syngenta

Event SYHT0H2 Soybean

Functional Element Copy Number Southern Blot Analysis

Final Report

DATA REQUIREMENTS: Not Applicable

AUTHOR: [redacted]

STUDY COMPLETION DATE: April 30, 2012

PERFORMING LABORATORY: Syngenta Crop Protection, LLC
3054 East Cornwallis Road
Research Triangle Park, NC 27709-2257 USA

LABORATORY PROJECT ID: Report Number: TK0059651
Task Number: TK0059651

SUBMITTER:
Syngenta Seeds, Inc.
3054 East Cornwallis Road
Post Office Box 12257
Research Triangle Park, NC 27709-2257 USA

SPONSOR:
Syngenta Crop Protection, LLC
410 Swing Road
Post Office Box 18300
Greensboro, NC 27419-8300 USA

STATEMENTS OF DATA CONFIDENTIALITY CLAIMS

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

Statement of No Data Confidentiality Claim

No claim of confidentiality is made for any 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).

Company: *Syngenta Seeds, Inc.*

Company Representative:

[redacted] April 30, 2012

Date

Manager, Regulatory Affairs

These data are the property of Syngenta Seeds, Inc. and, as such, are considered to be confidential for all purposes other than compliance with the regulations implementing FIFRA Section 10. Submission of these data in compliance with FIFRA does not constitute a waiver of any right to confidentiality that may exist under any other provision of common law or statute or in any other country.

The following statement applies to submissions to regulatory agencies and other competent authorities other than the US EPA and all other viewers.

This Document Contains Confidential Business Information

This document contains information that is proprietary to Syngenta and, as such, is considered to be confidential for all purposes other than compliance with the relevant registration procedures.

Without the prior written consent of Syngenta, this information may (i) not be used by any third party including, but not limited to, any other regulatory authority for the support of regulatory approval of this product or any other product, and (ii) not be published or disclosed to any third party including, but not limited to, any authority for the support of regulatory approval of any products.

Its submission does not constitute a waiver of any right to confidentiality that may exist in any other country.

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

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.
- Tissue samples were harvested outside of a protocol but harvesting was documented in accordance with FIFRA GLPS.

Study Director:

[redacted] April 30, 2012

Date

Technical Expert, Product Safety
Syngenta Crop Protection, LLC

Submitted by:

[redacted] April 30, 2012

Date

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

Sponsor:

[redacted] April 30, 2012

Date

Technical Leader, Product Safety
Syngenta Crop Protection, LLC
410 Swing Road
Post Office Box 18300
Greensboro, NC 27419-8300 USA

QUALITY ASSUNRANCE STATEMENT

Study Title: Event SYHT0H2 Soybean: Functional Element Copy Number Southern Blot Analysis

Study Director: [redacted]

Study Number: TK0059651

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 Dates
Audit Protocol	18-JUL-2011 - 18-JUL-2011	18-JUL-2011
Inspect Analytical	03-NOV-2011 - 03-NOV-2011	10-NOV-2011
Audit Study Data	15-MAR-2012 - 27-MAR-2012	27-MAR-2012
Audit Final Report	16-APR-2012 - 20-APR-2012	20-APR-2012

See page 82 in Appendix A of this report for additional Quality Assurance inspection/audit and reporting dates.

Prepared By [redacted] April 23, 2012

Staff Quality Assurance Auditor
Syngenta Crop Protection, LLC

GENERAL INFORMATION

Contributors

The following contributed to this report in the capacities indicated:

Name	Title
[redacted]	Study Director, Syngenta Crop Protection, LLC
	Analyst, Syngenta Crop Protection, LLC
	Analyst, Syngenta Crop Protection, LLC
	Analyst, Syngenta Crop Protection, LLC
	Analyst, Syngenta Crop Protection, LLC
	Analyst, Syngenta Crop Protection, LLC
	Principal Investigator, Beckman Coulter Genomics, Inc.
	Production Scientist, Beckman Coulter Genomics, Inc.
	Production Scientist, Beckman Coulter Genomics, Inc.
	Production Scientist, Beckman Coulter Genomics, Inc.
	Production Scientist, Beckman Coulter Genomics, Inc.
	Production Scientist, Beckman Coulter Genomics, Inc.
	Production Scientist, Beckman Coulter Genomics, Inc.

Study Dates

Study initiation date:	July 19, 2011
Experimental start date:	October 25, 2011
Experimental termination date:	March 30, 2012

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.

Additional Test Site

Beckman Coulter Genomics, Inc.
100 Perimeter Park, Suite C
Morrisville, NC 27560 USA

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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 pairs
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
g	gram
GLPS	Good Laboratory Practice Standards
HPPD	*p*-hydroxyphenylpyruvate dioxygenase enzyme
kb	kilobase pairs
l	liter
M	molar
mg	milligram
ml	milliliter
mM	millimolar
N	normal
NOS	nopaline synthase
PAT	phosphinothricin acetyltransferase enzyme
pat	phosphinothricin acetyltransferase gene
PCR	polymerase chain reaction
pg	picogram
S	Svedberg unit
SDS	sodium dodecyl sulfate
spec	spectinomycin resistance gene
SMP	synthetic minimal plant promoter
SSC	sodium chloride–sodium citrate
T-DNA	transferred deoxyribonucleic acid
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 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.

Southern blot analyses were performed to determine (1) the number of transferred deoxyribonucleic acid (T-DNA) integration sites in the SYHT0H2 soybean genome, (2) the number of copies in the SYHT0H2 soybean genome of each functional element of the transformation plasmid pSYN15954, and (3) the presence or absence of the plasmid backbone sequence in the SYHT0H2 soybean genome.

Southern blot analyses were performed according to standard molecular biology techniques. Two restriction enzyme digestion strategies were used. In the first strategy, soybean genomic DNA was digested with an enzyme that cuts 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 two different enzymes to determine the number of copies of the functional elements and the presence or absence of extraneous DNA fragments of the functional elements of plasmid pSYN15954 in other regions of the SYHT0H2 soybean genome. In the second strategy, soybean genomic DNA was digested with an enzyme, or enzymes, that cut in the insert on either side of the element, thus generating a DNA fragment of predictable size. This strategy was used to determine the presence or absence of any closely linked extraneous DNA fragments of the functional elements of plasmid pSYN15954 in the SYHT0H2 soybean genome.

Eight probes were used: (1) an *avhppd-03*-specific probe, (2) a *pat*-specific probe, (3) an *avhppd-03* enhancer complex-specific probe (consisting of tobacco mosaic virus [TMV] enhancer, synthetic minimal plant promoter [SMP], and 35S enhancer sequence), (4) a 35S promoter-specific probe, (5) a cestrum yellow leaf curling virus promoter (CMP) + TMV enhancer-specific probe, (6) a NOS terminator-specific probe, (7) a figwort mosaic virus (FMV) enhancer-specific probe, and (8) a plasmid pSYN15954 backbone-specific probe. Each element probe (except *pat*) covers every base of the element present in the plasmid pSYN15954 T-DNA, and the plasmid pSYN15954 backbone-specific probe covers every base pair outside of the pSYN15954 T-DNA. The pSYN15954 T-DNA consists of two PAT genes, differing by two base pairs. Since there was only a two base pair difference, only one *pat*-specific probe was used.

Each Southern blot contained a positive control and a negative control. The positive control represented one copy of a fragment of known size in the soybean genome; it was included to demonstrate the sensitivity of each experiment. The negative control was genomic DNA extracted from plants grown from nontransgenic soybean seed; it was included in order to identify possible endogenous DNA sequences that hybridized with the probes.

With the first digestion strategy, Southern blots probed with the *avhppd-03*-specific probe resulted in two hybridization bands of the expected sizes when SYHT0H2 soybean genomic DNA was digested with the first restriction enzyme (which cut once within *avhppd-03,* yielding two fragments containing *avhppd-03* sequence). Digestion of SYHT0H2 genomic DNA with the second restriction enzyme (which cut within a region of the insert outside of *avhppd-03*) resulted in one hybridization band of the expected size containing *avhppd-03* sequence. With the first digestion strategy, Southern blots probed with the *avhppd-03* enhancer complex-specific probe resulted in two hybridization bands of the expected size, corresponding to one copy of the *avhppd-03* enhancer complex and one copy of the 35S promoter, which contains sequence that is similar to the 35S enhancer and the SMP promoter. With the first digestion strategy, Southern blots probed with the *pat*-, 35S promoter-, CMP promoter + TMV enhancer-, and NOS terminator-specific probes, each analysis resulted in two hybridization bands of the expected size.

With the second digestion strategy, Southern blots probed with the *avhppd-03*-specific probe resulted in one hybridization band of the expected size. With the second digestion strategy, Southern blots probed with the *pat*-specific probe, the *avhppd-03* enhancer complex-specific probe, the 35S promoter-specific probe, the CMP promoter + TMV enhancer-specific probe, and the NOS terminator-specific probe all resulted in two hybridization bands of the expected size.

With both digestion strategies, Southern blots probed with the FMV enhancer- and plasmid pSYN15954 backbone-specific probes 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.

Insert sequence analysis determined that SYHT0H2 soybean contains a single copy of *avhppd-03*, four copies of *pat*, a single copy of the *avhppd-03* enhancer complex sequence, two copies of the 35S promoter, two copies of the CMP promoter, two copies of the TMV enhancer (contained in the *pat-03-02* cassette), and five copies of the NOS terminator, as expected for a single insertion site consisting of two truncated copies of the pSYN15954 T-DNA. Southern blot analysis of SYHT0H2 in this study supports this determination. The results also demonstrated that there are no extraneous DNA fragments from any of these functional elements elsewhere in the SYHT0H2 soybean genome, and the FMV enhancer and backbone sequence from transformation plasmid pSYN15954 are absent from SYHT0H2 soybean.

2.0 INTRODUCTION

The purpose of this study was to assess the number of transferred deoxyribonucleic acid (T-DNA) integration sites within the genome of soybean plants derived from transformation Event SYHT0H2, the number of copies of each functional element, and the presence or absence of plasmid backbone sequence.

2.1 Description of Event SYHT0H2 Soybean

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.

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 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 Functional Element Copy Number Southern Blot Analyses

This study employed Southern blot analyses to assess the number of T-DNA integration sites within the SYHT0H2 soybean genome, the number of copies of each functional element, and the presence or absence of the plasmid backbone sequence. Included in this report are data and information describing the genetic elements of plasmid pSYN15954 (the transformation plasmid used to generate SYHT0H2 soybean) and the Southern blot analyses of SYHT0H2 soybean.

3.0 MATERIALS AND METHODS

3.1 Genetic Elements in Plasmid pSYN15954

Table 1 lists the genetic elements in transformation plasmid pSYN15954 and their descriptions (including size in base pairs [bp] and position within the plasmid). Elements expected to be transferred to the plant cell and integrated into the plant genome during T-DNA transfer are categorized by the gene cassette in which they are contained. The elements of the plasmid necessary for its replication and selection in different bacterial hosts are categorized as plasmid backbone. These elements are not expected to be transferred to the plant cell and integrated into the plant genome during T-DNA transfer. The left and right borders are categorized as border regions since only a portion of each border is expected to be integrated into the plant genome (Tzfira *et al*. 2004). Figure 1 shows a map of plasmid pSYN15954.

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) transcriptional enhancer region (similar to Accession Number X06166.1 [NCBI 2012]) 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 transcriptional enhancer region (Ow *et al.* 1987).
Intervening sequence	20	801 to 820	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
SMP promoter	39	821 to 859	Synthetic minimal plant promoter (SMP) including the TATA box, an adenine-rich sequence involved in transcription initiation, from the cestrum yellow leaf curling virus promoter (Stavolone *et al*. 2003a), 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.
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 an 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 synthase (NOS) gene of *A. tumefaciens* (Accession Number V00087.1 [NCBI 2012]). 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 2012]). 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, similar to Accession Number AF364175.3 (NCBI 2012) (Stavolone *et al*. 2003b).
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 2012]). Provides a polyadenylation site (Depicker *et al*. 1982).
Intervening sequence	77	5494 to 5570	Intervening sequence with restriction sites used for cloning.
Border region			
Left Border	25	5571 to 5595	Left border region of T-DNA from *A. tumefaciens* nopaline Ti-plasmid (Accession Number J01825.1 [NCBI 2012]). 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).
Plasmid backbone			
Intervening sequence	349	5596 to 5944	Intervening sequence with restriction sites used for cloning.
spec	789	5945 to 6733	Aminoglycoside adenylyltransferase gene, *aadA*, from *Escherichia coli* transposon Tn7 (similar to Accession Number X03043.1 [NCBI 2012]). 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.

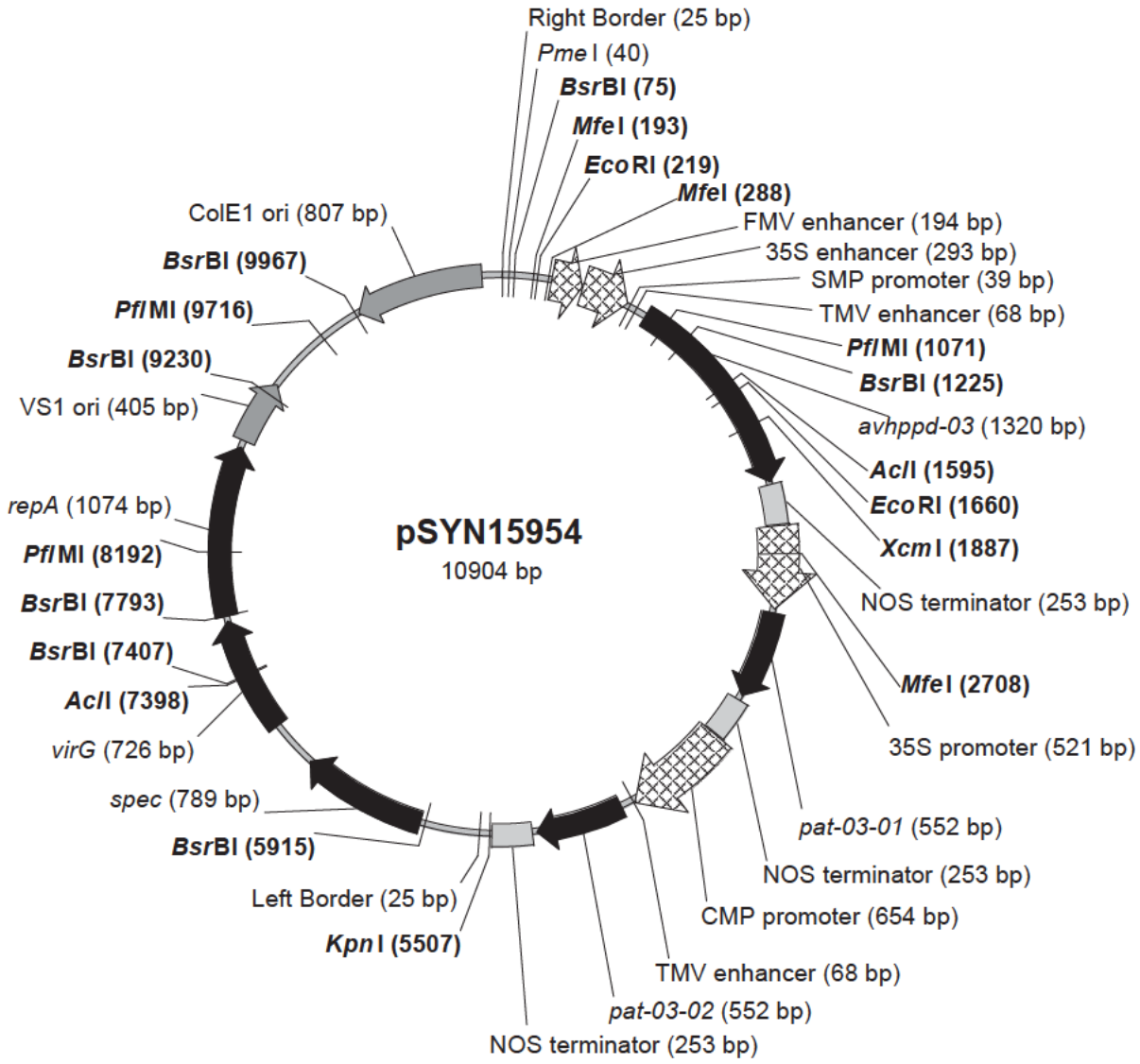
CMP = cestrum yellow leaf curling virus promoter
NCBI = National Center for Biotechnology Information

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 2012]). 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 *Pseudomonas aeruginosa* (similar to Accession Number AF133831.1 [NCBI 2012]), 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 2012]). 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 2012]) 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.
Border region			
Right Border	25	1 to 25	Right border region of T-DNA from *A. tumefaciens* nopaline Ti-plasmid (Accession Number J01826.1 [NCBI 2012]). 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
repA = replication gene
virG = part of the two-component regulatory system for the virulence regulon in *A.tumefaciens*

FIGURE 1 **Map of plasmid pSYN15954 indicating the restriction sites used in the Southern blot analyses**



CMP = cestrum yellow leaf curling virus promoter
FMV = figwort mosaic virus
NOS = nopaline synthase
ori = origin of replication
repA = replication gene
SMP = synthetic minimal plant promoter
TMV = tobacco mosaic virus
virG = part of the two-component regulatory system for the virulence regulon in *A.tumefaciens*

Restriction enzyme *Pme*I was used with *Kpn*I for digestion of pSYN15954 that served as a positive control. The restriction sites used in the Southern blot analyses are shown in bold type.

3.2 Test, Control, and Reference Substances

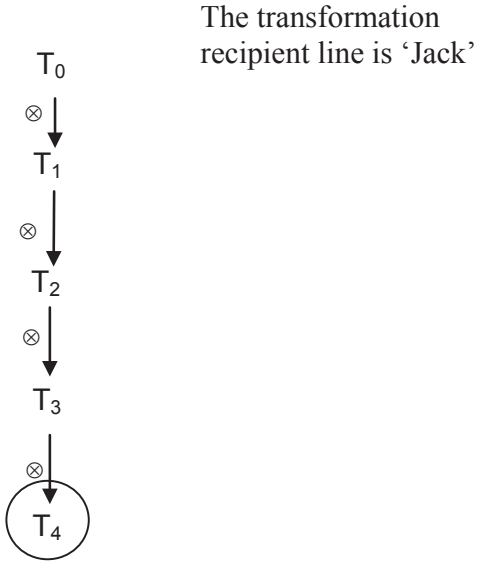
The test substance for this study was SYHT0H2 soybean seed in the genetic background 'Jack'. The control substance was nontransgenic, near-isogenic soybean seed of the same genetic background. Table 2 shows the identifications for the test and control substances. Figure 2 is a pedigree chart illustrating 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 identification	Entry	Material identification
SYHT0H2 T_4 soybean	test	09SG052316
Nontransgenic soybean ('Jack')	control	09SG050867
Nontransgenic soybean ('Jack')	control	'Jack'

The test and control substances were characterized by real-time PCR analysis (Ingham *et al.* 2001) to confirm identity and purity (Burgin 2009, Burgin 2011).

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



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

3.3 Plant Tissue for Genomic DNA Extraction

Seed of each test and control substance was grown in greenhouses located at Syngenta Biotechnology, Inc., Research Triangle Park, NC, USA or Syngenta Crop Protection, LLC, Greensboro, NC, USA. Following verification of the plants' identity by real-time PCR analysis, leaf tissue from plants grown from SYHT0H2 T_4 generation seed was pooled into a sampling bag and stored at –80°C ± 10°C. This process was repeated for nontransgenic 'Jack' soybean plants (Burgin 2009, Burgin 2011, Nesbitt 2012, and Testerman 2012).

3.4 Genomic DNA Extraction

Genomic DNA used for Southern blot analyses was isolated from the pooled leaf tissue using a modification of the method described by Saghai-Maroof *et al*. (1984).

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, approximately 5 g of this tissue and 25 ml of pre-warmed 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 approximately 60 to 120 minutes at 65°C ± 5°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 30 to 60 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* for 10 minutes, or collected with a sterile loop, and washed once with 70% (v/v) ethanol, and centrifuged at 7277 × *g* for 10 minutes. The DNA pellet was air-dried and dissolved in pre-warmed 0.1X tris-EDTA.

Genomic DNA extractions performed with lyophilized tissue were performed following the method above, with the following exceptions. Plant samples were ground individually into a fine powder in the presence of dry ice and/or liquid nitrogen. A subsample from each homogenous, powdered sample was lyophilized for extraction. Approximately 1 g of lyophilized tissue and 25 ml of pre-warmed CTAB buffer (50 mM tris pH 8.0, 10 mM EDTA pH 8.0, 0.7 M sodium chloride, 1% CTAB [w/v], and 0.1% β-mercaptoethanol [v/v]) were combined into a bottle.

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 triplicate using the Turner Biosystems TBS-380 Mini-Fluorometer.

3.6 Southern Blot Analyses

Southern blot analyses were performed according to 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 to seven hours.

In order to demonstrate the sensitivity of the analyses, each Southern blot analysis included a positive control representing one copy of a DNA fragment of known size in the soybean genome. The positive control 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 amount of positive control:

soybean genome size (bp)	1.10×10^{9}
soybean ploidy	2
DNA loaded in each lane (µg)	3
pSYN15954 plasmid digested with *Kpn*I + *Pme*I size (bp)	10904

The following amount of positive control was calculated:

pSYN15954 plasmid digested with *Kpn*I + *Pme*I (pg)	14.87

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

Following a 10 to 15 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 approximately 30 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 approximately 90 to 180 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 PCR-generated probes (each element-specific probe and 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 with the Invitrogen RadPrime or GE Healthcare Megaprime™ DNA labeling system. The NOS terminator-specific probe was also labeled with alpha-phosphorus-32-deoxyadenosine triphosphate ([α-^{32}P]-dATP). The unincorporated label ([α-^{32}P]-dCTP and α-^{32}P]-dATP) was removed through the use of Bio-Rad Micro Bio-Spin® chromatography columns or GE Healthcare ProbeQuant™ G-50 Micro Columns.

Membranes were incubated in 30 ml of Sigma-Aldrich PerfectHyb™ Plus hybridization buffer with or without denatured calf thymus DNA at 100 µg/ml for at least 30 minutes at 65°C ± 5°C. Both the molecular weight marker-specific probe and either the corresponding element-specific probe or the plasmid pSYN15954 backbone-specific probe were added to the hybridization solution. The membranes were incubated three hours to overnight at 65°C ± 5°C. Incubation was followed by a combination of washes at 65°C ± 5°C in 2X SSC buffer with 0.1% sodium dodecyl sulfate (SDS) buffer and washes at 65°C ± 5°C in 0.1X SSC buffer with 0.1% SDS buffer. Finally, the membranes were subjected to imaging with a Molecular Dynamics Storm 860™ phosphorimager or X-ray film.

3.7 Functional Element Copy Number Analyses Using Element-specific Probes

Seven element-specific probes were used with Southern blot analyses to determine the number of copies of each of the functional elements: (1) an *avhppd-03*-specific probe, (2) a *pat*-specific probe, (3) an *avhppd-03* enhancer complex-specific probe (consisting of tobacco mosaic virus [TMV] enhancer, synthetic minimal plant promoter [SMP], and 35S enhancer sequence), (4) a 35S promoter-specific probe, (5) a cestrum yellow leaf curling virus promoter (CMP) + TMV enhancer-specific probe, (6) a nopaline synthase (NOS) terminator-specific probe, and (7) a figwort mosaic virus (FMV) enhancer-specific probe. Each element probe (except *pat*) covers every base of element present in the plasmid pSYN15954 T-DNA. The pSYN15954 T-DNA consists of two PAT genes, differing by two base pairs. Since there was only a two base pair difference, only one *pat*-specific probe was used. Each Southern blot analysis was performed with genomic DNA extracted from SYHT0H2 T_4 soybean and from nontransgenic 'Jack' soybean, as a negative control to identify possible endogenous soybean DNA sequences that hybridized with the probes. Each analysis also included a positive control (pSYN15954 plasmid digested with *Kpn*I and *Pme*I plus digested nontransgenic 'Jack' soybean DNA).

The Southern blot analysis with the probe specific for the *avhppd-03* enhancer complex was performed by Beckman Coulter Genomics, Inc. (Morrisville, NC, USA). The report submitted by Beckman Coulter Genomics is provided as Appendix A. The Southern blot analyses with all other element specific probes were performed by Syngenta Crop Protection, LLC (Research Triangle Park, NC, USA).

Genomic DNA was analyzed using two restriction enzyme digestion strategies. In the first strategy, soybean genomic DNA was digested with an enzyme that cuts 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 two different enzymes to determine the numbers of copies of the functional elements and the presence or absence of extraneous DNA fragments of the functional elements in other regions of the SYHT0H2 soybean genome. The enzymes used were *Eco*RI, *Mfe*I, *Xcm*I, *Acl*I, or *Pfl*MI. In the second strategy, genomic DNA was digested with enzymes *Kpn*I or *Kpn*I + *Bsr*BI, which releases a DNA fragment(s) of predictable size. This strategy was used to determine the presence or absence of any closely linked extraneous DNA fragments of the functional elements. (Diagrams showing the locations of the restriction sites in the SYHT0H2 insert are provided in the Results and Discussion section.)

Table 3 shows the number of the expected hybridization bands for SYHT0H2 soybean in analyses with the *avhppd-03-*, *pat-*, *avhppd-03* enhancer complex-, 35S promoter-, CMP promoter + TMV enhancer-, NOS terminator-, and FMV enhancer specific probes. Additional, unexpected bands in any of these analyses would indicate the presence of additional copies of these functional elements in the SYHT0H2 soybean genome.

TABLE 3 Expected number of hybridization bands in Southern blot analysis of SYHT0H2 soybean

Probe	Restriction enzyme(s)	Expected number of bands
avhppd-03	*Eco*RI	2
	*Mfe*I	1
	*Kpn*I	1
pat	*Acl*I	2
	*Eco*RI	2
	*Kpn*I + *Bsr*BI	2
avhppd-03 enhancer complex	*Eco*RI	2
	*Xcm*I	2
	*Kpn*I + *Bsr*BI	2
35S promoter	*Eco*RI	2
	*Xcm*I	2
	*Kpn*I + *Bsr*BI	2
CMP promoter + TMV enhancer	*Eco*RI	2
	*Mfe*I	2
	*Kpn*I + *Bsr*BI	2
NOS terminator	*Acl*I	2
	*Eco*RI	2
	*Kpn*I + *Bsr*BI	2
FMV enhancer	*Acl*I	0
	*Pfl*MI	0
	*Kpn*I + *Bsr*BI	0

The FMV enhancer is not present in SYHT0H2 soybean (de Framond 2012); therefore no hybridization bands were expected. Unexpected bands would indicate the presence of this functional element in the SYHT0H2 soybean genome.

No hybridization bands were expected in any of the analyses of genomic DNA from nontransgenic 'Jack' soybean. The positive control (digested DNA from plasmid pSYN15954 equivalent to one copy of a fragment of known size in the soybean genome, plus digested DNA from the nontransgenic 'Jack' soybean) was expected to result in one hybridization band of approximately 5.5 kilobase pairs (kb) in all analyses.

3.8 Southern Blot Analyses Using a Plasmid Backbone-specific Probe

The presence or absence of plasmid backbone sequence in SYHT0H2 soybean was assessed through the use of the plasmid pSYN15954 backbone sequence as a probe in Southern blot analyses of DNA subjected to restriction enzyme digestion with *Acl*I, *Pfl*MI, and *Kpn*I.

The plasmid backbone-specific probe contained every base pair of the plasmid pSYN15954 backbone present outside of the T-DNA. No hybridization bands were expected in any of the analyses of DNA from SYHT0H2 T_4 soybean or nontransgenic 'Jack' soybean. The positive control was expected to result in one hybridization band of approximately 5.4 kb in this analysis.

3.9 Control of Bias Statement

The test and control substances were analyzed on the same Southern blot. A positive control 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 Statement

No statistical analysis was conducted during this study.

4.0 RESULTS AND DISCUSSION

4.1 Functional Element Copy Number: *avhppd-03*-specific Probe

A map of the insert in SYHT0H2 soybean indicating the locations of the *avhppd-03*-specific probe and the restriction sites *Eco*RI, *Mfe*I, and *Kpn*I is shown in Figure 3. Table 4 outlines the expected and observed hybridization band sizes, and Figure 4 depicts the results of the corresponding Southern blot analyses.

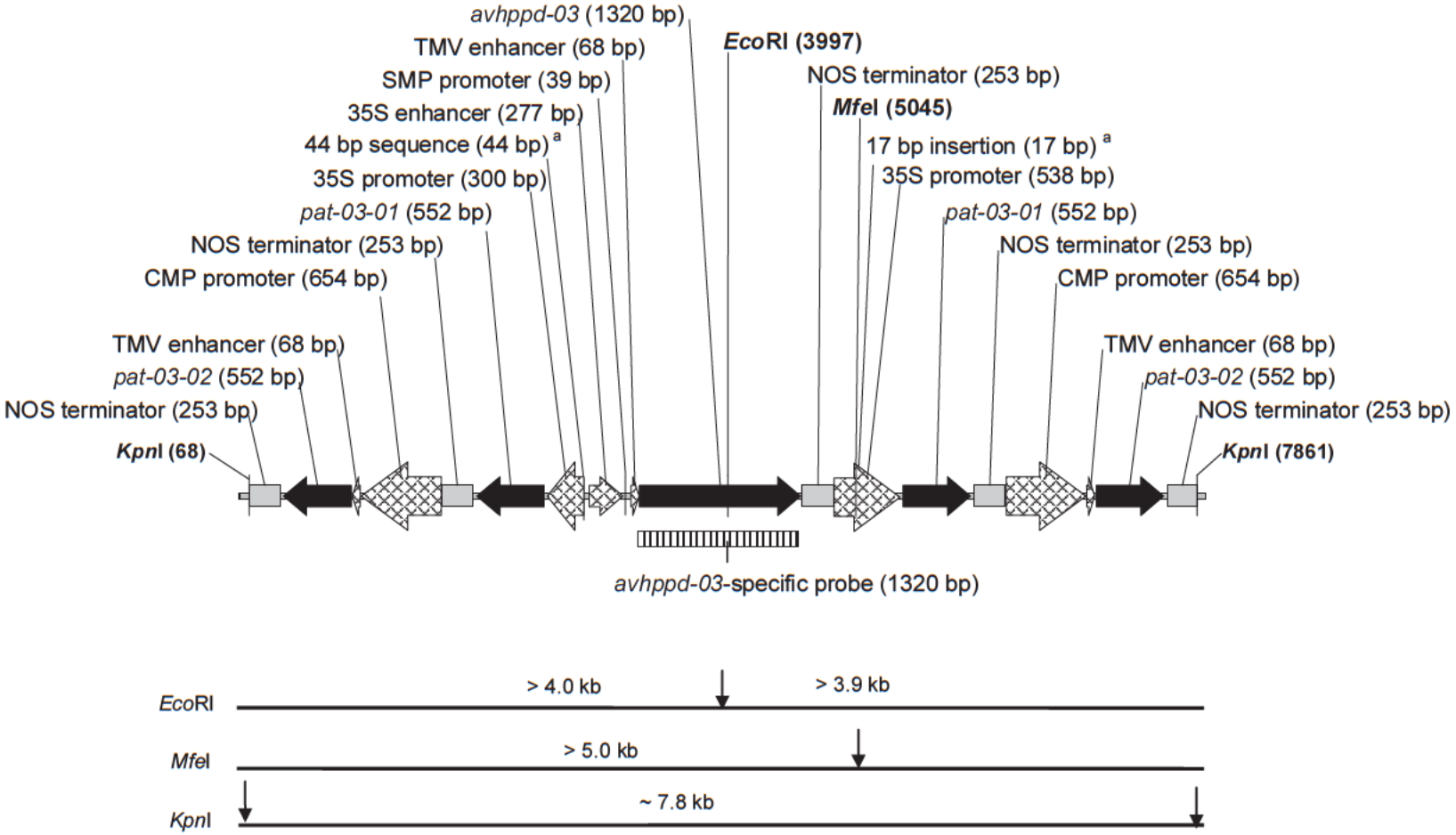
For Southern blot analysis with genomic DNA digested with *Eco*RI, and probed with the *avhppd-03*-specific probe, two hybridization bands of approximately 4.9 and 8.3 kb were observed in the lane containing DNA extracted from SYHT0H2 T_4 soybean (Table 4; Figure 4A, Lane 3). These hybridization bands were absent in the lane containing DNA extracted from nontransgenic 'Jack' soybean (Figure 4A, Lane 4) and were, therefore, specific to the SYHT0H2 insert. As expected, one hybridization band of approximately 5.5 kb was observed in the lane containing the positive control (14.87 pg of plasmid pSYN15954 DNA digested with *Kpn*I and *Pme*I and loaded with DNA extracted from nontransgenic 'Jack' soybean) (Figure 4A, Lane 5).

For Southern blot analysis with genomic DNA digested with *Mfe*I and probed with the *avhppd-03*-specific probe, one hybridization band of approximately 6.2 kb was observed in the lane containing DNA extracted from SYHT0H2 T_4 soybean (Table 4; Figure 4B, Lane 3). This hybridization band was absent in the lane containing DNA extracted from nontransgenic 'Jack' soybean (Figure 4B, Lane 4) and was, therefore, specific to the SYHT0H2 insert. As expected, one hybridization band of approximately 5.3 kb was observed in the lane containing the positive control (14.87 pg of plasmid pSYN15954 DNA digested with *Kpn*I and *Pme*I and loaded with DNA extracted from 'Jack' soybean) (Figure 4B, Lane 5).

For Southern blot analysis with genomic DNA digested with *Kpn*I and probed with the *avhppd-03*-specific probe, one hybridization band of approximately 7.8 kb was observed in the lane containing DNA from SYHT0H2 T_4 soybean (Table 4; Figure 4C, Lane 3). This hybridization band was absent in the lane containing DNA extracted from nontransgenic 'Jack' soybean (Figure 4C, Lane 4) and was, therefore, specific to the SYHT0H2 insert. As expected, one hybridization band of approximately 5.4 kb was observed in the lane containing the positive control (14.87 pg of plasmid pSYN15954 DNA digested with *Kpn*I and *Pme*I and loaded with DNA extracted from nontransgenic 'Jack' soybean) (Figure 4C, Lane 5).

For Southern blot analyses with the *avhppd-03*-specific probe, the expected numbers of hybridization bands were detected with both restriction enzyme digestion strategies. These results demonstrate that SYHT0H2 soybean contains a single copy of the *avhppd-03* sequence. No unexpected bands were detected, indicating that the SYHT0H2 soybean genome contains no extraneous DNA fragments of the *avhppd-03* sequence.

FIGURE 3 Locations of the 1320 bp *avhppd-03*-specific probe and the *Eco*RI, *Mfe*I, and *Kpn*I restriction sites in the SYHT0H2 soybean insert



The vertical arrows indicate the sites of restriction digestion. The sizes of the expected restriction fragments are indicated.
[a] The presence of a 44 bp sequence and a 17 bp insertion was determined during insert sequence analysis (de Framond 2012).

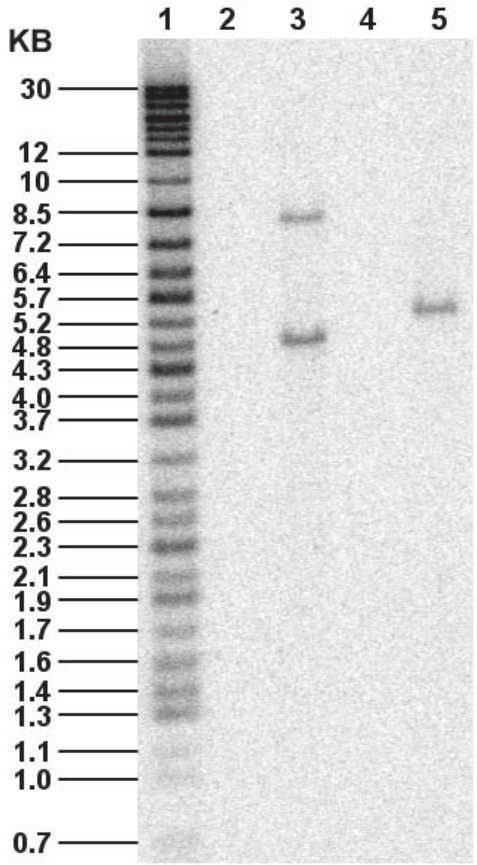
TABLE 4 Expected and observed hybridization bands in Southern blot analyses of SYHT0H2 soybean, with the *avhppd-03*-specific probe and restriction enzymes *Eco*RI, *Mfe*I, and *Kpn*I

Lane	Source of DNA	Restriction enzyme	Expected number of bands	Expected band size (kb)	Observed band size (kb)
Figure 4A, 3	SYHT0H2 T_4	*Eco*RI	2	>4.0, >3.9	~4.9, ~8.3
Figure 4A, 4	'Jack'	*Eco*RI	0	N/A	N/A
Figure 4A, 5	positive control ('Jack' digested with *Eco*RI and 14.87 pg of pSYN15954 digest [*Kpn*I + *Pme*I])	*Eco*RI	1	~5.5	~5.5
Figure 4B, 3	SYHT0H2 T_4	*Mfe*I	1	>5.0	~6.2
Figure 4B, 4	'Jack'	*Mfe*I	0	N/A	N/A
Figure 4B, 5	positive control ('Jack' digested with *Mfe*I and 14.87 pg of pSYN15954 digest [*Kpn*I + *Pme*I])	*Mfe*I	1	~5.5	~5.3[a]
Figure 4C, 3	SYHT0H2 T_4	*Kpn*I	1	~7.8	~7.8
Figure 4C, 4	'Jack'	*Kpn*I	0	N/A	N/A
Figure 4C, 5	positive control ('Jack' digested with *Kpn*I and 14.87 pg of pSYN15954 digest [*Kpn*I + *Pme*I])	*Kpn*I	1	~5.5	~5.4[a]

N/A = not applicable.
[a] The difference between the observed and expected size is within the accepted variability for Southern blot analysis.

FIGURE 4 **Functional element copy number Southern blot analysis of SYHT0H2 soybean with the 1320 bp *avhppd-03*-specific probe, using the restriction enzymes *Eco*RI, *Mfe*I, and *Kpn*I**

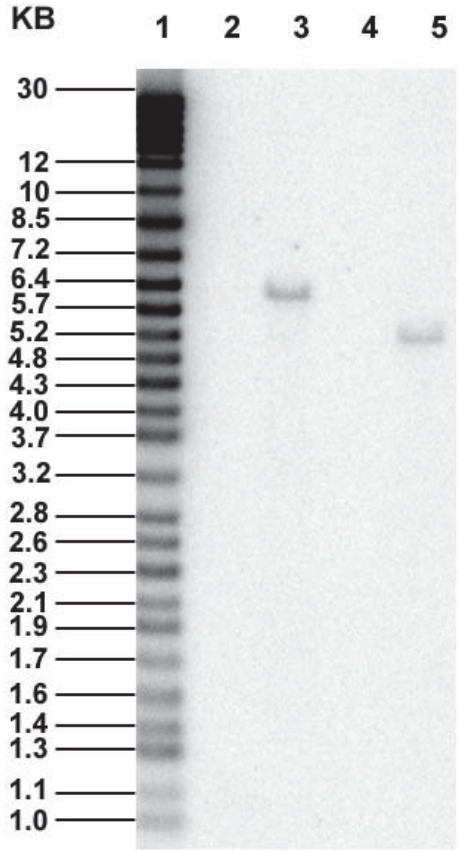
(A) ***Eco*RI**



Lane 1 = molecular weight markers.
Lane 2 = blank.
Lane 3 = SYHT0H2 T_4 digested with *Eco*RI.
Lane 4 = 'Jack' digested with *Eco*RI.
Lane 5 = positive control ('Jack' digested with *Eco*RI plus 14.87 pg of the pSYN15954 digest [*Kpn*I + *Pme*I]).

FIGURE 4 Functional element copy number Southern blot analysis of SYHT0H2 soybean with the 1320 bp *avhppd-03*-specific probe, using the restriction enzymes *Eco*RI, *Mfe*I, and *Kpn*I (Continued)

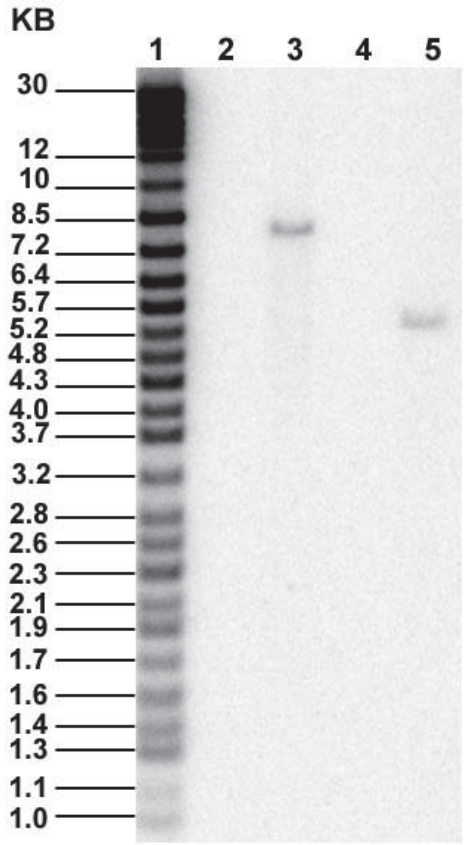
(B) *Mfe*I



Lane 1 = molecular weight markers.
Lane 2 = blank.
Lane 3 = SYTH0H2 T_4 digested with *Mfe*I.
Lane 4 = 'Jack' digested with *Mfe*I.
Lane 5 = positive control ('Jack' digested with *Mfe*I plus 14.87 pg of the pSYN15954 digest [*Kpn*I + *Pme*I]).

FIGURE 4 Functional element copy number Southern blot analysis of SYHT0H2 soybean with the 1320 bp *avhppd-03*-specific probe, using the restriction enzymes *Eco*RI, *Mfe*I, and *Kpn*I (Continued)

(C) *Kpn*I



Lane 1 = molecular weight markers.
Lane 2 = blank.
Lane 3 = SYHT0H2 T_4 digested with *Kpn*I.
Lane 4 = 'Jack' digested with *Kpn*I.
Lane 5 = positive control ('Jack' digested with *Kpn*I plus 14.87 pg of the pSYN15954 digest [*Kpn*I + *Pme*I]).

4.2 Functional Element Copy Number: *pat*-specific Probe

A map of the insert in SYHT0H2 soybean indicating the locations of the *pat*-specific probe and the restriction sites *Acl*I, *Eco*RI, *Kpn*I, and *Bsr*BI is shown in Figure 5. Table 5 outlines the expected and observed hybridization band sizes, and Figure 6 shows the results of the corresponding Southern blot analyses.

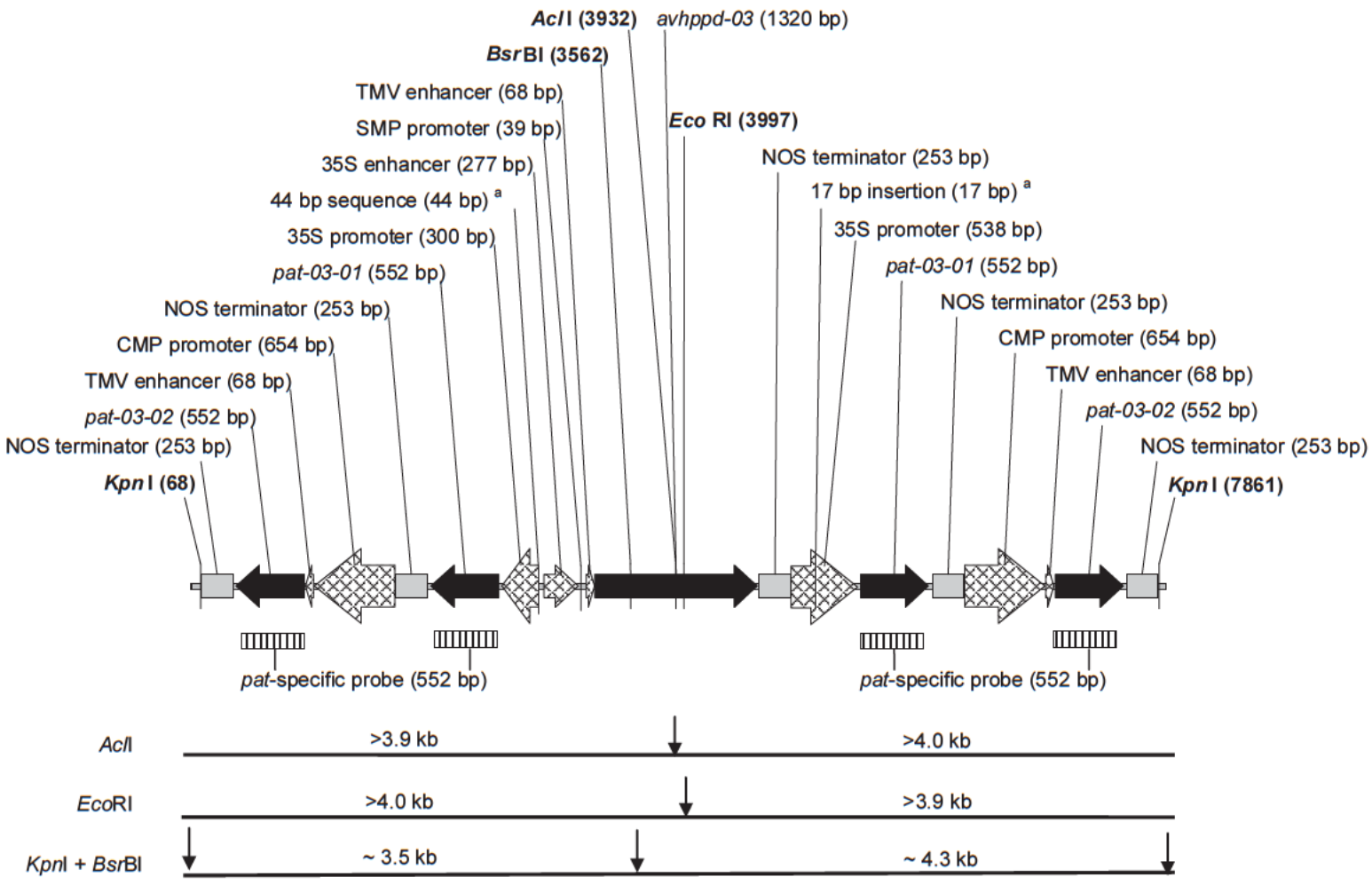
For Southern blot analysis with genomic DNA digested with *Acl*I, and probed with the *pat*-specific probe, two hybridization bands of approximately 7.6 kb and 10 kb were observed in the lane containing DNA extracted from SYHT0H2 T_4 soybean (Table 5; Figure 6A, Lane 3). These hybridization bands were absent in the lane containing DNA extracted from nontransgenic 'Jack' soybean (Figure 6A, Lane 4) and were, therefore, specific to the SYHT0H2 insert. As expected, one band of approximately 5.3 kb was observed in the lane containing the positive control (14.87 pg of plasmid pSYN15954 DNA digested with *Kpn*I and *Pme*I and loaded with DNA extracted from nontransgenic 'Jack' soybean) (Figure 6A, Lane 5).

For Southern blot analysis with genomic DNA digested with *Eco*RI and probed with the *pat*-specific probe, two hybridization bands of approximately 4.8 kb and 8.3 kb were observed in the lane containing DNA extracted from SYHT0H2 T_4 soybean (Table 5; Figure 6B, Lane 3). These hybridization bands were absent in the lane containing DNA extracted from nontransgenic 'Jack' soybean (Figure 6B, Lane 4) and were, therefore, specific to the SYHT0H2 insert. As expected, one band of approximately 5.3 kb was observed in the lane containing the positive control (14.87 pg of plasmid pSYN15954 DNA digested with *Kpn*I and *Pme*I and loaded with DNA extracted from nontransgenic 'Jack' soybean) (Figure 6B, Lane 5).

For Southern blot analysis with genomic DNA digested with *Kpn*I+ *Bsr*BI and probed with the *pat*-specific probe, two hybridization bands of approximately 3.5 kb and 4.3 kb were observed in the lane containing DNA extracted from SYHT0H2 T_4 soybean (Table 5; Figure 6C, Lane 3). These hybridization bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure 6C, Lane 4) and were, therefore specific to the SYHT0H2 insert. As expected, one band of approximately 5.4 kb was observed in the lane containing the positive control (14.87 pg of plasmid pSYN15954 DNA digested with *Kpn*I and *Pme*I and loaded with DNA extracted from nontransgenic 'Jack' soybean) (Figure 6C, Lane 5).

For Southern blot analyses with the *pat*-specific probe, the expected numbers of hybridization bands were detected with both restriction enzyme digestion strategies. These results support insert sequence analysis that determined SYHT0H2 soybean contains four copies of *pat* sequence (de Framond 2012). No unexpected bands were detected, indicating that the SYHT0H2 soybean genome contains no extraneous DNA fragments of *pat* sequence.

FIGURE 5 **Locations of the 552 bp *pat*-specific probe and the *Acl*I, *Eco*RI, *Kpn*I, and *Bsr*BI restriction sites in the SYHT0H2 soybean insert**



The vertical arrows indicate the sites of restriction digestion. The sizes of the expected restriction fragments are indicated.
[a] The presence of a 44 bp sequence and a 17 bp insertion was determined during insert sequence analysis (de Framond 2012).

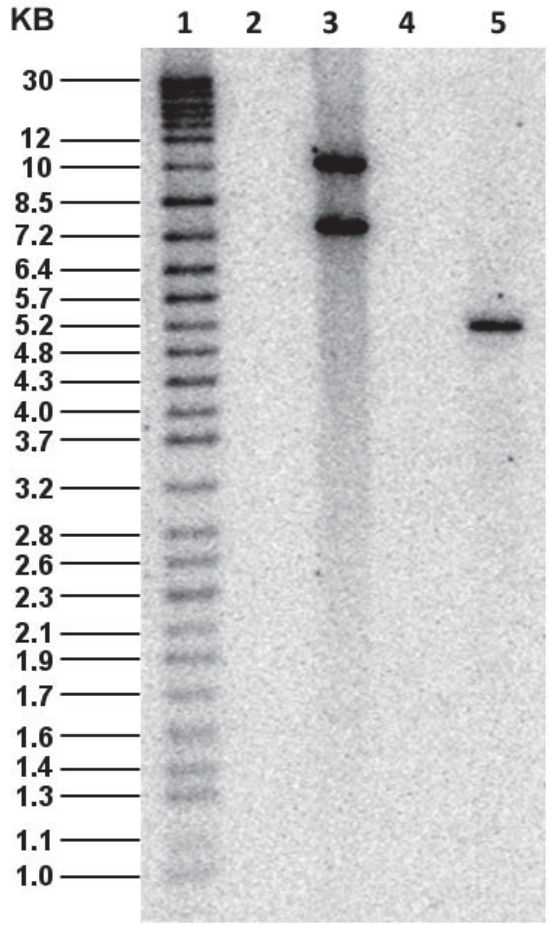
TABLE 5 Expected and observed hybridization bands in Southern blot analyses of SYHT0H2 soybean, with the *pat*-specific probe and restriction enzymes *Acl*I, *Eco*RI, and *Kpn*I + *Bsr*BI

Lane	Source of DNA	Restriction enzyme	Expected number of bands	Expected band size (kb)	Observed band size (kb)
Figure 6A, 3	SYHT0H2 T_4	*Acl*I	2	>3.9, >4.0	~7.6, ~10
Figure 6A, 4	'Jack'	*Acl*I	0	N/A	N/A
Figure 6A, 5	positive control ('Jack' digested with *Acl*I plus 14.87 pg of pSYN15954 digest [*Kpn*I+ *Pme*I])	*Acl*I	1	~5.5	~5.3[a]
Figure 6B, 3	SYHT0H2 T_4	*Eco*RI	2	>4.0, >3.9	~4.8, ~8.3
Figure 6B, 4	'Jack'	*Eco*RI	0	N/A	N/A
Figure 6B, 5	positive control ('Jack' digested with *Eco*RI plus 14.87 pg of pSYN15954 digest [*Kpn*I+ *Pme*I])	*Eco*RI	1	~5.5	~5.3[a]
Figure 6C, 3	SYHT0H2 T_4	*Kpn*I + *Bsr*BI	2	~3.5, ~4.3	~3.5, ~4.3
Figure 6C, 4	'Jack'	*Kpn*I+ *Bsr*BI	0	N/A	N/A
Figure 6C, 5	positive control ('Jack' digested with *Kpn*I + *Bsr*BI and 14.87 pg of pSYN15954 digest [*Kpn*I + *Pme*I])	*Kpn*I + *Bsr*BI	1	~5.5	~5.4[a]

N/A = not applicable.
[a] The difference between the observed and expected size is within the accepted variability for Southern blot analysis.

FIGURE 6 **Functional element copy number Southern blot analysis of SYHT0H2 soybean with the 552 bp *pat*-specific probe, using the restriction enzymes *Acl*I, *Eco*RI, and *Kpn*I + *Bsr*BI**

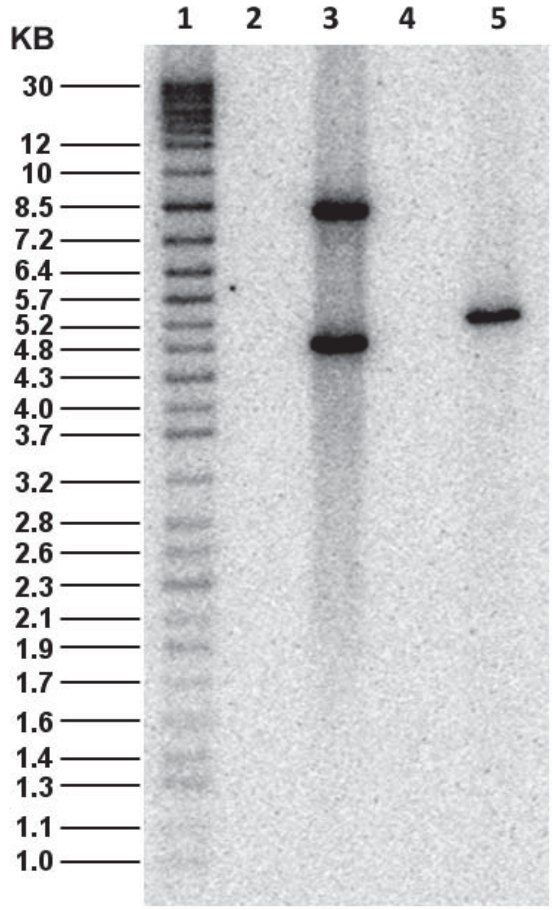
(A) ***Acl*I**



Lane 1 = molecular weight markers.
Lane 2 = blank.
Lane 3 = SYHT0H2 T_4 digested with *Acl*I.
Lane 4 = 'Jack' digested with *Acl*I.
Lane 5 = positive control ('Jack' digested with *Acl*I plus 14.87 pg of the pSYN15954 digest [*Kpn*I + *Pme*I]).

FIGURE 6 Functional element copy number Southern blot analysis of SYHT0H2 soybean with the 552 bp *pat*-specific probe, using the restriction enzymes *Acl*I, *Eco*RI, and *Kpn*I + *Bsr*BI (Continued)

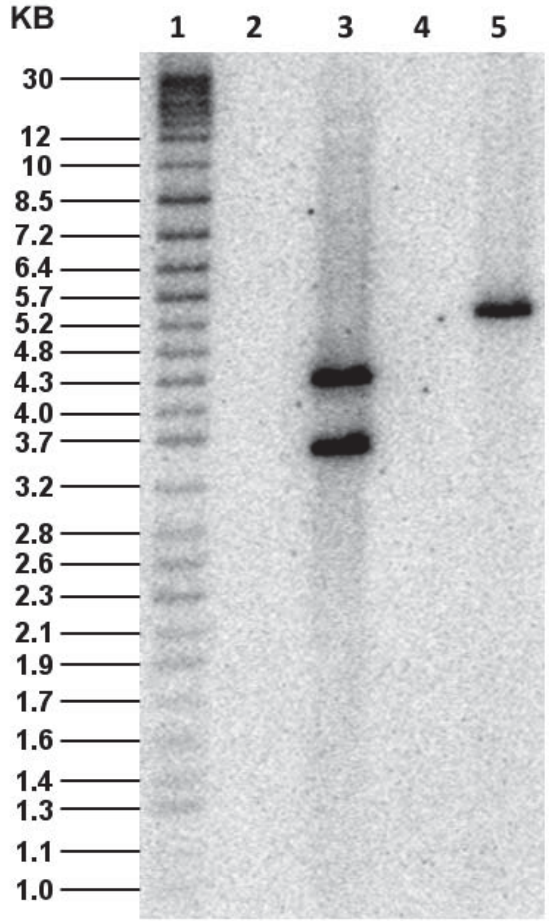
(B) *Eco*RI



Lane 1 = molecular weight markers.
Lane 2 = blank.
Lane 3 = SYHT0H2 T_4 digested with *Eco*RI.
Lane 4 = 'Jack' digested with *Eco*RI.
Lane 5 = positive control ('Jack' digested with *Eco*RI plus 14.87 pg of the pSYN15954 digest [*Kpn*I + *Pme*I]).

FIGURE 6 Functional element copy number Southern blot analysis of SYHT0H2 soybean with the 552 bp *pat*-specific probe, using the restriction enzymes *Acl*I, *Eco*RI, and *Kpn*I + *Bsr*BI (Continued)

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



Lane 1 = molecular weight markers.
Lane 2 = blank.
Lane 3 = SYHT0H2 T_4 digested with *Kpn*I + *Bsr*BI.
Lane 4 = 'Jack' digested with *Kpn*I + *Bsr*BI.
Lane 5 = positive control ('Jack' digested with *Kpn*I + *Bsr*BI plus 14.87 pg of the pSYN15954 digest [*Kpn*I + *Pme*I]).

4.3 Functional Element Copy Number: *avhppd-03* Enhancer Complex-specific Probe

A map of the insert in SYHT0H2 soybean indicating the locations of the *avhppd-03* enhancer complex-specific probe and the restriction sites *Eco*RI, *Xcm*I, *Kpn*I, and *Bsr*BI is shown in Figure 7. Table 6 outlines the expected and observed hybridization band sizes, and Figure 8 shows the results of the corresponding Southern blot analyses.

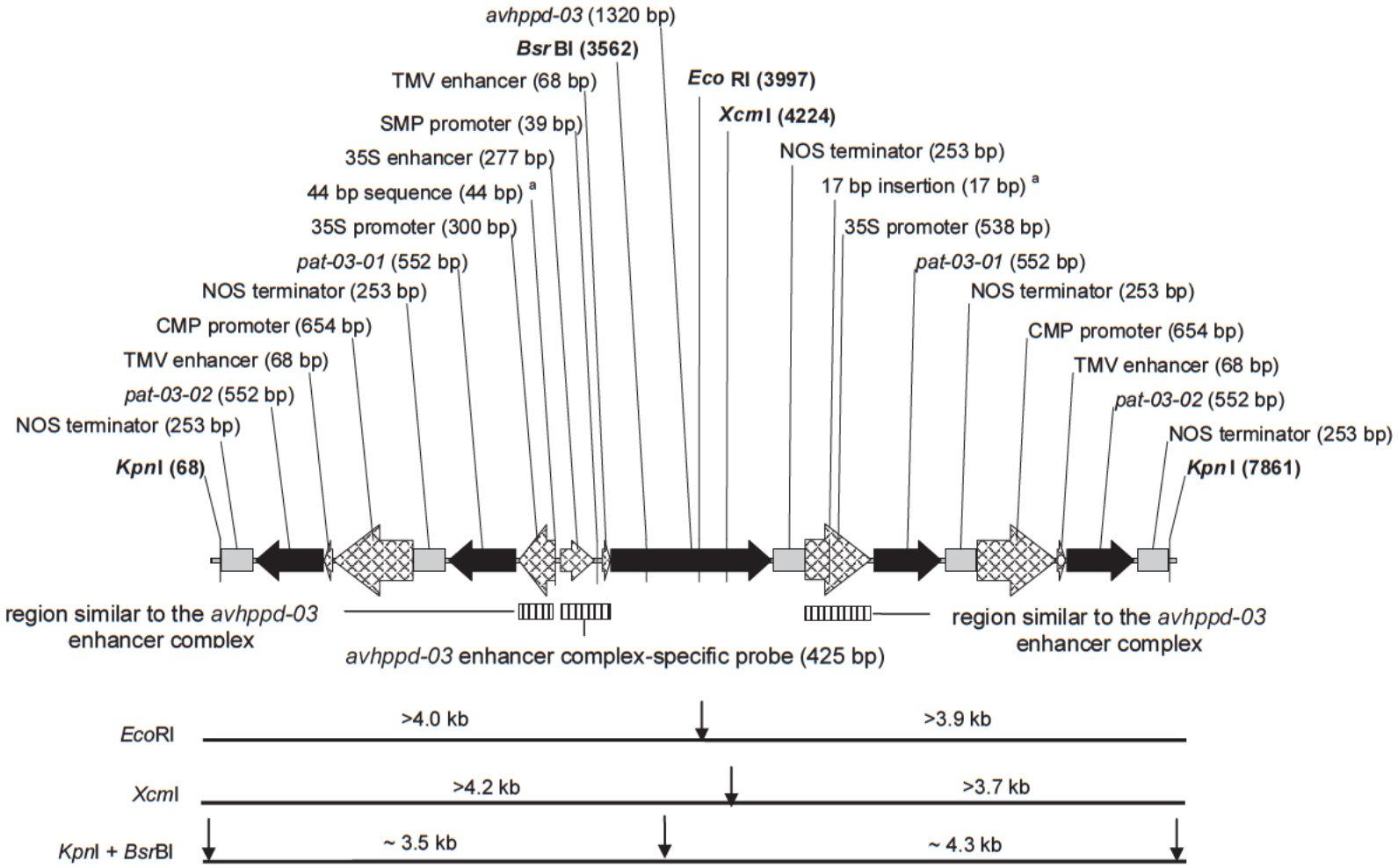
For Southern blot analysis with genomic DNA digested with *Eco*RI, and probed with the *avhppd-03* enhancer complex-specific probe, two hybridization bands of approximately 4.8 kb and 8.2 kb were observed in the lane containing DNA extracted from SYHT0H2 T_4 soybean (Table 6; Figure 8A, Lane 3). These hybridization bands were absent in the lane containing DNA extracted from nontransgenic 'Jack' soybean (Figure 8A, Lane 4) and were, therefore, specific to the SYHT0H2 insert. As expected, one band of approximately 5.3 kb was observed in the lane containing the positive control (14.87 pg of plasmid pSYN15954 DNA digested with *Kpn*I and *Pme*I and loaded with DNA extracted from nontransgenic 'Jack' soybean) (Figure 8A, Lane 5).

For Southern blot analysis with genomic DNA digested with *Xcm*I and probed with the *avhppd-03* enhancer complex-specific probe, two hybridization bands of approximately 4.3 kb and 5.7 kb were observed in the lane containing DNA extracted from SYHT0H2 T_4 soybean (Table 6; Figure 8B, Lane 3). These hybridization bands were absent in the lane containing DNA extracted from nontransgenic 'Jack' soybean (Figure 8B, Lane 4) and were, therefore, specific to the SYHT0H2 insert. As expected, one band of approximately 5.3 kb was observed in the lane containing the positive control (14.87 pg of plasmid pSYN15954 DNA digested with *Kpn*I and *Pme*I and loaded with DNA extracted from nontransgenic 'Jack' soybean) (Figure 8B, Lane 5).

For Southern blot analysis with genomic DNA digested with *Kpn*I + *Bsr*BI and probed with the *avhppd-03* enhancer complex-specific probe, two hybridization bands of approximately 3.5 kb and 4.3 kb were observed in the lane containing DNA extracted from SYHT0H2 T_4 soybean (Table 6; Figure 8C, Lane 3). These hybridization bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure 8C, Lane 4) and were, therefore specific to the SYHT0H2 insert. As expected, one band of approximately 5.3 kb was observed in the lane containing the positive control (14.87 pg of plasmid pSYN15954 DNA digested with *Kpn*I and *Pme*I and loaded with DNA extracted from nontransgenic 'Jack' soybean) (Figure 8C, Lane 5).

For Southern blot analyses with the *avhppd-03* enhancer complex-specific probe, two hybridization bands specific to SYHT0H2 soybean were detected with each restriction digestion enzyme strategy. Only one copy of the *avhppd-03* enhancer complex is present in SYHT0H2 soybean (de Framond 2012); however, because of sequence similarity between the 35S enhancer and SMP promoter (elements in the *avhppd-03* enhancer complex) and the 35S promoter, analyses with the *avhppd-03* enhancer complex-specific probe were expected to result in two hybridization bands, one corresponding to a copy of the 35S promoter in SYHT0H2 soybean. These results demonstrate that SYHT0H2 soybean contains a single copy of the *avhppd-03* enhancer complex sequence. No unexpected bands were detected, indicating that the SYHT0H2 soybean genome contains no extraneous DNA fragments of the *avhppd-03* enhancer complex sequence.

FIGURE 7 **Locations of the 425 bp *avhppd-03* enhancer complex-specific probe and the *Eco*RI, *Xcm*I, *Kpn*I, and *Bsr*BI restriction sites in the SYHT0H2 soybean insert**



The vertical arrows indicate the sites of restriction digestion. The sizes of the expected restriction fragments are indicated.
[a] The presence of a 44 bp sequence and a 17 bp insertion was determined during insert sequence analysis (de Framond 2012).

TABLE 6 Expected and observed hybridization bands in Southern blot analyses of SYHT0H2 soybean, with the *avhppd-03* enhancer complex-specific probe and restriction enzymes *Eco*RI, *Xcm*I, and *Kpn*I + *Bsr*BI

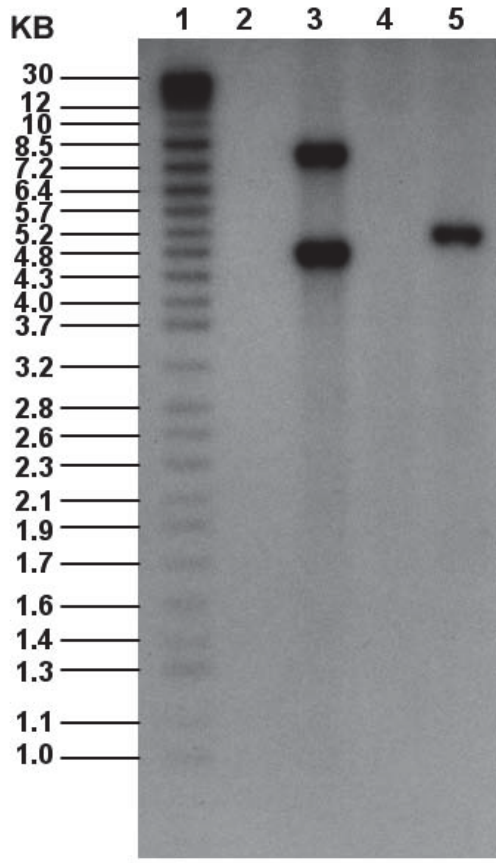
Lane	Source of DNA	Restriction enzyme	Expected number of bands	Expected band size (kb)	Observed band size (kb)
Figure 8A, 3	SYHT0H2 T_4	*Eco*RI	2	>4.0, >3.9	~4.8, ~8.2
Figure 8A, 4	'Jack'	*Eco*RI	0	N/A	N/A
Figure 8A, 5	positive control ('Jack' digested with *Eco*RI plus 14.87 pg of pSYN15954 digest [*Kpn*I + *Pme*I])	*Eco*RI	1	~5.5	~5.3[a]
Figure 8B, 3	SYHT0H2 T_4	*Xcm*I	2	>4.2, >3.7	~4.3, ~5.7
Figure 8B, 4	'Jack'	*Xcm*I	0	N/A	N/A
Figure 8B, 5	positive control ('Jack' digested with *Xcm*I plus 14.87 pg of pSYN15954 digest [*Kpn*I + *Pme*I])	*Xcm*I	1	~5.5	~5.3[a]
Figure 8C, 3	SYHT0H2 T_4	*Kpn*I + *Bsr*BI	2	~3.5, ~4.3	~3.5, ~4.3
Figure 8C, 4	'Jack'	*Kpn*I + *Bsr*BI	0	N/A	N/A
Figure 8C, 5	positive control ('Jack' digested with *Kpn*I + *Bsr*BI and 14.87 pg of pSYN15954 digest [*Kpn*I + *Pme*I])	*Kpn*I + *Bsr*BI	1	~5.5	~5.3[a]

N/A = not applicable.

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

FIGURE 8 **Functional element copy number Southern blot analysis of SYHT0H2 soybean with the 425 bp *avhppd-03* enhancer complex-specific probe, using the restriction enzymes *Eco*RI, *Xcm*I, and *Kpn*I + *Bsr*BI**

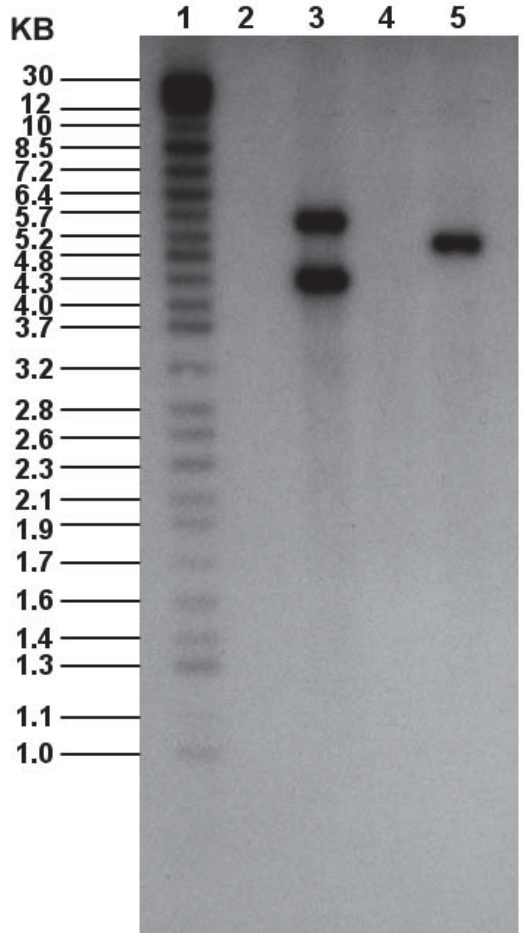
(A) ***Eco*RI**



Lane 1 = molecular weight markers.
Lane 2 = blank.
Lane 3 = SYHT0H2 T_4 digested with *Eco*RI.
Lane 4 = 'Jack' digested with *Eco*RI.
Lane 5 = positive control ('Jack' digested with *Eco*RI plus 14.87 pg of the pSYN15954 digest [*Kpn*I + *Pme*I]).

FIGURE 8 **Functional element copy number Southern blot analysis of SYHT0H2 soybean with the 425 bp *avhppd-03* enhancer complex-specific probe, using the restriction enzymes *Eco*RI, *Xcm*I, and *Kpn*I + *Bsr*BI (Continued)**

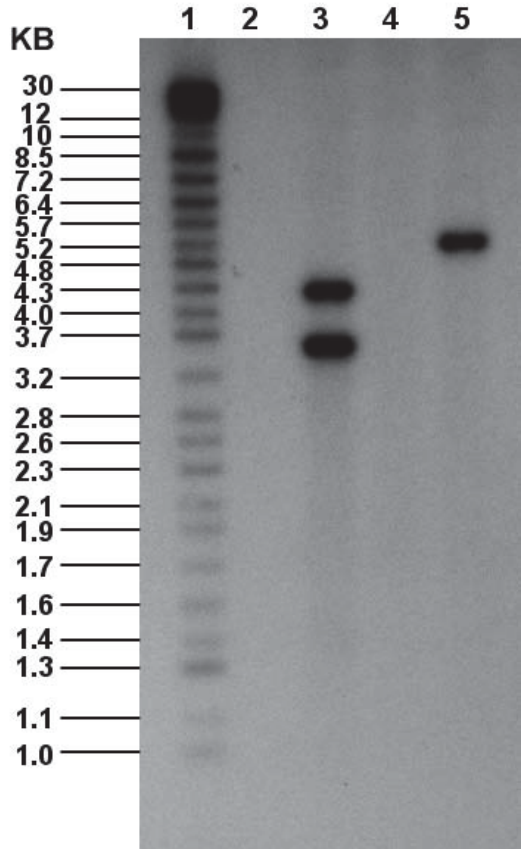
(B) ***Xcm*I**



Lane 1 = molecular weight markers.
Lane 2 = blank.
Lane 3 = SYHT0H2 T_4 digested with *Xcm*I.
Lane 4 = 'Jack' digested with *Xcm*I.
Lane 5 = positive control ('Jack' digested with *Xcm*I plus 14.87 pg of the pSYN15954 digest [*Kpn*I + *Pme*I]).

FIGURE 8 Functional element copy number Southern blot analysis of SYHT0H2 soybean with the 425 bp *avhppd-03* enhancer complex-specific probe, using the restriction enzymes *Eco*RI, *Xcm*I, and *Kpn*I + *Bsr*BI (Continued)

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



Lane 1 = molecular weight markers.
Lane 2 = blank.
Lane 3 = SYHT0H2 T_4 digested with *Kpn*I + *Bsr*BI.
Lane 4 = 'Jack' digested with *Kpn*I + *Bsr*BI.
Lane 5 = positive control ('Jack' digested with *Kpn*I + *Bsr*BI plus 14.87 pg of the pSYN15954 digest [*Kpn*I + *Pme*I]).

4.4 Functional Element Copy Number: 35S Promoter-specific Probe

A map of the insert in SYHT0H2 soybean indicating the locations of the 35S promoter-specific probe and the restriction sites *Eco*RI, *Xcm*I, *Kpn*I, and *Bsr*BI is shown in Figure 9. Table 7 outlines the expected and observed hybridization band sizes, and Figure 10 shows the results of the corresponding Southern blot analyses.

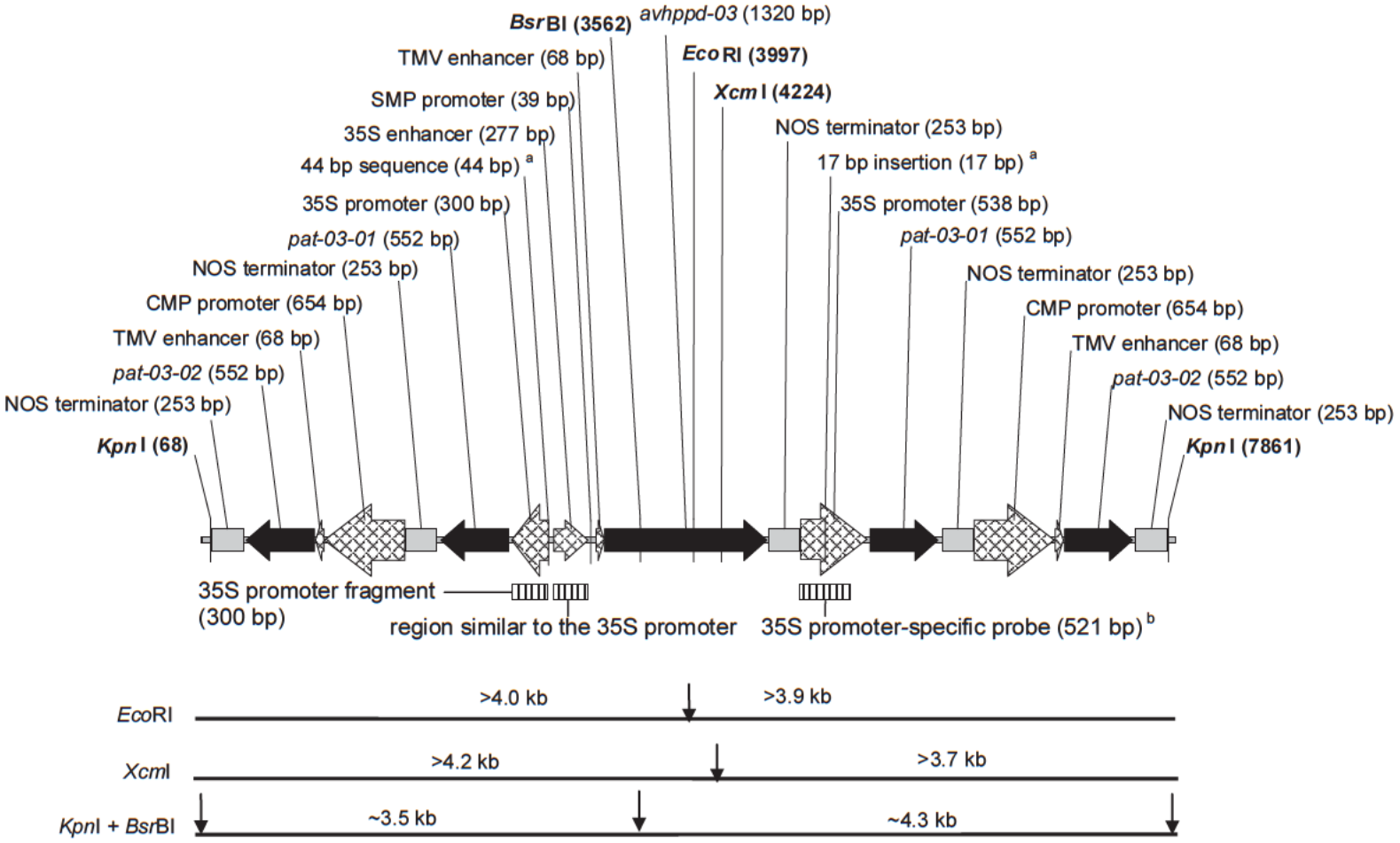
For Southern blot analysis with genomic DNA digested with *Eco*RI and probed with the 35S promoter-specific probe, two hybridization bands of approximately 4.8 kb and 8.3 kb were observed in the lane containing DNA extracted from SYHT0H2 T_4 soybean (Table 7; Figure 10A, Lane 3). These hybridization bands were absent in the lane containing DNA extracted from the nontransgenic 'Jack' soybean (Figure 10A, Lane 4) and were, therefore, specific to the SYHT0H2 insert. As expected, one hybridization band of approximately 5.3 kb was observed in the lane containing the positive control (14.87 pg of plasmid pSYN15954 DNA digested with *Kpn*I and *Pme*I and loaded with DNA extracted from nontransgenic 'Jack' soybean) (Figure 10A, Lane 5).

For Southern blot analysis with genomic DNA digested with *Xcm*I and probed with the 35S promoter-specific probe, two hybridization bands of approximately 4.3 kb and 5.7 kb were observed in the lane containing DNA extracted from SYHT0H2 T_4 soybean (Table 7; Figure 10B, Lane 3). These hybridization bands were absent in the lane containing DNA extracted from the nontransgenic 'Jack' soybean (Figure 10B, Lane 4) and were, therefore, specific to the SYHT0H2 insert. As expected, one hybridization band of approximately 5.3 kb was observed in the lane containing the positive control (14.87 pg of plasmid pSYN15954 DNA digested with *Kpn*I and *Pme*I and loaded with DNA extracted from nontransgenic 'Jack' soybean) (Figure 10B, Lane 5).

For Southern blot analysis with genomic DNA digested with *Kpn*I + *Bsr*BI and probed with the 35S promoter-specific probe, two hybridization bands of approximately 3.5 kb and 4.3 kb were observed in the lane containing DNA extracted from SYHT0H2 T_4 soybean (Table 7; Figure 10C, Lane 3). These hybridization bands were absent in the lane containing DNA extracted from the nontransgenic 'Jack' soybean (Figure 10C, Lane 4) and were, therefore, specific to the SYHT0H2 insert. As expected, one band of approximately 5.4 kb was observed in the lane containing the positive control (14.87 pg of plasmid pSYN15954 DNA digested with *Kpn*I and *Pme*I and loaded with DNA extracted from nontransgenic 'Jack' soybean) (Figure 10C, Lane 5).

For Southern blot analyses with the 35S promoter-specific probe, two hybridization bands specific to SYHT0H2 soybean were detected with each restriction digestion enzyme strategy. These results demonstrate that SYHT0H2 soybean contains two copies of 35S promoter sequence. No unexpected bands were detected, indicating that the SYHT0H2 soybean contains no extraneous DNA fragments of the 35S promoter sequence.

FIGURE 9 **Locations of the 521 bp 35S promoter-specific probe and the *Eco*RI, *Xcm*I, *Kpn*I, and *Bsr*BI restriction sites in SYHT0H2 soybean**



The vertical arrows indicate the sites of restriction digestion. The sizes of the expected restriction fragments are indicated.
[a] The presence of a 44 bp sequence and a 17 bp insertion was determined during insert sequence analysis (de Framond 2012).
[b] The 35S promoter-specific probe sequence is as it exists in the pSYN15954 T-DNA and does not contain the 17 base pair insertion that exists in SYHT0H2.

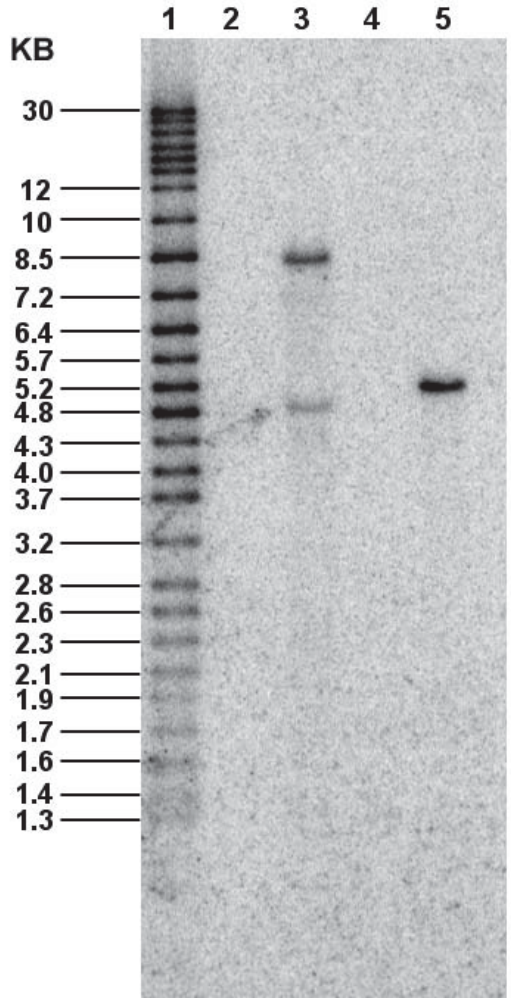
TABLE 7 Expected and observed hybridization bands in Southern blot analyses of SYHT0H2 soybean, with the 35S promoter-specific probe and restriction enzymes *Eco*RI, *Xcm*I, and *Kpn*I + *Bsr*BI

Lane	Source of DNA	Restriction enzyme	Expected number of bands	Expected band size (kb)	Observed band size (kb)
Figure 10A, 3	SYHT0H2 T_4	*Eco*RI	2	>4.0, >3.9	~4.8, ~8.3
Figure 10A, 4	'Jack'	*Eco*RI	0	N/A	N/A
Figure 10A, 5	positive control ('Jack' digested with *Eco*RI plus 14.87 pg of pSYN15954 digest [*Kpn*I + *Pme*I])	*Eco*RI	1	~5.5	~5.3[a]
Figure 10B, 3	SYHT0H2 T_4	*Xcm*I	2	>4.2, >3.7	~4.3, ~5.7
Figure 10B, 4	'Jack'	*Xcm*I	0	N/A	N/A
Figure 10B, 5	positive control ('Jack' digested with *Xcm*I plus 14.87 pg of pSYN15954 digest [*Kpn*I + *Pme*I])	*Xcm*I	1	~5.5	~5.3[a]
Figure 10C, 3	SYHT0H2 T_4	*Kpn*I + *Bsr*BI	2	~3.5, ~4.3	~3.5, ~4.3
Figure 10C, 4	'Jack'	*Kpn*I + *Bsr*BI	0	N/A	N/A
Figure 10C, 5	positive control ('Jack' digested with *Kpn*I + *Bsr*BI and 14.87 pg of pSYN15954 digest [*Kpn*I + *Pme*I])	*Kpn*I + *Bsr*BI	1	~5.5	~5.4[a]

N/A = not applicable.
[a] The difference between the observed and expected size is within the accepted variability for Southern blot analysis.

FIGURE 10 **Functional element copy number Southern blot analysis of SYHT0H2 soybean with the 521 bp 35S promoter-specific probe, using the restriction enzymes *Eco*RI, *Xcm*I, and *Kpn*I + *Bsr*BI**

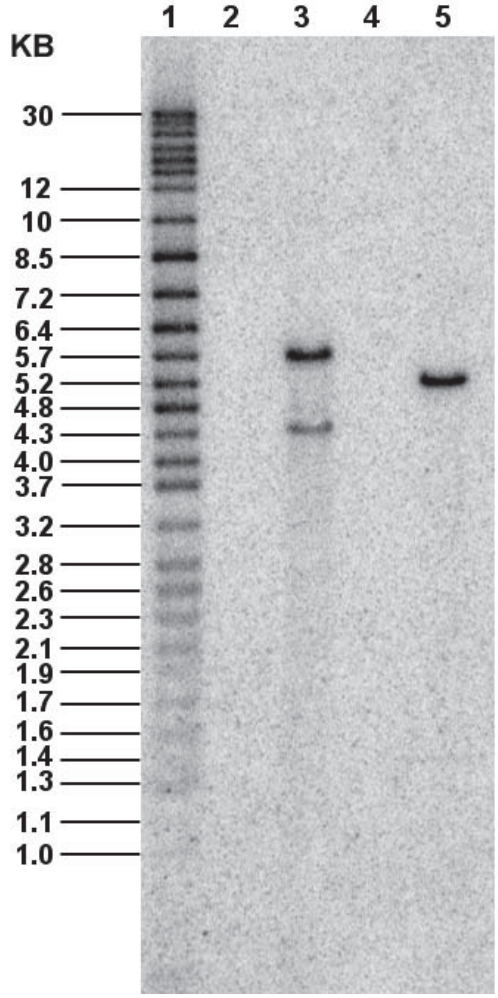
(A) ***Eco*RI**



Lane 1 = molecular weight markers.
Lane 2 = blank.
Lane 3 = SYHT0H2 T_4 digested with *Eco*RI.
Lane 4 = 'Jack' digested with *Eco*RI.
Lane 5 = positive control ('Jack' digested with *Eco*RI plus 14.87 pg of the pSYN15954 digest [*Kpn*I + *Pme*I]).

FIGURE 10 Functional element copy number Southern blot analysis of SYHT0H2 soybean with the 521 bp 35S promoter-specific probe, using the restriction enzyme *Eco*RI, *Xcm*I, and *Kpn*I + *Bsr*BI (Continued)

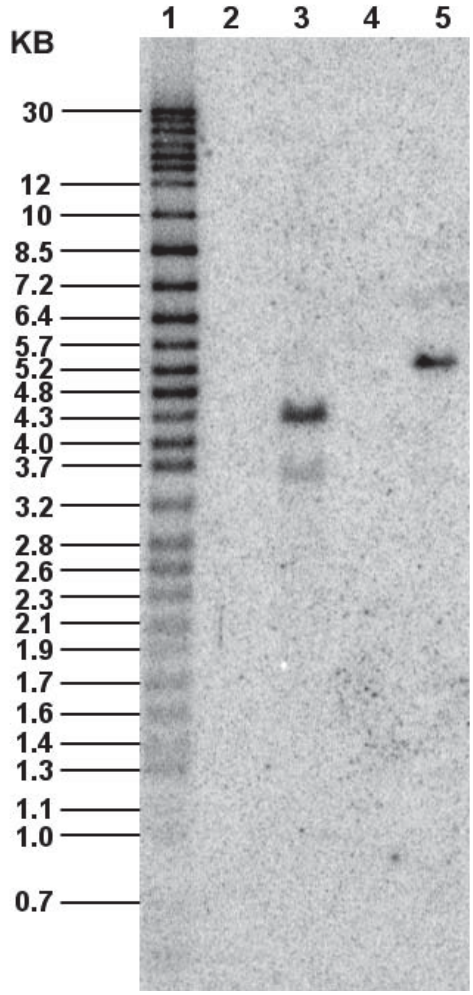
(B) *Xcm*I



Lane 1 = molecular weight markers.
Lane 2 = blank.
Lane 3 = SYHT0H2 T_4 digested with *Xcm*I.
Lane 4 = 'Jack' DNA digested with *Xcm*I.
Lane 5 = positive control ('Jack' digested with *Xcm*I plus 14.87 pg of the pSYN15954 digest [*Kpn*I + *Pme*I]).

FIGURE 10 Functional element copy number Southern blot analysis of SYHT0H2 soybean with the 521 bp 35S promoter-specific probe, using the restriction enzymes *Eco*RI, *Xcm*I, and *Kpn*I + *Bsr*BI (Continued)

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



Lane 1 = molecular weight markers.
Lane 2 = blank.
Lane 3 = SYHT0H2 T_4 digested with *Kpn*I + *Bsr*BI.
Lane 4 = 'Jack' DNA digested with *Kpn*I + *Bsr*BI.
Lane 5 = positive control ('Jack' digested with *Kpn*I + *Bsr*BI plus 14.87 pg of the pSYN15954 digest [*Kpn*I + *Pme*I]).

4.5 Functional Element Copy Number: CMP Promoter + TMV Enhancer-specific Probe

A map of the insert in SYHT0H2 soybean indicating the locations of the CMP promoter + TMV enhancer-specific probe and the restriction sites *Eco*RI, *Mfe*I, *Kpn*I, and *Bsr*BI is shown in Figure 11. Table 8 outlines the expected and observed hybridization band sizes, and Figure 12 shows the results of the corresponding Southern blot analyses.

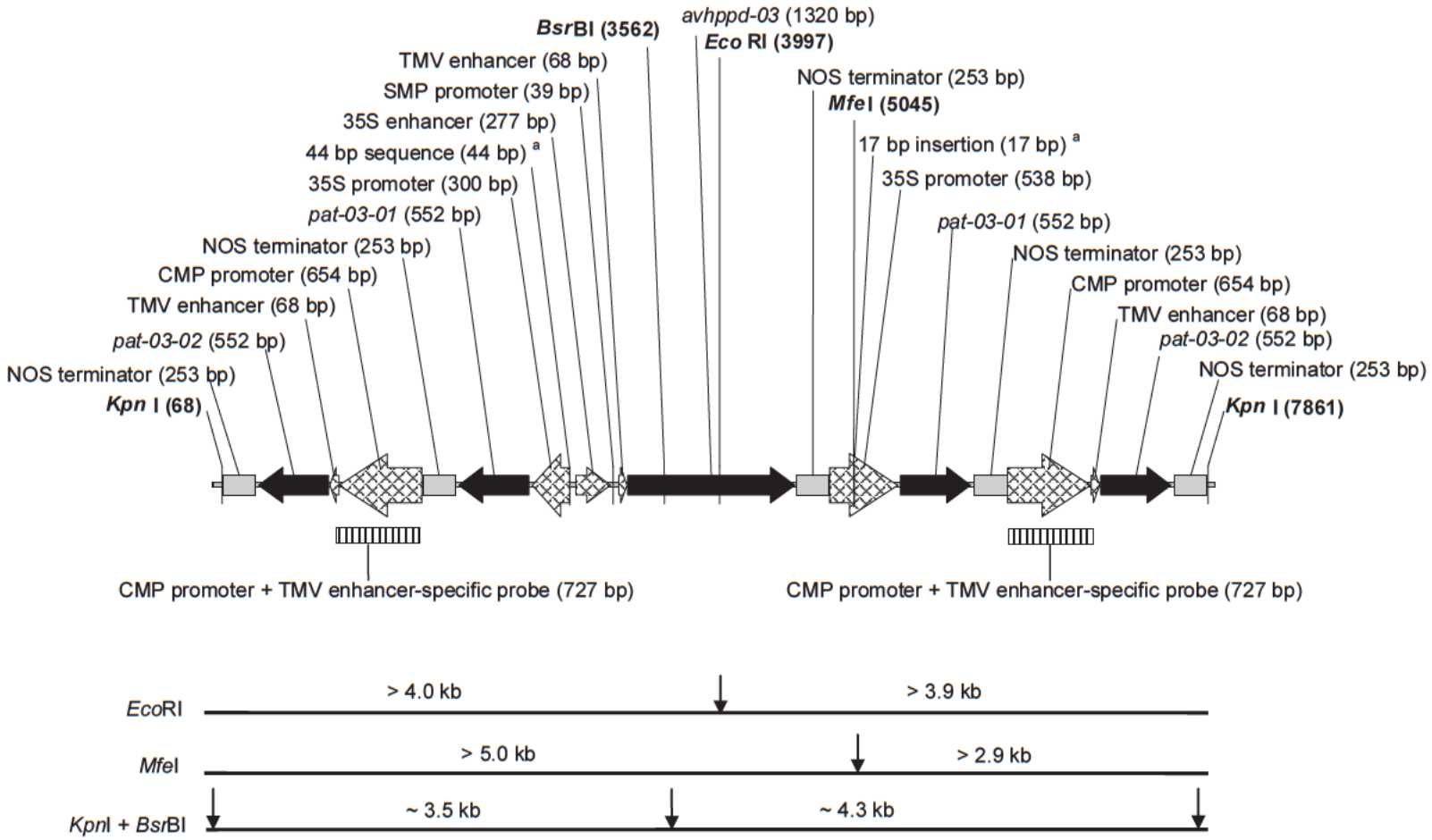
For Southern blot analysis with genomic DNA digested with *Eco*RI and probed with the CMP promoter + TMV enhancer-specific probe, two hybridization bands of approximately 4.8 kb and 8.3 kb were observed in the lane containing DNA extracted from SYHT0H2 T_4 soybean (Table 8; Figure 12A, Lane 3). These hybridization bands were absent in the lane containing DNA extracted from the nontransgenic 'Jack' soybean (Figure 12A, Lane 4) and were, therefore, specific to the SYHT0H2 insert. As expected, one hybridization band of approximately 5.3 kb was observed in the lane containing the positive control (14.87 pg of plasmid pSYN15954 DNA digested with *Kpn*I and *Pme*I and loaded with DNA extracted from nontransgenic 'Jack' soybean) (Figure 12A, Lane 5).

For Southern blot analysis with genomic DNA digested with *Mfe*I and probed with the CMP promoter + TMV enhancer-specific probe, two hybridization bands of approximately 5.2 kb and 6.2 kb were observed in the lane containing DNA extracted from SYHT0H2 T_4 soybean (Table 8; Figure 12B, Lane 3). These hybridization bands were absent in the lane containing DNA extracted from the nontransgenic 'Jack' soybean (Figure 12B, Lane 4) and were, therefore, specific to the SYHT0H2 insert. As expected, one hybridization band of approximately 5.3 kb was observed in the lane containing the positive control (14.87 pg of plasmid pSYN15954 DNA digested with *Kpn*I and *Pme*I and loaded with DNA extracted from nontransgenic 'Jack' soybean) (Figure 12B, Lane 5).

For Southern blot analysis with genomic DNA digested with *Kpn*I + *Bsr*BI and probed with the CMP promoter + TMV enhancer-specific probe, two hybridization bands of approximately 3.5 kb and 4.3 kb were observed in the lane containing DNA extracted from SYHT0H2 T_4 soybean (Table 8; Figure 12C, Lane 3). These hybridization band were absent in the lane containing DNA extracted from the nontransgenic 'Jack' soybean (Figure 12C, Lane 4) and were, therefore, specific to the SYHT0H2 insert. As expected, one band of approximately 5.4 kb was observed in the lane containing the positive control (14.87 pg of plasmid pSYN15954 DNA digested with *Kpn*I and *Pme*I and loaded with DNA extracted from nontransgenic 'Jack' soybean) (Figure 12C, Lane 5).

For Southern blot analyses with the CMP promoter + TMV enhancer-specific probe, two hybridization bands specific to SYHT0H2 soybean were detected with each restriction digestion enzyme strategy. These results demonstrate that SYHT0H2 soybean contains two copies of CMP promoter and TMV enhancer (contained in the *pat-03-02* cassette) sequence. No unexpected bands were detected, indicating that the SYHT0H2 soybean contains no extraneous DNA fragments of the CMP promoter and TMV enhancer sequence.

FIGURE 11 Locations of the 727 bp CMP promoter + TMV enhancer-specific probe and *Eco*RI, *Mfe*I, *Kpn*I, and *Bsr*BI restriction sites in the SYHT0H2 soybean insert



The vertical arrows indicate the sites of restriction digestion. The sizes of the expected restriction fragments are indicated.
[a] The presence of a 44 bp sequence and a 17 bp insertion was determined during insert sequence analysis (de Framond 2012).

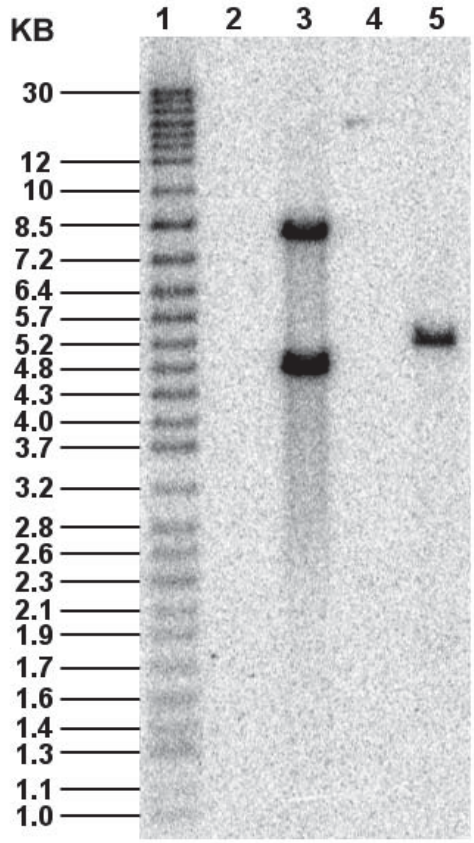
TABLE 8 Expected and observed hybridization bands in Southern blot analyses of SYHT0H2 soybean, with the CMP promoter + TMV enhancer-specific probe and the restriction enzymes *Eco*RI, *Mfe*I, and *Kpn*I + *Bsr*BI

Lane	Source of DNA	Restriction enzyme	Expected number of bands	Expected band size (kb)	Observed band size (kb)
Figure 12A, 3	SYHT0H2 T_4	*Eco*RI	2	>4.0, >3.9	~4.8, ~8.3
Figure 12A, 4	'Jack'	*Eco*RI	0	N/A	N/A
Figure 12A, 5	positive control ('Jack' digested with *Eco*RI plus 14.87 pg of pSYN15954 digest [*Kpn*I + *Pme*I])	*Eco*RI	1	~5.5	~5.3[a]
Figure 12B, 3	SYHT0H2 T_4	*Mfe*I	2	>5.0, >2.9	~5.2, ~6.2
Figure 12B, 4	'Jack'	*Mfe*I	0	N/A	N/A
Figure 12B, 5	positive control ('Jack' digested with *Mfe*I plus 14.87 pg of pSYN15954 digest [*Kpn*I + *Pme*I])	*Mfe*I	1	~5.5	~5.3[a]
Figure 12C, 3	SYHT0H2 T_4	*Kpn*I+*Bsr*BI	2	~3.5, ~4.3	~3.5, ~4.3
Figure 12C, 4	'Jack'	*Kpn*I+*Bsr*BI	0	N/A	N/A
Figure 12C, 5	positive control ('Jack' digested with *Kpn*I + *Bsr*BI plus 14.87 pg of pSYN15954 digest [*Kpn*I + *Pme*I])	*Kpn*I+*Bsr*BI	1	~5.5	~5.4[a]

N/A = not applicable.
[a] The difference between the observed and expected size is within the accepted variability for Southern blot analysis.

FIGURE 12 Functional element copy number Southern blot analysis of SYHT0H2 soybean with the 727 bp CMP promoter + TMV enhancer-specific probe using the restriction enzymes *Eco*RI, *Mfe*I, and *Kpn*I + *Bsr*BI

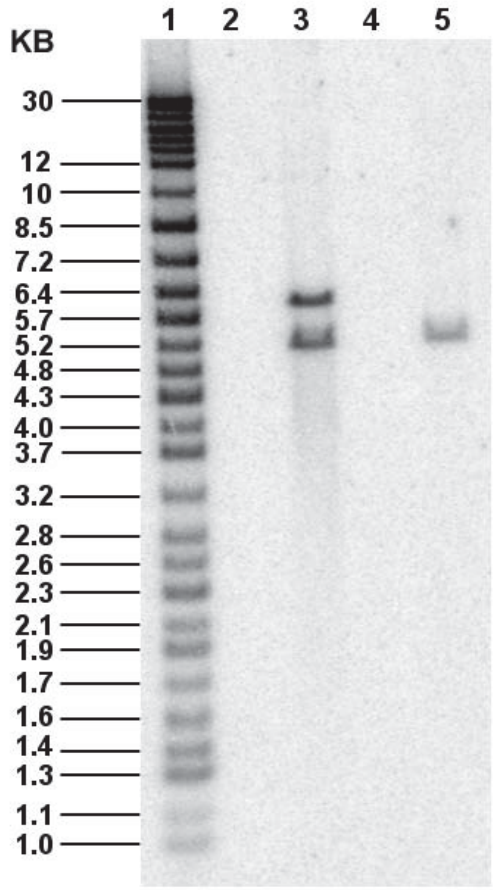
(A) ***Eco*RI**



Lane 1 = molecular weight markers.
Lane 2 = blank.
Lane 3 = SYHT0H2 T_4 digested with *Eco*RI.
Lane 4 = 'Jack' digested with *Eco*RI.
Lane 5 = positive control ('Jack' digested with *Eco*RI plus 14.87 pg of the pSYN15954 digest [*Kpn*I + *Pme*I]).

FIGURE 12 Functional element copy number Southern blot analysis of SYHT0H2 soybean with the 727 bp CMP promoter + TMV enhancer-specific probe using the restriction enzymes *Eco*RI, *Mfe*I, and *Kpn*I + *Bsr*BI (Continued)

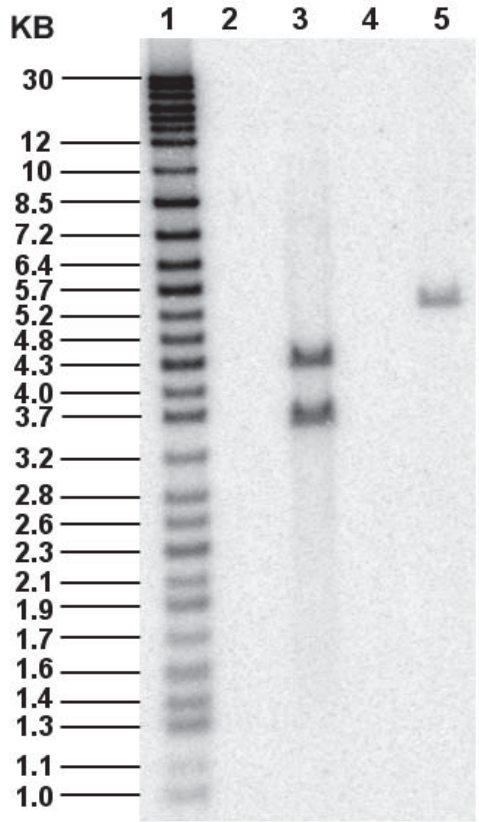
(B) *Mfe*I



Lane 1 = molecular weight markers.
Lane 2 = blank.
Lane 3 = SYHT0H2 T_4 digested with *Mfe*I.
Lane 4 = 'Jack' digested with *Mfe*I.
Lane 5 = positive control ('Jack' digested with *Mfe*I plus 14.87 pg of the pSYN15954 digest [*Kpn*I + *Pme*I]).

FIGURE 12 Functional element copy number Southern blot analysis of SYHT0H2 soybean with the 727 bp CMP promoter + TMV enhancer-specific probe using the restriction enzymes *Eco*RI, *Mfe*I, and *Kpn*I + *Bsr*BI (Continued)

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



Lane 1 = molecular weight markers.
Lane 2 = blank.
Lane 3 = SYHT0H2 T_4 digested with *Kpn*I + *Bsr*BI.
Lane 4 = 'Jack' digested with *Kpn*I + *Bsr*BI.
Lane 5 = positive control ('Jack' digested with *Kpn*I + *Bsr*BI plus 14.87 pg of the pSYN15954 digest [*Kpn*I + *Pme*I]).

4.6 Functional Element Copy Number: NOS Terminator-specific Probe

A map of the insert in SYHT0H2 soybean indicating the locations of the NOS terminator-specific probe and the restriction sites *Acl*I, *Eco*RI, *Kpn*I, and *Bsr*BI is shown in Figure 13. Table 9 outlines the expected and observed hybridization band sizes, and Figure 14 shows the results of the corresponding Southern blot analyses.

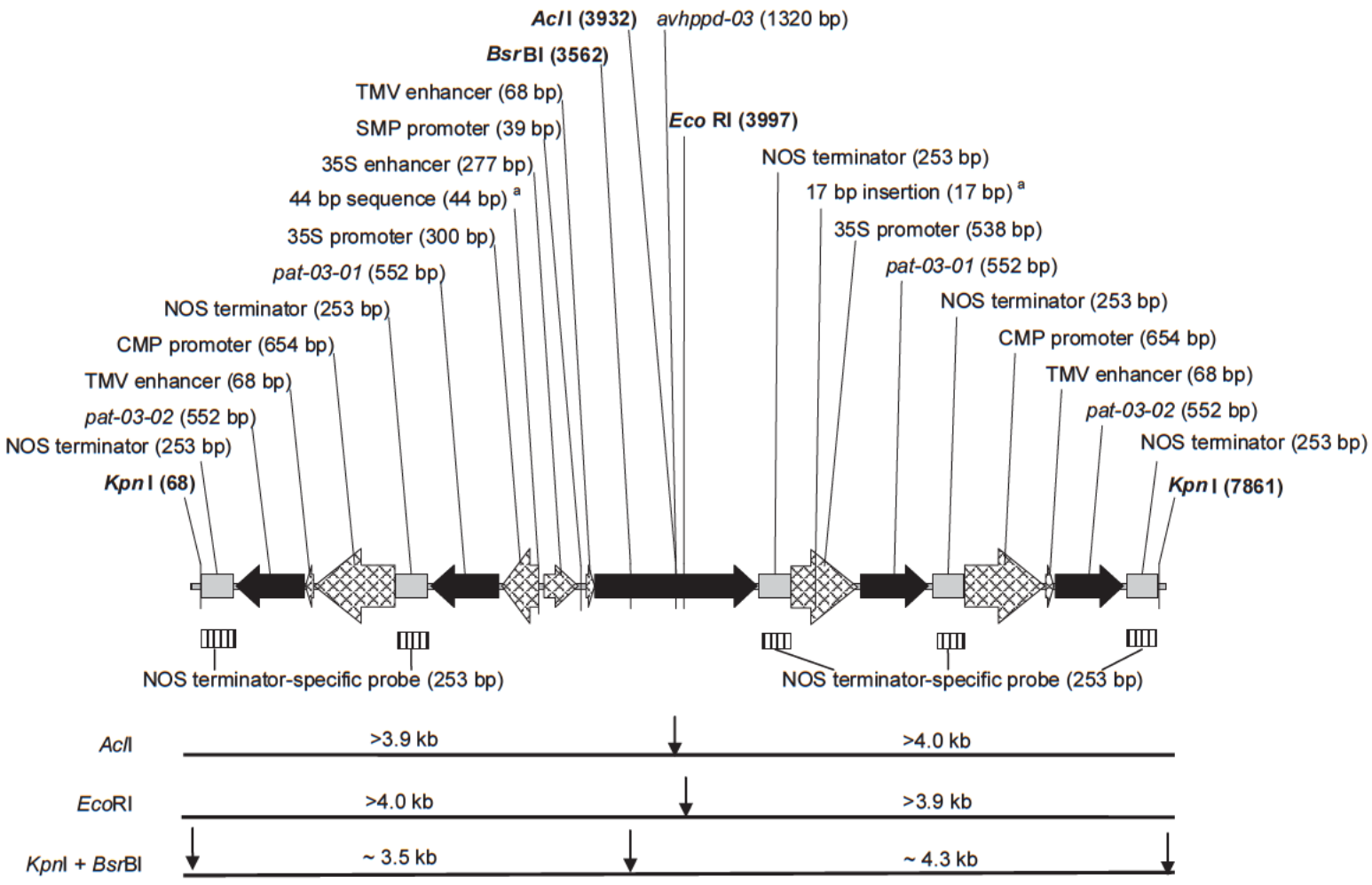
For Southern blot analysis with genomic DNA digested with *Acl*I, and probed with the NOS terminator-specific probe, two hybridization bands of approximately 7.6 kb and 10 kb were observed in the lane containing DNA extracted from SYHT0H2 T_4 soybean (Table 9; Figure 14A, Lane 3). These hybridization bands were absent in the lane containing DNA extracted from nontransgenic 'Jack' soybean (Figure 14A, Lane 4) and were, therefore, specific to the SYHT0H2 insert. As expected, one band of approximately 5.3 kb was observed in the lane containing the positive control (14.87 pg of plasmid pSYN15954 DNA digested with *Kpn*I and *Pme*I and loaded with DNA extracted from nontransgenic 'Jack' soybean) (Figure 14A, Lane 5).

For Southern blot analysis with genomic DNA digested with *Eco*RI and probed with the NOS terminator-specific probe, two hybridization bands of approximately 4.8 kb and 8.3 kb were observed in the lane containing DNA extracted from SYHT0H2 T_4 soybean (Table 9; Figure 14B, Lane 3). These hybridization bands were absent in the lane containing DNA extracted from nontransgenic 'Jack' soybean (Figure 14B, Lane 4) and were, therefore, specific to the SYHT0H2 insert. As expected, one band of approximately 5.5 kb was observed in the lane containing the positive control (14.87 pg of plasmid pSYN15954 DNA digested with *Kpn*I and *Pme*I and loaded with DNA extracted from nontransgenic 'Jack' soybean) (Figure 14B, Lane 5).

For Southern blot analysis with genomic DNA digested with *Kpn*I + *Bsr*BI and probed with the NOS terminator-specific probe, two hybridization bands of approximately 3.5 kb and 4.3 kb were observed in the lane containing DNA extracted from SYHT0H2 T_4 soybean (Table 9; Figure 14C, Lane 3). These hybridization bands were absent in the lane containing DNA from nontransgenic 'Jack' soybean (Figure 14C, Lane 4) and were, therefore specific to the SYHT0H2 insert. As expected, one band of approximately 5.5 kb was observed in the lane containing the positive control (14.87 pg of plasmid pSYN15954 DNA digested with *Kpn*I and *Pme*I and loaded with DNA extracted from nontransgenic 'Jack' soybean) (Figure 14C, Lane 5).

For Southern blot analyses with the NOS terminator-specific probe, two hybridization bands were detected with both restriction enzyme digestion strategies. These results support insert sequence analysis that determined SYHT0H2 soybean contains five copies of NOS terminator sequence (de Framond 2012). No unexpected bands were detected, indicating that the SYHT0H2 soybean genome contains no extraneous DNA fragments of NOS terminator sequence.

FIGURE 13 Locations of the 253 bp NOS terminator probe and *Acl*I, *Eco*RI, *Kpn*I, and *Bsr*BI restriction sites in the SYHT0H2 soybean insert



The vertical arrows indicate the sites of restriction digestion. The sizes of the expected restriction fragments are indicated.
[a] The presence of a 44 bp sequence and a 17 bp insertion was determined during insert sequence analysis (de Framond 2012).

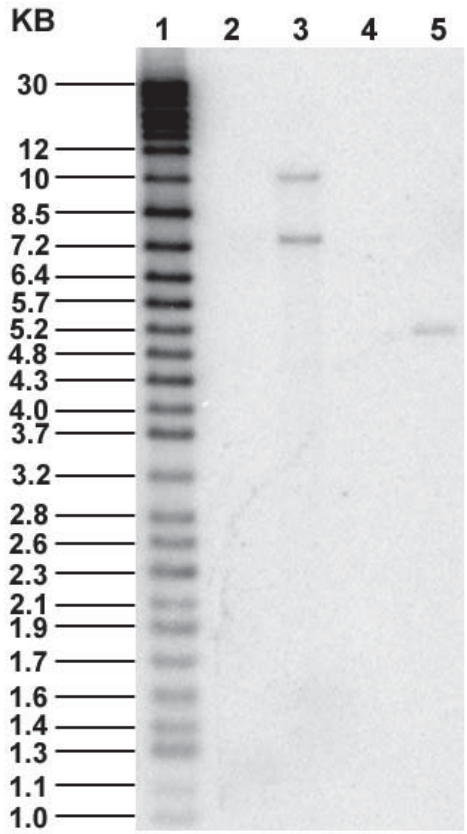
TABLE 9 Expected and observed hybridization bands in Southern blot analyses of SYHT0H2 soybean, with the NOS terminator-specific probe and restriction enzymes *Acl*I, *Eco*RI, and *Kpn*I + *Bsr*BI

Lane	Source of DNA	Restriction enzyme	Expected number of bands	Expected band size (kb)	Observed band size (kb)
Figure 14A, 3	SYHT0H2 T_4	*Acl*I	2	>3.9, >4.0	~7.6, ~10
Figure 14A, 4	'Jack'	*Acl*I	0	N/A	N/A
Figure 14A, 5	positive control ('Jack' digested with *Acl*I plus 14.87 pg of pSYN15954 digest [*Kpn*I+ *Pme*I])	*Acl*I	1	~5.5	~5.3[a]
Figure 14B, 3	SYHT0H2 T_4	*Eco*RI	2	>4.0, >3.9	~4.8, ~8.3
Figure 14B, 4	'Jack'	*Eco*RI	0	N/A	N/A
Figure 14B, 5	positive control ('Jack' digested with *Eco*RI plus 14.87 pg of pSYN15954 digest [*Kpn*I+ *Pme*I])	*Eco*RI	1	~5.5	~5.5
Figure 14C, 3	SYHT0H2 T_4	*Kpn*I + *Bsr*BI	2	~3.5, ~4.3	~3.5, ~4.3
Figure 14C, 4	'Jack'	*Kpn*I+ *Bsr*BI	0	N/A	N/A
Figure 14C, 5	positive control ('Jack' digested with *Kpn*I+ *Bsr*BI and 14.87 pg of pSYN15954 digest [*Kpn*I + *Pme*I])	*Kpn*I+ *Bsr*BI	1	~5.5	~5.5

N/A = not applicable.
[a] The difference between the observed and expected size is within the accepted variability for Southern blot analysis.

FIGURE 14 Functional element copy number Southern blot analysis of SYHT0H2 soybean with the 253 bp NOS terminator-specific probe, using restriction enzymes *Acl*I, *Eco*RI, and *Kpn*I + *Bsr*BI

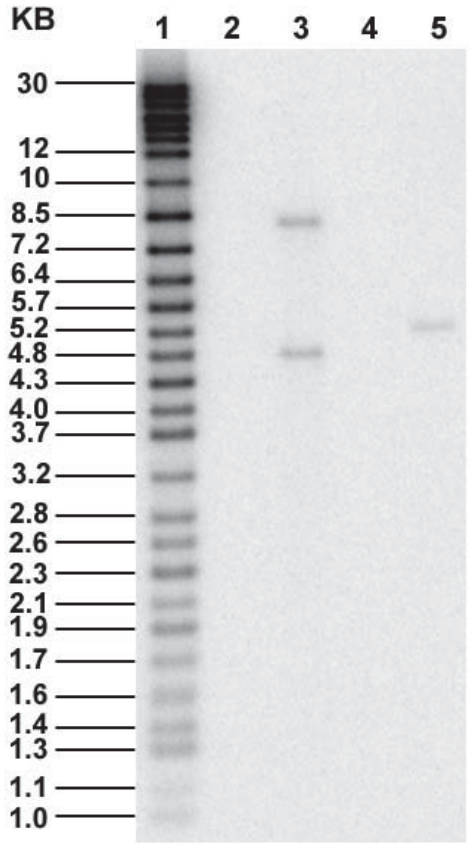
(A) *Acl*I



Lane 1 = molecular weight markers.
Lane 2 = blank.
Lane 3 = SYHT0H2 T_4 digested with *Acl*I.
Lane 4 = 'Jack' digested with *Acl*I.
Lane 5 = positive control ('Jack' digested with *Acl*I plus 14.87 pg of the pSYN15954 digest [*Kpn*I + *Pme*I]).

FIGURE 14 Copy number functional element Southern blot analysis of SYHT0H2 soybean with the 253 bp NOS terminator-specific probe, using restriction enzymes *Acl*I, *Eco*RI, and *Kpn*I + *Bsr*BI (Continued)

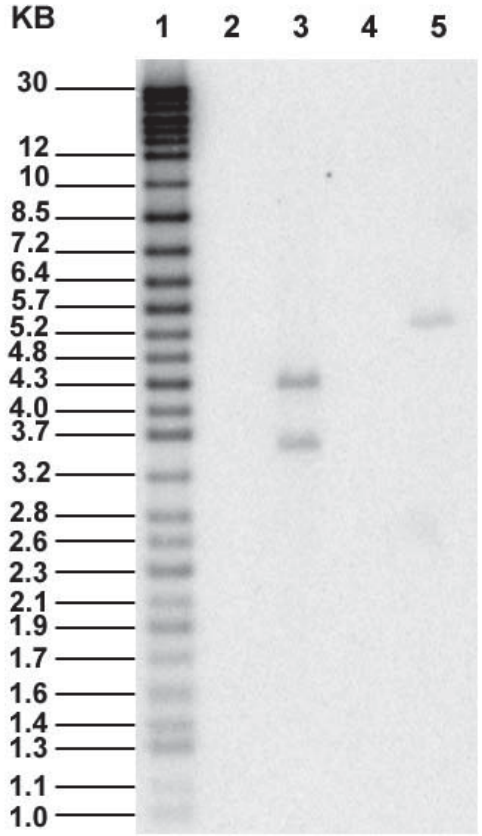
(B) *Eco*RI



Lane 1 = molecular weight markers.
Lane 2 = blank.
Lane 3 = SYHT0H2 T_4 digested with *Eco*RI.
Lane 4 = 'Jack' digested with *Eco*RI.
Lane 5 = positive control ('Jack' digested with *Eco*RI plus 14.87 pg of the pSYN15954 digest [*Kpn*I + *Pme*I]).

FIGURE 14 Copy number functional element Southern blot analysis of SYHT0H2 soybean with the 253 bp NOS terminator-specific probe, using restriction enzymes *Acl*I, *Eco*RI, and *Kpn*I + *Bsr*BI (Continued)

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



Lane 1 = molecular weight markers.
Lane 2 = blank.
Lane 3 = SYHT0H2 T_4 digested with *Kpn*I + *Bsr*BI.
Lane 4 = 'Jack' digested with *Kpn*I + *Bsr*BI.
Lane 5 = positive control ('Jack' digested with *Kpn*I + *Bsr*BI plus 14.87 pg of the pSYN15954 digest [*Kpn*I + *Pme*I]).

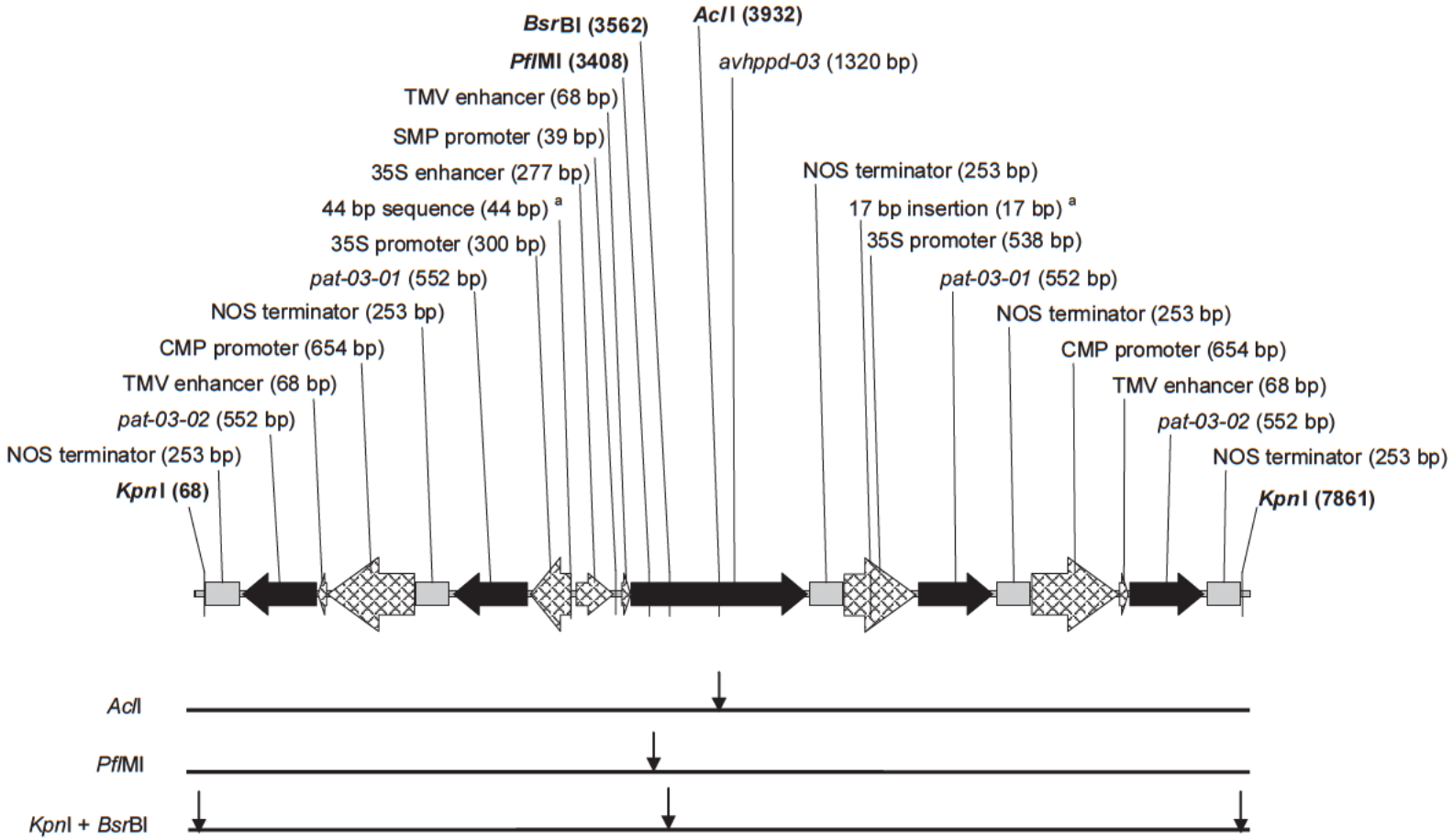
4.7 Functional Element Copy Number: FMV Enhancer-specific Probe

As discussed in Section 3.7, the FMV enhancer is not present in SYHT0H2 soybean. A map of the insert in SYHT0H2 soybean indicating the locations of the restriction sites *Acl*I, *Pfl*MI, *Kpn*I, and *Bsr*BI is shown in Figure 15. Figure 16 shows a map of the T-DNA region of the SYHT0H2 transformation plasmid pSYN15954 indicating the locations of the FMV enhancer-specific probe and the restriction sites *Acl*I, *Pfl*MI, *Kpn*I, and *Bsr*BI. Table 10 outlines the expected and observed hybridization band sizes, and Figure 17 shows the results of the corresponding Southern blot analyses.

In the analyses of genomic DNA digested with *Acl*I, *Pfl*MI, and *Kpn*I + *Bsr*BI, no hybridization bands were observed in the lanes containing DNA extracted from SYHT0H2 T_4 soybean (Table 10; Figures 17A through 17C, Lane 3) or in the lanes containing DNA extracted from nontransgenic 'Jack' soybean (Figures 17A through 17C, Lane 4). In all three analyses, one band of approximately 5.4 kb or 5.5 kb was observed in the lanes containing the positive control (14.87 pg of plasmid pSYN15954 DNA digested with *Kpn*I and *Pme*I and loaded with DNA extracted from nontransgenic 'Jack' soybean) (Figure 17A through 17C, Lane 5), as expected.

These results demonstrate that SYHT0H2 soybean does not contain sequence from the FMV enhancer from the transformation plasmid pSYN15954.

FIGURE 15 Locations of the *Acl*I, *Pfl*MI, *Kpn*I, and *Bsr*BI restriction sites in the SYHT0H2 soybean insert



The vertical arrows indicate the sites of restriction digestion. Because the FMV enhancer is not present in SYHT0H2 soybean, no restriction fragments are expected.

[a] The presence of a 44 bp sequence and a 17 bp insertion was determined during insert sequence analysis (de Framond 2012).

FIGURE 16 **Locations of the 194 bp FMV enhancer-specific probe and *Acl*I, *Pfl*MI, *Kpn*I, and *Bsr*BI restriction sites as in pSYN15954**

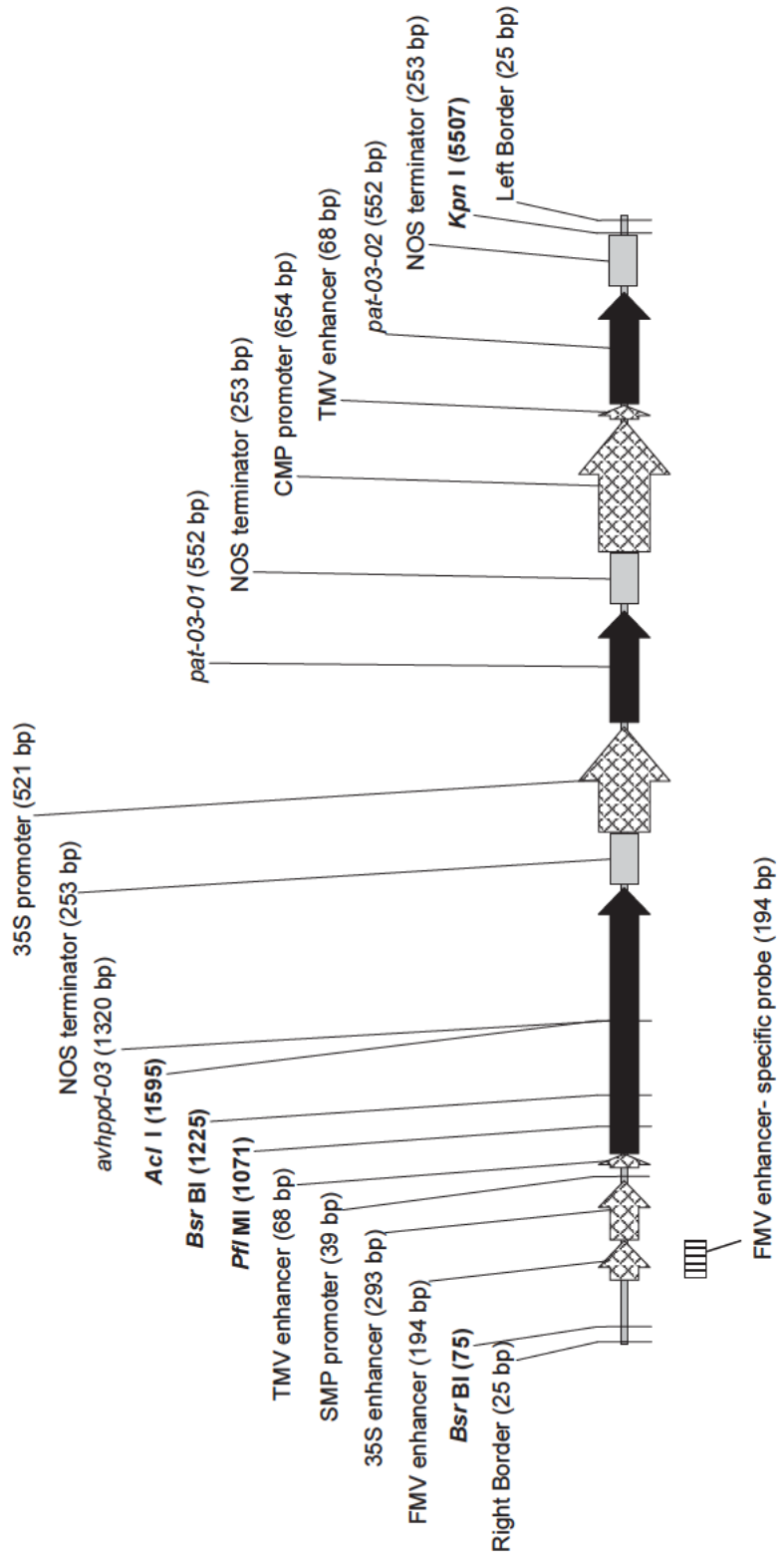


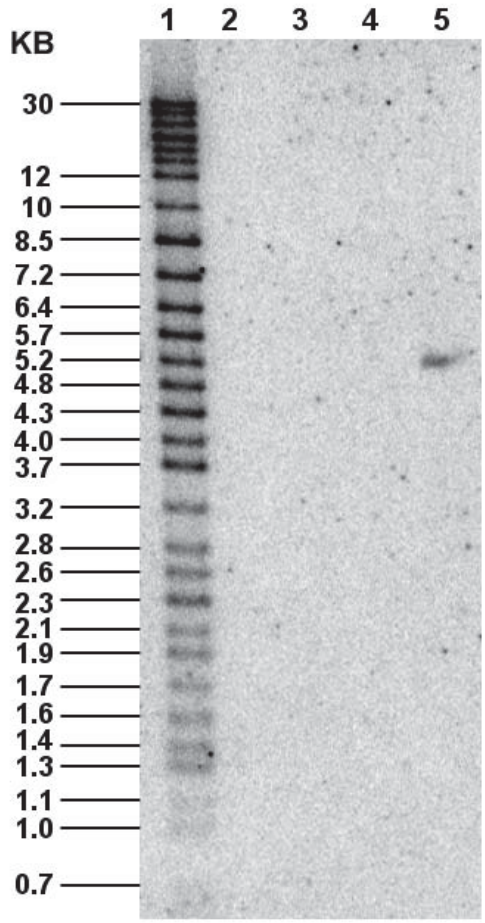
TABLE 10 Expected and observed hybridization bands in Southern blot analyses of SYHT0H2 soybean, with the FMV enhancer-specific probe and the restriction enzymes *Acl*I, *Pfl*MI, and *Kpn*I + *Bsr*BI

Lane	Source of DNA	Restriction enzyme	Expected number of bands	Expected band size (kb)	Observed band size (kb)
Figure 17A, 3	SYHT0H2 T_4	*Acl*I	0	N/A	N/A
Figure 17A, 4	'Jack'	*Acl*I	0	N/A	N/A
Figure 17A, 5	positive control ('Jack' digested with *Acl*I plus 14.87 pg of pSYN15954 digest [*Kpn*I + *Pme*I])	*Acl*I	1	~5.5	~5.5
Figure 17B, 3	SYHT0H2 T_4	*Pfl*MI	0	N/A	N/A
Figure 17B, 4	'Jack'	*Pfl*MI	0	N/A	N/A
Figure 17B, 5	positive control ('Jack' digested with *Pfl*MI plus 14.87 pg of pSYN15954 digest [*Kpn*I + *Pme*I])	*Pfl*MI	1	~5.5	~5.5
Figure 17C, 3	SYHT0H2 T_4	*Kpn*I + *Bsr*BI	0	N/A	N/A
Figure 17C, 4	'Jack'	*Kpn*I + *Bsr*BI	0	N/A	N/A
Figure 17C, 5	positive control ('Jack' digested with *Kpn*I + *Bsr*BI plus 14.87 pg of pSYN15954 digest [*Kpn*I + *Pme*I])	*Kpn*I + *Bsr*BI	1	~5.5	~5.4[a]

N/A = not applicable
[a] The difference between the observed and expected size is within the accepted variability for Southern blot analysis.

FIGURE 17 Functional element copy number Southern blot analysis of SYHT0H2 soybean with the 194 bp FMV enhancer-specific probe, using the restriction enzymes *Acl*I, *Pfl*MI, and *Kpn*I + *Bsr*BI

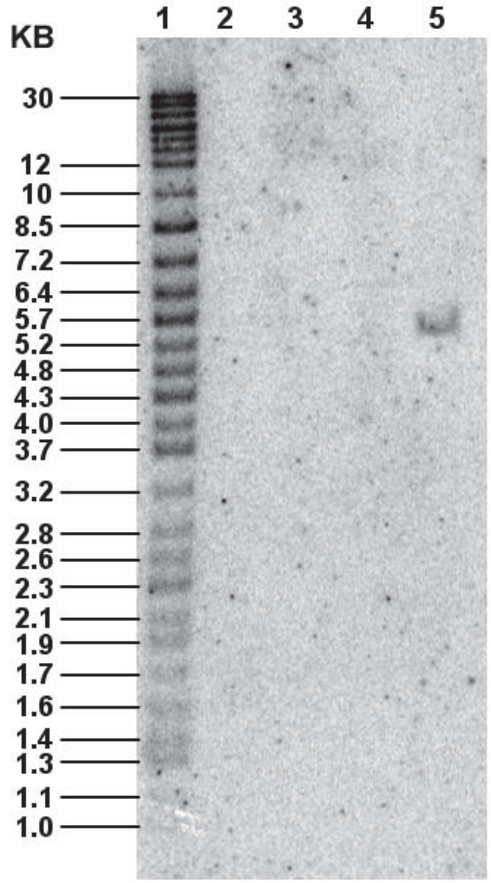
(A) *Acl*I



Lane 1 = molecular weight markers.
Lane 2 = blank.
Lane 3 = SYHT0H2 T_4 digested with *Acl*I.
Lane 4 = 'Jack' digested with *Acl*I.
Lane 5 = positive control ('Jack' digested with *Acl*I plus 14.87 pg of the pSYN15954 digest [*Kpn*I + *Pme*I]).

FIGURE 17 Functional element copy number Southern blot analysis of SYHT0H2 soybean with the 194 bp FMV enhancer-specific probe, using the restriction enzymes *Acl*I, *Pfl*MI, and *Kpn*I + *Bsr*BI (Continued)

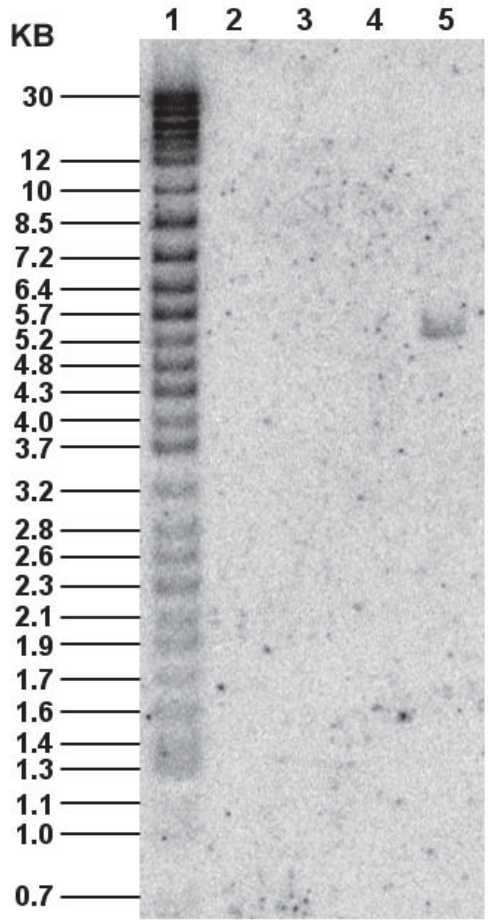
(B) *Pfl*MI



Lane 1 = molecular weight markers.
Lane 2 = blank.
Lane 3 = SYHT0H2 T_4 digested with *Pfl*MI.
Lane 4 = 'Jack' digested with *Pfl*MI.
Lane 5 = positive control ('Jack' digested with *Pfl*MI plus 14.87 pg of the pSYN15954 digest [*Kpn*I + *Pme*I]).

FIGURE 17 Functional element copy number Southern blot analysis of SYHT0H2 soybean with the 194 bp FMV enhancer-specific probe, using the restriction enzymes *Acl*I, *Pfl*MI, and *Kpn*I + *Bsr*BI (Continued)

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



Lane 1 = molecular weight markers.
Lane 2 = blank.
Lane 3 = SYHT0H2 T_4 digested with *Kpn*I + *Bsr*BI.
Lane 4 = 'Jack' digested with *Kpn*I + *Bsr*BI.
Lane 5 = positive control ('Jack' digested with *Kpn*I + *Bsr*BI plus 14.87 pg of the pSYN15954 digest [*Kpn*I + *Pme*I]).

4.8 Analyses for Plasmid pSYN15954 Backbone-specific Sequence

Figure 18 shows a map of the plasmid pSYN15954 indicating the locations of the plasmid pSYN15954 backbone-specific probe and the restriction sites for *Acl*I, *Pfl*MI, and *Kpn*I. Table 11 outlines the expected and observed hybridization band sizes, and Figure 19 shows the results of the corresponding Southern blot analyses.

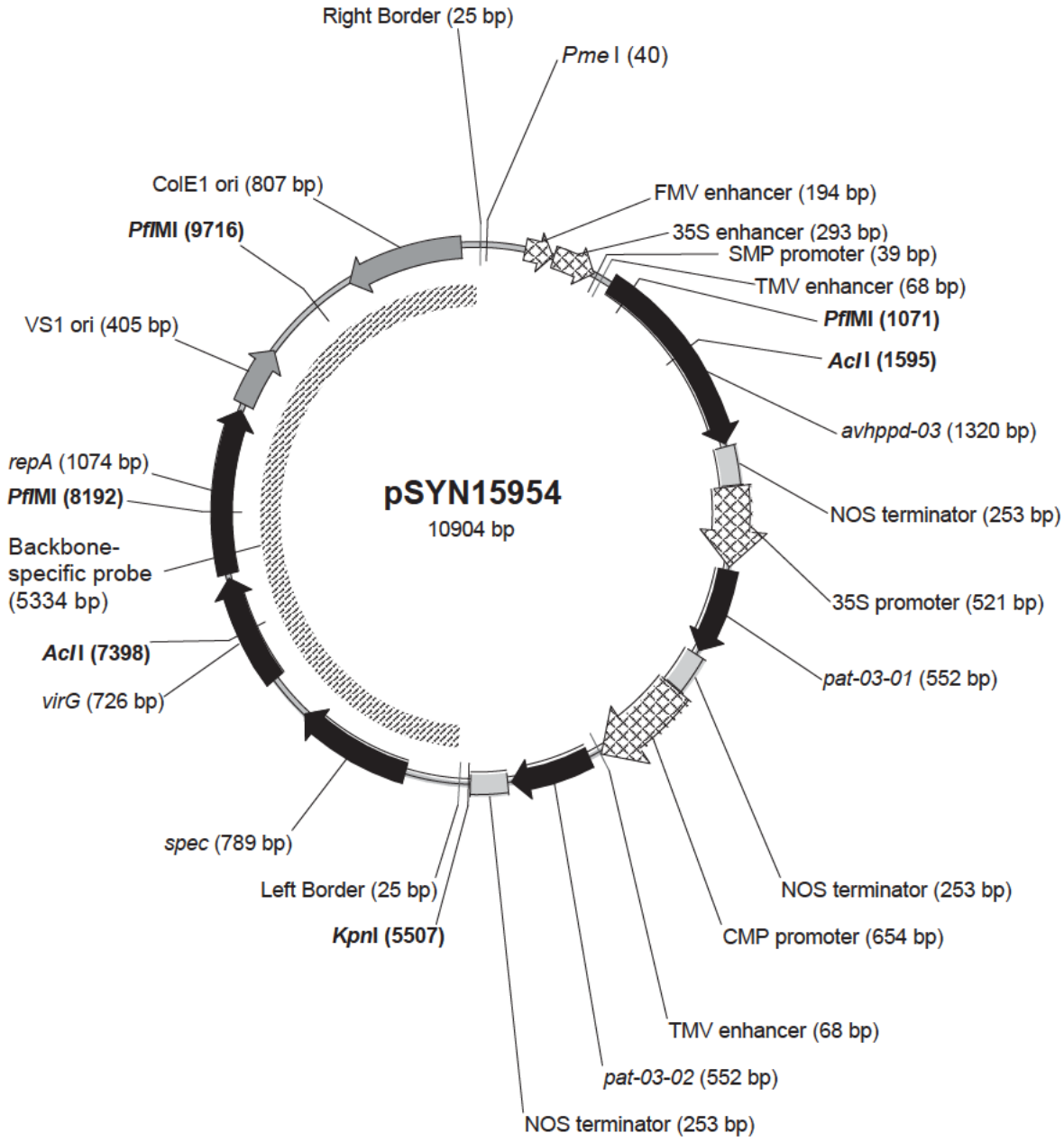
In the analyses of genomic DNA digested with *Acl*I, *Pfl*MI, or *Kpn*I, no hybridization bands were observed in the lanes containing DNA from extracted from SYHT0H2 T_4 soybean (Table 11; Figures 19A through 19C, Lane 3) or in the lanes containing DNA extracted from the nontransgenic 'Jack' soybean (Figures 19A through 19C, Lane 4).

In the analysis of genomic DNA digested with *Acl*I, three bands of approximately 5.5 kb, 3.5 kb, and 1.9 kb were observed in the lane containing the positive control (Figure 19A, Lane 5). The positive control, 14.87 pg of plasmid pSYN15954 DNA digested with *Kpn*I and *Pme*I, was loaded with DNA extracted from nontransgenic 'Jack' soybean that was digested with *Acl*I. After its addition to the DNA extracted from nontransgenic 'Jack' soybean, the positive control was also digested by *Acl*I. The 3.5 kb and 1.9 kb bands were expected for plasmid pSYN15954 digested with *Kpn*I, *Pme*I, and *Acl*I. An additional positive control without digested genomic DNA was included as a representation of the positive control that was not digested by *Acl*I; a hybridization band of approximately 5.5 kb was observed, as expected (Figure 19A, Lane 7).

In the analysis of genomic DNA digested with *Pfl*MI and *Kpn*I, one band of approximately 5.7 kb was observed in the lanes containing the positive control (Figures 19B and 19C, Lane 5), as expected.

These results demonstrate that SYHT0H2 soybean does not contain any backbone sequences from the transformation plasmid pSYN15954.

FIGURE 18 **Locations of the 5334 bp plasmid pSYN15954 backbone-specific probe and the *Acl*I, *Pfl*MI, and *Kpn*I restriction sites in the transformation plasmid pSYN15954**



ori = origin of replication
repA = replication gene
virG = part of the two-component regulatory system for the virulence regulon in *A.tumefaciens*

TABLE 11 Expected and observed hybridization bands in Southern blot analyses using the plasmid pSYN15954 backbone-specific probe and the restriction enzymes *Acl*I, *Pfl*MI, and *Kpn*I

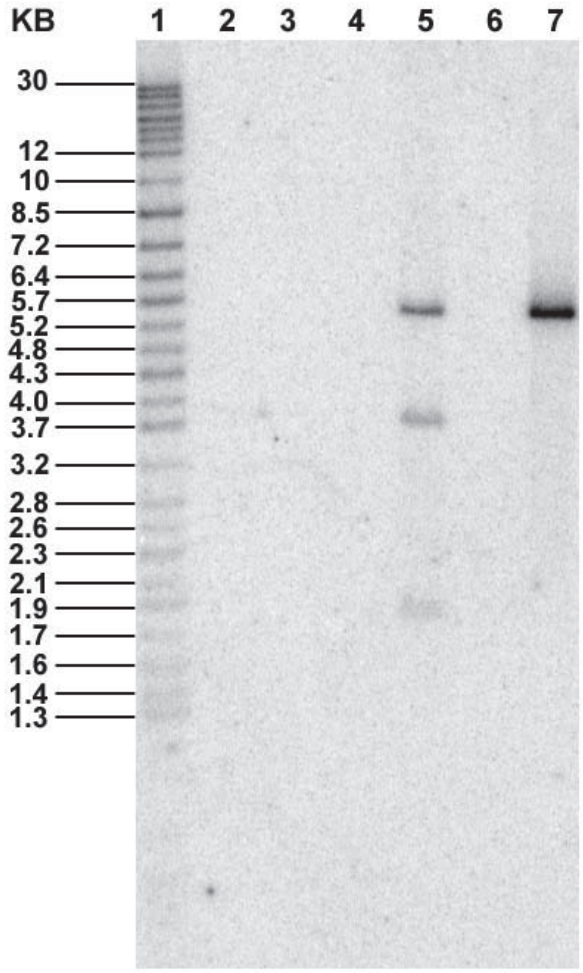
Lane	Source of DNA	Restriction enzyme	Expected number of bands	Expected band size (kb)	Observed band size (kb)
Figure 19A, 3	SYHT0H2 T_4	*Acl*I	0	N/A	N/A
Figure 19A, 4	'Jack'	*Acl*I	0	N/A	N/A
Figure 19A, 5	positive control ('Jack' digested with *Acl*I plus 14.87 pg of pSYN15954 digest [*Kpn*I + *Pme*I])	*Acl*I	1	~5.4	~1.9, ~3.5, ~5.5
Figure 19A, 7	positive control (14.87 pg of pSYN15954 digest [*Kpn*I + *Pme*I])	N/A	1	~5.4	~5.5
Figure 19B, 3	SYHT0H2 T_4	*Pfl*MI	0	N/A	N/A
Figure 19B, 4	'Jack'	*Pfl*MI	0	N/A	N/A
Figure 19B, 5	positive control ('Jack' digested with *Pfl*MI plus 14.87 pg of pSYN15954 digest [*Kpn*I + *Pme*I])	*Pfl*MI	1	~5.4	~5.7[a]
Figure 19C, 3	SYHT0H2 T_4	*Kpn*I	0	N/A	N/A
Figure 19C, 4	'Jack'	*Kpn*I	0	N/A	N/A
Figure 19C, 5	positive control ('Jack' digested with *Kpn*I plus 14.87 pg of pSYN15954 digest [*Kpn*I + *Pme*I])	*Kpn*I	1	~5.4	~5.7[a]

N/A = not applicable

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

FIGURE 19 Southern blot analysis of SYHT0H2 soybean with the 5334 bp plasmid pSYN15954 backbone-specific probe, using the restriction enzymes *Acl*I, *Pfl*MI, and *Kpn*I

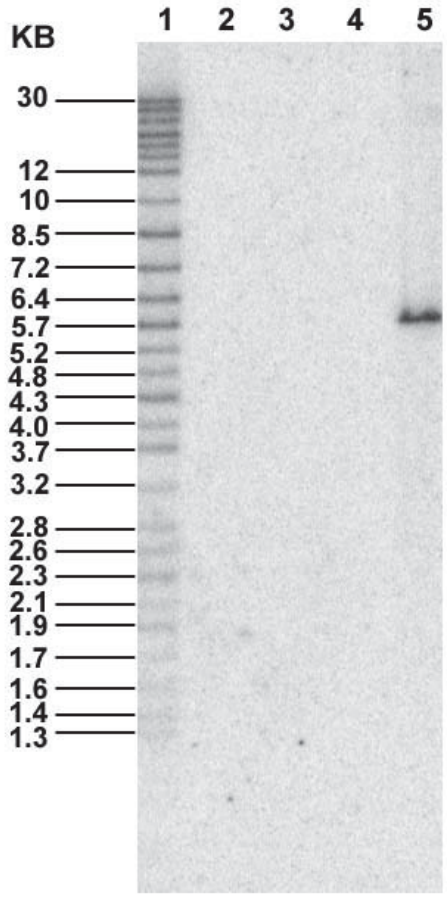
(A) *Acl*I



Lane 1 = molecular weight markers.
Lane 2 = blank.
Lane 3 = SYHT0H2 T_4 digested with *Acl*I.
Lane 4 = 'Jack' digested with *Acl*I.
Lane 5 = positive control ('Jack' digested with *Acl*I plus 14.87 pg of the pSYN15954 digest [*Kpn*I + *Pme*I]).
Lane 6 = blank.
Lane 7 = positive control (14.87 pg of the pSYN15954 digest [*Kpn*I + *Pme*I]).

FIGURE 19 Southern blot analysis of SYHT0H2 soybean with the 5334 bp plasmid pSYN15954 backbone-specific probe, using the restriction enzymes *Acl*I, *Pfl*MI, and *Kpn*I (Continued)

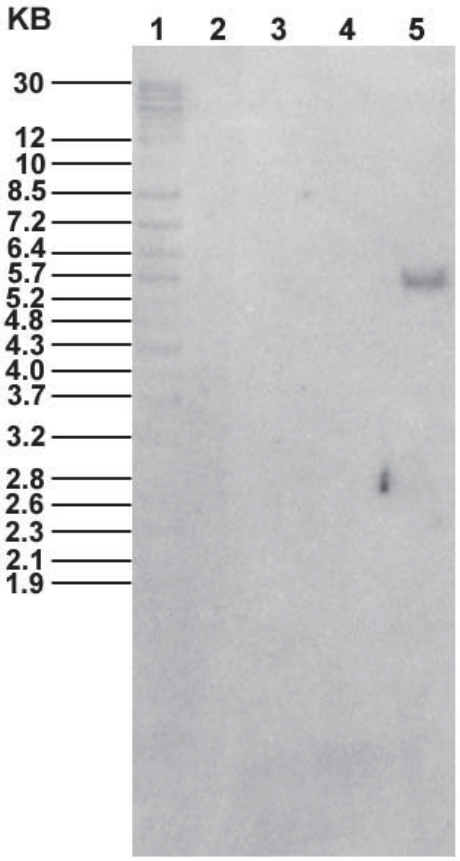
(B) *Pfl*MI



Lane 1 = molecular weight markers.
Lane 2 = blank.
Lane 3 = SYHT0H2 T_4 digested with *Pfl*MI.
Lane 4 = 'Jack' digested with *Pfl*MI.
Lane 5 = positive control ('Jack' digested with *Pfl*MI plus 14.87 pg of the pSYN15954 digest [*Kpn*I + *Pme*I]).

FIGURE 19 Southern blot analysis of SYHT0H2 soybean with the 5334 bp plasmid pSYN15954 backbone-specific probe, using the restriction enzymes *Acl*I, *Pfl*MI, and *Kpn*I (Continued)

(C) *Kpn*I



Lane 1 = molecular weight markers.
Lane 2 = blank.
Lane 3 = SYHT0H2 T_4 digested with *Kpn*I.
Lane 4 = 'Jack' digested with *Kpn*I.
Lane 5 = positive control ('Jack' digested with *Kpn*I plus 14.87 pg of the pSYN15954 digest [*Kpn*I+ *Pme*I]).

4.9 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 CONCLUSION

Insert sequence analysis determined that SYHT0H2 soybean contains a single copy of *avhppd-03*, four copies of *pat*, a single copy of the *avhppd-03* enhancer complex sequence, two copies of the 35S promoter, two copies of the CMP promoter, two copies of the TMV enhancer (contained in the *pat-03-02* cassette), and five copies of the NOS terminator, as expected for a single insertion site consisting two truncated copies of the pSYN15954 T-DNA (de Framond 2012). Southern blot analysis of SYHT0H2 in this study supports this determination. The results also demonstrated that there are no extraneous DNA fragments from any of these functional elements elsewhere in the SYHT0H2 soybean genome, and the FMV enhancer and backbone sequence from transformation plasmid pSYN15954 are absent from SYHT0H2 soybean.

6.0 REFERENCES

Arumuganathan K, Earle ED. 1991. Nuclear DNA content of some important plant species. *Plant Mol Biol Rep* 9:208–218.

Burgin K. 2009. *MT Soy Event SYHT04R: Test and Control Substance Characterization of T_3, T_4, and T_5 Generations and Jack Soy*. Report No. SSB-029-09 (unpublished). Research Triangle Park, NC: Syngenta Biotechnology, Inc.

Burgin K. 2011. *Event SYHT0H2 Soybean: Test and Control Substance Characterization of T4, T5, and T6 Generations and 'Jack' Soybean*. Report No. TK0055856 (unpublished). Research Triangle Park, NC: Syngenta Crop Protection, LLC.

Chomczynski P. 1992. One-hour downward alkaline capillary transfer for blotting of DNA and RNA. *Anal Biochem* 201:134–139.

de Framond A. 2012. *Event SYHT0H2 Soybean: Insert Sequence Analysis*. Report No. TK0059645 (unpublished). Research Triangle Park, NC: Syngenta Crop Protection, LLC.

Depicker A, Stachel S, Dhaese P, Zambryski P, Goodman HM. 1982. Nopaline synthase: transcript mapping and DNA sequence. *J Mol Appl Genet* 1:561–573.

Fling ME, Kopf J, Richards C. 1985. Nucleotide sequence of the transposon Tn7 gene encoding an aminoglycoside-modifying enzyme, 3″(9)-*O*-nucleotidyltransferase. *Nucleic Acids Res* 13:7095–7106.

Gallie DR, Sleat DE, Watts JW, Turner PC, Wilson TMA. 1987. The 5′-leader sequence of tobacco mosaic virus RNA enhances the expression of foreign gene transcripts *in vitro* and *in vivo*. *Nucleic Acids Res* 15(8):3257–3273.

Gallie DR. 2002. The 5′-leader of tobacco mosaic virus promotes translation through enhanced recruitment of eIF4F. *Nucleic Acids Res* 30(15):3401–3411.

Hansen G, Das A, Chilton M-D. 1994. Constitutive expression of the virulence genes improves the efficiency of plant transformation by *Agrobacterium*. *Proc Natl Acad Sci USA* 91:7603–7607.

Heeb S, Itoh Y, Nishijyo T, Schnider U, Keel C, Wade J, Walsh U, O'Gara F, Haas D. 2000. Small, stable shuttle vectors based on the minimal pVS1 replicon for use in Gram-negative, plant-associated bacteria. *Mol Plant Microbe In* 13:232–237.

Hwang YJ, Dawson J, Sigareva M, Que Q. 2008. Transformation of immature soybean seeds through organogenesis. Syngenta Biotechnology, Inc., assignee. Patent No. WO 08/112044. Geneva, Switzerland: World Intellectual Property Organization.

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.

Itoh T, Tomizawa J. 1979. Initiation of replication of plasmid ColE1 DNA by RNA polymerase, ribonuclease H and DNA polymerase I. *Cold Spring Harb Sym* 43:409–417.

Itoh Y, Watson JM, Dieter H, Leisinger T. 1984. Genetic and molecular characterization of the *Pseudomonas* plasmid pVS1. *Plasmid* 11:206–220.

Maiti IB, Gowda S, Kiernan J, Ghosh SK, Shepherd RJ. 1997. Promoter/leader deletion analysis and plant expression vectors with the figwort mosaic virus (FMV) full length transcript (FLt) promoter containing single or double enhancer domains. *Transgenic Res* 6:143–156.

Matringe M, Sailland A, Pelissier B, Rolland A, Zink O. 2005. *p*-Hydroxyphenylpyruvate dioxygenase inhibitor-resistant plants. *Pest Manag Sci* 61:269–276.

NCBI. 2012. Entrez Nucleotide Database. Bethesda, MD: National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide.

Nesbitt M. 2012. *Quantification of p-Hydroxyphenylpyruvate Dioxygenase and Phosphinothricin Acetyltransferase in Leaves and Seed from Five Generations of Soybean Event SYHT0H2*. Report No. TK0059699 (ongoing). Research Triangle Park, NC: Syngenta Crop Protection, LLC.

Nickell CD, Noel GR, Thomas DJ, Waller R. 1990. Registration of 'Jack' soybean. *Crop Sci* 30:1365.

Ow DW, Jacobs JD, Howell SH. 1987. Functional regions of the cauliflower mosaic virus 35S RNA promoter determined by use of the firefly luciferase gene as a reporter of promoter activity. *Proc Natl Acad Sci USA* 84:4870–4874.

Que Q, Dawson J, Sigareva M. 2008. Transformation of immature soybean seeds through organogenesis. Syngenta Biotechnology, Inc., assignee. Patent No. WO 08/112267. Geneva, Switzerland: World Intellectual Property Organization.

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW. 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *P Natl Acad Sci USA* 81:8014–8018.

Stavolone L, Ragozzino A, Hohn T. 2003a. Characterization of *Cestrum yellow leaf curling virus*: a new member of the family *Caulimoviridae*. *J Gen Virol* 84:3459–3464.

Stavolone L, Koronova M, Pauli S, Ragozzino A, De Haan P, Milligan S, Lawton K, Hohn T. 2003b. Cestrum yellow leaf curling virus (CmYLCV) promoter: a new strong constitutive promoter for heterologous gene expression in a wide variety of crops. *Plant Mol Biol* 53:703–713.

Testerman S. 2012. *Event SYHT0H2 Soybean: Test and Control Substance Characterization of T2, T3, T7, and T8 Generations*. Report No. TK0059659 (unpublished). Research Triangle Park, NC: Syngenta Crop Protection, LLC.

Tzfira T, Li J, Lacroix B, Citovsky V. 2004. *Agrobacterium* T-DNA integration: molecules and models. *Trends Genet* 20:375–383.

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

Wang K, Herrera-Estrella L, Van Montagu M, Zambryski P. 1984. Right 25 bp terminus sequence of the nopaline T-DNA is essential for and determines direction of DNA transfer from *Agrobacterium* to the plant genome. *Cell* 38:455–462.

Wohlleben W, Arnold W, Broer I, Hillemann D, Strauch E, Pühler A. 1988. Nucleotide sequence of the phosphinothricin *N*-acetyltransferase gene from *Streptomyces viridochromogenes* Tü494 and its expression in *Nicotiana tabacum*. *Gene* 70:25–37.

Zambryski P, Depicker A, Kruger K, Goodman HM. 1982. Tumor induction by *Agrobacterium tumefaciens*: analysis of the boundaries of T-DNA. *J Mol Appl Genet* 1:361–370.

APPENDICES SECTION

APPENDIX A Southern Analysis Phase Report

SOUTHERN BLOT ANALYTICAL FINAL PHASE REPORT

Event SYHT0H2 Soybean: Functional Element Copy Number Southern Blot Analysis

Phase Number: 288-009

Sponsor Study Number: TK0059651

For

[redacted]

Syngenta Crop Protection, LLC
Product Safety
3054 East Cornwallis Road
PO Box 12257
Research Triangle Park, NC 27709, USA

Prepared by

[redacted]

Beckman Coulter Genomics, Inc.
100 Perimeter Park Drive, Suite C
Morrisville, NC 27560

15 February 2012

Table of Contents of Southern Blot Analytical Final Phase Report

BECKMAN COULTER GENOMICS

QUALITY ASSURANCE STATEMENT

Sponsor: **Syngenta Crop Protection, LLC**

Phase Plan: **288-009**

Study Title: **Event SYHT0H2 Soybean: Functional Element Copy Number Southern Blot Analysis**

Sponsor Study Number: **TK0059651**

The following inspections were conducted during the course of this study phase by Quality Assurance and reported to the Principal Investigator, the Study Director and Study Director's Management as indicated below:

Date of Inspection	Type of Inspection	Date Reported Principal Investigator	Date Submitted to Study Director	Date Reported to Study Director's Management
22 December 2011	In process Audit	29 December 2011	08 February 2012	08 February 2012
09 February 2012	Data Audit	09 February 2012	15 February 2012	15 February 2012
09 February 2012	Draft Report Audit	09 February 2012	15 February 2012	15 February 2012
13 February 2012	Final Report Audit	13 February 2012	15 February 2012	15 February 2012

[redacted]
Quality Assurance

15 Feb 2012
Date

BECKMAN COULTER GENOMICS

GLP COMPLIANCE STATEMENT

This study phase was conducted in accordance with EPA Good Laboratory Practices (40 CFR Part 160) and approved Beckman Coulter Genomics, Inc. standard operating procedures. The reported data accurately reflects the raw data generated during the course of this study.

[redacted]

Principal Investigator
Melissa Barhoover, Ph.D.

15feb12

Date

BECKMAN COULTER GENOMICS

FINAL PHASE REPORT

1.0 Title: Event SYHT0H2 Soybean: Functional Element Copy Number Southern Blot Analysis

2.0 Phase Number: 288-009

3.0 Sponsor Study No.: TK0059651

4.0 Purpose: The purpose of this study was to assess the number of copies of the functional element, *avhppd-03* enhancer complex, within the genome of soybean plants derived from transformation Event SYHT0H2.

5.0 Test Site: Beckman Coulter Genomics, Inc.
100 Perimeter Park Drive, Suite C
Morrisville, NC 27560

Principal Investigator: [redacted]

6.0 Sponsor: Syngenta Crop Protection, LLC
Product Safety
3054 East Cornwallis Road
PO Box 12257
Research Triangle Park, NC 27709, USA

Sponsor Study Director: [redacted]

7.0 Study Dates:

Analytical Phase Start Date: 02 November 2011

Sample and Probe Receipt Dates: 04 November 2011, 15 November 2011, 08 December 2011, and 16 December 2011

Final Phase Report Date: 15 February 2012

8.0 Test and Control Substances:

8.1. Test and Control Substances:

BCG Accession ID	Test Materials	Quantity (concentration)	Receipt Date
ZZ176584	SYHT0H2 T4 DNA (test substance)	~480 µl (231.5 ng/µl)	16 December 2011
ZZ175963	'Jack' DNA (control substance)	~500 µl (306.7ng/µl)	04 November 2011

BECKMAN COULTER GENOMICS

8.2. Control Samples:

BCG Accession ID	Controls	Quantity (concentration)	Receipt Date
ZZ176182	Control 2 (15954 – KpnI + PmeI)	80 µl (2.98 pg/µl)	15 November 2011

BCG Accession ID	Reference	Quantity (concentration)	Receipt Date
ZZ176503	Analytical Wide Range ladder	20 ng (200pg/µl)	08 December 2011

8.3. Probes:

BCG Accession ID	Probes	Quantity (concentration)	Receipt Date
ZZ175972	*avhppd-03* enhancer complex (HPPD EC)	150 ng (5 ng/µl)	04 November 2011
ZZ175975	Ladder probe	580 ng (5 ng/µl)	04 November 2011

9.0 Test System: Soybean plants derived from Event SYHT0H2

10.0 Sample Information: Syngenta Crop Protection, LLC (SCP) provided DNA extracted from soybean plants grown from the test and control substances, the positive control, and probes to be used in the study.

11.0 Method of Work: Beckman Coulter Genomics, Inc. (BCG) analyzed the test substance using the probe provided by the Sponsor, according to a custom procedure requested by the Study Director and defined in Beckman Coulter Genomics, Inc. Working Practice Document WN.288.001.B.

11.1. gDNA Restriction Enzyme Digest:

Note: All reactions were 60 µL.

11.1.1 For one digest, the following was added to a micro centrifuge tube:
- 6 µL of appropriate 10x buffer to 1x final concentration
- 6.0 µL of 10X BSA to 1x final concentration
- 3 µg of gDNA
- x µL of sterile distilled water (to final desired volume)
- y µL of each restriction enzyme

11.1.2 The reaction tube was mixed gently by flicking the tube. The tubes were briefly centrifuged prior to the six hour incubation at the recommended incubation temperature for the specific enzyme(s) used.

11.1.3 The tubes were centrifuged briefly to remove condensate from walls and lid.

11.1.4 If necessary, the enzyme(s) were inactivated in the digests by incubating at the recommended temperature for heat-inactivation.

11.1.5 6 µL of gel loading dye was added to each digest.

11.2. Agarose Gel Electrophoresis:

11.2.1 A 450 mL 1% SeaKem Gold agarose gel in 1XTAE was prepared.

11.2.2 Molecular weight markers were prepared by mixing Analytical Marker DNA Wide Range with 1X TE in a final volume of 20 µL, plus 2 µL of gel-loading dye. Marker input was 1 ng as determined by Syngenta's Study Director.

11.2.3 1X TAE buffer was added to the gel rig.

11.2.4 25 µL of 10 mg/mL EtBr was added to the 1X TAE buffer located in the gel rig.

11.2.5 The positive control DNA was added to the appropriate gDNA digest corresponding to one copy per genome.

11.2.6 The samples and molecular weight markers were loaded.

11.2.7 The gel was run ~ 18 hours at a constant voltage (30V).

11.2.8 A picture of the gel was taken using the AlphaImager and the image was placed in the study file.

11.3. Southern Blotting:

11.3.1 The gel was placed in a glass dish, covered with depurination solution, and gently shaken on a rotary shaker for 15 minutes.

11.3.2 The depurination solution was replaced with E-Pure water, and gently shaken on a rotary shaker for 5 minutes.

11.3.3 The E-Pure water was replaced with denaturation solution, and gently shaken on a rotary shaker for 30 minutes.

11.3.4 The Zeta-Probe membrane was labeled appropriately using a pen.

11.3.5 The Zeta-Probe membrane was dipped in E-pure water, coating thoroughly.

11.3.6 The Zeta-Probe membrane was dipped in denaturation solution, coating thoroughly.

11.3.7 Using denaturation solution as transfer buffer, the downward alkaline Southern blotting transfer of the DNA from the gel onto a Zeta-Probe membrane was set up, using a vacuum blotter. The blotting proceeded for 3 hours at 5 in Hg.

11.3.8 The membrane was rinsed briefly in 2X SSC.

11.3.9 The DNA was cross-linked to the membrane using a UV Stratalinker by selecting the “auto-crosslink” function. Unnecessary edges were trimmed off the membrane with scissors or a safety scalpel.

Note: Cross-linked membranes were stored at -20°C between two filter papers in a plastic bag until ready to proceed to the hybridization step. The membranes were completely dry before placing them in the plastic bag.

11.4. Probe Labeling/Counting:

11.4.1 25 ng DNA for both the gene of interest (GOI) and the ladder were labeled with α-^{32}P.

11.4.2 Labeled probes were purified.

11.4.3 BioScan QC-4000 was used to determine the cpm.

11.4.4 The final concentration for gene-specific probes and marker-specific probes was 1,000,000 counts/mL and 500,000 counts/mL, respectively.

Note: Marker-specific probes were labeled and added separately from gene-specific probes.

11.5 Membrane Hybridization:

11.5.1 The entire PerfectHyb Plus Hybridization buffer bottle was warmed to 65°C to ensure that buffer was homogeneous.

11.5.2 For each hybridization, 30 mL of PerfectHyb Plus Hybridization buffer was aliquoted into a separate 50 mL conical tube and incubated in a water bath set at 65°C for at least 30 minutes.

11.5.3 The calf thymus DNA was denatured by placing the tube in a 95°C to 100°C heat block for at least 5 minutes.

11.5.4 The denatured calf thymus DNA was snap-cooled by removing the tube from the heat block and placing it immediately on ice for at least 5 minutes.

11.5.5 300 µL of the cooled denatured calf thymus DNA was added to each 30 mL aliquot of pre-warmed PerfectHyb Plus buffer, mixed well, and transferred to the radioactivity lab immediately.

11.5.6 The pre-warmed hybridization buffer with the calf thymus DNA was transferred into a 200 mL hybridization tube, and the tube was tilted to coat the sides.

11.5.7 Oven mesh was cut to be slightly larger than the membrane and rolled together so that membrane was completely covered by oven mesh.

11.5.8 Cross-linked membrane(s) in oven mesh were placed into the hybridization tube and the tube was rotated so that the membrane was completely and evenly coated with the hybridization buffer.

11.5.9 Pre-hybridization at 65°C was allowed for one hour.

11.5.10 The probe was denatured in 500 µL hybridization buffer immediately before incubation at 95°C to 100°C for approximately 5 minutes. A 2.0 mL screw-top cap was used for this process.

11.5.11 The probe was snap-cooled after incubating by removing it from the heat block and placing it immediately on ice for at least 5 minutes.

11.5.12 The probe was briefly spun down to remove contents from walls and lid.

11.5.13 Radiolabeled probe and marker were added.

11.5.14 The hybridization was allowed to proceed overnight at approximately 65°C.

11.5.15 The hybridization buffer was discarded into the high level radioactive waste container.

11.5.16 A sufficient amount of low stringency wash solution was added to fill approximately half of the tube, being careful not to pour it directly onto the membrane, and the tube was placed back into the hybridization oven for at least 15 minutes.

11.5.17 The low stringency wash solution was poured off into the high level radioactive waste container. The low stringency wash was repeated.

11.5.18 The low stringency wash solution was poured off into a high level radioactive waste container. High stringency wash solution was added and the tube was placed back into the hybridization oven for the desired amount of time.

11.5.19 The high stringency wash solution was poured off into a high level radioactive waste container.

11.5.20 The high stringency wash was repeated.

11.5.21 Wash solution was discarded into a high level radioactive waste container. Additional washes may have been performed following film development if deemed necessary by the SCP Study Director.

11.5.22 The moist membrane was wrapped in Saran Wrap for exposure to X-ray film.

11.5.23 The X-ray film was exposed and developed.

12.0 Chain-of-Custody Procedure: To ensure end-to-end chain-of-custody, the following procedures were established and followed for all samples:

12.1. Sample Receipt: Upon receipt at the Beckman Coulter Genomics, Inc. facility, each sample container was examined for condition. The sample condition was recorded for all samples.

12.2. Sample Processing: Samples were tracked during all procedures using a unique sample identifier in order to maintain chain-of-custody.

13.0 Results: See Appendix I results.

14.0 Unplanned Deviations/Phase Plan Amendment: There was one Phase Plan Amendment that occurred during the course of this study. The purpose section was changed to reflect only the data reported herein. There was no impact to the study.

There were two unplanned deviations that occurred during the course of this study. UD12-010 was initiated against SOP NC.SM.002, Receipt and Accessioning of Samples, for the sample being accessioned into the wrong study number. The sample was processed in the correct study and the study documentation and LIMS entry was corrected; therefore there is no impact to the data integrity. UD12-011 was initiated against SOP NC.GL.045 for the second review not being signed off on the Study Strategy prior to testing. The Study Strategy was reviewed by a Qualified Trainer, but the document was not signed at the time. There was no impact to the data integrity.

15.0 Sample Disposition and Record Retention: All project samples were stored under conditions appropriate for the sample types. Beckman Coulter Genomics, Inc. will retain project samples for at least 90 days beyond the completion of the study. At some point beyond 90 days, all samples will be destroyed unless an agreement has been established for a longer retention period. Beckman Coulter Genomics, Inc. will transfer the original phase plan, original phase report, and all raw data and records, except for the facility-specific records, to Syngenta Crop Protection, LLC for archiving within 90 days of study completion. Facility-specific records will be retained by BCG for no less than 10 years. At the end of this 10-year period, original facility-specific records, pertinent to this study phase, will be transferred to SCP for archival.

16.0 Key Laboratory Personnel

[redacted]

Principal Investigator
Production Scientist
Production Scientist
Production Scientist
Production Scientist
Production Scientist
Production Scientist

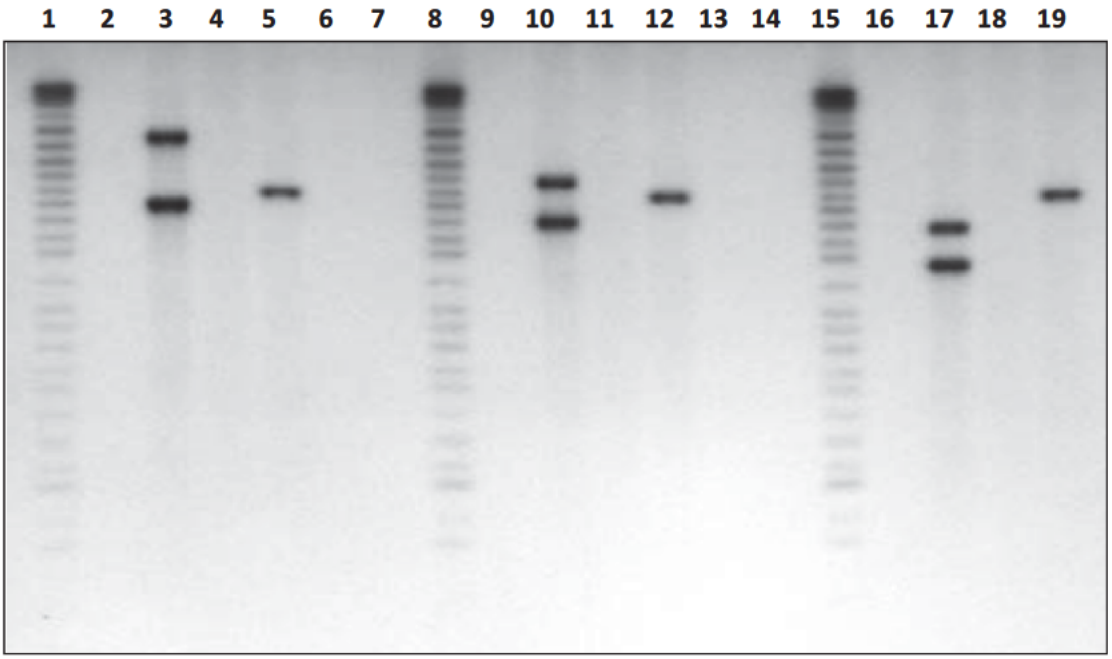
17.0 Appendices:

Appendix I *avhppd-03* Enhancer Complex

BECKMAN COULTER GENOMICS

Appendix I

avhppd-03 Enhancer Complex



Lane	Sample	Sample Tube Used
Lane 1	Analytical Wide Range Ladder	ZZ176503
Lane 2	Blank	NA
Lane 3	SYHT0H2T4 – *Eco* RI	ZZ176584
Lane 4	'Jack' - *Eco* RI	ZZ175963
Lane 5	'Jack' - *Eco* RI + Control 2	ZZ175963 + ZZ176182
Lane 6	Blank	NA
Lane 7	Blank	NA
Lane 8	Analytical Wide Range Ladder	ZZ176503
Lane 9	Blank	NA
Lane 10	SYHT0H2T4 – *Xcm* I	ZZ176584
Lane 11	'Jack' - *Xcm* I	ZZ175963
Lane 12	'Jack' - *Xcm* I + Control 2	ZZ175963 + ZZ176182
Lane 13	Blank	NA
Lane 14	Blank	NA
Lane 15	Analytical Wide Range Ladder	ZZ176503
Lane 16	Blank	NA
Lane 17	SYHT0H2T4 – *Kpn* I HF + *Bsr* BI	ZZ176584
Lane 18	'Jack' - *Kpn* I HF + *Bsr* BI	ZZ175963
Lane 19	'Jack' - *Kpn* I HF + *Bsr* BI + Control 2	ZZ175963 + ZZ176182