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Technology for Plants
Genective

Petition for the Determination of Non-regulated Status for Event VCO-Ø1981-5

OECD Unique Identifier: VCO-Ø1981-5

The undersigned submits this petition under 7 CFR 340.6 to request that the Administrator make a determination that the article should not be regulated under 7 CFR 340.

Submitted by:

Isabelle Coats. Ph.D.
US Registration Manager
Bayer CropScience LP

For:

GENECTIVE S.A.
1 rue Limagrain
BP1
63720 CHAPPES
FRANCE

Phone: +33 4 73 63 41 14
Fax: +33 4 73 64 67 37
e-mail: georges.freyssinet@limagrain.com

Contributors GENECTIVE S.A.:
Cristina Bernard, Laurent Beuf, Georges Freyssinet, Alain Toppan

Contributors Bayer CropScience:
Jayla Allen, Nicolas de Schrijver

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Revised: March 12, 2012

CONTAINS NO CONFIDENTIAL BUSINESS INFORMATION

COMPANY NAMES

GENECTIVE S.A., an affiliate of KWS and VCO Participation, an affiliate of Vilmorin & Cie, is a company organized and existing under the laws of France, having its head office at 1 rue Limagrain, 63720 Chappes, France, SIREN Number 513 533 612, RCS Clermont-Ferrand.

On July 1, 2008, Athenix Corp. and Vilmorin & Cie entered into an agreement for the development of maize tolerant to glyphosate and resistant to insects. Under this agreement, Athenix Corp. has licensed to Vilmorin & Cie the right to use worldwide the genes they discover and own for the above traits. On June 23, 2011 Vilmorin & Cie has transferred the above agreement to its affiliate GENECTIVE S.A.

On November 2, 2009 Athenix Corp., a biotechnology company that develops novel products and technology for agricultural applications, became an affiliate of Bayer CropScience LP.

GENECTIVE S.A. and Bayer CropScience are continuing the collaboration initiated in 2008 between Vilmorin & Cie and Athenix Corp. Some of the activities described in this petition were undertaken before the acquisition of Athenix Corp. by Bayer CropScience LP and based on the agreement between Athenix Corp. and Vilmorin & Cie.

RELEASE OF INFORMATION

The information in this petition is being submitted by GENECTIVE S.A. for review by the USDA as part of the regulatory process. By submitting this information, GENECTIVE S.A. does not authorize its release to any third party except to the extent it is requested under the Freedom of Information Act (FOIA), 5 U.S.C., Section 552 and 7 CFR 1, covering all or some of this information. Prior to releasing the information in this petition in accordance with FOIA, GENECTIVE S.A. kindly requests to be notified by the Agency's FOIA office. GENECTIVE S.A. does not authorize the release, publication or other distribution of this information without GENECTIVE S.A.'s prior notice and consent.

CERTIFICATION

The undersigned certify that to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes relevant data and information known to the petitioner, which is unfavorable to the petition.

Georges Freyssinet, CEO

GENECTIVE S.A.
1 rue Limagrain
BP1
63720 Chappes
FRANCE

Phone: +33 4 73 63 41 14
Fax: +33 4 73 64 67 37
e-mail: georges.freyssinet@limagrain.com

Isabelle Coats, Ph.D.
Global Regulatory Affairs Manager

Bayer CropScience LP
2 T.W. Alexander Dr.
Research Triangle Park, NC 27709

Phone: 919-549-2599
e-mail: isabelle.coats@bayer.com

SUMMARY

GENECTIVE S.A. is submitting a Petition for the Determination of Non-regulated Status under 7 CFR 340 to USDA Animal and Plant Health Inspection Service (APHIS) for glyphosate-tolerant maize event VCO-Ø1981-5, any progeny, and crosses of this event with other non-regulated maize lines.

Transformation event VCO-Ø1981-5 contains the stably integrated *epsps grg23ace5* gene expressing the EPSPS ACE5 protein, an improved EPSPS enzyme, which confers tolerance to the herbicide glyphosate. The gene was introduced into maize through *Agrobacterium*-mediated transformation. Southern blot analyses show maize event VCO-Ø1981-5 contains a single intact copy of the *epsps grg23ace5* gene.

The EPSPS ACE5 protein was derived using a directed evolution protein engineering strategy from the native GRG23 protein isolated from *Arthrobacter globiformis*. A total of ten amino acids were changed to produce EPSPS ACE5. The EPSPS ACE5 protein exhibits tolerance to glyphosate. The native GRG23 enzyme from *Arthrobacter* was optimized to more closely match the native maize EPSPS enzyme with regards to its temperature optimum activity under typical field-growing conditions. The native maize EPSPS enzymatic half-life is 86 hours while that of the native EPSPS GRG23 enzyme from *Arthrobacter* is 10 hours at 37ºC. The modified EPSPS ACE5 has an improved enzymatic half-life of 65 hours, bringing it closer to that of the native maize EPSPS and optimizing it for typical field-growing conditions.

The EPSPS ACE5 protein has a molecular weight of 45 kDa and is made of up of 413 amino acids. The EPSPS ACE5 protein does not share any characteristics with allergens or toxins.

Planting herbicide-tolerant maize varieties, containing event VCO-Ø1981-5, will provide growers with another option for weed control using glyphosate. Glyphosate is widely used in herbicide-tolerant maize and other agricultural production systems. In many instances, glyphosate replaces more harmful herbicides and simplifies weed control practices since the grower can rely on a single herbicide only when weed control is required. Herbicide-tolerant crops have allowed a rapid adoption of reduced tillage practices. Low-till and no-till farming is quickly dominating agricultural systems, since it conserves valuable topsoil, reduces fuel costs to the grower and reduces the amount of greenhouse gases in the environment.

Event VCO-Ø1981-5 has been field tested since 2007 in typical maize growing regions of the continental US and Puerto Rico. These tests have occurred at more than 20 locations under USDA APHIS environmental release authorizations. Data and results collected from these trials as well as laboratory analyses presented in this petition demonstrate that event VCO-Ø1981-5 maize:

- exhibits no plant pathogenic properties;
- is no more likely to become a weed than other maize varieties currently grown;
- is unlikely to increase the weediness potential of any other cultivated plant or native wild species;
- does not cause damage to processed agricultural commodities; and,
- is unlikely to harm other organisms that are beneficial to agriculture.

ACRONYMS AND SCIENTIFIC TERMS

ai	active ingredient
A	acre
AA	amino acid
ADF	Acid Detergent Fiber
ANOVA	Analysis Of Variance
APHIS	Animal and Plant Health Inspection Service
BC0N, BC1N, etc.	Back cross 0,1, etc. with recurrent line N
BCS	Bayer CropScience
BLASTP	Basic Local Alignment Search Tool Protein
bp	base pairs
bu/ac	bushels/acre
dw	Dry weight
DNA	DeoxyriboNucleic Acid
E. coli	*Escherichia coli*
ELISA	Enzyme Linked Immuno Sorbent Assay
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
FGENESH	Find GENES using Hidden Markov model
FIFRA	Federal Insecticide Fungicide and Rodenticide Act
fw	Fresh weight
g	gram
GM	Genetically Modified
GLY	glyphosate
HRP	Horseradish Peroxidase
ID	identification
kDa	kiloDalton
kg	kilogram
L	liter
LB	Left Border
lb	pound (1 pound = 0.454 kg)
LC/MS	Liquid Chromatography/Mass Spectroscopy
LD_{50}	lethal dose for 50% of animals
LOQ	Limit of Quantitation
LOD	Limit of Detection
M	million
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time of Flight
mg	milligram
mL	milliliter
µg	microgram
NA	Not Applicable
n/a	not available
ng	nanogram
ND	Not Detectable: Below the limit of detection
NDF	Neutral Detergent Fiber
nm	nanometer
nt	nucleotide
OECD	Organization for Economic Cooperation and Development
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
RB	Right Border
RCB	Randomized Complete Block
RM	Relative Maturity
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis
SGF	Simulated Gastric Fluid
ssp.	Subspecies
T0	Regenerated transformed plant
T-DNA	Transfer DNA from *Agrobacterium*
US	United States of America
USDA	United States Department of Agriculture
WHO	World Health Organization
wt	Wild type
Z. mays	*Zea mays*, maize

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I. RATIONALE

I.A. Basis for the request for determination of non-regulated status

The United States Department of Agriculture (UDSA) Animal and Plant Health Inspection Services (APHIS) is responsible for protection of the US agricultural infrastructure against noxious pests and weeds. Under the Plant Protection Act (7 USC § 7701-7772) APHIS considers plants altered or produced by genetic engineering as restricted articles under 7 CFR 340 , which cannot be released into the environment without appropriate approvals. APHIS provides that petitions may be filed under 7 CFR §340.6 to evaluate data to determine that a particular regulated article does not present a plant pest risk. Should APHIS determine that the submitted article does not present a plant pest risk, the article may be deregulated and released without further restrictions.

I.B. Herbicide-tolerant maize event VCO-Ø1981-5

GENECTIVE S.A. has developed, in collaboration with Athenix Corp., now an affiliate of Bayer CropScience LP, herbicide-tolerant maize event VCO-Ø1981-5. Event VCO-Ø1981-5 maize plants express an improved 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) protein, EPSPS ACE5, which confers tolerance to the common herbicide glyphosate.

I.C. Rationale for the development of event VCO-Ø1981-5 and benefits

I.C.1. Glyphosate-tolerant crops

N-phosphonomethylglycine, commonly referred to as glyphosate, is an important commercial herbicide. Glyphosate inhibits plant, fungal, and most bacterial EPSPS enzymes and thus prevents the shikimate pathway from producing chorismate (the precursor of many essential plant metabolites). As plant cells depend on the biosynthesis of aromatic amino acids for growth, herbicide sprays containing glyphosate can affect plant cells by effectively blocking the shikimate pathway. Because the shikimate pathway is not present in animals, glyphosate has a favorable toxicology profile and has become a very common non-selective herbicide (Franz *et al.*, 1997).

Glyphosate-tolerant crops have been commercially available since the mid-1990s, demonstrating a robust history of safe use (OECD, 1999; Gianessi, 2008) and their benefits have been well established (Nolte and Young, 2002; Cedeira and Duke, 2006; Gianessi, 2008). Post-emergent spray herbicides decrease farming costs by limiting the growth of weeds competing for water, soil nitrogen and nutrients. Herbicide sprays also minimize field labor costs associated with removing weeds during the growing season. In many instances, glyphosate replaces more harmful herbicides and simplifies weed control practices since the grower can rely on a single herbicide only when weed control is required. Historically, conventional maize production has utilized tillage (field ploughing) and multiple herbicide inputs (i.e., pre-season burndown, pre-emergent and post-emergent treatments). Herbicide-tolerant crops have allowed a rapid adoption of reduced tillage practices. Low-till and no-till farming is quickly dominating agricultural systems, since it conserves valuable topsoil, reduces fuel costs to the grower and reduces the amount of greenhouse gases in the environment (Brooks and Barfoot, 2011). All of these advantages have led to the widespread adoption of herbicide-tolerant

crops in modern agriculture with glyphosate-tolerant crops being the most widely-used of these technologies.

I.C.2. Glyphosate-tolerant event VCO-Ø1981-5 maize

Event VCO-Ø1981-5 has been produced with an expression cassette containing the *epsps grg23ace5* gene allowing for the synthesis of the EPSPS ACE5 protein. The intended effect of the modification is to confer tolerance to the herbicide glyphosate to maize and providing another option to simplifying weed control for growers.

The native EPSPS GRG23 protein isolated from the source organism, *Arthrobacter globiformis,* was modified to produce the EPSPS ACE5 protein using a directed evolution protein engineering strategy to more closely match it to the native maize EPSPS enzyme with regards to its temperature optimum under typical field-growing conditions.

The performance of an enzyme *in planta* can be directly related to the stability of that enzyme under field conditions. Frequently, commercial crops encounter hot weather conditions that can lead to enzyme denaturation and loss of enzymatic activity. Particularly when engineering a plant to be tolerant to herbicide spray treatment, it is critical that the enzyme functions under extreme environmental conditions.

The resulting tolerance to increased temperatures is illustrated with the following analysis. EPSPS ACE5, native EPSPS GRG23, and the native maize EPSPS enzymes were incubated at 37°C (to approximate extreme heat conditions potentially experienced in the field) for time periods ranging from 2 to 72 hours, and then the percentage of activity remaining was compared to protein incubated at 4°C in order to calculate the enzymatic half-life at 37°C. By this analysis (Table 1), the modified EPSPS ACE5 enzymatic half-life was greatly improved, bringing it closer to the native maize EPSPS temperature optimum under usual environmental conditions.

Table 1. Enzymatic half-life of EPSPS enzymes

	EPSPS ACE5	EPSPS GRG23	Maize EPSPS
Enzyme half-life @ 37°C	65 hours	10 hours	86 hours

The temperature stability of EPSPS ACE5 protein was further evaluated by examining the enzymatic stability of the protein by carrying out 30 min incubations at various temperatures that mimic industrial processing conditions for maize. Enzymatic activity was monitored by way of a real-time kinetic assay, which links the release of phosphate, a product of the EPSPS reaction, to the generation of a fluorescent substrate. A control sample without EPSPS ACE5 protein was assayed and baseline activity was subtracted from all recorded values. The average rate (relative fluorescent units per second) generated from samples incubated at 4°C minus the average rate of the buffer control was considered to represent 100% enzymatic activity. EPSPS ACE5 activity remained relatively stable after incubation at 25°C and 37°C, as observed above, but dropped to near zero after 30 minutes of incubation at 65°C and 95°C (Figure 1, Table 2).

Figure 1. EPSPS ACE 5 enzymatic activity under various temperature conditions

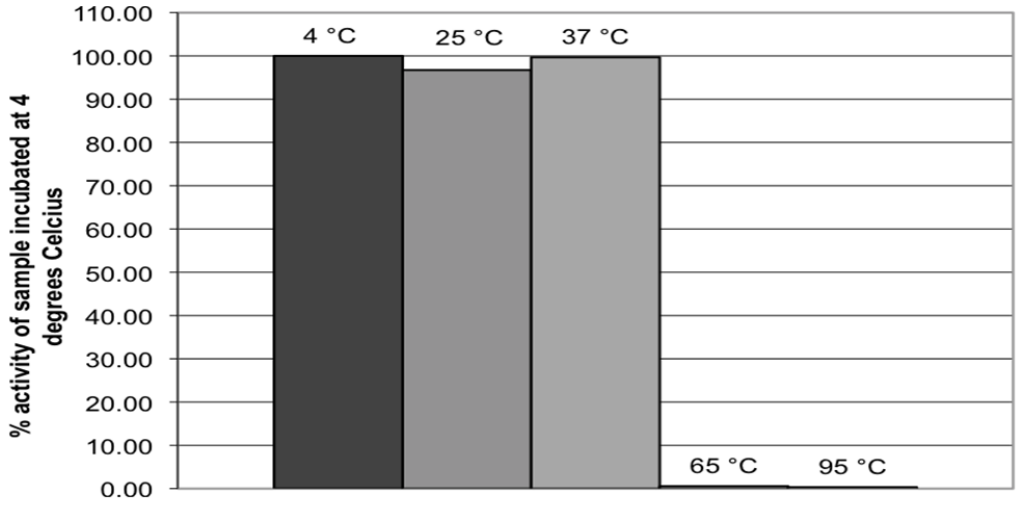


Table 2. Percent EPSPS ACE5 enzymatic activity after 30 min incubation

Sample	EPSPS ACE5 activity (%)				
	4°C	25°C	37°C	65°C	95°C
EPSPS ACE5 protein	100.00	96.72	99.67	0.55	0.33

I.D. Adoption of event VCO-Ø1981-5

US farmers have readily adopted genetically modified crops since their commercial introduction in 1996. Genetically modified maize is third to cotton and soybean in terms of the percentage of acres planted to genetically modified crops in the US. In 2011, 88% of all maize planted was genetically modified, with 23% being herbicide-tolerant only and 49% being stacked maize varieties expressing both insect control and herbicide tolerance traits; the remaining being insect control only. More than 90% of the herbicide-tolerant maize was planted to glyphosate-tolerant maize. The adoption of herbicide-tolerant maize, which had been slower in previous years, has accelerated, reaching 72% of US maize acreage in 2011 (ERS USDA, 2011).

The introduction of herbicide-tolerant crops, such as glyphosate and glufosinate ammonium-tolerant crops has brought many advantages to the grower, such as reduced crop injury, broader spectrum of weed control, more environmentally favorable profile and less herbicide carry-over. These tools, including event VCO-Ø1981-5 maize, will continue to provide the grower with greater flexibility and ease of use in their weed management systems. It is anticipated that event VCO-Ø1981-5 maize hybrids will be cultivated across the US Corn Belt without extending the current maize growing area.

I.E. Submissions to other regulatory agencies

Food and Drug Administration

Event VCO-Ø1981-5 maize is within the scope of the 1992 US FDA policy statement concerning regulation of products derived from new plant varieties, including those developed through biotechnology (FDA, 1992). In compliance with this policy, a food and feed safety and nutritional assessment summary for event VCO-Ø1981-5 will be submitted to the US FDA.

In 2009, Athenix Corp., now an affiliate of Bayer CropScience LP, submitted an Early Food Safety Evaluation to FDA. Their review was completed with no further questions in October, 2010 (FDA, 2010).

Foreign Governments

GENECTIVE S.A. or its local subsidiary as required by local authorities intends to submit dossiers to request import of event VCO-Ø1981-5 maize to the proper regulatory authorities of foreign countries that have regulatory processes in place. These may include submissions to the relevant Regulatory Authorities in Canada, EU, Japan, and others. Event VCO-Ø1981-5 maize has been, or is currently, in field trials in maize growing regions in the US, Canada and the European Union.

II. THE BIOLOGY OF MAIZE

II.A. The biology of maize

Maize is a member of the *Maydeae* tribe of the grass family, *Poaceae*.

Family: *Poaceae*

Subfamily: *Panicoideae*

(Western Hemisphere) Genus: *Zea*
Species: *Zea mays* L. (maize)
Subspecies: *Zea mays* subsp. *mays* (L.) Iltis (maize, 2n = 20)

The OECD consensus document on the biology of *Zea mays ssp. mays* (maize) (OECD, 2003) provides a thorough review of the following aspects of maize biology, taxonomy, history of use, and cultivation:

- General description (taxonomic status, morphology, use as a crop plant);
- Agronomic practices;
- Center of origin;
- Reproductive biology;
- Intra- and inter-species crosses and gene flow;
- Cultivation (volunteers, weediness potential, soil ecology and interactions with insects).

II.B. Characteristics of the recipient cultivar

The recipient maize line for generating event VCO-Ø1981-5 was Hi-II. Event VCO-Ø1981-5 was generated by the transfer of the T-DNA from plasmid pAG3541 into Hi-II via *Agrobacterium*-mediated transformation.

The Hi-II hybrid line was obtained from the crossing of two separate lines Hi-IIA and Hi-IIB (Armstrong *et al*., 1991). The resulting embryos of the hybrid are used as target tissue for maize transformation. Hi-IIA and Hi-IIB are partially inbred lines selected out of a cross between maize inbred lines A188 and B73. Hi-IIA and Hi-IIB were obtained from Maize Genetics COOP Stock Center (Urbana, IL, USA).

III. DEVELOPMENT OF HERBICIDE-TOLERANT CORN EVENT VCO-Ø1981-5

III.A. Description of the transformation system

Maize event VCO-Ø1981-5 was generated using a standard disarmed *Agrobacterium* mediated transformation protocol (Hiei and Komari, 1997). The presence and integrity of pAG3541 into the *Agrobacterium* strain was verified by Southern hybridization (data not shown). The verified *Agrobacterium* strain was then used to transform the hybrid maize line Hi-II (Maize Genetics Cooperation Stock Center) (Figure 2).

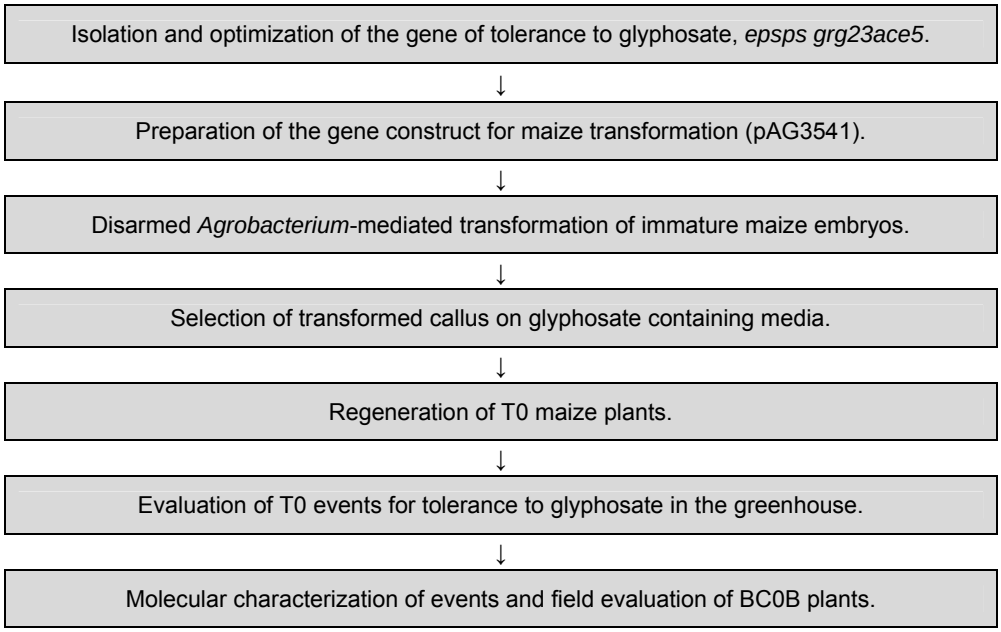
Cells from the dent type hybrid maize line Hi-II were transformed by the transfer of the T-DNA region of plasmid vector pAG3541 containing the *epsps grg23ace5* gene. Immature embryos of Hi-II corn were excised at approximately eight days after pollination and infected with *Agrobacterium tumefaciens* strain LBA4404 containing plasmid pAG3541. After three days of co-cultivation on solid culture media, the embryos were transferred to media containing the antibiotic timentin to eliminate *Agrobacterium* from the system post transformation (Cheng *et al.,* 1998).

Only the T-DNA existing between the right and left border (RB and LB) sequences is integrated into the maize genome. The DNA regions outside of the T-DNA borders such as the bacterial antibiotic resistant marker genes and *vir* genes are not typically transferred. The bacterial antibiotic resistant marker genes are required for the preparation of the transformation vector. The *vir* genes are required for the production of the T-DNA transfer complex (De la Riva *et al.,* 1998).

After approximately two weeks of culture, the embryos were transferred to selection media containing glyphosate. Transformed callus tolerant to glyphosate were identified and transferred to fresh selection media. Embryogenic callus was ultimately regenerated into whole plants and transferred to the greenhouse for further analysis (Vande Berg *et al.*, 2008). The regenerated plants (designated as T0) were evaluated further for tolerance to glyphosate spray and through molecular techniques for the presence of the gene of interest.

Figure 2 represents a schematic diagram of the steps involved in the development of event VCO-Ø1981-5 up to the molecular characterization.

Figure 2. Process for the development of event VCO-Ø1981-5



III.B. Parent line

Event VCO-Ø1981-5 was generated by the transfer of the T-DNA from plasmid pAG3541 into Hi-II via *Agrobacterium*-mediated transformation.

Following recovery from glyphosate selection during the plant transformation process, the T0 plantlet was transplanted into a germination soil mix and grown in a temperature-controlled greenhouse. At flowering, the plant was pollinated with inbred B110 (Committee for Agricultural Development, Iowa State University), and the seed were harvested and dried. These back-cross zero (BC0B, Figure 3) seeds were then planted in a winter nursery in Puerto Rico in 2007/2008.

III.C. Breeding diagram

The breeding diagram of event VCO-Ø1981-5 maize is shown in Figure 3.

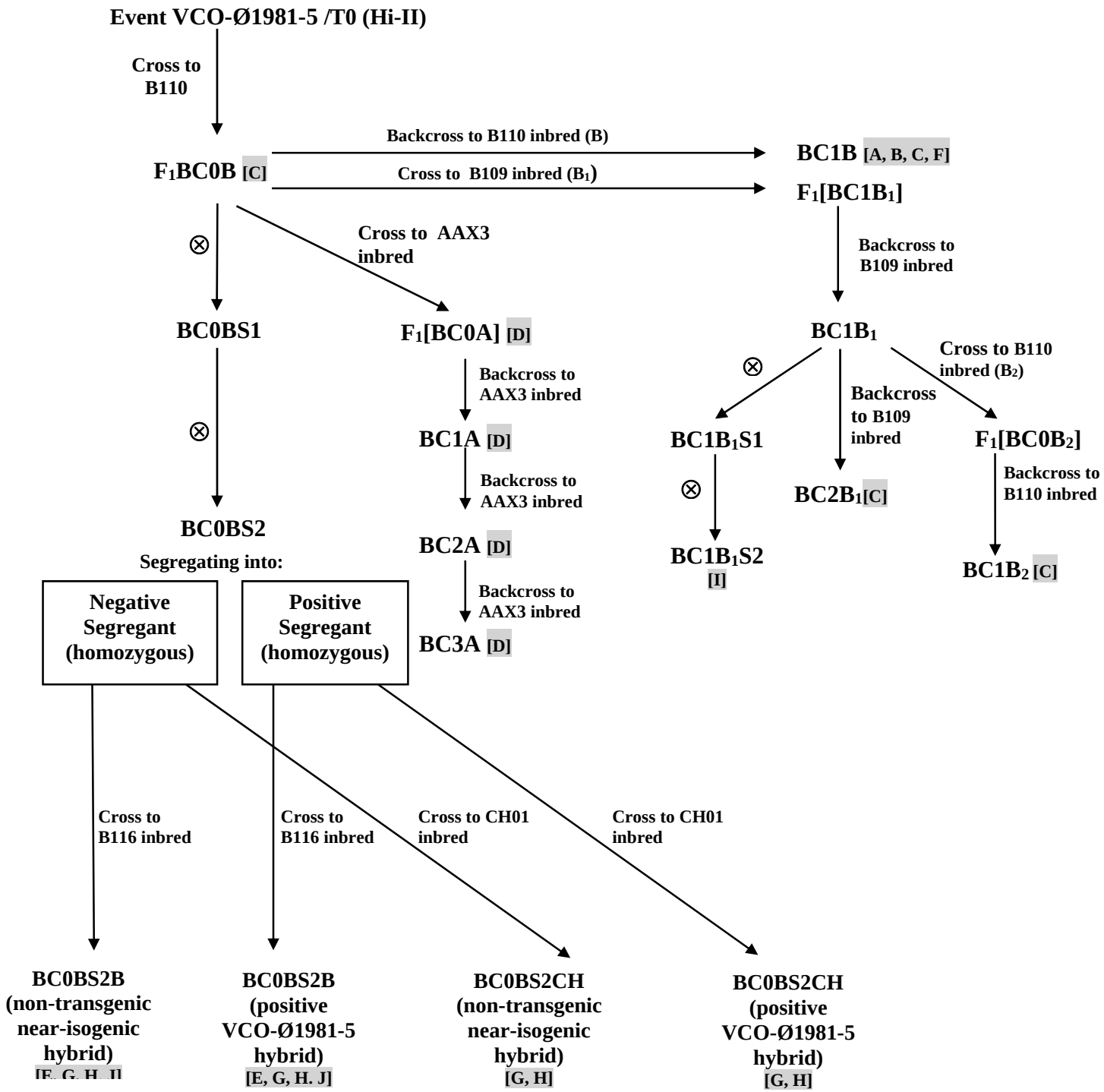
III.D. Generations used for analysis

The generations used for regulatory studies to analyze maize event VCO-Ø1981-5 are described in Table 3.

Table 3. Generations used for analysis of event VCO-Ø1981-5

Study	Letter code	Generation	Page # in petition
Insert characterization (copy #, stability within generation)	B	BC1B	26
Absence of vector backbone	A	BC1B	27
Mendelian inheritance	D	BC0A, BC1A, BC2A, BC3A	28
Structural stability (across generations)	C	BC0B, BC1B, BC2B, $BC1B_2$	29
Protein expression	E	BC0BS2B	36
Agronomic performance (2008)	F	BC1B	44
Agronomic performance, biotic and abiotic stressors (2009)	G	BC0BS2B, BC0BS2CH	44, 50
Field emergence	H	BC0BS2B, BC0BS2CH	50
Warm and cold germination	I	$BC1B_1S2$	52
Composition	J	BC0BS2B	53

Figure 3. Breeding diagram for event VCO-Ø1981-5



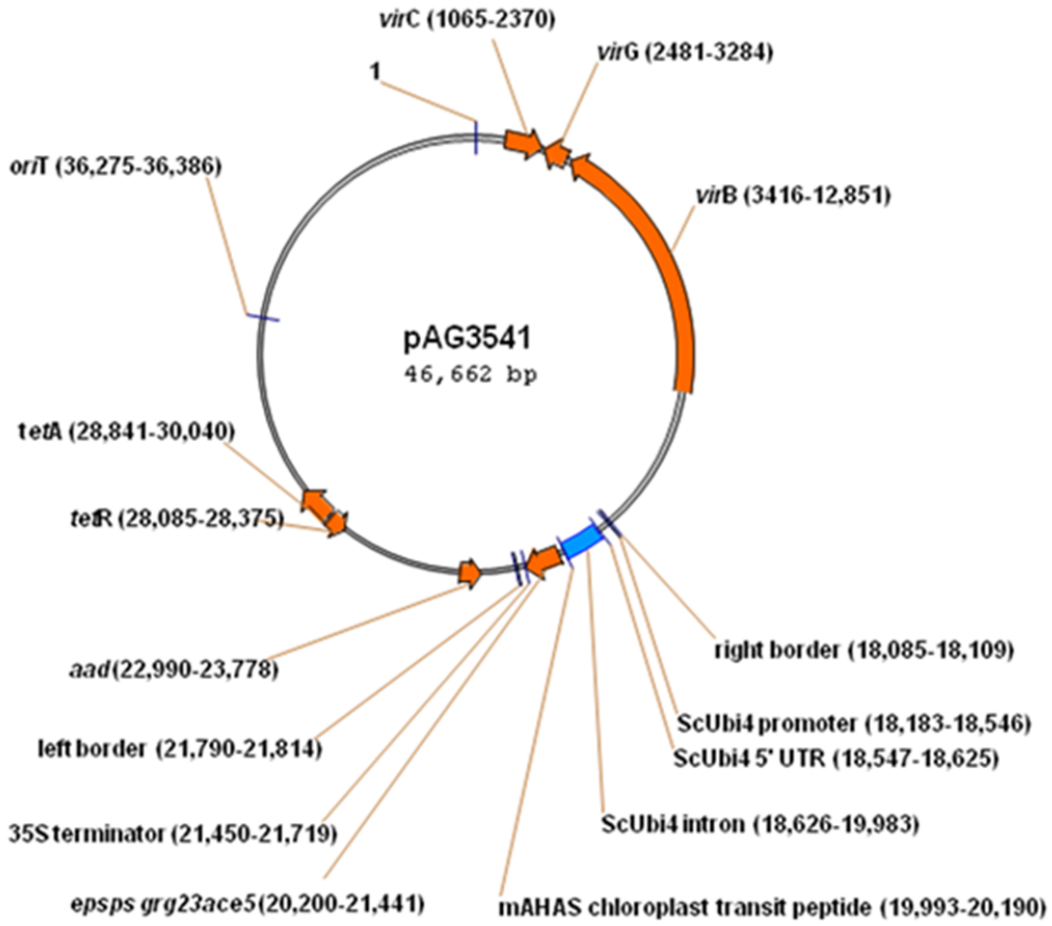
x; cross
⊗; selfing
[A, B, …]; letter code corresponding to regulatory studies listed in Table 3

IV. GENETIC MATERIAL USED FOR TRANSFORMATION OF EVENT VCO-Ø1981-5

IV.A. Construction of the plasmid used for transformation

A synthesized coding region comprising a maize chloroplast transit peptide from acetohydroxyacid synthase (Fang *et al.*, 1992) and the sequence encoding the *epsps grg23ace5* gene was generated. It was then subcloned downstream from a constitutive plant promoter (Ubiquitin4 promoter from *Saccharum officinarum L.*) (Albert and Wei, 2003) and upstream from a plant terminator sequence (3' non-coding sequence of 35S gene from Cauliflower mosaic virus) (Gardner *et al.*, 1981). The *promoter-coding region - terminator* fragment from this intermediate plasmid was subcloned into plasmid pSB11 (Japan Tobacco, Inc.) to create plasmid pAX3541 (Appendix 2, Figure 9). The plasmid was mobilized into *Agrobacterium tumefaciens* strain LBA4404, which also harbors the plasmid pSB1 (Japan Tobacco, Inc.), using triparental mating and plating on media containing spectinomycin, streptomycin, tetracycline and rifampicin. The cointegrate product of pSB1 and pAX3541 resulted in the final plasmid, pAG3541 (Figure 4).

Figure 4. Plasmid map of pAG3541 carrying the *epsps grg23ace5* gene



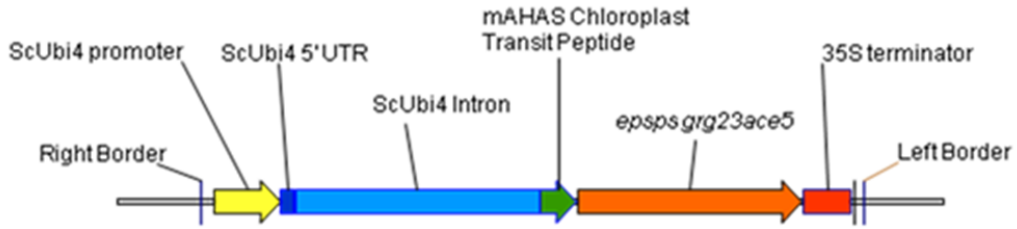
IV.B. Description of gene and regulatory sequences

Event VCO-Ø1981-5 was produced by disarmed *Agrobacterium*-mediated transformation using the plasmid pAG3541. This plasmid contains the 3730 bp T-DNA region (Figure 5) including the *epsps grg23ace5* gene expression cassette.

The T-DNA of plasmid pAG3541 contains a single gene cassette including a modified version of the 5-enolpyruvylshikimate-3-phosphate synthase nucleotide sequence from *Arthrobacter globiformis* (Schouten *et al.*, 2010) (*epsps grg23ace5* gene coding region) that encodes the EPSPS ACE5 enzyme. *Arthrobacter* species are part of the Gram-positive coryneform bacteria considered one of the major groups of aerobic soil bacteria and are found ubiquitously in soil and other natural habitats (Conn, 1948; Conn and Dimmick, 1947, Mulder *et al.*, 1966).

Regulatory sequences are comprised of the ScUbi4 promoter, the ScUbi4 intron, the mAHAS chloroplast transit peptide, and the 35S terminator from Cauliflower mosaic virus.

Figure 5. Diagram of the T-DNA region in plasmid pAG3541



IV.C. Identity and source of the genetic material

A detailed list of the genetic and regulatory elements, their position within the T-DNA and their origin is provided in Table 4.

Table 4. Genetic elements in the T-DNA of plasmid pAG3541

Genetic element	Size (bp)	Gene bank accession number	Vector nucleotide location	Description
Right border	25	AB027254.1	18,085-18,109	DNA sequence of right border sequence of nopaline type T-DNA derived from plasmid pTiT37. Used as the initiation point of T-DNA transfer from *Agrobacterium tumefaciens* to the plant genome (Zambryski *et al.*, 1980; Komari *et al.*, 1996).
Intervening sequence	73	NA	18,110-18,182	Sequences used in DNA cloning.
Promoter from sugarcane ubiquitin-4 gene	364	AF093504	18,183-18,546	Promoter region of the ubiquitin-4 gene from *Saccharum officinarum* L. (sugarcane) (Albert and Wei, 2003).
5' untranslated region (UTR)	79	NA	18,547-18,625	5' untranslated region of the ubiquitin-4 gene from *Saccharum officinarum* L. (sugarcane)(Albert and Wei, 2003).
Intron from sugarcane ubiquitin-4 gene	1358	NA	18,626-19,983	Intron region of the ubiquitin-4 gene from *Saccharum officinarum* L. (sugarcane)(Albert and Wei, 2003).
Intervening sequence	9	NA	19,984-19,992	Sequences used in DNA cloning.
Maize AHAS chloroplast transit peptide	198	X63553.1	19,993-20,190	N-terminal chloroplast transit peptide sequence derived from the *Zea mays* L. (maize) acetohydroxyacid synthase (*ahas*) gene (Fang *et al.*, 1992). The chloroplast transit peptide allows the expressed protein to be transported to the chloroplast.
Intervening sequence	9	NA	20,191-20,199	Sequences used in DNA cloning.
epsps grg23ace5 gene	1242	NA	20,200-21,441	Coding sequence of the modified 5-enolpyruvylshikimate-3-phosphate synthase from *Arthrobacter globiformis* (Schouten *et al.*, 2010)
Intervening sequence	8	NA	21,442-21,449	Sequences used in DNA cloning.
35S CaMV terminator	270	V00140	21,450-21,719	Terminator region of the 35S transcript of the cauliflower mosaic virus, which terminates mRNA transcription and induces polyadenylation (Gardner *et al.*, 1981).
Intervening sequence	70	NA	21,720-21,789	Sequences used in DNA cloning.
Left border	25	AB027254.1	21,790-21,814	DNA sequence of left border sequence from Ti plasmid pTiA6. Defines the termination point of T-DNA transfer from *A. tumefaciens* to the plant genome (Thomashow, *et al.*, 1980; Komari *et al.*, 1996).

V. GENETIC CHARACTERIZATION OF EVENT VCO-Ø1981-5

V.A. Overview

Event VCO-Ø1981-5 was generated using *Agrobacterium*-mediated transformation of a 3730 bp sized T-DNA fragment originating from vector plasmid pAG3541. The *epsps grg23ace5* gene expression cassette located on the T-DNA was inserted into event VCO-Ø1981-5 as a single copy intact insert. Molecular characterization verified that functional components of the expression cassette were inserted in the expected order. The insert has been shown to be stably inherited through four generations using both molecular (Southern blots) and phenotypic Mendelian segregation analysis that tracked tolerance to glyphosate. Southern blot analysis has confirmed the absence of the transformation vector components outside of the T-DNA borders. These results clearly indicate that the trait is inherited as expected for a single locus.

V.B. Copy number and insertion

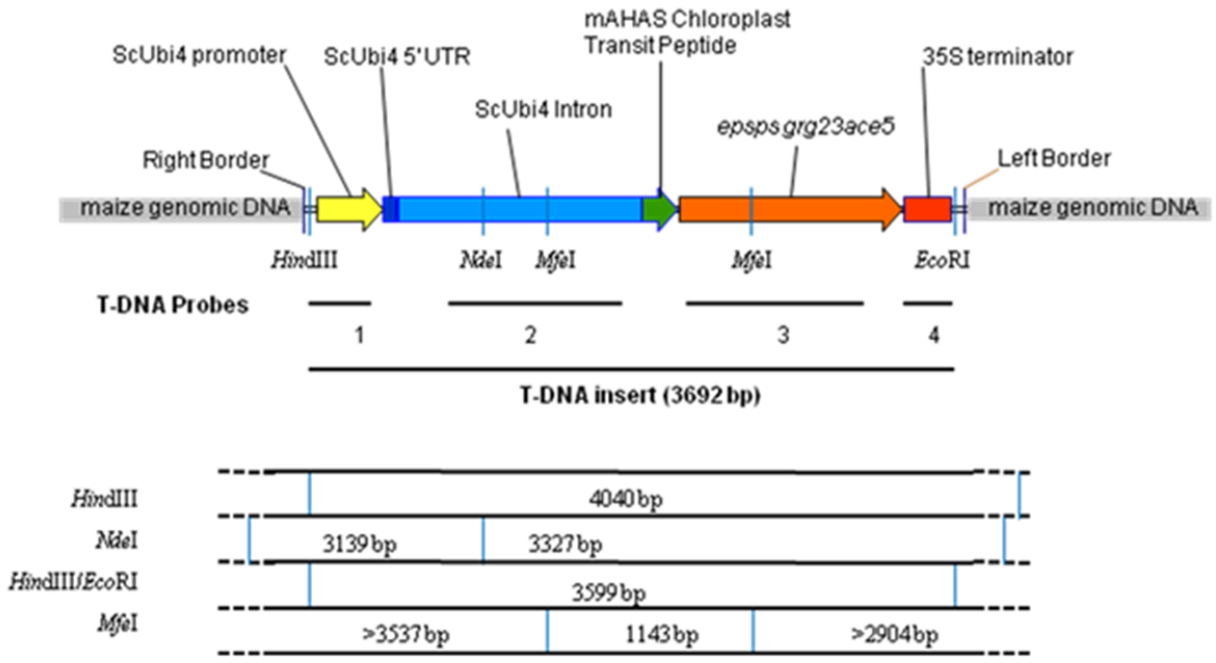
The number of copies of the inserted transgene was investigated by Southern blot analysis. Genomic DNA samples of event VCO-Ø1981-5 and appropriate control samples were digested with the restriction enzymes *Hin*dIII and *Nde*I independently (Figure 6). Each of these restriction enzymes cuts one time within the T-DNA region. When hybridized with the *epsps grg23ace5 gene* probe (Figure 6, probe #3), the resulting number of bands indicates the insert copy number within the maize genome.

Both digests, with *Hin*dIII and *Nde*I, probed with the *epsps grg23ace5* gene sequence produced a single band, indicating that a single copy of the insert is present in event VCO-Ø1981-5 (Appendix 3, Figure 12).

Southern blot analysis was also conducted to determine insert integrity. Maize genomic DNA from event VCO-Ø1981-5 and appropriate non-transgenic controls) was digested with a combination of *Hin*dIII and *Eco*RI, and independently with *Mfe*I. The locations of these restriction enzyme sites are shown in Figure 6. A set of four independent probes of the major genetic elements (ScUbi4 promoter, ScUbi4 intron, *epsps grg23ace5* gene, and 35S terminator; Figure 6, probes 1-4, respectively) was used to confirm the integrity of the expression cassette. The results indicate that the T-DNA segment that contains the *epsps grg23ace5* expression cassette is intact and the functional components were inserted in the expected order.

The predicted sizes of hybridization products for these analyses are shown in Figure 6. A description of the probes and their sizes is provided in Appendix 3, Table 34. Predicted and observed hybridization fragments are described in Appendix 3, Table 35. Southern blots are presented in Appendix 3, Figures 12-14.

Figure 6. Map of the T-DNA insertion site of event VCO-Ø1981-5



Note: The T-DNA size in plasmid pAG3541 is 3730 bp; it is 3692 bp after insertion into the Hi-II genome (Section V.D below).

V.C. Absence of vector backbone

Southern blot analysis was conducted to verify the absence of the transformation plasmid components outside of the transferred T-DNA region.

Southern blot analysis results (Appendix 3, Figures 17 and 18) indicate that none of the vector probes hybridized to genomic DNA of event VCO-Ø1981-5 confirming the absence of vector backbone. The same probes, however, did show hybridization with the plasmid vector control on each blot indicating that if the vector sequences were inadvertently transferred to event VCO-Ø1981-5, they would have been detected in this analysis.

V.D. The flanking regions of the inserted sequence

The maize genomic flanking regions of the insertion in event VCO-Ø1981-5 were identified using a PCR based approach that includes the Genome Walker™ strategy, followed by a bioinformatics analysis of the obtained sequences.

The sequences surrounding the insertion site have been identified by sequencing 2 Kb for the 5' flanking region and 1.7 Kb for the 3' flanking region. These sequences allowed for mapping the insertion site of event VCO-Ø1981-5 to the maize chromosome 1.

Comparison of the sequences from event VCO-Ø1981-5 with the Hi-II original sequence showed that only a short deletion (21 bp) in the maize genome was created by the

insertion of event VCO-Ø1981-5. The insertion process also created some short deletions at the extremities of the T-DNA borders - 22 bp and 16 bp on the right and left borders, respectively, which resulted in the insert being 3692 bp in size (T-DNA fragment originating from vector plasmid pAG3541 is 3730 bp). However, all regulatory sequences and the *epsps grg23ace5* gene were found to be inserted intact. This was also confirmed by Southern blot analysis as discussed in section V.B. above.

Bioinformatics analysis of the insertion region revealed the presence of a sequence potentially coding for an Acanthoscurrin-homolog protein. The insertion of the T-DNA seems to have occurred in the 5' untranslated region (UTR) of this gene, which has probably prevented its transcription in event VCO-Ø1981-5. Acanthoscurrin-2 and Acanthoscurrin-homolog transcripts have been found in maize (Yang *et al*., 2006), sorghum (Tanaka *et al*., 2008) and rice (Paterson *et al*., 2009). Acanthoscurrin has been described as an antimicrobial peptide (Lorenzini *et al*., 2003). The insertion, however, did not produce any phenotypic changes in event VCO-Ø1981-5 maize, nor were any differences in agronomic performance or disease incidence observed that would indicate a potential effect on the expression of an antimicrobial peptide. In addition, one can expect that the Acanthoscurrin-2 protein is still expressed in maize event VCO-Ø1981-5, since the Acanthoscurrin-2 and Acanthoscurrin-homolog proteins are encoded by two different genes in maize (Alexandrov *et al*., 2009; Yang *et al*., 2006).

Bioinformatics analysis also identified 12 potential open reading frames (ORFs, defined here as a sequence between two STOP codons) created by the T-DNA insertion in event VCO-Ø1981-5. No homologies with known allergen sequences were identified using a short (8) amino acid search algorithm when these potential ORFs were analyzed for their putative allergenicity. Using the 80 AA sliding window (> 35% homology) approach, two areas of potential homology were identified (AllergenOnline Database Version 11; http://www.allergenonline.org/databasefasta.shtml). However, these sequences are also present and are potential ORFs in the native maize genome, They were not a result of the insertion present in event VCO-Ø1981-5. No new ORFs were created by the insertion. No significant homologies with known toxins or other harmful proteins were found, when ORFs were analyzed against publically available databases using the BLASTP algorithm. All potential matches were manually inspected for homologies with known toxins. Moreover, it is highly unlikely that the identified genetic sequences would generate any translatable mRNA, since no START codons were found upstream.

V.E. Mendelian inheritance

Insert stability was confirmed using phenotypic Mendelian segregation analysis. For these experiments, the back-cross zero plants (BC0B) were crossed with the non-parental AAX3 inbred, to produce four successive generations: BC0A, BC1A, BC2A, BC3A (Figure 3).

Progeny plants were sprayed with glyphosate to identify plants that inherited the *epsps grg23ace5* gene and assess the segregation ratio in each generation. Positive segregants that survived the spray (little or no leaf damage) were scored as "tolerant", while negative segregants did not survive the spray and were scored as "sensitive" (Table 5). All plants were evaluated two weeks after spraying. A segregation ratio of 1:1, or 50% tolerant and 50% sensitive, was expected in each generation if the *epsps grg23ace5* gene was inserted at a single locus.

Observed segregation patterns were compared to the expected patterns and these data were compared using a chi-squared (CHI^2) distribution analysis, as follows: $\chi^2 = \Sigma [(|o - e|)2/e]$, where o = observed frequency of tolerance, and e = expected frequency of tolerance. A CHI^2 value of ≥ 0.05 was treated as the cutoff for statistical support of a 1:1 segregation in each generation, and this value was exceeded for each of the segregation analysis groups. The results of this analysis (Table 5) are consistent with the molecular results (see section V.F.) and confirm the stable inheritance of a single copy of *epsps grg23ace5* gene into the progeny of event VCO-Ø1981-5.

Table 5. Segregation data for the progeny of event VCO-Ø1981-5

Generation used	Number of plants	Observed tolerant	Observed sensitive	Tolerant (%)	CHI^2 test (p-value)
BC0A	28	12	16	42.9	0.450
BC1A	153	78	75	51.0	0.808
BC2A	58	29	29	50	1.000
BC3A	74	38	36	51.4	0.816

$p \geq 0.05$ in the CHI^2 test indicates no significant difference from expected ratio

V.F. Stability across generations

Stability was determined using molecular and phenotypic methodologies. The molecular methodology was based on Southern blot analysis, where gel banding patterns were predicted and observed using specific restriction enzymes and genetic probes. The phenotypic methodology evaluated the inheritance of the *epsps grg23ace5* gene through successive breeding generations. The tolerance to glyphosate application on plants grown under field conditions for event VCO-Ø1981-5 was used to evaluate the segregation ratios of this event (see section V.E.).

Southern blot analysis was conducted on multiple generations of event VCO-Ø1981-5 progeny to evaluate the stability of the T-DNA insertion site. Genomic DNA isolated from leaf material of event VCO-Ø1981-5 plants from four breeding generations resulting from crosses with non-transgenic inbred line B110 (BC0B, BC1B, BC2B, and $BC1B_2$) and negative controls were digested with the restriction enzyme *Hin*dIII, which as noted earlier, cuts once within the T-DNA region. When hybridized with probe 3, specific for the *epsps grg23ace5* gene coding region, genomic DNA from event VCO-Ø1981-5 digested with *Hin*dIII produces a single band 4040 bp in size, since one of the restriction sites is outside of the T-DNA, but within the maize genomic DNA (Figure 6). Plasmid pAG3541 was included as a hybridization control. All four generations analyzed showed an identical hybridization pattern producing the same single ~4000 bp band (Appendix 3, Figure 15). If the genetic insert was unstable within the maize genome through successive breeding of the event, one would expect to detect changes in the banding pattern produced. The data thus indicates a stable insertion site in event VCO-Ø1981-5 across four generations.

V.G. Conclusion

Results of the molecular characterization (Bernard and MacIntosh, 2011a) of event VCO-Ø1981-5 indicate that the event contains a single insert containing the *epsps grg23ace5* gene expression cassette. The results demonstrated that the T-DNA segment that contains the *epsps grg23ace5* gene expression cassette is intact and the functional components are in the expected order. The insert has been shown to be stably inherited in two different genetic backgrounds and through four generations using molecular analysis and Mendelian segregation analysis that tracked phenotypic tolerance to glyphosate. Southern blot analysis has confirmed the absence of the transformation vector components outside of the T-DNA borders. Those results clearly indicate that the trait is inherited as expected for a single locus. Genomic flanking sequences of 2 Kb and 1.7 Kb for the 5' and 3' junctions, respectively, of the insertion in event VCO-Ø1981-5 were identified using a PCR based approach. Bioinformatics analysis did not identify any homologies with known allergen sequences created by the insertion.

VI. CHARACTERIZATION OF THE INTRODUCED PROTEIN

VI.A. The EPSPS ACE5 protein

Maize event VCO-Ø1981-5 expresses an improved 5-enolpyruvyl-3-shikimate phosphate synthase (EPSPS) of bacterial origin, the EPSPS ACE5 protein, to confer tolerance to the herbicide glyphosate. The EPSPS ACE5 protein was derived from the native EPSPS GRG23 protein isolated from *Arthrobacter globiformis* using a directed evolution protein engineering strategy. The native EPSPS GRG23 enzyme from *Arthrobacter* was optimized by changing a total of ten amino acids to produce EPSPS ACE5, which more closely matches the native maize EPSPS enzyme. The changes made to create the EPSPS ACE5 protein did not alter its enzymatic profile with the exception of having greater temperature stability of the enzymatic activity under environmental conditions, yet still remaining sensitive to industrial processing temperatures and rapidly digesting in simulated gastric fluid. Therefore, EPSPS ACE5 protein is expected to have the same safety profile as other previously deregulated EPSPS proteins. The safety of EPSPS ACE5 protein was also evaluated as part of an Early Food Safety Evaluation submitted to FDA in 2009. FDA completed their review with no further questions in October, 2010 (FDA, 2010).

This section of the petition provides a compilation of the protein safety data (Bernard and MacIntosh, 2011b) for EPSPS ACE5 protein as it is expressed in maize event VCO-Ø1981-5.

VI.A.1. History and background

EPSPS enzymes are required for plant amino acid biosynthesis. This EPSPS enzymatic reaction is very important in plant biology, as it is a non-branching step in the shikimate pathway, leading to the biosynthesis of a large number of aromatic plant metabolites, including essential aromatic amino acids (phenylalanine, tyrosine, and tryptophan), tetrahydrofolate, ubiquinone and vitamin K (Franz *et al.*, 1997). It has been estimated that 35% or more of the plant dry mass is made up of molecules derived from the shikimate pathway (Franz *et al.*, 1997).

In addition to plants, EPSPS enzymes are also found in prokaryotic systems, and glyphosate is toxic to most bacterial species. However, certain bacteria are tolerant to glyphosate, and it has been found that the EPSPS enzymes isolated from these bacteria often have a high tolerance to glyphosate. Furthermore, genes encoding glyphosate-tolerant EPSPS enzymes have been transferred to recipient organisms, including plants, by means of recombinant DNA technology to confer glyphosate tolerance (OECD, 1999; Nolte *et al.*, 2002; Cedeira *et al.*, 2006; Gianessi, 2008; Vande Berg *et al.*, 2008). Indeed a number of crops (*e.g.*, maize, soybean, canola, alfalfa, etc.) expressing glyphosate tolerance have been granted non-regulated status (USDA-APHIS, 2011) and are commercially available in several countries (Biosafety Clearinghouse, 2011). EPSPS ACE5 is another example of a bacterial EPSPS that confers glyphosate tolerance in a plant system (Peters *et al*., 2010, Schouten *et al*., 2010).

EPSPS enzymes have a long history of safe use as they have been expressed in GM maize, cotton, soybean, canola, and sugarbeet and represent a large proportion of the total US acreage. According to Brookes and Barfoot (2009), 91% of the total US soybean crop in 2007, was planted to glyphosate-tolerant varieties, first introduced in

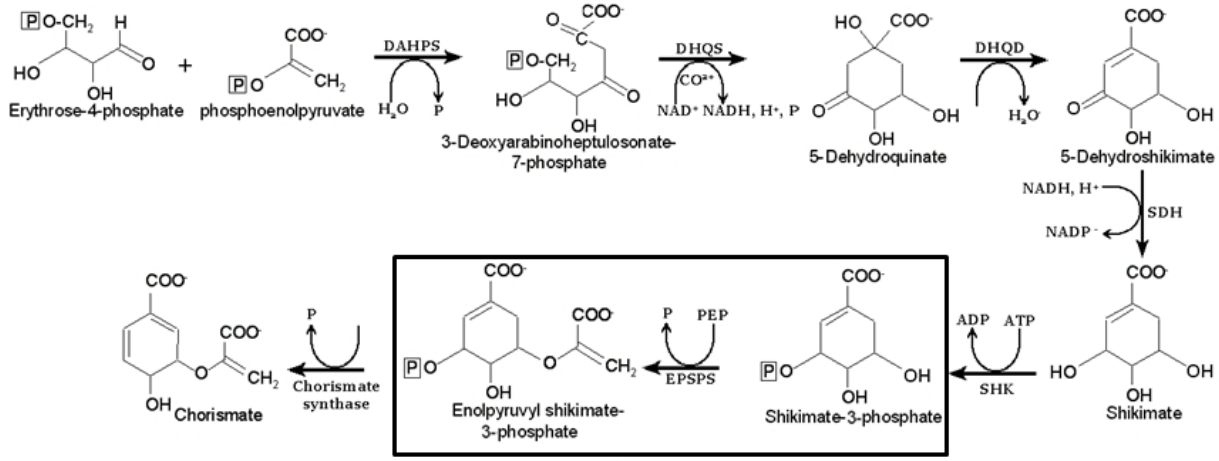
1996. Glyphosate-tolerant maize and cotton were first introduced in 1997 and 10 years later were planted on 52% and 70% of the US maize and cotton acres, respectively (Brookes and Barfoot, 2009). There have been no identified safety or environmental concerns raised for the glyphosate-tolerant technology, regardless of the crop in which they are expressed.

VI.A.2. Characterization of the EPSPS ACE5 protein

VI.A.2.1. Biochemistry and mode of action

The EPSPS enzymatic reaction is very important in plant biology, as it is a non-branching step in the shikimate pathway and the reaction mechanism has been very well-characterized. EPSPS enzymes catalyze the formation of 5-enolpyruvylshikimate-3-phosphate (EPSP) by linking phosphoenolpyruvate (PEP) to shikimate 3-phosphate (S3P) at a hydroxyl position on the S3P ring. During the reaction, PEP is hydrolyzed and an inorganic phosphate molecule (Pi) is released (Herrmann and Weaver, 1999). The final product of the shikimate pathway is chorismate (Figure 7), which is utilized for the biosynthesis of a large number of aromatic plant metabolites, including essential aromatic amino acids (phenylalanine, tyrosine, and tryptophan), tetrahydrofolate, ubiquinone and vitamin K (Franz *et al.,* 1997). The amino acid phenylalanine is further metabolized to produce several compounds needed for plant growth, including anthocyanins and lignin.

Figure 7. EPSPS functions in the shikimate pathway



The enzymatic function of EPSPS ACE5 protein was confirmed by carrying out enzymatic assays with purified *E.coli*-produced EPSPS ACE5 protein. An enzymatic assay was developed to quantify the production of inorganic phosphate (Pi) by EPSPS enzymes. The assay used enzyme coupling and resulted in the generation of a highly fluorescent product (Vazquez *et al*., 2003) (see Appendix 3, Section 3.D.).

Glyphosate inhibition of EPSPS enzymes

N-phosphonomethylglycine, commonly referred to as glyphosate, is an important commercial herbicide. Glyphosate inhibits plant, fungal, and most bacterial EPSPS enzymes and thus prevents the shikimate pathway from producing chorismate (the precursor of many essential plant metabolites). As plant cells depend on the biosynthesis of aromatic amino acids for growth, herbicide sprays containing glyphosate can affect plant cells by effectively blocking the shikimate pathway. Because the shikimate pathway is not present in humans and animals, glyphosate has a favorable toxicology profile and has become a very common non-selective herbicide (Franz *et al.,* 1997).

Glyphosate is a structural analog of PEP and competes with PEP for binding in the active site of EPSPS enzymes (Schonbrunn *et al.,* 2001). This mode of action is supported by enzymatic and structural studies. Treatment of plant cells with glyphosate leads to substantial accumulation of shikimate-5-phosphate (Amrhein *et al.,* 1980), and inhibits the incorporation of radiolabeled shikimate-3-phosphate into phenylalanine, tyrosine or tryptophan (Hollander and Amrhein, 1980). X-ray crystal studies have demonstrated that glyphosate is bound by EPSPS at the site normally occupied by PEP, and traps the enzyme in a non-productive state (Schonbrunn *et al.,* 2001). It is important to note that for plant EPSPS enzymes including maize, the binding affinity for glyphosate is much tighter than the binding affinity for PEP (Vande Berg *et al.,* 2008). Thus, relatively low concentrations of glyphosate in the plant cell are capable of effectively shutting down the shikimate pathway and producing cell death.

Enzymatic function in the presence of glyphosate

For a heterologous EPSPS enzyme to function well in plants, it is essential that it be similar to the native plant EPSPS in terms of binding affinity for the natural substrate phosphoenolpyruvate (PEP). However, a glyphosate-tolerant EPSPS must also possess very low affinity for glyphosate so that it can bind PEP and retain enzymatic activity.

The potential for EPSPS ACE5 to provide glyphosate tolerance in plants was evaluated by carrying out enzymatic assays in the presence and absence of glyphosate. Using the *in vitro* enzyme assay described in Appendix 3, the EPSPS activity of EPSPS ACE5 was measured over a range of PEP concentrations. The maize EPSPS and native EPSPS GRG23 were assayed alongside for comparison, and the *Km* and *Ki* values for the enzymes are shown in Table 6 (Peters *et al.,* 2010; Schouten *et al.,* 2010). While all three enzymes bind PEP with similar affinity, the native EPSPS GRG23 and EPSPS ACE5 proteins bind only weakly to glyphosate (*Ki* is over 900-fold higher than *Km*) and therefore will preferentially bind PEP even at high glyphosate concentrations (Peters *et al.,* 2010; Schouten *et al.,* 2010). This suggests that EPSPS ACE5 expression in maize can substantially reduce glyphosate-induced toxicity. In contrast, the maize EPSPS enzyme binds to glyphosate with 90-fold greater affinity than PEP, and is therefore inhibited by much lower concentrations of the herbicide. It should be noted that the *Km* value for EPSPS ACE5 is similar to published literature values for other bacterial EPSPS enzymes (Barry *et al.*, 1997; Priestman *et al.,* 2005; Funke *et al.,* 2006).

Table 6. Glyphosate binding affinities for EPSPS GRG23, EPSPS ACE5, and maize EPSPS proteins

Enzyme	K_m(PEP) µM	K_i µM	K_i/K_m
EPSPS GRG23	11	9,525	869
EPSPS ACE5	16.6	14,700	919
Maize EPSPS	18	0.2	0.01

VI.A.2.2. History of safe use

Recipient crop – *Zea mays*

Zea mays, the crop that was transformed with the expression cassette allowing the synthesis of an improved bacterial *epsps* gene is a crop widely used for food and feed that is unlikely to cause any pathogenic, toxic, allergenic or other adverse effects for humans or animals. Maize is a member of the *Maydeae* tribe of the grass family, which requires the help of man to disperse its seeds for survival. A large portion of the maize-produced in the US is utilized for animal feeds and biofuels, but maize is also processed for inclusion in many foodstuffs (*e.g.*, high fructose corn syrup, corn grits, corn meal, etc.). A comprehensive review of *Zea mays*, including detailed information on the biology, taxonomy, identification, diversity, reproductive characteristics and ecology, was published by OECD (2003).

Source organism and the *epsps grg23ace5* gene

DNA is the basis for all life, with the same nucleic acids and structure, and as such is generally recognized as safe. Humans and animals are exposed to DNA in everything they consume. The addition of a transgene, such as *epsps grg23ace5*, to maize is indistinguishable from any other DNA that is present in their diet.

The *epsps grg23ace5* gene is an optimized form of the native *epsps* gene (Schouten *et al.,* 2010) sourced from the common soil bacterium *Arthrobacter globiformis.*

Arthrobacter species are part of the Gram-positive coryneform bacteria considered one of the major groups of aerobic soil bacteria and are found ubiquitously in soil, water and other natural habitats (Conn, 1948; Conn and Dimmick, 1947; Mulder *et al.*, 1966). In addition to their prevalence in soil and water, *Arthrobacter* spp. (including *A. globiformis*) have been shown to be associated with the naturally occurring microflora of various produce including broccoli (Padaga *et al.*, 2000), strawberries (Krimm *et al.*, 2005), and sugarbeets (Bugbee *et al.*, 1975). *Arthrobacter* is one of the only organisms that can grow in the presence of - and reduce - hexavalent chromium (Lower, 1998).

A. globiformis works synergistically with *Streptomyces* to degrade agricultural pesticides such as organophosphate insecticide diazinon and also has the individual ability to degrade herbicides like glyphosate and pentachlorophenols (Lower, 1998; Microbewiki, 2011). *Arthrobacteria* are weakly motile and are nonsporulating. Most *Arthrobacter* species are obligate aerobes. As the upper layers of soil that they inhabit change oxygen concentration frequently, some *Arthrobacter* species, including *A. globiformis*, have developed adaptive oxygen independent growth strategies (Lower, 1998).

Arthrobacter sp. are not classified in many countries and are assigned the lowest risk group in others (Table 7). *Arthrobacter* strains are assigned biosafety level 1 (BSL1) for exposure control and personal protection. Human infections have been described for other *Arthrobacter* species, but not for *A. globiformis* (Mages *et al*., 2008). There are no known effects on animals.

Table 7. Risk group classification of *Arthrobacter globiformis*

U.S.A	**Not classified.** NIH Guidelines for Research Involving Recombinant DNA Molecules, Appendix B: Classification of human etiologic agents on the basis of hazard, January 2011.http://oba.od.nih.gov/oba/rac/Guidelines/NIH_Guidelines.pdf
Canada	**Not classified.** Pathogen Safety Data Sheets, Public Health Agency of Canada. http://www.phac-aspc.gc.ca/lab-bio/res/psds-ftss/index-eng.php
Belgium	**Not classified.** Revised lists of pathogens and their corresponding class of biological risk, Belgian Biosafety Server. http://www.biosafety.be/RA/Class/ClassBEL.html
Switzerland	**Risk group 1.** Classification of Organisms According to Risk Presented to People and the Environment. Bacteria (2005) and Viruses (2004). Swiss Department of the Environment, Transport, Energy and Communications. http://www.bafu.admin.ch/biotechnologie/01744/01753/index.html?lang=en
France	**Not classified.** Principles de Classement et Guides Officiels de la Commission de Génie Génétique, France, January 2000. ftp://trf.education.gouv.fr/pub/rechtec/commis/genetique/principe/guide.pdf
Germany	**Risk group 1.** Bundesanstalt für Arbeitsschutz und Arbeitsmedizin, Technische Regeln für Biologische Arbeitstoffe (TRBA) http://www.baua.de/cln_137/de/Themen-von-A-Z/Biologische-Arbeitsstoffe/TRBA/TRBA.html
UK	**Not classified.** HSE Advisory Committee on Dangerous Pathogens, The Approved List of Biological Agents, April 2004. http://www.hse.gov.uk/pubns/misc208.pdf
Singapore	**Not classified.** List of biological agents and toxins, Ministry of Health, Singapore, November 2009. http://www.biosafety.moh.gov.sg/home/uploadedFiles/Common/List_of_Biological_Agents_and_Toxins.pdf. Division into Schedules based on risk groups as defined by the WHO and other factors such as the potential to be weaponized, etc.

EPSPS ACE5 protein

EPSPS ACE5 is an improved glyphosate-tolerant EPSPS enzyme. It was derived using a directed evolution protein engineering strategy from the native EPSPS GRG23 protein isolated from *Arthrobacter globiformis* (Peters *et al.,* 2010; Schouten *et al.,* 2010). A total of ten amino acids were changed to produce the EPSPS ACE5 protein, which is 97.6% identical and 98.5% homologous at the amino acid level to the originally isolated EPSPS GRG23 enzyme.

As reviewed by ILSI (ILSI, 2010), several publicly available documents issued by regulatory authorities indicate that similar EPSPS protein family members expressed in glyphosate-tolerant crops are safe. The results of studies summarized in this petition are consistent with the published information, indicating that maize containing the EPSPS ACE5 protein can be safely used as food or feed.

Chloroplast transit peptide

Many nuclear-encoded proteins in plants are localized into the chloroplast to perform key metabolic functions, including amino acid biosynthesis and photosynthesis (Keegstra and Cline, 1999; Bruce, 2000). Included in this set of chloroplast-localized proteins are the enzymes that function in the shikimate pathway (Mousdale and Coggins, 1985). Thus, it is imperative that glyphosate-tolerant EPSPS enzymes become targeted to chloroplasts to function more directly in the shikimate pathway and confer more robust tolerance following glyphosate applications.

Chloroplast translocation is facilitated by addition of an N-terminal extension (referred to as a transit peptide) that guides the newly-translated proteins from the cytoplasm to the chloroplast outer membrane. Following translocation inside the chloroplast, the transit peptide is cleaved to yield the mature protein. The chloroplast transit peptide selected for the EPSPS ACE5 protein was cloned from the region upstream of the maize acetohydroxy acid synthase (AHAS) gene. The cleavage of the transit peptide was inferred by the verification of mature protein molecular weight and the N-terminal sequence of the EPSPS ACE5 protein isolated from event VCO-Ø1981-5 maize (Schouten, 2011).

VI.B. Expression of the EPSPS ACE5 protein in event VCO-Ø1981-5

The protein expression levels were determined by validated enzyme-linked immunosorbant assay (ELISA, Appendix 2, Section 2.C.) for various tissues of event VCO-Ø1981-5 and the non-transgenic near-isogenic control planted at three locations in Iowa during the 2009 growing season (Stauffer, 2011). At each location, three replicated plots of event VCO-Ø1981-5 and the non-transgenic control (non-transgenic near-isogenic hybrid) were planted using a randomized complete block field design. The plants were grown under conditions representative of commercial corn production in Iowa. Table 8 lists the growth stages and the plant tissue types collected.

Table 8. Sample collection per growth stage

Plant Tissue	# of plants per location and growth stage				
	V4	V8	R1	R4	R6
Leaf	6	3	3	3	3
Root	6	3	3	3	3
Whole Plant	-	3	3	3	3
Pollen	-	-	3	-	-
Grain	-	-	-	-	3

V4 (vegetative 4-leaf stage), V8 (vegetative 8-leaf stage),
R1 (reproductive silking stage), R4 (reproductive kernel dough stage),
R6 (physiological maturity)

The EPSPS ACE5 protein was detected at various life stages and in all tissues of event VCO-Ø1981-5 plants except in grain (Table 9). In general, the expression levels of the EPSPS ACE5 protein in leaf were highest at the V4 and V8 stages, dropped off approximately 3-fold at the R1 and R4 stages, and dropped to under the limit of detection (LOD) at R6. The protein expression levels in root were about 30% of leaf at the V4 stage and dropped to below the LOD at the reproductive growth stages (R1, R4, and R6). Whole plant expression levels of the EPSPS ACE5 protein were very similar to those of leaf, with the exception of the V4 stage as no whole plant samples were collected for that stage. Protein expression in the pollen was low to undetectable, and expression in grain was undetectable. All values for control tissue (leaf, root, whole plant, pollen and grain) were either 0.0 ng EPSPS ACE5 protein/mg dw or below the LOD, which is provided in Table 9.

Expression levels were in the tens of nanograms per milligram of dry tissue weight at the V4 and V8 stages, when the plant is undergoing rapid growth, and very low in the later reproductive stages when the plant's energy is going towards development of the grain and pollen. The *epsps grg23ace5* gene is driven by a constitutive promoter (Ubiquitin 4 promoter from sugarcane) and EPSPS ACE5 protein expression was detected in all tissues evaluated except for grain.

Table 9. EPSPS ACE5 expression in different tissues

Tissue	Growth Stage	Amount of protein (ng /mg dw)			Number of Samples <LOD/Total Number of Samples
		Average[1]	Range[1]	LOD	
Leaf	V4	17.77	7.47 - 24.86	1.34	0/18
	V8	18.59	8.63 - 24.01		0/9
	R1	4.75	2.95 - 7.63		0/9
	R4	6.20	1.40 - 8.70		0/9
	R6	0.00[2]	0.00	6.53	9/9
Root	V4	6.77	1.72 - 14.97	1.57	0/18
	V8	2.63	0.00 - 4.54		2/9
	R1	0.00	0.00 - 1.86		8/9
	R4	0.00	0.00		9/9
	R6	0.00	0.00	5.32	9/9
Whole Plant	V8	12.69	5.42 - 21.50	0.92	0/9
	R1	1.95	0.00 - 4.45		3/9
	R4	3.48	1.09 - 6.93		0/9
	R6	0.00	0.00	4.31	9/9
Pollen	R1	0.00	0.00 - 9.60	6.43	8/9
Grain	R6	0.00	0.00	1.79	9/9

[1] across locations
[2] any values below the LOD are represented by a value of zero

VI.C. Verification of biochemical and functional equivalence

The EPSPS ACE5 protein was produced in *E. coli* for use in studies to investigate the potential toxicity and allergenicity of the protein, since it is not feasible to produce an adequate amount of the protein for these studies from event VCO-Ø1981-5 plants. Therefore, it was necessary to demonstrate the equivalence between the *E. coli*-produced EPSPS ACE5 protein and the plant-produced protein in order to utilize the safety data. The *E. coli*-produced and plant-produced EPSPS ACE5 protein was compared, using the following six biochemical tests listed in Table 10.

Table 10. Biochemical analyses for demonstrating protein equivalence

Equivalence criteria	Methodology
Molecular weight	Protein mobility in SDS-PAGE
Peptide mass identification	MALDI-TOF mass spectroscopy
Immunoreactivity	Western blot analysis
Sequence comparison	N-terminal sequencing
Biological activity	Enzymatic activity
Glycosylation profile	Glycosylation analysis

These biochemical tests each confirmed the equivalency of the EPSPS ACE5 protein from *E. coli*- and plant-produced sources (Schouten, 2011). The analytical methods used are described in Appendix 2. The results are shown in Appendix 3.

VI.D. Summary of the food and feed safety assessment of the EPSPS ACE5 protein

VI.D.1. Homology searches to known allergens and toxins

One aspect of the protein safety assessment evaluates the amino acid similarity between the newly introduced protein and known allergens and toxins (Codex, 2003). These bioinformatics analyses are conducted *in silico* using different publically available protein databases.

Many different genomes have been sequenced over the last decade providing a vast resource of protein sequences and tools for analyzing sequence data. Several different databases are publicly available that contain thousands of protein sequences (*e.g.,* Uniprot-Swissprot, 2011; National Center for Biotechnology Information, 2011; Protein Informatics Resource, 2011). Search algorithms are used to compare the newly introduced protein with database proteins to classify and determine the relatedness to known protein families. A publicly available, curated genetic database of known protein allergen sequences, AllergenOnline (2011), has been developed allowing for characterization of the allergenic potential of unknown proteins in a consistent manner. This database (Version 11.0) contains a comprehensive list of unique proteins of known and putative allergenic proteins (food, airway, venom/salivary and contact) (Accessed March 31, 2011). Bioinformatic algorithms, FASTA and BLASTP, were utilized to evaluate the level of similarity of the EPSPS ACE5 protein to known allergens and toxins, respectively (Bernard and MacIntosh, 2011c).

The amino acid sequence of the EPSPS ACE5 protein was assessed for similarity to protein sequences in the National Center for Biotechnology Information Protein dataset by BLASTP bioinformatics analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The EPSPS ACE5 protein is highly homologous to other 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzymes. The top 100 hits from this search are all members of this class of enzymes (EC 2.5.1.19) and share greater than 50% protein sequence homology with EPSPS ACE5. All other resulting sequence matches, with an expectancy value of 1.0 or less, were manually inspected for sequences with known toxic or anti-nutritional effects as indicated in the EPA Code of Federal Regulations document 40 CFR 725.421. The EPSPS ACE5 protein exhibits no significant homology at >35% to known toxins or other harmful proteins (Table 11).

Three different similarity searches were conducted (Table 11):

- The entire EPSPS ACE5 protein sequence was compared to the toxin sequences using the BLASTP search algorithm. No homologies were identified;
- Contiguous EPSPS ACE5 peptides of 80 amino acids were compared to the allergen sequences using the FASTA search algorithm. No significant homologies were identified;
- The EPSPS ACE5 protein sequence was screened for eight contiguous amino acid identity matches with known or putative allergens. No matches were identified.

Table 11. Results of bioinformatics analyses

Type of *in silico* search	Results	Purpose
Full-length BLASTP	No hits at > 35% identity	Toxins
80-mer FASTA	No hits at > 35% identity	Allergens
8-mer exact match	No matches	Allergens

VI.D.2. Potential N-glycosylation sites

Glycosylation analysis was performed to determine if maize-produced EPSPS ACE5 was post-translationally modified by the addition of carbohydrate moieties. Both microbial- and maize-derived EPSPS ACE5 protein was separated by SDS-PAGE along with horseradish peroxidase (positive control protein) and soybean trypsin inhibitor (negative control protein). Gels were stained with Gelcode glycoprotein staining kit (Thermo Fisher Scientific, Rockford, IL) according to manufacturer's instructions. Glycoproteins were detected as bright magenta bands on the gel. The same gel was then stained with Imperial Coomassie Blue stain for detection of all proteins loaded on the gel (Appendix 3, Figure 23).

No glycosylation was detected for either the microbial-produced or maize-produced EPSPS ACE5 protein. The glycoprotein positive control, horseradish peroxidase, stained a bright magenta color while the glycoprotein negative control, soybean trypsin inhibitor, showed only very faint light pink color. These results show that neither maize- nor microbial-produced EPSPS ACE5 protein have been modified by the addition of detectable levels of carbohydrates.

VI.D.3. *In vitro* stability to human simulated gastric fluid

The resistance to enzymatic digestion of EPSPS ACE5 protein was analyzed using biochemical methods (Lautraite, 2011). The *E. coli*-produced EPSPS ACE5 protein was tested for stability in human simulated gastric fluid (SGF) with pepsin at pH 1.2 for incubation times ranging from 0.5 to 60 minutes using a standard pepsin digestion method (Thomas *et al*., 2004).

Test protein and reference protein (horseradish peroxidase and ovalbumin) solutions were incubated with human SGF (pH 1.2) at approximately 37°C and samples were taken for analysis at time-points of 0, 0.5, 2, 5, 10, 20, 30 and 60 minutes. The resultant protein solutions were analysed for presence of the test protein and potential stable protein fragments by Coomassie blue-stained SDS-PAGE and western blot (Appendix 3, Figures 24 and 25).

The EPSPS ACE5 protein was degraded very rapidly in human simulated gastric fluid, within 30 seconds of incubation, in presence of pepsin, at pH 1.2. Both reference proteins were digested as expected: HRP was rapidly digested and OVA was slowly digested (data not shown).

With such rapid SGF digestion, it is unlikely the EPSPS ACE5 protein could have any adverse effects on humans or animals that consume event VCO-Ø1981-5 maize. Moreover, EPSPS ACE5 protein was not detected in grain samples (Section VI.B.) further limiting any potential dietary exposure to humans.

It should be noted here that, while the optimized EPSPS ACE5 protein provides greater temperature stability under environmental conditions, the enzymatic activity remains sensitive to industrial processing temperatures (Section VI.A.2.1.) and its digestibility in SGF is comparable to that of other plant- expressed EPSPS proteins.

VI.D.4. Acute toxicity study in the mouse

To assess the toxic potential of the EPSPS ACE5 protein and confirm the safety assessment from data already collected (i.e., bioinformatics analysis, potential glycosylation sites, *in vitro* digestibility, etc.) an acute oral toxicity study was conducted in mice (Arulnesan, 2008). Two groups of CD-1 mice were tested with purified microbial-produced protein. Group 1 consisted of 3 male and 3 female mice that were each administered the negative buffer control. Group 2 consisted of 5 male and 5 female mice that were each administered EPSPS ACE5 protein at a dose of 2000 mg/kg. Both the negative buffer control and the EPSPS ACE5 protein were suspended in 3% methyl cellulose and administered by oral gavage at a dose volume of 25 ml/kg. The animals were observed for 14 days after dosing and body weights were recorded on Day 1 (prior to dosing), Day 7, Day 14 and Day 15, just prior to necropsy.

No mortality or evidence of toxicity were observed post dosing or during the 14-day observation period in any of the animals. Animals in each group gained body weight by the end of the study. After the animals were sacrificed (Day 15), they were submitted for gross necropsy and no gross pathological findings were observed. Based on these results, there is no evidence of toxicity when EPSPS ACE5 is administered orally at a dose level of 2000 mg/kg.

VI.E. Conclusion

EPSPS enzymes, a common class of enzymes found in plant, bacterial, and fungal species, have a long history of safe use. The EPSPS ACE5 protein was derived from the native EPSPS GRG23 protein isolated from *Arthrobacter globiformis* using a directed evolution protein engineering strategy. The native EPSPS GRG23 enzyme from *Arthrobacter* was optimized to produce the EPSPS ACE5 protein, which more closely matches the native maize EPSPS enzyme with regards to the temperature optimum for its activity under typical environmental conditions. The optimized EPSPS ACE5 protein provides greater temperature stability under environmental conditions but remains sensitive to industrial processing temperatures. A significant amount of safety and characterization data for the EPSPS ACE5 protein has been generated, which indicates this protein is not toxic or allergenic. The enzyme was isolated from a common soil bacterium, which is not known as a source of allergens or toxins. The recipient crop, *Zea mays*, is a crop widely used for food and feed and is unlikely to have any pathogenic, toxic, allergenic or other adverse effects in humans or animals.

The enzyme kinetics of the native maize EPSPS, the native EPSPS GRG23, and the EPSPS ACE5 proteins were evaluated and the *Km* and *Ki* values for the enzymes determined. While all three enzymes bind PEP with similar affinity, the native EPSPS GRG23 and EPSPS ACE5 proteins bind only weakly to glyphosate (*Ki* is over 900-fold higher than *Km*) and therefore will preferentially bind PEP even at very high glyphosate concentrations. This suggests that EPSPS ACE5 protein expression in maize can substantially reduce glyphosate-induced toxicity. In contrast, the native maize EPSPS enzyme binds to glyphosate with 90-fold tighter affinity than PEP, and is therefore inhibited by much lower concentrations of the herbicide. It should be noted that the *Km* value for the EPSPS ACE5 protein is similar to published literature values for other bacterial EPSPS enzymes.

The EPSPS ACE5 protein has no significant sequence homology to known allergens or toxins. It is readily digestible when subjected to simulated gastric fluid resulting in complete degradation in less than 30 seconds and has not been glycosylated *in planta*. This enzyme is completely inactivated by a thirty-minute treatment at 65°C, indicating that the EPSPS ACE5 protein would be inactivated with typical heat processing or cooking procedures. The protein expression level in various tissues of event VCO-Ø1981-5 plants harvested over an entire growing season, were determined and the pattern of expression was as expected given the genetic elements of the transformation cassette (*e.g.,* expression in maize grain was below the LOD). Additionally, an acute toxicity study has been conducted with the EPSPS ACE5 protein used as the test article in mice. The toxicity study was conducted using the maximum allowable total dose (*e.g.,* 2000 mg/kg) of a highly pure starting material and indicated no evidence of toxicity. In conclusion, the data described in this section indicate that the EPSPS ACE5 protein is likely safe for consumption and would pose little or no risk to human or animal health.

VII. AGRONOMIC AND PHENOTYPIC EVALUATION

VII.A. History of field activities

Event VCO-Ø1981-5 has been field tested in typical maize growing regions of the US and in winter nurseries in Puerto Rico since 2007. Table 12 lists the field trials and associated USDA notifications (see Appendix 1 for field trial reports from 2007-2009). Table 13 provides a list of field trials conducted in Europe and Canada.

Table 12. Summary of field trial activities

USDA notification #	Effective date	Type of trial	Number of locations planted	State
07-283-104n	12/03/2007	Efficacy	1	PR
08-059-104n	04/08/2008	Efficacy	1	IA
08-067-108n	03/20/2008	Efficacy	1	PR
08-073-108n	04/242008	Efficacy	1	IA
08-105-109n	05/08/2008	Efficacy	1	NE
08-254-111n	09/26/2008	Breeding	1	PR
08-280-103n	10/23/2008	Breeding	1	PR
08-309-103n	11/10/2008	Breeding	1	PR
09-047-108n	02/24/2009	Breeding	1	PR
09-091-101n	04/23/2009	Regulatory	19	IL, IN, SD, IA, NE, MN, WI
09-086-104n	04/14/2009	Efficacy	2	IA, IN
09-215-106n	08/22/2009	Breeding	1	PR
09-341-105n	12/11/2009	Breeding	1	PR
10-085-109n*	04/26/2010	Regulatory	3	IA, MN, WI
10-071-113n*	04/01/2010	Breeding	1	PR
11-013-102n*	02/11/2011	Breeding	1	PR

*; reports pending

Table 13. Summary of field trial activities outside the US

Field trial reference #	Effective date	Type of trial	Number of locations planted	Year of planting	Country
B/ES/09/01	03/13/2009	Efficacy	1	2009	Spain
B/ES/09/01	04/07/2009	Efficacy	1	2009	Spain
B/CZ/09/04	04/28/2010*	Regulatory	3	2010	Czech Republic
B/SK/10/03	04/16/2010*	Regulatory	2	2010	Slovak Republic
B/ES/10/47	05/17/2010	Regulatory	1	2010	Spain
B/ES/10/47	04/29/2010	Regulatory	1	2010	Spain
B/ES/10/47	05/12/2010	Regulatory	2	2010	Spain
B/RO/10/04	06/14/2010*	Regulatory	1	2011	Romania
B/CZ/09/04	04/28/2010*	Regulatory	3	2011	Czech Republic
B/SK/10/03	04/16/2010*	Regulatory	3	2011	Slovak Republic
B/ES/11/16	03/14/2011	Regulatory	3	2011	Spain
B/ES/11/16	04/04/2011	Regulatory	1	2011	Spain
B/ES/11/16	06/24/2011	Regulatory	2	2011	Spain
11-VMC1-426-COR	04/15/2011	Regulatory	2	2011	Canada

*Valid for five years

VII.B. Agronomic and phenotypic evaluation

Maize lines containing the *epsps grg23ace5* gene have been shown to be tolerant to applications of the broad-spectrum herbicide glyphosate. Transformation events were first tested under greenhouse and field environments to assess the level of tolerance to glyphosate. As part of the evaluation process, events were grown under field conditions to compare plant morphology and growth characteristics to the non-transgenic near-isogenic hybrid (non-transgenic control). Agronomic performance evaluations were conducted on event VCO-Ø1981-5 as compared to the non-transgenic control. These evaluations included both multi-location field trials and laboratory experiments aimed at determining if the GM crop has unanticipated effects that would render it phenotypically different from the appropriate non-transgenic control. While these field trials focused on agronomic performance, regular and frequent observations on non-target effects to weeds, insects and disease susceptibility were also evaluated.

Field trials in 2007 and 2008 were primarily focused on event selection and only a brief summary of the agronomic data from a single location in 2008 is provided here. Additional agronomic evaluations were carried out in several different locations in the 2009 growing season. The agronomic evaluations were conducted by scientists with

expertise in the production and evaluation of maize. Laboratory testing for germination under warm and cold conditions was also performed.

In 2009, a total of 17 field sites were utilized across six US states to collect agronomic data along with samples for other regulatory studies. The morphological and growth characteristics of event VCO-Ø1981-5 were evaluated in two genetic backgrounds, and compared to their respective non-transgenic near-isogenic hybrids (non-transgenic controls). Hybrids were characterized under diverse environmental and growing conditions representative of typical maize production in North America.

VII.C. Agronomic performance of event VCO-Ø1981-5

VII.C.1. Agronomic evaluation of event VCO-Ø1981-5 maize in 2008

The field trial conducted in 2008 was located in Polk County, Iowa, USA. Agronomic practices used were representative for maize production in highly productive soils in Central Iowa. As the test event is a segregating plant from the first backcross generation (BC1B, Figure 3), the plots were sprayed with a glyphosate solution (5lbs glyphosate per gallon or 600 g/L) to eliminate the negative segregants. The sprayer output rate was calibrated to apply 10.7 gallons of glyphosate solution per acre (100 L/ha) at a speed of 3.6 mph (5.8 km/h).

Parameters for the 2008 field trial focused on event selection and evaluated seedling and germination qualities, vegetative and reproductive parameters, and grain quality. The agronomic characteristics evaluated were: days to emergence, 50% pollen shed, 50% silking, 50% black layer, ear and plant height, ear girth and length, number of kernel rows/ear, kernels/row, kernel weight, pollen weight, and number of tassel branches. Four replicates were grown at a single location in 2008.

Overall, event VCO-Ø1981-5 performed similarly to the non-transgenic control. All data comparisons between event VCO-Ø1981-5 and the non-transgenic control were carried out using a two-sided Dunnett's analysis (Moore and McCabe, 1999). For all 13 of the agronomic traits no biologically significant differences were observed between the two groups. Event VCO-Ø1981-5 plants were phenotypically indistinguishable from the non-transgenic control in every measurement taken in the trial.

VII.C.2. Agronomic evaluation of VCO-Ø1981-5 maize in 2009

For the 2009 field trials, the back-cross zero (BC0B) generation was selfed two times to produce BC0BS2 (Figure 3). In order to evaluate agronomic performance characteristics of Event VCO-Ø1981-5 as compared to an appropriate non-transgenic control, two test-crosses were made and seed bulked for multiple location analysis in 2009 (Bernard and MacIntosh, 2011d). The test lines shown in Table 14 are maize hybrids of two genetic backgrounds: B116 (Committee for Agricultural Development, Iowa State University) and CH01 (private inbred line) containing the GM event or the non-transgenic near-isogenic comparator (non-transgenic control). Each of these hybrids was tested at each of the 17 separate locations.

Table 14. Maize hybrids tested in agronomic evaluations in 2009

Hybrid Tested	Pedigree of the hybrids
Event VCO-Ø1981-5 hybrid	BC0BS2 VCO-Ø1981-5 X B116 (BC0BS2B)
Non-transgenic near-isogenic hybrid	BC0BS2 negative segregant X B116 (BC0BS2B)
Event VCO-Ø1981-5 hybrid	BC0BS2 VCO-Ø1981-5 X CH01 (BC0BS2CH)
Non-transgenic near-isogenic hybrid	BC0BS2 negative segregant X CH01 (BC0BS2CH)

VII.C.2.1. Evaluation parameters, analysis and statistics

All trials were conducted using a randomized complete block design, with three replicated plots of each entry per location. Weed control was limited to conventional and cultural practices (hand hoeing); no broad-spectrum herbicides, such as glyphosate or glufosinate, were allowed except as a pre-plant or pre-emergence herbicide.

Typical agronomic evaluations used for maize were conducted (Table 15). Means for the event were compared to the appropriate non-transgenic control using a two-sided Dunnett's test with alpha = 0.05 (Moore and McCabe, 1999). The standard deviation and range of values collected across all locations are also reported (Tables 16 and 17).

Table 15. Traits evaluated in the 2009 field trials

General Characteristic	Trait	Growth Stage	Description	Scale
Emergence	Emergence	V2 - V4	Plant emergence	Percentage of plants to emerge after germination
Vegetative characteristics	Plant height	Maturity	Five plants per plot. Measure from ground to base of flag leaf.	Measured in inches
	Ear height	Maturity	Five plants per plot. Measure from ground to point of attachment of primary ear.	Measured in inches
	Final stand count	Pre-harvest	Total number of plants in middle two rows.	Number of plants
	Stalk lodging	Pre-harvest	Plants broken below ear.	Percentage of total
	Root lodging	Pre-harvest	Plants leaning at soil surface greater than 30° from vertical.	Percentage of total
	Grain moisture	Harvest	Moisture content of harvested, shelled grain.	Percent moisture
	Grain weight	Harvest	Total raw weight of grain harvested.	Pounds per plot
Reproductive parameters	Days to 50% pollen shed	Pollen-shed	Number of days from planting to the day 50% of plants are shedding pollen.	Number of days
	Days to 50% silking	Silking	Days from planting to the day 50% of the plants have silks emerged from the primary ear.	Number of days
	Dropped ears	Pre-harvest	Ears on the ground, no longer attached to the stalk.	Percentage of total
	Barrenness	Pre-harvest	Plants with no ears.	Percentage of total
	Yield	Harvest	Yield corrected to 15% moisture.	Bushels per acre

Evaluation Details for Certain Parameters

Pollination Data: Plots were evaluated daily during the pollination and tassel formation. Plots were considered at 50% pollen shed when 50% of the plants in each plot had dehisced anthers. Similarly, plots were recorded to be at 50% silking when 50% of the plants had emerged silks on the primary ear.

Plant and Ear Heights: Plant and ear heights were measured according to maize growing stage. Plant heights were measured from ground level to the base of the flag leaf. Ear heights were measured from the ground level to the base of the primary ear shank.

VII.C.2.2. Field locations, preparations and conditions

A total of 17 field sites were utilized in 2009 across six US states to collect agronomic data along with samples for other regulatory studies, such as compositional analysis. The locations include diverse environments within the major maize growing regions of the US. Off-season locations in Puerto Rico were used for breeding purposes (Table 12). The agronomic practices were representative of the location in, which the field trials were conducted.

VII.C.2.3. Results of agronomic performance evaluation

Vegetative and reproductive characteristics

As outlined in Table 15, a range of different vegetative and reproductive agronomic characteristics were measured across the 17 locations in the 2009 field trials. Ranges, standard deviations and p-values were recorded and calculated across all the locations (Tables 16 and 17).

With only minor exceptions, the evaluation of vegetative agronomic characteristics showed no statistical differences between event VCO-Ø1981-5 and its non-transgenic near-isogenic comparator (non-transgenic control). The grain moisture was slightly higher for the non-transgenic control than event VCO-Ø1981-5, but the ranges were large and overlapping, and the standard deviations also overlapped. In the B116 genetic background, differences were measured in the final stand count where the non-transgenic control had slightly less plants as compared to event VCO-Ø1981-5, but for the CH01 background there were no statistical differences in stand count.

There were no statistically significant differences in any of the reproductive measurements, with the exception of barrenness for CH01 background, but the numerical values were so low that these statistics are not meaningful (*e.g.*, the range is many times larger than the mean value).

Table 16. Vegetative characteristics

Agronomic characteristic (unit)	Genetic background	VCO-Ø1981-5 hybrid	Non-transgenic control	
Plant height (inches)	B116	116.9 ± 14.7	113.5 ± 14.2	Mean ± St Dev
		75.4-123.3	71.3-122.1	Range
		0.7918		p-value
	CH01	110.4 ± 12.1	106.8 ± 13.5	Mean ± St Dev
		77.1-128.7	63.8-130.7	Range
		0.7632		p-value
Ear height (inches)	B116	63.3 ± 7.7	59.9 ± 6.9	Mean ± St Dev
		34.7-65.7	33.2-61.1	Range
		0.4040		p-value
	CH01	56.8 ± 6.7	52.2 ± 7.4	Mean ± St Dev
		35.6-58.3	22.2-54.5	Range
		0.2095		p-value
Final stand count (# plants)	B116	48.8 ± 8.1	43.9 ± 9.2	Mean ± St Dev
		39.7-72	34.7-72	Range
		0.0067		p-value
	CH01	48.1 ± 7.7	45.6 ± 9.6	Mean ± St Dev
		38.0-72	36.3-72	Range
		0.1797		p-value
Stalk lodging (% of total)	B116	3.5 ± 3.0	3.1 ± 4.2	Mean ± St Dev
		0-10.0	0-12.9	Range
		0.6302		p-value
	CH01	4.5 ± 3.9	6.0 ± 7.3	Mean ± St Dev
		0-12.3	0-23.5	Range
		0.4118		p-value
Root lodging (% of total)	B116	12.4 ± 21.9	9.1 ± 21.6	Mean ± St Dev
		0-83.3	0-83.3	Range
		0.4992		p-value
	CH01	2.0 ± 4.0	0.8 ± 2.0	Mean ± St Dev
		0-15.4	0-8.1	Range
		0.1632		p-value
Grain moisture (moisture in % fw)	B116	25.3 ± 3.6	27.1 ± 5.3	Mean ± St Dev
		17.8-31.0	19.0-36.7	Range
		0.0923		p-value
	CH01	21.7 ± 3.9	24.2 ± 4.6	Mean ± St Dev
		16.5-29.2	14.4-29.5	Range
		0.0318		p-value
Grain weight (pounds per plot)	B116	19.5 ± 5.4	18.2 ± 4.7	Mean ± St Dev
		5.2-26.4	5.5-26.8	Range
		0.2292		p-value
	CH01	19.9 ± 5.2	18.6 ± 5.8	Mean ± St Dev
		6.2-27.5	3.9-28.0	Range
		0.3662		p-value

Table 17. Reproductive parameters

Agronomic characteristic	Genetic background	VCO-Ø1981-5 hybrid	Non-transgenic control	
Days to 50% pollen shed (# days)	B116	72.6 ± 8.6	73.3 ± 8.5	Mean ± St Dev
		59.0-94.0	59.0-92.7	Range
		0.6978		p-value
	CH01	72.3 ± 8.4	72.7 ± 8.8	Mean ± St Dev
		58.0-92.7	57.3-93.7	Range
		0.7365		p-value
Days to 50% silking (# days)	B116	74.6 ± 8.8	75.0 ± 8.2	Mean±St Dev
		59.7-96.3	59.3-94.7	Range
		0.8087		p-value
	CH01	72.6 ± 9.1	72.6 ± 9.2	Mean ± St Dev
		57.7-95.3	56.7-95.7	Range
		0.9003		p-value
Dropped ears (% of total)	B116	0.1 ± 0.3	0.2 ± 0.7	Mean ± St Dev
		0-0.7	0-2.6	Range
		0.5282		p-value
	CH01	0.4 ± 1.0	0.2 ± 0.5	Mean ± St Dev
		0-3.5	0-2.0	Range
		0.2648		p-value
Barrenness (% of total)	B116	1.3 ± 2.5	2.2 ± 3.5	Mean ± St Dev
		0-8.9	0-11.8	Range
		0.2497		p-value
	CH01	1.0 ± 1.0	4.0 ± 6.8	Mean ± St Dev
		0-5.5	0-25.1	Range
		0.0167		p-value
Yield (bushels per acre)	B116	143.0 ± 45.4	130.7 ± 39.4	Mean ± St Dev
		40.8-209.5	42.8-219.9	Range
		0.1584		p-value
	CH01	150.4 ± 38.9	137.9 ± 40.1	Mean ± St Dev
		58.7-224.5	31.8-208.0	Range
		0.1896		p-value

Emergence

Emergence data from the 2009 field trials are shown in Table 18. While the ranges for percent emergence overlap well between event VCO-Ø1981-5 and the non-transgenic control, means for the non-transgenic control are slightly lower. These differences in emergence may be attributed to the seed quality of the non-transgenic control. Since there was considerably more event VCO-Ø1981-5 seed than non-transgenic control seed produced in the winter nursery in Puerto Rico, the highest quality seed could be selected for the transgenic plots. For the non-transgenic comparator (non-transgenic control), some lower quality seed had to be used, in order to have sufficient seed for all 17 field locations, which may have impacted the emergence values. At 12 of the 17 locations, the emergence was numerically higher for event VCO-Ø1981-5 seeds, while 5 of the locations had split data (i.e., one background showed higher or equal germination than the non-transgenic control, and the other background showed lower germination than the non-transgenic control). The emergence was numerically lower than typically

(Tang et al., 2000) observed (65-78%), regardless of the genetic background or the presence or absence of the herbicide tolerance trait. Despite the statistically significant differences , the ranges for the percent emergence for the VCO-Ø1981-5 hybrids in both B116 and CH01 backgrounds (55.7 – 96.4 and 49.3-100) overlapped with the ranges for the respective non-transgenic controls (46.1-82.1 and 52.6-85.2). For all ranges, the minimum and maximum values were also spread out over a wide range of values. The same observation applies to the standard deviations, as they are relatively high in relation to their respective means and thus indicate that values had a wide distribution. Considering the wide variation observed for all the entries tested, regardless of genetic background or presence/absence of the introduced trait, the statistically significant differences based on the p-values are therefore not biologically significant.

Table 18. Emergence

Agronomic characteristic (unit)	Generation/Genetic background	VCO-Ø1981-5 hybrid	Non-transgenic control	
Emergence (%)	BC0BS2B/B116	77.8 ± 12.4	65.4 ± 10.6	Mean ± SD
		55.7-96.4	46.1-82.1	Range
		0.001		p-value
	BC0BS2CH/CH01	76.6 ± 13.9	68.9 ± 11.5	Mean ± SD
		49.3-100	52.6-85.2	Range
		0.0052		p-value

VII.D. Biotic and abiotic stress characteristics

Specific observations were recorded during the conduct of the 2009 agronomic performance field trials regarding ecological characteristics of event VCO-Ø1981-5 (BC0BS2 in B116 and CH01 backgrounds, Figure 3) and their respective non-transgenic controls. The rating scales for ecological evaluations are listed in Table 19. Late season disease ratings are summarized in Table 20 and the severity of a specific stressor is shown in Table 21. No differences between the event VCO-Ø1981-5 entries and the non-transgenic controls were identified.

Table 19. Disease rating scales for ecological evaluations

General characteristic	Trait	Growth Stage	Description	Scale
Ecological interactions	Late season disease rating	Maturity	Rating for disease pressure	% of leaf area with symptoms: 1 = no visible symptoms 2 = <1% of leaf area 3 = 1-5% of leaf area 4 = 6-20% of leaf area 5 = 21-50% of leaf area 6 = 51-75% of leaf area 7 = 76-90% of leaf area 8 = 91-99% of leaf area 9 = plant is dead
	Insect, disease, weed and abiotic stressors	Over season	Visual observations	Descriptive

Table 20. Disease ratings

Agronomic characteristic (unit)	Genetic background	VCO-Ø1981-5 hybrid	Non-transgenic control	
Late season disease rating (Scale 1-9)	B116	4	4	Mean
		2-6	2-6	Range
		0.8032		p-value
	CH01	4	4	Mean
		2-6	2-6	Range
		0.7805		p-value

Table 21. Ecological stressors

Stressor	State	County[1]	Severity[2]	Difference to non-transgenic control
Insects				
Fall armyworm *Spodoptera fugiperda*	IL	Clinton	Mild	No difference
Japanese beetles *Popillia japonica*	IL	Clinton	Mild	No difference
	WI	Walworth	Mild	No difference
European corn borer *Ostrinia nubilalis*	IL	Stark	Very mild	No difference
	MN	Stearns	Mild	No difference
	WI	Walworth	Mild	No difference
Aphids *Acyrthosiphon pisum*	MN	Stearns	Mild	No difference
Grasshoppers *Melanoplus* spp	MN	Stearns	Mild	No difference
Northern corn rootworm *Diabrotica barberi*	IA	Kossuth	Moderate	No difference
	IA	Wright	Mild	No difference
Black cutworm *Agrotis ipsilon*	IA	Wright	Mild	No difference
Common stalk borer *Papaipema nebris*	WI	Walworth	Mild	No difference
Western corn rootworm *Diabrotica virgifera virgifera*	WI	Walworth	Moderate	No difference
Diseases				
Gray leaf spot	IL	Clinton	Mild	No difference
	IL	Stark	Very mild	No difference
	MN	Stearns	Mild	No difference
	IA	Jasper	Mild	No difference

Table 21. Ecological stressors (continued)

Gray leaf spot	IA	Wright	Mild	No difference
	IA	Scott	Mild	No difference
	NE	Valley(1)	Mild	No difference
	NE	Valley(2)	Mild	No difference
Common rust	IL	Stark-=8	Mild to moderate	No difference
	IA	Scott	Mild	No difference
	NE	Valley(1)	Mild	No difference
	NE	Valley(2)	Mild	No difference
	WI	Walworth	Mild	No difference
Northern leaf blight	IA	Kossuth	Mild	No difference
Northern corn leaf spot	IA	Webster	Mild	No difference
Anthracnose	IA	Webster	Moderate	No difference
Common smut	IA	Wright	Mild	No difference
Abiotic				
Excess moisture	IL	Clinton	Severe	No difference
	IN	Parke	Moderate	No difference
	IA	Scott	Mild	No difference
Nitrogen deficiency	IA	Wright	Mild	No difference

[1] three replications per location
[2] based on visual, non-quantitative observations related to crop growth and development

VII.E. Seed dormancy evaluation

Seed dormancy and germination analysis were conducted using standard laboratory assays. Seeds of event VCO-Ø1981-5 and the non-transgenic control used in the germination studies were produced in the greenhouse and had a slightly different genetic background from the seed used to generate the emergence data from the 2009 field trials ($BC1B_1S2$, Figure 3). The warm germination test was conducted in accordance with the Association of Official Seed Analysts “Rules for Testing Seed” (AOSA, 2010).

The tetrazolium test was performed on the resulting ungerminated seeds at the end of each test to determine the percent non-viable seed. The germination and viability results provided below (Table 22) indicate that the germination and dormancy characteristics of event VCO-Ø1981-5 are not different as compared to the non-transgenic control.

Table 22. Seed germination

Line	Warm germination		Cold germination	
	Mean % germination	% Non-viable seed	Mean % germination	% Non-viable seed
Event VCO-Ø1981-5	99.6	0.4	97.0	3.0
Non-transgenic control	99.6	0.4	93.0	7.0

The laboratory studies show a high level of germination, with equivalent germination for the event VCO-Ø1981-5 and the non-transgenic control (99.6%) under warm conditions. A small difference in germination, 97% and 93%, respectively, was seen under cold conditions.

In conclusion, the observations and data collected from all the agronomic field trials conducted to evaluate agronomic performance and environmental effects found that event VCO-Ø1981-5 maize is equivalent to other maize hybrids with only a few minor differences. Event VCO-Ø1981-5 maize did not exhibit any plant pest characteristics.

VII.F. Composition analysis

VII.F.1. Introduction

Compositional analyses establish the nutritional value, and can identify any potential unintended effects of the transformation process on the plant composition, by measuring key nutrients, anti-nutrients and secondary plant metabolites. To evaluate the nutritional quality of event VCO-Ø1981-5, composition evaluations were carried out from field samples collected from five locations in three states in 2009 in the US (BC0BS2, Figure 3). As part of the evaluation of new maize varieties and to support the safety assessment, a range of compositional parameters were tested for event VCO-Ø1981-5 and compared to a non-transgenic near-isogenic hybrid (also referred to as the non-transgenic or negative control) and three commercial maize hybrids, representing the natural variation found in maize hybrids. The commercial hybrids were selected for their adaption to major US maize growing regions. Hybrids Ag5539 (RM: 105 days) and Ag58036 (RM: 103) are well adapted to the upper Midwest region. Hybrid Ag7584 (RM: 113) is well adapted to the lower Midwest. Hybrids Ag58036 and Ag7584 are currently sold in the US. Hybrid AgR5539 was last sold in 2010.

Detailed compositional analyses of 71 analytes were conducted for forage and grain samples of event VCO-Ø1981-5, a non-transgenic control and three reference hybrid varieties using standard methods. The analytes evaluated are the standard parameters by which maize varieties are assessed and are outlined in the OECD Consensus Document on Compositional Considerations for New Varieties of Maize (*Zea mays*) (OECD, 2002).

Results are presented and compared to the ranges of the references material grown, harvested, and analyzed simultaneously with the test material and to the published

values (ILSI, 2007) for nutritional content of maize forage (Table 23) and grain (Tables 24-29).

VII.F.2. Seed genetics, field trial design, and sampling

Seeds of event VCO-Ø1981-5 and the non-transgenic control had the same genetic background in order to better compare the nutritional data: BC0BS2 x B116 and BC0BS2 VCO-Ø1981-5 x B116.

Plant material derived from event VCO-Ø1981-5 and the non-transgenic control was produced and collected at five field locations across the US Corn Belt in 2009. The reference hybrids (AgR5539, AgR7584, and AgR58036) were handled the same as the event VCO-Ø1981-5 hybrids and their respective non-transgenic control. Data from the reference hybrids is used in conjunction with published data to help determine normal ranges and variability within the measured parameters.

VII.F.3. Analytical methods

The analytical methods were AOAC, AACC and AOCS International Methods or published methods as detailed in Appendix 2, Section 2.F.

VII.F.4. Nutritional composition

VII.F.4.1. Overview of the analysis

Detailed compositional analysis was conducted in accordance with the OECD Consensus Document on Compositional Considerations for New Varieties of Maize (*Zea mays*) (OECD, 2002; Bernard and MacIntosh, 2011e). Compositional analyses of forage samples included protein, fat, acid detergent fiber (ADF), neutral detergent fiber (NDF), crude fiber, ash, calcium, phosphorus, and carbohydrates. Compositional analyses of grain samples included protein, fat, ADF, NDF, crude fiber, ash, carbohydrates, fatty acids, amino acids, vitamins, minerals, key anti-nutrients, and secondary metabolites.
.
Data means, standard deviations, ranges and p-values were determined for the compositional data. Data were analyzed using JMP 8 software (SAS Institute, Cary, NC) and the equation Yij = U + Ti + Lj + LTij + eij. For each analyte an ANOVA model was fit using the site and line main effects and the site by line interaction. P-values (≤0.05%) were not reported where the means were below the LOD, or where missing data made the effect non-testable. When data points were at or below the LOQ, the LOQ value was used to calculate the averages, standard deviations and data ranges. The range of determined values for each of the analytes for the reference lines is also reported.

VII.F.4.2. Analysis of proximates and minerals in maize forage

The levels of proximates were measured in forage samples of event VCO-Ø1981-5, the non-transgenic control, and three reference hybrids. The results are reported in Table 23. No statistically significant differences were observed between event VCO-Ø1981-5 and non-transgenic control (p-values were >0.05).

Table 23. Proximates and minerals in event VCO-Ø1981-5 forage

Analyte (% of dry weight)		VCO-Ø1981-5 hybrid	Non-transgenic control	Range	
				Reference hybrids	Published[1]
Protein	Mean ± SD	6.1 ± 1.3	6.2 ± 1.6	3.6-9.1	3.9-11.6
	Range	3.9-8.3	3.4-8.9		
	p-value	0.640			
Fat	Mean ± SD	2.0 ± 0.3	2.1 ± 0.6	1.3-3.5	0.3-4.1
	Range	1.4-2.7	1.1-3.2		
	p-value	0.499			
Acid detergent fiber	Mean ± SD	34.3 ± 3.6	32.2 ± 3.1	19.1-41.0	18.8-47.4
	Range	27.8-43.0	23.0-35.6		
	p-value	0.168			
Neutral detergent fiber	Mean ± SD	55.5 ± 4.1	52.9 ± 4.6	34.4-61.8	26.4-57.9
	Range	49.5-64.6	40.8-61.0		
	p-value	0.125			
Crude fiber	Mean ± SD	28.0 ± 3.8	26.6 ± 1.6	14.8-32.5	n/a
	Range	22.8-38.3	23.6-30.6		
	p-value	0.234			
Ash	Mean ± SD	5.6 ± 0.8	5.4 ± 0.7	3.9-8.0	1.5-6.8
	Range	4.4-7.0	4.7-7.0		
	p-value	0.236			
Calcium	Mean ± SD	0.25 ± 0.04	0.25 ± 0.03	0.14-0.38	0.07-0.58
	Range	0.18-0.35	0.21-0.30		
	p-value	0.935			
Phosphorus	Mean ± SD	0.19 ± 0.04	0.19 ± 0.04	0.12-0.29	0.09-0.32
	Range	0.14-0.27	0.12-0.26		
	p-value	0.629			
Carbohydrates (calculated)	Mean ± SD	86.3 ± 1.9	86.3 ± 2.2	81.0-89.9	80.8-92.1
	Range	83.5-89.3	82.2-90.4		
	p-value	0.928			

[1] ILSI (2006) Crop Composition Database (Version 3.0)
n/a; not available

VII.F.4.3. Analysis of proximates in maize grain

The levels of proximates were measured in grain samples of event VCO-Ø1981-5, the non-transgenic control, and three reference hybrids. The results are reported in Table 24. No statistically significant differences were observed between event VCO-Ø1981-5 and the non-transgenic control (p-values were >0.05).

Table 24. Proximates in event VCO-Ø1981-5 grain

Analyte (% of fresh weight)		VCO-Ø1981-5 hybrid	Non-transgenic control	Range	
				Reference hybrids	Published[1]
Moisture	Mean ± SD	12.6 ± 1.6	13.0 ± 2.1	9.7-15.8	6.9-21.4
	Range	10.1-15.0	10.2-17.5		
	p-value	0.297			
Protein	Mean ± SD	10.3 ± 1.2	11.0 ± 1.2	5.6-13.0	6.7-14.7
	Range	7.1-11.8	7.4-12.3		
	p-value	0.227			
Fat	Mean ± SD	4.4 ± 0.9	4.4 ± 0.8	2.5-7.0	2.5-5.3
	Range	2.6-5.3	2.8-5.4		
	p-value	0.990			
Acid detergent fiber	Mean ± SD	3.5 ± 0.7	3.3 ± 0.7	2.3-4.2	1.8-11.3
	Range	2.3-4.4	2.0-4.1		
	p-value	0.154			
Neutral detergent fiber	Mean ± SD	12.0 ± 1.0	11.9 ± 1.1	7.4-15.0	6.2-20.6
	Range	10.6-14.4	10.1-14.3		
	p-value	0.716			
Crude fiber	Mean ± SD	2.8 ± 0.4	2.8 ± 0.6	1.4-3.5	1.4-3.3
	Range	1.5-3.4	1.1-3.3		
	p-value	0.562			
Total dietary fiber	Mean ± SD	9.9 ± 0.8	10.1 ± 0.7	7.5-11.1	9.0-35.3
	Range	8.5-11.4	8.6-11.6		
	p-value	0.289			
Starch	Mean ± SD	68.2 ± 1.6	67.0 ± 1.7	64.1-74.5	26.5-73.8
	Range	66.1-70.8	64.8-71.0		
	p-value	0.090			
Ash	Mean ± SD	1.3 ± 0.06	1.3 ± 0.04	1.1-1.7	0.6-6.3
	Range	1.2-1.4	1.2-1.4		
	p-value	0.373			
Carbohydrates (calculated)	Mean ± SD	84.0 ± 1.8	83.3 ± 1.8	79.3-90.5	77.4-89.5
	Range	82.0-88.7	81.9-88.5		
	p-value	0.282			

[1] ILSI (2006) Crop Composition Database (Version 3.0)

VII.F.4.4. Analysis of fatty acids in maize grain

The levels of twenty-four fatty acids were measured in event VCO-Ø1981-5 grain, the non-transgenic control, and three reference hybrids. The levels of the following fatty acids were below the level of quantitation for the assays used (data not shown): caprylic (C8:0), capric (C10:0), myristic (C14:0), myristoleic (C14:1), pentadecanoic (C15:0), pentadecenoic (C15:1), heptadecanoic (17:0), heptadecenoic (C17:1), gamma linolenic (C18:3), eicosadienoic (C20:2), eicosatrienoic (C20:3), arachidonic (C20:4), and erucic (C22:1). Results for those fatty acids that were measurable are shown in Table 25. In the majority of fatty acids, for which levels could be evaluated, no statistically significant differences were observed between event VCO-Ø1981-5 and the non-transgenic control (p-values were ≥0.05).

A statistical difference was observed for the analyte linoleic acid. The calculated means for both the event VCO-Ø1981-5 and the non-transgenic control were slightly above the data ranges for the three commercial varieties and the ILSI composition database. However, event VCO-Ø1981-5 values were lower than the non-transgenic control and closer to the commercial and published data ranges. For the non-essential fatty acid analytes palmitic and eicosenoic acid, the p-values determined showed a statistical difference in the calculated means; however, the standard deviations and ranges overlap, and the means and data ranges fell within the reference and published data ranges for those analytes. Therefore, it is unlikely that these differences indicate any biological significance.

Table 25. Fatty acids in event VCO-Ø1981-5 grain

Analyte (% Total fatty acids)		VCO-Ø1981-5 hybrid	Non-transgenic control	Range	
				Reference hybrids	Published[1]
Lauric (C12:0)	Mean ± SD	0.06 ± 0.6	0.06 ± 0.5	0-0.18	ND-0.3
	Range	0-0.15	0-0.130		
	p-value	0.802			
Palmitic (C16:0)	Mean ± SD	11.04 ± 0.16	10.80 ± 0.26	8.36-12.62	8.57-17.46
	Range	10.65-11.25	10.40-11.32		
	p-value	**0.016**[2]			
Palmitoleic (C16:1)	Mean ± SD	0.15 ± 0.01	0.14 ± 0.01	0-0.14	0.11-0.26
	Range	0.13-0.16	0.13-0.16		
	p-value	0.182			
Stearic (C18:0)	Mean ± SD	1.99 ± 0.18	2.05 ± 0.15	1.77-2.70	1.02-2.86
	Range	1.67-2.29	1.76-2.25		
	p-value	0.058			
Oleic (C18:1)	Mean ± SD	19.88 ± 1.16	19.06 ± 0.99	21.91-33.36	17.4-38.5
	Range	18.39-21.79	17.55-20.93		
	p-value	0.076			
Linoleic (C18:2)	Mean ± SD	64.76 ± 1.30	65.79 ± 1.20	51.61-62.64	47.7-65.6
	Range	62.37-66.34	63.42-67.70		
	p-value	**0.012**[2]			
Linolenic (C18:3)	Mean ± SD	1.12 ± 0.11	1.16 ± 0.11	0.950-1.60	0.77-2.25
	Range	0.97-1.29	1.00-1.37		
	p-value	0.111			
Arachidic (C20:0)	Mean ± SD	0.44 ± 0.03	0.44 ± 0.21	0.38-0.58	0.34-0.57
	Range	0.41-0.49	0.40-0.47		
	p-value	0.650			
Eicosenoic (C20:1)	Mean ± SD	0.21 ± 0.01	0.20 ± 0.01	0.23-0.35	0.17-0.45
	Range	0.20-0.24	0.18-0.23		
	p-value	**0.025**[2]			
Behenic (C22:0)	Mean ± SD	0.09 ± 0.09	0.06 ± 0.08	0-0.20	0.11-0.29
	Range	0-0.18	0-0.17		
	p-value	0.258			
Lignoceric (C24:0)	Mean ± SD	0.24 ± 0.03	0.23 ± 0.03	0-0.28	0.14-0.23
	Range	0.20-0.30	0.19-0.33		
	p-value	0.704			

[1] ILSI (2006) Crop Composition Database (Version 3.0)
[2] $p \leq 0.05$ indicates a statistical difference

VII.F.4.5. Analysis of amino acids in maize grain

The levels of essential and non-essential amino acids were measured in grain samples of event VCO-Ø1981-5, the non-transgenic control, and three reference hybrids. The results are reported in Table 26. In the majority of amino acids evaluated, no statistically significant differences were observed between event VCO-Ø1981-5 and the non-transgenic control (p-values were >0.05).

For the analytes arginine, phenylalanine, and tyrosine the p-values showed a statistical difference in the calculated means; however, the ranges and standard deviations overlap, and the means and data ranges fell within the reference and published data ranges for these analytes. Therefore, it is unlikely that these differences indicate any biological significance.

Table 26. Amino acids in event VCO-Ø1981-5 grain

Analyte (% dry weight)		VCO-Ø1981-5 hybrid	Non-transgenic control	Range	
				Reference hybrids	Published[1]
Alanine	Mean ± SD	0.796 ± 0.111	0.865 ± 0.112	0.378-1.097	0.489-1.20
	Range	0.527-0.934	0.529-1.003		
	p-value	0.164			
Arginine	Mean ± SD	0.395 ± 0.034	0.429 ± 0.038	0.267-0.524	0.119-0.637
	Range	0.307-0.445	0.334-0.479		
	p-value	**0.048**[2]			
Aspartic acid	Mean ± SD	0.749 ± 0.099	0.789 ± 0.099	0.381-0.998	0.335-0.963
	Range	0.478-0.907	0.521-0.906		
	p-value	0.344			
Cystine	Mean ± SD	0.128 ± 0.031	0.132 ± 0.022	0.071-0.216	0.125-0.325
	Range	0.070-0.173	0.071-0.165		
	p-value	0.690			
Glutamic acid	Mean ± SD	2.125 ± 0.297	2.320 ± 0.291	0.986-2.963	0.965-3.12
	Range	1.379-2.489	1.465-2.699		
	p-value	0.153			
Glycine	Mean ± SD	0.389 ± 0.030	0.400 ± 0.024	0.276-0.447	0.184-0.498
	Range	0.312-0.432	0.349-0.439		
	p-value	0.429			
Histidine	Mean ± SD	0.311 ± 0.030	0.330 ± 0.036	0.176-0.363	0.137-0.416
	Range	0.239-0.353	0.252-0.407		
	p-value	0.200			
Isoleucine	Mean ± SD	0.374 ± 0.047	0.411 ± 0.047	0.192-0.494	0.179-0.568
	Range	0.251-0.432	0.273-0.472		
	p-value	0.083			
Leucine	Mean ± SD	1.316 ± 0.210	1.465 ± 0.211	0.534-1.802	0.654-2.100
	Range	0.778-1.584	0.843-1.687		
	p-value	0.093			

Table 26. Amino acids in event VCO-Ø1981-5 grain (continued)

Analyte (% of dry weight)		VCO-Ø1981-5 hybrid	Non-transgenic control	Range	
				Reference hybrids	Published[1]
Lysine	Mean ± SD	0.305 ± 0.036	0.314 ± 0.044	0.197-0.418	0.172-0.597
	Range	0.257-0.372	0.260-0.378		
	p-value	0.524			
Methionine	Mean ± SD	0.128 ± 0.033	0.132 ± 0.019	0.065-0.200	0.129-0.326
	Range	0.075-0.184	0.077-0.148		
	p-value	0.653			
Phenylalanine	Mean ± SD	0.525 ± 0.075	0.587 ± 0.081	0.236-0.702	0.244-0.809
	Range	0.334-0.622	0.352-0.676		
	p-value	**0.050**[2]			
Proline	Mean ± SD	0.957 ± 0.132	1.021 ± 0.110	0.460-1.177	0.462-1.34
	Range	0.630-1.132	0.689-1.134		
	p-value	0.207			
Serine	Mean ± SD	0.497 ± 0.056	0.523 ± 0.060	0.272-0.644	0.254-0.728
	Range	0.345-0.560	0.361-0.600		
	p-value	0.171			
Threonine	Mean ± SD	0.372 ± 0.038	0.391 ± 0.037	0.225-0.445	0.231-0.666
	Range	0.273-0.420	0.289-0.441		
	p-value	0.253			
Tryptophan	Mean ± SD	0.075 ± 0.008	0.076 ± 0.005	0.050-0.097	0.027-0.090
	Range	0.060-0.087	0.063-0.082		
	p-value	0.656			
Tyrosine	Mean ± SD	0.234 ± 0.036	0.282 ± 0.081	0.135-0.337	0.103-0.534
	Range	0.165-0.281	0.157-0.441		
	p-value	**0.008**[2]			
Valine	Mean ± SD	0.509 ± 0.061	0.539 ± 0.050	0.275-0.629	0.266-0.723
	Range	0.351-0.578	0.394-0.595		
	p-value	0.222			

[1] ILSI (2006) Crop Composition Database (Version 3.0)
[2] $p \leq 0.05$ indicates a statistical difference

VII.F.4.6. Analysis of vitamins in maize grain

The levels of vitamins were measured in grain samples of event VCO-Ø1981-5, the non-transgenic control, and three reference hybrids. The results are reported in Table 27. For the majority of vitamins evaluated, no statistically significant differences were observed between event VCO-Ø1981-5 and the non-transgenic control (p-values were >0.05).

For the analytes beta carotene, vitamin B3, and total tocopherols, the p-values determined showed a statistical difference in the calculated means; however, the standard deviations and data ranges overlapped.

For total tocopherols, the means and the standard deviations are calculated on total tocopherols determined for each genotype from three replicates for each of the five field locations. The ranges reported in Table 27 are the minimum and the maximum results reported for the three replicates in all locations. As shown in Table 28 below, the standard deviation ranges overlap for the three entries, even though the mean for the non-transgenic control is below the value for the Mean-SD (26.5) of VCO-Ø1981-5 event. All means fell within the reference and published data ranges for these analytes. Therefore, it is unlikely that these differences indicate any biological significance.

Table 27. Vitamins in event VCO-Ø1981-5 grain

Analyte (mg/kg of dry weight)		VCO-Ø1981-5 hybrid	Non-transgenic control	Range	
				Reference hybrids	Published[1]
Beta carotene	Mean ± SD	4.79 ± 1.30	3.97 ± 0.71	2.50-15.62	0.53-17.03
	Range	2.50-6.42	3.11-5.43		
	p-value	**0.0072[2]**			
Vitamin B1	Mean ± SD	3.45 ± 0.62	3.24 ± 0.36	2.41-4.27	2.51-40.0
	Range	2.53-4.46	2.69-4.03		
	p-value	0.221			
Vitamin B2	Mean ± SD	0.91 ± 0.05	0.94 ± 0.10	<0.9 (all below LOQ)	0.8-2.36
	Range	0.90-1.10	0.90-1.26		
	p-value	not calculated[3]			
Folic acid	Mean ± SD	0.83 ± 0.18	0.85 ± 0.16	0.46-1.19	0.15-1.32
	Range	0.43-1.06	0.57-1.08		
	p-value	0.741			
Vitamin B3 (Niacin)	Mean ± SD	23.84 ± 4.90	19.64 ± 3.90	12.50-38.20	14.11-39.91
	Range	13.30-32.51	14.41-26.87		
	p-value	**0.0052[2]**			
Vitamin B5	Mean ± SD	5.54 ± 0.48	5.30 ± 0.67	4.23-10.12	n/a
	Range	4.56-6.31	4.16-6.20		
	p-value	0.201			
Vitamin B6	Mean ± SD	3.45 ± 1.09	3.06 ± 1.40	1.34-7.09	3.68-11.32
	Range	1.74-5.46	0.33-4.97		
	p-value	0.494			
Total tocopherols	Mean ± SD	37.7 ± 11.25	25.8 ± 8.81	22.4-65.30	4.1-41.7
	Range	20.8-58.6	17.6-50.9		
	p-value	**<0.00012[2]**			

[1] ILSI (2006) Crop Composition Database (Version 3.0)
[2] $p \leq 0.05$ indicates a statistical difference
[3] p value could not be calculated because there was no variability recorded for the test hybrid
n/a; not available

Table 28. Values for total tocopherols

	Mean	Mean - SD	Mean + SD	MIN	MAX
VCO-Ø1981-5	37.7	26.5	49.0	20.8	58.6
Non-transgenic control	25.8	17.0	34.6	17.6	50.9
Reference Hybrids	45.2	34.2	56.2	22.4	65.3

VII.F.4.7. Analysis minerals in maize grain

The levels of minerals were measured in grain samples of event VCO-Ø1981-5, the non-transgenic control, and three reference hybrids. The results are reported in Table 29. No statistically significant differences were observed between event VCO-Ø1981-5 and the non-transgenic control (p-values were ≥0.05) in the majority of minerals evaluated.

For the analytes calcium, copper, and potassium the p-values determined showed a statistical difference in the calculated means. However, for potassium the standard deviations and data ranges overlapped for event VCO-Ø1981-5 hybrids and the non-transgenic control, and both data ranges fell within the reference and published data ranges for these analytes. For copper and calcium, the standard deviations and data ranges overlapped for the event VCO-Ø1981-5 and the non-transgenic control, and the means and data ranges from both entries overlapped those generated from the reference hybrids. The data ranges for all 5 entries fell within the published data ranges for copper and calcium. Taken together, it is unlikely that these small differences indicate any biological significance.

Table 29. Minerals in event VCO-Ø1981-5 grain

Analyte (mg/kg of dry weight)		VCO-Ø1981-5 hybrid	Non-transgenic control	Range	
				Reference hybrids	Published[1]
Calcium	Mean ± SD	52.0 ± 10.8	60.0 ± 11.1	29.0-66.0	21.6-163.1
	Range	34.4-70.0	39.0-76.0		
	p-value	**0.00012**[2]			
Copper	Mean ± SD	1.6 ± 0.4	1.3 ± 0.3	0.6-1.6	0.8-7.1
	Range	0.8-2.3	0.6-1.7		
	p-value	**0.00052**[2]			
Iron	Mean ± SD	22.7 ± 5.2	22.4 ± 2.6	11.6-27.3	10.4-49.1
	Range	16.6-39.2	17.9-27.5		
	p-value	0.811			
Magnesium	Mean ± SD	1346 ± 107	1396 ± 150	895-1602	788-1940
	Range	1196-1550	1115-1591		
	p-value	0.338			
Phosphorus	Mean ± SD	2222 ± 188	2285 ± 180	1780-3084	1606-5330
	Range	1939-2569	2024-2536		
	p-value	0.485			
Potassium	Mean ± SD	3446 ± 317	3314 ± 284	2892-4252	2710-6030
	Range	2974-3840	2893-3773		
	p-value	**0.0462**[2]			
Sodium	Mean ± SD	2.7 ± 2.5	1.7 ± 0.7	0.6-7.7	1.0-731.5
	Range	0.6-10.7	0.6-3.4		
	p-value	0.140			
Zinc	Mean ± SD	23.0 ± 2.8	23.9 ± 3.5	15.6-28.4	6.5-33.8
	Range	17.9-26.8	16.6-31.8		
	p-value	0.316			

[1] ILSI (2006) Crop Composition Database (Version 3.0)
[2] p ≤ 0.05 indicates a statistical difference calculated.

VII.F.4.8. Analysis of secondary metabolites and anti-nutrients in maize grain

The levels of key secondary metabolites and known anti-nutrients were measured in grain samples of event VCO-Ø1981-5, the non-transgenic control, and three reference hybrids. The results are reported in Table 30. The levels of the furfural measured below the level of quantitation for the assay (data not shown). In all but one of the metabolites evaluated, no statistically significant differences were observed between event VCO-Ø1981-5 and the non-transgenic control (p-values were >0.05).

For the analyte ferulic acid, the p-value showed a statistical difference in the calculated means of event VCO-Ø1981-5 and the non-transgenic control; however, the standard deviations and data ranges overlapped and the level of ferulic acid was lower in event VCO-Ø1981-5. The data ranges of both entries overlapped with the data range of the reference hybrids and were well within the range of the published values. Therefore, it is unlikely that this difference indicates any biological significance.

Table 30. Secondary metabolites and anti-nutrients in event VCO-Ø1981-5 grain

Analyte (% of dry weight unless noted otherwise)		**VCO-Ø1981-5 hybrid**	**Non-transgenic control**	**Range**	
				Reference hybrids	**Published**[1]
SECONDARY METABOLITES					
Ferulic acid	Mean ± SD	0.272 ± 0.035	0.318 ± 0.034	0.175-0.309	0.09-0.390
	Range	0.206-0.332	0.276-0.385		
	p-value	**0.0001**[2]			
p-Coumaric acid	Mean ± SD	0.020 ± 0.005	0.021 ± 0.003	0.015-0.045	0.009-0.043
	Range	0.010-0.028	0.017-0.027		
	p-value	0.410			
Inositol	Mean ± SD	0.011 ± 0.003	0.010 ± 0.003	0.007-0.022	0.009-0.377
	Range	0.007-0.017	0.006-0.016		
	p-value	0.237			
ANTI-NUTRIENTS					
Raffinose	Mean ± SD	0.309 ± 0.082	0.279 ± 0.050	0.080-0.323	0.053-0.264
	Range	0.174-0.455	0.208-0.379		
	p-value	0.075			
Phytic acid	Mean ± SD	0.840 ± 0.122	0.864 ± 0.073	0.598-1.479	0.111-1.370
	Range	0.686-1.09	0.719-0.994		
	p-value	0.569			
Trypsin inhibitor (TIU/mg dry weight)[3]	Mean ± SD	2.79 ± 0.64	2.91 ± 0.63	1.05-4.55	1.45-7.18
	Range	1.58-3.53	1.48-3.68		
	p-value	0.325			

[1] ILSI (2006) Crop Composition Database (Version 3.0)
[2] $p \leq 0.05$ indicates a statistical difference calculated
[3] TIU = trypsin inhibitor units

VII.G. Conclusion

Event VCO-Ø1981-5 has been grown in the field under USDA notifications since 2007. In 2009, a total of 17 field sites across six US states were utilized to collect agronomic data. Hybrids were characterized under diverse environmental and growing conditions, which were representative for maize production across North America. Overall, these evaluations indicate that event VCO-Ø1981-5 does not show any unexpected changes in plant morphology as compared to conventional maize. Where small statistically significant differences were observed, they appeared unrelated to the introduced trait. The agronomic performance data indicate no biologically meaningful differences between event VCO-Ø1981-5 and the non-transgenic control (Bernard and MacIntosh, 2011d).

As part of the 2009 agronomic performance field trials, abiotic and biotic stress factors were also evaluated for event VCO-Ø1981-5 and compared to the non-transgenic control. Event VCO-Ø1981-5 did not differ in response to abiotic stressors. No differences between the event VCO-Ø1981-5 and the non-transgenic control were observed for disease occurrence or severity and response to insect pressure.

The germination test performed in the laboratory showed a high level of germination for event VCO-Ø1981-5 seeds. Under warm conditions, germination was equivalent to that of the non-transgenic control (99.6%), while there were only small differences between event VCO-Ø1981-5 and the non-transgenic control seeds under cold conditions, 97% and 93%, respectively.

Forage and grain samples of event VCO-Ø1981-5 were analyzed for their nutrient composition and compared to a non-transgenic control. To place the results in context of normal ranges of variability, the ranges for three commercial reference hybrids were determined. Published data ranges were provided as additional points of reference. Plant material was collected from five locations in 2009, analyzed using standards methods and statistically evaluated. A few statistically significant differences were noted for some analytes. However, the ranges and standard deviations overlapped for event VCO-Ø1981-5 and the non-transgenic control and none of the values determined were distinctly different from the normal range of maize variability for any particular analyte. Overall, event VCO-Ø1981-5 is comparable in nutrient composition to the non-transgenic control, reference hybrids and the publish data ranges.

In conclusion, no biologically meaningful differences between event VCO-Ø1981-5 and the non-transgenic control were identified with regards to its agronomic performance and nutrient composition.

VIII. ENVIRONMENTAL SAFETY AND IMPACT ON AGRONOMIC PRACTICES

VIII.A. Environmental assessment of the introduced protein

The EPSPS ACE5 protein was derived using a directed evolution strategy from the native EPSPS GRG23 protein and is 97.6% identical and 98.5% homologous at the amino acid level. The EPSPS GRG23 protein was isolated from *Arthrobacter globiformis*, a common soil bacterium. *Arthrobacter* species are part of the Gram-positive coryneform bacteria considered one of the major groups of aerobic soil bacteria and are found ubiquitously in soil and other natural habitats (Conn, 1948; Conn and Dimmick, 1947, Mulder *et al*., 1966). The EPSPS ACE5 protein is a glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme. EPSPS enzymes are found commonly in nature in microorganisms such as bacteria and fungi and in plants. The EPSPS ACE5 protein has no amino acid homology to proteins with known toxic or allergenic effects (Bernard and MacIntosh, 2011c). It is unlikely that the EPSPS ACE5 protein when expressed in maize will pose a safety risk to the environment.

EPSPS enzymes are found throughout the plant kingdom and are required for plant amino acid biosynthesis. In addition to plants, EPSPS enzymes are also found in prokaryotic systems, and glyphosate is toxic to most bacterial species. However, certain bacteria are tolerant to glyphosate, and it has been found that the EPSPS enzymes isolated from these bacteria often have a high tolerance to glyphosate. Furthermore, genes encoding glyphosate-tolerant EPSPS enzymes have been transferred to recipient organisms, including plants, to confer glyphosate tolerance (OECD, 1999; Nolte and Young, 2002; Cedeira and Duke, 2006; Gianessi, 2008; Vande Berg *et al*., 2008).

To date, EPSPS enzymes conferring glyphosate tolerance have been expressed in maize, cotton, soybean, canola, and sugarbeets and represent a large proportion of the US acreage. According to Brookes and Barfoot (2009), 91% of the total US soybean crop in 2007 was planted to glyphosate-tolerant varieties, first introduced in 1996. Glyphosate-tolerant maize and cotton were first introduced in 1997 and 10 years later were planted on 52% and 70% of the US maize and cotton acres, respectively (Brookes and Barfoot, 2009). Also in this time period, other herbicide-tolerant crops have been introduced into the marketplace (i.e., expressing the phosphinothricin acetyl transferase [PAT] protein). There have been no identified environmental concerns raised for herbicide-tolerant traits or the glyphosate-tolerant technology specifically, regardless of the crop in which they are expressed.

VIII.B. Potential for gene transfer

Maize is a highly domesticated crop that requires human intervention to produce high yielding results. Maize pollen does not drift large distances and the seed is heavy and not wind-born (Pleasants *et al*., 2001). Thus maize volunteers are rarely found outside of maize cultivation areas.

VIII.B.1. Vertical gene flow

Gene flow from maize to its wild relatives such as teosinte is not of particular concern in the US because the non-cultivated species are geographically restricted and occur only in Mexico and Central America. Teosinte species have co-existed with cultivated varieties of maize in these regions for thousands of years, yet they remain genetically

distinct with only a few cases of introgression (Baltazar *et al.,* 2005). While isolated populations of teosinte used to exist in the US in Florida and Texas, there have been no reports of these in the last 25 years (EPA, 2001). Some teosinte species are grown in botanical gardens, but pollen flow from these sources is highly unlikely (EPA, 2001).

The closest known relative of maize is *Tripsacum*, a genus of sixteen species, of which three can be found in the US: *T. dactyloides, T. floridanum* and *T. lanceolatum*. It is theorized that *Tripsacum* may be a progenitor of domesticated maize. Crosses between *Zea mays* L. subspecies (ssp.) *mays* and *T. dactyloides* are feasible, but only through human intervention and with difficulty. Moreover, the progeny of such crosses are frequently sterile or unstable (CFIA, 1994). However, maize breeders view *Tripsacum* as a good source for genes that could impart improved environmental traits, such as drought tolerance, and pest and disease resistance (OECD, 2003). The potential for *Z. mays* L. (ssp.) *mays* to introgress genes into *T. dactyloides* under field conditions was determined to be 'extremely remote' by the EPA (2001).

Weed spectrum shifts or glyphosate resistant weeds have been identified, but the incidence still remains low as compared to other herbicides (*e.g.,* ALS herbicides). To date, 13 weed species have been identified as resistant to glyphosate in the US or Canada, and 21 weed species globally (Brookes and Barfoot, 2011; Weed Science, 2011). Glyphosate provided growers unprecedented weed control in crops such as soybean and cotton, where chemical weed control options are limited. Consequently, rapid grower adoption of glyphosate-tolerant crops led to heavy selection pressure for glyphosate-resistant weeds. In light of the increased frequency of weed resistance, proactive integrated weed management systems are being promoted and recommended by the industry and being adopted by growers.

Taken together, it is unlikely that event VCO-Ø1981-5 would be found outside of maize cultivation areas or would introgress into wild relatives. Even if there were a rare introgression of the herbicide-tolerant trait from event VCO-Ø1981-5 into a wild maize relative, event VCO-Ø1981-5 has no selective advantage since glyphosate is just one of many different broad-spectrum herbicides that are commercially available. Likewise, management practices should be utilized to prevent, delay and/or minimize the development of glyphosate resistance in weeds.

VIII.B.2. Horizontal gene flow

The potential for horizontal gene transfer from plants to soil bacteria has been studied for many years with no such occurrences being observed in nature or in the field (EPA, 2001; Connor *et al.,* 2003) and only a few examples observed under laboratory or greenhouse conditions (De Vries *et al.,* 2001; Kay *et al.,* 2002). Event VCO-Ø1981-5 was developed to optimize expression in plants and not in bacteria, therefore even if such a genetic transfer did occur, expression in a bacterial strain, using the sugar cane ubiquitin 4 promoter, would not be expected to function in bacteria. The potential for event VCO-Ø1981-5 to be transferred beyond the typical maize cultivation regions by horizontal gene flow is negligible.

VIII.C. Weediness potential of maize event VCO-Ø1981-5

In the U.S., maize is not listed as a weed in the major weed references nor is it listed as a noxious weed species by the Federal Government, and maize has been grown throughout the world without any report that it is a serious weed. Maize has lost the ability to survive in the wild due to its long process of domestication and requires human intervention to disseminate its seeds. Although maize from a previous crop can sometimes overwinter and germinate, it cannot persist as a weed. Volunteers are common in many agronomic systems, but they can be controlled easily with mechanical or chemical methods (OECD, 2003).

Event VCO-Ø1981-5 has been evaluated carefully for agronomic performance and seed germination characteristics under field and laboratory conditions and did not show any biologically meaningful differences when compared to the non-transgenic control (Section VII.). The laboratory study yielded a level of germination for event VCO-Ø1981-5 equivalent to that of the non-transgenic control under warm conditions and only small differences under cold conditions (Section VII.E.). Differences in field emergence during the trials conducted in 2009 may be attributed to a difference in seed quality for event VCO-Ø1981-5 and the non-transgenic control (Section VII.C.2.3.). Overall, none of the agronomic parameters measured indicate that event VCO-Ø1981-5 has any more potential for weediness than maize varieties currently in the market.

Transformation event VCO-Ø1981-5 is tolerant to the broad-spectrum herbicide glyphosate only and remains sensitive to other herbicides registered for use on weeds in maize and other rotational crops. Any volunteers of event VCO-Ø1981-5 are as easily controlled with mechanical means or chemical treatments as other maize varieties used in current agricultural systems.

VIII.D. Current agronomic practices for maize

Maize is grown all across the US, with the majority (>80%) in the Midwest, from western New York to Nebraska and from the Canadian border south through Oklahoma and the northern panhandle of Texas. A recent history of maize planted acres is provided in Table 31 along with the yield, price and overall value of maize production (USDA-NASS, 2011a). It is anticipated that event VCO-Ø1981-5 maize hybrids will be cultivated across the US Corn Belt without extending the current maize growing area.

Table 31. History of maize production

Year	Planted (all purposes) [1]	Harvested [1]	Yield [2]	Production[3]	Price per unit [4]	Value of production [5]
2010	88,192	81,446	152.8	12,446,865	3.83	47,671,493
2009	86,482	79,620	164.9	13,130,632	3.70	48,588,665
2008	85,982	78,570	153.9	12,091,648	4.06	49,312,615
2007	93,527	86,520	150.7	13,037,875	4.20	54,666,959
2006	78,327	70,638	149.1	10,531,123	3.04	32,083,011
2005	81,779	75,117	147.9	11,112,187	2.00	22,194,287
2004	80,929	73,631	160.3	11,805,581	2.06	24,377,913

1 - thousand acres, 2 – bushel/acre, 3 - thousand bushels, 4 - dollars/bushel, 5 - thousand dollars

US growers produced the largest corn crop on record in 2009, 13.1 billion bushels at a value $48.6 billion (USDA-NASS, 2011a). Slightly less than half of all maize production is utilized for animal feeds, about 17% is exported and about 10% utilized for food (*e.g.*, sweeteners, cereal, starch, high fructose corn syrup, and alcohol (NCGA, 2011). These segments have been relatively stable over the last 10 years. The fastest growing segment is for ethanol production, growing to nearly 5 billion bushels in 2010 up from just under 1 billion bushels in 2002. The growth of maize production is at least partially explained by improved technology, such as biotechnology, which boosts agricultural productivity providing US producers a competitive edge globally.

In 2011, roughly 72% of the total maize acreage was planted with herbicide-tolerant maize in the US, out of approximately 87.8M total maize grain acres (USDA-NASS, 2011b). Of the 61.5 M acres planted with herbicide-tolerant varieties in 2011, 23% had only the herbicide-tolerant trait, while 49% were maize varieties stacked with both insect control and herbicide tolerance traits (USDA-NASS, 2011b). More than 90% of the herbicide-tolerant maize was planted to glyphosate-tolerant maize, with the balance planted to LibertyLink®[1] maize and non-GM Clearfield®[2] technology. Event VCO-Ø1981-5 will offer a new opportunity to US growers. It will be made available to them through our affiliate in the US and through licenses to third parties. It is difficult to predict the adoption rate of this new event compared to what is available on the market today and it is expected to be adopted by the market as a replacement product. As such, it is unlikely that the availability of this new event will significantly increase the maize acreage planted with by glyphosate-tolerant maize.

Herbicides were applied to 98% of the maize planted acreage in 2010 (USDA-NASS, 2011c). Glyphosate has surpassed atrazine as the most widely applied herbicide with 66% of the planted acreage being treated (USDA-NASS, 2011c). Atrazine was applied to 61% of the planted acres. These were followed closely by s-metolachlor and acetochlor, at 25% of the planted maize acreage reported.

Weeds cause significant yield reductions in maize by competing for light, water and nutrients, requiring careful management by producers. Weeds can also bring insect and fungal infestations. Many different weed species are found in maize growing regions, including perennials, annuals, grasses, broadleaf and sedge types. Early season weeds are perhaps the most damaging when the maize crop is becoming established. Reductions in maize yield are directly proportional to the amount of weeds present (Gianessi *et al.,* 2002). Prior to the 1960s, crop rotation, cultivation and tillage were the primary methods to control weeds in maize production fields. As herbicide use became widespread in the following decade, low and no-till farming were embraced and today almost every maize acre receives an herbicide treatment.

Weed management brings the best results when herbicides are applied on smaller weeds, early in the growing season. The uses of pre-emergent herbicides, such as atrazine, continue as a common weed management practice, even with the advent of herbicide-tolerant crops. Introduction of herbicide-tolerant crops, such as glyphosate- and glufosinate-tolerant crops has brought many advantages to the grower; better crop tolerance, broader spectrum of weed control, more environmentally favorable profile and more favorable crop rotation opportunities. These new tools, including event VCO-

[1] LibertyLink is a registered trademark of Bayer
[2] Clearfield is a registered trademark of BASF Corporation

Ø1981-5 maize, will continue to provide the grower with greater flexibility and ease of use in their weed management systems.

VIII.E. Potential impact of introduction on current agricultural practices

Weed management is a significant aspect of successful maize production for US growers. Until the advent of herbicide-tolerant maize, growers used a range of different herbicides pre-plant, pre- and post-emergence to manage weeds. Herbicide programs in maize can vary due to the geographic area, weed spectrum, and rotational versus continuous maize. Growers have traditionally relied upon triazine products in continuous maize where potential for carryover of the residual materials would not be a concern. Several weeds, however, have developed resistance to the triazines (LeBaron, 1991). Adverse weather conditions also reduce the effect of the triazines and other soil applied herbicides. Sulfonylurea and other herbicides are used to control grass and broadleaf weeds post-emergence in maize. In general, maize often receives a soil applied herbicide and a follow-up post-emergence application. Due to potential crop injury, rotational concerns and weed competition, multiple herbicide applications applied post-emergence are not widely used in maize. Also, many products are used in combination as premixes or as tank mixes, to widen the spectrum of control. The reasons for this are to prevent maize injury, reduce weed pressure on the crop, and reduce rotational restrictions to soybeans or other legumes. Perennials are difficult to control because they propagate by seed and/or underground plant parts. Control of these diverse species requires the use of multiple herbicide families and multiple applications, making weed management complex and difficult.

Aside from the material cost associated with traditional weed control programs in maize, there are the additional costs of fuel and time required to apply these herbicides. Utilization of event VCO-Ø1981-5 maize will facilitate fewer herbicide applications by offering broad-spectrum post-emergence weed control with a single chemical, glyphosate. Further, glyphosate is a suitable tank-mix partner for many selective herbicides making possible one pass weed control even where hard to control weeds may be present in the larger weed population. The ability to control weeds with a single broad-spectrum herbicide, or as a tank mix, allows for no- or minimum-till maize cropping systems. Reducing tillage has the effect of reducing fuel costs simply by reduction of equipment passes. No- or minimum-till maize cropping systems also have the added benefit of minimizing soil disturbance providing numerous advantages for soil and environmental quality (*e.g.*, greater carbon sequestration, less soil and nutrient run-off, soil moisture retention, etc.).

It is not surprising therefore that US maize growers have rapidly embraced glyphosate-tolerant maize products, now reported to be more than 70% of all maize-planted acres, based on the ease of use and excellent human, animal and environmental safety profiles of glyphosate. A grower utilizing herbicide-tolerant maize minimizes the need for multiple herbicide applications with more toxic herbicides, since weeds are controlled through precisely timed applications. The herbicide, in the herbicide-tolerant system, is only applied when weed pressure requires management, causing no crop damage to the herbicide-tolerant maize.

The potential impact of the introduction of event VCO-Ø1981-5 on current agricultural practices will not be significantly different than for the previously deregulated herbicide-tolerant maize lines. Growers have more than 15 years of experience with herbicide-

tolerant maize products. Aside from the tolerance to glyphosate herbicides that ease weed control, event VCO-Ø1981-5 maize will require the same management as any other maize variety. Cultivation practices for insect and nutrient management will not be any different.

No data has been generated for event VCO-Ø1981-5 that indicates an increase in fitness or weediness characteristics that would provide a competitive advantage or extend maize production beyond the current production areas. There have been no noted effects on insect or disease susceptibilities during multiple field trials. US agriculture has utilized herbicide-tolerant crops over the past 15 years and the cultivation of event VCO-Ø1981-5 maize will not change current agronomic practices.

VIII.F. Weed resistance management

Growers have had to manage resistance in weeds and insects since the advent of biological and chemical control measures. Theoretically, regular use of any pesticide could lead to resistance, but growers have ample options using a variety of techniques to prevent or delay the development of resistant weeds in maize fields.

Resistance management of event VCO-Ø1981-5 will be no different than the management practices used with other maize varieties. While glyphosate-tolerant maize provides the potential for a single weed control herbicide, there are many other effective herbicides in the marketplace that can and should be used in conjunction with glyphosate. Some of additional techniques for weed resistance management include crop rotation, rotating herbicide modes-of-action, tank mixing herbicides with different modes-of-action, and mechanical weed control.

Weed scientists agree that adopting and implementing best management practices that reduce weed resistance to herbicides is critical (Boerboom and Owen, 2006). A diversified and detailed integrated weed management plan not only improves overall weed control, it provides additional benefits such as improving the overall level and consistency of weed control, adding flexibility in scheduling applications and reducing the risk of yield loss due to weed competition.

Ideally, integrated weed management should utilize all available tools including herbicides in a well-balanced program, as the lower the diversity of weed control tools, the higher the risk of selecting resistant biotypes becomes. To ensure diversification is maintained in weed control methods, growers are encouraged to keep detailed records of weed management practices for each field. Integrated weed management guidelines promote an economically viable, environmentally sustainable and socially acceptable weed control program, which is fully detailed in Appendix 4.

The highlights of an integrated weed management include:
1) Correctly identify weeds and look for trouble areas within field to identify resistance indicators;
2) Rotate crops;
3) Start the growing season with clean fields;
4) Rotate herbicide modes of action by using multiple modes of action during the growing season and apply no more than two applications of a single herbicide mode of action to the same field in a two-year period. One method to accomplish this is to rotate herbicide-tolerant trait systems;

5) Apply recommended rates of herbicides to actively growing weeds at the correct time with the right application techniques;
6) Control any weeds that may have escaped the herbicide application;
7) Thoroughly clean field equipment between fields.

Glyphosate-tolerant event VCO-Ø1981-5 volunteers can be managed through crop rotation, using many different herbicides, and/or mechanical techniques. Volunteers can also be managed in continuous corn with mechanical methods, applications of burndown herbicides prior to crop emergence, or through different herbicide-tolerant maize systems, such as glufosinate-tolerant maize.

Today, growers have a wide range of weed control options. Information on weed resistance management will be provided to growers to assist them in making practical and useful choices to prevent, delay and/or minimize weed resistance development when using event VCO-Ø1981-5.

VIII.G. Potential impact on organic or conventional farming

Glyphosate-tolerant maize varieties have been in use for more than 15 years and the introduction of event VCO-Ø1981-5 maize will not change the potential impact on organic farming from the current agricultural situation. Organic and conventional growers may choose not to plant or sell glyphosate-tolerant maize. These growers will still be able to produce non-transgenic maize and will be able to coexist with biotechnology maize producers as they do now. Techniques have long been practiced to allow coexistence by minimizing or preventing cross-pollination. For maize, this is used for example when growing specialty corn, such as waxy or sweet, to keep the quality of products that can be affected by conventional maize pollination. Isolation distances between fields help to limit the effects of pollen flow. In addition to spatial isolation, growers can use reproductive isolation to minimize or eliminate cross-pollination (i.e., plant varieties of different maturity groups) or stagger planting dates for temporal isolation. Perhaps most importantly, growers need to communicate openly with each other in order to continue and maintain the historical practice of coexistence.

VIII.H. Potential impacts on raw or processed agricultural products

Data submitted in this petition in support of event VCO-Ø1981-5 maize with regards to agronomic performance evaluations, disease and insect susceptibility, and compositional analysis indicate no biological differences from the non-transgenic control that would be expected to cause a direct or indirect plant pest effect on raw or processed agricultural products. While the EPSPS ACE5 protein was the result of genetic modification to improve enzymatic activity under field conditions, thereby making it more similar to the native maize EPSPS protein, the introduced amino acid changes did not impact the EPSPS ACE5 protein stability under industrial conditions that are typically found in maize wet and dry milling processes. In addition, Athenix Corp., now an affiliate of Bayer CropScience LP, submitted an Early Food Safety Evaluation to FDA in 2009 and FDA completed their review with no further questions in October, 2010 (FDA, 2010).

VIII.I. Potential effects on non-target organisms

Maize production requires a highly managed environment in order to produce acceptable commercial level yields. Weeds and insect pests must be managed to keep below economic thresholds in order to prevent any loss of yield. Agronomic performance and environmental effects studies have been conducted across a wide range of environments with no adverse effects on non-target organisms.

Potential exposure to maize event VCO-Ø1981-5 would not have adverse effects to non-target organisms because the EPSPS ACE5 protein has no insecticidal or other pesticidal properties and as such does have target organisms. Time to bloom and other reproductive aspects are the same for event VCO-Ø1981-5 as for the non-transgenic control, therefore impacts to pollinators are not expected and have not been observed. Since the EPSPS ACE5 protein is expressed *in planta*, and the protein environmentally degrades as observed for all EPSPS enzymes, there is minimal exposure to non-target organisms. Agronomic performance evaluations, disease and insect susceptibility, and compositional analysis of event VCO-Ø1981-5 indicate no biological differences from the non-transgenic control maize. Furthermore, field trial reports prepared for the notifications listed in Table 11 (Section VII.) did not indicate any differences in insect pests or beneficial insect population occurrences (Appendix 1). As maize event VCO-Ø1981-5 and conventional maize are not different with respect to their phenotypic and agronomic characteristics and ecological interactions (except for the introduced trait), it can be concluded that the impact of event VCO-Ø1981-5 on non-target organisms will not be different from conventional corn.

EPSPS proteins and, specifically, the EPSPS ACE5 protein, have no known toxic or allergenic properties (Bannon *et al,* 2008; Delaney *et al.,* 2008; Bernard and MacIntosh, 2011c). An acute oral toxicity study in mice did not cause any toxicity, mortality or morbidity (Arulnesan, 2008). EPSPS ACE5 protein digested very quickly in simulated gastric fluids, did not match any toxic or allergenic protein sequences using a bioinformatics analysis and no glycosylation was detected (Bernard and MacIntosh, 2011b; Bernard and MacIntosh, 2011c; Shouten, 2011). A detailed compositional analysis of event VCO-Ø1981-5 forage and grain was conducted, and only minor statistical differences were noted as compared to the non-transgenic control maize (Bernard and MacIntosh, 2011e). However, almost all of those differences were within the normal published ranges for various maize composition analytes. Glyphosate-tolerant EPSPS enzymes have been previously assessed for safety and are widely used in commercially available maize products in production today (USDA, 2011). EPSPS GRG23, from which EPSPS ACE5 protein was derived, is a naturally occurring enzyme isolated from a common soil organism. No additional exposure risks are anticipated from this protein expressed in event VCO-Ø1981-5 maize.

VIII.J. Potential impact on biodiversity

Herbicide-tolerant crop systems have improved high production agriculture by providing valuable weed control without crop damage. Adoption of low or no till farming facilitated by glyphosate-tolerant technology further reduces disruptions to the soil environment. Plant expressed EPSPS ACE5 protein also limits environmental exposure and as noted above, no adverse effects on non-target organisms have been identified.

No increased weediness potential has been observed during the evaluation of event VCO-Ø1981-5. There are no expectations that an unconfined release of event VCO-Ø1981-5 should lead to increases in weediness of other sexually compatible species. The evaluation of event VCO-Ø1981-5 indicated no adverse effects on non-target organisms common to the agricultural ecosystem. Furthermore, glyphosate-tolerant maize varieties have been used in agricultural production for many years with no adverse effects reported. There is no reason to anticipate that this new event, with a similar mode of action, would cause negative effects to biodiversity. Glyphosate use and crop production practices are not expected to change significantly with the market introduction of event VCO-Ø1981-5, therefore there should be no indirect or cumulative impact on biodiversity. The effects of glyphosate on contamination of soil, water, and air are minimal (Cedeira and Duke, 2006); therefore, negative effects on soil microflora, aquatic organisms, arthropods, and mammals are not anticipated when the herbicide is applied according to label instructions.

VIII.K. Conclusion

Regulated field trials of event VCO-Ø1981-5 have been conducted since 2007. The agronomic performance data, collected during the 2009 season, indicate no biologically meaningful differences between event VCO-Ø1981-5 and the non-transgenic control. Ecological observations, conducted at the same time on a variety of stressors (*e.g.,* insects, diseases, abiotic), also showed no differences between event VCO-Ø1981-5 and the non-transgenic control regardless whether the stressor was measured to be mild to moderate in severity.

EPSPS enzymes are found throughout the plant kingdom, are required for plant amino acid biosynthesis and the corresponding transgenes have been expressed in maize, cotton, soybean, canola, and sugarbeet and represent a large proportion of the US acreage for the last 15 years, without any reports of adverse effects. There have been no identified environmental concerns raised for herbicide-tolerant traits or the glyphosate-tolerant technology specifically, regardless of the crop in which they are expressed.

Maize has been grown throughout the world without any report that it is a serious weed. Gene flow concerns are minimal, since teosinte is not found in the US or Canada and crosses with other wild relatives of maize, while possible, are difficult to produce and generally cause instability or sterility in the offspring. While glyphosate resistance has been found, it is easily managed using traditional methods such as other herbicides and crop rotation. Information on weed resistance management will be provided to growers to assist them in making practical and useful choices to prevent, delay and/or minimize weed resistance when planting event VCO-Ø1981-5 maize varieties.

Given the extensive experience growers have with herbicide-tolerant crops, there is no expectation that the introduction of event VCO-Ø1981-5 would alter current agronomic practices. The potential impact on organic farming should not change from the current agricultural situation, since techniques allowing coexistence are well established. Perhaps the most important measure to continue and maintain the historical practice of coexistence, is for growers to communicate openly. Given the results demonstrating that event VCO-Ø1981-5 is nutritionally equivalent to non-transgenic maize there should also be no impacts to raw or processed agricultural products. Agronomic performance evaluations, disease and insect susceptibility, and compositional analysis of event

VCO-Ø1981-5 showed no biological differences to the non-transgenic control that might indicate a direct or indirect effect on non-target organisms including beneficial, threatened or endangered species. These results also support the lack of impacts on biodiversity. Glyphosate effects on contamination of soil, water and air are minimal. Furthermore, the EPSPS ACE5 protein has no known toxic or allergenic properties that could impact non-target organisms.

IX. STATEMENT OF GROUNDS UNFAVORABLE

GENECTIVE S.A. knows of no study data and/or observations associated with glyphosate-tolerant event VCO-Ø1981-5 maize that will result in adverse environmental consequences for its introduction. Previously deregulated glyphosate-tolerant maize lines have been grown safely for more than 10 years with no adverse effects being observed. The only biologically relevant phenotypic difference between event VCO-Ø1981-5 and conventional maize is the expression of the EPSPS ACE5 protein, which provides tolerance to the application of glyphosate. Planting glyphosate-tolerant event VCO-Ø1981-5 maize will provide growers with another choice for using glyphosate-tolerant technology.

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APPENDIX 1

Field Trial Termination Reports 2007-2009

USDA Field Termination Report

USDA Notification/Permit Number: 07-283-104n

Applicant Internal Number: ATX-07-16

Applicant: Athenix Corporation
Research Triangle Park, NC 27709

Regulated Article: *Zea mays;* corn

Lines: 5234-5283, 5814-5816, 5766-5767, 5878-5886, 5764-5765, 5835-5869, 6022-6031, 6058-6059, 6038-6039, 6054-6057, 6103-6110, 6012-6021, 6135, 6136, 5402-5423, 5768-5784, 5090-5225, 5513-5562, 6046-6053, 6129-6131, 6040-6045, 6111-6128, 6148, 6149, 6032-6036, 6062, 5898-5922, 5563-5628, 1-100, 5946-5964, 6065-6094, 6132, 6133, 6145, 6146, 6096-6100, 6147, 6342, 6343, 6353-6370, 6402, 6403, 6419-6433, 6507-6522, 6598, 6599, 5685-5705, 5872-5877, 5424, 5425, 5427, 5436, 5443, 5445, 6663-6667, 6676-6684, 6671, 6672, 6742, 6743, 6371-6373, 6404-6418, 6441-6463, 6536-6560, 6622-6634, 6650-6652, 6304, 6306, 6307, 6750-6752, 6845-6916, 6933-6971, 6978-6993, 7045-7048, 6972, 6994-7013, 6791-6792, 6831, 6923-6925, 6794-6797, 6832-6844, 6926-6932, 7014, 5980-5982, 6001-6011

Section I. Site Release Information:

Trial sites were requested for one (1) location:

USDA-Notification Release Site No.	County / State	Acreage Planted	Date Planted	Date Terminated
1	1 Juana Diaz / PR	2.00	12/28/2007	4/22/2008

Section II. List of characteristic observations collected on the regulatory compliance *In-Season Forms* approximately every four weeks during the growing season between the transgenic plants/regulated & non-transgenic plants/non-regulated, insects and environment:

a	Plant Morphological - Observations
b	Plant Disease - Observations
c	Difference in Insect Occurrence - Observations
d	Unusual Occurrences During Flowering Stage -Observations
e	Unusual Occurrences During Pesticide Applications -Observations
f	Difference in Environmental Conditions - Observations

Section III. Summarized results from the regulatory In-Season Observation Forms:

1. Juana Diaz Co., PR :

a) Cooperator reported no observed differences in plant morphology.
b) Cooperator reported no unusual differences in plant disease.
c) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
d) Cooperator noted no unusual growth characteristics during flowering stage.
e) Cooperator reported no unusual interactions after pesticide applications.
f) Cooperator reported no unusual differences in environmental and/or weather conditions.

No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents

Section IV. Plant Disposition:

All plot locations were harvested or terminated, and all remaining material that was not sent to an Athenix Corporation facility or other laboratories for analysis was destroyed. Unplanted seed which was not utilized by the cooperator was returned to Athenix Corporation for storage or destroyed.

Preparation Date: May 12, 2010

USDA Field Termination Report

USDA Notification/Permit Number: 08-059-104n

Applicant Internal Number: ATX-07-17

Applicant: Athenix Corporation
Research Triangle Park, NC 27709

Regulated Article: *Zea mays;* corn

Lines: 5122, 6041, 6113-6117, 6127, 6033, 6035, 6062, 6068, 6078, 6081, 6091, 6093, 6342, 6359, 6421, 6423, 6427-6430, 6508, 6513, 6514, 6516, 6521, 6598, 6407-6418, 6447-6455, 6537-6560, 6626-6634, 6845-6916, 6933-6972, 6978-6993, 7045-7047, 7010, 7004, 7006, 7091-7101, 7148-7157, 7176-7183, 7214-7241, 7311-7317, 7613-7620, 7725-7727, 7754, 7622-7649, 7691-7693, 7722-7724, 7751-7753, 7792-7793, 7391-7400, 7433-7452, 7518-7535, 7610-7612, 7049, 7795, 7869-7873, 7728-7729, 7874-7879, 7884-7886

Section I. Site Release Information:

Trial sites were requested for one (1) location:

USDA-Notification Release Site No.	County / State	Acreage Planted	Date Planted	Date Terminated
1	Scott / IA	0.54	5/06/2008	11/28/2008

Section II. List of characteristic observations collected on the regulatory compliance *In-Season Forms* approximately every four weeks during the growing season between the transgenic plants/regulated & non-transgenic plants/non-regulated, insects and environment:

a	Plant Morphological - Observations
b	Plant Disease - Observations
c	Difference in Insect Occurrence - Observations
d	Unusual Occurrences During Flowering Stage -Observations
e	Unusual Occurrences During Pesticide Applications -Observations
f	Difference in Environmental Conditions - Observations

Section III. Summarized results from the regulatory In-Season Observation Forms:

1. **Scott Co., IA :**
   a) Cooperator reported no observed differences in plant morphology.
   b) Cooperator reported no unusual differences in plant disease.
   c) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
   d) Cooperator noted no unusual growth characteristics during flowering stage.
   e) Cooperator reported no unusual interactions after pesticide applications.
   f) Cooperator reported no unusual differences in environmental and/or weather conditions.

   No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

Section IV. Plant Disposition:

All plot locations were harvested or terminated, and all remaining material that was not sent to an Athenix Corporation facility or other laboratories for analysis was destroyed. Unplanted seed which was not utilized by the cooperator was returned to Athenix Corporation for storage or destroyed.

Preparation Date: May 12, 2010

USDA Field Termination Report

USDA Notification/Permit Number: 08-067-108n

Applicant Internal Number: ATX-07-18

Applicant: Athenix Corporation
Research Triangle Park, NC 27709

Regulated Article: *Zea mays;* corn

Lines: 6845-6916, 6933-6972, 6978-6993, 7045-7047

Section I. Site Release Information:

Trial sites were requested for one (1) location:

USDA-Notification Release Site No.	County / State	Acreage Planted	Date Planted	Date Terminated
1	1st Finca Florida-Santa Isabel / PR	0.33	4/25/2008	7/21/2008
1	2nd Finca Florida-Santa Isabel / PR	0.43	8/08/2008	10/27/2008

2 plantings same location

Section II. List of agronomic observations collected on the regulatory compliance *In-Season Forms* approximately once every month during the growing season between the transgenic plants/regulated & non-transgenic plants/non-regulated.:

a	Disease:	Resistance/susceptibility to disease.
b	Insects:	Abundance of non-target species and resistance/susceptibility to insect feeding not specifically engineered for resistance.
c	Plant Growth	Is plant morphology and growth similar for both transgenic and non-transgenic plants?
d	Weediness	Is germination, tasseling, seed production, etc. similar for both transgenic and non-transgenic plants?

Section III. Summarized results from the regulatory In-Season Observation Forms:

1. **Santa Isabel Co., PR (1st planting) :**
    a) Cooperator reported no unusual differences in plant disease.
    b) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
    c) Cooperator noted no unusual plant growth observations.
    d) Cooperator reported no unusual weediness characteristics.

    No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

1. **Santa Isabel Co., PR (2nd planting):**
    a. Cooperator reported no unusual differences in plant disease.
    b. Cooperator reported no unusual differences in insect pests or beneficial insect populations.
    c. Cooperator noted no unusual plant growth observations.
    d. Cooperator reported no unusual weediness characteristics.

    No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

Section IV. Plant Disposition:

All plot locations were harvested or terminated, and all remaining material that was not sent to an Athenix Corporation facility or other laboratories for analysis was destroyed. Unplanted seed which was not utilized by the cooperator was returned to Athenix Corporation for storage or destroyed.

Preparation Date: May 12, 2010

USDA Field Termination Report

USDA Notification/Permit Number: 08-073-108n

Applicant Internal Number: ATX-07-19

Applicant: Athenix Corporation
Research Triangle Park, NC 27709

Regulated Article: *Zea mays;* corn

Lines: 5122, 6041, 6113-6117, 6127, 6033, 6035, 6062, 6068, 6078, 6081, 6091, 6093, 6342, 6359, 6421, 6423, 6427-6430, 6508, 6513, 6514, 6516, 6521, 6598, 6407-6418, 6447-6455, 6537-6560, 6626-6634, 6845-6916, 6933-6972, 6978-6993, 7045-7047, 7010, 7004, 7006, 7091-7101, 7148-7157, 7176-7183, 7214-7241, 7311-7317, 7613-7620, 7725-7727, 7754, 7622-7649, 7691-7693, 7722-7724, 7751-7753, 7792-7793, 7391-7400, 7433-7452, 7518-7535, 7610-7612, 7049, 7795, 7869-7873, 7728-7729, 7874-7879, 7884-7886, 7887, 7888

Section I. Site Release Information:

Trials sites were requested for three (3) locations:

USDA-Notification Release Site No.	County / State	Acreage Planted	Date Planted	Date Terminated
1	Scott / IA	*N/A*	Not Planted	*N/A*
2	Madison / IA	*N/A*	Not Planted	*N/A*
3	Polk / IA	1.86	6/16/2008	12/07/2008

Section II. List of characteristic observations collected on the regulatory compliance *In-Season Forms* approximately every four weeks during the growing season between the transgenic plants/regulated & non-transgenic plants/non-regulated, insects and environment:

a	Plant Morphological - Observations
b	Plant Disease - Observations
c	Difference in Insect Occurrence - Observations
d	Unusual Occurrences During Flowering Stage -Observations
e	Unusual Occurrences During Pesticide Applications -Observations
f	Difference in Environmental Conditions - Observations

Section III. Summarized results from the regulatory In-Season Observation Forms:

1. **Scott Co., IA:** Not Planted.

2. **Madison Co., IA :** Not Planted

3. **Polk Co., IA :**
   a. Cooperator reported no observed differences in plant morphology.
   b. Cooperator reported no unusual differences in plant disease.
   c. Cooperator reported no unusual differences in insect pests or beneficial insect populations.
   d. Cooperator noted no unusual growth characteristics during flowering stage.
   e. Cooperator reported no unusual interactions after pesticide applications.
   f. Cooperator reported no unusual differences in environmental and/or weather conditions.

   No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

Section IV. Plant Disposition:

All plot locations were harvested or terminated, and all remaining material that was not sent to an Athenix Corporation facility or other laboratories for analysis was destroyed. Unplanted seed which was not utilized by the cooperator was returned to Athenix Corporation for storage or destroyed.

Preparation Date: May 12, 2010

USDA Field Termination Report

USDA Notification/Permit Number: 08-105-109n

Applicant Internal Number: ATX-07-23

Applicant: Athenix Corporation
Research Triangle Park, NC 27709

Regulated Article: *Zea mays;* Corn

Lines: 6845-6916, 6933-6972, 6978-6993, 7045-7047

Section I. Site Release Information:

Trial sites were requested for two (2) locations:

USDA-Notification Release Site No.	County / State	Acreage Planted	Date Planted	Date Terminated
1	1 Washington / NE	0.2	6/03/2008	09/26/2008
2	2 York / NE	N/A	Not Planted	N/A

Section II. List of agronomic observations collected on the regulatory compliance *In-Season Forms* approximately once every month during the growing season between the transgenic plants/regulated & non-transgenic plants/non-regulated:

a	Disease:	Resistance/susceptibility to disease.
b	Insects:	Abundance of non-target species and resistance/susceptibility to insect feeding not specifically engineered for resistance.
c	Plant Growth	Is plant morphology and growth similar for both transgenic and non-transgenic plants?
d	Weediness	Is germination, growth, flowering, seed production, etc. similar for both transgenic and non-transgenic plants?

Section III. Summarized results from the regulatory In-Season Observation Forms:

1. **Washington Co., NE (1st planting) :**
   a) Cooperator reported no unusual differences in plant disease.
   b) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
   c) Cooperator noted no unusual plant growth observations.
   d) Cooperator reported no unusual weediness characteristics.

   No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

2. **York Co., NE : Not Planted**

Section IV. Plant Disposition:

All plot locations were harvested or terminated, and all remaining material that was not sent to an Athenix Corporation facility or other laboratories for analysis was destroyed. Unplanted seed which was not utilized by the cooperator was returned to Athenix Corporation for storage or destroyed.

Preparation Date: May 12, 2010

USDA Field Termination Report

USDA Notification/Permit Number: 08-254-111n

Applicant Internal Number: ATX-07-24

Applicant: Athenix Corporation
Research Triangle Park, NC 27709

Regulated Article: *Zea mays;* Corn

Lines: 6845-6916, 6933-6972, 6978-6993, 7045-7047

Section I. Site Release Information:

Trial sites were requested for one (1) location:

USDA-Notification Release Site No.	County / State	Acreage Planted	Date Planted	Date Terminated
1	1st Santa Isabel / PR	0.87	12/10/2008	4/08/2009
1	2nd Santa Isabel / PR *	0.65	7/27/2009	10/21/2009

** 2nd planting at same location was planted under Notification 08-254-111n and Notification 09-215-106n.*

Section II. List of agronomic observations collected on the regulatory compliance *In-Season Forms* approximately once every month during the growing season between the transgenic plants/regulated & non-transgenic plants/non-regulated:

a	Disease:	Resistance/susceptibility to disease.
b	Insects:	Abundance of non-target species and resistance/susceptibility to insect feeding not specifically engineered for resistance.
c	Plant Growth	Is plant morphology and growth similar for both transgenic and non-transgenic plants?
d	Weediness	Is germination, tasseling, seed production, etc. similar for both transgenic and non-transgenic plants?

Section III. Summarized results from the regulatory In-Season Observation Forms:

1. **Santa Isabel Co., PR (1st planting) :**
   a) Cooperator reported no unusual differences in plant disease.
   b) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
   c) Cooperator noted no unusual plant growth observations.
   d) Cooperator reported no unusual weediness characteristics.

   No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

1. **Santa Isabel Co., PR (2nd planting):**
   a. Cooperator reported no unusual differences in plant disease.
   b. Cooperator reported no unusual differences in insect pests or beneficial insect populations.
   c. Cooperator noted no unusual plant growth observations.
   d. Cooperator reported no unusual weediness characteristics.

   No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

Section IV. Plant Disposition:

All plot locations were harvested or terminated, and all remaining material that was not sent to an Athenix Corporation facility or other laboratories for analysis was destroyed. Unplanted seed which was not utilized by the cooperator was returned to Athenix Corporation for storage or destroyed.

Preparation Date: May 12, 2010

USDA Field Termination Report

USDA Notification/Permit Number: 08-280-103n

Applicant Internal Number: ATX-07-25

Applicant: Athenix Corporation
Research Triangle Park, NC 27709

Regulated Article: *Zea mays;* Corn

Lines: 6845-6916, 6933-6972, 6978-6993, 7045-7047

Section I. Site Release Information:

Trial sites were requested for one (1) location:

USDA-Notification Release Site No.	County / State	Acreage Planted	Date Planted	Date Terminated
1	Barranquitas / PR	0.58	11/14/2008	3/02/2009

Section II. List of agronomic observations collected on the regulatory compliance *In-Season Forms* approximately once every month during the growing season between the transgenic plants/regulated & non-transgenic plants/non-regulated.:

a	Disease:	Resistance/susceptibility to disease.
b	Insects:	Abundance of non-target species and resistance/susceptibility to insect feeding not specifically engineered for resistance.
c	Plant Growth	Is plant morphology and growth similar for both transgenic and non-transgenic plants?
d	Weediness	Is germination, tasseling, seed production, etc. similar for both transgenic and non-transgenic plants?

Section III. Summarized results from the regulatory In-Season Observation Forms:

1. **Barranquitas Co., PR:**
   a) Cooperator reported no unusual differences in plant disease.
   b) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
   c) Cooperator noted no unusual plant growth observations.
   d) Cooperator reported no unusual weediness characteristics.

   No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

Section IV. Plant Disposition:

All plot locations were harvested or terminated, and all remaining material that was not sent to an Athenix Corporation facility or other laboratories for analysis was destroyed. Unplanted seed which was not utilized by the cooperator was returned to Athenix Corporation for storage or destroyed.

Preparation Date: May 12, 2010

USDA Field Termination Report

USDA Notification/Permit Number: 08-309-103n

Applicant Internal Number: ATX-07-27

Applicant: Athenix Corporation
Research Triangle Park, NC 27709

Regulated Article: *Zea mays;* Corn

Lines: 6750-6752, 6845-6916, 6933-6971, 6978-6993, 7045-7048, 6972

Section I. Site Release Information:

Trials sites were requested for one (1) location:

USDA-Notification Release Site No.	County / State	Acreage Planted	Date Planted	Date Terminated
1	Juana Diaz / PR	0.33	12/18/2008	4/03/2009

Section II. List of characteristic observations collected on the regulatory compliance *In-Season Forms* approximately every four weeks during the growing season between the transgenic plants/regulated & non-transgenic plants/non-regulated, insects and environment:

a	Plant Morphological - Observations
b	Plant Disease - Observations
c	Difference in Insect Occurrence - Observations
d	Unusual Occurrences During Flowering Stage -Observations
e	Unusual Occurrences During Pesticide Applications -Observations
f	Difference in Environmental Conditions - Observations

Section III. Summarized results from the regulatory In-Season Observation Forms:

1. **Juana Diaz Co., PR :**
   a) Cooperator reported no observed differences in plant morphology.
   b) Cooperator reported no unusual differences in plant disease.
   c) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
   d) Cooperator noted no unusual growth characteristics during flowering stage.
   e) Cooperator reported no unusual interactions after pesticide applications.
   f) Cooperator reported no unusual differences in environmental and/or weather conditions.

   No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

Section IV. Plant Disposition:

All plot locations were harvested or terminated, and all remaining material that was not sent to an Athenix Corporation facility or other laboratories for analysis was destroyed. Unplanted seed which was not utilized by the cooperator was returned to Athenix Corporation for storage or destroyed.

Preparation Date: May 12, 2010

USDA Field Termination Report

USDA Notification/Permit Number: 09-047-108n

Applicant Internal Number: ATX-07-29

Applicant: Athenix Corporation
Research Triangle Park, NC 27709

Regulated Article: *Zea mays;* corn

Lines: 6845-6916, 6933-6972, 6978-6993, 7045-7047

Section I. Site Release Information:

Trials were conducted at one (1) location:

USDA-Notification Release Site No.	County / State	Acreage Planted	Date Planted	Date Terminated
1	Santa Isabel/Corozal / PR	0.41	03/13/2009	06/19/2009

Section II. List of agronomic observations collected on the regulatory compliance *In-Season Forms* approximately once every month during the growing season between the transgenic plants/regulated & non-transgenic plants/non-regulated.:

a	Disease:	Resistance/susceptibility to disease.
b	Insects:	Abundance of non-target species and resistance/susceptibility to insect feeding not specifically engineered for resistance.
c	Plant Growth	Is plant morphology and growth similar for both transgenic and non-transgenic plants?
d	Weediness	Is germination, tasseling, seed production, etc. similar for both transgenic and non-transgenic plants?

Section III. Summarized results from the regulatory In-Season Observation Forms:

1. Santa Isabel/(Corozal) Co., PR:

a) Cooperator reported no unusual differences in plant disease.
b) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
c) Cooperator noted no unusual plant growth observations.
d) Cooperator reported no unusual weediness characteristics.

No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

Section IV. Plant Disposition:

All plot locations were harvested or terminated, and all remaining material that was not sent to an Athenix Corporation facility or other laboratories for analysis was destroyed. Unplanted seed which was not utilized by the cooperator was returned to Athenix Corporation for storage or destroyed.

Preparation Date: July 08, 2010

USDA Field Termination Report

USDA Notification/Permit Number: 09-091-101n

Applicant Internal Number: ATX-07-34

Applicant: Athenix Corporation
Research Triangle Park, NC 27709

Regulated Article: *Zea mays;* Corn

Lines: 6845-6916, 6933-6972, 6978-6993, 7045-7047

Section I. Site Release Information:

Trials were requested at twenty-two (22) locations:

USDA-Notification Release Site No.	City / State	Acreage Planted	Date Planted	Date Terminated
1	Bradford (Bureau Co.) / IL	N/A	Not Planted	N/A
2	Ivesdale (Champaign Co.) / IL	0.2	05/22/2009	10/28/2009
3	Carlyle (Clinton Co.) / IL	0.2	06/02/2009	11/10/2009
4	Mt Pulaski (Logan Co.) / IL	0.2	05/24/2009	10/21/2009
5	Wyoming (Stark Co.)/ IL	0.2	05/22/2009	11/13/2009
6	Lebanon (Boone Co.) / IN	0.4	05/30/2009	11/23/2009
7	Rockville (Parke Co.) / IN	0.2	05/23/2009	12/03/2009
8	Atlantic (Cass Co.) / IA	0.2	05/08/2009	10/09/2009
9	Colfax (Jasper Co.) / IA	0.5	05/21/2009	12/07/2009
10	Wesley (Kossuth Co.) / IA	0.2	05/19/2009	10/20/2009
11	Winterset (Madison Co.) / IA	N/A	Not Planted	N/A
12	Walcott (Scott Co.) / IA	0.2	05/16/2009	11/12/2009
13	Ottumwa (Wapello Co.) / IA	0.2	06/01/2009	07/07/2009
14	Gowrie (Webster Co.) / IA	0.2	05/11/2009	11/04/2009
15	Clarion (Wright Co.) / IA	0.2	05/20/2009	10/19/2009
16	Paynesville (Stearns Co.) / MN	0.2	05/19/2009	11/23/2009
17	Burwell (Valley Co.) / NE (site 1)	0.2	05/13/2009	11/10/2009
18	Burwell (Valley Co.) / NE (site 2)	0.2	05/14/2009	11/07/2009
19	Arlington (Washington Co.) / NE	0.2	05/06/2009	10/08/2009
20	Bruce (Brookings Co.) / SD	0.2	05/21/2009	07/31/2009
21	Delavan (Walworth Co.) / WI (site 1)	0.2	05/12/2009	11/10/2009
22	Delavan (Walworth Co.) / WI (site 2)	N/A	Not Planted	N/A

Section II. List of characteristic observations collected on the regulatory compliance *In-Season Forms* approximately every four weeks during the growing season between the transgenic plants/regulated & non-transgenic plants/non-regulated, insects and environment:

a	Plant Morphological - Observations
b	Plant Disease - Observations
c	Difference in Insect Occurrence - Observations
d	Unusual Occurrences During Flowering Stage -Observations
e	Unusual Occurrences During Pesticide Applications -Observations
f	Difference in Environmental Conditions - Observations

Section III. Summarized results from the regulatory In-Season Observation Forms:

1. **Bradford (Bureau Co.) , IL :**
   Not Planted

2. **Ivesdale (Champaign Co.) , IL :**
   a) Cooperator reported no observed differences in plant morphology.
   b) Cooperator reported no unusual differences in plant disease.
   c) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
   d) Cooperator noted no unusual growth characteristics during flowering stage.
   e) Cooperator reported no unusual interactions after pesticide applications.
   f) Cooperator reported no unusual differences in environmental and/or weather conditions.

   No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

3. **Carlyle (Clinton Co.) , IL :**
   a) Cooperator reported no observed differences in plant morphology.
   b) Cooperator reported no unusual differences in plant disease.
   c) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
   d) Cooperator noted no unusual growth characteristics during flowering stage.
   e) Cooperator reported no unusual interactions after pesticide applications.
   f) Cooperator reported no unusual differences in environmental and/or weather conditions.

   No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

4. **Mt. Pulaski (Logan Co.) , IL :**
   a) Cooperator reported no observed differences in plant morphology.
   b) Cooperator reported no unusual differences in plant disease.
   c) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
   d) Cooperator noted no unusual growth characteristics during flowering stage.
   e) Cooperator reported no unusual interactions after pesticide applications.
   f) Cooperator reported no unusual differences in environmental and/or weather conditions.

   No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

5. **Wyoming (Stark Co.) , IL :**
   a) Cooperator reported no observed differences in plant morphology.
   b) Cooperator reported no unusual differences in plant disease.
   c) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
   d) Cooperator noted no unusual growth characteristics during flowering stage.
   e) Cooperator reported no unusual interactions after pesticide applications.
   f) Cooperator reported no unusual differences in environmental and/or weather conditions.

   No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

6. **Lebanon (Boone Co.) , IN :**
   a) Cooperator reported no observed differences in plant morphology.
   b) Cooperator reported no unusual differences in plant disease.
   c) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
   d) Cooperator noted no unusual growth characteristics during flowering stage.
   e) Cooperator reported no unusual interactions after pesticide applications.
   f) Cooperator reported no unusual differences in environmental and/or weather conditions.

   No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

7. **Rockville (Parke Co.) , IN :**
   a) Cooperator reported no observed differences in plant morphology.
   b) Cooperator reported no unusual differences in plant disease.
   c) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
   d) Cooperator noted no unusual growth characteristics during flowering stage.
   e) Cooperator reported no unusual interactions after pesticide applications.
   f) Cooperator reported no unusual differences in environmental and/or weather conditions.

   No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

8. **Atlantic (Cass Co.) , IA :**
   a) Cooperator reported no observed differences in plant morphology.
   b) Cooperator reported no unusual differences in plant disease.
   c) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
   d) Cooperator noted no unusual growth characteristics during flowering stage.
   e) Cooperator reported no unusual interactions after pesticide applications.
   f) Cooperator reported no unusual differences in environmental and/or weather conditions.

   No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

9. **Colfax (Jasper Co.) , IA :**
   a) Cooperator reported no observed differences in plant morphology.
   b) Cooperator reported no unusual differences in plant disease.
   c) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
   d) Cooperator noted no unusual growth characteristics during flowering stage.
   e) Cooperator reported no unusual interactions after pesticide applications.
   f) Cooperator reported no unusual differences in environmental and/or weather conditions.

   No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

10. **Wesley (Kossuth Co.) , IA :**
    a) Cooperator reported no observed differences in plant morphology.
    b) Cooperator reported no unusual differences in plant disease.
    c) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
    d) Cooperator noted no unusual growth characteristics during flowering stage.
    e) Cooperator reported no unusual interactions after pesticide applications.
    f) Cooperator reported no unusual differences in environmental and/or weather conditions.

    No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

11. **Winterset (Madison Co.) , IA :**
    Not Planted

12. **Walcott (Scott Co.) , IA :**
    a) Cooperator reported no observed differences in plant morphology.
    b) Cooperator reported no unusual differences in plant disease.
    c) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
    d) Cooperator noted no unusual growth characteristics during flowering stage.
    e) Cooperator reported no unusual interactions after pesticide applications.
    f) Cooperator reported no unusual differences in environmental and/or weather conditions.

    No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

13. **Ottumwa (Wapello Co.) , IA :**
    a) Cooperator reported no observed differences in plant morphology.
    b) Cooperator reported no unusual differences in plant disease.
    c) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
    d) Cooperator noted no unusual growth characteristics during flowering stage.
    e) Cooperator reported no unusual interactions after pesticide applications.
    f) Cooperator reported no unusual differences in environmental and/or weather conditions.

    No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

14. **Gowrie (Webster Co.) , IA :**
    a) Cooperator reported no observed differences in plant morphology.
    b) Cooperator reported no unusual differences in plant disease.
    c) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
    d) Cooperator noted no unusual growth characteristics during flowering stage.
    e) Cooperator reported no unusual interactions after pesticide applications.
    f) Cooperator reported no unusual differences in environmental and/or weather conditions.

    No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

15. **Clarion (Wright Co.) , IA :**
    a) Cooperator reported no observed differences in plant morphology.
    b) Cooperator reported no unusual differences in plant disease.
    c) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
    d) Cooperator noted no unusual growth characteristics during flowering stage.
    e) Cooperator reported no unusual interactions after pesticide applications.
    f) Cooperator reported no unusual differences in environmental and/or weather conditions.

    No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

16. **Paynesville (Stearns Co.) , MN :**
    a) Cooperator reported no observed differences in plant morphology.
    b) Cooperator reported no unusual differences in plant disease.
    c) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
    d) Cooperator noted no unusual growth characteristics during flowering stage.
    e) Cooperator reported no unusual interactions after pesticide applications.
    f) Cooperator reported no unusual differences in environmental and/or weather conditions.

    No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

17. **Burwell (Valley Co.) , NE (site 1) :**
    a) Cooperator reported no observed differences in plant morphology.
    b) Cooperator reported no unusual differences in plant disease.
    c) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
    d) Cooperator noted no unusual growth characteristics during flowering stage.
    e) Cooperator reported no unusual interactions after pesticide applications.
    f) Cooperator reported no unusual differences in environmental and/or weather conditions.

    No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

18. **Burwell (Valley Co.) , NE (site 2) :**
    a) Cooperator reported no observed differences in plant morphology.
    b) Cooperator reported no unusual differences in plant disease.
    c) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
    d) Cooperator noted no unusual growth characteristics during flowering stage.
    e) Cooperator reported no unusual interactions after pesticide applications.
    f) Cooperator reported no unusual differences in environmental and/or weather conditions.

    No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

19. **Arlington (Washington Co.) , NE :**
    a) Cooperator reported no observed differences in plant morphology.
    b) Cooperator reported no unusual differences in plant disease.
    c) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
    d) Cooperator noted no unusual growth characteristics during flowering stage.
    e) Cooperator reported no unusual interactions after pesticide applications.
    f) Cooperator reported no unusual differences in environmental and/or weather conditions.

    No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

20. **Bruce (Brookings Co.) , SD :**
    a) Cooperator reported no observed differences in plant morphology.
    b) Cooperator reported no unusual differences in plant disease.
    c) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
    d) Cooperator noted no unusual growth characteristics during flowering stage.
    e) Cooperator reported no unusual interactions after pesticide applications.
    f) Cooperator reported no unusual differences in environmental and/or weather conditions.

    No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

21. Delavan (Walworth Co.) , WI :

a) Cooperator reported no observed differences in plant morphology.
b) Cooperator reported no unusual differences in plant disease.
c) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
d) Cooperator noted no unusual growth characteristics during flowering stage.
e) Cooperator reported no unusual interactions after pesticide applications.
f) Cooperator reported no unusual differences in environmental and/or weather conditions.

No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

22. Delavan (Walworth Co.) , WI :

Not Planted

Section IV. Plant Disposition:

All plot locations were harvested or terminated, and all remaining material that was not sent to an Athenix Corporation facility or other laboratories for analysis was destroyed. Unplanted seed which was not utilized by the cooperator was returned to Athenix Corporation for storage or destroyed.

Preparation Date: September 26, 2010

USDA Field Termination Report

USDA Notification/Permit Number: 09-086-104n

Applicant Internal Number: ATX-07-37

Applicant: Athenix Corporation
Research Triangle Park, NC 27709

Regulated Article: *Zea mays;* Corn

Lines: 6845-6916, 6933-6972, 6978-6993, 7045-7047

Section I. Site Release Information:

Trials sites were requested at three (3) locations:

USDA-Notification Release Site No.	City / State	Acreage Planted	Date Planted	Date Terminated
1	Bluffton (Wells Co.) / IN	0.5	05/25/2009	11/12/2009
2	Ames (Story Co.) / IA	1.3	05/09/2009	11/16/2009
3	Arlington (Washington Co.) / NE	N/A	Not Planted	N/A

Section II. List of agronomic observations collected on the regulatory compliance *In-Season Forms* approximately once every month during the growing season between the transgenic plants/regulated & non-transgenic plants/non-regulated:

a	Disease:	Resistance/susceptibility to disease.
b	Insects:	Abundance of non-target species and resistance/susceptibility to insect feeding not specifically engineered for resistance.
c	Plant Growth	Is plant morphology and growth similar for both transgenic and non-transgenic plants?
d	Weediness	Is germination, growth, flowering, seed production, etc. similar for both transgenic and non-transgenic plants?

Section III. Summarized results from the regulatory In-Season Observation Forms:

1. **Bluffton (Wells Co.) IN:**
   a) Cooperator reported no unusual differences in plant disease.
   b) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
   c) Cooperator noted no unusual plant growth observations.
   d) Cooperator reported no unusual weediness characteristics.

   No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

2. **Ames (Story Co.) IA:**
   a) Cooperator reported no unusual differences in plant disease.
   b) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
   c) Cooperator noted no unusual plant growth observations.
   d) Cooperator reported no unusual weediness characteristics.

   No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

Section IV. Plant Disposition:

All plot locations were harvested or terminated, and all remaining material that was not sent to an Athenix Corporation facility or other laboratories for analysis was destroyed. Unplanted seed which was not utilized by the cooperator was returned to Athenix Corporation for storage or destroyed.

Preparation Date: September 26, 2010

USDA Field Termination Report

USDA Notification/Permit Number: 09-215-106n

Applicant Internal Number: ATX-07-40

Applicant: Athenix Corporation
Research Triangle Park, NC 27709

Regulated Article: *Zea mays;* Corn

Lines: 6845-6916, 6933-6972, 6978-6993, 7045-7047

Section I. Site Release Information:

Trial was conducted at one (1) location:

USDA-Notification Release Site No.	County / State	Acreage Planted	Date Planted	Date Terminated
1	Santa Isabel / PR	0.42	4/06/2010	6/18/2010

Planted under Notification 08-254-111n and Notification 09-215-106n.

Section II. List of agronomic observations collected on the regulatory compliance *In-Season Forms* approximately once every month during the growing season between the transgenic plants/regulated & non-transgenic plants/non-regulated.:

a	Disease:	Resistance/susceptibility to disease.
b	Insects:	Abundance of non-target species and resistance/susceptibility to insect feeding not specifically engineered for resistance.
c	Plant Growth	Is plant morphology and growth similar for both transgenic and non-transgenic plants?
d	Weediness	Is germination, tasseling, seed production, etc. similar for both transgenic and non-transgenic plants?

Section III. Summarized results from the regulatory In-Season Observation Forms:

1. Santa Isabel Co., PR (1st planting) :

a) Cooperator reported no unusual differences in plant disease.
b) Cooperator reported no unusual differences in insect pests or beneficial insect populations.
c) Cooperator noted no unusual plant growth observations.
d) Cooperator reported no unusual weediness characteristics.

No unauthorized environmental releases occurred during the testing/growing phase of this regulated test. A Potential Compliance Incident (PCI) did occur during the volunteer monitoring period of this regulated field site, soil movement reported on August 10, 2010 (BCS PCI-10-005).

Section IV. Plant Disposition:

All plot locations were harvested or terminated, and all remaining material that was not sent to an Athenix Corporation facility or other laboratories for analysis was destroyed. Unplanted seed which was not utilized by the cooperator was returned to Athenix Corporation for storage or destroyed.

Preparation Date: February 7, 2011

USDA Field Termination Report

USDA Notification/Permit Number: 09-341-105n

Applicant Internal Number: ATX-07-43

Applicant: Athenix Corporation
Research Triangle Park, NC 27709

Regulated Article: *Zea mays;* corn

Lines: 6845-6916, 6933-6972, 6978-6993, 7045-7047

Section I. Site Release Information:

Trial was conducted at one (1) location:

USDA-Notification Release Site No.	City / State	Acreage Planted	Date Planted	Date Terminated
1	1 Juana Diaz / PR	0.32	01/26/2010	05/05/2010

Section II. List of characteristic observations collected on the regulatory compliance *In-Season Forms* during the growing season (*approximately: emergence, ~60, ~90 and ~120 Days After Planting – Crop Dependent*) between the transgenic plants & non-transgenic plants, insects and environment:

a	Overall Crop/Test Condition Observations
b	Isolation Condition Observations
c	Phenotypic Plant Characteristic (Includes Weediness) Observations
d	Plant Disease Observations
e	Insect Beneficial Observations
f	Insect Pest Observations
g	Difference in Environmental Conditions Observations

Section III. Summarized results from the regulatory In-Season Observation Forms:

1. **Juana Diaz, PR :**
   a) Cooperator reported overall crop condition observations as good.
   b) Cooperator reported overall isolation condition observations as good.
   c) Cooperator noted no unusual phenotypic plant characteristics.
   d) Cooperator reported no unusual differences in plant disease.
   e) Cooperator reported no unusual differences with beneficial insects.
   f) Cooperator reported no unusual differences with pest insects.
   g) Cooperator reported no unusual differences in environmental and/or weather conditions.

   No plot damage occurred. Trial was properly conducted and completed without any unauthorized environmental releases or compliance incidents.

Section IV. Plant Disposition:

All plot locations were harvested or terminated, and all remaining material that was not sent to a Bayer CropScience/Athenix Corporation facility or other laboratories for analysis was destroyed. Unplanted seed which was not utilized by the cooperator was returned to Bayer CropScience/Athenix Corporation for storage or destroyed.

Signature: _____ Date: May 19, 2011

Randy J. Wegener
Regulatory Compliance Specialist
Bayer CropScience – Regulatory Affairs BioScience

APPENDIX 2

Materials and Methods – Product Characterization

2.A. Materials and methods for molecular characterization

Materials

Leaf material was generated from various backcross generations of event VCO-Ø1981-5 with the B110 and B109 inbred lines (BC0B, BC1B, BC2B, and $BC1B_2$) or the AAX3 inbred line (BC0A, BC1A, BC2A, BC3A) and appropriate non-transgenic control material for molecular characterization. Young leaves from 10 to 20 plants were harvested from greenhouse grown plants. Control materials were either non-transgenic control maize of the BC1B generation or the B110 inbred line.

Reference materials

DNA of plasmid pAG3541 was included on all Southern blots as a positive control for probe hybridization. The *Agrobacterium* transformation plasmid pAG3541 (Figure 8) and *E. coli* plasmid pAX3541 (Figure 9) were used when appropriate to verify expected hybridization product sizes. The plasmid pAX3541 was prepared as a precursor in the cloning process of pAG3541 and contains the entire *epsps grg23ace5* gene expression cassette. DNA molecular weight markers were used for Southern blot analysis. A 1 Kb DNA Extension Ladder (Life Technologies, Carlsbad, CA) was included as a size standard for hybridizing fragments on each gel and represented by kb line markers in this report.

Plant processing and genomic DNA extraction

All plants produced for these analyses were grown in the greenhouse and individually analyzed by event specific PCR. Leaf tissue of 10 to 20 plants from PCR positive plants of the same generation were combined, and genomic DNA was extracted and quantified for use in Southern blot analyses (Dellaporta *et al.,* 1983**).**

Quantitation of genomic DNA

The concentration of each DNA sample was measured using a Quant-iT™ PicoGreen® dsDNA kit (Life Technologies, Carlsbad, CA) with a spectrofluorometer following the manufacturer's instructions. A Lambda DNA standard was used to calibrate the instrument prior to quantification.

Figure 8. Transformation vector pAG3541 for event VCO-Ø1981-5

Coding sequences are shown as orange arrows and regulatory elements as a blue box. Restriction enzyme locations are identified for the entire vector and Southern probes (5-10) are indicated for the vector backbone.

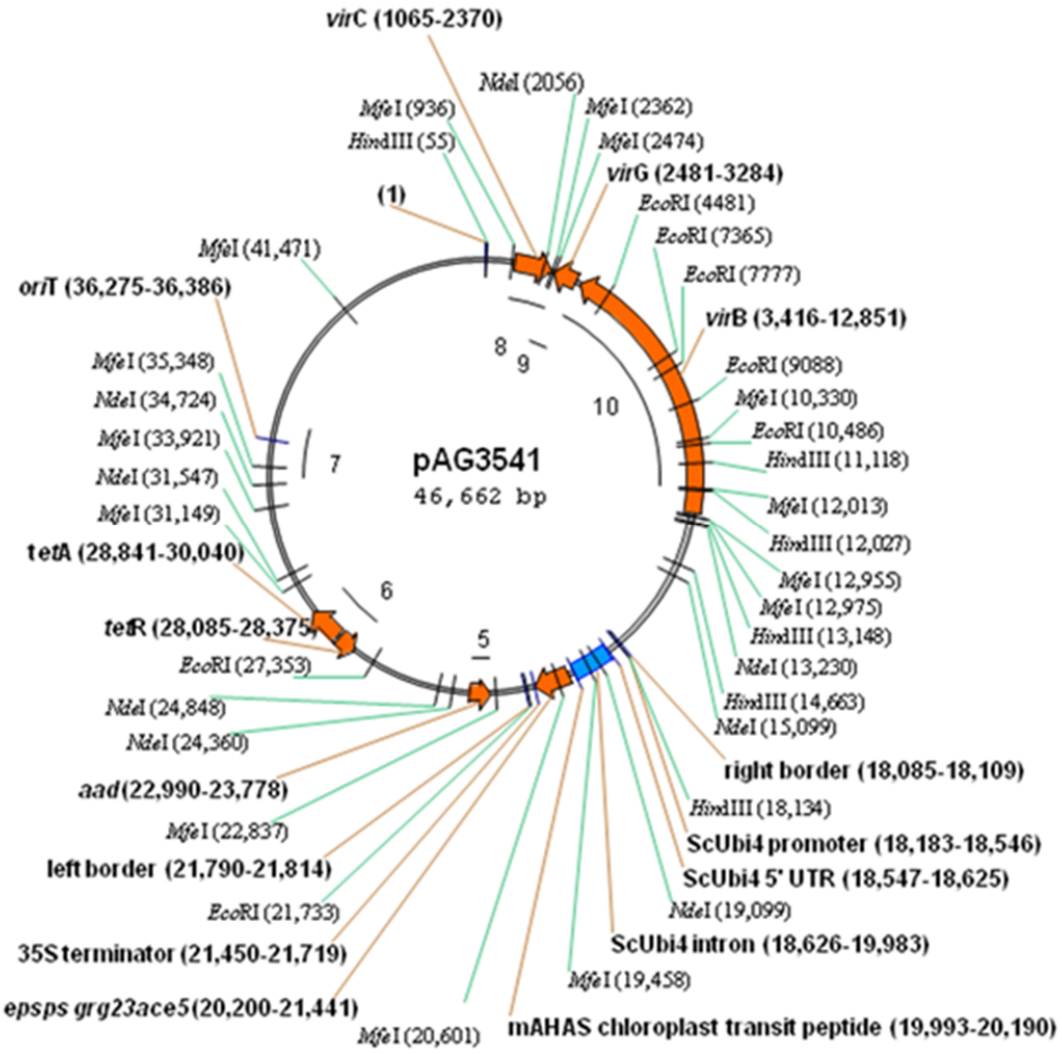
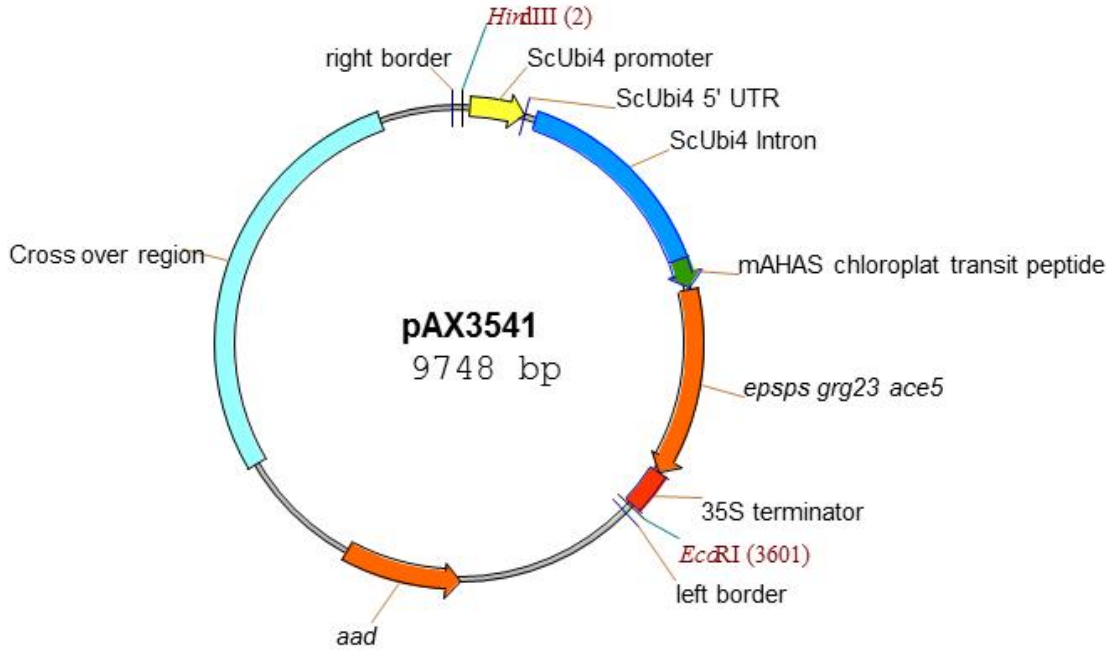


Figure 9. Plasmid map of pAX3541



Southern blot analysis

Southern blot analyses were performed using standard molecular biology techniques (Southern, 1975; Sambrook *et al.,* 1989). Genomic DNA (15 µg) was digested with the appropriate restriction enzymes overnight at the optimal temperature for each enzyme. Digested DNA was loaded onto 0.65% agarose gels and separated electrophoretically in 2.5X TAE (100 mM Tris-acetate, 2.5 mM EDTA pH 8.3) buffer along with DNA molecular weight markers. Agarose gels containing the separated genomic DNA were submerged in 0.25 N HCl for 10 minutes to depurinate the DNA. Depurination was followed by denaturation in 0.5 M NaOH and 1.5 M NaCl for 30 minutes. Finally, the gel was neutralized in 0.5 M Tris-HCl at pH 7.0 and 1.5 M NaCl for 30 minutes, and soaked in 20X SSC (3M NaCl, 0.3 M sodium citrate pH 7.0) for 30 minutes. The DNA was transferred to a Nytran SuPerCharge nylon (Whatman, Sanford, ME) membrane and assembled as a part of the TurboBlotter™ (Whatman, Sanford, ME) system. The DNA was allowed to transfer onto the membrane overnight in a 20X SSC buffer. Following transfer to the membrane, the DNA was bound to the membrane using a Stratalinker UV Crosslinker (Stratagene, La Jolla, CA).

DNA probe preparation

Element-specific DNA probes were labeled with dCTP-^{32}P via random priming using the Ready-To-Go DNA labeling beads (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Two of the probes with low GC content (ScUbi4 intron and 35S terminator) were labeled with dTTP-^{32}P via random priming using the RadPrime DNA (Life Technologies, Carlsbad, CA) labeling system. For all element-specific probes, 25-30 ng of DNA were used for labeling. The radiolabeled probes were added to fresh hybridization solution,

and the membranes were incubated overnight. Hybridization was carried out at 65°C for approximately 16 hours, followed by two washes each in a) 40 mM $NaPO_4$, pH 7.0, 1 mM EDTA, 5% SDS, 0.5% BSA (Fraction V) and b) 40 mM $NaPO_4$, pH 7.0, 1 mM EDTA, 1% SDS. The membranes were then subjected to autoradiography (Kodak AR X-OMAT film using a Kodak intensifying screen at -80ºC).

Confirmation of the absence of vector backbone components and insert integrity

Southern blot analysis was conducted to verify the absence of the transformation plasmid components outside of the transferred T-DNA region and the integrity of the insert. Genomic DNA (BC1B generation was used in the B110 inbred background), appropriate negative controls (both the non-transgenic control (BC1B) and the non-transgenic inbred (B110) were digested with a combination of *Hin*dIII and *Eco*RI, and independently with *Mfe*I and *Nde*I.

The probes employed were designed to hybridize to the functional components of the plasmid outside of the T-DNA. The *Agrobacterium* plasmid pAG3541 was included as a positive control for hybridization of the transformation plasmid components and loaded as genomic equivalent of 0.5, 1 and 3 copies.

2.B. Materials and methods for protein characterization tests

Studies on potential toxicology and allergenicity for food, feed and the environment are conducted with purified EPSPS ACE5 protein expressed by *Escherichia coli*. In order to utilize the safety data of the proteins produced in the microorganism for the safety assessment of the same protein produced in a genetically modified plant, it is important to confirm that the protein produced in a microorganism is representative of the protein produced in the modified plant. The analytical tests show that the protein produced in *E. coli* is representative of EPSPS ACE5 protein produced in event VCO-Ø1981-5.

Materials

EPSPS ACE5 protein was produced in *E. coli* strain BL21-DE3 star (Life Technologies, Carlsbad, CA) using a T7 inducible expression plasmid with IPTG induction and was purified using ammonium sulfate fractionation (38-80% saturation) followed by hydrophobic interaction and anion exchange chromatography. The purified EPSPS ACE5 protein was dialyzed into phosphate buffered saline (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4/KH_2PO_4 pH 7.4) lyophilized, and stored at -80ºC. For protein analysis, lyophilized protein was dissolved in ultrapure water to original volume yielding purified EPSPS ACE5 protein in PBS buffer for subsequent analysis.

EPSPS ACE5 protein was purified from event VCO-Ø1981-5 leaf material in extraction buffer (40mM Tris-HCl, pH 8.0, 80mM NaCl, including complete protease inhibitor cocktail [Roche Diagnostics, Mannheim, Germany]) followed by ammonium sulfate fractionation (38-80% saturation), treatment with activated charcoal, and filtration through 0.22 micron polyester filters (Corning Life Sciences, Lowell, MA). Soluble extracted proteins were then further purified utilizing fast protein liquid chromatography (FPLC column) using hydrophobic interaction, anion exchange, and size exclusion chromatography. The protein fractions enriched for maize-produced EPSPS ACE5 protein were concentrated using Biomax Ultrafree 5,000 dalton nominal molecular weight limit centrifugation tubes and stored at 4ºC in 40mM Tris, pH 7.5, 80mM NaCl, 10% glycerol buffer prior to analysis.

In vitro enzyme assay

The function of EPSPS ACE5 protein was confirmed by carrying out enzymatic assays with purified *E.coli*-produced EPSPS ACE5 protein. An enzymatic assay was developed to quantify the production of inorganic phosphate (Pi) by EPSPS enzymes. The assay used enzyme coupling and resulted in the generation of a highly fluorescent product (Vazquez *et al.,* 2003). This purified EPSPS ACE5 protein was mixed with shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) and the presence of product (inorganic phosphate) was quantified using a fluorimeter.

Analysis of the molecular weight comparison by SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was used to separate proteins based on their molecular weight. This technique was employed prior to Coomassie Blue gel staining for molecular weight determination and mass spectroscopy analysis, as well as prior to electroblotting onto nitrocellulose or polyvinylidene fluoride (PVDF) membranes for western blot analysis or N-terminal sequencing, respectively. Proteins were prepared for SDS PAGE analysis by mixing with Laemmli buffer and heating for approximately 5 minutes to 100ºC. Proteins were then loaded into the wells of NuPage 4-12% Bis-tris gels (Life Technologies, Carlsbad, CA) with either NuPage MOPs or MES running buffer in an XCell Surelock MiniCell electrophoresis chamber (Life Technologies, Carlsbad, CA). Novex Sharp prestained protein molecular weight markers (Life Technologies, Carlsbad, CA) were included as a visual reference for molecular weight determination.

For molecular weight determination, total protein amounts of ~450 ng maize-produced EPSPS ACE5 and ~400 ng *E. coli*-produced EPSPS ACE5 were separated on a 4-12% Bis-Tris polyacrylamide gel in 1X MES buffer alongside prestained protein molecular weight marker. The separated proteins were visualized by Imperial Coomassie protein stain (Life Technologies, Carlsbad, CA) and photographed.

MALDI-TOF mass spectrophotometry protein identification

Protein bands were excised from Coomassie Blue stained SDS-Page gels and submitted to Alphalyse, Inc. (Palo Alto, CA) for analysis. The protein samples were reduced and alkylated with iodoacetamide and digested with trypsin that cleaves after lysine and arginine residues. The resulting peptides were concentrated on a ZipTip micropurification column and eluted onto an anchorchip target for analysis on a Bruker Autoflex III MALDI TOF/TOF instrument. The peptide mixture was analyzed in positive reflector mode for accurate peptide mass determination. MALDI MS/MS was performed on approximately 15 peptides for partial peptide sequencing. The MS and MS/MS spectra were combined and used for database searching using the Mascot software. Peptide masses and peptide fragment masses were matched against the expected EPSPS ACE5 sequence.

Western blot analysis

Immunoreactivity was examined by western blot analysis. Purified maize- and *E. coli*-produced EPSPS ACE5 proteins were separated on a 4-12% Bis-Tris polyacrylamide gel along with protein molecular weight markers. Separated proteins were blotted onto nitrocellulose membrane in an XCell Surelock MiniCell (Life Technologies, Carlsbad, CA) system with a constant current of 30V for approximately 2 hours. Successive applications of primary antibody (affinity purified rabbit anti-EPSPS ACE5), and donkey anti-rabbit IgG linked horseradish peroxidase secondary antibody were applied. Electrochemiluminescencent substrate was then applied to allow visualization of immobilized antibody complexes by developing on x-ray film.

Analysis of enzymatic activity of EPSPS ACE5 protein

EPSPS ACE5 is a 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme, which releases inorganic phosphate as a by-product of its catalytic reaction. Released phosphate was detected by way of a real-time, coupled fluorescent kinetic assay. The final product of this assay was a red fluorescent marker with an excitation wavelength of 555 nm and emission wavelength of 590 nm detectable by way of a spectrofluorometer (Molecular Devices).

Samples containing both required substrates for the synthesis reaction as well as various dilutions of maize-produced EPSPS ACE5 samples were assayed to determine appropriate dilutions of the enzyme to yield a rate of product formation within the dynamic or linear range of the assay. Additional samples were assayed in triplicate containing an appropriate dilution of plant-purified EPSPS ACE5 along with specific reaction substrates as well as the following negative controls: buffer only, buffer plus substrate without enzyme, and buffer plus enzyme but without substrate.

The enzymatic activity of *E. coli*-produced EPSPS ACE5 protein was analyzed following the same methods as described above (data not shown).

N-terminal sequencing analysis

Proteins were prepared for N-terminal sequencing by SDS Page separation followed by electroblotting onto a polyvinylidene fluoride (PVDF) membrane. Electroblotting onto PVDF membrane was performed exactly as described for blotting onto nitrocellulose for western blotting except that the PVDF membrane was equilibrated in 100% methanol for approximately 30 seconds prior to equilibration in transfer buffer. Proteins were transferred to the PVDF membrane by applying a constant charge of 30V for 120 minutes. Blotted EPSPS ACE5 proteins were stained with Imperial Coomassie Blue for 5 minutes and then destained for approximately 3 minutes in 50% methanol, 10% acetic acid until bands were clearly visible. The PVDF membrane was then washed extensively in ultrapure water and bands corresponding to EPSPS ACE5 were carefully excised for further analysis. Excised bands were packaged and shipped to Alphalyse, Inc (Palo Alto, CA) for sequence analysis.

The analysis is performed on an ABI Procise 494 sequencer. The procedure determines the N-terminal amino acid sequence of proteins and peptides by the Edman degradation chemistry. The sequencing takes place on an acid-etched glass fiber disk or on a PVDF membrane. The Edman degradation is a cyclic procedure where amino acid residues

are cleaved off one at a time and identified by chromatography. There are three steps in the cyclic procedure. In step one the phenylisothiocyanate (PITC) reagent is coupled to the N-terminal amino group under alkaline conditions. In step two the N-terminal residue is cleaved in acidic media. In step three, the PITC coupled residue is transferred to a flask, converted to a PTH-residue and identified by HPLC chromatography. The next cycle is then started for identification of the next N-terminal residue. The cyclic process is not always 100% and therefore carryover from previous cycle can be observed. The identity of each single residue is detected by subtracting the previous chromatogram from the present chromatogram.

Glycosylation Assay

Glycosylation analysis was performed to determine if maize-expressed EPSPS ACE5 was modified post-translation by the addition of carbohydrate moieties. Both microbial-expressed and maize-expressed EPSPS ACE5 proteins were separated by SDS-PAGE along with horseradish peroxidase (positive control protein) and soybean trypsin inhibitor (negative control protein). Gels were stained with Gelcode glycoprotein staining kit from Pierce Biotechnology, Inc. according to manufacturer's instructions. The gel containing separated proteins was treated with periodate solution, which oxidizes cis-diol sugar groups in glycoproteins. The resulting aldehyde groups were detected through the formation of Schiff-base bonds with a proprietary reagent that produces magenta bands, traditionally called the periodic acid-Schiff (PAS) reagent method. Glycoproteins were detected as bright magenta bands on the gel. The same gel was then stained with Imperial Coomassie Blue stain for detection of all proteins loaded on the gel.

Protein digestibility in simulated gastric fluid

The *E. coli*-produced EPSPS ACE5 protein was evaluated for *in vitro* gastric digestibility using a standard pepsin digestion method (Thomas *et al.,* 2004).

Test protein and reference protein solutions were incubated with human SGF (a pepsin solution at pH 1.2) at approximately 37°C and samples were taken for analysis at time-points ranging from 0 to 60 minutes. Reference proteins, horseradish peroxidase (HRP) and ovalbumin (OVA), known to be digested rapidly and slowly, respectively, were tested in parallel.

The protein incubation for the test and reference materials was made in 2 ml microcentrifuge tubes in a waterbath at approximately 37°C. For each test and reference protein solution, 80 µl was added to 1,520 µl of SGF and mixed. Samples of 200 µl were taken at 0.5, 2, 5, 10, 20, 30 and 60 minutes. The tubes were agitated after each sampling and at approximately 45 minutes. A dilution of the test protein solution at 1/10 in phosphate buffered saline (PBS 1X, 137 mM NaCl, 2.7 mM KCl, 10 mM Phosphate buffer, pH 7.4) supplemented with 20% glycerol was prepared for the 10% loading control. As soon as samples were taken, the reaction was terminated by adding the 200 µl sample to a tube containing 70 µl 200 mM $NaHCO_3$ (pH 11.0).

Additional control samples were prepared:
- a zero minute incubation of protein (10 µl) with 'SGF without pepsin' (190 µl);
- a zero minute incubation of the 1/10 diluted protein (10 µl) with 'SGF without pepsin' (190 µl) (10% loading control);
- a 60 minutes incubation of protein (10 µl) with 'SGF without any pepsin' (190 µl);

- a 'time zero' sample was produced by adding the protein (10 µl) to SGF (190 µl) after the reaction was terminated as above;
- a sample of SGF alone before incubation and the reaction terminated as above;
- a sample of SGF alone after 60 minutes incubation and the reaction terminated as above.

SDS-PAGE was carried out following the method of Laemmli (1970) using a BioRad Mini-Protean III cell (BioRad, France). Prior to running SDS-PAGE, 5 µl of 5X Leammli solution was added to 20 µl of digestion samples and heated for 10 minutes at more than 90°C before loading the gel. Samples of 15 µl were added to wells of an SDS-PAGE gel (15 wells, 1 mm 10-20% gradient polyacrylamide Tris/Tricine)(BioRad, France). A suitable marker solution (2.5-200 kDa) was used to provide reference points of known molecular weights on the gel (Mark 12, Invitrogen, France). Gels were stained with Coomassie blue (Invitrogen).

For western blot analysis, a polyvinylidene fluoride (PVDF) membrane was placed on the SDS-PAGE gel in a Tris/Glycine buffer and an electrical current applied in order to transfer the protein bands onto the membrane. To detect the EPSPS ACE5 protein band and/or its potential fragments, the membrane was incubated in the presence of a specific rabbit polyclonal anti-EPSPS ACE5 protein antibody. The hybridization of the antibody with the proteins immobilized on the membrane was revealed using a goat anti-rabbit polyclonal antibody coupled with a peroxidase. The hybridization bands were visualized using enhanced chemiluminescence (ECL) detection system (Amersham ECL detection system, GE Healthcare Life Sciences, France).

2.C. Materials and methods for protein levels in plant parts and during the life cycle

Materials

The field production of test materials (maize event VCO-Ø1981-5) was initiated in the 2009 growing season to generate test and control materials at three locations in the US cornbelt. The field sites were as follows: Wright County, IA, Kossuth County, IA, and Webster County, IA. At each site, three replicated plots of event VCO-Ø1981-5 and the non-transgenic control hybrid were planted using a randomized complete block design. For each replicate, two plants each for event VCO-Ø1981-5 and the non-transgenic control were collected per growth stage. Leaf, root, and whole plant samples were collected at five different growth stages, pollen and mature grain were collected at the appropriate stages:

V4 (vegetative 4-leaf stage) (leaf, root)
V8 (vegetative 8-leaf stage) (leaf, root, whole plant)
R1 (reproductive silking stage) (leaf, root, whole plant, pollen)
R4 (reproductive kernel dough stage) (leaf, root, forage)
R6 (physiological maturity) (leaf, root, whole plant, grain)

All samples were packed and shipped on wet ice to the Athenix processing site in Research Triangle Park, NC. Plants were separated into parts, labeled, and frozen at minus 80°C until the time of processing.

Previous generations of the seed planted in this trial had been characterized by PCR and Southern blot and shown to be positive for the *epsps grg23ace5* gene sequence. Representative tissues from event VCO-Ø1981-5 were characterized by event-specific PCR after receiving plants from the field to confirm correct planting, sampling, and labeling.

The control material was a non-transgenic control produced through the same breeding scheme as event VCO-Ø1981-5. The identities of the test and control corn were confirmed by event-specific PCR prior to analysis.

The protein reference standard (batch # GRG23ACE5-0108) was produced through over expression in *E. coli*. The purity of the standard was determined to be 89.15% by SDS-PAGE and densitometry.

Tissue processing and protein extraction

Leaf, root, and grain samples were processed by grinding in dry ice in a Grindomix 200 (Retsch). Ground tissues were sub-sampled and lyophilized frozen under vacuum until dry and stored at - 80°C until protein extracts were made. Whole plant and forage samples were processed by coarsely chopping the tissue and then grinding in dry ice in a VCM12 industrial blender (Sympak). Ground tissues were sub-sampled and lyophilized frozen under vacuum until dry and stored at -80°C until protein extracts were made. Pollen samples were sub-sampled and processed by grinding with stainless steel beads in 1.5 ml tubes in a MiniBeadbeater-96 (Bio Spec Products Inc.). Pollen samples were processed immediately before protein extracts were made.

A sub-sample of each lyophilized sample was weighed into a 1.5 ml tube containing 4-8 stainless steel beads. Using buffer ratios of 30:1 (leaf, root, forage, whole plant, and grain) and 120:1 (pollen), an appropriate volume of ELISA extraction buffer was added to each sample. Tissues were crushed in a MiniBeadbeater-96 for approximately 2.5 minutes at room temperature. Extracts were spun down at 4°C, diluted at 1:10, 1:25, or 1:50 in ELISA extraction buffer, and added to the ELISA wells at a volume of 50 µl. All experimental samples were run in triplicate wells on the ELISA plate.

Antibodies

The rabbit polyclonal antibody specific for EPSPS ACE5 was purified using immunoaffinity chromatography. The concentration and optimal dilution for plate-coating were determined, and the antibody stored at -20°C prior to analysis. A goat polyclonal antibody specific for EPSPS ACE5 was purified using immunoaffinity chromatography. The antibody was conjugated to horseradish peroxidase (HRP), its concentration and optimal dilution for use as a detection (secondary) antibody were determined, and the antibody conjugate was stored at -20°C prior to analysis.

Enzyme linked immunosorbent assay (ELISA)

Rabbit anti-EPSPS ACE5 antibody was diluted to a concentration of 0.5 µg/ml in coat buffer (0.01 M phosphate buffered saline, pH 7.4), added to medical grade polystyrene 96-well EIA/RIA plates at a volume of 50 µl/well, and incubated overnight at 4°C. Plates were washed in wash buffer (0.01 M phosphate buffered saline and TWEEN® 20 0.05%, pH 7.4) and blocked for 1 hour at room temperature in 100 µl/well block buffer (0.01 M phosphate buffered saline, TWEEN 20 0.05%, pH 7.4 and 1% Bovine Serum Albumin).

Plates were washed in wash buffer. Samples and standards were diluted to appropriate concentrations in extraction buffer (0.01 M phosphate buffered saline (NaCl 0.138 M; KCl 0.0027 M); TWEEN 20 0.55%, pH 7.4), added to the plates at 50 µl/well, and incubated at room temperature for 1 hour. Plates were washed in wash buffer. HRP-conjugated goat anti-EPSPS ACE5 antibody was diluted 1:2000 in coat buffer and added to plates. Plates were incubated at room temperature for 1 hour. Plates were washed in wash buffer. Tetramethyl benzidine (TMB) detection substrate was added to plates at 100 µl/well, and incubated at room temperature for 10 minutes. 1 N HCl was added to plates at 100 µl/well to stop the color reaction, and plates were read on a spectrophotometer (Spectromax 190 microplate reader, Molecular Devices, Sunnyvale, CA). Quantification of EPSPS ACE5 was determined by interpolation from the standard curve, whose linear range was 3 to 60 ng/ml.

ELISA validation

The ELISA assay for EPSPS ACE5 was validated under GLP by determining the Limit of Detection (LOD) of the assay in each tissue (matrix). Limit of Detection is defined as a concentration of protein detected in a matrix, which is statistically different from a non-transgenic control. LOD was determined in the following tissue types and ages: lyophilized leaf (V8 and R6), root (V8 and R6), whole plant (V8 and R6), pollen, and grain from non-transgenic control plants. Twelve replicate samples per tissue type and age were run *vs*. the standard curve in the EPSPS ACE5 ELISA assay.

Calculations
The Limit of Detection (LOD) for the GRG23 ACE5 protein was calculated as follows:

a) For each tissue, an optical density (OD) value was calculated by performing 12 replicate measurements and determining the arithmetic mean of those 12 values. The replicate values were corrected for background using a buffer blank.
b) OD values of multiple non-transgenic tissue samples were measured as described in step a.
c) The arithmetic mean (average) of the tissue OD values from step b was calculated.
d) The standard deviation (sd) of the tissue OD values from step c was calculated.
e) The standard deviation from step d was multiplied by 3 (3*sd) and added to the average OD value from step c.
f) The value from step e (average + 3*sd) was converted to ng EPSPS ACE5/ml sample volume using a linear standard curve obtained with Microsoft Excel software.
g) The ng/ml value from step e was corrected for dilution (multiplied by a dilution factor) and converted to ng/mg dry weight of tissue (using tissue weight and total sample volume) to provide the LOD for EPSPS ACE5 in plant tissue.

The value in step g. was corrected for the percent purity of the EPSPS ACE5 standard by multiplying by 0.8915. The resulting values represent the LOD of EPSPS ACE5 in each tissue. Any values from event VCO-Ø1981-5 which fell below the LOD for that tissue were considered to be below the LOD of the assay, and were assigned a value of zero for calculation purposes.

Statistics

Data are presented as ng of EPSPS ACE5/mg dw tissue. SoftMax Pro software (Molecular Devices, Sunnyvale, CA) calculated the concentration of EPSPS ACE5 in each sample in micrograms/milliliter (µg/ml) by comparison to a standard curve. Calculations to determine ng/mg dry weight were performed as follows:

- The concentration of EPSPS ACE5 in ng/ml was multiplied by 0.05 ml (the volume of extract added to each well), which gave a value for the total ng of EPSPS ACE5 per well.
- The dry tissue (dw) weight, volume of buffer used to create the tissue extract, and the volume of extract added to the ELISA were used to determine the mg dw tissue added per well.
- Finally, the total ng EPSPS ACE5 per well was divided by the total mg dw tissue added to the well to calculate the expression level in ng EPSPS ACE5/mg dw tissue.

Means and standard deviations of expression level (ng EPSPS ACE5/mg dw) were determined for each growth stage per tissue type across all three locations.

2.D. Materials and methods for agronomic studies

Genetics of event VCO-Ø1981-5 for field trial evaluations

Following recovery from glyphosate selection during the plant transformation protocol, the T0 plantlet of event VCO-Ø1981-5 was transplanted into a germination soil mix and grown in a temperature-controlled greenhouse (daytime temperature = 27°C; night time temperature = 22°C). At flowering, the plant was pollinated with inbred B110 (Committee for Agricultural Development, Iowa State University), and the seeds were harvested and dried. These backcross zero (BC0B) seeds were then planted in a winter nursery (2007/2008) in Puerto Rico. Positive segregants were further identified by glyphosate spraying and crossed with inbred B110 line to produce back-cross one (BC1B) seeds used for the agronomic study in 2008. For the 2009 field trials, the back-cross zero (BC0B) were selfed two times to produce BC0BS2. In order to evaluate agronomic performance characteristics of event VCO-Ø1981-5 as compared to an appropriate non-transgenic control, two test-crosses were made and seed bulked for multiple location analysis in 2009.

Trial design

All trials were conducted using a randomized complete block design, with three replicated plots of each entry per location for 2009. Each plot consisted of four, 30 in (76.2 cm) rows by 17.5 to 20 ft (5.25-6.0 meters) long. Plants were thinned prior to reaching the V8 leaf stage resulting in a uniform number of plants in each row. Weed control was limited to conventional and cultural practices (hand hoeing); no broad-spectrum herbicides, such as glyphosate or glufosinate are allowed except as a pre-plant or pre-emergence herbicide. Data on all parameters were collected on the middle two rows of each four-row plot.

Statistics

All data collected was analyzed using Statistix Version 8.1 (Analytical Software, Tallahassee, FL). Within and "across location" analyses were conducted using an Analysis of Variance (ANOVA) for a Randomized Block Design. Means for the event were compared to the appropriate non-transgenic control using a two-sided Dunnett's test with alpha = 0.05 (Moore and McCabe, 1999). The standard deviation and range of values collected across all locations are also reported. The following equation, Yij = U + Ti + Lj + LTij + eij, was used for the multi-location analysis where T was the presence or absence of the transgene, and L was the location.

Field Locations, Preparations and Conditions

The field trial conducted in 2008 was located in Polk County, Iowa, USA. As event VCO-Ø1981-5 was in the segregating backcross 1 (BC1B) generation, the plots were sprayed with a glyphosate solution to identify the negative segregants.

A total of 17 field sites were utilized in 2009 across six US states to collect agronomic data (Figure 10 and Table 32). The locations include diverse environments within the major maize growing regions of the US. Off-season locations in Puerto Rico were also used for breeding purposes (not included in Figure 10). Table 32 lists the state, county and type of trial data collected. Color markers link the trial data to the location from which it was collected. The agronomic practices were representative of the location in which the field trials were conducted.

Figure 10. Map of 2009 agronomic field trial locations

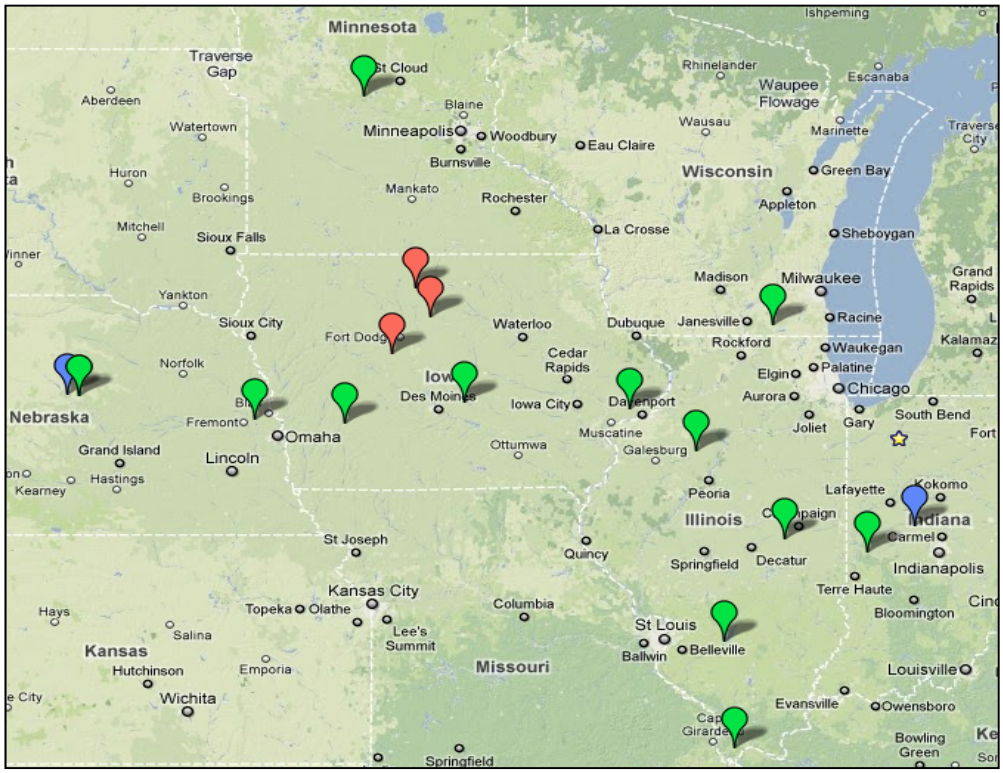


Table 32. 2009 Field trial location details

State	County	Color Marker on Map	Protocol
Iowa	Cass	Green	agronomic performance
	Jasper	Green	agronomic performance
	Kossuth	Red	agronomic performance, protein expression, compositional analysis
	Webster	Red	agronomic performance, protein expression, compositional analysis
	Wright	Red	agronomic performance, protein expression, compositional analysis
	Scott	Green	agronomic performance
Illinois	Clinton	Green	agronomic performance
	Stark	Green	agronomic performance
	Champaign	Green	agronomic performance
	Logan	Green	agronomic performance
Indiana	Parke	Green	agronomic performance
	Boone	Blue	agronomic performance and compositional analysis
Nebraska	Washington	Green	agronomic performance
	Valley	Green	agronomic performance
	Valley	Blue	agronomic performance and compositional analysis
Minnesota	Stearns	Green	agronomic performance
Wisconsin	Walworth	Green	agronomic performance

2.E. Materials and methods for seed germination studies

Five replicates of 50 seeds were planted on moistened germination paper and placed at 25°C for seven days and percent emergence was determined. The cold germination test was conducted in accordance with AOSA, Seed Vigor Testing Handbook (2009). Four replications of 50 seeds were planted on moist creped cellulose paper and covered with ½ to ¾ inch of sand, placed at 10°C for 7 days and then moved to 25°C for 4 days at which time percent emergence was determined.

The tetrazolium test was performed on the resulting ungerminated seeds at the end of each test to determine the percent non-viable seed. Seeds were bisected longitudinally and placed into a 0.1% tetrazolium solution to stain. After staining the seed was evaluated for viability determined by the appearance of red stain on living viable tissue and the location of the stain.

The equation used to calculate percent germination was:
100 – [(number of non-viable seed / germinated seeds) x 100] = % germinated

2.F. Materials and methods for composition analysis

Materials

Plant material derived from event VCO-Ø1981-5 and the non-transgenic control was produced and collected at five field locations across the USA corn belt in 2009 (DM Crop Research Group, Inc., Granger, IA 50109). The reference hybrids (AgR5539, AgR7584, and AgR58036) were handled identically as the GM hybrids and the non-transgenic controls. Data from the reference hybrids is used in conjunction with published data to help determine normal ranges and variability within the measured parameters.

Field Trial Description

The field sites were planted in a randomized block design with 3 replications per site. The sites included five locations across three states (Boone County, IN; Valley County, NE; Kossuth County, IA; Webster County, IA; and Wright County, IA). No herbicide sprays (conventional or glyphosate) were used post-emergence. The planting population and all agronomic practices were representative of those used for maize production in each study location.

Pollination Procedures

In order to produce grain, hand pollinations were conducted within each replicate. Location personnel made five pollinations in each row. Five separate pollinates were carried out per replicate. Plants were crossed within the row (sib mating), but were not selfed. Standard breeding procedures for hand pollination of regulated material were followed.

Sampling Procedures

For the forage samples, one whole plant from each plot was collected at the dough (R4) stage. Each plant was placed in a large plastic bag and shipped on ice directly from the field to the laboratory for analysis (EPL Bio-Analytical Services). For the grain samples, two ears were harvested at physiological maturity (R6) and 150 grams of grain were collected from center portion of each ear for analysis. Sampled grain was shipped at ambient temperature to the laboratory for analysis (EPL Bio-Analytical Services).

Analytical Methods

The analytical methods were AOAC, AACC and AOCS International Methods or published methods as detailed in Table 33 for maize forage and Table 34 for maize grain.

Table 33. Analytical methods for maize forage

Maize Forage	
Analyte	**Method**
Ash	AOAC International Method 923.03, 2000.
Carbohydrates	100 – Ash (%Dry Base) - Fat (%DB) - Protein (%DB) USDA, 1973. Energy Value of Food, *Agriculture Handbook No. 74,* pp. 2-11.
Crude Fat	AOAC International Method 922.06, 2000.
Moisture	AOAC International Method 930.15, 2000.
Crude Protein	Foss-Tecator, 1999. In *Foss-Tecator Kjeltec 2300 Site Preparation, Installation, and Operating Guide*, Foss-Tecator AB, Box 70, S-263 21 Hoganas, Sweden.
Acid Detergent Fiber	Ankom Technology, 1999. ANKOM^{200} Fiber Analyzer Operator's Manual, Ankom Technology, 140 Turk Hill Park, Fairport, NY 14450.
Neutral Detergent Fiber	Ankom Technology, 1999. ANKOM^{200} Fiber Analyzer Operator's Manual, Ankom Technology, 140 Turk Hill Park, Fairport, NY 14450.
Crude Fiber	Ankom Technology, 1999. ANKOM^{200} Fiber Analyzer Operator's Manual, Ankom Technology, 140 Turk Hill Park, Fairport, NY 14450.; Ankom Technology, 2006. ANKOM^{2000} Fiber Analyzer Operator's Manual, Ankom Technology, 140 Turk Hill Park, Fairport, NY 14450.
Minerals	AOAC International Method 999.11, 2000.

Table 34. Analytical methods for maize grain

Maize Grain Analytical Methods	
Analyte	**Method**
Ash	AOAC International Method 923.03, 2000.
Carbohydrates	100 – Ash (%DryBasis) - Fat (%DB) - Protein (%DB) USDA (1973).Merrill A.L. and Watt B.K. "Energy Value of Foods," *Agriculture Handbook No. 74,* pp. 2-11.
Crude Fat	Ankom Technology, 2008. ANKOM^{HCl} Hydrolysis System Operator's Manual, Ankom Technology, 2052 O'Neil Road, Macedon, NY 14502; Ankom Technology (2008). ANKOM^{XT15} Extraction System Operator's Manual, Ankom Technology, 2052 O'Neil Road, Macedon, NY 14502.
Moisture	AOAC International Method 925.09, 2000.
Crude Protein	Foss-Tecator, 1999. *Foss-Tecator Kjeltec 2300 Site Preparation, Installation, and Operating Guide*, Foss-Tecator AB, Box 70, S-263 21 Hoganas, Sweden.
Acid Detergent Fiber	Ankom Technology, 1999. ANKOM^{200} Fiber Analyzer Operator's Manual, Ankom Technology, 140 Turk Hill Park, Fairport, NY 14450.
Neutral Detergent Fiber	Ankom Technology, 1999. ANKOM^{200} Fiber Analyzer Operator's Manual, Ankom Technology, 140 Turk Hill Park, Fairport, NY 14450.
Crude Fiber	Ankom Technology, 1999. ANKOM^{200} Fiber Analyzer Operator's Manual, Ankom Technology, 140 Turk Hill Park, Fairport, NY 14450; Ankom Technology, 2006. ANKOM^{2000} Fiber Analyzer Operator's Manual, Ankom Technology, 140 Turk Hill Park, Fairport, NY 14450.
Amino Acids	Liu, H. J. 1994. Determination of amino acids by precolumn derivatization with 6-aminoquinolyl-n-hydroxysuccinimidyl carbamate and ultra-performance liquid chromatography with ultraviolet detection. *J. Chrom. A, 670*:59-66; Waters Method, Analysis of amino acids in feeds and foods using modification of the accq•tag method ^{tm} for amino acid analysis.
Tryptophan	Rogers, S.R.; Pesti, G.M. 1990. Determination of Tryptophan from Feedstuffs Using Reverse Phase High-performance liquid chromatography. *J. Micronutr Anal*. 7:27-35.
Fatty Acid	AOAC International Method 939.05, 2000. AOCS, Ce 2-66.; AOCS, Ce 1e-91.
Vitamin B_1/B_2 (Thiamine/Riboflavin)	AACC International Method 86-80, 2000.
Vitamin E	Weber, E.J., 1984. High performance liquid chromatography of the tocols in corn grain. *JAOCS, 61*:1231-1234.
Folic Acid	AACC International Method 86-47, 2000.
Phytic Acid	AOAC International Method 986.11, 2000.
Raffinose/Inositol	AACC International Method 80-04, 2000.
Minerals	AOAC International Method 999.11, 2000.
Vitamin A (Beta-carotene)	AOAC International Method 941.15, 2000.
Niacin	AACC International Method 86-51, 2000.
Vitamin B_6 (Pyridoxine)	AACC International Method 86-31, 2000.

Maize Grain Analytical Methods	
Trypsin inhibitor	Anonymous 1997. Trypsin Inhibitor Activity. AOCS, Ba 12-75.
p-Coumaric Acid and Ferulic Acid (phenolics)	Figueroa-Espinoza, M-C, Morel, M-H, Rouau, X. 1998. Effect of lysine, tyrosine, cysteine, and glutathione on the oxidative cross-linking of feruloylated arabinoxylans by a fungal laccase. *J. Agric. Food Chem., 46*, 2583-2589; Classen, D., Arnason, J.T., Serratos, J.A., Lambert, J.D.H., Nozzolillo, C., Philogene, B.J.R., 1990. Correlation of phenolic acid content of maize to resistance to *Sitophilus zeamais*, the maize weevil, *J. Chem. Ecol. 16*: 301-315; Krygier, K., Sosulski, F., Hogge, L. 1982. Free, esterified, and insoluble-bound phenolic acids. 1. Extraction and purification procedure. *J. Agric. Food Chem. 30*: 330-334; Sosulski, F., Krygier, K., Hogge, L. 1982. Free, esterified, and insoluble-bound phenolic acids. 3. Composition of phenolic acids in cereals and potato flours. *J. Agric. Food Chem. 30*: 337-340.
Furfural	Bredie, W.L.P., Mottram, D.S., Guy, R.C.E. 1998. Aroma volatiles generated during extrusion cooking of maize flour. *J.Agric. Food. Chem. 46:*1479-1487.; Buttery, R.G., Stern, D.J., Ling, L.C. 1994. Studies on flavor volatiles of some sweet corn products. *J. Agric. Food. Chem. 42*: 791-795.
Pantothenic Acid	AOAC International Method 945.74, 1960.
Total Dietary Fiber	AOAC International Method 991.43, 2000.
Starch	Corn Refiners Association Method A-20, 2nd Rev, 1985.

Statistical Analysis

Data means, standard deviations, ranges and p-values were determined for the compositional data. Data were analyzed using JMP 8 software package from SAS Institute, Cary NC. For each analyte an ANOVA model was fit using the site and line main effects and the site by line interaction. Single degree of freedom contrasts were used to test for differences between the experimental line (VCO-Ø1981-5) and the non-transgenic control. P-values (≤0.05%) are not reported where the means were below the LOD, or where missing data made the effect non-testable. When data points were at or below the LOQ, the LOQ value was used to calculate the averages, standard deviations and data ranges.

APPENDIX 3

Characterization of event VCO-Ø1981-5 maize

3.A. Insert Characterization

Transgene copy number was investigated by Southern blot analysis. Genomic DNA samples of event VCO-Ø1981-5 and appropriate control samples were digested with the restriction enzymes *Hin*dIII and *Nde*I independently (Figure 11). Each of these restriction enzymes cuts one time within the T-DNA region. When hybridized with the *epsps grg23ace5* gene probe (Figure 11 and Table 34, probe #3), the resulting number of bands indicates the insert copy number within the maize genome.

The predicted sizes for the hybridization products are shown in Figure 11. The *Hin*dIII cleavage site located within the maize genome, adjacent to the left border region of the T-DNA insert, was further identified through isolation and sequencing of the flanking DNA regions (data not shown). Predicted and observed results are described in Table 35 below. Southern blots are presented in Figure 12.

Southern blot analysis was also conducted to determine insert integrity. Maize genomic DNA (event VCO-Ø1981-5 and appropriate non-transgenic controls) was digested with a combination of *Hin*dIII and *Eco*RI, and independently with *Mfe*I. The locations of these restriction enzyme sites are shown in Figure 11. A set of four independent probes of the major genetic elements, namely, ScUbi4 promoter, ScUbi4 intron, *epsps grg23ace5* gene and the 35S terminator (Figure 11, Tables 35 and 36, probes # 1-4) was used to confirm the integrity of the expression cassette. The predicted sizes for the hybridization products are shown in Figure 11 and Table 36. Southern blots are presented in Figures 13 and 14 along with the predicted and observed results.

Figure 11. Map of the T-DNA insertion site of event VCO-Ø1981-5

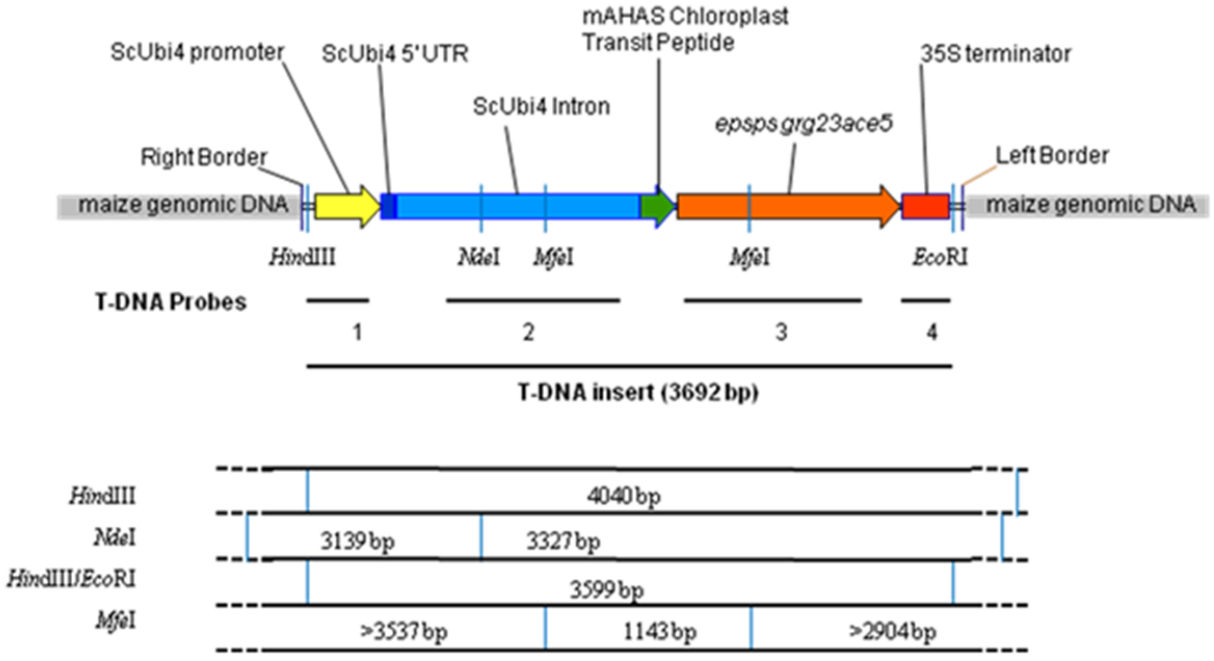


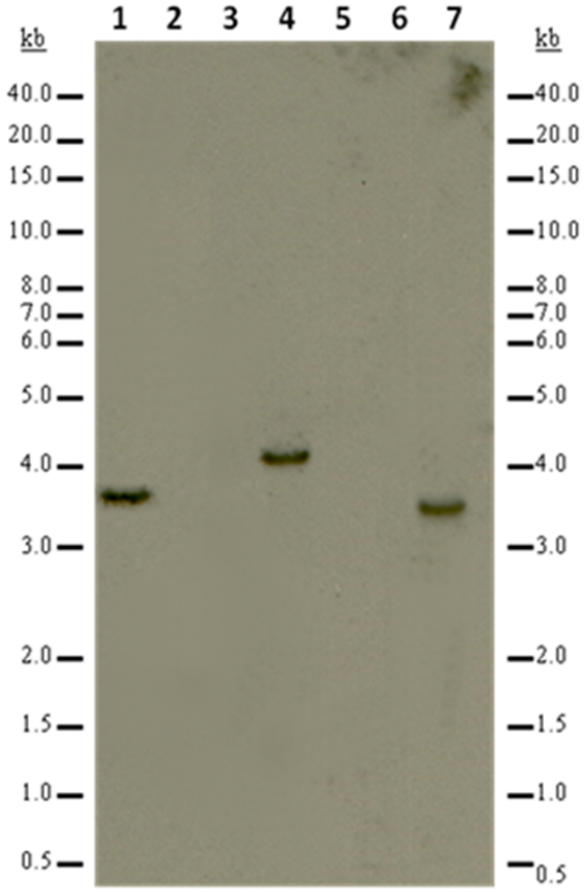
Table 35. Description of DNA probes used in Southern analysis

Probe N°	Description	Probe size (bp)
1	ScUbi4 promoter probe	351
2	ScUbi4 intron probe	969
3	*epsps grg23ace5* probe	988
4	35S terminator probe	280
5	*aad* probe	784
6	*tet*A/*tet*R probe	1956
7	oriT probe	2001
8	*vir*C probe	1526
9	*vir*G probe	925
10	*vir*B probe	Region probed: 8513; 3 overlapping probes of 2985, 2757 and 2900 bp were used simultaneously.

Table 36. Predicted and observed hybridization band sizes for insert copy number and insert integrity

Probe Nº	Probe	Restriction Enzyme	Predicted Fragment Size	Observed Fragment Size	Figure Nº
3	*epsps grg23ace5* gene probe	*Hin*dIII	4040 bp	~4000 bp	12
3	*epsps grg23ace5* gene probe	*Nde*I	3327 bp	~3300 bp	12
1	ScUbi4 promoter probe	*Hin*dIII/*Eco*RI	3599 bp	~3600 bp	13
1	ScUbi4 promoter probe	*Mfe*I	>3537 bp	~3800 bp	13
2	ScUbi4 intron probe	*Hin*dIII/*Eco*RI	3599 bp	~3600 bp	13
2	ScUbi4 intron probe	*Mfe*I	>3537 bp,and 1143 bp	~3800 bp, ~1100 bp	13
3	*epsps grg23ace5* gene probe	*Hin*dIII/*Eco*RI	3599 bp	~3600 bp	14
3	*epsps grg23ace5* gene probe	*Mfe*I	1143 bp and >2904 bp	~1100 bp, ~4000 bp	14
4	35S terminator probe	*Hin*dIII/*Eco*RI	3599 bp	~3600 bp	14
4	35S terminator probe	*Nde*I	>3327 bp	~3400 bp	14

Figure 12. Determination of insert copy number using probe 3 (*epsps grg23ace5* gene)

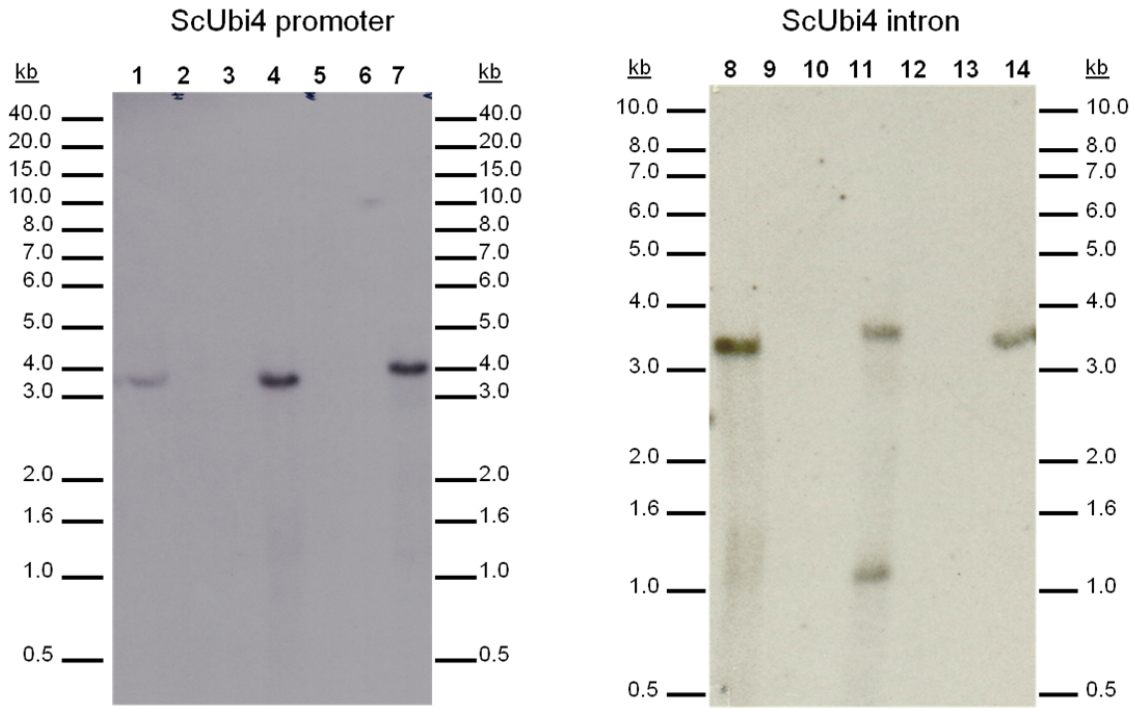


The lane numbers, sample ID (restriction enzyme), and predicted and observed fragment sizes are indicated in the table below. The blot was probed with *epsps grg23ace5* gene (probe 3).

Lane	Sample ID (Restriction Enzyme)	Predicted Fragment Size	Observed Fragment Size
1	pAX3541 *(Hin*dIII/*Eco*RI*)*	3599 bp	~3600 bp
2	B110 (*Hin*dIII)	--	--
3	BC1B non-transgenic control (*Hin*dIII)	--	--
4	VCO-Ø1981-5 (*Hin*dIII)	4040 bp	~4000 bp
5	B110 (*Nde*I)	--	--
6	BC1B non-transgenic control (*Nde*I)	--	--
7	VCO-Ø1981-5 (*Nde*I)	3327 bp	~3500 bp

--; no hybridization

Figure 13. Analysis of insert integrity using probe1 (ScUbi4 promoter) and probe 2 (intron)

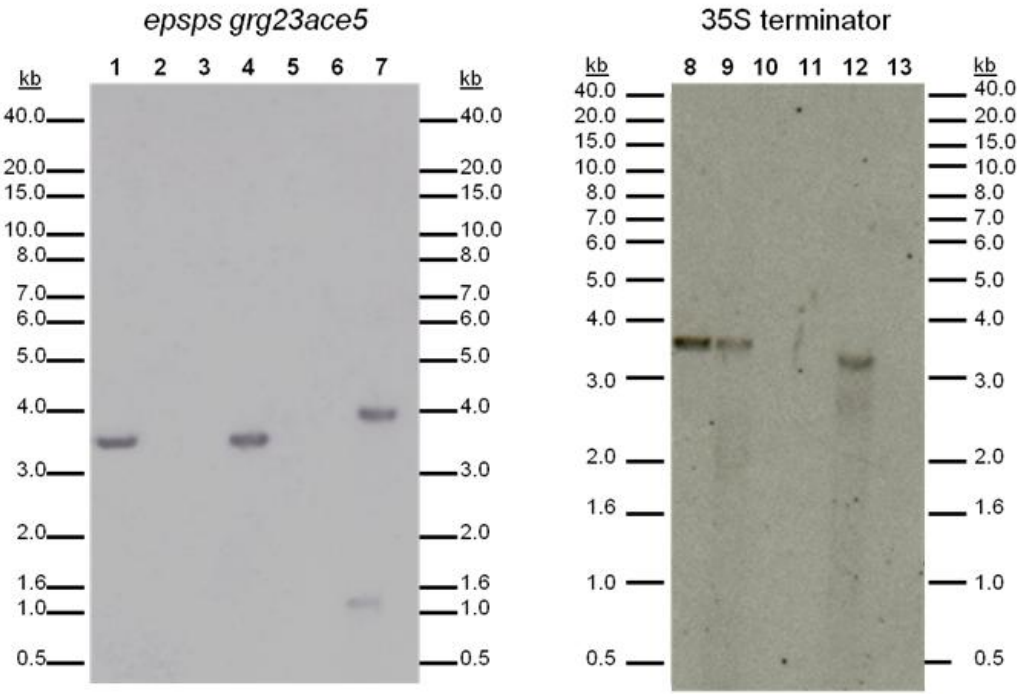


The lane numbers, sample ID (restriction enzyme), and predicted and observed fragment sizes are presented in the table below.

Lane	Sample ID (Restriction Enzyme)	Predicted Fragment Size	Observed Fragment Size	Lane	Sample ID (Restriction Enzyme)	Predicted Fragment Size	Observed Fragment Size
1	pAX3541 (*Hin*dIII/*Eco*RI)	3599 bp	~3600 bp	8	VCO-Ø1981-5 (*Hin*dIII/*Eco*R1)	3599 bp	~3600 bp
2	B110 (*Hin*dIII/*Eco*RI)	--	--	9	BC1B non-transgenic control (*Hin*dIII/*Eco*R1)	--	--
3	BC1B non-transgenic control (*Hin*dIII/*Eco*RI)	--	--	10	B110 (*Hin*dIII/*Eco*R1)	--	--
4	VCO-Ø1981-5 (*Hin*dIII/*Eco*RI)	3599 bp	~3600 bp	11	VCO-Ø1981-5 (*Mfe*I)	>3537 bp, 1143 bp	~ 3800 bp, ~1100 bp
5	B110 (*Mfe*I)	--	--	12	BC1B non-transgenic control (*Mfe*I)	--	--
6	BC1B non-transgenic control (*Mfe*I)	--	--	13	B110 (*Mfe*I)	--	--
7	VCO-Ø1981-5 (*Mfe*I)	>3537 bp	~3800bp	14	pAX3541 (*Hin*dIII/*Eco*RI)	3599 bp	~3600 bp

--; no hybridization

Figure 14. Analysis of insert integrity probe 3 (*epsps grg23ace5* gene) and probe 4 (35S terminator)



The lane numbers, sample ID (restriction enzyme), and predicted and observed fragment sizes are presented in the table below.

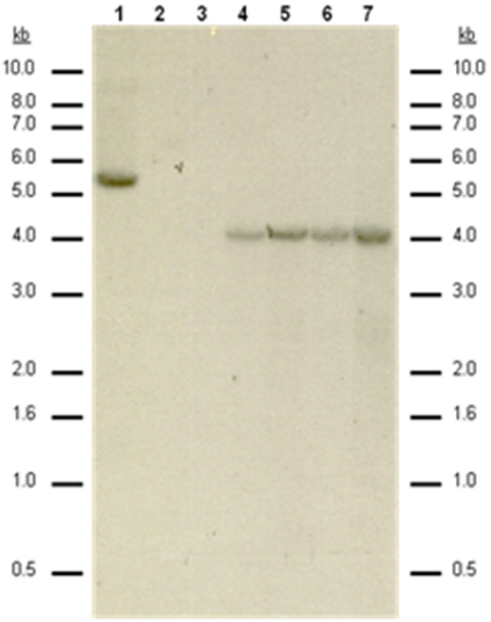
Lane	Sample ID (Restriction Enzyme)	Predicted Fragment Size	Observed Fragment Size	Lane	Sample ID (Restriction Enzyme)	Predicted Fragment Size	Observed Fragment Size
1	pAX3541 (*Hin*dIII/*Eco*RI)	3599 bp	~3600 bp	8	pAX3541 (*Hin*dIII/*Eco*RI)	3599 bp	~3600 bp
2	B110 (*Hin*dIII/*Eco*RI)	--	--	9	VCO-Ø1981-5 (*Hin*dIII/*Eco*RI)	3599 bp	~3600 bp
3	BC1B non-transgenic control (*Hin*dIII/*Eco*RI)	--	--	10	BC1B non-transgenic control (*Hin*dIII/*Eco*RI)	--	--
4	VCO-Ø1981-5 (*Hin*dIII/*Eco*RI)	3599 bp	~3600 bp	11	B110 (*Hin*dIII/*Eco*RI)	--	--
5	B110 (*Mfe*I)	--	--	12	VCO-Ø1981-5 (*Nde*I)	3327 bp	~3400 bp
6	BC1B non-transgenic control (*Mfe*I)	--	--	13	BC1B non-transgenic control (*Nde*I)	--	--
7	VCO-Ø1981-5 (*Mfe*I)	1143 bp, >3527 bp	~1100 bp, ~4000bp				

--; no hybridization

3.B. Insert stability across generations

Southern blot analysis was conducted on multiple generations of event VCO-Ø1981-5 progeny to evaluate the stability of the T-DNA insertion site. Genomic DNA isolated from leaf material of event VCO-Ø1981-5 plants from four breeding generations resulting from crosses with non-transgenic inbred line B110 (BC0B, BC1B, BC2B, and $BC1B_2$) and non-transgenic controls were digested with the restriction enzyme *Hin*dIII. When hybridized with probe 3, specific for the *epsps grg23ace5* coding region, genomic DNA from event VCO-Ø1981-5 digested with *Hin*dIII produces a single band >3600 bp in size (Figure 11). The transformation plasmid pAG3541 was included as a hybridization control. All four generations analyzed showed an identical hybridization pattern producing the same single ~4000 bp band (Figure 15).

Figure 15. Insert stability across generations



The lane numbers, sample ID (generation) (restriction enzyme), and predicted and observed fragment sizes are presented in the table below.

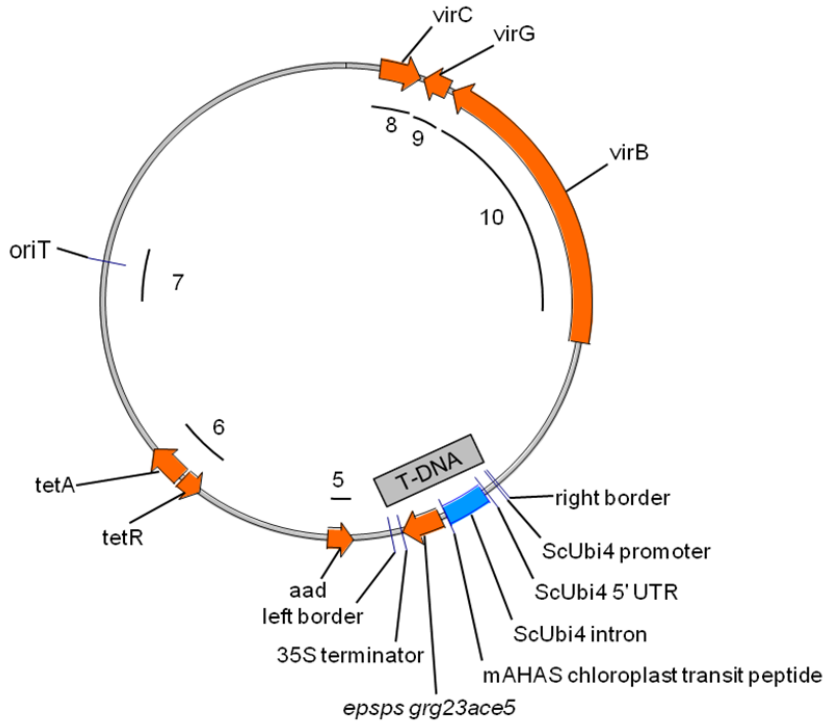
Lane	Sample ID	Predicted Fragment Size	Observed Fragment Size
1	pAG3541 (*Nde*I)	5261 bp	~5300 bp
2	B110 (*Hin*dIII)	--	--
3	BC0B non-transgenic control (*Hin*dIII)	--	--
4	VCO-Ø1981-5 Event (BC0B) (*Hin*dIII)	4040 bp	~4000 bp
5	VCO-Ø1981-5 Event (BC1B) (*Hin*dIII)	4040 bp	~4000 bp
6	VCO-Ø1981-5 Event (BC2B) (*Hin*dIII)	4040 bp	~4000 bp
7	VCO-Ø1981-5 Event ($BC1B_2$) (*Hin*dIII)	4040 bp	~4000 bp

--; no hybridization

3.C. Confirmation of the absence of vector backbone

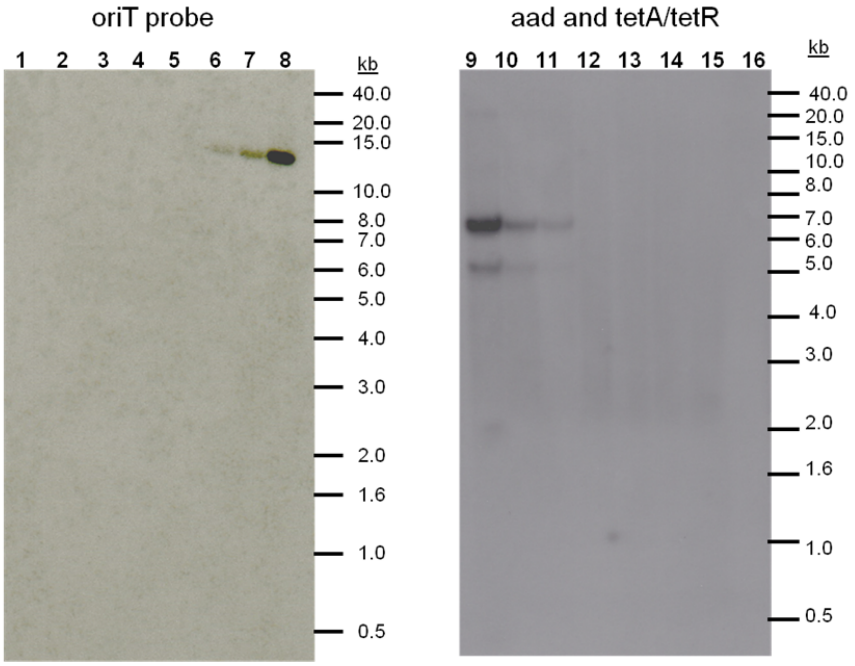
DNA (event VCO-Ø1981-5 and appropriate non-transgenic controls) was digested with a combination of *Hin*dIII and *Eco*RI, and independently with *Mfe*I and *Nde*I. The locations of these restriction enzyme sites are shown in Figures 11 and 16. The probes employed were designed to hybridize to the functional components of the plasmid outside of the T-DNA (Probe 5: *aad*, Probe 6: *tet*R/*tet*A, Probe 7: *ori*T, Probe 8: *vir*C, Probe 9: *vir*G, and Probe 10: *vir*B; Figure16 and Table 33). The *Agrobacterium* plasmid pAG3541 was included as a positive control for hybridization of the transformation plasmid components and loaded as genomic equivalent of 0.5, 1 and 3 copies (Figures 17 and 18). The *tet*A/*tet*R probe hybridizes with a 6700 bp fragment in the control and the *aad* probe hybridizes with a 5300 bp fragment in the control, while the *ori*T probe hybridizes with a 14,000 bp fragment. Southern blot analysis results are shown in Figures 17 and 18.

Figure 16. Map of plasmid pAG3541 with vector backbone probes



Southern Probe #	Description
5	*aad* probe
6	*tet*A/*tet*R probe
7	oriT probe
8	*vir*C probe
9	*vir*G probe
10	*vir*B probe

Figure 17. Absence of the transformation plasmid components (oriT, *aad*, *tet*A, and *tet*R probes)

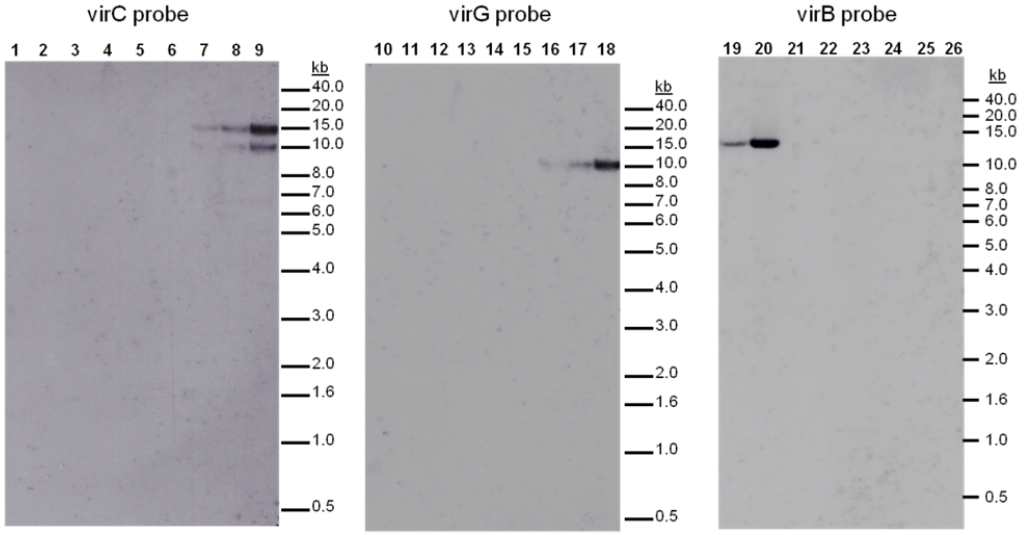


The lane numbers, sample ID (restriction enzyme), and predicted and observed fragment sizes are indicated in the table below. Brackets indicate the copy number related to maize genomic equivalent.

Lane	Sample ID (Restriction Enzyme)	Predicted Fragment Size	Observed Fragment Size	Lane	Sample ID (Restriction Enzyme)	Predicted Fragment Size	Observed Fragment Size
1	VCO-Ø1981-5 (*Hin*dIII/*Eco*R1)	--	--	9	pAG3541 (*Nde*I) **[3 copies]**	5261 bp, 6699 bp	~5300 bp, ~6700 bp
2	BC1B non-transgenic control (*Hin*dIII/*Eco*R1)	--	--	10	pAG3541 (*Nde*I) **[1 copy]**	5261 bp, 6699 bp	~5300 bp, ~6700 bp
3	B110 (*Hin*dIII/*Eco*R1)	--	--	11	pAG3541 (*Nde*I) **[0.5 copy]**	5261 bp, 6699 bp	~5300 bp, ~6700 bp
4	VCO-Ø1981-5 (*Hin*dIII/*Eco*R1)	--	--	12	VCO-Ø1981-5 (*Hin*dIII/*Eco*R1)	--	--
5	BC1B non-transgenic control (*Hin*dIII/*Eco*R1)	--	--	13	BC1B non-transgenic control (*Hin*dIII/*Eco*R1)	--	--
6	pAG3541 (*Nde*I) **[0.5 copy*]**	13,994 bp	~14,000 bp	14	B110 (*Hin*dIII/*Eco*R1)	--	--
7	pAG3541 (*Nde*I) **[1 copy]**	13,994 bp	~14,000 bp	15	VCO-Ø1981-5 (*Mfe*I)	--	--
8	pAG3541 (*Nde*I) **[3 copies]**	13,994 bp	~14,000 bp	16	BC1B non-transgenic control (*Mfe*I)	--	--

--; no hybridization
*; considered as genome equivalent

Figure 18. Absence of the transformation plasmid components (*vir*C, *vir*G, and *vir*B probes)



The lane numbers, sample ID (restriction enzyme), and predicted and observed fragment sizes are presented in the table below. Brackets indicate the copy number related to maize genomic equivalent**.**

Lane	Sample ID (Restriction Enzyme)	Predicted Fragment Size	Observed Fragment Size	Lane	Sample ID (Restriction Enzyme)	Predicted Fragment Size	Observed Fragment Size
1	B110 (*Hin*dIII/*Eco*R1)	--	--	14	BC1B non-transgenic control (*Mfe*I)	--	--
2	BC1 non-transgenic control (*Hin*dIII/*Eco*R1)	--	--	15	VCO-Ø1981-5 (*Mfe*I)	--	--
3	VCO-Ø1981-5 (*Hin*dIII/*Eco*R1)	--	--	16	pAG3541 (*Nde*I) [**0.5 copy**]	11,174 bp	~11,500 bp
4	B110 (*Mfe*I)	--	--	17	pAG3541 (*Nde*I) [**1 copy**]	11,174 bp	~11,500 bp
5	BC1B non-transgenic control (*Mfe*I)	--	--	18	pAG3541 (*Nde*I) [**3 copies**]	11,174 bp	~11,500 bp
6	VCO-Ø1981-5 (*Mfe*I)	--	--	19	pAG3541 (*Nde*I) [**1 copy**]	11,174 bp	~11,500 bp
7	pAG3541 (*Nde*I) [**0.5 copy***]	11,174 bp, 13,994 bp	~11,500 bp, ~14,000 bp	20	pAG3541 (*Nde*I) [**3 copies**]	11,174 bp	~11,500 bp
8	pAG3541 (*Nde*I) [**1 copy**]	11,174 bp, 13,994 bp	~11,500 bp, ~14,000 bp	21	B110 (*Hin*dIII/*Eco*R1)	--	--
9	pAG3541 (*Nde*I) [**3 copies**]	11,174 bp, 13,994 bp	~11,500 bp, ~14,000 bp	22	BC1B non-transgenic control (*Hin*dIII/*Eco*R1)	--	--
10	B110 (*Hin*dIII/*Eco*R1)	--	--	23	VCO-Ø1981-5 (*Hin*dIII/*Eco*R1)	--	--
11	BC1B non-transgenic control (*Hin*dIII/*Eco*R1)	--	--	24	B110 (*Mfe*I)	--	--
12	VCO-Ø1981-5 (*Hin*dIII/*Eco*R1)	--	--	25	BC1B non-transgenic control (*Mfe*I)	--	--
13	B110 (*Mfe*I)	--	--	26	VCO-Ø1981-5 (*Mfe*I)	--	--

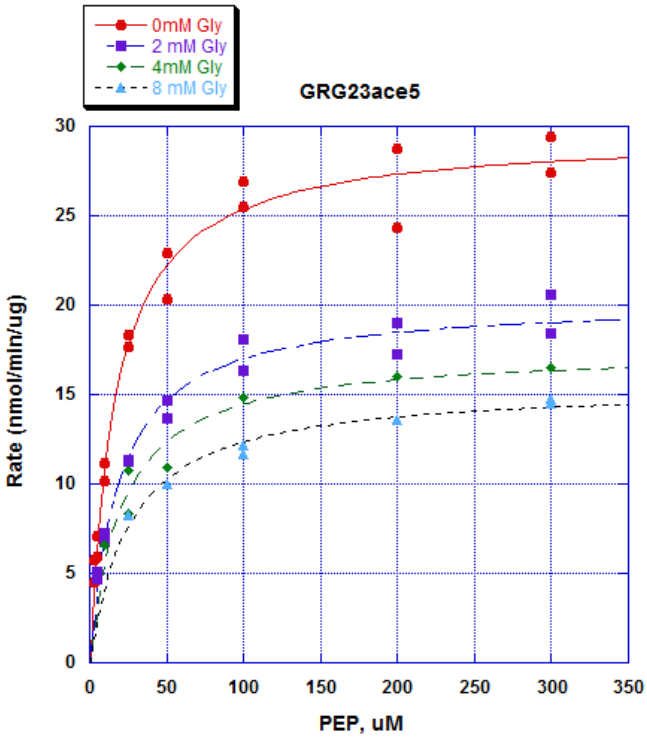
--; no hybridization
*; considered as genome equivalent

3.D. *In vitro* enzyme assay

The function of EPSPS ACE5 protein was confirmed by carrying out enzymatic assays with purified *E.coli*-produced EPSPS ACE5 protein. An enzymatic assay was developed to quantify the production of inorganic phosphate (Pi) by EPSPS enzymes. The assay used enzyme coupling and resulted in the generation of a highly fluorescent product (Vazquez *et al.,* 2003). This purified EPSPS ACE5 protein was mixed with shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) and the presence of product (inorganic phosphate) was quantified using a fluorimeter. As anticipated, addition of EPSPS ACE5 protein led to the steady-state production of inorganic phosphate (Figure 19), while control reactions without EPSPS ACE5, PEP or S3P did not generate a fluorescent signal.

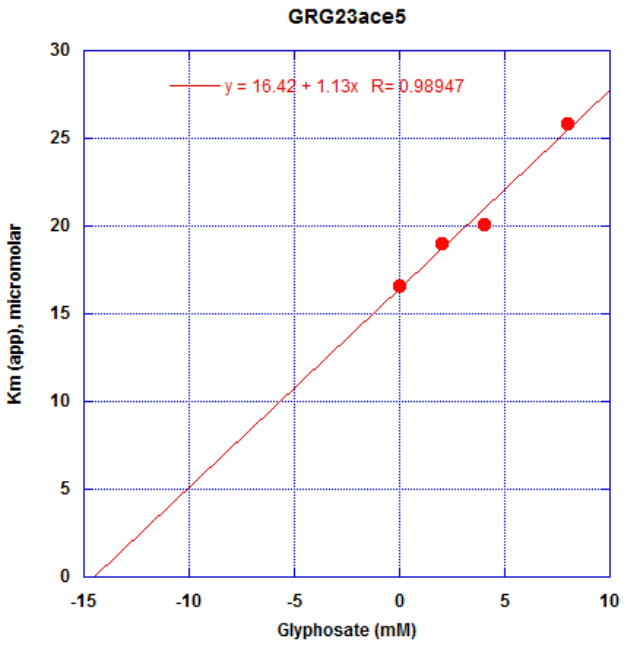
Figure 19. Kinetic characterization of EPSPS ACE5 protein

Panel A



Panel A: A scatter plot of EPSPS ACE5 (designated as GRG23ace5 in the above plot) enzymatic activity as a function of the concentration of PEP at different glyphosate concentrations. The apparent Michaelis – Menten binding constant for PEP [K_m (app)] and maximal rate (V_{max}) were calculated from the half-maximal and maximal rates, respectively.

Panel B



Panel B: The K_m (app) was measured at glyphosate concentrations ranging from 0 to 8 mM, and was plotted against the glyphosate concentration to derive the K_i for the enzyme (designated as GRG23ace5 in the above plot) (x intercept= 14,700 µM glyphosate).

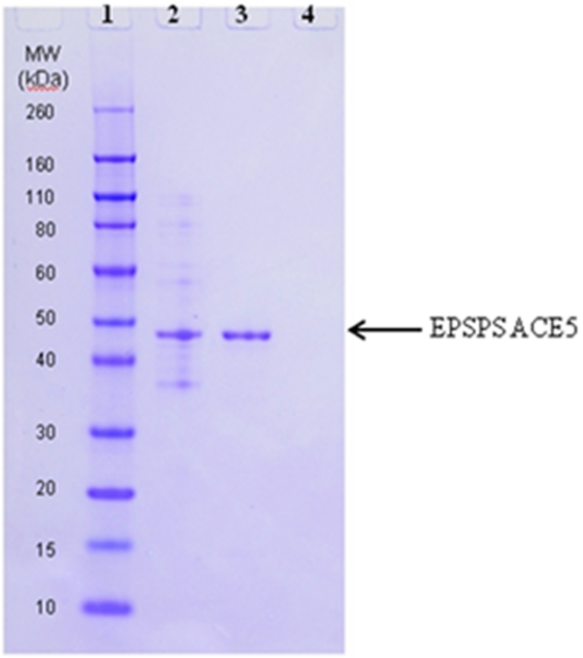
3.E. Protein equivalency

3.E.1. SDS-PAGE of EPSPS ACE5

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was used to separate proteins based on their molecular weight.

The dominant protein bands for both the maize- and *E. coli*-produced EPSPS ACE5 preparations migrated at a rate consistent with a molecular weight of approximately 45,000 Daltons when compared to the molecular weight markers (Figure 20). This data indicates that the molecular weight of event VCO-Ø1981-5 maize partially purified EPSPS ACE5 protein is consistent with both the molecular weight of the *E. coli* -produced protein and the expected molecular weight of 44.3 kDa.

Figure 20. SDS PAGE



Lane #	Sample ID
1	Novex Sharp Prestained Protein Marker
2	~ 450 ng EPSPS ACE5 (maize expressed)
3	~ 400 ng EPSPS ACE5 (microbial-produced)
4	Blank

3.E.2. MALDI-TOF Mass spectrophotometry protein identification

Peptides confirmed by MS/MS sequencing are indicated in Table 37 and were mapped onto the expected EPSPS ACE5 amino acid sequence. These matching peptides constitute 45% coverage of the microbial-expressed EPSPS ACE5 protein and 42% coverage of the maize-derived protein when mapped onto the expected EPSPS ACE5 amino acid sequence.

The degree of coverage for both microbial-expressed EPSPS ACE5 protein and the maize derived EPSPS ACE5 protein confirms their identity and indicates that both proteins were expressed as intended.

Table 37. Comparison of EPSPS ACE5 peptides analyzed by MALDI MS/MS

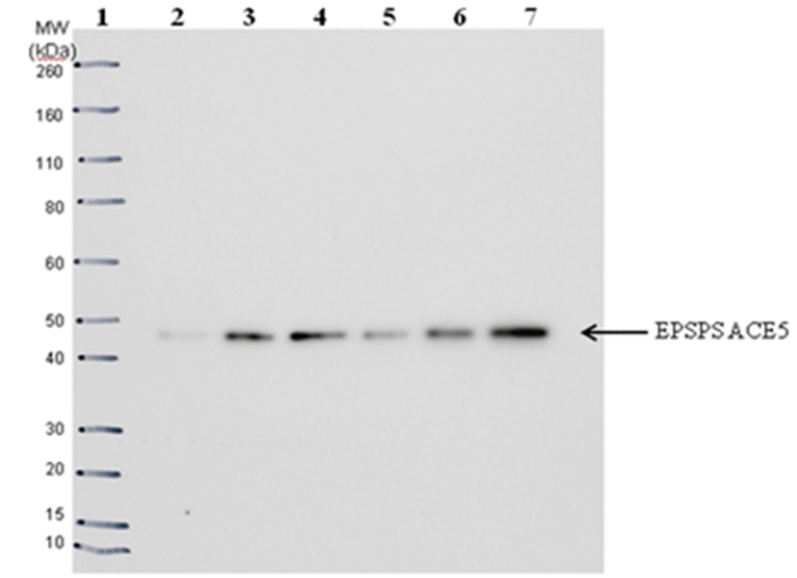
Residue #	Observed plant-derived protein mass	Observed bacterial-derived protein mass	Theoretical Mass (calculated)	Sequence
1-12	1361.67 [1]	1361.68 [1]	1360.70	METDRLVIPGSK
27-44	1848.00	1848.02	1847.02	GTSVLVRPLVSA
87-98	1231.63	1231.65	1230.68	FLPPFVAAGQGK
87-108	2364.20	ND	2363.23	FLPPFVAAGQGKFTVDGSEQLR
99-108	1151.55	1151.56	1150.56	FTVDGSEQLR
99-109	1307.63	1307.64	1306.66	FTVDGSEQLRR
109-120	1433.85	1433.87	1432.87	RRPLRPVVDGIR
110-120	1277.75	1277.76	1276.77	RPLRPVVDGIR
172-188	1973.06 [1]	1973.09 [1]	1972.08	VKIPNPVSQPYLTMTLR
174-188	1745.88 [1]	1745.90 [1]	1744.92	IPNPVSQPYLTMTLR
189-210	2356.06 [1]	2356.09	2355.08	MMRDFGIETSTDGATVSVPPGR
192-210	1905.89	1905.92	1904.91	DFGIETSTDGATVSVPPGR
192-214	2397.14	2397.17	2396.16	DFGIETSTDGATVSVPPGRYTAR
215-237	2419.14	2419.17	2418.15	RYEIEPDASTASYFAAASAVSGR
216-237	2263.02	2263.06	2262.04	YEIEPDASTASYFAAASAVSGR
335-348	1630.72 [1]	1630.74 [1]	1629.76	TLGVQTDVGHDWMR
349-358	1084.54	1084.54	1083.55	IYPSTPHGGR
367-377	1207.65 [1]	1207.66 [1]	1206.68	IAMAFSILGLR
392-402	1319.62	1319.63	1318.63	TFPGFFDYLGR

1; Peptide identified following correction of observed mass for the oxidation of methionine.
ND = not detected

3.E.3. Western blot analysis

As shown in Figure 21, defined bands of the expected molecular weight of approximately 45,000 Daltons were present for all three lanes containing partially purified maize-produced EPSPS ACE5 protein (Lanes 2, 3, and 4) and all three lanes containing *E. coli*-produced EPSPS ACE5 protein (Lanes 5, 6, and 7). The rabbit anti-EPSPS ACE5 antibody used in this analysis has been previously shown to be specific only for EPSPS ACE5 protein, and therefore immunoreactivity of both EPSPS ACE5 proteins confirms their identity.

Figure 21. Western blot analysis of microbial-expressed and plant-expressed EPSPS ACE5 protein



Lane #	Sample ID
1	Novex Sharp Prestained Protein Marker
2	~ 0.45 ng EPSPS ACE5 (maize-expressed)
3	~ 0.90 ng EPSPS ACE5 (maize-expressed)
4	~ 1.80 ng EPSPS ACE5 (maize-expressed)
5	~ 0.40 ng EPSPS ACE5 (*E. coli* -expressed)
6	~ 0.80 ng EPSPS ACE5 (*E. coli* -expressed)
7	~ 1.60 ng EPSPS ACE5 (*E. coli* -expressed)

3.E.4. Enzymatic activity

Maize-expressed EPSPS ACE5 protein showed enzymatic activity only in the presence of its substrates as shown in Table 38. This result is comparable to the activity observed for *E. coli*-produced EPSPS ACE5 protein (data not shown).

Table 38. Enzymatic activity of maize-expressed EPSPS ACE5 protein

Sample Identification	Average Rate (RFU/sec)
Buffer Control	1.17
Buffer + PEP	2.614
Buffer + Event VCO-Ø1981-5 leaf	1.985
Buffer + Event VCO-Ø1981-5 leaf + PEP	17.662

RFU; relative fluorescence units
PEP; phosphoenolpyruvate

3.E.5. N-terminal sequencing

The N-terminal analysis of maize- and *E. coli*-produced EPSPS ACE5 protein was consistent with the expected sequence (Table 39). The parentheses around the methionine for the maize-derived EPSPS ACE5 protein sequence indicate low signal strength for that cycle.

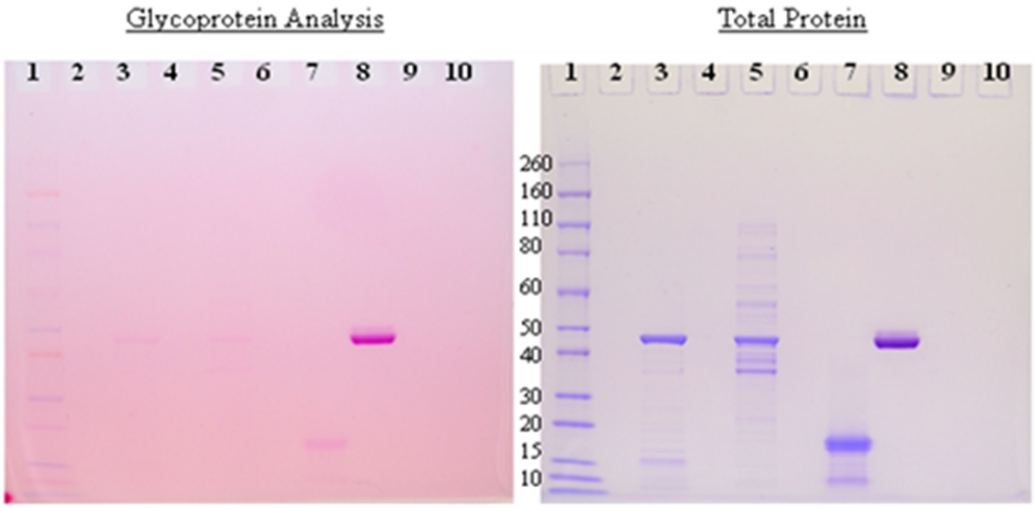
Table 39. N-terminal amino acid sequence of maize- and *E. coli*-produced EPSPS ACE5 protein

Expected sequence	M E T D R L V I P G S K S I T N
Maize-expressed EPSPS ACE5 sequence	(M)E T D R L V I P G S K S I T N
Microbial-expressed EPSPS ACE5 sequence	M E T D R L V I P G S K S I T N

3.E.6. Glycosylation assay

No glycosylation was detected for either the microbial- or maize-expressed EPSPS ACE5 protein. As shown in Figure 22, the glycoprotein positive control, horseradish peroxidase, stained a bright magenta color while the glycoprotein negative control, soybean trypsin inhibitor, showed only very faint light pink color. These results show that neither maize- nor microbial-expressed EPSPS ACE5 protein have been modified by the addition of detectable levels of carbohydrates.

Figure 22. Glycosylation analysis of *E. coli*- and plant-expressed EPSPS ACE5 protein



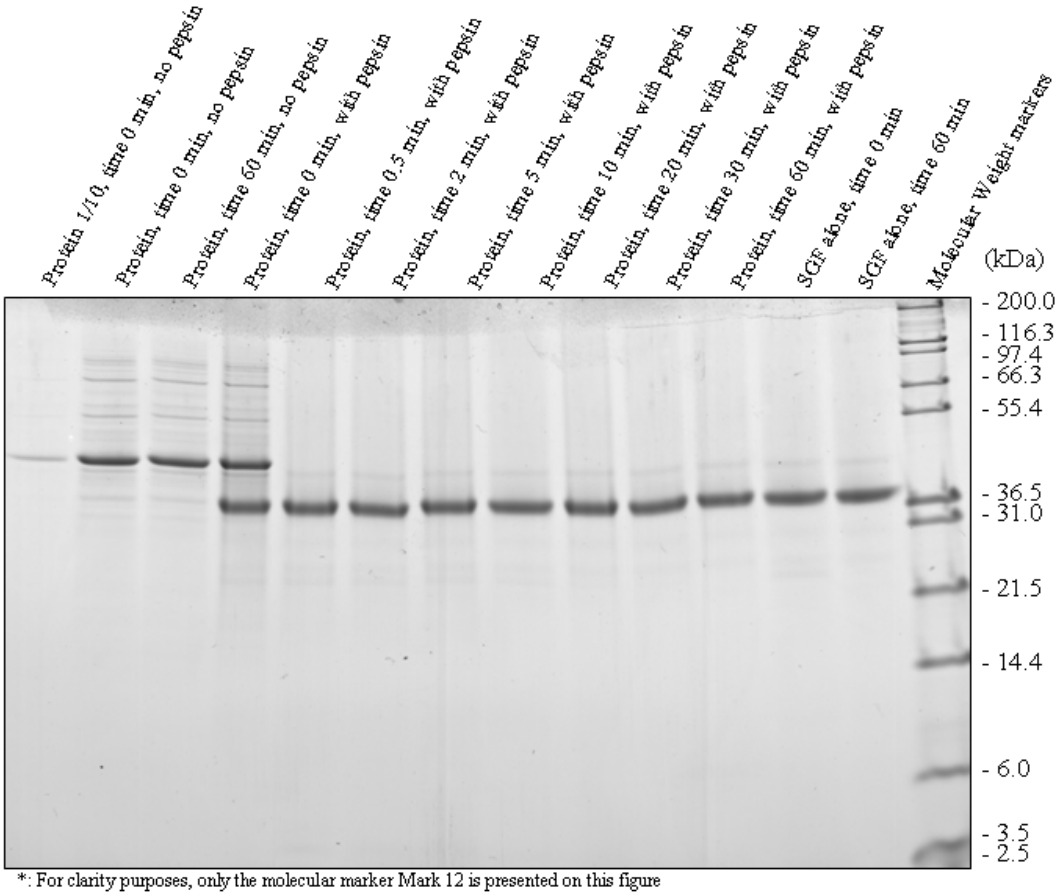
Lane #	Sample ID
1	Novex Sharp Prestained Protein Marker
2	blank
3	~ 4µg microbial expressed EPSPS ACE5
4	blank
5	~ 4.5µg maize expressed EPSPS ACE5
6	blank
7	5 µg Soybean Trypsin Inhibitor (negative control)
8	5 µg Horseradish Peroxidase (positive control)
9	blank
10	blank

3.F. Protein digestibility in simulated gastric fluid

It was shown that the pepsin was active, and that the two reference proteins, HRP and OVA, were rapidly and slowly digested, respectively. The results of the reference proteins are in line with the results obtained in an international ring trial organized by ILSI (Thomas *et al*., 2004). These quality control procedures confirm that the study procedures and reagents were adequate to detect the rate of digestion of proteins in this SGF study.

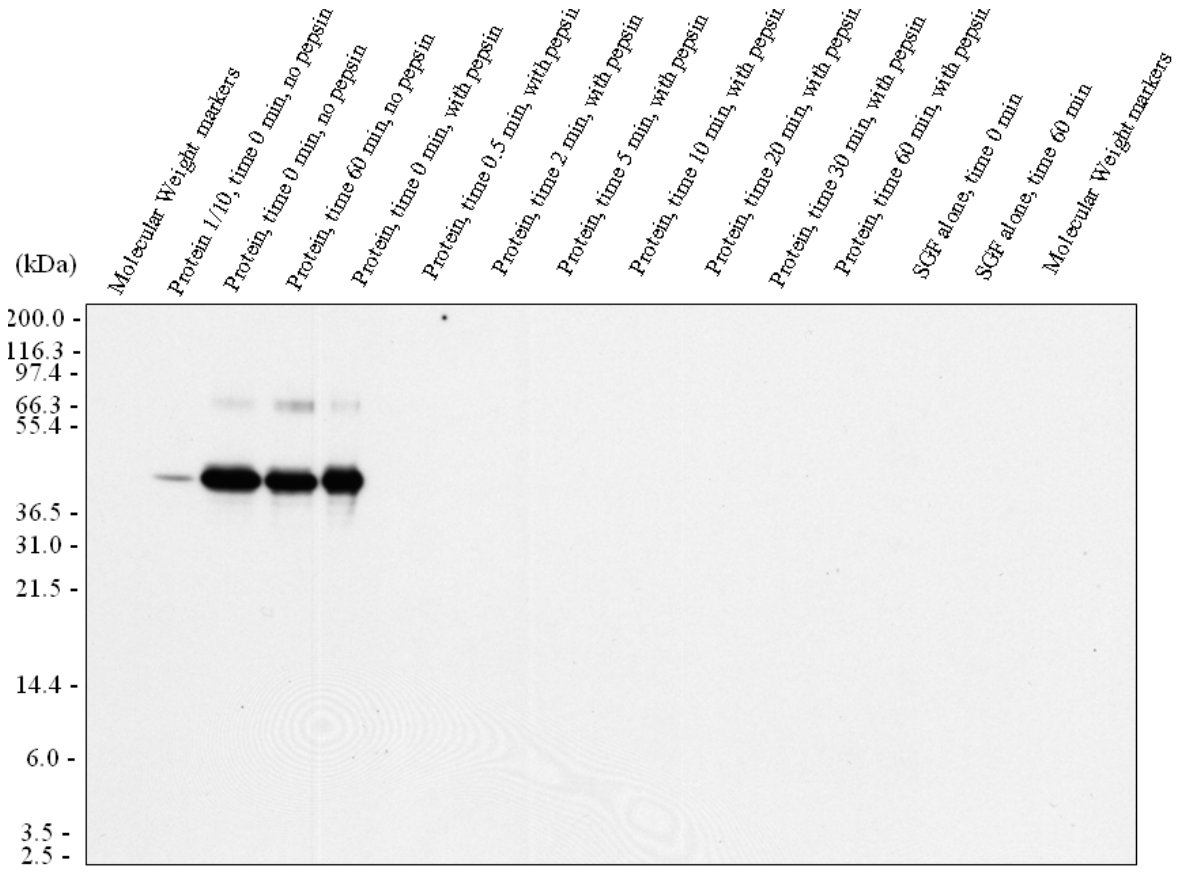
The EPSPS ACE5 protein was degraded very rapidly with no residual protein visible at 30 seconds of incubation with SGF, in presence of pepsin, at pH 1.2. There was no significant digestion at pH 1.2 in the absence of pepsin, showing that digestion requires pepsin.

Figure 23. SDS-PAGE gel of EPSPS ACE5 protein digested in a standardized SGF



*: For clarity purposes, only the molecular marker Mark 12 is presented on this figure

Figure 24. Western blot of EPSPS ACE5 protein digested in a standardized SGF



APPENDIX 4

Herbicide Resistance and Product Stewardship

4.A. Evolution of herbicide resistant weeds

Herbicides are the most economical, effective and reliable method of weed control in most crop production systems. Herbicides act by targeting and inhibiting specific plant biochemical processes or pathways. The process of specific activity is termed “mode of action” (MOA). Herbicides are classified into groups based on their MOA (HRAC, 2011).

During the past several decades, diversity in weed control methods has been declining. Consolidation of agriculture has occurred at all levels including combining smaller farms to form larger farms. The resulting economic pressures have led to the selection of the most profitable crops and have driven the adoption of monocultures. Tillage, a key cultural practice contributing to a diversified weed management program, has also been severely reduced through the adoption of conservation tillage systems such as no-till and minimum tillage to combat the widespread problem of soil erosion (Anderson, 1996).

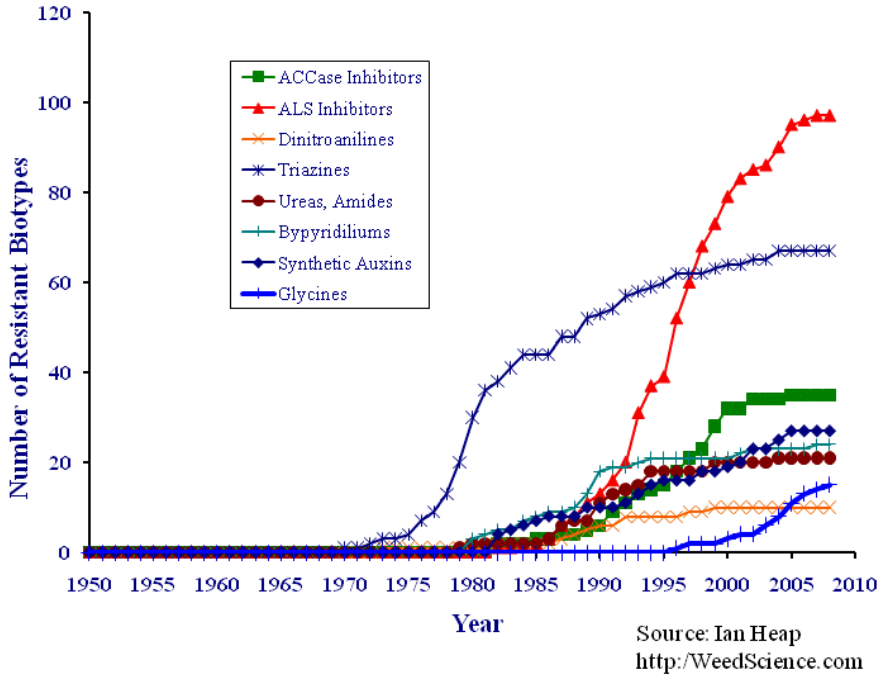
Weed control in the absence of complementary cultural control practices has resulted in the use of herbicides as the only weed control tactic. With this decline in use of alternative weed control methods, extensive use of herbicides with a single MOA has not only resulted in weed shifts but also high selection pressure for herbicide resistant weeds. Plants have the ability to adapt to ensure survival, which includes adapting to survive an herbicide application. The development of herbicide resistance is a function of time and exposure and also the genetic capability of the weed population present in a field (HRAC, 2011).

Herbicide resistance is the naturally-occurring inheritable ability of some weed biotypes within a given population to survive an herbicide treatment that should, under normal use conditions, effectively control that weed population (HRAC, 2011).

The first herbicide resistant weed was identified in 1964 (HRAC, 2011). An increase in the number of documented herbicide resistant weeds began a steep incline after the ALS inhibiting herbicides were introduced in the 1980’s. ALS herbicides inhibit the plant enzyme acetolactate synthase (ALS) and provide effective control of many grass and broadleaf weed species (Anderson, 1996; Whaley *et al*., 2007). ALS herbicides were available for a broad number of crops for both postemergence and residual weed control. Farming practices shifted, as use of ALS inhibitors enable a reduction in the amount of tillage needed for weed control benefitting soil conservation efforts. The lack of diversified weed control methods lead to the selection of populations of ALS herbicide-resistant weed species or biotypes.

There are more than 40 weed species resistant to the ALS class of chemistry in the US today and 112 ALS-resistant weed species reported worldwide (Heap, 2011). Virtually all waterhemp (*Amaranthus rudis*) is considered by university weed scientists to be resistant to ALS inhibiting herbicides, resulting in the conclusion that ALS inhibiting herbicides are considered “obsolete” technology for weed control in soybean (Nordby *et al*., 2007). In addition to weeds resistant to ALS inhibiting herbicides, resistance has also developed to many other herbicide modes of action as evident in Figure 26 (Heap, 2011).

Figure 25. Timeline of the development of herbicide resistant weeds classified by herbicide mode of action



In addition to the increasing frequency of weed resistance, there has been a steady decline in herbicide discovery (Duke, 2005). We must maintain the utility of the current herbicides in the marketplace to continue to enable the economic and environmental advancements in agriculture that have been enabled by chemical weed control. The use of detailed, diversified integrated weed management plans will be needed to deter resistance and to continue to enable conservation tillage practices, fewer herbicide applications, and the use of herbicides with more favorable environmental profiles.

4.B. Managing herbicide resistant weeds

Ideally integrated weed management should utilize all available tools including herbicides in a well-balanced program as the lower the diversity of weed control tools, the higher the risk of selecting a resistant biotype becomes. To ensure diversification is maintained in weed control methods, we also encourage growers to keep detailed records of weed management practices for each field. The following are our integrated weed management guidelines to promote an economically viable, environmentally sustainable, and socially acceptable weed control program:

1. Know your weeds, know your fields

Today's herbicides control a broad spectrum of weed species, minimizing the importance of weed identification to a grower. However, identification of weed species will help identify an herbicide program that works best for every acre. Equally important is for the grower to understand the weed pressure and history within each field. Problematic areas like difficult-to-control weeds or dense weed populations should be closely monitored. There are several indications for a grower to consider with weed escapes to identify resistant weeds.

Resistance indicators

- The field has been sprayed repeatedly with the same herbicide (mode of action), particularly if there was no mode of action diversity in the weed management system;
- A patch of weeds occurs in the same area year after year and is spreading;
- Many weed species are managed, but one particular weed species is no longer controlled. For example, following a glyphosate application, actively growing marestail can still be seen, in the absence of other weeds;
- Surviving weeds of the problem species may be in a patch where some are dead and some exhibit variable symptoms, but all are approximately the same age.

2. Crop rotation

Crop rotation is one of the most important factors in an Integrated Weed Management (IWM) program. Crop rotation adds weed management diversity through the inherent use of herbicides with different modes of action. In addition, crops vary in their ability to compete for sunlight, water and nutrients with weeds. Different planting times and seedbed preparation techniques can lead to a variety of cultural methods, which employ diversity in a weed management program. Reliance on a monoculture crop leads to weed population shifts of fewer weed species but at overall higher densities of such weeds, which increases the selection pressure for herbicide resistant weeds.

3. Start with clean fields

Yields can be significantly reduced by early season weed competition. Proper tillage or the use of a burndown herbicide program should be used to control all emerged weeds prior to planting. Not only does the control of weeds prior to planting aid in the ease of planting, it also eliminates weed competition for soil moisture, light and nutrients.

Regardless of the tillage system (conventional, minimal, or no-till), a pre or early post-emergent soil-applied residual herbicide should be a part of every weed control program. A soil-applied herbicide provides residual weed control allowing the crop to get a head start. Residual herbicides minimize the weed pressure and allow a wider post-emergent herbicide application window. Generally, soil-applied herbicides can be included in the burndown herbicide program for residual weed control on no-till acres. A residual herbicide also introduces another mode of action into weed resistance management programs (Nordby *et al.*, 2007).

4. Rotate herbicide modes of action

There are three key factors in using herbicides to promote good resistant weed management:

Use multiple modes of action during the growing season
The use of multiple modes of action during the growing season increases the diversity within the weed control program by reducing the selection pressure of a single mode of action. A planned two pass herbicide (pre- followed by post-emergence) program implements multiple modes of action in weed management systems for delaying weed resistance.

Apply no more than two applications of a single herbicide mode of action to the same field in a two-year period
Repeated, successive use of herbicides with the same mode of action increases the likelihood that resistant plants will reproduce and become dominant in the population. The best way to manage resistant weeds is to prevent them from spreading or populating. Herbicide-resistant weeds become problematic due to overuse of a single herbicide mode of action. To preserve an herbicide's efficacy, maintain its use, and reap its benefits, growers should not use more than two applications of a single herbicide mode of action on the same field in a two-year period (Boerboom and Owen, 2006). In addition, rotating crops generally allows additional modes of actions to be used in a weed management program.

Rotate herbicide-tolerant trait systems
To ensure the viability of all traits for the future, rotate the herbicide tolerant trait used in each field each year to increase the chemical diversity used in each field.

5. Correct herbicide application

Product efficacy can be influenced by a multitude of factors. Ensuring correct use rates, weed growth stage and crop growth stage, and application technique will maximize weed control (Boerboom and Owen, 2006).

Apply to actively growing weeds
Herbicides provide peak performance when applied to actively growing weeds. Weeds that are actively growing absorb more herbicide. Conditions that provide peak growing environment for weeds are adequate soil moisture, optimal soil nutrients and temperature, and sunlight.

Timing
The use of pre-emergent residual herbicides will provide key control of early season weeds that result in the greatest crop yield reduction and open a wider application window for post-emergence applications. Post-emergence herbicides should be applied after crop emergence when weeds are within the growth stage range specified on the label for optimal performance. Applying post-emergence herbicides to smaller weeds increases crop yield again by eliminating early season weed competition.

Application technique
Herbicides differ in the optimal application technique. Read and follow all label instructions to ensure proper application technique is achieved. Factors affecting weed control include: spray coverage, carrier volume, application speed, adjuvants, and tankmix partners.

Product rate
The rate listed on the product label has been researched and tested by manufacturers and university researchers to provide the optimal control of the weeds at the height/growth stage listed on the label. The application of an herbicide at a rate less than listed on the label can result in insufficient control and will have a significant impact on the immediate weed control and therefore the weed seed bank by allowing partially controlled weeds to reproduce and set seed.

6. Control weed escapes

Weeds that escape the herbicide applications should be controlled to reduce weed seed production. A grower should consider spot herbicide applications, row wicking, cultivation or hand removal of weeds to improve weed management for the subsequent growing seasons.

7. Clean equipment

To prevent the spread of herbicide-resistant weeds and potentially introduce new invasive weeds on to the farm, avoid moving equipment that has not been thoroughly cleaned.

4.C. Glyphosate resistant weeds

There are currently 13 glyphosate-resistant weeds in the United States. These weeds include palmer amaranth (*Amaranthus palmeri*), marestail *(Conyza canadensis)*, waterhemp (*Amaranthus rudis*), giant ragweed *(Ambrosia trifida)*, common ragweed (*Ambrosia artemisifolia*), Johnsongrass (*Sorghum halepense*), Italian ryegrass (*Lolium multiflorum*), hairy fleabane (*Conyza bonariensis*), jungle rice (*Echinochloa colona*), annual bluegrass (*Poa annua*), kochia (*Kochia scoparia*), goosegrass (*Eleusine indica*), and rigid ryegrass (*Lolium rigidum*). There are an additional 8 glyphosate resistant weeds that can be found in other parts of the world (Heap, 2011).

Although the list of herbicide resistant weeds continues to grow, corn growers still have many effective conventional chemical options for weed control both with soil-applied residual herbicides and post-emergence herbicides. The various modes-of-action available to a corn grower include triazines, photosystem II inhibitors, chloroacetamides, HPPD's (4-hydroxyphenyl-pyruvate-dioxygenase), PPO's (protoporphyrinogen oxidase), dinitroanilines, growth regulators, and ALS inhibitors. The conventional chemistry options for weed control in corn offer a grower multiple choices when rotating herbicide modes of action even when growing continuous corn. In addition to conventional chemistry, glufosinate (a glutamine synthease inhibitor) and the LibertyLink trait system gives growers the option to continue to use a herbicide-tolerant trait system and rotate herbicide MOA's for weed resistance management.

4.D. Characteristics of glyphosate herbicide

Glyphosate is a non-selective, broad spectrum systemic herbicide introduced to the marketplace in the 1970's. Glyphosate can be formulated in multiple ways: glyphosate isopropylamine salt (Roundup®[3]), glyphosate trimethylsulfonium salt (Touchdown®[4]), or glyphosate diammonium salt (Touchdown4®[4] or Touchdown Pro®[4]). Glyphosate is the only member of the glycine herbicide family. Glyphosate inhibits the biosysnthesis of the aromatic amino acids in the shikimic acid pathway by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Anderson, 1996; Vencill, 2002).

Glyphosate is labeled for the control of 113 annual broadleaf and grass weeds and additional 62 perennial weeds (Roundup Weathermax®[3] label 2006). Glyphosate is likely the most broad spectrum herbicide available today for weed control in row crops. The effectiveness of glyphosate is well established; around 70% of the corn acres planted were herbicide-tolerant in 2010 (National Academies, 2010).

[3] Roundup and Roundup Weathermax are registered trademarks of Monsanto Company
[4] Touchdown, Touchdown4, and Touchdown Pro are registered trademarks of Syngenta Group Company

4.E. Stewardship of glyphosate-tolerant event VCO-Ø1981-5 maize

Maintaining the sustainability of this technology is of high importance and the adoption of a life-cycle approach to product stewardship is a must. This means that appropriate stewardship principles are applied at every stage of development of such products, from research to product discontinuation. Our commitment to stewardship extends to corporate relationships and to the application of these stewardship and quality assurance standards that are required in those relationships. This commitment is also proven through all third party agreements related to these products, which include the following:

> **"**We are committed to apply the proper stewardship for these products and expects those with whom we contract to handle material containing our technology in an appropriate manner. This includes without limitation adherence to the stewardship and quality assurance provisions described in this Agreement".

All the teams involved in the development of such products in our organization as well as our subcontractors are committed to apply the stewardship guidelines and are aware of the procedures derived from these guidelines in order to communicate the appropriate information within the team matrix to correctly and rapidly respond to issues that may develop from the handling of such technologies. Field development and market support teams are provided with the corresponding tools necessary to support and help growers as a local and direct contact for any questions they may have and, which are related to our technologies with regards to product performance, product integrity or any eventual impacts on human and environmental health and safety.

The procedures derived from these guidelines are audited regularly to check the conformity between procedures and their applications on site. Any non-conformity will be promptly corrected.

4.F. Customer outreach

We have a commitment to stewardship on all of our products, including herbicide-tolerant trait (HTT) technology. We strive to provide best management practices of HTT technology, which includes integrated weed management to our customers. Education of integrated weed management is the only practical method for its success. Education starts internally with our own field development, technical service, and seed salesmen. Externally, we collaborate with key influencers to help growers understand the long term economic viability of integrated weed management. Those key influencers include university extension agents, agronomists, consultants, and local retail seed and chemical salesmen. In addition, we directly provide the integrated weed management message to growers through grower meetings, trade shows, and web and mail communications.

A Technology Use Agreement or similar agreement will be developed and provided to each grower at the time of seed purchase. By signing the agreement, the grower will agree to best management strategies that are indicated in the agreement. The agreement will contain company contact information including a website for the best

management practices and product information. In addition, a toll free hotline for growers to obtain live technical product support will be provided. We are committed to our stewardship principles and procedures to communicate appropriate information to rapidly respond to any issue that may develop.

Growers may also contact the seed company for product support. The seed company name and contact information will be provided on the label of each bag of seed sold. Each grower purchase of event VCO-Ø1981-5 maize will be recorded by seed company partners. This information will be provided to us to maintain a database of all growers utilizing products derived from event VCO-Ø1981-5. This database could be used to disseminate updated stewardship information.

4.G. Additional customer support

Product information
There are a number of ways that a grower can obtain product information. The product label is the formal legal method of communicating directions for use of a herbicide.

Screening for Herbicide Resistance
Currently, confirmation of weed resistance is commonly conducted by collecting seed of suspected resistant plants. Those seeds are replanted in a greenhouse environment and sprayed with various rates of the herbicide to which resistance is suspected. The survival of the weeds confirms resistance.

4.H. Monitoring of effectiveness of the stewardship plan

Each grower purchase of event VCO-01981-5 maize will be recorded by the individual seed companies selling this event. This information will be provided to us to enable us to maintain a database of all growers utilizing event VCO-01981-5 products. We regularly utilize market research surveys to determine market share and adoption of technology.

Seed company partners will have direct contact with growers and will be able to provide feedback to us regarding the stewardship effectiveness. Our field representatives, or those from our affiliates or licensees will also interact with growers and will be a source of information.

We will continue to support ongoing efforts to understand weed resistance to herbicides and to apply learning to product labels and provide information to growers.

APPENDIX 5

References Appendices 1-4

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