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Event SYHT0H2 Soybean: Mendelian Inheritance Analysis

Final Report

Amended Report No. 1

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DATA REQUIREMENT(S): Not Applicable

AUTHOR: [redacted]

STUDY COMPLETION DATE: October 31, 2011

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Task Number: TK0055859

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VOLUME 1 OF 2 OF STUDY

STATEMENTS OF DATA CONFIDENTIALITY CLAIMS

The following statement applies to submissions to the United States Environmental Protection Agency (US EPA).

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A claim of confidentiality is being made for information contained in this report on the basis of its falling within the scope of Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Section 10 (d) (1) (A), (B), or (C). The information claimed confidential has been removed to a confidential volume and is cited by cross reference number in the body of this volume.

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Manager, Regulatory Affairs

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted in compliance with the relevant provisions of the Good Laboratory Practice Standards (GLPS) (40 CFR Part 160, US EPA 1989) pursuant to the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) with the following exceptions:

- Due to the limited supply, a sample of each test substance was not retained in accordance with EPA FIFRA GLPS (40 CFR Part 160).
- The reference substances used for real-time polymerase chain reaction analysis were not characterized in accordance with EPA FIFRA GLPS (40 CFR Part 160).
- The recording of some of the data was not in accordance with EPA FIFRA GLPS (40 CFR Part 160).

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QUALITY ASSURANCE STATEMENT

Study Title: Event SYHT0H2 Soybean: Mendelian Inheritance Analysis

Study Director: [redacted]

Study Number: TK0055859

Pursuant to Good Laboratory Practice Regulations (40 CFR Part 160), this statement verifies that the aforementioned study was inspected and/or audited and the findings reported to Management and to the Study Director by the Quality Assurance Unit on the dates listed below.

Inspection/Audit Type	Inspection/Audit Dates	Reporting Date
Audit Protocol	April 12, 2011	April 12, 2011
Inspect Analytical	April 20, 2011	April 20, 2011
Audit Study Data	August 5, 2011	August 5, 2011
Audit Final Report (1st Audit)	September 13, 2011	September 13, 2011
Audit Final Report (2nd Audit)	September 21, 2011	September 21, 2011

Prepared by: [redacted] Date: Sept. 22, 2011

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Study dates

Study initiation date:	April 13, 2011
Experimental start date:	April 18, 2011
Experimental termination date:	May 6, 2011

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.

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LIST OF ACRONYMS AND ABBREVIATIONS

3′	three prime
5′	five prime
adh1	alcohol dehydrogenase gene 1
avhppd-03	*p*-hydroxyphenylpyruvate dioxygenase gene derived from oat
AvHPPD-03	*p*-hydroxyphenylpyruvate dioxygenase enzyme encoded by *avhppd-03*
BC	backcross
bp	base pair
DNA	deoxyribonucleic acid
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
GmADH	soybean native alcohol dehydrogenase gene
GLPS	Good Laboratory Practice Standards
HPPD	*p*-hydroxyphenylpyruvate dioxygenase enzyme
pat	phosphinothricin acetyltransferase gene
PAT	phosphinothricin acetyltransferase enzyme
PCR	polymerase chain reaction
T_0	original transformant
T-DNA	transferred DNA
US EPA	United States Environmental Protection Agency
χ^2	chi-square
×	cross
®	registered trademark
⊗	self-pollination

AMENDED REPORT No. 1

31 October, 2011 This report is an amended version of the original Syngenta Crop Protection Report No. TK0055859. This amended report serves to correct information regarding the starting material for the test substances used in this study. The text in section 3.1 and the pedigree chart in Figure 1 have to been corrected to indicate "T_2" as starting material, not "T_3". The corrected pages in this report are indicated as "*REVISED*".

1.0 EXECUTIVE SUMMARY

Soybean (*Glycine max* [L.] Merrill) has been genetically modified to express the genes *avhppd-03* derived from oat *(Avena sativa* L.*)* and *pat* from *Streptomyces viridochromogenes*. The gene *avhppd-03* encodes a *p*-hydroxyphenylpyruvate dioxygenase (HPPD) enzyme, designated AvHPPD-03, that catalyzes the formation of homogentisic acid, the aromatic precursor in plastoquinone and vitamin E biosynthesis. In comparison with the native soybean HPPD, the AvHPPD-03 isozyme from oat has lower binding affinity for mesotrione, an herbicide that inhibits HPPD. Expression of *avhppd-03* in the transgenic Event SYHT0H2 soybean plants confers a mesotrione-tolerance phenotype. The gene *pat* encodes the enzyme phosphinothricin acetyltransferase (PAT), which inactivates the herbicide glufosinate, an inhibitor of glutamine synthetase, an enzyme in the nitrogen assimilation pathway. Expression of *pat* confers a glufosinate-tolerance phenotype, which was used as a selectable marker in the development of Event SYHT0H2 soybeans.

Individual plants from three SYHT0H2 soybean generations were tested for the presence of *avhppd-03* and *pat* by real-time polymerase chain (PCR) analysis. The results from real-time PCR analysis were used to determine the segregation ratios of *avhppd-03* and *pat*. Chi-square analysis of this segregation data was performed to test the hypothesis that the SYHT0H2 soybean insert is inherited according to Mendelian principles, and consistent with insertion into a chromosome within the soybean nuclear genome.

The statistical analysis of segregation data from three SYHT0H2 soybean generations (F_2, $BC2F_2$, and $BC3F_2$) confirmed that the observed segregation ratios for *avhppd-03* and *pat* were as expected for a gene that is inherited according to Mendelian principles. This indicates that the SYHT0H2 soybean insert segregated according to Mendelian principles and supports the conclusion that the SYHT0H2 soybean insert integrated into a chromosome within the soybean nuclear genome.

2.0 INTRODUCTION

2.1 Description of the Event SYHT0H2 Soybean

Soybean (*Glycine max* [L.] Merrill) has been genetically modified to express the genes *avhppd-03* derived from oat *(Avena sativa* L.*)* and *pat* from *Streptomyces viridochromogenes*. The gene *avhppd-03* encodes a *p*-hydroxyphenylpyruvate dioxygenase (HPPD) enzyme, designated AvHPPD-03, that catalyzes the formation of homogentisic acid, the aromatic precursor in plastoquinone and vitamin E biosynthesis. In comparison with the native soybean HPPD, the AvHPPD-03 isozyme from oat has lower binding affinity for mesotrione, an herbicide that inhibits HPPD. Expression of *avhppd-03* in the transgenic Event SYHT0H2 soybean plants confers a mesotrione-tolerance phenotype. The gene *pat* encodes the enzyme phosphinothricin acetyltransferase (PAT), which inactivates the herbicide glufosinate, an inhibitor of glutamine synthetase, an enzyme in the nitrogen assimilation pathway. Expression of *pat* confers a glufosinate-tolerance phenotype, which was used as a selectable marker in the development of Event SYHT0H2 soybeans.

2.2 Mendelian Inheritance Analysis

The purpose of this study is to determine the segregation patterns and confirm Mendelian inheritance ratios for *avhppd-03* and *pat* in three generations of SYHT0H2 soybean.

Event SYHT0H2 soybean plants were tested for *avhppd-03* and *pat* by real-time polymerase chain reaction (PCR) analysis. The results from real-time PCR analysis were used to determine the segregation ratios of *avhppd-03* and *pat*. Chi-square analysis of this segregation data was performed to test the hypothesis that the SYHT0H2 insert is inherited in a predictable manner according to Mendelian principles, and consistent with insertion into a chromosome within the soybean nuclear genome.

3.0 MATERIALS AND METHODS

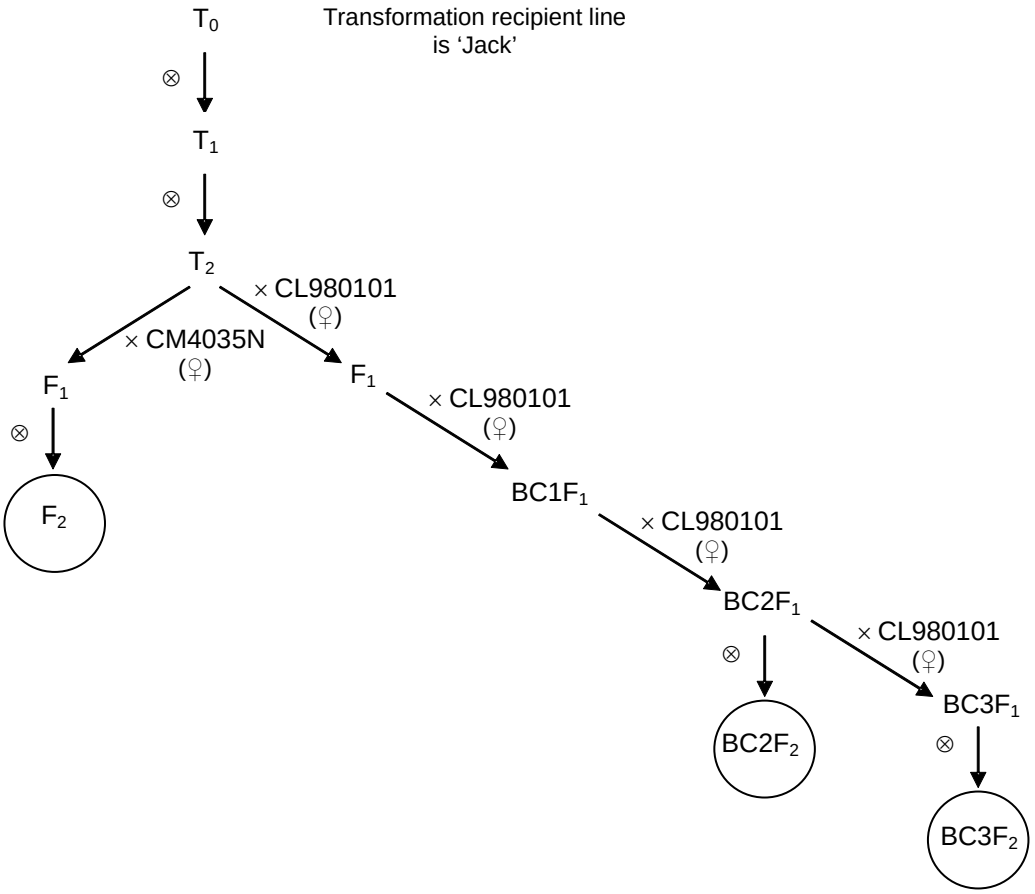
3.1 Test and Reference Substances

Prior to this study, homozygous SYHT0H2 plants of the T_2 generation were crossed with nontransgenic soybean line CM4035N creating an F_1 generation. This F_1 generation was self-pollinated creating an F_2 generation.

Homozygous SYHT0H2 plants of the T_2 generation were also crossed with nontransgenic soybean line CL980101 creating a different F_1 generation. This F_1 generation was backcrossed to the nontransgenic recurrent parent (CL980101) to yield the $BC1F_1$ generation. SYHT0H2 plants from the $BC1F_1$ generation were backcrossed to the nontransgenic recurrent parent (CL980101) to yield the $BC2F_1$ generation. The $BC2F_1$ generation was self-pollinated creating the $BC2F_2$ generation. SYHT0H2 plants from the $BC2F_1$ generation were also backcrossed to the nontransgenic recurrent parent (CL980101) to yield the $BC3F_1$ generation. The $BC3F_1$ generation was self-pollinated creating the $BC3F_2$ generation. Positive segregants, as determined by mesotrione application and real-time PCR anlaysis,

were utilized in each backcross. Figure 1 contains the pedigree for SYHT0H2 soybean, which displays the lineage of the test substances used in this analysis.

Figure 1 Pedigree chart for SYHT0H2 soybean indicating the lineage of the test substances used in this study



The generations used in this study are denoted with a circle.
T_0 = original transformant
⊗ = self-pollination
× = cross
BC = backcross
♀ = female parent

The test substances for this study were SYHT0H2 F_2, $BC2F_2$, and $BC3F_2$ soybean seed. Table 1 shows the descriptions for the test substances.

Table 1 Test substances

Seed identification	Entry	Material identification number
SYHT0H2 F_2	Test	10CG000115 10CG000116 10CG000119 10CG000122
SYHT0H2 $BC2F_2$	Test	10CG001681 10CG001772 10CG001917 10CG002001 10CG002098 10CG002329 10CG002380 10CG002403
SYHT0H2 $BC3F_2$	Test	10CG003285 10CG003286 10CG003287 10CG003288 10CG003289 10CG003290 10CG003292 10CG003298 10CG003302 10CG003304 10CG003305 10CG003306 10CG003307

The test substances were characterized by real-time PCR analysis (Ingham *et al.* 2001) to confirm the identity, purity, and stability (Appendix 1).

Deoxyribonucleic acid (DNA) containing at least one copy per genome of the amplicon of interest was used as a reference substance for real-time PCR analysis.

3.2 Plant Material

Event SYHT0H2 soybean seed from F_2, $BC2F_2$, and $BC3F_2$ generations were grown in greenhouses at Syngenta Biotechnology, Inc., Research Triangle Park, NC, USA in 2011. At least 100 plants were grown for each generation. For each individual plant, genomic DNA was isolated from leaf discs using a method adapted from the Wizard® Magnetic 96 DNA Plant System.

3.3 Real-time PCR Analysis

All plants grown from the test substances were individually analyzed for the presence of *avhppd-03* and *pat* by real-time PCR analysis (Ingham *et al.* 2001). A control assay targeting soybean native alcohol dehydrogenase 1 (*adh1*) gene, referred to as *GmADH* in this study, was used to confirm the presence of DNA in each reaction and to confirm that the real-time PCR reactions were functioning as expected. Table 2 lists the primers and probes used to detect *avhppd-03*, *pat,* and the endogenous gene *GmADH*. Figure 2 shows the locations of the *avhppd-03*-specific and *pat*-specific primers and probes in the transferred DNA (T-DNA) of the pSYN15954 plasmid, the transformation plasmid used to generate Event SYHT0H2 soybean.

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.

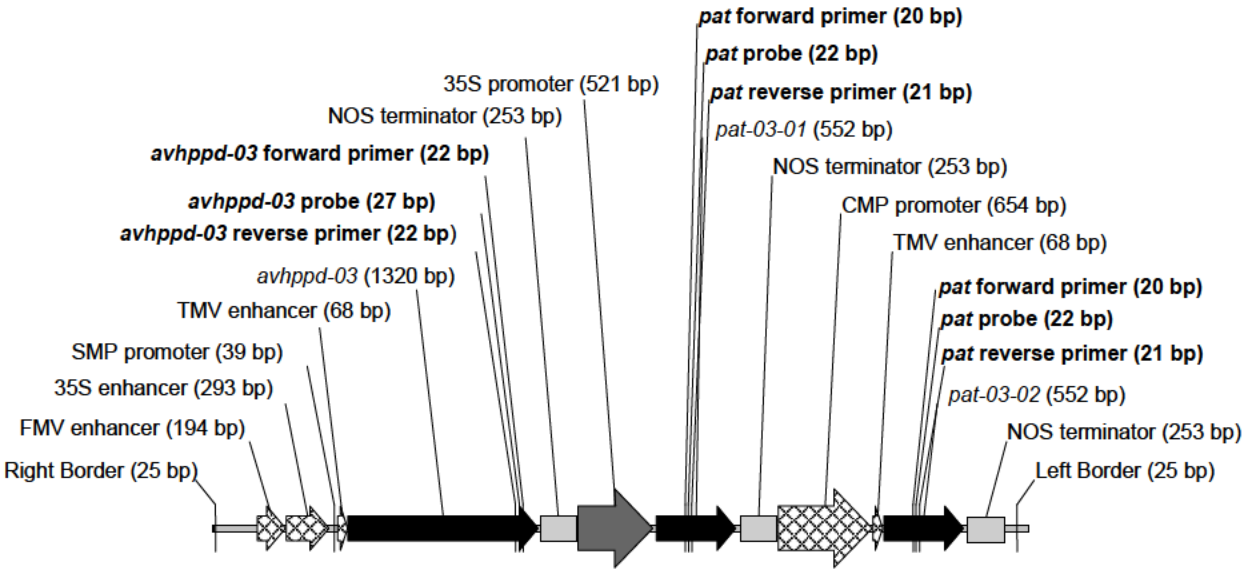
Table 2 Real-time PCR primers and probes used for the detection of *avhppd-03*, *pat*, and *GmADH*

Amplicon of interest	**Forward primer 5′ to 3′**	**Reverse primer 5′ to 3′**	**Probe 5′ to 3′**
avhppd-03	CTGGTACTCTTGCCC AACTTCA	TCCCACTTTTTTCCTC GAAATG	CCTTCTCCATGCATC CTATTCGCTGAA
pat	TGAGGGTGTTGTGGC TGGTA	TGTCCAATCGTAAGC GTTCCT	CTTCCAGGGCCCAGC GTAAGCA
GmADH	AGGTGTGGATCGGG CTGTT	CATCGTGGACGCATT CGA	ACTGGCAGCATCCAA GCCATGGTCT

3′ = three prime

5′ = five prime

Figure 2 Real-time PCR primer and probe locations in the pSYN15954 plasmid T-DNA



bp = base pair
FMV = figwort mosaic virus
NOS = terminator sequence from the nopaline synthase gene
SMP = synthetic minimal plant promoter
TMV = tobacco mosaic virus

3.4 Statistical Analysis

Chi-square analysis values were calculated using Microsoft® Office Excel® 2007.

For each generation tested, the expected segregation ratio of positive to negative plants was 3:1.

Genotypic data (Tables 3 and 4) were used to assess the goodness-of-fit of the observed segregation ratios to the expected segregation ratios using chi-square (χ^2) analysis (Strickberger 1976) with Yates correction factor as in Armitage and Berry (1987).

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

4.0 RESULTS

4.1 Real-time PCR Analysis

Real-time PCR analysis confirmed the presence of *avhppd-03* and *pat* in a portion of the test substance plants. Since the population is segregating, some plants were positive for *avhppd-03* and *pat*, while some plants were negative for *avhppd-03* and *pat*, as expected (Tables 3 and 4). In addition, all plants tested positive for the control assay targeting the endogenous gene *GmADH*, indicating that DNA was present in all reactions.

4.2 Chi-square Analysis

Chi-square analysis of the segregation data covering three generations of SYHT0H2 soybean was performed to test the hypothesis that the insert was inherited according to Mendelian principles.

The expected and observed segregation ratios of *avhppd-03* and *pat* are presented in Tables 3 and 4, respectively. 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 *avhppd-03* and *pat* are inherited in a predictable manner, according to Mendelian principles.

Table 3 Observed versus expected genotype for *avhppd-03* for three SYHT0H2 generations as determined by real-time PCR analysis

Trait	F_2		$BC2F_2$		$BC3F_2$	
	Observed	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**
Positive	115	123	99	104.25	134	131.25
Negative	49	41	40	34.75	41	43.75
Total	164	164	139	139	175	175
X^2 value	1.83		0.87		0.15	

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

Table 4 Observed versus expected genotype for *pat* for three SYHT0H2 generations as determined by real-time PCR analysis

Trait	F_2		$BC2F_2$		$BC3F_2$	
	Observed	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**
Positive	115	123	99	104.25	134	131.25
Negative	49	41	40	34.75	41	43.75
Total	164	164	139	139	175	175
X^2 value	1.83		0.87		0.15	

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

4.3 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.

5.0 CONCLUSIONS

Statistical analysis of segregation data from three generations of SYHT0H2 soybean confirmed that the observed segregation ratios for *avhppd-03* and *pat* were as expected for a gene inherited according to Mendelian principles. The data indicate that the insert is inherited according to Mendelian principles and, thus, integrated into a chromosome within the nuclear genome of SYHT0H2 soybean.

6.0 REFERENCES

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

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

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

APPENDICES SECTION

APPENDIX 1 Test Substance Characterization

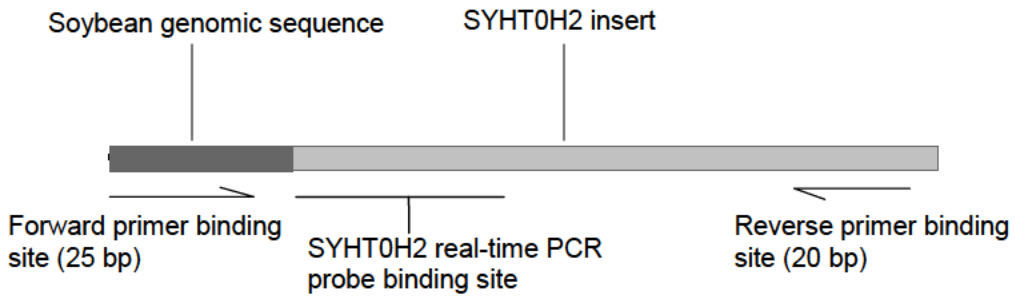
Test Substance Characterization

Real-time PCR data were also analyzed to characterize the test substances, SYHT0H2 soybean generations F_2, $BC2F_2$, and $BC3F_2$ seed. Event SYHT0H2 soybean seed was cultivated, and each plant was individually analyzed for the presence of the SYHT0H2 event by real-time PCR analysis as described in the Material and Methods section of this report. The primers and probes used to detect the SYHT0H2 event are indicated in Table A1. The forward primer binding site is located in the soybean genomic sequence, the reverse primer binding site is located in the SYHT0H2 insert, and the probe binding site is located in the SYHT0H2 insert (Figure A1).

Table A1 The SYHT0H2 real-time, event-specific PCR primers and probe sequences

{Volume 2: Confidential Business Information (CBI) Cross-reference Number 1}

Figure A1 Location of the Event SYHT0H2 real-time, event-specific PCR primer and probe binding sites



bp = base pair

The results were used to confirm the identity, purity, and stability which were defined as follows:

Identity: The expected number of seed in a lot contains the expected event

Purity: A percentage of seed in a lot contains the expected identity

Stability:

- SYHT0H2 F_2: Presence of the expected event, in the expected ratio, in a seed lot demonstrates stable transmission of the expected event in this generation
- SYHT0H2 $BC2F_2$: Presence of the expected event, in the expected ratio, in a seed lot demonstrates stable transmission of the expected event in this generation
- SYHT0H2 $BC3F_2$: Presence of the expected event, in the expected ratio, in a seed lot demonstrates stable transmission of the expected event in this generation

For each generation tested, the expected ratio of positive to negative plants was 3:1.

Following verification of the plants' identity by real-time PCR analysis, leaf tissue was collected from 10 positive plants per generation, to be used in subsequent studies. Leaf tissue was stored at -80°C ± 10°C.

Results

Results from real-time PCR analysis confirmed the presence of the SYHT0H2 event in a portion of the test substance plants. Since the population is segregating, some plants were positive for the SYHT0H2 event, while some plants were negative, as expected. For the F_2 generation, 115 plants (70%) were positive for the SYHT0H2 event, and 49 plants (30%) were negative, as expected. For the $BC2F_2$ generation, 99 plants (71%) were positive for the SYHT0H2 event, and 40 plants (29%) were negative, as expected. For the $BC3F_2$ generation, 134 plants (77%) were positive for the SYHT0H2 event, and 41 plants (23%) were negative, as expected.

Conclusions

Results of real-time PCR analysis confirmed the presence of the SYHT0H2 event in a portion of the following test substances: SYHT0H2 F_2, $BC2F_2$, and $BC3F_2$ soybean. The results confirmed the identity, purity, and stability of the test substances analyzed in this study.