syngenta

Event SYHT0H2 Soybean

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
EDTA	ethylenediaminetetraacetic acid
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
HPPD	*p*-hydroxyphenylpyruvate dioxygenase enzyme
pat	phosphinothricin acetyltransferase gene
PAT	phosphinothricin acetyltransferase enzyme
PCR	polymerase chain reaction
tris	2-amino-2(hydroxymethyl)-1,3-propanediol
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 SYHT0H2 soybean deoxyribonucleic acid (DNA). This method uses two oligonucleotide primers to amplify a 140 base pair DNA fragment that spans one of the junctions between the soybean genome and the SYHT0H2 insert.

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.

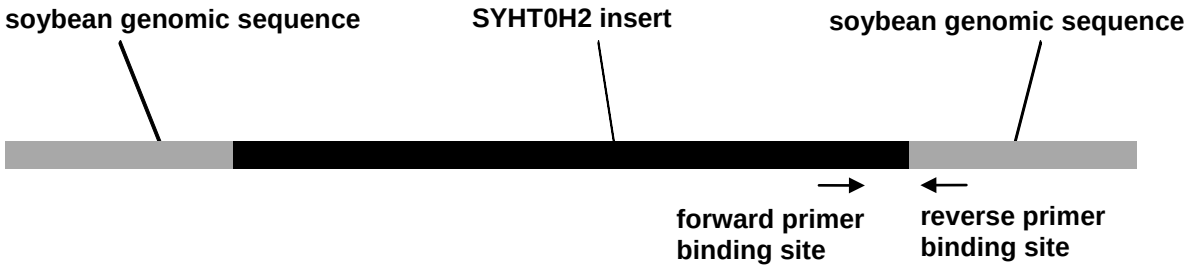
A gel-based, event-specific polymerase chain reaction (PCR) method was developed to detect SYHT0H2 soybean deoxyribonucleic acid (DNA). This method uses two oligonucleotide primers to amplify a 140 base pair (bp) DNA fragment that spans one of the junctions between the soybean genome and the SYHT0H2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

3.0 METHOD

3.1 Principle of the Method

For specific detection of SYHT0H2 genomic DNA, a 140 bp fragment that spans the 3′ insert-to-plant genome junction in SYHT0H2 soybean is amplified using two primers. The forward primer binding site is located in the SYHT0H2 insert and the reverse primer binding site is located in the soybean genomic sequence (Figure 1).

FIGURE 1 Location of the SYHT0H2 Gel-based, Event-specific PCR Primer Binding Sites



3.2 Reagents and Equipment

All materials (e.g., vials, containers, and pipette tips) should be suitable for PCR and molecular biology applications. Table 1 contains a list of equipment and materials needed to perform the PCR method. Materials should be deoxyribonuclease-free, DNA-free, sterile, and unable to absorb protein or DNA. To avoid contamination, materials for use in this method should be

stored separately from materials used in other laboratory procedures, benches and pipettes should be regularly cleaned with 70% ethanol, filter tips should be used with all pipettes, and disposable gloves should be used and changed often. The use of an electronic, repeat pipette is recommended to reduce sample to sample variability and to reduce the time needed to set up the reactions. Genomic DNA can be extracted from plants using a cetyltrimethyl ammonium bromide-based extraction method (JRC 2009) excluding steps 24 through 40 (i.e., "Wizard DNA Clean-up" and "MicroSpin Column" steps) and quantitated using Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen™ Catalog Number P11496). Table 2 contains a list of reagents and solutions needed to perform the PCR method.

TABLE 1 Equipment and Materials

Equipment and materials	Specification
96-Well GeneAmp® PCR System 9700	Applied Biosystems™ Part Number N8050200
Thermo-Fast® 96 PCR Detection Plate MKII	Abgene® Catalog Number AB-1400 or equivalent
Clear Seal Diamond Heat Sealing Film	Abgene® Catalog Number AB-0812 or equivalent
Thermo-Sealer	Abgene® Catalog Number AB-0384/240 or equivalent
Pipettes with adjustable volume	Eppendorf Research®, 2 to 20 µl, 20 to 200 µl, 100 to1000 µl or equivalent
Aerosol resistant tips	Molecular BioProducts, Catalog Number 2149P
Microcentrifuge tubes 1.5 ml	Roth Catalog Number 4182.1 or equivalent

TABLE 2 Reagents, Buffers, and Solutions

Reagents, buffers, and solutions	Specification
JumpStart™ REDTaq® ReadyMix™ Reaction Mix for High Throughput PCR	Sigma-Aldrich® Catalog Number P1107
Nuclease-free Water	Ambion® Catalog Number AM9932
Low DNA Mass™ Ladder	Invitrogen™ Catalog Number 10068-013

3.3 Primers and Amplicon

For the specific detection of SYHT0H2 genomic DNA, two primers (Table 3) are used to amplify a 140 bp fragment (Figure 2) that spans the 3′ insert-to-plant genome junction. 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 3 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

FIGURE 2 Sequence of the 140 bp Amplicon Generated by PCR Amplification with SYHT0H2 Gel-based, Event-specific PCR Primers

FE5313
CGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCGGGAATTGGGTACC

FE08317
ATGCCCGGGCGGCCAGCATGGCCGTATCCGCAATGTGTTATTAAGTTGTCTAAACCCTAAACCAAT

GGCACACA
Primer binding sites are underlined.

3.4 Master Mix

All reagents should be thawed, as necessary, and thoroughly mixed before each use. A master mix that contains all components of the PCR reaction except DNA (Table 4) can be prepared in sufficient quantities before the reactions are performed.

TABLE 4 Master Mix Components for the SYHT0H2 Gel-based, Event-specific PCR Method

Components	Volume per reaction (µl)[a]	Final concentration
JumpStart™ REDTaq® ReadyMix™, 2X	10	1X
FE5031313, 10 µM	1	0.5 µM
FE08317, 10 µM	1	0.5 µM
Nuclease-free water	4	Not applicable
Total volume of master mix	16	Not applicable

[a]Total PCR reaction is 20 µl (16 µl master mix and 4 µl genomic DNA at 10 ng/µl concentration).

3.5 PCR Method Controls

The following controls are recommended for this method:

- negative control 1: nuclease-free water substituted for DNA
- negative control 2: genomic DNA from transgenic SYHT0H2-free soybean
- negative control 3: genomic DNA from nontransgenic soybean
- positive control: 0.1% SYHT0H2 DNA prepared from either mixed DNA consisting of 0.1% SYHT0H2 DNA and 99.9% DNA from SYHT0H2-free soybean or DNA extracted from mixed ground seed containing 0.1% SYHT0H2 soybean and 99.9% of SYHT0H2-free soybean

3.6 Cycling Parameters

The method should be performed with the cycling parameters shown in Table 5.

TABLE 5 Cycling Parameters

Cycle	Step	Temperature (°C)	Time (seconds)	Number of cycles
A	1	94	120	1
B	1	94	30	40
	2	60	30	
	3	72	30	
C	1	72	180	1
D	1	4	Hold	Not applicable

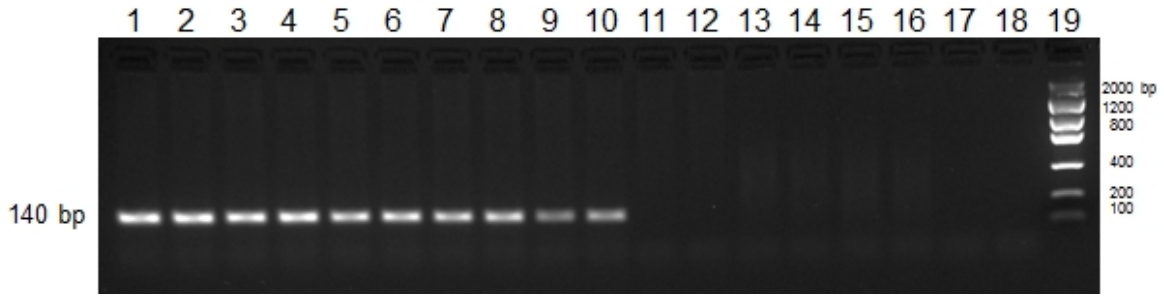
4.0 STEP-BY-STEP INSTRUCTIONS FOR PERFORMING THE METHOD

1. At room temperature, prepare a master mix of all reagents except DNA.
2. Mix the solution gently using a standard pipette.
3. Aliquot the appropriate amount of master mix into individual tubes (i.e., total volume of PCR reaction minus the DNA volume to be added [e.g., in the above example 20 µl minus 4 µl equals 16 µl master mix per well]).
4. Add DNA samples and controls in the following order:
   4 µl nuclease-free water to the negative control 1 well
   4 µl genomic DNA (10 ng/µl) from SYHT0H2-free soybean to the negative control 2 well
   4 µl genomic DNA (10 ng/µl) from nontransgenic soybean to the negative control 3 well
   4 µl genomic DNA (10 ng/µl) extracted from the test samples
   4 µl of 0.1% Event SYHT0H2 DNA (10 ng/µl) to the positive control well.
5. Seal the PCR plates with adhesive PCR sealing film.
6. Centrifuge the plates at 4000 × g for approximately 20 seconds.
7. Perform PCR using the cycling parameters in Table 5.
8. Following completion of PCR, store the PCR products at 4°C until further analysis.
9. Load the molecular weight marker and 5 to 10 µl of each PCR reaction onto a 2% agarose gel in 1X tris-acetate-EDTA (TAE) or tris-borate-EDTA (TBE) buffer containing 0.5 µg/ml ethidium bromide and electrophorese at 100 volts for 20 to 30 minutes. (Tris = 2-amino-2-(hydroxymethyl)-1,3-propanediol and EDTA = ethylenediaminetetraacetic acid).
10. Capture the image and score the results.

5.0 EXPECTED RESULTS

The agarose gel image in Figure 3 is an example of the expected results. A 140 bp PCR product is expected for all reactions containing SYHT0H2 DNA (Lanes 1 through 10). The PCR product is expected to be absent in all reactions that do not contain SYHT0H2 DNA (Lanes 11 through 18).

FIGURE 3 Agarose Gel Image of the PCR Products Generated by Amplification with SYHT0H2 Gel-based, Event-specific Primers



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
Lanes 11 and 12: Negative control 3 (genomic DNA from nontransgenic soybean)
Lanes 13 through 16: Negative control 2 (genomic DNA from transgenic SYHT0H2-free soybean)
Lanes 17 and 18: Negative control 1 (water substituted for DNA)
Lane 19: Low DNA Mass™ Ladder

6.0 PCR INHIBITION

The absence of the expected PCR product may indicate that the DNA extract is inhibiting the PCR analysis. In this case, re-extraction of DNA and further purification may be necessary.

To determine if a DNA extract is inhibiting the PCR analysis, add 1 µl of 0.04 ng/µl 100% SYHT0H2 DNA into a well containing the reaction mixture and the DNA extract that did not show the expected PCR product. Perform PCR analysis (as described in this method). If the expected PCR product is still absent, the DNA extract is inhibiting the PCR.

7.0 REFERENCES

JRC. 2009. *Report on the Validation of a DNA Extraction Method for Soybean Seeds.* Report number CRLVL04/07XP. Ispra, Italy: Joint Research Centre Institute for Health and Consumer Protection Biotechnology and GMOs Unit. 14 pp.

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