Event 5307 Maize:

Mendelian Inheritance Analysis

AMENDED REPORT NO.1

Data Requirement:	Not applicable
Author:	Stephen New
Study Completion Date:	March 22, 2011
Performing Laboratory:	Syngenta Crop Protection, LLC Product Safety 3054 East Cornwallis Road PO Box 12257 Research Triangle Park, NC 27709-2257, USA
Syngenta Study No.:	Not applicable
Report No.:	SSB-203-10 A1

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Company: *Syngenta Seeds, Inc.*

Company Representative:

Demetra Vlachos — March 22, 2011

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:

March 22, 2011

Stephen New
Technical Expert I
Product Safety
Syngenta Crop Protection, LLC

Date

Submitted by:

March 22, 2011

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

Date

Sponsor:

March 22, 2011

Hope Hart
Technical Leader I
Product Safety
Syngenta Crop Protection, LLC

Date

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LIST OF ACRONYMS AND ABBREVIATIONS

3′	three prime
5′	five prime
adh1	*Zea mays* alcohol dehydrogenase 1 gene
BC	backcross
CMP	cestrum yellow leaf curling virus promoter
Cry1Ab	Cry1Ab protein
Cry3A	Cry3A protein
DNA	deoxyribonucleic acid
ecry3.1Ab	eCry3.1Ab gene
eCry3.1Ab	eCry3.1Ab protein
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
GLPS	Good Laboratory Practices Standards
LB	left border
manA	phosphomannose isomerase gene
mCry3A	modified Cry3A protein
NOS	nopaline synthase
PCR	polymerase chain reaction
pmi	phosphomannose isomerase gene
PMI	phosphomannose isomerase protein
RB	right border
T_0	original transformant
T-DNA	transferred DNA
US EPA	United States Environmental Protection Agency
ZmUbiInt	*Zea mays* ubiquitin promoter with intron
°C	degrees Celsius
×	cross
X^2	chi-square; X^2 = sum [|(observed - expected)| - 0.5]^2 / expected
⊗	self-pollination

REPORT AMENDMENTS

Amendment No. 1: March 22, 2011

This amended report has the following changes or corrections:

On page 1, the company name was updated to Syngenta Crop Protection, LLC.

On page 2, the Regulatory Affairs Manager name was updated.

On page 3, the Regulatory Affairs Manager was updated, and position and company name for the Study Director and the Sponsor were updated.

On page 4, the Table of Contents was updated.

On page 5, the page numbers in the List of Figures and List of Tables were updated.

On page 7, a new section was added listing the Report Amendments.

On pages 8 and 12, the word "hemizygous" was corrected to "homozygous" in the statement describing how the NP2171 × $BC5F_3$ generation was generated.

On page 14, the position and company name for the Study Director and the Sponsor were updated.

Each amended page is indicated in the page header 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 analysis was performed to determine whether the 5307 maize insert integrated into a chromosome within the maize nuclear genome.

Individual plants from four 5307 maize generations were tested for the presence of *ecry3.1Ab* and *pmi* by real-time polymerase chain reaction (PCR) analysis. The results from real-time PCR analysis were used to determine the segregation ratios of *ecry3.1Ab* and *pmi*. Chi-square analysis of the segregation data for three of the generations was performed to test the hypothesis that the 5307 maize insert is inherited in a predictable manner, according to Mendelian principles and consistent with insertion into a chromosome within the maize nuclear genome.

Plants for one generation were grown from seed that was produced by crossing between plants from a nontransgenic maize line and plants that were homozygous for *ecry3.1Ab* and *pmi*. These plants were all expected to be positive for *ecry3.1Ab* and *pmi*. Because the expected ratio of positive to negative plants is 1:0, Chi-square analysis could not be applied to data for this generation. Still, the observed ratio and the expected ratio for this generation were identical. This suggest that the 5307 insert is inherited according to Mendelian principles.

Segregation data from four 5307 maize generations confirmed the expected segregation ratio for *ecry3.1Ab* and *pmi*, indicating that the 5307 maize insert is inherited according to Mendelian principles. This supports the conclusion that the 5307 maize insert integrated into a chromosome within the maize nuclear genome.

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 segregation ratios of *ecry3.1Ab* and *pmi* in multiple generations of 5307 maize and to confirm that the 5307 maize insert is inherited in a predictable manner, according to Mendelian principles. Plants from four generations of 5307 maize were tested for the presence of *ecry3.1Ab* and *pmi* by real-time polymerase chain reaction (PCR) analysis. The results from real-time PCR analysis were used to determine the segregation ratios of *ecry3.1Ab* and *pmi*. Chi-square analysis of the segregation data from three generations was performed to test the hypothesis that the 5307 maize insert is inherited in a predictable manner, according to Mendelian principles and consistent with insertion into a chromosome within the maize nuclear genome. Chi-square analysis could not be applied to data from one generation because all of the plants were expected to be positive for *ecry3.1Ab* and *pmi*.

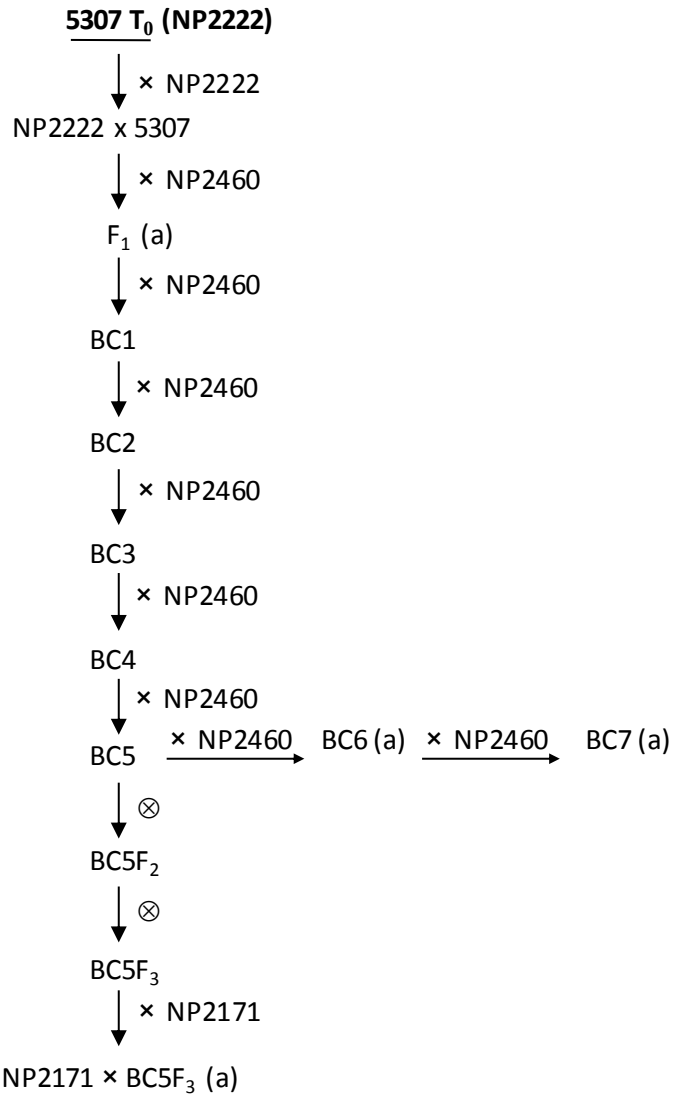
MATERIALS AND METHODS

Test Substances

Prior to this study, the initial 5307 maize plant (T_0 transformant) was crossed with maize inbred line NP2222 generating NP2222 × 5307. NP2222 × 5307 plants were crossed with maize inbred line NP2460, creating the F_1 generation. Event 5307 maize plants from the F_1 generation were then repeatedly backcrossed to maize inbred line NP2460 yielding the corresponding backcross (BC) lines BC1, BC2, BC3, BC4, BC5, BC6, and BC7. Positive segregants were utilized in each backcross. Event 5307 maize plants from BC5 were self-pollinated, resulting in the $BC5F_2$ generation. Event 5307 maize plants from this $BC5F_2$ generation were self-pollinated, resulting in the $BC5F_3$ generation. Event 5307 maize plants from this $BC5F_3$ generation were crossed with the maize inbred line NP2171, resulting in the NP2171 × $BC5F_3$ generation. Figure 1 illustrates a pedigree chart demonstrating the production of the test substances.

The test substances for this study were 5307 maize seed from generations F_1, BC6, BC7, and NP2171 × $BC5F_3$. Table 1 shows the descriptions and pedigree codes for the test substances.

Figure 1. Pedigree history for 5307 maize indicating the generations used in the study presented in this report



(a) = Mendelian Inheritance Analysis
T_0 = original transformant
× = cross
BC = backcross
⊗ = self-pollination

Table 1. Test 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++)-

Plant Material

Ninety-two plants from 5307 F_1 seed, 91 plants from 5307 BC6 seed, 90 plants from 5307 BC7 seed, and 100 plants from 5307 NP2171 × $BC5F_3$ seed were grown in a Syngenta Biotechnology, Inc. greenhouse in Research Triangle Park, North Carolina, USA. Leaf discs were sampled from each individual plant. Deoxyribonucleic acid (DNA) was isolated from leaf discs of each individual plant using a method adapted from the Wizard® Magnetic 96 DNA Plant System for real-time PCR analysis.

Real-Time PCR Analysis

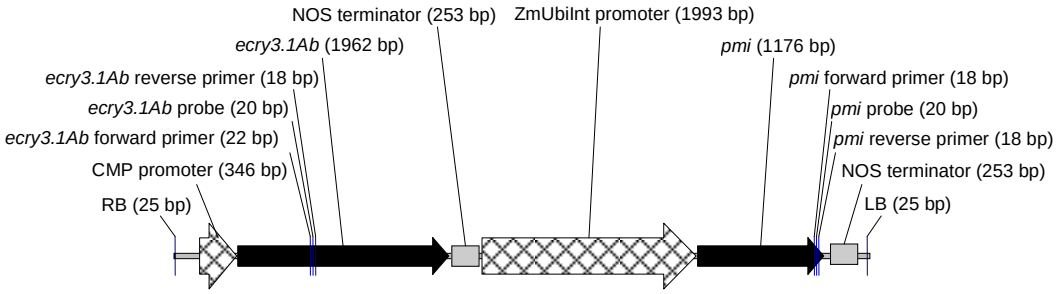
All plants grown from the test substances were individually analyzed for the presence of *ecry3.1Ab* and *pmi* by real-time PCR analysis (Ingham *et al.* 2001). A control assay targeting the endogenous maize alcohol dehydrogenase gene 1 (*adh1*) was used to confirm the presence of DNA in each reaction. Table 2 lists the primers and probes used to detect these genes. Figure 2 shows the locations of the *ecry3.1Ab*-specifc and *pmi*-specific primers and probes in the transferred DNA (T-DNA) of plasmid pSYN12274, the transformation plasmid used to generate 5307 maize.

The following cycling parameters were used for this reaction: 95°C for five minutes, followed by 40 cycles of 95°C for five seconds and 60°C for 30 seconds.

Table 2. Real-time PCR primers and probes used for the detection of *ecry3.1Ab, pmi,* and *adh1*

Amplicon of interest	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Probe sequence (5' to 3')
ecry3.1Ab	TACGAGAGCTGGGTG AACTTCA	CGATCAGGTCCAGCA CGG	CCGCTACCGCCGCG AGATGA
pmi	CCGGGTGAATCAGCG TTT	GCCGTGGCCTTTGAC AGT	TGCCGCCAACGAATC ACCGG
adh1	GAACGGTGTTGGGTTT GCAT	TGCAGCCTAACCATG CGCAGGGTA	TCCAGCAATCCTTGC ACCTT

Figure 2. Real-time PCR primers and probes locations in the plasmid pSYN12274 T-DNA



bp = base pair

Statistical Analysis Statement

Chi-square analysis values were calculated using Microsoft Excel.

For the 5307 F_1, 5307 BC6, and 5307 BC7 generations, the expected segregation ratio of positive to negative plants for the hemizygous trait was 1:1. For the 5307 NP2171 × $BC5F_3$ generation, all plants were expected to be positive for *ecry3.1Ab* and *pmi.*

Genotypic data generated for the 5307 F_1, 5307 BC6, and 5307 BC7 generations (Tables 3 and 4) were used to assess the goodness-of-fit of the observed genotypic ratios to the expected genotypic ratios using Chi-square (X^2) analysis with Yates' correction factor as in Armitage and Berry (1987).

$$X^2 = \text{sum } [|(\text{observed} - \text{expected})| - 0.5]^2 / \text{expected}$$

The 5307 NP2171 × $BC5F_3$ material was produced by crossing between plants from a nontransgenic maize line and plants that were homozygous for *ecry3.1Ab* and *pmi*. Therefore, all plants grown from 5307 NP2171 × $BC5F_3$ seed were expected to be positive for *ecry3.1Ab* and *pmi*. Because the expected ratio of positive to negative plants is 1:0, Chi-square analysis could not be applied to data for this generation.

RESULTS

Real-Time PCR Analysis

Results of real-time PCR analysis indicated that *ecry3.1Ab* and *pmi* were present in 44 plants grown from 5307 F_1 seed, 40 plants grown from 5307 BC6 seed, and 43 plants grown from 5307 BC7 seed (Tables 3 and 4). These results also indicated that *ecry3.1Ab* and *pmi* were absent from 48 plants grown from 5307 F1 seed, 51 plants grown from the 5307 BC6 seed, and 47 plants grown from 5307 BC7 seed. All 100 plants grown from the 5307 NP2171 × $BC5F_3$ seed were positive for *ecry3.1Ab* and *pmi* as expected. All plants tested positive for the control assay targeting the maize endogenous gene *adh1*, indicating that DNA was present in all real-time PCR reactions.

Chi-Square Analysis

Chi-square analysis of the segregation data obtained from real-time PCR analysis was performed to test the hypothesis that the insert is inherited according to Mendelian principles.

For plants grown from 5307 F_1 seed, 5307 BC6 seed, and 5307 BC7 seed, the expected and observed segregation ratios of *ecry3.1Ab* and *pmi* are presented in Tables 3 and 4. The critical value to reject the hypothesis at the 5% level is 3.84 (Strickberger 1976). The Chi-square value is less than 3.84 for each generation tested indicating that *ecry3.1Ab* and *pmi* are inherited in a predictable manner, according to Mendelian principles.

Because plants grown from the 5307 NP2171 × $BC5F_3$ seed are all positive for *ecry3.1Ab* and *pmi*, the ratio of positive to negative plants is 1:0. Therefore, Chi-square analysis could not be conducted on the data obtained for this generation. Still, the observed ratio and the expected ratio for this generation are identical. This suggest that the 5307 insert is inherited according to Mendelian principles.

Table 3. Observed versus expected genotype for *ecry3.1Ab* for 5307 F_1, 5307 BC6, and 5307 BC7 as determined by real-time PCR analysis

Trait	F_1		BC6		BC7	
	Observed	Expected	Observed	Expected	Observed	Expected
Positive	44	46	40	45.5	43	45
Negative	48	46	51	45.5	47	45
Total	92	92	91	91	90	90
X^2 value	0.098		1.099		0.100	

X^2 = sum [|(observed - expected)| - 0.5]^2 / expected
NA= not applicable

Table 4. Observed versus expected genotype for *pmi* for 5307 F_1, 5307 BC6, and 5307 BC7 as determined by real-time PCR analysis

Trait	F_1		BC6		BC7	
	Observed	Expected	Observed	Expected	Observed	Expected
Positive	44	46	40	45.5	43	45
Negative	48	46	51	45.5	47	45
Total	92	92	91	91	90	90
X^2 value	0.098		1.099		0.100	

X^2 = sum [|(observed - expected)| - 0.5]^2 / expected
NA = not applicable

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

Segregation ratios determined for four generations of 5307 maize confirmed that *ecry3.1Ab* and *pmi* are inherited in a predictable manner. This data is consistent with Mendelian principles and supports the conclusion that the 5307 maize insert integrated into a chromosome within the maize nuclear 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. and Annick de Framond, PhD. This work was conducted at Syngenta Crop Protection, LLC.

Reported by: _____ March 22, 2011

Stephen New
Technical Expert I
Product Safety
Syngenta Crop Protection, LLC

Date

Approved by: Hope Hart March 22, 2011

Hope Hart
Technical Leader I
Product Safety
Syngenta Crop Protection, LLC

Date

REFERENCES

Armitage P, Berry G. 1987. *Statistical Methods in Medical Research*, 2nd ed. Oxford: Blackwell Scientific Publications. pp. 129-130.

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.

Strickberger MW. 1976. In *Probability and statistical testing. Genetics*, 2nd ed. New York: Macmillan Publishing Company. pp. 140-163.

US EPA. 1989. Good Laboratory Practices Standards. 40 CFR Part 160.