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Event SYHT0H2 Soybean: Genetic Stability Analysis

Final Report

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This study was conducted in compliance with the relevant provisions of 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:

- Characterization of the reference substance was not conducted in accordance with FIFRA GLPS.
- A late entry was made on 7/20/11 pertaining to DNA extraction.

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QUALITY ASSURANCE STATEMENT

Study Title: Event SYHT0H2 Soybean: Genetic Stability Analysis

Study Director: [redacted]

Study Number: TK0055857

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	March 29, 2011	March 29, 2011
Inspect Analytical	August 9, 2011	August 9, 2011
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Records Retention

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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avhppd-03	*p*-hydroxyphenylpyruvate dioxygenase gene derived from oat
AvHPPD-03	*p*-hydroxyphenylpyruvate dioxygenase enzyme encoded by *avhppd-03*
bp	base pair
CMP	cestrum yellow leaf curling virus promoter
CTAB	cetyltrimethyl ammonium bromide
dCTP	deoxycytidine triphosphate
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
EDTA	ethylenediaminetetraacetic acid
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
FMV	figwort mosaic virus
GLPS	Good Laboratory Practice Standards
HPPD	*p*-hydroxyphenylpyruvate dioxygenase enzyme
kb	kilobase pair
l	liter
M	molar
mg	milligram
ml	milliliter
mM	millimolar
N	normal
NOS	noplaline synthase gene
pat	phosphinothricin acetlytransferase gene
PAT	phosphinothricin acetyltransferase enzyme
PCR	polymerase chain reaction
pg	picogram
S	Svedberg unit
SDS	sodium dodecyl sulfate
spec	spectinomycin resistance gene
SSC	sodium chloride–sodium citrate
SMP	synthetic minimal plant promoter
T-DNA	transferred DNA
TMV	tobacco mosaic virus
tris	2-amino-2(hydroxymethyl)-1,3-propanediol
US EPA	United States Environmental Protection Agency
v/v	volume to volume
w/v	weight to volume
× *g*	times gravity
α-^{32}P	alpha-phosphorus-32 radioisotope
µg	microgram

1.0 EXECUTIVE SUMMARY

Soybean (*Glycine max* [L.] Merrill) has been genetically modified to express the novel gene *avhppd-03* derived from oat (*Avena sativa* L.) which encodes a *p*-hydroxyphenylpyruvate dioxygenase (HPPD) enzyme. 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. Event SYHT0H2 also contains *pat,* from *Streptomyces viridochromogenes,* which encodes the enzyme phosphinothricin acetyltransferase (PAT). Expression of *pat* confers a glufosinate-tolerance phenotype, which was used as a selectable marker in the development of Event SYHT0H2 soybeans.

Southern blot analyses were performed using standard molecular biology techniques. Two restriction enzyme digestion strategies were used in these Southern blot analyses. In the first strategy, soybean genomic deoxyribonucleic acid (DNA) was digested with an enzyme that cuts at least once within the SYHT0H2 insert; the other recognition sites for this enzyme were located in the soybean genome flanking the SYHT0H2 insert. This first strategy was used twice, with different enzymes, to determine the copy number of the SYHT0H2 insert and the presence or absence of extraneous DNA fragments of plasmid pSYN15954 in other regions of the SYHT0H2 soybean genome. In the second strategy, soybean genomic DNA was digested with two enzymes that cut within the insert such that two DNA fragments of predictable size were generated. This strategy was used to determine the intactness of the SYHT0H2 insert and the presence or absence of any closely linked extraneous DNA fragments of plasmid pSYN15954. The following probes were used: two transferred DNA (T-DNA)-specific probes that, when combined, cover every base of the pSYN15954 T-DNA and a plasmid pSYN15954 backbone-specific probe that covers every base of plasmid pSYN15954 present outside of the T-DNA region. Each Southern blot contained a positive control and a negative control. One or more positive controls, representing one copy of a fragment of known size in the soybean genome, were included to demonstrate the sensitivity of each experiment; the negative control, DNA extracted from plants grown from nontransgenic soybean seed, was included in order to identify possible endogenous DNA sequences that hybridize with the probe(s).

With the first digestion strategy, Southern blots probed with the two T-DNA-specific probes resulted in (1) two hybridization bands of the expected size when SYHT0H2 soybean genomic DNA was digested with the first restriction enzyme (which cut once within the SYHT0H2 insert, yielding two fragments containing SYHT0H2 insert sequence) and (2) four hybridization bands of the expected size when SYHT0H2 genomic DNA was digested with the second restriction enzyme (which cut three times within the SYHT0H2 insert yielding four fragments containing SYHT0H2 insert sequence). Detection of the expected number hybridization bands, of the expected size, indicated that the T-DNA integrated into a single location in the genome, and that there were no extraneous DNA fragments of the plasmid pSYN15954 T-DNA in other regions of the SYHT0H2 soybean genome.

With the second digestion strategy, Southern blots probed with the two T-DNA-specific probes resulted in two hybridization bands associated with the SYHT0H2 insert, as expected. Detection of only two hybridization bands 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 pSYN15954 T-DNA present in the SYHT0H2 soybean genome.

With each of these digestion strategies, Southern blots probed with the plasmid pSYN15954 backbone-specific probe resulted in no hybridization bands, as expected. No hybridization bands were observed in any of the analyses of genomic DNA from the nontransgenic soybean, and the expected bands were observed in lanes containing the positive control.

Southern blot analyses of SYHT0H2 soybean demonstrated that (1) SYHT0H2 soybean carries a single insert consisting of two partial copies of the pSYN15954 T-DNA with no extraneous T-DNA fragments of plasmid pSYN15954 inserted elsewhere in the soybean genome, (2) the transgenic locus is stable across all the SYHT0H2 generations analyzed, and (3) every generation of SYHT0H2 soybean examined is free of backbone sequence from the transformation plasmid pSYN15954.

2.0 INTRODUCTION

The purpose of this study was to determine the copy number of the SYHT0H2 insert, the presence or absence of extraneous fragments of plasmid pSYN15954 T-DNA, the stability of the SYHT0H2 insert over multiple generations, and the presence or absence of plasmid pSYN15954 backbone sequence in the soybean genome.

2.1 Description of 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 Description of the Transformation System and Method

Transformation of soybean to produce mesotrione-tolerant soybean plants was accomplished through the use of immature seed of variety 'Jack' (Nickell *et al.* 1990) via *Agrobacterium tumefaciens*–mediated transformation as described in Hwang *et al.* (2008) and Que *et al.* (2008). By this method, genetic elements within the left and right border regions of the transformation plasmid are transferred and integrated into the genome of the plant cell, while genetic elements outside these border regions generally are not transferred.

Maturing soybean pods were harvested from greenhouse-grown plants, sterilized with diluted bleach solution, and rinsed with sterile water. Immature seeds were then excised from the seed pods, sterilized, and rinsed with sterile water briefly. The explants were prepared from sterilized immature seeds as described in Hwang *et al.* (2008), infected with *A. tumefaciens* strain EHA101 harboring the transformation binary plasmid pSYN15954, and allowed to incubate for an additional 30 to 240 minutes. Excess *A. tumefaciens* suspension was then removed by aspiration, and the explants were moved to plates containing a non-selective co-culture medium. The explants were co-cultured with the remaining *A. tumefaciens* at 23°C for four days in the dark. The explants were then transferred to regeneration medium supplemented with an antibiotic mixture to kill *A. tumefaciens,* consisting of ticarcillin, cefotaxime, and vancomycin (75 mg/l each), and incubated in the dark for seven days. The explants were then transferred to cell-culture medium containing glufosinate (6 to 8 mg/l) and the antibiotic mixture. The gene *pat* was used as a selectable marker during the transformation process. The glufosinate selection concentration was kept low enough to allow for optimal shoot growth.

The regenerated plantlets were tested for the presence of the genes *pat* and *avhppd-03* and for the absence of the spectinomycin resistance gene (*spec*) present on the transformation plasmid backbone by real-time polymerase chain reaction (PCR) analysis (Ingham *et al*. 2001). This screen allowed for the selection of transgenic events that carry the transferred deoxyribonucleic acid (T-DNA) and were free of plasmid backbone DNA. Plants positive for *avhppd-03* and *pat,* and negative for *spec* were transferred to the greenhouse for seed setting.

2.3 Southern Blot Analysis

Southern blot analyses were performed to characterize the SYHT0H2 insert. Included in this report are data describing the genetic elements of plasmid pSYN15954 (the transformation plasmid used to generate SYHT0H2 soybean) and the molecular characterization of multiple generations of SYHT0H2 soybean.

3.0 MATERIALS AND METHODS

3.1 Genetic Elements for SYHT0H2 Soybean in Plasmid pSYN15954

Table 1 lists the genetic elements in plasmid pSYN15954 and their descriptions (including size in base pairs [bp] and position within the plasmid). Figure 1 shows a map of plasmid pSYN15954 and the positions of the restriction sites used in the Southern blot analyses.

TABLE 1 Genetic elements in plasmid pSYN15954

Genetic element	Size (bp)	Position	Description
***avhppd-03* cassette**			
Intervening sequence	282	26 to 307	Intervening sequence with restriction sites used for cloning.
FMV enhancer	194	308 to 501	Figwort mosaic virus (FMV) enhancer region (similar to Accession Number X06166.1 [NCBI 2011]) which increases gene expression (Maiti *et al.* 1997).
Intervening sequence	6	502 to 507	Intervening sequence with restriction sites used for cloning.
35S enhancer	293	508 to 800	Cauliflower mosaic virus 35S enhancer region (Ow *et al.* 1987).
Intervening sequence	20	801 to 820	Intervening sequence with restriction sites used for cloning.
SMP promoter	39	821 to 859	Synthetic minimal plant (SMP) promoter including the TATA box, adenine-rich sequence involved in transcription initiation, from the cestrum yellow leaf curling virus promoter (Stavolone *et al*. 2003), linked to a sequence taken from the region that is 3′ to the TATA box of the 35S promoter (Ow *et al.* 1987).
Intervening sequence	5	860 to 864	Intervening sequence with restriction sites used for cloning.

NCBI = National Center for Biotechnology Information

TABLE 1 Genetic elements in plasmid pSYN15954 (*continued*)

Genetic element	Size (bp)	Position	Description
TMV enhancer	68	865 to 932	The 5′ non-coding leader sequence (called omega) from tobacco mosaic virus (TMV) (Gallie *et al.* 1987) functions as a translational enhancer in plants (Gallie 2002).
Intervening sequence	3	933 to 935	Intervening sequence with restriction sites used for cloning.
avhppd-03	1320	936 to 2255	The gene *avhppd-03*, derived from oat, encodes a AvHPPD-03 enzyme. This enzyme catalyzes the formation of homogentisic acid, the aromatic precursor of plastoquinone and vitamin E biosynthesis (Matringe *et al.* 2005). 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 plant cells confers a mesotrione-tolerance phenotype.
Intervening sequence	16	2256 to 2271	Intervening sequence with restriction sites used for cloning.
NOS terminator	253	2272 to 2524	Terminator sequence from the nopaline synthanse (NOS) gene of *A. tumefaciens* (Accession Number V00087.1 [NCBI 2011]). Provides a polyadenylation site (Depicker *et al*. 1982).
Intervening sequence	8	2525 to 2532	Intervening sequence with restriction sites used for cloning.
***pat-03-01* cassette**			
35S promoter	521	2533 to 3053	Promoter region of cauliflower mosaic virus (Ow *et al.* 1987).
Intervening sequence	24	3054 to 3077	Intervening sequence with restriction sites used for cloning.
pat-03-01	552	3078 to 3629	*Streptomyces viridochromogenes* strain Tü494 gene encoding the selectable marker PAT. The native coding sequence (Wohlleben *et al*. 1988) was codon-optimized for enhanced expression. The synthetic *pat-03-01* gene was obtained from AgrEvo, Germany. PAT confers resistance to herbicides containing glufosinate (phosphinothricin).
Intervening sequence	33	3630 to 3662	Intervening sequence with restriction sites used for cloning.
NOS terminator	253	3663 to 3915	Terminator sequence from the NOS gene of *A. tumefaciens* (Accession Number V00087.1 [NCBI 2011]). Provides a polyadenylation site (Depicker *et al*. 1982).
Intervening sequence	8	3916 to 3923	Intervening sequence with restriction sites used for cloning.

NCBI = National Center for Biotechnology Information

TABLE 1 Genetic elements in plasmid pSYN15954 (*continued*)

Genetic element	Size (bp)	Position	Description
***pat-03-02* cassette**			
CMP promoter	654	3924 to 4577	Promoter and leader sequence from the cestrum yellow leaf curling virus.
Intervening sequence	5	4578 to 4582	Intervening sequence with restriction sites used for cloning.
TMV enhancer	68	4583 to 4650	The 5′ non-coding leader sequence (called omega) from tobacco mosaic virus (Gallie *et al.* 1987) functions as a translational enhancer in plants (Gallie 2002).
Intervening sequence	10	4651 to 4660	Intervening sequence with restriction sites used for cloning.
pat-03-02	552	4661 to 5212	*S. viridochromogenes* strain Tü494 gene encoding the selectable marker PAT. The native coding sequence (Wohlleben *et al*. 1988) was codon-optimized for enhanced expression and altered to remove restriction sites. PAT confers resistance to herbicides containing glufosinate (phosphinothricin).
Intervening sequence	28	5213 to 5240	Intervening sequence with restriction sites used for cloning.
NOS terminator	253	5241 to 5493	Terminator sequence from the nopaline synthase gene of *A. tumefaciens* (Accession Number V00087.1 [NCBI 2011]). Provides a polyadenylation site (Depicker *et al*. 1982).
Intervening sequence	77	5494 to 5570	Intervening sequence with restriction sites used for cloning.
Plasmid backbone			
Left border	25	5571 to 5595	Left border region of T-DNA from *A. tumefaciens* nopaline Ti-plasmid (Accession Number J01825.1 [NCBI 2011]). 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	5596 to 5944	Intervening sequence with restriction sites used for cloning
spec	789	5945 to 6733	Aminoglycoside adenylyltransferase gene, *aadA* gene (*spec*) from *Escherichia coli* transposon Tn7 (similar to Accession Number X03043.1 [NCBI 2011]). Confers resistance to streptomycin and spectinomycin and is used as a bacterial selectable marker (Fling *et al*. 1985).
Intervening sequence	299	6734 to 7032	Intervening sequence with restriction sites used for cloning.

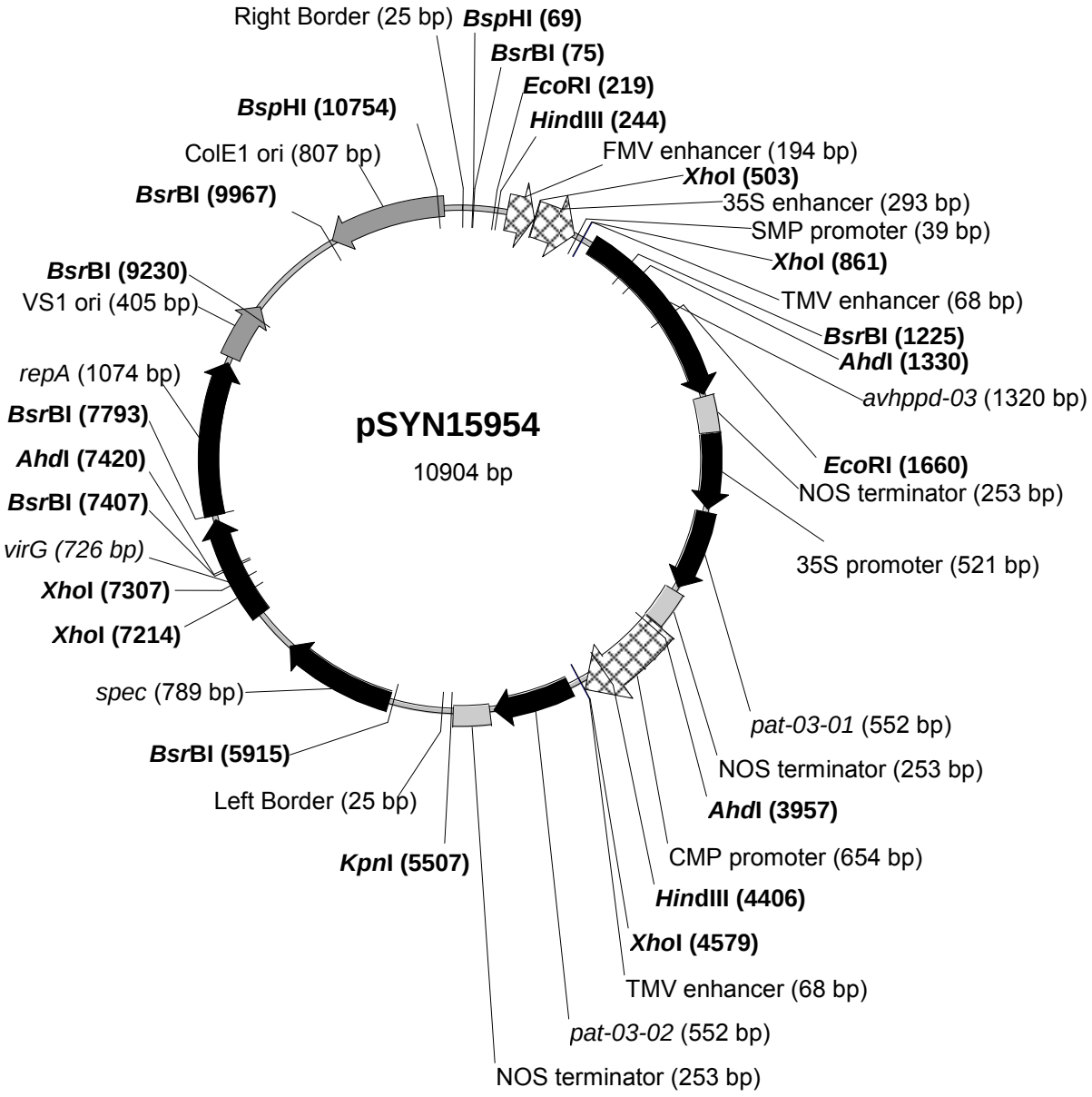
CMP = cestrum yellow curling leaf virus promoter
NCBI = National Center for Biotechnology Information

TABLE 1 Genetic elements in plasmid pSYN15954 (*continued*)

Genetic element	Size (bp)	Position	Description
virG	726	7033 to 7758	The VirGN54D gene (*virG*) from pAD1289 (similar to Accession Number AF242881.1 [NCBI 2011]). The N54D substitution results in a constitutive *virG* phenotype. The gene *virG* is part of the two-component regulatory system for the virulence regulon in *A. tumefaciens* (Hansen *et al*. 1994).
Intervening sequence	29	7759 to 7787	Intervening sequence with restriction sites used for cloning.
repA	1074	7788 to 8861	Gene encoding the pVS1 replication protein from *P. aeruginosa* (similar to Accession Number AF133831.1 [NCBI 2011]), 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	8862 to 8903	Intervening sequence with restriction sites used for cloning.
VS1 ori	405	8904 to 9308	Consensus sequence for the origin of replication (ori) and partitioning region from plasmid pVS1 of *P. aeruginosa* (Accession Number U10487.1 [NCBI 2011]). Serves as origin of replication in *A. tumefaciens* host (Itoh *et al*. 1984).
Intervening sequence	677	9309 to 9985	Intervening sequence with restriction sites used for cloning.
ColE1 ori	807	9986 to 10792	Origin of replication (similar to Accession Number V00268.1 [NCBI 2011]) that permits replication of plasmids in *E. coli* (Itoh and Tomizawa 1979).
Intervening sequence	112	10793 to 10904	Intervening sequence with restriction sites used for cloning.
Right border	25	1 to 25	Right border region of T-DNA from *A. tumefaciens* nopaline Ti-plasmid (Accession Number J01826.1 [NCBI 2011]). 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).

NCBI = National Center for Biotechnology Information
ori = origin of replication
repA = replication gene
virG = virulence gene

FIGURE 1 Map of plasmid pSYN15954 indicating the restriction sites used in the Southern blot analyses (shown in bold type)



CMP = cestrum yellow leaf curling virus promoter
FMV = figwort mosaic virus
TMV = tobacco mosaic virus
ori = origin of replication
repA = replication gene
virG = virulence gene

3.2 Test, Control, and Reference Substances

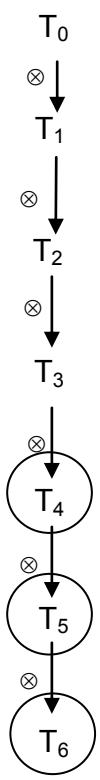
The test substances for this study were SYHT0H2 soybean seed from generations T_4, T_5, and T_6 in the genetic background 'Jack'. The control substance was nontransgenic soybean seed of the same genetic background ('Jack'). Table 2 shows the descriptions of the test and control substances. Figure 2 is a pedigree chart illustrating the production of the test substance seed. The reference substance for Southern blot analyses was the Promega Analytical Marker DNA Wide Range molecular weight marker.

TABLE 2 Test and Control Substances

Seed	Entry	Material identification number
Event SYHT0H2 T_4	test	09SG052316
Event SYHT0H2 T_5	test	10SG900011
Event SYHT0H2 T_6	test	10SG900903
'Jack'	control	09SG050867

The test and control substances were characterized by real-time PCR analysis (Ingham *et al.* 2001) to confirm the identity and purity (Burgin 2011).

FIGURE 2 Pedigree chart for SYHT0H2 soybean illustrating the production of the test substances used in this study



The transformation recipient line is 'Jack'

T_0 = original transformant
⊗ = self-pollination
The generations used in this study are denoted with a circle.

3.3 Plant Tissue for Genomic DNA Extraction

Seed of each test and control substance were grown in greenhouses located at Syngenta Biotechnology, Inc. in Research Triangle Park, North Carolina, USA. Following verification of the plants' identity by real-time PCR analysis, leaf tissue from the SYHT0H2 T_4 generation was pooled into a sampling bag and stored at -80°C ± 10°C. This process was repeated for SYHT0H2 T_5 and T_6 generations and Jack soybean plants. For each test and control substance, plant tissue was collected from 10 to 20 plants. This process took place in study TK0055856.

3.4 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). Additional genomic DNA was extracted as needed.

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, tissue and prewarmed cetyltrimethyl ammonium bromide (CTAB) buffer (100 mM 2-amino-2[hydroxymethyl]-1,3-propanediol [tris] pH 8.0, 20 mM ethylenediaminetetraacetic acid [EDTA] pH 8.0, 1.4 M sodium chloride, 2% CTAB [w/v], 0.2% β-mercaptoethanol [v/v]) were combined in a bottle; the sample was then mixed gently and incubated for 72 to 90 minutes at 60°C to 65°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 A per ml of aqueous phase was added. The sample was mixed and incubated for 31 to 53 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 0.7 volume of isopropanol. The DNA was then pelleted by centrifugation at 291 × *g*. The DNA was washed once with 70% ethanol and centrifuged at 7277 × *g* for ten minutes. The DNA pellet was air-dried and dissolved in prewarmed 0.1X tris-EDTA.

3.5 DNA Quantitation

The concentration of DNA was measured using an Invitrogen Quant-iT™ PicoGreen® dsDNA kit. A two-point standard curve was generated using a lambda DNA standard. The linear attribute of the standard curve was verified with samples generated from a serial dilution of lambda DNA standard in 1X tris-EDTA. Genomic DNA was quantified by interpolation from the two-point standard curve, and each genomic DNA was assayed in duplicate or triplicate using the Turner Biosystems TBS-380 Mini-Fluorometer.

3.6 Southern Blot Analyses

Southern blot analyses were performed using standard molecular biology techniques (Chomczynski 1992). Each lane contained 3 µg of genomic DNA that was digested with the appropriate restriction enzyme(s) for approximately six hours.

In order to demonstrate the sensitivity of the analyses, each Southern blot analysis included one or more positive controls representing one copy of a DNA fragment of known size in the soybean genome. The positive controls for Southern blot analyses using the two T-DNA probes were PCR-amplified DNA fragments containing sequence specific to the two T-DNA probes (*avhppd-03*-specific and CMP promoter-specific DNA fragments). The positive control for Southern blot analyses using the pSYN15954 backbone-specific probe was digested DNA from plasmid pSYN15954. The positive control was loaded in a well together with 3 µg of digested DNA from nontransgenic 'Jack' soybean, so that the migration speed of the positive control DNA would more accurately reflect the migration speed of the restriction fragment containing the target sequence in the soybean genome.

The amount of positive control (picograms for one copy) was calculated by the following formula (Arumuganathan and Earle 1991):

$$\left\{\left(\frac{\text{positive control size (bp)}}{\text{genome size (bp)} \times \text{ploidy}}\right) \times \mu\text{g loaded}\right\} \times 1 \times 10^{6} = \text{pg for 1 copy}$$

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

soybean genome size (bp)	1.10×10^{9}
soybean ploidy	2
DNA loaded in each lane (µg)	3
CMP promoter-specific DNA fragment (bp)	654
avhppd-03-specific DNA fragment (bp)	1320
pSYN15954 plasmid digest (*Kpn*I + *Bsp*HI) (bp)	10904

The following amount of positive control was calculated:

CMP promoter-specific DNA fragment (pg)	0.89
avhppd-03-specific DNA fragment (pg)	1.80
pSYN15954 plasmid digest (*Kpn*I + *Bsp*HI) (pg)	14.87

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

Following a 10 to 12 minute depurination in 0.25 N hydrochloric acid, the DNA in the gel was denatured in 0.5 M sodium hydroxide, 1.5 M sodium chloride, and 2 mM EDTA for 30 to 35 minutes. A Bio-Rad Appligene vacuum blotter was then used to transfer the DNA to a Bio-Rad Zeta-Probe® GT membrane by downward alkaline transfer for 102 to 141 minutes. The membrane was rinsed briefly in 2X sodium chloride–sodium citrate (SSC) buffer, and ultraviolet light was used to crosslink the DNA to the membrane.

All probes (the two T-DNA-specific probes, the plasmid pSYN15954 backbone-specific probe, and the molecular weight marker-specific probe) were labeled with alpha-phosphorus-32-deoxycytidine triphosphate ([α-^{32}P]-dCTP) by random priming using the GE Healthcare Megaprime™ DNA labeling system. The unincorporated label ([α-^{32}P]-dCTP) was removed using the Bio-Rad Micro Bio-Spin® Chromatography Columns.

Membranes were incubated in 30 ml of Sigma-Aldrich PerfectHyb™ Plus Hybridization Buffer, which contained 100 µg/ml denatured calf thymus DNA, for at least 15 to 140 minutes at 65°C ± 5°C. The molecular weight marker-specific probe and either the two T-DNA-specific probes or the plasmid pSYN15954 backbone-specific probe were added to the hybridization solution, and the membranes were incubated for 20 to 22 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% sodium dodecyl sulfate (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™.

3.7 Genetic Stability Southern Blot Analysis of SYHT0H2 Soybean Using T-DNA-specific Probes

Genetic stability of the insert during conventional breeding of SYHT0H2 soybean was determined by Southern blot analyses using two T-DNA-specific probes, that when combined cover every base pair of the pSYN15954 T-DNA. Genomic DNA from the T_4, T_5, and T_6 generations and negative control ('Jack' soybean) were included in these analyses (see breeding pedigree in Figure 2).

For the experiments described in this study, genomic DNA was analyzed using two restriction enzyme digestion strategies. In the first strategy, the soybean genomic DNA was digested with an enzyme that cuts at least once within the SYHT0H2 insert. The other recognition sites for this enzyme were located in the soybean genome flanking SYHT0H2 insert. This first strategy was used twice, with different enzymes (*Eco*RI and *Xho*I), to determine the copy number of the SYHT0H2 insert and the presence or absence of extraneous plasmid pSYN15954 T-DNA fragments in other regions of the SYHT0H2 soybean genome. The *Eco*RI digest was expected to result in two hybridization bands and the *Xho*I digest was expected to result in four hybridization bands when the T-DNA-specific probes were used.

In the second strategy, the soybean genomic DNA was digested with two enzymes (*Kpn*I + *Bsr*BI), which cut within the SYHT0H2 insert such that two DNA fragments of predictable size were released. This strategy was used to determine the intactness of the insert and the presence of any closely linked extraneous plasmid pSYN15954 T-DNA fragments. Additional bands with either of the digest strategies would have indicated that there were multiple copies of the insert in the plant genome.

3.8 Southern Blot Analysis of SYHT0H2 Soybean Using a Plasmid Backbone-specific Probe

The absence of plasmid backbone sequence in SYHT0H2 soybean was assessed by Southern blot analyses using plasmid pSYN15954 backbone sequence as a probe on Southern blots of DNA subjected to the two restriction enzyme digestion strategies described above. For the first enzyme strategy, *Eco*RI and *Hin*dIII were used, and *Ahd*I was used for the second enzyme strategy. The plasmid backbone-specific probe contained every base pair of the plasmid pSYN15954 present outside of the T-DNA region (*i.e.* the backbone region). With both restriction enzyme digestion strategies, no hybridization bands were expected.

3.9 Control of Bias Statement

The test and control substances were analyzed on the same Southern blot. One or more positive controls representing one copy of a fragment of known size in the soybean genome was included in each Southern blot to demonstrate the sensitivity of the experiment. Any rejected data, and the documented reasons for the rejection of those data, were retained in the study file.

3.10 Statistical Analysis

No statistical analysis was conducted during this study.

4.0 RESULTS AND DISCUSSION

4.1 Genetic Stability Southern Blot Analysis of SYHT0H2 Soybean Using T-DNA-specific Probes

Genetic stability of the insert during conventional breeding of SYHT0H2 soybean was determined by Southern blot analyses using T-DNA-specific probes. Southern blot analyses included genomic DNA extracted from plants grown from the test and control substances (T_4, T_5, and T_6 generations and 'Jack' soybean). A map of the insert in SYHT0H2 soybean, indicating the locations of the T-DNA-specific probes and restriction sites *Eco*RI, *Xho*I, *Kpn*I, *and Bsr*BI, is shown in Figure 3. Figure 4 shows a map of full T-DNA region in the SYHT0H2 transformation plasmid pSYN15954 indicating the locations of the T-DNA-specific probes and restriction enzymes. Table 3 outlines the expected and observed sizes of the hybridization bands.

For Southern blot analysis with genomic DNA digested with *Eco*RI and probed with the T-DNA-specific probes, two hybridization bands of approximately 4.8 kb and 8.5 kb were observed in lanes containing DNA extracted from SYHT0H2 T_4, T_5, and T_6 soybean (Table 3; Figure 5A, Lanes 2 through 4). These hybridization bands were absent in the lane containing DNA extracted from nontransgenic 'Jack' soybean (Figure 5A, Lane 5) and were, therefore, specific to the SYHT0H2 insert. Two hybridization bands of approximately 0.7 kb and 1.3 kb were observed in the lane containing the positive controls (0.89 pg of CMP promoter-specific and 1.80 pg of *avhppd-03*-specific DNA fragments loaded with DNA extracted from nontransgenic 'Jack' soybean) (Figure 5A, Lane 6). Hybridization intensity of the CMP promoter-specific positive control and the *avhppd-03*-specific positive control correlate to one copy of a DNA fragment of 0.7 kb and 1.3 kb respectively.

For Southern blot analysis with genomic DNA digested with *Xho*I and probed with the T-DNA-specific probes, four hybridization bands of approximately 2.2 kb, 3.7 kb, 6.6 kb, and 20 kb were observed in lanes containing DNA extracted from SYHT0H2 T_4, T_5, and T_6 soybean (Table 3; Figure 5B, Lanes 2 through 4). These hybridization bands were absent in the lane containing DNA extracted from nontransgenic 'Jack' soybean (Figure 5B, Lane 5) and were, therefore, specific to the SYHT0H2 insert. Two hybridization bands of approximately 0.7 kb and 1.3 kb were observed in the lane containing the positive controls (0.89 pg of CMP promoter-specific and 1.80 pg of *avhppd-03*-specific DNA fragments

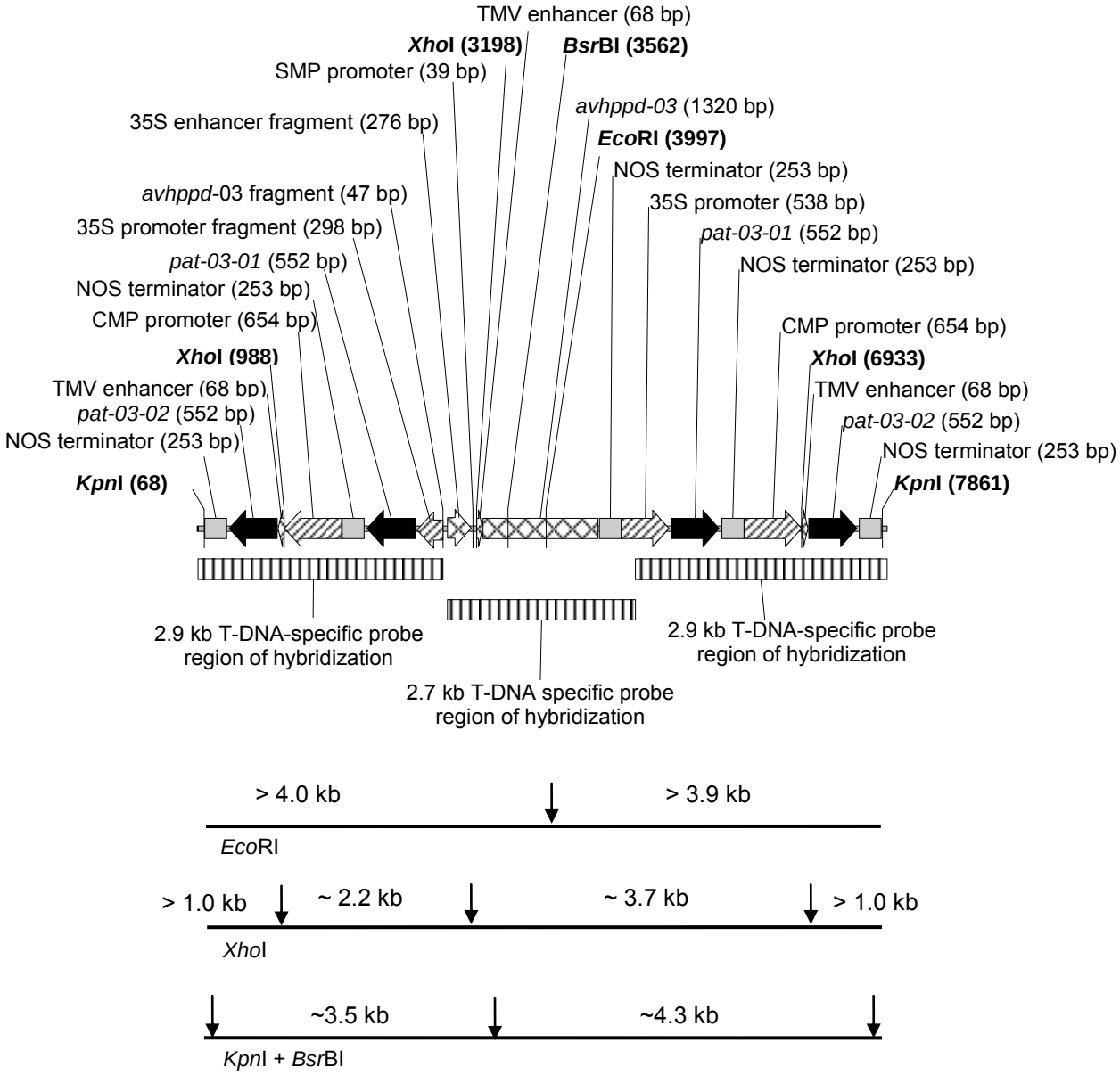
loaded with DNA extracted from 'Jack' soybean) (Figure 5B, Lane 6). Hybridization intensity of the CMP promoter-specific positive control and the *avhppd-03*-specific positive control correlate to one copy of a DNA fragment of 0.7 kb and 1.3 kb respectively.

For Southern blot analysis with genomic DNA digested with *Kpn*I + *Bsr*BI and probed with the T-DNA-specific probes, two hybridization bands of approximately 3.5 kb and 4.3 kb were observed in lanes containing DNA extracted from SYHT0H2 T_4, T_5, and T_6 soybean (Table 3; Figure 5C, Lanes 2 through 4). These hybridization bands were absent in the lane containing DNA extracted from nontransgenic 'Jack' soybean (Figure 5C, Lane 5) and were, therefore, specific to the SYHT0H2 insert. Two hybridization bands of approximately 0.7 kb and 1.3 kb were observed in the lane containing the positive controls (0.89 pg of CMP promoter-specific and 1.80 pg of *avhppd-03*-specific DNA fragments loaded with DNA extracted from 'Jack' soybean) (Figure 5C, Lane 6). Hybridization intensity of the CMP promoter-specific positive control and the *avhppd-03*-specific positive control correlate to one copy of a DNA fragment of 0.7 kb and 1.3 kb respectively.

For Southern blot analyses with the T-DNA-specific probes, the expected number of hybridization bands, which were of the expected size, was detected with both restriction enzymes strategies. These results demonstrate that two partial copies of the SYHT0H2 insert integrated into a single locus in the soybean genome. No unexpected bands were observed; Southern blot analyses indicated that the SYHT0H2 soybean genome contains no extraneous DNA fragments of SYHT0H2 sequence.

The data depicted in the Southern blot analyses demonstrated that the hybridization bands specific to the insert were identical in lanes containing DNA extracted from plants grown from all generations T_4, T_5, and T_6; these results indicate that the SYHT0H2 insert is stably inherited from one generation to the next.

FIGURE 3 **Location of the 2.7 kb and 2.9 kb T-DNA-specific probes and the restrictions sites *Eco*RI, *Xho*I, *Kpn*I, and *Bsr*BI in the SYHT0H2 soybean insert**

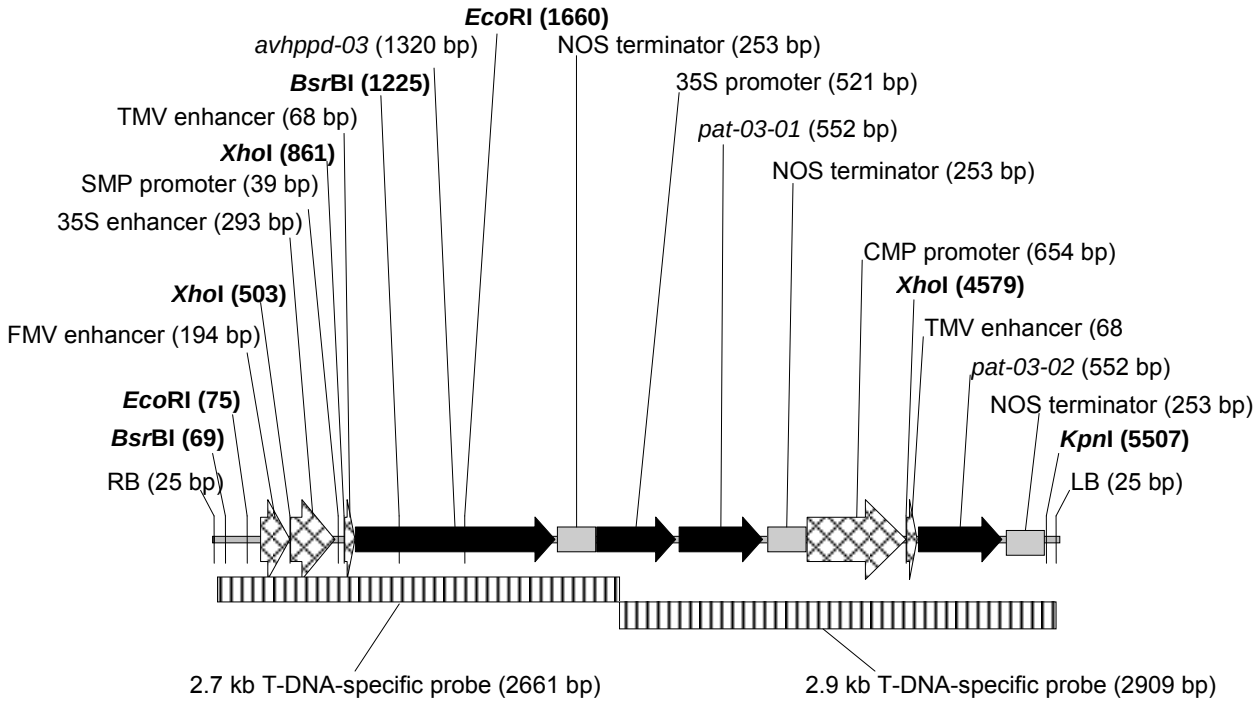


The locations of the restriction sites are based on their locations in this specific map, and will differ from their locations in plasmid pSYN15954 as shown in Figure 4.

The vertical arrows indicate the site of restriction digestion.

Sizes of the expected restriction fragments are indicated.

FIGURE 4 Location of the 2.7 kb and 2.9 kb T-DNA-specific probes and restrictions sites *Eco*RI, *Xho*I, *Kpn*I, and *Bsr*BI restriction sites in the T-DNA region of the transformation plasmid pSYN15954



LB = left border
RB = right border

TABLE 3 Expected and observed hybridization bands in Southern blot analysis of SYHT0H2 soybean, with T-DNA-specific probes and restriction enzymes *Eco*RI, *Xho*I, and *Kpn*I + *Bsr*BI

Lane	Source of DNA	Restriction enzyme	Expected number of bands	Expected band size (kb)	Observed band size (kb)
Figure 5A, 2	SYHT0H2 T_4	*Eco*RI	2	>3.9 >4.0	~4.8 ~8.5
Figure 5A, 3	SYHT0H2 T_5	*Eco*RI	2	>3.9 >4.0	~4.8 ~8.5
Figure 5A, 4	SYHT0H2 T_6	*Eco*RI	2	>3.9 >4.0	~4.8 ~8.5
Figure 5A, 5	'Jack'	*Eco*RI	0	N/A	N/A
Figure 5A, 6	Positive control ('Jack' digested with *Eco*RI plus 0.89 pg of CMP promoter-specific and 1.80 pg of *avhppd-03*-specific DNA fragments)	*Eco*RI	2[a]	~0.7, ~ 1.3	~0.7, ~ 1.3
Figure 5B, 2	SYHT0H2 T_4	*Xho*I	4	>1.0 >1.0 ~2.2 ~3.7	~2.2 ~3.7 ~6.6 ~20
Figure 5B, 3	SYHT0H2 T_5	*Xho*I	4	>1.0 >1.0 ~2.2 ~3.7	~2.2 ~3.7 ~6.6 ~20
Figure 5B, 4	SYHT0H2 T_6	*Xho*I	4	>1.0 >1.0 ~2.2 ~3.7	~2.2 ~3.7 ~6.6 ~20
Figure 5B, 5	'Jack'	*Xho*I	0	N/A	N/A
Figure 5B, 6	Positive control ('Jack' digested with *Xho*I plus 0.89 pg of CMP promoter-specific and 1.80 pg of *avhppd-03*-specific DNA fragments)	*Xho*I	2[a]	~0.7, ~ 1.3	~0.7, ~ 1.3

N/A = not applicable

[a]The two expected bands in this lane correspond to the two positive controls added to this digest; 0.7 kb for the CMP promoter-specific positive control and 1.3 kb for the *avhppd*-03-specific positive control.

TABLE 3 Expected and observed hybridization bands in Southern blot analysis of SYHT0H2 soybean, with T-DNA-specific probes and restriction enzymes *Eco*RI, *Xho*I, and *Kpn*I + *Bsr*BI (*continued*)

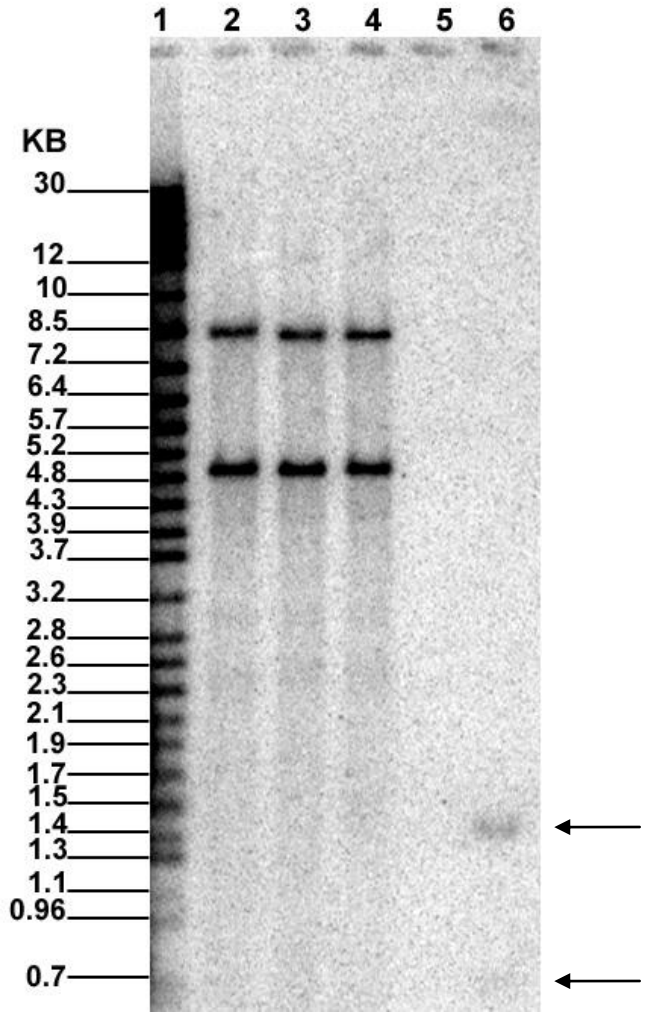
Lane	Source of DNA	Restriction enzyme	Expected number of bands	Expected band size (kb)	Observed band size (kb)
Figure 5C, 2	SYHT0H2 T_4	*Kpn*I + *Bsr*BI	2	~3.5 ~4.3	~3.5 ~4.3
Figure 5C, 3	SYHT0H2 T_5	*Kpn*I + *Bsr*BI	2	~3.5 ~4.3	~3.5 ~4.3
Figure 5C, 4	SYHT0H2 T_6	*Kpn*I + *Bsr*BI	2	~3.5 ~4.3	~3.5 ~4.3
Figure 5C, 5	Jack	*Kpn*I + *Bsr*BI	0	N/A	N/A
Figure 5C, 6	Positive control (Jack digested with *Kpn*I + *Bsr*BI plus 0.89 pg of CMP promoter-specific and 1.80 pg of *avhppd-03*-specific DNA fragments)	*Kpn*I + *Bsr*BI	2 [a]	~0.7, ~ 1.3	~0.7, ~ 1.3

N/A = not applicable

[a] The two expected bands in this lane correspond to the two positive controls added to this digest; 0.7 kb for the CMP promoter-specific positive control and 1.3 kb for the *avhppd*-03-specific positive control.

FIGURE 5 **Genetic stability Southern blot analysis of SYHT0H2 soybean with the 2.7 kb and 2.9 kb T-DNA-specific probes, using restriction enzymes *Eco*RI, *Xho*I, *Kpn*I + *Bsr*BI**

(A) ***Eco*RI**

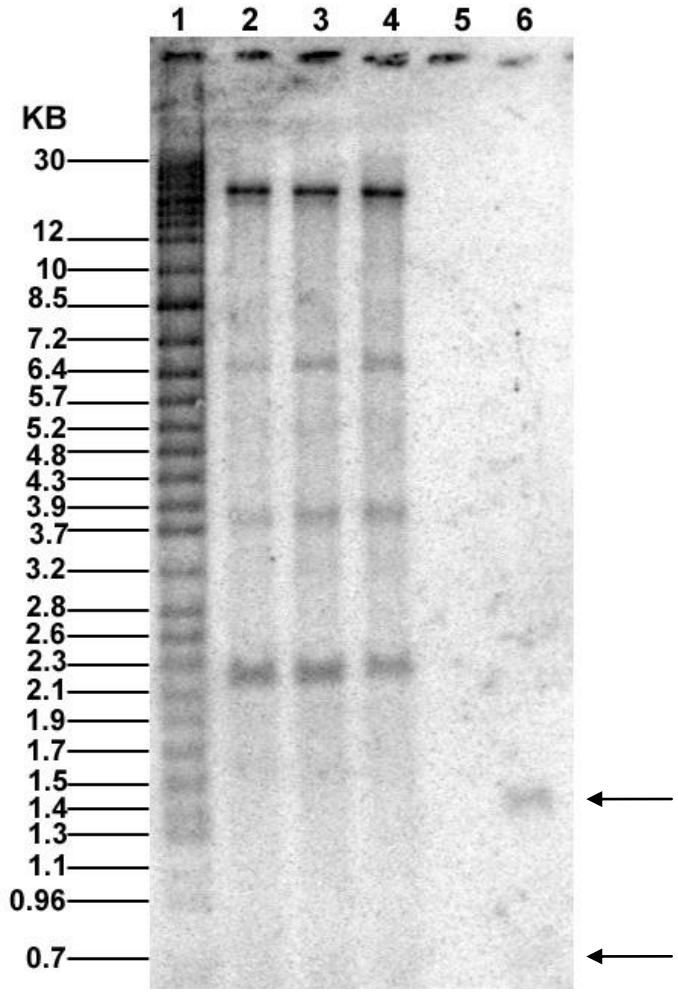


The horizontal arrow notes the location of the *avhppd-03*-specific and CMP promoter-specific positive control.

Lane 1 = molecular weight markers.
Lane 2 = SYHT0H2 T_4 digested with *Eco*RI.
Lane 3 = SYHT0H2 T_5 digested with *Eco*RI.
Lane 4 = SYHT0H2 T_6 digested with *Eco*RI.
Lane 5 = 'Jack' digested with *Eco*RI.
Lane 6 = positive control ('Jack' digested with *Eco*RI plus 0.89 pg of CMP promoter-specific and 1.80 pg of *avhppd-03*-specific DNA fragments).

FIGURE 5 Genetic stability Southern blot analysis of SYHT0H2 soybean with the 2.7 kb and 2.9 kb T-DNA-specific probes, using restriction enzymes *Eco*RI, *Xho*I, *Kpn*I + *Bsr*BI (*continued*)

(B) *Xho*I

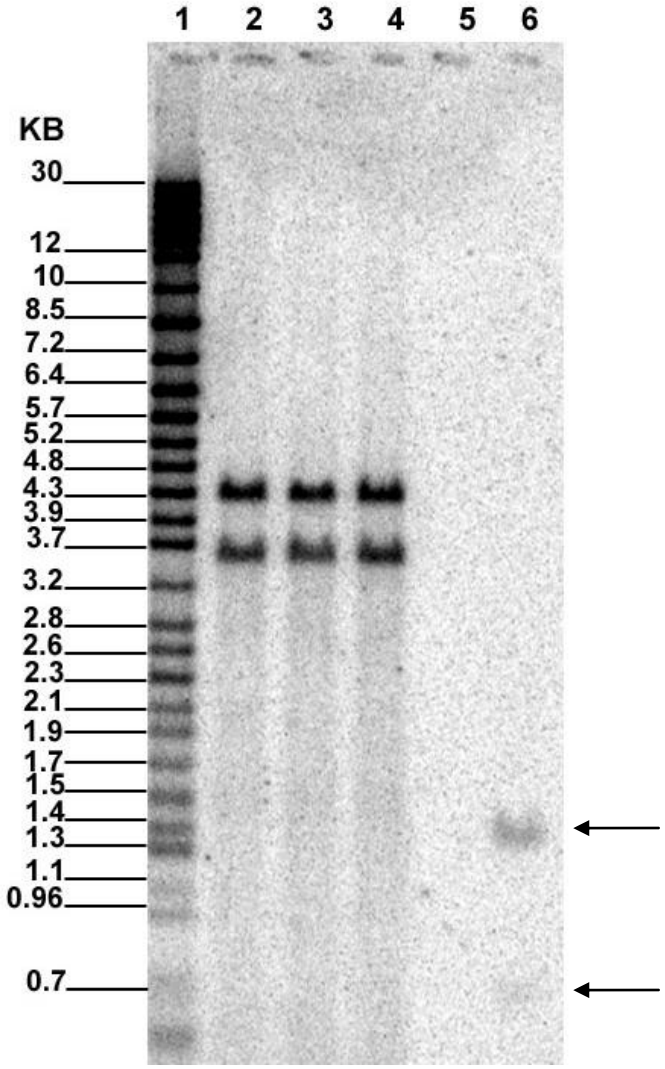


The horizontal arrow notes the location of the *avhppd-03*-specific and CMP promoter-specific positive control.

Lane 1 = molecular weight markers.
Lane 2 = SYHT0H2 T_4 digested with *Xho*I.
Lane 3 = SYHT0H2 T_5 digested with *Xho*I.
Lane 4 = SYHT0H2 T_6 digested with *Xho*I.
Lane 5 = Jack digested with *Xho*I.
Lane 6 = positive control ('Jack' digested with *Xho*I plus 0.89 pg of CMP promoter-specific and 1.80 pg of *avhppd-03*-specific DNA fragments).

FIGURE 5 Genetic stability Southern blot analysis of SYHT0H2 soybean with the 2.7 kb and 2.9 kb T-DNA-specific probes, using restriction enzymes *Eco*RI, *Xho*I, *Kpn*I + *Bsr*BI (*continued*)

(C) *Kpn*I + *Bsr*BI



The horizontal arrow notes the location of the *avhppd-03*-specific and CMP promoter-specific positive control..

Lane 1 = molecular weight markers.
Lane 2 = SYHT0H2 T_4 digested with *Kpn*I + *Bsr*BI.
Lane 3 = SYHT0H2 T_5 digested with *Kpn*I + *Bsr*BI.
Lane 4 = SYHT0H2 T_6 digested with *Kpn*I + *Bsr*BI.
Lane 5 = 'Jack' digested with *Kpn*I + *Bsr*BI.
Lane 6 = positive control ('Jack' digested with *Kpn*I + *Bsr*BI plus 0.89 pg of CMP-specific and 1.80 pg of *avhppd-03*-specific DNA fragments).

4.2 Southern Blot Analysis of SYHT0H2 Soybean Using a Plasmid Backbone-specific Probe

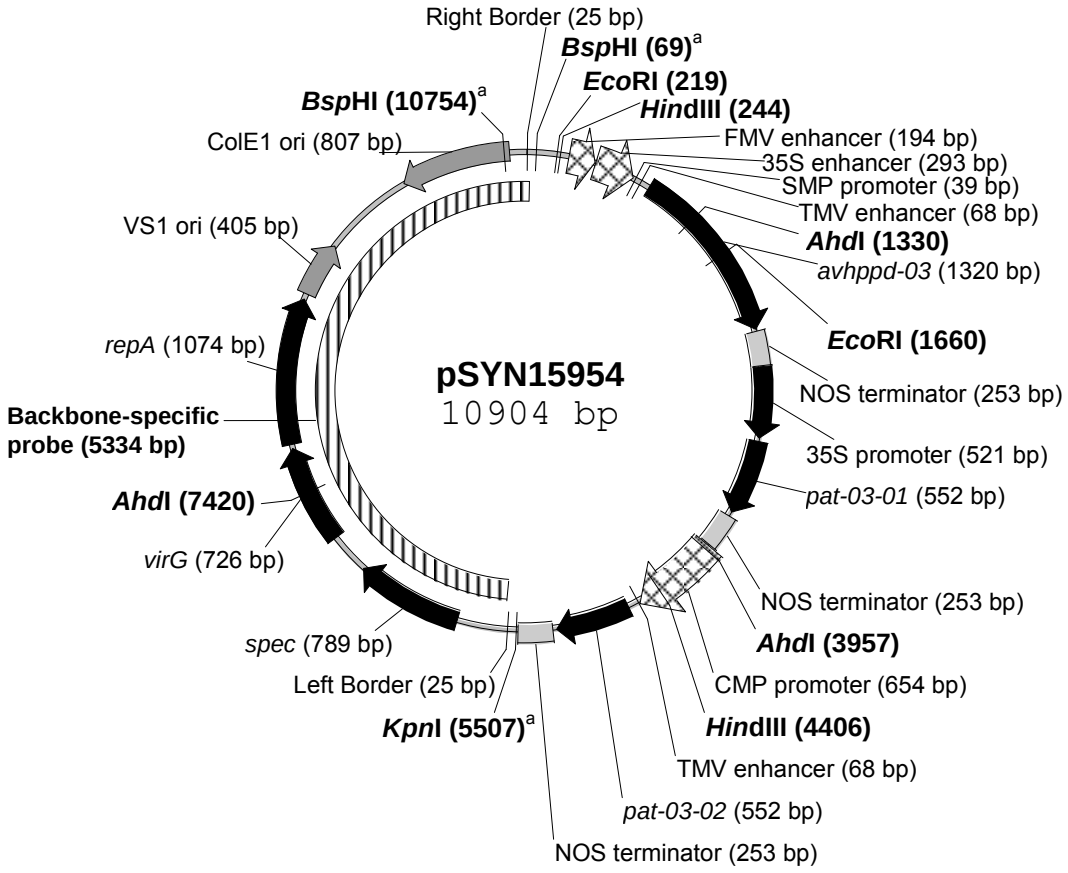
Absence of the pSYN15954 backbone was determined by Southern blot analysis using a plasmid backbone-specific probe. The Southern blot analyses included genomic DNA from plants grown from the test and control substances (SYHT0H2 generations T_4, T_5, T_6, and nontransgenic 'Jack' soybean).

Figure 6 shows a map of the plasmid pSYN15954 indicating the location of the plasmid pSYN15954 backbone-specific probe and restriction sites for *Eco*RI, *Hin*dIII, and *Ahd*I. Table 4 outlines the expected and observed sizes of the hybridization bands, and Figure 7 depicts the results of the corresponding Southern blot analyses.

In the analyses of genomic DNA digested with *Eco*RI, *Hin*dIII, or *Ahd*I, no hybridization bands were observed in the lanes containing DNA from extracted from SYHT0H2 T_4, T_5, or T_6 soybean (Table 4; Figures 7A through 7C, Lanes 2 through 4) or in the lanes containing DNA extracted from the nontransgenic 'Jack' soybean (Figures 7A through 7C, Lane 5). In all three analyses, one band of approximately 5.3 kb was observed in the lanes containing the positive control (Figures 7A through 7C, Lane 6), as expected.

These results demonstrate that all SYHT0H2 generations analyzed are free of plasmid pSYN15954 backbone sequence.

FIGURE 6 Location of the 5334 bp pSYN15954 plasmid backbone-specific probe and restriction sites *Eco*RI, *Hin*dIII, and *Ahd*I in the transformation plasmid pSYN15954



ori = origin of replication

[a]The restriction enzymes *Kpn*I and *Bsp*HI were used to digest pSYN15954 plasmid DNA for use as the positive control.

TABLE 4 Expected and observed hybridization bands in Southern blot analysis of SYHT0H2 soybean, using a pSYN15954 plasmid backbone-specific probe and restriction enzymes *Eco*RI, *Hin*dIII, and *Ahd*I

Lane	Source of DNA	Restriction enzyme	Expected number of bands	Expected band size (kb)	Observed band size (kb)
Figure 7A, 2	SYHT0H2 T_4	*Eco*RI	0	N/A	N/A
Figure 7A, 3	SYHT0H2 T_5	*Eco*RI	0	N/A	N/A
Figure 7A, 4	SYHT0H2 T_6	*Eco*RI	0	N/A	N/A
Figure 7A, 5	'Jack'	*Eco*RI	0	N/A	N/A
Figure 7A, 6	Positive control ('Jack' digested with *Eco*RI plus 14.87 pg of pSYN15954 digested with *Kpn*I + *Bs*pHI)	*Eco*RI	1 [a]	~5.2	~5.3 [b]
Figure 7B, 2	SYHT0H2 T_4	*Hin*dIII	0	N/A	N/A
Figure 7B, 3	SYHT0H2 T_5	*Hin*dIII	0	N/A	N/A
Figure 7B, 4	SYHT0H2 T_6	*Hin*dIII	0	N/A	N/A
Figure 7B, 5	'Jack'	*Hin*dIII	0	N/A	N/A
Figure 7B, 6	Positive control ('Jack' digested with *Hin*dIII plus 14.87 pg of pSYN15954 digested with *Kpn*I + *Bs*pHI)	*Hin*dIII	1 [a]	~5.2	~5.3 [b]
Figure 7C, 2	SYHT0H2 T_4	*Ahd*I	0	N/A	N/A
Figure 7C, 3	SYHT0H2 T_5	*Ahd*I	0	N/A	N/A
Figure 7C, 4	SYHT0H2 T_6	*Ahd*I	0	N/A	N/A
Figure 7C, 5	'Jack'	*Ahd*I	0	N/A	N/A
Figure 7C, 6	Positive control ('Jack' digested with *Ahd*I plus 14.87 pg of pSYN15954 digested with *Kpn*I + *Bs*pHI)	*Ahd*I	1 [a]	~5.2	~5.3 [b]

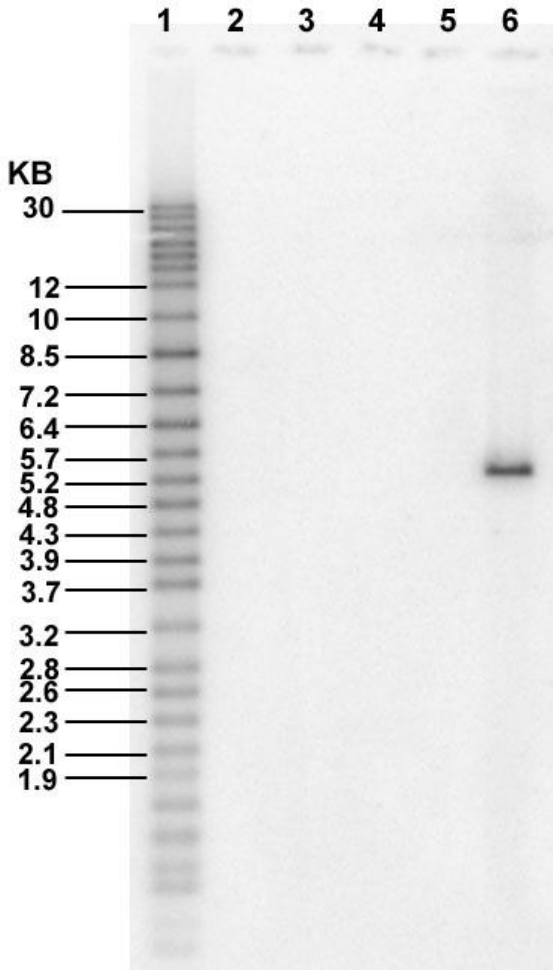
N/A = not applicable

[a] The expected band in this lane corresponds to the positive control added to this digest (pSYN15954 digested with *Kpn*I + *Bs*pHI).

[b] The difference between the observed and expected size is within the accepted variability for Southern blot analysis.

FIGURE 7 **Southern blot analysis of SYHT0H2 soybean with the 5334 bp pSYN15954 plasmid backbone-specific probe, using restriction enzymes *Eco*RI, *Hin*dIII, *Ahd*I**

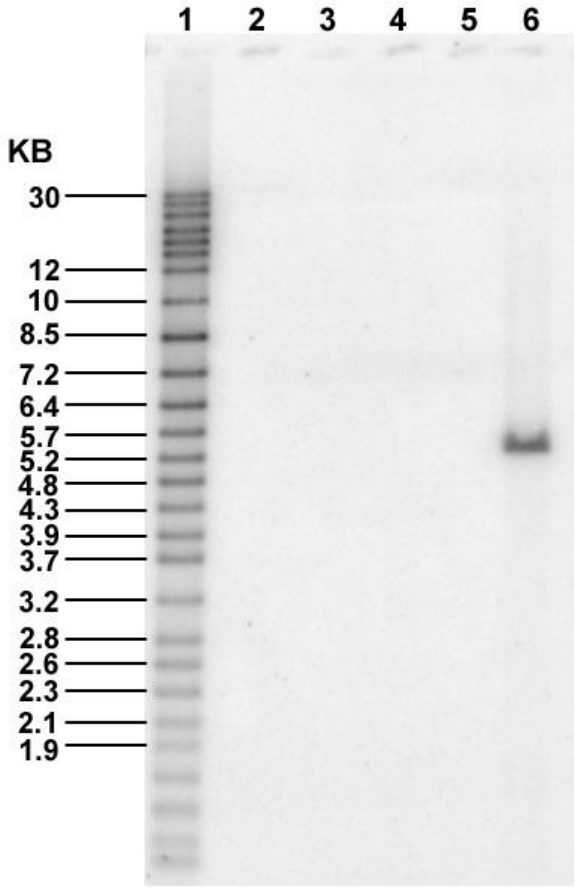
(A) ***Eco*RI**



Lane 1 = molecular weight markers.
Lane 2 = SYHT0H2 T_4 digested with *Eco*RI.
Lane 3 = SYHT0H2 T_5 digested with *Eco*RI.
Lane 4 = SYHT0H2 T_6 digested with *Eco*RI.
Lane 5 = 'Jack' digested with *Eco*RI.
Lane 6 = positive control ('Jack' digested with *Eco*RI plus 14.87 pg of pSYN15954 digested with *Kpn*I + *B*spHI).

FIGURE 7 **Southern blot analysis of SYHT0H2 soybean with the 5334 bp pSYN15954 plasmid backbone-specific probe, using restriction enzymes *Eco*RI, *Hin*dIII, *Ahd*I (*continued*)**

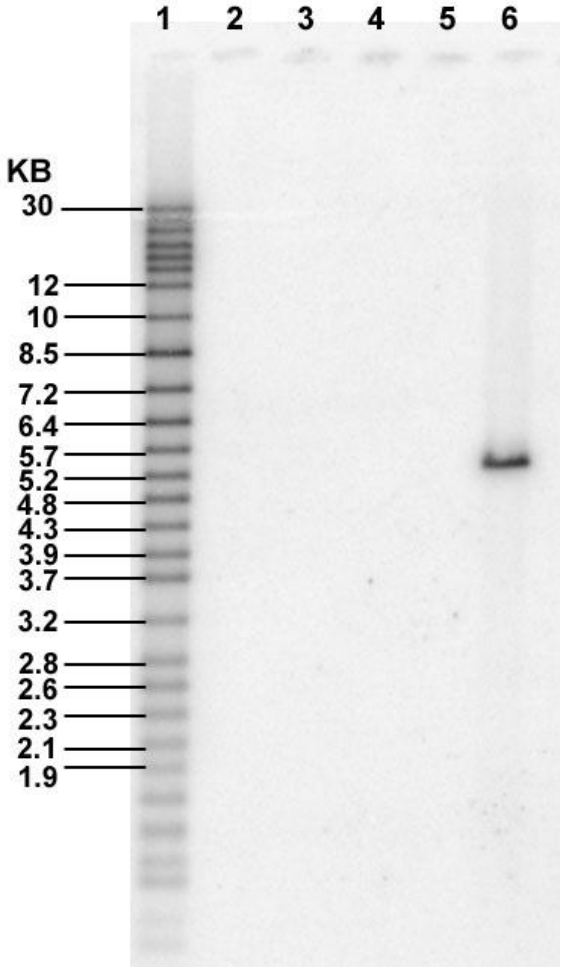
(B) ***Hin*dIII**



Lane 1 = molecular weight markers.
Lane 2 = SYHT0H2 T_4 digested with *Hin*dIII.
Lane 3 = SYHT0H2 T_5 digested with *Hin*dIII.
Lane 4 = SYHT0H2 T_6 digested with *Hin*dIII.
Lane 5 = 'Jack' digested with *Hin*dIII.
Lane 6 = positive control ('Jack' digested with *Hin*dIII plus 14.87 pg of pSYN15954 digested with *Kpn*I + *Bs*pHI).

FIGURE 7 **Southern blot analysis of SYHT0H2 soybean with the 5334 bp pSYN15954 plasmid backbone-specific probe, using restriction enzymes *Eco*RI, *Hin*dIII, *Ahd*I (*continued*)**

(C) ***Ahd*I**



Lane 1 = molecular weight markers.
Lane 2 = SYHT0H2 T_4 digested with *Ahd*I.
Lane 3 = SYHT0H2 T_5 digested with *Ahd*I.
Lane 4 = SYHT0H2 T_6 digested with *Ahd*I.
Lane 5 = 'Jack' digested with *Ahd*I.
Lane 6 = positive control ('Jack' digested with *Ahd*I plus 14.87 pg of pSYN15954 digested with *Kpn*I + *Bs*pHI).

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

Southern blot analyses of SYHT0H2 soybean demonstrated that (1) SYHT0H2 soybean carries a single insert consisting of two partial copies of the pSYN15954 T-DNA with no extraneous T-DNA fragments of plasmid pSYN15954 inserted elsewhere in the soybean genome, (2) the transgenic locus is stable across all the SYHT0H2 generations analyzed, and (3) every generation SYHT0H2 soybean examined is free of backbone sequence from the transformation plasmid pSYN15954.

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