SUMMARY

(In accordance with 40 CFR part 152, this summary is available for public release after registration)

STUDY TITLE

Molecular Characterization of DAS-44406-6 Soybean within a Single Segregating Generation

DATA REQUIREMENTS

Not Applicable

AUTHOR(S)

J. Mo

STUDY COMPLETED ON

22-June-2011

PERFORMING LABORATORY

Regulatory Sciences and Government Affairs—Indianapolis Lab
Dow AgroSciences LLC
9330 Zionsville Road
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LABORATORY STUDY ID

102097

Molecular Characterization of DAS-44406-6 Soybean within a Single Segregating Generation

SUMMARY

Soybean (*Glycine max* L.) DAS-44406-6 was generated by the integration of the T-DNA from plasmid pDAB8264 through *Agrobacterium* transformation method. The T-DNA of plasmid pDAB8264 contains three plant transcription units (PTUs); 1) Histone H4A748 promoter, 2m*EPSPS* gene, and Histone H4A748 3′ UTR, 2) AtUbi10 promoter, *aad*-12 gene, and AtuORF23 3′ UTR, 3) CsVMV promoter, *pat* gene, and AtuORF1 3′ UTR. In addition, a RB7 matrix attachment region (RB7 MAR) is located at the 5′ end of the T-DNA insert.

A segregating F2 generation of DAS-44406-6 soybean was used to characterize the inheritance of the T-DNA insert. Genomic DNA from the individual plant leaf tissue was extracted and used to determine the presence or absence of the T-DNA insert by event-specific PCR analysis. Based on the PCR results, statistical analysis indicated that the ratio of 96 positive to 23 null segregants in the F2 generation matched a segregation ratio of 3:1 based on a single locus. The typical Mendelian inheritance pattern for a single independent locus was observed in the segregating F2 generation.

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DATA REQUIREMENTS

Not Applicable

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Title: Molecular Characterization of DAS-44406-6 Soybean within a Single Segregating Generation

- STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS:

No claim of confidentiality, on any basis whatsoever, is made for any information contained in this document. I acknowledge that information not designated as within the scope of FIFRA sec. 10(d)(1)(A), (B), or (C) and which pertains to a registered or previously registered pesticide is not entitled to confidential treatment and may be released to the public, subject to the provisions regarding disclosure to multinational entities under FIFRA sec. 10(g).

Company: Dow AgroSciences LLC

Company Agent: M. Krieger

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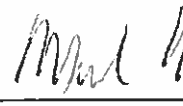
Signature: 

Date: 15 June 2011

THIS DATA MAY BE CONSIDERED CONFIDENTIAL IN COUNTRIES OUTSIDE THE UNITED STATES.

STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Title: Molecular Characterization of DAS-44406-6 Soybean within a Single Segregating Generation

Study Initiation Date: 16 Dec 2010

This report represents data generated after the effective date of the EPA FIFRA Good Laboratory Practice Standards.

United States Environmental Protection Agency
Title 40 Code of Federal Regulations Part 160
FEDERAL REGISTER, August 17, 1989

All aspects of this study were conducted in accordance with the requirements for Good Laboratory Practice Standards, 40 CFR 160.135 (b). The chain of custody of commercial products, the statistical analyzing software SAS, and the SynergyMx plate reader were not monitored under GLP.

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Study Completion Date

Dow AgroSciences Quality Assurance Unit
Good Laboratory Practice Statement Page

Study ID: 102097

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GLP Quality Assurance Inspections

Date of GLP Inspection(s)	Date Reported to the Study Director and to Management	Phases of the Study which received a GLP Inspection by the Quality Assurance Unit
16-Dec-2010	17-Dec-2010	Protocol Review
17-Dec-2010	23-Dec-2010	Planting
15-Apr-2011	15-Apr-2011	Event Specific Taqman Assay
6, 7, 8-Jun-2011	8-Jun-2011	Report and Raw Data Review; Test Substance Container Verification

QUALITY ASSURANCE STATEMENT:

The Quality Assurance Unit has reviewed the final study report and has determined that the report reflects the raw data generated during the conduct of this study.

Tracey Froggatt
Dow AgroSciences, Quality Assurance

22-Jun-2011
Date

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Title: Molecular Characterization of DAS-44406-6 Soybean within a Single Segregating Generation

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TABLE OF CONTENTS

Molecular Characterization of DAS-44406-6 Soybean within a Single Segregating Generation

ABSTRACT

Soybean (*Glycine max* L.) DAS-44406-6 was generated by the integration of the T-DNA from plasmid pDAB8264 through *Agrobacterium* transformation method. The T-DNA of plasmid pDAB8264 contains three plant transcription units (PTUs); 1) Histone H4A748 promoter, 2m*EPSPS* gene, and Histone H4A748 3′ UTR, 2) AtUbi10 promoter, *aad*-12 gene, and AtuORF23 3′ UTR, 3) CsVMV promoter, *pat* gene, and AtuORF1 3′ UTR. In addition, a RB7 matrix attachment region (RB7 MAR) is located at the 5′ end of the T-DNA insert.

A segregating F2 generation of DAS-44406-6 soybean was used to characterize the inheritance of the T-DNA insert. Genomic DNA from the individual plant leaf tissue was extracted and used to determine the presence or absence of the T-DNA insert by event-specific PCR analysis. Based on the PCR results, statistical analysis indicated that the ratio of 96 positive to 23 null segregants in the F2 generation matched a segregation ratio of 3:1 based on a single locus. The typical Mendelian inheritance pattern for a single independent locus was observed in the segregating F2 generation.

ABBREVIATIONS

2mEPSPS	Double-mutant 5-enolpyruvylshikimate 3-phosphate synthase
AAD-12	Aryloxyalkanoate dioxygenase-12
PAT	Phosphinothricin acetyl transferase
LFS	Lateral flow strip
bp	Base pair
DNA	Deoxyribonucleic acid
kb	Kilobase
µL	Microliter
µg	Microgram
ng	Nanogram
mL	Milliliter
PCR	Polymerase chain reaction
PTU	Plant transcription unit
TBE	Buffer solution containing a mixture of Tris base, boric acid and EDTA, pH 8.3

INTRODUCTION

Soybean (*Glycine max* L.) event DAS-44406-6 was generated by the integration of the T-DNA from plasmid pDAB8264 through *Agrobacterium* transformation method. The T-DNA of plasmid pDAB8264 contains three plant transcription units (PTUs); 1) Histone H4A748 promoter, *2mEPSPS* gene, and Histone H4A748 3′ UTR, 2) AtUbi10 promoter, *aad*-12 gene, and AtuORF23 3′ UTR, 3) CsVMV promoter, *pat* gene, and AtuORF1 3′ UTR. In addition, a RB7 matrix attachment region (RB7 MAR) is located at the 5′ end of the T-DNA insert.

The purpose of this study is to demonstrate the typical Mendelian inheritance pattern of a single independent transgene locus in a segregating F2 generation. Expression of PAT protein in the individual plants was determined using PAT protein specific LFS assay. Genomic DNA from the individual plant leaf tissue was extracted and analyzed by event-specific PCR method to determine the presence or absence of the T-DNA insert specifically for DAS-44406-6 soybean.

MATERIALS AND METHODS

Test Substance/Test System

The test substances in the study were DAS-44406-6 soybean event segregating F2 generation. Soybean seeds from F2 generations of DAS-44406-6, along with the source identification, were obtained from the Dow AgroSciences Trait Product Development department (Table 1).

Reference and Control Materials

The negative control substance used in this study was the unmodified soybean variety Maverick. The unmodified Maverick variety has a genetic background representative of the test substance line, but does not contain genes for the 2mEPSPS, AAD-12, or PAT proteins. The control soybean seed(s) were provided by the Dow AgroSciences Trait Product Development (TPD) department (Table 1).

Genomic DNA from the T6 generation of DAS-44406-6 soybean was previously characterized by Southern blot analysis (Poorbaugh, 2011 unpublished) and was used as a positive control in event-specific PCR analysis.

Lateral Flow Strip Testing

Leaf punches were taken from each plant to test for PAT protein expression using a rapid LFS kit specific to PAT protein (Envirologix) according to the manufacturer's recommended procedure. Each plant sample was given a score of "+" or "–" for the presence or absence of PAT protein, respectively (Table 2).

Soybean Leaf Sample Collection

Soybean leaf samples from the individual DAS-44406-6 and Maverick plants, which grew big enough for genomic DNA isolation, were harvested and quickly frozen in liquid nitrogen. Leaf samples were then stored at approximately -80°C until usage.

Genomic DNA Extraction

Genomic DNA from Maverick and individual F2 plants of DAS-44406-6 soybean was extracted from leaf tissue using the modified CTAB method (Richards *et al.*, 2001). Following extraction, the DNA was quantified spectrofluorometrically using PicoGreen (Invitrogen). The DNA was then visualized on agarose gels to check for quality.

Event-Specific PCR Analysis

Event-specific PCR was conducted following the modified End point event-specific TaqMan® assay developed by the Trait Product Development department (Zhou and Novak, 2010) to specifically amplify a 97 bp fragment at the 5' integration junction region of DAS-44406-6 soybean. The assay was further validated by the Quality Control group in BRS (Biotechnology Regulatory Science) (Cramer *et al.*, 2011 unpublished). Briefly, TaqMan® assay primers and probes were designed using the Primer Express 3.0 software (Applied Biosystems). End point PCRs were performed with three replicates of each positive control (T6 generation), negative control (Maverick soybean), non-template control (water control), and test samples (DAS-44406-6 soybean F2 generation). The fluorescence signal was measured at the end of PCR and the results were recorded as either a positive "+" or negative "–" for DAS-44406-6 soybean. The generated data was subject to Chi-Square test.

RESULTS AND DISCUSSION

PAT Protein Expression

In this study, among the 119 plants tested for event-specific PCR analysis, 96 plants were positive and 23 were negative (segregated nulls) (Table 2) for PAT protein expression by PAT specific LFS testing (Envirologix). Maverick plants were negative for PAT protein expression as well. All of the plants that were big enough for sampling were subjected to event-specific PCR analysis to test for the presence or absence of DAS-44406-6 insert. Some plants were tested for PAT expression but not preceded with event-specific PCR analysis due to the individual plants did not grow big enough to provide sufficient leaf tissue for genomic DNA isolation.

Event-Specific PCR Analysis

A total of 119 samples for DAS-44406-6 plus one non-transgenic control soybean variety Maverick were analyzed by event-specific PCR. Among the 119 tested plants, 96 plants were positive and 23 plants were negative. The samples which showed positive for DAS-44406-6 transgene insert in event-specific PCR analysis were positive for PAT expression in LFS test. The tested plants that were negative for DAS-44406-6 insert by event-specific PCR were negative for PAT expression in LFS test. The non-transgenic control Maverick showed negative in both LFS test and event-specific PCR analysis (Table 2). Since the results also confirmed that the phenotype matches the genotype in all tested plants, only one Chi-Square test was performed for the generated data. Statistically analysis confirmed that the ratio of 96 positive to 23 null segregants in the F2 generation matched the expected segregating ratio of 3:1 based on Mendelian inheritance pattern for a single independent locus.

CONCLUSION

The plants with positive results from event-specific PCR assay were positive for PAT protein expression in LFS test, confirming that the phenotypic and genotypic results are consistent. The ratio of 96 positive to 23 null segregants in the F2 generation matched the expected segregating ratio of 3:1 based on a single independent locus, demonstrating that the inheritance of the T-DNA insert in DAS-44406-6 soybean followed the typical Mendelian inheritance pattern of a single locus.

ARCHIVING

The protocol, raw data, and the original version of the final report will be filed in the Dow AgroSciences LLC archives at 9330 Zionsville Road in Indianapolis, IN 46268-1054.

STATISTICAL TREATMENT OF DATA

A chi-square test for specified proportions was used to compare the observed segregation data to the hypothesized ratio of 3:1. The analysis was carried out using the FRET procedure in SAS Version 9.2.

Event-specific PCR Analysis	Null	Transgenic Positive
Observed Sample Number	23	96

The SAS System

Obs	event	gen	Status	Count
1	a	a	null	23
2	a	a	trans	96

The SAS System

---------- event=a gen=a ----------

The FREQ Procedure

Status	Frequency	Percent	Test Cumulative Percent	Cumulative Frequency	Cumulative Percent
null	23	19.33	25.00	23	19.33
trans	96	80.67	75.00	119	100.00

Chi-Square Test
for Specified Proportions

Chi-Square	2.0420
DF	1
Pr > ChiSq	0.1530

Sample Size = 119

REFERENCES

1. Richards, E., Reichardt, M., Rogers, S., 2001. Preparation of genomic DNA from plant tissue. Curr Protoc Mol Biol Chapter 2, Unit2 3.
2. Zhou, N.; Novak, S.; (2010) End-Point Event-Specific TaqMan® Assay for Soybean Herbicide Tolerance Event pDAB8264.44.06.1 (I) Indianapolis, Dow AgroSciences LLC
3. Cramer, M.; Gampala, S.; Lee, W.; Zhou, N.; (2011) Validation of an event specific TaqMan® assay for detection of 2mEPSPS event pDAB8264.44.06.1 from Herbicide Tolerant (HT) molecular stack in soybean. Indianapolis, Dow AgroSciences LLC
4. Poorbaugh, J. (2011) Molecular Caracterization of DAS-44406-6 Soybean (Study ID: 101947). Indianapolis, Dow AgroSciences LLC

Table 1. Description of Soybean Samples

Plant	Source ID	Purpose
DAS-44406-6 F2	YX10BX026010.0007.001	Test substance
Maverick	YW10EW000012.0001	Control substance

Table 2. PAT Protein Expression and Event-Specific PCR Analysis of DAS-44406-6 Soybean

Test Sample Number	Plant/ Sample ID	Event-specific PCR	PAT LFS	Test Sample Number	Plant/ Sample ID	Event-specific PCR	PAT LFS
	Maverick-01	-	-	30	F2-034	+	+
1	F2-001	+	+	31	F2-035	+	+
2	F2-002	+	+	32	F2-036	+	+
3	F2-003	+	+	33	F2-037	-	-
4	F2-005	+	+	34	F2-038	-	-
5	F2-006	+	+	35	F2-039	-	-
6	F2-007	+	+	36	F2-040	-	-
7	F2-008	+	+	37	F2-041	+	+
8	F2-009	+	+	38	F2-042	-	-
9	F2-010	-	-	39	F2-043	+	+
10	F2-011	+	+	40	F2-044	+	+
11	F2-012	+	+	41	F2-045	-	-
12	F2-013	+	+	42	F2-049	+	+
13	F2-014	+	+	43	F2-050	+	+
14	F2-015	+	+	44	F2-051	-	-
15	F2-016	+	+	45	F2-052	+	+
16	F2-017	+	+	46	F2-053	+	+
17	F2-018	-	-	47	F2-055	+	+
18	F2-020	+	+	48	F2-056	+	+
19	F2-021	+	+	49	F2-057	-	-
20	F2-022	+	+	50	F2-058	+	+
21	F2-023	+	+	51	F2-060	+	+
22	F2-024	+	+	52	F2-061	+	+
23	F2-026	+	+	53	F2-062	+	+
24	F2-027	+	+	54	F2-063	+	+
25	F2-029	+	+	55	F2-064	+	+
26	F2-030	+	+	56	F2-065	+	+
27	F2-031	+	+	57	F2-066	+	+
28	F2-032	+	+	58	F2-067	+	+
29	F2-033	+	+	59	F2-068	+	+

Table 2 (cont.)

Test Sample Number	Plant/ Sample ID	Event-specific PCR	PAT LFS	Test Sample Number	Plant/ Sample ID	Event-specific PCR	PAT LFS
60	F2-069	+	+	90	F2-109	+	+
61	F2-071	+	+	91	F2-110	+	+
62	F2-072	+	+	92	F2-111	+	+
63	F2-073	+	+	93	F2-112	+	+
64	F2-075	-	-	94	F2-113	-	-
65	F2-076	-	-	95	F2-114	+	+
66	F2-077	+	+	96	F2-115	+	+
67	F2-078	+	+	97	F2-116	-	-
68	F2-079	+	+	98	F2-117	+	+
69	F2-080	+	+	99	F2-120	-	-
70	F2-083	+	+	100	F2-122	+	+
71	F2-084	+	+	101	F2-126	+	+
72	F2-085	+	+	102	F2-127	+	+
73	F2-086	-	-	103	F2-130	+	+
74	F2-087	+	+	104	F2-131	+	+
75	F2-090	+	+	105	F2-137	+	+
76	F2-092	+	+	106	F2-138	+	+
77	F2-093	+	+	107	F2-139	-	-
78	F2-095	+	+	108	F2-143	-	-
79	F2-097	+	+	109	F2-145	-	-
80	F2-098	+	+	110	F2-150	+	+
81	F2-099	+	+	111	F2-151	-	-
82	F2-100	+	+	112	F2-152	+	+
83	F2-101	+	+	113	F2-153	-	-
84	F2-102	+	+	114	F2-154	+	+
85	F2-103	-	-	115	F2-156	+	+
86	F2-104	+	+	116	F2-157	-	-
87	F2-105	+	+	117	F2-158	+	+
88	F2-106	+	+	118	F2-166	+	+
89	F2-108	+	+	119	F2-168	+	+

Note: All F2 samples listed in the table were DAS-44406-6 soybean.