bst*EII	2 5307 insert x endogenous	>2.4 >4 unknown	~2.9 ~7.2 ~8.4 (endogenous) ~12 (endogenous)
Figure 4A, 5	5307 BC7	*Bst*EII	2 5307 insert x endogenous	>2.4 >4 unknown	~2.9 ~7.2 ~8.4 (endogenous) ~12 (endogenous)
Figure 4A, 6	5307 NP2171 × $BC5F_3$	*Bst*EII	2 5307 insert x endogenous	>2.4 >4 unknown	~2.9 ~7.2 ~12 (endogenous) ~18 (endogenous)
Figure 4A, 7	NP2171/NP2460	*Bst*EII	x endogenous	unknown	~8.4 (endogenous) ~18 (endogenous)
Figure 4A, 8	NP2222	*Bst*EII	x endogenous	unknown	~12 (endogenous)
Figure 4A, 9	NP2460	*Bst*EII	x endogenous	unknown	~8.4 (endogenous)
Figure 4A, 10	NP2171	*Bst*EII	x endogenous	unknown	~18 (endogenous)
Figure 4A, 11	5307 F_1	*Spe*I	2 5307 insert x endogenous	>0.6 >5.8 unknown	~2.6 ~7 ~20 (endogenous) ~25 (endogenous)
Figure 4A, 12	5307 BC6	*Spe*I	2 5307 insert x endogenous	>0.6 >5.8 unknown	~2.6 ~7 ~20 (endogenous) ~25 (endogenous)
Figure 4A, 13	5307 BC7	*Spe*I	2 5307 insert x endogenous	>0.6 >5.8 unknown	~2.6 ~7 ~20 (endogenous) ~25 (endogenous)
Figure 4A, 14	5307 NP2171 × $BC5F_3$	*Spe*I	2 5307 insert x endogenous	>0.6 >5.8 unknown	~2.6 ~7 ~14 (endogenous) ~25 (endogenous)
Figure 4A, 15	NP2171/NP2460	*Spe*I	x endogenous	unknown	~14 (endogenous) ~20 (endogenous)
Figure 4A, 16	NP2222	*Spe*I	x endogenous	unknown	~25 (endogenous)
Figure 4A, 17	NP2460	*Spe*I	x endogenous	unknown	~20 (endogenous)
Figure 4A, 18	NP2171	*Spe*I	x endogenous	unknown	~14 (endogenous)
Figure 4A, 19	Positive control (NP2171/NP2460 and 16.53 pg of pSYN12274)	*Spe*I	1 pSYN12274 x endogenous	11.8 unknown	~11.8 ~14 (endogenous) ~20 (endogenous)

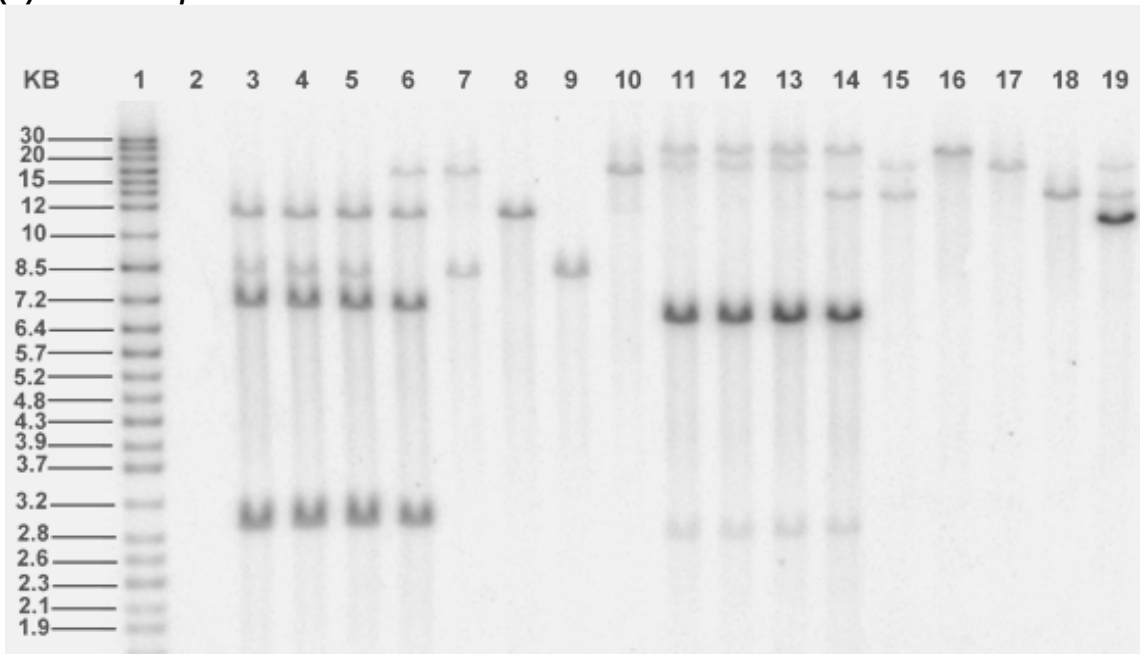
Table 3. Expected and observed hybridization band sizes in Southern blot analysis of 5307 maize, using a full length T-DNA-specific probe and restriction enzymes *Bst*EII, *Spe*I, and *Sma*I + *Pme*I (*Continued*)

Lane	Source of DNA	Restriction enzyme	Expected number of bands	Expected band size (kb)	Observed band size (kb)
Figure 4B, 3	5307 F_1	*Sma*I + *Pme*I	1 5307 insert x endogenous	6.3 unknown	~6.3 ~18 (endogenous) >30 (endogenous)
Figure 4B, 4	5307 BC6	*Sma*I + *Pme*I	1 5307 insert x endogenous	6.3 unknown	~6.3 ~18 (endogenous) >30 (endogenous)
Figure 4B, 5	5307 BC7	*Sma*I + *Pme*I	1 5307 insert x endogenous	6.3 unknown	~6.3 ~18 (endogenous) >30 (endogenous)
Figure 4B, 6	5307 NP2171 × $BC5F_3$	*Sma*I + *Pme*I	1 5307 insert x endogenous	6.3 unknown	~6.3 ~18 (endogenous) >30 (endogenous)
Figure 4B, 7	NP2171/NP2460	*Sma*I + *Pme*I	x endogenous	unknown	>30 (endogenous)
Figure 4B, 8	NP2222	*Sma*I + *Pme*I	x endogenous	unknown	~18 (endogenous)
Figure 4B, 9	NP2460	*Sma*I + *Pme*I	x endogenous	unknown	>30 (endogenous)
Figure 4B,10	NP2171	*Sma*I + *Pme*I	x endogenous	unknown	>30 (endogenous)
Figure 4B, 11	Positive control (NP2171/NP2460 and 16.53 pg of pSYN12274)	*Sma*I + *Pme*I	1 pSYN12274 x endogenous	6.3 unknown	~6.3 >30 (endogenous)

x = unknown

Figure 4. Genetic stability Southern blot analysis of 5307 maize with the 6423 bp full length T-DNA-specific probe, using restriction enzymes *Bst*EII, *Spe*I, and *Sma*I + *Pme*I

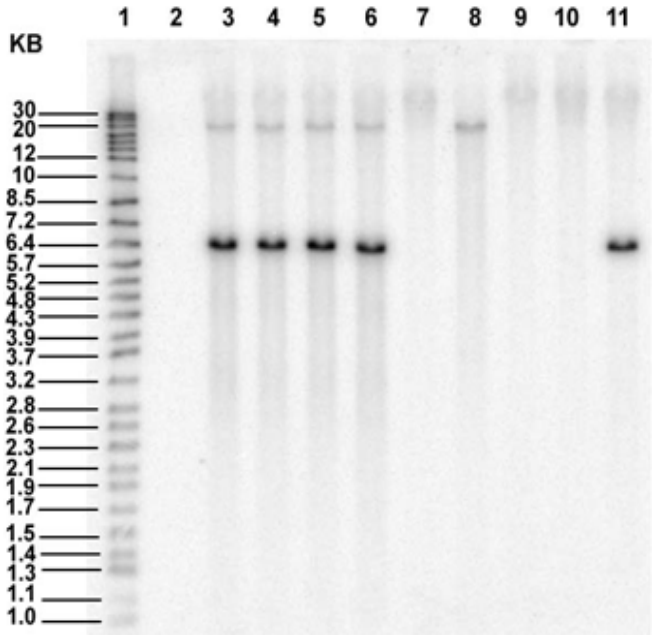
(A) *Bst*EII or *Spe*I



Lane A1 = molecular weight markers
Lane A2 = blank
Lane A3 = 5307 F_1 digested with *Bst*EII
Lane A4 = 5307 BC6 digested with *Bst*EII
Lane A5 = 5307 BC7 digested with *Bst*EII
Lane A6 = 5307 NP2171 × $BC5F_3$ digested with *Bst*EII
Lane A7 = NP2171/NP2460 digested with *Bst*EII
Lane A8 = NP2222 digested with *Bst*EII
Lane A9 = NP2460 digested with *Bst*EII
Lane A10 = NP2171 digested with *Bst*EII
Lane A11 = 5307 F_1 digested with *Spe*I
Lane A12 = 5307 BC6 digested with *Spe*I
Lane A13 = 5307 BC7 digested with *Spe*I
Lane A14 = 5307 NP2171 × $BC5F_3$ digested with *Spe*I
Lane A15 = NP2171/NP2460 digested with *Spe*I
Lane A16 = NP2222 digested with *Spe*I
Lane A17 = NP2460 digested with *Spe*I
Lane A18 = NP2171 digested with *Spe*I
Lane A19 = Positive control (NP2171/NP2460 and 16.53 pg of pSYN12274 digested with *Spe*I)

Figure 4. Genetic stability Southern blot analysis of 5307 maize with the 6423 bp full length T-DNA-specific probe, using restriction enzymes *Bst*EII, *Spe*I, and *Sma*I + *Pme*I *(Continued)*

(B) *Sma*I + *Pme*I



Lane B1 = molecular weight markers
Lane B2 = blank
Lane B3 = 5307 F_1 digested with *Sma*I + *Pme*I
Lane B4 = 5307 BC6 digested with *Sma*I + *Pme*I
Lane B5 = 5307 BC7 digested with *Sma*I + *Pme*I
Lane B6 = 5307 NP2171 × $BC5F_3$ digested with *Sma*I + *Pme*I
Lane B7 = NP2171/NP2460 digested with *Sma*I + *Pme*I
Lane B8 = NP2222 digested with *Sma*I + *Pme*I
Lane B9 = NP2460 digested with *Sma*I + *Pme*I
Lane B10 = NP2171 digested with *Sma*I + *Pme*I
Lane B11 = Positive control (NP2171/NP2460 and 16.53 pg of pSYN12274 digested with *Sma*I + *Pme*I)

Genetic Stability of 5307 Maize Using a Plasmid Backbone-specific Probe

Genetic stability of the insert during conventional breeding of 5307 maize was determined by Southern blot analysis using a plasmid pSYN12274 backbone-specific probe. The Southern blot analyses included genomic DNA from plants grown from the test and control substances (5307 F_1, 5307 BC6, 5307 BC7, 5307 NP2171 × $BC5F_3$, NP2171/NP2460, NP2222, NP2460, and NP2171).

Figure 5 shows a map of the plasmid pSYN12274 indicating the location of the plasmid pSYN12274 backbone-specific probe and restriction sites for *Bst*EII, *Spe*I, *Sma*I, and *Pme*I. Figure 6 depicts the results of the corresponding Southern blot analyses, and Table 4 outlines the expected and observed sizes of the hybridization bands.

For Southern blot analyses with genomic DNA digested with *Bst*EII and probed with the plasmid pSYN12274 backbone-specific probe, no hybridization bands were observed in

the lanes containing DNA extracted from plants grown from the test and control substances (5307 F_1, 5307 BC6, 5307 BC7, 5307 NP2171 × $BC5F_3$, NP2171/NP2460, NP2222, NP2460, and NP2171) (Figure 6A, Lanes 3, 4, 5, 6, 7, 8, 9, and 10) as expected. The positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Spe*I and loaded with DNA extracted from NP2171/NP2460 plants) produced the expected 11.8 kb band (Figure 6A, Lane 19).

For Southern blot analyses with genomic DNA digested with *Spe*I and probed with the plasmid pSYN12274 backbone-specific probe, no hybridization bands were observed in the test and control substances (5307 F_1, 5307 BC6, 5307 BC7, 5307 NP2171 × $BC5F_3$, NP2171/NP2460, NP2222, NP2460, and NP2171) (Figure 6A, Lanes 11, 12, 13, 14, 15, 16, 17, and 18) as expected. The positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Spe*I and loaded with DNA extracted from NP2171/NP2460 plants) produced the expected 11.8 kb band (Figure 6A, Lane 19).

For Southern blot analyses with genomic DNA digested with *Sma*I + *Pme*I and probed with the backbone-specific probe, no hybridization bands were observed in the test and control substances (5307 F_1, 5307 BC6, 5307 BC7, 5307 NP2171 × $BC5F_3$, NP2171/NP2460, NP2222, NP2460, and NP2171) (Figure 6B, Lanes 3, 4, 5, 6, 7, 8, 9, and 10). The positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Sma*I + *Pme*I and loaded with DNA extracted from NP2171/NP2460 plants) produced the expected 4.2 kb and 1.2 kb bands (Figure 6B, Lane 11).

The data from the three Southern blot analyses demonstrated that all the 5307 maize generations analyzed are free of plasmid pSYN12274 backbone sequence.

Figure 5. Location of the *Bst*EII, *Spe*I, *Sma*I, and *Pme*I restriction sites and position of the 5312 bp plasmid backbone-specific probe in the transformation plasmid pSYN12274

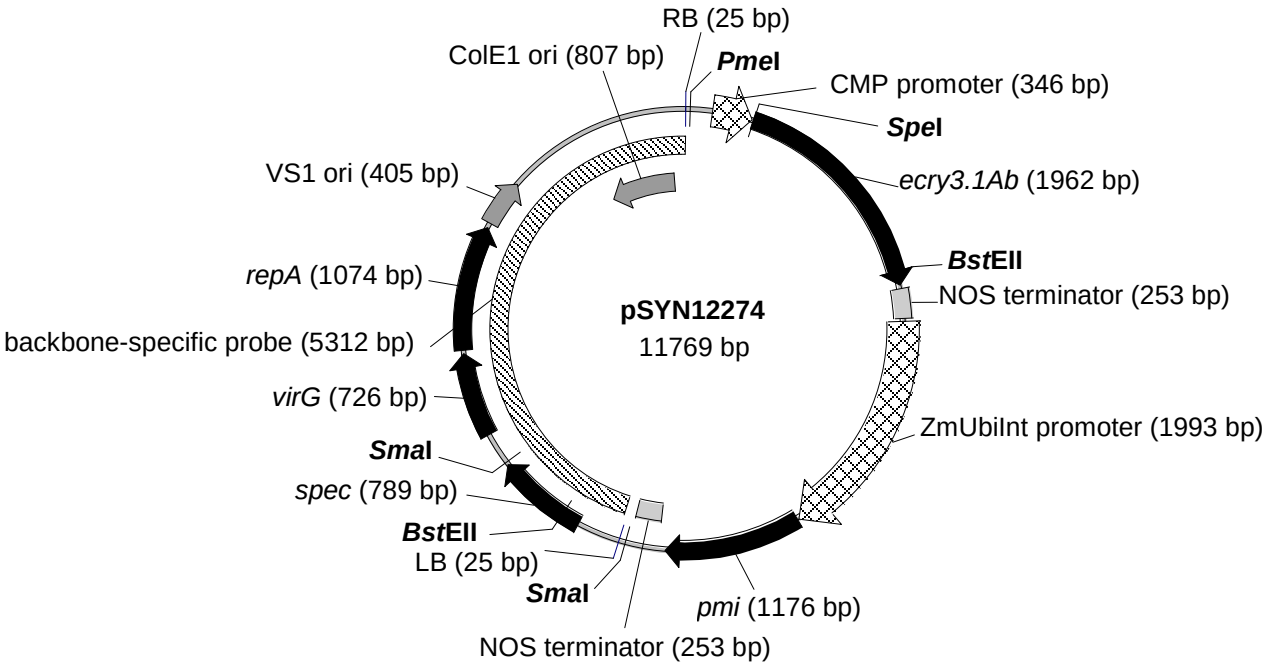


Table 4. Expected and observed hybridization band sizes in Southern blot analysis of 5307 maize, using a plasmid backbone-specific probe and restriction enzymes *Bst*EII, *Spe*I, and *Sma*I + *Pme*I

Lane	Source of DNA	Restriction enzyme	Expected number of bands	Expected band size (kb)	Observed band size (kb)
Figure 6A, 3	5307 F_1	*Bst*EII	none	none	none
Figure 6A, 4	5307 BC6	*Bst*EII	none	none	none
Figure 6A, 5	5307 BC7	*Bst*EII	none	none	none
Figure 6A, 6	5307 NP2171 × $BC5F_3$	*Bst*EII	none	none	none
Figure 6A, 7	NP2171/NP2460	*Bst*EII	none	none	none
Figure 6A, 8	NP2222	*Bst*EII	none	none	none
Figure 6A, 9	NP2460	*Bst*EII	none	none	none
Figure 6A, 10	NP2171	*Bst*EII	none	none	none
Figure 6A, 11	5307 F_1	*Spe*I	none	none	none
Figure 6A, 12	5307 BC6	*Spe*I	none	none	none
Figure 6A, 13	5307 BC7	*Spe*I	none	none	none
Figure 6A, 14	5307 NP2171 × $BC5F_3$	*Spe*I	none	none	none
Figure 6A, 15	NP2171/NP2460	*Spe*I	none	none	none
Figure 6A, 16	NP2222	*Spe*I	none	none	none
Figure 6A, 17	NP2460	*Spe*I	none	none	none
Figure 6A, 18	NP2171	*Spe*I	none	none	none
Figure 6A, 19	Positive control (NP2171/NP2460 and 16.53 pg of pSYN12274)	*Spe*I	1	11.8	~11.8
Figure 6B, 3	5307 F_1	*Sma*I + *Pme*I	none	none	none
Figure 6B, 4	5307 BC6	*Sma*I + *Pme*I	none	none	none
Figure 6B, 5	5307 BC7	*Sma*I + *Pme*I	none	none	none
Figure 6B, 6	5307 NP2171 × $BC5F_3$	*Sma*I + *Pme*I	none	none	none

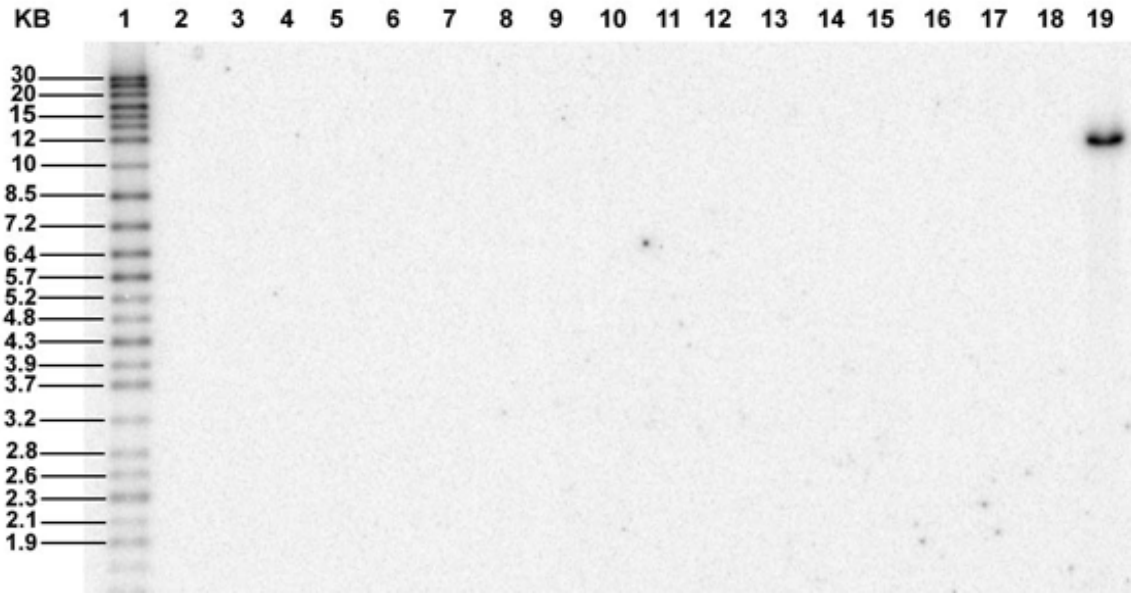
Table 4. Expected and observed hybridization band sizes in Southern blot analysis of 5307 maize, using a plasmid backbone-specific probe and restriction enzymes *Bst*EII, *Spe*I, and *Sma*I + *Pme*I *(Continued)*

Lane	Source of DNA	Restriction enzyme	Expected number of bands	Expected band size (kb)	Observed band size (kb)
Figure 6B, 7	NP2171/NP2460	*Sma*I + *Pme*I	none	none	none
Figure 6B, 8	NP2222	*Sma*I + *Pme*I	none	none	none
Figure 6B, 9	NP2460	*Sma*I + *Pme*I	none	none	none
Figure 6B,10	NP2171	*Sma*I + *Pme*I	none	none	none
Figure 6B, 11	Positive control (NP2171/NP2460 and 16.53 pg of pSYN12274)	*Sma*I + *Pme*I	2	4.2 1.2	~4.2 ~1.2

x = unknown

Figure 6. Genetic stability Southern blot analysis of 5307 maize with the 5312 bp plasmid backbone-specific probe, using restriction enzymes *Bst*EII, *Spe*I, and *Sma*I + *Pme*I

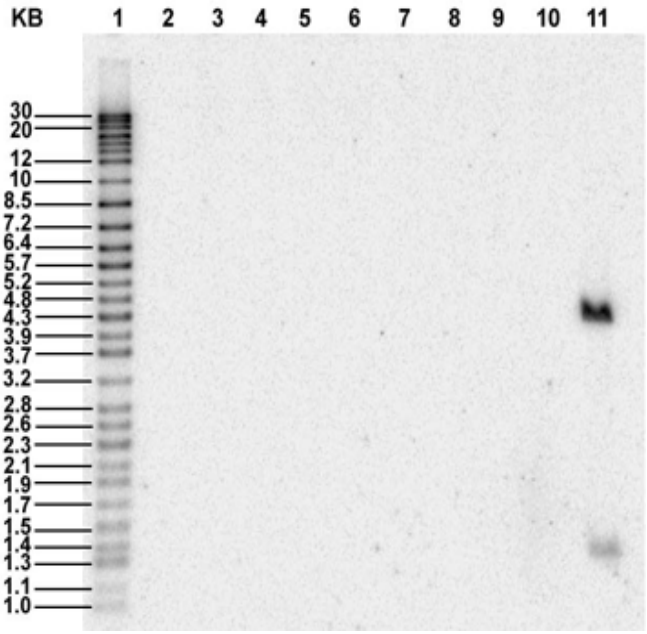
(A) *Bst*EII or *Spe*I



Lane A1 = molecular weight markers
Lane A2 = blank
Lane A3 = 5307 F_1 digested with *Bst*EII
Lane A4 = 5307 BC6 digested with *Bst*EII
Lane A5 = 5307 BC7 digested with *Bst*EII
Lane A6 = 5307 NP2171 × $BC5F_3$ digested with *Bst*EII
Lane A7 = NP2171/NP2460 digested with *Bst*EII
Lane A8 = NP2222 digested with *Bst*EII
Lane A9 = NP2460 digested with *Bst*EII
Lane A10 = NP2171 digested with *Bst*EII
Lane A11 = 5307 F_1 digested with *Spe*I
Lane A12 = 5307 BC6 digested with *Spe*I
Lane A13 = 5307 BC7 digested with *Spe*I
Lane A14 = 5307 NP2171 × $BC5F_3$ digested with *Spe*I
Lane A15 = NP2171/NP2460 digested with *Spe*I
Lane A16 = NP2222 digested with *Spe*I
Lane A17 = NP2460 digested with *Spe*I
Lane A18 = NP2171 digested with *Spe*I
Lane A19 = Positive control (NP2171/NP2460 and 16.53 pg of pSYN12274 digested with *Spe*I)

Figure 6. Genetic stability Southern blot analysis of 5307 maize with the 5312 bp plasmid backbone-specific probe, using restriction enzymes *Bst*EII, *Spe*I, and *Sma*I + *Pme*I *(Continued)*

(B) *Sma*I + *Pme*I



Lane B1 = molecular weight markers
Lane B2 = blank
Lane B3 = 5307 F_1 digested with *Sma*I + *Pme*I
Lane B4 = 5307 BC6 digested with *Sma*I + *Pme*I
Lane B5 = 5307 BC7 digested with *Sma*I + *Pme*I
Lane B6 = 5307 NP2171 × $BC5F_3$ digested with *Sma*I + *Pme*I
Lane B7 = NP2171/NP2460 digested with *Sma*I + *Pme*I
Lane B8 = NP2222 digested with *Sma*I + *Pme*I
Lane B9 = NP2460 digested with *Sma*I + *Pme*I
Lane B10 = NP2171 digested with *Sma*I + *Pme*I
Lane B11 = Positive control (NP2171/NP2460 and 16.53 pg of pSYN12274 digested with *Sma*I + *Pme*I)

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

Southern blot analyses of 5307 maize demonstrated that (1) the 5307 maize carries a single, complete copy of the insert with no extraneous DNA fragments of plasmid pSYN12274 T-DNA inserted elsewhere in the maize genome, (2) the transgenic locus is stable across all the 5307 maize generations analyzed, and (3) every generation of 5307 maize examined is free of backbone sequence from the transformation plasmid pSYN12274.

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., and Annick deFramond, PhD. This work was conducted at Syngenta Biotechnology, Inc.

Reported by: _____ November 3, 2010

Stephen New
Technical Expert I
Product Safety
Syngenta Biotechnology, Inc.

Date

Approved by: _____ November 3, 2010

Linda Meyer
Technical Leader III
Product Safety
Syngenta Biotechnology, Inc.

Date

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