syngenta

Event SYHT0H2 Soybean

Validation of a Gel-based, Event-specific Polymerase Chain Reaction Method

Assessment

DATA REQUIREMENTS: Not Applicable

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This study was not subject to the provisions of Good Laboratory Practice Standards (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 the relevant study records (including raw data) have been retained.

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GENERAL INFORMATION

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Retention of Records

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

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LIST OF ACRONYMS AND ABBREVIATIONS

avhppd-03	*p*-hydroxyphenylpyruvate dioxygenase gene derived from oat
AvHPPD-03	*p*-hydroxyphenylpyruvate dioxygenase enzyme encoded by *avhppd-03*
bp	base pair
DNA	deoxyribonucleic acid
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
HPPD	*p*-hydroxyphenylpyruvate dioxygenase enzyme
NA	not applicable
neg	negative
pat	phosphinothricin acetyltransferase gene
PAT	phosphinothricin acetyltransferase enzyme
PCR	polymerase chain reaction
pos	positive
rep	replicate
US EPA	United States Environmental Protection Agency

Definitions of International System of Units (SI) base units and derived units may be found in NIST (2011).

1.0 EXECUTIVE SUMMARY

Soybean (*Glycine max* [L.] Merrill) has been genetically modified to express the novel 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 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 SYHT0H2 soybean.

A gel-based, event-specific polymerase chain reaction method was developed to detect Event SYHT0H2 deoxyribonucleic acid (DNA). This method uses two oligonucleotide primers to amplify a 140 base pair DNA fragment that spans one of the specific junctions between the soybean genome and the Event SYHT0H2 insert.

This method was validated using SYHT0H2 soybean DNA and nontransgenic soybean DNA. The method demonstrated specificity, sensitivity, repeatability, and reproducibility at all concentrations of Event SYHT0H2 DNA tested and can be used to detect Event SYHT0H2 DNA in samples containing at least 0.01% Event SYHT0H2 DNA. The method is suitable for detecting Event SYHT0H2 DNA in DNA extracted from soybean samples.

2.0 INTRODUCTION

Soybean (*Glycine max* [L.] Merrill) has been genetically modified to express the novel 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 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 SYHT0H2 soybean.

A gel-based, event-specific polymerase chain reaction (PCR) method was developed to detect SYHT0H2 deoxyribonucleic acid (DNA) (Carlin 2012). This method uses two oligonucleotide primers to amplify a 140 base pair (bp) DNA fragment that spans one of the junctions between the soybean genome and the SYHT0H2 insert. This report describes the validation of the SYHT0H2 gel-based, event-specific PCR method.

3.0 MATERIALS AND METHODS

3.1 Sample Materials

Genomic DNA extracted from ground SYHT0H2 soybean leaf and/or seed was mixed with nontransgenic soybean DNA to various concentrations of SYHT0H2 DNA. Genomic DNA samples containing 1.0%, 0.5%, 0.1%, 0.05%, and 0.01% SYHT0H2 DNA were used in experiments to test the sensitivity, repeatability, and reproducibility of the SYHT0H2 soybean gel-based, event-specific method. The specificity of the SYHT0H2 soybean gel-based, event-specific PCR method was tested with DNA from SYHT0H2 soybean, transgenic soybean and maize events that do not contain the SYHT0H2 insert, and nontransgenic soybean, maize, and rice.

3.2 DNA Extraction and Quantification

Genomic DNA was extracted from ground leaf and/or seed using a cetyltrimethyl ammonium bromide-based method (JRC 2009) excluding steps 24 through 40 (i.e., "Wizard DNA Clean-up" and "MicroSpin Column" steps). DNA concentrations were determined using the Invitrogen Quant-iT™ PicoGreen® dsDNA Assay Kit following the manufacturer's instructions. The concentrations of all DNA samples were adjusted with 1X 2-amino-2-(hydroxymethyl)-1,3-propanediol ethylenediaminetetraacetic acid (TE) buffer to a final concentration of 10 ng/µl.

3.3 PCR Amplification

Detailed instructions for performing the SYHT0H2-specific method can be found in the Syngenta report by Carlin (2012). The conditions for the *adh1*-specific PCR method were identical to the SYHT0H2-specific method. For specific detection of Event SYHT0H2 genomic DNA, a 140 bp fragment that spans the 3′ insert-to-plant genome junction was amplified using two primers (Table 1). The forward primer (FE5313_F) binding site is located within the Event SYHT0H2 insert, and the reverse primer (FE08317_R) binding site is located in the soybean genomic sequence.

TABLE 1 Primers Used with the SYHT0H2 Gel-based, Event-specific PCR Method

Primer name	Length (bp)	Primer sequence 5′ to 3′
FE5313 F	25	CGCGCAAACTAGGATAAATTATCGC
FE08317 R	21	TGTGTGCCATTGGTTTAGGGT

A soybean-specific PCR method, which amplifies a 148 bp fragment of the soybean native alcohol dehydrogenase gene 1 (*adh1*) (Accession Number AF079058.1; NCBI 2012) was used to monitor DNA quality and PCR performance. Table 2 lists the *adh1*-specific primers used in this validation.

TABLE 2 Primers Used with the *adh1*-specific PCR Method

Primer name	Length (bp)	Primer sequence 5′ to 3′
Gm Adh1 primer F	26	ATTATTACTCATTGCATTGGTTGGTG
Gm Adh1 primer R	25	AGGTGTAGAGGATCTTCAAACGGAC

3.4 Specificity

To determine the specificity of the SYHT0H2 gel-based, event-specific PCR method, PCR analysis was performed with DNA extracted from SYHT0H2 soybean, transgenic soybean and maize plants that do not contain the SYHT0H2 insert, and nontransgenic soybean plants. A negative control containing no DNA was also included in the PCR analysis. Table 3 lists the DNA samples used in this experiment. PCR analysis using the *adh1*-specific PCR method was included to monitor soybean DNA quality and performance of PCR components common to both the SYHT0H2-specific and the *adh1*-specific methods (i.e., buffers, reagents, and equipment).

TABLE 3 DNA Samples for Specificity Test

DNA samples	Crop	Transgenic or nontransgenic
SYHT0H2 soybean	Soybean	Transgenic
SY-soy6 soybean	Soybean	Transgenic
SY-soy7 soybean	Soybean	Transgenic
SY-soy8 soybean	Soybean	Transgenic
SY-soy9 soybean	Soybean	Transgenic
3272 maize	Maize	Transgenic
Bt11 maize	Maize	Transgenic
Nontransgenic soybean	Soybean	Nontransgenic
Nontransgenic maize	Maize	Nontransgenic
Nontransgenic rice	Rice	Nontransgenic
Negative control containing no DNA	NA	NA

NA = not applicable.

3.5 Limit of Detection

To approximate the lowest detectable level of SYHT0H2 DNA that will result in greater than 95% positive results using the SYHT0H2 gel-based, event-specific PCR method, samples containing 1.0%, 0.5%, 0.1%, 0.05%, and 0.01% SYHT0H2 DNA diluted in nontransgenic soybean DNA were analyzed. Twenty-two replicate reactions were analyzed for each sample.

3.6 Repeatability and Reproducibility

To determine the repeatability and reproducibility of the SYHT0H2 gel-based, event-specific PCR method, experiments were conducted in two Syngenta laboratories at different times over different days. One operator in one laboratory conducted three experiments, and a second operator in a second laboratory conducted two experiments. Each experiment was done in replicates of two, and included DNA samples containing 1.0%, 0.5%, 0.1%, 0.05%, and 0.01% SYHT0H2 DNA diluted in nontransgenic soybean DNA. Repeatability is determined by comparing results obtained in one laboratory and by one operator at different times. Reproducibilty is determined by comparing results obtained from different laboratories by different operators.

3.7 Control of Bias

Any rejected data, and the documented reasons for the rejection of those data, will be retained in the study file.

3.8 Statistical Analysis

No statistical analysis was required for any parameter evaluated in this study.

4.0 RESULTS

4.1 Data Quality and Integrity

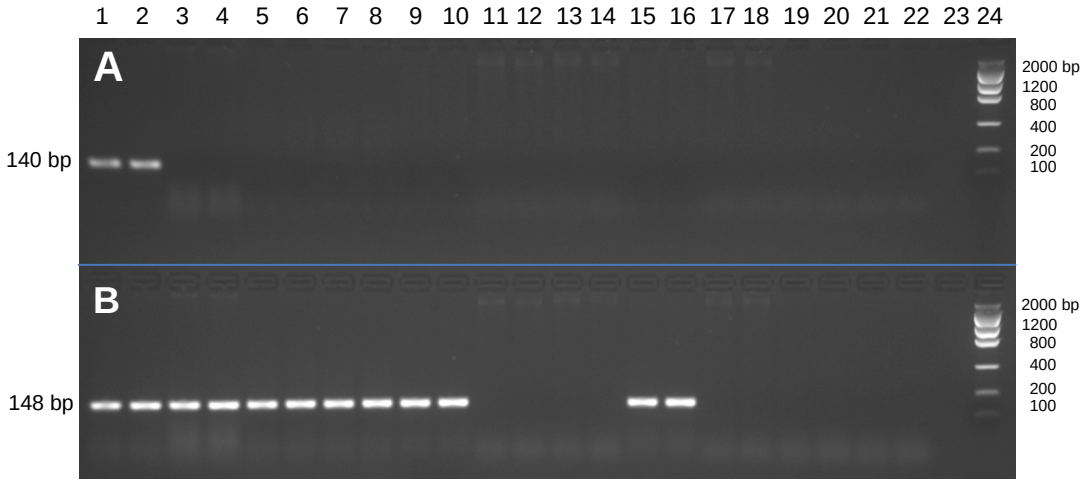
No circumstances occurred during conduct of this method validation that would have adversely affected the quality or integrity of the data generated.

4.2 Specificity

Panel A of Figure 1 shows the results of the PCR analysis using the SYHT0H2 gel-based, event-specific PCR method. The SYHT0H2-specific 140 bp amplicon was detected only in the samples containing SYHT0H2 DNA (Figure 1A, Lanes 1 and 2). The SYHT0H2-specific 140 bp amplicon was not detected in DNA samples extracted from plants that did not contain the SYHT0H2 insert (Figure 1A, Lanes 3 through 22).

Panel B of Figure 1 shows the results of the PCR analysis using the *adh1*-specific PCR method. PCR analysis using the *adh1*-specific PCR method was included to monitor soybean DNA quality and performance of PCR components common to both the SYHT0H2-specific and the *adh1*-specific methods (i.e., buffers, reagents, and equipment). The *adh1*-specific 148 bp amplicon was detected in all soybean samples as expected (Figure 1B, Lanes 1 through 10, 15, and 16). The *adh1*-specific 148 bp amplicon was not detected in the transgenic maize samples (Figure 1B, Lanes 11 through 14) or the nontransgenic maize and nontransgenic rice samples (Figure 1B, Lanes 17 through 20). Results of the specificity experiment are summarized in Table 4.

FIGURE 1 Agarose Gel Image of Specificity Test Results



Panel A: SYHT0H2 gel-based, event specific PCR method.
Panel B: Soybean *adh1*-specific PCR method.

Lanes 1 and 2: SYHT0H2 soybean DNA.
Lanes 3 and 4: SY-soy6 soybean DNA.
Lanes 5 and 6: SY-soy7 soybean DNA.
Lanes 7 and 8: SY-soy8 soybean DNA.
Lanes 9 and 10: SY-soy9 soybean DNA.
Lanes 11 and 12: 3272 maize DNA.
Lanes 13 and 14: Bt11 maize DNA.

Lanes 15 and 16: nontransgenic soybean DNA.
Lanes 17 and 18: nontransgenic maize DNA.
Lanes 19 and 20: nontransgenic rice DNA.
Lanes 21 and 22: negative control containing no DNA.
Lane 23: blank.
Lane 24: Invitrogen Low DNA Mass™ Ladder .

TABLE 4 Specificity Test Results

DNA samples	Crop	Transgenic or nontransgenic	Event SYHT0H2 event-specific PCR	*adh1*-specific PCR
SYHT0H2 soybean	Soybean	Transgenic	Positive	Positive
SY-soy6 soybean	Soybean	Transgenic	Negative	Positive
SY-soy7 soybean	Soybean	Transgenic	Negative	Positive
SY-soy8 soybean	Soybean	Transgenic	Negative	Positive
SY-soy9 soybean	Soybean	Transgenic	Negative	Positive
3272 maize	Maize	Transgenic	Negative	Negative
Bt11 maize	Maize	Transgenic	Negative	Negative
Nontransgenic soybean	Soybean	Nontransgenic	Negative	Positive
Nontransgenic maize	Maize	Nontransgenic	Negative	Negative
Nontransgenic rice	Rice	Nontransgenic	Negative	Negative
Negative control containing no DNA	NA	NA	Negative	Negative

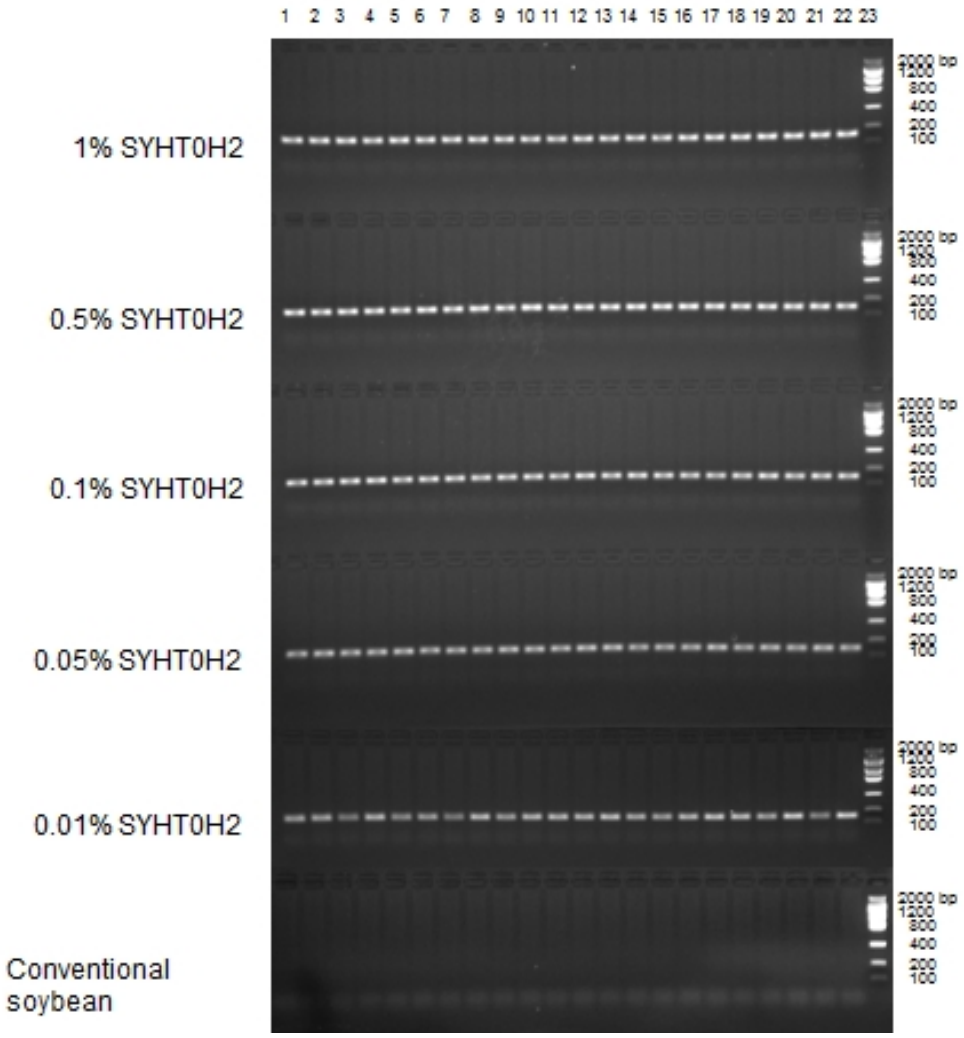
NA = not applicable.

Acceptance criterion: The detection of the SYHT0H2-specific 140 bp PCR product should only occur in samples containing SYHT0H2 DNA. Acceptance criterion was met.

4.3 Limit of Detection

The SYHT0H2 DNA was detected in all 22 replicates of PCR reactions with samples containing 1.0%, 0.5%, 0.1%, 0.05%, and 0.01% SYHT0H2 DNA (Figure 2). As expected, SYHT0H2 DNA was not detected in any of the 22 replicates of PCR reactions with nontransgenic soybean DNA (Figure 2). Table 5 indicates the number of replicates in which Event SYHT0H2 DNA was detected at each concentration of SYHT0H2 DNA. These data demonstrate that the SYHT0H2 gel-based, event-specific PCR method can detect SYHT0H2 DNA in samples containing at least 0.01% SYHT0H2 DNA.

FIGURE 2 Agarose Gel Image of Limit of Detection Experiment



1% SYHT0H2:
Lanes 1 though 22: Replicates of PCR reactions containing 1.0% SYHT0H2 DNA.
Lane 23: Invitrogen Low DNA Mass™ Ladder.

0.5% SYHT0H2:
Lanes 1 though 22: Replicates of PCR reactions containing 0.5% SYHT0H2 DNA.
Lane 23: Invitrogen Low DNA Mass™ Ladder.

0.1% SYHT0H2:
Lanes 1 though 22: Replicates of PCR reactions containing 0.1% SYHT0H2 DNA.
Lane 23: Invitrogen Low DNA Mass™ Ladder.

0.05% SYHT0H2:
Lanes 1 though 22: Replicates of PCR reactions containing 0.05% SYHT0H2 DNA.
Lane 23: Invitrogen Low DNA Mass™ Ladder.

0.01% SYHT0H2:
Lanes 1 though 22: Replicates of PCR reactions containing 0.01% SYHT0H2 DNA.
Lane 23: Invitrogen Low DNA Mass™ Ladder.

TABLE 5 Limit of Detection Experiment Results

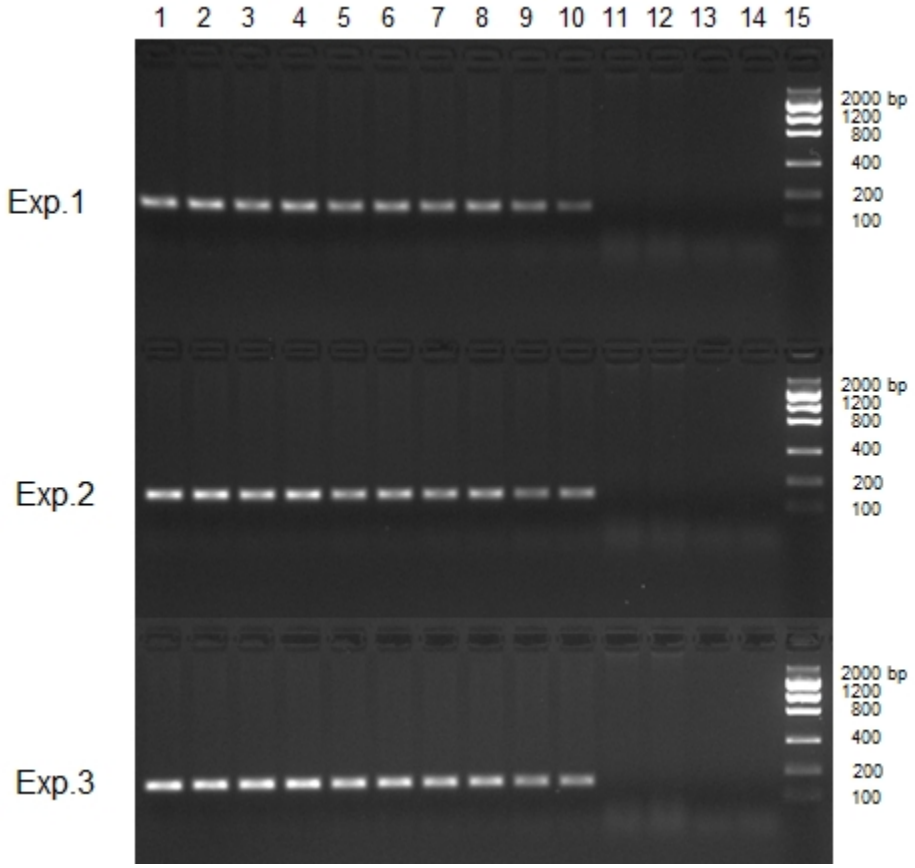
Sample	Number of replicates tested	Number of replicates in which Event SYHT0H2 DNA was detected
1.0% SYHT0H2 DNA	22	22
0.5% SYHT0H2 DNA	22	22
0.1% SYHT0H2 DNA	22	22
0.05% SYHT0H2 DNA	22	22
0.01% SYHT0H2 DNA	22	22
nontransgenic soybean DNA	22	0

Acceptance criterion: The limit of detection is at least 0.01% SYHT0H2 DNA under the conditions described in the method. Acceptance criterion was met.

4.4 Repeatability and Reproducibility

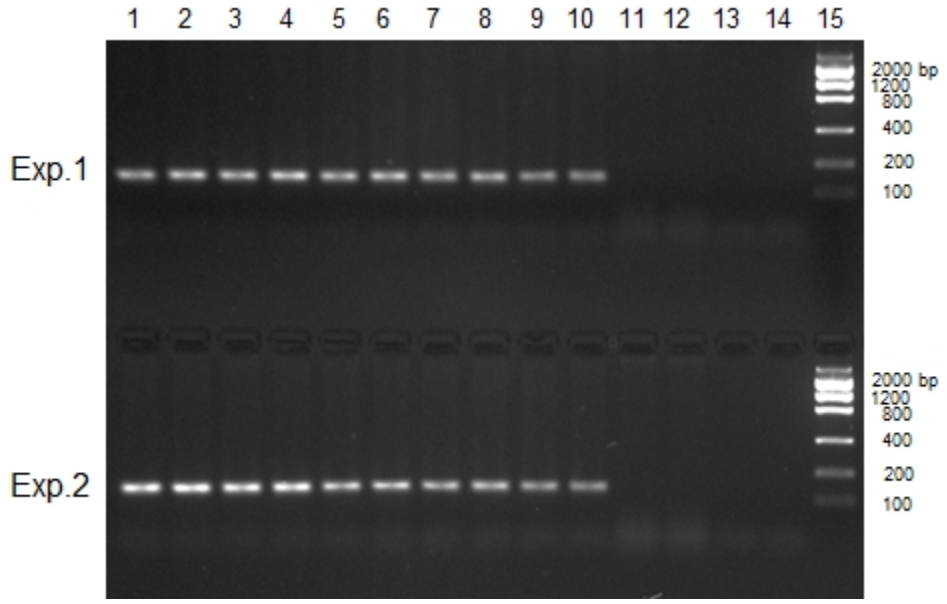
The experiments conducted in two Syngenta research laboratories at different times over different days indicated that the SYHT0H2 gel-based, event-specific method is repeatable and reproducible (Figure 3, Figure 4, and Table 6). In all experiments to test repeatability and reproducibility, the SYHT0H2 DNA was detected in all samples containing 1.0%, 0.5%, 0.1%, 0.05%, and 0.01% SYHT0H2 DNA (Figure 3, Lanes 1 through 10; Figure 4, Lanes 1 through 10). As expected, SYHT0H2 DNA was not detected in the nontransgenic soybean DNA samples (Figure 3, Lanes 11 and 12; Figure 4, Lanes 11 and 12) or the samples containing no DNA (Figure 3, Lanes 13 and 14; Figure 4, Lanes 13 and 14).

FIGURE 3 Agarose Gel Images of Repeatability and Reproducibility Experiments Conducted in Laboratory 1



Lanes 1 and 2: 1.0% SYHT0H2 DNA.
Lanes 3 and 4: 0.5% SYHT0H2 DNA.
Lanes 5 and 6: 0.1% SYHT0H2 DNA.
Lanes 7 and 8: 0.05% SYHT0H2 DNA.
Lanes 9 and 10: 0.01% SYHT0H2 DNA.
Lanes11 and 12: nontransgenic soybean DNA.
Lane 13 and 14: negative control containing no DNA.
Lane 15: Invitrogen Low DNA Mass™ Ladder.

FIGURE 4 Agarose Gel Images of Repeatability and Reproducibility Experiments Conducted in Laboratory 2



Lanes 1 and 2: 1.0% SYHT0H2 DNA.
Lanes 3 and 4: 0.5% SYHT0H2 DNA.
Lanes 5 and 6: 0.1% SYHT0H2 DNA.
Lanes 7 and 8: 0.05% SYHT0H2 DNA.
Lanes 9 and 10: 0.01% SYHT0H2 DNA.
Lanes11 and 12: nontransgenic soybean DNA.
Lane 13 and 14: negative control containing no DNA.
Lane 15: Invitrogen Low DNA Mass™ Ladder.

TABLE 6 Repeatability and Reproducibility Results

Sample	Laboratory 1						Laboratory 2			
	Experiment 1		Experiment 2		Experiment 3		Experiment 1		Experiment 2	
	Rep1	Rep 2	Rep1	Rep 2	Rep1	Rep 2	Rep1	Rep 2	Rep1	Rep 2
1.0% SYHT0H2 DNA	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
0.5% SYHT0H2 DNA	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
0.1% SYHT0H2 DNA	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
0.05% SYHT0H2 DNA	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
0.01% SYHT0H2 DNA	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
Nontransgenic soybean DNA	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Negative control containing no DNA	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

Neg = negative.
Pos = positive.
Rep = replicate.

Acceptance criterion for repeatability: The expected results should be obtained in the same laboratory with the same operator at different times. Acceptance criterion was met.

Acceptance criterion for reproducibility: The expected results should be obtained in different laboratories by different operators. Acceptance criterion was met.

5.0 CONCLUSIONS

A gel-based, event-specific PCR method was developed to detect SYHT0H2 DNA. This method uses two oligonucleotide primers to amplify a 140 bp DNA fragment that spans one of the specific junctions between the soybean genome and the SYHT0H2 insert.

Syngenta tested the sensitivity, repeatability, and reproducibility of this method using SYHT0H2 soybean DNA and nontransgenic soybean DNA mixed to various final concentrations of SYHT0H2 DNA: 1%, 0.5%, 0.1%, 0.05%, and 0.01%. The method demonstrated sensitivity, repeatability, and reproducibility at all concentrations of SYHT0H2 DNA tested, and the limit of detection of the method was determined to be at least 0.01% SYHT0H2 DNA. The repeatability and reproducibility of this method were confirmed by an inter-laboratory validation. The method was specific to SYHT0H2 soybean DNA. The method described by Carlin (2012) is suitable for detecting SYHT0H2 DNA in DNA extracted from soybean samples.

6.0 REFERENCES

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