Petition for the Determination of Non-regulated Status for Event FG72

OECD Unique Identifier MST-FGØ72-2

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.

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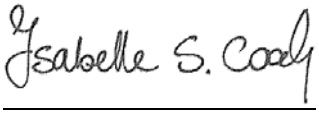

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Submitted: November 20, 2009
(Revised)

COMPANY NAMES

On June 3, 2002, Bayer CropScience was formed by the acquisition of Aventis CropScience by Bayer AG. From that date, Bayer CropScience is the agricultural business unit of Bayer that is engaged in the research, development, and marketing of crop protection, seed technology, turf and ornamentals, professional pest and vector control, and home and garden products.

On December 15, 1999, Aventis S.A. was formed by the completion of the merger between Hoechst AG and Rhône-Poulenc S.A. Aventis CropScience was formed as part of a worldwide merger between Rhone-Poulenc S.A. and Hoechst AG. A portion of that merger created Aventis CropScience Holding S.A. that included interests from Hoechst AG and Schering AG. Hoechst AG and Schering AG were the parent companies of AgrEvo USA Company which were all merged into the Aventis companies.

Some of the activities described in this petition were undertaken before the merger and acquisition. Consequently, the names Aventis CropScience, AgrEvo USA Company, AgrEvo, and Hoechst Schering AgrEvo GmbH may appear throughout this petition.

M.S. Technologies, LLC, is an Iowa limited liability company, with offices at 103 Avenue D, West Point, Iowa 52656, U.S.A. The FG72 transformation event is owned by M.S. Technologies, LLC.

In November of 2007, M.S. Technologies, LLC and Bayer CropScience AG entered into an agreement for the joint development of herbicide tolerant soybeans, including the FG72 transformation event.

Some of the activities described in this report were undertaken in the context of the agreement between Bayer CropScience AG and M.S. Technologies, LLC. For example, some of the field activities, described in this petition were conducted by M.S. Technologies, LLC.

RELEASE OF INFORMATION

The information in this petition is being submitted by Bayer CropScience and M.S. Technologies, LLC for review by the USDA as part of the regulatory process. By submitting this information, Bayer CropScience and M.S. Technologies, LLC do 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. Except in accordance with FOIA, Bayer CropScience and M.S. Technologies, LLC do not authorize the release, publication or other distribution of this information without Bayer CropScience's and M.S. Technologies' prior notice and consent.

CERTIFICATION

The undersigned certifies, 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.

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SUMMARY

Bayer CropScience (BCS) and M.S. Technologies, LLC (MSTech) are submitting a Petition for the Determination of Non-regulated Status under 7 CFR 340 to USDA Animal and Plant Health Inspection Service (APHIS) for double-herbicide-tolerant soybean event FG72 (Event FG72), any progeny, and crosses of this event with other non-regulated soybean lines.

Transformation event FG72 contains the stably integrated *2mepsps* gene which confers tolerance to the herbicide glyphosate and the *hppdPfW336* gene which confers tolerance to HPPD inhibitors such as the herbicide isoxaflutole (IFT). The genes were introduced into soybean via direct-gene transfer. Southern blot analyses show soybean event FG72 contains two copies of the *2mepsps* and *hppdPfW336* genes.

The *2mepsps* gene was generated by introducing mutations into the wild-type *epsps* (*wt epsps*) gene from maize, leading to a modified EPSPS protein with two amino acid substitutions (2mEPSPS). This modification confers a decreased binding affinity of the protein for glyphosate, allowing it to maintain sufficient enzymatic activity in the presence of the herbicide. Therefore, the plants expressing the 2mEPSPS protein (encoded by the *2mepsps* gene) are tolerant to glyphosate herbicides.

The wild-type (wt) *hppd* gene was isolated and cloned from *Pseudomonas fluorescens.* A single amino acid substitution introduced in the wt *hppd* gene resulted in the modified *hppdPf W336* gene. The expressed protein, HPPD W336, has a molecular weight of 40 kDa and is made of up of 358 amino acids. The modified protein possesses greater than 99.5% homology to the native HPPD protein from *P. fluorescens* and is tolerant to isoxaflutole (IFT).

Planting double-herbicide-tolerant soybean varieties, containing transformation event FG72, provides growers with new options for weed control using IFT herbicide in combination with a glyphosate herbicide. Glyphosate is widely used in herbicide-tolerant soybean and other agricultural production systems. IFT herbicide offers an alternative weed control option for the soybean grower. IFT controls weeds via a new herbicide mode of action for soybeans that is efficacious against many of the herbicide resistant weeds currently found in soybean fields. IFT has the flexibility to be applied pre-plant, pre-emergence, or post emergence to FG72 soybeans.

Event FG72 has been field tested beginning in 2001 in adapted growing regions of the U.S. These tests have occurred at more than 10 locations under USDA APHIS field release authorizations. Data and results collected from these trials as well as laboratory analyses presented herein demonstrate that FG72 soybeans: 1) exhibit no plant pathogenic properties; 2) are no more likely to become a weed than non-modified soybeans; 3) are unlikely to increase the weediness potential of any other cultivated plant or native wild species; 4) do not cause damage to processed agricultural commodities; and 5) are unlikely to harm other organisms that are beneficial to agriculture.

ACRONYMS AND SCIENTIFIC TERMS

ai	active ingredient
A	acre
ADF	Acid Detergent Fiber
ANOVA	Analysis Of Variance
APHIS	Animal and Plant Health Inspection Service
BCS	Bayer CropScience
BLASTP	Basic Local Alignment Search Tool
BLASTx	BLAST search of protein databases using a translated nucleotide query
BLOSUM	BLOcks SubstitUtion Matrix
bp	base pairs
bu/ac	bushels/acre
CAC	Codex Alimentarius Commission
DAD	DDBJ Amino acid sequence Database
DDBJ	DNA Data Bank of Japan
dw	Dry weight
DNA	DeoxyriboNucleic Acid
E. coli	*Escherichia coli*
ELISA	Enzyme Linked Immunosorbent Assay
EMBOSS	European Molecular Biology Open Software Suite
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
2mEPSPS	modified 5-enolpyruvylshikimate-3-phosphate synthase
2mepsps	modified 5-enolpyruvylshikimate-3-phosphate synthase gene
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
FRAC	Fractionated Raw Agricultural Commodity
fw	Fresh weight
INCAP	Institution Of Nutrition Of Central America And Panama
g	gram
GetORF	EMBOSS database for ORFs
G. max	*Glycine max*
GM	Genetically Modified
GLY	glyphosate
HPPD	p-hydroxyphenylpyruvate dioxygenase
HPPD W336	modified p-hydroxyphenylpyruvate dioxygenase
hppdPfW336	modified p-hydroxyphenylpyruvate dioxygenase gene
HRP	Horseradish Peroxidase
ID	identification
IFT	isoxaflutole
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
M	million
mg	milligram
mL	milliter
µg	microgram
NA	Not Applicable
ng	nanogram
ND	Not Detectable: Below the limit of detection
NDF	Neutral Detergent Fiber
nm	nanometer
nt	nucleotide
OECD	Organization for Economic Co operation and Development
ORF	Open Reading Frame
P. fluorescens, Pf	*Pseudomonas fluorescens*
PCR	Polymerase Chain Reaction
PDB	Protein DataBase
PIR	Protein Identification Resources
RAC	Raw Agricultural Commodity
RB	Right Border
RCB	Randomized complete block
RBS	Ribosome Binding Site
RR	Roundup Ready
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SIM	Selected Ion Monitoring
Subsp.	Subspecies
T_1, T_2, etc	generations after T_0 (transformation)
T-DNA	transfer DNA from Agrobacterium
TDN	Total Digestible Nutrients
TEP	Total Extractable Protein
TrEMBL	Translated Sequences from the European Molecular Biology Laboratory Nucleotide Sequence Database
US	United States of America
USDA	United States Department of Agriculture
WHO	World Health Organization
wt	Wild type
Z. mays	*Zea mays*, corn

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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 article 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 risk as a noxious pest or weed to agricultural infrastructure. 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. Double-herbicide-tolerant soybean event FG72

Bayer CropScience (BCS) and M.S.Technologies, LLC (MSTech) have developed double-herbicide-tolerant soybean event FG72 which produces the 2mEPSPS and HPPD W336 proteins which confer tolerance to the herbicides glyphosate and isoxaflutole (IFT), respectively. The combination of the two herbicide tolerances in a single plant provides an effective, broad spectrum weed control option using glyphosate and IFT.

I.C. Rationale for the development of event FG72 and benefits

Planting double-herbicide-tolerant soybean FG72 varieties, containing transformation event FG72, provides growers with new options for weed control using IFT herbicide (registered in North America as Balance Pro®) in combination with glyphosate herbicide. Glyphosate is widely used in herbicide-tolerant soybean and other agricultural production systems and over the years, resistance to the chemical has been reported for certain weed species (Heap, 2011). IFT herbicide offers an alternative weed control option for the soybean grower to assist in controlling weeds already resistant to glyphosate as well as providing growers an alternative herbicide for soybean production, thus providing a tool for weed resistance management. IFT controls weeds via a new herbicide mode of action for soybeans that is efficacious against many of the herbicide resistant weeds currently found in soybean fields as well as offering an alternative time of application. IFT has the flexibility to be applied pre-plant incorporation, pre-emergence, or post emergence to FG72 soybeans which allows more flexibility to the user. When used as a pre-emergent herbicide, along with glyphosate as a post-emergent herbicide, the combination potentially offers a one-pass application for season long control of weeds.

Efficacious Weed Control

Both IFT and glyphosate have been shown to be efficacious weed control systems when utilized with FG72 herbicide tolerant soybean. Broad spectrum weed control is usually achieved with a single co-application that controls the weeds in the field as well as germination of new weeds for 6-8 weeks (Loux *et al*., 2011). This flexibility is key in timing herbicide sprays at the ideal time in weed development, rather than at a specific time during the development of the soybean plant.

Simplicity and Convenience
FG72 herbicide-tolerant soybean provides an easy-to-use system that allows a highly efficient weed control in the crop. High efficiency allows for the cultivation of additional acreage and expansion of production operations with the existing level of infrastructure. Additionally, some equipment costs and labor may be eliminated in situations where cultivation equipment is no longer necessary, such as no-till practices (Carson, 2008).

Economic Benefit to Growers
BCS believes that the use of glyphosate-tolerant soybeans will increase grower returns in the form of higher yields and reduced overhead production costs.

Environmental Benefits
The main environmental benefit of FG72 herbicide-tolerant soybean is the use of reduced and no-till production systems. These cultivation practices contribute to reductions in soil erosion from water and wind. Reduced tillage also contributes to reduced fossil fuel use, less air pollution from dust, improved soil moisture retention, and reduced soil compaction (Reicosky, 2008; Duiker and Myers, 2006).

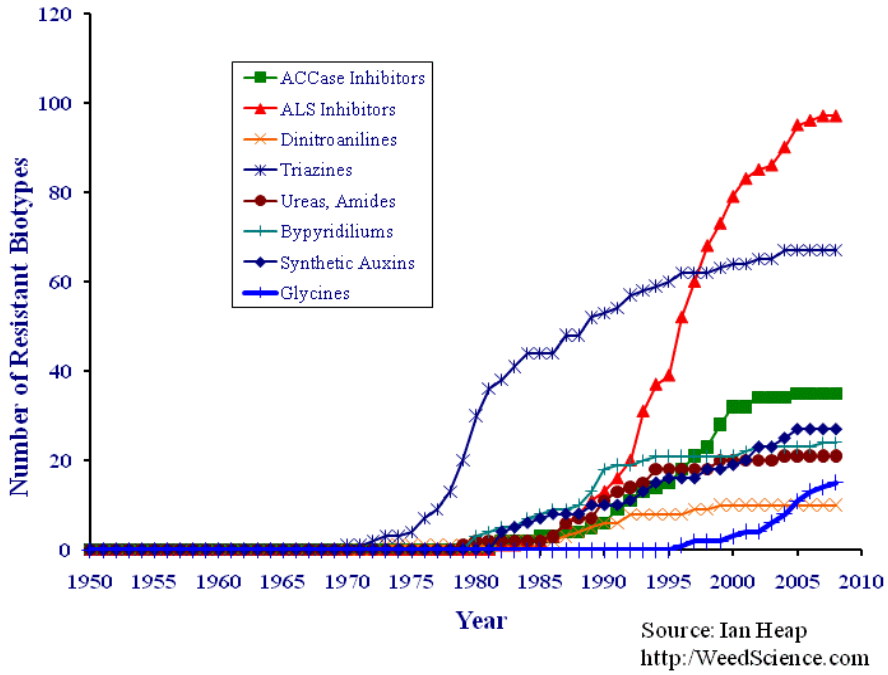
I.D. Adoption of event FG72

Soybeans are an important oilseed crop. In 2010, soybeans represented 58% of the world oilseed production, with 35% of those soybeans being produced in the U.S. (http://www.soystats.com/2011/Default-frames.htm). In 2009, 77% of the world's soybean crop was genetically modified, a higher percentage than for any other crop. The United States (91%) and Argentina (99%) produce almost exclusively GM soybeans (http://www.gmo-compass.org/eng/agri_biotechnology/gmo_planting/342.genetically_modified_soybean_global_area_under_cultivation.html).

Adoption of this technology is expected to progress in a manner similar to the adoption rate for other genetically modified soybean technologies. Where allowed around the world, growers are expected to adopt FG72 soybean at a swift rate based on the real problem of glyphosate resistant weeds as well as proactive crop management to avoid the eventual development of glyphosate resistance. The rise in glyphosate resistance among different biotypes is demonstrated in Figure 1.

This strategy will prolong the effective use of these excellent herbicides well into the future.

Figure 1. Rise in glyphosate resistance among different biotypes



I.E. Submissions to other regulatory agencies

Food and Drug Administration

FG72 soybean 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, BCS and MSTech will submit a food and feed safety and nutritional assessment summary for FG72 soybean to the US FDA.

Environmental Protection Agency

The United States Environmental Protection Agency (US EPA) has authority over the use of pesticidal substances under the Federal Insecticide Fungicide and Rodenticide Act (FIFRA) as amended (7 USA §136 *et. Seq*.). A submission for the use of an isoxaflutole formulation to be used on FG72 soybean will be presented to the Agency.

Foreign Governments

BCS and MSTech intend to submit dossiers to request import of FG72 soybean to the proper regulatory authorities of foreign governments that have regulatory processes in place. These may include submissions to the relevant Regulatory Authorities in Canada, Mexico, EU, Japan, China and others. FG72 soybean has been, or is currently, in field trials in soybean growing regions around the world.

II. THE BIOLOGY OF SOYBEAN

II.A. Biology of soybean

The scientific name of soybean is *Glycine max* (L.) Merr. The genus *Glycine* is classified under the tribe *Phaseoleae*, subfamily *Papilionoideae*, and the family *Leguminosae (Fabaceae)*.

The OECD consensus document (OECD, 2000) and the CFIA biology document (CFIA, 1996) provide information pertaining to the following aspects of soybean biology:

- General description, including taxonomy and morphology and use as a crop plant;
- Agronomic practices;
- Centers of origin of the species;
- Reproductive biology;
- Cultivated *Glycine max* as a volunteer weed;
- Inter-species/genus crosses, introgression into relatives
- Interactions with other organisms;
- Summary of the ecology of *Glycine max*.

II.B. Characteristics of the recipient soybean cultivar

The publicly available cultivar, Jack, was used as the recipient line for the generation of soybean event FG72. The variety was originally developed at the Illinois Agricultural Experimental Station and commercially released in 1989 (Nickell *et al*., 1990). Jack is classified as maturity group II and is best adapted to approximately 40 to 42 degrees of Northern latitude. It has white flowers, gray pubescence, brown pods at maturity, and seeds with dull yellow coat and yellow hila. Jack was developed and released because of its resistance to soybean cyst nematode (Races 3 and 4) and higher yield when compared with cultivars of similar maturity. It is susceptible to *Phytophthora sp.* rot (Races 1, 4, and 7) (Nickell *et al*., 1990).

Jack is extensively used in soybean transformation because of its high embryogenic capacity (Stewart *et al*., 1996; Santarem *et al*., 1998; Yan *et al*., 2000). Somatic embryos can be induced from immature cotyledons, proliferated, and maintained in liquid medium until transformation.

III. DEVELOPMENT OF DOUBLE-HERBICIDE-TOLERANT SOYBEAN EVENT FG72

III.A. Description of the transformation system

Soybean (*Glycine max* (L.) Merr.) plants of the variety Jack were genetically modified by means of direct gene transfer of the purified *Sal*I fragment from plasmid pSF10 into an embryogenic cell line. The transformed cells were selected on IFT, and after a round of multiplication cycles, regenerated into embryos and shoots in the absence of the selective agent. The regenerated plantlets were then transferred to the greenhouse and sprayed with glyphosate for evaluation. Surviving plantlets were allowed to flower and set seeds.

III.B. Parent line

The publicly available cultivar, Jack, was used as the recipient line for the generation of event FG72 soybean (See Section II.B).

III.C. Breeding Diagram

The breeding diagram of soybean event FG72 is shown in Figure 2.

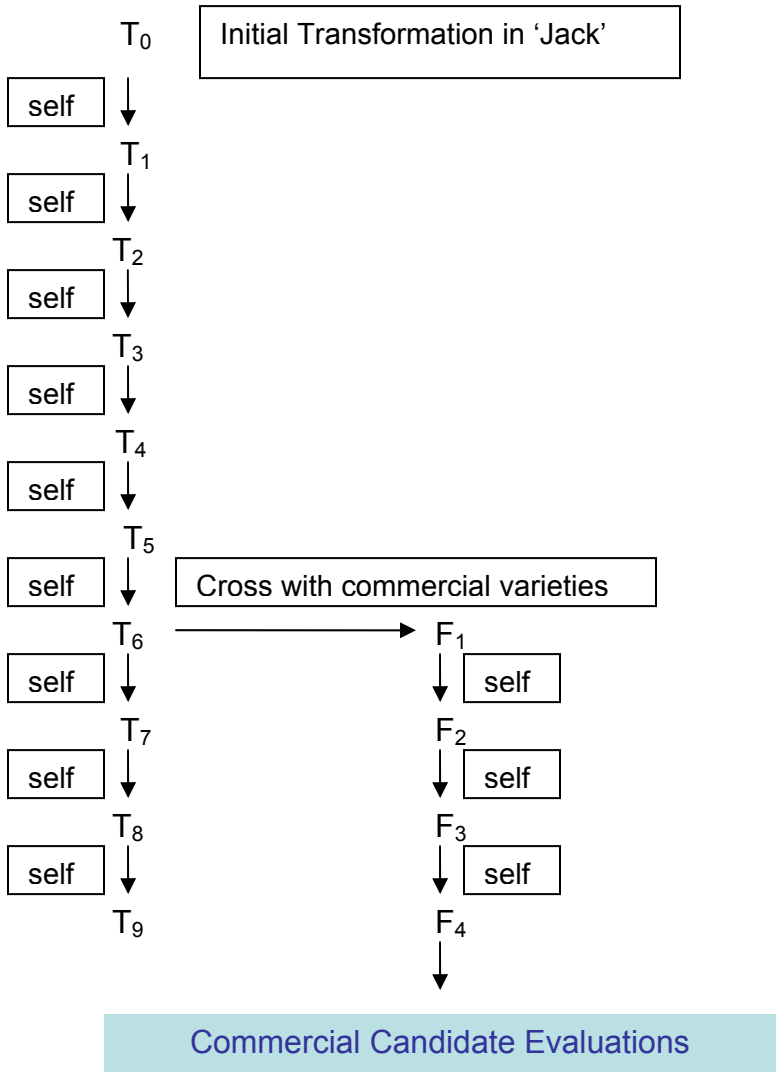
III.D. Generations Used for Analysis

The generations used for the studies to analyze soybean event FG72 are described in Table 1.

Table 1. Generations used for analysis of event FG72

Generation	Study
T2, F4	Inheritance study
T2, T7, T9, F4	Structural stability study
T5	Yield, agronomic, herbicide evaluations
T7	Absence of vector backbone sequences, detailed insert characterization, full expression analysis
T8	Nutrient composition assessment, protein expression in grain, phenotypic and agronomic assessment, grain production
F4	Full DNA sequence

Figure 2. Breeding Diagram of event FG72

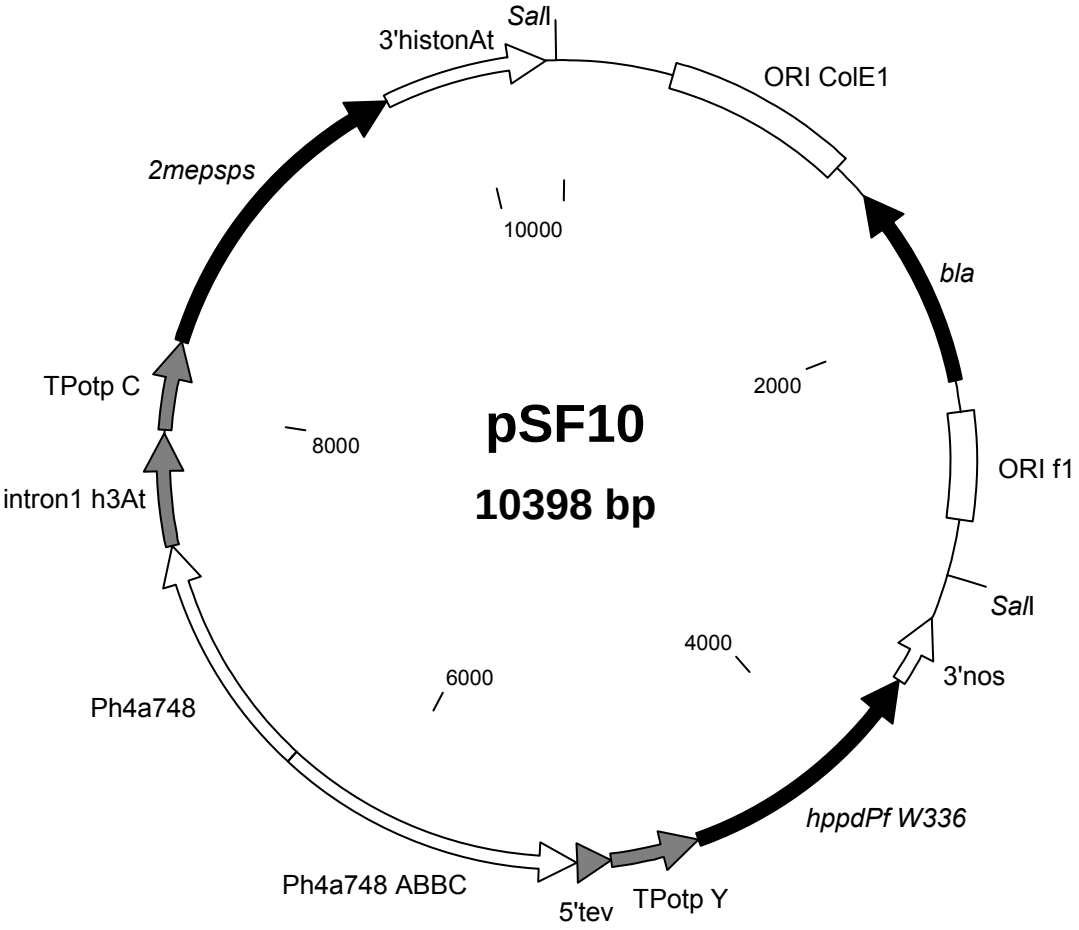


IV. GENETIC MATERIAL USED FOR TRANSFORMATION OF EVENT FG72

IV.A. Construction of the plasmid used for transformation

The double-herbicide tolerance was introduced to the plant by direct DNA integration of a linear DNA fragment isolated from the plasmid pSF10 (Figure 3). Plasmid pSF10 was constructed by inserting the *2mepsps* and *hppdPfW336* genes into an *E.coli* pBR322-derived cloning plasmid (Bolivar *et al.*, 1977). The fragment of interest was cleaved and isolated from the plasmid by means of the restriction enzyme *Sal*I and HPLC purification.

Figure 3. Map of plasmid pSF10



IV.B. Donor genes and associated regulatory regions

IV.B.1. The *2mepsps* gene expression cassette

The *2mepsps* gene expression cassette borne by pSF10 is represented by the following string: "Ph4a748-intron1 h3At-TPotpC::*2mepsps*::3'histonAt". In this cassette, the *2mepsps* gene coding sequence is under the control of the H4 promoter of *Arabidopsis thaliana* (Ph4A748, Chabouté *et al.*, 1987), followed by the first intron of gene II of the histone H3.III variant of *Arabidopsis thaliana* (Chaubet *et al*., 1992), and by the optimized transit peptide as described by Lebrun *et al*. (1996), and terminated by the 3' untranslated region of the histone H4 gene of *Arabidopsis thaliana* (Chabouté *et al.*, 1987).

Ph4a748 promoter and intron 1 h3At
The Ph4a748 promoter sequence is derived from the histone H4 gene of *Arabidopsis thaliana* (Chabouté *et al*., 1987) and controls expression of the *2mepsps* gene. The Ph4a748 promoter, combined with the first intron of gene II of the histone H3.III variant of *Arabidopsis thaliana* (Chaubet *et al*., 1992) directs high level constitutive expression, especially in rapidly growing plant tissues.

TPotp C transit peptide
The optimized transit peptide, which contains sequences from the RuBisCO small subunit genes of corn and sunflower, targets the mature protein to the plastids, which is where the wild-type protein would be located (Lebrun *et al*., 1996).

2mepsps coding sequence
The wild type *epsps* gene isolated from maize (*Zea mays*) was mutated using site-directed mutagenesis. Two point mutations resulted in the double mutant *2mepsps* gene (Lebrun *et al*., 1997). A methionine codon is added to the N-terminal of the 2mEPSPS protein sequence in order to restore the cleavage site of the optimized plastid transit peptide. The *2mepsps* gene encodes a 47 kDa protein consisting of 445 amino acids.

3'histonAt terminator
The 3' untranslated region of the histone H4 gene of *Arabidopsis thaliana* (Chabouté *et al*., 1987) is a polyadenylation signal.

IV.B.2. The *hppdPfW336* gene expression cassette

The *hppdPfW336* gene expression cassette is represented by the following string: "Ph4a748 ABBC-5'tev -TPotpY::*hppdPfW336*::3'nos". In this cassette, the *hppdPfW336* gene coding sequence is under the control of the duplicated H4 promoter of Arabidopsis thaliana (Ph4A748, Chabouté *et al*., 1987), followed by the enhancer sequence of the tobacco etch virus (Carrington and Freed, 1990), and by the optimized transit peptide as described by Lebrun *et al.* (1996), and terminated by the 3' untranslated region of the nopaline synthase from *Agrobacterium tumefaciens*.

Ph4a748 ABBC promoter and 5'tev enhancer
The same Ph4a748 promoter was used to drive the expression of the *hppdPfW336* gene, but an internal portion of the promoter sequence (referred to as "B") was duplicated to increase the promoter activity in plant cells. In combination with the leader sequence of the tobacco etch virus (5'tev), this promoter brings the level of expression of *hppdPfW336* gene to an appropriate level that enables tolerance at agronomic doses of IFT.

TPotp Y transit peptide
The optimized transit peptide, which contains sequences from the RuBisCO small subunit genes of corn and sunflower, targets the mature protein to the plastids, which is where the wild-type protein would be located (Lebrun *et al*., 1996).

hppdPfW336 gene coding sequence
The wild type *hppd* gene isolated from *Pseudomonas fluorescens* was mutated using site-directed mutagenesis. A point mutation resulted in the *hppdPfW336* gene (Boudec *et al.*, 2001). The *hppdPfW336* gene encodes a 40 kDa protein consisting of 358 amino acids.

3' nos terminator
The 3' untranslated region of the nopaline synthase from *Agrobacterium tumefaciens* is a polyadenylation signal.

IV.C. Identity and source of the genetic material

Table 2 summarizes the identity and source of the genetic elements of the *Sal*I insert excised from plasmid pSF10 (Figure 3) and their regulatory sequences.

Table 2. Genetic elements located on insert

Nt Positions	Orientation	Origin
3262 - 3553	Counter clockwise	3´nos: sequence including the 3' untranslated region of the nopaline synthase gene from the T-DNA of pTiT37 (Depicker *et al.*, 1982)
3554 - 4630	Counter clockwise	*hppdPfW336*: the coding sequence of the 4-hydroxyphenylpyruvate dioxygenase of *Pseudomonas fluorescens* strain A32 modified by the replacement of the amino acid glycine with a tryptophane, as described by Boudec *et al*. (2001)
4631 - 5002	Counter clockwise	TPotp Y: coding sequence of an optimized transit peptide derivative (position 55 changed into tyrosine), containing sequence of the RuBisCO small subunit genes of *Z. mays* (corn) and *Helianthus annuus* (sunflower), as described by Lebrun *et al.* (1996)

Table 2. Genetic elements located on *Sal*I insert (continued)

Nt Positions	Orientation	Origin
5003 - 5143	Counter clockwise	5'tev: sequence including the leader sequence of the tobacco etch virus as described by Carrington and Freed (1990)
5144 - 6433	Counter clockwise	Ph4a748 ABBC: sequence including the promoter region of the histone H4 gene of *Arabidopsis thaliana*, containing an internal duplication (Chabouté *et al*., 1987)
6434 - 7448	Clockwise	Ph4a748: sequence including the promoter region of the histone H4 gene of *Arabidopsis thaliana* (Chabouté *et al*., 1987)
7449 - 7929	Clockwise	intron1 h3At: first intron of gene II of the histone H3.III variant of *Arabidopsis thaliana* (Chaubet *et a*l., 1992)
7930 - 8301	Clockwise	TPotp C: coding sequence of the optimized transit peptide, containing sequence of the RuBisCO small subunit genes of *Z. mays* (corn) and *Helianthus annuus* (sunflower), as described by Lebrun *et al.* (1996)
8302 - 9639	Clockwise	*2mepsps*: the coding sequence of the double-mutant 5-enol-pyruvylshikimate-3-phosphate synthase gene of *Z. mays* (corn) (Lebrun *et al*., 1997)
9640 - 10326	Clockwise	3´histonAt: sequence including the 3´ untranslated region of the histone H4 gene of *Arabidopsis thaliana* (Chabouté *et al*., 1987)

V. GENETIC CHARACTERIZATION OF EVENT FG72

V.A. Overview

In order to obtain the double-herbicide-tolerant soybean event FG72, a linear fragment of DNA originating from the plasmid pSF10 and bearing the *2mepsps* and the *hppdPfW336* expression cassettes was introduced into the soybean genome by means of direct gene transfer. Event FG72 contains a single insert of two consecutive complete copies of the linear fragment. These two copies were integrated into the soybean genome and are flanked on their 5' side by two partial fragments corresponding to the 3'histonAt element. At the insertion point of the two full copies, a stretch of soybean genomic DNA was excised and translocated downstream of the 3'end of the insertion point in association with another partial fragment corresponding to the H4 promoter. This molecular modification is genetically stable and shows a typical Mendelian segregation pattern. The materials and methods for the molecular characterization of event FG72 are provided in Appendix 2.A.

V.B. Copy number and Insertion

The inserted transgenic sequence in soybean event FG72 consists of two partial 3'histonAt sequences in a head to head orientation followed by 2 complete copies of the linear fragment excised from plasmid pSF10 arranged in a head to tail orientation. Upon integration of the FG72 insert into the soybean genome, a genomic region translocated to a new position, which is joined at the 3' junction by 158 bases of Ph4a748 promoter sequences (Figure 4).

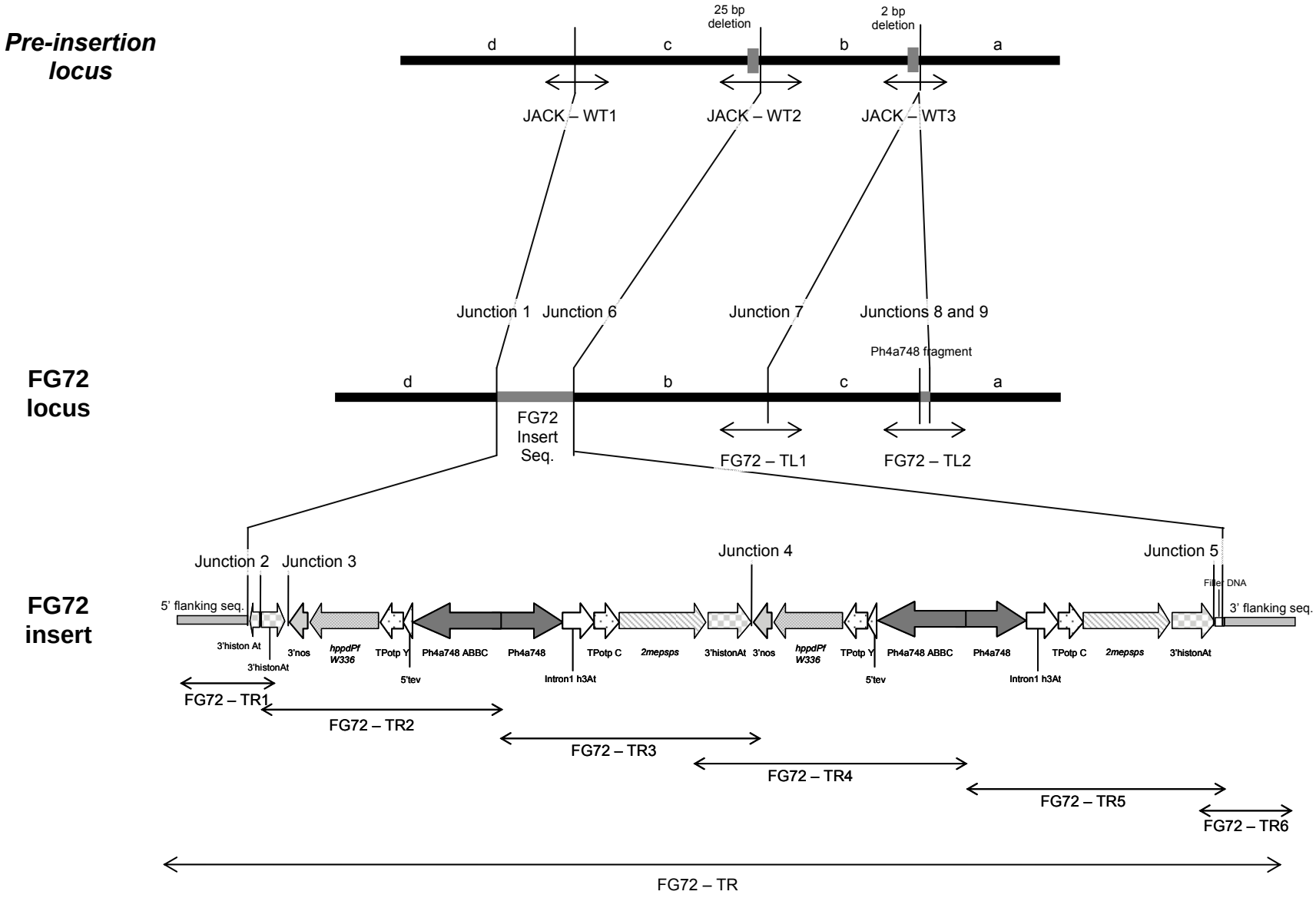
Genomic DNA prepared from FG72 soybean plants was subjected to Southern blot analysis using eight probes, each containing a single genetic element present in the pSF10 vector used for the transformation (*2mepsps*, 3'histonAt, 3'nos, Ph4a748, Intron1 h3At, 5'tev + TPotp Y, Ph4a748B, and *hppdPfW336*), and the complete insert probe. The expected and observed hybridization fragments, as well as the hybridization strategy, are described in Appendix 3, Tables 44 and 45. Hybridization results support the model of the FG72 insert organization as described above (Appendix 3, Figures 12-20).

V.C. Absence of vector backbone

For the molecular verification of the absence of pSF10 vector backbone sequences in event FG72 soybean, Southern blot analysis was performed using two overlapping probes covering the complete vector backbone sequences of the pSF10 transformation vector. Afterwards, the membranes were stripped of the vector backbone probes and re-hybridized with an insert probe, in order to demonstrate that ample FG72 soybean genomic DNA was loaded on the gels. Information on the probes and a schematic overview of the Southern blot strategy is presented in Appendix 3, Tables 46 and 47.

Since both vector backbone probes contain a number of regions also present in the insert sequence, several fragments originating from inserted transgenic DNA hybridize with the vector backbone probes. Based on sequence homology, only the expected fragments were obtained when hybridizing with vector backbone probes, demonstrating the absence of vector backbone sequences in event FG72 soybean (Appendix 3, Figures 21-23). The *hppdPf W366* gene was used as the selectable marker, therefore the same gene of interest acts as a marker. No other marker genes were present.

Figure 4. Event FG72 insert diagram



V.D. The flanking regions of the inserted sequence(s)

The flanking regions of the insert and the translocated fragment have been determined by means of PCR-based sequencing experiments. The corresponding sequences have been determined in parallel on the original, non-transformed genome of the parent line Jack. The obtained sequences are exactly identical, which demonstrates that the flanking sequences of the inserted DNA correspond to the original soybean genome in its original organization. For a diagram of event FG72 and its flanking regions refer to Figure 4 above.

The junctions between the inserted sequences and the original genome, as well as the junction created at the translocation point, have been analyzed by means of bioinformatics tools (open reading frames, promoter and gene predictions). No evidence of any potential unintended genes or any disruption of pre-existing genes was found.

V.E. Mendelian inheritance

Transformation event FG72 was derived from the transformation of soybean cells. Seed harvested from T_0 plants in the greenhouse in Lyon, France was sent to Puerto Rico for the winter season of 2001-2002. Three blocks were planted and sprayed with 0, 2, or 4 kg/ha glyphosate (GLY). Seed was harvested from plants demonstrating the desired level of tolerance to the glyphosate herbicide.

Seed harvested from T_1 plants grown in Puerto Rico (2001-2002 season) were planted "plant to row" in the US (Benton, IN) in 2002. Six progeny rows of event FG72 (T_2) were planted and the census of surviving plants was taken in August, 2002. Of the six rows, one row had no plants sensitive to glyphosate. Of the 172 individual plants, 124 were tolerant and 48 were scored as sensitive to glyphosate. The expected ratio for a single locus is 1 fully resistant to 2 partially resistant rows and for the individual plants, the expected ratio is 3 tolerant for each sensitive plant. Chi square analysis of segregation data for rows (fully or partially tolerant) and of individual plants within rows (tolerant or sensitive) demonstrates the expected Mendelian inheritance of a single insertion (Table 3).

Selection and seed increase continued until the T_4 generation which was determined to be homozygous for transformation event FG72 and selected for core seed production in the fourth generation. In 2004, T_5 generation seed of event FG72 was transferred to MSTech as a candidate for variety development. In the summer 2007 season, plants in the sixth generation were crossed with conventional soybean breeding lines in the introgression program designed to move event FG72 into a broader base of commercial soybean germplasm. F_1 hybrid plants (FG72 x conventional lines) were grown to maturity and the F_2 seed was planted. Leaf samples of 901 F_2 plants were analyzed by PCR probes designed to identify the zygosity of the event FG72 insert. The expected ratio of 1:2:1 for a single insertion segregating by the rules of Mendel was observed (Table 4).

Table 3. Segregation data for progeny rows and individuals of self-pollinated event FG72

Parents and zygosity for the FG72 locus	Progeny	Fully Tolerant[a] Rows/ Tolerant Plants	Partially Tolerant Rows/ Sensitive Plants	Expected Ratio	χ^2 calculated[b]
Tolerant T_1 progenies of the self-pollinated T_0 transformants (1/4 FG72/FG72 ; 2/4 FG72/-)	T_2 Rows	1	5	1 to 2	0.485
Hemizygous T_1 plants (FG72/-) resulting in the partially resistant rows	T_2 Individual Plants	124	48	3 to 1	0.194

[a] Based upon survival to herbicide (glyphosate) application.
[b] Assumes a single locus model. There was no significant difference (p=0.05) for the χ^2 goodness-of-fit test for the hypothesis of a single locus. To reject the null hypothesis, the χ^2 value must be greater than 3.84, with one degree of freedom.

Table 4. Segregation data for individual F2 progeny of the FG72 x conventional line cross using zygosity PCR probes designed to identify the FG72 insert

Zygosity of the FG72 locus	PCR result[a]	total	Ratio	Expected ratio
Homozygous (null / null)	nn	212	0.24	0.25
Heterozygous (FG72 / null)	pn	471	0.52	0.5
Homozygous (FG72 / FG72)	pp	218	0.24	0.25
	# plants tested:	901		χ^2 value[b] = 0.172

[a] PCR result; n = negative, p = positive
[b] Assumes a single locus model. There was no significant difference (p=0.05) for the χ^2 goodness-of-fit test for the hypothesis of a single locus. To reject the null hypothesis, the χ^2 value must be greater than 3.84, with one degree of freedom.

V.F. Stability across and within generations

In order to demonstrate the stability of event FG72, genomic DNA was prepared from several individual plants of three generations and three different genetic backgrounds. The impact of environment was assessed by analyzing the progeny of transgenic plants cultivated at 4 different field locations. The isolated DNA was digested with the restriction enzyme *Hin*dIII, which provides a unique pattern for event FG72.

Successive hybridization of these samples with the Ph4a748B probe and the insert probe revealed the expected profile in all tested samples (Appendix 3, Tables 49 and 50). These findings demonstrate the stability of event FG72 at the genomic level in different environments (Appendix 3, Figures 24-27), different backgrounds (Appendix 3, Figures 28 and 29), and different generations (Appendix 3, Figures 30 and 31).

V.G. Conclusion

The double-herbicide-tolerant soybean event FG72 contains two consecutive complete copies of the pSF10 DNA fragment that bears the *2mepsps* and the *hppdPfW336* expression cassettes. The two copies integrated in the soybean genome at a single locus and are flanked on their 5' side by two partial fragments of the 3'histonAt element. At the insertion point of the two copies, a stretch of soybean genomic DNA was excised and translocated downstream of the 3' end of the insertion point, in association with a partial fragment originating from the H4 promoter. The absence of vector backbone was demonstrated by Southern blot analysis.

The molecular modification is genetically stable and shows a typical Mendelian segregation pattern. Bioinformatics analyses of the junctions between the original genome and inserted sequences did not reveal any potential creation of unintended genes or any disruption of pre-existing genes.

VI. CHARACTERIZATION OF THE INTRODUCED PROTEINS

VI.A. The 2mEPSPS protein

VI.A.1. History and background

In the early 1970s, it was demonstrated that inhibitors of the aromatic amino acid biosynthetic pathway can have an herbicidal activity (Jaworski, 1972; Baillie *et al.*, 1972). In particular, the work published by Jaworski's group opened the path for the development of herbicides, such as glyphosate.

In plants, as much as 20% of all fixed carbon flows through the shikimate pathway leading to the formation of the aromatic amino acids tyrosine (tyr), phenyalanine (phe) and tryptophan (trp), as well as tetrahydrofolate, ubiquinone, and vitamins K and E (Haslam, 1993; Franz *et al*., 1997). The aromatic amino acids, in turn, serve as precursors for an array of secondary metabolites including lignin, flavanoids and alkaloids (Herrmann, 1995). The shikimate pathway exists exclusively in plants and microorganisms including fungi. In contrast, mammals, fish, birds, reptiles, and insects must derive their aromatic compounds from their diet. For this reason, there has been interest over the last three decades in the shikimate pathway enzymes as potential targets for non-toxic herbicides and anti-microbial compounds.

Glyphosate is the active ingredient of a non-selective, broad-spectrum, systemic, post-emergence herbicide that has been used extensively throughout the world over the past three decades. It has a very low mammalian toxicity and low soil persistence. It is used to inhibit weeds in conservation tillage systems just prior to planting (Rueppel *et al*., 1977; Williams *et al*., 2000; Andréa *et al*., 2003). It is also applied as a non-selective herbicide with direct spraying in orchards (Pacific Northwest Weed Management Handbook, 2010; Willamette Valley Pest Management Guide, 2011). Given the importance of this compound, considerable effort has been made in attempts to engineer glyphosate tolerance in various crops.

Study of the shikimate pathway led to the discovery of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) by Amrhein *et al*., (1980). The mode of action of glyphosate [N-(phosphonomethyl)glycine], a simple amino acid analog, was determined to be the selective inhibition of EPSP synthase (EPSPS; EC 2.5.1.19), the sixth and penultimate enzyme of the shikimate pathway (Steinrücken and Amrhein, 1980). The reaction catalyzed by EPSPS is the reversible transfer of the phosphoenolpyruvate (PEP) to shikimate-3-phosphate (S3P), leading to the formation of 5-enolpyruvyl-3-shikimate phosphate (EPSP). Substrate binding to the enzyme is sequential, with S3P binding first, followed by PEP (Boocock and Coggins, 1983). The reaction catalyzed by EPSPS proceeds via C-O bond cleavage of PEP (Walsh *et al.*, 1996).

VI.A.2. Characterization of the 2mEPSPS protein

VI.A.2.1. Biochemistry and mode of action

The enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) is involved in the shikimic acid pathway (Williams *et al*., 2000). This pathway produces an important branch point intermediate,

chorismate, for aromatic amino acid and aromatic metabolites biosynthesis in plants and microorganisms (Williams *et al*., 2000; Steinruecken and Amrhein, 1980). The shikimate pathway is not present in animals, a fact that contributes to the selective effect of glyphosate to plants (Hermann, 1995).

The family of EPSPS proteins is wide-spread in nature, specifically in plant, fungi and microbial sources. In higher plants, EPSPS is synthesized from a nuclear gene in the form of a cytoplasmic precursor and then imported into the plastids where it accumulates in its mature form (Kishore and Shah, 1988; Forlani *et al.*, 1994; Lebrun *et al.*, 1997). Transit peptides are typically cleaved from the mature protein following delivery to the plastids (Della-Cioppa *et al*., 1986).

Since the 1980s, several attempts have been made to identify and characterize glyphosate-insensitive EPSPS enzyme variants from various organisms with the ultimate aim to engineer glyphosate tolerance in crop plants (Kishore and Shah, 1988). Lebrun *et al.* (1997) selected the *2mepsps* gene, a double mutant gene from maize which, when fused to a chimeric optimized transit peptide, generates optimal glyphosate tolerance in various crops without any pleiotropic effects. The *2mepsps* gene has been introduced as the source of glyphosate tolerance in the maize transgenic event GA21 which has been approved by different agencies worldwide for environment, food, and feed (OECD unique identifier MON-ØØØ21-9) (CERA, 2011). Glyphosate tolerance was also achieved in rice by mutagenesis of the rice *epsps* gene (Zhou *et al.*, 2006).

VI.A.2.2. 2mEPSPS protein safety

The *2mepsps* gene was generated by introducing mutations into the wt *epsps* gene from maize (*Z. mays* L.), leading to a double mutant EPSPS protein with two amino acid substitutions (2mEPSPS). These modifications confer to the protein a decreased binding affinity for glyphosate, allowing it to maintain sufficient enzymatic activity in the presence of the herbicide. Therefore, the plants bearing this gene become tolerant to glyphosate herbicides (Lebrun *et al.*, 1997).

In order to assess any potential adverse effects to humans or animals resulting from the environmental release of crops containing the 2mEPSPS protein, BCS has conducted a detailed safety evaluation based on the Codex Alimentarius Commission (Codex; Alinorm 03/34A). As a basis, BCS performed a series of safety studies with the 2mEPSPS protein, including homology searches of the amino acid sequence to all known allergens and toxins from large public databases, an *in vitro* digestibility assay of the protein, and an acute toxicity test in the mouse. As reviewed in ILSI (2010), several publicly available documents issued by regulatory authorities indicate that similar EPSPS protein family members are safe. The results of studies conducted by BCS are consistent with the published information, confirming that the crops containing this protein can be safely used as food or feed.

Assessments of the maize (*Zea mays* L.) source organism, the *2mepsps* gene, and the 2mEPSPS protein indicate that they are not pathogenic, allergenic, or toxic to mammals:

History of safe use

The maize source organism is a safe crop plant widely used for food and feed with little pathogenic, toxic, or allergenic effects on humans and animals (OECD, 2003).

The 2*mepsps* gene is composed of the same essential nucleic acids found in any food or feed DNA, which is commonly consumed as part of human or animal diets. Decades of research have indicated that dietary DNA poses no direct toxicity to human health.

The EPSPS proteins are ubiquitous in nature, widely expressed in food and feed crops (*e.g.* soybean, tomato, maize) (ILSI, 2010). No health-related adverse effects have been associated with these proteins. Since the 2mEPSPS protein is derived from maize and has only two amino acid modifications, the safety profile of the novel protein is expected to remain unchanged relative to its wild-type counterpart.

The 2mEPSPS protein is highly homologous to, and shares similar molecular weight and functionalities with other shikimate synthase proteins which have been demonstrated to be non-toxic and non-allergenic over the years through consumption. Its identity with the wt EPSPS enzyme is greater than 99.5% (Herouet-Guicheney, 2009).

The EPSPS proteins have a very well known and specific biochemical role in plants. The biochemical properties of the 2mEPSPS enzyme have been well characterized in comparison to the wt EPSPS protein. Except for the insensitivity to glyphosate herbicide, the change in the two amino acids results in comparable biochemical properties. The metabolic effects of the 2mEPSPS activity in plants are comparable to those of endogenous EPSPS proteins except for the insensitivity to glyphosate (Herouet-Guicheney, 2009).

The 2mEPSPS protein is present in glyphosate tolerant maize event GA21 (MON-ØØØ21-9), which has been approved for cultivation and for food/feed use in many countries (CERA, 2011).

Lack of allergenic potential

The 2mEPSPS protein has no amino acid sequence similarity to known allergens, as demonstrated by overall amino acid and epitope homology searches (refer to Section VI.E.2.1. of this petition for details).

As expected, the 2mEPSPS protein has high structural similarity only to the non-allergenic *Z. mays* wt EPSPS protein and other non-allergenic EPSPS enzymes (refer to Section VI.D.1. of this petition for details).

The 2mEPSPS shares the same potential N-glycosylation sites as the endogenous *Z. mays* EPSPS enzyme, and both proteins are targeted to the same plastid cellular compartment. Therefore, it is unlikely that post-translational glycosylation occurs on the 2mEPSPS protein, which could potentially lead to allergenic characteristics different from the wild-type enzyme (refer to Section VI.E.2.2. of this petition for details).

The 2mEPSPS protein is rapidly and completely degraded in human simulated gastric and intestinal fluids. This minimizes the likelihood that this protein could survive in the human digestive tract and be absorbed (refer to Sections VI.E.2.3. and VI.E.2.4. of this petition for details).

Lack of toxic potential
The 2mEPSPS protein has no amino acid sequence similarity to known toxins, as demonstrated by overall amino acid and epitope homology searches (refer to Section VI.E.3. of this petition for details).

As expected, the 2mEPSPS protein only has high structural similarity to the non-toxic *Z. mays* wt EPSPS protein and other non-toxic EPSPS enzymes (refer to Section VI.D.1. of this petition for details).

The 2mEPSPS protein is rapidly and completely degraded in human simulated gastric and intestinal fluids (refer to Sections VI.E.2.3. and VI.E.2.4. of this petition for details). This minimizes the likelihood that this protein could survive in the human digestive tract and be absorbed.

There were no mortalities, clinical signs, or treatment-related effects on OF1 mice after an acute oral administration by gavage of 2mEPSPS protein at 2,000 mg protein/kg body weight (refer to Section VI.E.4. of this petition for details).

In conclusion, it is considered that the source organism, maize, is non-pathogenic and the *2mepsps* gene as well as the 2mEPSPS protein are not toxic to mammals and do not possess any of the characteristics associated with food allergens. Therefore, no adverse effects on animal and human health are to be expected from the consumption of the *2mepsps* gene and the 2mEPSPS protein.

VI.B. The HPPD protein

VI.B.1. History and background

The coding sequence of the 4-hydroxyphenylpyruvate dioxygenase (HPPD) protein was isolated from the *Pseudomonas fluorescens* strain A32 by a DNA amplification technique (PCR). The primers in the amplification were based on the amino acid sequence of the HPPD protein present in *Pseudomonas* strain P.J. 874. The isolated DNA sequence was modified to improve the tolerance against HPPD inhibitors. The modified protein is designated as HPPD W336.

VI.B.2. The function of the gene product

VI.B.2.1. Biochemistry and mode of action

The biochemical pathways in which HPPD is involved differ between plants and non-photosynthetic organisms. In bacteria and animals, it merely serves catabolic purposes by catalyzing the first committed step in tyrosine degradation that in the end yields energetically exploitable glucogenic and ketogenic products (Brownlee *et al.*, 2004). In plants, however, it is also involved in several anabolic pathways; its reaction product homogentisate (2,5-dihydroxyohenylacetate) being the aromatic precursor of tocopherol and plastoquinone, which are essential to the photosynthetic transport chain and antioxidative systems (Fritze *et al*.,

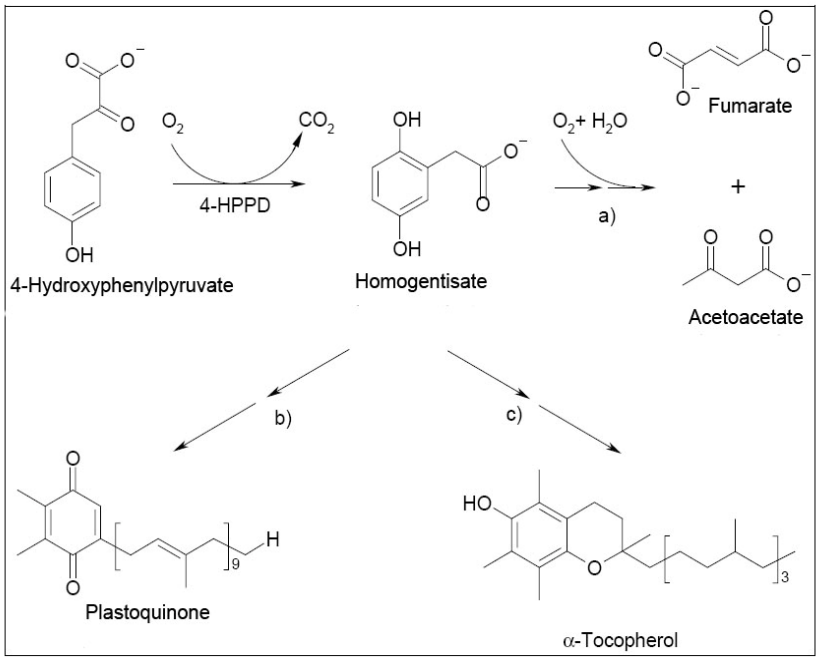
2004). Figure 5 shows a diagram of the different metabolic pathways in plants and non-photosynthetic organisms.

HPPD enzymes require a α-keto acid and molecular oxygen to oxidize or oxygenate a third molecule. The activity of HPPD is suppressed by benzoylisoxazoles bleaching herbicides, such as IFT, and by β-triketones such as sulcotrione and mesotrione (Pallett *et al.*, 2001; Dayan *et al.*, 2007). The inhibitor of HPPD is the diketonitrile (DKN) derivative of IFT formed by the opening of the isoxazole ring. DKN is formed rapidly in plants following uptake of IFT by roots and shoots. HPPD enzyme inhibition results in the disruption of the biosynthesis of carotenoids, which destabilizes photosynthesis and leads to bleaching of the foliage and death of the plant (Figure 6).

In order to create a form of the HPPD enzyme with tolerance to IFT herbicide, a single amino acid substitution, glycine (G) to tryptophan (W) at position 336, was introduced to the native HPPD protein from *Pseudomonas fluorescens* (Boudec *et al.*, 2001), resulting the modified IFT-tolerant HPPD W336 protein.

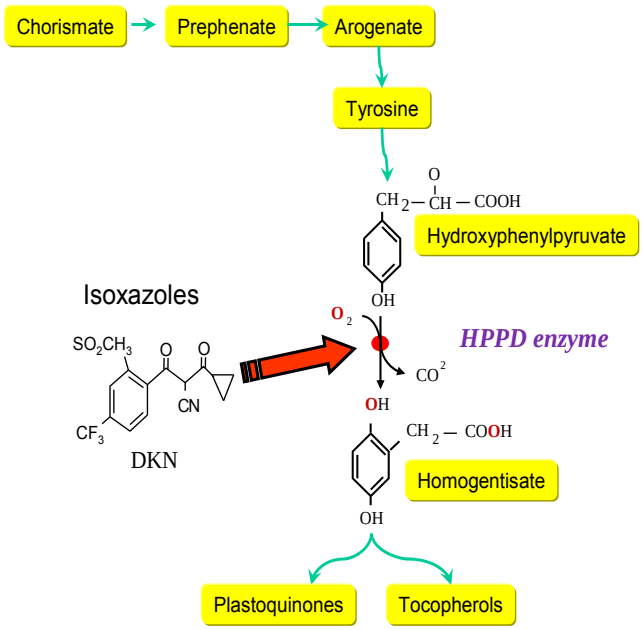
Several different HPPD variants, including the wild type HPPD and modified HPPD W336 enzymes were tested for their activity in the presence or absence of the inhibitor IFT. When compared to the wild type HPPD enzyme, HPPD W336 enzyme was significantly less inhibited by IFT (Fischer, 2008).

Figure 5. Biochemical pathways of HPPD proteins



a) catabolism of tyrosine, b) biosynthesis of plastoquinone (plants)
c) biosynthesis of tocopherol (plants)

Figure 6. Interaction of HPPD and isoxazole herbicides



VI.B.2.2. Source of the gene

The *hppd* gene was isolated from the bacterium *Pseudomonas fluorescens*, strain A32 – Genebank A69533 (McKellar, 1982). *P. fluorescens* is a non-pathogenic bacterium, ubiquitous in nature, with a good history of safe use.

P. fluorescens (Migula 1895, type strain ATCC 13525; taxonomy ID: 136843) belongs to the *Pseudomonadaceae* family, order of *Pseudomonadales*, class of *Gammaproteobacteria* (Skerman, 1980). *P. fluorescens, P. putida* and *P. chlororaphis* are closely related to each other and are seen as forming a complex within the fluorescent subgroup of the *Pseudomonas* genus. In addition, *P. fluorescens* is a heterogeneous species comprising several biovars, each of which may deserve species rank, but which are so interconnected that adequate methods have not been devised to clearly separate them (OECD, 1997).

P. fluorescens is a Gram-negative, rod-shaped, motile, asporogenous, aerobic bacteria. This species produces fluorescent pigments and is catalase and oxidase-positive. *P. fluorescens* strains are generally not able to grow above 42°C, but grow at 5°C (OECD, 1997, Palleroni, 1981). This organism is a nonpathogenic saprophyte which inhabits soil, water and plant surface environments. It is able to produce a soluble, greenish fluorescent pigment, which relates to its name.

VI.B.2.3. History of safe use of the source organism

Risk Group Classification
P. fluorescens (*Pf*) strains are generally classified as non-pathogenic bacteria in several national classifications for microorganisms (Table 5).

Table 5. Risk group classification of *P. fluorescens*

USA	Not classified. http://www.cdc.gov/OD/ohs/biosfty/bmbl5/bmbl5toc.htm, accessed on March 02, 2009.
Canada	Non-pathogenic organism. http://www.phac-aspc.gc.ca/ols-bsl/pathogen/organism-eng.php, accessed on March 02, 2009.
European Union	Not classified. Directive 2000/54/EC
Belgium	Risk Group 2 plant pathogen. Belgian Monitor 01.04.2004 18362-18442. 2004
Switzerland	1 + opportunistic pathogen. http://www.bafu.admin.ch/publikationen/index.html?action=show_publ&lang=de&id_thema=6&series=VU&nr_publ=4401; accessed on March 02, 2009.
France	Not classified. Commission de Genie Génétique
Germany	Risk Group 1 + - opportunistic pathogen. Classification of bacteria and archaea bacteria into risk groups – TRBA 466. 2005

Pathogenicity to humans
P. fluorescens can be an opportunistic pathogen in immunocompromised patients (McKellar, 1982). Some cases of septicemia have been reported due to *P. fluorescens* contamination of transfused blood and blood products, given its ability to grow at 5°C (Gibb *et al*., 1995, Puckett *et al*., 1992). Some *P. fluorescens* strains were also reported to create biofilms on compounded sterile products like catheters and have led to rare infections in immunocompromised populations (Gershman *et al.,* 2008). However, the general virulence of *P fluorescens* is low, due to its inability to multiply rapidly at body temperature and having to compete with defense mechanisms of the host (Liu, 1964).

Pathogenicity to animals
P. fluorescens can infect a wide range of animals including horses, chickens, marine turtles, and many fish and invertebrate species. However, since it is unable to grow at elevated temperatures, it is probably only an opportunistic pathogen for warm-blooded animals (OECD, 1997).

Pathogenicity to plants
Generally *P. fluorescens* is considered saprophytic but it may be an opportunistic pathogen causing soft rot in plants (OECD, 1997).

Allergenicity

In general fluorescent pseudomonads have not been described as allergens. However, they do possess an endotoxin (lipopolysaccharide) which may induce an allergic response in some individuals (OECD, 1997).

History of safe use

P. fluorescens is a ubiquitous bacterium frequently present in water, soil and the plant rhizosphere (Bossis *et al*., 2000). It can be isolated from water, animals, human clinical specimens, the hospital environment, and spoiled foodstuffs such as fish and meat. The survival of *P. fluorescens* is affected by number of biotic and abiotic factors such as soil density, temperature, pH, humidity (OECD, 1997).

P. fluorescens is used in agriculture as growth-promoting agent (Fliessbach *et al*., 2009; OECD, 1997). It can enhance plant growth through production of siderophores, which efficiently complex environmental iron, making it unavailable to other components of the soil microflora. In addition, *P. fluorescens* is used as a biopesticide on certain crops and fruits to prevent the growth of frost-forming bacteria on leaves and blossoms (Compant *et al*., 2005; Raaijmakers *et al*., 2006; EPA, 2011). It is also used as seed treatment agent for damping off diseases caused by fungi (Haas and Defago, 2005; Thrane *et al*., 2001; Voisard *et al*., 1989) and nematodes (Hamid *et al*., 2003). This pesticide activity of *P. fluorescens* is attributed to three mechanisms: competition for an ecological niche or a substrate, production of inhibitory chemicals, and induction of systemic resistance in host plants to a broad spectrum of pathogens (Compant *et al*., 2000; Haas and Defago, 2005).

Naturally occurring strains of *P. fluorescens* have been registered commercially for the control of frost injury and fire blight on pear (Wilson and Lindow, 1993). Since 1992, 4 end products containing *P. fluorescens* strains as active ingredients have been approved by US-EPA (EPA, 2011). US-EPA has recognized that this bacterial active ingredient is not expected to have any adverse health effects on humans, based on various studies that found no evidence that these *P. fluorescens* strains are harmful to mammals (EPA, 2011). In addition, US-EPA has established a tolerance exemption for residues of *P. fluorescens* in or on raw agricultural commodity mushrooms (EPA, 1994).

Moreover, strains of *P. fluorescens* have been genetically modified to encapsulate crystal δ-endotoxins (Cry proteins) from the bacterium *Bacillus thuringiensis* (*Bt*) (Downing *et al*., 2000, Peng *et al*., 2003). The Cry proteins encapsulated by *P. fluorescens* showed high insecticidal activity and retained their activity for two to three times longer than conventional *Bt* formulations (Peng *et al*., 2003).

In pharmaceutical uses, *P. fluorescens* produces the antibiotic pseudomonic acid (also called mupirocin), which is used to prevent *Staphylococcus aureus* infections (Hothersall *et al*., 2007; Tacconelli *et al*., 2003).

Finally, due to the metabolic diversity of *P. fluorescens*, it may be used in bioremediation applications. *P. fluorescens* is able to degrade a wide variety of compounds, including 3-chlorobenzoic acid, naphthalene, phenathrene, fluorene and fluoranthene, chlorinated aliphatic hydrocarbons, styrene, pure hydrocarbons and crude oil (OECD, 1997).

The source organism of the *hppd* gene, *P. fluorescens*, is ubiquitous in the environment, including soil, water and food. It has many beneficial uses in agriculture, human health and bioremediation. Despite this widespread presence, it is not described as allergenic, toxic or pathogenic to healthy humans and animals and has an overall history of safe use.

VI.B.2.4. Familiarity of the gene product

History of safe consumption
HPPD is ubiquitous in nature across all kingdoms: bacteria, fungi, plants and animals including mammals. For instance, HPPD amino acid sequences have been determined in bacteria such as *Streptomyces avermitilis* (Accession number Q53586), in fungi such as *Aspergillus fumigatus* (Accession number Q4WPV8), in plants such as *Arabidopsis thaliana* (Accession number P93836), and in animals such as *Caenorhabditis elegans* (Accession number Q22633), mouse (*Mus musculus*, Accession number P49429), and human (*Homo sapiens*, Accession number P32754).

In particular, HPPD has been characterized in organisms present in human food, such as carrot (*Daucus carota*, Accession number O23920; Garcia *et al*., 1997), barley (*Hordeum vulgare* Accession number O48604; Falk *et al*., 2002), pork (*Sus scrofa*, Accession number Q02110; Endo, 1992) and beef (*Bos Taurus*, Accession number Q5EA20; Harhay, 2005).

No toxicity or allergenicity findings were found associated with HPPD proteins.

In conclusion, HPPD proteins are present in food from plant, fungal or animal origin, with good safety records. Therefore, HPPD have a history of safe consumption.

VI.C. Expression of the 2mEPSPS and HPPD W336 proteins in event FG72

VI.C.1. Expression in grain

Expression of the 2mEPSPS (Currier and Harbin, 2011) and HPPD W366 (Poe, 2009) proteins was determined from field-grown event FG72 soybean plants. The trial design applied at all 10 locations was a randomized complete block (RCB) design including three plots planted with the non-transgenic variety Jack and six plots planted with event FG72. Three additional plots were planted with the non-transgenic commercial soybean lines Stine® 2686-6, Stine® 2788 and Stine® 3000-0. There were a total of 12 plots at each of the 10 trial locations.

The plants were grown under conditions typical of production practices. Transgenic and non-transgenic plants were treated identically, except for the IFT and GLY treatments of some transgenic plots. Fertilization and normal cultural practices were carried out by MSTech test site personnel.

Treatment with IFT (at a target rate of 70 grams ai/hectare) and GLY herbicide (at 1060 grams ai/hectare) was done as a foliar spray at about the V4-V5 growth stage. Ammonium sulfate at 2850 grams/hectare was added to the spray mixture. Plots not treated with these herbicides were conventionally treated.

Soybean seed samples were harvested from the center two rows of each six-row plot for each field trial. The soybean plots were harvested at normal maturity by mechanical means. The samples were stored after harvest and shipped to the BCS, BioAnalytics, RTP, North Carolina at ambient temperatures. Within one week of arrival at BCS, the seed samples were sub-sampled and transferred to frozen storage. Appendix 2.C. describes the materials and methods for the 2mEPSPS and HPPD W336 protein levels in grain.

The expression results of the 2mEPSPS and HPPD W336 protein levels in soybean seeds were obtained for the conventionally treated and GLY + IFT herbicide treated entries. The across location averages as well as the range of protein expression are presented in Table 6.

A fairly broad range of expression was observed for the 2mEPSPS protein in the FG72 soybean grain from entries B (conventional treated) and C (treated with GLY + IFT herbicides). The expression level was between 87 and 180 µg/g fw in seeds treated with a conventional herbicide (mean value: 130 µg/g fw) and between 87 and 240 µg/g fw in the seeds treated with GLY + IFT (mean value: 140 µg/g fw).

The dry weight and % crude protein analyte amounts were calculated using the average 2mEPSPS value of samples from each plot. The respective amounts for the 2mEPSPS protein expressed on a dry weight basis were150 µg/g dw in seeds from conventionally treated and in seeds from GLY + IFT treated soybean plants.

The HPPD W336 protein content ranged from 0.46 to 1.32 µg/g fw in the seeds from soybean plants treated with a conventional herbicide (mean value: 0.85 µg/g fw) and from 0.41 to 1.31 µg/g fw in the seeds from soybean plants treated with GLY + IFT (mean value: 0.8 µg/g fw). When converted to a dry weight basis, the expression levels were 0.62 to 1.26 µg/g dw (mean value: 0.94 µg/g dw) and 0.54 to 1.40 µg/g dw (mean value: 0.89 µg/g dw).

Table 6. Protein expression levels in event FG72 soybean seeds

Protein	Treatment/Entry		Fresh Weight (µg/g)[a]	Dry Weight (µg/g)	Content as % Crude Protein[b]
2mEPSPS	Conventional Treated/B	Range	87 – 180	150 [b]	0.039 [b]
		Mean ± SD	130 ± 22		
2mEPSPS	GLY + IFT Treated/C	Range	87 – 240	150 [b]	0.041 [b]
		Mean ± SD	140 ± 33		
HPPD W336	Conventional Treated/B	Range	0.46– 1.32	0.94 [c]	0.00024 [c]
		Mean ± SD	0.85 ± 0.20		
HPPD W336	GLY + IFT Treated/C	Range	0.41 – 1.31	0.89 [c]	0.00023 [c]
		Mean ± SD	0.80 ± 0.22		

[a] Range and overall mean ± standard deviation in µg/g for the fresh weight protein content in grain samples from the 10 sites.
[b] The dry weight (dw) and % crude protein analyte amounts were calculated using the average 2mEPSPS value of samples from each plot.
[c] The dry weight (dw) and % crude protein analyte amounts were determined using the average of four individual results per sample, therefore no standard deviation or range is given for these amounts.

In summary, the mean concentration of 2mEPSPS protein measured in soybean seeds was 150 µg/g dw for both entries B an C. The mean concentration of HPPD W336 measured in soybean seeds on a dry weight basis was 0.94 µg/g dw and 0.89 µg/g dw for entries B an C, respectively.

VI.C.2. Expression in plant parts and during the life cycle

The HPPD W336 and 2mEPSPS protein expression levels were determined in different tissues at different growth stages of event FG72 soybean (Habex and Debaveye, 2009). Event FG72 and wt plants were grown under greenhouse conditions. Leaf samples were taken at three different growth stages (V4, V6 and V8) and samples of stem and root were taken at two different growth stages (V4 and V8). Samples of the wt soybean line (Jack) were also sampled at the same stages for the same tissues. Seeds were collected for both event FG72 and wt soybean plants. Samples were analyzed for HPPD W336 and 2mEPSPS protein content by ELISA. HPPD W336 and 2mEPSPS proteins were detected in all event FG72 samples for all growth stages and tissue types analyzed. Expressions levels for HPPD W336 and 2mEPSPS proteins are summarized in Tables 7 and 8, respectively. Appendix 2.D. describes the materials and methods for the 2mEPSPS and HPPD W336 protein levels in different plants parts and during the life cycle.

Table 7. HPPD W336 protein expression in different tissues and growth stages

Matrix	Growth stage	HPPD W336 protein content			
		µg/g fresh weight (fw)		µg/g dry weight (dw)	
		Average ± SD	Range	Average ± SD	Range
Leaf	V4	6.10 ± 2.78	2.65 – 10.4	38.4 ± 17.5	16.7 – 65.7
	V6	6.48 ± 4.08	2.31 – 17.4	35.8 ± 22.5	12.8 – 96.0
	V8	4.69 ± 1.87	2.00 – 8.91	27.2 ± 10.9	11.6 – 51.8
Stem	V4	1.48 ± 0.42	0.74 – 2.20	16.6 ± 4.65	8.29 – 24.6
	V8	0.69 ± 0.35	0.29 – 1.49	6.04 ± 3.10	2.49 – 13.0
Root	V4	0.87 ± 0.35	0.45 – 1.66	5.81 ± 2.30	2.98 – 11.0
	V8	0.84 ± 0.50	0.20 – 1.64	6.42 ± 3.82	1.51 – 12.4
Seed	NA	1.27 ± 0.42	0.71 – 2.68	1.41 ± 0.47	0.79 – 2.96

Table 8. 2mEPSPS protein expression in different tissues and growth stages

Matrix	Growth stage	2mEPSPS protein content			
		µg/g fresh weight (fw)		µg/g dry weight (dw)	
		Average ± SD	Range	Average ± SD	Range
Leaf	V4	90.4 ± 26.1	44.9 – 152	569 ± 164	283 – 958
	V6	79.1 ± 29.6	39.2 – 136	437 ± 163	216 – 753
	V8	115 ± 38.2	60.5 – 203	668 ± 222	351 – 1180
Stem	V4	18.8 ± 6.16	6.08 – 31.3	211 ± 68.9	68.0 – 350
	V8	13.4 ± 2.62	8.71 – 17.3	117 ± 22.9	76.1 – 151
Root	V4	4.89 ± 1.99	1.63 – 8.21	32.5 ± 13.2	10.8 – 54.5
	V8	5.75 ± 2.31	2.62 – 10.7	43.7 ± 17.6	19.9 – 81.2

VI.D. Biochemical and functional equivalence of the expressed proteins

The 2mEPSPS and HPPD W336 proteins were produced in *E. coli* for use in studies to investigate the toxicity and potential allergenicity of the protein, since it is not feasible to produce an adequate amount of the respective proteins for these studies from event FG72 soybean plants. Therefore, it was necessary to demonstrate the equivalence between the *E.coli* produced proteins and the plant-produced proteins in order to utilize the safety data. The *E.coli*-produced, and plant-produced 2mEPSPS and HPPD W336 proteins, were compared, using the 5 following criteria and associated methods listed in Table.

Table 9. Criteria and methodologies for demonstrating protein equivalence

Equivalence criteria	Methodology[a]
Confirm identity of 2mEPSPS and HPPD W336 proteins	Edman degradation
Comparable molecular weight	Protein mobility in SDS-PAGE
Comparable immuno-reactivity	Western blot analysis
Comparable peptide masses	HPLC/Electrospray Mass Spectrometry (LC/MS) of peptides
Comparable biological activity	Enzymatic activity

[a] N-terminal amino acid sequencing and enzyme activity analyses could not be performed on the HPPD W336 protein produced in plants because of the small amount of protein isolated from the plant.

VI.D.1. 2mEPSPS protein

Equivalence of *E.coli* produced 2mEPSPS protein and plant produced protein was established via SDS-PAGE, western blot, N-terminal amino acid sequencing, enzyme activity and LC/MS methods.

The SDS-PAGE (Appendix 3, Figures 33 and 34) and western blot (Appendix 3, Figure 35) demonstrated that the molecular weight and mobility of the two proteins are the same. The western blot also indicated that both proteins have the same immuno-reactivity.

The activity assay indicated that both proteins were active.

The N-terminal amino acid sequence data suggests that both the N-terminal of the *E. coli* produced 2mEPSPS protein and the FG72 soybean produced 2mEPSPS protein is missing the N-terminal methionine. The loss of a methionine is not unusual, post-translational modifications, such as removal of a methionine are often found in proteins from both prokaryotic and eukaryotic organisms (Bradshaw *et al*., 1998). Only a small amount of the N-terminal peptide with the methionine was detected for the *E. coli* produced 2mEPSPS protein by N-terminal amino acid sequencing. The N-terminal amino acid sequence data also suggests that some of the plant produced 2mEPSPS protein may be blocked at the N-terminus. The mass spectrometry data confirms the N-terminal amino acid sequencing data.

The mass spectrometry results indicate that 98% of the *E. coli*-produced 2mEPSPS sequence coverage and 71% sequence coverage of the FG72 soybean produced 2mEPSPS protein was detected (Appendix 3, Tables 51 and 52). The mass spectrometry data presents direct evidence that 72% of the protein sequence of the FG72 soybean produced 2mEPSPS protein is identical to the protein sequence of the *E. coli* produced 2mEPSPS protein. Taken together, the analytical results demonstrate equivalence of the FG72 soybean produced 2mEPSPS protein to the *E. coli* produced 2mEPSPS protein.

VI.D.2. HPPD W336 protein

Equivalence of *E. coli* produced HPPD W336 protein and the plant produced HPPD W336 protein was established via SDS-PAGE, western blot and LC/MS methods. N-terminal amino acid sequencing and enzyme activity analyses could not be performed on the plant produced HPPD W336 protein because of the small amount of protein isolated from the plant.

The SDS-PAGE (Appendix 3, Figure 36) and western blot (Appendix 3, Figure 37) demonstrated that the molecular weight and mobility of the two proteins are the same. The western blot also indicated that both proteins have the same immuno-reactivity.

The activity assay indicates that the protein from the *E. coli*-produced protein was active; but the concentration of the protein in event FG72 soybean leaves was below the limit of detection of the assay.

An N-terminal amino acid sequence analysis could not be performed on the plant produced HPPD W336 protein because the amount of protein isolated from the leaves was insufficient for analysis. However, an N-terminal amino acid sequence analysis was performed on the *E. coli* - produced HPPD W336 protein. The N-terminal amino acid sequencing results indicated that the methionine was missing from the N-terminal of the *E. coli* produced HPPD W336 protein. Mass spectrometry analysis results of the *E. coli* produced HPPD W336 protein confirmed that the methionine was missing from the N-terminal peptide.

Mass spectrometry analysis results of the plant produced HPPD W336 protein also indicate that the methionine is missing from the N-terminal peptide. A 95.2 % sequence coverage of the *E. coli* produced HPPD W336 protein and 70.1% sequence coverage of the plant produced HPPD W336 protein was detected by mass spectrometry (Appendix 3, Tables 53 and 54). The mass spectrometry results indicate that 71% of the protein sequence of plant produced HPPD W336 protein is equivalent to the protein sequence of *E. coli* produced HPPD W336 protein. Taken together, the analytical results demonstrate equivalence of the plant produced HPPD W336 protein to the *E. coli* produced HPPD W336 protein.

VI.E. Summary of the Food and Feed Safety Assessment of the 2mEPSPS Protein

VI.E.1. Familiarity to the protein

EPSPS is the 6th enzyme of the shikimate pathway, the metabolic pathway for the biosynthesis of aromatic compounds found in microorganisms and in plants (Herrmann *et al*., 1995). As such, it has been shown that EPSPS enzymes are ubiquitous in nature and are present in foods derived from plant and microbial sources.

In addition, insensitivity of some EPSPS enzymes to glyphosate also exists in nature at various levels and has been specifically studied for the development of the glyphosate tolerance trait in plants (Van der Klis *et al*., 2006).

It is apparent that these proteins have a long history of safe use as endogenous components of food and feed. There is no evidence suggesting that these proteins may be related to any type of allergenicity or toxicity to humans or other animals (Herouet-Guicheney, 2009). Thus, exposure to the known EPSPS proteins can be deemed as innocuous as exposure to other naturally occurring proteins without inducing adverse effects (ILSI, 2011).

The 2mEPSPS, which contains only two amino acid substitutions of the maize wt EPSPS protein, was modified in such a way that the enzymatic characteristics remain as much as possible unchanged with the exception of the insensitivity to glyphosate (Schultz *et al*., 1985). Therefore, it is expected to have the same safety profile as the wild-type protein.

VI.E.2. Potential allergenicity

VI.E.2.1. Homology search to known allergens

The overall amino acid sequence homology search was carried out by using FASTA algorithm, which compares the complete amino acid sequence of the 2mEPSPS protein with all protein sequences present in the public allergen database AllergenOnline (www.allergenonline.com; release 8.0, 1313 sequences) (Capt, 2008 a and b). The criterion indicating potential allergenicity was 35% identity over at least 80 consecutive amino acids with an allergenic protein.

In addition, an allergenic identity search (80-mer amino acid sequence homology) was performed to compare the query sequence subdivided into 80 amino acid blocks, with all known allergens present in the AllergenOnline database. The criterion indicating potential allergenicity was 35 % identity with an allergenic protein.

Furthermore, the amino acid sequence of the 2mEPSPS protein, subdivided into 8 amino acid blocks, was compared with all known allergens present in the allergen database (epitope search). The algorithm used was FindPatterns and the criterion indicating potential allergenicity was 100 % identity on a window of 8 amino acids with an allergenic protein.

The overall and 80-mer identity searches showed no relevant similarity between the 2mEPSPS sequences and any known allergenic sequences from the allergen database. In addition, the

epitope search showed no identity between all the blocks (8 amino acids) of the 2mEPSPS protein and known allergens.

Although very conservative, this homology search confirms that it is unlikely that the 2mEPSPS protein possesses any allergenic properties.

VI.E.2.2. Potential N-glycosylation sites

Potential N-glycosylation sites were determined using *in silico* search of the 2mEPSPS protein sequence for the presence of the consensus epitope Asn-Xaa-Ser/Thr (N-X~P-S/T), where Xaa = any amino acid except Pro (P), and Asn-Xaa-Cys (N-X-C) (Capt, 2008 a, Larsen *et al*., 1998).

Two potential N-glycosylation sites were identified on the amino acid sequence of the 2mEPSPS protein. However, the biological relevance of those potential N-glycosylations in eliciting allergenic response is not proven. The 2mEPSPS protein is not expected to be glycosylated, since chloroplastic proteins targeted directly to the chloroplast do not transit through the Endoplasmic Reticulum (ER) where glycosylation occurs in eukaryotes (Mousdale and Coggins, 1985, Pattison and Amtmann, 2008). In bacteria, protein glycosylation is rare (Sherlock *et al*., 2006).

Furthermore, in the specific case of event FG72, it has been shown that the 2mEPSPS protein is not glycosylated (see Section D.1.). Therefore, potential allergenicity triggered by the presence of N-glycosylation sites is a remote possibility.

VI.E.2.3. *In vitro* digestibility in human simulated gastric fluid

The 2mEPSPS protein was assayed for digestibility in SGF containing pepsin at pH 1.2 for incubation times from 0.5 to 60 minutes (Rouquié, 2006a).

The test protein was incubated at 37°C in SGF with pepsin at a final concentration of 10 units of pepsin per µg test protein, at pH 1.2, and samples were taken for analysis at time-points of 0, 0.5, 2, 5, 10, 20, 30 and 60 minutes. The resulting protein solution was analyzed for presence of the test proteins and potential stable protein fragments by SDS-PAGE followed by Coomassie blue staining. Appropriate controls included the test protein at pH 1.2 without pepsin and SGF without the test protein.

Coomassie blue staining analysis showed that the 2mEPSPS protein was very rapidly digested in pepsin at pH 1.2, within 30 seconds of incubation. No fragment bands were found to result from digestion of the 2mEPSPS protein.

In conclusion, the 2mEPSPS protein is very rapidly degraded in SGF. This minimizes the likelihood that this protein could survive in the human digestive tract and cause an allergic reaction.

VI.E.2.4. *In vitro* digestibility in human simulated intestinal fluid

The 2mEPSPS protein was further tested for stability in SIF with pancreatin at pH 7.5 for incubation times from 0.5 to 60 minutes, using a protocol adapted from the SGF assay (Rouquié, 2006b). A solution of the test protein was incubated with SIF, a porcine pancreatin solution at pH 7.5, at approximately 37°C. Then samples were analyzed at time-points of 0, 0.5, 2, 5, 10, 20, 30 and 60 minutes for the presence of the 2mEPSPS protein or potential stable protein fragments by western blot. The immunodetection was performed using a polyclonal antibody directed against the 2mEPSPS protein. Appropriate controls included 2mEPSPS protein in buffer without pancreatin and SIF without 2mEPSPS protein.

Western blot analysis showed that the 2mEPSPS protein band was not visible anymore at time 0 and all subsequent incubation times, indicating that the 2mEPSPS protein was degraded within a few seconds in the presence of pancreatin.

In conclusion, a complete digestion of the 2mEPSPS protein was observed within a few seconds of incubation with SIF, in presence of pancreatin, at pH 7.5.

Rapid degradation of the 2mEPSPS protein in the SGF and SIF indicates a minimal likelihood that the protein could survive and be absorbed through the gastrointestinal system. In case the protein survives in the stomach, 2mEPSPS would be rapidly degraded in the intestine.

VI.E.2.5. *In vitro* stability to heat

Highly purified (>99%) 2mEPSPS protein produced in *E. coli* (batch LEJ5837) was tested for structural stability at temperatures of 60, 75 or 90°C for periods of 10, 30 or 60 minutes. The protein was examined by SDS-PAGE followed by Coomassie blue staining or by western blot analysis (Rouquié, 2007). The immunodetection was performed using a polyclonal antibody directed against 2mEPSPS protein.

The Coomassie blue-stained SDS-PAGE showed no visible changes of the band intensity at 60°C and 75°C from 10 up to 60 minutes. After 30 minutes of incubation at 90°C, the band was visible with a lower intensity than other heated samples at 60°C and 75°C. After 60 minutes at 90°C, the band was still visible, with a marked decrease in intensity compared to all other samples, including the unheated sample.

The western blot analysis showed an unchanged intensity of the intact 2mEPSPS band after incubation at 60°C or 75°C for 10 up to 60 minutes. At 90°C, the intensity of the intact 2mEPSPS band was unchanged after 10 and 30 minutes, but was decreased after 60 minutes, in accordance with the results obtained by SDS-PAGE analysis after Coomassie blue staining.

In conclusion, the 2mEPSPS protein is partially heat-stable up to 90°C for 60 minutes.

VI.E.3. Homology search to known toxins

The overall amino acid sequence identity search was carried out by using BLASTP algorithm, which compared the complete amino acid sequence of the 2mEPSPS protein with all protein sequences present in the following large reference databases: Uniprot_Swissprot, Uniprot_TrEMBL, PDB, DAD and GenPept (Capt, 2008c). The scoring matrix used was BLOSUM62. The overconservative criterion for selecting similar proteins was a threshold E-value of 1.0. Matched sequence proteins were further examined for potential toxicity records in literature in order to assess their biological relevance.

The results showed no sequence identity of the 2mEPSPS protein with known toxins.

In conclusion, it is unlikely that the 2mEPSPS protein would exhibit any toxic properties.

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

A group of 5 female OF-1 mice were treated by oral gavage with the 2mEPSPS protein produced in *E. coli* (>99% purity) at a dose level of 2000 mg/kg body weight (Rouquié, 2006c). Another group of 5 female OF-1 mice were treated by oral gavage with bovine serum albumin at the same dose level as the negative control. All animals were observed for clinical signs daily for 15 days, with special attention given during the first 4 hours. Their body weights were measured weekly. At study termination, animals were subjected to a necropsy including a macroscopic examination and the spleen, liver, kidney and brain were weighed.

There were no mortalities, no clinical signs or treatment-related effects in female OF1 mice.

In conclusion, a single administration of the 2mEPSPS protein at 2000 mg/kg body weight *via* the oral route did not produce signs of systemic toxicity in the OF1 female mouse. The acute oral LD_{50} of 2mEPSPS was found to be greater than 2000 mg/kg body weight in mice.

These results taken together with the results of the homology search with known toxins indicate that it is unlikely that the 2mEPSPS protein would exhibit any toxic properties.

VI.F. Summary of the Food and Feed Safety Assessment of the HPPD Protein

VI.F.1. Familiarity to the protein

HPPD is ubiquitous in nature across all kingdoms: bacteria, fungi, plants and animals including mammals. For instance, HPPD amino acid sequences have been determined in bacteria such as *Streptomyces avermitilis* (Accession number Q53586), in fungi such as *Aspergillus fumigatus* (Accession number Q4WPV8), in plants such as *Arabidopsis thaliana* (Accession number P93836), and in animals such as *Caenorhabditis elegans* (Accession number Q22633), mouse

(*Mus musculus*, Accession number P49429), and human (*Homo sapiens*, Accession number P32754).

In particular, HPPD has been characterized in organisms present in human food, such as carrot (*Daucus carota*, Accession number O23920; Garcia *et al*., 1997), barley (*Hordeum vulgare* Accession number O48604; Falk *et al*., 2002), pork (*Sus scrofa*, Accession number Q02110; Endo, 1992)) and beef (*Bos Taurus*, Accession number Q5EA20; Harhay, 2005).

In conclusion, HPPD proteins are present in food from plant, fungal or animal origin, with good safety records. Therefore, HPPD proteins have a history of safe consumption.

VI.F.2. Potential allergenicity

VI.F.2.1. Homology search to known allergens

The overall amino acid sequence homology search was carried out by using FASTA algorithm, which compares the complete amino acid sequence of the HPPD W336 protein with all protein sequences present in the public allergen database AllergenOnline (www.allergenonline.com; release 9.2, 1386 sequences) (Capt, 2009a). The criterion indicating potential allergenicity was 35% identity over at least 80 consecutive amino acids with an allergenic protein.

In addition, an allergenic identity search (80-mer amino acid sequence homology) was performed to compare the query sequence subdivided into 80 amino acid blocks, with all known allergens present in the AllergenOnline database. The criterion indicating potential allergenicity was 35 % identity with an allergenic protein.

Furthermore, the amino acid sequence of the HPPD W336 protein, subdivided into 8 amino acid blocks, was compared with all known allergens present in the AllergenOnline allergen database (epitope search). The algorithm used was FindPatterns and the criterion indicating potential allergenicity was 100 % identity on a window of 8 amino acids with an allergenic protein.

The overall and 80-mer identity searches showed no relevant similarity between the HPPD W336 sequences and any known allergenic sequences from the allergen database. In addition, the epitope search showed no identity between all the blocks (8 amino acids) of the HPPD W336 protein and known allergens.

Although very conservative, this homology search confirms that it is unlikely that the HPPD W336 protein possesses any allergenic properties.

VI.F.2.2. Potential N-glycosylation sites

Potential N-glycosylation sites were determined using an *in silico* search of the HPPD W336 protein sequence for the presence of the consensus epitope Asn-Xaa-Ser/Thr (N-X~P-S/T), where Xaa = any amino acid except Pro (P), and Asn-Xaa-Cys (N-X-C) (Capt, 2009a).

No potential N-glycosylation sites were identified on the amino acid sequence of the HPPD W336 protein.

VI.F.2.3. *In vitro* digestibility in human simulated gastric fluid

The test protein was incubated at 37°C in SGF with pepsin at a final concentration of 10 units of pepsin per µg test protein, at pH 1.2, and samples were taken for analysis at time-points of 0, 0.5, 2, 5, 10, 20, 30 and 60 minutes (Rascle, 2009a). The resulting protein solution was analyzed for presence of the test proteins and potential stable protein fragments by SDS-PAGE followed by Coomassie blue staining or by western blot. The immunodetection was performed using a polyclonal antibody directed against HPPD W336 protein. Appropriate controls included the test protein at pH 1.2 without pepsin, SGF without the test protein, and a 10% loading control (1/10 dilution of the test protein). This 10% loading control was used to estimate the time to reach 90% digestion of the protein, *i.e.* the first sample time having less than 10% residual protein.

Both Coomassie blue staining and western blot analysis showed that the HPPD W336 protein was very rapidly digested in pepsin at pH 1.2, with more than 90% of the protein being digested in less than 30 seconds. No fragments were found as a result of the digestion of the HPPD W336 protein in SGF.

In conclusion, the HPPD W336 protein is very rapidly degraded in simulated gastric fluid. This minimizes the likelihood that this protein could survive in the human digestive tract and cause an allergic reaction.

VI.F.2.4. *In vitro* digestibility in human simulated intestinal fluid

The HPPD W336 protein was further tested for stability in SIF with pancreatin at pH 7.5 for incubation times from 0.5 to 60 minutes, using a protocol adapted from the SGF assay (Rascle, 2009b). A solution of the test protein was incubated with SIF, a porcine pancreatin solution at pH 7.5, at approximately 37°C. Samples were analyzed at time-points of 0, 0.5, 2, 5, 10, 20, 30 and 60 minutes for the presence of the HPPD W336 protein or potential stable protein fragments by SDS-PAGE coupled with Coomassie blue staining and western blot analyses. The immunodetection was performed using a polyclonal antibody directed against the HPPD W336 protein. Appropriate controls included HPPD W336 protein in buffer without pancreatin, the corresponding 10% loading control (to verify the sensitivity of the detection procedure) and SIF without HPPD W336 protein.

Both Coomassie blue staining and western blot analysis showed that the HPPD W336 protein band was not visible anymore at time 0 and all subsequent incubation times, indicating that more than 90% of the HPPD W336 protein was degraded within a few seconds in presence of pancreatin.

In conclusion, a complete digestion of the HPPD W336 protein was observed within a few seconds of incubation with SIF, in presence of pancreatin, at pH 7.5.

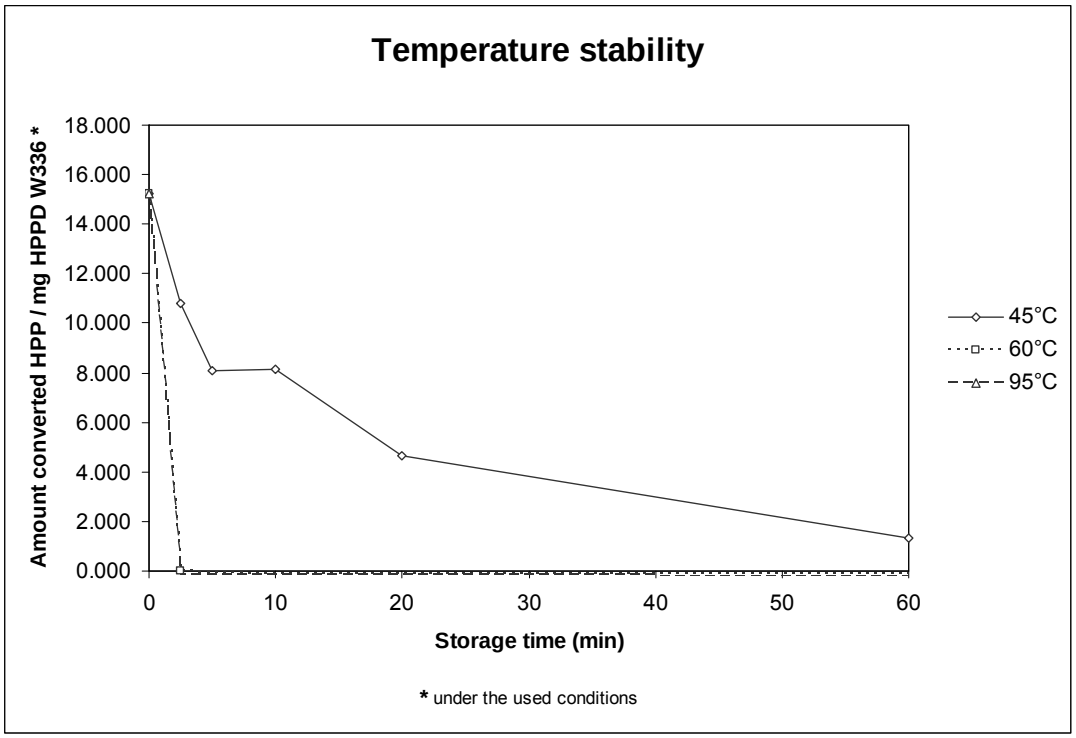
Rapid degradation of the HPPD W336 protein in the SGF and SIF indicates a minimal likelihood that the protein could survive and be absorbed through the gastrointestinal system. In case the protein survives in the stomach, HPPD W336 would be rapidly degraded in the intestine.

VI.F.2.5. *In vitro* stability to heat

The effect of heat on the enzyme activity and structural stability of the protein was investigated. For enzymatic activity, an absorbance assay was developed which monitored the production of homogentisate and CO_2 from 4-HPP and O_2 catalyzed by HPPD (Habex, 2009).

To assess the temperature stability of the HPPD W336 protein, the protein was incubated at 45°C, 60°C and 95°C for 2.5, 5, 10, 20 and 60 minutes (Habex, 2009). Subsequently the activity of the protein was assessed under standard conditions (room temperature). The activity drops below 50% after the protein was incubated at 45°C for 20 minutes. At more elevated temperatures (60°C and 95°C) HPPD activity is abolished after 2.5 minutes.

Figure 7. The effect of temperature on the activity of the HPPD W336 enzyme



Highly purified HPPD W336 protein produced in *E. coli* (96% purity) was also tested for structural stability at temperatures of 60, 75 or 90°C for periods of 10, 30 or 60 minutes (Rascle, 2009c). The protein was examined by SDS-PAGE followed by Coomassie blue staining and by western blot analysis. The immunodetection was performed using a polyclonal antibody directed against HPPD W336 protein. A 10% loading control was included in gels, to verify the sensitivity of the staining procedures.

The Coomassie blue-stained SDS-PAGE showed no significant changes in the HPPD W336 protein after heat treatment at 60, 75 or 90°C from 10 to 60 minutes, with intensities similar to the unheated sample. Similar results were obtained with western blot analyses.

These findings illustrate that while the HPPD W336 protein may retain its structural stability, its enzymatic activity decreases rapidly at 45°C and is completely lost at higher temperatures (60°C and above) after 2.5 minutes.

VI.F.3. Homology search to known toxins

An overall amino acid sequence identity search was carried out by using BLASTP algorithm, which compared the complete amino acid sequence of the HPPD W336 protein with all protein sequences present in the following large reference databases: Uniprot_Swissprot, Uniprot_TrEMBL, PDB, DAD and GenPept (Capt, 2009b). The scoring matrix used was BLOSUM62 (block substitution matrix at ≥62% alignment). The overconservative criterion for selecting similar proteins was a threshold E-value of 0.1. Matched sequence proteins were further examined for potential toxicity records in the scientific literature in order to assess their biological relevance.

As expected, the query sequence matched with the HPPD proteins (also called HPPDase; MelA; enzyme classification E.C. 1.13.11.27) from *P. fluorescens* and other bacterial origins. No records were found on the potential toxicity associated with these proteins.

An identity of 54% was observed with the VLLY protein (Uniprot_SwissProt entry: O06695) from *Vibrio vulnificus*, a pathogenic bacterium present in sea waters and able to infect humans who consume seafood (Chang *et al*., 1997). Similarities with lower percentages of identity (49-50%) were also found between HPPD W336 and LLY, also called legiolysin, from strains of *Legionella pneumophila*. Both VLLY protein and LLY are suspected to be hemolysins, and are also annotated to belong to the HPPD family.

Because VLLY and LLY proteins are also annotated as HPPD proteins, the hypothesis was raised that the similarity between these proteins and the HPPD W336 protein was caused by the presence of HPPD domains on the VLLY and LLY proteins. To test this hypothesis, a multiple alignment of the HPPD W336, VLLY, LLY and 27 other HPPD sequences extracted from the Uniprot_Swissprot database from bacteria, plants, vertebrates, invertebrates and fungi was performed. Refined analysis of this multiple alignment showed that similarities between HPPD W336 and either VLLY or LLY proteins correspond to the domains conserved among all HPPD proteins.

These observations support the hypothesis that the VLLY and LLY proteins are HPPDs and therefore, share the typical HPPD structure with HPPD W336. This is corroborated by several authors who demonstrated the HPPD activity of the LLY protein (Wintermeyer *et al*., 1994; Steinert *et al*., 2001). In addition, although VLLY or LLY protein expression was shown to be necessary for the hemolytic activity of bacteria, the direct hemolytic activity of these proteins was not observed (Wintermeyer *et al.*, 1994; Chang *et al.*, 1997; Steinert *et al.*, 2001).

In conclusion, the HPPD W336 is similar to other HPPD proteins from various origins. The list includes the HPPD-like hemolysin (e.g. from *Vibrio vulnificus*), which is described as a bacterial toxin. However, a refined bioinformatics analysis demonstrated that this specific homology was unlikely to be relevant. This match is due to the domains of proteins that possess the HPPD activity and that are conserved between all the HPPD proteins.

Since all other HPPD proteins from various organisms, although sharing the typical HPPD conserved domains, display no toxic properties, and since no limitation cut-off criteria have been determined for defining relevant homologies to toxins (Delaney *et al*., 2008), this homology is highly unlikely to be biologically relevant.

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

A group of 5 female OF1 mice were administered a single dose of purified *E.coli* produced HPPD W336 protein (97% purity) by oral gavage at the dose level of 2000 mg/kg body weight (Rascle, 2009d). A similarly constituted group of 5 female mice received bovine serum albumin (BSA) at the same dose level and acted as a negative control. Both proteins were administered in two doses of 1000 mg/kg body weight each administered within a 4 hours period on the day of treatment. All animals were observed for clinical signs daily for fifteen days. Their body weights were measured weekly. At termination of the study period, all animals were subjected to a necropsy including a macroscopic examination, and the spleen, liver, kidney and brain were weighed. Microscopic examination of the spleen was performed.

There were no mortalities, no clinical signs or treatment-related effects on body weight, body weight gain, organ weights, gross and microscopic examinations.

In conclusion, an acute oral dose of 2000 mg/kg body weight of the HPPD W336 protein did not induce any evidence of systemic toxicity in the OF1 female mouse.

VII. AGRONOMIC AND PHENOTYPIC EVALUATION

VII.A. History of field activities

Event FG72 has been field tested in adapted growing regions of the United States and Canada, and in winter nurseries (Argentina, Brazil, Chile and Puerto Rico). The field activities were managed by BCS from 2001 to 2003 and by MSTech beginning in 2007. Field activities in Canada are managed by BCS. Table 10 presents a summary of the field trials and associated USDA notifications (see Appendix 1 for field trial termination reports). Table 11 lists the field trials and associated CFIA permits granted for Canada. The activities in Argentina are summarized in Table 12.

Table 10. Summary of field activities under USDA notifications for event FG72

USDA Notification #	Planting / Harvest Dates	Number of Locations	Type of Trial	Locations
01-268-11n	Dec 2001 / April 2002	1	Herbicide efficacy, Progeny advanced	PR
02-071-03n	June 2002 / Nov 2002	1	Herbicide efficacy, Progeny advanced	IN
02-071-03n	July 2002 / November 2002	1	Herbicide efficacy, Progeny advanced	PR
02-274-09n	Jan 2003 / May 2003	1	Herbicide efficacy, Seed increase	PR
03-080-05n	May 2003 / Oct 2003	7	Herbicide efficacy, Yield, Breeding	IL, IA, NE
07-065-121n	May 2007 / Sept-Nov 2007	1	Breeding, Seed increase	IA
07-211-105n	Dec 2007 / May 2008	1	Breeding	FL
08-057-116n	May 2008 / Oct 2008	10	Registration studies and Breeding	AR, IA, IL, IN, MI, MN, MO
08-219-102n	Oct 2008/ Feb 2009	1	Breeding, Seed increase	FL

Table 11. Summary of field activities under CFIA permits for event FG72

CFIA permit #	Year	Trial # (Type)	Location in Canada
08-MTS1-3110SOY01-0864-ON101-01	2008	HD08NARKBP (Efficacy & Tolerance)	Rockwood, Ontario
2009-MST1-387-SOY01-0864-ON016-01	2009	HD09NARKBN (Efficacy & Tolerance)	Breslau, Ontario
		HD09NARKBO (Efficacy & Tolerance)	
2009-MST1-387-SOY01-0864-ON101-01	2009	HD08NARKBP (Efficacy & Tolerance)	Rockwood, Ontario
		HD09NARKBN (Efficacy & Tolerance)	
		HD09NARKBO (Efficacy & Tolerance)	
		RAISP006 (Residue Analysis on RAC's)	
		RAISP008 (Residue Analysis on RAC's)	
		RAISP010 (Residue Analysis on RAC's)	

Table 12. Summary of field activities under CONABIA permits for event FG72

CONABIA Permit #	Planting / Harvest Dates	Number of Locations	Type of Trial	Location in Argentina (Facility)
800-9541/01	Dec 2001 / April 2002	1	Basic observations, progeny advanced	Balcarce (Agrar del Sur)
247377/02	16 Dec 2002 / 3 April 2003	1	Basic observations, progeny advanced	Chacabuco (Don Mario)
182500/03	31 Dec 2003/ 25 April 2004			Chacabuco (Don Mario)
182500/03	12 Jan 2004/ 26 April 2004			Chacabuco (Don Mario)
295033/07	19 Dec 2007 / 5 April 2008		Seed increase and breeding	Chacabuco (Don Mario)
107155/08	10 Dec 2008 / 27 March 2009		Seed increase and breeding	Chacabuco (Don Mario)

VII.B. Agronomic and phenotypic evaluation

Agronomic evaluations of the event FG72 were conducted in field tests in 2003 and 2008. Evaluations included key agronomic parameters to assess the growth habit and phenotype of the transformed lines, their reactions to biotic and abiotic stressors in their respective environments, and analyze soybean meal and oil factors. These parameters were designed to evaluate event FG72 in soybean plants to ensure commercial herbicide tolerance and agronomic performance. Other field activities, such as rating herbicide tolerance and efficacy, planting seed increases and breeding allowed for the material to be assessed for stability and performance of the introduced trait and the agronomic characteristics of event FG72.

Event FG72 was created by transformation of the soybean variety Jack (maturity group II) to express the 2mEPSPS protein which will convey tolerance to the herbicide glyphosate (GLY) and the HPPD W336 protein which will impart tolerance to the herbicide IFT. Event FG72 was selected based on demonstrated tolerance to the herbicide glyphosate, the herbicide IFT and agronomic performance.

Glyphosate is widely used in herbicide-tolerant soybean and other agricultural production systems. IFT herbicide offers an alternative weed control option for the soybean grower. It controls weeds via a new herbicide mode of action for soybeans that is effective against many of the herbicide resistant weeds currently found in soybean fields. IFT has the flexibility to be applied pre-plant, pre-emergence, or post emergence to event FG72 soybeans.

Event FG72 was evaluated by comparison to the non-transgenic counterpart Jack in different growing regions of the mid-western United States (Table 10). Agronomic performance field studies were managed in a manner representative of normal agricultural practices, including; conventional herbicide applications, both pre- and post- planting. In addition, event FG72 was evaluated for herbicide tolerance to GLY and IFT. Thus, comparisons of agronomic properties and performance of event FG72 were made under conventional herbicide and GLY and/or IFT herbicide regimens. Appendix 2.E. describes the materials and methods for the agronomics studies.

Event FG72 was evaluated at the T_8 generation with Jack for equivalence testing (Table 13) to assess seed characteristics that may contribute to weediness potential of a plant such as increased seed dormancy, disease susceptibility or pest preference. No changes in fitness characteristics (reproduction, disease resistance, fecundity, seed dispersal, dormancy, and persistence) were found that could contribute to increased weediness potential of event FG72. Comparison of pollen from FG72 and Jack found no difference in viability or germination (Haas, 2009).

Morphological parameters necessary for the filing of a Plant Variety Protection (PVP) certificate were measured. These parameters are described in the objective description of the variety for soybean and include plant and seed descriptive characteristics, oil profile, and seed protein content among others. In all these parameters, event FG72 and Jack were found to be similar.

Table 13. Summary of performance characteristics evaluated for event FG72 – US (2008)

Type of study	Parameters analyzed	Comparator	Findings
Self and cross pollination	Segregation analysis for herbicide tolerance	Jack	No change in self or cross fertility
Plant phenotype comparison using PVP [a] standards	Flower color, pubescence color, pod color, seed coat, hilum color, canopy architecture, leaf shape, growth habit	Jack, Stine® 2686-6, Stine 2788, Stine 3000-0	FG72 was identical to Jack, but some differences compared to the other varieties
Seed phenotype comparison using PVP [a] standards	Seed coat color and luster, hilum color, seed coat mottling Shape class defined by L/W, L/T and T/W ratios	Jack, Stine 2686-6, Stine 2788, Stine 3000-0	FG72 was identical to Jack, but some differences compared to the other varieties
Comparison of grain characters using PVP [a] standards	Fatty Acid profile Total Oil content Protein content	Jack, Stine 2686-6, Stine 2788, Stine 3000-0	No change in grain qualities
Field performance	Emergence, stand, vigor, height, yield	Jack, Stine 2686-6, Stine 2788, Stine 3000-0	FG72 and the other varieties are shorter at maturity than Jack. Plant stand and yield were reduced in FG72.
Reproduction	Pollen viability, date of emergence, date of 50% flowering, date of maturity	Jack	No change in reproductive potential
Disease resistance	Severity rating for naturally occurring pathogens	Jack	No change in susceptibility
Fecundity	100 seed weight	Jack	No change
Seed dispersal	Pod shattering, lodging rating of mature plants	Jack	FG72 has less lodging than Jack
Dormancy	Germination ,survival of imbibed seed	Jack	No difference
Persistence	Census of volunteers in subsequent season	Jack	No difference observed

[a] Characters described by the Plant Variety Protection office of the USDA for Soybean

VII.C. Agronomic performance of event FG72

VII.C.1. Agronomic evaluation of event FG72 (2003 data)

VII.C.1.1. Yield evaluation of event FG72 under conventional weed control practices

After field trial evaluations performed in 2003 in the US, BCS and MSTech selected event FG72 as the event best suited for potential commercial development. The planting seed was derived from homozygous lines in the T_4 generation. The evaluation included agronomic performance under conventional weed control practices and tolerance evaluation to GLY and IFT herbicides. As a result, event FG72 was selected for tolerance to both herbicides and acceptable agronomic characteristics.

For the 2003 yield data summarized below (Table 14), fields were planted and managed using conventional weed control at nine locations. There were six sites, three of which had an early and a late planting. All trial locations were grown with conventional weed control (commercial rate of imazethapyr, 196 g ai/ha). Data provided are the mean of three replicated plots at each location.

The yield of event FG72 and the parent line Jack did not differ at any of the 9 locations. Event FG72 had a very consistent performance across locations when compared to all entries receiving the conventional herbicide treatment. The yield of event FG72 follows closely the mean yield of all the entries (location mean) at each of the nine locations (Figure 8).

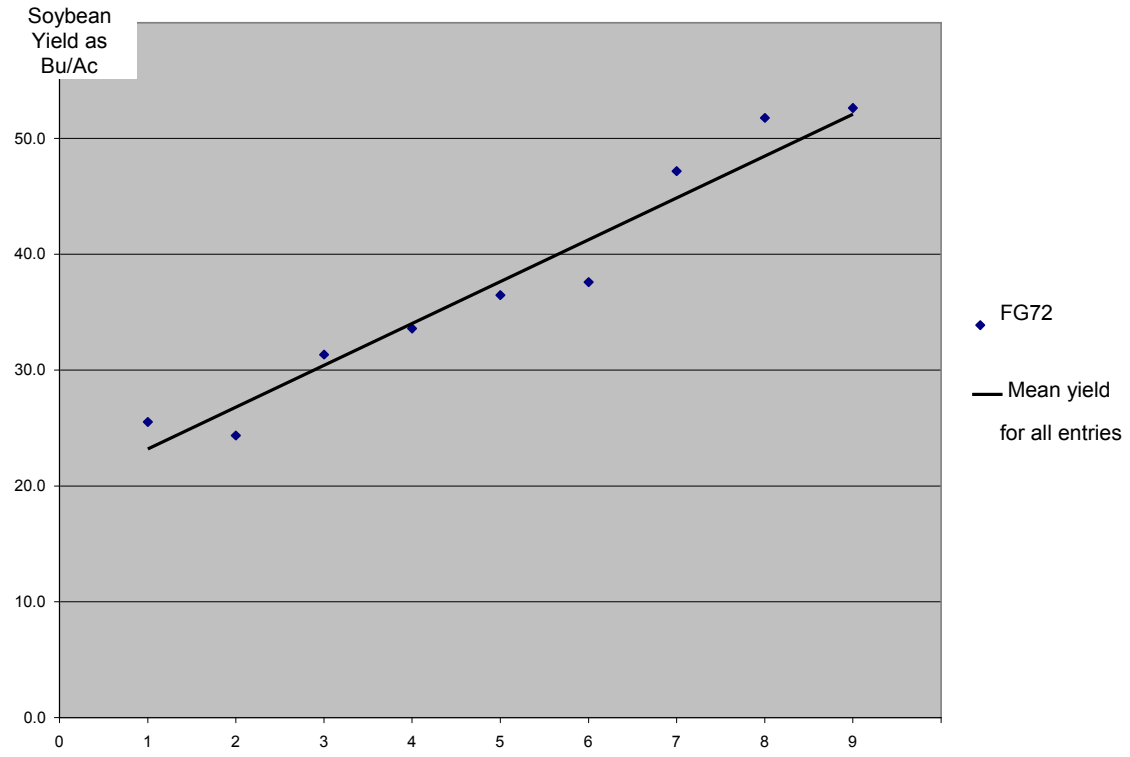
Table 14. Yield means for FG72 -US (2003)

Location	County/State	Loc #[a]	Event FG72[b]	Jack[b]	Location Mean[c]
Minburn (Late)	Dallas/IA	1	25.5	24.0	25.1
Minburn (Early)	Dallas/IA	2	24.4	26.2	27.8
Winterset (Late)	Madison/IA	3	31.4	32.8	33.5
Winterset (Early)	Madison/IA	4	33.6	36.8	37.0
Linden (Late)	Dallas/IA	5	36.5	38.7	39.1
Linden (Early)	Dallas/IA	6	37.6	42.3	40.7
West IA	Carroll/IA	7	47.2	48.8	47.5
South East IA	Des Moines/IA	8	51.8	54.4	52.2
Marshalltown	Marshall/IA	9	52.6	48.3	53.4

[a] Loc #; location # corresponds to x axis of Figure 8.
[b] Yield is reported as bu/ac
[c] Location mean is the average yield of all the entries in the trial, including other conventional soybean varieties.

Figure 8. Yield of event FG72 – Event FG72 and location means

Yield data is ranked by location mean, and best fit regression line for location mean yield of all entries (individual data points not shown).



VII.C.1.2. Intended herbicide tolerance evaluations of event FG72

Field tests at three locations (Champaign, IL; Seymour, IL; and Adel, IA) were designed to evaluate tolerance to the intended herbicides; GLY and IFT in 13 different herbicide treatments. Event FG72 performed well as illustrated by the data in Table 15 which summarizes the mean yield data (bu/ac) from the three locations with three replicates at each location. The conventional soybean variety, Jack, showed extreme crop injury when sprayed with the intended herbicides for the event, GLY, IFT, and IFT + GLY, which resulted in a substantial yield loss. The crop injury values for FG72 were recorded as 0.7% in the GLY treatment and 5.7% in the IFT+GLY treatment. Crop injury is a standard weed science rating, where 0% indicates no injury and a 100% indicates complete plant death.

Table 15. Yield of event FG72 and Jack for selected herbicide treatments

	Conventional herbicide	GLY	IFT	GLY+ IFT	IFT Pre
Jack	47.6	0.5	18.5	0.5	3.2
FG72	43.1	34.1	38.0	30.7	37.5

Standard error for comparison = 3.4
GLY; glyphosate at 2800grams of ai/Ha
IFT; isoxaflutole at 210 grams ai/Ha
GLY+IFT; tank mix of the same herbicide amounts
IFT Pre; IFT applied pre-emergence at 315 grams ai/Ha

In the 2003 field season, FG72 soybean demonstrated agronomic performance equivalent to an appropriate comparator, the parent line, Jack, and was tolerant to more than twice the anticipated commercial application rates of both glyphosate and IFT herbicides.

VII.C.2. Agronomic evaluation of event FG72 (2008 data)

VII.C.2.1. Agronomic performance and yield evaluation of event FG72 under conventional weed control practices

Field trials were conducted in the states of Iowa, Illinois, Indiana and Missouri, which are typical soybean growing regions of the Midwestern United States (Table 16). The plants in this study were grown under conditions typical of production practices for Group II maturity soybeans. Plants were observed through out the season, harvested at maturity and samples were reserved for the analysis of nutritional composition.

The trial design was a RCB design (Kowite, 2009a). Each trial contained three replicate plots of Jack soybean (Regimen A), three replicate plots of event FG72 soybeans (Regimen B), and three replicate plots of sprayed (IFT + GLY) event FG72 soybeans (Regimen C). Three non-replicated plots of commercial conventional soybean lines were also grown at each trial location. The non-replicated commercial soybean plots are not included in the statistical analysis of the quantitative agronomic data. These lines were Stine® 2686-6 (Regimen D), Stine® 2788 (Regimen E) and Stine® 3000-0 (Regimen F). Thus there were a total of 12 plots (9 randomized) established at each field trial site. The plot sizes were 15 x 20 feet, with 6 rows per plot and 30 inch row spacing.

The entire trial site received a conventional, pre-emergence soil herbicide application of pendimethalin (1060 gm ai /Ha) with the exception of trial #05, which was treated with metolachlor (1880 gm ai/Ha). Regimen C represents the intended weed control practice in which event FG72 plots were sprayed with IFT at a target rate of 70 grams ai/Ha and GLY at a target rate of 1060 grams ai/Ha. Herbicide applications were made to the Regimen C plants as a foliar spray at about the V4-V5 plant growth stage. The trial treatments are summarized in Table 17.

Table 16. Trial site location for the equivalence field tests

Trial Number	County	State	Sowing date	Seedling emergence	Harvest date
01	Cherokee	IA	May 17	May 30	November 3
02	Hardin	IA	May 20	June 1	November 2
03	Greene	IA	May 19	May 30	October 10
04	Dallas	IA	May 21	June 3	October 3
05	Dallas	IA	May 8	May 18	October 3
06	Madison	IA	May 20	June 1	October 6
07	Clinton	MO	May 29	June 5	October 5
08	Vermillion	IL	June18	June 24	October 22
09	Tipton	IN	May 28	June 6	September 28
10	Des Moines	IA	May 22	June 4	October 4

Table 17. Treatments for the 2008 season field studies

Regimen	Designation	rDNA status	Soil herbicide treatment [a]	Foliar herbicide treatment [b]	Reps
A	Jack	Non-transgenic	Conventional	-	3
B	FG72	Transgenic	Conventional	-	3
C	FG72	Transgenic	Conventional	GLY + IFT	3
D	Stine 2686-6	Non-transgenic	Conventional	-	1
E	Stine 2788	Non-transgenic	Conventional	-	1
F	Stine 3000-0	Non-transgenic	Conventional	-	1

[a] The entire trial site received a conventional, pre-emergence soil herbicide application of pendimethalin (1060 gm ai /Ha) with the exception of trial #05, which was treated with metolachlor (1880 gm ai/Ha).

[b] In one location (Trial #09), a foliar application of imazethapyr (68.6 gm ai/Ha) was made for Regimen A, B, D, E and F at the same time as the Regimen C application.

Agronomic observations were made in the early, mid and late season (Table 18). To evaluate some of the parameters important for soybean yield, a census of all the plants in two 10 ft sections of each plot were made. The plant density (parameter; stand count) was higher for the Jack and the conventional line plots than in the event FG72 plots. The early stand count difference may be the result of seed lot quality, as the FG72 planting seed was produced at the counter season nursery in South America, while the seed of the conventional lines were produced in the US during the normal production season. However, the number of days to achieve 50% emergence and the plant vigor ratings were the same, indicating that the seed lots were comparable for these performance parameters. In late season stand counts, Jack and the

conventional lines remained different from event FG72. Plot yields of FG72 event plants were also lower than those of Jack, perhaps a result of the lower plant density of the FG72 event plots. The yield for the conventional lines was higher than for Jack; a demonstration of the advancement in yield potential to be found in the current commercial varieties. Agronomic raw data for all 10 locations of the 2008 season is provided in Appendix 4.

Table 18. Summary of agronomic performance for event FG72 and Jack

		Regimen								
		Jack			FG72			FG72 Treated		
Agronomic characteristic	**Unit[a]**	**MEAN**	**±**	**STD**	**MEAN**	**±**	**STD**	**MEAN**	**±**	**STD**
Early Season										
Emergence (50% of plants)	N of days	9.7	±	2.4	10.0	±	2.3	10.0	±	2.4
Stand count (20 ft section of row in each plot)	N of plants	111.1	±	21.4	82.1	±	21.7	81.2	±	20.6
Plant vigor	rating 1-9	8.1	±	0.6	7.8	±	0.5	7.8	±	0.5
Plant health at V4-5	rating 0-5	4.8	±	0.5	4.6	±	0.7	3.7	±	0.7
Mid Season										
Flowering date (50% of plants)	N of days	46.5	±	5.3	46.7	±	5.2	47.4	±	5.6
Plant health at R1	rating 0-5	4.9	±	0.3	4.6	±	0.8	3.6	±	0.8
Late Season at Crop Maturity										
Days to maturity (95% of pods turning color)	N of days #	127.7	±	7.2	127.7	±	7.0	128.8	±	7.1
Yield	bu/ac	53.18	±	8.96	46.41	±	11.00	45.24	±	10.79
Plant height (mean from 12 points in each plot)	inch	42.03	±	5.94	37.20	±	6.08	32.52	±	5.47
Plant lodging	rating 1-9	4.4	±	1.7	5.1	±	1.6	6.1	±	1.2
Final Stand count (20 ft section of row in each plot)	N of plants	93.0	±	19.1	73.5	±	19.0	73.6	±	21.4
Pod shattering	rating 1-9	8.3	±	0.5	8.0	±	0.6	7.9	±	0.6
Plant health at maturity	rating 0-5	5.0	±	0.0	5.0	±	0.0	5.0	±	0.0

Mean values and standard deviations are over all trial sites.
[a] N; Number of days from the date of planting or number of plants
Plant vigor rating; 1 is no emergence and 9 is complete emergence
Plant health rating, 0 is death, 5 is no injury
Plant lodging score; 1 is plants are flat on the ground, 5 representing that plant stems are 45 degrees to the ground, and 9 representing that all plant stems are straight up and down with minimal branching. Lodging scores were taken just prior to harvest.
Pod shattering score; 1 is all pods are open, 5 being 50% of pods are open, and 9 representing no pods are open. Shattering scores were taken at maturity and approximately 2 weeks after maturity (on border rows).

Field agronomists made plant health ratings at three growth stages; V4-5, R1 and full maturity. The first evaluation was shortly after the intended herbicide application. In Regimen C, the health ratings reflect the bleaching characteristic of the IFT herbicidal action. By the final plant health evaluation, the plants in Regimen C had the same score as Regimen A (Jack) and B (FG72). In ratings by the agronomic staff, Regimen C (FG72 treated) plants received a health rating of 3-4 (moderate injury) at the V4-5 and R1 plant growth stages. Plants in the other Regimen were rated as 4.6-4.8 (rating of 5 indicates no injury). At the final plant health rating, all plots received the same rating of 5 (no injury).

Independent ratings were made by experienced weed scientists at 8 of the 10 locations using a standard rating for herbicide-related crop injury in which 0% is no injury and 100% is complete plant death within the experimental plot. The target evaluation intervals were 6-10 days, 11-18 days and 25-44 days following the foliar herbicide treatment. The 2008 season was one of exceptional rainfall and crop injury in the event FG72 plants following the intended herbicide was more obvious than observed in previous seasons. The crop damage ratings ranged from 10% to 30% in Regimen C, where the event FG72 plots were treated with IFT + GLY. Unlike the experience in the 2003 season, event FG72 plants consistently showed crop injury following foliar application of the intended herbicides.

The field evaluations included monitoring of the fitness characters (reproduction, disease resistance, fecundity, seed dispersal, dormancy, persistence) that could contribute to increased weediness potential in soybean. For the reproductive characteristics; days to emergence, days to 50% flowering and days to 95% pods maturing, the event FG72 and Jack plants were not different. The Jack plots were higher in seed production than those plots containing event FG72. Reaction to natural infestations of plant diseases and insect pests were monitored, and no differences were noted (see Section VII.D.). Although event FG72 produced less ultimate yield than Jack, no difference in fecundity (100 seed weight) was found. The assessment of seed dispersal parameters (pod shattering and plant lodging) found that event FG72 and Jack have the same pod shattering score, but found event FG72 to be less prone to lodging. Evaluation of seed harvested from the 10 locations found no concerns raised by germination or dormancy testing (see Section VII.F.). Volunteer monitoring found no evidence of persistence in the following season.

In the 2008 field season, a 10 location study was designed to compare the agronomic performance of event FG72 soybean to the parent line, Jack. Using a RCB design, event FG72 soybean was grown in replicated plots with either conventional weed control or with the intended herbicides, GLY and IFT. The final yield of event FG72 soybean, regardless of the weed control treatment, was less than the yield of Jack. Crop injury (bleaching in 10-30% of the crop area) was reported for event FG72 plots up to six weeks following foliar application of the GLY and IFT herbicides. However, at time of maturity, “no injury” plant health ratings were assigned to all plots.

VII.C.2.2. Objective variety description

USDA Plant Variety Protection Office has published a guidance for the description of soybean varieties (Objective Description of Variety, Soybean, *Glycine max* (L.) Merr.). It includes the

following qualitative traits of flower color, pubescence color, pod color, hilum color, canopy architecture, leaf shape and growth habit, which were recorded for the plots from ten field trials in 2008 (Table 19). For these characters, observations of the variety Jack were in agreement with the variety description and event FG72 was not different in any of these traits (Nickell *et al*., 1990).

Table 19. General plant descriptors comparison for event FG72 and Jack

Morphology characters	Jack	FG72
Flower color	White	White
Pubescence color	Gray	Gray
Pod color	Brown	Brown
Seed coat	Dull Yellow	Dull Yellow
Hilum color	Yellow	Yellow
Canopy architecture	Medium	Medium
Leaf shape	Oval	Oval
Growth habit	Indeterminate	Indeterminate

VII.C.2.3. Seed characteristics

Observations of the phenotypic characteristics of soybean event FG72 and the parent variety Jack included seed characteristics that are commonly used to describe soybean varieties (Objective Description of Variety, Soybean, *Glycine max* (L.) Merr.). Following the convention described by the USDA, National Genetic Resources Program, the following observations for event FG72 and Jack seed were made: seed size hilum color, mottling score, seed coat color, seed coat luster, seed quality and seed shape. The measurement of seed size was made using four independent samples of 100 seed each from each of the ten locations. The four independent samples were also examined for other seed characteristics. In all locations and for all characters, Jack and FG72 are identical (Table 20).

In addition to the morphological characteristics, the USDA Plant Variety Protection Office requests information concerning variety maturity, height, fatty acid profile and total oil and protein content when applying for registration of a new soybean variety. The information compiled in Table 21 follows the template provided by the USDA Plant Variety Protection (PVP) Office. The comparison demonstrates that Jack and event FG72 are similar in all characteristics, with the exception of height. There have been eight generations of selection of lines and seed increase since the transformation of Jack to create event FG72. Modern soybean varieties are shorter than the older variety Jack, and we can see evidence of the breeder's eye at work in the 12 cm difference in plant height.

FG72 soybean is similar to Jack for all parameters considered by Objective Variety Description, with the exception of plant height. The only distinction between event FG72 and Jack is the addition of the *2mepsps* and *hppd* genes to confer double herbicide tolerance to GLY and IFT.

Table 20. Seed phenotypic characteristics

Seed Characteristics	FG72 (mean ± std)[a]	Jack (mean ± std)[a]
Seed size [b]	13.1 ± 1.4	12.3 ± 1.0
Hilum color	Yellow	Yellow
Mottling [c]	1.9% ± 1.6	1.5% ±1.7
Seed coat color	Yellow	Yellow
Seed coat luster	Dull	Dull
Seed quality[d]	7.3 ± 0.9	7.0 ± 0.9
Seed shape[e]	Spherical	Spherical
L/W	1.1	1.2
L/T	1.0	1.1
T/W	1.1	1.1

[a] Mean and standard deviation for the replicate measurements from 10 locations (2008).
[b] Seed size was recorded as the weight in grams of 100 seed.
[c] Mottling is the number of mottled seeds in each 100 seed reported as %
[d] Seed quality rating is a numerical score of 1-9 based on visual appearance of the seed (9 = best quality, 1 = worst quality)
[e] The seed shape is measured as the length and width of the seed with the hilum facing up (L and W) and the width with the hilum on the side (T). A seed shape is scored as spherical when the L/W, L/T and T/W ratio is less than or equal to 1.2.

Table 21. Paired comparison of variety characteristics

Paired comparison	# Days to maturity	Plant height in cm	% Linoleic acid (18:2)	% Oleic acid (18:1)	% Linolenic acid (18:3)	% Palmitic acid (16:0)	Total oil % dw	Crude protein % dw
Jack	128	106	54%	22%	8%	10%	19%	38%
FG72	128	94	54%	24%	8%	9%	19%	38%

VII.D. Biotic and abiotic stress characteristics

Insect pests of soybean encountered at the trial sites included aphid and bean leaf beetle. The presence of beneficial or non-pest insects, such as lady beetles and leafhoppers was noted at some of the sites. In all cases, no preference was observed by the insects for any of the soybean varieties or herbicide regimen (Kowite, 2009b).

Natural infestations of common soybean plant diseases were observed at most of the sites (Table 22). In most cases, the symptoms were uniform in all the plots. In locations where the incidence (% of plants with symptoms) and severity (% of plant tissue showing symptoms) could be scored, ratings by plot were recorded (Kowite, 2009b).

Table 22. Plant diseases and syndromes observed

Phytopathology observed	Causal agent
Downy mildew	*Peronospora manshurica*
Bacterial blight	*Pseudomonas syringae pv. Glycinea*
Cercosopora leaf blight	*Cercospora kikuchii*
Brown spot	*Septoria glycines*
Frogeye leafspot	*Cercospora sojina*
Powdery mildew	*Microsphaera diffusa*
Top die back	cause unknown, syndrome described by Iowa State University as plants dying from the top down
Sudden death syndrome	*Fusarium virguliforme*

The variety registration of Jack, claims resistance to soybean cyst nematode (SCN) (Races 3 and 4) *(Heterodera glycines* Ichinohe) and susceptibility to *Phytophthora* rot (Races 1, 4, and 7) caused by *Phytophthora megasperma* (Drechs.) f. sp. *glycinea* T. Kuan & D.C. Erwin. Neither of these phytopathologies was observed consistently in the trials, so it was not possible to confirm expression of these variety traits. At the Perry location, one plant of event FG72 was presumed to have died of *Phytophthora* root rot.

No insect susceptibility or disease susceptibility or resistance differences were observed between the event FG72 soybean plants and Jack.

There was no evidence of a change in characteristics that would enhance survival of event FG72 soybean plants when compared to Jack.

VII.E. Equivalence between event FG72 and commercial varieties

A comparison of event FG72 soybean and the commercial varieties were used to evaluate any potential agronomic effect of the transformation on the plant parameters of event FG72. The early plant density and plant vigor ratings were lower in event FG72 plots than in the commercial variety plots (Table 23). These early season differences may again be the result of

seed lot quality (see Section VII.C.2.1.). In the late season stand counts, event FG72 and the commercial varieties remained different. Plot yields of event FG72 plants were also lower than those of the commercial varieties, perhaps a result of the lower plant density of the event FG72 plots. Agronomic raw data for all 10 locations of the 2008 season is provided in Appendix 4.

Plant health ratings showed similar trends, as the at the three growth stages; V4-5, R1 and full maturity. At the final plant health rating, all the plots received the same rating of 5.

Table 23. Summary of agronomic performance for FG72 and commercial varieties

Agronomic characteristic	Unit[a]	FG72 unsprayed			Commercial varieties		
		MEAN	±	STD	MEAN	±	STD
Early Season							
Stand count (20 ft section of row in each plot)	N of plants	82.1	±	21.7	113.0	±	22.4
Plant vigor	rating 1-9	7.8	±	0.5	8.1	±	0.6
Plant health at V4-5	rating 0-5	4.6	±	0.7	4.9	±	0.3
Mid Season							
Flowering date (50% of plants)	N of days	46.7	±	5.2	48.5	±	6.1
Plant health at R1	rating 0-5	4.6	±	0.8	4.8	±	0.5
Late Season at Crop Maturity							
Days to maturity (95% of pods turning color)	N of days #	127.7	±	7.0	127.3	±	6.7
Yield	bushel/acre	46.41	±	11.00	60.36	±	11.00
Plant lodging	rating 1-9	5.1	±	1.6	7.0	±	0.9
Final Stand count (20 ft section of row in each plot)	N of plants	73.5	±	19.0	104.4	±	21.1
Pod shattering	rating 1-9	8.0	±	0.6	8.8	±	0.4
Plant health at maturity	rating 0-5	5.0	±	0.0	5.0	±	0.0

Mean values and standard deviations are over all trial sites.
[a] N; Number of days from the date of planting or number of plants
Plant vigor rating; 1 is no emergence and 9 is complete emergence
Plant health rating, 0 is death, 5 is no injury
Plant lodging score; 1 is plants are flat on the ground, 5 representing that plant stems are 45 degrees to the ground, and 9 representing that all plant stems are straight up and down with minimal branching. Lodging scores were taken just prior to harvest.
Pod shattering score; 1 is all pods are open, 5 being 50% of pods are open, and 9 representing no pods are open. Shattering scores were taken at maturity and approximately 2 weeks after maturity (on border rows).

For the reproductive characteristics; days to 50% flowering were shorter for event FG72 than for the conventional varieties, but for days to 95% pods maturing, event FG72 and commercial variety plants were not different. The conventional plots were higher in seed production than event FG72, probably due to the higher plant stand count. The assessment of seed dispersal parameters (pod shattering and plant lodging) found event FG72 and the commercial varieties to have the same pod shattering score, but found the commercial varieties to be less prone to lodging.

The yield of the conventional varieties was higher than that of event FG72; a demonstration of the advancement in yield potential to be found in the current varieties.

In the 2008 field season, double-herbicide-tolerant soybean event FG72 was compared to a series of commercial varieties. Overall, no differences were observed that could be attributed to any pleiotropic effects of the transformation process, or the presence of the introduced proteins, 2mEPSPS and HPPD W336, but rather to the advancement of the new varieties.

VII.F. Seed dormancy evaluation

To provide an evaluation of seed dormancy potential of event FG72 and the variety Jack, measurements of seed germination and dormancy were conducted. The hypothesis was to test seed samples harvested from plants grown to maturity at 10 locations. These seed samples would represent the physiological state of seed that might fall into a field at the end of the season. The seed tests were completed by the Iowa State Seed Lab using the standard test (warm germination) which is used for seed lot evaluations of field emergence under favorable conditions. Seeds are rolled into germination towels, placed into germination trays and incubated at 25°C and 90%relative humidity for five days. Germination is scored on day 6. A minimum of 400 seed were evaluated from each location. Appendix 2.F. describes the materials and methods for the germination studies.

A very small difference in germination (94% vs. 96%) at day 6 was observed (Table 24). In cases where hard seed were observed on day 6, the germination study was extended to 13 days, and in every case, the hard seed germinated. The percent of viable seed from each of the samples (total viable; 95% vs. 96%) was the same.

No dormant seed were identified. Although small difference was observed between the event FG72 and the parent line Jack, the differences were not more than one standard deviation. No impact of the production environment or harvest conditions was observed.

Table 24. Seed germination test results

ID	Location	Germination[a] (%)	Hard seed	Dormant seed	Total viable	Abnormal seedlings	Dead seed	Days tested
FG72	Mediapolis	92	2	0	94	3	3	13
FG72	Perry	89	3	0	92	8	0	13
FG72	Sharpsville	82	6	0	88	10	2	13
FG72	Adel	97	1	0	98	2	0	6
FG72	Fithian	90	0	0	90	9	1	13
FG72	Marcus	97	0	0	97	3	0	6
FG72	Glidden	95	0	0	95	5	0	13
FG72	Winterset	99	0	0	99	0	1	6
FG72	Osborn	97	2	0	99	1	0	13
FG72	Iowa Falls	99	0	0	99	1	0	7
	Mean	**93.7**	**1.4**	**0**	**95.1**	**4.2**	**0.7**	
	StDev[b]	**5.4**	**2.0**	**0.0**	**4.0**	**3.6**	**1.1**	
Jack	Mediapolis	97	0	0	97	1	2	
Jack	Perry	96	0	0	96	3	1	6
Jack	Sharpsville	93	3	0	96	4	0	13
Jack	Adel	94	0	0	94	5	1	6
Jack	Fithian	94	0	0	94	6	0	6
Jack	Marcus	98	0	0	98	2	0	6
Jack	Glidden	98	0	0	98	2	0	6
Jack	Winterset	99	0	0	99	1	0	6
Jack	Osborn	98	0	0	98	2	0	6
Jack	Iowa Falls	95	0	0	95	4	1	6
	Mean	**96.2**	**0.3**	**0**	**96.5**	**3**	**0.5**	
	StDev[b]	**2.1**	**0.9**	**0.0**	**1.8**	**1.7**	**0.7**	

[a] Warm germination – 8 reps of 50 seed

[b] StDev; standard deviation

VII.G. Composition analysis

VII.G.1. Introduction

Analysis of the nutritional composition of the double-herbicide-tolerant soybean event FG72 was performed for soybean grain harvested from 10 different locations in the soybean growing areas of North America (Mackie, 2009). The study was conducted during the 2008 growing season using seed of the T_8 generation. Planted at each of the 10 locations were three entries:

Entry A; the control counterpart variety Jack, which was treated with conventional herbicides registered for use on soybean; (designated as Jack in Tables 25-29)
Entry B; the test entry event FG72 treated with conventional herbicides (designated as FG72 in Tables 25-29)
Entry C; the test entry event FG72 treated with the intended herbicides (IFT + GLY) (designated as FG72 treated in Tables 25-29).

Each of the three entries was planted in a RBC design with three replications per location. Three commercial soybean varieties were planted along side the test and control entries at the same locations. These three commercial soybean varieties provided reference values to establish ranges of natural variation for the nutritional components analyzed in this study.

The nutritional composition analysis conducted was based on the OECD guidance document for soybean (OECD, 2001). The nutritional endpoints selected were proximates, fiber compounds, total amino acids, fatty acids, anti-nutrients and isoflavones.

For comparative purposes, the values obtained for the commercial reference lines were used to establish in-study ranges in addition to the ranges reported in the published literature (OECD 2001; ILSI 2007). Together, these two sets of ranges were used to evaluate the nutritional composition results of event FG72 soybean. Nutrient component means that fell within the limits of the commercial or literature reference ranges were considered to be within the normal variation for commercial soybeans.

The test plots were each 15 ft by 20 ft in size and contained 6 rows spaced 30 inches apart. At maturity, grain samples were harvested from the two interior rows of each plot.

VII.G.2. Nutritional composition of soybean grain

Tables 25-29 show the comparisons of the pooled results of the two test and counterpart control entries from all locations, with reference ranges calculated from three commercial soybean varieties. Appendix 2.G. describes the materials and methods for the composition analysis. All mean values typically fell within the respective commercial variety or literature reference ranges and are not considered to be of biological concern or due to the intended modification of event FG72 (Rattemeyer, 2009).

The analysis of proximates and fiber between the test and the counterpart control entries were similar. All mean values were within the calculated commercial variety and literature reference ranges (Table 25).

Analysis of the total amino acid profile for all 18 amino acids between the two test and counterpart control entries were found to be similar (Table 26).

Levels of 24 fatty acids were measured for the two test entries, the counterpart control Jack, and the three commercial varieties. Seventeen of these fatty acids; C08:0, C10:0, C12:0, C14:0, C14:1, C15:0, C15:1, C16:1, C17:1, C18:3 (gamma), C18:4, C20:2, C20:3, C20:4, C20:5, C22:1, C22:5 and C22:6 were below the limit of quantification (LOQ = 0.02 % fw) in all soybean seed samples. These minor fatty acids of soybean were not statistically analyzed and are not reported in Table 26.

The results of the fatty acid analysis of the two test entries and Jack are shown in Table 27. All mean values for the fatty acids listed in Table 27 fell within both the calculated commercial variety and literature reference ranges. The sum of all detected fatty acids was 99.9% and accounts for nearly all fatty acids present in the oil.

The level of the anti-nutrient phytic acid in Jack and event FG72 soybean grain entries fell within the commercial variety and the literature reference ranges (Table 28).

The levels of the two low molecular weight carbohydrates raffinose and stachyose in the two test and control entries were within the range of the commercial lines tested (Table 28).

The analysis of the levels of lectins in the two test and isoline control entries were found to be similar, and fell within the commercial variety and literature reference ranges (Table 28).

The trypsin inhibitor mean values for the two test and control entries fell within the commercial variety and reference literature ranges (Table 28).

Soybeans contain isoflavones which are glucosides and esters of three aglycones (daidzein, genistein and glycitein). The mean values and range reported for isoflavone content in the test and counterpart entries were very similar in numerical value, and all mean values fell within the ranges for the commercial lines and literature references (Table 29).

In summary, no safety related issues were identified in the analysis of the nutrient composition of event FG72 soybean grain. All components measured were comparable to either the commercial soybean varieties grown at the same locations as the test and control entries, or were within the cited literature reference ranges.

Table 25. Proximate and fiber components

Component		Jack	FG72	FG72 Treated	Commercial Lines [b]	Literature Reference[c]
Moisture % fw	Mean [a]	9.51	9.65	9.45		
	Range	6.57-10.50	7.90-11.50	6.51-10.90	8.00 – 10.60	5.6-12
Protein % dw	Mean	38.2	38.2	38.1		
	Range	36.2-40.3	36.8-39.8	36.5-39.6	35.8 – 40.1	32 – 45.5
Fat % dw	Mean	19.3	18.9	19.2		
	Range	17.9-21.4	16.6-21.0	17.1-21.6	15.1 – 21.4	8.1 – 24.7
Ash % dw	Mean	5.24	5.07	5.06		
	Range	4.38-6.07	4.17-5.56	4.50-5.68	4.89 – 5.73	3.9 – 7.0
Carb. % dw	Mean	37.3	37.9	37.6		
	Range	34.3-39.3	35.6-39.7	35.3-40.0	34.8 – 41.6	29.6- 50.2
ADF % dw	Mean	17.8	18.1	17.9		
	Range	14.2-22.4	14.1-23.5	15.2-21.4	13.6 – 23.5	7.8 – 18.6
NDF % dw	Mean	19.8	20.3	20.0		
	Range	16.8-24.5	16.9-25.4	17.4-23.0	16.1 – 24.8	5.0 – 21.3

[a] Least square mean
[b] Reference ranges of the 3 analyzed commercial soybean lines
[c] Literature ranges from OECD (2001) and ILSI (2007)

Table 26. Amino acids

Amino acid % dw		Jack	FG72	FG72 Treated	Commercial Lines [b]	Literature Reference [c]
Alanine	Mean [a]	1.68	1.68	1.68		
	Range	1.60-1.75	1.60-1.74	1.60-1.72	1.55 – 1.78	1.51 – 2.10
Arginine	Mean	2.94	2.97	2.95		
	Range	2.71-3.20	2.77-3.14	2.74-3.10	2.69 – 3.13	2.17 – 3.40
Aspartic acid	Mean	4.40	4.38	4.37		
	Range	4.15-4.70	4.08-4.60	4.13-4.55	4.06 – 4.67	3.81 – 5.12
Cystine	Mean	0.58	0.58	0.59		
	Range	0.53-0.63	0.51-0.62	0.49-0.63	0.50 – 0.63	0.37 – 0.81
Glutamic acid	Mean	6.75	6.77	6.74		
	Range	6.30-7.24	6.30-7.21	6.34-7.03	6.32 – 7.23	5.84 – 8.20
Glycine	Mean	1.68	1.68	1.68		
	Range	1.60-1.76	1.60-1.75	1.60-1.74	1.53 – 1.76	1.46 – 2.27
Histidine	Mean	1.05	1.05	1.05		
	Range	1.00-1.10	0.99-1.09	0.98-1.09	0.93 – 1.07	0.84 – 1.22
Isoleucine	Mean	1.81	1.80	1.79		
	Range	1.73-1.92	1.69-1.87	1.67-1.86	1.62 – 1.96	1.54 – 2.32
Leucine	Mean	2.99	2.99	2.98		
	Range	2.84-3.18	2.84-3.13	2.81-3.09	2.71 – 3.13	2.2 – 4.0
Lysine	Mean	2.48	2.48	2.47		
	Range	2.37-2.62	2.34-2.58	2.33-2.56	2.34 – 2.64	1.55 – 2.84
Methionine	Mean	0.54	0.54	0.54		
	Range	0.49-0.60	0.49-0.58	0.46-0.58	0.50 – 0.58	0.43 – 0.76
Methionine	Mean	0.54	0.54	0.54		
	Range	0.49-0.60	0.49-0.58	0.46-0.58	0.50 – 0.58	0.43 – 0.76
Phenylalanine	Mean	1.97	1.98	1.96		
	Range	1.89-2.13	1.87-2.09	1.83-2.05	1.83 – 2.08	1.60 – 2.39
Proline	Mean	1.82	1.83	1.82		
	Range	1.68-1.97	1.72-1.98	1.65-1.94	1.71 – 1.94	1.69 – 2.33
Serine	Mean	1.97	1.98	1.99		
	Range	1.82-2.14	1.75-2.10	1.83-2.11	1.77 – 2.13	1.11 – 2.48
Methionine	Mean	0.54	0.54	0.54		
	Range	0.49-0.60	0.49-0.58	0.46-0.58	0.50 – 0.58	0.43 – 0.76
Phenylalanine	Mean	1.97	1.98	1.96		
	Range	1.89-2.13	1.87-2.09	1.83-2.05	1.83 – 2.08	1.60 – 2.39
Proline	Mean	1.82	1.83	1.82		
	Range	1.68-1.97	1.72-1.98	1.65-1.94	1.71 – 1.94	1.69 – 2.33
Serine	Mean	1.97	1.98	1.99		
	Range	1.82-2.14	1.75-2.10	1.83-2.11	1.77 – 2.13	1.11 – 2.48
Threonine	Mean	1.55	1.54	1.53		
	Range	1.48-1.66	1.45-1.61	1.44-1.62	1.44 – 1.62	1.14 – 1.89
Tryptophan	Mean	0.45	0.44	0.44		
	Range	0.40-0.50	0.38-0.48	0.39-0.50	0.39 – 0.54	0.36 – 0.67
Tyrosine	Mean	1.40	1.40	1.40		
	Range	1.28-1.49	1.33-1.46	1.30-1.46	1.32 – 1.48	0.10 – 1.61
Valine	Mean	1.89	1.88	1.87		
	Range	1.80-2.01	1.78-1.98	1.75-1.95	1.66 – 2.03	1.50 – 2.44

[a] Least square mean; [b] Reference ranges of the 3 analyzed commercial soybean lines, [c] Reference ranges from OECD (2001) and ILSI (2007)

Table 27. Fatty acids

Fatty Acid % relative		Jack	FG72	FG72 Treated	Commercial Lines [b]	Literature Reference[c]
Saturated						
C16:0 (palmitic)	Mean [a] Range	10.1 9.75-10.9	9.34 9.02-9.58	9.38 9.03-10.4	9.78 – 11.40	7 – 16
C18:0 (stearic)	Mean Range	4.28 4.07-4.70	4.52 4.23-5.05	4.51 3.80-5.08	3.49 – 4.81	2 – 5.9
C20:0 (arachidic)	Mean Range	0.31 0.28-0.36	0.32 0.30-0.37	0.32 0.27-0.38	0.25 – 0.35	< 0.10 - 0.48
C22:0 (behenic)	Mean Range	0.32 0.30-0.34	0.33 0.31-0.35	0.33 0.26-0.36	0.25 – 0.35	0.28 – 0.60
C24:0 (lignoceric)	Mean Range	0.113 < 0.10 -0.16	0.119 < 0.10 -0.17	0.122 < 0.10 -0.17	< 0.10 – 0.15	0.15
Sum of the saturated		**14.9**	**14.5**	**14.5**	**13.8 – 17.2**	**9.43 – 23.55**
Mono-unsaturated						
C18:1 (oleic)	Mean Range	21.97 20.10-25.00	24.65 23.20-27.20	24.12 22.40-26.30	21.10 –24.10	14 – 34
C20:1 (eicosenoic)	Mean Range	0.16 0.14- 0.19	0.16 0.15-0.19	0.17 0.15-0.19	< 0.10 – 0.18	0.14 – 0.35
Sum of mono-unsaturated		**22.13**	**24.81**	**24.29**	**21.10 – 24.28**	**14.14 – 34.83**
Poly-unsaturated						
C18:2 (linoleic)	Mean Range	54.56 51.70-55.90	52.65 50.60-53.70	53.08 51.20-54.70	51.50 –55.40	48 – 60
C18:3 (α-linolenic)	Mean Range	8.27 7.37-9.14	7.94 7.24-8.65	8.01 7.22-8.82	7.59– 10.30	2 – 10
Sum of poly-unsaturated		**62.83**	**60.59**	**61.09**	**59.09 – 65.70**	**50 - 70**
Sum of all the fatty acids		**99.93**	**99.91**	**99.92**		

[a] Least mean square
[b] Reference ranges of the 3 analyzed commercial soybean lines
[c] Reference ranges from OECD (2001) and ILSI (2007)

Table 28. Anti-nutrients

Anti-nutrients (dw)		Jack	FG72	FG72 Treated	Commercial Lines [b]	Literature Reference[c]
Phytic Acid (%)	Mean [a] Range	1.40 1.03-1.70	1.37 0.89-1.91	1.35 0.79-1.87	0.96 – 1.50	0.63 – 2.74
Raffinose (%)	Mean Range	0.361 0.286-0.428	0.378 0.280-0.526	0.379 0.295-0.511	0.290 – 0.504	0.11 – 1.28
Stachyose (%)	Mean Range	2.49 2.04-2.91	2.42 2.09-2.88	2.50 2.06-2.90	2.23 – 2.96	1.21 – 6.30
Lectin (HU/mg)	Mean Range	1.74 0.91-4.29	1.40 0.66-3.08	1.54 0.88-2.63	0.46 – 8.63	0.11 - 129
Trypsin inhibitor	Mean Range	33.0 23.3-47.6	30.1 19.6-42.4	33.9 23.6-43.4	23.5 – 60.1	19.59-118

[a] Least mean square
[b] Reference ranges of the 3 analyzed commercial soybean lines
[c] Reference ranges from OECD (2001) and ILSI (2007)

Table 29. Isoflavones

Isoflavones mg/kg dw		Jack	FG72	FG72 Treated	Commercial Lines [b]	Literature Reference[c]
Daidzin	Mean Range	1035 480-1850	1034 416-1690	994 400-1810	568 – 2530	60.0 – 2454
Genistin	Mean Range	1817 839-2760	1682 627-2460	1640 609-2400	1130 – 3290	144 – 2837
Glycitin	Mean Range	365 298-445	414 345-511	400 169-492	142 – 315	15.3 – 1070
Daidzein [e]	Mean Range [d]	---------- < 10 – 17.5	---------- < 10 – 15.1	---------- < 10 – 14.6	< 10 – 14.0	5 – 35
Genistein [e]	Mean Range [d]	---------- < 10 – 17.2	---------- < 10 – 15.7	---------- < 10 – 12.2	< 10 – 20.6	0.3 – 46
Glycitein	Mean Range [d]	<LOQ < 10	<LOQ < 10	<LOQ < 10	<LOQ < 10	1.1 – 80
Total Isoflavones	**Mean [a] Range**	**2010 1040-3130**	**1953 930-2860**	**1891 881-2890**	**1160 - 3390**	**679 – 3733**

[a] Least mean square
[b] Reference ranges of the 3 analyzed commercial soybean lines
[c] Reference ranges from ILSI (2007)
[d] some or all values reported below the limit of quantification
[e] Mean not calculated as some samples were below LOQ

VII. H. Poultry feeding study

A 42-day broiler chicken feeding study was conducted using diets containing 20% toasted soybean meal from event FG72 soybean, Jack soybean and a non-commercial soybean line (Stafford, 2009). Broiler chicken is very sensitive to minor differences in nutrient quality, since it undergoes an approximate 15-fold increase in body weight during the first 21 days of life.

All chickens were monitored at least daily for health status, overt signs of toxicity, and mortality. Effects of diets on health, survival, live body weight, total weight gain, feed consumption, food conversion, marketable carcass weight and muscle tissue weight and yield (breast, thigh, leg, wing), and abdominal fat pad weight were compared among groups. Gross post-mortem examination findings were reported as appropriate.

After 42 days of daily exposure, no differences were observed between the event FG72 group and the control groups. Minor statistical differences were recorded and were considered not treatment-related. Overall, the growth and health of chickens were similar in all groups.

In conclusion, there was no evidence that the group of broiler chickens fed event FG72 soybean toasted meal were adversely affected in any manner. The toasted meal with event FG72 soybean incorporated at 20 % was as safe and nutritious as the meals made with 20 % of control group soybeans.

VII.I. Conclusion for agronomic evaluation of event FG72

A thorough review of double-herbicide-tolerant soybean event FG72 was conducted over the 2003 and 2008 crop seasons. During these field studies, more than 20 different agronomic parameters were identified and evaluated to assess the impact of event FG72 on the soybean plant. Development and maturity, environmental susceptibility to biotic and abiotic stressors, and the yield potential and quality of the soybean grain were all evaluated to determine if event FG72 differed from the parent line Jack and other conventional soybean varieties of the same type.

In addition to the agronomic evaluation, event FG72 was analyzed for its main nutritional components and compared to the parent line Jack and commercial soybean varieties. The compositional analysis demonstrated that the intended modification in event FG72 did not change the compositional make-up and the nutritional profile of event FG72 is similar to that of the Jack and within the range of commercial soybeans lines and the established literature ranges.

The overall conclusion is that there are no agronomically meaningful differences between the transformed double-herbicide-tolerant soybean event FG72 and other soybean varieties evaluated. The resulting conclusion is that the introduction of event FG72 soybean poses no new agronomic plant pest risks.

VIII. ENVIRONMENTAL SAFETY AND IMPACT ON AGRONOMIC PRACTICES

VIII.A. Environmental assessment of the introduced proteins

The presence of the 2mEPSPS and HPPD W336 proteins introduced in FG72 soybean will not present adverse environmental effects, as both are derived from common, naturally occurring proteins and differ by only 2 and 1 amino acid substitutions, respectively.

The *2mepsps* gene was generated by introducing two point mutations into the wild-type *epsps* (wt *epsps*) gene cloned from maize (*Zea mays*). These changes to the gene result in the production of a double mutant EPSPS (2mEPSPS) protein which has a lower binding affinity for glyphosate, thus allowing sufficient enzyme activity for the plants to grow normally in the presence of this herbicide.

The naturally occurring EPSPS protein is universally expressed in plants and microorganisms, and has been a safe component of food and feed for a long history of consumption. In addition, the substitution of two amino acids was not expected to change the protein's safety or its potential for toxicity and allergenicity. And recent studies conducted by BCS have confirmed the safety profile of the 2mEPSPS protein (Section VI).

Moreover, the safety of the 2mEPSPS protein, which is also present in the genetically modified herbicide tolerant maize Event GA21 and in GlyTol™ cotton, has been evaluated by several regulatory agencies.

HPPD proteins have been isolated from many different organisms, such as bacteria, fungi, plants, animals, and humans. HPPD proteins from diverse origins share a common structure and have several amino acids that are completely conserved among all HPPD proteins. They are present in food of plant, fungal and animal origin and have a history of safe consumption.

In plants, the inhibition of HPPD by IFT herbicide leads to the disruption of photosynthesis and subsequent bleaching of foliage and eventually plant death. To take advantage of this interaction for purposes of weed control, an HPPD protein with increased tolerance to IFT, was developed by introducing a single amino acid change to the native HPPD protein isolated from *Pseudomonas fluorescens*. A simple mutant has been made to introduce a glycine (G) to tryptophan (W) substitution at position 336 of the native enzyme, resulting in the HPPD W336 protein. This mutation reduces the sensitivity of the enzyme to the herbicide isoxaflutole. A single mutation is not expected to change the safety aspects of the HPPD protein. Studies conducted by BCS have established the safety of the HPPD protein (Section VI).

VIII.B. Potential for horizontal or vertical gene transfer

Soybean is a self pollinating crop. Anthers mature in the floral buds and directly pollinate the stigma of the same flower (cleistogamy). Natural cross-pollination with near-by soybean plants is reported to be less than 1% (OECD 2000). The extent of outcrossing can be influenced by the distance between individual plants, floral characteristics of different varieties, environmental conditions and insect activities. In seed production fields, the occurrence of cross pollination is

so low that the standards of certified seed production require isolation distances to prevent mechanical mixture (7CFR 201.76) and are based upon the width of the harvest machinery.

Soybean is a non-native crop of the Americas. The origin of the *Glycine* species is Asia and there are no wild or native soybean relatives in the Americas which could be considered to be potential targets for gene flow.

VIII.C. Weediness potential of double-herbicide-tolerant soybean event FG72

Commercial soybean varieties in the United States are neither problematic volunteer weeds in other cropping systems nor are they found as feral populations on unmanaged lands (OECD 2000). The potential fate of soybean seeds remaining in the field after harvest includes; rot, predation, herbicides from rotational crops, and winter weather. Soybeans generally do not survive over the winter season. When climatic conditions are permissive, volunteer soybeans provide minimal interference in the rotational crop and are not recognized as an economic problem in soybean production. Volunteer soybeans are not competitive and can be managed by existing agronomic practices.

Double-herbicide-tolerant soybean transformation event FG72 is tolerant to two herbicides with different modes of action[i]; class G (glyphosate) and class F (isoxaflutole), and remains sensitive to herbicides registered for pre-plant and pre-emergence use for weed control in soybean and other crops which are common in rotation with soybean. Volunteer soybeans can be treated with a pre-emergence or post-emergence herbicides such as 2,4-D, atrazine, glufosinate, mesotrione, acetochlor, dicamba, and others. These products are also widely used for weed control in the rotational crops of soybean.

As soybean are not difficult to control as volunteers in a subsequent crop, and as FG72 has been shown to be no different from cultivated soybean in any of the traits that might impact weediness, the current practice to control volunteers will be effective.

VIII.D. Current agronomic practices for soybean

The introduction of glyphosate tolerant soybeans in 1996 significantly changed the way growers manage weed control in soybeans. Glyphosate tolerant soybeans were rapidly adopted by growers due to many unique properties of glyphosate enabling the grower to simply, effectively and economically manage their weeds.

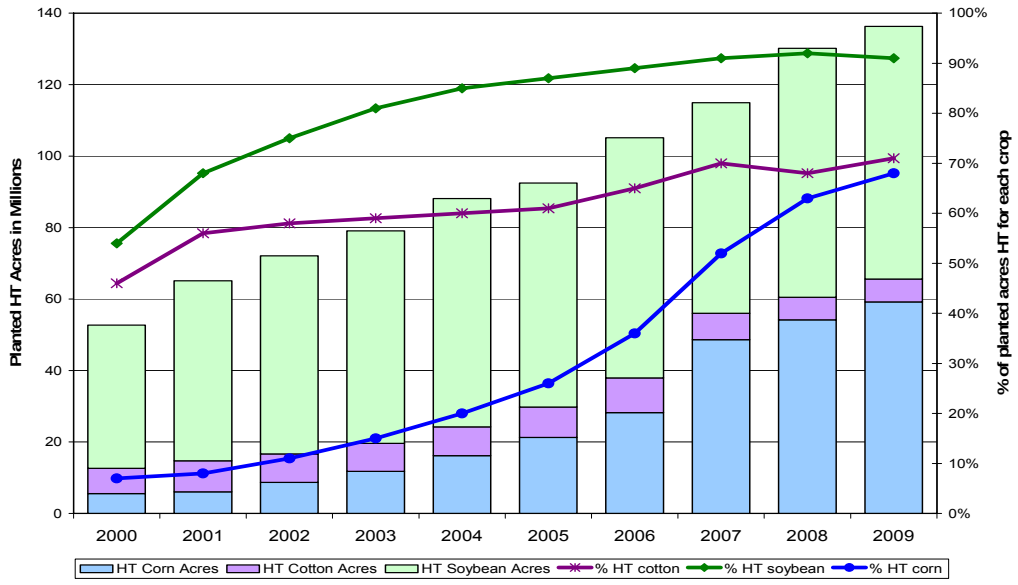
Glyphosate herbicide is exceptionally effective for controlling a broad spectrum of weeds, including many difficult to control weeds, in glyphosate tolerant soybeans with virtually no crop damage. Weed management with glyphosate is also exceptionally simple as the application technique (spray nozzles, spray pattern, carrier volume, and speed of application) has little impact on weed control. Glyphosate herbicide has flexible use rates and patterns and now is more economical since becoming available from generic manufacturers. The use of glyphosate has also resulted in reduction in tillage both prior to planting and in crop cultivation due to its effective weed control (Boerboom and Owen, 2006). This allows growers to forgo tillage which

improves soil conservation, saves labor, and reduces fuel consumption. In addition, the glyphosate tolerant system allows growers to reduce field scouting as effective herbicide applications can be made to large weeds with a wide application window. Furthermore, late season weed control methods for weed escapes such as hand labor, rope wicking, and spot spraying have been virtually eliminated. Additional weed management benefits with glyphosate include no carryover concerns to the following crop, no replanting restrictions, low environmental and human health risks and it is not a restricted-use pesticide (Boerboom and Owen 2006). The glyphosate tolerant trait is widely available and can be found in the highest yielding soybean varieties available on the market.

The simple, effective and economical management of weeds with glyphosate in soybean has led to the adoption of a solitary chemical weed control practice at the sacrifice of chemical diversity. By 2007, more than 90% of the US soybean production area was planted to glyphosate herbicide tolerant soybean (USDA 2009). The grower of glyphosate tolerant soybeans makes an average of 1.7 applications of glyphosate to the crop per growing season (USDA 2007). The rapid adaptation of this new technology and the exclusion of other weed control measures set the stage for weed population shifts and the evolution of weeds resistant to glyphosate herbicide. With the application of glyphosate herbicide over most of the soybean production areas, many weeds were exposed to the herbicide and resistant biotypes were enriched

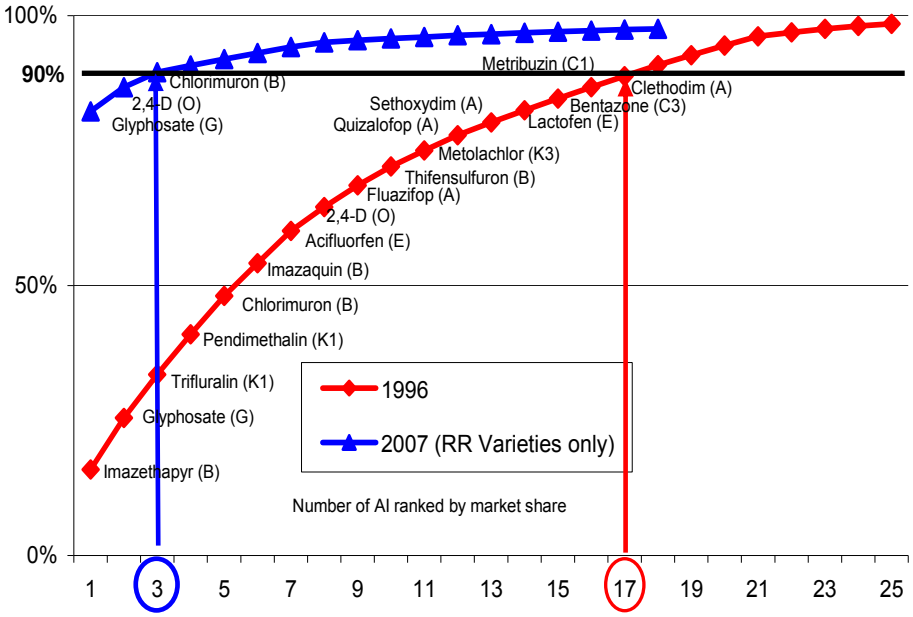
The extensive use of glyphosate was encouraged by the availability of glyphosate tolerance in other crops. Glyphosate tolerant corn is grown on 68% of the 87 million acres of corn in the US. Glyphosate tolerant cotton is grown on 71% of the 9.5 million acres of cotton (Figure 9, USDA 2009). Also available in the market are glyphosate tolerant canola and sugar beet varieties thus, creating unprecedented selection pressure for resistant biotypes. In 1996, seventeen herbicides composed 90% of the market. In 2007, only three herbicides comprised 90% of the market with glyphosate at 80% (Figure 10) for use in soybean production in the USA.

Figure 9. Growth of herbicide tolerant traits



Adapted from USDA, NASS Acreage 2001-2009

Figure 10. Comparison of herbicide use for the years 1996 and 2007



The declining use of herbicide options lead to the loss of herbicide diversity in soybean weed control in the USA. Indicated in the figure by the red diamond line, seventeen herbicides composed 90% of the market in 1996. In 2007, only three herbicides comprised 90% of the market with glyphosate at 80% and 2,4-D and chlorimuron in minor use. Letters in brackets indicate the mode of action (HRAC 2009).

The over-reliance on a single weed control method can lead to the eventual development of resistant weeds and consequential loss of that particular production system and perhaps even eventually jeopardize the ability to grow a specific crop in a specific field. Because of cost considerations and the additional workload, IWM tactics generally have not been employed until herbicidal efficacy starts to fail and herbicide resistance becomes a problem threatening the economic viability of the farmer. Working preemptively through incorporating integrated weed management measures can lead to the successful prevention of the development of a resistant weed population.

There are currently 189 species of resistant weeds worldwide, 16 of which are resistant to glyphosate (Heap 2009). Growers have been reluctant to alter their weed management practices and return to conventional herbicides in light of the advent of glyphosate resistant weeds. Even with weed resistance, growers will continue to produce glyphosate tolerant crops as glyphosate herbicide remains effective for a number of weeds that are difficult to control with other herbicides. Conventional herbicides generally have a narrower application window, narrower weed spectrum, increased risk of crop injury, various application techniques required, additional time for sprayer cleanout between fields, carryover concerns, replanting restrictions and are considered to be less economical.

However, growers are beginning to alter their farming practices to gain better control of glyphosate resistant weeds. Some growers are utilizing tankmixes with conventional herbicides to help control herbicide-resistant weeds. Conventional tankmix partners have limitations however such as increased cost, increase the risk of injury to the soybeans and limit the application window due to weed size restrictions. Some growers have moved away from reduced or no-till practices as their burndown program no longer provides effective control of glyphosate resistant marestail. Uncontrolled weeds result in soybean yield loss as the weeds compete for soil nutrients, moisture, and sunlight. The impact of glyphosate-resistant weeds firmly impacts a grower's available time and financial resources.

There is an urgency to produce viable alternatives to glyphosate weed control programs in soybeans. There are several HT soybean products available to the US soybean grower (Table 3) however most provide crop tolerance to herbicides for which herbicide resistant weeds are already identified (ALS inhibitors and glyphosate).

Today, the LibertyLink soybean system is the only nonselective alternative to the glyphosate system available for growers. Launched in 2009, there were more than 300,000 acres of LibertyLink soybeans planted in the US. LibertyLink soybeans also must be managed correctly to prevent the development of herbicide-resistant weeds.

VIII.E. Potential impact on agricultural practices for soybean

In the near future, soybean growers will have additional options. Table 30 identifies several new soybean events in the process of development and registration. A new herbicide mode of action 4-hydroxy-phenyl-pyruvate-deoxygenase enzyme by specific inhibitors (HPPD inhibitors) was developed during the 1980's. The double-herbicide-tolerant soybean event, FG72 which combines glyphosate tolerance with isoxaflutole tolerance is the first genetic source of crop tolerance to the HPPD inhibiting herbicides.

Table 30. Sources of genetic-based herbicide tolerance

Applicant(s)	Event / Trade Name	Trait Description(s)
Bayer CropScience 98-014-01p	A5547-127 / LibertyLink™	Glufosinate tolerant
Pioneer Hi-Bred International 06-271-01p	356042 / Optimum™ GAT™	Glyphosate and ALS inhibitor tolerant
BASF Plant Science 09-015-01p	BPS-CV127-9	ALS tolerant
Bayer CropScience and MS Tech 09-328-01p	FG72/ Double-Herbicide-Tolerant soybean	Glyphosate and HPPD tolerant
Monsanto Company	Not announced	Dicamba tolerant

The use of isoxaflutole, based on the FG72 tolerance trait, will enable growers to proactively manage resistant weeds with a new herbicide mode of action for soybeans. Growers will still be able to use glyphosate with FG72, but they will not be dependent upon it as their only broad spectrum herbicide choice. Managed appropriately, glyphosate will be able to maintain its utility for post-emergence control of many difficult to control weeds in soybeans.

FG72 will offer growers unmatched flexibility in their weed control programs for both conservation and conventional till acres. IFT herbicide can be applied either pre-emergence to the soybean or in a post-emergence tankmix. IFT can provide control of a broad spectrum of weeds including herbicide-resistant weed species. IFT not only introduces a new MOA into soybean production for resistance management, it also provides a residual tool to prevent early season weed competition. Early season weed control is key in preventing soybean yield loss due to weed competition.

Growers utilizing conservation tillage practices will be able to tankmix isoxaflutole with their burndown program prior to planting. A post-emergence application of glyphosate can be applied if needed. Growers in conventional tillage practices have two base options for weed control with FG72 soybeans. Isoxaflutole can be applied prior to planting, pre-plant incorporated, or pre-emergence to the soybean crop. Again, a post-emergence application of glyphosate can be applied if needed. Growers that prefer a total post-emergence weed control program due to the critical time management needed at planting will be able to apply glyphosate for emerged weed control in a tankmix with isoxaflutole for residual weed control.

VIII.F. Weed resistance management

Weed scientists agree that adopting and implementing best management practices that reduce weed resistance to herbicides is critical (Boerboom and Owen, 2006). We have developed detailed methods for integrated weed management that includes diverse farming practices. Integrated weed management not only improved 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 biotype becomes. To ensure diversification is maintained in weed control methods, we will also encourage growers to keep detailed records of weed management practices for each field. Our integrated weed management guidelines promote an economically viable, environmentally sustainable and socially acceptable weed control program is fully detailed in Appendix 5. The highlights of our 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.

VIII.G. Potential impact on farming practices

Although more than 90% of the soybean acres planted today are glyphosate-tolerant, conventional and organic farming continue to be an important sect of the soybean market. Conventional and organic soybean growers will find no adverse effect on their farming practices with the introduction of FG72 soybeans.

It is not likely that organic farmer or other farmers who choose not to grow FG72 soybeans will be significantly impacted by the expected commercial use of this product. Nontransgenic soybeans varieties will still be available for conventional and organic soybean producers. Soybean is mostly a self-fertilized plant and therefore limits the chance of hybridization to conventional soybean varieties. In addition to the National Organic Program administered by USDA's Agricultural Marketing Service which requires organic production operations to have distinct, defined boundaries and buffer zones to prevent unintended contact with prohibited substances from adjoining land that is not under organic management.

VIII.H. Potential effects on non-target organisms, including beneficial organisms

No adverse effect on non-target organisms from either the transgenic or non-transgenic plants was observed during any of the trials. Refer to Section VII D for biotic and abiotic stress characteristics.

The FDA issued a finding of "No Concern" for glyphosate tolerant soybeans. As the presence of the 2mEPSPS and the HPPD proteins are the only difference found in FG72 that is not found in conventional soybean, FG72 and its progeny should have no indirect or direct plant pest effects.

VIII.I. Threatened and endangered species considerations

The US Fish & Wildlife Service (FWS) has accountability for endangered species under the Endangered Species Act (ESA), (16 USC 1531). Section 6 of the ESA requires federal agencies who conduct activities which may affect listed species to consult with the FWS to ensure that listed species are protected should there be a potential impact.

It is not anticipated that the use of FG72 soybean will impact any currently listed species of concern. Species of concern that may inhabit areas close to commercial soybean operations would not be impacted by the use of FG72 soybean. Commercial agriculture routinely disturbs the ground in which crops are currently planted. As a result, perennial vegetative species would not grow in these areas. Additionally, because horizontal gene flow to sexually incompatible species is not an issue, there is negligible potential for exposure to the transgenes contained in FG72 soybean through sexual reproduction.

Isoxaflutole is currently registered for weed control use in corn in 18 of the primary corn producing states in the US. Collectively, these states represent approximately 75 % of the planted acres for corn and soybean (a four year average through 2009 - http://www.doane.com/research.php). End use products containing the active ingredient isoxaflutole are listed as “Restricted Use” and are for sale and use only by certified applicators. The approved and proposed end use product labels (e.g., tolerant soybean) also have extensive precautionary and restrictive language statements addressing handling and use of the product including specific endangered species protection requirements. The EPA “Registration Review” process for isoxaflutole is scheduled to be initiated in fiscal year 2011.

Glyphosate is currently supplied to US growers by numerous generic sources of the active ingredient and generic end use products. This active is registered for use on tolerant soybean, corn and cotton as well as on specific non-tolerant crops and non-crop uses. The current Bayer understanding is that future endangered species assessments will be addressed in the “Registration Review” process for this active which was initiated by EPA in July of 2009.

VIII.J. Potential impact on biodiversity

Soybean is considered a self-pollinated species, propagated commercially by seed. The soybean flower stigma is receptive to pollen approximately 24 hours before anthesis and remains receptive 48 hours after anthesis. The anthers mature in the bud and directly pollinate the stigma of the same flower. As a result, soybeans exhibit a high level of self-fertilization and cross pollination is usually less than one percent (Beckie 2007, Palmer *et al*., 2001).

There is no evidence of genetic transfer and exchange with organisms other than those with which soybean is able to produce fertile crosses through sexual reproduction (Beckie 2007, Stewart *et al*., 2003). There are no wild *Glycine* species in the United States, nor are their wild or weedy species with which soybeans can produce fertile crosses.

VIII.K. Conclusion

It has been demonstrated that the presence of the 2mEPSPS and HPPD W336 proteins introduced in FG72 soybean will not present adverse environmental effects. The lack of wild type soybean species or relatives in the Americas in addition to the self pollinating nature of soybean prevents gene transfer into unintended targets. The current practice to control volunteer soybean plants will not be altered by FG72. Current agronomic practices limit weed control diversity tactics. The introduction of FG72 will provide a new mode of action for weed control in soybean to improve resistant weed management. It is expected that growers who choose not to grow FG72 will not be impacted by the commercial use of this product. It is also not anticipated that the commercial use of FG72 will have any potential impacts on non-target organisms or on threatened or endangered species.

IX. STATEMENT OF GROUNDS UNFAVORABLE

Bayer CropScience and M.S. Technologies know of no study data and/or observations associated with Event FG72 soybean that will result in adverse environmental consequences for its introduction. The only biologically relevant phenotypic difference between Event FG72 soybean and conventional soybean is the expression of the 2mEPSPS and HPPD W336 proteins which provide tolerance to the application of glyphosate herbicide and isoxaflutole herbicide, respectively. Planting double-herbicide-tolerant soybean varieties, containing transformation event FG72, will provide growers with new options for weed control using IFT herbicide in combination with a glyphosate herbicide. Glyphosate is widely used in herbicide-tolerant soybean and other agricultural production systems. IFT herbicide offers an alternative weed control option for the soybean grower via a new herbicide mode of action for soybeans that is efficacious against many of the herbicide resistant weeds currently found in soybean fields.

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[1] Roundup is a registered trademark of Monsanto.

Appendix 1

FIELD TRIAL TERMINATION REPORTS 2001-2008

2001 USDA Termination Report for Herbicide Tolerant Soybean

Bayer CropScience LP

Trials Conducted: State (County)

01-087-08n: IL (Champaign)
01-268-11n: PR (Juana Diaz)

1. PLANTING DATES

May 25, 2001 (Champaign Co., IL) through December 15, 2001 (Juana Diaz, PR).

2. HARVEST/DESTRUCT DATES

September 28, 2001 (Champaign Co., IL) through April 12, 2002 (Juana Diaz, PR) with all plants tilled and disked under at the termination of the studies.

3. PURPOSE

Field trials utilizing herbicide tolerant transgenic soybean lines were established for breeding, efficacy testing and the generation of analytical data.

General Field Observations

Experienced personnel qualified in soybean cultivation performed all plot observations. Records for the transgenic and non-transgenic control plots were provided from VE (emergence) through final stand counts prior to harvest and crop destruction.

Plant emergence measured at four to five days averaged 50% at both sites. Stand counts taken at the V2 and V3 stages ranged from 70% (IL) to 80% (PR). Final stand counts of transgenic plants at the V3 and V4 stages of development at both sites ranged from 40 - 60% while non-transgenic controls average greater than 70 – 80%.

The only phenotypic difference recorded between the plant types were their respective levels of herbicide tolerance.

Insect pest species recorded from the Puerto Rico plot were bean leaf beetles (Coleoptera: Chrysomelidae), whitefly (Homoptera: Aleyrididae), and loopers (Lepidoptera: Noctuidae). Only bean leaf beetles were recorded at the Illinois site.

Beneficial species recorded in Illinois were ladybugs (*Hippodamia convergens*), earthworms (*Lumbricus sp.*), and honey bees (*Apis melifera*). The Puerto Rico plot had ladybugs (*Hippodamia convergens*) and honey bees (*Apis melifera*). No differences in density or diversity of these species were noted between the two plot types.

No diseases were found affecting any of the plants at either site. Both sites listed the growing season as typical.

Final Disposition and Volunteer Monitoring

All plant materials remaining at the completion of the studies were tilled under. Volunteer plants were found at the Puerto Rico study site on May 20, June 15, and June 28, 2002. A second “clean” count for this plot was achieved by August 5, 2002.

USDA Field Termination Report

Notification No.: 02-071-03n

Applicant No.: GLY/ISX-2A-Soybean-MR

Permittee: Aventis CropScience (Now Bayer CropScience LP)
Research Triangle Park, NC; 919-549-2655

Regulated Article: Herbicide-tolerant, glyphosate and isoxazole-tolerant, Soybean (*Glycine max*)

Site Release Information: As shown below, five (5) of seven (7) sites were planted:

County/Parish/District	State or Territory	Release Status
Hamilton	IA	Planted
Webster	IA	Not Planted
Champaign	IL	Planted
Benton	IN	Planted
York	NE	Planted
Juana Diaz	PR	Planted
Sabana Grande	PR	Not Planted

Information on each release follows:

County/ State	Acreage Planted	Date Planted	Germination Data Transgenic *vs.* Non-transgenic	Date Terminated
Hamilton/IA	0.248	6/6/02	**% Emergence/Seedling Vigor** Not provided **Initial Stand Count Percentage** 80.28% *vs*>88.06% on 6/28/02 **Final Stand Count Percentage** 80.28% *vs* >88.06% on 7/17/02	Harvested 11/1/02. Plant material was chopped 11/4/02.
Champaign/IL	0.5	6/7/02	**% Emergence/Seedling Vigor** >70% *vs* >75% on 6/14/02 **Initial Stand Count Percentage** >60% *vs* >70% on 7/3/02 **Final Stand Count Percentage** 0-60+% *vs* 0-70+% on 7/22/02	Harvested 10/14/02 and 10/15/02. Plot area disked 11/15/02.

County/ State	Acreage Planted	Date Planted	Germination Data Transgenic *vs.* Non-transgenic	Date Terminated
Benton/IN	0.3	6/8/02	**% Emergence/Seedling Vigor** >70% *vs* >70% on 6/14/02 **Initial Stand Count Percentage** >60% *vs* >60% on 7/3/02 **Final Stand Count Percentage** 0-60+% *vs* 0-60+% on 7/22/02	Harvested 10/21/02. Plot area mowed and disked 10/25/02.
York/NE	0.37	6/10/02	**% Emergence/Seedling Vigor** % not provided. Good vigor. **Initial Stand Count Percentage** Not provided **Final Stand Count Percentage** Not provided	Harvested 10/21/02. Plot area tilled 11/8/02.
Juana Diaz/PR	0.33	7/18/02	**% Emergence/Seedling Vigor** >50% *vs* >50% on 7/23/02 **Initial Stand Count Percentage** >80% *vs* >80% on 8/1/02 **Final Stand Count Percentage** 40-60% *vs* >80% on 8/15/02	Harvested 11/11/02 and 11/12/02. Plot area mechanically cultivated 12/4/02.

Purpose of Release: The trials were established for the purpose evaluating efficacy, generating seed and providing analytical data. The performance of the herbicide-tolerant soybean with respect to the nontransgenic counterpart was also evaluated, as were the overall agronomic characteristics.

Observations: The test sites were inspected multiple times during the growing season for agronomic growth characteristics and disease and insect pest infestation.

Plant emergence patterns were uniform and vigorous within the transgenic and nontransgenic plots for the plots located in Hamilton Co., Benton Co., York Co. and Juana Diaz District. Plant emergence of the transgenic soybeans at the Champaign Co. site was not as good as the non-transgenic counterpart due to the poor seed quality produced in Puerto Rico.

Observations were recorded at various stages throughout the growing season, ranging from plant emergence through full maturity. Insect species categorized as pests and beneficials were noted among the plots, as was phytopathology. No difference in susceptibility was noted between transgenic and nontransgenic plants.

County/State	Observations/Dates		
	Fungi/Diseases	Insect Pests	Beneficial Insects
Hamilton/IA	Iron chlorosis was evident 7/19/02 on both plant types.	Bean leaf beetles and occasional grasshoppers were noted 6/17/02, 7/19/02, 8/19/02 on both plant types. Damage was very minor.	Ladybugs were observed 7/19/02 and 8/19/02 on both plant types.
Champaign/IL	Bacterial blight and brown spot were observed 7/22/02 and 8/9/02 on both plant types. Damage was minor.	Woolly bear, caterpillar, grasshoppers and bean leaf beetles were observed 7/22/02 and 8/28/02 on both plant types. Damage was mild.	Ladybugs were seen 7/19/02 and 7/30/02 on both plant types.
Benton/IN	Bacterial blight and brown spot were noted 7/22/02 and 8/9/02 on both plant types. Damage was minor.	The Japanese beetle was seen during flowering on 7/31/02 on both plant types. Damage was minor.	Ladybugs were seen on 7/16/02 and 7/31/02 on both plant types.
York/NE	No phytopathology noted 6/17/02, 6/27/02, 7/18/02 or 10/21/02 on either plant type.	No pests were observed on 6/17/02, 6/27/02, 7/18/02 or 10/21/02 on either plant type.	Ladybugs were seen 6/27/02 and 7/18/02 on both plant types.
Juana Diaz/PR	No phytopathology noted 9/3/02, 9/6/02 or 9/11/02 on either plant type.	Whiteflies and loopers were evident 7/20/02, 8/15/02 and 9/3/02 on both plant types. Damage levels ranged from mild to moderately severe.	Ladybugs were seen 8/15/02 and 9/6/03 on both plant types.

Results: No agronomic, insect susceptibility or disease susceptibility or resistance differences were observed between the transgenic soybean plants and the nontransgenic counterpart. The only difference noted between the transgenic and nontransgenic plants was in the desired trait – tolerance to glyphosate herbicide – in the transgenic soybean plants, whereas the nontransgenic plants were susceptible to treatment with glyphosate.

Plant Disposition: Crops were harvested from mid October (10/14/02) until about mid November (11/12/02). Methods used for the destruction of remaining plant material included chopping, mowing, mechanical cultivation and under-tilling.

Volunteer Monitoring: The plot areas were visually inspected for volunteer soybean plants during the following growing season. The table below summarizes observations made and remedial action(s) taken, if needed, at each site.

Post-Season Volunteer Monitoring			
County/State	**Date**	**No. Plants Observed/Stage**	**Method of Destruction**
Hamilton/IA	6/5/03	None	
	7/1/03	None	
	7/30/03	None	
	8/27/03	None	
Champaign/IL	7/3/03	1 to 10 plants/V5-V6	Tillage
	8/8/03	None	
	8/19/03	None	
	8/25/03	None	
Benton/IN	6/25/03	None	
	7/25/03	None	
	8/12/03	None	
	8/26/03	None	
York/NE	5/17/03	11 to 50 plants/V1	Mechanically cultivated
	6/20/03	11 to 50 plants/V1-V2	Hand-weeded
	7/18/03	None	
	7/31/03	None	
	8/28/03	None	
Juana Diaz/PR	12/18/02	>50 plants/V2	Mechanically cultivated
	1/3/03	>50 plants/V3	Mechanically cultivated
	1/17/03	11 to 50 plants/V3	Mechanically cultivated
	1/31/03	None	Field mechanically cultivated
	2/7/03	None	Field mechanically cultivated

Weediness Characteristics: There was no evidence of change in characteristics that would enhance survival of the herbicide-tolerant soybean plants as compared to nontransgenic soybean plants. No difference in weediness characteristics between the transgenic and nontransgenic soybean lines was observed.

Field Management: Conventional field practices were utilized at all sites.

Non-Target Organisms: No adverse effect on non-target organisms from either the transgenic or nontransgenic plants was observed in any of the trials.

Weather Synopsis: Weather notations indicate the Hamilton Co., Benton Co. and Juana Diaz sites experienced normal/typical climatic conditions. The Champaign and York Co. sites were noted as being hotter and drier than normal.

Containment Measures: Isolation methods included the separation of soybean plants by distances of 5 to 10 feet. Additionally, border rows were used to prevent the flow of pollen. The border rows were not harvested but destroyed at the conclusion of the trial. The test plots and border areas were monitored the following growing season for volunteer soybean plants.

USDA Field Termination Report

Notification No.: 02-274-09n

Applicant No.: GLY/ISX-2E-Soybean-MR

Permittee: Bayer CropScience LP
Research Triangle Park, NC; 919-549-2655

Regulated Article: Herbicide-tolerant, glyphosate and isoxazole-tolerant, Soybean (*Glycine max*)

Site Release Information:

As follows:

District/ Territory	Acreage Planted	Date Planted	Germination Data Transgenic *vs.* Non-transgenic	Date Terminated
Sabana Grande/ PR	1.10	1/5/03	**% Emergence/Seedling Vigor** >50% *vs* >50% on 1/10/03 **Initial Stand Count Percentage** >80% *vs* >80% on 1/20/03 **Final Stand Count Percentage** 40-60% *vs* >80% on 2/4/03	Harvest occurred on 5/6/03 and 5/12/03. The field was mechanically cultivated on 5/16/03.

Purpose of Release: The purpose of the release was to test the efficacy as well as breeding of transgenic soybean plants. The performance of the herbicide-tolerant soybean with respect to the nontransgenic counterpart plant was also evaluated, as were the overall agronomic characteristics.

Observations: The test site was inspected eleven (11) times during the growing season (1/10/03, 1/20/03, 2/4/03, 3/3/03, 3/24/03, 4/1/03, 4/16/03, 5/1/03, 5/5/03, 5/6/03 and 5/12/03) for agronomic growth characteristics and disease and insect pest infestation.

Plant emergence patterns were uniform and vigorous within the transgenic and nontransgenic plots; germination rate was noted as >80%. Observations were recorded from first node through beginning pod stages. Two (2) species of insect pests were noted: whiteflies and loopers. Whiteflies were seen on 1/20/03, 2/4/03, 3/24/03 and 5/1/03. Loopers were observed on 3/24/03. Damage levels ranged from mild to moderately severe. Two beneficial insect species were observed: ladybug and ant. The ladybugs were seen on 2/4/03 and 4/1/03. The ants were observed on 3/24/03. Observations were made on both the transgenic and nontransgenic plots.

No disease susceptibility was noted on the transgenic or nontransgenic plants during any of the visits made on 3/3/03, 3/24/03, 4/16/03 and 5/5/03.

Results: No agronomic, insect susceptibility or disease susceptibility or resistance differences were observed between the transgenic soybean plants and its nontransgenic counterpart.

Plant Disposition: The crop was harvested on 5/6/03 and 5/12/03. All remaining vegetative material was mechanically cultivated on 5/16/03.

Volunteer Monitoring: The plot area was visually inspected for volunteer soybean plants five (5) times during the following growing season, until no volunteers had been observed for two (2) consecutive monitoring visits.

Post-Season Volunteer Monitoring

Date	No. Plants Observed/Stage	Method of Destruction
5/22/03	>50 plants/V2	Mechanically Cultivated
6/6/03	>50 plants/V3	Mechanically Cultivated
6/27/03	11 to 50 plants/V3	Mechanically Cultivated
7/6/03	None	Field Mechanically Cultivated
7/21/03	None	Field Mechanically Cultivated

Weediness Characteristics: There was no evidence of change in characteristics that would enhance survival of the herbicide-tolerant transgenic soybean plants as compared to nontransgenic soybean plants. No difference in weediness characteristics between the transgenic and non-transgenic soybean lines was observed.

Non-Target Organisms: No adverse effect on non-target organisms from either the transgenic or nontransgenic plants was observed in the trial.

Weather Synopsis: Weather notations indicate the site experienced normal/typical climatic conditions.

Containment Measures: The Sabana Grande test site is a 59-acre farm. The site produces no commercial crops. Transgenic soybean plants were separated by a distance of 10 feet. Border rows surrounded the perimeter of the test plot to minimize the flow of pollen. Border rows were not harvested but destroyed at trial conclusion. The test plot and border area were monitored for volunteer soybean plants during the period that followed.

USDA Field Termination Report

Notification No.: 03-080-05n

Applicant No.: GLY/ISX-3C-Soybean-MR

Permittee: Bayer CropScience LP
Research Triangle Park, NC; 919-549-2655

Regulated Article: Herbicide-tolerant, Glyphosate and Isoxazole-tolerant, Soybean (*Glycine max*)

Site Release Information: Eight (8) of twenty (20) sites were planted:

County/Parish/District	State or Territory	Release Status
Boone	IA	Not Planted
Carroll	IA	Planted
Dallas	IA	Planted
Des Moines	IA	Planted
Madison	IA	Planted
Marshall	IA	Planted
Polk	IA	Not Planted
Webster	IA	Not Planted
Champaign (Bayer)	IL	Planted
Champaign (Mertec)	IL	Not Planted
Hamilton	IL	Not Planted
Logan	IL	Not Planted
Warren	IL	Not Planted
Boone	IN	Not Planted
Clinton	IN	Not Planted
Tipton	IN	Not Planted
Clinton	MO	Not Planted
Saline	MO	Not Planted
York	NE	Planted
Sabana Grande	PR	Planted

Acreage and planting and termination dates follow:

County or District/ State or Territory	Acreage Planted	Date Planted	Date Terminated
Carroll/IA	0.45	5/22/03	10/1/03
Dallas/IA	4.19	5/19/03, 5/20/03, 5/29/03 & 6/4/03	10/4/03
Des Moines/IA	0.45	5/17/03	9/28/03
Madison/IA	1.59	5/23/03 & 6/5/03	10/7/03
Marshall/IA	0.45	5/17/03	10/5/03
Champaign/IL	3.25	5/22/03, 5/23/03, 8/14/03	10/10/03
York/NE	2.04	5/28/03 & 5/30/03	9/4/03
Sabana Grande/PR	4.93	5/24/03, 7/11/03 & 8/21/03	9/1/03 & 12/3/03

Purpose of Release: The trials were established for the purpose of breeding, evaluating efficacy and agronomic characteristics, and to obtain analytical data on the herbicide-tolerant soybean plants. The performance of the transgenic soybean with respect to the nontransgenic counterpart was also evaluated, as were the overall agronomic characteristics.

Observations: The test sites were visually inspected multiple times during the growing season for agronomic growth characteristics and disease and insect pest infestation. Observations were recorded for the transgenic and nontransgenic plants from emergence through harvest.

County or District/ State or Territory	Germination Data Transgenic *vs.* Non-transgenic
Carroll/IA	**% Emergence/Seedling Vigor** 88.5% *vs.* 88.5% on 6/3/03 **Initial Stand Count Percentage** 88.5% *vs.* 88.5% on 6/21/03 **Final Stand Count Percentage** 88.5% *vs.* 88.5% on 10/1/03
Dallas/IA (1st Location)	**% Emergence/Seedling Vigor** 88.5% *vs.* 88.5% on 5/31/03 **Initial Stand Count Percentage** 88.5% *vs.* 92.0% on 6/14/03 **Final Stand Count Percentage** 96.4% *vs.* 88.5% on 10/2/03
Dallas/IA (2nd Location)	**% Emergence/Seedling Vigor** 76.9% *vs.* 76.9% on 5/31/03 **Initial Stand Count Percentage** 76.9% *vs.* 76.9% on 6/14/03 **Final Stand Count Percentage** 76.9% *vs.* 76.9% on 10/3/03
Dallas/IA (3rd Location)	**% Emergence/Seedling Vigor** 88.5% *vs.* 88.5% on 6/10/03 **Initial Stand Count Percentage** 88.5% *vs.* 88.5% on 6/25/03 **Final Stand Count Percentage** 88.5% *vs.* 88.5% on 10/4/03
Des Moines/IA	**% Emergence/Seedling Vigor** 84.6% *vs.* 84.6% on 5/29/03 **Initial Stand Count Percentage** 84.6% *vs.* 84.6% on 6/16/03 **Final Stand Count Percentage** 84.6% *vs.* 84.6% on 9/28/03
Madison/IA	**% Emergence/Seedling Vigor** 92.3% *vs.* 92.3% on 6/5/03 **Initial Stand Count Percentage** 92.3% *vs.* 92.3% on 6/20/03 **Final Stand Count Percentage** ~92.3% *vs.* 92.3% on 9/20/03

County or District/ State or Territory	Germination Data Transgenic *vs.* Non-transgenic
Marshall/IA	**% Emergence/Seedling Vigor** 88.5% *vs.* 88.5% on 6/1/03 **Initial Stand Count Percentage** 88.5% *vs.* 88.5% on 6/18/03 **Final Stand Count Percentage** 88.5% *vs.* 88.5% on 7/19/03
Champaign/IL (1st and 2nd Plantings)	**% Emergence/Seedling Vigor** >60% *vs.* >60% on 6/4/03 **Initial Stand Count Percentage** >60% *vs.* >60% on 6/16/03 **Final Stand Count Percentage** 60% *vs.* 0% on 7/14/03
Champaign/IL (3rd Planting)	**% Emergence/Seedling Vigor** >70% *vs.* >70% on 9/3/03 **Initial Stand Count Percentage** >70% *vs.* >70% on 9/18/03 **Final Stand Count Percentage** >70% *vs.* >70% on 10/2/03
York/NE	**% Emergence/Seedling Vigor** 50-90% *vs.* 50-90% on 6/17/03 **Initial Stand Count Percentage** 50-90% *vs.* 50-90% on 6/17/03 **Final Stand Count Percentage** 50-90% *vs.* 0-90% on 9/4/03
Sabana Grande/PR (1st Planting)	**% Emergence/Seedling Vigor** >50% *vs.* >50% on 6/3/03 **Initial Stand Count Percentage** >80% *vs.* >80% on 6/9/03 **Final Stand Count Percentage** 40-60% *vs.* >80% on 6/23/03
Sabana Grande/PR (2nd and 3rd Plantings)	**% Emergence/Seedling Vigor** >50% *vs.* >50% on 7/16/03 & 8/27/03 **Initial Stand Count Percentage** >80% *vs.* >80% on 7/28/03 & 9/8/03 **Final Stand Count Percentage** 40-60% *vs.* >80% on 8/11/03 & 9/22/03

All sites reported normal growth and development of the soybean plants from seedling emergence through flowering, pod development and seed fill. The Champaign Co. site reported delayed emergence due to dry soil conditions. A killing frost occurred 10/2/03.

County or District/State or Territory	Observations/Dates		
	Fungi/Diseases	**Insect Pests**	**Beneficial Insects**
Carroll/IA	None observed 6/3/03, 6/21/03, 7/18/03 and 10/1/03.		
Dallas/IA-Loc.1	None observed 5/31/03, 6/14/03, 7/10/03, 9/3/03 and 10/2/03.		
Dallas/IA-Loc.2	None observed 5/31/03, 6/14/03, 7/10/03, 9/3/03 and 10/3/03.	Light infestation of bean leaf beetle observed 5/31/03.	None observed 5/31/03, 6/14/03, 7/10/03, 9/3/03 and 10/3/03.
Dallas/IA-Loc.3	Moderate levels of iron deficiency chlorosis seen 6/25/03, 7/14/03, 9/7/03 and 10/4/03 on both plant types.	None observed 6/25/03, 7/14/03, 9/7/03 and 10/4/03.	
Des Moines/IA	None observed 5/29/03, 6/16/03, 7/6/03, 8/27/03 and 9/28/03.		
Madison/IA	None observed 6/20/03, 7/10/03, 9/20/03 and 10/7/03.	None observed 6/5/03, 6/20/03, 9/20/03 and 10/7/03.	
Marshall/IA	None observed 6/1/03, 6/18/03, 7/19/03 and 10/5/03.		
Champaign/IL (1st and 2nd Plantings)	Light infestation of brown spot and bacterial blight observed 7/23/03 and 8/21/03 on both plant types.	Light to moderate infestations of leafhoppers, bean leaf beetles, Japanese beetles and soybean aphids observed 6/16/03, 7/23/03 and 8/21/03.	Ladybugs and spiders observed 7/23/03 and 8/21/03.
Champaign/IL (3rd Planting)	None observed 9/3/03, 9/18/03 and 10/2/03.	Bean leaf beetles and Western corn rootworms observed on both plant types 9/18/03 and 10/2/03 but no damage evident.	Ladybugs observed 9/3/03, 9/18/03 and 10/2/03.
York/NE	None observed 6/17/03, 7/8/03, 8/1/03 and 9/4/03.	Light populations of bean leaf beetles, loopers, grasshopper and blister beetles observed 6/17/03, 7/8/03, 8/1/03 and 9/4/03.	Ladybugs observed 7/8/03, 8/1/03 and 9/4/03.
Sabana Grande/PR (1st Planting)	None observed 6/9/03, 6/23/03, 7/15/03 and 8/14/03.	Light populations of loopers and whiteflies seen 6/23/03, 7/9/03 and 8/15/03.	Ladybugs seen 7/15/03.
Sabana Grande/PR (2nd and 3rd Plantings)	None observed 7/28/03, 8/11/03, 9/4/03, 9/8/03, 9/22/03 and 11/17/03.	Light populations of loopers and whiteflies observed 8/11/03, 8/29/03, 9/22/03, 10/9/03 and 11/17/03.	Ladybugs observed 8/11/03, 9/10/03, 9/22/03 and 10/21/03.

Results: No insect susceptibility or disease susceptibility or resistance differences were observed between the transgenic soybean plants and its nontransgenic counterpart. Some phenotypic differences were observed between the transgenic and nontransgenic plants in terms of the level of tolerance to glyphosate and isoxazole herbicide treatments.

Plant Disposition: See below:

County or District/ State or Territory	Harvest or Crop Destruct Date(s)	Disposition of Remaining Plant Material
Carroll/IA	10/1/03	Plot area mechanically cultivated.
Dallas/IA	10/2/03, 10/3/03 & 10/4/03	Plot area disked under 10/4/03.
Des Moines/IA	9/28/03	Plot area mechanically cultivated.
Madison/IA	10/7/03	Plot area mechanically cultivated.
Marshall/IA	10/5/03	Plot area disked under 10/5/03.
Champaign/IL	9/15/03, 10/2/03, 10/7/03 & 10/10/03	Trial areas mowed and disked under 10/10/03.
York/NE	9/4/03	Plot area disked under.
Sabana Grande/PR	8/15/03, 11/17/03, 11/20/03 & 12/3/03	Trial areas mechanically cultivated 9/1/03 and 12/3/03.

Volunteer Monitoring: The plot areas were visually inspected for volunteer soybean plants during the following growing season. The table below summarizes observations made and actions taken to eliminate volunteer plants.

Post-Season Volunteer Monitoring			
County or District/State or Territory	**Date**	**No. Plants Observed/Stage**	**Method of Destruction**
Carroll/IA	4/15/04	None	
	5/12/04	11 to 50 plants	Mechanically cultivated
	6/9/04	>50 plants/V1	Removed plants by hand.
	7/6/04	None	
	8/11/04	None	
Dallas/IA-Loc.1	4/17/04	None	
	5/15/04	1 to 10 plants	Mechanically cultivated
	6/11/04	>50 plants	Atrazine applied.
	7/6/04	None	
	8/11/04	None	
Dallas/IA-Loc.2	4/17/04	None	
	5/15/04	1 to 10 plants	Mechanically cultivated
	6/11/04	11 to 50 plants	Atrazine applied.
	7/6/04	None	
	8/11/04	None	
Dallas/IA-Loc.3	4/17/04	None	
	5/15/04	1 to 10 plants	Mechanically cultivated
	6/11/04	11 to 50 plants	Atrazine applied.
	7/6/04	None	
	8/11/04	None	
Des Moines/IA	4/19/04	None	
	5/17/04	1 to 10 plants	Mechanically cultivated
	6/10/04	11 to 50 plants	Removed plants by hand.
	7/8/04	None	
	8/12/04	None	

Post-Season Volunteer Monitoring			
County or District/State or Territory	**Date**	**No. Plants Observed/Stage**	**Method of Destruction**
Madison/IA	4/16/04	1 to 10 plants	Removed plant by hand.
	5/14/04	11 to 50 plants	Mechanically cultivated
	6/11/04	>50 plants	Removed plants by hand.
	7/6/04	None	
	8/11/04	None	
Marshall/IA	4/15/04	None	
	5/12/04	1 to 10 plants	Removed plants by hand and field mechanically cultivated.
	6/9/04	11 to 50 plants	Removed plants by hand.
	7/8/04	None	
	8/12/04	None	
*Champaign/IL (1st and 2nd Plantings)	5/20/04	>50 plants/V3	Callisto herbicide applied and field mechanically cultivated.
	6/28-30/04	>50 plants/V1-V5	Removed plants by hand.
	7/13/04	None	
	9/7/04	None	
	9/13/04	>50 plants/V1-R1	Removed plants by hand
Champaign/IL (3rd Planting)	5/28/04	None	Callisto herbicide applied and the field was mechanically cultivated, despite no presence of volunteers.
	6/11/04	None	
	6/25/04	None	
	6/29/04	None	
	7/13/04	None	
York/NE	5/3/04	None	
	6/4/04	None	
	7/1/04	None	
	7/30/04	None	
Sabana Grande/PR (1st Planting)	9/16/03	>50 plants/V2	Mechanically cultivated
	9/30/03	11 to 50 plants/V3	Mechanically cultivated
	10/17/03	1 to 10 plants/V4	Mechanically cultivated
	10/31/03	None	
	11/14/03	None	
Sabana Grande/PR (2nd & 3rd Plantings)	11/24/03	>50 plants/V2	Mechanically cultivated
	12/10/03	>50 plants/V2	Mechanically cultivated
	12/22/03	11 to 50 plants/V2	Mechanically cultivated
	1/2/04	None	
	1/16/04	None	
	2/6/04	None	
	2/26/04	None	
	3/24/04	None	

*Volunteer monitoring of this plot will continue in 2005 to ensure the elimination of all volunteer soybean plants.

Weediness Characteristics: There was no evidence of change in characteristics that would enhance survival of the transgenic soybean plants as compared to the nontransgenic soybean plants. No difference in weediness characteristics was observed between the transgenic and nontransgenic soybean lines.

Non-Target Organisms: No adverse effect on non-target organisms from either the transgenic or nontransgenic plants was observed during any of the trials.

Field Management/Experimental Treatments: As follows:

County or District/ State or Territory	Field Management/Experimental Treatments
Carroll/IA	Pursuit herbicide applied ~6/20/03.
Dallas/IA	For two locations, Pursuit herbicide was applied to half of the plot, while Roundup was applied to the other half. Only Pursuit herbicide was applied at the third location.
Des Moines/IA	Pursuit herbicide applied ~6/20/03.
Madison/IA	Pursuit herbicide was applied to half of the plot, while Roundup was applied to the other half on 6/20/03.
Marshall/IA	Pursuit herbicide applied ~6/20/03.
Champaign/IL	Made multiple herbicide treatments containing glyphosate and HPPD inhibiting herbicides.
York/NE	No special field management techniques.
Sabana Grande/PR	Roundup applied.

Weather Synopsis: Climatic conditions were as follows:

County or District/ State or Territory	Weather Conditions
Carroll/IA Dallas/IA Des Moines/IA Madison/IA Marshall/IA	Spring was wet and cool, while August was very hot and dry.
Champaign/IL	Initially dry, but later becoming normal
York/NE	Weather was dry and hot.
Sabana Grande/PR	Typical

Containment Measures: Transgenic soybean plants were separated from other soybean plants not a part of the trial by distances up to 100 feet. In addition, some sites planted border rows that were destroyed at the conclusion of the trial.

The test plots including border areas were monitored the next growing season for volunteer soybean plants.

Bayer CropScience

USDA Termination Report for Herbicide Tolerant Soybean

M.S. Technologies, LLC

Notification#: 07-065-121n
07-211-105n

Applicant: M.S. Technologies, LLC

Regulated Article: Glyphosate and Isoxazole Tolerant Soybean designated FG72

Release Site Information:

Notification #	Release Site	Planting Date	Area Planted	Harvest Date
07-065-121n	Dallas County, IA	5-15-07	.1 acre	9-24-07
07-211-105n	Miami Dade County, FL	10-15-07	2.7	5-30-08

Purpose: 07-065-121n: The purpose of this planting was for seed increase and breeding.
07-211-105n: The purpose of this planting was for seed increase and breeding.

Notes:

		FG72	Jack (non transgenic)
07-065-121n (observations taken September 2007)	Herbicide Treatments	Glyphosate applied at least twice during season	Non applied
	Hilum Color	Yellow	Yellow
	Pod Color	Brown	Brown
	Pubescence Color	Gray	Gray
	Diseases Observed	None observed	None observed
07-211-105n (observations taken May 2008)	Herbicide Treatments	Glyphosate applied at least once during season	Non applied
	Hilum Color	Yellow	Yellow
	Pod Color	Brown	Brown
	Pubescence Color	Gray	Gray
	Diseases Observed	None observed	None observed

At both locations, field sites were visited multiple times per week through the end of harvest. All plants exhibited normal growth characteristics throughout the growing season at both locations. Pollinations and seed development occurred normally. No differences in insect or disease resistance or susceptibility were observed at either location.

Containment:

Equipment used in the trial areas was cleaned of plant materials while inside the trial areas. Remaining plant materials at the completion of the studies were tilled into the soil. Field sites were monitored for volunteer plants monthly for a period of one growing season after termination. No volunteer plants were observed at either site.

_____ Date 7/14/09

Justin T. Mason
M.S. Technologies, LLC

Bayer CropScience

USDA Termination Report

Notification # 08-057-116n

Glyphosate and Isoxazole tolerant Soybean

Release Site Information:

Release Site	County, State	Planting Date	Acreage Planted	Termination Date
Stuttgart	Arkansas, Arkansas	Not planted	0	n/a
Fithian	Vermillion, Illinois	6-18-08	2.2	10-22-08
Sheridan	Hamilton, Indiana	Not planted	0	n/a
Sharpsville	Tipton, Indiana	5-29-08	4.0	9-28-08
Marcus	Cherokee, Iowa	5-17-08	3.2	11-3-08
Perry and Adel	Dallas, Iowa	5-8-08	37.4	10-6-08
Mediapolis	Des Moines, Iowa	5-22-08	2.4	10-4-08
Scranton	Greene, Iowa	5-19-08	4.0	10-10-08
Iowa Falls	Hardin, Iowa	5-20-08	3.5	11-2-08
Winterset	Madison, Iowa	5-20-08	4.0	10-6-08
Corunna	Shiawasse, Michigan	5-30-08	.5	~9-1-08
Vernon Center	Blue Earth, Minnesota	5-13-08	1.2	~9-1-08
Osborn	Clinton, Missouri	5-29-08	4.0	10-5-08

Purpose: Release was for the purpose of breeding, evaluating efficacy, collecting analytical data on the herbicide tolerant plants, and evaluating agronomic characteristics. Performance of transgenic soybean plants in comparison to the non transgenic counterpart was also evaluated along with agronomic characteristics.

Observations:

Release Site		Planting Date	Avg Days to Emergence	Avg Days to Flowering	Avg Days to Maturity
Fithian, IL	Transgenic	6-18-08	6	37	118
	Non-transgenic	6-18-08	6	36	118
Sharpsville, IN	Transgenic	5-29-08	6	46	118
	Non-transgenic	5-29-08	6	46	118
Marcus, IA	Transgenic	5-17-08	13	53	137
	Non-transgenic	5-17-08	13	52	137
Adel, IA	Transgenic	5-8-08	11	54	138
	Non-transgenic	5-8-08	11	54	136
Perry, IA	Transgenic	5-21-08	11	45	128
	Non-transgenic	5-21-08	11	45	128
Mediapolis, IA	Transgenic	5-22-08	10	45	124
	Non-transgenic	5-22-08	9	46	124
Scranton, IA	Transgenic	5-19-08	11	45	131
	Non-transgenic	5-19-08	11	45	131
Iowa Falls, IA	Transgenic	5-20-08	12	52	134
	Non-transgenic	5-20-08	12	52	135
Winterset, IA	Transgenic	5-20-08	12	49	130
	Non-transgenic	5-20-08	12	49	129
Osborn, MO	Transgenic	5-29-08	8	41	120
	Non-transgenic	5-29-08	7	40	119

Normal growth and development of the transgenic soybean plants was observed at all sites and at all stages of growth and development.

Release Site	Observation Date	Insects Observed	Diseases Observed
Fithian, IL	7-26-08	Less than 1 bean leaf beetle per 10 ft of row, evidence of Japanese beetles was present but no beetles were observed, leafhoppers averaged less than 3 per 10 ft of row	none
Fithian, IL	9-11-08	<50 aphids per plant in all plots, white flies present in low numbers in all plots, 3 Japanese beetles seen in entire trial, bean leaf beetles present at 1 beetle per ft of row	Brown Spot lesions covered 5% of total leaf area in all plots, rare Frogeye Leaf Spot observed with no difference between plots, Cercospora Leaf Blight was seen in one transgenic plot and one non-transgenic plot, Downy Mildew and Bacterial Blight were also observed throughout the trial
Sharpsville, IN	7-26-08	Bean leaf beetles at 6 beetles per 10 ft of row, a few leafhoppers were also observed	Brown Spot was present in all plots on the lowest trifoliate leaves
Sharpsville, IN	9-11-08	Heavy infestation of bean leaf beetles	Sudden Death Syndrome observed in all plots, Brown spot observed on all plants
Marcus, IA	5-27-08	None observed	None observed
Marcus, IA	6-24-08	None observed	Brown Spot observed on nearly all unifoliate leaves
Adel, IA	7-14-08	<1 bean leaf beetle per ft of row	Brown Spot present on bottom leaves of plants
Adel, IA	9-3-08	Consistent aphids in all plots, one lady beetle per plant in all plots, evidence of bean leaf beetle feeding in all plots but none were observed	Downy Mildew, Bacterial Blight, Cercospora Leaf Spot, Brown Spot, and Frogeye Leaf Spot found in all plots
Perry, IA	7-14-08	A few leafhoppers observed	Brown Spot on bottom trifoliates, occasional leaves with Bacterial Blight
Perry, IA	9-10-08	Bean leaf beetles at 1-3 per ft of row	Downy Mildew, Bacterial Blight, Cercospora Leaf Spot, Brown Spot,and Frogeye Leaf Spot observed in all plots

Release Site	Observation Date	Insects Observed	Diseases Observed
Scranton, IA	6-25-08	None observed	A few Brown Spot lesions observed
Scranton, IA	9-15-08	Aphids in all plots, a few bean leaf beetles seen in all plots	Cercospora Leaf Spot, Bacterial Leaf Blight, Brown Spot, and Frogeye Leaf Spot found throughout all plots
Winterset, IA	7-14-08	2 bean leaf beetles observed in entire trial, a few leafhoppers found in all plots	None observed
Winterset, IA	9-3-08	Extreme infestation of aphids in all plots, a few lady beetles in all plots	Brown Spot on lowest leaves of all plants, Bacterial Blight found at same occurrence in all plots
Osborn, MO	8-1-08	<1 bean leaf beetle per ft of row	Brown Spot present on bottom leaves in all plots, Bacterial Blight observed at low incidence
Osborn, MO	9-17-08	<1 bean leaf beetle per ft of row, stink bugs at 1 per 6 ft of row	Cercospora Leaf Spot and Brown Spot consistent throughout plots, a few Frogeye Leaf Spot lesions observed throughout plots

No differences in insect or disease susceptibility or resistance were observed between transgenic plants and non transgenic plants.

Disposition of Remaining Plant Material:

Release Site	County, State	Harvest Date/Destruction Date	Disposition of Remaining Plant Material
Fithian	Vermillion, Illinois	10-22-08	Plot area tilled under
Sharpsville	Tipton, Indiana	9-28-08	Plot area tilled under
Marcus	Cherokee, Iowa	11-3-08	Plot area tilled under
Perry and Adel	Dallas, Iowa	10-6-08	Plot area tilled under
Mediapolis	Des Moines, Iowa	10-4-08	Plot area tilled under
Scranton	Greene, Iowa	10-10-08	Plot area tilled under
Iowa Falls	Hardin, Iowa	11-2-08	Plot area tilled under
Winterset	Madison, Iowa	10-6-08	Plot area tilled under
Corunna	Shiawasse, Michigan	~9-1-08	Plot tilled under and destroyed before harvest
Vernon Center	Blue Earth, Minnesota	~9-1-08	Plot tilled under and destroyed before harvest
Osborn	Clinton, Missouri	10-5-08	Plot area tilled under

Summary:

Field sites were visited at least once every two weeks during the duration of the growing season for purposes of monitoring and evaluation of transgenic and non transgenic plants. Normal growth and development of both the transgenic and non transgenic soybeans was observed at all locations throughout the entire growing season. No differences in insect or disease resistance or susceptibility were observed between transgenic and non transgenic plots. No changes or differences in weediness characteristics were observed in the transgenic plants versus non transgenic controls. No adverse effects on non-target organisms were observed in any of the trials. All plant material was handled in accordance with regulations.

Bayer CropScience

USDA Termination Report for Herbicide Tolerant Soybean

Permit #: 08-219-102n

Permittor: USDA

Permittee: M.S. Technologies, LLC

Regulated Article: Glyphosate and Isoxazole tolerant soybean designated FG72

Release Site Information:

Release Site	Planting Date	Area Planted	Harvest/Destruct Dates
Miami Dade County, FL	10-20-08	0.4 acres	~02-07-09
Miami Dade County, FL	02-14-09	0.7 acres	05-30-09

Purpose: The purpose of this planting was for seed increase and breeding.

Notes:

Observations taken 4/1/09	FG72	Non transgenic
Herbicide Treatments	Dual used at planting and Roundup applied prior to flowering	Dual used at planting
Insects Observed	Low to no damage, clean of insects.	Low to no damage, clean of insects.
Diseases Observed	None	None
Plant Height	9" to 10"	9" to 10"
Stands	15 to 18 plants per meter of row	15 to 18 plants per meter of row
Pod Wall Color	Brown	Brown
Pubescence Color	Gray	Gray
Hilum Color	Yellow	Yellow
General Description	Plants look good with good leaf development. Weather- hot and sunny 85 degrees during the day and 72 degrees at night	Plants look good with good leaf development. Weather- hot and sunny 85 degrees during the day and 72 degrees at night

There were no unanticipated or abnormal occurrences observed in either the transgenic or the non-transgenic soybeans. Plant growth and development and insect or disease susceptibility or resistance did not show any variance between the transgenic and non-transgenic soybeans. No adverse effect on non-target organisms was observed in either the transgenic or the non-transgenic plants. The field was visited at least every 5 days throughout the growing season.

Containment: The field site was surrounded by fallow isolation of at least 10 ft. Equipment used at the field site was cleaned while inside the regulated area. Remaining plant materials were burned on site after field work was complete. The site will be monitored for volunteer plants.

Justin T. Mason

Date 7/7/09

Biotechnology and Regulatory Affairs, M.S. Technologies, LLC.

Appendix 2

MATERIALS AND METHODS- PRODUCT CHARACTERIZATION

2.A. Materials and methods for molecular characterization - DNA tests

Materials

DNA for the analyses was isolated from leaves of event FG72 soybean and the control (Jack) produced in the greenhouse. The references included the plasmid pSF10 that was used to produce soybean event FG72. For Southern blot analysis of soybean genomic DNA, digested plasmid pSF10 DNA (approximately 0.1 or 1 genomic copies equivalent) was mixed with Jack genomic DNA (negative control) and separated by electrophoresis on agarose gels. Phage Lambda (*Pst*I or *Hind*III digested) was used as a molecular weight marker for size estimation of the DNA fragments.

Identity of the materials

The identity of the greenhouse produced material was confirmed by PCR prior to use in order to verify the presence or absence of FG72, as appropriate. The zygosity status of the harvested plants was determined by means of zPCR when necessary. The integrity of the isolated DNA was verified in each Southern analysis by observation of the DNA samples on an ethidium bromide stained agarose gel. The identity of the materials used in generational stability analyses was confirmed by chain-of-custody documents and by PCR analysis.

DNA preparation for Southern blot and PCR analyses

Harvested plant tissues were directly transferred and frozen in liquid nitrogen, then stored in an ultralow freezer until DNA extraction. Genomic DNA was isolated following standard procedures, and stored at 4°C. Plasmid DNA was prepared from an *E. coli* cell strain containing plasmid pSF10. Concentration of the DNA extracts was determined by measurement with PicoGreen®. DNA was digested with restriction enzymes following the procedure indicated by the manufacturer.

Southern blot analysis of genomic DNA

Digested genomic DNA samples were subjected to electrophoresis on 1% TAE agarose gels and transferred to nylon membranes following standard procedures (Sambrook *et al*., 1989).

An appropriate dilution of the restriction enzyme digested pSF10 was prepared, and an amount representing 0.1 or 1 plasmid copies per soybean genome was added to a digested non-transgenic DNA sample. This reconstitution sample served as a positive control and was used to show that the hybridizations were performed under conditions allowing hybridization of the probe with target sequences. Phage Lambda DNA digested with *Pst*I or *Hind*III was included as a size standard.

DNA probe preparation

The T-DNA probe template was prepared by means of a SacI/SmaI restriction digestion of the pSF10 plasmid DNA, according to the conditions proposed by the manufacturer. To purify the probe template, the complete reaction mixture of the restriction digestion was loaded on a 1% TAE agarose gel, the fragment of 7204 bp was cut out of the gel and the probe template was isolated from the gel slice using the QIAquick Gel Extraction Kit (Qiagen).

The Ph4a748B probe template was synthesized by means of PCR amplification using the Expand™ High Fidelity PCR system and pSF10 plasmid. Each primer has two priming sites in plasmid pSF10; therefore, two amplicons were obtained. After loading the complete reaction mixture on a 1% TAE agarose gel, the 430 bp amplicon was cut out of the gel and the probe template was isolated from the gel slice using the QIAquick Gel Extraction Kit (Qiagen).

The DNA templates were labeled with [α-^{32}P]-dCTP using the Ready-to-go DNA labeling beads (GE Healthcare). Unincorporated nucleotides were removed by separation on a micro Bio-Spin® 30 column (Bio-Rad).

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 2mEPSPS and HPPD W336 proteins expressed by *Escherichia coli* (*E.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 proteins produced in *E.coli* are representative of 2mEPSPS and HPPD W336 proteins produced in FG72 soybean plants.

2mEPSPS

Equivalency of *E.coli*-produced 2mEPSPS protein and plant-produced protein was established via SDS-PAGE, western blot, N-terminal amino acid sequencing, enzyme activity and LC/MS methods.

Materials

The plant-produced 2mEPSPS protein was isolated from greenhouse-grown plants of double-herbicide-tolerant soybean. The identity of the plants was confirmed by PCR of the planted seed. Leaf extract was purified on an antibody affinity column, and the purified protein solution was stored at -20°C or lower until further analyses were performed. The antibody affinity column used for this purification was purchased from Pierce (Rockford, IL, product number 44894), and was prepared using a covalently attached polyclonal antibody specific for 2mEPSPS.

The 2mEPSPS protein reference standard (BCS reference standard, Batch N° LEJ5837, purity >99%) was produced in *E. coli*, and purified following a modification of the method of Priestman *et al.* (2005). The protein solution is stored in an Ultrafreezer.

Analysis by N-terminal sequencing

The affinity purified FG72 soybean produced 2mEPSPS protein, the non-transgenic (NT) Jack soybean extract and the *E.coli* produced 2mEPSPS *protein* were loaded onto the PVDF membrane at Catalent in San Diego, CA for analysis of the N-terminal amino acid sequence of the proteins by Edman degradation. The protein bands of interest were excised from the PVDF membranes and placed into labeled Eppendorf tubes. The PVDF strips of *E. coli* produced 2mEPSPS protein, FG72 soybean produced 2mEPSPS and NT Jack soybean controls were placed into the center of the Procise Sequencing cartridge blocks. Sequencing was performed on the first 5 residues using a pulsed liquid method for PVDF on an Applied Biosystems Procise 494 protein sequencer. Amino acids were identified by comparison with the retention times of PTH amino-acid standards analyzed at the beginning of the sequence.

Analysis by SDS-PAGE

SDS-PAGE was performed using a Novex Bis-Tris 10% polyacrylamide gel (InVitrogen, CA, product number NP0301) and a MOPS SDS running buffer (cat# NP0001) according to the manufacturer's instructions. The 2mEPSPS protein isolated from FG72 soybean leaves, the

corresponding protein from *E.coli* and the NT Jack soybean sample were denatured and analyzed by electrophoresis on a denaturing polyacrylamide gel where mobility can be correlated to molecular weight. The gel was then stained with Pierce Imperial™ Stain to visualize the protein bands. The gel was stained with Pierce Imperial™ Protein Stain (cat# 24615) for 1 hour and then destained in water over-night. For the first 2 hours of destaining, the water was changed every 30 minutes. BenchMark™ molecular weight markers from InVitrogen Life Technologies (product number 100747-012) were used.

Analysis by western blotting

Western blotting was performed in the same electrophoresis system used for SDS-PAGE and the gel was blotted to a PVDF membrane (Bio-Rad, Immun-Blot PVDF Membrane, cat# 162-0174) according to the instructions provided by Bio-Rad and InVitrogen. The proteins in the gel were transferred out of the gel perpendicular to the direction of the first electrophoresis. They were adsorbed to the membrane giving an exact replica of the positions of all the proteins in the gel. The membrane was washed with TBS (Tris buffer saline, Bio-Rad, cat # 170-6435) for 10 minutes and blocked for 1 hour with 5% non-fat milk in TBS. The membrane was washed twice with TTBS (Tween Tris-buffer saline, Bio-Rad, cat #170-6531 and cat #170-6435) and once with TBS and incubated for 2 hours with rabbit polyclonal antibodies raised against the 2mEPSPS protein. The membrane was washed twice with TTBS and once with TBS and incubated over-night with the enzyme conjugate, a second antibody with a horse radish peroxidase (HRP) linked anti-rabbit antibody. After the second antibody incubation, the membrane was washed with TTBS and TBS. The color was developed according to the manufacture's instructions (BIO-RAD, LIT178RevC). All reagents except the rabbit polyclonal anti-2mEPSPS antibodies used for western blotting were obtained from Bio-Rad as the HRP Conjugate Substrate kit (cat # 170-6431) which included the blotting grade goat anti-rabbit IgG HRP conjugate (cat# 170-6515). The rabbit polyclonal anti-2mEPSPS antibodies were obtained from Bayer BioScience in Gent, Belgium.

Analysis by HPLC/Electrospray Mass Spectrometry

This analysis was performed at Catalent in San Diego, CA, USA.The 2mEPSPS protein from *E. coli* and the 2mEPSPS protein from the NT Jack soybean extracts were denatured for 1 hour at 37 °C in Rapid Gest (Waters Corporation, 1 mg/mL) containing 10 mM dithiothreitol (DTT). The denatured protein disulfide bonds were alkylated with iodoacetamide for 30 minutes in the dark and digested with trypsin for 1 hour at 37°C. A full scan analysis was performed on the *E. coli* produced 2mEPSPS protein trypsin digest, the trypsin digest of the NT extract and the trypsin digest of the FG72 soybean produced 2mEPSPS protein to determine the location and identification of the peptides.

Analysis of enzymatic activity

The enzymatic activity of the FG72 soybean produced 2mEPSPS protein was determined using the Gent SOP BBS 07/74/02 with modifications to adjust for sample concentration. The activity is determined by measuring the release of inorganic phosphate using a malachite green dye at a wavelength of 660 nm. 2mEPSPS catalyzes the reaction of shikimate-3-phospate and phosphoenolpyruvate to form 5-enolpyruvylshikimate-3-phosphate and phosphoric acid. The 5-enolpyruvylshikimate-3-phosphate then eliminates phosphoric acid to form chorismate. An increase in the detection of inorganic phosphate release above 10% indicates the protein is active.

HPPD W366

Equivalence of *E.coli*-produced HPPD W336 protein and plant-produced protein was established via SDS-PAGE, western blot, N-terminal amino acid sequencing, enzyme activity and HPLC/Electrospray Mass Spectrometry methods.

Materials

The plant-produced HPPD W336 protein was isolated from greenhouse-grown plants of double herbicide-tolerant-soybean for event FG72. The identity of the plants was confirmed by PCR. Leaf extract was purified using HPPD W336 monoclonal antibody affinity column, and the purified protein solution was stored at 4°C or lower until further analyses were performed. The antibody affinity column used for this purification was purchased from Pierce (Rockford, IL, product number 44894), and was prepared using a covalently attached monoclonal antibody specific for HPPD W336.

The HPPD W336 protein reference standard (BCS reference standard, Batch N° LB18050, purity >96%) was produced in *E. coli*, and purified following a modification of the method of Priestman *et al.* (2005). The protein solution is stored in an Ultralowfreezer.

Analysis by N-terminal sequencing

The affinity purified *E.coli* produced HPPD W336 protein was loaded onto the PVDF membrane of a sample preparation cartridge according to manufacturer's instructions. The completed analysis of the N-terminal amino acid sequence of the protein by Edman degradation was performed at Catalent, San Diego, CA, USA.

Analysis by SDS-PAGE

SDS-PAGE was performed using a InVitrogen 10% BisTris polycarylamide gel (InVitrogen, CA, cat# NP0001) and a MOPS SDS running buffer according to the manufacturer's instructions. The gel was stained with Pierce Imperial™ stain (cat# 24615) for 1 hour and then destained in water over-night. For the first 2 hours of destaining, the water was changed every 30 minutes. BenchMark™ molecular weight markers from InVitrogen Life Technologies (product number 100747-012) were used.

Analysis by western blotting

Western blotting was performed in the same electrophoresis system as used for SDS-PAGE and the gel was blotted to PVDF membranes (Bio-Rad, Immun-Blot PVDF Membrane, cat#162-0174) according to the instructions provided by BioRad. The proteins in the gel were transferred out of the gel perpendicular to the direction of the first electrophoresis using a 10mM CAP buffer, pH 11. The proteins were adsorbed to the membrane giving an exact replica of the positions of all the proteins in the gel. The membrane was washed with TBS (Tris buffer saline, Bio-Rad, cat # 170-6435) for 10 minutes and blocked for 1 hour with 5% non-fat milk in TBS. The membrane was washed twice with TTBS (Tween Tris-buffer saline, Bio-Rad, cat #170-6531 and cat #170-6435) and once with TBS and incubated for 2 hours with mouse monoclonal antibodies raised against the HPPD W336 protein. The membrane was washed twice with TTBS and once with TBS and incubated over-night with the enzyme conjugate, a second antibody with a horse radish peroxidase (HRP) linked anti-mouse antibody. After the second

antibody incubation, the membrane was washed twice with TTBS and once with TBS. The color was developed according to the manufacture's instructions (BIO-RAD, LIT178RevC). All reagents except the mouse monoclonal anti-HPPD antibodies used for western blotting were obtained from BIO-RAD as the HRP Conjugate Substrate kit (cat # 170-6431) which contains the blotting grade goat anti-mouse IgG HRP conjugate (cat# 170-6516). The mouse monoclonal anti-HPPD antibody was obtained from M.S. Technologies (US Patent application USSN 12/609,200)

Analysis by HPLC/Electrospray Mass Spectrometry

The *E. coli* produced HPPD W336 protein, the NT Jack soybean extract and the FG72 soybean produced HPPD W336 protein were denatured for 1 hour at 37 °C in Rapid Gest (Waters Corporation, 1mg/mL) containing 10 mM dithiothreitol at Catalent in San Diego, CA, USA. The denatured protein disulfide bonds were alkylated with iodoacetamide for 30 minutes in the dark and digested with trypsin for 2 hour at 37°C. A full scan analysis was performed on the *E. coli* produced HPPD W336 protein trypsin digest, the trypsin digest of the NT extract and the trypsin digest of the FG72 soybean produced HPPD W336 protein to determine the location and identification of the peptides.

Analysis of enzymatic activity

HPPD catalyzes the reaction of 4-hydroxyphenylpyruvate (HPP) with oxygen to form homogentisate. The activity assay, which is a colorimetric method, measures the amount 2,4-dinitrophenylhydrazine (DNP) derivatized HPP remaining in the assay mixture at the end of the incubation and derivitization period. The wavelength monitored for the derivatized HPP is 405 nm. An activity of at least 10% is considered to be active.

2.C. Materials and methods for protein levels in grain

Seed samples analyzed in this study were produced under field conditions in 2008. The ten field trials supplying samples for this study were performed under M.S. Technologies, LLC. The seed for all soybean grown, analyzed, and reported herein was supplied by M.S. Technologies LLC, Adel, Iowa. The field-produced seed samples were assayed by PCR testing to confirm their identity.

An *E.coli* produced 2mEPSPS protein standard (batch # LEJ5838) and HPPD W336 standard (batch# LB020309) were used as reference materials for analysis, and to fortify non-transgenic samples for validation and recovery studies.

Field design

Soybean plants containing the double-herbicide-tolerant soybean event FG72 and soybean plants representing the non-transgenic (non-transformed) counterpart "Jack" were grown in the field for Bayer CropScience by M.S. Technologies, LLC. Trials were conducted in EPA Regions V in the following locations: Trial number 01-Cherokee county, Iowa; Trial number 02-Hardin County, Iowa; Trial number 03-Greene county, Iowa, Trial number 04-Dallas County, Iowa; Trial number 05-Dallas County, Iowa; Trial number 06-Madison County, Iowa; Trial number 07-Clinton County, Missouri; Trial number 08-Vermillion County, Illinois;

Trial number 09-Tipton County, Indiana and Trial Number 10-Des Moines County, Iowa, which are typical soybean growing regions of the United States. The plants in this study were grown under conditions typical of production practices for Group II maturity soybeans. There were three non-transgenic plots (Regimen A) and six transgenic plots (Regimen B and C) at each test site. The plots were randomized at each trial site. The Regimen A plots were planted with the non-transgenic counterpart variety "Jack" soybeans. The Regimen B and C plots were planted with the transgenic event FG72 soybeans. Three additional plots (Regimen D, E and F) were planted with commercial conventional (non-transgenic) soybeans for reference (Stine® 2686-6, Stine 2788, and Stine 3000-0, respectively). Thus there were 12 plots total at each trial site.

The Regimen C transgenic event FG72 plots were sprayed with isoxaflutole herbicide at a target rate of 70 grams ai/Ha and with glyphosate herbicide at a target rate of 1060 grams ai/Ha, and the other plots were not treated with these herbicides. Ammonium sulfate at 2850 grams/Ha was added to the spray mixture for the Regimen C herbicides. Application of the herbicides was made to the Regimen C plants as a foliar spray at about the V4-V5 growth stage. The herbicide applications were made by Bayer CropScience personnel according to Bayer CropScience protocol HD08NARJX5. To keep the site weed-free, a conventional soil-applied herbicide was sprayed after planting but prior to soybean emergence to all the plots. This herbicide treatment was pendimethalin (1060 grams ai per ha), except for trial 05 in which metolachlor (1880 gm ai/Ha) was used.

Soybean grain was obtained from each test plot at maturity by M.S. Technologies, LLC field personnel. A representative sample of grain from each plot was shipped to the BioAnalytics laboratories of Bayer CropScience in Research Triangle Park, NC, USA.

Certificates of analysis (COA) were produced by the BCS BioAnalytics Laboratory in Research Triangle Park, NC for seed shipped to the nine field test sites for planting. The data showed that the transgenic soybean seed that was planted in the field was indeed double-herbicide-tolerant soybean event FG72.

Sample preparation

Grain samples were ground in a blender pre-chilled with dry ice. Small amounts of dry ice were added to the blender periodically to ensure the samples remained frozen during preparation. A separate blender was used for each sample. The ground samples were stored in a freezer at approximately -20°C for overnight or longer to allow the dry ice to sublimate before extraction.

Protein extraction

2mEPSPS

Total protein was extracted from the raw agricultural product of soybean grain using an optimized extraction procedure using '2mEPSPS Extraction Buffer' (0.2% SDS in EPSPS (SDIX Catalog number 7000102)) as the extraction/dilution buffer.

A representative fraction (approximately 0.1 g) of ground sample was mixed with 4 mL extraction buffer in a 50 mL polypropylene centrifuge tube, and then shaken for 30 minutes at ~ 4°C on a shaker (IKA-SCHÜTTLER MTS 4) at 250 rpm. The liquid extract was transferred to a clean centrifuge tube and centrifuged at approximately 18000 x g for 10 minutes at ~ 4°C. The clear supernatant was used for 2mEPSPS analyses. Duplicate extracts were prepared for each sample. Total extractable protein was determined as a relative measure of extraction efficiency.

HPPD W336

PBST Extraction/Dilution Buffer, Agdia, Inc. Catalog Number ACC 00501A, was used. A representative fraction (approximately 0.1 g) of ground sample was mixed with 4 mL extraction buffer in a 50 mL polypropylene centrifuge tube, and then shaken for 30 minutes at ~ 4°C on a shaker (IKA-SCHÜTTLER MTS 4) at 250 rpm. The liquid extract was transferred to a clean centrifuge tube and centrifuged at approximately 18000 x g for 10 minutes at ~ 4°C. The clear supernatant was then used for HPPD W336 analyses. Duplicate extracts were prepared for each sample. Total extractable protein was determined as a relative measure of extraction efficiency.

Bioassay

All quantitative determinations of 2mEPSPS protein were conducted at Bayer CropScience, Morrisville, NC. All quantitative determinations of HPPD W336 protein were conducted at Bayer CropScience, Research Triangle Park, NC.

The levels of HPPD W336 and 2mEPSPS proteins were determined by an Enzyme Linked ImmunoSorbent Assay (ELISA) using antibodies specific for each protein.

2mEPSPS

The levels of 2mEPSPS protein were determined by an Enzyme Linked ImmunoSorbent Assay (ELISA) using antibodies specific for the protein. The sample to buffer ratio was 0.1 g of matrix per 4 mL extraction buffer. All ELISA assays were conducted at Morrisville, NC.

Protein standards were included in duplicate on each 2mEPSPS ELISA plate at the following concentrations: 32, 16, 8, 4, 2, 1, and 0.5 ng/mL.

HPPD W336

The amount of HPPD W336 in the total protein extracts was measured using a quantitative ELISA developed by M.S. Technologies (US Patent application USSN 12/609,200). Before the analysis was performed the Limit of Detection (LOD) was determined for seeds.

Serially-diluted sample extracts were applied to ELISA plates at 100 µL/well. This was followed by about 2 hours of incubation on a shaker at 200 rpm at room temperature. After the first incubation, the plate was washed four times using the BIO-TEK EL404 microplate washer. One hundred µL of diluted (1:12500) HPPD antibody conjugate was added to each well and incubated on ELISA plate shaker at approximately 200 rpm for about 2 hours at room temperature. This allowed the protein that was present in the samples to bind to the capture antibody. Unbound material was removed by rinsing the wells 4 times with wash solution. One hundred µL of substrate solution per well was added and the plate was incubated on a shaker at 200rpm for about 30 minutes.

Validation

The ELISA procedures were validated with a non-transgenic sample “Jack”. The standards were added to the extraction buffer at the indicated concentrations prior to extraction in five replicates. Each replicate was analyzed using duplicate wells. A summary of the validation data for each analyte is shown in Table 31.

Table 31. Validation of sample extraction with fortified non-transgenic soybean grain

2mEPSPS ELISA Validation			
2mEPSPS fortified (ng/mL)	2mEPSPS detected (ng/mL)[a] Mean ± SD	% 2mEPSPS Recovery Mean ± SD	2mEPSPS Recovery %CV
100	85.3 ± 3.4	85.3 ± 3.4	3.93
32	26.6 ± 1.5	83.1 ± 4.7	5.63
16	11.9 ± 1.2	74.3 ± 7.7	10.3
8	6.41 ± 0.71	80.1 ± 8.9	11.1
4	3.13 ± 0.37	78.2 ± 9.2	11.7
2[b]	0.09 ± 0.14	4.68 ± 7.0	151
1[b]	-0.16 ± 0.17	-15.8 ± 17	-109
0.5[b]	-0.03 ± 0.33	-5.36 ± 66	-1230

HPPD W336 ELISA Validation			
HPPD W336 fortified (ng/mL)	HPPD W336 detected (ng/mL)[a] Mean ± SD	% HPPD W336 Recovery Mean ± SD	HPPD W336 Recovery %CV
100	123 ± 4	123 ± 4	3.01
32	31.4 ± 1.3	98.2 ± 3.9	4.01
16	18.0 ± 0.9	112 ± 6	5.26
8	8.34 ± 0.54	104 ± 7	6.47
4	3.51 ± 0.24	87.8 ± 6.0	6.78
2	1.43 ± 0.26	71.6 ± 13.1	18.2
1[b]	0.43 ± 0.19	43.0 ± 19.2	44.6

[a] The protein analyte detected and its recovery are expressed as the average of 10 data points from duplicate extracts of 5 samples at each fortification level using non-transgenic matrix. Averages, standard deviations and %CV values are calculated with full precision and then rounded to 2 or 3 significant figures.
[b] Validity criteria were not met at indicated concentration.

Limit of detection and limit of quantification

The limit of detection (LOD) is determined for each matrix using the average standard curve and the concentration derived from the background optical density (OD) of the negative control samples. The LOD is the concentration corresponding to an OD value three standard deviations above the mean background OD.

The LOD is expressed in the unit of concentration (ng/mL) and the unit of weight ratio (ng/g matrix, i.e. ppb) calculated based on the extraction of an amount of the matrix with a known volume of extraction buffer, *e.g.*, 0.1 g of matrix per 4 mL extraction buffer. The estimated LOD are summarized in Table 32 below. An absorbance reading giving rise to a protein analyte concentration above the LOD is assumed to be greater than the zero dose reading.

Table 32. Limits of detection and quantification of 2mEPSPS and HPPD W336 proteins in fortified non-transgenic soybean grain

Protein Analyte	LOD		LOQ	
2mEPSPS	1.50 ng/mL	60.1 ng/g [a]	4 ng/mL	160 ng/g [a]
HPPD W336	0.52 ng/mL	20.8 ng/g [a]	2 ng/mL	80 ng/g [a]

[a] Calculated based on the extraction of 0.1 g matrix per 4 mL of extraction buffer.

The limit of quantification (LOQ) is defined as the lowest concentration of the standard that meets the validity criteria for the LOQ. Validity criteria are a) analyte recoveries from fortified

matrix samples are ≥ 60 % and ≤ 130 % and b) the coefficient of variance (relative standard deviation) is less than 25%. When a lower recovery is caused by the nature of a specific matrix or the effect of a process, the lowest concentration of the standard that gives a coefficient of variance equal to or less than 25% is used as the LOQ. Values below the LOD are reported as zero and values below the LOQ but above the LOD are reported at the LOQ. In calculations, values below the LOD are treated as zero. Values below the LOQ but above the LOD are assumed to be at the LOQ.

Protein Analyte Content

Protein determinations were made in order to confirm that protein was extracted from the samples. SoftMax Pro™ software (Molecular Devices, Version 4.0) was used to derive the concentration of 2mEPSPS and HPPD W336 proteins from the ELISA data. Absorbance units were adjusted for the buffer blank. A set of wells containing samples of the corresponding non-transgenic matrix was included on each plate for background subtraction. The appropriate background corrections for the transgenic grain samples were obtained from background values of a non-transgenic grain sample, which was diluted on the same plate and to the same extent as the transgenic grain sample. Thus the dilution of the non-transgenic sample used for background subtraction was the same as the dilution of the transgenic sample that was required in order to place the OD reading in the center portion of the standard curve. The absorbance readings corrected for both buffer blank and non-transgenic background were converted to the protein concentration using the standard curve.

The ELISA assays give results in units of ng of analyte per milliliter of extract that are then converted into µg of analyte per gram of fresh sample. As different tissues have different protein and water contents, the results are also expressed in dry weight concentrations as well as percent of crude protein. Percent moisture and crude protein (fresh and dry weight basis) data for these samples were obtained from study DQ08B009. Results presented are rounded to 2 or 3 significant figures.

Analyte as percent crude protein is obtained by dividing the fresh weight content of analyte by the % crude protein value, then dividing by 100 for unit conversion, as shown in the following formula.

$$analyte\ as\ \%\ crude\ protein = \frac{\mu g\ analyte\ /\ g}{\%\ crude\ protein\ x\ 100}$$

Dry weight analyte content is obtained by dividing the fresh weight analyte content by the calculated percent dry matter and multiplying by 100. Percent dry matter is obtained by subtracting the percent moisture from 100. The following is the formula used.

$$analyte\ dry\ weight\ content\ \ \mu g / g = \left(\frac{\mu g\ analyte\ /\ g}{(100 - \%\ moisture)} \right) \times 100$$

A summary of the results is given in Table 33.

Table 33. Amounts of 2mEPSPS and HPPD W336 proteins

Protein	Treatment/Entry		Fresh Weight (µg/g)[a]	Dry Weight (µg/g)	Content as % Crude Protein[b]
2mEPSPS	Conventional Treated/B	Range	87 – 180	150 [b]	0.039 [b]
		Mean ± SD	130 ± 22		
2mEPSPS	GLY + IFT Treated/C	Range	87 – 240	150 [b]	0.041 [b]
		Mean ± SD	140 ± 33		
HPPD W336	Conventional Treated/B	Range	0.46– 1.32	0.94 [c]	0.00024 [c]
		Mean ± SD	0.85 ± 0.20		
HPPD W336	GLY + IFT Treated/C	Range	0.41 – 1.31	0.89 [c]	0.00023 [c]
		Mean ± SD	0.80 ± 0.22		

[a] Range and overall mean ± standard deviation in µg/g for the fresh weight protein content in grain samples from the 10 sites.
[b] The dry weight (dw) and % crude protein analyte amounts were calculated using the average 2mEPSPS value of samples from each plot.
[c] The dry weight (dw) and % crude protein analyte amounts were determined using the average of four individual results per sample, therefore no standard deviation or range is given for these amounts.

Statistical analysis

Descriptive statistics (mean, standard deviation, and coefficient of variance) were calculated for each sample matrix and treatment (Devore and Peck, 1986).

Results

The 2mEPSPS and HPPD W336 proteins were detected in all transgenic soybean grain samples. The results show that the expression of the 2mEPSPS and HPPD W336 proteins is similar between sprayed and unsprayed FG72 soybean plants.

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

FG72 transgenic and Jack wild type plants were grown under greenhouse conditions. Leaf samples taken at three different growth stages (V4, V6 and V8), stem and root samples taken at two different growth stages (V4 and V8) and seeds used to grow the plants were analyzed for the HPPD W336 and 2mEPSPS protein content.

The transgenic plants were selected by spraying with glyphosate before harvesting.
The test items used in this study are different tissues from FG72 plants, the reference items are different tissues from WT soybean plants, variety Jack. The samples were directly frozen in liquid nitrogen and shipped on dry ice. Samples were stored at -70°C. Seeds were collected from the same seedlots and were stored at room temperature until crushing.

For all FG72 plants, the FG72 identity and zygosity (homozygous) was confirmed by means of PCR. As control items, the HPPD W336 protein produced in bacteria (Batch no. LB020309) and

the 2mEPSPS protein produced in bacteria (Batch no. LEJ5838) were used to generate a standard curve for the ELISAs.

In order to analyze the presence of HPPD W336 and 2mEPSPS protein in these tissues, samples were crushed, extracted and the total amount of extractable protein (TEP) was determined using the Bradford method. The amount of HPPD W336 in the protein extracts was measured using a quantitative ELISA developed by Agdia (Elkhart, IN 46514, USA). The amount of 2mEPSPS in the protein extracts was measured using an ELISA developed by Strategic Diagnostics Inc. (SDI, Newark, DE, USA).

Extraction

For the expression analysis of HPPD W336 and 2mEPSPS protein, ten separate samples per tissue and per growth stage were chosen randomly and the complete samples were crushed with dry ice in a blender, resulting in homogeneous tissue samples. The powders were collected in pre-cooled 50 mL Falcon tubes, stored at -20 °C overnight to let the dry ice evaporate, and subsequently stored at -70 °C.

The amount of approximately 100 mg crushed material was weighed in pre-cooled 15 mL tubes. A volume of 4 mL extraction buffer was added. Samples were vortexed for 15 seconds or until all powder was in suspension, incubated on a rotary mixer at 30 rpm for 10 minutes at 4 °C and centrifuged at 4000 g for 10 minutes at 4°C. Supernatants were partly transferred to a 1.5 mL eppendorf tube and centrifuged at 20000 g for 5 minutes at 4°C. Clear supernatants were used as such or in dilutions to load on the ELISA plate.

Determination of Total Extractable Protein

After extraction, the total extractable protein (TEP) was measured using the Bradford protein assay with bovine serum albumin (BSA) as reference protein and by measuring the optical density (OD) at 595 nm. Clear supernatants of the extracts were diluted in MQ water so that the total protein concentration of all samples could be fit to the standard curve. The BSA concentrations used to generate the standard curve (36-25-20-15-12-10-5 µg/mL) were prepared using PBST diluted with MQ water to the same extend as the samples, in order to compensate background signal from the PBST buffer. The value of the TEP measurement was used as an internal control to quality check the protein extraction process. The value was not used in any calculation of ELISA results.

The TEP data, determined to assess the quality of the extractions, was per tissue for all samples always in the same range. Therefore, no ELISA results coming from samples having a poor TEP content were excluded.

Determination of Dry Weight

Per soybean tissue and per growth stage, 2 pooled samples were used to determine dry weight. The analysis was performed by SGS Belgium, Agro Food Services, CTS, Antwerpen. Crushed samples were weighed, dried overnight in an oven at 103 ± 2 °C and weighed again. This weight data was used to calculate the % moisture and the % dry weight of the samples. The conversion of the expression data from fresh weight to dry weight was done using the values shown in Table 34.

Table 34 : Results of dry weight determination on FG72 samples

Matrix	Dry weight, as % of Fresh weight		
	Growth stage 1 (V4)	Growth stage 2 (V6)	Growth stage 3 (V8)
Leaf	15.88	18.11	17.23
Stem	8.94	NA	11.44
Root	15.05	NA	13.15
Seed	90.54		

HPPD and 2mEPSPS ELISA

The HPPD ELISA kit from Agdia and the EPSPS ELISA kit commercialized by Strategic Diagnostics Inc. were used to determine respectively the HPPD W336 and the 2mEPSPS content in the protein extracts of FG72 tissues.

Per tissue and growth stage, each of the 10 samples was assayed 3 times on the ELISA plate. In order to fit the concentrations of HPPD W336 and 2mEPSPS to the respective standard curves, the protein extracts had to be diluted as described in Table 35.

Table 35. Final dilutions for protein extracts of the soybean FG72 tissues used for the HPPD and the EPSPS ELISA

Matrix	Final dilutions used for (fold dilution)	
	HPPD ELISA	EPSPS ELISA
Leaf	32	128
Stem	8	16
Root	4 or 8	16
Seed	8	4

Validation

To validate an ELISA for a certain matrix, different concentrations of a standard dilution series are spiked into extraction buffer, which is subsequently used to prepare 5 independent extracts of each soybean WT tissue. Each of the 5 extracts is loaded on the ELISA plate in triplicate, resulting in 15 values for each concentration of the standard curve. An average of all 15 values per spike, the recovery and the coefficient of variance (CV) are calculated.

Validity criteria for the determination of the LOQ are a) analyte recoveries from spiked matrix samples are ≥ 60 % and ≤ 130 % and b) the coefficient of variance is less than 25%. The LOQ is defined as the lowest spiked concentration that meets these criteria.

LOD values were not determined since the expression levels of HPPD W336 and 2mEPSPS were in all transgenic soybean tissues and developmental stages higher than the LOQ. The OD values of the non-transgenic controls were in the range of the buffer blanks. Therefore, the measured absorbances for all samples were corrected with the average signal of at least 2 buffer-only samples (blanks).

The LOQs were expressed as the concentration in ELISA of HPPD W336and 2m EPSPS (ng/ml) and as the amount of HPPD W336 and 2m EPSPS per gram fresh weight (ng/g fw) and per gram dry weight (ng/g dw). The LOQ results are summarized in Tables 36 and 37.

Table 36. LOQ for the HPPD W336 ELISA in different soybean tissues

Matrix	LOQ ng/mL	LOQ ng/g Fresh weight	LOQ ng/g Dry weight
Leaf	1.00	40	252
Stem	1.00	40	447
Root	1.00	40	266
Seed	1.00	40	44

Table 37. LOQ for the EPSPS ELISA in different soybean tissues

Matrix	LOQ ng/mL	LOQ ng/g Fresh weight	LOQ ng/g Dry weight
Leaf	1.88	75.2	474
Stem	1.88	75.2	841
Root	0.94	37.6	250
Seed	1.88	75.2	83

An ELISA absorbance resulting in a HPPD W336 or 2mEPSPS concentration equal to or above the LOQ level is assumed to represent a reliable concentration. Reliable results for the HPPD ELISA were only obtained at concentrations between 1 ng/ml and 8 ng/ml. Concentration above 8 ng/ml had to be diluted before measurement.

The HPPD ELISA plate validation indicated that the measurement of HPPD W336 protein using these plates had an LOQ of 40 ng/g fw for leaf, stem, root and seed. This corresponds to an LOQ of 252 ng/g dw for leaf, 447 ng/g dw for stem, 266 ng/g dw for root and 44 ng/g dw for seed.

The 2mEPSPS ELISA plate validation indicated that the measurement of 2m EPSPS protein using these plates had an LOQ of 75.2 ng/g fw for soybean leaf, stem and seed and 37.6 ng/g fw for root tissue. This corresponds to an LOQ of 474 ng/g dw for leaf, 841 ng/g dw for stem, 250 ng/g dw for root and 83 ng/g dw for seed.

Results

The averages and the range of the HPPD W336 and the 2mEPSPS protein contents per gram fresh weight and per gram dry weight in the different transgenic tissues are given per growth stage in Tables 7 and 8 (Section VI.C.2.). The ranges as well as the averages and standard deviations were derived and calculated from all 30 data points. All values were calculated with full precision and then rounded to 2 or 3 significant figures. The average and SD were calculated on triplicate measurements of the individual samples.

2.E. Materials and methods for agronomic studies

Materials

Materials for efficacy evaluation were created at field sites in 2008 in the mid-western region of the United States. Ten locations in the states of Iowa, Illinois, Indiana and Missouri were used to produce the reference material for seed characteristic analysis, and the plants used in agronomic performance. Material was obtained from the three treatment regimes of the transformed soybean and their corresponding non-transgenic counterpart in the Jack variety.

Characterization of the materials

Identity of the materials was preserved through chain of custody documentation. Chain of custody documentation was utilized to identify the materials shipped to their respective field sites for proper identification of the evaluated plots in the field. Harvested materials contained chain of custody documentation for samples sent from the field to analytical laboratories to preserve identity.

Performing facility and experimental methods

Trials in 2008 were utilized to characterize and evaluate agronomic performance of the selected event, and develop materials for nutritional and compositional testing. Trials were conducted in one geographic region of the United States.

Field studies were managed in a manner representative of normal agricultural practices for inputs including, but not limited to:

- Conventional herbicide treatments, both pre- and post- planting
- Granular insecticide and/or fungicide application at planting
- Fertilizer applications
- Necessary in-season insecticide and/or fungicide applications
- Irrigation if needed to maintain adequate moisture
- Additional hand weeding or cultivation as necessary

All trials received similar agronomic treatments for the care and upkeep of the plots. Field studies utilized an experimental treatment regime which compared the transformed event FG72 sprayed and unsprayed compared to the non-transformed counterpart of the Jack soybean variety.

Trials were performed using a randomized complete block design using six row plots with three replications and three treatments (Table 38). In addition, three non-replicated plots of conventional non-transgenic soybeans were grown at each location to provide comparative data. A total of 17 agronomic parameters were used to measure the growth and development of the plant, and provide visual observations on the effect of any biotic and abiotic stressors upon the field plots across locations. Of the agronomic parameters observed eight determined qualitative measurement characteristics and nine determined quantitative measurement characteristics. These parameters were selected as key indicators of commercial and agronomic importance to commercial soybean growers, and the ability of the crop to perform under a variety of stresses from the different growing locations within the region.

Table 38. Treatment schedule for agronomic field tests in 2008

Label	Treatment	Description
UTC	Not sprayed	Non-transgenic unsprayed
Control	Not sprayed	Transgenic FG72 unsprayed
Sprayed	Glyphosate and Isoxaflutole	1060 g a.i. glyphosate per hectare and 70 g a.i. isoxaflutole per hectare

Evaluation of seed phenotypic characteristics of seed produced at the 10 different locations was conducted. Following the convention described by the USDA, National Genetic Resources Program, the following observations for FG72 and Jack seed were collected: seed size, hilum color, mottling score, seed coat color, seed coat luster, seed quality and seed shape.

Genotypic traits that can be assessed using qualitative measures, including flower color, pubescence color, pod color, hilum color and shapes, canopy architecture, leaf shape, growth habit, and susceptibility to pests and diseases were collected and analyzed.

Agronomic traits that can be measured quantitatively, including emergence, stand count, plant vigor and health rating, flowering date, plant height, days to maturity, plant lodging, pod shattering and yield were recorded and evaluated statistically.

Statistical analysis

Analysis of variance (ANOVA) between groups was calculated to analyze data for significant differences. All treatments were analyzed in comparison to their non-transgenic counterpart across locations and locally. Data were reviewed using a confidence interval of 95%.

2.F. Materials and methods for seed germination studies

Materials

Materials were created in efficacy trials conducted at ten locations in 2008. Samples were taken from each plot replicate from locations in the mid-western United States.

Characterization of the materials

Identity of the materials was preserved through chain of custody documentation. Chain of custody documentation was utilized to identify the materials shipped to their respective field sites for proper identification of the evaluated plots in the field. Harvested materials contained chain of custody documentation for samples sent from the field to analytical laboratories to preserve identity.

Performing facility and experimental methods

To provide an evaluation of seed dormancy potential of event FG72 and the variety Jack, measurement of seed germination and dormancy were conducted. Seed samples from plants grown to maturity at 10 difference locations were taken. These seed represent the physiological state of seed that might fall into a field at the end of the season. The seed tests were conducted by the Iowa State Seed Lab using the standard test (warm germination) which is used for seed lot evaluations of field emergence under favorable conditions. A minimum of 400 seed were evaluated from each location. A very small difference in germination (94% vs. 96%) at day 6 was observed. In cases where hard seed were observed on day 6, the germination study was extended to 13 days, and in every case, the hard seed germinated so that the Total Viable score (95% vs. 96%) was the same. No dormant seed were identified.

Statistical analysis

The mean and standard deviation for each of the measured parameters was determined.

2.G. Materials and methods for composition analysis

Field design

Field trials were conducted by M.S. Technologies, LLC in EPA Region V in Iowa, Illinois, Indiana and Missouri, which are typical soybean growing regions of the Midwestern United States. The plants in this study were grown under conditions typical of production practices for Group II maturity soybeans. There were three non-transgenic plots (Regimen A) and six transgenic plots (Regimen B and C) at each test site. The plots were randomized at each trial site. The Regimen A plots were planted with the non-transgenic counterpart variety "Jack" soybeans. The Regimen B and C plots were planted with the transgenic event FG72 soybeans. Three additional plots (Regimen D, E and F) were planted with commercial conventional (non-transgenic) soybeans for reference (Stine® 2686-6, Stine 2788, and Stine 3000-0, respectively). Thus there were 12 plots total at each trial site.

The Regimen C transgenic event FG72 plots were sprayed with isoxaflutole herbicide at a target rate of 70 grams ai/Ha and with glyphosate herbicide at a target rate of 1060 grams ai/Ha, and the other plots were not treated with these herbicides. Ammonium sulfate at 2850 grams/Ha was added to the spray mixture for the Regimen C herbicides. Application of the herbicides was made to the Regimen C plants as a foliar spray at about the V4-V5 growth stage. The herbicide applications were made by Bayer CropScience personnel. To keep the site weed-free, a conventional soil-applied herbicide was sprayed after planting but prior to soybean emergence to all the plots. This herbicide treatment was pendimethalin (1060 grams ai per ha), except for one trial in which metolachlor (1880 gm ai/Ha) was used.

One sample of soybean grain was obtained from each test plot at maturity by M.S. Technologies, LLC field personnel. A representative sub-sample of grain from each plot was shipped to the BioAnalytics laboratories of Bayer CropScience in Research Triangle Park, NC, USA. Samples of the received soybean grain were shipped in a frozen state from Bayer CropScience to the analytical laboratory, Covance Laboratories Inc., 3301 Kinsman Blvd, Madison, WI, USA.

Characterization of the material

A Certificate of Analysis (COA) was prepared by MS Technologies, LLC for the FG72 seed lots used for planting in this study. The Jack seed used was certified seed lot number 1297. Identity and purity of the transgenic event FG72 seed and the corresponding non-transgenic seed were confirmed to be acceptable.

Processed fractions

Portions of the sprayed and unsprayed transgenic event FG72 soybeans and non-transgenic "Jack" soybeans were shipped from Adel, IA by M.S. Technologies to the processing facility. Processing of the soybeans into toasted meal was performed by GLP Technologies, with Dick Dusek serving as the Processing Principal Investigator. The samples were processed at: GLP Technologies, 22723 State Highway 6, South, Navasota, TX 77868, USA

After receipt at the processing facility, the soybeans were placed into frozen storage. The processing of the soybean samples took place between June 23, 2009, and July 14, 2009. The non-transgenic "Jack" grain was processed first, followed by the unsprayed transgenic event FG72 grain, and finally the GLY and IFT herbicide sprayed transgenic event FG72 grain. Processing of each Regimen was done in cleaned equipment using simulated industry standard protocols. The nutritional composition of the soybean processed commodities (fractions) was determined at Covance Laboratories Inc. The following samples were obtained in the processing study for composition analysis and are identified in Table 39.

Table 39. Sample identification

Sample	Regimen A	Regimen B	Regimen C	Total
	Sample Code: HT08SOY001-01-			
Soybean Seed (Grain used for processing)	01	02	03	3
	Sample Code: DQ09B002 -			
Hulls (H)	AH	BH	CH	3
Meal (M)	AM	BM	CM	3
Toasted Meal (T)	AT	BT	CT	3
Protein Isolate (P)	AP	BP	CP	3
Crude Oil (C)	AC	BC	CC	3
Refined, Bleached, Deodorized (RBD) Oil (D)	AD	BD	CD	3
Crude Lecithin (L)	AL	BL	CL	3
Total Samples	8	8	8	24

Analytical procedures

The analyses performed and methods used are detailed in Tables 40 to 45.

Table 40. Methods used for analysis of soybean grain

Parameter (Analyte)	Method Mnemonic	Covance Method Reference
Proximates		
Ash	ASHM	AOAC 923.03
Fat	FSOX	AOAC 960.39 and 948.22
Moisture	M100	AOAC 926.08 and 925.09
Protein	PGEN	AOAC 955.04 and 979.09
Carbohydrate (Calculated)	CHO	Difference between 100 and the sum of moisture, crude protein, fat and ash. Agric. Handbook No. 74
Acid detergent fiber	ADF	Agric. Handbook No. 379
Neutral detergent fiber	NDFE	AACC 32.20 and Agric. Handbook No. 379
Calcium, Iron, Magnesium, Phosphorus, Potassium, Sodium	ICPS	AOAC 984.27 and 985.01
Vitamin A (β-carotene)	BCLC	AOAC 941.15
Vitamin B_1 (Thiamin)	BIDE	AOAC 942.23, 953.17 and 957.17
Vitamin B_2 (Riboflavin)	B2FV	AOAC 940.33 and 960.46
Folic Acid	FOAN	AOAC 960.46 and 992.05
Vitamin K	VKLC	AOAC 992.27
Tocopherols (single and total)	TTLC/TOIL	HPLC method (see references)
Raffinose and Stachyose	SUGT	Gas-Liquid Chromatography (see references)
Phytic Acid	PHYT	HPLC method (see references)
Trypsin Inhibitor	TRIP	AOCS Ba 12-75
Lectins	LECT	Photometric methods (see references)
Isoflavones	ASOF	AOAC 2001.10
Total Amino Acids	TAA5	AOAC 982.30
Total Fatty Acids	FALC	AOCS Ce 1-62 and Ce 1b-89

Table 41. Methods used for analysis of soybean hulls

Analyte	Method Mnemonic	Covance Method Reference
Proximates		
Ash	ASHM	AOAC 923.03
Fat	FSOX	AOAC 960.39 and 948.22
Moisture	M100	AOAC 926.08 and 925.09
Protein	PGEN	AOAC 955.04 and 979.09
Carbohydrate (Calculated)	CHO	Difference between 100 and the sum of moisture, crude protein, fat and ash. Agric. Handbook No. 74
Acid detergent fiber	ADF	Agric. Handbook No. 379
Neutral detergent fiber	NDFE	AACC 32.20 and Agric. Handbook No. 379

Table 42. Methods used for analysis of soybean meal and toasted meal

Analyte	Method Mnemonic	Covance Method Reference
Proximates		
Ash	ASHM	AOAC 923.03
Fat	FSOX	AOAC 960.39 and 948.22
Moisture	M100	AOAC 926.08 and 925.09
Protein	PGEN	AOAC 955.04 and 979.09
Carbohydrate (Calculated)	CHO	Difference between 100 and the sum of moisture, crude protein, fat and ash. Agric. Handbook No. 74
Acid detergent fiber	ADF	Agric. Handbook No. 379
Neutral detergent fiber	NDFE	AACC 32.20 and Agric. Handbook No. 379
Raffinose and Stachyose	SUGT	Gas-Liquid Chromatography (see references)
Phytic Acid	PHYT	HPLC method (see references)
Trypsin Inhibitor	TRIP	AOCS Ba 12-75
Lectins	LECT	Photometric methods (see references)
Isoflavones	ASOF	AOAC 2001.10
Total Amino Acids	TAA5	AOAC 982.30

Table 43. Methods used for analysis of soybean protein isolate

Analyte	Method Mnemonic	Covance Method Reference
Proximates		
Moisture	M100	AOAC 926.08 and 925.09
Protein	PGEN	AOAC 955.04 and 979.09
Trypsin Inhibitor	TRIP	AOCS Ba 12-75
Lectins	LECT	Photometric methods (see references)
Total Amino Acids	TAA5	AOAC 982.30

Table 44. Methods used for analysis of soybean crude oil and RBD oil

Analyte	Method Mnemonic	Covance Method Reference
Vitamin A (β-carotene)	BCLC	AOAC 941.15
Vitamin K	VKLC/VKLP	AOAC 992.27 and 999.15
Tocopherols (single and total)	TTLC/TOIL	HPLC method (see references)
Total Fatty Acids	FALC	AOCS Ce 1-62 and Ce 1b-89

Table 45. Method used for analysis of soybean lecithin

Analyte	Method Mnemonic	Covance Method Reference
Phosphatides	LPLC	HPLC method (see reference)

Appendix 3

CHARACTERIZATION OF EVENT FG72 SOYBEAN

3.A. Verification of the insert

Several aliquots of soybean event FG72 genomic DNA were digested with the restriction enzymes *Hinc*II, *Sac*I, *Hin*dIII, *Bsp*HI, *Apa*I, *Stu*I, *Nco*I, *Sca*I, *Eco*RI and *Bsu36*I. Wild type genomic DNA (variety Jack) digested with *Hin*dIII was used as negative control, and an aliquot was supplemented with *Hin*dIII digested pSF10 plasmid DNA for use as a positive control. After separation by agarose gel-electrophoresis, the DNA was transferred to a nylon membrane and hybridized with eight probes, each containing a single genetic element present in the pSF10 vector used for the transformation, and the complete insert probe (Table 44). Figure 11 provides a schematic drawing of the event FG72 insert indicating restriction enzymes, probes, and expected fragment lengths. The membranes were visualized by autoradiography, and electronic scans (Figures 12 through 17).

The expected and obtained hybridization fragments for each of the 10 different restriction patterns are listed in Table 45. Some expected fragments were not visualized on the membranes due to the small overlap between the DNA fragments and the probe used.

Event FG72 genomic DNA digested with *Apa*I shows an additional fragment of 8570 bp (Table 45). This fragment is also visible after hybridization with the 3' nos probe, indicating that it comes from incomplete digestion of the *Apa*I restriction site between the internal 558 bp fragment and the 7900 bp 3' integration fragment. Hybridization results of *Bsu*36I digested event FG72 genomic DNA with probes PT059-1 and PT062-1 show the presence of very weak bands of 11590 bp and >14 kb (Figure 16 lane 13, Figure 17 lane 13, Table 45), also from incomplete digestion.

The inserted sequence in event FG72 consists of two partial 3'histonAt sequences in a head to head orientation, followed by two complete T-DNA copies arranged in a head to tail orientation. Upon integration of the event FG72 insert into the *Glycine max* genome, a non-transgenic region translocated to a new position, which is joined at the 3' junction by 158 bases of Ph4a748 promoter sequences. Figure 11 shows a schematic presentation of this model with indication of the restriction enzymes, probes used, and the expected hybridization fragments. The hybridization results support this model of the event FG72 insert organization.

Table 46. Probes used in Southern hybridization of event FG72

Probe template ID	Description	Size (bp)	Position in pSF10 (bp)
PT015-1	*2mepsps*	1351	8309 → 9659
PT016-1	3'histonAt	753	9614 → 10366
PT024-2	3'nos	214	3265 → 3478
PT058-4	insert probe	7204	3142 → 10345
PT059-1	Ph4a748	959	6491 → 7449
PT060-1	Intron1 h3At	507	7446 → 7952
PT061-1	5'tev + TPotp Y	460	4650 → 5109
PT062-1	Ph4a748B	430	6866 → 7295
PT063-1	*hppdPf W336*	1055	3558 → 4612

Figure 11. Schematic drawing of the event FG72 insert indicating restriction enzymes, probes, and expected fragment lengths

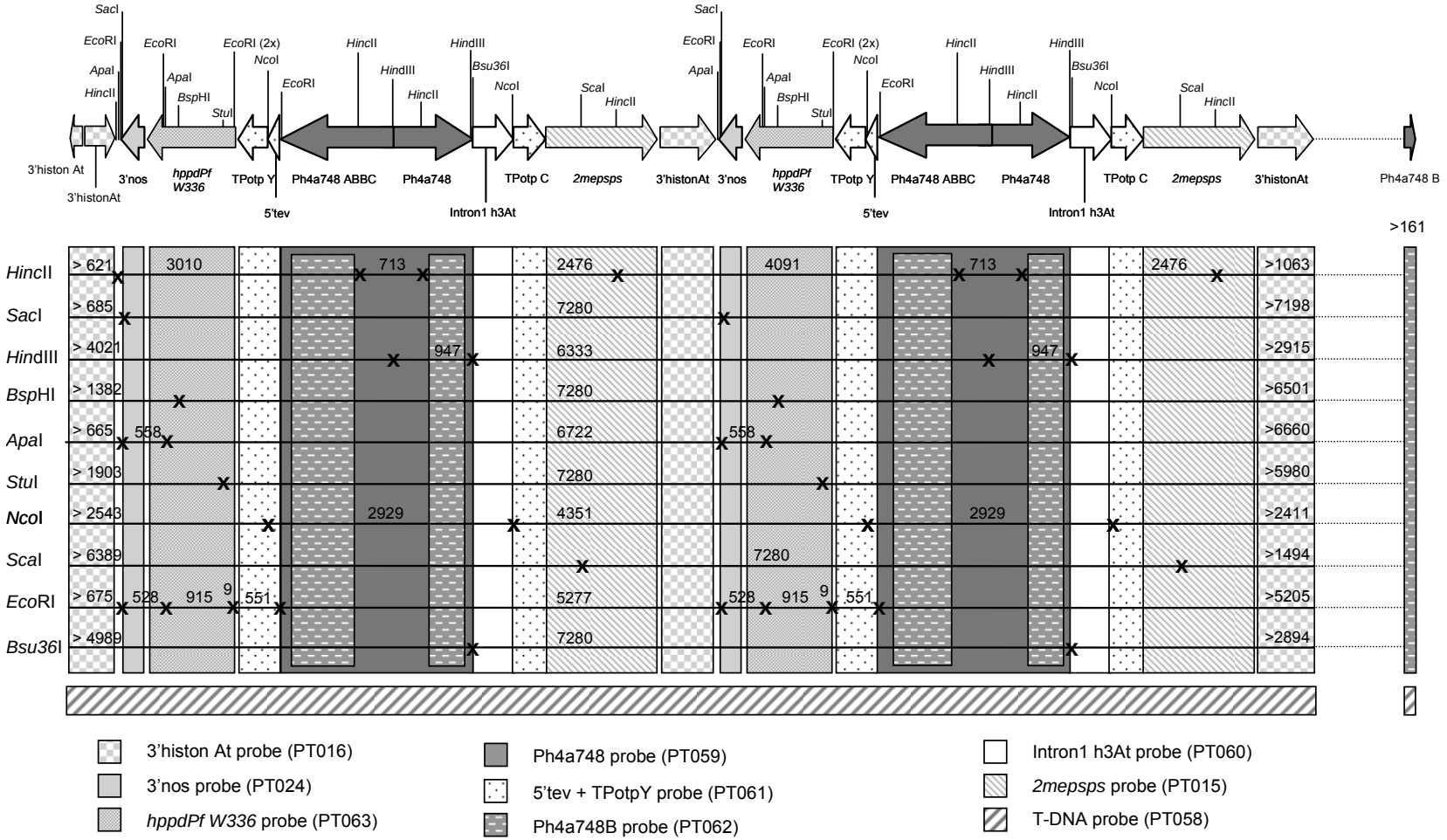


Table 47 . Expected and obtained hybridization fragments

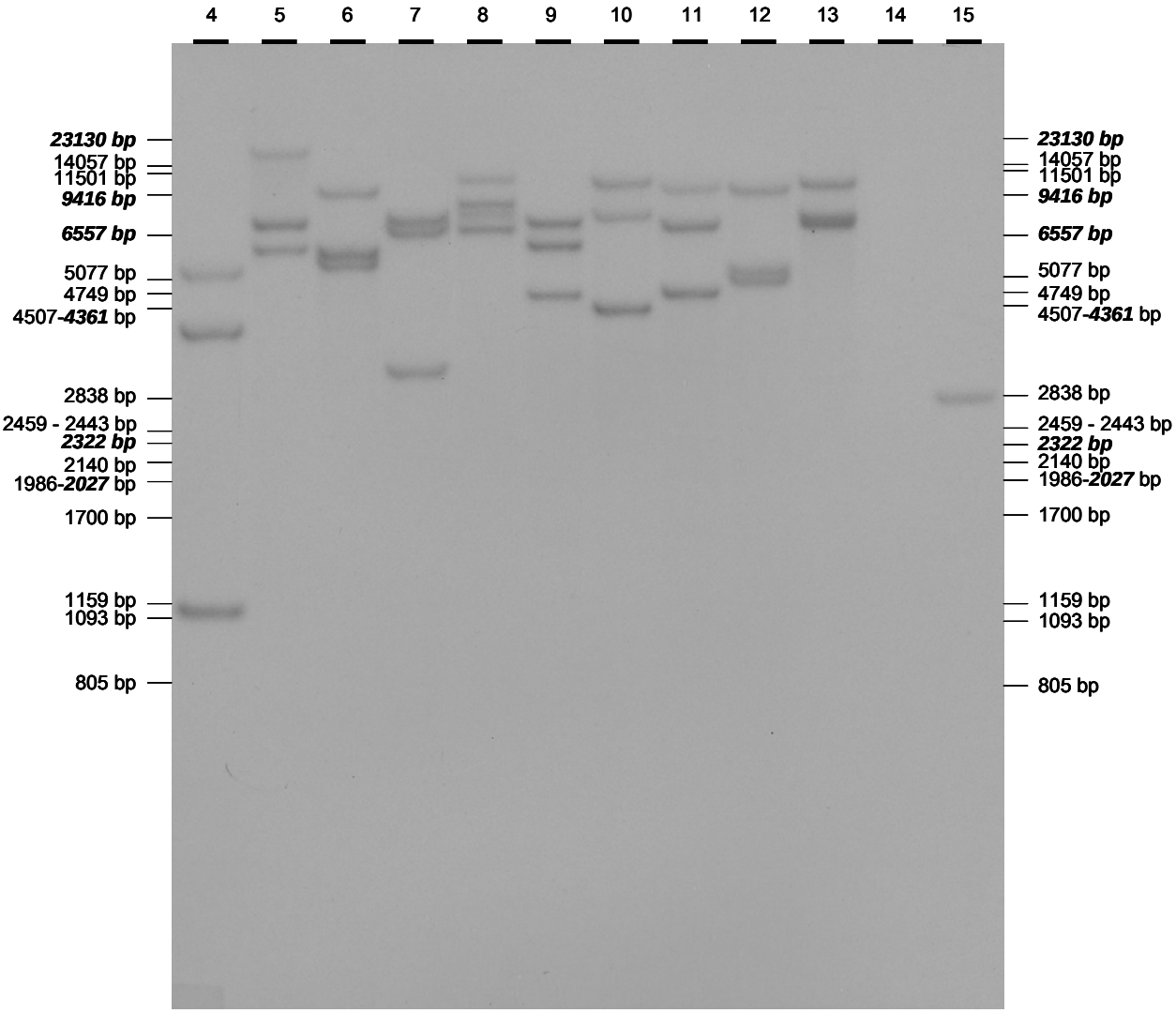
Digest	Description	Expected fragment sizes (bp)	Obtained fragment sizes (bp)	PT016-1: 3'histonAt		PT024-2: 3'nos		PT063-1: hppdPfW336		PT061-1: 5'tev+TPotp Y		PT059-1: Ph4a748		PT062-1: Ph4a748B		PT060-1: Intron1 h3At		PT015-1: 2mepsps		PT058-4: T-DNA probe	
				Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.
HincII	5' integration fr.	> 621	5250	Yes	Yes	No	No	No	No	No	No	No	No	No	No	No	No	No	No	Yes	No[a]
	internal fr.	3010	3010	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	No	Yes	Yes
	internal fr.	4091	4091	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Yes	Yes
	internal fr.	713	713	No	No	No	No	No	No	No	No	Yes	Yes	No	No	No	No	No	No	Yes	No[a]
	internal fr.	2476	2476	No	No	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	3' integration fr.	> 1063	1130	Yes	Yes	No	No	No	No	No	No	No	No	No	No	No	No	Yes	Yes	Yes	Yes
	3' junction translocation	> 158	1300	No	No	No	No	No	No	No	No	Yes	Yes	Yes	Yes	No	No	No	No	Yes	No[a]
SacI	5' integration fr.	> 685	6060	Yes	Yes	No	No	No	No	No	No	No	No	No	No	No	No	No	No	Yes	Yes, weak
	internal fr.	7280	7280	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	3' integration fr.	> 7198	>14 kb	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	3' junction translocation	> 158	>14 kb	No	No	No	No	No	No	No	No	Yes	No[a]	Yes	Yes	No	No	No	No	Yes	No[a]
HindIII	5' integration fr.	> 4021	9550	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	No	Yes	Yes
	internal fr.	6333	6333	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	internal fr.	947	947	No	No	No	No	No	No	No	No	Yes	Yes	Yes	Yes	No	No	No	No	Yes	Yes
	3' integration fr.	> 2915	5500	Yes	Yes	No	No	No	No	Yes	Yes	Yes	No[a]	No	No	Yes	Yes	Yes	Yes	Yes	Yes
	3' junction translocation	> 158	1480	No	No	No	No	No	No	No	No	Yes	Yes	Yes	Yes	No	No	No	No	Yes	No[a]
BspHI	5' integration fr.	> 1382	3200	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	No	No	No	No	No	No	No	Yes	Yes
	internal fr.	7280	7280	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	3' integration fr.	> 6501	7480	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	3' junction translocation	> 158	4260	No	No	No	No	No	No	No	No	Yes	Yes	Yes	Yes	No	No	No	No	Yes	No[a]
ApaI	5' integration fr.	> 665	10760	Yes	Yes	No	No	No	No	No	No	No	No	No	No	No	No	No	No	Yes	Yes, weak
	internal fr.	6722	6722	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	internal fr.	558	558	No	No	Yes	Yes	Yes	Yes	No	No	No	No	No	No	No	No	No	No	Yes	Yes

Digest	Description	Expected fragment sizes (bp)	Obtained fragment sizes (bp)	PT016-1: 3'histonAt		PT024-2: 3'nos		PT063-1: hppdPfW336		PT061-1: 5'tev+TPotp Y		PT059-1: Ph4a748		PT062-1: Ph4a748B		PT060-1: Intron1 h3At		PT015-1: 2mepsps		PT058-4: T-DNA probe	
				Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.
	3' integration fr.	> 6660	7900	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	Partial fr.	/	8570	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes
	3' junction translocation	> 158	>14 kb	No	No	No	No	No	No	No	No	Yes	No[a]	Yes	Yes, weak	No	No	No	No	Yes	No[a]
StuI	5' integration fr.	> 1903	4710	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	No	No	No	No	No	No	No	Yes	Yes
	internal fr.	7280	7280	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	3' integration fr.	> 5980	6210	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	3' junction translocation	> 158	>14 kb	No	No	No	No	No	No	No	No	Yes	No[a]	Yes	Yes, weak	No	No	No	No	Yes	No[a]
NcoI	5' integration fr.	> 2543	7660	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	No	No	No	No	No	Yes	Yes
	internal fr.	2929	2929	No	No	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Yes
	internal fr.	4351	4351	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	No	Yes	No[a]	Yes	Yes	Yes	Yes
	3' integration fr.	> 2411	10270	Yes	Yes	No	No	No	No	Yes	Yes	No	No	No	No	Yes	No[a]	Yes	Yes	Yes	Yes
	3' junction translocation	> 158	>14 kb	No	No	No	No	No	No	No	No	Yes	Yes, weak	Yes	Yes	No	No	No	No	Yes	No[a]
ScaI	5' integration fr.	> 6389	9900	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	internal fr.	7280	7280	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	3' integration fr.	> 1494	4730	Yes	Yes	No	No	No	No	No	No	No	No	No	No	No	No	Yes	Yes	Yes	Yes
	3' junction translocation	> 158	11430	No	No	No	No	No	No	No	No	Yes	No[a]	Yes	Yes	No	No	No	No	Yes	No[a]
EcoRI	5' integration fr.	> 675	5110[d]	Yes	Yes	No	No	No	No	No	No	No	No	No	No	No	No	No	No	Yes	Yes
	internal fr.	5277	5277[d]	Yes	Yes	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	internal fr.	528[c]	528[c]	No	No	Yes	Yes	Yes	Yes	No	No	No	No	No	No	No	No	No	No	Yes	Yes
	internal fr.	915	915	No	No	No	No	Yes	Yes	No	No	No	No	No	No	No	No	No	No	Yes	Yes
	internal fr.	9[b]	/	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
	internal fr.	551[c]	551[c]	No	No	No	No	Yes	Yes	Yes	Yes	No	No	No	No	No	No	No	No	Yes	Yes
	3' integration fr.	> 5205	9610	Yes	Yes	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	3' junction translocation	> 158	4670	No	No	No	No	No	No	No	No	Yes	Yes	Yes	Yes	No	No	No	No	Yes	No[a]
Bsu36I	5' integration fr.	> 4989	7650[e]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	No	Yes	Yes

Digest	Description	Expected fragment sizes (bp)	Obtained fragment sizes (bp)	PT016-1: 3'histonAt		PT024-2: 3'nos		PT063-1: hppdPfW336		PT061-1: 5'tev+TPotp Y		PT059-1: Ph4a748		PT062-1: Ph4a748B		PT060-1: Intron1 h3At		PT015-1: 2mepsps		PT058-4: T-DNA probe	
				Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.
	internal fr.	7280	7280[e]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	3' integration fr.	> 2894	10350	Yes	Yes	No	No	No	No	Yes	Yes	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes
	3' junction translocation	> 158	7670[e]	No	No	No	No	No	No	No	No	Yes	Yes	Yes	Yes	No	No	No	No	Yes	Yes
	Partial fr.	/	11590	No	No	No	No	No	No	No	No	No	Yes, weak	No	Yes, weak	No	No	No	No	No	No
	Partial fr.	/	>14 kb	No	No	No	No	No	No	No	No	No	Yes, weak	No	Yes, weak	No	No	No	No	No	No
WT genomic DNA-HindIII				/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
WT genomic DNA - HindIII + pSF10 - HindIII	Positive control	3420	3420	No	No	Yes	Yes	Yes	Yes	Yes	Yes, weak	Yes	Yes	Yes	Yes	No	No	No	No	Yes	Yes
	Positive control	947	947	No	No	No	No	No	No	No	No	Yes	Yes	Yes	Yes	No	No	No	No	Yes	No[a]
	Positive control	2961	2961	Yes	Yes	No	No	No	No	Yes	Yes, weak	Yes	No[a]	No	No	Yes	Yes	Yes	Yes	Yes	Yes
	Positive control	3070	/	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No

[a] Not visualized due to the small overlap between the fragments and the T-DNA probe.
[b] This fragment is too small to be visualized.
[c,d, e] These fragments can appear as a single fragment.

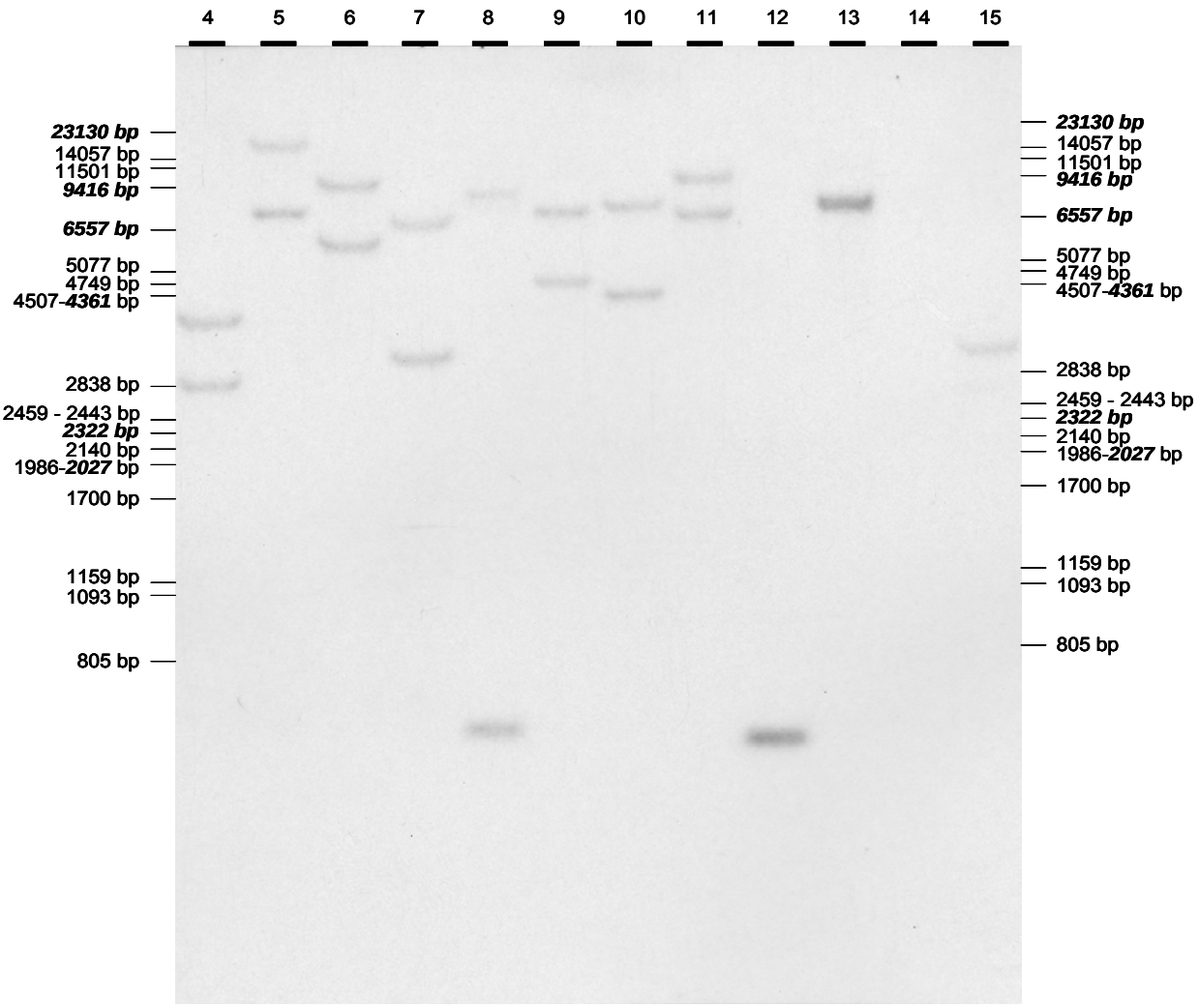
Figure 12. Southern blot analysis of event FG72 – 3'histonAt probe



Genomic DNA samples (8 µg) were digested with different restriction enzymes and probed with the 3'histonAt probe (PT016-1: 753 bp DPA207-SMP086 fragment of pTEM2).

Lane 1: Phage Lambda – *Hind*III digested
Lane 2: Phage Lambda – *Pst*I digested
Lane 3: empty
Lane 4: Event FG72 – *Hinc*II digested
Lane 5: Event FG72 – *Sac*I digested
Lane 6: Event FG72 – *Hind*III digested
Lane 7: Event FG72 – *BspH*I digested
Lane 8: Event FG72 – *Apa*I digested
Lane 9: Event FG72 – *Stu*I digested
Lane 10: Event FG72 – *Nco*I digested
Lane 11: Event FG72 – *Sca*I digested
Lane 12: Event FG72 – *Eco*RI digested
Lane 13: Event FG72 – *Bsu36*I digested
Lane 14: WT variety Jack – *Hind*III digested
Lane 15: WT variety Jack – *Hind*III digested + half of an equimolar amount of pSF10 – *Hind*III digested
Lane 16: Phage Lambda – *Pst*I digested
Lane 17: Phage Lambda – *Hind*III digested

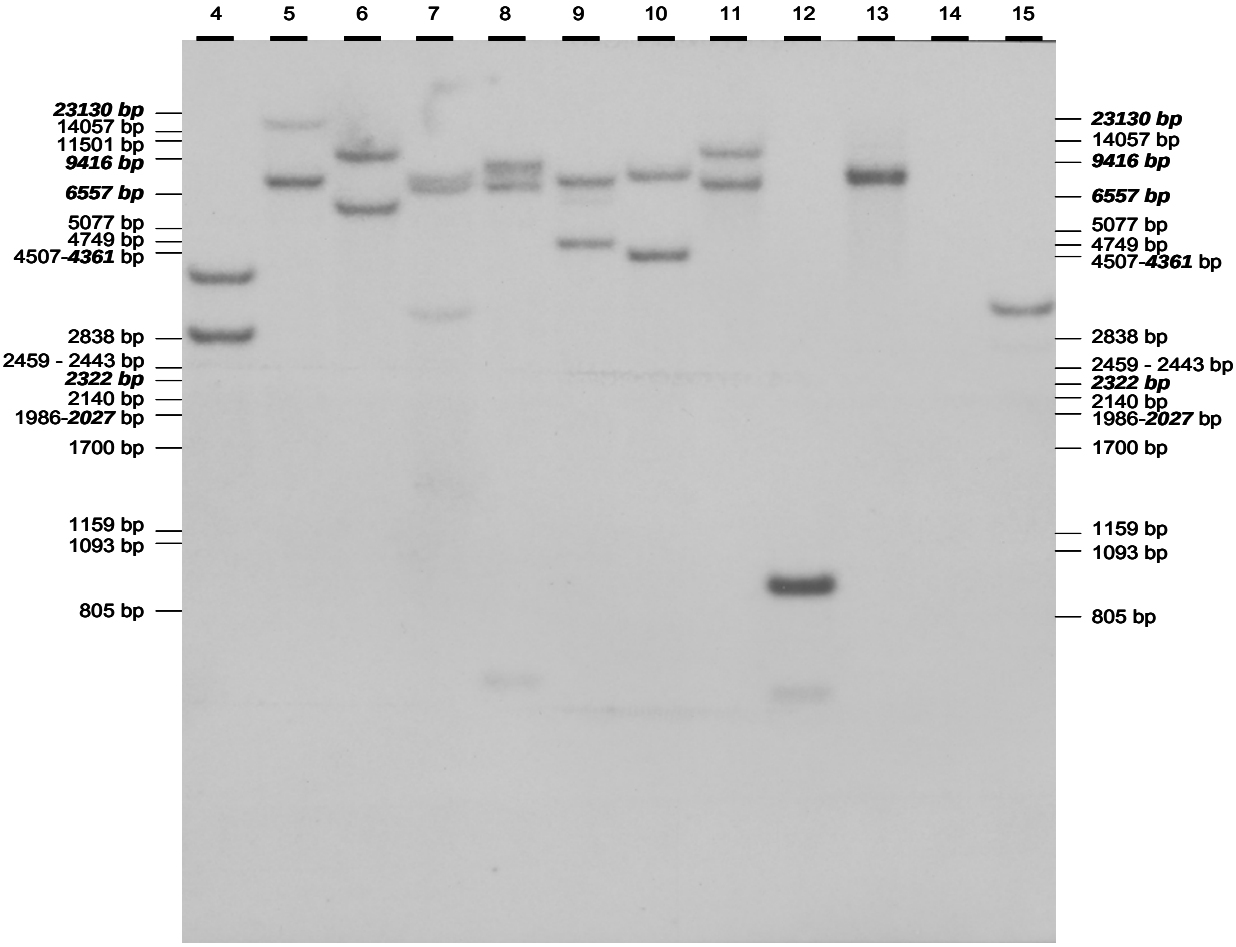
Figure 13. Southern blot analysis of event FG72 – 3'nos probe



Genomic DNA samples (8 µg) were digested with different restriction enzymes and probed with the 3'nos probe (PT024-2: 214 bp MDB355-MDB055 fragment of pTDL004).

Lane 1: Phage Lambda – *Hind*III digested
Lane 2: Phage Lambda – *Pst*I digested
Lane 3: empty
Lane 4: Event FG72 – *Hinc*II digested
Lane 5: Event FG72 – *Sac*I digested
Lane 6: Event FG72 – *Hind*III digested
Lane 7: *Event* FG72 – *BspH*I digested
Lane 8: Event FG72 – *Apa*I digested
Lane 9: Event FG72 – *Stu*I digested
Lane 10: Event FG72 – *Nco*I digested
Lane 11: Event FG72 – *Sca*I digested
Lane 12: Event FG72 – *Eco*RI digested
Lane 13: Event FG72 – *Bsu36*I digested
Lane 14: WT variety Jack – *Hind*III digested
Lane 15: WT variety Jack – *Hind*III digested + an equimolar amount of pSF10 – *Hind*III digested
Lane 16: Phage Lambda – *Pst*I digested
Lane 17: Phage Lambda – *Hind*III digested

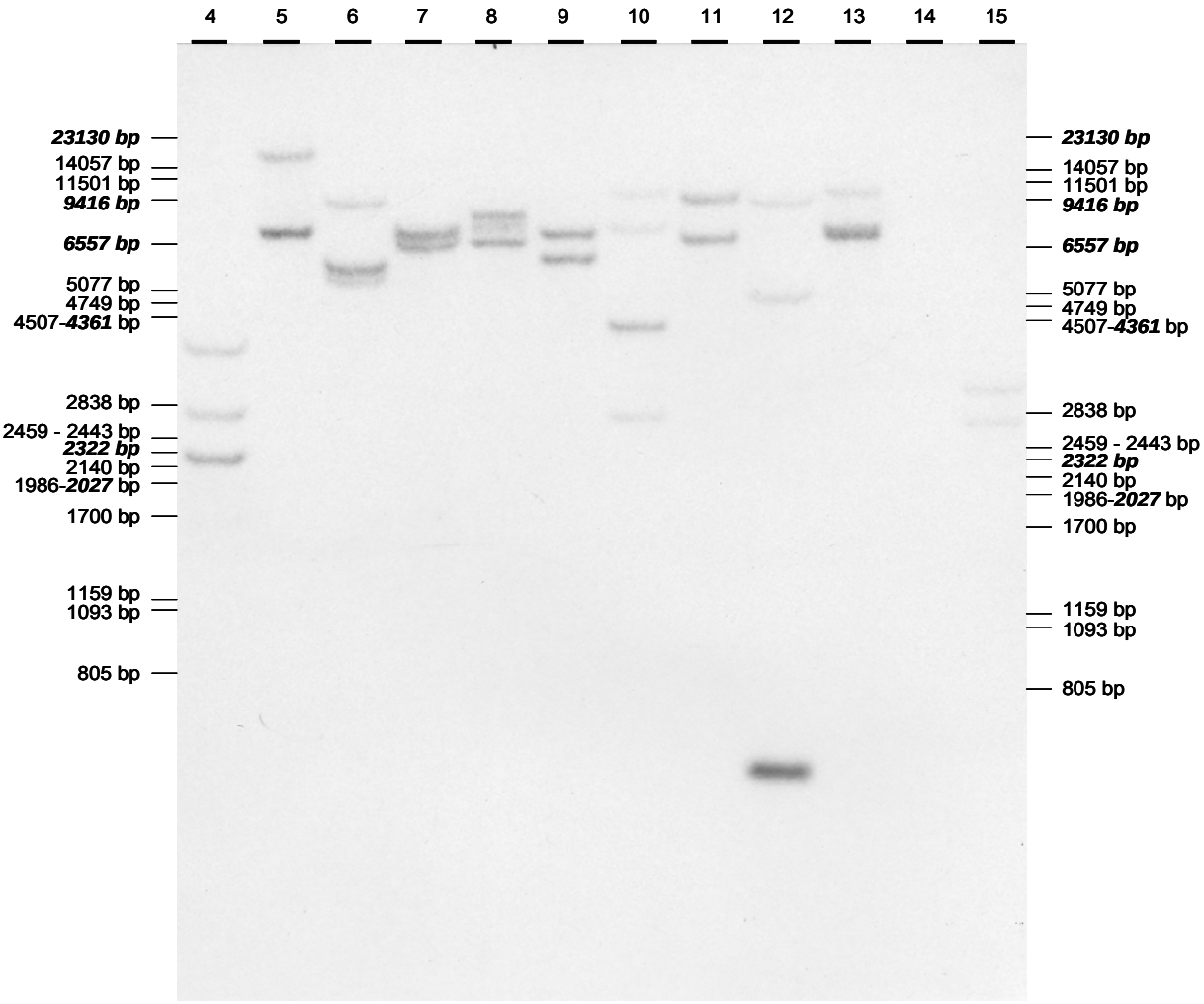
Figure 14. Southern blot analysis of event FG72 – *hppdPfW336* probe



Genomic DNA samples (8 µg) were digested with different restriction enzymes and probed with the hppdPf W336 probe (PT063-1: 1055 bp SMP083-SMP082 fragment of pSF10).

Lane 1: Phage Lambda – *Hind*III digested
Lane 2: Phage Lambda – *Pst*I digested
Lane 3: empty
Lane 4: Event FG72 – *Hinc*II digested
Lane 5: Event FG72 – *Sac*I digested
Lane 6: Event FG72 – *Hind*III digested
Lane 7: Event FG72 – *BspH*I digested
Lane 8: Event FG72 – *Apa*I digested
Lane 9: Event FG72 – *Stu*I digested
Lane 10: Event FG72 – *Nco*I digested
Lane 11: Event FG72 – *Sca*I digested
Lane 12: Event FG72 – *Eco*RI digested
Lane 13: Event FG72 – *Bsu36*I digested
Lane 14: WT variety Jack – *Hind*III digested
Lane 15: WT variety Jack – *Hind*III digested + half of an equimolar amount of pSF10 – *Hind*III digested
Lane 16: Phage Lambda – *Pst*I digested
Lane 17: Phage Lambda – *Hind*II

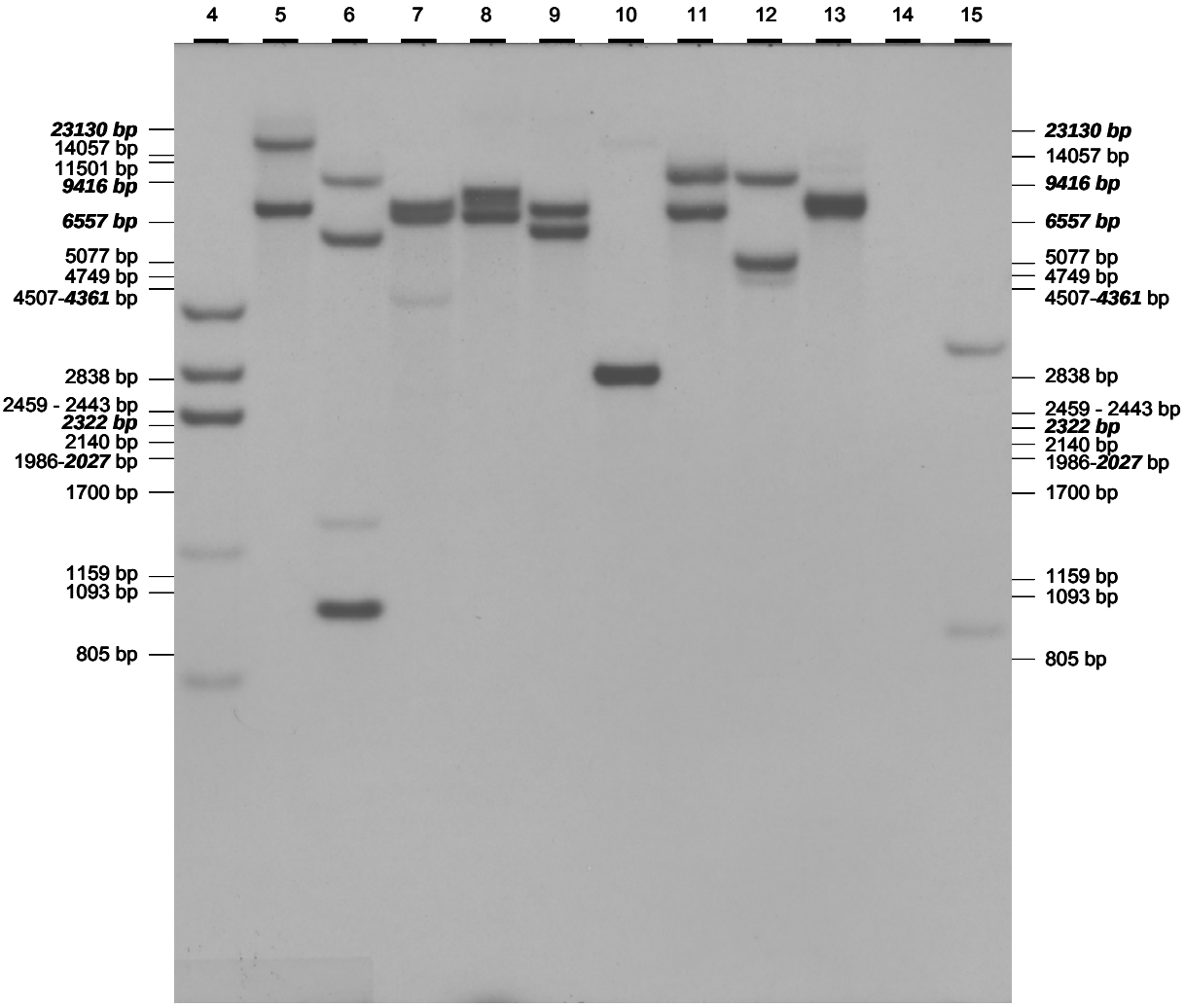
Figure 15. Southern blot analysis of event FG72 – 5'tev+TPotp Y probe



Genomic DNA samples (8 µg) were digested with different restriction enzymes and probed with the 5'tev+TPotp Y probe (PT061-1: 460 bp MLD123-STV129 fragment of pSF10).

Lane 1: Phage Lambda – *Hind*III digested
Lane 2: Phage Lambda – *Pst*I digested
Lane 3: empty
Lane 4: Event FG72 – *Hinc*II digested
Lane 5: Event FG72 – *Sac*I digested
Lane 6: Event FG72 – *Hind*III digested
Lane 7: Event FG72 – *BspH*I digested
Lane 8: Event FG72 – *Apa*I digested
Lane 9: Event FG72 – *Stu*I digested
Lane 10: Event FG72 – *Nco*I digested
Lane 11: Event FG72 – *Sca*I digested
Lane 12: Event FG72 – *Eco*RI digested
Lane 13: Event FG72 – *Bsu36*I digested
Lane 14: WT variety Jack – *Hind*III digested
Lane 15: WT variety Jack – *Hind*III digested + an equimolar amount of pSF10 – *Hind*III digested
Lane 16: Phage Lambda – *Pst*I digested
Lane 17: Phage Lambda – *Hind*III digested

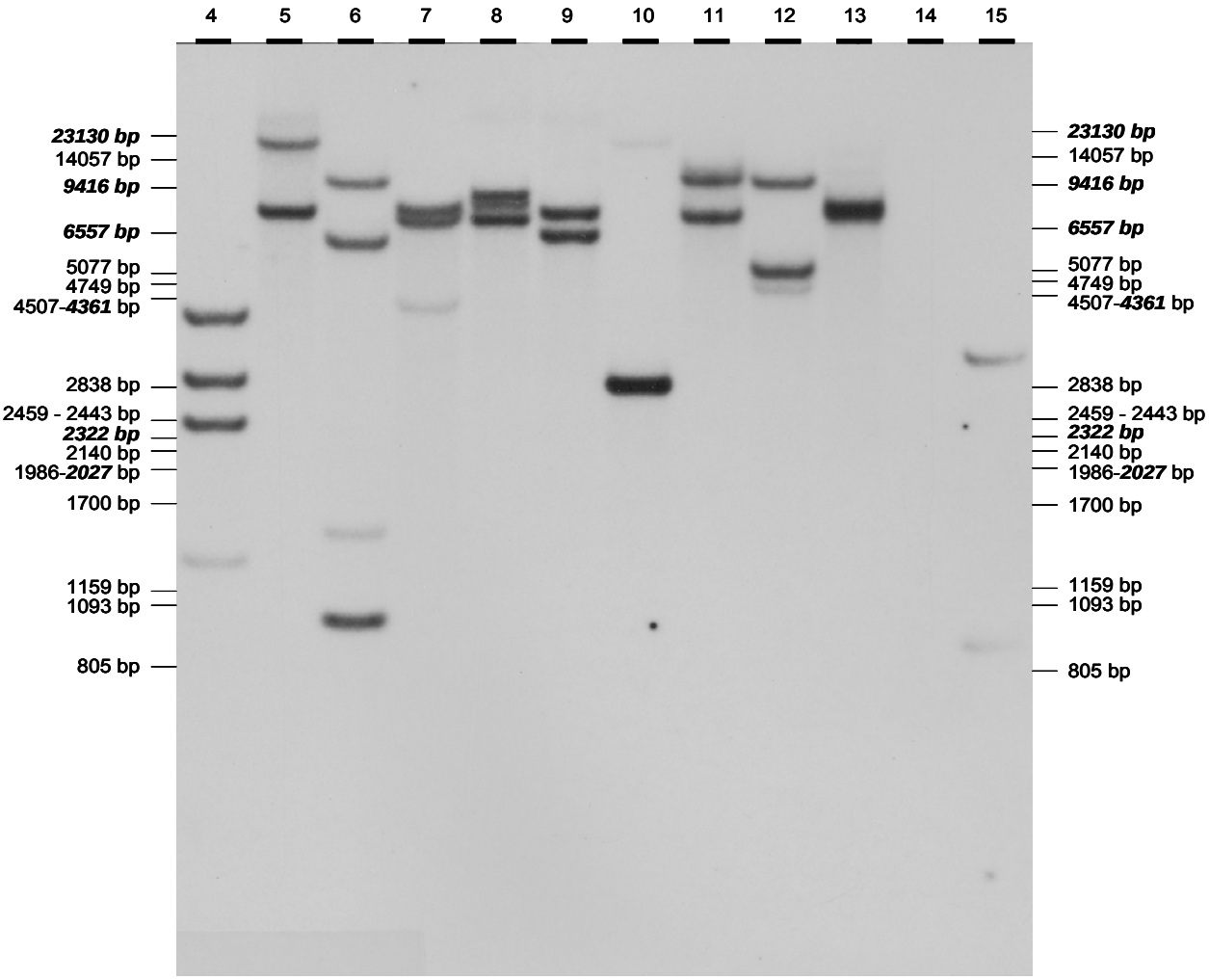
Figure 16. Southern blot analysis of event FG72 – Ph4a748 probe



Genomic DNA samples (8 µg) were digested with different restriction enzymes and probed with the Ph4a748 probe (PT059-1: 959 bp SMP131-KM008 fragment of pSF10).

Lane 1: Phage Lambda – *Hind*III digested
Lane 2: Phage Lambda – *Pst*I digested
Lane 3: empty
Lane 4: Event FG72 – *Hinc*II digested
Lane 5: Event FG72 – *Sac*I digested
Lane 6: Event FG72 – *Hind*III digested
Lane 7: Event FG72 – *BspH*I digested
Lane 8: Event FG72 – *Apa*I digested
Lane 9: Event FG72 – *Stu*I digested
Lane 10: Event FG72 – *Nco*I digested
Lane 11: Event FG72 – *Sca*I digested
Lane 12: Event FG72 – *Eco*RI digested
Lane 13: Event FG72 – *Bsu36*I digested
Lane 14: WT variety Jack – *Hind*III digested
Lane 15: WT variety Jack – *Hind*III digested + half of an equimolar amount of pSF10 – *Hind*III digested
Lane 16: Phage Lambda – *Pst*I digested
Lane 17: Phage Lambda – *Hind*III digested

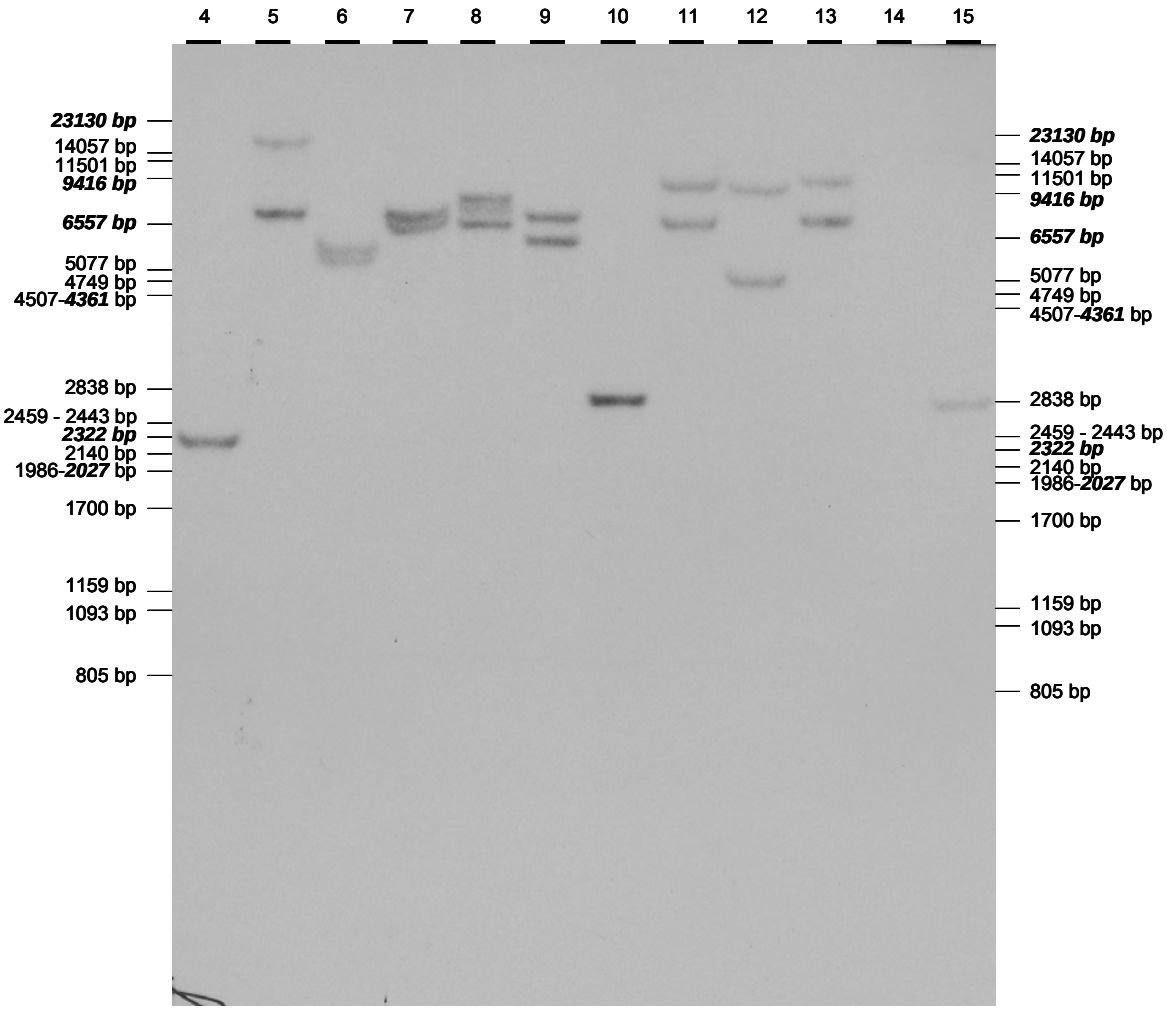
Figure 17. Southern blot analysis of event FG72 – Ph4a748B probe



Genomic DNA samples (8 µg) were digested with different restriction enzymes and probed with the Ph4a748B probe (PT062-1: 430 bp JDB018-JDB019 fragment of pTEM2).

Lane 1: Phage Lambda – *Hind*III digested
Lane 2: Phage Lambda – *Pst*I digested
Lane 3: empty
Lane 4: Event FG72 – *Hinc*II digested
Lane 5: Event FG72 – *Sac*I digested
Lane 6: Event FG72 – *Hind*III digested
Lane 7: Event FG72 – *BspH*I digested
Lane 8: Event FG72 – *Apa*I digested
Lane 9: Event FG72 – *Stu*I digested
Lane 10: Event FG72 – *Nco*I digested
Lane 11: Event FG72 – *Sca*I digested
Lane 12: Event FG72 – *Eco*RI digested
Lane 13: Event FG72 – *Bsu36*I digested
Lane 14: WT variety Jack – *Hind*III digested
Lane 15: WT variety Jack – *Hind*III digested + half of an equimolar amount of pSF10 – *Hind*III digested
Lane 16: Phage Lambda – *Pst*I digested
Lane 17: Phage Lambda – *Hind*III digested

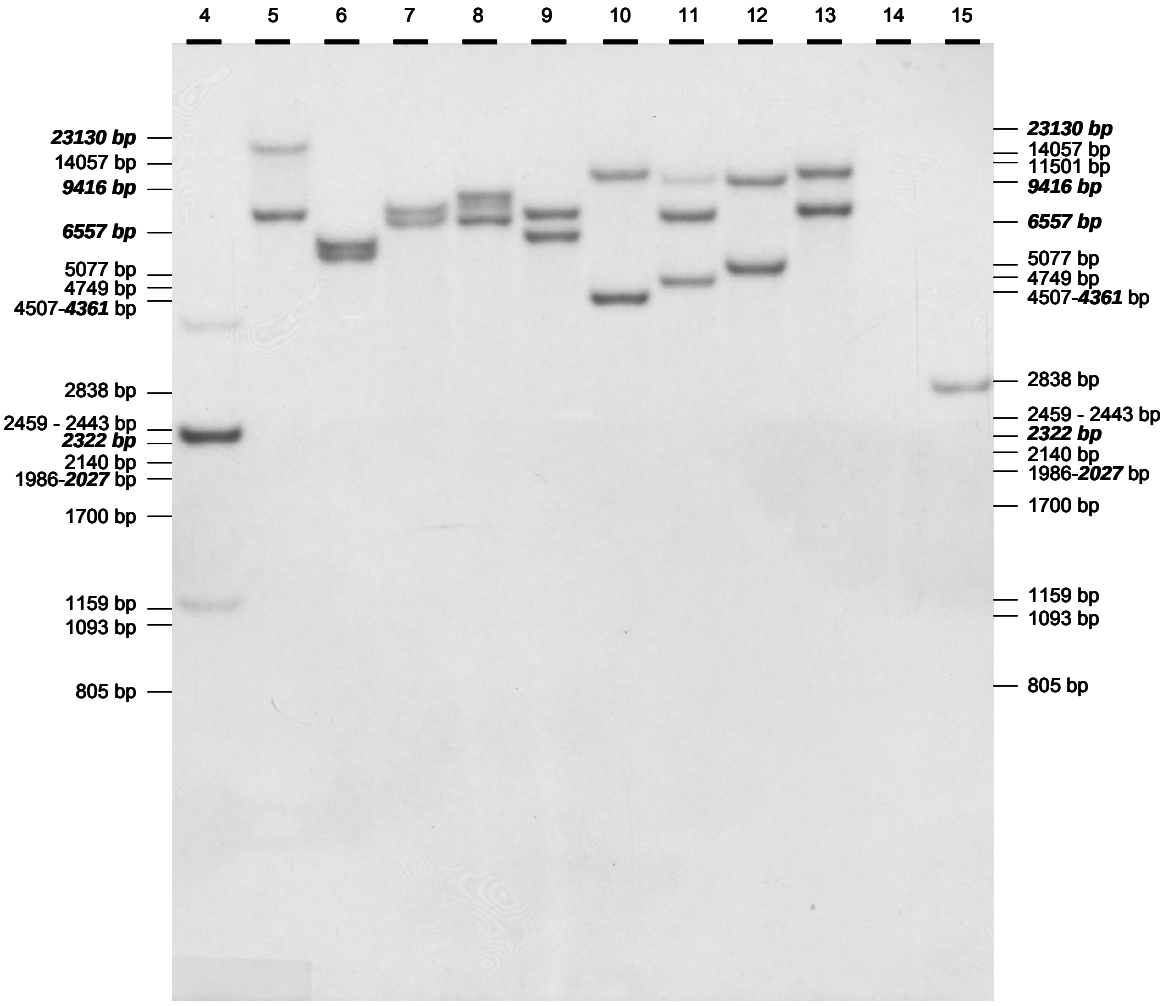
Figure 18. Southern blot analysis of event FG72 – Intron1 h3At probe



Genomic DNA samples (8 µg) were digested with different restriction enzymes and probed with the Intron1 h3At probe (PT060-1: 507 bp MLD148-SMP052 fragment of pSF10).

Lane 1: Phage Lambda – *Hind*III digested
Lane 2: Phage Lambda – *Pst*I digested
Lane 3: empty
Lane 4: Event FG72 – *Hinc*II digested
Lane 5: Event FG72 – *Sac*I digested
Lane 6: Event FG72 – *Hind*III digested
Lane 7: Event FG72 – *Bsp*HI digested
Lane 8: Event FG72 – *Apa*I digested
Lane 9: Event FG72 – *Stu*I digested
Lane 10: Event FG72 – *Nco*I digested
Lane 11: Event FG72 – *Sca*I digested
Lane 12: Event FG72 – *Eco*RI digested
Lane 13: Event FG72 – *Bsu36*I digested
Lane 14: WT variety Jack – *Hind*III digested
Lane 15: WT variety Jack – *Hind*III digested + an equimolar amount of pSF10 – *Hind*III digested
Lane 16: Phage Lambda – *Pst*I digested
Lane 17: Phage Lambda – *Hind*III digested

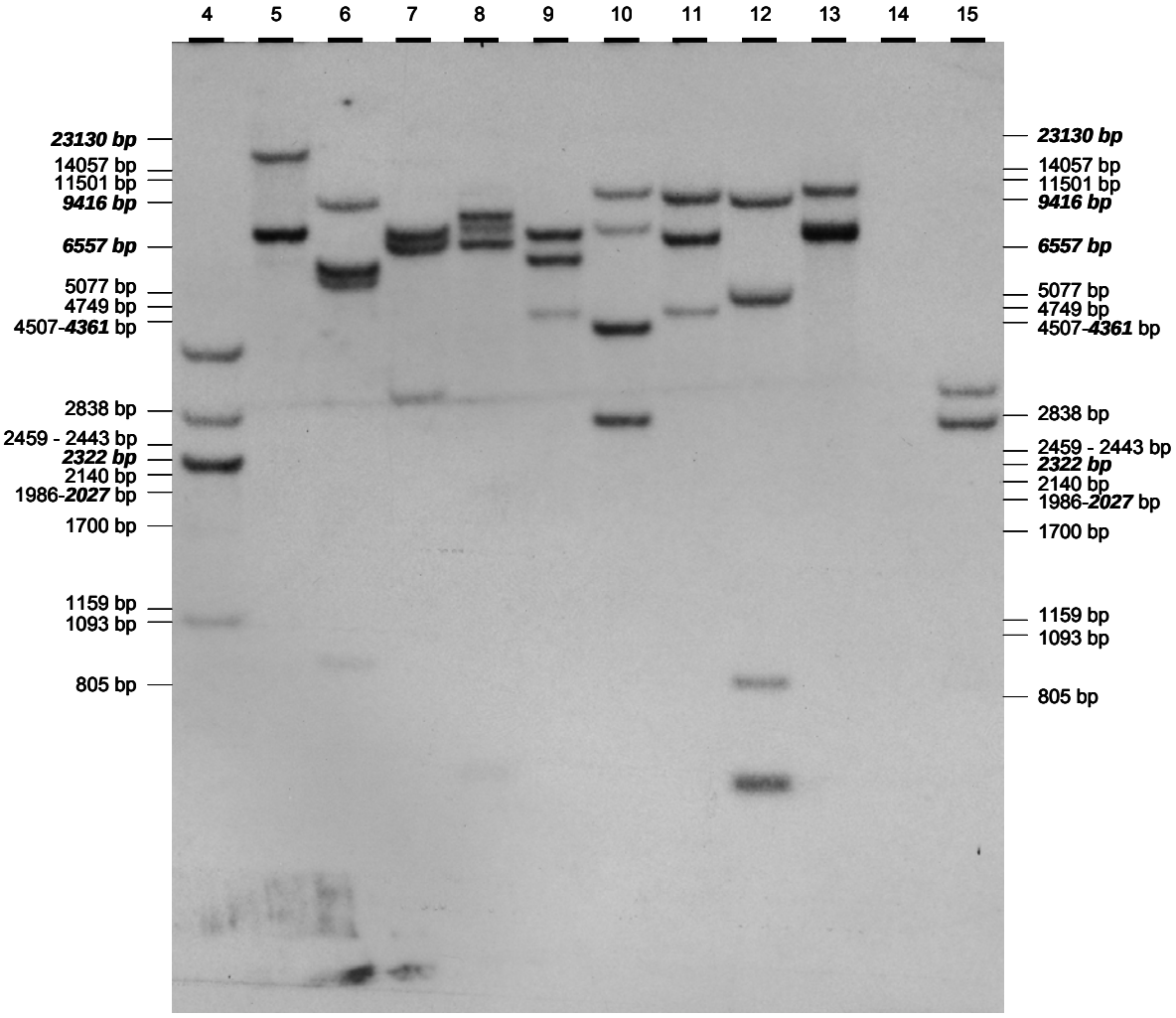
Figure 19. Southern blot analysis of event FG72 – 2*mepsps* probe



Genomic DNA samples (8 µg) were digested with different restriction enzymes and probed with the *2mepsps* probe (PT015-1: 1351 bp SMP084-MLD090 fragment of pTEM2).

Lane 1: Phage Lambda – *Hind*III digested
Lane 2: Phage Lambda – *Pst*I digested
Lane 3: empty
Lane 4: Event FG72 – *Hinc*II digested
Lane 5: Event FG72 – *Sac*I digested
Lane 6: Event FG72 – *Hind*III digested
Lane 7: Event FG72 – *BspH*I digested
Lane 8: Event FG72 – *Apa*I digested
Lane 9: Event FG72 – *Stu*I digested
Lane 10: Event FG72 – *Nco*I digested
Lane 11: Event FG72 – *Sca*I digested
Lane 12: Event FG72 – *Eco*RI digested
Lane 13: Event FG72 – *Bsu36*I digested
Lane 14: WT variety Jack – *Hind*III digested
Lane 15: WT variety Jack – *Hind*III digested + pSF10 – *Hind*III digested
Lane 16: Phage Lambda – *Pst*I digested
Lane 17: Phage Lambda – *Hind*III digested

Figure 20. Southern blot analysis of event FG72 – insert-DNA probe



Genomic DNA samples (8 µg) were digested with different restriction enzymes and probed with the insert-DNA probe (PT058-4: 7204bp SacI/SmaI fragment of pSF10).

Lane 1: Phage Lambda – *Hind*III digested
Lane 2: Phage Lambda – *Pst*I digested
Lane 3: empty
Lane 4: Event FG72 – *Hinc*II digested
Lane 5: Event FG72 – *Sac*I digested
Lane 6: Event FG72 – *Hind*III digested
Lane 7: Event FG72 – *BspH*I digested
Lane 8: Event FG72 – *Apa*I digested
Lane 9: Event FG72 – *Stu*I digested
Lane 10: Event FG72 – *Nco*I digested
Lane 11: Event FG72 – *Sca*I digested
Lane 12: Event FG72 – *Eco*RI digested
Lane 13: Event FG72 – *Bsu36*I digested
Lane 14: WT variety Jack – *Hind*III digested
Lane 15: WT variety Jack – *Hind*III digested + an equimolar amount of pSF10 – *Hind*III digested
Lane 16: Phage Lambda – *Pst*I digested
Lane 17: Phage Lambda – *Hind*III digested

3.B. Absence of Vector Backbone

For the molecular verification of the absence of pSF10 vector backbone, event FG72 genomic DNA was digested with the restriction enzymes *Hin*dIII and *Hinc*II. The resulting DNA fragments were separated by agarose gel electrophoresis, transferred to a membrane and subjected to Southern blot analysis using two overlapping vector backbone probes covering the complete vector backbone sequences of the pSF10 transformation vector. Afterwards, the vector backbone probes were removed from the membranes and they were re-hybridized with the insert DNA probe.

Information on the probes used is presented in Table 48. A schematic overview of the Southern blot strategy is presented in Figure 21.

Table 48. Probes used for demonstration of absence of vector backbone sequences

Probe template ID	Description	Size (bp)	Probe Overlap (bp)
PT056	Vector backbone probe	1730	573
PT057	Vector backbone probe	1982	
PT058	T-DNA probe	7204	N.A.

An overview of the expected and obtained hybridization fragments is presented in Table 49. The obtained Southern blot results are presented in Figures 22 and 23.

Since both vector backbone probes contain a number of regions also present in the T-DNA sequence, several fragments originating from inserted transgenic DNA hybridize with the vector backbone probes. However, based on sequence homology, only the expected fragments were obtained when hybridizing with vector backbone probes.

After hybridization with the insert-DNA probe, the expected internal 947 bp *Hin*dIII fragment was visible for the genomic event FG72 DNA samples, while this fragment was not visible in the positive control. Also the 0.1x equimolar amount of *Hin*dIII digested pSF10 plasmid DNA could not be visualized after hybridization with the insert-DNA probe while it could be visualized after hybridization with the vector backbone probes. However, these results have no influence on the final interpretation of the Southern blot analyses.

The expected Southern blot profile was obtained in the event FG72 samples after hybridization with the insert-DNA probe. This demonstrated that an adequate amount of a sufficient quality of event FG72 genomic DNA was loaded on the gels for the detection of the presence of vector backbone sequences in event FG72.

In conclusion, Southern blot analysis using two overlapping vector backbone probes covering the complete vector backbone sequence of the pSF10 transformation vector confirmed the absence of vector backbone sequences in the genome of the soybean transformation event FG72.

Figure 21. Schematic drawing of plasmid pSF10 indicating the relevant restriction sites and probes

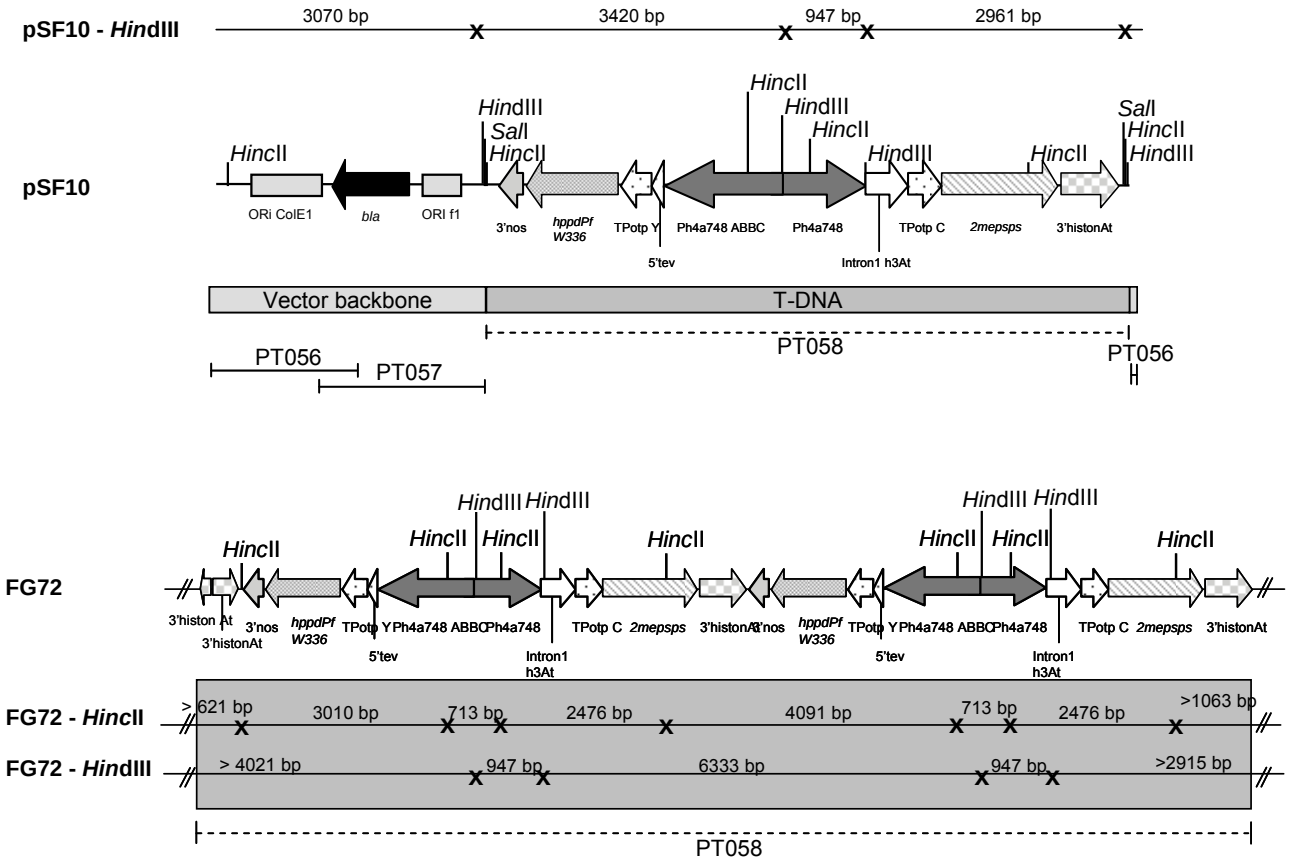
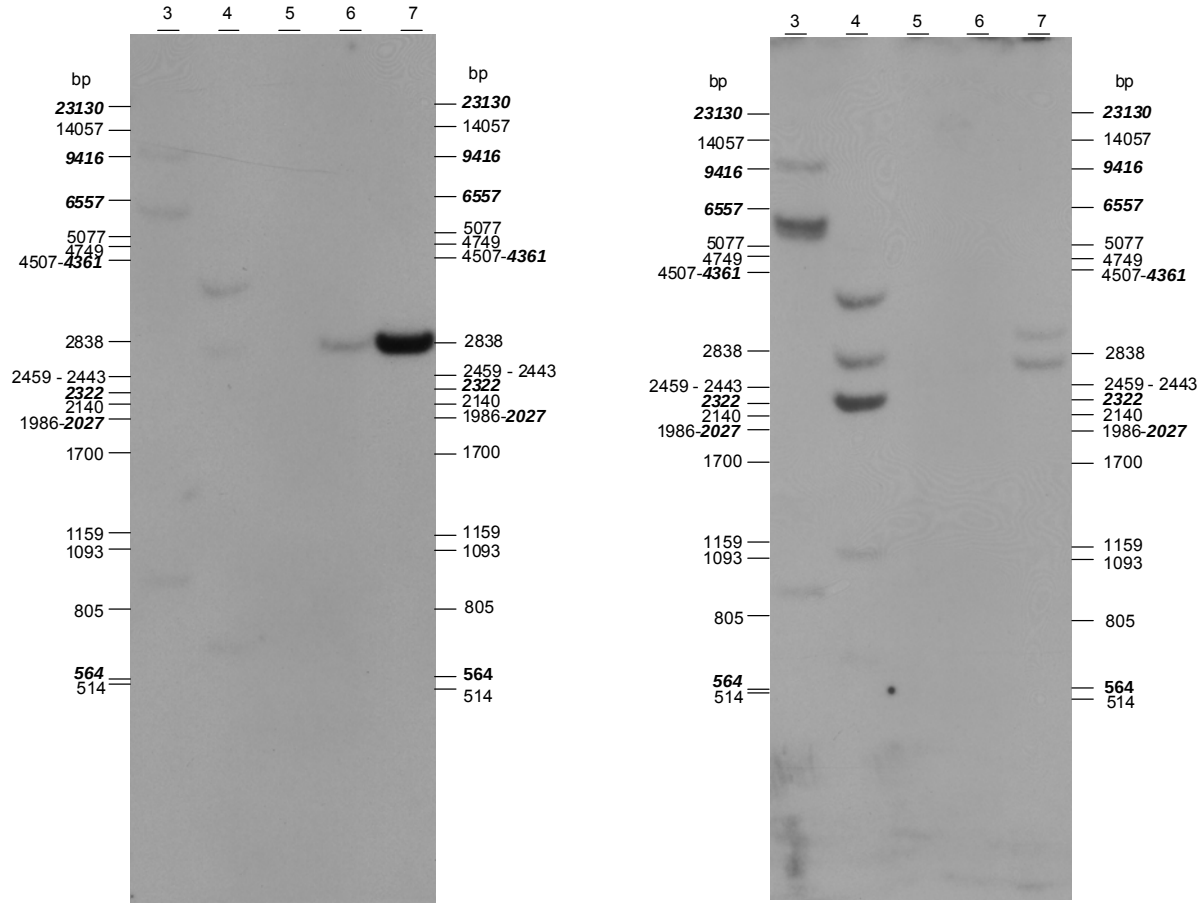


Table 49. Expected and obtained hybridization fragments

Sample	Expected T-DNA or plasmid fragment sizes	Fragment description	Figure 1				Figure 2			
			PT056-1 Vector backbone probe		PT058-6 T-DNA probe		PT057-1 Vector backbone probe		PT058-6 T-DNA probe	
			Exp.	Obt.	Exp.	Obt.	Exp.	Obt.	Exp.	Obt.
FG72 - *Hin*dIII	9550 bp [c]	5' integration fr.	Yes [d]	Yes	Yes	Yes	Yes [d]	Yes	Yes	Yes
	947 bp	internal fragment	Yes [d]	Yes	Yes	Yes	Yes [d]	No	Yes	Yes
	6333 bp	internal fragment	Yes [d]	Yes	Yes	Yes	Yes [d]	Yes	Yes	Yes
	5500 bp [c]	3' integration fr.	No	No	Yes	Yes	No	No	Yes	Yes
	1480 bp [c]	3' junction translocation	No	No	Yes [b]	No	No	No	Yes [b]	No
FG72 – *Hinc*II	5250 bp [c]	5' integration fr.	Yes [d]	No	Yes [b]	No	No	No	Yes [b]	No
	3010 bp	internal fragment	Yes [d]	Yes	Yes	Yes	Yes [d]	Yes	Yes	Yes
	713 bp	internal fragment	Yes [d]	Yes	Yes	Yes, weak	Yes [d]	No	Yes	Yes, weak
	2476 bp	internal fragment	No	No	Yes	Yes	No	No	Yes	Yes
	4091 bp	internal fragment	Yes [d]	Yes	Yes	Yes	Yes [d]	Yes	Yes	Yes
	1130 bp [c]	3' integration fr.	No	No	Yes	Yes	No	No	Yes	Yes
	1300 bp [c]	3' junction translocation	No	No	Yes [b]	No	No	No	Yes [b]	No
WT - *Hin*dIII	/	/	/	/	/	/	/	/	/	/
WT - *Hin*dIII + 0.1 equimolar amount pSF10 - *Hin*dIII	3420 bp	positive control	Yes [d]	No	Yes	No [a]	Yes [b]	No	Yes	No [a]
	947 bp	positive control	Yes [d]	No	Yes	No [a]	Yes [d]	No	Yes	No [a]
	2961 bp	positive control	Yes [b]	No	Yes	No [a]	No	No	Yes	No [a]
	3070 bp	positive control	Yes	Yes	Yes [b]	No	Yes	Yes	Yes [b]	No
WT - *Hin*dIII + 1 equimolar amount pSF10 - *Hin*dIII	3420 bp	positive control	Yes [d]	No	Yes	Yes	Yes [b]	No	Yes	Yes
	947 bp	positive control	Yes [d]	No	Yes	No [a]	Yes [d]	No	Yes	No [a]
	2961 bp	positive control	Yes [b]	No	Yes	Yes	No	No	Yes	Yes
	3070 bp	positive control	Yes	Yes	Yes [b]	No	Yes	Yes	Yes [b]	No

[a] These fragments of the positive control were weak or could not be visualized after hybridization with the T-DNA probe. This has no impact on the interpretation of the results.
[b] The overlap between the probe and the fragment can be too small to visualize this fragment.
[c] Expected fragment sizes as determined in the detailed insert characterization study.
[d] Since part of the sequence of both vector backbone probes is present in the T-DNA sequences, fragments originating from inserted transgenic sequences hybridize with vector backbone probes.

Figure 22. Southern blot analysis of event FG72 – Absence of vector backbone – PT056-1 probe and insert-DNA probe



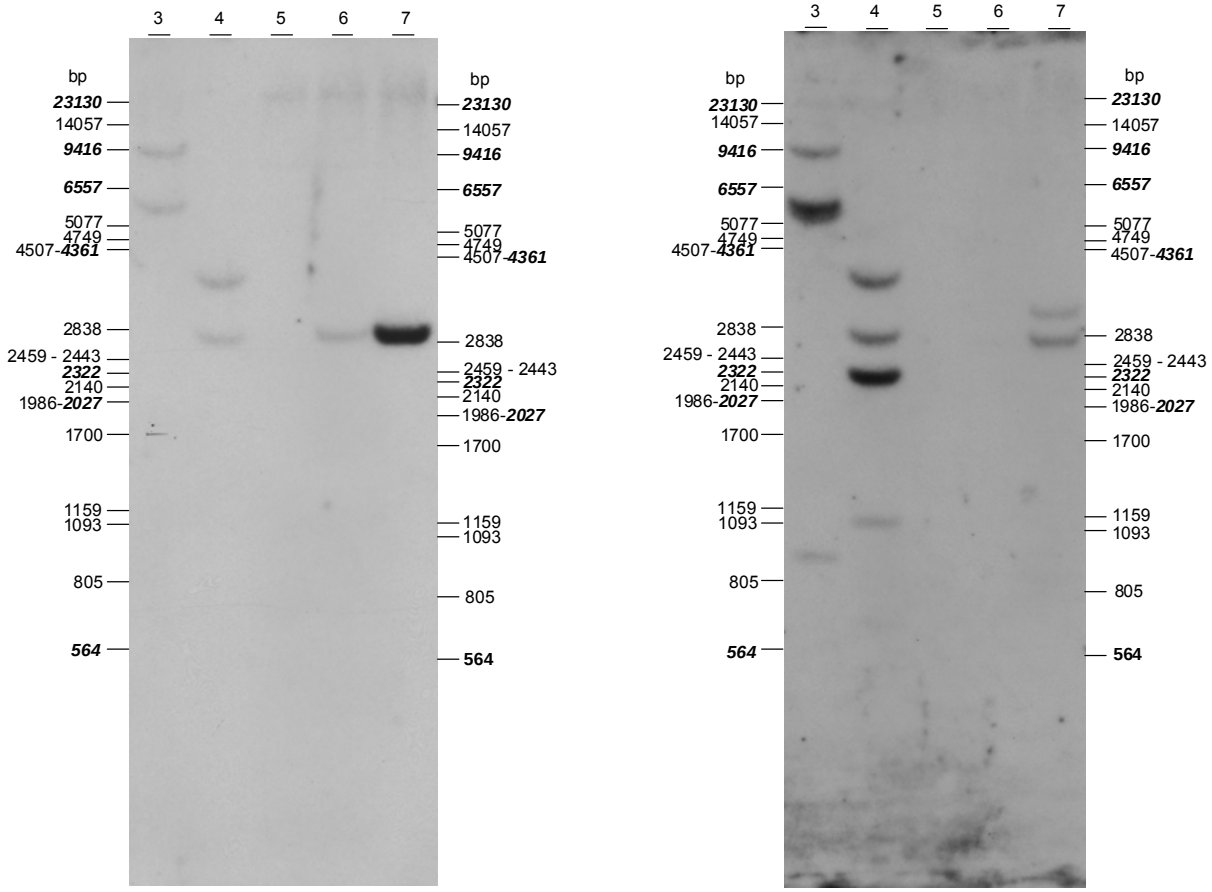
Panel A: PT056-1 Probe

Panel B: PT058-6 Probe

Genomic DNA samples (10 µg) were digested with different restriction enzymes and hybridized sequentially with a vector backbone probe (PT056-1: 1730 bp, KM033 – DPA010 fragment of pSF10) and with the insert-DNA probe (PT058-6: 7204 bp, *Sac*I/*Sma*I fragment of pSF10).

Lane 1: λ-*Pst*I digested (not shown)
Lane 2: λ-*Hin*dIII digested (not shown)
Lane 3: Event FG72 – *Hin*dIII digested
Lane 4: Event FG72 – *Hin*cII digested
Lane 5: WT variety Jack – *Hin*dIII digested
Lane 6: WT variety Jack – *Hin*dIII digested + a 10-fold dilution of an equimolar amount of pSF10 – *Hin*dIII digested
Lane 7: WT variety Jack – *Hin*dIII digested + an equimolar amount of pSF10 – *Hin*dIII digested
Lane 8: λ-*Hin*dIII digested (not shown)
Lane 9: λ-*Pst*I digested (not shown)

Figure 23. Southern blot analysis of event FG72 – Absence of vector backbone – PT057-1 probe and insert-DNA probe



Panel A: PT057-1 Probe

Panel B: PT058-6 Probe

Genomic DNA samples (10 µg) were digested with different restriction enzymes and hybridized sequentially with a vector backbone probe (PT056-1: 1982 bp, VH055 – STV039 fragment of pSF10) and with the insert-DNA probe (PT058-6: 7204 bp, *Sac*I/*Sma*I fragment of pSF10).

Lane 1: λ-*Pst*I digested (not shown)
Lane 2: λ-*Hin*dIII digested (not shown)
Lane 3: Event FG72 – *Hin*dIII digested
Lane 4: Event FG72 – *Hin*cII digested
Lane 5: WT variety Jack – *Hin*dIII digested
Lane 6: WT variety Jack – *Hin*dIII digested + a 10-fold dilution of an equimolar amount of pSF10 – *Hin*dIII digested
Lane 7: WT variety Jack – *Hin*dIII digested + an equimolar amount of pSF10 – *Hin*dIII digested
Lane 8: λ-*Hin*dIII digested (not shown)
Lane 9: λ-*Pst*I digested (not shown)

3.C. Stability across and within generations

To demonstrate the structural stability of event FG72, genomic DNA was prepared from several individual plants of three generations and three different genetic backgrounds (Table 48). The impact of different environments was assessed by analyzing the progeny of transgenic plants cultivated at 4 different field locations (Table 50). The isolated DNA was digested with the restriction enzyme *Hin*dIII, which provides a unique pattern for transformation event FG72.

Successive hybridization of these samples with the Ph4a748B probe and the insert-DNA probe revealed the expected profile in all tested samples. These findings demonstrated the stability of event FG72 at the genomic level in different generations, different environments and different backgrounds.

Information on the probes is given in Table 51. A schematic presentation of the hybridization strategy is shown in Figure 32. Hybridization results are shown in Figures 24 – 31 and are summarized in Table 52.

Table 50. Overview of the tested seed lots of event FG72

Different locations for seed lot production	Generation	Seed lot n°
Adel, Iowa	T9	HT08SOY002-05-32
Osborn, Missouri	T9	HT08SOY002-07-32
Fithian, Illinois	T9	HT08SOY002-08-32
Sharpsville, Indiana	T9	HT08SOY002-09-32
Different generations	**Generation**	**Seed lot n°**
T2	T2	FG72a-T2, FG72b-T2, FG72c-T2, FG72d-T2 **
T7	T7	FG72-x-x-14-5-1-6-x T7
T9	T9	All seed lots of different locations
Different backgrounds	**Generation**	**Seed lot n°**
3068115-48 x Jack *	F4	7BD60018
3066617-48 x Jack *	F4	7BD60008
Jack		All seed lots of different locations and generations tested

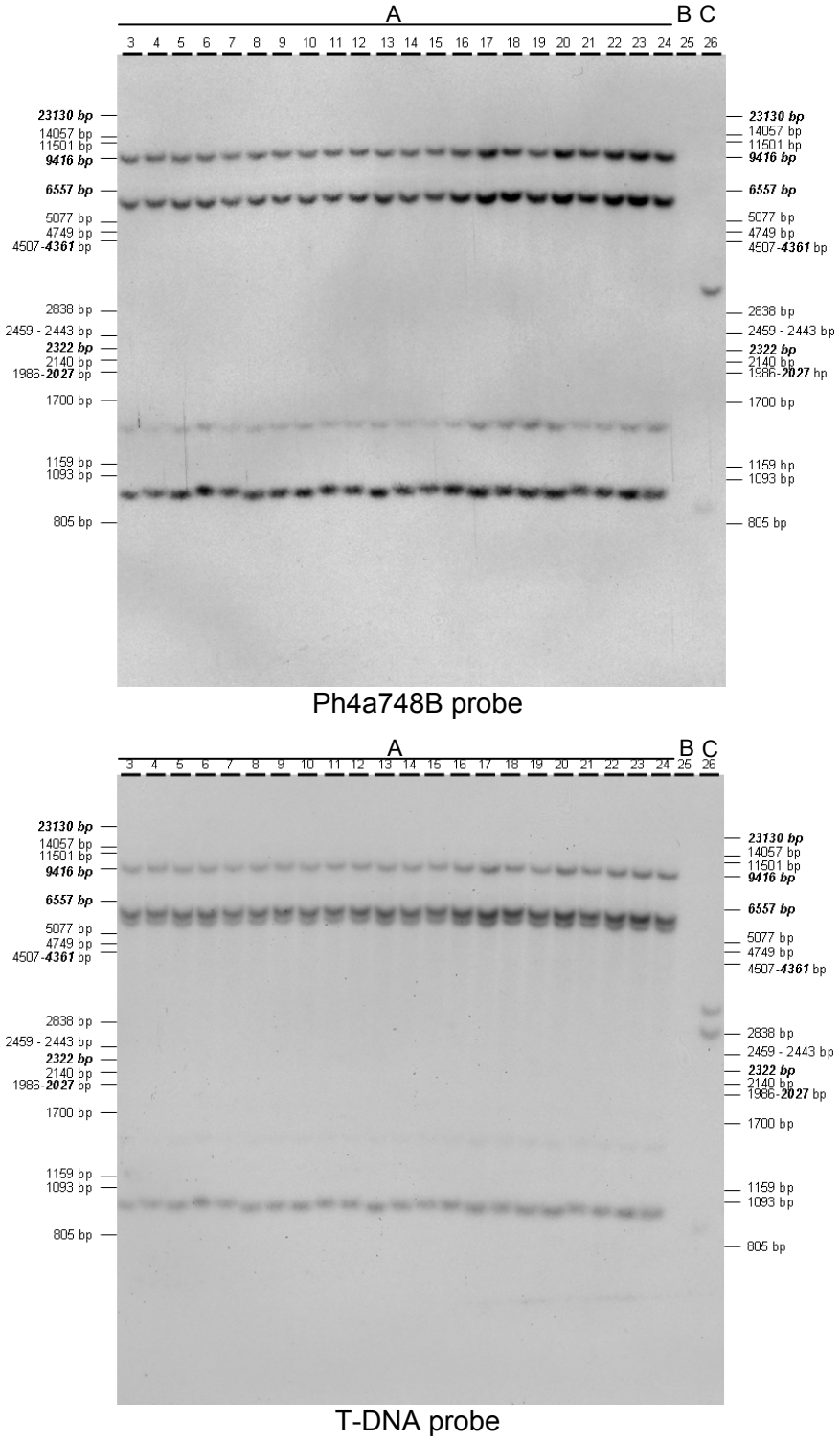
* Conventional lines 3068115-48 and 3066617-48 were crossed with event FG72 in Jack genetic background one time and then selfed 3 times.

** Seeds lots FG72a-T2, FG72b-T2, FG72c-T2 and FG72d-T2 are each the progeny of 1 individual seed of event FG72 T1 generation. Leaf material grown from these T2 seeds was randomly collected from individual plants and used in this study as T2 generation.

Table 51. Probes used for stability studies

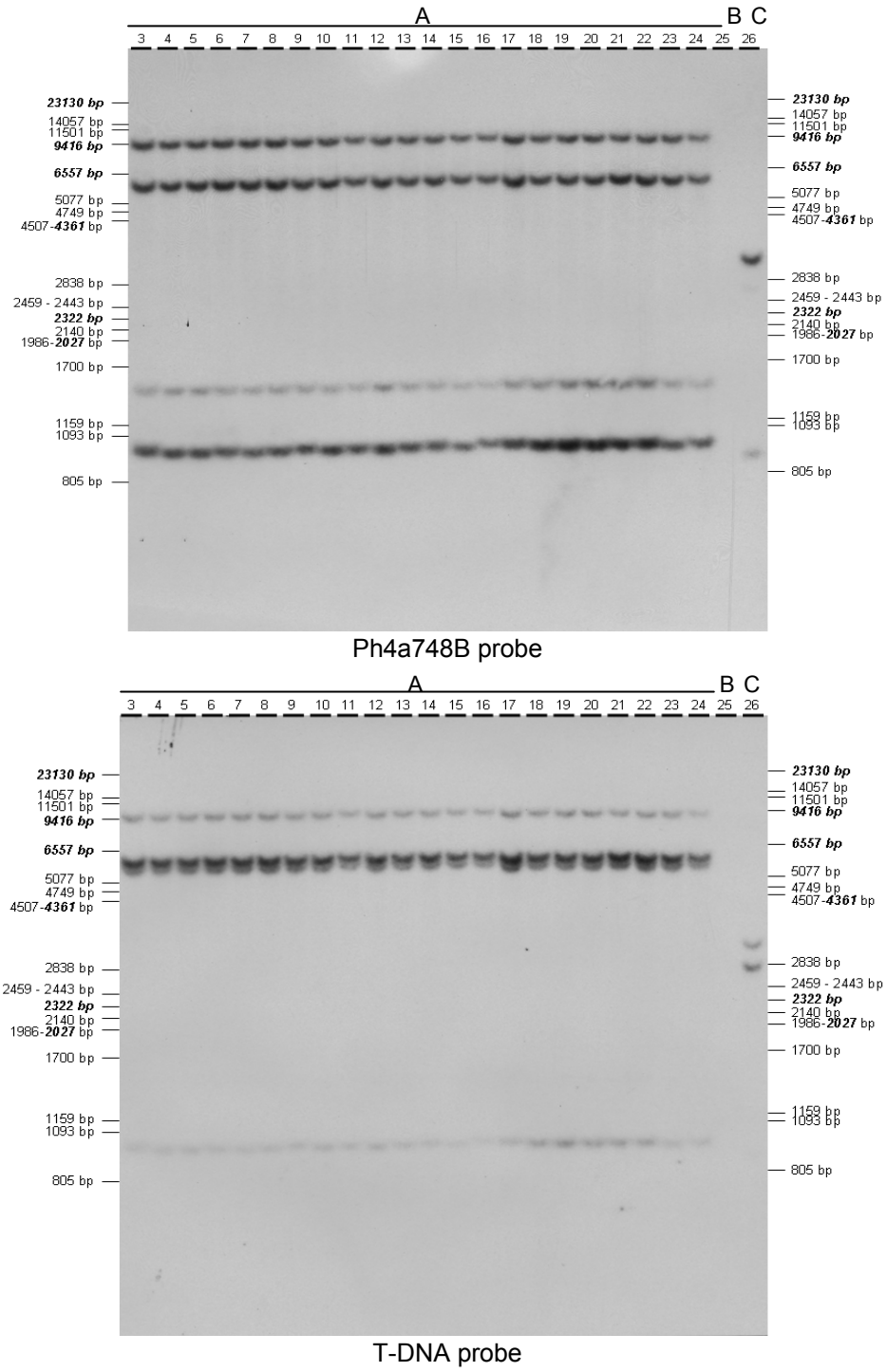
Probe	Restriction digest or Primer pair	Position in pSF10	Probe size
FG72 T-DNA	*Sac*I/*Sma*I	3142 bp → 10345 bp	7204 bp
Ph4a748B	JDB018	6866 bp → 7295 bp	430 bp
	JDB019		

Figure 24. Environment Adel



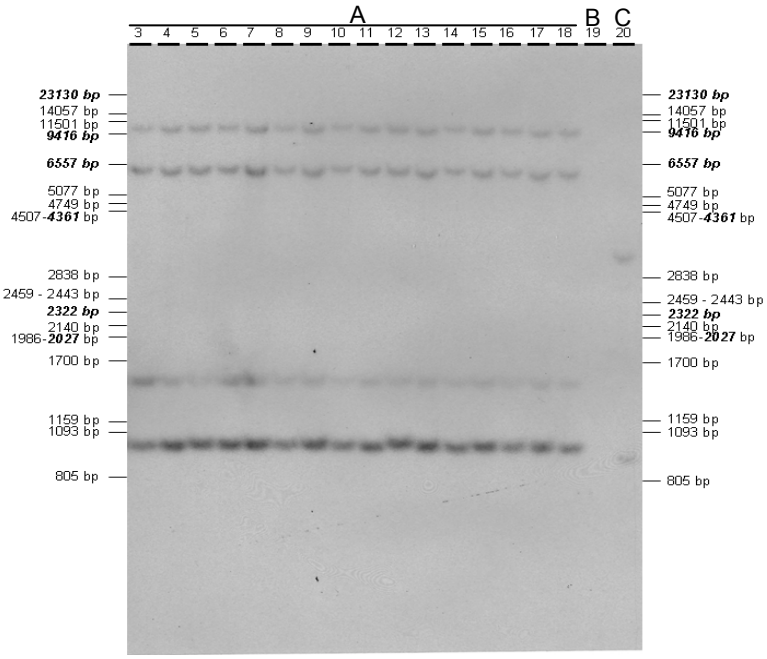
A: event FG72 samples; B: Negative control; C: positive control

Figure 25. Environment Osborn

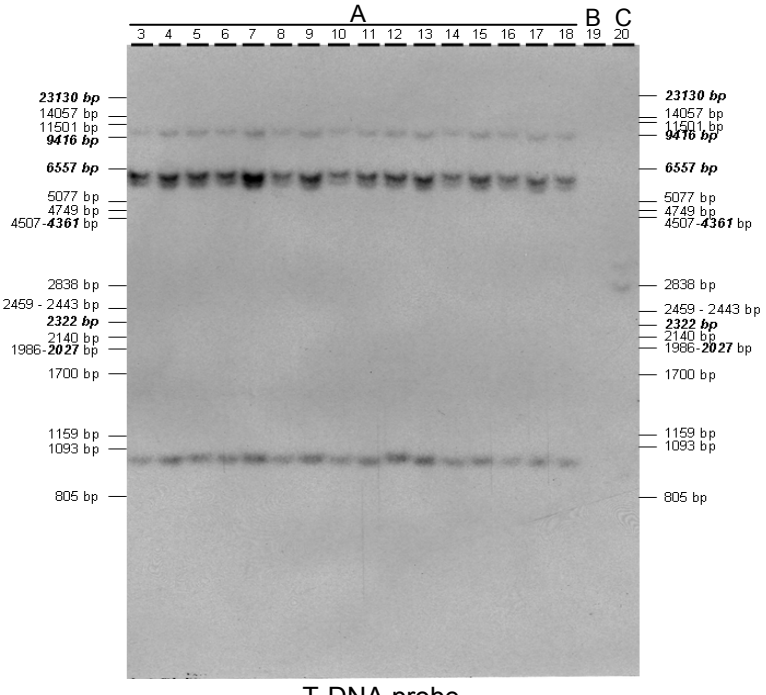


A: event FG72 samples; B: Negative control; C: positive control

Figure 26. Environment Sharpsville



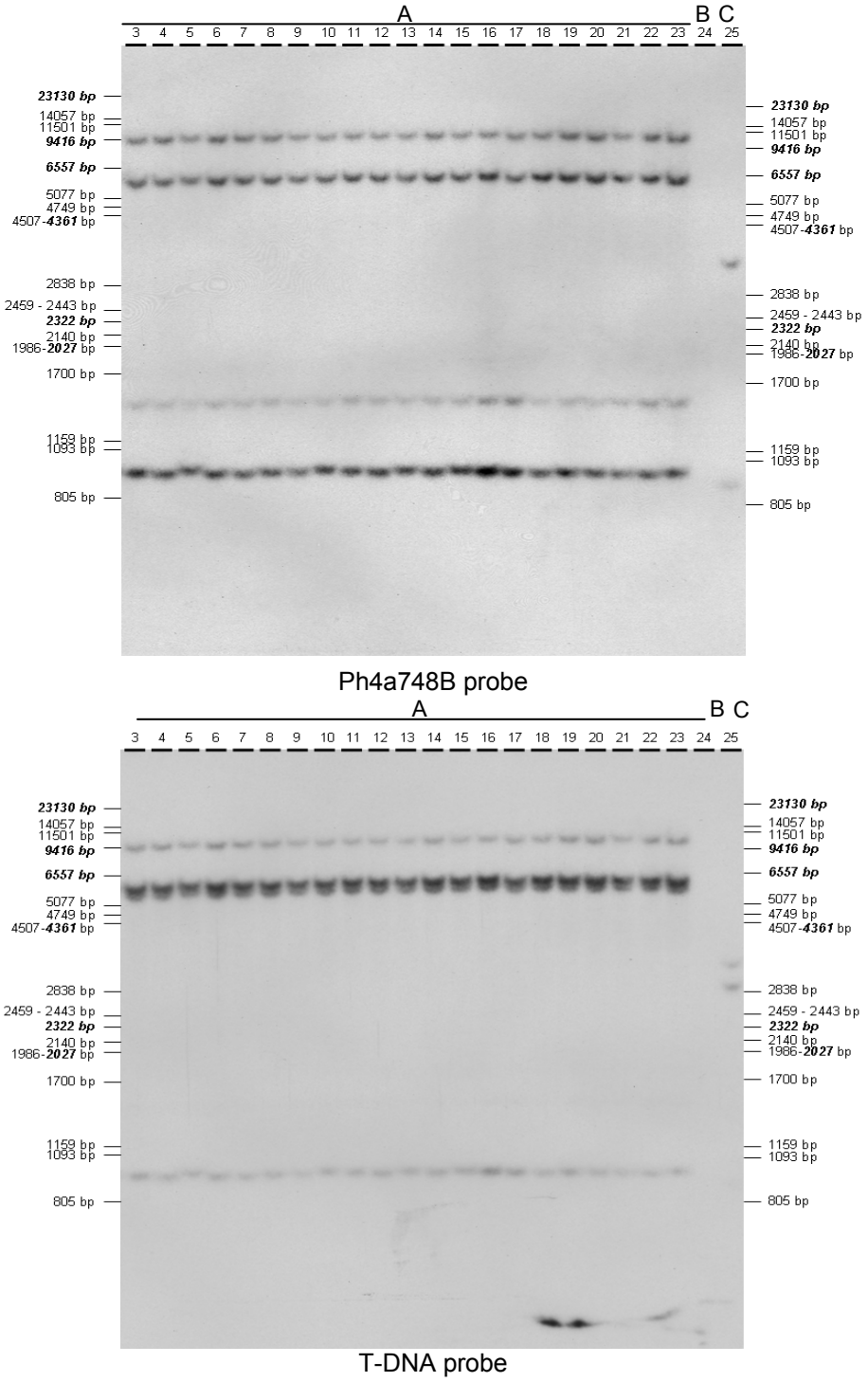
Ph4a748B probe



T-DNA probe

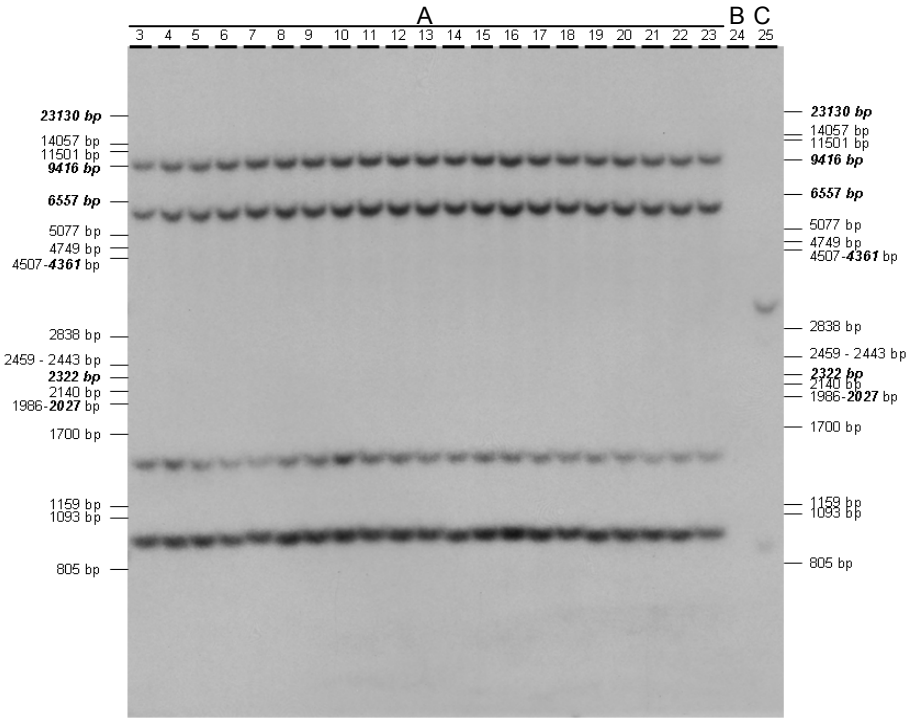
A: event FG72 samples; B: Negative control; C: positive control

Figure 27. Environment Fithian

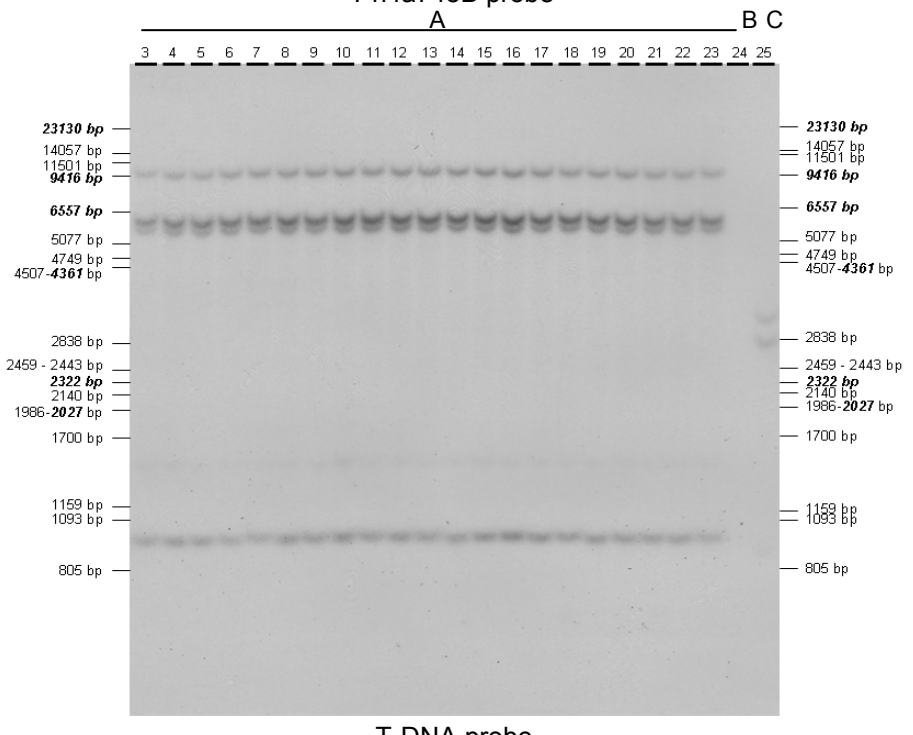


A: event FG72 samples; B: Negative control; C: positive control

Figure 28. Background 3068115-48 X Jack



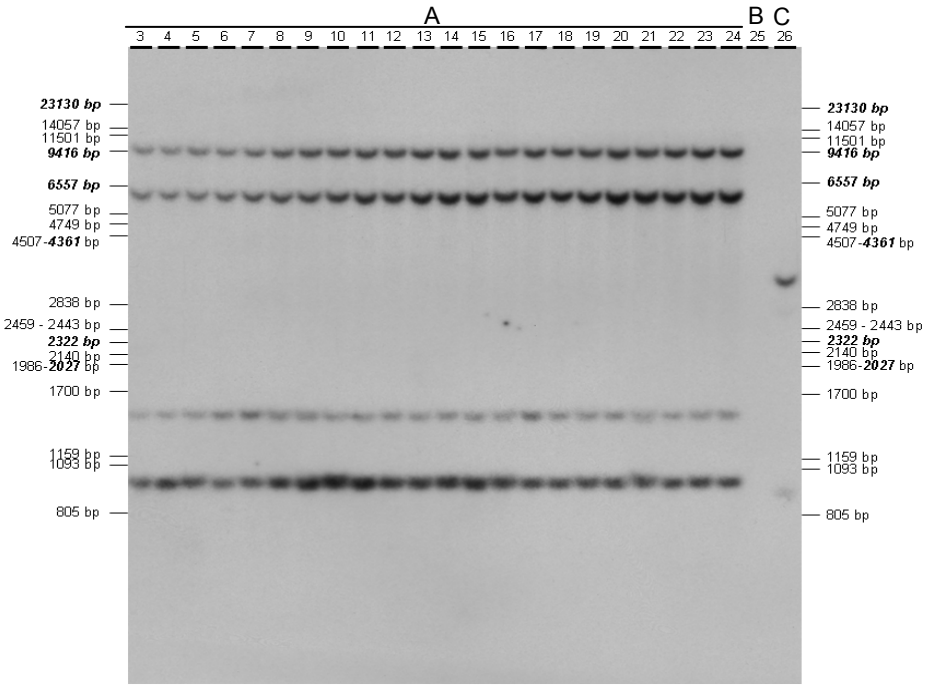
Ph4a748B probe



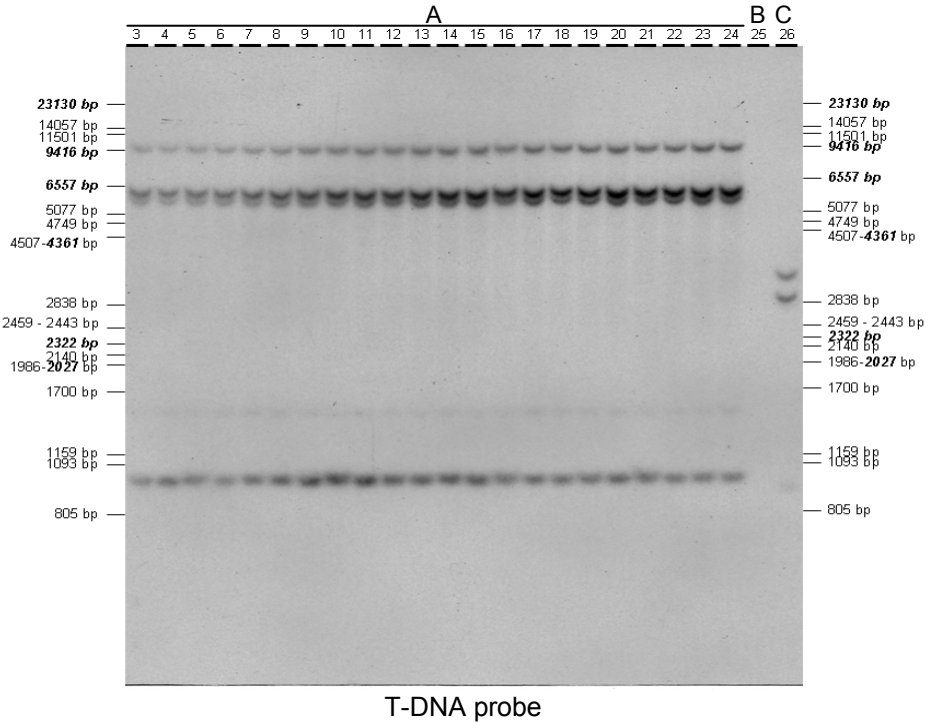
T-DNA probe

A: event FG72 samples; B: Negative control; C: positive control

Figure 29. Background 3066617-48 X Jack



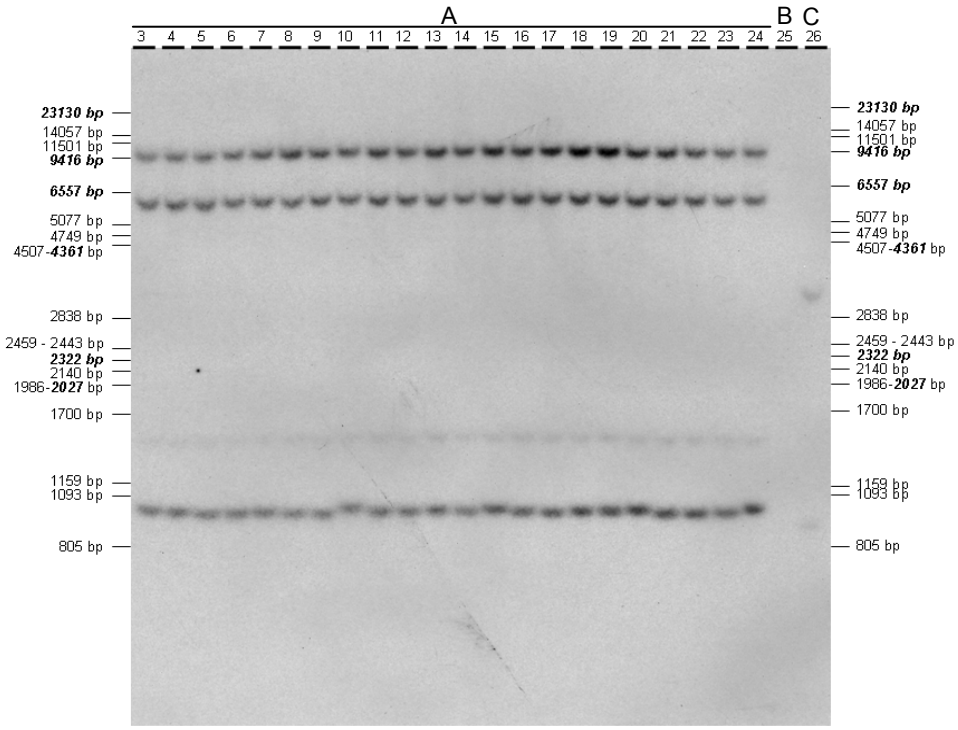
Ph4a748B probe



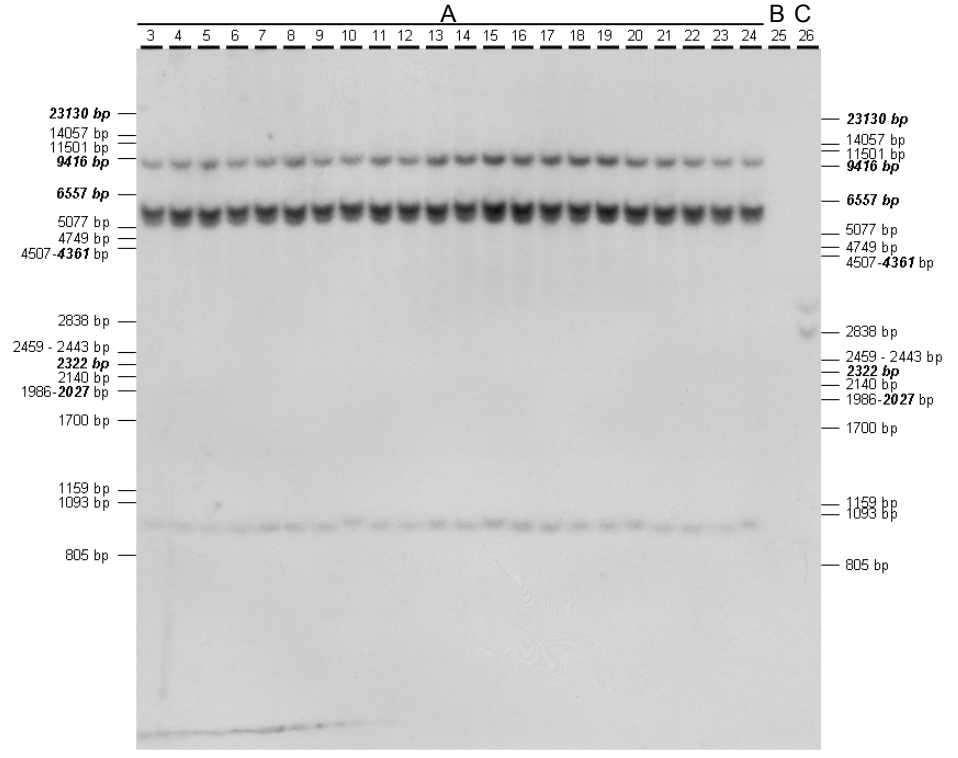
T-DNA probe

A: event FG72 samples; B: Negative control; C: positive control

Figure 30. Generation T2



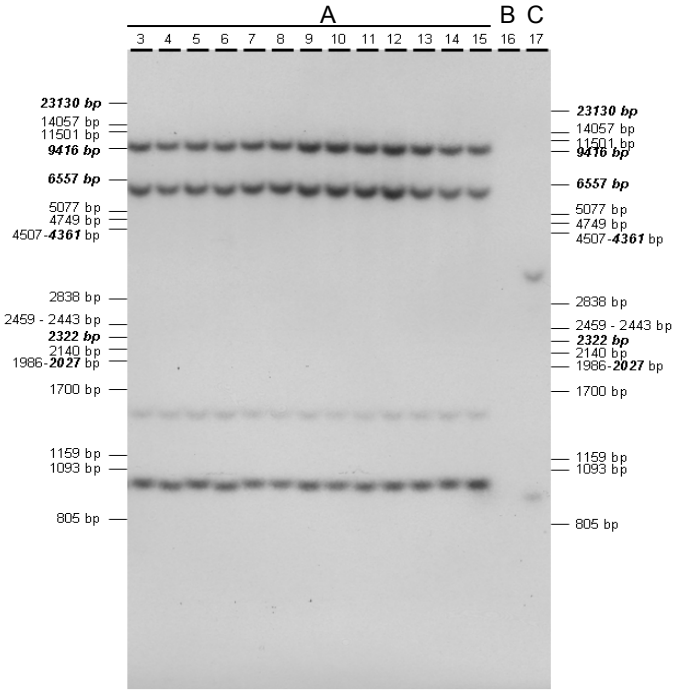
Ph4a748B probe



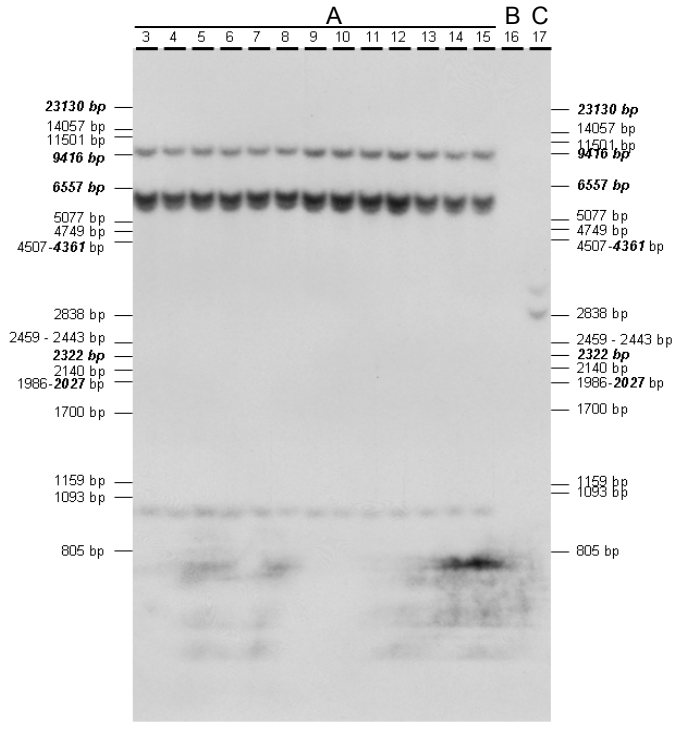
T-DNA probe

A: event FG72 samples; B: Negative control; C: positive control

Figure 31. Generation T7



Ph4a748B probe



T-DNA probe

A: event FG72 samples; B: Negative control; C: positive control

Figure 32. Schematic drawing of hybridization strategy for the demonstration of structural stability

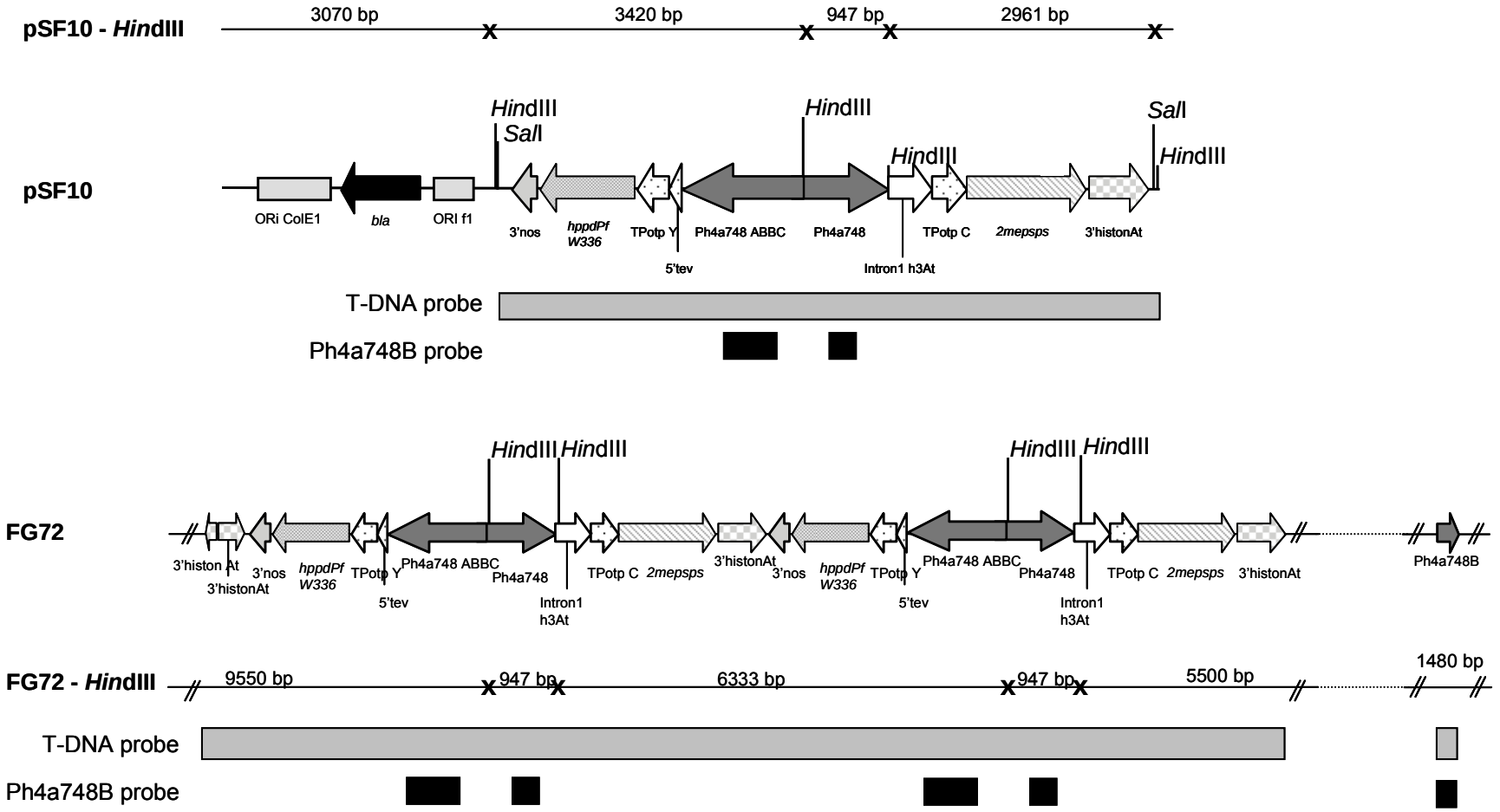


Table 52. Expected and obtained hybridization fragments

Samples	Condition tested	Seed lot	N° of plants	Expected T-DNA or plasmid fragment sizes	Fragment description	Ph4a748B probe		T-DNA probe	
						Exp.	Obt.	Exp.	Obt
FG72 - *Hin*dIII	Location Adel, Iowa	HT08SOY002-05-32	22						
	Location Osborn, Missouri	HT08SOY002-07-32	22						
	Location Fithian, Illinois	HT08SOY002-08-32	21	9550 bp [d]	5' integration fr.	Yes	Yes	Yes	Yes
	Location Sharpsville, Indiana	HT08SOY002-09-32	16	947 bp	internal fragment	Yes	Yes	Yes	Yes
	Background 3068115-48 x Jack	7BD60008	21	6333 bp	internal fragment	Yes	Yes	Yes	Yes
	Background 3066617-48 x Jack	7BD60018	22	5500 bp [d]	3' integration fr.	No	No	Yes	Yes
	Generation T2	FG72a-T2, FG72b-T2, FG72c-T2, FG72d,T2	22	1480 bp [d]	3' junction translocation	Yes	Yes [a]	Yes [b]	No [c]
	Generation T7	FG72-x-x-14-5-1-6-x T7	13						
Non transgenic Jack - *Hin*dIII	Non-transgenic variety Jack			/	Negative control	/	/	/	/
Non transgenic Jack - *Hin*dIII + pSF10 - *Hin*dIII	Non-transgenic variety Jack + equimolar amount pSF10			3420 bp	positive control	Yes	Yes	Yes	Yes
				947 bp	positive control	Yes	Yes	Yes	No [c]
				2961 bp	positive control	No	No	Yes	Yes
				3070 bp	positive control	No	No	No	No

[a] In some hybridizations this fragment is very weak, but present for all samples

[b] The overlap between the probe and the fragment can be too small to visualize this fragment

[c] Not always visible after hybridization with T-DNA probe but presence is confirmed after hybridization with probe Ph4a748B.

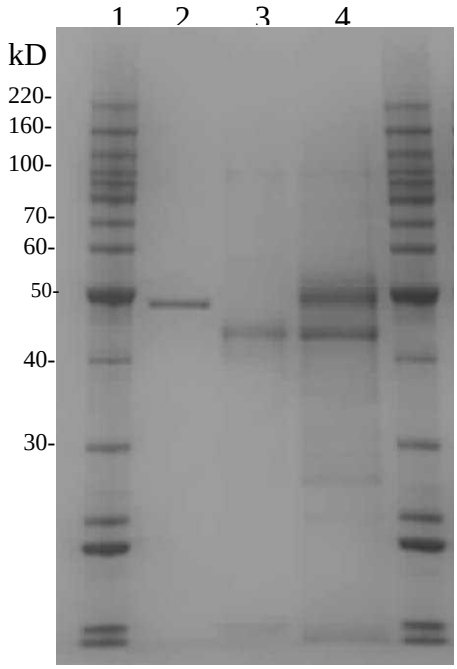
[d] Expected fragment sizes as determined in the detailed insert characterization study

3.D. Demonstration of protein equivalence

3.D.1 2mEPSPS protein

The SDS-PAGE and western blots demonstrated that the molecular weight, mobility, and immuno-reactivity of the plant-produced and microbially-produced 2mEPSPS proteins are the same. The western blot also indicated that the non-transgenic Jack soybean control sample did not have immunoreactive proteins. The band appearing below the 2mEPSPS band in the plant-produced protein did not appear on the western blot, indicating that the band is not related to the 2mEPSPS protein.

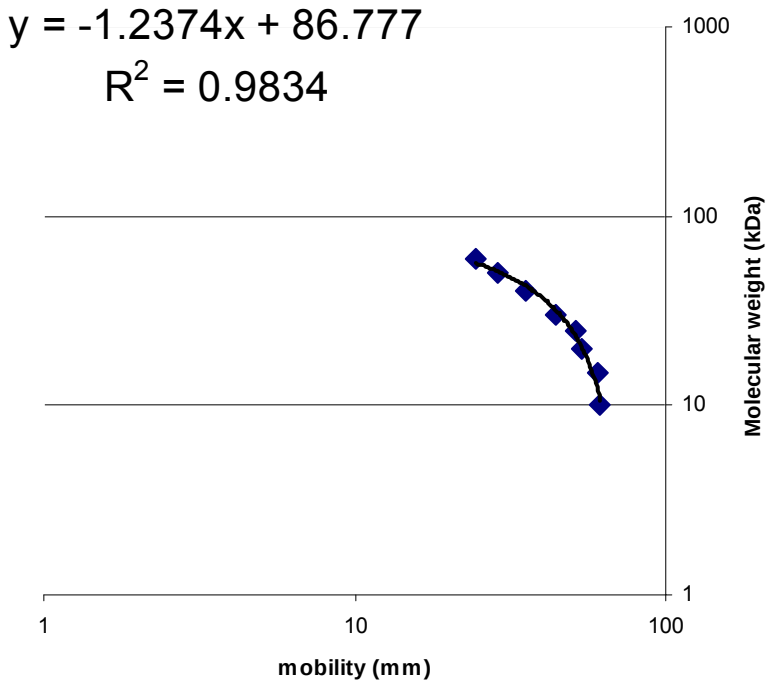
Figure 33. Comparison of the plant-produced and microbially-produced 2mEPSPS protein



The SDS-PAGE gel was stained with Pierce Imperial Protein Stain. Lane 2 contains approximately 300 ng of 2mEPSPS protein produced in E. coli. Lane 3 contains the non-transgenic Jack soybean leaves control sample. Lane 4 contains approximately 430 ng 2mEPSPS protein isolated from FG72 soybean leaves. Lanes 1 and 5 contain molecular weight markers of 220, 160, 120, 100, 90, 80, 70, 60, 50, 40, 30, 25, 20, 15 and 10 kDa. Underlined molecular weights are shown on the gel. A band below the FG72 soybean produced 2mEPSPS band in lane 4 appears to be coming from the soybean matrix.

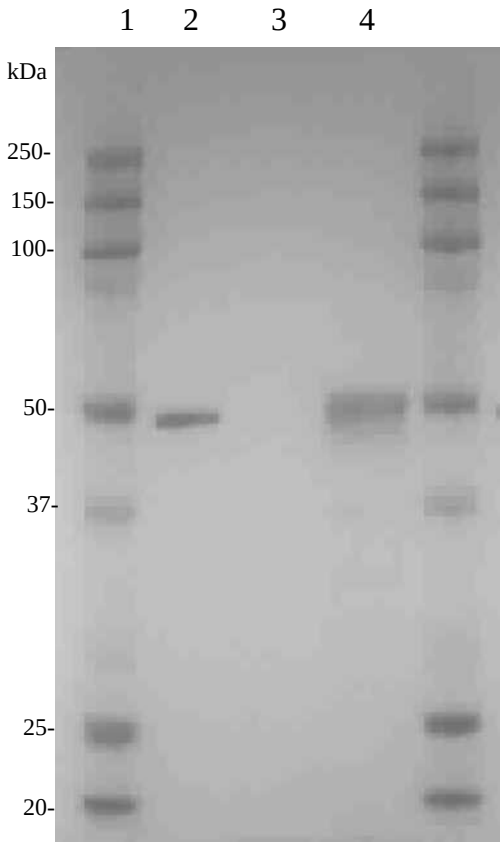
Lane 1. BenchMark Protein Ladder
Lane 2. *E.coli* produced 2mEPSPS Protein (Batch LEJ5838)
Lane 3. Non-transgenic Jack soybean leaves control sample
Lane 4. 2mEPSPS isolated from FG72 soybean leaves
Lane 5. BenchMark Protein Ladder

Figure 34. Standard curve of electrophoretic mobility versus molecular weight



The electrophoretic mobility of the protein standards for the SDS-PAGE gel shown in Figure 3.23 were plotted against their respective molecular weights bracketing the 2mEPSPS protein. The equation defining the curve is y = -1.2374x + 86.777. The R^2 value for this curve is 0.9834. The equation defining this curve was used to calculate an approximate molecular weight of 50.3 kDa for 2mEPSPS isolated from soybean, event FG72. The molecular weight of the *E. coli* produced 2mEPSPS protein calculated from the equation is 49.7 kDa.

Figure 35. Western blot comparison of the plant-produced and microbially-produced 2mEPSPS protein



Lane 2 contains the 2mEPSPS protein produced in *E. coli.* Lane 3 contains the Jack soybean leaves control sample. Lane 4 contains the 2mEPSPS protein isolated from FG72 soybean leaves. Lanes 1 and 5 contain molecular weight markers of 250, 150, 100, 75, 50, 37, 25 and 20 kDa. Underlined molecular weights are shown on the gel.

Lane 1. Kaleidoscope Precision Plus Protein Standards
Lane 2. *E. coli* produced 2mEPSPS Protein (Batch LEJ5838)
Lane 3. Non-transgenic Jack soybean leaves control sample
Lane 4. 2mEPSPS isolated from FG72 soybean leaves
Lane 5. Kaleidoscope Precision Plus Protein Standards

The full scan mass spectrometry results and amino acid coverage are given in Tables 53 and 54. The full scan analysis obtained 98% sequence coverage of the *E. coli* produced 2mEPSPS protein. The analysis of the tryptic digest of the plant-produced 2mEPSPS protein by full scan analysis obtained 71% sequence coverage of the protein. The peptides not being detected by full scan mass spectrometry could be caused by a missed cleavage, modification or matrix. The mass spectrometry results indicate that the methionine is missing from the N-terminal peptide, residue 1 to 13, for both the *E. coli* produced 2mEPSPS protein and the plant-produced 2mEPSPS protein. The loss of a methionine is not unusual, as post-translational modifications such as removal of a methionine are often found in proteins from both prokaryotic and eukaryotic organisms (Bradshaw *et al.*, 1998).

Table 53. Electrospray LC/MS peptide mapping of the 2mEPSPS protein

2mEPSPS Residue	Sequence	Theoretical [M+H]	98% cov. *E. coli* 2mEPSPS protein*	71% cov. FG72 Soybean Leaf*	Non-transgenic Jack Soybean Leaf*
1 to 13	MAGAEEIVLQPIK		700.9 [M+2H]	ND	ND
2 to 13	AGAEEIVLQPI	1268.7	635.4 [M+2H]	635.3 [M+2H]	ND
14 to 20	K EISGTVK	733.8	734.4 [M+H]	734.4 [M+H]	ND
1 to 20	MAGAEEIVLQPIKEISGTV	2113.2	705.4 [M+3H]	ND	ND
21 to 25	K LPGSK	501.6	502.4 [M+H]	502.3 [M+H]	ND
26 to 30	SLSNR	576.6	577.4 [M+H]	577.5 [M+H]	ND
31 to 61	ILLLAALSEGTTVVDNLLNSEDV HYMLGALR	3342.9	1115.3 [M+3H]	ND	ND
62 to 71	TLGLSVEAD	1033	517.6 [M+2H]	517.5 [M+2H]	ND
72 to 74	K AAK	289	289.5 [M+H]	289.7 [M+H]	289.9[M+H]
75 to 75	R	175	ND**	ND	ND
76 to 84	AVVVGCGG	790	424.5 [M+2H]	424.5 [M+2H]	ND
85 to 91	K FPVEDAK	805.9	ND**	ND**	ND
92 to 106	EEVQLFLGNAGIAMR	1648.9	ND**	ND**	ND
85 to 106	FPVEDAKEEVQLFLGNAGIAMR	2434.2	812.4 [M+3H]	812.5 [M+3H]	ND
107 to 128	SLTAAVTAAGGNATYVLDGVPR	2105	702.9 [M+3H]	702.8 [M+3H]	702.7 [M+3H]
129 to 130	MR	306	307.2 [M+H]	307.1 [M+H]	ND
131 to 142	ERPIGDLVVGLK	1296	649.2 [M+2H]	649.4 [M+2H]	ND
143 to 160	QLGADVDCFLGTDCPPVR	1907	1011.7 [M+2H]	1011.7 [M+2H]	ND
161 to 171	VNGIGGLPGGK	969	485.6 [M+2H]	485.5 [M+2H]	ND
172 to 173	VK	246	246.8 [M+H]	247.2 [M+H]	ND
174 to 204	LSGSISSQYLSALLMAAPLALGD VEIEIIDK	3219.8	ND**	ND	ND
205 to 216	LISIPYVEMTL	1435.8	ND**	ND	ND
217 to 220	R LMER	548.7	ND**	ND	ND
221 to 224	FGVK	450.6	ND**	ND**	ND
225 to 233	AEHSDSWDR	1103	ND**	ND**	ND
174 to 233	LSGSISSQYLSALLMAAPLALGD VEIEIIDKLISIPYVEMTLRLMERF GVKAEHSDSWDR	**6681.8**	1114.5 [M+6H]	ND	ND
234 to 237	FYIK	570.7	286.2 [M+2H]	286.3 [M+2H]	ND
238 to 241	GGQK	389	390.3 [M+H]	390.1 [M+H]	ND
242 to243	YK	310	310.8 [M+H]	310.7 [M+H]	ND
244 to 246	SPK	331	332.3 [M+H]	332.2 [M+H]	ND
247 to 286	NAYVEGDASSASYFLAGAAITG GTVTVEGCGTTSLQGDVK	3870	1309.8 [M+3H]	1309.8 [M+3H]	1310 [M+3H]
287 to 297	FAEVLEMMGAK	1226	614.6 [M+2H]	614.3 [M+2H]	ND
298 to 312	VTWTETSVTVTGPPR	1631.8	817 [M+2H]	816.9 [M+2H]	ND
313 to 317	EPFGR	605.6	ND	ND	ND
318 to 318	K	147	ND	ND	ND
319 to 321	HLK	397	398.3 [M+H]	398.2 [M+H]	ND
322 to 329	AIDVNMNK	905	453.6 [M+2H]	453.4 [M+2H]	ND
330 to 351	MPDVAMTLAVVALFADGPTAIR	2260.7	ND**	ND	ND
352 to 357	DVASWR	733.8	733.9 [M+H]	734.3 [M+H]	ND
330 to 357	MPDVAMTLAVVALFADGPTAIR DVASWR	**2975.5**	992.5 [M+3H]	ND	ND
358 to 359	VK	246	246.8 [M+H]	247.2 [M+H]	246.2 [M+H]
360 to 363	ETER	534	535.3 [M+H]	534.8 [M+H]	ND
364 to 368	MVAIR	589.8	590.4 [M+H]	590.3 [M+H]	ND
369 to 373	TELT	591.7	592.4 [M+H]	592.3 [M+H]	ND
374 to 392	LGASVEEK PDYCIITPPEK	2019	1039.2 [M+2H]	1039.2 [M+2H]	ND
393 to 405	LNVTAIDTYDDHR	1533.6	767.9 [M+2H]	768.1 [M+2H]	767.4 [M+2H]
406 to 423	MAMAFSLAACAEVPVTIR	1882	ND**	ND**	876.3 [M+3H]
424 to 429	DPGCTR	648.7	ND**	ND**	ND
406 to 429	MAMAFSLAACAEVPVTIRDPGC TR (Cys_CAM mod)	2624.2	875.9 [M+3H]	875.9 [M+3H]	876.3 [M+3H]
430 to 430	K	147	ND**	ND**	ND
431 to 444	TFPDYFDVLSTFVK	1679.9	841 [M+2H]	840.9 [M+2H]	ND
445 to 445	N	133	ND**	ND**	ND

* = Average mass reported. ND= Not detected. ND** = Missed cleavage, peptide not detected by full scan analysis.

Table 54. Amino acid coverage of the 2mEPSPS protein

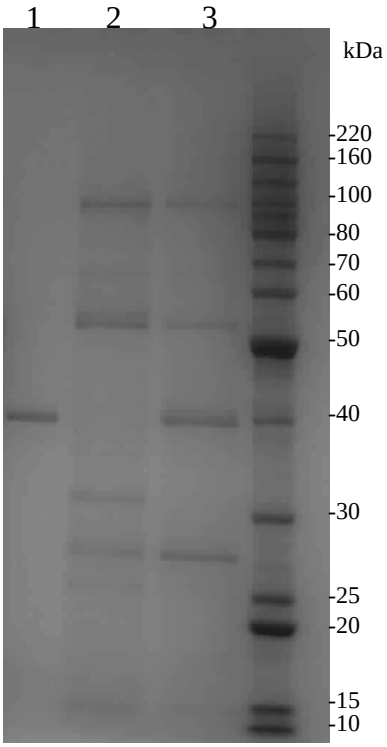
Calculation of % Amino Acid coverage	Number of Amino Acids Not Detected		Residue Number
	E. coli 2mEPSPS	Plant-produced 2mEPSPS	
		1	1-13
		31	31-61
	1	1	75-75
		60	174-233
	5	5	313-317
	1	1	318-318
		28	330-357
	1	1	430-430
	1	1	445-445
Total	9	129	NA[a]
Total number amino Acids	445	445	
% Amino Acid Not Detected or Analyzed	2	29	
% Amino Acid Sequence Coverage	98	71	
% Amino Acid Coverage to 2mEPSPS from *E. coli.*	100	72	

[a] NA = Not Applicable

3.D.2. HPPD W366 protein

The SDS-PAGE and western blots demonstrated that the molecular weight, mobility, and immuno-reactivity of the microbially-produced and plant-produced HPPD W336 proteins are the same. The western blot also indicated that the non-transgenic Jack soybean sample did not contain immunoreactive proteins. The plant-produced HPPD W336 protein had other bands due to non-specific binding of proteins from the soybean matrix as shown in the SDS-PAGE (Figure 36). These non-specifically bound matrix proteins are not immunoreactive and were not detected in the western blot (Figure 37). The *E. coli*-produced HPPD W336 protein batch LB020309 had higher molecular weight bands appearing in the western blot, most likely due to protein dimers. A band below the *E. coli* produced HPPD W336 protein was also detected and is believed to be due to protein degradation.

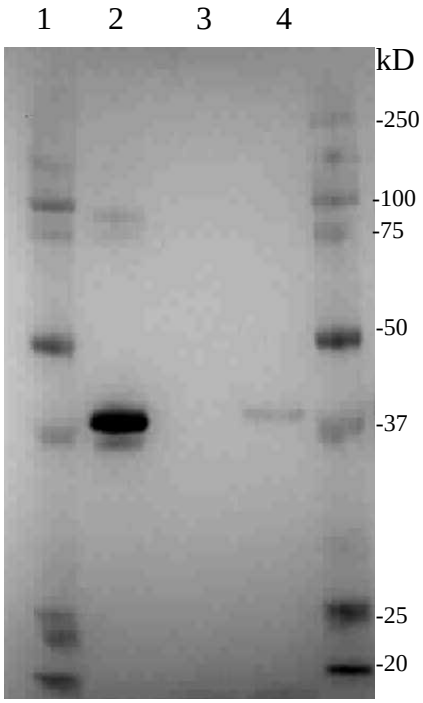
Figure 36. Comparison of the plant-produced and microbially-produced HPPD W336 protein



The SDS-PAGE gel was stained with Pierce Imperial Protein Stain. Lane 1 contains approximately 300 ng of HPPD W336 batch LB020309 protein produced in E. coli. Lane 2 contains the non-transgenic Jack soybean leaves control sample. Lane 3 contains the HPPD W336 protein isolated from FG72 soybean leaves. Lane 4 contains molecular weight markers of 220, 160, 120, 100, 90, 80, 70, 60, 50, 40, 30, 25, 20, 15 and 10 kDa. Underlined molecular weights are shown on the gel.

Lane 1. E. coli produced HPPD W336 Protein (Batch LB020309)
Lane 2. Non-transgenic Jack soybean leaves control sample
Lane 3. HPPD W336 isolated from FG72 soybean leaves
Lane 4. BenchMark Protein Ladder

Figure 37. Western blot comparison of the plant-produced and microbially-produced HPPD W336 protein



Lane 2 contains the HPPD W336 batch LB020309 protein produced in E. coli. Lane 3 contains the non-transgenic Jack soybean leaves control sample. Lane 4 contains the HPPD W336 protein isolated from FG72 soybean leaves. Lane 5 contains molecular weight markers of 250, 150, 100, 75, 50, 37, 25 and 20 kDa. Underlined molecular weights are shown on the gel.

Lane 1. Kaleidoscope Precision Plus Protein Standard
Lane 2. *E. coli* produced HPPD W336 Protein (Batch LB020309)
Lane 3. Non-transgenic Jack soybean leaves control sample
Lane 4. HPPD W336 isolated from FG72 soybean leaves
Lane 5. Kaleidoscope Precision Plus Protein Standard

The full scan mass spectrometry results are shown in Table 55. The full scan analysis obtained 95.2% sequence coverage of the *E. coli* produced HPPD W336 protein (Table 56). The analysis of the tryptic digest of the FG72 soybean produced HPPD W336 protein by full scan analysis obtained 70.1% sequence coverage of the protein (Table 56). Peptides not identified may be due to matrix effects or incomplete digestion.

Table 55. Electrospray LC/MS peptide mapping of the HPPD W336 protein

HPPD W336 Residue	Sequence	Theoretical [M+H]	95.2% cov *E. coli* HPPD W336 Protein *	70.1% cov. FG72 Soybean Leaf*	Non-transgenic Jack Soybean Leaf*
1 to 38	MADLYENPMGLMGFEFIEFASP TPGTLEPIFEIMGFTK	4274	ND	ND	ND
2 to 38	ADLYENPMGLMGFEFIEFASPT PGTLEPIFEIMGFTK	4143	1382 [M+3H]	1381.6 [M+3H]	ND
39 to 43	VATHR	583.7	584.4 [M+H]	584.3 [M+H]	ND
44 to 45	SK	234.3	ND	**234.1 [M+H]**	ND
46 to 51	NVHLYR	801.9	402 [M+2H]	401.7 [M+2H]	ND
52 to 84	QGEINLILNNEPNSIASYFAEHG PSV**C**GMAFR (cys_CAM mod)	3609	1203.7 [M+3H]	1204 [M+3H]	ND
85 to 86	VK	246.3	247.2 [M+H]	ND	ND
87 to 90	DSQK	477.5	ND	ND	ND
91 to 94	AYNR	523.6	524.3 [M+H]	524.2 [M+H]	ND
95 to 118	ALELGAQPIHIDTGPMELNLPAI K	2543	848.8 [M+3H]	848.7 [M+3H]	ND
119 to 130	GIGGAPLYLIDR	1245.5	623.9 [M+2H]	623.6 [M+2H]	ND
131 to 150	FGEGSSIYDIDFVYLEGVER	2296.5	1149.4 [M+2H]	ND	ND
151 to 158	NPVGAGLK	755.9	378.7 [M+2H]	ND	ND
159 to 169	VIDHLTHNVYR	1367.5	456.9 [M+3H]	456.5 [M+3H]	ND
170 to 171	GR	232.3	232.6 [M+H]	232.6 [M+H]	ND
172 to 181	MVYWANFYEK	1351.6	676.9 [M+2H]	676.5 [M+2H]	ND
182 to 186	LFNFR	696.8	ND	**697.4 [M+H]**	ND
187 to 189	EAR	375.4	375.7 [M+H]	375.5 [M+H]	ND
190 to 194	YFDIK	685.8	343.7 [M+2H]	343.4 [M+2H]	ND
195 to 203	GEYTGLTSK	956.0	479.0 [M+2H]	478.7 [M+2H]	ND
204 to 213	AMSAPDGMIR	1049.2	525.8 [M+2H]	525.7 [M+2H]	ND
214 to 222	IPLNEESSK	1017.1	509.5 [M+2H]	509.2 [M+2H]	ND
223 to 251	GAGQIEEFLMQFNGEGIQHVAF	3208.6	1070.8 [M+3H]	ND	ND
252 to 257	TWDALK	733.8	ND**	ND	ND
258 to 258	K	147.2	ND**	ND	ND
252 to 258	TWDALKK	862.0	432.0 [M+2H]	ND	ND
259 to 262	IGMR	476.6	477.3 [M+H]	ND	ND
263 to 278	FMTAPPDTYYEMLEGR	1922.2	962.2 [M+2H]	ND	ND
279 to 292	LPDHGEPVDQLQAR	1575.7	526.3 [M+3H]	526.1 [M+3H]	ND
293 to 305	GILLDGSSVEGDK	1290.4	ND**	ND**	ND
306 to 306	R	175.2	ND**	ND**	ND
293 to 306	GILLDGSSVEGDKR	1446.6	724.4 [M+2H]	724.1 [M+2H]	ND
307 to 327	LLLQIFSETLMGPVFFEFIQR	2530.0425	ND	ND	ND
328 to 328	K	147.1968	ND	ND	ND
329 to 339	GDDGFGEWNFK	1272.3154	637.1 [M+2H]	ND	ND
340 to 347	ALFESIER	965.0936	483.5 [M+2H]	483.2 [M+2H]	ND
348 to 351	DQVR	517.5621	ND**	ND**	ND
352 to 352	R	175.2102	ND**	ND**	ND
348 to 352	DQVRR	673.7496	337.7 [M+2H]	337.6 [M+2H]	ND
353 to 358	GVLTAD	575.6391	576.3 [M+H]	576.3 [M+H]	ND

Table 56. Amino acid coverage of the HPPD W336 protein

Calculation of % Amino Acid overage	Number of Amino Acids Not Detected		Residue Number
	E. coli HPPD W336	Plant-produced HPPD W336	
	1	1	1-38
	2[b]		44-45
		2	85-86
	4	4	87-90
		20	131-150
		8	151-158
	5[b]		182-186
		29	223-251
		7	252-258
		4	259-262
		16	263-278
		11	329-339
	4	4	348-351
	1	1	352-352
Total	17	107	NA[a]
Total number amino Acids	358	358	
% Amino Acid Not Detected or Analyzed	5	30	
% Amino Acid Sequence Coverage	95	70	
% Amino Acid Coverage to HPPD W336 from *E. coli*	100	71	

[a] NA = Not Applicable. [b]The amino acids were detected in the FG72 soybean produced HPPD W336 sample protein; but not in the *E. coli* produced HPPD W336 protein. [c]The sequence coverage was adjusted to account for the aminoacids 44-45 and 182-186 that were detected in the FG72 HPPD W336 sample; but not in the *E. coli* produced HPPD W336 protein.

Appendix 4

RAW AGRONOMIC DATA FOR 2008

Table 57. Raw agronomic data for 2008

Trial Number	Description	Early SC	Plant Vigor	Days to flowering	Flower Color	Leaf Shape	PH V4-5	PH R1	PH M	Pb	Pd	Hilum	Canopy	Days to Maturity	Yield bu/acre	Lodging	Final SC	Pod Shatter	Growth Habit
01	Jack	109	8	51	W	Oval	5	5	5	G	B	Y	Medium	138	50.17	5	97	8	I
01	Jack	100	8	52	W	Oval	5	5	5	G	B	Y	Medium	136	50.31	5	86	8	I
01	Jack	96	8	52	W	Oval	5	5	5	G	B	Y	Medium	138	52.78	5	92	8	I
02	Jack	110	8	52	W	Oval	5	5	5	G	B	Y	Medium	134	64.40	4	90	8	I
02	Jack	112	8	52	W	Oval	5	5	5	G	B	Y	Medium	135	67.52	4	104	8	I
02	Jack	117	8	52	W	Oval	5	5	5	G	B	Y	Medium	136	69.62	5	94	8	I
03	Jack	113	8	45	W	Oval	5	5	5	G	B	Y	Medium	131	71.87	2	89	8	I
03	Jack	106	8	45	W	Oval	5	5	5	G	B	Y	Medium	131	59.75	2	94	8	I
03	Jack	115	8	45	W	Oval	5	5	5	G	B	Y	Medium	131	67.23	2	92	8	I
04	Jack	150	8	45	W	Oval	5	5	5	G	B	Y	Medium	128	57.43	3	97	8	I
04	Jack	147	9	45	W	Oval	5	5	5	G	B	Y	Medium	128	47.77	3	98	7	I
04	Jack	146	9	45	W	Oval	5	5	5	G	B	Y	Medium	129	50.38	2	90	8	I
05	Jack	100	8	54	W	Oval	5	5	5	G	B	Y	Medium	136	60.84	3	74	8	I
05	Jack	98	8	54	W	Oval	5	5	5	G	B	Y	Medium	136	56.70	4	77	9	I
05	Jack	114	8	54	W	Oval	5	5	5	G	B	Y	Medium	137	56.27	4	89	9	I
06	Jack	90	8	48	W	Oval	5	5	5	G	B	Y	Medium	128	44.36	4	82	9	I
06	Jack	81	8	50	W	Oval	5	5	5	G	B	Y	Medium	129	40.51	4	60	9	I
06	Jack	95	8	48	W	Oval	5	5	5	G	B	Y	Medium	130	51.98	4	85	9	I
07	Jack	90	8	40	W	Oval	5	5	5	G	B	Y	Medium	119	44.21	6	78	8	I
07	Jack	106	9	40	W	Oval	5	5	5	G	B	Y	Medium	120	51.69	5	77	9	I
07	Jack	89	8	40	W	Oval	5	5	5	G	B	Y	Medium	119	48.64	5	69	9	I
08	Jack	123	8	36	W	Oval	5	5	5	G	T	Y	Medium Bushy	118	58.37	4	124	9	I
08	Jack	135	7	37	W	Oval	4	5	5	G	T	Y	Medium Bushy	118	53.29	4	134	8	I
08	Jack	129	9	36	W	Oval	4	5	5	G	T	Y	Medium Bushy	118	45.88	3	129	9	I
09	Jack	52	6	46	W	Oval	3	4	5	G	T	Y	Medium Bushy	118	39.78	9	51	8	I
09	Jack	101	8	46	W	Oval	5	4	5	G	T	Y	Medium Bushy	118		8	101	8	I
09	Jack	127	8	46	W	Oval	5	5	5	G	T	Y	Medium Bushy	119	39.78	8	128	8	I
10	Jack	118	8	46	W	Oval	4	5	5	G	B	Y	Medium	124	46.54	5	104	8	I
10	Jack	139	9	46	W	Oval	4	5	5	G	B	Y	Medium	124	45.81	5	106	8	I
10	Jack	126	8	46	W	Oval	4	5	5	G	B	Y	Medium	124	48.35	5	100	8	I
Mean		**111.1**	**8.1**	**46.47**			**4.8**	**4.9**	**5**					**127.7**	**53.18**	**4.4**	**93.0**	**8.3**	
SD		**21.4**	**0.6**	**5.28**			**0.5**	**0.3**	**0**					**7.2**	**8.96**	**1.7**	**19.1**	**0.5**	

Trial Number	Description	Early SC	Plant Vigor	Days to flowering	Flower Color	Leaf Shape	PH V4-5	PH R1	PH M	Pb	Pd	Hilum	Canopy	Days to Maturity	Yield bu/acre	Lodging	Final SC	Pod Shatter	Growth Habit
01	FG72 unsprayed	99	8	52	W	Oval	5	5	5	G	B	Y	Medium	137	42.11	4	94	8	I
01	FG72 unsprayed	91	8	53	W	Oval	5	5	5	G	B	Y	Medium	137	45.59	5	86	8	I
01	FG72 unsprayed	97	8	53	W	Oval	5	5	5	G	B	Y	Medium	136	46.61	5	87	8	I
02	FG72 unsprayed	89	8	52	W	Oval	5	5	5	G	B	Y	Medium	133	55.10	5	84	8	I
02	FG72 unsprayed	86	8	53	W	Oval	5	5	5	G	B	Y	Medium	134	59.97	4	79	8	I
02	FG72 unsprayed	79	8	52	W	Oval	5	5	5	G	B	Y	Medium	134	59.82	4	70	8	I
03	FG72 unsprayed	95	8	45	W	Oval	5	3	5	G	B	Y	Medium	131	59.90	4	81	8	I
03	FG72 unsprayed	101	8	45	W	Oval	5	5	5	G	B	Y	Medium	131	58.01	4	79	8	I
03	FG72 unsprayed	92	8	45	W	Oval	5	5	5	G	B	Y	Medium	130	60.91	4	83	8	I
04	FG72 unsprayed	94	8	46	W	Oval	5	3	5	G	B	Y	Medium	128	44.14	4	71	7	I
04	FG72 unsprayed	88	8	45	W	Oval	5	5	5	G	B	Y	Medium	127	55.76	4	76	7	I
04	FG72 unsprayed	95	8	45	W	Oval	5	5	5	G	B	Y	Medium	128	48.86	4	71	7	I
05	FG72 unsprayed	95	8	54	W	Oval	5	5	5	G	B	Y	Medium	137	51.33	5	74	8	I
05	FG72 unsprayed	87	8	54	W	Oval	5	5	5	G	B	Y	Medium	138	63.23	4	67	8	I
05	FG72 unsprayed	109	8	54	W	Oval	5	5	5	G	B	Y	Medium	138	70.13	3	74	8	I
07	FG72 unsprayed	23	7	40	W	Oval	3	3	5	G	B	Y	Medium	120	31.07	6	23	7	I
07	FG72 unsprayed	26	7	41	W	Oval	3	3	5	G	B	Y	Medium	120	33.32	6	30	8	I
07	FG72 unsprayed	39	7	42	W	Oval	3	3	5	G	B	Y	Medium	120	34.05	6	35	8	I
06	FG72 unsprayed	70	8	50	W	Oval	3	5	5	G	B	Y	Medium	131	43.49	6	63	8	I
06	FG72 unsprayed	87	8	48	W	Oval	5	5	5	G	B	Y	Medium	129	49.88	5	74	8	I
06	FG72 unsprayed	79	8	49	W	Oval	5	5	5	G	B	Y	Medium	129	48.06	5	79	8	I
08	FG72 unsprayed	102	8	37	W	Oval	5	5	5	G	T	Y	Medium Bushy	118	40.66	4	102	9	I
08	FG72 unsprayed	95	9	37	W	Oval	5	4	5	G	T	Y	Medium Bushy	119	36.66	5	93	9	I
08	FG72 unsprayed	103	7	37	W	Oval	5	5	5	G	T	Y	Medium Bushy	118	39.35	4	102	9	I
09	FG72 unsprayed	89	7	46	W	Oval	4	4	5	G	T	Y	Medium Bushy	118	41.09	9	88	9	I
09	FG72 unsprayed	89	7	46	W	Oval	4	4	5	G	T	Y	Medium Bushy	118	35.14	9	88	9	I
09	FG72 unsprayed	76	9	46	W	Oval	5	5	5	G	T	Y	Medium Bushy	119	27.15	9	76	8	I
10	FG72 unsprayed	70	8	45	W	Oval	4	5	5	G	B	Y	Medium	124	36.66	6	68	7	I
10	FG72 unsprayed	52	7	45	W	Oval	4	5	5	G	B	Y	Medium	124	37.75	5	47	7	I
10	FG72 unsprayed	67	8	45	W	Oval	4	5	5	G	B	Y	Medium	124	36.45	4	62	8	I
Mean		**82.1**	**7.8**	**46.7**			**4.6**	**4.6**	**5.0**					**127.7**	**46.4**	**5.1**	**73.5**	**8.0**	
SD		**21.7**	**0.5**	**5.2**			**0.7**	**0.8**	**0.0**					**7.0**	**11.0**	**1.6**	**19.0**	**0.6**	

Trial Number	Description	Early SC	Plant Vigor	Days to flowering	Flower Color	Leaf Shape	PH V4-5	PH R1	PH M	Pb	Pd	Hilum	Canopy	Days to Maturity	Yield bu/acre	Lodging	Final SC	Pod Shatter	Growth Habit
01	FG72 sprayed	79	8	53	W	Oval	3	3	5	G	B	Y	Medium	137	43.05	6	78	8	I
01	FG72 sprayed	99	8	54	W	Oval	3	3	5	G	B	Y	Medium	138	45.96	6	95	8	I
01	FG72 sprayed	95	8	53	W	Oval	3	3	5	G	B	Y	Medium	138	48.28	6	87	8	I
02	FG72 sprayed	109	8	54	W	Oval	3	3	5	G	B	Y	Medium	134	57.50	6	99	8	I
02	FG72 Sprayed	82	8	54	W	Oval	3	3	5	G	B	Y	Medium	137	49.95	6	76	8	I
02	FG72 sprayed	72	8	58	W	Oval	3	3	5	G	B	Y	Medium	137	51.40	7	75	8	I
03	FG72 sprayed	85	8	45	W	Oval	3	3	5	G	B	Y	Medium	133	52.49	6	74	8	I
03	FG72 sprayed	78	8	46	W	Oval	3	3	5	G	B	Y	Medium	133	56.70	6	74	8	I
03	FG72 sprayed	84	8	45	W	Oval	3	3	5	G	B	Y	Medium	133	60.19	6	83	8	I
04	FG72 sprayed	89	8	45	W	Oval	4	3	5	G	B	Y	Medium	127	49.08	6	70	7	I
04	FG72 sprayed	69	8	46	W	Oval	3	3	5	G	B	Y	Medium	129	43.12	5	58	7	I
04	FG72 sprayed	74	8	47	W	Oval	3	3	5	G	B	Y	Medium	130	44.94	6	50	7	I
05	FG72 sprayed	89	8	54	W	Oval	4	5	5	G	B	Y	Medium	138	58.30	5	74	8	I
05	FG72 sprayed	85	8	54	W	Oval	4	5	5	G	B	Y	Medium	137	62.80	6	69	8	I
05	FG72 sprayed	99	8	54	W	Oval	4	5	5	G	B	Y	Medium	137	63.31	4	81	8	I
06	FG72 sprayed	89	8	49	W	Oval	5	3	5	G	B	Y	Medium	130	44.58	5	63	8	I
06	FG72 sprayed	91	8	49	W	Oval	5	3	5	G	B	Y	Medium	130	50.75	6	83	8	I
06	FG72 sprayed	92	8	49	W	Oval	5	3	5	G	B	Y	Medium	131	42.40	6	81	8	I
07	FG72 sprayed	33	7	41	W	Oval	3	3	5	G	B	Y	Medium	121	29.04	6	28	8	I
07	FG72 sprayed	39	8	42	W	Oval	3	3	5	G	B	Y	Medium	121	34.78	6	32	7	I
07	FG72 sprayed	40	7	42	W	Oval	3	3	5	G	B	Y	Medium	121	31.00	6	38	7	I
08	FG72 sprayed	127	7	37	W	Oval	4	4	5	G	T	Y	Medium Bushy	120	43.34	5	127	9	I
08	FG72 sprayed	106	6	37	W	Oval	4	4	5	G	T	Y	Medium Bushy	120	55.54	5	109	9	I
08	FG72 sprayed	95	7	37	W	Oval	4	4	5	G	T	Y	Medium Bushy	119	36.30	5	92	9	I
09	FG72 sprayed	80	8	46	W	Oval	5	5	5	G	T	Y	Medium Bushy	119		9	78	8	I
09	FG72 sprayed	72	7	46	W	Oval	4	4	5	G	T	Y	Medium Bushy	119	28.10	9	72	8	I
09	FG72 sprayed	93	8	46	W	Oval	4	4	5	G	T	Y	Medium Bushy	119	33.83	9	92	8	I
10	FG72 sprayed	68	8	46	W	Oval	4	5	5	G	B	Y	Medium	125	32.89	6	57	8	I
10	FG72 sprayed	54	8	46	W	Oval	4	3	5	G	B	Y	Medium	125	27.23	6	54	7	I
10	FG72 sprayed	68	8	46	W	Oval	4	5	5	G	B	Y	Medium	125	35.21	7	60	7	I
Mean		**81.2**	**7.8**	**47.4**			**3.7**	**3.6**	**5.0**					**128.8**	**45.2**	**6.1**	**73.6**	**7.9**	
SD		**20.6**	**0.5**	**5.6**			**0.7**	**0.8**	**0.0**					**7.1**	**10.8**	**1.2**	**21.4**	**0.6**	

Trial Number	Description	Early SC	Plant Vigor	Days to flowering	Flower Color	Leaf Shape	PH V4-5	PH R1	PH M	Pb	Pd	Hilum	Canopy	Days to Maturity	Yield bu/acre	Lodging	Final SC	Pod Shatter	Growth Habit
01	2686-6	123	8	57	P	Ovate	5	5	5	G	B	Y	Medium Bushy	135	58.59	6	112	8	I
01	2788	139	8	55	P	Ovate	5	5	5	T	B	Bl	Medium Bushy	136	66.43	6	126	8	I
01	3000-0	127	8	59	P	Ovate	5	5	5	t	T	Br	Medium Bushy	138	73.62	6	112	8	I
02	2686-6	135	8	53	P	Ovate	5	5	5	G	B	Y	Medium Bushy	129	53.51	7	124	8	I
02	2788	109	8	54	P	Ovate	5	5	5	T	B	Bl	Medium Bushy	135	71.00	7	109	8	I
02	3000-0	100	8	57	P	Ovate	5	5	5	t	T	Br	Medium Bushy	139	78.84	7	90	8	I
03	2686-6	132	8	47	P	Ovate	5	5	5	G	B	Y	Medium Bushy	127	57.79	7	114	9	I
03	2788	129	8	47	P	Ovate	5	5	5	T	B	Bl	Medium Bushy	128	66.07	7	115	9	I
03	3000-0	106	8	50	P	Ovate	5	5	5	t	T	Br	Medium Bushy	131	67.81	7	100	9	I
04	2686-6	100	8	47	P	Ovate	5	5	5	G	B	Y	Medium Bushy	129	65.12	8	79	9	I
04	2788	121	9	46	P	Ovate	5	5	5	T	B	Bl	Medium Bushy	128	66.21	5	99	9	I
04	3000-0	118	8	48	P	Ovate	5	5	5	t	T	Br	Medium Bushy	129	65.63	6	97	9	I
05	2686-6	137	8	55	P	Ovate	5	5	5	G	B	Y	Medium Bushy	134	71.00	7	117	9	I
05	2788	101	8	55	P	Ovate	5	5	5	T	B	Bl	Medium Bushy	134	75.14	6	92	9	I
05	3000-0	103	8	57	P	Ovate	5	5	5	t	T	Br	Medium Bushy	134	79.93	6	93	9	I
06	2686-6	115	8	50	P	Ovate	5	5	5	G	B	Y	Medium Bushy	128	56.63	8	104	9	I
06	2788	124	8	51	P	Ovate	5	5	5	T	B	Bl	Medium Bushy	130	56.85	7	117	9	I
06	3000-0	116	8	53	P	Ovate	5	5	5	t	T	Br	Medium Bushy	131	71.15	6	103	9	I
07	2686-6	93	8	40	P	Ovate	5	5	5	G	B	Y	Medium Bushy	116	48.93	8	84	9	I
07	2788	54	8	40	P	Ovate	5	3	5	T	B	Bl	Medium Bushy	118	51.11	8	55	9	I
07	3000-0	65	8	42	P	Ovate	5	3	5	t	T	Br	Medium Bushy	120	63.89	7	60	9	I
08	2686-6	134	7	36	P	Ovate	5	5	5	G	T	Y	Medium Bushy	118	49.01	8	134	9	I
08	2788	133	9	37	P	Ovate	5	5	5	T	B	Bl	Bushy	120	34.85	6	132	9	I
08	3000-0	123	6	44	P	Ovate	5	4	5	T	B	Br	Bushy	120	58.08	7	124	9	I
09	2686-6	128	9	46	W	Ovate	5	5	5	G	T	Y	Medium Bushy	119	47.55	9	127	9	I
09	2788	133	9	46	W	Ovate	5	5	5	T	B	Bl	Bushy	119	58.23	8	128	9	I
10	3000-0	120	8	46	W	Ovate	4	5	5	T	B	Br	Bushy	119	48.35	8	120	9	I
10	2686-6	124	9	45	P	Ovate	4	5	5	G	B	Y	Medium Bushy	123	53.72	8	115	8	I
10	2788	65	8	46	P	Ovate	4	5	5	T	B	Bl	Medium Bushy	125	41.75	8	68	9	I
10	3000-0	84	8	47	P	Ovate	4	5	5	t	T	Br	Medium Bushy	127	54.16	7	82	9	I
Mean		**113.0**	**8.1**	**48.5**			**4.9**	**4.8**	**5.0**					**127.3**	**60.4**	**7.0**	**104.4**	**8.8**	
SD		**22.4**	**0.6**	**6.1**			**0.3**	**0.5**	**0.0**					**6.7**	**11.0**	**0.9**	**21.1**	**0.4**	
Legends																			
	Early SC = Early Stand Count;			PH V4-5 = Plant Health Rating Stage V4-5;						PH R1 = Plant Health Rating Stage R1;				PH M = Plant Health Rating Mature plants;					
	Pb = Pubescence color;			Pd = Pod color;		Final SC = Final Stand Count													

Appendix 5

HERBICIDE RESISTANCE AND STEWARDSHIP

5.A. 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 2009).

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).

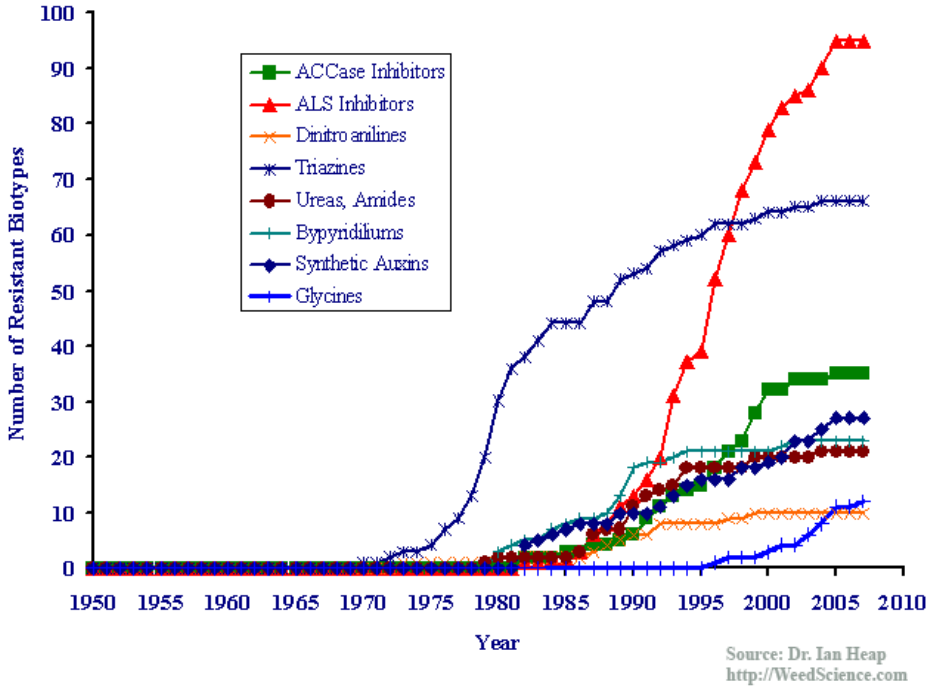
Weed control in the absence of complementary cultural control practices has resulted in the use of herbicides as the only weed control tactic. With this decline in use of alternative weed control methods, extensive use of herbicides with a single MOA has not only resulted in weed shifts but also high 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.

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, 2009).

The first herbicide resistant weed was identified in 1964 (HRAC, 2009). 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 post-emergence and residual weed control. Farming practices shifted, as use of ALS inhibitors reduced the amount of tillage needed for weed control. The lack of diversified weed control methods lead to the selection of populations of ALS herbicide-resistant weed species or biotypes.

There are 39 weed species resistant to the ALS class of chemistry in the US today and more than 90 resistant weed species reported world wide (Heap, 2009). 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 too many other herbicide modes of action as evident in Figure 38.

Figure 38. Timeline of the development of herbicide resistant weeds



5.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 will 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:

<u>Know your weeds, know your fields</u>
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.

Crop rotation

Crop rotation is one of the most important factors in an 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 to fewer weed species but to overall higher densities, which increases the selection pressure for herbicide resistant weeds.

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 spray 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).

Rotate herbicide modes of action

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

1. 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.

2. 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 et al. 2006). In addition, rotating crops generally allows additional modes of actions to be used in a weed management program.

3. 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.

Correct herbicide application
Product efficacy can be influenced by a multitude of factors. Ensuring correct use rates, weed stage and crop growth, and application technique will maximize weed control (Boerboom *et al.,* 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, sunlight and optimal soil nutrients.

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 3 inches to 4 inches tall 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 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.

Control weed escapes
Problematic 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.

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.

5.C. Evolution of herbicide resistant weeds

There are currently 9 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 artemisiifolia*), Johnsongrass (*Sorghum halepense*), Italian ryegrass (*Lolium multiflorum*), hairy fleabane (*Conyza bonariensis*), and rigid ryegrass (*Lolium rigidum*). There are an additional 7 glyphosate resistant weeds that can be found in other parts of the world (Heap 2009). Giant ragweed, common ragweed, and waterhemp are 3 of the top 10 most frequently sprayed for weeds in soybeans (Bayer CropScience, 2009).

Marestail, also known as horseweed, is the most widely spread glyphosate-resistant weed in the U.S. Marestail can produce up to 200,000 seeds per plant. In a management study conducted in Michigan, soybean yields could be reduced up to 83% by marestail in untreated check treatments (Bruce and Kells, 1990). Some populations of marestail have become resistant to other available herbicides including atrazines, simazines, diurons, and ALS inhibiting herbicides (Loux *et al.,* 2006; Heap, 2009).

Another glyphosate-resistant weed of concern is giant ragweed. Glyphosate-resistant giant ragweed isn't as widespread today as glyphosate-resistant waterhemp; however, it can be just as difficult to control with alternative herbicides. Giant ragweed can grow up to 17 feet tall and produces allergenic pollen. One giant ragweed plant per 110 square foot can reduce soybean yield 50%. There are also populations of giant ragweed that are resistant to ALS inhibiting herbicides (Johnson *et al.*, 2007).

Waterhemp is likely the weed of most concern in terms of control to soybean growers and university researchers. Waterhemp can produce more than 1 million seeds per plant. Waterhemp can reduce soybean yields by 37 to 44% in 7.5" and 30" rows, respectively (Nordby *et al.*, 2007). Moreover, nearly all populations of waterhemp are also resistant to ALS inhibiting herbicides and some populations are resistant to triazines and PPO inhibiting herbicides (Boerboom and Owen, 2006; Heap,2009).

Today there are few choices for conventional herbicides that are rated as "good" by University Extension programs for waterhemp control in glyphosate tolerant soybeans. Of those that are rated as good, their use is complicated as described in the following discussion.

<u>Soil-applied residual herbicides</u>
Growers applied pre-emergence or pre-plant incorporated herbicides on less than 5% of soybean acres in 2006 (USDA, 2007). The use of residual herbicides declined due to the efficacy and ease of glyphosate use. The seedling growth inhibitors or microtubule inhibitors such as pendimethalin (Prowl®), trifluralin (Treflan®), and ethalfluralin (Sonalan®) which inhibit cell division, provide residual control of waterhemp, however, these herbicides need to be incorporated into the soil for maximum efficacy. Products that contain chloroacetamide herbicides and control waterhemp in soybean include s- metolachlor (Dual II Magnum®), s-metolachlor + fomesafen, dimethenamid-P (Outlook®) and alachlor (Intrro®, Micro-tech®). Another pre-emergence herbicide for the control of waterhemp is metribuzin (Sencor®), which is in the triazinone family but can result in crop damage in certain environmental conditions.

Post-applied herbicides
The only conventional herbicide mode of action that provides "good" control of waterhemp post-emergence as rated by University Weed Scientists are the PPO inhibitors. These herbicides inhibit the protoporphyrinogen oxidase (PPO) enzyme which is involved in the heme-pigment synthesis pathway. Products that contain PPO inhibitors, such as lactofen (Cobra®, Phoenix™), fomesafen (Flexstar®, Reflex®) and s-metolachlor + fomesafen (Prefix™) have potential to injure the soybean crop. Applying PPO inhibitors under high temperature and humidity increases the potential crop injury. Also, there are populations of waterhemp that are resistant to PPO inhibiting herbicides (Boerboom and Owen 2006; Heap 2009). In addition, there conventional herbicides have stringent limitations on the size of waterhemp and other weeds that they can control. Environmental situations prevent timely application of conventional herbicides, weed control will be sacrificed.

5.D. Characteristics of glyphosate and isoxaflutole herbicides

5.D.1. 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®), glyphosate trimethylsulfonium salt (Touchdown®), or glyphosate diammonium salt (Touchdown® 4 or Touchdown Pro®). 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® label 2006). Glyphosate is likely the most broad spectrum herbicide available today for weed control in row crops. The effectiveness of glyphosate is established; more than 90% of the soybean acres in 2006 were treated with glyphosate at an average use rate of 0.802 lb/A with an average of 1.7 applications per season (USDA, 2007). The lack of effective alternatives is illustrated by the fact that the second most commonly used herbicide in 2006 was 2,4-D 2-EHE which was sprayed on only 7% of the US soybean acres (USDA, 2007). In addition to soybean, glyphosate was applied to 85% of the planted cotton acres in 2007 (USDA, 2008) although resistance to glyphosate has developed in several weed species, the chemical is extremely effective on the vast array of weeds in commercial crops.

5.D.2. Isoxaflutole herbicide

Isoxaflutole (IFT), the base active ingredient for several broad spectrum residual herbicides, is an isoxazole which inhibits the enzyme p-hydroxylphenyl pyruvate deoxygenase (HPPD) in plants resulting in inhibition of carotenoid biosynthesis which gives rise to a bleached appearance of new growth (Vencill, 2002). Isoxaflutole has been commercially available in the US since 1999 as Balance® Pro registered for use in field corn. Other herbicides registered for use in the US based upon the active ingredient IFT include Balance® Flexx (IFT + cyprosulfamide), Corvus™ (IFT + thiencarbazone + cyprosulfamide) and Radius™ (IFT + flufenacet). There are two IFT containing products available for use on field corn in Canada: Converge® Pro (IFT) and Converge® XT (IFT + cyprosulfamide).

To date, there is no documented resistance of any weed species to isoxaflutole or any other HPPD inhibitor in the world (Heap, 2009). The herbicidal mode of action of IFT, class F (4-hppd inhibitors), is unique to soybean production systems. Soil applied herbicides, like IFT, increase the consistency of weed control, reduce early season weed competition which results in significant yield losses, and introduces a new mode of action for decreased selection pressure for glyphosate resistance (Nordby *et al.*, 2007).

IFT herbicide can be applied either pre-emergence to the soybean or post emergence in a tankmix with a post-emergence herbicide. IFT can provide control of a broad spectrum of weeds including herbicide-resistant weed species. IFT provides pre-emergence control of 54 grass and broadleaf weed species important to soybean production including weeds resistant to glyphosate such as marestail, palmer amaranth, waterhemp, giant ragweed, common ragweed, and seedling Johnsongrass (Balance® Pro herbicide label).

5.E. Stewardship of double-herbicide-tolerant soybean event FG72

Bayer CropScience (BCS) places a high importance on the sustainability of its technology and has adopted a life-cycle approach to product stewardship. This means that appropriate stewardship principles are applied at every stage of biotechnology development from research through to product discontinuation and as a founding member of Excellence Through Stewardship®, BCS is helping advance stewardship best practices throughout the industry. BCS commitment to stewardship extends to our corporate relationships and is evidenced by the stewardship and quality assurance standards that are required in those relationships and is also indicated in the following clause that is now included in third party agreements related to BCS biotechnology traits:

> **"**BAYER is committed to the proper stewardship of its products and expects those with whom it contracts to handle material containing BAYER technology in an appropriate manner. This includes without limitation adherence to the stewardship and quality assurance provisions of this Agreement. BAYER supports and has affirmed its commitment to the Excellence Through Stewardship® industry stewardship initiative. Further information relating to this initiative can be found at www.excellencethroughstewardship.org."

In the BCS organization, our crop market area teams are committed to BCS stewardship principles and are aware of procedures to communicate appropriate information within the BCS crop team matrix to rapidly respond to issues that may develop from use of our technologies. Field development and market support teams are provided the tools necessary to serve the grower as a local and direct contact for any questions related to BCS technologies with regards to product performance or impacts on human and environmental health and safety.

BCS participates in several industry and professional initiatives in support of stewardship:

- Herbicide Resistance Action Committee (HRAC)
  HRAC is an industry initiative which fosters co-operation between plant protection manufacturers, government, researchers, advisors and farmers. The objective of the working group is to facilitate the effective management of herbicide resistance. Weed scientists employed by Bayer CropScience participate as members of the Herbicide Resistance Action Committee and BCS supports the work of this group.

- CropLife America - US
  BCS is active in CropLife, serving on committees and working groups that develop industry-wide approaches to regulatory and technology management issues.

- American Seed Trade Association (ASTA)
  M.S. Technologies, LLC (MSTech) and BCS are active in serving on committees and working groups that set industry standards for seed quality and purity, and product stewardship.

- BIO
  BCS is active in the Biotechnology Industry Organization, serving on committees and working groups that develop industry-wide approaches to regulatory and technology management issues.

- Excellence Through Stewardship
  BCS is active in Excellence Through Stewardship, serving on the board of directors, committees and working groups that develop industry best practices for stewardship.

- Weed Science professional societies
  BCS is active participant in a number of organizations. We maintain active memberships in the Weed Science Society of America, North Central Weed Science Society, Northeastern Weed Science Society, Southern Weed Science Society, and Western Weed Science Society, all of which are professional, non-profit societies, established to promote research, education, and extension outreach activities related to weeds; provide science-based information to the public and policy makers; and foster awareness of weeds and their impacts on managed and natural ecosystems (WSSA 2009).

5.E.1. Customer outreach

BCS and MSTech have a commitment to stewardship of 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 (see section 5.B.). Education of integrated weed management is the only practical method for its success. Education starts internally with our own field development, technical service, chemical sales representatives, 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 that will 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. BCS and MSTech are committed to stewardship principles and procedures, and to communicating appropriate information in order to rapidly respond to any issues 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 FG72 Soybeans will be recorded by seed company partners. This information will be provided to MSTech which will enable MSTech to maintain a database of all growers utilizing event FG72 products. This database could be used to disseminate updated stewardship information.

5.E.2. 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 an herbicide. BCS's history of including recommendations on product labels for integrated weed management. Here is an example of a BCS product label on this topic.

> BALANCE® FLEXX Herbicide is also recommended as the first herbicide applied in an integrated weed control program that includes sequential post-emergence herbicide applications.
>
> CORVUS™ Herbicide may be applied as the first herbicide in an Integrated weed control program that includes sequential post-emergence herbicide applications with products such as LAUDIS™ Herbicide, or IGNITE® 280 SL Herbicide or glyphosate in transgenic field corn.

BCS is committed to supporting research by university institutions to generate local grower recommendations. University Extension Weed Control Handbooks (*2008 Guide for Iowa Corn and Soybean Production, Illinois Agricultural Pest Management Handbook, 2009 Weed Control Guide for Ohio and Indiana*) contain use directions and product information on many BCS herbicides.

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.

BCS invests a significant amount of resources to inform and train our own employees, customers and stakeholders so that they can develop sustainable programs to manage both their resistant and susceptible weed populations. Modern testing conducted in the laboratory such as those employed by Bayer CropScience will in the future allow faster and more reliable herbicide resistance diagnosis. Such methods include testing for metabolic resistance by following the degradation of an active substance in a plant and testing for target-site resistance through PCR analysis coupled with pyrosequencing.

5.E.3. Monitoring of effectiveness of the stewardship plan

Each grower purchase of event FG72 soybeans will be recorded by the individual seed company making the sale. This information will be provided to MSTech which will enable MSTech to maintain a database of all growers utilizing event FG72 products. BCS regularly utilizes market research surveys to determine market share and adaptation of technology.

Seed company partners will have direct contact with growers and will be able to provide feed back to MSTech regarding the stewardship effectiveness. BCS field representatives will also interact with growers and will be a source of information.

BCS will continue to support ongoing efforts to understand weed resistance to herbicides, to apply up-to-date information to product labels, and to provide information to growers.

Appendix 6

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