Appendix 5

Molecular Bridging Study for Herbicide-tolerant Soybean BPS-CV127-9

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MOLECULAR BRIDGING STUDY FOR HERBICIDE-TOLERANT SOYBEAN BPS-CV127-9

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA §10(d) (1) (A), (B), or (C).

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Company Agent: [redacted] Date: 12 Dec 2007

Title: Regulatory Affairs Manager

Signature: [redacted]

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STATEMENT OF COMPLIANCE

This study was not conducted in compliance with the requirements of 40 CFR Part 160.

The data generated by BASF Plant Science in support of product safety comply with generally accepted scientific procedures. Record-keeping is consistent with procedures used throughout the research community. This report accurately presents the raw data developed during the study.

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ABBREVIATIONS AND DEFINITIONS

AHAS	acetohydroxyacid synthase enzyme
ahasl	imidazolinone-tolerant alleles of acetohydroxyacid synthase large subunit gene; includes the *ahasl* (S653N) allele found in plasmid pAC321 and the *ahasl* (R272K, S653N) allele found in BPS-CV127-9
AHASL	wild-type *Arabidopsis thaliana* acetohydroxyacid synthase large subunit gene; also referred to in the literature as *CSR1*
bp	base pairs
CDS	coding sequence
csr1-2	allele of *Arabidopsis thaliana* acetohydroxyacid synthase large subunit gene; contains a single mutation (S653N) which confers resistance to imidazolinone herbicides
DNA	deoxyribonucleic acid
F_5	fifth filial generation
F_8	eighth filial generation
gDNA	genomic DNA
PCR	polymerase chain reaction
R272K	arginine residue at position 272 of *Arabidopsis thaliana* acetohydroxyacid synthase large subunit replaced with lysine
S653N	serine residue at position 653 of *Arabidopsis thaliana* acetohydroxyacid synthase large subunit replaced with asparagine
T_0	generation resulting from regenerated transformed cells
T_4	fourth transgenic generation
UTR	untranslated region

MOLECULAR BRIDGING STUDY FOR HERBICIDE-TOLERANT SOYBEAN BPS-CV127-9

SUMMARY

Soybean [*Glycine max* (L.) Merr.] plants have been developed that are tolerant to the imidazolinone class of agricultural herbicides. The herbicide-tolerant soybean plants, referred to as BPS-CV127-9, were produced by the introduction of an imidazolinone-tolerant acetohydroxyacid synthase large subunit (*ahasl*) gene from *Arabidopsis thaliana* (L.) Heynh. into the soybean plant genome. Previously, a complete molecular characterization of the transgene insert in the eighth filial (F_8) generation of BPS-CV127-9 line 603 was conducted (Shen, 2007). Shen demonstrated that the novel *ahasl* expression cassette was integrated at a single genetic locus in the soybean genome and that the *ahasl* expression cassette was stably inherited across the nine generations studied (Shen, 2007). BPS-CV127-9 line 127 was derived from BPS-CV127-9 line 603 by traditional breeding methods and used to generate regulatory safety data in regulatory field trials in Brazil in 2006-2007. This current study extends the previous intergenerational stability data. This study demonstrates by Southern blot analysis that the transgene insert in BPS-CV127-9 line 603, from which some commercial varieties of BPS-CV127-9 will be derived, is identical to the transgene insert in line 127 and thus, the transgene insert is stably inherited between these lines and the regulatory safety data generated from line 127 is equally applicable to line 603.

INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] plants have been developed that are tolerant to the imidazolinone class of agricultural herbicides. The herbicide-tolerant soybean plants, referred to as BPS-CV127-9, are derived from a single transformation event and were produced by the introduction of an imidazolinone-tolerant acetohydroxyacid synthase large subunit (*ahasl*) gene from *Arabidopsis thaliana* (L.) Heynh. into the soybean plant genome via biolistics using the PvuII fragment of transformation vector pAC321 (Table 1 and Figure 1). The PvuII fragment includes the wild-type arabidopsis *AHASL* promoter, the herbicide-tolerant arabidopsis *ahasl* coding sequence, and the wild-type arabidopsis *AHASL* terminator. This promoter, coding sequence, and terminator cassette is referred to herein as the *ahasl* cassette.

Acetohydroxyacid synthase (AHAS) is found ubiquitously in the plant kingdom. The AHAS enzyme catalyzes the first step in the biosynthesis of branched-chain amino acids in plants. In conventional plants, inhibition of the AHAS enzyme by imidazolinone herbicides leads to a deficiency in branched-chain amino acids and other compounds derived from this pathway that are needed for plant survival. The *ahasl* gene from arabidopsis confers tolerance to imidazolinone herbicides by encoding an AHAS catalytic subunit with altered herbicide-binding properties, while retaining its normal biosynthetic function in the soybean plant.

The herbicide tolerance of BPS-CV127-9 allows growers to treat the soybean crop with imidazolinone herbicides with no significant injury at normal field application rates. Therefore, introduction of herbicide-tolerant BPS-CV127-9 soybean varieties offers soybean growers an additional tool for improved weed control. Furthermore, it is expected that growers planting BPS-CV127-9 soybeans will be able to reduce the number of herbicides used to control weeds in their soybean fields and benefit from reduced weed-control costs. The reduction in herbicide use is also expected to benefit the environment.

The purpose of this study was to demonstrate that the transgene insert in BPS-CV127-9 line 127 is the same as the transgene insert in a related line, line 603. Line 603 was used for the molecular characterization of BPS-CV127-9 (Shen, 2007) and also as a donor source for introgression of the herbicide tolerance trait into commercial soybean varieties by traditional breeding methods. BPS-CV127-9 line 127 was derived by breeding from BPS-CV127-9 line 603 and used to generate regulatory safety data in regulatory field trials in Brazil in 2006-2007.

MATERIALS AND METHODS

Source of plant materials. Leaf tissue from the fifth filial (F_5) generation of BPS-CV127-9 line 127, as well as non-transgenic soybean variety 'Conquista', was provided to BASF Plant Science Limburgerhof by BASF SA for genomic DNA isolation and characterization. These leaf samples were harvested from field-grown plants in Brazil during the 2006-2007 season regulatory field trials, frozen on dry ice, and shipped to the BASF Plant Science analytical laboratory in Limburgerhof, Germany. Leaf tissue from the F_8 generation of BPS-CV127-9 line 603, as well as non-transgenic soybean variety 'Conquista', was collected from greenhouse-grown plants in Limburgerhof. Furthermore, the BPS-CV127-9 line 603 F_8 generation and non-transgenic 'Conquista' genomic DNA preparations used in the original molecular characterization (Shen, 2007) were analyzed side-by-side with the freshly-isolated genomic DNA from the plant material just described. The breeding history/pedigree of plant material used within this study is shown in Figure 2.

DNA isolation and quantitation methods. DNA was isolated from soybean leaf tissue via the DNeasy Plant Mini Kit (Qiagen GmbH; Hilden, Germany). Frozen leaf tissue (50-100 mg) was powdered with tungsten beads in a MM 300 mixer mill (Retsch GmbH; Haan, Germany) for 1 minute (frequency 20) and incubated with preheated extraction Buffer AP1 (including RNase) at 65°C for 10 minutes. After adding Buffer AP2 and incubating for 5 minutes on ice, the lysate was loaded on a QIAshredder Mini spin column and centrifuged at 16,000 x g for two minutes. The flow-through fraction was mixed with 1.5 volumes of Buffer AP3/E and bound to a DNeasy Mini spin column, washed two times with Buffer AW, and eluted two times with 100 µl double-distilled water. Quantification was performed by measuring the optical density at 260 nm using a NanoDrop ND-100 (NanoDrop Technologies; Wilmington, Delaware).

Probe isolation and labeling methods. The DNA fragment used as a transgene-specific probe is indicated in Figure 1B. Specifically, the probe spans the *ahasl* coding sequence plus an additional 215 bp of the *AHASL* promoter and 5' untranslated region and 125 bp of the *AHASL* 3' untranslated region. The probe DNA fragment was generated by polymerase chain reaction (PCR) amplification using 20 pg of plasmid pAC321 as a template. The probe was labeled with digoxygenin according to the PCR DIG Probe Synthesis Kit (Roche Diagnostics GmbH; Mannheim, Germany) instruction manual. The PCR DIG Probe Synthesis Mix was diluted with an equal volume of the provided dNTP stock solution when preparing the amplification reaction to reduce the DIG-dUTP concentration. The thermal cycling conditions recommended in the instruction manual were utilized. The labeled probe was checked by electrophoresis on an agarose gel. Digoxigenin-labeled fragments migrated at a higher molecular weight due to incorporated digoxigenin.

Primers Used to Generate Probe for Southern Blot Analysis

Probe	Direction	Primer Sequence	Position (Fig. 1)
ahasl CDS	Forward	CGAAGGCTCAATCACAAATAC	2269-2289
	Reverse	AGCAGGCAGATCAACAAC	4604-4621

Southern blot analysis. Southern blot analysis was used to confirm that the fingerprint of the transgene insert in BPS-CV127-9 was the same in lines 127 and 603. Restriction enzymes NcoI and SpeI (New England Biolabs; Ipswich, Massachusetts) were used to digest the genomic DNA of lines 127 and 603 and the non-transgenic control 'Conquista'. A single NcoI restriction site in the *ahasl* cassette is located at the 5' end of the *ahasl* coding sequence and digestion of genomic DNA of BPS-CV127-9 soybean lines with NcoI was expected to generate two fragments that contain DNA from the *ahasl* cassette. Both fragments are defined by the NcoI site in the *ahasl* cassette and by the nearest NcoI sites in the flanking soybean genomic sequence. There is one SpeI restriction site in the 5' flanking soybean genomic sequence, one SpeI site downstream of the *AHASL* 3' untranslated region (UTR), and a third SpeI site in the 3' flanking sequence in BPS-CV127-9 soybean lines. The number and sizes of the DNA fragments expected to be detected by Southern hybridization are listed in Table 2.

Genomic DNA (7 µg) from BPS-CV127-9 lines 603 and 127, as well as the non-transgenic control 'Conquista', was digested overnight at 37°C under the conditions specified by the manufacturer of the restriction enzymes. Two additional non-transgenic 'Conquista' genomic DNA samples were spiked with one- and two-copy equivalents[1] of pAC321 plasmid DNA (27 and 54 pg, respectively) and used as positive controls. Restriction digests were precipitated by glycogen and ethanol and separated by electrophoresis in a ten-centimeter-long 1% agarose gel without ethidium bromide. The DNA was further fragmented by soaking the gel in 0.25 M hydrochloric acid for about 10 minutes and then denatured with 0.5 M sodium hydroxide/1.5 M sodium chloride for about 30 minutes. The gel was rinsed with 1.5 M sodium chloride/0.5 M Tris, pH 7.5 for 15 minutes and the denatured DNA transferred onto Hybond N+ nylon membrane (GE Healthcare; Munich, Germany) using 10X SSC (150 mM sodium citrate, pH 7.0; 1.5 M sodium chloride) as a transfer buffer. The blot was crosslinked with 120,000 microjoules using a Stratalinker 1800 UV Crosslinker (Stratagene; La Jolla, California). Southern hybridization was carried out as specified by the manufacturer's manual for DIG Easy Hyb (Roche Diagnostics GmbH). The membrane was prehybridized at 42°C for 4 hours in a hybridization bottle in a HB-1D Hybridization Oven (Techne Inc.; Burlington, New Jersey) and hybridized at 42°C overnight with a probe concentration of 50 ng/ml in a volume of 50-100 ml. After hybridization, the membrane was washed twice with 2X SSC, 0.1% sodium dodecyl sulfate at room temperature for 5 minutes, twice in 0.5X SSC, 0.1% sodium dodecyl sulfate at 65°C for 15 minutes, and rinsed 1 minute with 100 ml 1X Washing Buffer from the DIG Wash and Block Buffer Set (Roche Diagnostics GmbH). All further steps were performed according to the DIG Wash and Block Buffer Set product instructions. After blocking for 60 minutes in 100 ml Blocking solution (DIG Wash and Block Buffer Set) and incubating for 30 minutes with 100 ml of alkaline phosphatase-conjugated digoxigenin antibody (Roche Diagnostics GmbH) diluted 1:20,000 in Blocking solution, the membrane was washed three times for 10 minutes with 100 ml Washing buffer and incubated for two minutes in 100 ml of Detection buffer (DIG Wash and Block Buffer Set). CDP-Star solution (Roche Diagnostics GmbH) was added to completely cover the membrane and the membrane was incubated for approximately 5 minutes at room temperature. Chemiluminescense was detected using a LAS-3000 imaging system (Raytest GmbH; Straubenhardt, Germany).

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[1] ***CALCULATION OF COPY NUMBER EQUIVALENTS***

Assumptions:

- The haploid content of the soybean genome is 1.115×10^9 bp (Arumuganathan and Earle, 1991).
- Plasmid pAC321 is 8,669 bp.

Since the insert in BPS-CV127-9 is homozygous and 7 µg of soybean genomic DNA is used per digest in the Southern blot analysis, the mass of one copy equivalent of pAC321 is:

$$\frac{\text{mass of pAC321 DNA}}{\text{7 µg genomic DNA}} = \frac{\text{8,669 bp transgene DNA}}{(1.115 \times 10^9 \text{ bp genomic DNA}) \times 2}$$

Mass of one–copy equivalent of pAC321 DNA = 27 pg

RESULTS AND DISCUSSION

Insert Stability Confirmation. The profile of the hybridizing bands associated with the transgene insert in BPS-CV127-9 was evaluated by Southern blot analysis of genomic DNA from lines 127 and 603 digested with restriction enzymes NcoI and SpeI. The Southern blot (Figure 4) was hybridized with a probe which included 215 bp of the *AHASL* promoter and 5' UTR along with the entire *ahasl* coding sequence and 125 bp of the *AHASL* 3' UTR (Figure 1, Panel B). Genomic DNA from the non-transgenic variety 'Conquista' as well as 'Conquista' genomic DNA spiked with one- and two-copy equivalents of pAC321 plasmid DNA served as controls in the Southern blot.

Non-transgenic 'Conquista' DNA digested with either NcoI or SpeI and hybridized with the *ahasl* coding sequence probe showed hybridizing bands at approximately 2,500 and 1,700 bp for NcoI digests and 2,900 bp for SpeI digests indicating that, at the Southern blot stringency conditions used, there was cross-hybridization with an endogenous gene (Figure 4, lanes 13 through 18). These bands were detected regardless of the source of 'Conquista' material: field-grown in Brazil (Figure 4, lanes 15 and 16), greenhouse-grown in Germany (Figure 4, lanes 13 and 14), or genomic DNA isolated in Canada for the BPS-CV127-9 molecular characterization report BPS-001-06 (Figure 4, lanes 17 and 18). These cross-reactive bands are also visible in the non-transgenic 'Conquista' DNA spiked with plasmid pAC321 DNA at one- and two-copy equivalents (Figure 4, lanes 1 through 4).

Non-transgenic 'Conquista' DNA spiked with plasmid pAC321 at one- and two-copy equivalents and digested with NcoI shows a unique hybridizing band, relative to unspiked 'Conquista', at approximately the expected size of linearized pAC321, 8,669 bp (Figure 4, lanes 1 and 3, and expected size from Table 2). SpeI digests of pAC321-spiked 'Conquista' show a hybridizing band, close to the predicted pAC321 SpeI hybridizing fragment size of 8,292 bp (Figure 4, lanes 2 and 4, and expected size from Table 2).

The same BPS-CV127-9 line 603 F_8 generation genomic DNA which was subjected to Southern blot analysis in the event molecular characterization (Shen, 2007) was also digested with NcoI or SpeI and subjected to Southern blot analysis in this study (Figure 4, lanes 19 and 20). Two unique hybridizing bands were observed in NcoI digests of line 603 at approximately 4,600 bp and 9,800 bp. The Southern blot was hybridized with a probe which included 215 bp of the *AHASL* promoter and 5' UTR along with the entire *ahasl* coding sequence and 125 bp of the *AHASL* 3' UTR. The solitary NcoI site within the BPS-CV127-9 transgene insert exists at the 5' end of the *ahasl* coding sequence (Figure 3). Thus, the 4,600 bp observed hybridizing band most likely corresponds to a DNA fragment produced from the NcoI restriction site within the insert and a site in the 5' flanking soybean genomic DNA and contains the *AHASL* promoter and 5' UTR. Similarly, the 9,800 bp observed hybridizing band most likely corresponds to a DNA fragment produced from the NcoI restriction site within the insert and a site in the 3' flanking soybean genomic DNA and contains the *ahasl* coding sequence (Table 2). Two unique hybridizing bands were observed in Spe I digests of line 603 at approximately 4,500 bp and 890 bp. Three SpeI sites have been identified in the BPS-CV127-9

transgene insert and flanking sequence. The first SpeI site is in the 5' flanking sequence, the second SpeI site is downstream of the *AHASL* 3' UTR, and the third SpeI site is in the 3' flanking sequence (Figure 3). Thus, the observed hybridizing bands would correspond to an expected 4,352 bp fragment containing the *AHASL* promoter and 5' UTR as well as the *ahasl* coding sequence and an expected 885 bp fragment containing a small portion of the *ahasl* coding sequence which was duplicated immediately upstream of the 3' transgene integration site (Table 2).

Southern blot analysis of NcoI or SpeI digests of genomic DNA extracted from the F_8 generation of BPS-CV127-9 line 603 grown in a greenhouse in Germany (Figure 4, lanes 6 and 7) showed the same pattern of unique hybridizing bands as was observed with the line 603 F_8 generation genomic DNA prepared in Canada (Figure 4, lanes 19 and 20). The BPS-CV127-9 line 603 NcoI digest in lane 6 of Figure 4 shows weaker hybridizing bands than observed in other lanes. This is likely due to a gel-loading error, as evident by the faint band observed in the adjacent "blank" lane, lane 5.

BPS-CV127-9 line 127 NcoI and SpeI digests (Figure 4, lanes 8 and 9) show a pattern of unique hybridizing bands that is identical to those observed with BPS-CV127-9 line 603 (Figure 4; lanes 6, 7, 19, and 20). BPS-CV127-9 line 603 was analyzed in the event molecular characterization report (Shen, 2007) and used as a donor source for introgression of the herbicide tolerance trait into commercial soybean varieties by traditional breeding methods. BPS-CV127-9 line 127 was derived by breeding from BPS-CV127-9 line 603 and used to generate regulatory safety data in regulatory field trials in Brazil in 2006-2007. This study demonstrates that the Southern blot profile of the transgene insert in BPS-CV127-9 line 603, from which some commercial varieties of BPS-CV127-9 will be derived, is identical to the transgene insert in line 127. Thus, the transgene insert is stably inherited between these lines and the regulatory safety data generated from line 127 is equally applicable to line 603.

CONCLUSIONS

BPS-CV127-9 line 603 was previously analyzed in the event molecular characterization report (Shen, 2007). BPS-CV127-9 line 127 was derived by traditional breeding methods from BPS-CV127-9 line 603 and used to generate regulatory safety data in regulatory field trials in Brazil in 2006-2007. This current study demonstrates that the Southern blot profile of hybridizing bands for the transgene insert in BPS-CV127-9 line 603, from which some commercial varieties of BPS-CV127-9 will be derived, is identical to the Southern blot profile of hybridizing bands for the transgene insert in line 127. Thus, the transgene insert is stably inherited between these lines and the regulatory safety data generated from BPS-CV127-9 line 127 is equally applicable to BPS-CV127-9 line 603.

RECORDS RETENTION

Raw data, the original copy of this report, and other relevant records are archived at BASF, 26 Davis Drive, Research Triangle Park, North Carolina 27709 U. S. A.

STUDY PERSONNEL

Analytical work reported herein conducted by [redacted], Ph.D., BASF Plant Science GmbH, D-67117 Limburgerhof, Germany.

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Shen, Y. (2007) Molecular characterization of Cultivance Soybean Event 127. BASF Plant Science Report No. BPS-001-06.

Short, J. M., Fernandez, J. M., Sorge, J. A., and Huse, W. D. (1988) λ ZAP: a bacteriophage λ expression vector with *in vivo* excision properties. Nucl. Acids Res. 16:7583-7600.

Table 1. DNA Components of Plasmid pAC321 (8,669 bp)

Genetic Element	Range (bp)	Function
Arabidopsis gDNA, unannotated	1-1051	*Arabidopsis thaliana* genomic DNA: no genes currently annotated in this region
Arabidopsis locus At3g48570	1052-2113	Protein translocation complex SEC61 GAMMA CHAIN-LIKE protein from *Arabidopsis thaliana*
At3g48570 5' UTR	1052-1113	5' untranslated region for putative arabidopsis *SEC61 GAMMA CHAIN*
At3g48570 CDS	1114-1207, 1307-1422	Putative arabidopsis *SEC61 GAMMA CHAIN* coding sequence
At3g48570 intron 1	1208-1306	Putative arabidopsis *SEC61 GAMMA CHAIN* intron 1, interrupts CDS
At3g48570 3' UTR	1423-1442, 1916-2113	3' untranslated region for putative arabidopsis *SEC61 GAMMA CHAIN*
At3g48570 intron 2	1443-1915	Putative arabidopsis *SEC61 GAMMA CHAIN* intron 2
AHASL putative promoter and 5' UTR	2114-2483	Putative promoter and 5' untranslated region for arabidopsis *ACETOHYDROXYACID SYNTHASE LARGE SUBUNIT*; arabidopsis locus At3g48560
ahasl (S653N) CDS	2484-4496	Coding sequence for *Arabidopsis thaliana acetohydroxyacid synthase large subunit* with (S653N) point mutation (also known as *csr1-2*) which confers tolerance to imidazolinones (Sathasivan *et al*., 1990); accession number X51514
AHASL 3' UTR	4497-4714	3' untranslated region for arabidopsis *ACETOHYDROXYACID SYNTHASE LARGE SUBUNIT*; arabidopsis locus At3g48560
Arabidopsis gDNA, unannotated	4715-5717	*Arabidopsis thaliana* genomic DNA: no genes currently annotated in this region[1]
pBluescript SK(-) phagemid	5718-8669	Stratagene Corporation; La Jolla, CA. (Short *et al*., 1988); accession number X52324 [2]
T7 promoter	5805	Bacteriophage T7 promoter transcription initiation site; allows *in vitro* synthesis of RNA from DNA cloned in phagemid by T7 RNA polymerase
phage f1 (-) ori	6118-6424	Bacteriophage f1 origin of replication; allows single-strand DNA production in *Escherichia coli* strains containing the F' episome when a helper phage is present
bla CDS	6573-7433	*E. coli* β-lactamase coding sequence; confers resistance to β-lactam antibiotics such as ampicillin and carbenicillin

Genetic Element	Range (bp)	Function
ColE1 ori	7581-8248	*E. coli* plasmid replication origin ColE1; derived from pUC19
lacZ promoter	8468-8589	*E. coli lacZ* promoter; drives transcription of the alpha fragment of β-galactosidase (*lacZ'*)
lacZ' CDS, interrupted	8590-8669, 5718-5994	*E. coli* β-galactosidase alpha fragment coding sequence, interrupted by arabidopsis genomic DNA in pAC321; allows blue-white screening for DNA insertions in pBluescript SK(-) multiple cloning site by alpha-complementation
T3 promoter	8632	Bacteriophage T3 promoter transcription initiation site; allows *in vitro* synthesis of RNA from DNA cloned in phagemid by T3 RNA polymerase

1. The sequence of pAC321 differs from the arabidopsis genome sequence data available at www.Arabidopsis.org by a single nucleotide within the unannotated region at nucleotide 5,073 of pAC321. pAC321 is missing an A residue relative to the public sequence.
2. The pBluescript SK(-) sequence in pAC321 differs from accession number X52324 at nucleotide 7,751 of pAC321. Nucleotide 7,751 of pAC321 is T (in agreement with the pUC19 origin of replication sequence, accession number L09137) while the X52324 sequence contains a C residue at this position. This difference does not occur within the region of pAC321 that was used for transformation (the 6,156 bp PvuII fragment).

Table 2. Predicted and Observed Hybridizing Bands in Southern Blot Analysis of BPS-CV127-9 Genomic DNA

Figure	Probe	Restriction Enzyme	Predicted Fragment Size from BPS-CV127-9 Insert (bp)[a]	Observed BPS-CV127-9 Fragment Size (bp)	Predicted Fragment Size from Plasmid pAC321 (bp)
4	***ahasl* CDS**	NcoI	>2,760 >7,896	~4,600[b] ~9,800	8,669
		SpeI	4,352 885	~4,500 ~890[c]	8,292

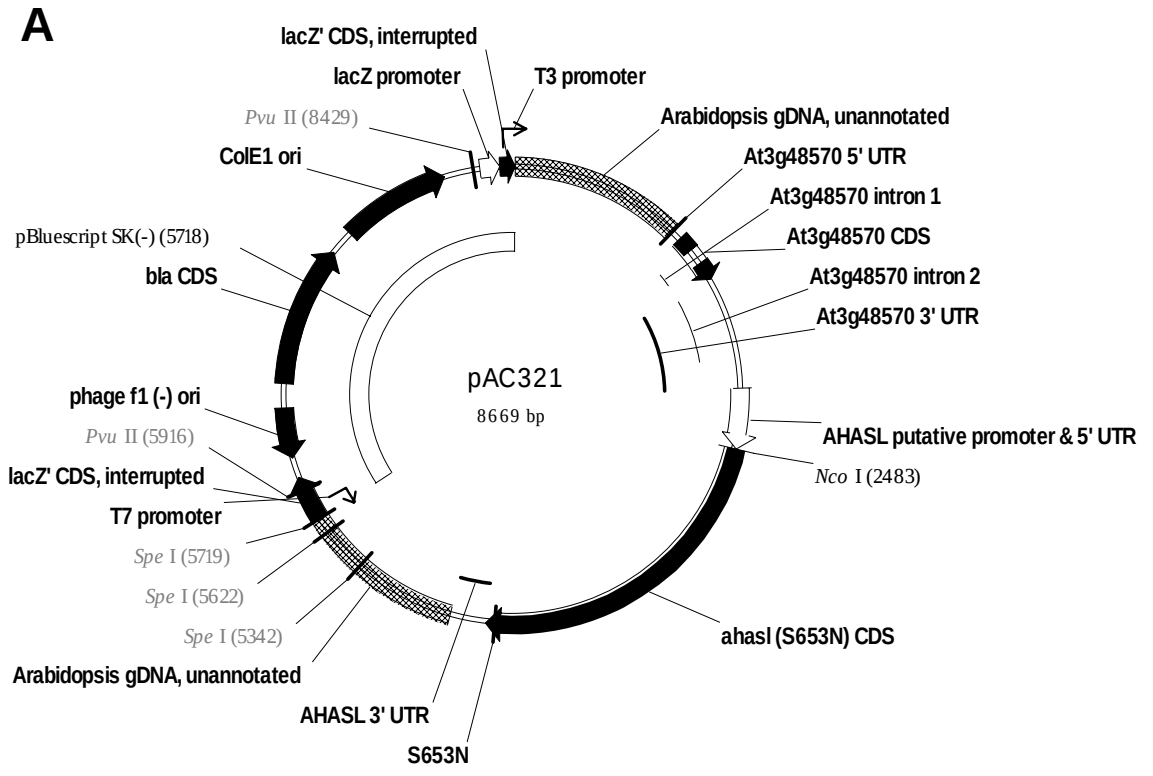
a. The predicted fragment size is estimated based on the cloned insert and flanking sequences in BPS-CV127-9.

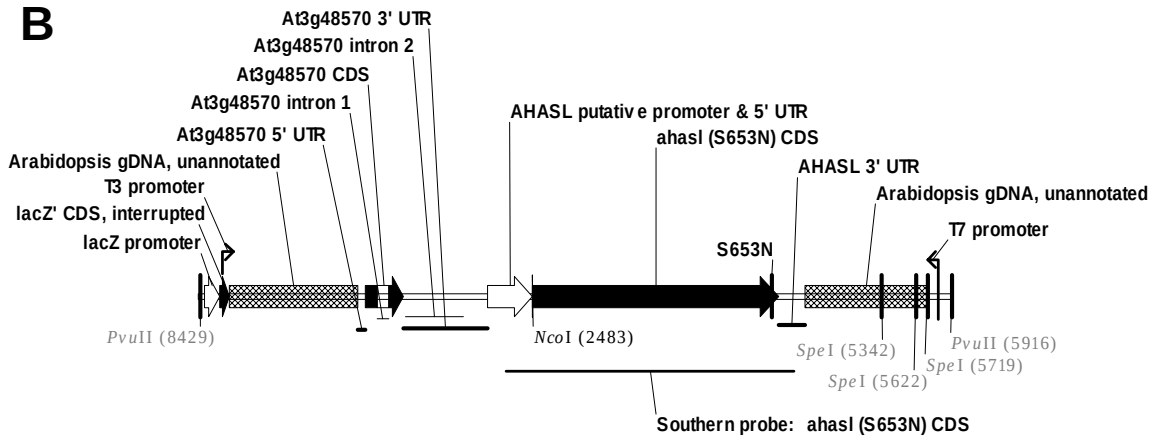
b. The *ahasl* coding sequence probe extended 215 bp into the *AHASL* promoter and 5' UTR and, under the hybridization conditions utilized, also detected the *AHASL* promoter and 5' UTR fragment.

c. Sequence analysis of the BPS-CV127-9 insert indicates that a small portion of the *ahasl* coding sequence was duplicated immediately upstream of the 3' transgene integration site, confirming the identity of the 890 bp band seen in these Southern blots.

Figure 1. Plasmid pAC321

The graphical representation of plasmid pAC321 is pictured in Panel A. For a complete description of the genetic annotations see Table 1. Panel B depicts the pAC321 PvuII fragment which was used for transformation to produce BPS-CV127-9. The restriction sites of the enzymes (NcoI and SpeI) used for Southern blot analyses are indicated. Region of pAC321 amplified to generate the Southern blot probe is also indicated.





Fragment of pAC321

6162 bp (molecule 8669 bp)

Figure 2. Breeding History of BPS-CV127-9

Breeding history of BPS-CV127-9 showing elite event and variety development of line 603 and line 127. The original transformed event is designated as T_0 and the fourth self-fertilized generation as T_4. Key molecular and inheritance data were collected at the indicated points in the history: ⊗ = self-pollination, ● = intergenerational stability (Shen, 2007), ▲= DNA sequence analysis (Shen, 2007), ◊ = Southern blot analysis for molecular characterization (Shen, 2007), and ○ = Southern blot analysis, this report.

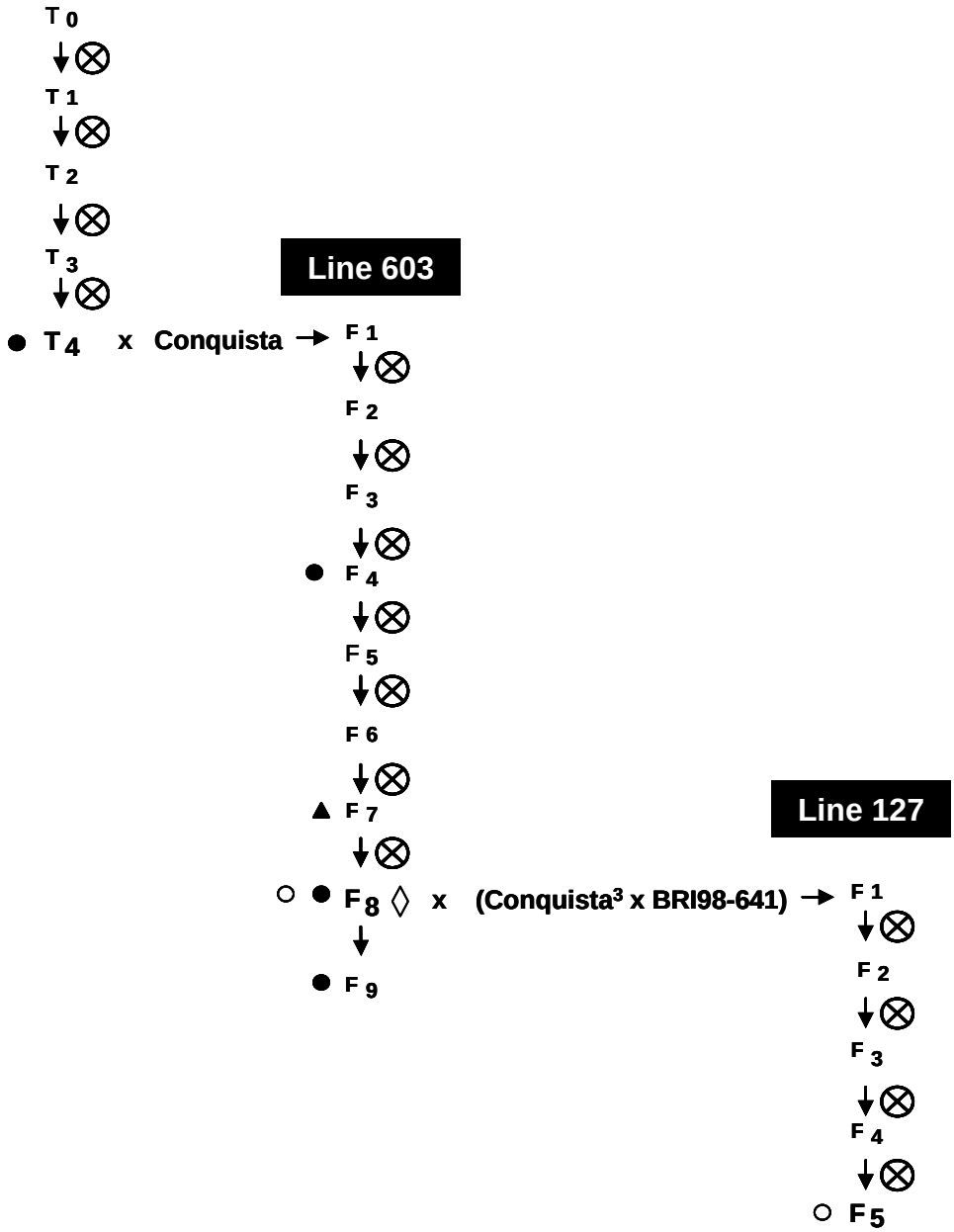


Figure 3. Alignment of pAC321 PvuII Transformation Fragment with BPS-CV127-9 Insert

The PvuII transformation fragment is shown with the portions of the fragment absent from the BPS-CV127-9 insert indicated (diagonally striped boxes). The region within the vertical dotted lines is common to both DNA fragments. Restriction sites relevant to the Southern blot analysis are indicated. The numbering system of the PvuII transformation fragment corresponds to that of the pAC321 plasmid map in Figure 1. The numbering system for the BPS-CV127-9 insert corresponds to that in Figure 8 of report BPS-001-06, where #1 is the first nucleotide at the 5' end of the reported soybean genomic flanking sequence (flanking sequences indicated by gray boxes).

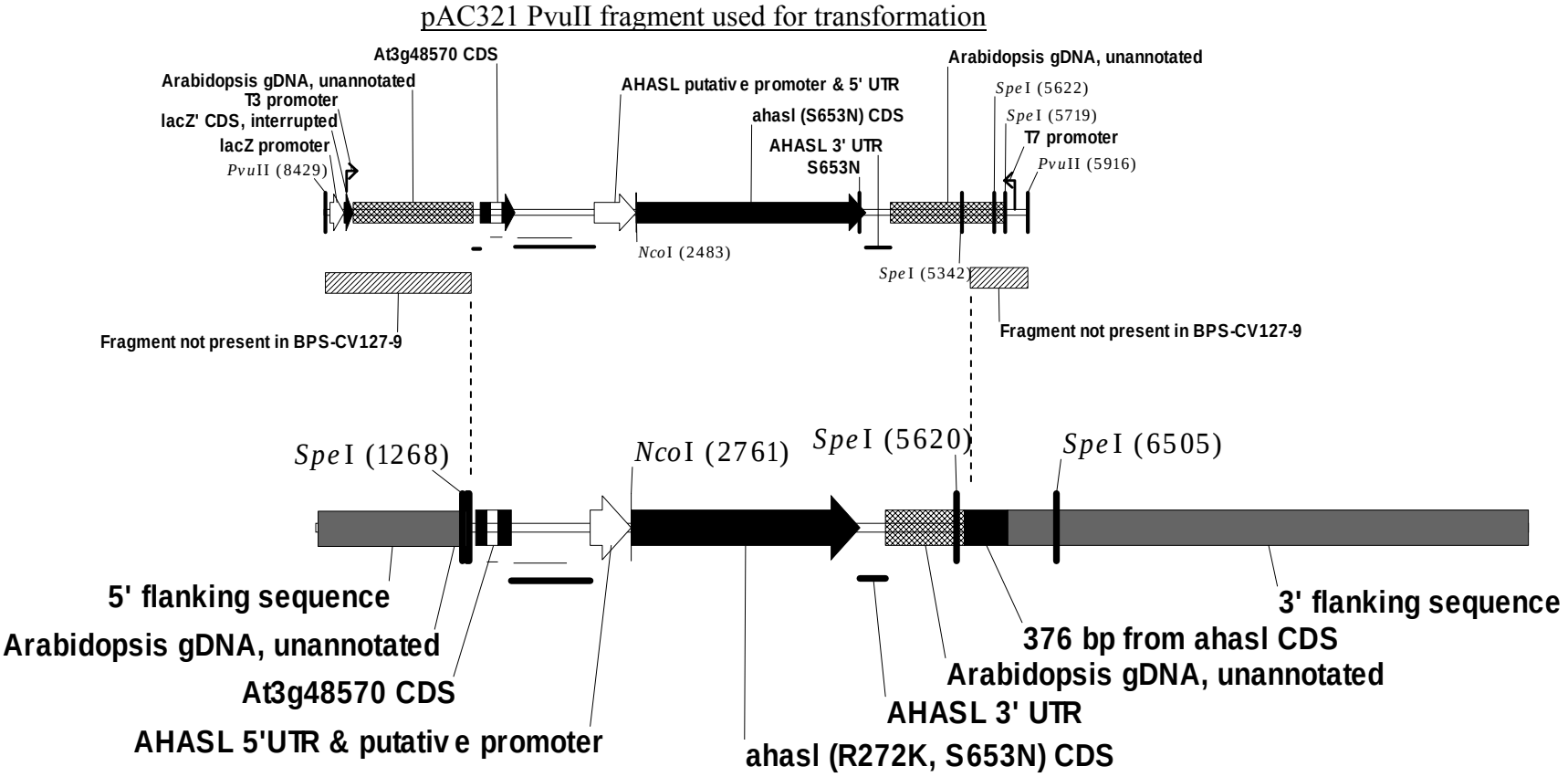


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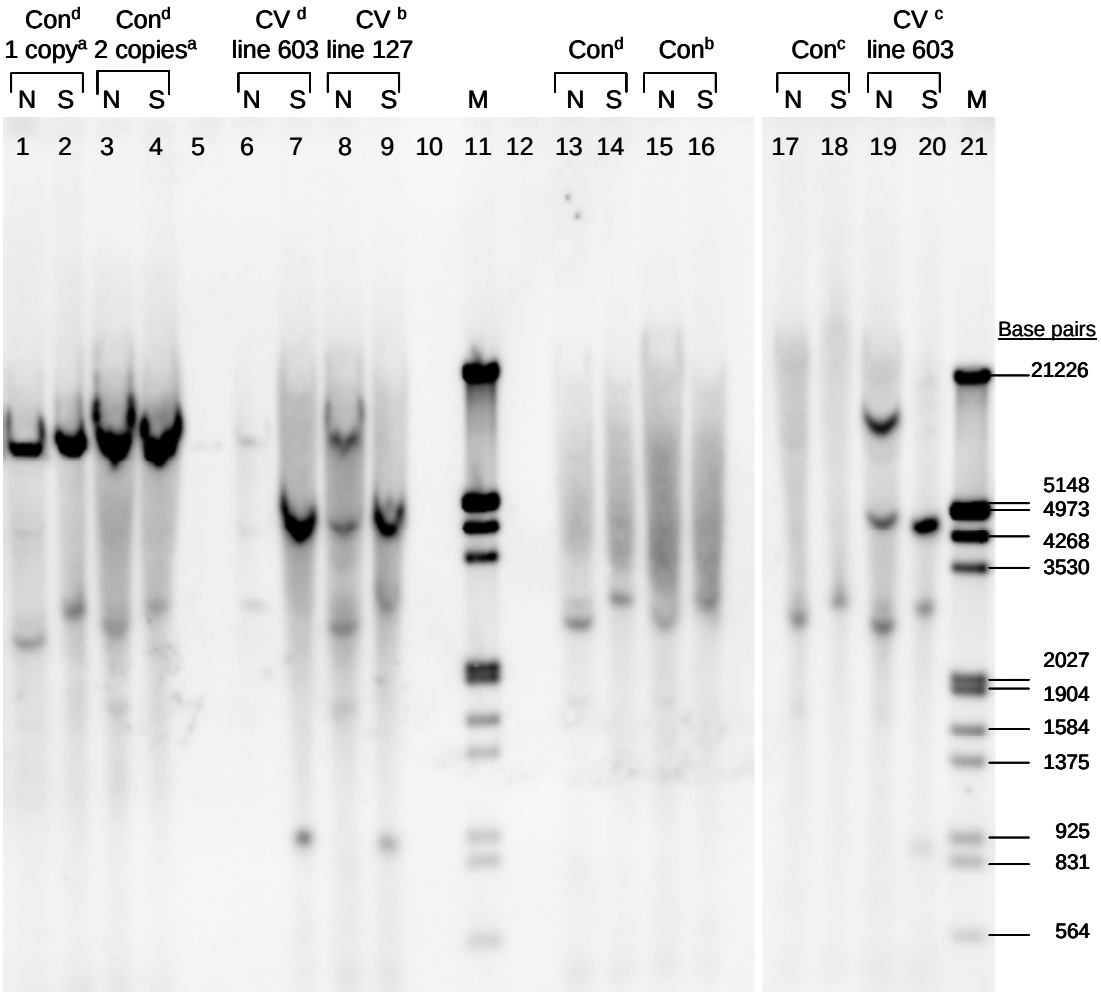
Figure 4. Southern Blot Analysis of BPS-CV127-9: *ahasl* Coding Sequence Probe

Genomic DNA from non-transgenic soybean variety 'Conquista' was spiked with one- (lanes 1 and 2) or two-genome copy equivalents of pAC321 (lanes 3 and 4) and digested with NcoI (N) or SpeI (S). Genomic DNA from the F_8 generation of BPS-CV127-9 line 603 (lanes 6 and 7), the F_5 generation of BPS-CV127-9 line 127 (lanes 8 and 9), and non-transgenic 'Conquista' (lanes 13 through 16) was also digested with NcoI and SpeI. Finally, the non-transgenic 'Conquista' (lanes 17 and 18) and F_8 generation of BPS-CV127-9 line 603 (lanes 19 and 20) genomic DNA which was utilized in the original molecular characterization (Shen, 2007) was also digested with NcoI and SpeI. The Southern blot was hybridized with the *ahasl* coding sequence probe. Lanes labeled "M" each contain 30 ng of DIG-labeled DNA Molecular Weight Marker III (Roche Diagnostics GmbH).



a. The number of genome copy equivalents of pAC321 added to each digest.
b. Genomic DNA isolated from plants grown in Brazil 2006-2007 season regulatory field trials.
c. Genomic DNA also used in BPS-CV127-9 molecular characterization (Shen, 2007).
d. Genomic DNA isolated from plants grown in greenhouse in Limburgerhof, Germany.