Study Title

Assessment of Delta 6 and Delta 15 Desaturase Protein Levels in Tissues from MON 87769 Soybean Grown in 2006 U.S. Field Trials

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Sponsor and Performing Laboratory

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REG-07-181
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Statement of Compliance

This study meets the U.S. EPA Good Laboratory Practice requirements as specified in 40 CFR Part 160 with the following exception: stability for Delta 6 and Delta 15 desaturase proteins for the duration of study is not known.

Submitter Date

Cheriaw George 12-4-2008

Sponsor Representative Date

12/04/2008

Study Director Date

Quality Assurance Statement

Reviews conducted by the Quality Assurance Unit confirm that the final report accurately describes the methods and standard operating procedures followed and accurately reflects the raw data of the study.

Following is a list of reviews conducted by the Monsanto Regulatory Quality Assurance Unit on the study reported herein.

Dates of Inspection/Audit	Phase	Date Reported to Study Director	Date Reported to Management
10/29/2007	Protein Extraction and/or Quantitation	11/02/2007	11/02/2007
11/16/2007	Western Blot	11/16/2007	11/16/2007
02/25/2008	Moisture Analysis	03/10/2008	03/10/2008
02/29/2008	Raw Data Audit	04/01/2008	04/01/2008
10/17/2008	Draft Report Review	10/23/2008	10/23/2008

Mary Stephen 12/4/08

Quality Assurance Unit Date
Monsanto Regulatory, Monsanto Company

Study Certification

This report is an accurate and complete representation of the study/project activities.

Signatures of Approval:

12/04/2008

Study Director Date

Dec 4, 2008

Protein Quantification Team Lead Date

Study Information

Study Number:	REG-07-181
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Facility:	Monsanto Company Biotechnology Regulatory Sciences 800 North Lindbergh Boulevard Saint Louis, Missouri 63167
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Records Retention:	All study specific raw data (including rejected data and data not reported), protocols, final reports, and facility records will be retained at Monsanto, St. Louis.

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Abbreviations[1] and Definitions

CV	coefficient of variation
DWCF	dry weight conversion factor
dwt	dry weight of tissue
fwt	fresh weight of tissue
HRP	horseradish peroxidase
IgG	immunoglobulin G
LOD	Limit of Detection
LOQ	Limit of Quantitation
OSL	over season leaf
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with Tween 20
PCR	polymerase chain reaction
QAU	Quality Assurance Unit
SD	standard deviation
SDA	stearidonic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOP	standard operating procedure
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween 20
V	volts of electricity applied to SDS-PAGE

[1] Standard abbreviations, e.g., units of measure, were used in this report according to format described in "Instructions to Authors" in the Journal of Biological Chemistry.

1.0 Summary

Monsanto has developed soybean, MON 87769, which produces stearidonic acid (SDA), an omega-3 fatty acid. Production of SDA in soybean seed was achieved through the introduction of genes encoding the production of Δ6 and Δ15 desaturases from *Primula juliae* and *Neurospora crassa*, respectively, via *Agrobacterium* mediated transformation. The Δ6 and Δ15 genes are driven by 7sα' and 7sα promoters, respectively, which are known to be seed-enhanced.

The purpose of this study was to determine the levels of the Δ6 and Δ15 desaturase proteins in MON 87769 soybean tissues collected in 2006 U.S. field trials by using optimized western blot methods. Tissue samples were collected from plants grown at five sites in the U.S. in 2006 under Production Plan 06-01-83-06. In this study, over season leaf (OSL-1, OSL-2, OSL-3, and OSL-4), root, forage, immature seed, and mature seed tissues were analyzed by western blot analysis. All protein levels for all tissue types were calculated on a microgram (μg) per gram (g) fresh weight (fwt) basis. Moisture content was then measured for all tissue types displaying detectable Δ6 and Δ15 desaturase protein expression, and the protein levels were converted and reported on a microgram (μg) per gram (g) dry weight (dwt) basis.

Results showed that the mean protein level for Δ6 desaturase across all sites was 16 μg/g dwt in forage, 100 μg/g dwt in immature seed, and 1.8 μg/g dwt in mature seed. The Δ6 desaturase protein was not detected in over season leaf (OSL-1, OSL-2, OSL-3, and OSL-4) and root.

Results showed that the mean protein level for Δ15 desaturase across all sites was 14 μg/g dwt in forage, 200 μg/g dwt in immature seed, and 10 μg/g dwt in mature seed. The Δ15 desaturase protein was not detected in over season leaf (OSL-1, OSL-2, OSL-3, and OSL-4) and root.

2.0 Introduction

2.1 Background

Monsanto has developed soybean, MON 87769, which produces stearidonic acid (SDA), an omega-3 fatty acid. Production of SDA in soybean seed was achieved through the introduction of genes encoding the production of Δ6 and Δ15 desaturases from *Primula juliae* and *Neurospora crassa*, respectively, via *Agrobacterium* mediated transformation. The Δ6 and Δ15 genes are driven by 7sα' and 7sα promoters, respectively, which are known to be seed-enhanced.

Δ6 and Δ15 desaturase protein levels were determined in tissues collected from soybean plants grown at five U.S. field sites in 2006. Field sites were selected to represent geographical regions representing commercial soybean production.

2.2 Purpose

The purpose of this study was to determine the levels of Δ6 and Δ15 desaturase proteins expressed in MON 87769 soybean tissues. Tissue samples were collected from plants grown in the U.S. at five field sites in 2006 under Production Plan 06-01-83-06.

3.0 Materials

3.1 Test, Control, and Reference Substances

3.1.1 Test Substance

The test substance was MON 87769 grown in 2006 U.S. field trials. Tissue samples were collected as outlined in Production Plan 06-01-83-06 from plants grown from starting seed lot GLP-0604-17267-S.

3.1.2 Control Substances

The negative control substance was conventional soybean A3525, with a similar genetic background to the test plants grown in 2006 U.S. field trials. Tissue samples were collected as outlined in Production Plan 06-01-83-06 from plants grown from starting seed lot GLP-0604-17278-S.

3.1.3 Characterization of Test and Control Substances

The identities of the test and control substances were confirmed by analysis of the starting seed DNA by an event-specific polymerase chain reaction (PCR) method and were archived under the seed lot numbers. The identities of the mature seed samples harvested from the field were verified by event specific PCR and assigned a Verification of Identity that was archived under the starting seed lot numbers. Test or control substances, and their associated tissues, which had three or more pools that tested unexpectedly during the PCR verification, were not analyzed in this study.

3.1.4 Reference Substances

E. coli-produced Δ6 and Δ15 desaturase protein standards were used in this study and copies of the certificates of analyses are archived with the study data.

A Δ6 desaturase protein standard (lot 20-100109) was used as the reference substance for the determination of Δ6 protein levels. The protein concentration of the purified standard was determined to be 1.5 mg/ml by amino acid composition analysis. The purity was 89% as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and densitometric analysis. The purity-corrected concentration was calculated to be 1.3 mg/ml.

A Δ15 desaturase protein standard (lot 20-100127) was used as the reference substance for the determination of Δ15 protein levels. The total protein concentration of the purified standard was determined to be 0.8 mg/ml by amino acid composition analysis. The purity was 91% as determined by SDS-PAGE and

densitometric analysis. The purity-corrected concentration was calculated to be 0.7 mg/ml.

4.0 Methods

4.1 Generation of Plant Samples

4.1.1 Summary of Field Design

Production Plan 06-01-83-06 was initiated during the 2006 planting season to generate test and control substances at various soybean-growing locations in the U.S (Colyer, 2008). The field sites were as follows: Jefferson County, IA; Guthrie County, IA; Clinton County, IL; Ottawa County, MI; Fayette County, OH. These field sites were located within the major soybean-growing regions of the U.S. and provided a variety of environmental conditions. At each site, three replicated plots of MON 87769, as well as the conventional control, were planted using a randomized complete block field design. Over-season leaf (OSL-1, OSL-2, OSL-3, and OSL-4), root, forage, immature seed, and mature seed tissues were collected from each replicated plot at all field sites. The over-season leaf 1-4 samples were collected at the V3-V4, V6-V8, V10-V12, and V14-V16 growth stages, respectively (Pedersen, 2004). Forage samples were collected at the R6 growth stage, which contain all above ground portions of the plants including developing seed. Root samples were collected at the R6 growth stage. Immature seed samples were collected at the R5- R6 growth stage. Mature seed samples were collected at the R8 growth stage. Throughout the field production, sample identity was maintained by using unique sample identifiers and proper chain-of-custody documentation. All tissue samples, except mature seed, were stored and shipped on dry ice to the Monsanto Sample Processing facility in Saint Louis, Missouri. Mature seed samples were stored and shipped at ambient temperature.

4.2 Tissue Processing and Protein Extraction Methods

4.2.1 Processing Method

All tissue samples produced at the field sites were shipped to the Monsanto Sample Processing facility. Processed tissue samples were stored at -80°C until shipped on dry ice to Monsanto's analytical facility. All processed tissue samples were stored at -80°C during the study.

4.2.2 Extraction Methods

The Δ6 and Δ15 desaturase proteins were extracted from soybean tissues as described in Appendix 2. All processed tissues were kept on dry ice during extract preparation. All tissues were extracted using a Harbil mixer and insoluble material was removed from the extracts by centrifugation. The supernatants were aliquoted and stored at -80°C until Western Blot analyses.

4.3 Western Blot Reagents and Methods

4.3.1 Δ6 Antibodies

Goat polyclonal antibodies (lot 7580971) specific to a peptide fragment of the Δ6 desaturase protein were produced and affinity purified by Invitrogen Life Sciences (Carlsbad, CA). The concentration of the purified IgG was determined to be 2.2 mg/ml by spectrophotometric methods. The purified antibody was stored in 25 mM sodium borate, 100mM boric acid, 75 mM NaCl, 5mM EDTA, pH 8.2 to 8.4 (Borate Buffered Saline).

4.3.2 Δ15 Antibodies

Goat polyclonal antibodies (lot 7580965) specific to a peptide fragment of the Δ15 desaturase protein were produced and affinity purified by Invitrogen Life Sciences. The concentration of the purified IgG was determined to be 2.6 mg/ml by spectrophotometric methods. The purified antibody was stored in Borate Buffered Saline.

4.3.3 Δ6 Desaturase Western Blot Method

Supernatants from extraction were analyzed by SDS-PAGE on a 4-20% Tris-HCl gradient gel (Hercules, CA). Prior to loading, the samples were diluted in 2X Laemmli Buffer (Bio-Rad). Sample extracts along with the appropriate reference standards were loaded onto gels. The reference standards were prepared in tissue-specific non-transgenic controls for each tissue type, and they were diluted in 2X Laemmli buffer and spiked with 1, 2.5, 5, 10 and 20 ng of reference standards. The non-transgenic controls used for the reference standards for each tissue type were pooled from all sites. Except for the reference standards, all samples were loaded in triplicate and a minimum of two were analyzed by densitometry for quantification. Additionally, Precision Plus Protein Standards (Bio-Rad) were loaded on the gel to demonstrate the transfer of protein to membrane and for the approximate molecular weight determination.

Electrophoresis was conducted according to SOP BR-ME-0388-02 at 200 V for approximately 5 minutes and at 120 V for approximately 80 minutes in 1X Tris-Glycine-SDS running buffer (Bio-Rad). Electrotransfer and Western Blot analysis were conducted according to SOPs BR-ME-0924-01 and BR-ME-0392-01 respectively. Proteins separated by SDS-PAGE were electrophoretically transferred to 0.45 µm Criterion Nitrocellulose membrane (Bio-Rad) using 1X Tris-glycine transfer buffer (Bio-Rad) containing 20% methanol. After transfer, non-specific sites on the membrane were blocked using 5% (w/v) non-fat dried milk (NFDM, Bio-Rad) in 1X Phosphate-Buffered Saline with 0.05% (v/v) Tween-20 (1X PBST).

The membrane was probed for the presence of the Δ6 desaturase protein with a 1:2500 dilution of purified goat antibodies, anti-PjD6d6-3 antibody (Lot 7580971) in 1X PBST with 1% (w/v) NFDM. Unbound antibodies were removed by rinsing the membrane briefly and then washing three times for 10 minutes each in

1X PBST. Bound antibodies were probed with a 1:5000 dilution of anti-goat IgG antibody conjugated to horseradish peroxidase (Pierce, Rockford, IL) in 1X PBST with 1% (w/v) NFDM. Unbound anti-goat IgG-HRP antibodies were removed by rinsing the membrane briefly and then washing four times for 10 minutes each in 1X PBST. The Enhanced Chemiluminescence (ECL) substrate (Amersham, Piscataway, NJ) was added to the membrane according to the manufacturers' instructions. The membrane was exposed to Hyperfilm ECL (Amersham) to record an image of the immunoreactive bands. Quantification of the bands was performed according to SOP BR-ME-0932-03. To meet acceptance criteria, at least three standards must be present on the X-ray film used to generate the standard curve for sample quantification. For those tissues that did not show detectable presence of the Δ6 desaturase, only the standard curve was generated. For the tissues that did show specific protein expression, at least two sample replicates were analyzed by densitometry for quantification. The coefficient of variation (CV) for the sample replicates must meet pre-defined criteria of $\leq$ 33%.

4.3.4 Δ15 Desaturase Western Blot Method

Supernatants from extraction were analyzed by SDS-PAGE on a Novex 4-20% gradient gel (Invitrogen). Prior to loading, the samples were diluted in 2X Laemmli Buffer (Bio-Rad). Sample extracts along with the appropriate reference standards were loaded onto gels. The reference standards were prepared in tissue-specific non-transgenic controls for each tissue type, and they were diluted in 2X Laemmli buffer and spiked with 1, 2, 5, 10, 25, 50 and 75 ng of reference standards. The non-transgenic controls used for the reference standards for each tissue type were pooled from all sites. Except for the reference standards, all samples were loaded in triplicate and a minimum of two were analyzed by densitometry for quantification. Additionally, Precision Plus Protein Standards (Bio-Rad) was loaded on the gel to demonstrate the transfer of protein to membrane and for the approximate molecular weight determination.

Electrophoresis was conducted according to SOP BR-ME-0388-02 at 120 V for approximately 120 minutes in 1X Novex Tris-Glycine SDS running buffer (Invitrogen). Electrotransfer and Western Blot analysis were conducted according to SOPs BR-ME-0924-01 and BR-ME-0392-01, respectively. Proteins separated by SDS-PAGE were electrophoretically transferred to Invitrolon PVDF membrane (Invitrogen) using 1X Novex Tris-Glycine transfer buffer (Invitrogen) containing 20% methanol. After transfer, non-specific sites on the membrane were blocked using 5% (w/v) non-fat dried milk (NFDM, Bio-Rad) in 1X Tris-Buffered Saline with 0.1% (v/v) Tween-20 (1X TBST).

The membrane was probed for the presence of the Δ15 desaturase protein with a 1:2500 dilution of purified goat antibodies, anti-Ncd15d 15-1 antibody (Lot 7580965) in 1X TBST with 5% (w/v) NFDM. Unbound antibodies were removed by rinsing the membrane briefly and then washing three times for 10 minutes each in 1X TBST. Bound antibodies were probed with a 1:5000 dilution of anti-goat

IgG antibody conjugated to horseradish peroxidase (Pierce, Rockford, IL) in 1X TBST with 5% (w/v) NFDM. Unbound anti-goat IgG-HRP antibodies were removed by rinsing the membrane briefly and then washing four times for 10 minutes each in 1X TBST. The SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) was added to the membrane according to the manufacturers' instructions. The membrane was exposed to Hyperfilm ECL (Amersham) to record an image of the immunoreactive bands. Quantification of the bands was performed according to SOP BR-ME-0932-03. At least three standards must be present on the film and used to generate the standard curve for sample quantification. For those tissues that did not show detectable presence of the Δ15 desaturase, only the standard curve was generated. For those tissues that did show specific protein expression, at least two sample replicates were analyzed by densitometry for quantification. The coefficient of variation (CV) must meet pre-defined criteria of $\leq$ 33%.

4.4 Control of Bias

The test and control substances were planted in a non-systematic manner at all field sites using a randomized complete block design as described in Production Plan 06-01-83-06. Representative tissues from each plot were collected as described in the production plan. All tissues were processed by thoroughly grinding before extraction to minimize sampling bias. The Western Blot methods used were optimized to minimize method bias.

4.5 Moisture Analysis

For each of the tissues in which protein expression was detected, one tissue-specific site pool (TSSP) was prepared using the test and control samples of a given tissue type grown at a given site. All TSSP samples were analyzed for moisture content using an IR-200 Moisture Analyzer (Denver Instrument Company, Arvada, CO) under the program Soybean Forage according to SOP AG-EQ-1023-01. The average percent moisture for each TSSP was calculated from triplicate analyses. A TSSP Dry Weight Conversion Factor (DWCF) was calculated as follows:

$$DWCF = 1 - [Mean\ \%\ TSSP\ Moisture / 100]$$

The DWCF was used to convert protein levels assessed on a µg/g fresh weight (fwt) basis into levels reported on a µg/g dry weight (dwt) basis using the following calculation:

$$Protein\ Level\ in\ Dry\ Weight = \frac{(Protein\ Level\ Fresh\ Weight)}{(DWCF)}$$

4.6 Data Analyses

All Delta 6 and Delta 15 Western Blots were analyzed on a GS-800 densitometer (Bio-Rad) with Quantity One software (version 4.4.0). Following the interpolation from the standard curve, the amount of protein in the tissue was calculated and

converted to a "µg/g fwt" basis utilizing a sample dilution factor and a tissue-to-buffer ratio. The protein values in "µg/g fwt" were also converted to "µg/g dwt" by applying the DWCF. Microsoft Excel 2002 (Version 10.6834.6830 SP3, Microsoft, Redmond, WA) was used in the process of calculation and conversion.

4.7 Protocol Deviations

One test substance from the OH field site was inadvertently left out of this study, though its identity was confirmed by PCR. As a result, no data was generated or reported for this test substance. Tissue and extract storage stability was not determined due to unavailability of samples.

5.0 Results

Summaries of mean, standard deviation (SD), and range are reported for Δ6 and Δ15 desaturase protein levels on µg/g fwt and µg/g dwt basis in soybean tissues collected from five U.S. field sites in 2006.

5.1 Limit of Quantitation (LOQ)

The Limit of Quantitation (LOQ) for the Δ6 and Δ15 desaturase Western Blot is listed in Table 1. All visible protein bands at the expected molecular weight were quantified. The calculation of the LOQ was based on the lowest amount on fwt basis of protein standard visualized and recorded from the Western Blot in the study. In the Δ6 desaturase Western Blot, the LOQ was 1.0 µg/g for OSL-1, OSL-2, OSL-3, OSL-4, and root, 1.0 µg/g for forage, 4.0 µg/g for immature seed, and 2.0 µg/g for mature seed. In the Δ15 desaturase Western Blot, the LOQ was 2.0 µg/g for OSL-1, OSL-2, OSL-3, OSL-4, and root, 1.0 µg/g for forage, 10.0 µg/g for immature seed, and 2.0 µg/g for mature seed.

5.2 Δ6 Desaturase Protein Levels in Soybean

The Δ6 desaturase protein levels for MON 87769 are presented in Table 2. The mean Δ6 desaturase protein levels in MON 87769 across all sites were 16 µg/g dwt in forage, 100 µg/g dwt in immature seed, and 1.8 µg/g dwt in mature seed. The Δ6 desaturase protein was not detected in over season leaf (OSL-1, OSL-2, OSL-3, and OSL-4) and root of MON 87769. As expected, Δ6 desaturase protein was not detected in all tissues of conventional control soybean.

5.3 Δ15 Desaturase Protein Levels in Soybean

The Δ15 desaturase protein levels for MON 87769 are presented in Table 2. The mean Δ15 desaturase protein levels in MON 87769 across all sites were 14 µg/g dwt in forage, 200 µg/g dwt in immature seed, and 10 µg/g dwt in mature seed. The Δ15 desaturase protein was not detected in over season leaf (OSL-1, OSL-2, OSL-3, and OSL-4) and root of MON 87769. As expected, Δ15 desaturase protein was not detected in all tissues of conventional control soybean.

6.0 Conclusions

MON 87769 was grown in U.S. field trials at five field sites in 2006. Tissue samples were collected at various growth stages throughout the growing season and analyzed for Δ6 and Δ15 desaturase protein levels using optimized Western Blot methods. These data provide an estimation of the protein levels of Δ6 and Δ15 desaturase on a fresh weight and dry weight basis in three of eight tissue types for MON 87769 soybean.

7.0 Acknowledgments

The authors would like to acknowledge Jack Milligan and the Agronomy and Sample Processing Center for processing the tissue samples and Andre Van Oyen, Jr., John Lake, and Abdul Fabellar from the Sample Dispensary for the sample distributions.

8.0 References

Colyer, J. 2008. Field Production of Tissues from Omega-3 Soybean MON 87769 Grown in the United States during 2006. Monsanto Technical Report, St. Louis, MO, MSL0021075.

Pedersen, P. 2004. Soybean Growth and Development PM 1945. Iowa State University of Science and Technology Cooperative Extension Service, Ames, IA.

Table 1. Limit of Quantitation (LOQ) for the Δ6 and Δ15 desaturase Western Blot

	Limit of Quantitation (µg/g fwt)[1]	
Tissue Type[2]	Δ6 Western Blot	Δ15 Western Blot
OSL-1	1.0	2.0
OSL-2	1.0	2.0
OSL-3	1.0	2.0
OSL-4	1.0	2.0
Root	1.0	2.0
Forage	1.0	1.0
Immature Seed	4.0	10.0
Mature Seed	2.0	2.0

1. For each tissue type, the Limit of Quantitation (LOQ) was calculated based on the lowest amount of protein standard visualized and recorded from the Western Blot in the study.
2. Tissues were collected at the following growth stages (Pedersen, 2004):
a. OSL-1: V3 – V4 c. OSL-3: V10 – V12 e. Root: R6 g. Immature Seed: R5 – R6
b. OSL-2: V6 – V8 d. OSL-4: V14 – V16 f. Forage: R6 h. Mature Seed: R8

Table 2. Summary of Δ6 and Δ15 Desaturase Protein Levels in MON 87769 Soybean Tissues Collected from 2006 U.S. Field Trials

Tissue Type[1]	Δ6 Desaturase Levels		Δ15 Desaturase Levels	
	Mean (SD)[2,4] Range[5] (µg/g fwt)	Mean (SD)[3] Range (µg/g dwt)	Mean (SD) Range (µg/g fwt)	Mean (SD) Range (µg/g dwt)
OSL-1	Not detected[6]		Not detected	
OSL-2	Not detected		Not detected	
OSL-3	Not detected		Not detected	
OSL-4	Not detected		Not detected	
Root	Not detected		Not detected	
Forage	4.3 (2.4) 1.0 – 7.4	16 (9.5) 3.6 – 28	3.7 (1.7) 1.3 – 7.9	14 (6.8) 4.6 – 30
Immature Seed	27 (15) 5.6 – 45	100 (63) 19 – 210	55 (21) 20 – 85	200 (89) 66 – 330
Mature Seed[7]	1.7 (0.86) 0.45 – 3.0	1.8 (0.95) 0.50 – 3.2	9.5 (5.9) 4.3 – 23	10 (6.5) 4.8 – 25

1. Tissues were collected at the following growth stages (Pedersen, 2004):
a. OSL-1: V3 – V4 c. OSL-3: V10 – V12 e. Root: R6 g. Immature Seed: R5 – R6
b. OSL-2: V6 – V8 d. OSL-4: V14 – V16 f. Forage: R6 h. Mature Seed: R8
2. Protein levels are expressed as microgram (µg) of protein per gram (g) of tissue on a fresh weight (fwt) basis.
3. Protein levels are expressed as µg/g on a dry weight (dwt) basis. The dry weight values were calculated by dividing the fwt values by the dry weight conversion factors obtained from moisture analysis data.
4. The mean and standard deviation were calculated across sites (n=14).
5. Minimum and maximum values were determined for each tissue type across sites.
6. Protein signal was not observed in Western Blot.
7. The Δ6 desaturase level in mature seed reported here is below the LOQ (Table 1). This level that is below the LOQ was calculated from the curve generated by extrapolating the standard curve beyond the LOQ and up to LOD (from 2 µg/g fwt to 0.4 µg/g fwt). The LOD (0.4 µg/g fwt) for the Δ6 desaturase was determined in RAR-08-354 by serially diluting protein standard to the lowest amount that produced a visible band in the Western Blot.

Appendices

Appendix 1. Standard Operating Procedures

AG-EQ-1023-01	Denver Instrument IR-200 Moisture Analyzer
BR-ME-0388-02	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
BR-ME-0392-01	Western Blot Analysis (Immunoblotting)
BR-ME-0924-01	Electrotransfer of Proteins to Membranes
BR-ME-0932-03	Assessment of Immunoreactive Bands from Western Blots Exposed to X-Ray Films Using the Bio-Rad GS-800 Densitometer

Appendix 2. Summary of the Extraction Method for Δ6 and Δ15 Desaturase Proteins from Soybean Tissues

Tissue	Extraction Buffer	Tissue-to-Buffer Ratio	Number of Beads	Shake Time	Clarification Method
Leaf	SDA Extraction Buffer[1]	1:10	2	3.5 min. x 2	Centrifugation[2]
Root	SDA Extraction Buffer	1:10	2	3.5 min. x 2	Centrifugation
Forage	SDA Extraction Buffer	1:10	2	3.5 min. x 2	Centrifugation
Immature Seed	SDA Extraction Buffer	1:20	2	3.5 min. x 2	Centrifugation
Mature Seed	SDA Extraction Buffer	1:20	2	3.5 min. x 2	Centrifugation

1. SDA extraction buffer: 100mM Tris-HCl pH 8.0, 500 mM NaCl, 10% glycerol, 2% Triton X-100, 1X protease inhibitors (Roche Diagnostics, Indianapolis, IN).
2. Approximately 14, 000 rpm x 15 minutes at 4°C.