Appendix 1

Molecular Characterization of Cultivance Soybean Event 127

BASF
The Chemical Company
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MOLECULAR CHARACTERIZATION OF CULTIVANCE SOYBEAN EVENT 127

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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 the 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
AHASL	acetohydroxyacid synthase large subunit
At *SEC61* γ	*Arabidopsis thaliana* Sec 61 γ subunit
BLAST	basic local alignment tool
bp	base pair
csr1-2	this is the designation for the imidazolinone-tolerant (S653N mutation) acetohydroxyacid synthase large subunit gene from *Arabidopsis thaliana*
CTAB	cetyl trimethyl ammonium bromide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EST	expressed sequence tag
Gm *Sec61* γ	*Glycine max* Sec 61 γ subunit
kb	kilobase
nt	nucleotide
ORF	open reading frame
PCR	polymerase chain reaction
PVP	polyvinylpyrrolidone
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
S653N	serine at position 653 replaced by asparagine
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SSC	sodium chloride/sodium citrate solution
TE	10 mM Tris-HCl, 1 mM EDTA, pH 8.0
UTR	untranslated region
VB	vector backbone

MOLECULAR CHARACTERIZATION OF CULTIVANCE SOYBEAN EVENT 127

SUMMARY

Soybean (*Glycine max* L.) plants have been developed that are tolerant to the imidazolinone class of agricultural herbicides. The herbicide-tolerant soybean plants derived from a single transformation event, referred to as Cultivance Soybean Event 127, were produced by introduction of an imidazolinone-tolerant acetohydroxyacid synthase large subunit (*csr1-2*) gene from *Arabidopsis thaliana* into the soybean plant genome. A complete molecular characterization of the transgenic insert in Cultivance Soybean Event 127 was conducted. The novel *csr1-2* expression cassette was integrated at a single genetic locus in the soybean genome. No DNA sequences from the backbone of the transformation vector were detected in the event. The *csr1-2* cassette in Event 127 contains three point mutations relative to the transformation plasmid with one mutation in the *Arabidopsis thaliana* acetohydroxyacid synthase large subunit (AHASL) coding sequence and the other two downstream of the *AHASL* 3' untranslated region (UTR). The G to A mutation in the coding sequence, which caused an amino acid change of R_{272} to $K_{272,}$ occurred before the T4 generation in the event development. This mutation does not affect the desired phenotype conferred by the *ahasl* gene. Southern blot analysis and sequence verification of the point mutation indicate that the insert is stable across the eight generations studied. In the insert, there is also a 376 base pair (bp) duplication of a portion of the *csr1-2* coding sequence directly before the 3' integration point. This duplicated 376 bp segment creates a 501 bp open reading frame (ORF) that extends into the 3' flanking sequence. Reverse transcription-polymerase chain reaction (RT-PCR) results suggest that this 501 bp ORF is not transcribed. The insert also contains the majority of the Arabidopsis *SEC61* γ subunit gene locus (At3g48570), which is a component of the DNA fragment used for transformation. RT-PCR experiments show that the Arabidopsis *SEC61* γ subunit gene is weakly transcribed in Event 127 leaf tissue. A total of 1.3 kilobases (kb) of 5' flanking soybean DNA has been sequenced together with 4.6 kb of 3' flanking soybean DNA. The flanking sequence information was used in the development of an Event 127-specific qualitative PCR detection method.

INTRODUCTION

Soybean (*Glycine max* L.) plants have been developed that are tolerant to the imidazolinone class of agricultural herbicides. The herbicide-tolerant soybean plants are derived from a single transformation event, referred to as Cultivance Soybean Event 127, and were produced by introduction of an imidazolinone-tolerant acetohydroxyacid synthase large subunit (*ahasl*) gene (*csr1-2*) from *Arabidopsis thaliana* into the soybean plant genome via biolistics using the PvuII fragment of transformation vector pAC321 (Table 1 and Figure 1). This designation, *csr1-2*, has been applied to the *ahasl* gene

which contains a mutation resulting in a single amino acid substitution, (S653N), to provide the imidazolinone herbicide tolerance. The PvuII fragment includes the Arabidopsis *AHASL* promoter, the herbicide-tolerant Arabidopsis *ahasl* (*csr1-2*) coding sequence and the Arabidopsis *AHASL* terminator. This promoter, coding sequence and terminator cassette is referred to herein as the *csr1-2* cassette.

The Arabidopsis AHASL protein is a member of the class of AHAS proteins found ubiquitously in plants. 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 *csr1-2* 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 in Cultivance Soybean Event 127 allows growers to treat the soybean crop with imidazolinone herbicides with no significant injury at normal field application rates. Therefore, introduction of Cultivance soybean varieties offers soybean growers an additional tool for improved weed control. Furthermore, it is expected that growers planting Cultivance 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 characterize the transgene insert and native genomic DNA flanking the transgene insert in Cultivance Soybean Event 127. Specifically, the event was characterized for 1) the number of DNA integration sites in the plant genome and transgene copy number; 2) DNA insert integrity; 3) stability of the insert across breeding generations; 4) sequence of genomic DNA flanking the 5' and 3' ends of the DNA insert, and confirmation that the flanking DNA sequences are native to the plant genome; 5) development of an event-specific PCR detection method; 6) confirmation of lack of vector backbone present in the plant genome; 7) complete sequence of the DNA insert; and 8) identification of any new open reading frame (ORF) created as a result of the DNA insertion in the plant genome and investigation of its potential expression.

MATERIALS AND METHODS

Source of plant materials. Young leaf tissue of Cultivance Soybean Event 127 was provided to DNA Landmarks, Inc., by BASF SA for DNA and RNA isolation and characterization. Control DNA and RNA was isolated from leaf tissue of the non-transgenic soybean variety Conquista. All plants were grown under greenhouse conditions in Londrina, Brazil, and young, fully-expanded trifoliate leaves were harvested for molecular analyses. The breeding history/pedigree of plant material used within this study is shown in Figure 2.

DNA and RNA isolation and quantitation methods. DNA was isolated from soybean leaf tissue via a modified cetyl trimethyl ammonium bromide (CTAB) method (Carlson *et al*., 1991). Silica gel-desiccated leaf tissue was frozen with liquid nitrogen and ground with an Autogrinder (Autogen; Holliston, MA). The ground tissue was incubated with preheated extraction buffer consisting of 2% (w/v) CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 1% (w/v) polyvinylpyrrolidone (PVP), 20 mM ethylenediamine tetraacetic acid (EDTA), pH 9.5 (5 ml/60 mg dried leaf tissue) and β-mercaptoethanol (10 µl/ml buffer) at 74°C for 20 min. After centrifugation at 2440 x *g* for 10 min, the supernatant was extracted twice with an equal volume of chloroform/isoamyl alcohol (24:1). DNA was precipitated with 0.7 volume of isopropanol and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) with 0.5 mg/ml RNase A (Invitrogen; Carlsbad, CA) added to a final concentration of about 500 ng/µl. The isolated DNA was quantified with Hoechst 33258 dye (Invitrogen) with calf thymus DNA (Invitrogen) used as the DNA standard on a Packard FluoroCount™ BF10000 Microplate Fluorometer (Packard Instrument Company; Meriden, CT) according to the fluorometer user manual.

Total RNA was extracted from silica gel-desiccated young leaves derived from F7 and F8 generation plants of Event 127 and from leaves of the non-transgenic parental soybean variety Conquista with the Qiagen RNeasy Mini Kit (Qiagen; Valencia, CA). About 25 mg of silica gel-desiccated leaf tissue was frozen with liquid nitrogen and ground with an Autogrinder. The total RNA isolation procedure was carried out according to the manufacturer's directions. On-column DNase digestion was performed with RNase-Free DNase (Qiagen) to eliminate any soybean genomic DNA from the total RNA preparation according to the recommendation in the RNeasy Mini Kit user manual. The isolated RNA was quantitated by measuring the absorbance at 260 nm using a BioMate™ 3 spectrophotometer (Thermo Electron Corporation; Waltham, MA).

Probe isolation and labeling methods. The DNA fragments used as transgene-specific probes are indicated in Figure 1B. The vector backbone probes are indicated in Figure 5C. Together these 5 overlapping probes span the entire plasmid. Specifically, probe 1 spans the *AHASL* promoter region, probe 2 the *csr1-2* coding sequence, probe 3 the *AHASL* terminator region, and probes 4 and 5 together cover the complete vector backbone (VB). The probe DNA fragments were generated by PCR amplification using plasmid pAC321 as a template. The probes (25 – 50 ng each) were radiolabeled with 50 µCi of (α-^{32}P)-dCTP (3000 Ci/mmol) (MP Biomedicals; Irvine, CA) using the Rediprime™ II DNA Labeling System (Amersham; Piscataway, NJ) according to the manufacturer's instructions. The labeled probes were purified with a Spin-X® Centrifuge Tube Filter (Corning Costar Corporation; Acton, MA).

Primers Used to Generate Probes for Southern Blot Analysis

Purpose	Direction	Primer Sequence	Position (Fig. 1)
Probe 1	Forward	TGCGTTATCCCCTGATTCTG	8261-8280
5' UTR	Reverse	TGTTGGGGTTTAGGGAG	2597-2613
Probe 2	Forward	CGAAGGCTCAATCACAAATAC	2269-2289
AHASL	Reverse	AGCAGGCAGATCAACAAC	4604-4621
Probe 3	Forward	GAACATGTGTTGCCGATGAT	4416-4435
3' UTR	Reverse	CGCAACTGTTGGGAAGGG	5949-5966
Probe 4	Forward	GTTTTACAACGTCGTGACTG	5839-5858
VP1	Reverse	CGGTTAGCTCCTTCGGTC	6997-7014
Probe 5	Forward	CACTGCGGCCAACTTACT	6962-6979
VP2	Reverse	CTTGGCGTAATCATGGTC	8592-8609

Restriction digestions and Southern blot analyses. Southern blot analyses were used to determine the number of copies and the integrity of the *csr1-2* expression cassette as well as to confirm the absence of plasmid backbone in Event 127. Restriction enzymes NcoI, SpeI and XbaI were used to digest the genomic DNA of Event 127 and non-transgenic control Conquista. The pAC321 PvuII transformation fragment is aligned with the Cultivance Soybean Event 127 insert in Figure 3. A single NcoI restriction site in the *csr1-2* cassette is located at the 5' end of the *csr1-2* coding sequence and digestion of genomic DNA of Event 127 with NcoI was expected to generate two fragments that contain DNA from the *csr1-2* cassette. Both fragments are defined by the NcoI site in the *csr1-2* 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 and two SpeI restriction sites downstream of the *AHASL* 3' UTR in Event 127. The XbaI restriction sites flank the complete *csr1-2* expression cassette. 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 the F8 generation of Event 127 and from the non-transgenic control Conquista was digested overnight in a volume of 40 µl with the restriction enzymes listed above (8 units/µg DNA) under the conditions specified by the enzyme manufacturers (New England Biolabs; Ipswich, MA; or Amersham). Two additional non-transgenic Conquista genomic DNA samples were spiked with one- and two-genome

copy equivalents[1] of pAC321 plasmid DNA (27 and 54 pg, respectively) and used as positive controls (Camper, 1987). Restriction digests were separated by electrophoresis in 10 cm long 0.8% agarose gels. The DNA was further fragmented by soaking the gels in 0.25 N HCl for about 20 min and were denatured with 0.4 N NaOH for about 30 min. The gels were rinsed with 2X NaCl/sodium citrate solution (SSC) and the denatured DNA was transfered onto Hybond N+ nylon membrane (Amersham) using 0.4 N NaOH as a transfer buffer. Southern hybridization was carried out according to Sambrook *et al*. (1989). The membranes were prehybridized at 65°C for 2-4 h and hybridized at 65°C overnight in 20 – 30 ml (about 0.2 ml/cm^2) of hybridization buffer (2x SSC, 0.6% SDS, 50 mM Na_2HPO_4, 1x Denhardt's solution, 2.5 mM EDTA, 5% dextran sulfate, pH 7.2) in a Hybaid MAXI 14 Hybridization Oven (Thermo Electron Corporation). After hybridization, the membranes were washed with 2x SSC, 0.5% SDS (1 ml/cm^2) at room temperature for 15 min, 2x SSC, 0.1% SDS (4 ml/cm^2) at 65°C for 30 min, and finally with 0.1x SSC, 0.1% SDS (4 ml/cm^2) at 65°C for 15 min. After washing, the membranes were wrapped in plastic wrap and exposed to Hyperfilm™ MP film (Amersham) for 2-5 days, depending on the radioactive signal intensity, in cassettes with intensifying screens at –80ºC.

Stability of the DNA insert across breeding generations. Southern blot analyses were also conducted as described above to monitor the stability of the insert across multiple generations. Plant material was obtained from the T4, F4, F8 and F9 generations (Figure 2). Genomic DNA from these samples was digested with NcoI and SpeI (as described above) and Southern blot analysis was carried out as described above.

Genomic DNA sequence flanking the 5' and 3' ends of the DNA insert. Inverse-PCR was used to obtain the sequence of the soybean genomic DNA flanking the inserted *csr1-2* cassette (Triglia *et al*., 1988). Genomic DNA (1 µg) from the Event 127 F7 generation was digested with 15 units of XbaI, SpeI, HindIII, NcoI, EcoRI, BamHI or BglII in 20 µl reaction volumes for 3 h. The XbaI, HindIII, NcoI and EcoRI digests were incubated at 65°C for 20 min to inactivate the enzymes while the BamHI and BglII digests were subjected to isopropanol precipitation. T4 DNA ligase (800 units, New England Biolabs) was directly added to each digestion reaction. Water was also added to bring the reaction volume to 200 µl. The reactions were incubated at 16°C overnight and the circularized DNA was directly used as a template for inverse-PCR. The transgene

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

Assumptions:

- The haploid content of the soybean genome is 1.115 X 10^9 bp (Arumuganathan and Earle, 1991).
- Plasmid pAC321 is 8669 bp.

Since the insert in Cultivance Event 127 is homozygous and 7 µg of soybean DNA is used per digest in the Southern blot analysis, the mass of one copy equivalent of pAC321 is:

$$\frac{\text{mass of pAC321 DNA}}{\text{7 µg genomic DNA}} = \frac{\text{8669 bp transgene DNA}}{(1.115 \text{ X } 10^9 \text{ bp genomic DNA}) \text{ x } 2}$$

mass of pAC321 DNA = 27 pg

flanking sequences were amplified with the GeneAmp® XL PCR kit (Applied Biosystems; Foster City, CA). The 100 µl primary PCR contained 1x manufacturer-supplied PCR buffer, 200 µM of each dNTP, 25 ng of the circularized genomic DNA fragments, 1.2 mM magnesium acetate, 2 units of rTth DNA polymerase XL, and 0.2 µM of each primary PCR primer. The 100 µl nested PCR contained the same components as the primary PCR except that 10 µl of a 1:50 dilution of the primary PCR was used as a template. The primary and nested PCRs were carried out on the GeneAmp PCR System 9700 (Applied Biosystems). After an initial one-minute denaturing step at 94ºC, 30 cycles of 94ºC for 15 sec, 60ºC for 8 min and 72ºC for 2 min were performed followed by a final 10 min extension step at 72ºC.

Primers Used for Obtaining Flanking DNA Sequence Data

Purpose	Direction	Primer Sequence	Position (Fig. 8)
5' flank NcoI digest	Forward 1˚ PCR	GCAGCTTGTATCCATTCTCTTAACC	2450-2474
	Reverse 1˚ PCR	TTGTTGATTGGGATGAAAACGA	1657-1678
	Forward 2˚PCR	Same as Forward 1˚ PCR primer (NcoI digest IPCR)	
	Reverse 2˚ PCR	ACGAAGAATCCAACGAATCCC	1631-1651
3' flank XbaI digest	Forward 1˚ PCR	AAGGAAATCCAGAAGCACTAATCA	5517-5540
	Reverse 1˚ PCR	TAATGCGAGATCAATTACCTC	1718-1738
	Forward 2˚ PCR	Same as Forward 1˚ PCR primer (XbaI digest IPCR)	
	Reverse 2˚ PCR	CAATTACCTCGTAAAGAAAGTACTA	1703-1727

After the PCR reactions were complete, the products were purified with Zymo DNA Clean & Concentrator™-5 (Zymo Research; Orange, CA). PCR products were either directly sequenced or sequenced after cloning. When the PCR products or cloned fragments were longer than 1 kb, primer walking was employed to obtain full-length sequence. Both DNA strands were sequenced to obtain a sequence quality of greater than Phred 40 at each base. Sequencing was performed with the BigDye™ Terminator v3.1 Ready Reaction Cycle Sequencing Kit and ABI 3730 DNA Analyzer from Applied Biosystems.

The 3' flanking PCR product amplified from the XbaI digest was about 6 kb and the amplification was too weak to obtain enough DNA for direct sequencing. Therefore, the PCR product was digested with SpeI and the resulting restriction fragments, one of about 800 bp and the other of 5.2 kb, were treated with DNA Polymerase I, Large (Klenow) Fragment (New England Biolabs) to generate blunt ends and cloned into the pCR®-Blunt II-TOPO cloning vector (Zero Blunt® TOPO® PCR Cloning Kit; Invitrogen). Ten clones of each fragment were verified by restriction digestion and were sequenced by primer walking. The junction of the restriction fragments was confirmed by PCR amplification and sequencing across the junction.

Complete sequence of the DNA insert. Six PCR-generated amplicons were designed to span the entire insert as well as the junctions with the adjacent soybean genomic sequences (Figure 7). The complete sequence of the inserted DNA was obtained by PCR

amplification of these six overlapping fragments followed by DNA sequence analysis. PCR amplicons containing sequence discrepancies relative to the sequence of the transformation fragment were re-amplified with rTth DNA polymerase XL. PCR products were purified with the Zymo DNA Clean & Concentrator™-5 and were sequenced on both strands to a quality level of Phred 40 by direct sequencing and primer walking. DNA sequencing was performed as described above.

Primers Used for Event 127 DNA Insert Amplification

Purpose	Direction	Primer Sequence	Position (Fig. 8)
PCR1	Forward	GCTTGATATGCCTTTTGGTTC	5265-5285
	Reverse	TTGTCTTCCCTCATTGGAC	6150-6168
PCR2	Forward	GACGAGATATTCCCGAAC	4544-4561
	Reverse	GTCTGATTAGTGCTTCTGG	5525-5543
PCR3	Forward	CCCTGTTGCGAGTACGTTGA	3739-3758
	Reverse	CTTCCGTTATGACATCGTTG	4732-4751
PCR4	Forward	AACCACTCCCTCTCCAAC	2980-2997
	Reverse	CTGATGATAGCCACTGCC	4266-4283
PCR5	Forward	TTCGTTCGCTCTGGTGTC	2062-2079
	Reverse	ACGGTTTCTACGCCTTG	3089-3105
PCR6	Forward	GAAAATAGGAAGTTTAGGCTTG	1000-1021
	Reverse	GGGCTGATAATGTCGTTTG	2229-2247

Event-specific PCR. Event-specific PCR was developed using the information obtained from both the DNA flanking sequence and the insert sequence. Four pairs of primers were designed with one primer of each pair in the 5' soybean flanking sequence and the other in the *csr1-2* cassette. These are described below.

Primers Used for Event-Specific PCR

Purpose	Direction	Primer Sequence	Position (Fig. 8)
Event PCR1	Forward	GAAAATAGGAAGTTTAGGCTTG	1000-1021
	Reverse	CACTGCTCTTAGCGAAATCTC	1426-1446
Event PCR2	Forward	GCCCTCCTTATTTATCCCCTTA	1210-1231
	Reverse	GCCGTACGCACAGCTACTTTC	1592-1612
Event PCR3	Forward	ATAGGAAAGCGCAAACTG	1128-1145
	Reverse	CGAACACTGCTCTTAGCGAAAT	1429-1450
Event PCR4	Forward	GCCCTCCTTATTTATCCCCTTA	1210-1231
	Reverse	AGGATCGATTGCGGAATCA	1403-1421

The PCR products were designed to be 200 to 400 bp in length. Genomic DNA from both Event 127 and the non-transgenic variety Conquista was used as template. PCRs were performed in 25 µl total volume with 25 ng of the template DNA, 200 µM of each dNTP, 0.4 µM of each primer and 1 unit Taq DNA polymerase per reaction. After an initial 4 min denaturation at 94°C, 30 cycles of 94°C for 30 sec, 60°C (Event PCR1 and 3) or 66°C (Event PCR2 and 4) for 30 sec, and 72°C for 45 sec were performed, followed

by a final 10 min extension at 72°C. Four plants each of Cultivance Soybean Event 127 and the non-transgenic variety Conquista from six different planting locations were analyzed by qualitative PCR using the "Event PCR3" primer set.

Reverse Transcription-PCR. RT-PCR was conducted to determine if either the 376 bp duplication of a portion of the *csr1-2* coding sequence or the At *SEC61 γ* gene present in Cultivance Soybean Event 127 was transcribed. Total RNA was used as template for RT-PCR using the Qiagen OneStep RT-PCR Kit (Qiagen). For RT-PCR analysis of the At *SEC61 γ* coding sequence, *Arabidopsis* total RNA samples from leaves and roots were also used as positive controls. The Arabidopsis total RNA samples were provided by BASF Plant Science (Research Triangle Park, NC). The Arabidopsis total RNA samples were prepared with TRIzol reagent (Invitrogen) without DNase treatment. The RT-PCR reactions contained 1x Qiagen OneStep RT-PCR Buffer, 400 µM of each dNTP, 0.6 µM of each primer, 2 µl of Qiagen OneStep RT-PCR Enzyme mix, and 500 ng or 125 ng of total RNA in a total volume of 50 µl. The RT-PCR was conducted using the GeneAmp PCR System 9700. Following a 30 min reverse transcription step at 50°C, PCR amplification was carried out under the following conditions: one 15 min denaturation step at 95ºC; 30 cycles at 94ºC for 30 sec, 64ºC for 30 sec, and 72ºC for one min; and one ten-min extension at 72ºC.

The endogenous soybean *Sec61γ* subunit and *Iota* genes were used as positive controls. The soybean *Iota* subunit gene is expressed constitutively and ubiquitously in soybean (Yamamoto and Knap, 2001).

Primers Used for RT-PCR Analysis

Purpose	Direction	Primer Sequence	Position (Fig. 8)
RT-PCR At SEC61	Forward	ACGAACCTGCTGAAACCCTAAT	1338-1359
	Reverse	TAAGAATGGAGAATTTGGCTACA	2280-2302
RT-PCR Iota	Forward	TGAAGCAGCAGCTGAGTTTCGC	N/A*
	Reverse	GGCAGTCTGAACCGTCTCCTC	N/A
RT-PCR Gm Sec61	Forward	GCTTGGGAGACAGAGAAAGAGA	N/A*
	Reverse	CCTTTTGCTTGACAACCTGAAT	N/A
RT-PCR ORF501	Forward	TTGGAATGCATGGGACTGT	3807-3825, 5733-5751
	Reverse	TGTCTTCCCTCATTGGACTG	6148-6167

*N/A – not applicable. This is a positive control; primer set is expected to amplify cDNA derived from an endogenous soybean transcript unrelated to the Event 127 insert.

PCR analysis of the integration site. Three PCR reactions were carried out to characterize the insertion site in the non-transgenic soybean variety Conquista. The PCR primers used in this study were derived from the DNA sequence that was determined for the 5' and 3' genomic regions flanking the novel expression cassette in the genome of Event 127.

PCR primers for PCRA (see table below) were designed such that the forward primer would bind in the 5' flanking sequence and the reverse primer would bind in the 3' flanking sequence immediately after the 3' integration point. Primers for PCRB were

designed so that the forward primer would bind in the 5' flanking sequence while the reverse primer would bind close to the distal end of the 3' flanking sequence. Both primers for PCRC were designed to bind within the 3' flanking sequence.

Primers Used for Analysis of Integration Site

Purpose	Direction	Primer Sequence	Position (Fig. 8)
PCRA	Forward	CCACAATGTGCCAATTAAGT	936-955
	Reverse	GCGTGTTTCTTTAGCATCA	6,319-6,337
PCRB	Forward	CTCCTTCGCCGTTTAGTGTA	1,109-1,128
	Reverse	GTTTCGCGTTTAGGGTTCC	10,098-10,116
PCRC	Forward	ATAAGCCAATTTGGGTCTGCC	8,312-8,332
	Reverse	GTTTCGCGTTTAGGGTTCC	10,098-10,116

PCRA and PCRC were carried out with Qiagen Taq DNA polymerase and PCR2 with GeneAmp® XL PCR kit. Twenty-five ng of either Event 127 or Conquista DNA was used in all PCR amplifications. For the PCR with Qiagen Taq DNA polymerase, the reaction contained 1x Qiagen PCR buffer, 200 μM of each dNTP, 0.4 μM of each primer and 1 unit of Taq DNA polymerase in 25 μl total volume. After an initial 4 minute denaturation step at 94ºC, 30 cycles at 94ºC for 30 sec, 66ºC for 45 sec and 72ºC for 2 min were performed followed by a final 10 minute extension step at 72ºC.

For PCR with the GeneAmp® XL PCR kit, the reaction contained the same components as used in the cloning of flanking sequence except for the template DNA. After an initial 1 min denaturation step at 94ºC, 30 cycles at 94ºC for 1 min and 66ºC for 10 min were performed followed by a final 10 minute extension at 72ºC.

Bioinformatics analysis. DNA sequence assembly was carried out with Staden Pregap4 and Gap4 (Staden, 1996). Alignment of the cloned and expected insert sequences was conducted with LI-COR AlignIR software (Licor Biotechnology; Lincoln, NE). The flanking sequences were queried against available public DNA databases (all GenBank+EMBL+DDBJ+PDB sequences) and BASF Plant Science proprietary DNA databases via BLAST analysis (Altschul *et al.*, 1997). Open reading frames of 30 codons or more were identified with the ORF analysis function of Vector NTI v9.0 (Invitrogen).

RESULTS AND DISCUSSION

Copy Number and Insert Integrity.
The copy number of the insert in Event 127 was evaluated by Southern blot analysis of genomic DNA from Event 127 F8 generation plants digested with NcoI, SpeI and XbaI restriction enzymes. Blots were hybridized separately with three different probes (the At *AHASL* 5' UTR region, the At *ahasl* coding sequence, and the At *AHASL* 3' UTR region) that spanned the entire DNA fragment used for transformation (Figure 1B). Genomic DNA from the non-transgenic cultivar Conquista as well as Conquista genomic DNA spiked with one- and two-genome copy equivalents of pAC321 plasmid DNA was included in the Southern blots as controls.

Non-transgenic Conquista DNA digested with all three restriction enzymes and hybridized with the three probes did not show any signal, indicating that neither the endogenous soybean *Ahasl* nor the endogenous soybean *Sec61* γ subunit gene are detected at the Southern blot stringency conditions used (Figure 4, lanes 1, 5 and 9). The pAC321 DNA, spiked at one- and two-genome copy equivalents into non-transgenic Conquista DNA (Figure 4, lanes 2, 3, 6, 7, 10 and 11) , was detected at the expected band sizes (Table 2) with a fairly strong intensity suggesting that the Southern method employed was sensitive enough to detect a single copy insert in the transgenic line. DNA samples from Event 127 F8 generation treated with the different enzyme and probe combinations all gave single bands (Figure 4, lanes 4, 8 and 12) except for the SpeI digest hybridized with the *ahasl* coding sequence probe, which had an additional small band of about 800 bp (Figure 4B, lane 8). This *ahasl*-hybridizing 800 bp SpeI fragment is consistent with the observation that a small fragment of the *ahasl* coding sequence was repeated at the 3' flanking sequence junction in Event 127 (see section on Complete Insert Sequence). All major bands had signal intensities similar to the one-genome copy equivalent of pAC321. Due to the homozygous state of the material analyzed, it would be reasonable to expect band intensities closer to the two genome copy equivalent control. The quantitation of plasmid DNA versus genomic DNA coupled with the large dilution required for loading the plasmid DNA, can result in variations of intensity. However, the DNA sequence information for the insert as well as genomic flanking DNA presented in a subsequent section, and the number of bands observed in the Southern blot substantiate the conclusion that a single copy of the insert is present in Event 127.

Genomic DNA from Event 127 that was digested with NcoI and probed with At *AHASL* 5' UTR produced a hybridizing band approximately 4.5 kb in size. The size of this band is consistent with the production of a single DNA fragment defined by the NcoI site within the insert (nt 2761, Figure 4D) and an NcoI site approximately 4.5 kb upstream in the 5' genomic soybean flanking sequence. The same digest probed with either the At *ahasl* coding sequence or At *AHASL* 3'UTR produced a hybridizing band approximately 9.0 kb in size. The size of this band is consistent with a single DNA fragment defined by the NcoI site in the insert (nt 2761, Figure 4D) and an NcoI site approximately 9.0 kb downstream in the 3' soybean genomic flanking sequence.

Digestion of Event 127 genomic DNA with SpeI and probed with At *AHASL* 5' UTR produced a hybridizing band of approximate size 4.4 kb. This is consistent with the production of a single DNA fragment from a SpeI site in the insert (nt 5620, Figure 4, panel D) and a SpeI restriction site approximately 4.4 kb upstream in the 5' DNA flanking sequence of the soybean genome (nt 1268, Figure 4, panel D). The presence of this upstream SpeI site was confirmed in the analysis of the 5' flanking sequence of Event 127 (refer to Results and Discussion section entitled "Flanking sequence"). The same digest probed with either the At *ahasl* coding sequence or the At *AHASL* 3' UTR also produced a 4.4 kb hybridizing band corresponding to the same fragment described above. In addition, a hybridizing band of approximate size 0.8 kb was detected when the SpeI digest was probed with At *ahasl* coding sequence, consistent with a single SpeI DNA fragment containing the 376 bp segment of the *csr1-2* gene at the 3' flanking DNA sequence junction. This hybridizing fragment was produced from the SpeI site in the DNA insert and a SpeI site 0.8 kb downstream in the soybean genome (nt 5620 – 6505, Figure 4, panel D). The 0.8 kb hybridizing band was not detected by the At *AHASL* 3' UTR probe, indicating that At *AHASL* 3' UTR DNA was not included in the 0.8 kb fragment, and the SpeI nt 5620 site in the insert is adjacent to the 376 bp segment of the *csr1-2* gene. Therefore, SpeI restriction enzyme sites at nucleotide 5622 and 5719 in the linear PvuII fragment of plasmid pAC321 used for transformation (shown in Figure 1B) were not included in the DNA insert in the Event 127 genome. This was confirmed by DNA sequence analysis of the DNA insert (refer to Results and Discussion section entitled "Complete Insert Sequence"). The smaller predicted SpeI fragments of 280 and 97 bp in the pAC321-spiked controls would produce signal below the level of detection.

Event 127 genomic DNA when digested with XbaI and probed with At *AHASL* 5' UTR shows a single hybridizing band of approximate size 10 kb. Based on the positions of the XbaI restriction sites in the linear DNA used for transformation (Figure 1B), a hybridizing band of approximately 5.7 kb was expected, produced from within the DNA insert in the Event 127 genome. However, DNA sequence analysis of the DNA insert in Event 127 showed that neither of the XbaI restriction sites in the linear transformation DNA was included in the DNA insert (refer to section entitled "Complete Insert Sequence"). Therefore, the 10 kb hybridizing band was produced from XbaI restriction sites within the 5' and 3' DNA sequences flanking the insert (nt 410 and 10652, Figure 4, panel D). Accordingly, the same digest probed with either the At *ahasl* coding sequence or At *AHASL* 3' UTR produced the same 10 kb hybridizing band corresponding to the same DNA fragment described above.

Analysis of the number and size of all hybridizing bands on the Southern blots shown in Figure 4 is consistent with the integration of a single DNA insert in the Cultivance Soybean Event 127 genome containing a single functional copy of the *csr1-2* gene, as well as coding sequences for the protein SEC61γ on the 5' end of the *csr1-2* gene, and a single DNA fragment containing a 376 bp segment of the *csr1-2* gene at the 3' end of the insert.

Analysis for plasmid backbone.

Although the transformation was carried out with the PvuII restriction fragment of pAC321 that did not include vector backbone DNA, Southern blot studies were conducted to confirm the absence of plasmid pAC321 vector DNA in the Event 127 genome. In order to determine whether there was any vector backbone integrated in Event 127, the same set of blots used for Southern analysis described above (Figure 4) was hybridized with two vector backbone-specific probes (Figure 5). As expected, no hybridizing bands were detected in lanes containing non-transgenic Conquista genomic DNA. Non-transgenic Conquista genomic DNA spiked with one- or two-genome copy equivalents of transformation plasmid pAC321 showed hybridizing bands of the expected sizes (Table 2). No hybridizing bands were detected in Event 127 F8 generation DNA, clearly indicating that no vector backbone DNA was integrated into the soybean genome in this event.

Intergenerational stability.

In order to determine the stability of the insert in Event 127, DNA samples from four different generations, T4, F4, F8 and F9, (Figure 2) were subjected to Southern blot analysis. Genomic DNA samples were digested with NcoI and SpeI and probed with either the At *AHASL* 5' UTR, At *ahasl* coding sequence or At *AHASL* 3' UTR probes spanning the entire DNA fragment used for transformation (Figure 1B). The combination of these restriction enzymes and probes provides a unique fingerprint for the DNA insert in Event 127 (Figure 4). Non-transgenic Conquista genomic DNA was used as a negative control and Conquista spiked with one- and two-genome copy equivalents of pAC321 was used as a positive control (Figure 6). Multiple bands from Event 127 T4 generation DNA digested with either NcoI or SpeI were detected with all three probes, indicating that the T4 generation contains multiple copies of the *csr1-2* cassette. However, DNA from the F4, F8 and F9 generations all showed the same Southern pattern (Figure 6) previously observed in the insert and copy number analyses (Figure 4). This result clearly indicates that the multiple copies of the insert in the T4 generation segregated in the progeny of the cross between T4 and Conquista and that only a single copy is retained in the segregant selected. Moreover, this single copy is stably inherited in subsequent generations.

Flanking Sequence.

A 3 kb DNA fragment was amplified by inverse PCR from the intramolecularly circularized NcoI digest of Event 127 F7 generation genomic DNA. Sequencing of both ends of the fragment indicated that it was specifically amplified from the 5' side of the transgene insert. The fragment was further sequenced to obtain 1.3 kb of the 5' soybean flanking genomic sequence. A 6 kb DNA fragment was amplified by inverse PCR from the XbaI digest of Event 127 F7 generation genomic DNA and was entirely sequenced after subcloning. The obtained sequence indicates that it flanks the insert on the 3' side. PCR analysis of non-transgenic variety Conquista DNA using primers from the 5' and 3'

flanking regions was conducted to confirm that the flanking sequence was native to the plant genome (data not shown).

The entire Cultivance Soybean Event 127 transgene insert sequence with 5' and 3' flanking sequence is displayed in Figure 8. BLAST analysis of the 5' flanking sequence queried against available public DNA databases (all GenBank + EMBL + DDBJ + PDB sequences) and BASF Plant Science proprietary DNA databases revealed a region of sequence identity with a proprietary soybean expressed sequence tag (EST), confirming that the origin of the identified flanking sequence is native soybean DNA. The sequence was further analyzed for predicted open reading frames. Results indicated that there is a 315 bp ORF, from nucleotides 941 to 1255 of the flanking sequence, upstream of the 5' end of the insertion. Alignment of the 5' flanking sequence with the transformation sequence revealed that the integration point is at nucleotide 1312 (Figure 8), which is 60 bp downstream from the stop codon of the predicted ORF.

Analysis of the 3' flanking sequence showed that, before the 3' integration point, there is a 376 bp segment of sequence which differs from a portion of the *csr1-2* coding sequence (nucleotides 3768-4143 of Figure 8) by only a single nucleotide. The insertion of this 376 bp sequence at the 3' flanking sequence junction created an ORF of 501 bp that extends from the transgene insert into the 3' flanking sequence. The potential transcription of this ORF was investigated by RT-PCR (refer to section entitled "RT-PCR Analysis of Open Reading Frame Associated with Cultivance Soybean Event 127 Insert"). BLAST analysis of the 3' flanking sequence queried against available public DNA databases (all GenBank + EMBL + DDBJ + PDB sequences) and BASF Plant Science proprietary DNA databases revealed a region of sequence similarity in the proximal 3' flanking sequence to a soybean catalase gene (accession No. Z12021). However, the integration point is about 500 bp upstream of the potential gene homolog and the putative coding sequence is about 2.4 kb downstream of the integration point. Therefore even if the possible catalase homolog is an active gene, it is unlikely to be affected by the insertion. In addition, a region of the distal 3' flanking sequence shared sequence identity with a proprietary soybean EST.

Studies were conducted to PCR-amplify the Event 127 integration site from the genome of non-transgenic Conquista. PCR primer sets A and B that include one primer in the 5' flanking region and a second primer in the 3' flanking region (see section entitled "PCR analysis of the integration site") in order to amplify the insertion site did not produce an amplified DNA product using genomic DNA from the non-transgenic variety Conquista as a template. PCR primer set C with primers specific to the 3' flanking sequence did not produce an amplification product from non-transgenic variety Conquista DNA while the expected amplicon was produced with genomic DNA from Event 127 (data not shown). This demonstrates that the DNA fragment amplified by primer set C is present in Event 127 but does not exist in the same context in the genome of Conquista, which suggests that a DNA rearrangement at the insertion site has occurred in Event 127. This is consistent with the discovery of a 376 bp segment of duplicated sequence from the *csr1-2* coding region near the junction of the inserted DNA and the genomic soybean DNA.

Complete Insert Sequence.

Although Southern blot analysis suggested that the transgene insert contained the complete *csr1-2* expression cassette, cloning and sequencing of the insert was performed to confirm insert integrity. The complete sequence of the inserted DNA was obtained by PCR amplification of six overlapping amplicons with Taq DNA polymerase (Figure 7). The complete Cultivance Soybean Event 127 insert sequence is 4758 bp in length and other than the insertion of the 376 bp fragment from *csr1-2* at the 3' integration point, the sequence is identical to the sequence of the transformation fragment except for three point mutations (Figure 8). One of the point mutations is a G to A mutation in the *ahasl* coding sequence, which results in an amino acid change from R_{272} to $K_{272.}$ This is a conservative amino acid substitution and has no impact on the herbicide tolerance or enzymatic properties of the At AHAS protein. The other two mutations include a G to A mutation and a G to C mutation, both of which are located downstream of the 3'UTR of the *csr1-2* gene and so are genetically silent.

Experiments were carried out to determine at what point in the production and breeding development of Cultivance Soybean Event 127 the G to A mutation in the *ahasl* coding sequence occurred. Initially, the PCR4 reaction (Figure 7) used in sequencing of the insert was set up with genomic DNA from both Event 127 T4 and F8 generations as template and PCR products were sequenced. Sequence from the Event 127 T4 generation did not differ from the expected (pAC321) sequence. Considering that the T4 generation contains multiple copies of the insert and the PCR4 product is likely a mix of sequences from various copies of the insert, an Event 127 locus-specific PCR amplicon of 2.5 kb was designed with a forward primer (5'-GCCCTCCTTATTTATCCCCTTA-3') in the 5' flanking sequence and a reverse primer (5'- ACAAACCTACCCAATTCATCGC-3') in the *csr1-2* coding sequence. PCR products were sequenced directly. Sequence comparison revealed that the G to A mutation also exists in the Event 127 T4 generation, indicating that the mutation occurred before the T4 generation (data not shown) and has been maintained for the subsequent eight generations. It is not known exactly when the mutation occurred but the possibility that it was present in the linear DNA molecule that integrated in the Cultivance Soybean Event 127 genome cannot be discounted.

RT-PCR Analysis of Arabidopsis *SEC61* γ Transcription in Soybean Cultivance Event 127.

The transformation sequence contains a 2.5 kb segment which was originally annotated as the *AHASL* promoter and 5' UTR. Recent sequence analysis has revealed that this sequence segment also contains a previously unannotated Arabidopsis gene encoding the gamma subunit of SEC61, a multimeric transport protein. The Event 127 insert sequence contains the majority of the At *SEC61* γ subunit gene including the complete coding sequence. The At *SEC61*γ 5' UTR, as annotated by The Arabidopsis Information Resource, begins 18 nucleotides downstream from the 5' transgene integration site. As such, it is extremely unlikely that the insert contains the complete native promoter for the At *SEC61*γ gene.

The possible transcription of the Arabidopsis At *SEC61 γ* subunit gene found in the insert in Cultivance Soybean Event 127 was evaluated using RT-PCR. RT-PCR was carried out using DNase-treated total RNA extracted from the F7 generation of Event 127 as a template. Primers specific to two endogenous soybean genes, *Iota* and Gm *Sec61* γ, were used as positive controls to confirm the quality of template RNA. Total RNA from Arabidopsis leaf and root tissues without DNase treatment was also used as a positive control. Results showed that both endogenous soybean positive controls, *Iota* and Gm *Sec61* γ, were strongly transcribed in young soybean leaf tissue while the At *SEC61 γ* subunit gene in the Event 127 F7 generation was only weakly transcribed. The amplified 393 bp At *SEC61 γ* subunit DNA band from the F7 generation of Event 127 is the same size as that amplified from Arabidopsis leaves and roots (Figure 9). The same pair of primers also amplified a band of the expected size, 965 bp, from contaminating genomic DNA in Arabidopsis leaf and root samples. To confirm the identity of the Event 127 RT-PCR product, it was sequenced and compared with the predicted mRNA sequence of the At *SEC61 γ* subunit (data not presented). Both sequences matched, indicateing that the At SEC61 γ subunit is weakly transcribed in leaves of Event 127.

RT-PCR Analysis of Open Reading Frame Associated with Cultivance Soybean Event 127 Insert.

The insertion of a 376 bp portion of the *csr1-2* coding sequence near the 3' flanking sequence junction (Figure 7) created a 501 bp ORF. The possible transcription of this ORF was investigated by RT-PCR analysis. RT-PCR was carried out with two different amounts of RNA template, 500 ng and 125 ng. Event 127 F8 generation genomic DNA was also used in a positive control reaction with ORF-specific primers. Primers specific for the soybean *Iota* gene were used in positive control reactions to confirm the quality of the template RNA. The ORF-specific primers amplify a 435 bp fragment from Event 127 genomic DNA. However, no detectable RT-PCR product was observed using total RNA from young leaf tissue as a template, suggesting that the ORF is not expressed in Event 127 (Figure 10).

Event-Specific PCR.

Four event-specific PCRs for Cultivance Soybean Event 127 were designed with one primer anchored in the 5' flanking sequence and the other in the insert. The expected PCR product size ranged from 212 bp to 447 bp. All four PCRs generated products of the expected size specifically from Cultivance Soybean Event 127 (Figure 11A), suggesting that any of the four primer sets may be used for detection of Cultivance Soybean Event 127. Event-specific PCR product 3 was further validated with samples of Cultivance Soybean Event 127 and non-transgenic variety Conquista collected from six different planting locations. Results showed that PCR product 3 was amplified specifically in all 24 samples of Cultivance Soybean Event 127 but not in non-transgenic control variety Conquista (Figures 11 B and C).

CONCLUSIONS

Cultivance Soybean Event 127 contains a single copy insert of 4758 bp that includes the complete expression cassette of *csr1-2*, as well as the 5' UTR, the entire coding sequence, and the 3' UTR of the At *SEC61 γ* subunit gene. A 376 bp repeat of coding sequence from the *csr1-2* gene is also integrated at the 3' flanking sequence junction. No vector backbone sequence was found to be integrated in the soybean genome. The insert was stably inherited across eight breeding generations, demonstrating that the insert is stably integrated in the soybean genome. There are three point mutations in the *csr1-2* expression cassette: one conservative mutation in the *ahasl* coding sequence that has no impact on the herbicide tolerance or enzymatic properties of the mutant AHASL protein, and two mutations downstream of the 3' UTR. It appears that a rearrangement of the soybean genomic DNA occurred in DNA flanking the 3' end of the insert, and this most likely occurred during the DNA integration process. The insert contains the coding sequence of the At *SEC61 γ* subunit gene, which was included in the DNA fragment used for transformation. This gene is only weakly transcribed. A 376 bp fragment of the *csr1-2* coding sequence created a new ORF of 501 bp. RT-PCR experiments indicated no detectable transcription of this ORF.

Contributing Scientists

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Date

REFERENCES

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389-3402.

Arumuganathan, K. and Earle, E. D. (1991) Nuclear DNA content of some important plant species. Plant Molecular Biology Reporter 9(3):211-215.

Camper, S. A. (1987) Research applications of transgenic mice. Biotechniques 5:638-650.

Carlson, J. E., Tulsieram, L. K., Glaubitz, J. C, Luk, V. W. K., Kauffeldt, C. and Rutledge, R. (1991) Segregation of random amplified DNA markers in F1 progeny of conifers. Theor. Appl. Genet. 83:194-200.

Sambrook, J., Fritsch, E. and Maniatis, T. (1989) Molecular cloning: A laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Sathasivan, K., Haughn, G. W., and Murai, N. (1990) Nucleotide sequence of a mutant acetolactate synthase gene from an imidazolinone-resistant *Arabidopsis thaliana* var. Columbia. Nucl. Acids Res. 18:2188.

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

Staden, R. (1996) The Staden sequence analysis package. Mol. Biotechnol. 5:233-241.

Triglia, T., Peterson, M. G. and Kemp, D. J. (1988) A procedure for *in vitro* amplification of DNA segments that lie outside the boundaries of known sequences. Nucleic Acids Res. 16:8186.

Yamamoto, E. and Knap, H. T. (2001) Soybean receptor-like protein kinase genes: paralogous divergence of a gene family. Mol. Biol. Evol. 18:1522-1531.

Table 1: DNA Components of Plasmid pAC321 (8669 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	1052-2119	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, 1916-2119	3' untranslated region for putative Arabidopsis *SEC61 GAMMA CHAIN*
At3g48570 intron 2	1443-1915	Putative Arabidopsis *SEC61 GAMMA CHAIN* intron 2
At *AHASL* 5' UTR and putative promoter	2120-2483	Putative promoter and 5' untranslated region for Arabidopsis *ACETOHYDROXYACID SYNTHASE LARGE SUBUNIT*
csr1-2 CDS	2484-4496	Coding sequence for *Arabidopsis thaliana acetohydroxyacid synthase large subunit* with (S653N) point mutation (csr1-2) which confers tolerance to imidazolinones (Sathasivan *et al*., 1990)
At *AHASL* 3' UTR	4497-4714	3' untranslated region for Arabidopsis *ACETOHYDROXYACID SYNTHASE LARGE SUBUNIT*
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) [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	5986-6442	Bacteriophage f1 origin of replication; allows single-strand DNA production in *E. 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
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'*).

Genetic Element	Range (bp)	Function
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

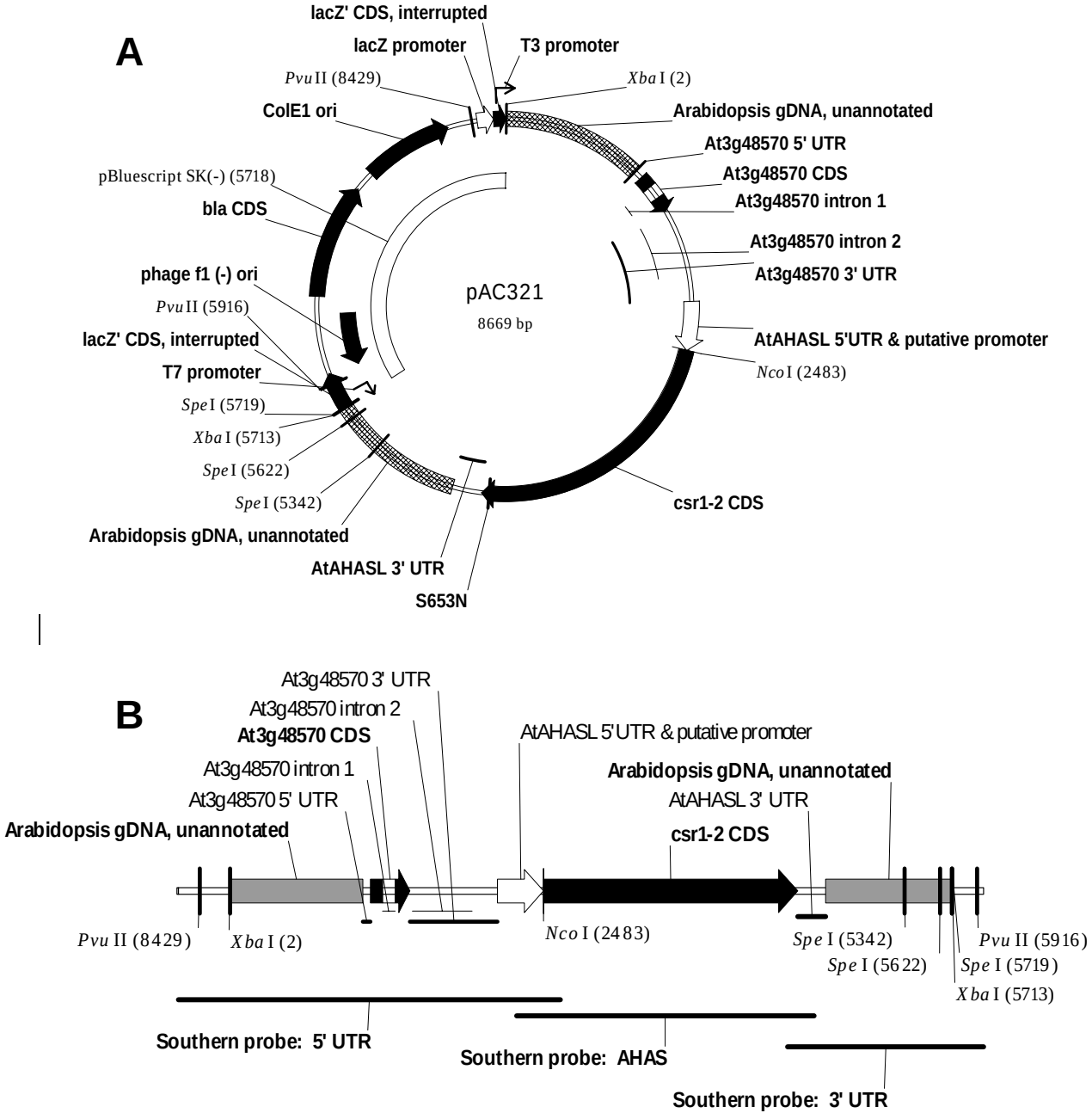
1. The sequence of pAC321 differs from the Arabidopsis genome sequence data available at www.Arabidopsis.org by a single nucleotide within the unannotated region at nucleotide 5073 of pAC321. pAC321 is missing an A residue relative to the public sequence.
2. The pBluescript SK(-) sequence in pAC321 differs from accession number X52324 at nucleotide 7751 of pAC321. Nucleotide 7751 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 6156 bp PvuII fragment).

Table 2. Predicted and Observed Hybridizing Bands on Southern Blots of Cultivance Soybean Event 127 Genomic DNA.

Figure	Probe	Restriction Enzyme	Predicted Fragment Size from Event 127 Insert (bp)[a]	Observed Event 127 Fragment Size	Predicted Fragment Size from Plasmid pAC321 (bp)	Observed Plasmid Fragment Size
4A	**5' UTR**	NcoI	>2760	~4500	8669	~9000
		SpeI	4352	~4400	8292	~8500
		XbaI	10242	~10000	5711[b] 2958	~5500 ~3000
4B	**AHAS**	NcoI	>7896	~9000	8669	~9000
		SpeI	4352 885	~4400 ~800[c]	8292	~8500
		XbaI	10242	~10000	5711	~5500
4C	**3' UTR**	NcoI	>7896	~9000	8669	~9000
		SpeI	4352	~4400	8292 280 97	~8500
		XbaI	10242	~10000	5711[b] 2958	~5500 ~3000
5A	**VP1**	NcoI	none	none	8669	~9000
		SpeI	none	none	8292	~8500
		XbaI	none	none	2958	~3000
5B	**VP2**	NcoI	none	none	8669	~9000
		SpeI	none	none	8292	~8500
		XbaI	none	none	2958	~3000

[a]The predicted fragment size is estimated based on the cloned insert and flanking sequences in Event 127.
[b]The 5' UTR probe and the 3' UTR probe each overlap a XbaI site and therefore hybridize to both XbaI fragments of the plasmid.
[c]Sequence analysis of the Event 127 insert indicates that a small portion of the *csr1-2* coding region was duplicated immediately upstream of the 3' transgene integration site, confirming the identity of the 800 bp band seen in these Southern blots.

Figure 1. Plasmid pAC321. A. Plasmid map, for a complete description of annotations see Table 1. B. The PvuII fragment of pAC321 containing the *AHASL* 5' UTR, *csr1-2* coding sequence and *AHASL* 3' UTR that was used for transformation. The restriction sites of the enzymes (NcoI, XbaI, SpeI) used for Southern blot analyses of copy number, absence of backbone and intergenerational stability are indicated.



Fragment of pAC321 with probes

Figure 2. Breeding History of Cultivance Soybean Event 127

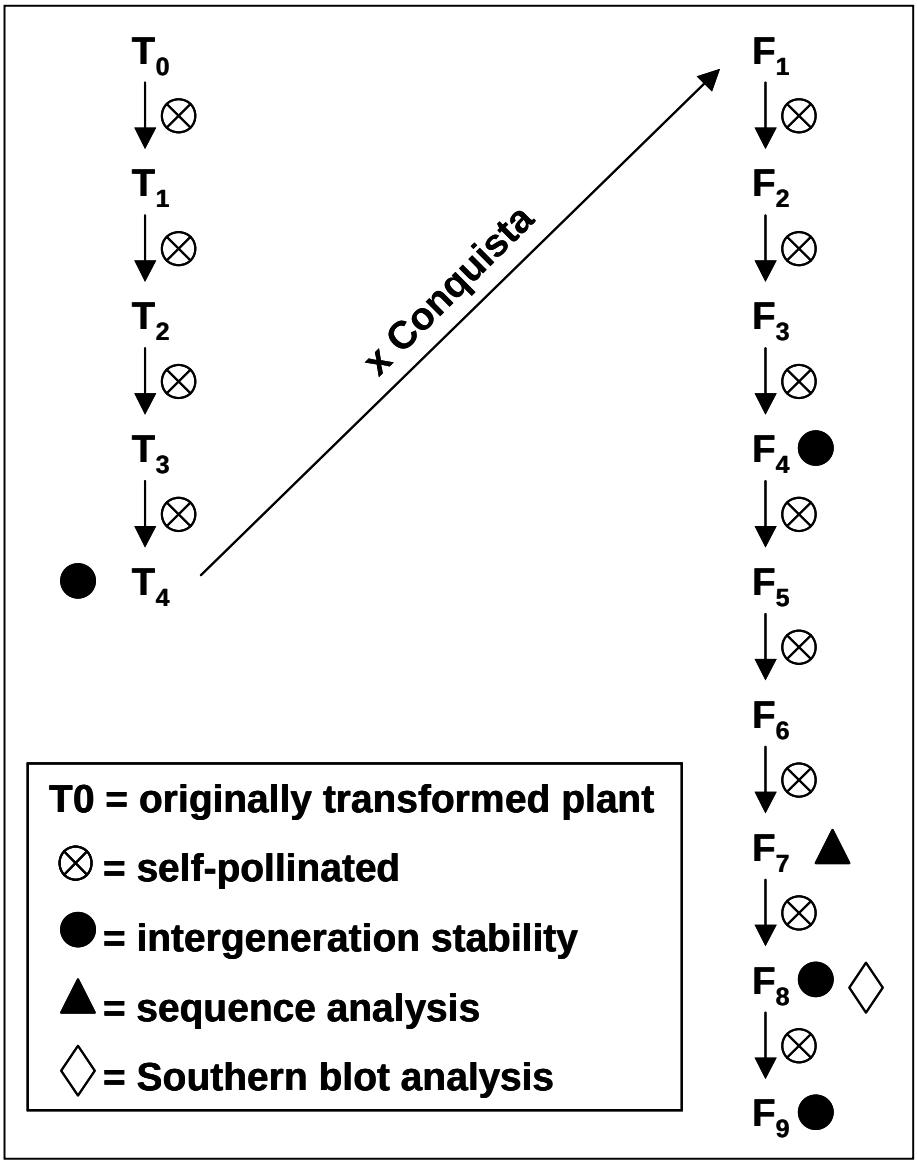


Figure 3. Alignment of pAC321 PvuII Transformation Fragment with Cultivance Soybean Event 127 Insert. The PvuII fragment from plasmid pAC321 that was used to transform soybean is shown in the upper portion of the figure. Parts of this fragment that are not contained within the transgene insert in Cultivance Soybean Event 127 are indicated by boxes filled with diagonal stripes. Characteristics of the transgene insert and the flanking genomic soybean DNA in Cultivance Soybean Event 127 are shown in the lower portion of the figure. The DNA between the vertical dotted lines that are drawn between the maps of the PvuII transformation fragment and the transgene insertion region 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 Cultivance Soybean Event 127 insert corresponds to that in Figure 8, where #1 is the first nucleotide at the 5' end of the soybean genomic flanking sequence (flanking sequences indicated by gray boxes).

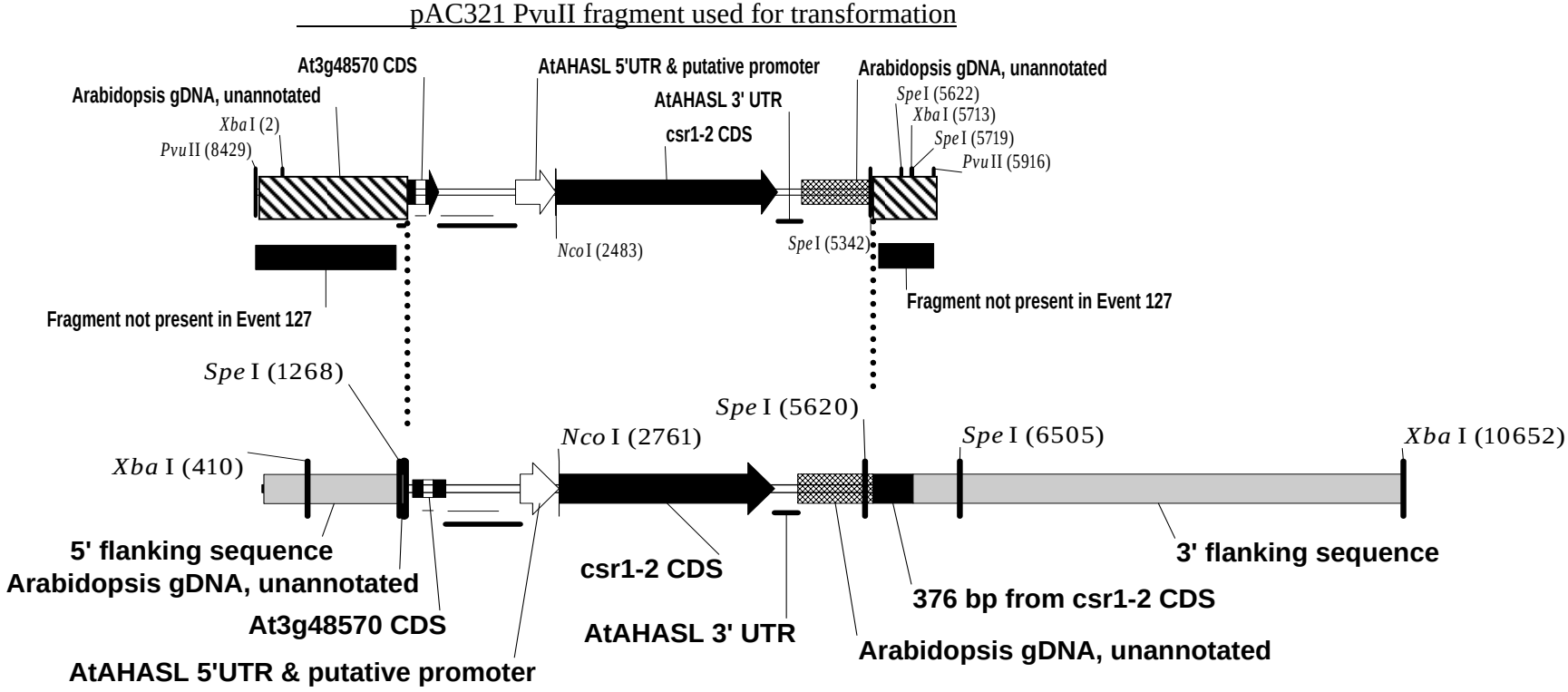
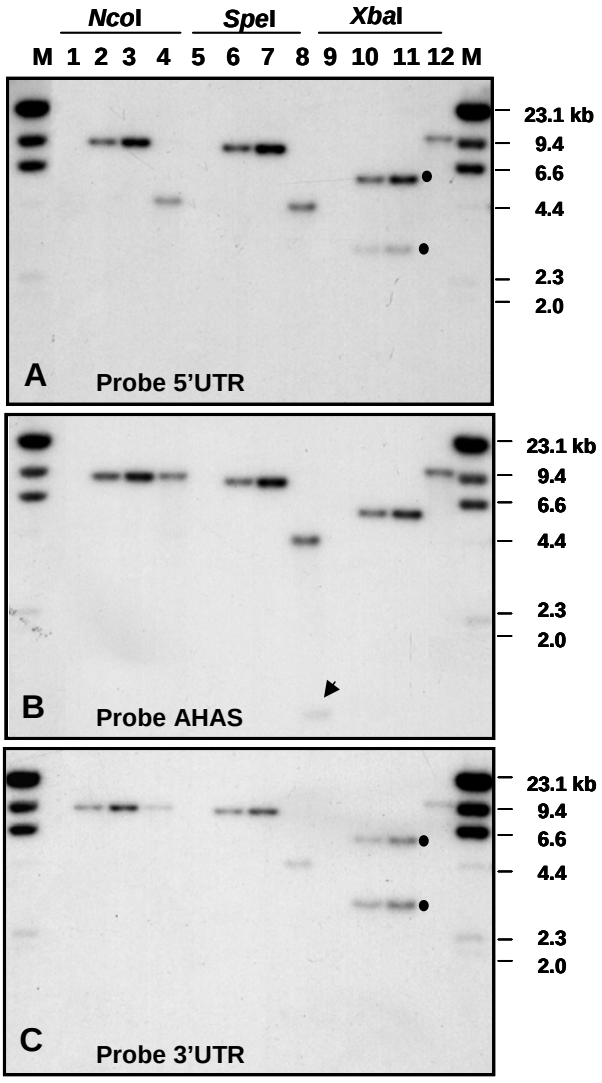


Figure 4. Southern Blot Analysis of Insert Copy Number in Cultivance Soybean Event 127.



Genomic DNA of non-transgenic soybean variety Conquista (lanes 1, 5 and 9); Conquista spiked with 1- (lanes 2, 6 and 10); or 2-genome copy equivalents of pAC321 (lanes 3, 7 and 11); and genomic DNA of Cultivance Soybean Event 127 from the F8 generation (lanes 4, 8 and 12) were digested with NcoI (1-4), SpeI (5-8) and XbaI (9-12) restriction enzymes. Blots were hybridized with probe 5' UTR (panel A), probe AHAS (panel B) and probe 3' UTR (panel C). The first and last lanes (labeled M) contain a λ/HindIII ladder; band sizes are indicated in kilobases. Panel D indicates regions of homology between the Southern hybridization probes and the Event 127 insert. The arrow in panel B indicates a 885 bp SpeI fragment containing an additional 376 bp fragment of *csr1-2* present in Event 127 at the 3' flanking sequence junction.

Note: The 5' UTR probe and the 3' UTR probe each overlap a XbaI site in pAC321 and therefore hybridize to both XbaI fragments of the plasmid. These two bands are marked with a dot to the right of them in lane 11 (Panels A and C).

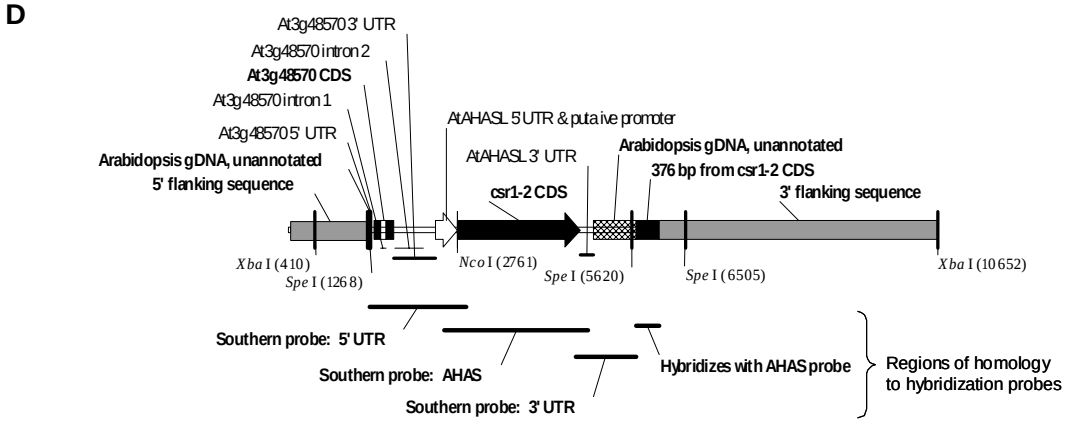


Figure 5. Southern Blot Analysis of Absence of Vector Backbone Sequence in Cultivance Soybean Event 127. Genomic DNA of non-transgenic soybean variety Conquista (lanes 1, 5 and 9); Conquista spiked with 1- (lanes 2, 6 and 10); or 2-genome copy equivalents of pAC321 (lanes 3, 7 and 11); and genomic DNA of Cultivance Soybean Event 127 from the F8 generation (lanes 4, 8 and 12) were digested with NcoI (lanes 1-4), SpeI (lanes 5-8) and XbaI (lanes 9-12) restriction enzymes. The blot was hybridized with probe VP1 (panel A) and probe VP2 (panel B). The first and last lanes (labeled M) contain a λ/HindIII ladder; sizes are indicated in kilobases. Panel C indicates the origin of probes VP1 and VP2 in the sequence of pAC321.

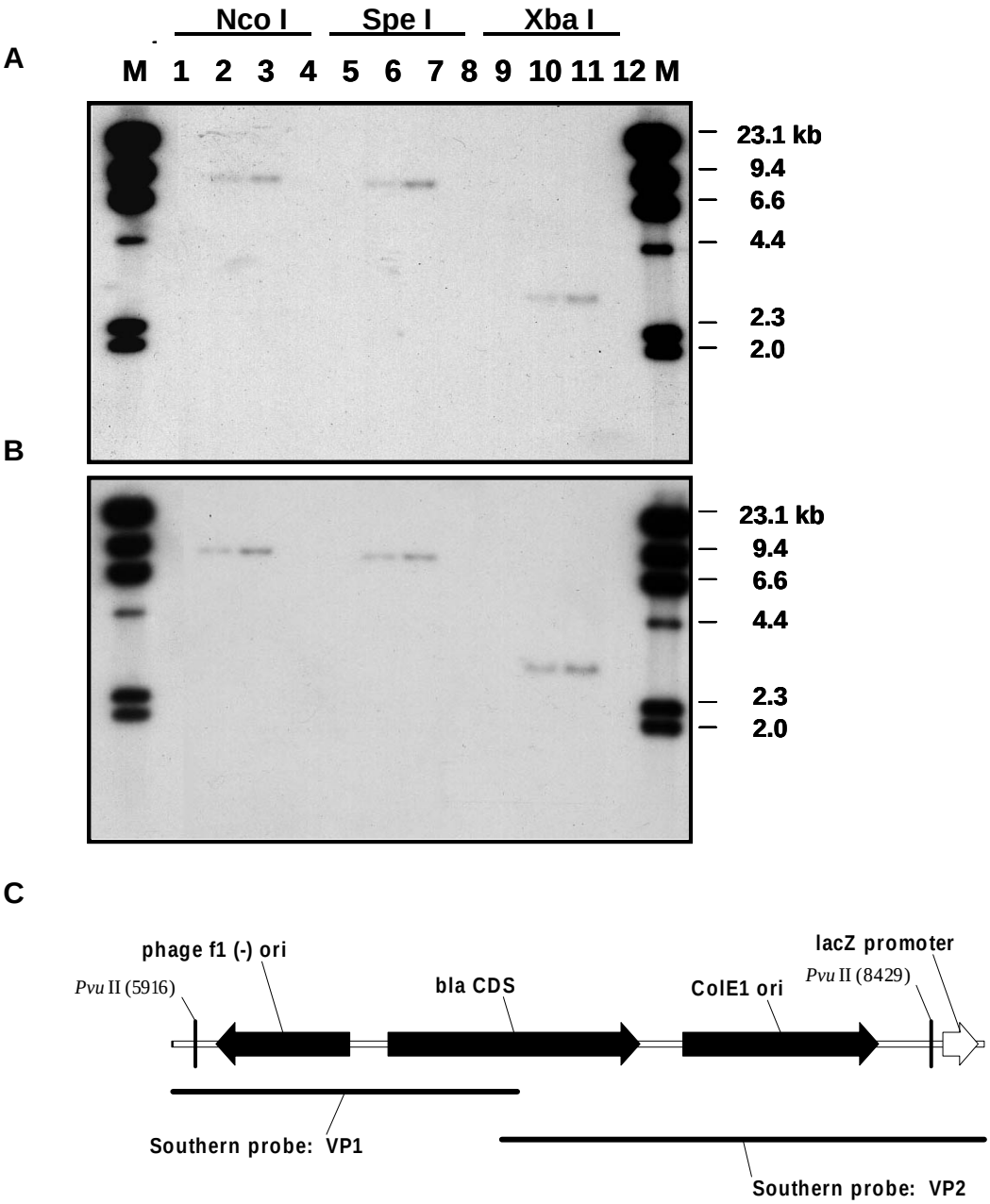
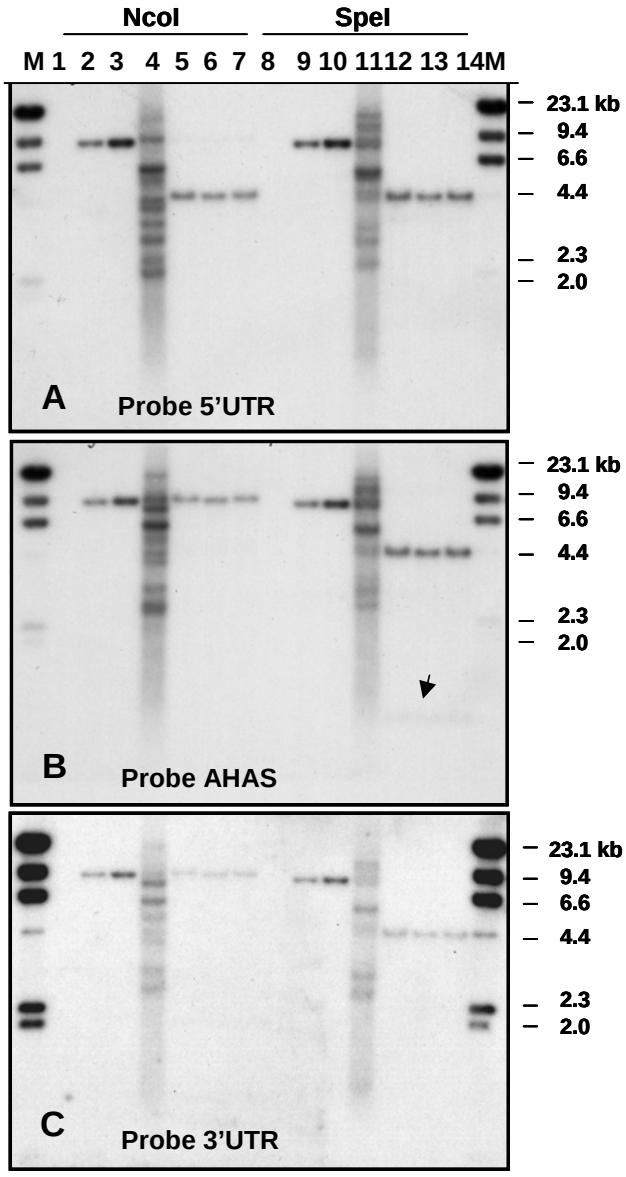


Figure 6. Southern Blot Analysis of Intergenerational Stability of Cultivance Soybean Event 127 Insert



Genomic DNA of non-transgenic soybean variety Conquista (lanes 1 and 8); Conquista spiked with 1-copy (lanes 2 and 9), or 2-copy equivalents of pAC321 (lane 3 and 10); and genomic DNA of Cultivance Soybean Event 127 of either the T4 (lanes 4 and 11), F4 (lanes 5 and 12), F8 (lanes 6 and 13) or F9 (lanes 7 and 14) generation was digested with either NcoI (lanes 1- 7) or SpeI (lanes 8- 14) restriction enzymes. The blot was hybridized with either probe 5' UTR (panel A); probe AHAS (panel B); or probe 3' UTR (panel C). The first and last lanes (labeled M) contain a λ/HindIII ladder; sizes are indicated in kilobases. Panel D indicates the origin of the Southern hybridization probes in the PvuII fragment of pAC321 used for transformation. The arrow in panel B indicates the 885 bp SpeI fragment containing the 376 bp fragment of *csr1-2* coding sequence inserted near the 3' flanking sequence junction.

D

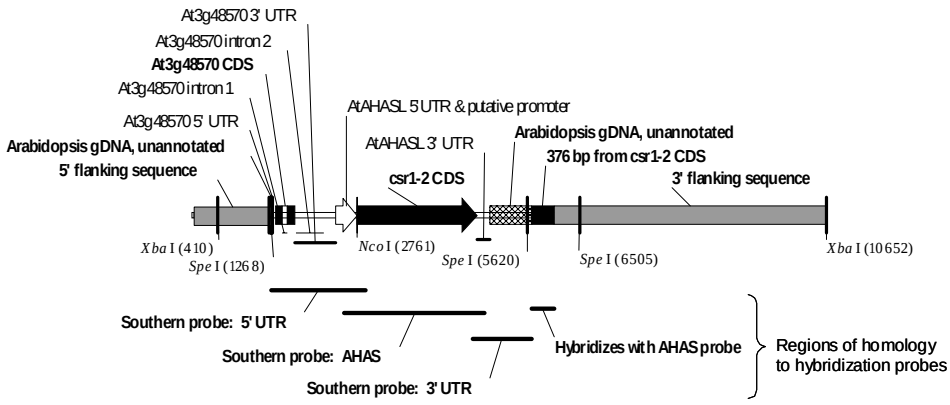


Figure 7. **Diagram of Insert and Flanking Sequence in Cultivance Soybean Event 127**. The insert and flanking sequences are displayed. Six amplicons used for sequencing of the insert are also indicated.

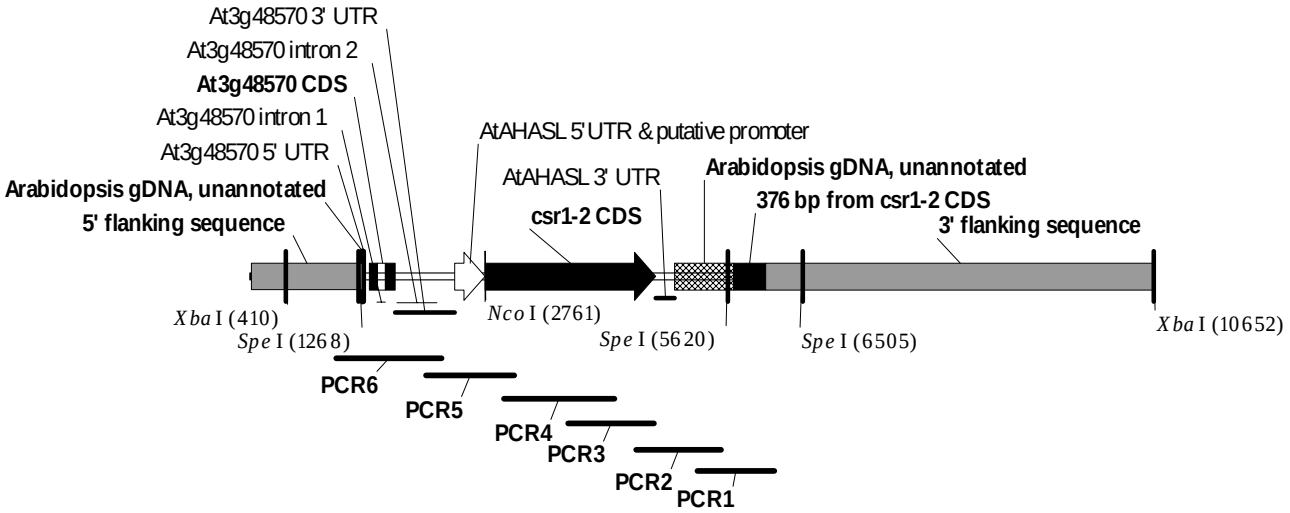


Figure 8. Sequence of the Insert Containing the *csr1-2* Expression Cassette and Flanking Soybean Genomic DNA in Cultivance Soybean Event 127.

General Description

DNA Cultivance Event 127 transgene insert 10.26.2005
Entire molecule length: 10656 bp

Feature Map

CDS (2 total)

At3g48570 CDS
Start: 1392 End: 1700

csr1-2 CDS
Start: 2762 End: 4774

Intron (2 total)

At3g48570 intron 1
Start: 1485 End: 1583

At3g48570 intron 2
Start: 1721 End: 2193

Misc. Feature (8 total)

5' flanking sequence
Start: 1 End: 1311

Arabidopsis gDNA, unannotated
Start: 1312 End: 1329

nt differs from pAC321
Start: 3576 End: 3576

Arabidopsis gDNA, unannotated
Start: 4993 End: 5693

nt differs from pAC321
Start: 5022 End: 5022

nt differs from pAC321
Start: 5065 End: 5065

376 bp from csr1-2 CDS
Start: 5694 End: 6069

3' flanking sequence
Start: 6070 End: 10656

Promoter Eukaryotic (1 total)

AtAHASL 5'UTR & putative promoter
Start: 2398 End: 2761

3' UTR (2 total)

At3g48570 3' UTR
Start: 1701 End: 2397

AtAHASL 3' UTR
Start: 4775 End: 4992

5' UTR (1 total)

At3g48570 5' UTR
Start: 1330 End: 1391

Sequence

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10651 tctaga
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Figure 9. RT-PCR Analysis of Transcription of the At *SEC61* γ Subunit in Cultivance Soybean Event 127. Total RNA from Event 127 F7 generation plant leaf tissue was extracted with Qiagen RNeasy Mini Kit and treated with DNase and total RNA from Arabidopsis leaf (At-L) and root (At-R) was extracted with TRIzol reagent without DNase treatment. Primers specific to the soybean proteasomal *Iota* subunit gene and the endogenous soybean *Sec61* γ subunit gene (Gm *Sec61* γ) are used in positive control reactions. Reactions without template RNA using At *SEC61* γ subunit-specific primers and Gm *Sec61* γ subunit-specific primers are used as negative controls. M: 1 kb DNA ladder. The arrow indicates the faint RT-PCR product corresponding to the At *SEC61* γ subunit amplified from Event 127.

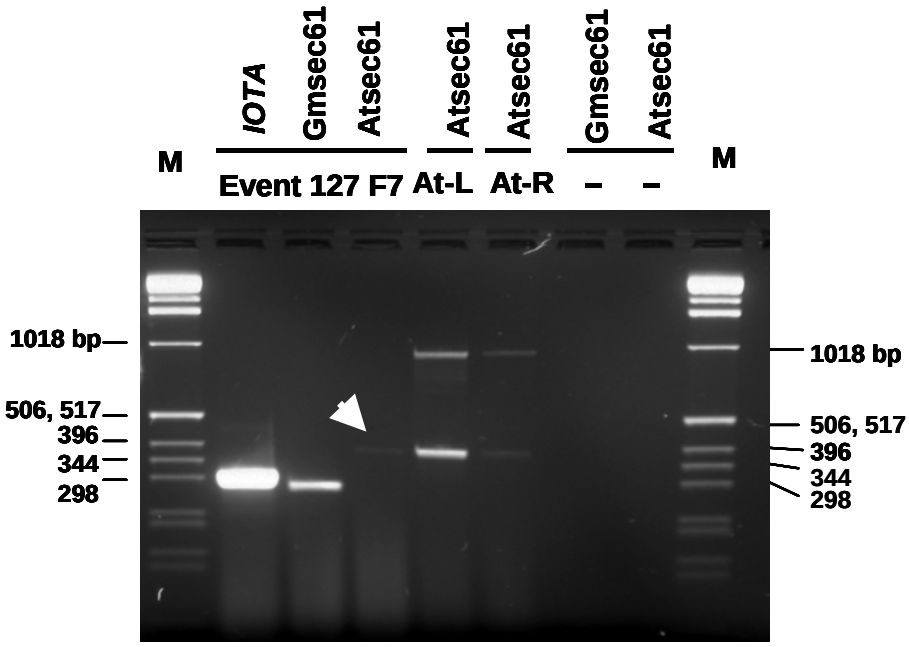

Figure 10. RT-PCR Analysis of a 501 bp ORF Created by Insertion of a 376 bp Portion of the *csr1-2* Coding Sequence at the 3' Flanking Sequence Junction in Cultivance Soybean Event 127. Total RNA was extracted from Event 127 F8 generation and non-transgenic Conquista leaf tissues with the Qiagen RNeasy Mini Kit and treated with DNase. Primers specific for the soybean proteasomal subunit *Iota* gene were used in positive control reactions. Reactions without RNA using both *Iota*- and ORF501-specific primers are used as negative controls (panel A). Genomic DNA from the Event 127 F8 generation was used as template in a PCR to test the primers for ORF501 (panel B). Of the 25 µl reaction, 5 µl was loaded on the gel (panel B). The band with an expected size of 435 bp is indicated with an arrow in panel B. M1: 1 kb ladder and M2: 100 bp ladder.

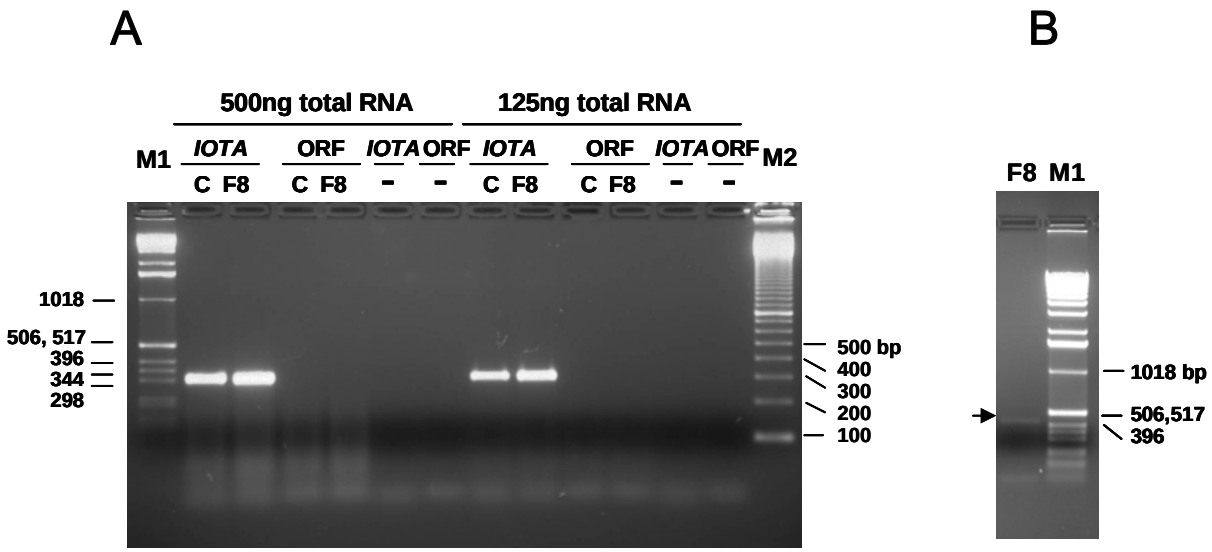

Figure 11. Event-Specific PCR Detection Method for Cultivance Soybean Event 127. Four pairs of primers were tested with Cultivance Soybean Event 127 F7 generation (F7) and non-transgenic Conquista (C) (Panel A). "Event PCR3" was validated with Cultivance Soybean Event 127 samples (T) and non-transgenic Conquista samples (C) collected from six different planting locations, Loc 1 - 6 (Panels B and C).