Appendix 10

AtSec61γ Subunit Protein Expression in Cultivance Soybean Event 127

AtSEC61γ SUBUNIT PROTEIN EXPRESSION IN CULTIVANCE SOYBEAN EVENT 127

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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. This report was amended to reflect corrected terminology and identity of control materials. The study was completed on February 26, 2007, but the amended report was issued September 2, 2008.

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ABBREVIATIONS AND DEFINITIONS

ahasl	imidazolinone-tolerant acetohydroxyacid synthase large subunit
AHASL	acetohydroxyacid synthase large subunit
GST	Glutathione S-transferase
KLH	Keyhole limpet hemacyanin, used as a carrier protein for immunizations
PBS	phosphate buffered saline
PVDF	polyvinylidene fluoride
RT-PCR	Reverse transcriptase-polymerase chain reaction
S653N	serine residue at position 653 of *Arabidopsis thaliana* acetohydroxyacid synthase large subunit replaced with asparagine

AtSEC61γ SUBUNIT PROTEIN EXPRESSION IN CULTIVANCE SOYBEAN EVENT 127

SUMMARY

Cultivance Soybean Event 127 (CV127) was produced by the introduction of an imidazolinone-tolerant acetohydroxyacid synthase large subunit (*ahasl*) gene from *Arabidopsis thaliana* into the soybean plant genome, resulting in soybean plants that are resistant to imidazolinone herbicides. The majority of the Arabidopsis *SEC61γ* subunit gene, including the entire Arabidopsis *SEC61γ* coding sequence, was also introduced into the soybean genome in Cultivance Soybean Event 127. This Arabidopsis *SEC61γ* subunit gene has been shown by reverse transcription-polymerase chain reaction (RT-PCR) experiments to be weakly transcribed in CV127 leaves (Shen, 2007). In this study, the presence of the Arabidopsis SEC61γ subunit protein (AtSEC61) was monitored by western blot analysis of microsomal membrane protein fractions prepared from leaf and grain samples of CV127. No AtSEC61 protein was detected in these tissues meaning that the protein was not present in CV127 tissues at a level above 5 ng/g leaf or 15 ng/g grain tissue (AtSEC61 protein was therefore not present at levels > 5 or >15 ppb, respectively for leaf and grain).

INTRODUCTION

Soybean (*Glycine max* L.) plants have been developed that are tolerant to the imidazolinone class of agricultural herbicides. The herbicide-tolerant soybean plants, referred to as Cultivance Soybean Event 127 (CV127), were produced by introduction of an imidazolinone-tolerant acetohydroxyacid synthase large subunit (*ahasl*) gene from *Arabidopsis thaliana* into the soybean plant genome via biolistics using the PvuII fragment of transformation vector pAC321 (Figure 1). This fragment includes what, at the time of transformation, was defined as the Arabidopsis *AHASL* promoter, the herbicide-tolerant Arabidopsis *ahasl* coding sequence, and the Arabidopsis *AHASL* terminator. Also included in the region originally annotated as the *AHASL* promoter was the majority of the Arabidopsis *SEC61γ* subunit gene (Arabidopsis Genome Initiative locus code At3g48570), including the entire Arabidopsis *SEC61γ* coding sequence. This Arabidopsis *SEC61γ* subunit gene has since been shown by reverse transcription-polymerase chain reaction experiments to be weakly transcribed in CV127 leaves (Shen, 2007) and by 5' race experiments to be the expected size transcript and encoding the predicted polypeptide sequence (McKean, 2007). SEC61γ subunit, together with the α and β subunits, is part of the protein translocation complex associated with the endoplasmic reticulum and is ubiquitous and highly conserved in eukaryotes as well as being structurally related to analogous proteins in prokaryotes (Hartman, *et al.*, 1994). Close amino acid sequence homology (86%) exists between the soybean SEC61γ subunit and that from Arabidopsis (see Figure 2 for the alignment). The purpose of this study was to determine if the Arabidopsis SEC61γ subunit protein (AtSEC61) is detectable in

microsomal membrane protein preparations from leaf and grain tissue of Cultivance Soybean Event 127 plants by western blot analysis.

MATERIALS AND METHODS

Test material. The test materials for this study were microsomal membrane protein preparations from leaf tissues (greenhouse and field grown) and grain derived from CV 127 lines. Microsomal membranes include the endoplasmic reticulum and were selected to enrich the SEC61γ subunit in the samples to be analyzed.

Control material. The control material for this study included microsomal membrane protein preparations from leaf tissue of field-grown conventional isoline control (null segregant of CV127), as well as leaf and grain tissues of the parental control variety Conquista (greenhouse grown plants).

Reference material. The reference material for this study was a microsomal membrane protein preparation from *Arabidopsis thaliana* leaves.

AtSEC61 protein standard. The At3g48570 gene, encoding the AtSEC61γ subunit protein, was cloned into the expression cassette pGEX-6P (GE Healthcare Bio-Sciences Corp, Pistcaway, NJ) and transformed into *Escherchia coli* strain Origami B (DE3)pLysS (EMD Biosciences). This expression cassette encodes a glutathione S-transferase (GST) – SEC61γ subunit fusion protein (mol. wt. *ca.* 35,000). The GST-SEC61γ subunit fusion protein was purified from *E. coli* using an immobilized glutathione column according to the manufacturer's instructions (GE Healthcare). On column digestion using PreScission Protease™ (GE Healthcare) was performed to release the SEC61γ subunit protein (mol. wt. *ca.* 7000). This was the SEC61γ subunit protein that was used as a standard on the western blots.

Source of plant materials. Leaf tissue of CV127 lines 127 and 603[1] as well as greenhouse-produced grain from line 603 were provided to BASF Plant Science by BASF SA for microsomal membrane protein isolation. Microsomal membrane protein was isolated from leaf tissue harvested from field grown plants of CV127 lines 127 and 603 as well as from the nontransgenic isoline control soybean produced at the same locations. These leaf samples were harvested from field grown plants at two locations, Ponte Grossa and Sete Lagoas in Brazil during the 2006/2007 regulatory field trials, lyophilized and shipped to the BASF Plant Science analytical laboratory in Limburgerhof, Germany. Leaf samples of greenhouse grown (Limburgerhof, Germany) plants of CV127 line 603 and parental control soybean variety Conquista were also used in this study. Grain was harvested from greenhouse grown CV127 line 603 and parental control soybean variety Conquista plants produced in Londrina, Brazil. Arabidopsis

[1] Cultivance Soybean Event 127 (CV127) line 127 was produced by traditional breeding from CV127 line 603, which is an earlier generation of the same event. Specifically, CV127 line 127 was produced by crossing the F8 generation of CV127 line 603 with BR1 x Conquista.

leaves were produced in Limburgerhof, Germany as well as Research Triangle Park, North Carolina, United States.

Microsomal Membrane Protein Preparations. Arabidopsis and greenhouse grown soybean leaf extracts were prepared by homogenizing 3 g frozen powdered leaves in 9 ml extraction buffer [8 mM sodium phosphate, 2 mM potassium phosphate, 0.14 M sodium chloride, 10 mM potassium chloride, pH 7.4 (PBS) with HALT™ protease inhibitor cocktail (Pierce Biotechnology, Inc., Rockford, NJ) and 2 mM EDTA]. The homogenates were filtered through miracloth and centrifuged at 10,000 x g for 15 min. The supernatant was then centrifuged at 100,000 x g for 1 hour. The resulting pellet was the microsomal membrane pellet and was resuspended in 80 µl 2 X Laemmli sample buffer. Lyophilized soybean leaf extracts (from field grown plants) were prepared by repeated vortexing of powdered leaves (0.2 g) with 6 ml extraction buffer; for soybean grain extract, 0.4 g powdered grain was extracted with 6 ml extraction buffer. The extracts were filtered through miracloth and microsomal membranes were isolated. The pellets were resuspended in 2X Laemmli buffer.

Antibody generation. Two peptides (aa 1 - 30 and 31 – 59) spanning the entire 59 amino acid sequence of the SEC61γ subunit protein were synthesized, conjugated with keyhole limpet hemacyanin (KLH) and subsequently used as antigen for sensitizing rabbits (Virusys, Inc., Sykesville, MD). The IgGs in the sera were purified using protein A sepharose.

Western blot analysis. Aliquots of the microsomal membrane preparations were subjected to SDS-polyacrylamide gel electrophoresis on a 4 - 20% polyacrylamide gradient gel followed by electroblotting onto a PVDF membrane (Invitrogen; SOP BPS 510.03). The membrane was probed with rabbit anti-AtSEC61 polyclonal antibody. Donkey anti-rabbit IgG linked to horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), diluted 1:3000 in blocking buffer (3% nonfat dry milk in 0.1% Tween-20, 10 mM Tris-HCl, 150 mM NaCl, pH 7.5), was used to bind to the primary antibody and was visualized by development with the ECL Plus Western Blotting Detection Reagents (GE Healthcare). The limit of detection of this development method with this antibody was found to be approximately 2.5 ng SEC61γ subunit protein.

RESULTS AND DISCUSSION

In companion studies (Shen 2007; McKean 2007), it was shown by reverse transcription-polymerase chain reaction (RT-PCR) and by RNA-ligase mediated rapid amplification of 5' complementary DNA ends (RLM-5'-RACE) that a transcript corresponding to the Arabidopsis *SEC61γ* subunit gene is detected at very low levels in CV127 leaves. Since the transcript was highly amplified by RT-PCR to maximize detection capability, and only a faint band corresponding to the Arabidopsis *SEC61γ* subunit transcript was detected, it was presumed that either no AtSEC61 protein is produced in CV127, or if produced the protein would be present at extremely low levels in the plant. For this reason, methods were used to maximize the probability of detection of the AtSEC61

protein in leaf and grain tissues of CV127. First, the leaf and grain extracts were loaded on the SDS-PAGE gels at high levels, and secondly an over-exposure of the ECL Plus Western Blotting Detection method was used for protein detection on the western blot. As a result of these methods used to maximize sensitivity for detection of the Arabidopsis SEC61γ subunit protein in tissues of CV127, detection of cross-reactivity of the antibody to other proteins was enhanced, and perturbations of the protein bands on the SDS-PAGE gels were also increased because of the high sample loading procedures and compounded by the fact that samples were microsomal membrane preparations (Figure 3).

The AtSEC61γ protein was readily detected by these methods in the standard protein lanes at a mol. wt. of approximately 7000 (Figure 3). However, western blot analysis detected no Arabidopsis SEC61γ subunit protein in leaf or grain microsomal membrane protein preparations of CV127 (Figure 3A and 3B) even when exaggerated levels of sample were loaded on the gels (e.g. Figure 3A, lane 7). Because of the high amino acid sequence homology of SEC61γ subunit protein across species (e.g., 86% homology between soybean and Arabidopsis) it was expected that the antibody would detect the endogenous soybean SEC61γ subunit protein, however no protein band corresponding to the SEC61γ subunit protein was detected in tissue extracts of the control soybeans. Furthermore, endogenous expression levels of the SEC61γ subunit protein in Arabidopsis appear to be so low that even in microsomal membrane protein preparations from Arabidopsis, no SEC61γ subunit protein was detected (Figure 3A, lane 10, Figure 3B, lanes 7 and 8). Therefore, AtSEC61γ subunit protein was not expressed at levels greater than 5 ng/g leaf issue or 15 ng/g grain tissue or greater than 5 and 15 ppb for leaf and grain of CV127, respectively. This result is not unexpected given the extremely low levels of the transcript corresponding to the *AtSEC61γ* subunit gene detected in CV127.

STUDY PERSONNEL

Analytical work reported herein conducted b[redacted]M.S., BASF Plant Science GmbH, D-67117 Limburgerhof, Germany.

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McKean, A. (2007) Determination of the 5' end of the *Arabidopsis thaliana SEC61*γ subunit transcript in Cultivance Soybean Event 127. BASF Plant Science Report No. BPS-002-06.

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

Figure 1. Plasmid pAC321

The pAC321 PvuII fragment containing the *AHASL* 5' UTR, *csr1-2* coding sequence (AHASL), and *AHASL* 3' UTR was used for transformation to produce Cultivance Soybean Event 127. *AHASL* 5' UTR includes the coding sequence designated At3g48570, SEC61γ subunit.

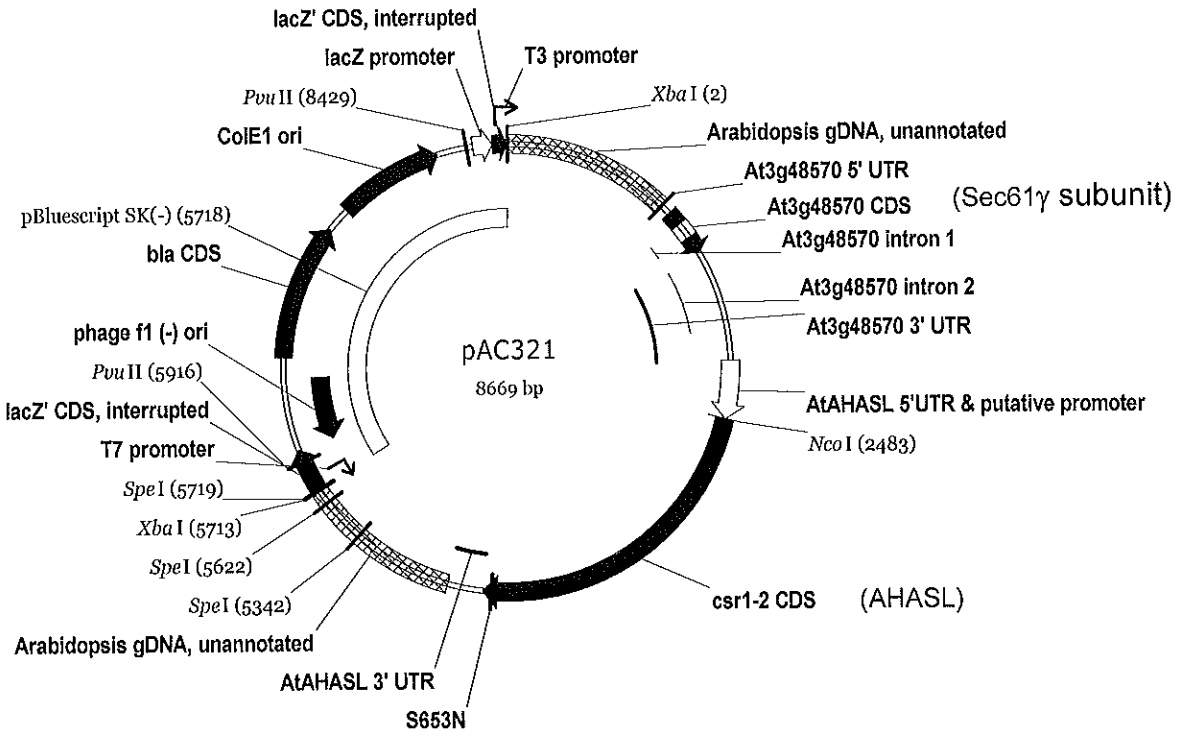

Figure 2. Sequence comparison of the SEC61γ subunit-like protein from Arabidopsis with SEC61γ subunit-like proteins encoded by four genes from Soybean.

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                  1                                                  50
At3g48570/Sec61   MEAIDSAIDP LRDFAKSSVR LVQRCHKPDR KEFTKVAVRT AIGFVVMGFV
   Soy_Sec61γ_1   MDAIDSVFDP LREFAKDSVR LVKRCHKPDR KEFSKVAVRT AIGFVVMGFV
   Soy_Sec61γ_2   MDAIDSVFDP LREFAKDSVR LVKRCHKPDR KEFSKVAVRT AIGFVVMGFV
   Soy_Sec61γ_3   MDAIDSVFDP LREFAKDSVR LVKRCHKPDR KEFSKVAVRT AMGFVVMGFV
   Soy_Sec61γ_4   MDAIDSVFDP LLEFAKDSVR LVKRCHKPDR KEFSKVAVRT AIGFVVMGFV

                  51                  69
At3g48570/Sec61   GFFVKLVFIP INNIIVGSS
   Soy_Sec61γ_1   GFFVKLIFIP INNIIVGSG
   Soy_Sec61γ_2   GFFVKIIFIP INNIIVGSG
   Soy_Sec61γ_3   GFFVKLIFIP INNIIVGSG
   Soy_Sec61γ_4   GFFVKLIFIP INNIIVGSG
```

Percent Identical Amino Acids

	At3g48570/SEC61	Soy SEC61γ 1	Soy SEC61γ 2	Soy SEC61γ 3	Soy SEC61γ 4
At3g48570/SEC61	100	87	86	86	86
Soy SEC61γ 1		100	98	98	98
Soy SEC61γ 2			100	97	97
Soy SEC61γ 3				100	97
Soy SEC61γ 4					100

Figure 3. Western blot analysis for SEC61γ Subunit Protein in Leaf and Grain Microsomal Membrane Protein Preparations from Cultivance Soybean Event 127 and Conquista.

Microsomal membrane proteins were prepared from leaf and grain samples and subjected to electrophoresis on a 4 – 20% polyacrylamide gel. Western blot analysis was conducted using rabbit anti-AtSEC61γ subunit polyclonal antibodies and developed using a chemiluminiscent substrate. The molecular weight of SEC61γ subunit protein is approximately 7000. All samples except for the AtSEC61γ subunit protein standard are from microsomal membrane protein preparations. Panel A. Lanes 1, 2, and 3, isoline control, CV127 line 603 and CV127 line 127 leaves from Ponta Grossa; Lanes 4 and 11, 236 and 407 ng standard AtSEC61γ subunit protein, respectively; Lanes 5, 6 and 7, isoline control, CV127 line 603 and CV127 line 127 leaves from Sete Lagoas; Lanes 8 and 9, Conquista and CV603, leaves from greenhouse grown plants in Limburgerhof; Lane 10, Arabidopsis leaves from greenhouse-grown plants in Limburgerhof. Panel B, Lanes, 1, 4, and 9, 236 ng standard AtSEC61γ subunit protein; Lanes 2 and 3, Conquista and CV127 line 603 grain from Londrina; Lanes 5 and 6, Conquista and CV127 line 603 leaves from Limburgerhof; Lanes 7 and 8, Arabidopsis leaves from Limburgerhof and Research Triangle Park, respectively.