SAFETY AND NUTRITIONAL SUMMARY ASSESSMENT FOR HERBICIDE-TOLERANT MZHG0JG CORN

OECD Unique Identifier: SYN-ØØØJG-2

Applicant

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Submission Date

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Executive Summary

Syngenta Crop Protection LLC, on behalf of Syngenta AG and its affiliates, has developed MZHG0JG corn (maize; *Zea mays* L.), a new cultivar that has been genetically modified to tolerate glyphosate and glufosinate-ammonium herbicides. Most corn currently grown in the United States and Canada consists of herbicide-tolerant transgenic varieties. MZHG0JG corn will offer growers much-needed flexibility to use herbicides with two alternative modes of action in their weed management programs and will help mitigate and manage the evolution of herbicide resistance in weed populations.

MZHG0JG corn plants contain the transgene *mepsps-02*, which encodes the enzyme mEPSPS, and the transgene *pat-09*, which encodes the enzyme phosphinothricin acetyltransferase (PAT). The native 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) from *Z. mays* is involved in the synthesis of aromatic amino acids and is inhibited by glyphosate. The mEPSPS produced by MZHG0JG corn has low affinity for glyphosate, thus conferring tolerance to glyphosate in herbicide products. The transgene *pat-09* was derived from the soil bacterium *Streptomyces viridochromogenes*. PAT acetylates glufosinate-ammonium, thus inactivating it and conferring tolerance to glufosinate-ammonium in herbicide products. PAT was used as a selectable marker in the development of MZHG0JG corn.

MZHG0JG corn was produced by transformation of immature embryos of proprietary variety NP2222 via *Agrobacterium tumefaciens*–mediated transformation. The region of the plasmid vector, pSYN18857, intended for insertion into the corn genome included gene-expression cassettes for *mepsps-02* and *pat-09*. The *mepsps-02* expression cassette consisted of the *mepsps-02* coding region regulated by a corn ubiquitin promoter (Ubi58-02) and terminator (Ubil58-02), as well as the figwort mosaic virus (FMV-05), cauliflower mosaic virus 35S (35S-05), and tobacco mosaic virus (TMV-03) enhancer sequences and an optimized transit peptide (OTP-02). The *pat-09* expression cassette consisted of the *pat-09* coding region regulated by a 35S promoter from cauliflower mosaic virus (35S-19) and the nopaline synthase (NOS) terminator sequence from *A. tumefaciens* (NOS-05-01).

Genetic characterization studies demonstrate that MZHG0JG corn contains, at a single locus within the corn genome, a single copy of each of the following functional elements: *mepsps-02*, *pat-09*, FMV-05 enhancer, 35S-05 enhancer, OTP-02 transit peptide, Ubi158-02 promoter, TMV-03 enhancer, Ubi158-02 terminator, 35S-19 promoter, and NOS-05-01 terminator. No extraneous DNA fragments of these functional elements occur elsewhere in the MZHG0JG corn genome. Similarly, plasmid backbone sequence from transformation plasmid pSYN18857 is not present in the MZHG0JG corn genome. Analyses comparing the corn genomic sequence flanking the MZHG0JG insert with sequences in public databases indicate that the inserted DNA does not disrupt any known endogenous corn gene.

Southern blot analyses demonstrated that the MZHG0JG T-DNA insert is stably inherited from one generation to the next and that the MZHG0JG corn genome contains a single T-DNA insert. The observed segregation ratios for *mepsps-02* and *pat-09* in three generations of MZHG0JG corn plants indicated that they are inherited in a predictable manner, according to Mendelian principles. Analyses of grain and forage demonstrate that MZHG0JG corn is nutritionally and compositionally similar to, and as safe and nutritious as, conventional corn.

Well-characterized modes of action, physicochemical properties, and a history of safe use demonstrate that the mEPSPS and PAT proteins present in MZHG0JG corn present no risk of harm to humans or livestock that consume corn products or to wildlife potentially exposed to MZHG0JG corn.

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Table of Abbreviations, Acronyms, and Symbols

35S-05 enhancer	transcriptional enhancer region of the cauliflower mosaic virus
35S-19 promoter	promoter region of the cauliflower mosaic virus
aadA-03	spectinomycin resistance gene
acetyl CoA	acetyl coenzyme A
ADF	acid detergent fiber
ANOVA	analysis of variance
BC	backcross
BLASTP	Basic Local Alignment Search Tool for Proteins
BLASTX	Basic Local Alignment Search Tool for Translated Nucleotides
bp	base pair
CFIA	Canadian Food Inspection Agency
CoASH	coenzyme A
CTP	chloroplast transit peptide
DNA	deoxyribonucleic acid
DTNB	5,5′-dithiobis(2-nitrobenzoic acid)
DW	dry weight
ECCB	Exclusive Capturable Commercial Benefit
ELISA	enzyme-linked immunosorbent assay
EPSP	5-enolpyruvylshikimate-3-phosphate
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
F_1	first filial generation
FMV05 enhancer	transcriptional enhancer region of the figwort mosaic virus
FW	fresh weight
FSANZ	Food Standards Australia New Zealand
HC	Health Canada
HRP	horseradish peroxidase
ILSI	International Life Sciences Institute
kb	kilobase pairs
kDa	kilodalton
LB	left border
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LOD	limit of detection
LOQ	limit of quantitation
mEPSPS	modified EPSPS
mepsps-02	double-mutated 5-enolpyruvylshikimate-3-phosphate synthase gene
N	sample size
N/A	not applicable
NCBI	National Center for Biotechnology Information
NDF	neutral detergent fiber
ng	nanogram
NOS	nopaline synthase
NOS-05-01 terminator	nopaline synthase terminator sequence from *A. tumefaciens*
OECD	Organisation for Economic Co-operation and Development
ORF	open reading frame
ori	origin of replication
OTP-02	optimized transit peptide
P	probability
PAGE	polyacrylamide gel electrophoresis

PAT	phosphinothricin acetyltransferase
pat-09	phosphinothricin acetyltransferase gene
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
RB	Right border
repA-03	replication-protein gene from *Pseudomonas aeruginosa* plasmid VS1
S3P	shikimate-3-phosphate
SDS	sodium dodecyl sulfate
SEM	atandard error of the mean
SGF	simulated mammalian gastric fluid
SIF	simulated mammalian intestinal fluid
T_0, T_1, T_2, T_3, etc.	T_0 refers to the original transformed plant, and T_1, T_2, T_3, etc., refer to successive self-pollinated generations
T-DNA	transferred DNA
TIU	trypsin inhibitor unit
TMV-03 enchancer	transcription enhancer region of the tobacco mosaic virus
TNB	2-nitro-5-thiobenzoic acid
TNB^{2-}	2-nitro-5-thiobenzoate anion
U.S.C.	United States Code
Ubi158-02	corn ubiquitin promoter and terminator
v.	version
virG-01	part of the regulatory system for the virulence regulon in *Agrobacterium tumefaciens*
WHO	World Health Organization
×	cross, cross-pollination
⊗	self-pollination
χ^2	chi squared

Corn Growth Stages (Abendroth *et al.* 2011)

Vegetative:	
V2	first two leaves collared
V3	first three leaves collared
V4	first four leaves collared
V5	first five leaves collared
V6	first six leaves collared
V7	first seven leaves collared
V8	first eight leaves collared
V9	first nine leaves collared
V10	first ten leaves collared
V11	first eleven leaves collared
V12	first twelve leaves collared
V13	first thirteen leaves collared
VT	tassel
Reproductive:	
R1	silking
R2	blister
R3	milk
R4	dough
R5	dent
R6	physiological maturity

Amino Acids

Ala, A	alanine
Arg, R	arginine
Arn, N	asparagine
Asp, D	aspartic acid
Cys, C	cysteine
Gln, Q	glutamine
Glu, E	glutamic acid
Gly, G	glycine
His, H	histidine
Ile, I	isoleucine
Leu, L	leucine
Lys, K	lysine
Met, M	methionine
Phe, F	phenylalanine
Pro, P	proline
Ser, S	serine
Thr, T	threonine
Trp, W	tryptophan
Tyr, Y	tyrosine
Val, V	valine

Part I. General Information

I.A. Name, Address, and Contact Information for Applicant

Australia: Food Standards Australia New Zealand (FSANZ)

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[redacted]

I.B. Purpose of the Application

The purpose of the application is to seek regulatory approvals in Australia, New Zealand, and Canada to permit the sale and use of food derived from MZHG0JG.

Australia and New Zealand

This application seeks to vary FSANZ Standard 1.5.2 to allow the use of genetically modified corn (maize; *Zea mays* L.) derived from Event MZHG0JG corn (hereafter MZHG0JG corn) in Australia and New Zealand food industries.

Canada

This application seeks authorization for use of food from HC and feed from CFIA derived from genetically modified MZHG0JG corn in Canada.

This application has been written by Syngenta Australia Pty Ltd and Syngenta Canada, Inc. (Syngenta), and is aimed to be reviewed in parallel by FSANZ and HC. This *parallel review* approach may be considered as a first step towards achieving and implementing Mutual Recognition.

I.C. Justification for the Application

Crops improved through modern biotechnology have brought significant benefits to agriculture in the form of improved yields, pest management, and crop quality. Continued innovation in this area will benefit growers, consumers, and the environment.

Adoption of genetically engineered crops with herbicide tolerance and insect resistance traits has increased dramatically since the first commercial introductions of transgenic corn, cotton, and soybean in 1996. Net economic benefits at the farm level have been substantial (Brookes and Barfoot 2006, Hutchison *et al.* 2010). Improved weed and insect control have led to increased crop yields, reductions in conventional pesticide applications, and environmental benefits (Brooks and Barfoot 2010).

MZHG0JG corn will offer growers increased flexibility in using herbicides with two different modes of action in their weed-management programs, thus helping to mitigate and manage the evolution of herbicide resistance in weed populations. As most corn currently grown in the United States consists of transgenic varieties that are glyphosate and/or glufosinate-ammonium tolerant, introduction of MZHG0JG corn to commercial cultivation is not expected to significantly alter corn agronomic practices or the use of these two herbicide products. By providing dual modes of herbicide tolerance at single breeding locus, MZHG0JG corn will also increase the efficiency of trait conversion into elite genetic lines, thus increasing the speed with which multiple traits can be combined in commercial corn products to meet growers' needs.

I.D. Costs and Benefits and Impact on Trade

Australia and New Zealand

The costs and benefits and impact on trade are the same as those described in previous corn applications submitted to FSANZ (A1060; A1001).

Canada

Not applicable

I.E. Exclusive Capturable Commercial Benefit (ECCB)

Australia and New Zealand

This application is likely to result in an amendment to the FSANZ Food Standards Code. Approval for use of MZHG0JG as food in Australia is likely to provide an ECCB for Syngenta, and therefore Syngenta will pay the full cost of processing this application.

Canada

Not applicable.

I.F. Confidential Commercial Information

Syngenta will request that FSANZ, HC, and CFIA treat parts of the information supplied in this application as confidential information. This information will be clearly marked as Confidential Business Information.

I.G. References Citied in Part I

Brookes G, Barfoot P. 2006. Global impact of biotech crops: Socio-economic and environmental effects in the first ten years of commercial use. *AgBioForum* 9:139–151.

Brookes G, Barfoot P. 2010. Global impact of biotech crops: Environmental effects, 1996-2008. *AgBioForum* 13:76–94.

Hutchison WD, Burkness EC, Mitchell PD, Moon RD, Leslie TW, Fleischer SJ, Abrahamson M, Hamilton KL, Steffey KL, Gray ME, Hellmich RL, Kaster LV, Hunt TE, Wright RJ, Pecinovsky K, Rabaey TL, Flood BR, Raun ES. 2010. Areawide suppression of European corn borer with Bt maize reaps savings to non-Bt maize growers. *Science* 330:222–225. DOI: 10.1126/science.1190242.

Part II. Submissions

II.A. Submissions for Cultivation Approvals

Syngenta is pursuing regulatory approvals for MZHG0JG corn cultivation in the United States and Canada, and may seek cultivation approvals in other countries in the future.

Submissions requesting approvals of MZHG0JG corn for cultivation in Canada will soon be made to the Canadian Food Inspection Agency.

Syngenta does not plan to cultivate MZHG0JG corn in Australia or New Zealand. Food products derived from MZHG0JG corn will therefore enter the Australian and New Zealand food supply as only imported and largely processed food ingredients.

II.B. International Submissions for Food, Feed, and Processing Import Approvals

Submissions requesting approvals of MZHG0JG corn for cultivation and importation will be sought on an as-needed basis.

II.C. International Standards

Syngenta reports and studies included in the information supporting this application have been conducted according to international standards. In the safety assessment of biotechnology products, Syngenta referred primarily to the *Codex Alimentarius* Commission Foods Derived from Modern Biotechnology (CAC 2009), and the relevant Codex Standard is as follows:

> Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants. CAC/GL 45-2003.

II.D. References Citied in Part II

CAC. 2009. *Foods Derived from Modern Biotechnology*, 2nd ed. Codex Alimentarius Commission. Rome, Italy: World Health Organization, Food and Agriculture Organization of the United Nations. 85 pp. ftp://ftp.fao.org/docrep/fao/011/a1554e/a1554e00.pdf.

Part III. Technical Information on the Genetically Modified Food

III.A. Nature and Identity of the Genetically Modified Food

III.A.1. Description of the GM Organism (including the nature and purpose of the genetic modification)

Syngenta transformed corn (maize; *Zea mays* L.) to produce MZHG0JG corn, which exhibits tolerance to herbicides with two different modes of action. Specifically, MZHG0JG corn is tolerant to the herbicides glyphosate and glufosinate-ammonium.

MZHG0JG corn plants contain the transgene *mepsps-02*, which encodes the enzyme mEPSPS, and the transgene *pat-09*, which encodes the enzyme phosphinothricin acetyltransferase (PAT).

The native 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) from *Z. mays* is involved in the synthesis of aromatic amino acids and is inhibited by glyphosate. The mEPSPS enzyme is a variant of the native maize EPSPS, and has a lower affinity for glyphosate, thus conferring tolerance to glyphosate in herbicide products. The mEPSPS enzyme produced by MZHG0JG corn includes two amino acid substitutions, at amino acid position 102 (threonine to isoleucine) and 106 (proline to serine), that were introduced specifically to confer tolerance to the herbicide glyphosate.

The transgene *pat-09* was derived from the soil bacterium *Streptomyces viridochromogenes*. PAT acetylates glufosinate-ammonium, thus inactivating it and conferring tolerance to glufosinate-ammonium in herbicide products. PAT was used as a selectable marker in the development of MZHG0JG corn.

The transgenic proteins mEPSPS and PAT produced in MZHG0JG corn are identical to the transgenic proteins produced in other approved biotechnology-derived products, which have been commercially available for almost two decades.

III.A.2. Designation of Transformation Event

The designation of the transformant is Event MZHG0JG corn, which has been assigned the OECD Unique Identifier SYN-ØØØJG-2.

III.A.3. The Types of Products Likely to Include the Food or Food Ingredient

MZHG0JG corn, and the food and feed derived from it, are not materially different from conventional corn. The uses of MZHG0JG corn are expected to be the same as conventional corn.

Domestic production of corn in Canada, Australia, and New Zealand is supplemented by the import of corn-based products from places such as the United States, which is one of largest producers of corn (FAOSTAT 2015). Corn grown in the United States is predominantly of the yellow dent type, a commodity crop largely used to feed domestic animals, as either grain or silage. The remainder of the crop is exported for food, feed, or industrial uses or processed by wet milling, dry milling, or alkali treatment to yield products such as high-fructose corn syrup and starch or oil, grits, and flour. These processed products are used extensively in the food industry. For example, corn starch serves as a raw material for an array of processed foods and is also used in industrial manufacturing processes. Since the early 1980s, a significant amount of

corn grain has also been used for fuel ethanol production. The by-products from these distilling processes are often used in animal feeds.

III.B. History of Use of the Host and Donor Organism(s)

III.B.1. Description of Host Organism into Which the Genes Were Transferred and Its History of Safe Use for Food

III.B.1.a. Recipient Corn Line

The recipient germplasm for transformation to produce MZHG0JG corn was an elite Syngenta inbred corn line, NP2222 (Plant Variety Protection certificate 200200071, issued November 2004; USDA-AMS 2010). This inbred line was used because it is well-suited to *Agrobacterium tumefaciens*–mediated transformation and regeneration from tissue culture. NP2222 is a Stiff-Stalk family, yellow dent inbred.

III.B.1.b. Biology of Corn

The Consensus Document on the Biology of *Zea mays* subsp. *mays* (Maize), published by the Organisation for Economic Co-operation and Development (OECD 2003), provides comprehensive information regarding the biology of corn. This Consensus Document is referenced in support of MZHG0JG corn, and includes the following information:

- Uses of corn as a crop plant
- Taxonomic status of the genus *Zea*
- Identification methods among races of *Zea mays* and wild species
- Centers of origin and diversity of corn
- Reproductive biology of corn
- Intra-specific and inter-specific crosses of corn and gene flow
- Agro-ecology of corn, including cultivation, volunteers, weediness, soil ecology, and corn-insect interactions
- Corn biotechnology
- Common diseases and insect pests of corn

III.B.1.c. Parts and/or Processing of Corn for Food or Feed

Kernels from MZHG0JG corn are the most likely tissue to enter the food supply, either as grain or grain by-products. Humans would potentially consume corn at the senescence stage of development, whereas livestock would be more likely to consume the kernels at maturity.

As mentioned previously, corn is typically processed by wet milling, dry milling, or alkali treatment to yield products such as high-fructose corn syrup and starch or oil, grits, and flour, although no special processing is required to render the food safe to eat.

III.B.1.d. Significance of Corn to the Diet in Australia, New Zealand, and Canada of Food Derived from the Host Organism

Corn or corn for grain is the number one produced cereal crop worldwide with 872.8 million tonnes produced in 2012, according to the Food and Agricultural Organization estimates

(FAOSTAT 2014). In 2012, the top producer continued to be the U.S. with 273.8 million tonnes or 31.4% of global production. The U.S was followed by China which produced 205.6 million tonnes (23.6% of world production). Canada placed 10th in world production of corn with 13.1 million tonnes of grain corn produced in 2012. Australia produced 0.45 million tonnes and New Zealand produced 0.21 million tonnes. Domestic production of corn in Australia and New Zealand is supplemented by the import of corn based products.

III.B.2. Description of the Donor Organisms from Which the Genetic Elements are Derived

MZHG0JG corn contains the transgene *mepsps-02* derived from *Zea mays* L. spp. *mays.* No significant native toxins or allergens are reported to be associated with the genus *Zea*, the source organism for mEPSPS (OGTR 2008). Additional information regarding the safety, history of use, and extent of corn in the diet is described in Part III.B.1.

MZHG0JG corn contains the transgene *pat-09*, derived from *Streptomyces viridochromogenes,* a common nonpathogenic soil bacterium. Bacteria are not known to be sources of allergenic proteins (Taylor and Hefle 2001).

Part V of this application demonstrate that the proteins mEPSPS and PAT produced in MZHG0JG corn are identical to the mEPSPS and PAT proteins that have been present in commercial corn products that contain GA21 corn and Bt11 corn.

GA21 was first approved by CFIA in 1998 (GA21 DD 1999-33), HC in 1999, and FSANZ in 2000 (GA21-Approval no. 362).

Bt11 was first approved by CFIA in 1996 (Bt11 DD96-12), HC in 1996, and FSANZ in 2001 (Bt11-Approval no. 386).

III.C. References Citied in Part III

FAOSTAT. 2014. Food and Agriculture Organization of the United Nations, FAOSTAT database. http://faostat.fao.org/site/567/default.aspx#ancor (accessed April 16, 2015).

FAOSTAT. 2015. Food and Agriculture Organization of the United Nations Statistics Division. http://faostat3.fao.org/home/E (accessed April 16, 2015).

OECD. 2003. *Consensus Document on the Biology of* Zea mays *subsp.* mays *(maize).* Series on Harmonisation of Regulatory Oversight in Biotechnology, No. 27. Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology, Environment Directorate, Organisation for Economic Co-operation and Development. http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?doclanguage=en&cote=env/jm/mono(2003)11.

OGTR. 2008. *The Biology of* Zea mays *L spp* mays *(maize or corn)*, version 1, September 2008. Office of the Gene Technology Regulator, Department of Health and Ageing, Australian Government. http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/content/maize-3/$FILE/biologymaize08_2.pdf.

Taylor SL, Hefle SL. 2001. Will genetically modified foods be allergenic? *Journal of Allergy and Clinical Immunology* 107:765–771.

USDA-AMS. 2010. Plant Variety Protection Office, Agricultural Marketing Service, United States Department of Agriculture. http://apps.ams.usda.gov/CMS/ (accessed November 29, 2010).

Part IV. The Nature of the Genetic Modification

This section describes the method by which corn was transformed to produce herbicide-tolerant corn plants, the development of MZHG0JG corn, including the production of test and control seed lots for use in the studies described in this application, and the genetic characterization of MZHG0JG corn.

IV.A. Description of the Transformation Method

Transformation of *Z. mays* to produce MZHG0JG corn was accomplished through the use of immature embryos of a proprietary corn line via *Agrobacterium tumefaciens*–mediated transformation, as described by Negrotto *et al*. 2000. By this method, genetic elements within the left and right border regions of the transformation plasmid were efficiently transferred and integrated into the genome of the target plant cell, while genetic elements outside these border regions were not transferred.

Immature embryos were excised from corn ears (NP2222) that were harvested 8 to 12 days after pollination. The embryos were rinsed with fresh medium and mixed with a suspension of *A. tumefaciens* strain LBA4404 harboring plasmids pSB1 (Komari *et al.* 1996) and pSYN18857. The embryos in suspension were vortexed for 30 seconds and allowed to incubate for an additional 5 minutes. Excess *A. tumefaciens* suspension was removed by aspiration, and the embryos were moved to plates containing a nonselective culture medium. The embryos were co-cultured with the remaining *A. tumefaciens* at 22°C for 2 to 3 days in the dark. The embryos were then transferred to culture medium supplemented with ticarcillin (200 mg/l) and silver nitrate (1.6 mg/l) and incubated in the dark for 10 days. The *pat-09* gene was used as a selectable marker during the transformation process (Negrotto *et al*. 2000). The embryos producing embryogenic calli were transferred to a cell culture medium containing glufosinate-ammonium as a selection agent. The transformed tissue was transferred to a selective medium containing the broad-spectrum antibiotic cefotaxime at 500 mg/l (a concentration known to kill *A. tumefaciens* [Xing *et al*. 2008]) and grown for four months, ensuring that the *A. tumefaciens* was cleared from the transformed tissue.

The regenerated plantlets were tested for the presence of *mepsps-02* and *pat-09* and for the absence of the spectinomycin resistance gene (*aadA-03*) present on the vector backbone by real-time polymerase chain reaction (PCR) analysis (Ingham *et al*. 2001). This screen allowed for the selection of transgenic events that carried the transferred deoxyribonucleic acid (T-DNA) and were free of plasmid backbone DNA. Plants that tested positive for *mepsps-02* and *pat-09* and negative for *aadA-03* were transferred to the greenhouse for further propagation.

IV.B. Intermediate Host Organisms Used for All Laboratory Manipulations Prior to Plant Transformation

Standard strains of *Escherichia coli* were used for laboratory manipulations prior to plant transformation using disarmed *Agrobacterium tumefaciens*. Such strains of *E. coli* used in molecular biology are considered non-pathogenic (Muhldorfer and Hacker 1994).

IV.C. Development of MZHG0JG Corn

Progeny of the original transformants (T_0 plants) were field tested for tolerance to glyphosate, tolerance to glufosinate-ammonium, and agronomic performance in multiple elite lines of corn. Event MZHG0JG corn was selected as the lead commercial candidate and underwent further field testing and development. Figure IV-1 shows the steps in the development of MZHG0JG corn.

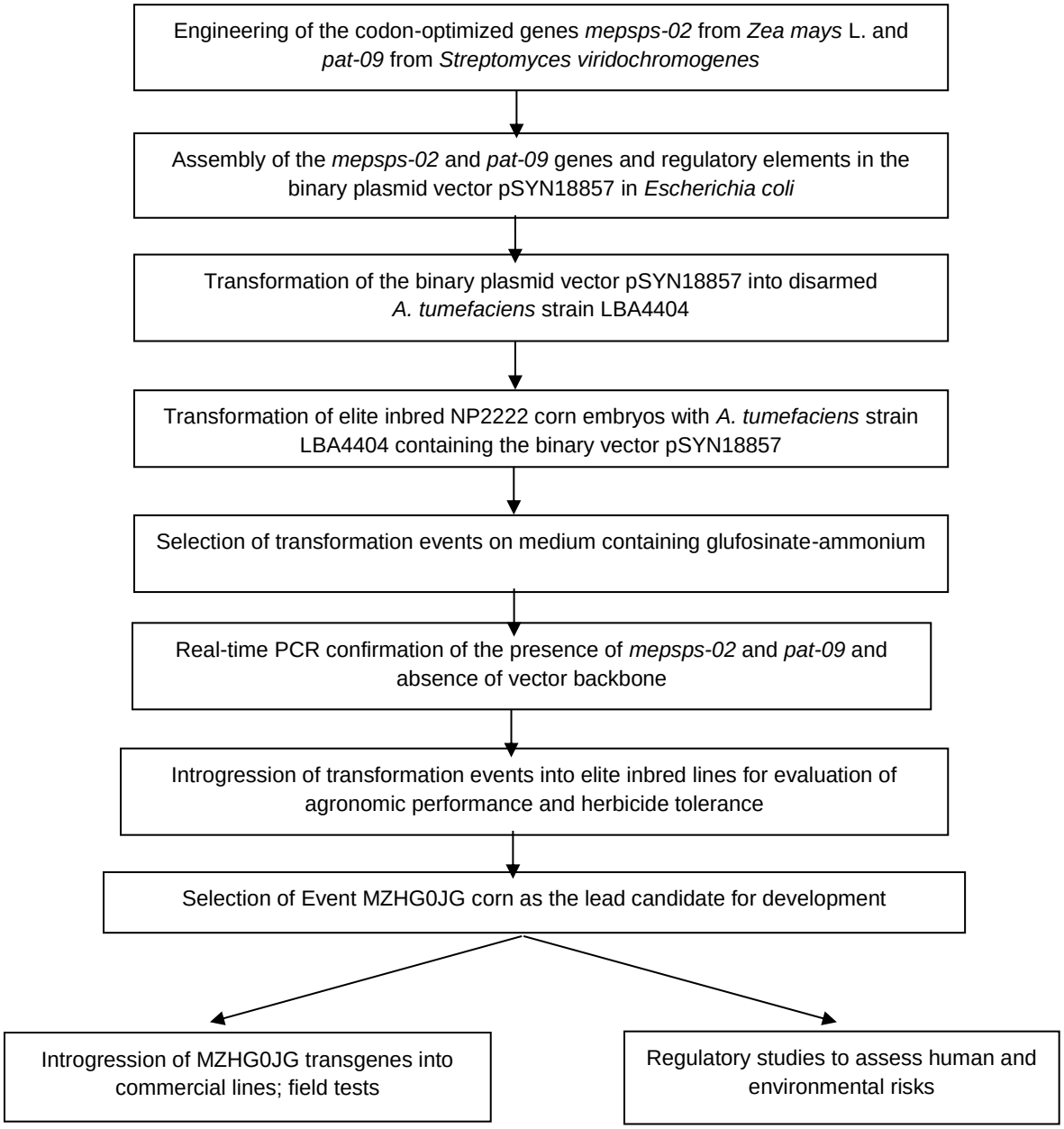
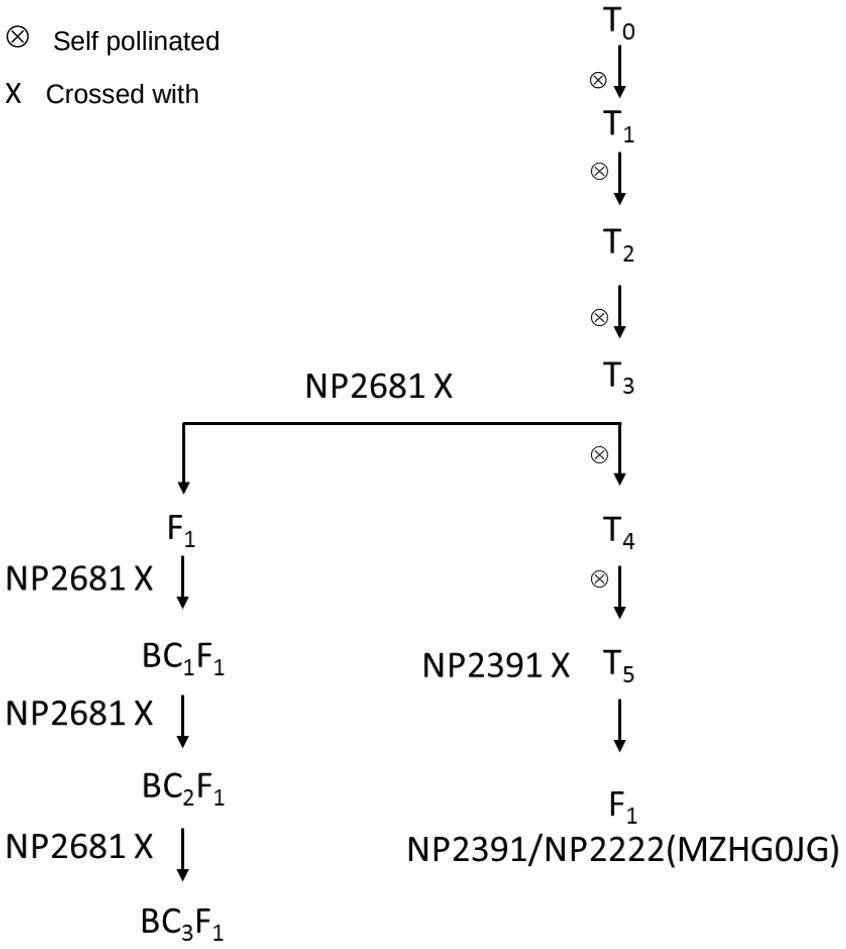


Figure IV-1. Steps in the development of MZHG0JG corn

IV.D. Production of Test and Control Seed

Production of all MZHG0JG corn and nontransgenic, near-isogenic control corn seed lots used in the studies described in this application were carried out under controlled and isolated conditions under the direction of Syngenta breeders and field researchers. Figure IV-2 shows the pedigree of MZHG0JG corn seed materials.



The recipient germplasm for transformation to produce MZHG0JG corn was an elite Syngenta inbred corn line, NP2222.

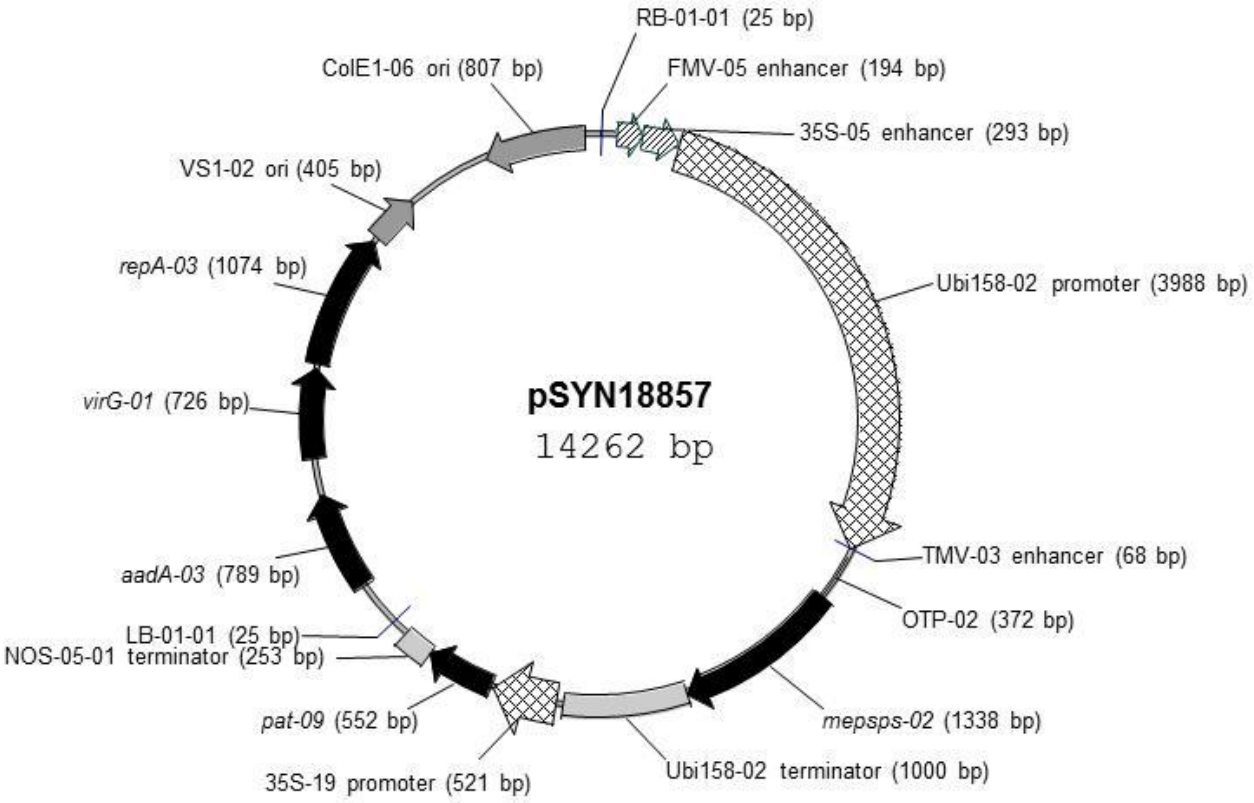
Figure IV-2. Pedigree of the MZHG0JG plant materials used in regulatory studies

IV.E. Quality Control of Test and Control Materials

All MZHG0JG and nontransgenic, near-isogenic control corn seed lots were analyzed by real-time PCR for the presence of MZHG0JG DNA and the absence of adventitious DNA from other transformation events. All MZHG0JG corn seed lots were confirmed to contain Event MZHG0JG-specific DNA. Event MZHG0JG DNA was not detected in any nontransgenic, near-isogenic control corn seed lots. None of the MZHG0JG or nontransgenic, near-isogenic control corn seed lots contained any detectable sequences indicative of DNA from other events.

IV.F. Description of the Gene Construct and the Transformation Vector

The transformation plasmid pSYN18857 was used to produce MZHG0JG corn by *A. tumefaciens*–mediated transformation of immature corn embryos. The DNA region between the left and right borders of the transformation plasmid included gene-expression cassettes for *mepsps-02* and *pat-09*. The *mepsps-02* expression cassette consisted of the *mepsps-02* coding region regulated by a corn ubiquitin promoter (Ubi158-02) and terminator (Ubi158-02), as well as the figwort mosaic virus (FMV-05), cauliflower mosaic virus 35S (35S-05), and tobacco mosaic virus (TMV-03) enhancer sequences and an optimized transit peptide (OTP-02). The *pat-09* expression cassette consisted of the *pat-09* coding region regulated by a 35S promoter from cauliflower mosaic virus (35S-19) and the nopaline synthase (NOS) terminator sequence from *A. tumefaciens* (NOS-05-01). A map of the transformation plasmid is shown in Figure IV-3, and each genetic element in the transformation plasmid is described in Table IV-1.



bp = base pairs

Figure IV-3. Plasmid map for the vector pSYN18857

Table IV-1. Description of the genetic elements in vector pSYN18857

Genetic element	Size (bp)	Position	Description
***mepsps*-02 cassette**			
Region-01	102	26 to 127	Region used for cloning.
FMV-05 enhancer	194	128 to 321	Figwort mosaic virus (FMV) enhancer region (similar to National Center for Biotechnology Information [NCBI] accession number X06166.1), which increases gene expression (Maiti *et al*. 1997).
Region-02	6	322 to 327	Region used for cloning.
CaMV 35S-05 enhancer	293	328 to 620	Cauliflower mosaic virus (CaMV) 35S enhancer region, which can activate heterologous core promoters (Ow *et al*. 1987).
Region-03	10	621 to 630	Region used for cloning.
Ubi158-02 promoter	3988	631 to 4618	Corn constitutive promoter based on the corn Ubiquitin ZmU29158-3 gene. Similar to the corn polyubiquitin (Ubi) promoter (NCBI accession number S94466.1; Christensen *et al*. 1992). The original Ubi158 promoter was altered by 6 bp to eliminate unintended open reading frames (ORFs).
TMV-03 enhancer	68	4619 to 4686	The reverse orientation of the 5′ non-coding leader sequence (called omega) from tobacco mosaic virus (TMV) (Gallie *et al*. 1987) functions as a translational enhancer in plants (Gallie 2002).
Optimized transit peptide (OTP-02)	372	4687 to 5058	N-terminal chloroplast transit peptide (CTP) sequences based on CTP sequences from *Helianthus annus* (sunflower) and corn. Directs the mEPSPS protein to the chloroplast (Lebrun *et al*. 1996).
mepsps-02	1338	5059 to 6396	Sequence encoding the modified corn mEPSPS, which confers tolerance to glyphosate (Lebrun *et al*. 2003).
Region-04	7	6397 to 6403	Region used for cloning.
Ubi158-02 terminator	1000	6404 to 7403	The terminator based on the corn Ubiquitin ZmU29158-3 gene. It is similar to the corn polyubiquitin terminator (NCBI accession number S94466.1; Christensen *et al*. 1992). The original Ubi158 terminator was altered by 1 bp to eliminate an unintended ORF.
Region-05	57	7404 to 7460	Region used for cloning.
***pat-09* cassette**			
35S-19 promoter	521	7461 to 7981	Promoter region of cauliflower mosaic virus (Odell *et al*. 1985). Provides constitutive expression in plants.
Region-06	13	7982 to 7994	Region used for cloning.
pat-09	552	7995 to 8546	*S. viridochromogenes* strain Tü494 gene encoding the selectable marker PAT. The native coding sequence (Wohlleben *et al*. 1988) was codon-optimized for enhanced expression. The synthetic *pat* gene was obtained from AgrEvo, Germany (NCBI accession number DQ156557.1). The gene *pat-09* encodes the same amino acid sequence as *pat* from AgrEvo, but several nucleotide changes were made to remove a cryptic splice site, a restriction site, and unintended ORFs. PAT confers resistance to herbicides containing glufosinate-ammonium (phosphinothricin).

Genetic element	Size (bp)	Position	Description
Region-07	4	8547 to 8550	Region used for cloning.
NOS-05-01 terminator	253	8551 to 8803	Terminator sequence from the nopaline synthase (NOS) gene of *A. tumefaciens* (NCBI accession number V00087.1). Provides a polyadenylation site (Bevan *et al*. 1983).
Region-08	125	8804 to 8928	Region used for cloning.
Border Region			
LB-01-01	25	8929 to 8953	Left border region of T-DNA from *A. tumefaciens* nopaline Ti plasmid (NCBI accession number J01825.1). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell (Yadav *et al*. 1982).
Plasmid backbone			
Region-09	349	8954 to 9302	Region used for cloning.
aadA-03	789	9303 to 10091	Aminoglycoside adenylyltransferase gene from *Escherichia coli* transposon Tn7 (similar to NCBI accession number X03043.1). Confers resistance to streptomycin and spectinomycin and is used as a bacterial selectable marker (Fling *et al*. 1985).
Region-10	299	10092 to 10390	Region used for cloning.
virG-01	726	10391 to 11116	The VirGN54D gene from pAD1289 (similar to NCBI accession number AF242881.1). The N54D substitution results in a constitutive virG phenotype. The gene *virG* is part of the two-component regulatory system for the virulence regulon in *A. tumefaciens* (Hansen *et al*. 1994).
Region-11	29	11117 to 11145	Region used for cloning.
repA-03	1074	11146 to 12219	Gene encoding the pVS1 replication protein from *Pseudomonas aeruginosa* (similar to NCBI accession number AF133831.1), which is a part of the minimal pVS1 replicon that is functional in Gram-negative, plant-associated bacteria (Heeb *et al*. 2000).
Region-12	42	12220 to 12261	Region used for cloning; contains sequence from the pVS1 replicon from *P. aeruginosa*.
VS1-02 ori	405	12262 to 12666	Consensus sequence for the origin of replication (ori) and partitioning region from plasmid pVS1 of *P. aeruginosa* (NCBI accession number U10487.1). Serves as origin of replication in *A. tumefaciens* host (Itoh *et al*. 1984).
Region-13	677	12667 to 13343	Region used for cloning.
ColE1-06 ori	807	13344 to 14150	Origin of replication (similar to NCBI accession number V00268.1) that permits replication of plasmids in *E. coli* (Itoh and Tomizawa 1979).
Region-14	112	14151 to 14262	Region used for cloning.
Border region			
RB-01-01	25	1 to 25	Right border region of T-DNA from *A. tumefaciens* nopaline Ti plasmid (NCBI accession number J01826.1). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell (Wang *et al*. 1984).

IV.G. Molecular Characterization of MZHG0JG Corn

An extensive genetic characterization of the T-DNA insert in Event MZHG0JG corn was performed by means of Southern blot analyses and nucleotide sequencing. The genetic stability of the insert was assessed both by Southern blot analyses and by examining the inheritance patterns of the transgenes over at least three generations of MZHG0JG corn. Sequencing results confirmed the expected copy number of each of the functional elements in the T-DNA. In addition, the corn genomic sequences flanking the MZHG0JG insert were identified and characterized. Finally, it was determined that the MZHG0JG insert did not disrupt the function of any known corn gene. These data collectively demonstrate that no deleterious changes occurred in the MZHG0JG corn genome as a result of the T-DNA insertion.

IV.G.1. Nucleotide Sequence of the T-DNA Insert and Copy Number of the Functional Elements

Three overlapping DNA fragments that covered the entire MZHG0JG insert were amplified via PCR from genomic DNA extracted from MZHG0JG T_3 generation corn. These fragments were cloned, and the sequences of the clones were assembled to generate a consensus sequence for the MZHG0JG insert. This sequence was then compared with the sequence of the T-DNA in plasmid pSYN18857, the transformation plasmid used to create MZHG0JG corn. The nucleotide sequence analysis demonstrated that the MZHG0JG insert contains a single copy of each of the functional elements (*mepsps-02*, *pat-09*, FMV-05 enhancer, 35S-05 enhancer, OTP-02 transit peptide, Ubi158-02 promoter, TMV-03 enhancer, Ubi158-02 terminator, 35S-19 promoter, and NOS-05-01 terminator).

Comparison of the MZHG0JG insert sequence with the transformation plasmid pSYN18857 showed that the 8910 bp MZHG0JG insert was intact, with no rearrangements or base pair changes. Some truncation occurred at the right and left border regions of the T-DNA during the transformation process that resulted in MZHG0JG corn; 22 bp of the right border and 21 bp of the left border were truncated. These deletions have no effect on the functionality of the T-DNA insert.

The copy number and sequence of each of the functional elements in MZHG0JG corn is as expected based on the pSYN18857 T-DNA sequence. The MZHG0JG insert contains a single copy of each of the functional elements (*mepsps-02*, *pat-09*, FMV-05 enhancer, 35S-05 enhancer, OTP-02 transit peptide, Ubi158-02 promoter, TMV-03 enhancer, Ubi158-02 terminator, 35S-19 promoter, and NOS-05-01 terminator). A map of the MZHG0JG insert and flanking sequence is shown in Figure IV-4.

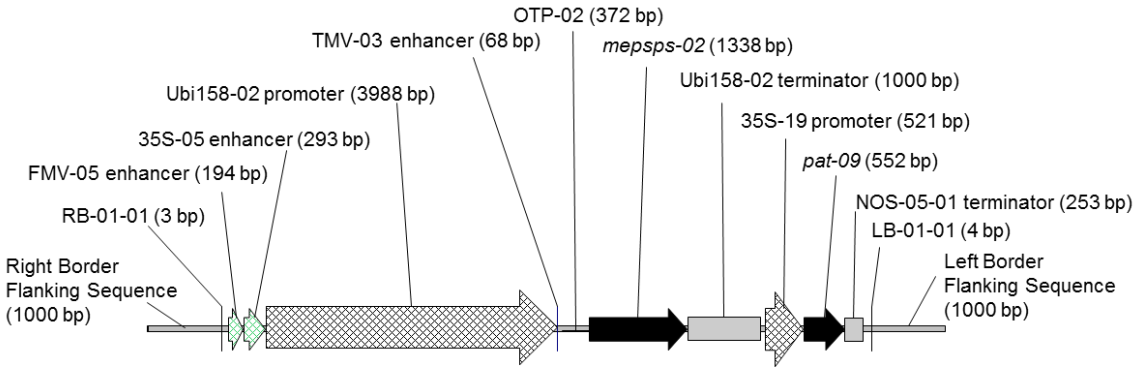


Figure IV-4. Map of the MZHG0JG insert and flanking sequences

IV.G.2. MZHG0JG Insertion Site Analysis

PCR analysis was used to determine the genomic sequence in nontransgenic, near-isogenic corn at the point of integration of the MZHG0JG insert and the genomic sequences flanking the 5′ and 3′ ends of the MZHG0JG insert. Comparison of these two sequences showed that 22 bp from the corn genomic sequence were deleted during the integration of the MZHG0JG insert, and 43 bp of DNA were inserted into the integration site: a 4-bp DNA sequence was present at the junction between the MZHG0JG insert and the 5′ genomic sequence flanking the insert, and a 39-bp DNA sequence was present at the junction between the MZHG0JG insert and the 3′ flanking region. Basic Local Alignment Search Tool for Translated Nucleotides (BLASTX) analyses (Altschul *et al.* 1997) comparing the corn genomic sequence flanking the MZHG0JG insert with sequences in public databases indicated that the insert does not disrupt any known endogenous corn gene. A bioinformatics analysis indicated that no potential open reading frames (ORFs) ≥ 30 amino acids (based on the presence of start and stop codons) span the junction between the corn genome and the MZHG0JG insert.

IV.G.3. Insert Copy Number and Absence of Plasmid of Backbone Sequence Across Multiple Generations by Southern Blot Analysis

Southern blot analyses were performed to characterize the transgenic insert of MZHG0JG corn by determining the number of plasmid pSYN18857 T-DNA integration sites and the presence or absence of pSYN18857 backbone sequence or additional extraneous fragments of T-DNA. In addition, this characterization established the genetic identity of the MZHG0JG T_2 generation used to create commercial MZHG0JG corn lines and the MZHG0JG corn generations used in regulatory and safety studies and the stable inheritance of the MZHG0JG insert over five generations of MZHG0JG corn.

IV.G.3.a. Southern Blot Analysis Methods

The MZHG0JG corn generations used in Southern blot analysis included T_2 (two samples, from ear 4 and ear 35), T_3, T_4, T_5, and F_1. The T_2 through T_5 generations were in the genetic background NP2222. The F_1 generation was in the background NP2391/NP2222 and was representative of a commercial corn hybrid. The control substances were nontransgenic, near-isogenic NP2222, NP2391, and NP2222/NP2391 corn. The genomic DNA used for Southern blot analyses was isolated from leaf tissue by a method modified from that described by Murray and Thompson (1980).

In the Southern blot analyses, the number of integration sites within the MZHG0JG corn genome and number of copies of the T-DNA at each location within the MZHG0JG corn genome were determined through the use of three T-DNA-specific probes that together covered every base pair of the pSYN18857 T-DNA expected to be transferred and integrated into the corn genome. The templates for the probes were segments of the pSYN18857 T-DNA corresponding to (A) the right border sequence to the end of TMV-03 enhancer, (B) the OTP-02 transit peptide and the *mepsps-02* coding sequence, and (C) the Ubi158-02 terminator sequence to the left border (as shown in Figure IV-5 and Table IV-2). The left border and right border are categorized as "border regions" because only a portion of each border was expected to be integrated into the corn genome (Tzfira *et al*. 2004).

The elements of the plasmid necessary for its replication and selection in different bacterial hosts are categorized as "plasmid backbone" (the region outside of the T-DNA). In the Southern blot analyses, the presence or absence of plasmid backbone was determined through the use of two backbone-specific probes that together covered every base pair of pSYN18857 outside of the T-DNA. These elements (shown as probes D and E in Figure IV-5 and Table IV-2) were not expected to be transferred to the plant cell or integrated into the plant genome during T-DNA transfer.

Each Southern blot analysis was performed with genomic DNA extracted from MZHG0JG corn and from nontransgenic, near-isogenic corn, which was used as a negative control to identify any endogenous corn DNA sequences that hybridized with the probes. To demonstrate the sensitivity of the analyses, each analysis also included two positive assay controls representing 1 copy and 1/7 copy per genome of a DNA fragment of known size in the corn genome. The positive assay controls were PCR-amplified fragments that corresponded to each of the five probes used in characterization of the MZHG0JG corn insert.

The positive assay controls for T-DNA-specific probes 2 and 3 and backbone-specific probes 1 and 2 were loaded in a well together with 7.5 µg of digested DNA from nontransgenic, near-isogenic NP2222/NP2391 corn, in order to more accurately reflect their migration speeds in the corn genome matrix. The positive assay control for T-DNA-specific probe 1 was analyzed in the absence of nontransgenic corn genomic DNA, so that endogenous bands would not obscure the positive assay control.

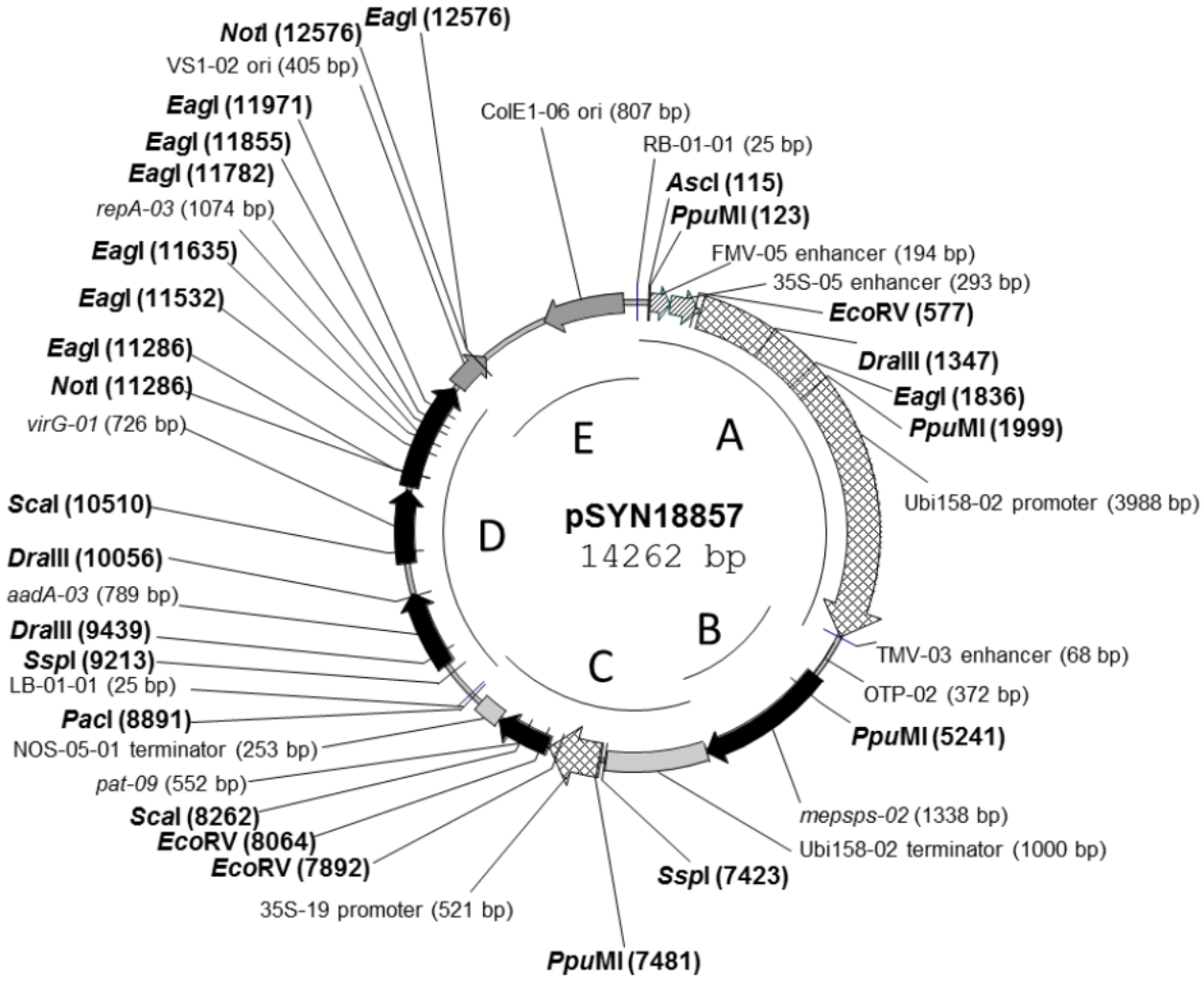


Figure IV-5. Map of plasmid pSYN18857 indicating the restriction sites and probes used in the MZHG0JG Southern blot analyses

Table IV-2. Probes used in the MZHG0JG Southern blot analyses

Probe	Name	T-DNA elements contained	Size (bp)	Position
A	T-DNA-specific probe 1	FMV-05 enhancer, 35S-05 enhancer, Ubi158-02 promoter, and TMV-03 enhancer	4673	23 to 4695
B	T-DNA-specific probe 2	OTP-02 transit peptide and *mepsps-02*	1710	4687 to 6396
C	T-DNA-specific probe 3	Ubi158-02 terminator, 35S-19 promoter, *pat-09*, NOS-05-01 terminator	2554	6397 to 8950
D	Backbone-specific probe 1	none	3311	8951 to 12261
E	Backbone-specific probe 2	none	2065	12220 to 22

The amount of positive assay control (in picograms for one copy) was calculated by the following formula (Arumuganathan and Earle 1991):

$$\left\{\left(\frac{\text{positive assay control size (bp)}}{\text{genome size (bp)} \times \text{ploidy}}\right) \times \mu\text{g loaded}\right\} \times 1 \times 10^{6} = \text{pg for 1 copy}$$

The following factors were used to calculate the amounts of the positive assay controls:

corn genome size (bp)	2.67×10^{9}
corn ploidy	2
DNA loaded in each lane (µg)	7.5
T-DNA-specific DNA fragment 1 (bp)	4673
T-DNA-specific DNA fragment 2 (bp)	1710
T-DNA-specific DNA fragment 3 (bp)	2554
backbone-specific DNA fragment 1 (bp)	3311
backbone-specific DNA fragment 2 (bp)	2065

Table IV-3 shows the calculated amounts of the positive assay controls used in each Southern blot analysis.

Table IV-3. Positive assay control amounts

	Control amount (pg)	
Positive assay control name	**1 copy**	**1/7 copy**
T-DNA-specific DNA fragment 1	6.56	0.94
T-DNA-specific DNA fragment 2	2.40	0.34
T-DNA-specific DNA fragment 3	3.59	0.51
Backbone-specific DNA fragment 1	4.65	0.66
Backbone-specific DNA fragment 2	2.90	0.41

Corn genomic DNA was analyzed via two restriction enzyme digestion strategies. In the first strategy, the genomic DNA was digested with an enzyme that cut within the MZHG0JG insert and in the corn genome flanking the MZHG0JG insert. This first strategy was used twice, with two different enzymes, to determine the number of pSYN18857 T-DNA inserts within the MZHG0JG corn genome and the presence or absence of extraneous DNA fragments of the insert in other regions of the MZHG0JG corn genome. The enzymes used were *Ppu*MI, *Eco*RV, *Dra*III, *Ssp*I, *Eag*I, *Sca*I, and *Not*I. In the second strategy, the genomic DNA was digested with restriction enzymes that cut within the insert to release DNA fragments of predictable size. This strategy was used to determine the number of copies of the T-DNA at each location within the MZHG0JG corn genome, the intactness of the insert, and the presence or absence of any closely linked extraneous T-DNA fragments. The enzymes used were *Asc*I + *Pac*I. The locations of the restriction sites are shown in Figure IV-5, above.

For analyses with T-DNA-specific probe 2 using the second strategy mentioned above, the genomic DNA was further digested with *Kpn*I, for a total of three enzymes used in digestion (*Asc*I + *Pac*I + *Kpn*I). The limited numbers of restriction sites for *Asc*I + *Pac*I resulted in large

genomic DNA fragments that migrated slowly and poorly through the agarose gel, obscuring visualization of the banding pattern. *Kpn*I does not cut within the MZHG0JG insert, and its use did not affect the results of the insert analysis.

Table IV-4 shows the expected numbers of hybridization bands for MZHG0JG corn in the analyses with the three T-DNA-specific probes. For the analyses with T-DNA-specific probe 1 and restriction enzymes *Ppu*MI and *Eco*RV, an additional fragment was possible, based on the locations of the restriction sites; however, the target sequence was too small to bind the probe under the conditions used in these analyses, so additional bands were not expected (and are not shown in Table IV-4). Additional, unexpected bands in any of these analyses would indicate the presence of more than one copy of the T-DNA at more than one location within the MZHG0JG corn genome. No hybridization bands were expected in the analyses with either of the backbone-specific probes or in any of the analyses of genomic DNA from nontransgenic, near-isogenic corn. In the analyses of NP2222, NP2391, and NP2222/NP2391 corn genomic DNA, the observation of bands that were also present in genomic DNA from the various generations of MZHG0JG corn was the result of cross-hybridization of the T-DNA-specific probe sequence with the endogenous corn sequence.

Table IV-4. Expected number of hybridization bands in Southern blot analyses of MZHG0JG corn with the T-DNA-specific probes

Probe	Restriction enzyme(s)	Expected no. of bands
T-DNA-specific probe 1	*Ppu*MI	3[a]
	*Eco*RV	2[a]
	AscI + *Pac*I	1
T-DNA-specific probe 2	*Dra*III	1
	*Ssp*I	1
	*Asc*I + *Pac*I + *Kpn*I	1
T-DNA-specific probe 3	*Eag*I	2
	ScaI	2
	AscI + *Pac*I	1

[a]Based on the restriction sites, an additional fragment was possible; however, the target sequence was too small to bind the probe under the conditions used in these analyses, so additional bands were not expected.

IV.G.3.b. Results of Southern Blot Analysis with T-DNA-Specific Probe 1

Figure IV-6 shows the digestion strategy used with T-DNA-specific probe 1, Table IV-5 shows the insert-specific hybridization bands expected and observed in Southern blot analyses of MZHG0JG corn DNA with T-DNA-specific probe 1, and Figures IV-7 through IV-9 show the results of the Southern blot analyses with T-DNA-specific probe 1.

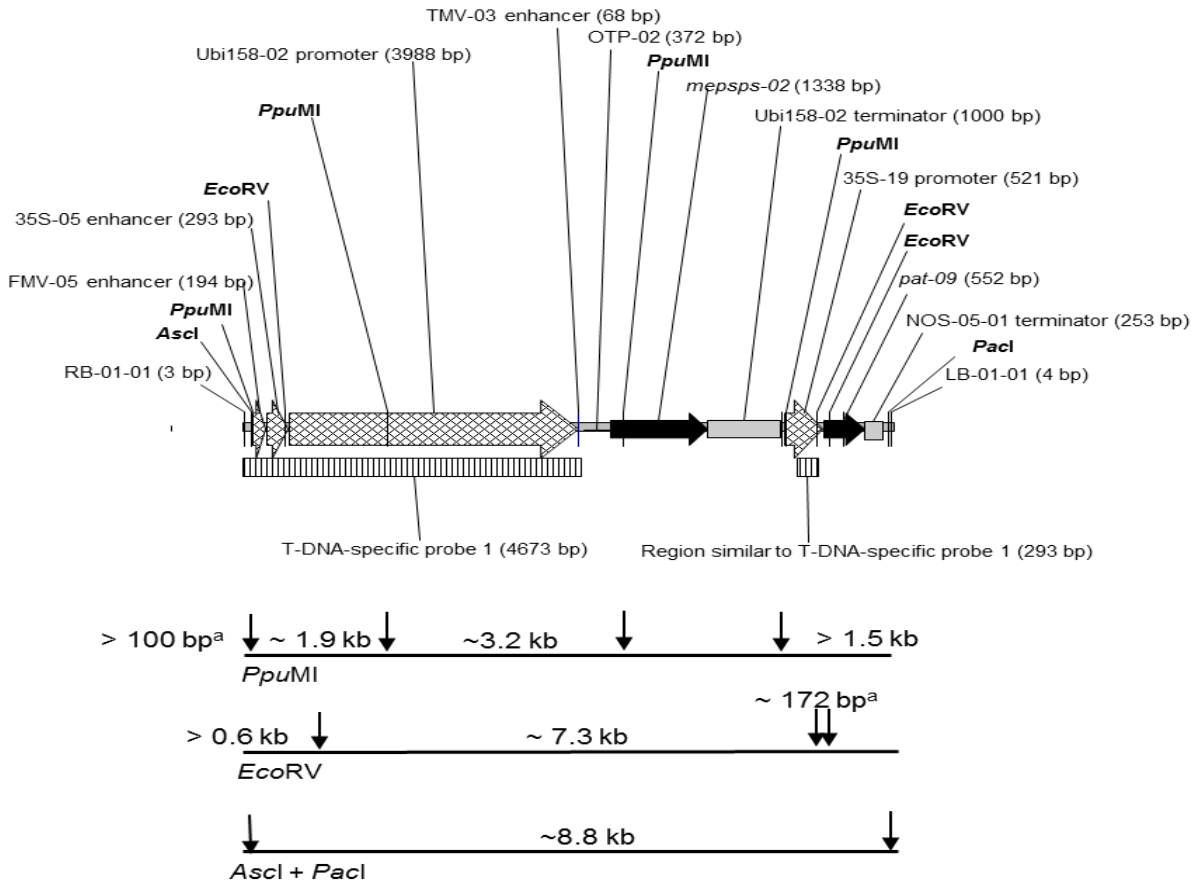
In the analysis of genomic DNA digested with *Ppu*MI, three bands of approximately 1.9, 3.2, and 7.2 kb were observed in the lanes containing DNA from MZHG0JG T_2 (ear 4), T_2 (ear 35), T_3, T_4, T_5, or F_1 corn (Figure IV-7, Lanes 2 through 7). These bands were absent from the lanes containing DNA from the nontransgenic NP2222, NP2391, or NP2222/NP2391 corn (Figure IV-7, Lanes 8 through 10) and were, therefore, specific to the MZHG0JG insert. As

expected, one band of approximately 4.7 kb was observed in the lanes containing the positive controls (Figure IV-7, Lanes 11 and 12).

In the analysis of genomic DNA digested with *Eco*RV, two bands of approximately 2.7 and 7.3 kb were observed in the lanes containing DNA from MZHG0JG T_2 (ear 4), T_2 (ear 35), T_3, T_4, T_5, or F_1 corn (Figure IV-8, Lanes 2 through 7). These bands were absent from the lanes containing DNA from nontransgenic NP2222, NP2391, or NP2222/NP2391 corn (Figure IV-8, Lanes 8 through 10) and were, therefore, specific to the MZHG0JG insert. As expected, one band of approximately 4.7 kb was observed in the lanes containing the positive controls (Figure IV-8, Lanes 11 and 12).

In the analysis of genomic DNA digested with *Asc*I + *Pac*I, one band of approximately 8.8 kb was observed in lanes containing DNA extracted from MZHG0JG T_2 (ear 4), T_2 (ear 35), T_3, T_4, T_5, or F_1 corn (Figure IV-9, Lanes 2 through 7). This band was absent from the lanes containing DNA from nontransgenic NP2222, NP2391, or NP2222/NP2391 corn (Figure IV-9, Lanes 8 through 10) and was, therefore, specific to the MZHG0JG insert. As expected, one band of approximately 4.7 kb was observed in the lanes containing the positive controls (Figure IV-9, Lanes 11 and 12).

In the analyses with *Ppu*MI digestion, an additional band was detected because of sequence similarity between the 35S-05 enhancer (an element in the *mepsps-02* cassette and covered by T-DNA-specific probe 1) and the 35S-19 promoter (an element in the *pat-09* cassette and covered by T-DNA-specific probe 3). As a result, three hybridization bands, one corresponding to a copy of the 35S-19 promoter in MZHG0JG corn and two corresponding to the portion of the T-DNA covered by the probe, were seen in this analysis. No additional bands were seen with *Eco*RV digestion, because the 35S-19 promoter and the portion of the T-DNA covered by the probe were on the same fragment. No unexpected bands were detected, indicating that the MZHG0JG corn genome contains no extraneous DNA fragments of the T-DNA-specific probe 1 sequence.



[a]The target sequence is too small for the probe to bind to in the conditions used in this Southern analysis.
The vertical arrows indicate the site of restriction digestion.
Sizes of the expected restriction fragments are indicated.

Figure IV-6. Locations of the 4.7-kb T-DNA-specific probe 1 and the restriction sites *Ppu*MI, *Eco*RV, and *Asc*I + *Pac*I in the MZHG0JG corn insert

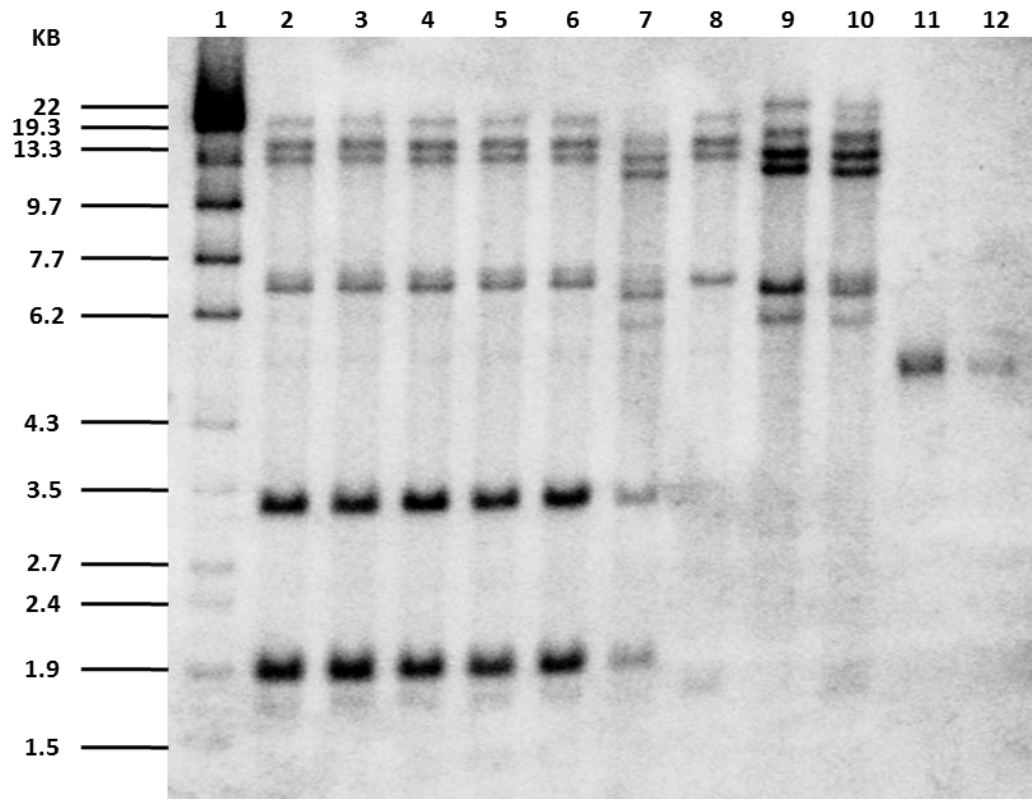
Table IV-5. Expected and observed insert-specific hybridization bands in Southern blot analyses of MZHG0JG corn DNA with T-DNA-specific probe 1 and restriction enzymes *Ppu*MI, *Eco*RV, and *Asc*I + *Pac*I

Figure & lane	Source of DNA	Restriction enzymes	Expected no. of bands[a]	Approximate band size (kb)	
				Expected	Observed[a]
IV-7, 2	MZHG0JG T_2 (ear 4) corn	*Ppu*MI	3	>1.5	1.9
				1.9	3.2
				3.2	7.2
IV-7, 3	MZHG0JG T_2 (ear 35) corn	*Ppu*MI	3	>1.5	1.9
				1.9	3.2
				3.2	7.2
IV-7, 4	MZHG0JG T_3 corn	*Ppu*MI	3	>1.5	1.9
				1.9	3.2
				3.2	7.2
IV-7, 5	MZHG0JG T_4 corn	*Ppu*MI	3	>1.5	1.9
				1.9	3.2
				3.2	7.2
IV-7, 6	MZHG0JG T_5 corn	*Ppu*MI	3	>1.5	1.9
				1.9	3.2
				3.2	7.2
IV-7, 7	MZHG0JG F_1 corn	*Ppu*MI	3	>1.5	1.9
				1.9	3.2
				3.2	7.2
IV-7, 8	NP2222 corn (negative control)	*Ppu*MI	0	N/A	N/A
IV-7, 9	NP2391 corn (negative control)	*Ppu*MI	0	N/A	N/A
IV-7, 10	NP2222/NP2391 corn (negative control)	*Ppu*MI	0	N/A	N/A
IV-7, 11	1-copy positive control	N/A	1	4.7	4.7
IV-7, 12	1/7-copy positive control	N/A	1	4.7	4.7
IV-8, 2	MZHG0JG T_2 (ear 4) corn	*Eco*RV	2	>0.6	2.7
				7.3	7.3
IV-8, 3	MZHG0JG T_2 (ear 35) corn	*Eco*RV	2	>0.6	2.7
				7.3	7.3
IV-8, 4	MZHG0JG T_3 corn	*Eco*RV	2	>0.6	2.7
				7.3	7.3
IV-8, 5	MZHG0JG T_4 corn	*Eco*RV	2	>0.6	2.7
				7.3	7.3
IV-8, 6	MZHG0JG T_5 corn	*Eco*RV	2	>0.6	2.7
				7.3	7.3
IV-8, 7	MZHG0JG F_1 corn	*Eco*RV	2	>0.6	2.7
				7.3	7.3
IV-8, 8	NP2222 corn (negative control)	*Eco*RV	0	N/A	N/A
IV-8, 9	NP2391 corn (negative control)	*Eco*RV	0	N/A	N/A
IV-8, 10	NP2222/NP2391 corn (negative control)	*Eco*RV	0	N/A	N/A
IV-8, 11	1-copy positive control	N/A	1	4.7	4.7
IV-8, 12	1/7-copy positive control	N/A	1	4.7	4.7

Figure & lane	Source of DNA	Restriction enzymes	Expected no. of bands[a]	Approximate band size (kb)	
				Expected	Observed[a]
IV-9, 2	MZHG0JG T_2 (ear 4) corn	*Asc*I + *Pac*I	1	8.8	8.8
IV-9, 3	MZHG0JG T_2 (ear 35) corn	*Asc*I + *Pac*I	1	8.8	8.8
IV-9, 4	MZHG0JG T_3 corn	*Asc*I + *Pac*I	1	8.8	8.8
IV-9, 5	MZHG0JG T_4 corn	*Asc*I + *Pac*I	1	8.8	8.8
IV-9, 6	MZHG0JG T_5 corn	*Asc*I + *Pac*I	1	8.8	8.8
IV-9, 7	MZHG0JG F_1 corn	*Asc*I + *Pac*I	1	8.8	8.8
IV-9, 8	NP2222 corn (negative control)	*Asc*I + *Pac*I	0	N/A	N/A
IV-9, 9	NP2391 corn (negative control)	*Asc*I + *Pac*I	0	N/A	N/A
IV-9, 10	NP2222/NP2391 corn (negative control)	*Asc*I + *Pac*I	0	N/A	N/A
IV-9, 11	1-copy positive control	N/A	1	4.7	4.7
IV-9, 12	1/7-copy positive control	N/A	1	4.7	4.7

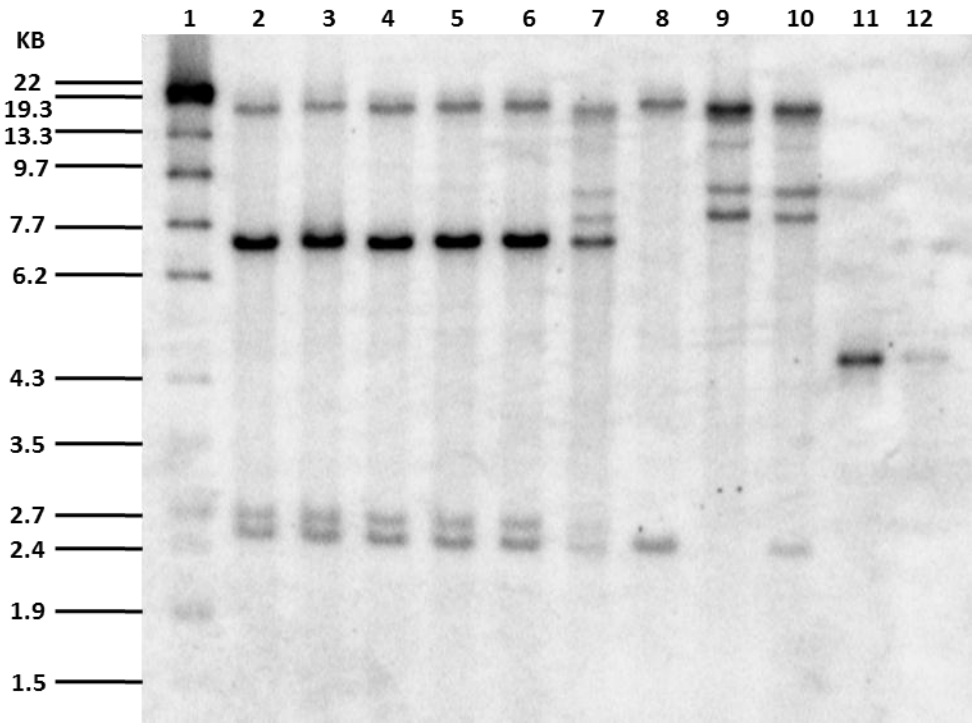
N/A = not applicable.

[a]Bands resulting from cross-hybridization to endogenous corn elements that are not specific to the MZHG0JG insert are not included.



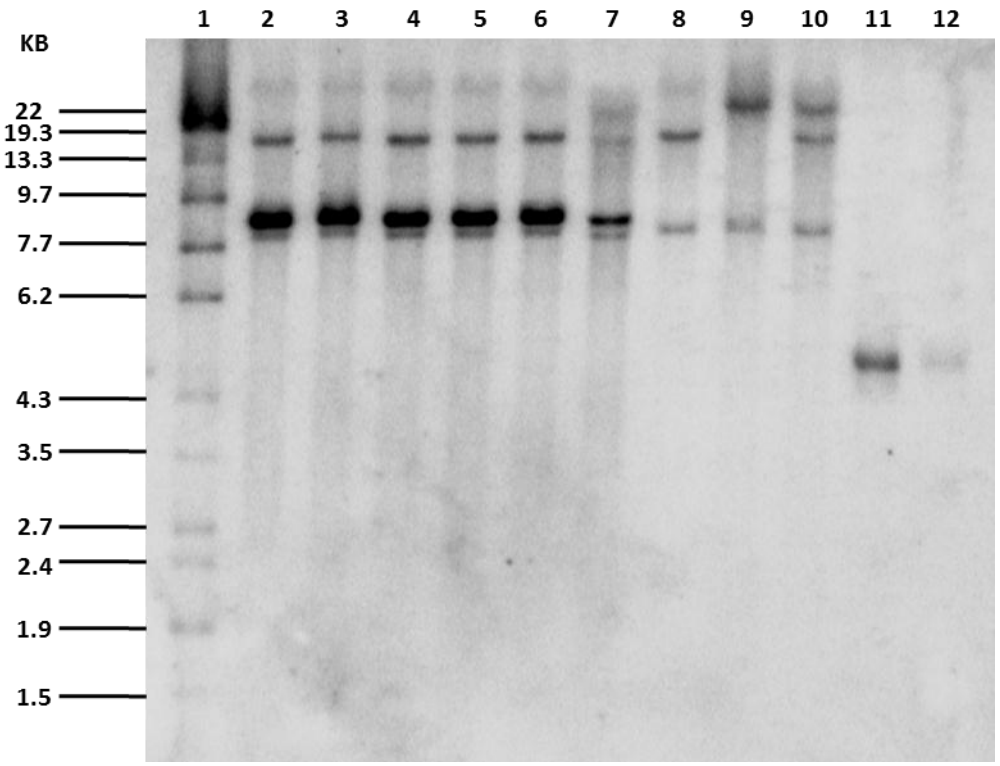
Lane 1 = molecular weight markers
Lane 2 = MZHG0JG T_2 (ear 4) corn
Lane 3 = MZHG0JG T_2 (ear 35) corn
Lane 4 = MZHG0JG T_3 corn
Lane 5 = MZHG0JG T_4 corn
Lane 6 = MZHG0JG T_5 corn
Lane 7 = MZHG0JG F_1 corn
Lane 8 = NP2222 corn (negative control)
Lane 9 = NP2391 corn (negative control)
Lane 10 = NP2222/NP2391 corn (negative control)
Lane 11 = 1-copy positive control (6.56 pg of T-DNA fragment 1)
Lane 12 = 1/7-copy positive control (0.94 pg of T-DNA fragment 1)

Figure IV-7. Southern blot analysis of MZHG0JG corn with the 4.7-kb T-DNA-specific probe 1 and restriction enzyme *PpuMI*



Lane 1 = molecular weight markers
Lane 2 = MZHG0JG T_2 (ear 4) corn
Lane 3 = MZHG0JG T_2 (ear 35) corn
Lane 4 = MZHG0JG T_3 corn
Lane 5 = MZHG0JG T_4 corn
Lane 6 = MZHG0JG T_5 corn
Lane 7 = MZHG0JG F_1 corn
Lane 8 = NP2222 corn (negative control)
Lane 9 = NP2391 corn (negative control)
Lane 10 = NP2222/NP2391 corn (negative control)
Lane 11 = 1-copy positive control (6.56 pg of T-DNA fragment 1)
Lane 12 = 1/7-copy positive control (0.94 pg of T-DNA fragment 1)

Figure IV-8. Southern blot analysis of MZHG0JG corn with the 4.7-kb T-DNA-specific probe 1 and restriction enzyme *Eco*RV



Lane 1 = molecular weight markers
Lane 2 = MZHG0JG T_2 (ear 4) corn
Lane 3 = MZHG0JG T_2 (ear 35) corn
Lane 4 = MZHG0JG T_3 corn
Lane 5 = MZHG0JG T_4 corn
Lane 6 = MZHG0JG T_5 corn
Lane 7 = MZHG0JG F_1 corn
Lane 8 = NP2222 corn (negative control)
Lane 9 = NP2391 corn (negative control)
Lane 10 = NP2222/NP2391 corn (negative control)
Lane 11 = 1-copy positive control (6.56 pg of T-DNA fragment 1)
Lane 12 = 1/7-copy positive control (0.94 pg of T-DNA fragment 1)

Figure IV-9. Southern blot analysis of MZHG0JG corn with the 4.7-kb T-DNA-specific probe 1 and restriction enzymes *Asc*I + *Pac*I

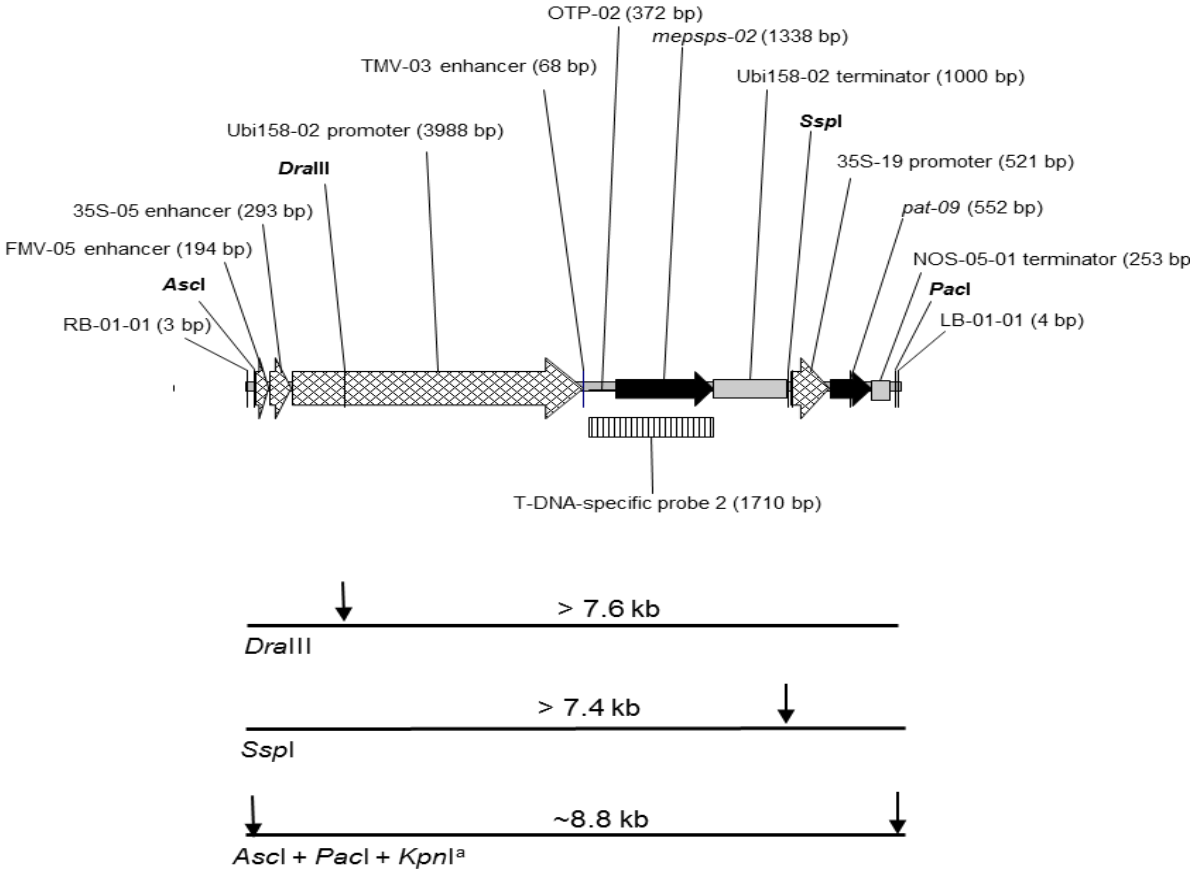
IV.G.3.c. Results of Southern Blot Analysis with T-DNA-Specific Probe 2

Figure IV-10 shows the digestion strategy used with T-DNA-specific probe 2, Table IV-6 shows the insert-specific hybridization bands expected and observed in Southern blot analyses of MZHG0JG corn DNA with T-DNA-specific probe 2, and Figures IV-11 through IV-13 show the results of the Southern blot analyses with T-DNA-specific probe 2.

In the analysis of genomic DNA digested with *Dra*III, one band of approximately 20 kb was observed in the lanes containing DNA from MZHG0JG T_2 (ear 4), T_2 (ear 35), T_3, T_4, T_5, or F_1 corn (Figure IV-11, Lanes 2 through 7). This band was absent from the lanes containing DNA from the nontransgenic NP2222, NP2391, or NP2222/NP2391 corn (Figure IV-11, Lanes 8 through 10) and was, therefore, specific to the MZHG0JG insert. As expected, one band of approximately 1.7 kb was observed in the lanes containing the positive controls (Figure IV-11, Lanes 11 and 12).

In the analysis of genomic DNA digested with *Ssp*I, one band of approximately 9.6 kb was observed in the lanes containing DNA from MZHG0JG T_2 (ear 4), T_2 (ear 35), T_3, T_4, T_5, or F_1 corn (Figure IV-12, Lanes 2 through 7). This band was absent from the lanes containing DNA from nontransgenic NP2222, NP2391, or NP2222/NP2391 corn (Figure IV-12, Lanes 8 through 10) and was, therefore, specific to the MZHG0JG insert. As expected, one band of approximately 1.7 kb was observed in the lanes containing the positive controls (Figure IV-12, Lanes 11 and 12).

In the analysis of genomic DNA digested with *Asc*I + *Pac*I + *Kpn*I, one band of approximately 8.8 kb was observed in the lanes containing DNA from MZHG0JG T_2 (ear 4), T_2 (ear 35), T_3, T_4, T_5, or F_1 corn (Figure IV-13, Lanes 2 through 7). This band was absent from the lanes containing DNA from nontransgenic NP2222, NP2391, or NP2222/NP2391 corn (Figure IV-13, Lanes 8 through 10) and was, therefore, specific to the MZHG0JG insert. As expected, one band of approximately 1.7 kb was observed in the lanes containing the positive controls (Figure IV-13, Lanes 11 and 12).



[a]*Kpn*I was used to more efficiently digest the genomic DNA.
*Kpn*I does not cut within the insert and is therefore not represented in the figure.
The vertical arrows indicate the site of restriction digestion.
Sizes of the expected restriction fragments are indicated.

Figure IV-10. Locations of the 1.7-kb T-DNA-specific probe 2 and the restriction sites *Dra*III, *Ssp*I, and *Asc*I + *Pac*I + *Kpn*I in the MZHG0JG corn insert

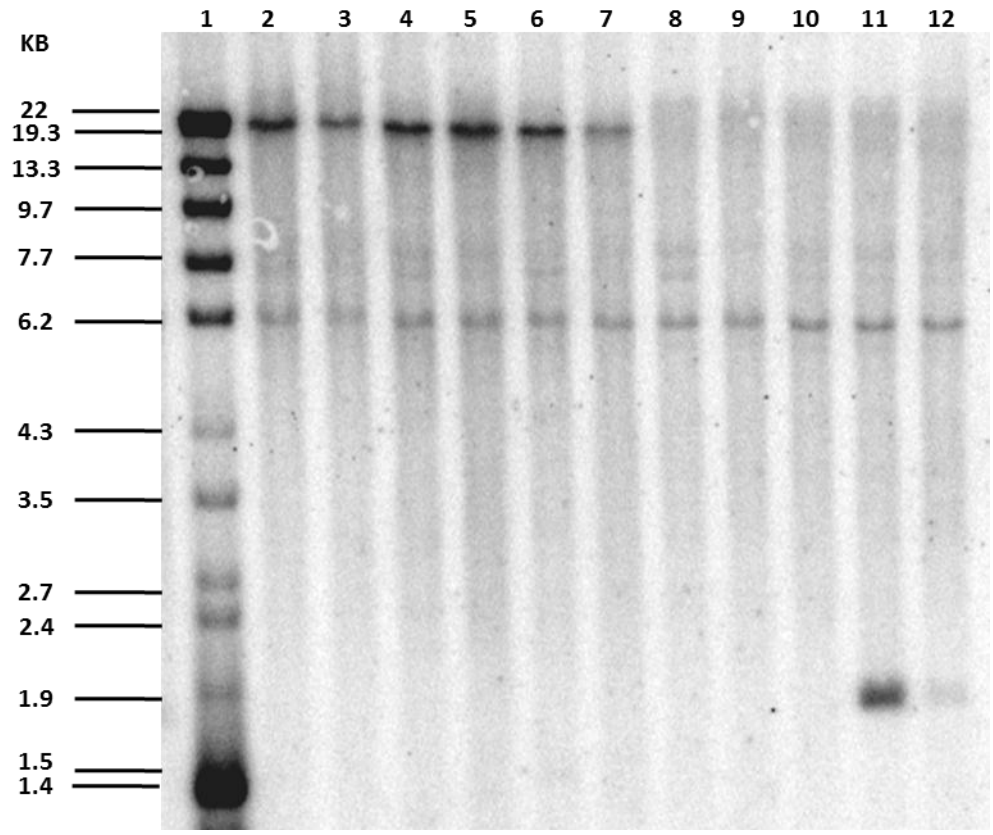
Table IV-6. Expected and observed insert-specific hybridization bands in Southern blot analyses of MZHG0JG corn DNA with T-DNA-specific probe 2 and restriction enzymes *Dra*III, *Ssp*I, and *Asc*I + *Pac*I + *Kpn*I

Figure & lane	Source of DNA	Restriction enzymes	Expected no. of bands[a]	Approximate band size (kb)	
				Expected	Observed[a]
IV-11, 2	MZHG0JG T_2 (ear 4) corn	*Dra*III	1	>7.6	20
IV-11, 3	MZHG0JG T_2 (ear 35) corn	*Dra*III	1	>7.6	20
IV-11, 4	MZHG0JG T_3 corn	*Dra*III	1	>7.6	20
IV-11, 5	MZHG0JG T_4 corn	*Dra*III	1	>7.6	20
IV-11, 6	MZHG0JG T_5 corn	*Dra*III	1	>7.6	20
IV-11, 7	MZHG0JG F_1 corn	*Dra*III	1	>7.6	20
IV-11, 8	NP2222 corn (negative control)	*Dra*III	0	N/A	N/A
IV-11, 9	NP2391 corn (negative control)	*Dra*III	0	N/A	N/A
IV-11, 10	NP2222/NP2391 corn (negative control)	*Dra*III	0	N/A	N/A
IV-11, 11	1-copy positive control	*Dra*III	1	1.7	1.7
IV-11, 12	1/7-copy positive control	*Dra*III	1	1.7	1.7
IV-12, 2	MZHG0JG T_2 (ear 4) corn	*Ssp*I	1	>7.4	9.6
IV-12, 3	MZHG0JG T_2 (ear 35) corn	*Ssp*I	1	>7.4	9.6
IV-12, 4	MZHG0JG T_3 corn	*Ssp*I	1	>7.4	9.6
IV-12, 5	MZHG0JG T_4 corn	*Ssp*I	1	>7.4	9.6
IV-12, 6	MZHG0JG T_5 corn	*Ssp*I	1	>7.4	9.6
IV-12, 7	MZHG0JG F_1 corn	*Ssp*I	1	>7.4	9.6
IV-12, 8	NP2222 corn (negative control)	*Ssp*I	0	N/A	N/A
IV-12, 9	NP2391 corn (negative control)	*Ssp*I	0	N/A	N/A
IV-12, 10	NP2222/NP2391 corn (negative control)	*Ssp*I	0	N/A	N/A
IV-12, 11	1-copy positive control	*Ssp*I	1	1.7	1.7
IV-12, 12	1/7-copy positive control	*Ssp*I	1	1.7	1.7

Figure & lane	Source of DNA	Restriction enzymes	Expected no. of bands[a]	Approximate band size (kb)	
				Expected	Observed[a]
IV-13, 2	MZHG0JG T_2 (ear 4) corn	*Ascl* + *Pacl* + *Kpnl*	1	8.8	8.8
IV-13, 3	MZHG0JG T_2 (ear 35) corn	*Ascl* + *Pacl* + *Kpnl*	1	8.8	8.8
IV-13, 4	MZHG0JG T_3 corn	*Ascl* + *Pacl* + *Kpnl*	1	8.8	8.8
IV-13, 5	MZHG0JG T_4 corn	*Ascl* + *Pacl* + *Kpnl*	1	8.8	8.8
IV-13, 6	MZHG0JG T_5 corn	*Ascl* + *Pacl* + *Kpnl*	1	8.8	8.8
IV-13, 7	MZHG0JG F_1 corn	*Ascl* + *Pacl* + *Kpnl*	1	8.8	8.8
IV-13, 8	NP2222 corn (negative control)	*Ascl* + *Pacl* + *Kpnl*	0	N/A	N/A
IV-13, 9	NP2391 corn (negative control)	*Ascl* + *Pacl* + *Kpnl*	0	N/A	N/A
IV-13, 10	NP2222/NP2391 corn (negative control)	*Ascl* + *Pacl* + *Kpnl*	0	N/A	N/A
IV-13, 11	1-copy positive control	*Ascl* + *Pacl* + *Kpnl*	1	1.7	1.7
IV-13, 12	1/7-copy positive control	*Ascl* + *Pacl* + *Kpnl*	1	1.7	1.7

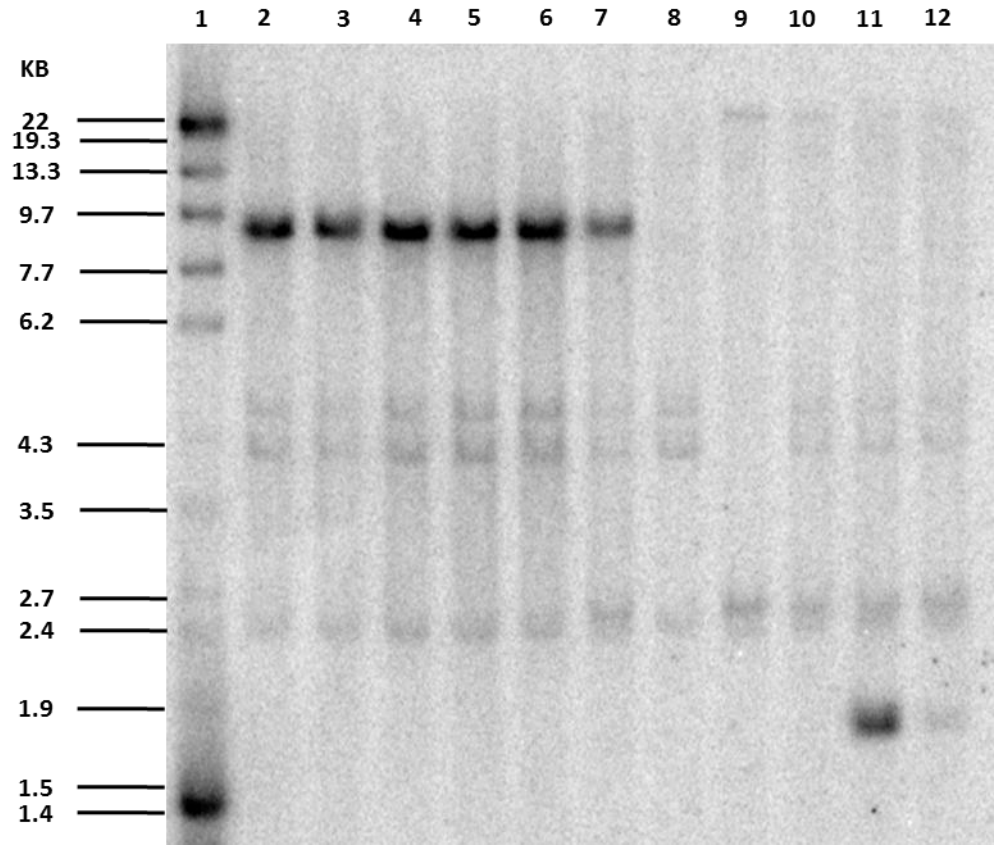
N/A = not applicable.

[a]Bands resulting from cross-hybridization to endogenous corn elements that are not specific to the MZHG0JG insert are not included.



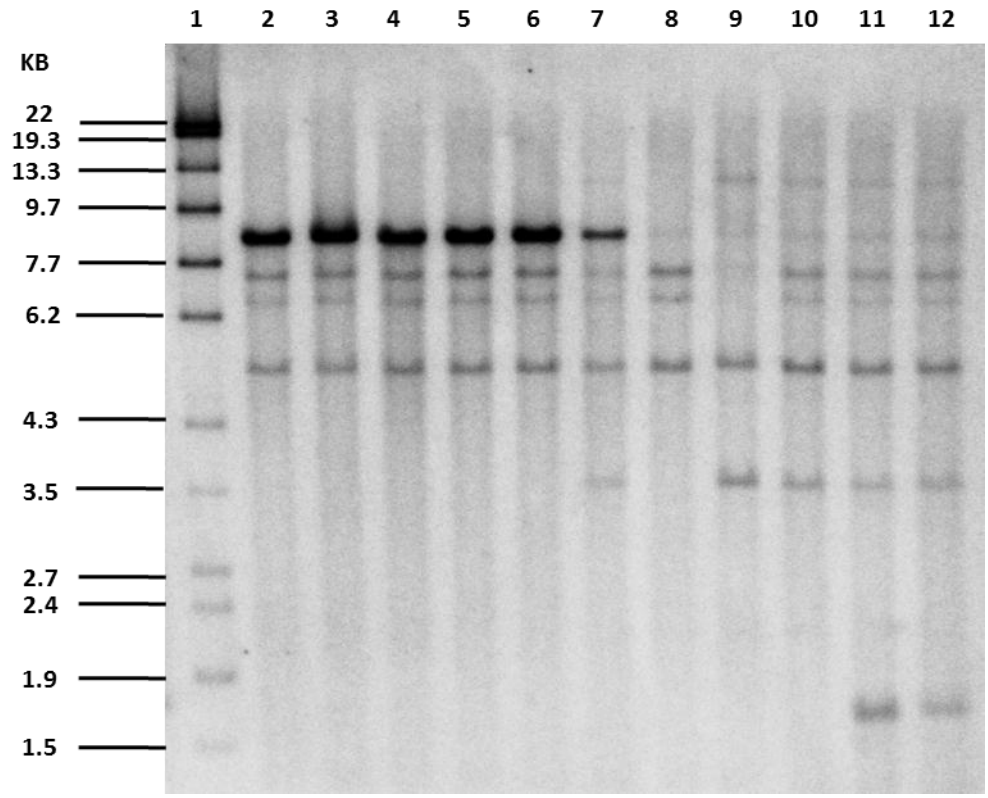
Lane 1 = molecular weight markers
Lane 2 = MZHG0JG T_2 (ear 4) corn
Lane 3 = MZHG0JG T_2 (ear 35) corn
Lane 4 = MZHG0JG T_3 corn
Lane 5 = MZHG0JG T_4 corn
Lane 6 = MZHG0JG T_5 corn
Lane 7 = MZHG0JG F_1 corn
Lane 8 = NP2222 corn (negative control)
Lane 9 = NP2391 corn (negative control)
Lane 10 = NP2222/NP2391 corn (negative control)
Lane 11 = 1-copy positive control (NP2222/NP2391 corn + 2.40 pg of T-DNA fragment 2).
Lane 12 = 1/7-copy positive control (NP2222/NP2391 corn + 0.34 pg of T-DNA fragment 2)

Figure IV-11. Southern blot analysis of MZHG0JG corn with the 1.7-kb T-DNA-specific probe 2 and restriction enzyme *Dra*III



Lane 1 = molecular weight markers
Lane 2 = MZHG0JG T_2 (ear 4) corn
Lane 3 = MZHG0JG T_2 (ear 35) corn
Lane 4 = MZHG0JG T_3 corn
Lane 5 = MZHG0JG T_4 corn
Lane 6 = MZHG0JG T_5 corn
Lane 7 = MZHG0JG F_1 corn
Lane 8 = NP2222 corn (negative control)
Lane 9 = NP2391 corn (negative control)
Lane 10 = NP2222/NP2391 corn (negative control)
Lane 11 = 1-copy positive control (NP2222/NP2391 corn + 2.40 pg of T-DNA fragment 2)
Lane 12 = 1/7-copy positive control (NP2222/NP2391 corn + 0.34 pg of T-DNA fragment 2)

Figure IV-12. Southern blot analysis of MZHG0JG corn with the 1.7-kb T-DNA-specific probe 2 and restriction enzyme *Ssp*I



Lane 1 = molecular weight markers
Lane 2 = MZHG0JG T_2 (ear 4) corn
Lane 3 = MZHG0JG T_2 (ear 35) corn
Lane 4 = MZHG0JG T_3 corn
Lane 5 = MZHG0JG T_4 corn
Lane 6 = MZHG0JG T_5 corn
Lane 7 = MZHG0JG F_1 corn
Lane 8 = NP2222 corn (negative control)
Lane 9 = NP2391 corn (negative control)
Lane 10 = NP2222/NP2391 corn (negative control)
Lane 11 = 1-copy positive control (NP2222/NP2391 corn + 2.40 pg of T-DNA fragment 2)
Lane 12 = 1/7-copy positive control (NP2222/NP2391 corn + 0.34 pg of T-DNA fragment 2)

Figure IV-13. Southern blot analysis of MZHG0JG corn with the 1.7-kb T-DNA-specific probe 2 and restriction enzymes *Asc*I + *Pac*I + *Kpn*I

IV.G.3.d. Results of Southern Blot Analysis with T-DNA-Specific Probe 3

Figure IV-14 shows the digestion strategy used with T-DNA-specific probe 3, Table IV-7 shows the insert-specific hybridization bands expected and observed in Southern blot analyses of MZHG0JG corn DNA with T-DNA-specific probe 3, and Figures IV-15 through IV-17 show the results of the Southern blot analyses with T-DNA-specific probe 3.

In the analysis of genomic DNA digested with *Eag*I, two bands of approximately 7.7 and 20 kb were observed in the lanes containing DNA from MZHG0JG T_2 (ear 4), T_2 (ear 35), T_3, T_4, T_5, or F_1 corn (Figure IV-15, Lanes 2 through 7). These bands were absent from the lanes containing DNA from the nontransgenic NP2222, NP2391, or NP2222/NP2391 corn (Figure IV-15, Lanes 8 through 10) and were, therefore, specific to the MZHG0JG insert. As expected, one band of approximately 2.6 kb was observed in the lanes containing the positive controls (Figure IV-15, Lanes 11 and 12).

In the analysis of genomic DNA digested with *Sca*I, two bands of approximately 3.7 and 22 kb were observed in the lanes containing DNA from MZHG0JG T_2 (ear 4), T_2 (ear 35), T_3, T_4, T_5, or F_1 corn (Figure IV-16, Lanes 2 through 7). These bands were absent from the lanes containing DNA from nontransgenic NP2222, NP2391, or NP2222/NP2391 corn (Figure IV-16, Lanes 8 through 10) and were, therefore, specific to the MZHG0JG insert. As expected, one band of approximately 2.6 kb was observed in the lanes containing the positive controls (Figure IV-16, Lanes 11 and 12).

In the analysis of genomic DNA digested with *Asc*I + *Pac*I, one band of approximately 8.8 kb was observed in the lanes containing DNA from MZHG0JG T_2 (ear 4), T_2 (ear 35), T_3, T_4, T_5, or F_1 corn (Figure IV-17, Lanes 2 through 7). This band was absent in the lanes containing DNA from nontransgenic NP2222, NP2391, or NP2222/NP2391 corn (Figure IV-17, Lanes 8 through 10) and was, therefore, specific to the MZHG0JG insert. As expected, one band of approximately 2.6 kb was observed in the lanes containing the positive controls (Figure IV-17, Lanes 11 and 12).

In the analysis with *Eag*I digestion, an additional band was detected because of sequence similarity between the 35S-05 enhancer (an element in the *mepsps-02* cassette and covered by T-DNA-specific probe 1) and the 35S-19 promoter (an element in *pat-09* cassette and covered by T-DNA-specific probe 3). As a result, two hybridization bands, one corresponding to a copy of the 35S-05 enhancer in MZHG0JG corn and one corresponding to the portion of the T-DNA covered by the probe, were seen in this analysis. No additional bands were seen with *Sca*I digestion, because the 35S-05 enhancer and the portion of the T-DNA covered by the probe were on the same fragment. No unexpected bands were detected, indicating that the MZHG0JG corn genome contains no extraneous DNA fragments of the T-DNA-specific probe 3 sequence.

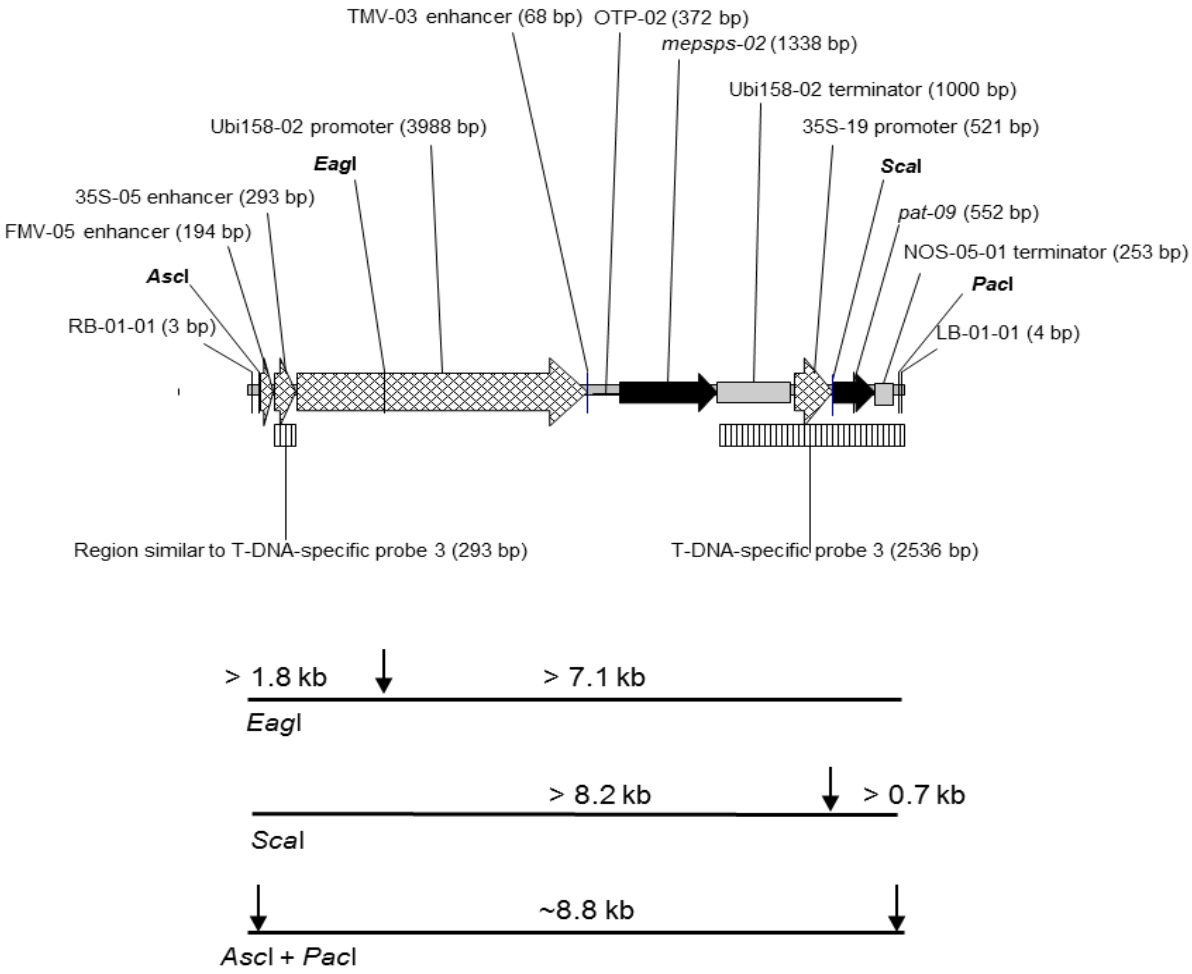


Figure IV-14. Locations of the 2.6-kb T-DNA-specific probe 3 and the restriction sites *Eag*I, *Sca*I, and *Asc*I + *Pac*I in the MZHG0JG corn insert

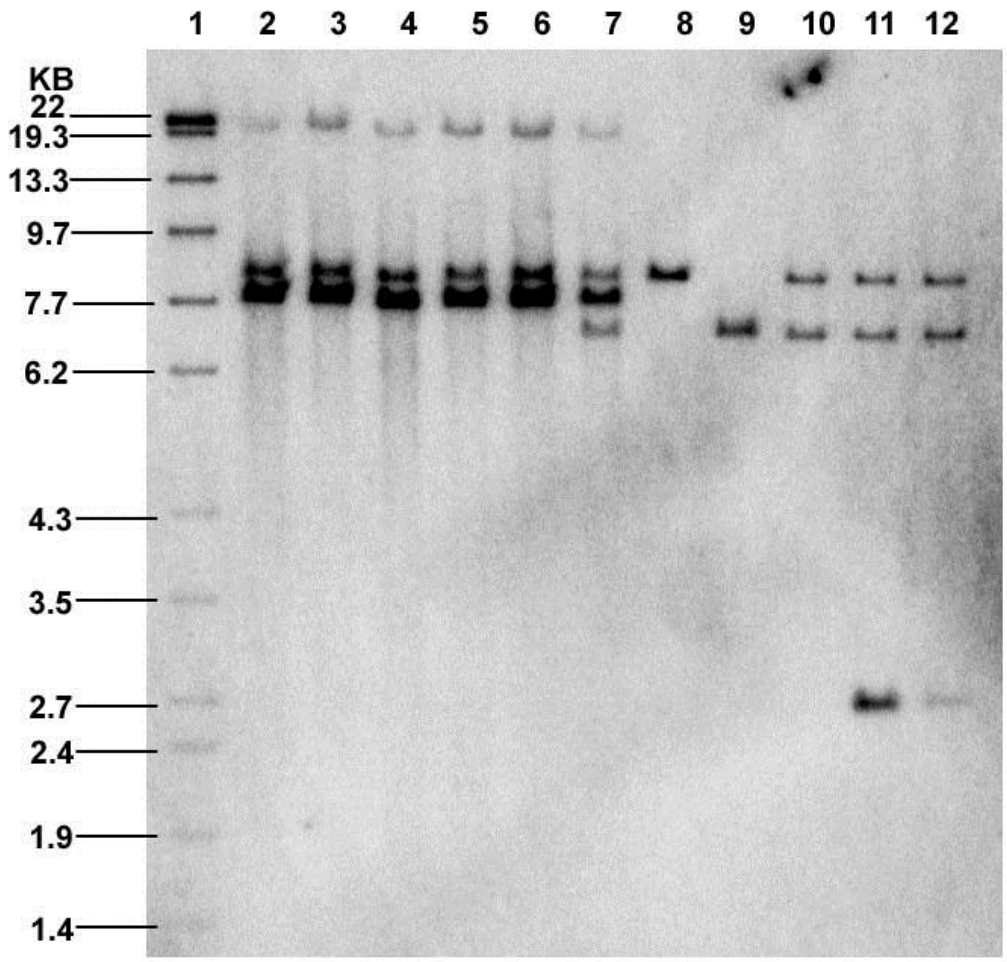
Table IV-7. Expected and observed insert-specific hybridization bands in Southern blot analyses of MZHG0JG corn DNA with T-DNA-specific probe 3 and restriction enzymes *Eag*I, *Sca*I, and *Asc*I + *Pac*I

Figure & lane	Source of DNA	Restriction enzymes	Expected no. of bands[a]	Approximate band size (kb)	
				Expected	Observed[a]
IV-15, 2	MZHG0JG T_2 (ear 4) corn	*Eag*I	2	>1.8	7.7
				>7.1	20
IV-15, 3	MZHG0JG T_2 (ear 35) corn	*Eag*I	2	>1.8	7.7
				>7.1	20
IV-15, 4	MZHG0JG T_3 corn	*Eag*I	2	>1.8	7.7
				>7.1	20
IV-15, 5	MZHG0JG T_4 corn	*Eag*I	2	>1.8	7.7
				>7.1	20
IV-15, 6	MZHG0JG T_5 corn	*Eag*I	2	>1.8	7.7
				>7.1	20
IV-15, 7	MZHG0JG F_1 corn	*Eag*I	2	>1.8	7.7
				>7.1	20
IV-15, 8	NP2222 corn (negative control)	*Eag*I	0	N/A	N/A
IV-15, 9	NP2391 corn (negative control)	*Eag*I	0	N/A	N/A
IV-15, 10	NP2222/NP2391 corn (negative control)	*Eag*I	0	N/A	N/A
IV-15, 11	1-copy positive control	*Eag*I	1	2.6	2.6
IV-15, 12	1/7-copy positive control	*Eag*I	1	2.6	2.6
IV-16, 2	MZHG0JG T_2 (ear 4) corn	*Sca*I	2	>0.7	3.7
				>8.2	22
IV-16, 3	MZHG0JG T_2 (ear 35) corn	*Sca*I	2	>0.7	3.7
				>8.2	22
IV-16, 4	MZHG0JG T_3 corn	*Sca*I	2	>0.7	3.7
				>8.2	22
IV-16, 5	MZHG0JG T_4 corn	*Sca*I	2	>0.7	3.7
				>8.2	22
IV-16, 6	MZHG0JG T_5 corn	*Sca*I	2	>0.7	3.7
				>8.2	22
IV-16, 7	MZHG0JG F_1 corn	*Sca*I	2	>0.7	3.7
				>8.2	22
IV-16, 8	NP2222 corn (negative control)	*Sca*I	0	N/A	N/A
IV-16, 9	NP2391 corn (negative control)	*Sca*I	0	N/A	N/A
IV-16, 10	NP2222/NP2391 corn (negative control)	*Sca*I	0	N/A	N/A
IV-16, 11	1-copy positive control	*Sca*I	1	2.6	2.6
IV-16, 12	1/7-copy positive control	*Sca*I	1	2.6	2.6

Figure & lane	Source of DNA	Restriction enzymes	Expected no. of bands[a]	Approximate band size (kb)	
				Expected	Observed[a]
IV-17, 2	MZHG0JG T_2 (ear 4) corn	*Asc*I + *Pac*I	1	8.8	8.8
IV-17, 3	MZHG0JG T_2 (ear 35) corn	*Asc*I + *Pac*I	1	8.8	8.8
IV-17, 4	MZHG0JG T_3 corn	*Asc*I + *Pac*I	1	8.8	8.8
IV-17, 5	MZHG0JG T_4 corn	*Asc*I + *Pac*I	1	8.8	8.8
IV-17, 6	MZHG0JG T_5 corn	*Asc*I + *Pac*I	1	8.8	8.8
IV-17, 7	MZHG0JG F_1 corn	*Asc*I + *Pac*I	1	8.8	8.8
IV-17, 8	NP2222 corn (negative control)	*Asc*I + *Pac*I	0	N/A	N/A
IV-17, 9	NP2391 corn (negative control)	*Asc*I + *Pac*I	0	N/A	N/A
IV-17, 10	NP2222/NP2391 corn (negative control)	*Asc*I + *Pac*I	0	N/A	N/A
IV-17, 11	1-copy positive control	*Asc*I + *Pac*I	1	2.6	2.6
IV-17, 12	1/7-copy positive control	*Asc*I + *Pac*I	1	2.6	2.6

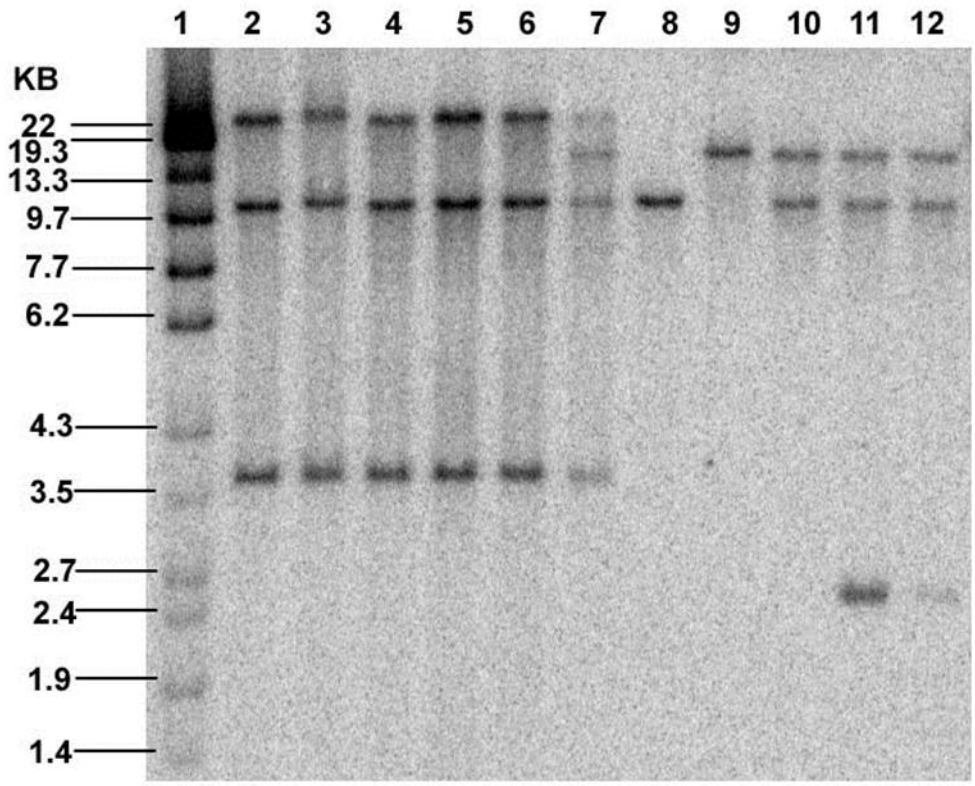
N/A = not applicable.

[a]Bands resulting from cross-hybridization to endogenous corn elements that are not specific to the MZHG0JG insert are not included.



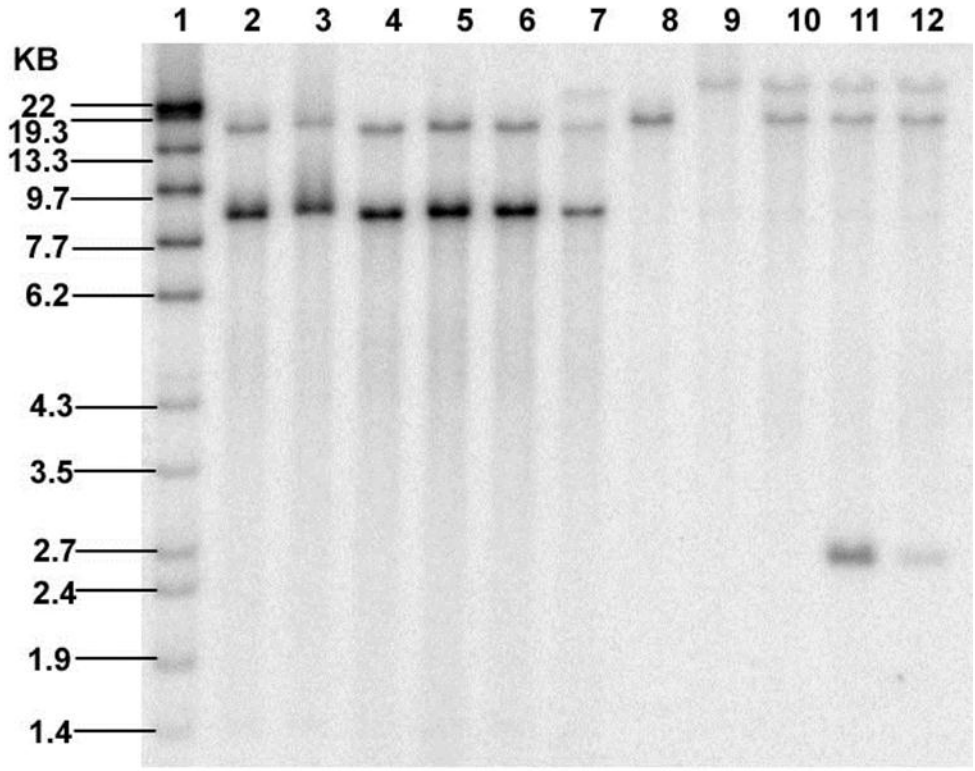
Lane 1 = molecular weight markers
Lane 2 = MZHG0JG T_2 (ear 4) corn
Lane 3 = MZHG0JG T_2 (ear 35) corn
Lane 4 = MZHG0JG T_3 corn
Lane 5 = MZHG0JG T_4 corn
Lane 6 = MZHG0JG T_5 corn
Lane 7 = MZHG0JG F_1 corn
Lane 8 = NP2222 corn (negative control)
Lane 9 = NP2391 corn (negative control)
Lane 10 = NP2222/NP2391 corn (negative control)
Lane 11 = 1-copy positive control (NP2222/NP2391 corn + 3.59 pg of T-DNA fragment 3)
Lane 12 = 1/7-copy positive control (NP2222/NP2391 corn + 0.51 pg of T-DNA fragment 3)

Figure IV-15. Southern blot analysis of MZHG0JG corn with the 2.6-kb T-DNA-specific probe 3 and restriction enzyme *EagI*



Lane 1 = molecular weight markers
Lane 2 = MZHG0JG T_2 (ear 4) corn
Lane 3 = MZHG0JG T_2 (ear 35) corn
Lane 4 = MZHG0JG T_3 corn
Lane 5 = MZHG0JG T_4 corn
Lane 6 = MZHG0JG T_5 corn
Lane 7 = MZHG0JG F_1 corn
Lane 8 = NP2222 corn (negative control)
Lane 9 = NP2391 corn (negative control)
Lane 10 = NP2222/NP2391 corn (negative control)
Lane 11 = 1-copy positive control (NP2222/NP2391 corn + 3.59 pg of T-DNA fragment 3)
Lane 12 = 1/7-copy positive control (NP2222/NP2391 corn + 0.51 pg of T-DNA fragment 3)

Figure IV-16. Southern blot analysis of MZHG0JG corn with the 2.6-kb T-DNA-specific probe 3 and restriction enzyme *Sca*I



Lane 1 = molecular weight markers
Lane 2 = MZHG0JG T_2 (ear 4) corn
Lane 3 = MZHG0JG T_2 (ear 35) corn
Lane 4 = MZHG0JG T_3 corn
Lane 5 = MZHG0JG T_4 corn
Lane 6 = MZHG0JG T_5 corn
Lane 7 = MZHG0JG F_1 corn
Lane 8 = NP2222 corn (negative control)
Lane 9 = NP2391 corn (negative control)
Lane 10 = NP2222/NP2391 corn (negative control)
Lane 11 = 1-copy positive control (NP2222/NP2391 corn + 3.59 pg of T-DNA fragment 3)
Lane 12 = 1/7-copy positive control (NP2222/NP2391 corn + 0.51 pg of T-DNA fragment 3)

Figure IV-17. Southern blot analysis of MZHG0JG corn with the 2.6-kb T-DNA-specific probe 3 and restriction enzymes *Asc*I + *Pac*I

IV.G.3.e. Results of Southern Blot Analysis with Plasmid-Backbone-Specific Probe 1

Figure IV-18 shows the digestion strategy used with backbone-specific probe 1, and Figures IV-19 through IV-21 show the results of the Southern blot analyses with backbone-specific probe 1.

In the analyses of genomic DNA digested with *Dra*III, *Not*I, or *Asc*I + *Pac*I, no bands were observed in any of the lanes containing DNA from MZHG0JG corn of any generation tested (Figures IV-19 through IV-21, Lanes 2 through 7) or in the lanes containing DNA from nontransgenic NP2222, NP2391, and NP2222/NP2391 corn (Figures IV-19 through IV-21, Lanes 8 through 10). One band of approximately 3.3 kb was observed in the lanes containing the 1-copy and 1/7-copy positive controls (Figures IV-19 through IV-21, Lanes 11 and 12), as expected.

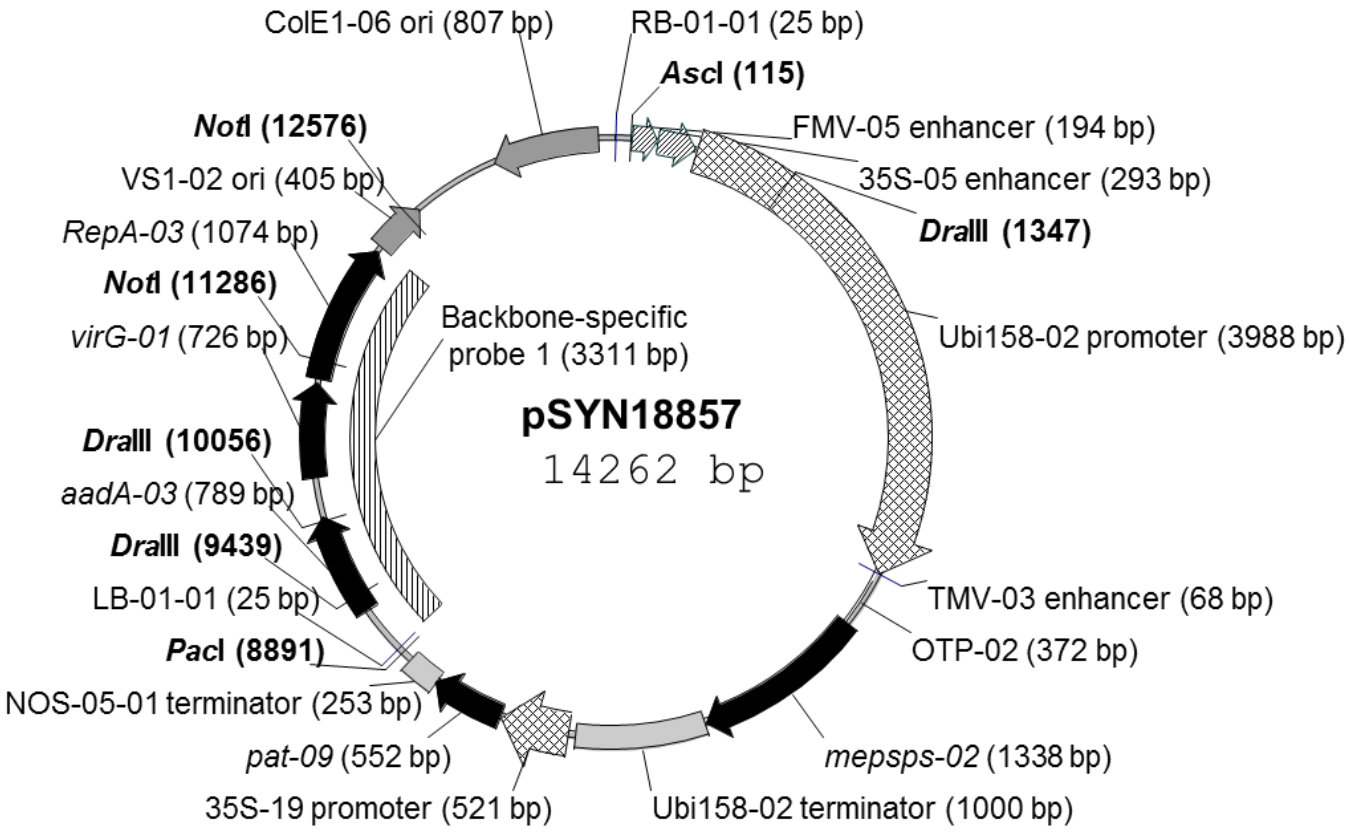
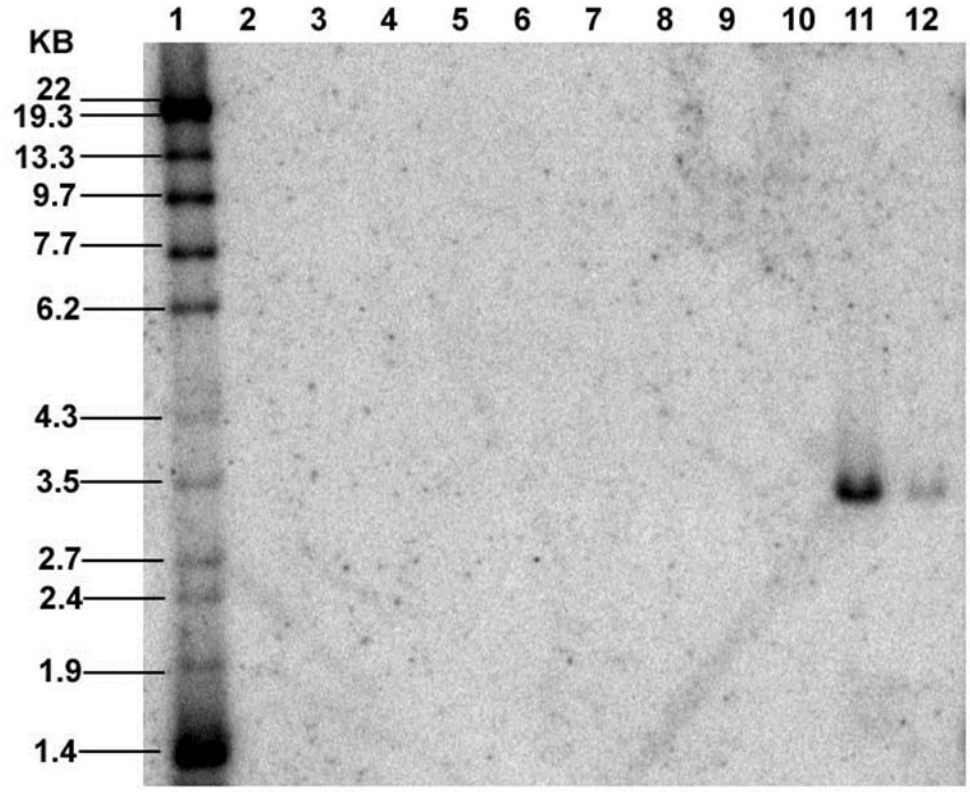
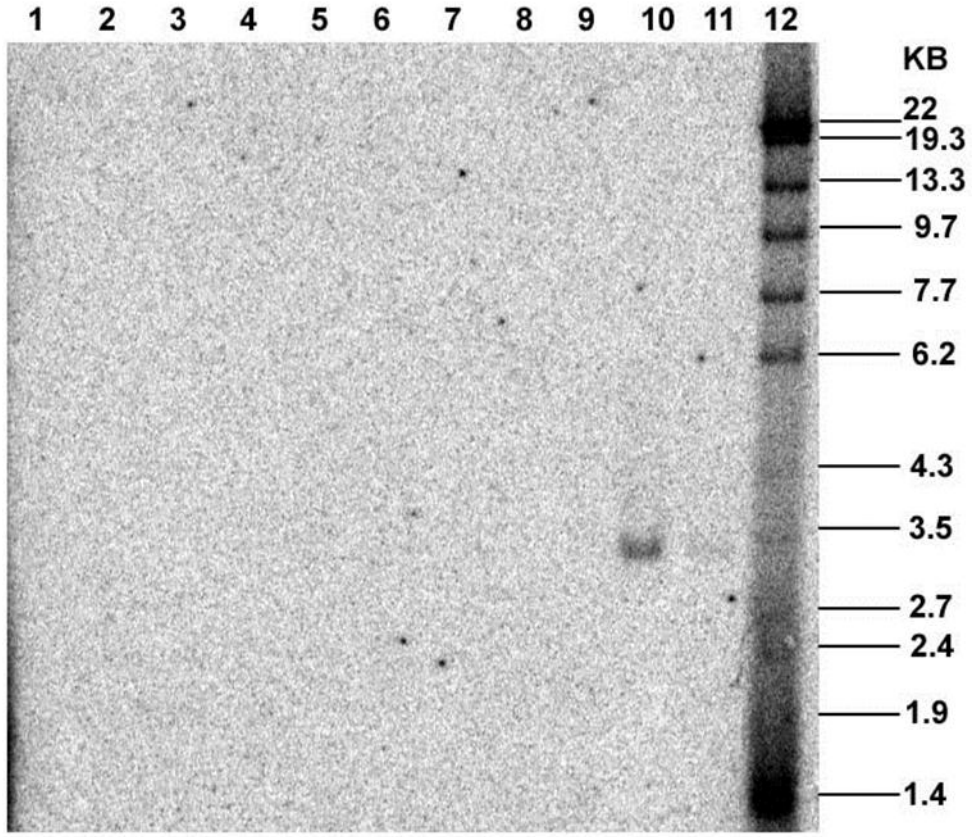


Figure IV-18. Locations of the 3.3-kb backbone-specific probe 1 and the restriction sites *Dra*III, *Not*I, and *Asc*I + *Pac*I in the transformation plasmid pSYN18857



Lane 1 = molecular weight markers
Lane 2 = MZHG0JG T_2 (ear 4) corn
Lane 3 = MZHG0JG T_2 (ear 35) corn
Lane 4 = MZHG0JG T_3 corn
Lane 5 = MZHG0JG T_4 corn
Lane 6 = MZHG0JG T_5 corn
Lane 7 = MZHG0JG F_1 corn
Lane 8 = NP2222 corn (negative control)
Lane 9 = NP2391 corn (negative control)
Lane 10 = NP2222/NP2391 corn (negative control)
Lane 11 = 1-copy positive control (NP2222/NP2391 corn + 4.65 pg of backbone-specific fragment 1)
Lane 12 = 1/7-copy positive control (NP2222/NP2391 corn + 0.66 pg of backbone-specific fragment 1)

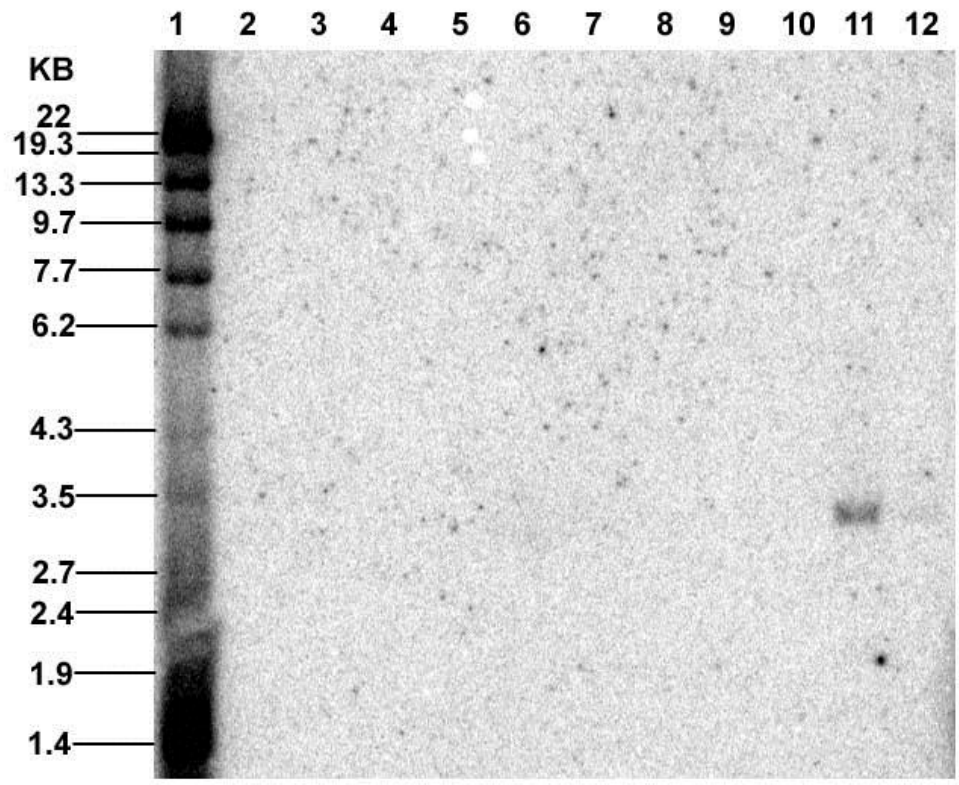
Figure IV-19. Southern blot analysis of MZHG0JG corn with the 3.3-kb plasmid pSYN18857 backbone-specific probe 1 and restriction enzyme *Dra*III



Lane 1 = MZHG0JG T_2 (ear 4) corn
Lane 2 = MZHG0JG T_2 (ear 35) corn
Lane 3 = MZHG0JG T_3 corn
Lane 4 = MZHG0JG T_4 corn
Lane 5 = MZHG0JG T_5 corn
Lane 6 = MZHG0JG F_1 corn
Lane 7 = NP2222 corn (negative control)
Lane 8 = NP2391 corn (negative control)
Lane 9 = NP2222/NP2391 corn (negative control)
Lane 10 = 1-copy positive control (NP2222/NP2391 corn + 4.65 pg of backbone-specific fragment 1)
Lane 11 = 1/7-copy positive control (NP2222/NP2391 corn + 0.66 pg of backbone-specific fragment 1) [a]
Lane 12 = molecular weight markers

[a]Because of limitations in printer resolution, the faint band visible at approximately 3.3 kb in lane 11 may not be visible on the printed copy.

Figure IV-20. Southern blot analysis of MZHG0JG corn with the 3.3-kb plasmid pSYN18857 backbone-specific probe 1 and restriction enzyme *Not*I



Lane 1 = molecular weight markers
Lane 2 = MZHG0JG T_2 (ear 4) corn
Lane 3 = MZHG0JG T_2 (ear 35) corn
Lane 4 = MZHG0JG T_3 corn
Lane 5 = MZHG0JG T_4 corn
Lane 6 = MZHG0JG T_5 corn
Lane 7 = MZHG0JG F_1 corn
Lane 8 = NP2222 corn (negative control)
Lane 9 = NP2391 corn (negative control)
Lane 10 = NP2222/NP2391 corn (negative control)
Lane 11 = 1-copy positive control (NP2222/NP2391 corn + 4.65 pg of backbone-specific fragment 1)
Lane 12 = 1/7-copy positive control (NP2222/NP2391 corn + 0.66 pg of backbone-specific fragment 1) [a]

[a]Because of limitations in printer resolution, the faint band visible at approximately 3.3 kb in lane 12 may not be visible on the printed copy.

Figure IV-21. Southern blot analysis of MZHG0JG corn with the 3.3-kb plasmid pSYN18857 backbone-specific probe 1 and restriction enzymes *Asc*I + *Pac*I

IV.G.3.f. Results of Southern Blot Analysis with Plasmid-Backbone-Specific Probe 2

Figure IV-22 shows the digestion strategy used with backbone-specific probe 2, and Figures IV-23 through IV-25 show the results of the Southern blot analyses with backbone-specific probe 2.

In the analyses of genomic DNA digested with *Dra*III, *Not*I, or *Asc*I + *Pac*I, no bands were observed in any of the lanes containing DNA from MZHG0JG corn of any generation tested (Figures IV-23 through IV-25, Lanes 2 through 7) or in the lanes containing DNA from nontransgenic NP2222, NP2391, and NP2222/NP2391 corn (Figures IV-23 through IV-25, Lanes 8 through 10). One band of approximately 2.1 kb was observed in the lanes containing the one-copy and 1/7-copy positive controls (Figures IV-23 through IV-25, Lanes 11 and 12), as expected.

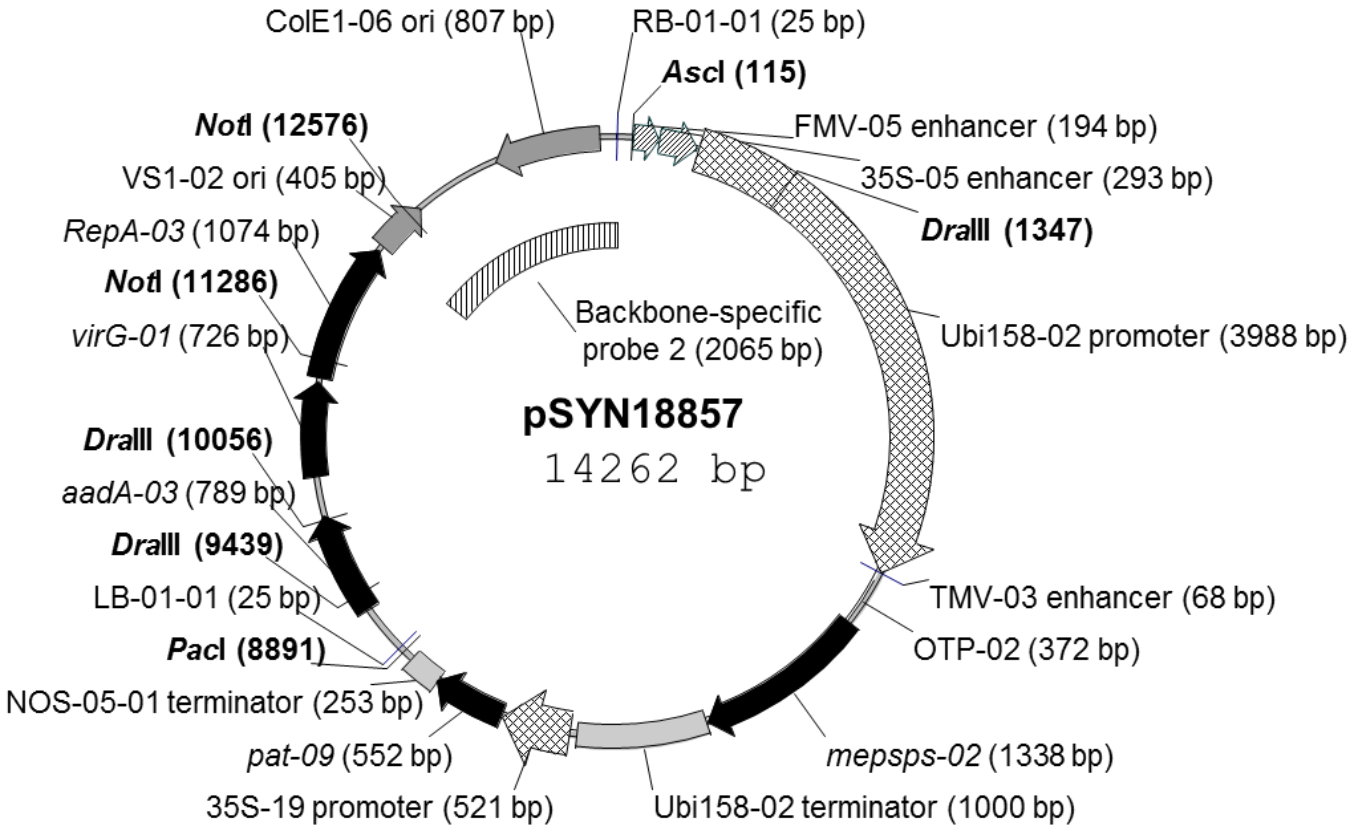
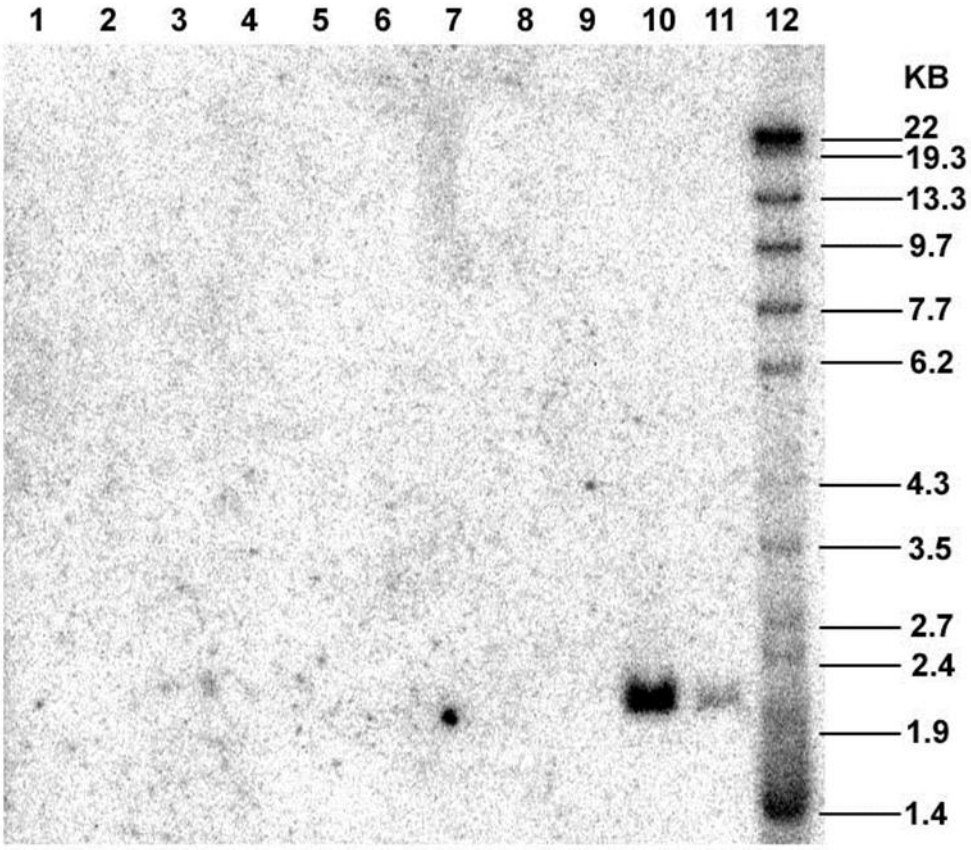
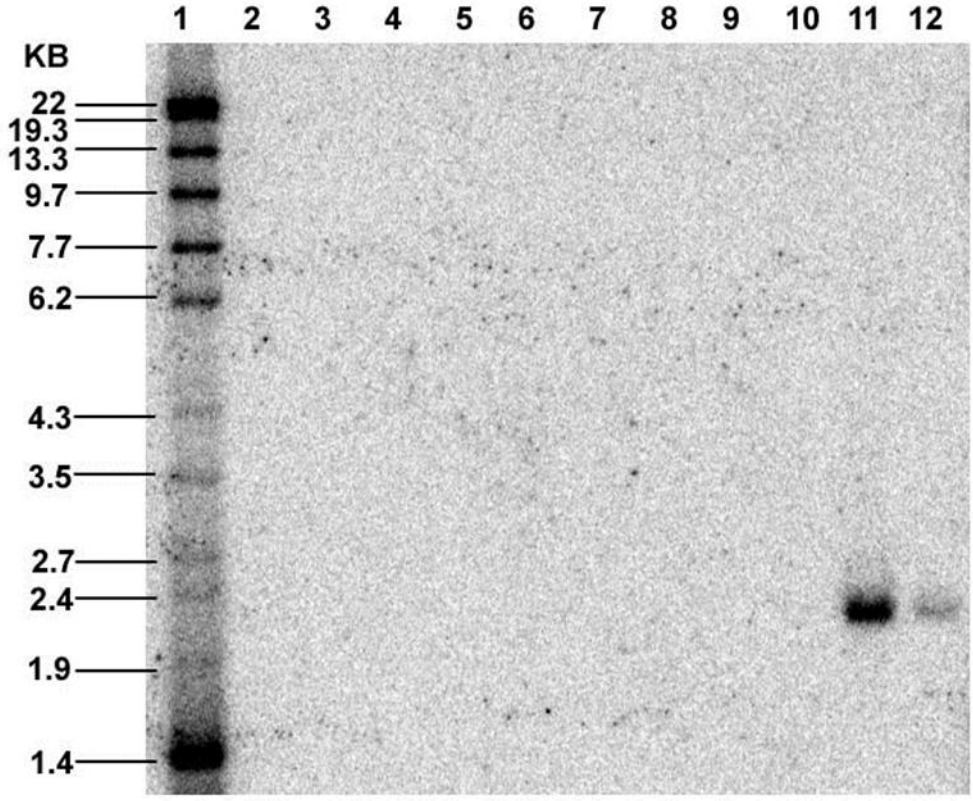


Figure IV-22. Locations of the 2.1-kb backbone-specific probe 2 and the restriction sites *Dra*III, *Not*I, and *Asc*I + *Pac*I in the transformation plasmid pSYN18857



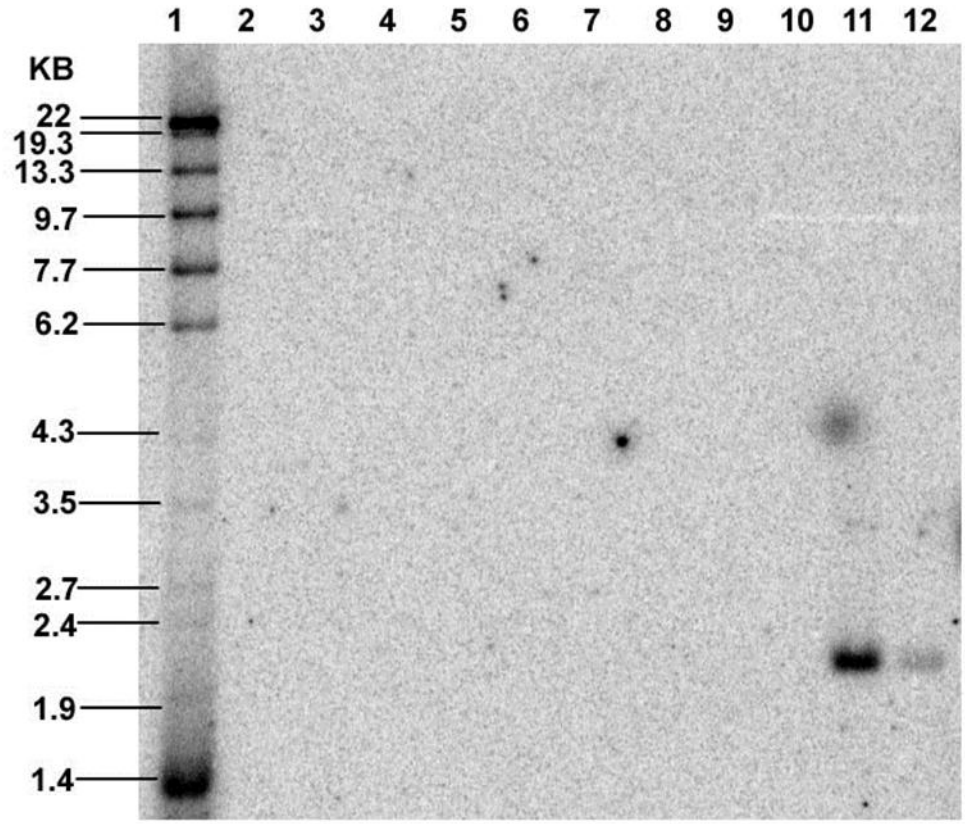
Lane 1 = MZHG0JG T_2 (ear 4) corn
Lane 2 = MZHG0JG T_2 (ear 35) corn
Lane 3 = MZHG0JG T_3 corn
Lane 4 = MZHG0JG T_4 corn
Lane 5 = MZHG0JG T_5 corn
Lane 6 = MZHG0JG F_1 corn
Lane 7 = NP2222 corn (negative control)
Lane 8 = NP2391 corn (negative control)
Lane 9 = NP2222/NP2391 corn (negative control)
Lane 10 = 1-copy positive control (NP2222/NP2391 corn + 2.9 pg of backbone-specific fragment 2)
Lane 11 = 1/7-copy positive control (NP2222/NP2391 corn + 0.41 pg of backbone-specific fragment 2)
Lane 12 = molecular weight marker

Figure IV-23. Southern blot analysis of MZHG0JG corn with the 2.1-kb plasmid pSYN18857 backbone-specific probe 2 and restriction enzyme *Dra*III



Lane 1 = molecular weight markers
Lane 2 = MZHG0JG T_2 (ear 4) corn
Lane 3 = MZHG0JG T_2 (ear 35) corn
Lane 4 = MZHG0JG T_3 corn
Lane 5 = MZHG0JG T_4 corn
Lane 6 = MZHG0JG T_5 corn
Lane 7 = MZHG0JG F_1 corn
Lane 8 = NP2222 corn (negative control)
Lane 9 = NP2391 corn (negative control)
Lane 10 = NP2222/NP2391 corn (negative control)
Lane 11 = 1-copy positive control (NP2222/NP2391 corn + 2.9 pg of backbone-specific fragment 2)
Lane 12 = 1/7-copy positive control (NP2222/NP2391 corn + 0.41 pg of backbone-specific fragment 2)

Figure IV-24. Southern blot analysis of MZHG0JG corn with the 2.1-kb plasmid pSYN18857 backbone-specific probe 2 and restriction enzyme *Not*I



Lane 1 = molecular weight markers
Lane 2 = MZHG0JG T_2 (ear 4) corn
Lane 3 = MZHG0JG T_2 (ear 35) corn
Lane 4 = MZHG0JG T_3 corn
Lane 5 = MZHG0JG T_4 corn
Lane 6 = MZHG0JG T_5 corn
Lane 7 = MZHG0JG F_1 corn
Lane 8 = NP2222 corn (negative control)
Lane 9 = NP2391 corn (negative control)
Lane 10 = NP2222/NP2391 corn (negative control)
Lane 11 = 1-copy positive control (NP2222/NP2391 corn + 2.9 pg of backbone-specific fragment 2)
Lane 12 = 1/7-copy positive control (NP2222/NP2391 corn + 0.41 pg of backbone-specific fragment 2)

Figure IV-25. Southern blot analysis of MZHG0JG corn with the 2.1-kb plasmid pSYN18857 backbone-specific probe 2 and restriction enzyme *Asc*I + *Pac*I

IV.G.3.g. Conclusions from the Results of the Southern Blot Analyses

The Southern blot analyses demonstrated that the hybridization bands specific to the MZHG0JG insert were identical in all lanes containing genomic DNA extracted from MZHG0JG corn plants of generation T_2 (ear 4), T_2 (ear 35), T_3, T_4, T_5, or F_1. These results support the conclusion that the MZHG0JG insert is stably inherited from one generation to the next and that MZHG0JG corn contains a single T-DNA insert. No unexpected bands were detected, indicating that the MZHG0JG corn genome contains no extraneous DNA fragments of the insert. The Southern blot analyses also demonstrated that MZHG0JG corn does not contain any backbone sequence from the transformation plasmid pSYN18857.

IV.G.4. Mendelian Inheritance of the T-DNA Insert

Three generations of MZHG0JG corn were individually analyzed for the presence of *mepsps-02* and *pat-09* by real-time PCR analysis (Ingham *et al.* 2001). The results from real-time PCR analysis were used to determine the segregation ratios of *mepsps-02* and *pat-09*. Hemizygous MZHG0JG corn plants of the T_3 generation were crossed with nontransgenic corn line NP2681. The resulting F_1 generation was backcrossed with the nontransgenic recurrent parent (NP2681) to yield the BC_1F_1 generation. MZHG0JG corn plants from the BC_1F_1 generation were backcrossed two more times with the nontransgenic recurrent parent (NP2681) to yield the BC_2F_1 and BC_3F_1 generations analyzed in this study. The expected segregation ratio for each gene was 1:1 in each generation (i.e., 50% of the plants in each generation were expected to carry the gene). Chi-square analysis of the segregation data was performed to test the hypothesis that the MZHG0JG insert is inherited in a predictable manner according to Mendelian principles and consistent with insertion into a chromosome within the corn nuclear genome. The goodness-of-fit of the observed to the expected segregation ratios was tested by chi-square analysis (Strickberger 1976):

$$\chi^2 = \text{sum } (\text{observed} - \text{expected})^2 \div \text{expected}$$

The expected and observed segregation ratios are shown in Table IV-8. The genes *mepsps-02* and *pat-09* co-segregated (i.e., when one gene was present, the other gene was also present). The critical value for rejection of the hypothesis of segregation according to Mendelian inheritance at $\alpha = 0.05$ was 3.84 (Strickberger 1976). All of the chi-square values were less than 3.84 for each generation tested, indicating that *mepsps-02* and *pat-09* were inherited in a predictable manner, according to Mendelian principles. These results support the conclusion that the MZHG0JG insert integrated into a chromosome within the corn nuclear genome.

Table IV-8. Observed and expected frequencies of *mepsps-02* and *pat-09* in three generations of MZHG0JG corn

	BC_1F_1		BC_2F_1		BC_3F_1	
Trait[a]	Observed	Expected	Observed	Expected	Observed	Expected
Positive	115	110	100	108	97	88.5
Negative	105	110	116	108	80	88.5
Total	220	220	216	216	177	177
χ^2	0.455*		1.185*		1.633*	

[a] The observed frequencies of *mepsps-02 and pat-09* were identical; the two genes segregated as one locus. *$P < 0.05$ ($\chi^2 < 3.84$).

IV.G.5. Summary of the Genetic Characterization of MZHG0JG Corn

Genetic characterization studies demonstrated that MZHG0JG corn contains, at a single locus within the corn genome, a single copy of each of the following functional elements: *mepsps-02*, *pat-09*, FMV-05 enhancer, 35S-05 enhancer, OTP-02 transit peptide, Ubi158-02 promoter, TMV-03 enhancer, Ubi158-02 terminator, 35S-19 promoter, and NOS-05-01 terminator. It does not contain any extraneous DNA fragments of these functional elements elsewhere in the MZHG0JG corn genome, and it does not contain the plasmid backbone sequence from transformation plasmid pSYN18857.

Nucleotide sequence analysis determined that the MZHG0JG insert consists of the intact T-DNA region of pSYN18857. The results of the Southern blot analyses are consistent with the results of the nucleotide sequence analysis.

Sequence analysis of the MZHG0JG insertion site demonstrated that 22 bp from the corn genomic sequence were deleted during the integration of the MZHG0JG insert, and 43 bp of DNA were inserted into the integration site: a 4-bp DNA sequence was present at the junction between the MZHG0JG insert and the 5′ flanking region, and a 39-bp DNA sequence was present at the junction between the MZHG0JG insert and the 3′ flanking region.

BLASTX analyses comparing the corn genomic sequence flanking the MZHG0JG insert with sequences in public databases indicated that the insert does not disrupt any known endogenous corn gene. Bioinformatics analysis indicated that no ORFs ≥ 30 amino acids (based on start to stop codons) span the junction between the corn genome and the MZHG0JG insert.

The observed segregation ratios for *mepsps-02* and *pat-09* in three generations of MZHG0JG corn plants were as expected for a gene inherited according to Mendelian principles. The data indicate that the insert is inherited as a single locus in the corn nuclear genome. These data and the results of Southern blot analyses of five generations of MZHG0JG corn indicate that the transgenic locus is stably inherited during conventional breeding.

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Part V. Absence of Genes that Encode Antibiotic Resistance

V.A. Absence of Genes that Encode Antibiotic Resistance in MZHG0JG Corn

The aminoglycoside adenyltransferase gene (*aadA-03*) from *E. coli* transposon Tn7 (similar to Accession No. X03043.1 [NCBI 2012]) was a component in the plasmid backbone used to generate MZHG0JG corn. Its presence conferred resistance to streptomycin and spectinomycin, and it was used as a bacterial selectable marker. This gene is located outside of the right and left borders of the T-DNA and therefore is not incorporated into the transformed corn genome. Part IV.G.3, above, describes the analyses used to confirm the absence of any plasmid backbone sequence in MZHG0JG corn.

V.B. References Citied in Part V

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Part VI. Substances in the Food

VI.A. Identity and Characterization of mEPSPS Produced in MZHG0JG corn

The native 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) from *Z. mays* is involved in the synthesis of aromatic amino acids and is inhibited by glyphosate. The EPSPS enzymes catalyze the synthesis of 5-enolpyruvylshikimate-3-phosphate (EPSP) from shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) (Figure VI-1). The mEPSPS produced in MZHG0JG corn differs from the native corn EPSPS in that mEPSPS contains two amino acid substitutions that were introduced specifically to confer tolerance to the herbicide glyphosate. The peptide sequence of the mEPSPS produced in MZHG0JG corn was confirmed to encode a 47.4-kD protein consisting of a single polypeptide of 445 amino acids. The amino acid homology between the mEPSPS produced in MZHG0JG corn and native EPSPS from corn is greater than 99.6%. Therefore, these proteins are expected to be and functionally equivalent except for their affinity to glyphosate.

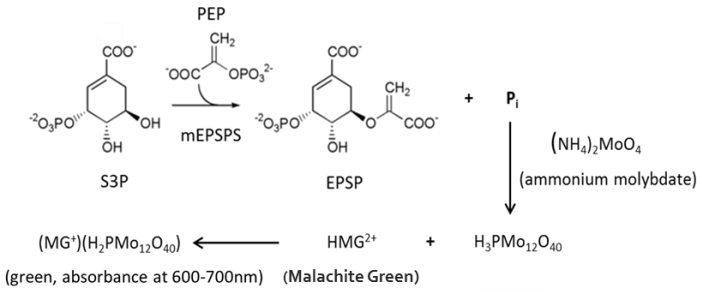


Figure VI-1. The reaction catalyzed by mEPSPS

The mEPSPS enzyme has significantly lower affinity for glyphosate than native corn EPSPS. When corn plants are treated with glyphosate, the endogenous EPSPS is inhibited, preventing synthesis of aromatic amino acids and leading to plant death. When corn plants producing mEPSPS are treated with glyphosate, the endogenous EPSPS is inhibited by glyphosate; however, mEPSPS is not inhibited, allowing synthesis of aromatic amino acids to continue and the plants to survive the herbicide treatment.

VI.A.1. Deduced Amino Acid Sequence Alignment of mEPSPS

Event GA21 corn was first introduced to the market in 1998. The mEPSPS produced in MZHG0JG corn (SYN-ØØØJG-2) is identical to the mEPSPS produced in GA21 corn (OECD identifier MON-ØØØ21-9). The nucleotide sequences of the *mepsps-02* in MZHG0JG corn encoding mEPSPS and the *mepsps* in GA21 corn encoding mEPSPS were confirmed by nucleotide sequencing of the inserts. The deduced amino acid sequence of mEPSPS in MZHG0JG corn and GA21 corn is identical (as shown in Figure VI-2).

```
Translation of Event GA21 mepsps           (1) MAGAEEIVLQPIKEISGTVKLPGSK
Translation of Event MZHG0JG mepsps-02     (1) MAGAEEIVLQPIKEISGTVKLPGSK

Translation of Event GA21 mepsps          (26) SLSNRILLLAALSEGTTVVDNLLNS
Translation of Event MZHG0JG mepsps-02    (26) SLSNRILLLAALSEGTTVVDNLLNS

Translation of Event GA21 mepsps          (51) EDVHYMLGALRTLGLSVEADKAAKR
Translation of Event MZHG0JG mepsps-02    (51) EDVHYMLGALRTLGLSVEADKAAKR

Translation of Event GA21 mepsps          (76) AVVVGCGGKFPVEDAKEEVQLFLGN
Translation of Event MZHG0JG mepsps-02    (76) AVVVGCGGKFPVEDAKEEVQLFLGN

Translation of Event GA21 mepsps         (101) AGIAMRSLTAAVTAAGGNATYVLDG
Translation of Event MZHG0JG mepsps-02   (101) AGIAMRSLTAAVTAAGGNATYVLDG

Translation of Event GA21 mepsps         (126) VPRMRERPIGDLVVGLKQLGADVDC
Translation of Event MZHG0JG mepsps-02   (126) VPRMRERPIGDLVVGLKQLGADVDC

Translation of Event GA21 mepsps         (151) FLGTDCPPVRVNGIGGLPGGKVKLS
Translation of Event MZHG0JG mepsps-02   (151) FLGTDCPPVRVNGIGGLPGGKVKLS

Translation of Event GA21 mepsps         (176) GSISSQYLSALLMAAPLALGDVEIE
Translation of Event MZHG0JG mepsps-02   (176) GSISSQYLSALLMAAPLALGDVEIE

Translation of Event GA21 mepsps         (201) IIDKLISIPYVEMTLRLMERFGVKA
Translation of Event MZHG0JG mepsps-02   (201) IIDKLISIPYVEMTLRLMERFGVKA

Translation of Event GA21 mepsps         (226) EHSDSWDRFYIKGGQKYKSPKNAYV
Translation of Event MZHG0JG mepsps-02   (226) EHSDSWDRFYIKGGQKYKSPKNAYV

Translation of Event GA21 mepsps         (251) EGDASSASYFLAGAAITGGTVTVEG
Translation of Event MZHG0JG mepsps-02   (251) EGDASSASYFLAGAAITGGTVTVEG

Translation of Event GA21 mepsps         (276) CGTTSLQGDVKFAEVLEMMGAKVTW
Translation of Event MZHG0JG mepsps-02   (276) CGTTSLQGDVKFAEVLEMMGAKVTW

Translation of Event GA21 mepsps         (301) TETSVTVTGPPREPFGRKHLKAIDV
Translation of Event MZHG0JG mepsps-02   (301) TETSVTVTGPPREPFGRKHLKAIDV

Translation of Event GA21 mepsps         (326) NMNKMPDVAMTLAVVALFADGPTAI
Translation of Event MZHG0JG mepsps-02   (326) NMNKMPDVAMTLAVVALFADGPTAI

Translation of Event GA21 mepsps         (351) RDVASWRVKETERMVAIRTELTKLG
Translation of Event MZHG0JG mepsps-02   (351) RDVASWRVKETERMVAIRTELTKLG

Translation of Event GA21 mepsps         (376) ASVEEGPDYCIITPPEKLNVTAIDT
Translation of Event MZHG0JG mepsps-02   (376) ASVEEGPDYCIITPPEKLNVTAIDT

Translation of Event GA21 mepsps         (401) YDDHRMAMAFSLAACAEVPVTIRDP
Translation of Event MZHG0JG mepsps-02   (401) YDDHRMAMAFSLAACAEVPVTIRDP

Translation of Event GA21 mepsps         (426) GCTRKTFPDYFDVLSTFVKN-
Translation of Event MZHG0JG mepsps-02   (426) GCTRKTFPDYFDVLSTFVKN-
```

Figure VI-2. Alignment of the deduced amino acid sequence for mEPSPS encoded by *mepsps* in GA21 corn and *mepsps-02* in MZHG0JG corn

VI.A.2. Peptide Mass Coverage Analysis of mEPSPS Produced in MZHG0JG Corn

Peptide mass coverage analysis was used to determine the identity of purified mEPSPS from MZHG0JG corn extract by liquid chromatography–tandem mass spectrometry (LC-MS/MS) using an Ultra-Performance Liquid Chromatography system.

The collective analysis of the three proteolytic digests of the purified mEPSPS preparation from MZHG0JG corn extract yielded coverage of 88% of the total predicted mEPSPS amino acid sequence. Evidence for 52%, 70%, and 40% of mEPSPS protein amino acid sequence was obtained for trypsin, chymotrypsin, and endoproteinase Asp-N, respectively. The sequence coverage map is shown in Figure VI-3.

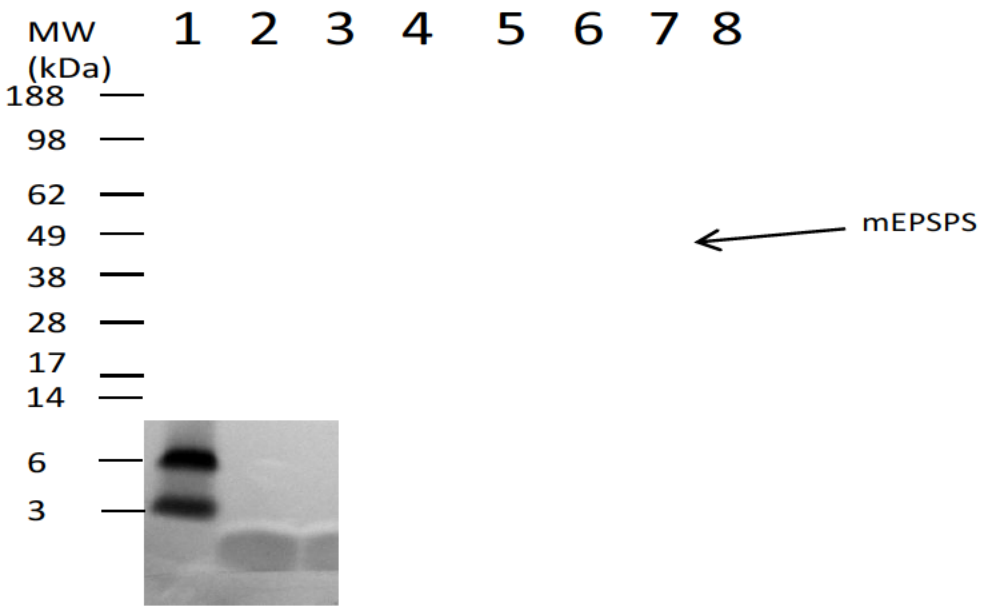
1 **AGAEEIVLQPIK**EISGTVKLPGSKSLSNRILLLAALSEGT 40
41 TVV**DNLLNSE**DVHYMLGALRTLGLSVEA**DKAAKRAVVVGC** 80
81 **GGKFPVE**DAKEEVQLFLGNAGIAMRSLTAAVTAAGGNATY 120
121 VLDGVPRMRERPIG**DLVVGLKQLGADV**DCFLGTDCPPVRV 160
161 NGIGGLPGGKVKLSGSISSQY*LSALLMAAPL*ALG**DVEIEI** 200
201 **IDKLISIPYV**EMTL*RLMER*FGVKAEHSDSW**DRFYIKGGQK** 240
241 **YKSPKNAYVEG**DASSASYF*LAGAAITGGTVTVEGCGTTSL* 280
281 QG**DVKFAEVL**EMMGAKVTWTETSVTVTGPPREPFGRK*HLK* 320
321 *AIDVNMNKMPDVAMTL*AVVALFADGPTAIR**DVASWRVKET** 360
361 ERMVAIRT**ELTKLGASVEEGPDYCIITPPEKLNVTAI**DTY 400
401 **DDHRMAMAFSLAACAEVPVTIRDPGCTRKTFPDYFDVLST** 440
441 **FVKN** 444

Trypsin-detected
Chymotrypsin-detected
Endoproteinase Asp-N-detected
Italics indicate amino acids not identified.

Figure VI-3. Amino acid sequence identified for mEPSPS from MZHG0JG corn by peptide mass coverage analysis

VI.A.3. Immunoreactivity and Molecular Weight of mEPSPS Produced in MZHG0JG Corn

Western blot analysis demonstrated that the apparent molecular weight of mEPSPS produced in MZHG0JG corn was consistent with the predicted molecular weight of 47.4 kDa, and the protein cross-reacted with mEPSPS-specific antibody (as shown in Figure VI-4).



Lane 1: Molecular weight standard
Lane 2: Nontransgenic corn leaf extract (10 µg total protein)
Lane 3: Crude MZHG0JG corn leaf extract (5ng mEPSPS, 10 µg total protein)
Lane 4: Nontransgenic corn leaf extract fortified with microbially produced mEPSPS (5 ng mEPSPS, 10 µg total protein)
Lane 5: mEPSPS purified preparation from MZHG0JG extract (5ng mEPSPS)
Lane 6: Microbially produced mEPSPS (5 ng mEPSPS) [a]
Lane 7: Microbially produced mEPSPS (5 ng mEPSPS)[a]
Lane 8: Molecular weight standard

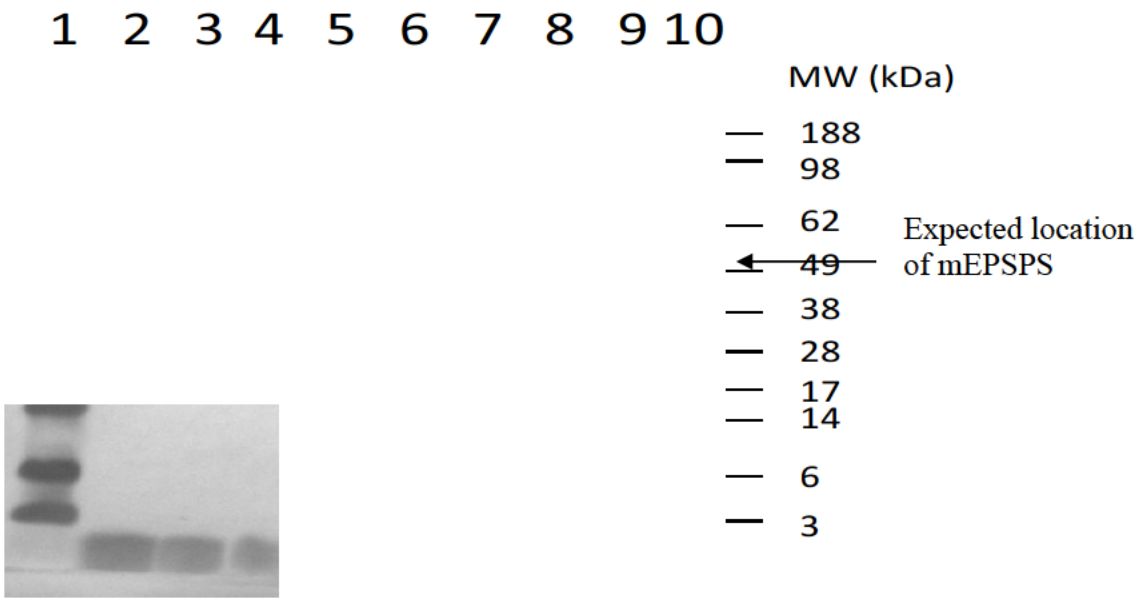
[a]Microbially produced protein was used as a positive assay control.

Figure VI-4. Western blot analysis of mEPSPS produced in MZHG0JG corn

VI.A.4. Glycosylation Analysis of mEPSPS produced in MZHG0JG corn

The mEPSPS produced in MZHG0JG corn was analyzed to ensure that no post-translational glycosylation of the protein had occurred *in planta*. As shown in Figure VI-5, this analysis demonstrated the absence of post-translational glycosylation of mEPSPS produced in MZHG0JG corn.

No bands corresponding to the presence of glycosylated mEPSPS were visible in plant-produced mEPSPS samples derived from MZHG0JG corn (Figure VI-5, Lane 8). The positive control, horseradish peroxidase (HRP), generated a visible band when applied to the gel at 25, 10, 5, 2.5, and 1 pmol (Figure VI-5, Lanes 2 through 6). The negative control, soybean trypsin inhibitor was not detected (Figure VI-5, Lane 7). Therefore, these results support the conclusion that mEPSPS produced in MZHG0JG corn is not glycosylated.



Lane 1: Molecular weight standard
Lane 2: HRP (positive control), 25 pmol
Lane 3: HRP (positive control), 10 pmol
Lane 4: HRP (positive control), 5 pmol
Lane 5: HRP (positive control), 2.5 pmol
Lane 6: HRP (positive control), 1 pmol
Lane 7: Soybean trypsin inhibitor (negative control), 25 pmol
Lane 8: mEPSPS purified preparation from MZHG0JG corn leaf extract, 25 pmol
Lane 9: Microbially produced mEPSPS, 25 pmol
Lane 10: Molecular weight standard

Figure VI-5. Glycosylation analysis of mEPSPS produced in MZHG0JG corn

VI.B. Identity and Characterization of the PAT Produced in MZHG0JG Corn

MZHG0JG corn contains the transgene *pat-09*, derived from the soil bacterium *Streptomyces viridochromogenes,* which encodes the enzyme PAT. PAT acetylates glufosinate-ammonium, thus inactivating it and conferring tolerance to glufosinate-ammonium in herbicide products. Specifically, PAT catalyzes the transfer of the acetyl group from acetyl coenzyme A to phosphinothricin. The released free thiol reacts with 5,5′-dithiobis(2-nitrobenzoic acid) to form 2-nitro-5-thiobenzoate anion under mild alkaline conditions (pH 7 to 8) (Habeeb 1972) (as shown in Figure VI-6). Glufosinate-ammonium (L-phosphinothricin) inhibits glutamine synthetase, an enzyme in the nitrogen assimilation pathway.

PAT is a highly specific enzyme for acetylation of glufosinate-ammonium. It does not acetylate glutamate (the closest structural analog to glufosinate-ammonium) or other L-amino acids and only poorly recognizes analogues such as methionine sulfoximine and hydroxylysine (Wehrmann *et al.* 1996, Hérouet *et al.* 2005).

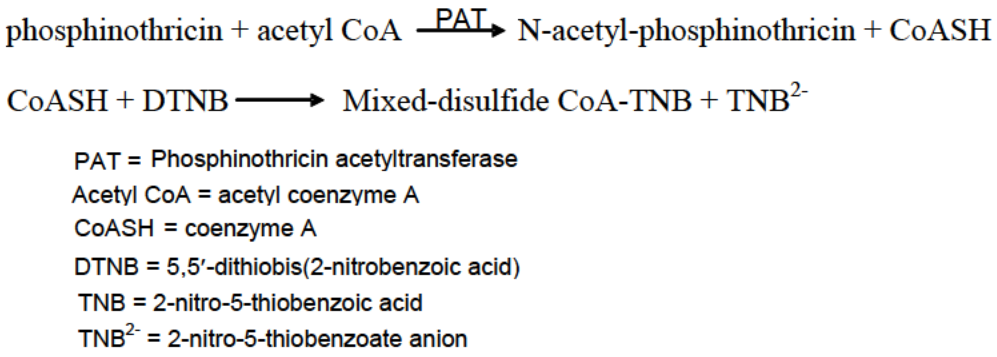


Figure VI-6. The reaction catalyzed by PAT

VI.B.1. Deduced Amino Acid Sequence Alignment for PAT

Event Bt11 corn was first introduced to the marketplace in 1997. The PAT produced in MZHG0JG corn (SYN-ØØØJG-2) is identical to the PAT produced in Bt11 corn (OECD identifier SYN-BTØ11-1). The nucleotide sequence of *pat-09* encoding the PAT in MZHG0JG corn and the nucleotide sequence of *pat* encoding the PAT in Bt11 corn were confirmed by nucleotide sequencing of the inserts. The deduced amino acid sequence of the PAT protein in MZHG0JG corn and Bt11 corn is identical (Figure VI-7).

```
Translation of Event Bt11 pat            (1) MSPERRPVEIRPATAADMAAVCDIV
Translation of Event MZHG0JG pat-09      (1) MSPERRPVEIRPATAADMAAVCDIV

Translation of Event Bt11 pat           (26) NHYIETSTVNFRTEPQTPQEWIDDL
Translation of Event MZHG0JG pat-09     (26) NHYIETSTVNFRTEPQTPQEWIDDL

Translation of Event Bt11 pat           (51) ERLQDRYPWLVAEVEGVVAGIAYAG
Translation of Event MZHG0JG pat-09     (51) ERLQDRYPWLVAEVEGVVAGIAYAG

Translation of Event Bt11 pat           (76) PWKARNAYDWTVESTVYVSHRHQRL
Translation of Event MZHG0JG pat-09     (76) PWKARNAYDWTVESTVYVSHRHQRL

Translation of Event Bt11 pat          (101) GLGSTLYTHLLKSMEAQGFKSVVAV
Translation of Event MZHG0JG pat-09    (101) GLGSTLYTHLLKSMEAQGFKSVVAV

Translation of Event Bt11 pat          (126) IGLPNDPSVRLHEALGYTARGTLRA
Translation of Event MZHG0JG pat-09    (126) IGLPNDPSVRLHEALGYTARGTLRA

Translation of Event Bt11 pat          (151) AGYKHGGWHDVGFWQRDFELPAPPR
Translation of Event MZHG0JG pat-09    (151) AGYKHGGWHDVGFWQRDFELPAPPR

Translation of Event Bt11 pat          (176) PVRPVTQI-
Translation of Event MZHG0JG pat-09    (176) PVRPVTQI-
```

Figure VI-7. Alignment of the deduced amino acid sequence for PAT encoded by *pat* in Bt11 corn and *pat-09* in MZHG0JG corn

V.B.2. Peptide Mass Coverage Analysis of PAT Produced in MZHG0JG Corn

Peptide mass coverage analysis was used to determine the identity of the purified PAT preparation from MZHG0JG corn extract by LC-MS/MS using an Ultra-Performance Liquid Chromatography system.

The collective analysis of the three proteolytic digests of the purified PAT preparation from MZHG0JG corn extract resulted in coverage of 90% of the total predicted PAT amino acid sequence. Evidence for 52%, 74%, and 45% of the PAT protein amino acid sequence was obtained for trypsin, chymotrypsin, and endoproteinase Asp-N, respectively. The sequence coverage map is shown in Figure VI-8.

1 *M***SPERRPVEIRPATAA***DMAAVC***DIVNHYIETSTVNFRTEP** 40

41 **QTPQ**EWI**DDLERLQDRYPWLVA**EVEGVVAGIAY*AGPW*KAR 80

81 NAYDWTVESTVYVSHRHQR*LGLGSTLY*THLLKSMEAQGFK 120

121 SVVAVIGLPN**DPSVRLH**EALGYTARGTLRAAGYKHGGWH**D** 160

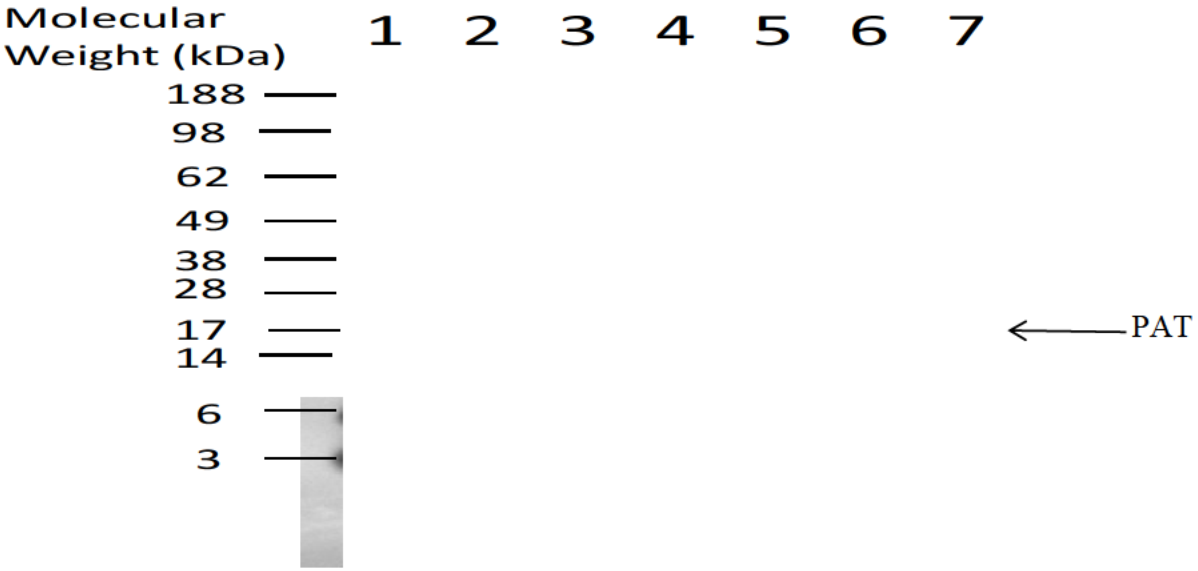
161 **VGFWQRDFELPAPPRPVRPVTQI** 183

Trypsin-detected
Chymotrypsin-detected
Endoproteinase Asp-N-detected
Italics indicate amino acids not identified.

Figure VI-8. Amino acid sequence identified for PAT from MZHG0JG corn by peptide mass coverage analysis

VI.B.3. Immunoreactivity and Molecular Weight of PAT Produced in MZHG0JG Corn

Western blot analysis demonstrated that the apparent molecular weight of PAT in MZHG0JG corn was consistent with the predicted molecular weight of 20.5 kDa, and the protein cross-reacted with the PAT-specific antibody (as shown in Figure VI-9).



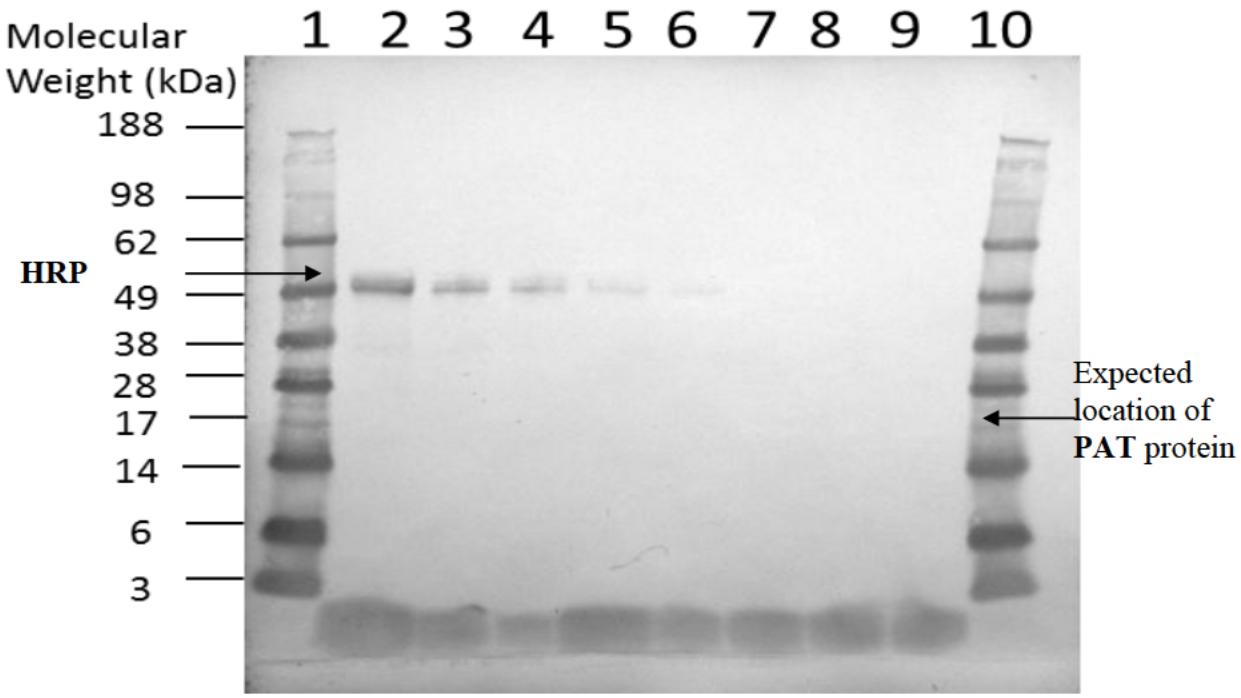
Lane 1: Molecular weight standard
Lane 2: Nontransgenic corn leaf extract (80 µg total protein)
Lane 3: Nontransgenic corn leaf extract fortified with microbially produced PAT(5 ng PAT, 80 ug total protein)
Lane 4: MZHG0JG corn leaf extract (5 ng PAT, 80 µg total protein)
Lane 5: PAT purified preparation from MZHG0JG extract (5 ng PAT)
Lane 6: Microbially produced PAT (5 ng PAT)
Lane 7: Molecular weight standard

Figure VI-9. Western blot analysis of PAT produced in MZHG0JG corn

VI.B.4. Glycosylation Analysis of PAT Produced in MZHG0JG Corn

The PAT produced in MZHG0JG corn was analyzed to ensure that no post-translational glycosylation of the protein had occurred *in planta*. As shown in Figure VI-10, this analysis demonstrated the absence of post-translational glycosylation of PAT produced in MZHG0JG corn.

No bands corresponding to the presence of glycosylated PAT were visible in the plant-produced PAT samples derived from MZHG0JG corn (Figure VI-10, Lane 8). The positive control, HRP, generated a visible band when applied to the gel at 25, 10, 5, 2.5, and 1 pmol (Figure VI-10, Lanes 2 through 6). The negative control, soybean trypsin inhibitor, was not detected (Figure V-10, Lane 7). Therefore, these results support the conclusion that PAT produced in MZHG0JG corn is not glycosylated.



Lane 1: Molecular weight standard
Lane 2: HRP (positive control), 25 pmol
Lane 3: HRP (positive control), 10 pmol
Lane 4: HRP (positive control), 5 pmol
Lane 5: HRP (positive control), 2.5 pmol
Lane 6: HRP (positive control), 1 pmol
Lane 7: Soybean trypsin inhibitor (negative control), 25 pmol
Lane 8: PAT purified preparation from MZHG0JG corn leaf extract, 25 pmol
Lane 9: Microbially produced PAT, 25 pmol
Lane 10: Molecular weight standard

Figure VI-10. Glycosylation of PAT produced in MZHG0JG corn

VI.C. Levels of mEPSPS and PAT Produced in MZHG0JG Corn Tissue

The concentrations of mEPSPS and PAT in various MZHG0JG corn tissues were quantified by enzyme-linked immunosorbent assay (ELISA) to establish an expression profile for these proteins as produced in MZHG0JG corn. The tissues analyzed were leaves and roots at four growth stages (V6, R1, R6, and senescence), whole plants at three stages (V6, R1, and R6), kernels at two stages (R6 and senescence), and pollen at one stage (R1). The tissues were collected from MZHG0JG corn and a nontransgenic, near-isogenic control corn grown concurrently according to local agronomic practices at four U.S. locations in 2013. The MZHG0JG corn used in these studies were NP2391 × NP2222(MZHG0JG) and NP2391 × NP2222 (pedigree shown in Figure IV-2, above).

At each location, one plot was planted with MZHG0JG corn, and one plot was planted with nontransgenic corn. Five replicate samples of each tissue type except pollen were collected from each plot. For pollen, a pooled sample was collected from 10 to 15 tassels per plot. All tissue

samples except pollen were ground to a powder, and all samples were then lyophilized. The percent dry weight (DW) of each sample was determined from the sample weight before and after lyophilization.

Protein was extracted from representative aliquots of the lyophilized tissue samples. The sample extracts were analyzed by ELISA in duplicate or triplicate, and a standard curve was generated for each ELISA plate with known amounts of the corresponding reference protein. Concurrent analysis of tissues from the nontransgenic corn confirmed the absence of plant-matrix effects on the analysis methods. All protein concentrations were adjusted for extraction efficiency.

Table VI-1 shows the ranges of protein concentrations observed in each tissue type across all growth stages and locations on a fresh-weight (FW) and dry-weight basis for MZHG0JG corn.

Table VI-1. Ranges of concentrations of mEPSPS and PAT in tissues of MZHG0JG corn across four growth stages and across four locations

Tissue	No. of stages	Fresh-weight concentration (µg/g)		Dry-weight concentration (µg/g)	
		mEPSPS	PAT	mEPSPS	PAT
Leaves	4	<LOD–936	<LOD–3.47	<LOD–3203	<LOD–17.03
Roots	4	7.02–110	0.05–0.65	57.58–707	0.37–3.91
Whole plants	3	51.75–437	<LOD–1.45	94.78–2347	<LOD–9.76
Pollen	1	<LOQ	<LOD	<LOD	<LOD
Kernels	2	16.30–58.68	<LOD–0.02	19.94–86.15	<LOD–0.04

Limit of detection (LOD) for mEPSPS in leaves = 2.00 µg/g DW; LOD for mEPSPS in pollen = 37.50 µg/g DW.
Limit of quantitation (LOQ) for mEPSPS in pollen = 75.0 µg/g DW.
LOD for PAT in leaves, whole plants, pollen, and kernels = 0.025 µg/g DW.

Kernels from MZHG0JG corn are the most likely tissue to enter the food supply, as either grain or grain by-products. Humans would potentially consume corn at the senescence stage of development, whereas livestock would be more likely to consume the kernels at maturity. The average mEPSPS concentration measured in kernels from MZHG0JG corn was 36.89 µg/g dry weight at senescence and 58.23 µg/g dry weight at maturity. The PAT concentration measured in kernels from MZHG0JG corn was below the LOD for the assay (0.025 µg/g dry weight) at senescence and ranged from the LOD to 0.02 µg/g dry weight at maturity.

VI.D. Existing Safety Data and History of Safe Exposure

MZHG0JG corn produces the proteins mEPSPS (a modified EPSPS) and PAT. The modified corn EPSPS is a variant of the native EPSPS from *Z. mays* with two amino acid substitutions that were introduced specifically to confer tolerance to the herbicide glyphosate. Both the native EPSPS and the modified corn EPSPS have a long history of safe use, as they are widely consumed in corn crop commodities. PAT belongs to the class of acetyltransferase enzymes common in plants and animals, and it shares similar three-dimensional structure, molecular weight, and functional properties with other acetyltransferase enzymes, which are present as natural components of human and animal diets. There are no reports of toxicity or allergenicity associated with the acetyltransferase class of enzymes.

Because of the ubiquitous occurrence of EPSPS and PAT proteins in microorganisms and plants (ILSI 2011a,b), it is likely that small amounts of EPSPS and PAT from various sources have always been present in the food and feed supply. Humans have a long history of dietary exposure to EPSPS and PAT from the endogenous proteomes of microorganisms and certain plant species and their presence in many commercially available transgenic crop plants, including corn, cotton, and soybean. No adverse effects associated with intake of EPSPS or PAT have been reported.

As demonstrated in Part V, the proteins mEPSPS and PAT produced in MZHG0JG corn are identical to the mEPSPS and PAT that have been present in commercial corn products that contain GA21 corn and Bt11 corn. Variants of EPSPS (the mutated form encoded by genes from either *A. tumefaciens* strain CP4 or corn) and PAT (encoded by either *pat* or a similar gene, *bar*) have been extensively studied over the years and several publications demonstrating their safety are available, including peer reviewed journal articles, and regulatory agency approvals and technical reports.

The following references on GA21 corn and Bt11 corn are of particular relevance to this application as they refer to the safety of the mEPSPS and PAT proteins, including Syngenta generated data.

- U.S. Environmental Protection Agency permanent exemption from food tolerances for EPSPS and PAT (US EPA 2007a and US EPA 2007b, respectively). Based on review of toxicity and exposure data, the EPA concluded that EPSPS and PAT are safe at any level and can therefore be exempt from setting tolerances for food safety.

- The European Food Safety Authority (EFSA) opinion on GA21 corn (EFSA 2007). Based on data provided by Syngenta, this report concluded that GA21 corn and derived products are unlikely to have any adverse effect on human and animal health in the context of the intended uses.

- The Organisation for Economic Co-operation and Development (OECD) consensus document on genes that confer tolerance to glyphosate and glufosinate-ammonium (OECD 1999a and OECD 1999b, respectively). These reports support the conclusions that EPSPS and PAT lack oral toxicity and allergenicity.

- A comprehensive characterization and safety assessment of the PAT protein is available in a 2005 article published in *Regulatory Toxicology and Pharmacology* (Hérouet *et al.* 2005), which concludes that PAT proteins are safe in human and animal feed.

Biotechnology-derived corn products that produce EPSPS and PAT have been available to farmers and in the food and feed supply for almost two decades. According to a survey by the United States Department of Agriculture National Agricultural Statistics Service, approximately 91 million acres of corn were planted in the United States in 2014, of which 93% was biotechnology-derived (USDA 2015). Most of the biotechnology-derived corn currently grown in the United States and Canada consists of transgenic varieties that are glyphosate and/or glufosinate-ammonium tolerant. Therefore, variants of EPSPS and PAT occur in numerous commercial transgenic corn varieties. There are no scientific reports of concern about EPSPS,

mEPSPS, or PAT as they exist in commercially available transgenic food crops. A list of transgenic crops containing EPSPS and/or PAT proteins is shown in Appendix A.

VI.F. Conclusions on the Characterization and Safety of mEPSPS and PAT Produced in MZHG0JG Corn

The safety of mEPSPS and PAT have been thoroughly assessed by Syngenta. Numerous regulatory agencies globally have assessed the safety of these proteins in the context of GA21 corn and Bt11 corn. The conclusions of safety of mEPSPS and PAT are supported by the following:

- Genetic characterization demonstrates that mEPSPS and PAT produced in MZHG0JG are identical to mEPSPS and PAT produced in GA21 corn and Bt11 corn, respectively.
- mEPSPS and PAT have been safely used and consumed in commercial transgenic crops for almost two decades.
- Both proteins have a very specific and well-characterized mode of action and are ubiquitous in nature.
- Given the low levels of mEPSPS and PAT in MZHG0JG kernels, dietary exposure can be considered minimal.
- No adverse effects associated with intake of EPSPS or PAT have been reported.

The weight of evidence from the data presented in this application, a history of safe use either through their presence in transgenic crops or abundance in nature, and the weight of evidence in the publicly available literature on the safety of mEPSPS and PAT proteins for consumption, demonstrate that the mEPSPS and PAT proteins present in MZHG0JG corn present no risk of harm to humans or livestock that consume corn products. Additional safety assessments of these proteins are not warranted for this application; however, a summary of laboratory studies, including the full study reports, conducted by Syngenta to assess the potential toxicity and allergenicity of mEPSPS and PAT are provided in Appendix B.

VI.G. References Cited in Part V

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Hérouet C, Esdaile DJ, Mallyon BA, Debruyne E, Schulz A, Currier T, Hendrickx K, van der Klis R-J, Rouan D. 2005. Safety evaluation of the phosphinothricin acetyltransferase proteins encoded by the *pat* and *bar* sequences that confer tolerance to glufosinate-

ammonium herbicide in transgenic plants. *Regulatory Toxicology and Pharmacology* 41:134–149.

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US EPA. 2007a. CP4 Enolpyruvylshikimate-3-phosphate (CP4 EPSPS) synthase in all plants; exemption from the requirement of a tolerance. 40 CFR 174.523.

US EPA. 2007b. Phosphinothricin acetyltransferase (PAT); exemption from the requirement of a tolerance. 40 CFR 174.522.

Wehrmann A, van Vliet A, Opsomer C, Botterman J, Schulz A. 1996. The similarities of *bar* and *pat* gene products make them equally applicable for plant engineers. *Nature Biotechnology* 14:1274-1278.

Part VII. Absence of Other Novel Substances Produced as a Result of the Genetic Modification.

MZHG0JG corn produces the intended proteins mEPSPS and PAT. The two genes expressed in MZHG0JG corn, *mepsps-02* and *pat-09*, are functional as intended, suggesting there is no gene silencing due to the expression of either transgene. These proteins are identical to the transgenic proteins produced in other approved biotechnology-derived products, which have been commercially available for almost two decades and have a history of safe use.

No other novel metabolites were identified or expected as a result of the genetic modification. Specifically, no new herbicide metabolites are expected from spraying MZHG0JG corn with glyphosate or glufosinate-ammonium herbicides. The herbicide metabolic profiles resulting from the transgenic protein–herbicide interaction in corn have been established for mEPSPS and PAT through a significant history of use. Corn grown in the United States (and exported to many countries, including Australia, New Zealand, and Canada) has primarily consisted of transgenic varieties that are glyphosate and/or glufosinate-ammonium tolerant for a number of years.

Part VIII. Compositional Analysis of the GM Food

Corn grown in the U.S. is predominantly of the yellow dent type, a commodity crop. Roughly 60% of the crop is fed to livestock either as grain or silage. Livestock that feed on corn include cattle, pigs, poultry, sheep, and goats. The remainder of the crop is exported or processed by wet milling, dry milling, or alkali treatment to yield products such as high-fructose corn syrup, starch, oil, grits, and flour. These processed products are used extensively in the food industry. For example, corn starch serves as a raw material for an array of processed foods and is also used in industrial manufacturing processes. Since the early 1980s, a significant amount of grain has also been used for fuel ethanol production. The by-products from these processes are often used in animal feeds. Described below is a study conducted to measure and compare key nutrients and anti-nutrients in forage and grain from MZHG0JG and conventional corn.

VIII.A. Composition Study Design and Methods

Compositional analyses of MZHG0JG corn, the corresponding nontransgenic, near-isogenic control corn, and six nontrangenic corn reference varieties were performed to assess nutritional equivalence. This assessment consisted of quantitative analyses of 73 nutritional components of grain and nine nutritional components in forage, including key food and feed nutrients, secondary plant metabolites, and anti-nutrients.

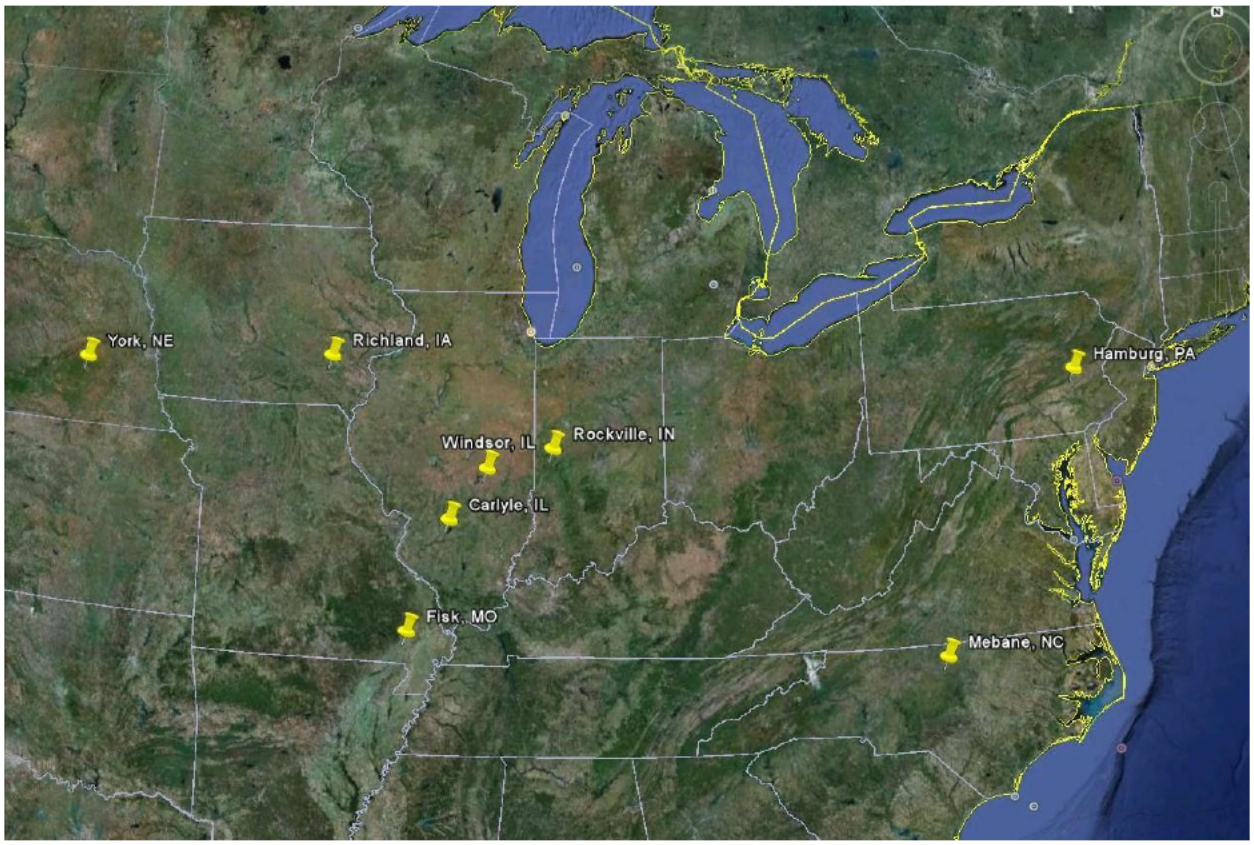
Compositional analyses were conducted on corn forage and grain samples harvested from replicated field trials planted at eight locations in the United States in 2013. The test substance was MZHG0JG corn, and the control substance was nontransgenic, near-isogenic corn. Six nontransgenic commercial corn varieties were included in the study design as reference entries to establish a range of natural variation in germplasm with a history of production in the area of cultivation. The test, control, and reference entries are listed in Table VIII-1.

Table VIII-1. Identification of test, control, and reference corn varieties

Entry Identification	Seed description	Hybrid genotype	Material Identification
E01	Nontransgenic, near-isogenic (control)	NP2391/NP2222	12MG044982
E03	MZHG0JG + trait-specific herbicide (test + TSH)	NP2391/NP2222(MZHG0JG)	12MG044984
E09	Reference variety 1	H-7191	09BIC000006
E10	Reference variety 2	H-7540	09BIC000007
E11	Reference variety 3	SY Genoroso	12PS804310
E12	Reference variety 4	NK Lucius	12PS804303
E13	Reference variety 5	NK Cisko	12PS804304
E14	Reference variety 6	SY Provial	12PS804307

The locations selected were representative of agricultural regions suitable for the cultivation of the hybrid corn varieties. At each location, the entries were grown in a randomized complete block design with four replicate plots. The plots were six rows spaced 30 inches (~76 cm) apart and 17 feet long (~5.2 m), planted with approximately 40 seeds per row. The locations are listed in Table VIII-2 and shown on a map in Figure VIII-1.

The plots were managed according to local agricultural practices, and all plots at a given location were managed identically with regard to irrigation, fertilization, and pest control. Seed and forage samples were taken from rows 4 and 5 of each plot. A satellite view of the composition trial locations is shown in Figure VIII-1. The locations, with soil type, previous year's crop, and planting date for each location, are listed in Table VIII-2.



The location designated is the city nearest to the field plots.

Figure VIII-1. Satellite view of composition trial locations in the United States

Table VIII-2. Field-trial locations

Location		Soil type	Previous crop	Planting date (2013)
L01	Richland, Iowa	silty clay loam	soybean	June 4
L02	York, Nebraska	silty clay loam	soybean	June 3
L03	Seymour, Illinois	silty clay loam	corn	June 20
L04	Bagley, Iowa	clay loam	field corn	June 13
L05	Larned, Kansas	loam	sorghum	June 12
L06	Stewardson, Illinois	silt loam	corn	June 10
L09	Wyoming, Illinois	silt loam	corn	June 8
L10	Germansville, Pennsylvania	clay loam	general vegetables	June 20

Forage samples collected from each plot consisted of the entire above-ground portions of five plants harvested at dough stage (R4 growth stage, as defined by Abendroth *et al.* 2011). The

plants were chopped and pooled to create a composite sample for each plot. After the plants reached physiological maturity (R6 growth stage), 15 ears were collected from each plot for grain samples. The ears were dried mechanically or in the field until the grain contained not more than 17% moisture.

The nutritional components measured in corn forage and grain were chosen based on recommendations of the OECD (2002) for comparative assessment of the composition of new varieties of corn. The components analyzed in forage and grain are listed in Tables VIII-3 and VIII-4.

Table VIII-3. Nutritional components analyzed in corn forage

Proximates		Minerals
moisture	carbohydrates	calcium
protein	ADF[a]	phosphorus
fat	NDF[b]	
ash		

[a]Acid detergent fiber.
[b]Neutral detergent fiber.

Table VIII-4. Nutritional components analyzed in corn grain

Proximates and starch	Minerals	Vitamins	Amino acids	
moisture	calcium	A (β-carotene)	alanine	lysine
protein	copper	B_1 (thiamine)	arginine	methionine
fat	Iron	B_2 (riboflavin)	aspartic acid	phenylalanine
ash	magnesium	B_3 (niacin)	cystine	proline
carbohydrates	manganese	B_6 (pyridoxine)	glutamic acid	serine
ADF	phosphorus	B_9 (folic acid)	glycine	threonine
NDF	potassium	E (α-tocopherol)	histidine	tryptophan
TDF[a]	selenium		isoleucine	tyrosine
starch	sodium		leucine	valine
	zinc			

Fatty acids		Secondary metabolites	Anti-nutrients
8:0 caprylic	18:0 stearic	*p*-coumaric acid	phytic acid
10:0 capric	18:1 oleic	ferulic acid	raffinose
12:0 lauric	18:2 linoleic	furfural	trypsin inhibitor
14:0 myristic	18:3 gamma linolenic	inositol	
14:1 myristoleic	18:3 linolenic		
15:0 pentadecanoic	20:0 arachidic		
15:1 pentadecenoic	20:1 eicosenoic		
16:0 palmitic	20:2 eicosadienoic		
16:1 palmitoleic	20:3 eicosatrienoic		
17:0 heptadecanoic	20:4 arachidonic		
17:1 heptadecenoic	22:0 behenic		

[a]Total detergent fiber.

The component levels were converted to equivalent units of dry weight based on the moisture content of each sample. All compositional analyses were conducted according to methods published and approved by AOAC International, or were other industry-standard methods, or were based on literature references and developed and validated by the analytical laboratory.

VIII.B. Data Analysis

The mean levels of each component across locations were computed. The data for each quantifiable component were subjected to analysis of variance (ANOVA) using the following mixed model:

$$Y_{ijk} = U + T_i + L_j + B(L)_{jk} + LT_{ij} + e_{ijk}$$

In this model, Y_{ijk} is the observed response for entry i at location j block k, U is the overall mean, T_i is the entry effect, L_j is the location effect, $B(L)_{jk}$ is the effect of block within location, LT_{ij} is the location-by-entry interaction effect, and e_{ijk} is the residual error. Entry was regarded as a fixed effect, while the effects of location, block within location, and location-by-entry interaction were regarded as random. In the across-location ANOVA, only the control and test entries were included, to avoid inflation of the residual error by any interaction that may have been present between location and the reference varieties.

For each component, *t*-tests were used to assess the statistical significance of the comparison of interest (MZHG0JG vs. control). Significance was based on an alpha level of 0.05, and denominator degrees of freedom were determined by the Kenward-Roger method (Kenward and Roger 1997). The standard error of the mean (SEM) was also determined for each component.

In cases where some or all values for a component were below the limit of quantitation and substitution of the LOQ was not appropriate because of the number or distribution of substitutions required, calculation of the mean and ANOVA could not be performed, and only the range is reported.

The across-location means for the components of MZHG0JG corn were also compared nonstatistically with the ranges of component levels from the nontransgenic corn reference varieties and with the ranges for conventional corn published in the International Life Sciences Institute (ILSI) Crop Composition Database (ILSI 2014).

VIII.C. Compositional Analysis Results

Sections VIII.C.1 and VIII.C.2 describe the compositional analysis results for MZHG0JG corn forage and grain and compare them with the results for the nontransgenic, near-isogenic control corn, as well as the reference-variety and ILSI database ranges. The conclusions from the compositional analysis are presented in Section VIII.C.3.

VIII.C.1. Forage

Across-location statistics for proximate and mineral composition of corn forage are shown in Table VIII-5. In statistical comparisons between MZHG0JG corn and the nontransgenic control corn, no significant differences were observed in the levels of moisture, protein, fat, ash, ADF, NDF, or calcium. The level of carbohydrates was significantly higher in MZHG0JG corn than in the control corn, and the level of phosphorus was significantly lower.

In both MZHG0JG corn and the nontransgenic control corn, the mean levels of all proximates and minerals were within the ranges for the reference varieties and the ranges reported in the ILSI database.

Table VIII-5. Proximate and mineral composition of forage from MZHG0JG corn and nontransgenic corn

Data source	Statistic	Moisture	Protein	Fat	Ash	Carbohydrates	ADF	NDF	Calcium	Phosphorus
MZHG0JG	mean	68.6	7.15	1.85	3.83	87.2	25.4	42.5	1833	1723
(*N* = 32)	range	61.2–75.2	5.86–8.32	0.789–2.88	2.63–5.97	84.5–90.3	20.4–29.7	33.8–49.3	964–2550	1060–2230
Control	mean	69.1	7.53	1.80	4.06	86.6	25.1	42.7	1914	1817
(*N* = 32)	range	63.7–74.0	5.92–9.94	0.622–2.79	2.43–6.40	82.2–89.8	20.1–29.7	34.9–50.4	958–2780	1260–2450
		ANOVA (*t*-test) entry effect and SEM								
	P	0.290	0.054	0.639	0.151	***0.007***	0.624	0.865	0.244	***0.027***
	SEM	0.88	0.215	0.083	0.241	0.40	0.59	0.89	113	84
Reference	mean	69.8	7.19	2.18	3.79	86.8	24.1	40.3	1920	1817
(*N* = 192)	range	61.2–80.0	4.54–9.54	0.587–3.79	2.41–5.98	82.0–90.7	15.8–32.8	28.8–57.0	1010–3300	1090–2800
ILSI (2014)	mean	69.9	7.68	2.063	4.30	86.0	25.80	41.88	1902.87	1938.01
	range	48.8–82.0	3.14–15.20	<LOQ–6.755	0.66–13.20	74.3–92.9	9.90–47.39	20.29–67.80	582.00–5767.90	689.78–4385.20
	N[a]	4316	3897	3873	4316	3897	4116	4116	3650	3650

Proximate levels are shown as percent dry weight except for moisture, which is shown as percent fresh weight.
Calcium and phosphorus levels shown as milligrams per kilogram of dry weight.
P-values for significantly different results ($P < 0.05$) are shown in bold italic type.
[a]The number of ILSI values used to calculate the mean; excludes values < LOQ.

VIII.C.2. Grain

VIII.C.2.a. Proximates, Starch, Minerals, and Vitamins

Across-location statistics for proximate and starch components of corn grain are shown in Table VIII-6. In statistical comparisons between MZHG0JG corn and the nontransgenic control corn, no significant differences were observed in the levels of protein, fat, ash, carbohydrates, ADF, TDF, or starch. The level of NDF was significantly lower in MZHG0JG corn than in the control corn. Moisture levels were adjusted by drying, either mechanically or in the field, and therefore were not compared statistically.

Across-location statistics for mineral components of corn grain are shown in Table VIII-7. In statistical comparisons between MZHG0JG corn and the nontransgenic control corn, no significant differences were observed in the levels of calcium, magnesium, manganese, phosphorus, potassium, or zinc. The levels of copper and iron were significantly lower in MZHG0JG corn than in the control corn. For selenium and sodium, levels below the LOQ for all corn varieties precluded calculation of the means and statistical comparisons across locations.

Across-location statistics for vitamin components of corn grain are shown in Table VII-8. In statistical comparisons between MZHG0JG corn and the nontransgenic control corn, no significant differences were observed in the levels of vitamins B_1, B_2, B_3, or B_9. The level of vitamin A (β-carotene) was significantly higher in MZHG0JG corn than in the control corn, and the levels of vitamins B_6 (pyridoxine) and E (α-tocopherol) were significantly lower.

In both MZHG0JG corn and the nontransgenic control corn, the mean levels of all proximates, starch, minerals, and vitamins were within the ranges for the reference varieties and the ranges reported in the ILSI database.

Table VIII-6. Proximate and starch composition of grain from MZHG0JG corn and nontransgenic corn

Data source	Statistic	Moisture[a]	Protein	Fat	Ash	Carbohydrates	ADF	NDF	TDF	Starch
MZHG0JG	mean	12.1	10.2	3.80	1.42	84.6	3.98	11.0	16.0	65.0
(*N* = 32)	range	9.18–15.3	9.29–11.7	3.16–4.40	1.21–1.64	83.0–86.0	3.23–4.60	9.71–12.3	13.6–20.1	58.0–78.1
Control	mean	12.4	10.5	3.85	1.41	84.3	4.06	11.5	16.5	65.4
(*N* = 32)	range	8.89–16.3	8.53–13.2	3.31–4.45	1.11–1.67	81.6–86.1	3.12–4.88	10.2–14.2	13.6–19.7	59.6–70.6
ANOVA (*t*-test) entry effect and SEM										
	P	–	0.218	0.560	0.902	0.187	0.408	***0.031***	0.094	0.608
	SEM	–	0.32	0.083	0.034	0.33	0.104	0.21	0.32	0.70
Reference	mean	12.2	10.3	3.40	1.48	84.8	3.40	9.54	13.6	66.4
(*N* = 192)	range	7.99–17.4	7.68–13.9	2.39–4.41	1.18–1.87	81.3–88.0	2.43–4.48	7.42–12.2	11.2–20.0	53.3–79.6
ILSI (2014)	mean	14.5	10.31	3.829	1.415	84.5	3.72	10.31	13.90	66.6
	range	5.1–40.5	5.72–17.26	1.363–7.830	0.616–6.282	77.4–89.7	1.41–11.34	4.28–22.64	8.73–35.31	26.5–83.7
	N[b]	6616	5790	5790	6190	5765	5942	5941	3763	1931

Proximate and starch levels shown as percent dry weight except for moisture, which is shown as percent fresh weight.
P-values for significantly different results ($P < 0.05$) are shown in bold italic type.
[a]Grain was dried in the field or mechanically after harvest, so moisture levels were not subjected to ANOVA.
[b]The number of ILSI values used to calculate the mean; excludes values < LOQ.

Table VIII-7. Mineral composition of grain from MZHG0JG corn and nontransgenic corn

Data source	Statistic	Ca	Cu[a]	Fe	Mg	Mn	P	K	Se[b]	Na[c]	Zn
MZHG0JG	Mean	34.8	1.75	18.5	1161	5.73	3012	3517	–	–	20.4
(*N* = 32)	Range	26.3–45.3	1.27–2.44	15.4–22.5	984–1300	3.62–8.44	2490–3570	3050–4120	<LOQ–0.586	<LOQ–139	16.2–24.0
Control	Mean	36.0	2.06	19.3	1177	6.02	3033	3593	–	–	20.9
(*N* = 32)	Range	26.9–48.2	1.46–3.11	15.9–23.0	994–1380	3.62–10.1	2590–3790	3280–4070	<LOQ–0.695	<LOQ–182	14.6–25.2
ANOVA (*t*-test) entry effect and SEM											
	P	0.086	***<0.001***	***0.008***	0.305	0.111	0.649	0.089	–	–	0.152
	SEM	2.06	0.113	0.47	23	0.558	77	66	–	–	0.78
Reference	Mean	41.2	2.09	20.3	1168	5.80	3053	3807	–	–	21.3
(*N* = 192)	Range	27.4–59.1	1.33–3.20	13.4–28.8	867–1400	3.15–9.10	2410–3750	3170–4640	<LOQ–0.802	<LOQ–185	12.7–29.3
ILSI (2014)	Mean	44.2	1.71	20.56	1217.0	6.45	3142.0	3690.6	0.28	24.94	22.8
	Range	<LOQ–1010.0	<LOQ–21.20	9.51–191.00	594.0–1940.0	1.69–14.30	1300.0–5520.0	1810.0–6030.0	<LOQ–1.51	<LOQ–731.54	6.5–42.6
	N[d]	5932	5650	5819	5823	5822	5938	5823	973	1110	5823

Mineral levels shown as milligrams per kilogram of dry weight.

P-values for significantly different results ($P < 0.05$) are shown in bold italic type. When some or all values were < LOQ and substitution with the LOQ was not appropriate because of the number or distr bution of substitutions required, calculation of the mean and ANOVA could not be performed, and only the range is shown.

[a]For copper, two outlying values for the nontransgenic control were included in the analyses.

[b]Original units of parts per billion were converted to milligrams per kilogram. The LOQ for selenium was 0.033–0.036 mg/kg dry weight.

[c]The LOQ for sodium was 109–121 mg/kg dry weight.

[d]The number of ILSI values used to calculate the mean; excludes values < LOQ.

Table VIII-8. Vitamin composition of grain from MZHG0JG corn and nontransgenic corn

Data source	Statistic	Vitamin A[a] (β-carotene)	Vitamin B_1 (thiamine)	Vitamin B_2 (riboflavin)	Vitamin B_3 (niacin)	Vitamin B_6 (pyridoxine)	Vitamin B_9 (folic acid)	Vitamin E[b] (α-tocopherol)
MZHG0JG	Mean	0.169	0.370	0.204	2.11	0.521	0.0459	0.0117
(*N* = 32)	Range	0.126–0.201	0.288–0.442	0.121–0.329	1.83–2.42	0.404–0.651	0.0337–0.0615	0.00785–0.0161
Control	Mean	0.145	0.377	0.220	2.04	0.552	0.0432	0.0121
(*N* = 32)	Range	0.116–0.165	0.295–0.490	0.119–0.351	1.67–2.36	0.414–0.666	0.0313–0.0539	0.00830–0.0155
ANOVA (*t*-test) entry effect and SEM								
	P	***<0.001***	0.291	0.255	0.065	***0.030***	0.120	***0.013***
	SEM	0.0051	0.0128	0.0119	0.053	0.0169	0.00227	0.00072
Reference	Mean	0.134	0.368	0.214	2.45	0.632	0.0414	0.0132
(*N* = 192)	Range	0.064–0.318	0.249–0.506	0.114–0.375	1.55–4.17	0.365–0.910	0.0232–0.0640	0.00762–0.0221
ILSI (2014)	Mean	0.481	0.383	0.190	2.094	0.601	0.0575	0.0106
	Range	<LOQ–4.990	<LOQ–4.000	<LOQ–0.735	<LOQ–4.694	<LOQ–1.214	<LOQ–0.3500	<LOQ–0.0687
	N[c]	4373	4981	4061	4999	4998	5460	4480

Vitamin levels are shown as milligrams per 100 grams of dry weight except for vitamin E, which is shown as milligrams per gram.
P-values for significantly different results ($P < 0.05$) are shown in bold italic type.
[a]β-carotene is measured in this study, as vitamin A is not produced in plants.
[b]The original units of milligrams per 100 grams were converted to milligrams per gram.
[c]The number of ILSI values used to calculate the mean; excludes values < LOQ.

VIII.C.2.b. Amino Acids, Fatty Acids, Secondary Metabolites, and Anti-nutrients

Across-location statistics for amino acid components of corn grain are shown in Table VIII-9. In statistical comparisons between MZHG0JG corn and the nontransgenic control corn, no significant differences were observed in the levels of 15 amino acids. The levels of aspartic acid, arginine, and tryptophan were significantly lower in MZHG0JG corn than in the control corn.

The across-location statistics for the ten quantifiable fatty acids in corn grain are shown in Table VIII-10. In statistical comparisons between MZHG0JG corn and the nontransgenic control corn, no significant differences were observed in the proportions of 16:0 palmitic, 16:1 palmitoleic, 18:0 stearic, 18:1 oleic, 18:2 linoleic, 20:0 arachidic, 20:1 eicosenoic, or 22:0 behenic acid. The proportions of 17:0 heptadecanoic and 18:3 linolenic acid were significantly higher in MZHG0JG corn than in the control corn.

The levels of twelve fatty acids were below the LOQ in all replicates at all locations and could not be analyzed; these included 8:0 caprylic, 10:0 capric, 12:0 lauric, 14:0 myristic, 14:1 myristoleic, 15:0 pentadecanoic, 15:1 pentadecenoic, 17:1 heptadecenoic, 18:3 gamma linolenic, 20:2 eicosadienoic, 20:3 eicosatrienoic, and 20:4 arachidonic acids.

Across-location statistics for secondary metabolite and anti-nutrient components of corn grain are shown in Table VIII-11. In statistical comparisons between MZHG0JG corn and the nontransgenic control corn, no significant differences were observed in the levels of ferulic acid, inositol, phytic acid, raffinose, or trypsin inhibitor. The level of *p*-coumaric acid was significantly higher in MZHG0JG corn than in the control corn. For furfural, levels below the LOQ precluded calculation of the means and statistical comparisons across locations.

In both MZHG0JG corn and the nontransgenic control corn, the mean levels of all amino acids and quantifiable fatty acids were within the ranges for the reference varieties and the ranges reported in the ILSI database.

In both MZHG0JG corn and the nontransgenic control corn, the mean levels of ferulic acid were above the range for the reference varieties, but were within the range reported in the ILSI database. The mean levels of all other quantifiable secondary metabolites and anti-nutrients were within the ranges for the reference varieties and the ranges reported in the ILSI database.

Table VIII-9. Amino acid composition of grain from MZHG0JG corn and nontransgenic corn

Data source	Statistic	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Cys	Val
MZHG0JG	mean	6.42	3.51	4.57	18.6	8.70	3.77	7.73	2.01	4.48
(*N* = 32)	range	5.76–7.30	3.22–3.93	4.03–5.16	15.5–21.3	7.73–10.0	3.36–4.13	6.67–8.93	1.70–2.32	3.97–5.03
Control	mean	6.69	3.61	4.75	19.4	9.08	3.80	8.05	2.02	4.66
(*N* = 32)	range	5.64–8.12	3.03–4.37	3.86–6.11	15.7–25.3	7.55–11.2	3.16–4.36	6.42–10.4	1.67–2.46	4.04–5.64
ANOVA (*t*-test) entry effect and SEM										
	P	***0.038***	0.123	0.086	0.109	0.055	0.505	0.110	0.793	0.074
	SEM	0.179	0.100	0.149	0.69	0.256	0.087	0.272	0.045	0.121
Reference	mean	6.79	3.58	4.74	19.1	9.10	3.83	7.81	2.06	4.67
(*N* = 192)	range	4.87–8.94	2.56–4.74	3.33–7.04	12.8–28.9	5.97–12.6	2.70–4.82	5.42–11.4	1.52–2.59	3.27–6.23
ILSI (2014)	mean	6.82	3.68	4.97	19.70	9.19	3.88	7.89	2.14	4.83
	range	3.35–12.08	2.19–6.66	1.82–7.69	9.65–35.40	4.62–17.50	1.84–6.85	4.39–14.80	1.16– 5.14	2.66–8.55
	N	5918	5918	5918	5918	5918	5918	5918	5917	5918

Data source	Statistic	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Trp
MZHG0JG	mean	2.22	3.44	12.5	4.02	4.98	2.89	2.52	4.80	0.835
(*N* = 32)	range	1.92–2.47	3.00–3.94	10.3–14.5	3.50–4.66	4.21–5.86	2.66–3.16	2.25–2.75	4.14–5.44	0.717–0.912
Control	mean	2.19	3.57	13.1	4.09	5.26	2.96	2.60	4.95	0.859
(*N* = 32)	range	1.77–2.73	2.98–4.45	10.2–17.7	3.13–5.34	4.20–6.87	2.51–3.31	2.19–3.12	3.92–5.85	0.730–0.988
ANOVA (*t*-test) entry effect and SEM										
	P	0.431	0.127	0.114	0.414	0.055	0.095	0.106	***0.021***	***0.009***
	SEM	0.060	0.116	0.51	0.134	0.191	0.048	0.065	0.132	0.0170
Reference	mean	2.02	3.55	12.8	4.03	5.19	2.92	2.68	5.02	0.859
(*N* = 192)	range	1.51–2.49	2.38–5.18	8.30–20.7	2.69–6.09	3.52–7.92	1.88–3.85	1.95–3.58	3.47–6.54	0.639–1.02
ILSI (2014)	mean	2.10	3.68	13.03	3.54	5.30	2.94	2.87	4.65	0.712
	range	1.05–4.68	1.79–6.92	6.42–24.92	1.03–7.34	2.44–9.30	1.29–6.68	1.37–4.56	1.19–7.08	0.271–2.150
	N[a]	5915	5918	5918	5918	5918	5909	5918	5918	5916

Amino acid levels are shown as milligrams per gram of dry weight.
P-values for significantly different results ($P < 0.05$) are shown in bold italic type.
[a]The number of ILSI values used to calculate the mean; excludes values < LOQ.

Table VIII-10. Fatty acid composition of grain from MZHG0JG corn and nontransgenic corn

Data source	Statistic	16:0 Palmitic	16:1 Palmitoleic	17:0 Heptadecanoic	18:0 Stearic	18:1 Oleic	18:2 Linoleic	18:3 Linolenic	20:0 Arachidic	20:1 Eicosenoic	22:0 Behenic
MZHG0JG	mean	14.1	0.130	0.0866	2.13	26.6	54.3	1.81	0.425	0.227	0.182
(*N* = 32)	range	13.4–14.7	0.113–0.144	0.0743–0.0975	1.76–2.45	23.1–28.9	52.6–58.5	1.69–1.94	0.356–0.486	0.202–0.242	0.131–0.209
Control	mean	14.3	0.129	0.0834	2.12	26.8	54.0	1.78	0.427	0.229	0.182
(*N* = 32)	range	13.7–14.8	0.108–0.141	0.0677–0.0994	1.76–2.34	23.3–29.3	51.3–58.3	1.67–1.92	0.360–0.494	0.198–0.249	0.148–0.218
ANOVA (*t*-test) entry effect and SEM											
	P	0.051	0.430	***0.004***	0.439	0.359	0.191	***0.045***	0.580	0.096	0.876
	SEM	0.09	0.0026	0.00226	0.062	0.54	0.62	0.020	0.0117	0.0039	0.0054
Reference	mean	15.1	0.127	0.0871	2.06	24.9	55.1	1.73	0.415	0.256	0.187
(*N* = 192)	range	13.2–17.0	0.0876–0.200	0.0698–0.121	1.59–2.48	16.5–31.1	47.5–64.1	1.39–2.12	0.329–0.485	0.178–0.348	0.0977–0.247
ILSI (2014)	mean	12.55	0.147	0.089	1.90	26.52	56.72	1.38	0.419	0.270	0.185
	range	6.81– 26.55	<LOQ–0.453	<LOQ–0.203	1.02– 3.83	17.40–42.81	34.27–67.68	0.55–2.33	0.267–0.993	<LOQ–1.952	<LOQ–0.417
	N[a]	4682	2119	265	4682	4682	4682	4682	4344	4322	3858

Fatty acids are shown as percent of total fatty acids.

P-values for significantly different results ($P < 0.05$) are shown in bold italic type. When some or all values were < LOQ and substitution with the LOQ was not appropriate because of the number or distr bution of substitutions required, calculation of the mean and ANOVA could not be performed, and only the range is shown.

[a]The number of ILSI values used to calculate the mean; excludes values < LOQ. Levels < LOQ were observed for all replicates at all locations for 8:0 caprylic, 10:0 capric, 12:0 lauric, 14:0 myristic acid, 14:1 myristoleic, 15:0 pentadecanoic, 15:1 pentadecenoic, 17:1 heptadecenoic, 18:3 gamma linolenic, 20:2 eicosadienoic, 20:3 eicosatrienoic, and 20:4 arachidonic fatty acids.

Table VIII-11. Secondary metabolite and anti-nutrient composition of grain from MZHG0JG corn and nontransgenic corn

Data source	Statistic	*p*-Coumaric acid (mg/kg)	Ferulic acid (mg/kg)	Furfural[a] (mg/kg)	Inositol (ppm)	Phytic acid (%)	Raffinose[b] (%)	Trypsin inhibitor (TIU[c]/mg)
MZHG0JG	mean	347	3409	–	2481	0.840	0.116	4.05
(*N* = 32)	range	304–401	3000–3900	<LOQ	1560–3260	0.559–0.985	<LOQ–0.175	2.26–5.33
Control	mean	303	3387	–	2528	0.883	0.113	3.87
(*N* = 32)	range	239–352	2920–4040	<LOQ	1920–3850	0.609–1.10	<LOQ–0.195	2.35–4.85
ANOVA (*t*-test) entry effect and SEM								
	P	***<0.001***	0.501	–	0.659	0.108	0.482	0.291
	SEM	10.4	74	–	104	0.0268	0.0142	0.119
Reference	mean	222	2249	–	2606	0.893	0.172	4.04
(*N* = 192)	range	113–435	1700–2920	<LOQ	1720–3890	0.503–1.34	<LOQ–0.386	1.67–6.09
ILSI (2014)	mean	224.2	2254.93	3.697	1737.1	0.861	0.174	3.51
	range	<LOQ–820.0	291.93–4397.30	<LOQ–6.340	<LOQ–4750.0	<LOQ–1.570	<LOQ–0.443	<LOQ–8.42
	N[d]	5371	5378	14	4003	5762	4585	4089

Units for anti-nutrients are shown in the column headings; all are expressed on a dry weight basis.

P-values for significantly different results ($P < 0.05$) are shown in bold italic type. When some or all values were < LOQ and substitution with the LOQ was not appropriate because of the number or distr bution of substitutions required, calculation of the mean and ANOVA could not be performed, and only the range is shown.

[a] The LOQ for furfural was 0.543–0.605 mg/kg DW.

[b] The LOQ for raffinose was 0.057–0.060 mg/kg DW. Levels for one test sample and two control samples were replaced with the LOQ to perform ANOVA.

[c] Trypsin inhibitor units.

[d] The number of ILSI values used to calculate the mean; excludes values < LOQ.

VIII.C.3. Conclusions from Compositional Analysis

In the compositional assessment of MZHG0JG corn forage and grain, the levels of the majority of nutritional components did not differ significantly between MZHG0JG corn and nontransgenic, near-isogenic control corn.

Across-location mean levels of all quantifiable components except ferulic acid were within the ranges observed in the nontransgenic commercial corn reference varieties grown in the same field trials. The levels of ferulic acid did not differ significantly between the MZHG0JG and nontransgenic control corn. The across-location mean levels of all components of MZHG0JG corn were within the ranges published in the ILSI Crop Composition Database.

These results indicate that the levels of the majority of nutritional components did not differ between MZHG0JG corn and near-isogenic, nontransgenic control corn, and that those levels that did differ fell within ranges considered to be normal for conventional corn. Based on the conclusions from the compositional analysis, there no reason to perform any additional nutritional impact studies.

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Appendix A. Transgenic Crops Approved for Use Globally that Contain EPSPS and PAT Proteins

This appendix contains all biotechnology-derived traits containing transgenic EPSPS and PAT proteins with the potential to currently be in commerce. Companies listed are only those that contribute to biotradestatus.com. BCS = Bayer CropScience, Dow = Dow AgroSciences LLC. Product commercial names may vary by region, the list of products and approving countries may be incomplete, and therefore, this list may not be comprehensive.

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Table A-1. Transgenic crops approved for use globally that contain EPSPS proteins

Crop	Company	Product name	Event/ stacked event	OECD unique identifier	Source of EPSPS gene	Countries with approvals
Corn	Dow	HERCULEX® I × Roundup Ready® Corn 2	TC1507 × NK603	DAS-Ø15Ø7-1 × MON-ØØ6Ø3-6	*A. tumefaciens* strain CP4	Argentina, Australia, Brazil, Canada, China, Colombia, European Union, Japan, Korea, Mexico, New Zealand, Philippines, Taiwan, United States, Uruguay
Corn	Dow	HERCULEX® XTRA × Roundup Ready® Corn 2	TC1507 × 59122 × NK603	DAS-Ø15Ø7-1 × DAS-59122-7 × MON-ØØ6Ø3-6	*A. tumefaciens* strain CP4	Australia, Canada, China, European Union, Japan, Korea, Mexico, New Zealand, Philippines, South Africa, Taiwan, United States
Corn	Dow	POWERCORE™	MON89034 × TC1507 × NK603	MON-89Ø34-3 × DAS-Ø15Ø7-1 × MON-ØØ6Ø3-6	*A. tumefaciens* strain CP4	Argentina, Australia, Brazil, Canada, China, Colombia, European Union, Japan, Korea, Mexico, New Zealand, Paraguay, Philippines, South Africa, Taiwan, United States, Uruguay
Corn	Dow	SmartStax® Corn	MON89034 × TC1507 × MON88017 × 59122	MON-89Ø34-3 × DAS-Ø15Ø7-1 × MON-88Ø17-3 × DAS-59122-7	*A. tumefaciens* strain CP4	Australia, Canada, Colombia, European Union, Japan, Korea, Mexico, New Zealand, Philippines, South Africa, Taiwan, United States
Corn	DuPont Pioneer	HERCULEX® I Insect Protection × Roundup Ready® Corn 2	TC1507 × NK603	DAS-Ø15Ø7-1 × MON-ØØ6Ø3-6	*A. tumefaciens* strain CP4	Argentina, Australia, Brazil, Canada, China, Colombia, European Union, Honduras, Japan, Korea, Mexico, New Zealand, Philippines, Taiwan, United States
Corn	DuPont Pioneer	HERCULEX® RW Rootworm Protection × Roundup Ready® Corn 2	59122-7 × NK603	DAS-59122-7 × MONØØ6Ø3-6	*A. tumefaciens* strain CP4	Canada, China, European Union, Japan, Korea, Mexico, Philippines, Taiwan, United States
Corn	DuPont Pioneer	HERCULEX® XTRA Insect Protection × Roundup Ready® Corn 2	TC1507 × 59122 × NK603	DAS-Ø15Ø7-1 × DAS-59122-7 × MON-ØØ6Ø3-6	*A. tumefaciens* strain CP4	Australia, Canada, China, Colombia, European Union, Japan, Korea, Mexico, New Zealand, Philippines, Taiwan, United States
Corn	DuPont Pioneer	Optimum® Intrasect®	TC1507 × MON810 × NK603	DAS-Ø15Ø7-1 × MON-ØØ81Ø-6 × MON-ØØ6Ø3-6	*A. tumefaciens* strain CP4	Australia, Brazil, Canada, China, Colombia, Japan, Korea, Mexico, New Zealand, Philippines, Taiwan, United States
Corn	DuPont Pioneer	Optimum® Intrasect® Xtra	TC1507 × DAS-59122 × MON810 × NK603	DAS-Ø15Ø7-1 × DAS-59122-7 × MON-ØØ81Ø-6 × MON-ØØ6Ø3-6	*A. tumefaciens* strain CP4	Australia, Canada, China, Japan, Korea, Mexico, New Zealand, Taiwan, United States

Crop	Company	Product name	Event/ stacked event	OECD unique identifier	Source of EPSPS gene	Countries with approvals
Corn	DuPont Pioneer	Optimum® Intrasect® XTreme	TC1507 × 59122 × MON810 × MIR604 × NK603	DAS-Ø15Ø7-1 × DAS-59122-7 × MON-ØØ81Ø-6 × SYN-IR6Ø4-5 × MON-ØØ6Ø3-6	*A. tumefaciens* strain CP4	Australia, Canada, China, Japan, Korea, Mexico, New Zealand, Taiwan United States
Corn	DuPont Pioneer	Optimum® Leptra®	TC1507 × MON810 × MIR162 × NK603	DAS-Ø15Ø7-1 × MON-ØØ81Ø-6 × SYN-IR162-4 × MON-ØØ6Ø3-6	*A. tumefaciens* strain CP4	Australia, Canada, Japan, Korea, Mexico, New Zealand, Taiwan, United States
Corn	DuPont Pioneer	Optimum® TRIsect®	TC1507 × MIR604 × NK603	DAS-Ø15Ø7-1 × SYN-IR6Ø4-5 × MON-ØØ6Ø3-6	*A. tumefaciens* strain CP4	Australia, Canada, China, Japan, Korea, Mexico, New Zealand, Taiwan, United States
Corn	Monsanto	Genuity® DroughtGard™ with Roundup Ready® Corn 2	MON87460 × NK603	MON-8746Ø-4 × MON-ØØ6Ø3-6	*A. tumefaciens* strain CP4	Australia, Canada, China, Japan, Korea, Mexico, New Zealand, Taiwan, United States
Corn	Monsanto	Genuity® DroughtGard™ with VT Double PRO® Corn	MON87460 × MON89034 × NK603	MON-8746Ø-4 × MON-89Ø34-3 × MON-ØØ6Ø3-6	*A. tumefaciens* strain CP4	Australia, Canada, China, Japan, Korea, Mexico, New Zealand, Taiwan, United States
Corn	Monsanto	Genuity® DroughtGard™ with VT Triple PRO® Corn	MON87460 × MON89034 × MON88017	MON-8746Ø-4 × MON-89Ø34-3 × MON-88Ø17-3	*A. tumefaciens* strain CP4	Australia, Canada, China, Japan, Korea, Mexico, New Zealand, Taiwan, United States
Corn	Monsanto	Genuity® SmartStax® Corn	MON89034 × TC1507 × MON88017 × DAS-59122-7	MON-89Ø34-3 × DAS-Ø15Ø7 × MON-88Ø17-3 × DAS-59122-7	*A. tumefaciens* strain CP4	Australia, Canada, Colombia, European Union, Japan, Korea, Mexico, New Zealand, Philippines, Taiwan, United States
Corn	Monsanto	Genuity® VT Double PRO® Corn	MON89034 × NK603	MON-89Ø34-3 × MON-ØØ6Ø3-6	*A. tumefaciens* strain CP4	Argentina, Australia, Brazil, Canada, Colombia, European Union, Honduras, Japan, Korea, Mexico, New Zealand, Philippines, South Africa, United States, Vietnam
Corn	Monsanto	Genuity® VT Triple PRO® Corn	MON89034 × MON88017	MON-89Ø34-3 × MON-88Ø17-3	*A. tumefaciens* strain CP4	Argentina, Brazil, Canada, Colombia, European Union, Japan, Korea, Mexico, Philippines, Taiwan, United States

Crop	Company	Product name	Event/ stacked event	OECD unique identifier	Source of EPSPS gene	Countries with approvals
Corn	Monsanto	POWERCORE™	MON89034 × TC1507 × NK603	MON-89Ø34-3 × DAS-Ø15Ø7-1 × MON-ØØ6Ø3-6	*A. tumefaciens* strain CP4	Argentina, Brazil, Canada, Japan, Korea, Philippines, South Africa
Corn	Monsanto	Roundup Ready® Corn 2/Glufosinate Tolerant Corn	NK603 × T25	MON-ØØ6Ø3-6 × ACS-ZMØØ3-2	*A. tumefaciens* strain CP4	Canada, Colombia, Japan, Korea, Mexico, Philippines, Taiwan, United States
Corn	Monsanto	Roundup Ready® Corn 2	NK603	MON-ØØ6Ø3-6	*A. tumefaciens* strain CP4	Argentina, Australia, Brazil, Canada, China, Colombia, European Union, Honduras, Indonesia, Japan, Korea, Malaysia, Mexico, New Zealand, Philippines, Russian Federation, Singapore, South Africa, Taiwan, United States, Uruguay, Vietnam
Corn	Monsanto	YieldGard® Corn Borer with Roundup Ready® Corn 2	NK603 × MON 810	MON-ØØ6Ø3-6 × MON-ØØ81Ø-6	*A. tumefaciens* strain CP4	Argentina, Australia, Brazil, Canada, Colombia, European Union, Honduras, Japan, Korea, Mexico, New Zealand, Philippines, South Africa, Taiwan, United States, Uruguay
Corn	Monsanto	YieldGard® Plus with Roundup Ready® Corn 2	MON863 × MON810 × NK603	MON-ØØ863-5 × MON-ØØ81Ø-6 × MON-ØØ6Ø3-6	*A. tumefaciens* strain CP4	Australia, Canada, European Union, Japan, Mexico, New Zealand, United States
Corn	Monsanto	YieldGard® Rootworm with Roundup Ready® Corn 2	MON863 × NK603	MON-ØØ863-5 × MON-ØØ6Ø3-6	*A. tumefaciens* strain CP4	Australia, Canada, European Union, Japan, Mexico, New Zealand, United States
Corn	Monsanto	YieldGard® VT Rootworm	MON88017	MON-88Ø17-3	*A. tumefaciens* strain CP4	Argentina, Australia, Brazil, Canada, China, Colombia, European Union, Japan, Korea, Mexico, New Zealand, Philippines, Russian Federation, Singapore, Taiwan, United States
Corn	Monsanto	YieldGard VT Triple®	MON810 × MON88017	MON-ØØ81Ø-6 × MON-88Ø17-3	*A. tumefaciens* strain CP4	Australia, Canada, European Union, Japan, Korea, Mexico, New Zealand, Philippines, Taiwan, United States
Corn	Syngenta	Agrisure® 3000GT	Bt11 × MIR604 × GA21	SYN-BTØ11-1 × SYN-IR6Ø4-5 × MON-ØØØ21-9	*Z. mays*	Argentina, Australia, Canada, European Union, Japan, Korea, Mexico, New Zealand, Philippines, South Africa, Taiwan, United States

Crop	Company	Product name	Event/ stacked event	OECD unique identifier	Source of EPSPS gene	Countries with approvals
Corn	Syngenta	Agrisure® 3122	Bt11 × 59122 × MIR604 × TC1507 × GA21	SYN-BTØ11-1 × DAS-59122-7 × SYN-IR6Ø4-5 × DAS-Ø15Ø7-1 × MON-ØØØ21-9	*Z. mays*	Canada, Japan, Korea, Mexico, Philippines, South Africa, Taiwan, United States
Corn	Syngenta	Agrisure Duracade™ E-Z Refuge™ 5122	Bt11 × MIR604 × TC1507 × 5307 × GA21	SYN-BTØ11-1 × SYN-IR6Ø4-5 × DAS-Ø15Ø7-1 × SYN-Ø53Ø7-1 × MON-ØØØ21-9	*Z. mays*	Australia, Canada, Japan, Mexico, New Zealand, South Africa, Taiwan, United States
Corn	Syngenta	Agrisure Duracade™ E-Z Refuge™ 5222	Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21	SYN-BTØ11-1 × SYN-IR162-4 × SYN-IR6Ø4-5 × DAS-Ø15Ø7-1 × SYN-Ø53Ø7-1 × MON-ØØØ21-9	*Z. mays*	Australia, Canada, Japan, Mexico, New Zealand, South Africa, Taiwan, United States
Corn	Syngenta	Agrisure® GT	GA21	MON-ØØØ21-9	*Z. mays*	Argentina, Australia, Brazil, Canada, China, Colombia, European Union, Indonesia, Japan, Korea, Mexico, New Zealand, Philippines, Russian Federation, South Africa, Taiwan, United States, Uruguay
Corn	Syngenta	Agrisure® GT/CB/LL	Bt11 × GA21	SYN-BTØ11-1 × MON-ØØØ21-9	*Z. mays*	Argentina, Australia, Brazil, Canada, Colombia, European Union, Japan, Korea, Mexico. New Zealand, Philippines, South Africa, Taiwan, Turkey, United States, Uruguay
Corn	Syngenta	Agrisure® GT/RW	MIR604 × GA21	SYN-IR6Ø4-5 × MON-ØØØ21-9	*Z. mays*	Argentina, Australia, Canada, Colombia, European Union, Japan, Korea, Mexico, New Zealand, Philippines, South Africa, Taiwan, United States
Corn	Syngenta	Agrisure Viptera® 3110	Bt11 × MIR162 × GA21	SYN-BTØ11-1 × SYN-IR162-4 × MON-ØØØ21-9	*Z. mays*	Argentina, Australia, Brazil, Canada, Colombia, Japan, Korea, Mexico, New Zealand, Philippines, South Africa, Taiwan, United States, Uruguay
Corn	Syngenta	Agrisure Viptera® 3111	Bt11 × MIR162 × MIR604 × GA21	SYN-BTØ11-1 × SYN-IR162-4 × SYN-IR6Ø4-5 × MON-ØØØ21-9	*Z. mays*	Argentina, Australia, Canada, Colombia, Japan, Korea, Mexico, New Zealand, Philippines, South Africa, Taiwan, United States

Crop	Company	Product name	Event/ stacked event	OECD unique identifier	Source of EPSPS gene	Countries with approvals
Corn	Syngenta	Agrisure Viptera® 3220	Bt11 × MIR162 × TC1507 × GA21	SYN-BTØ11-1 × SYN-IR162-4 × DAS-Ø15Ø7-1 × MON-ØØØ21-9	*Z. mays*	Canada, Japan, Korea, Mexico, Philippines, South Africa, Taiwan United States
Corn	Syngenta	Enogen® Agrisure® 3000GT	3272 × Bt11 × MIR604 × GA21	SYN-E3272-5 × SYN-BTØ11-1 × SYN-IR6Ø4-5 × MON-ØØØ21-9	*Z. mays*	Australia, Canada, Japan, Korea, Mexico, New Zealand, Philippines, South Africa, Taiwan, United States
Cotton	BCS	GlyTol®	GHB614	BCS-GHØØ2-5	*Z. mays*	Argentina, Australia, Brazil, Canada, China, Colombia, European Union, Japan, Korea, Mexico, New Zealand, United States
Cotton	BCS	GlyTol® × LibertyLink® Cotton	GHB614 × LLCotton25	BCS-GHØØ2-5 × ACS-GHØØ1-3	*Z. mays*	Argentina, Australia, Brazil, Canada, Colombia, Japan, Korea, Mexico, New Zealand, United States
Cotton	BCS	GlyTol® × TwinLink®	GHB614 × T304-40 × GHB119	BCS-GHØØ2-5 × BCS-GHØØ4-7 × BCS-GHØØ5-8	*Z. mays*	Australia, Brazil, Canada, Japan, Korea, Mexico, New Zealand, United States
Cotton	Dow	WideStrike® × Roundup Ready® Flex Cotton	281-24-236 × 3006-210-23 × MON88913	DAS-24236-5 × DAS-21Ø23-5 × MON-88913-8	*A. tumefaciens* strain CP4	Australia, Canada, Japan, Korea, Mexico, New Zealand, United States
Cotton	Dow	WideStrike® × RR1445 Cotton	281-24-236 × 3006-210-23 × MON1445	DAS-24236-5 × DAS-21Ø23-5 × MON-Ø1445-2	*A. tumefaciens* strain CP4	Australia, Canada, Japan, Korea, Mexico, New Zealand, United States
Cotton	Monsanto	Genuity® Bollgard II® with Roundup Ready® Flex Cotton	MON15985 × MON1445	MON-15985-7 × MON-Ø1445-2	*A. tumefaciens* strain CP4	Australia, Canada, European Union, Japan, Korea, Mexico, New Zealand, Philippines, United States
Cotton	Monsanto	Genuity® Bollgard II® with Roundup Ready® Flex Cotton	MON88913 × MON15985	MON-88913-8 × MON-15985-7	*A. tumefaciens* strain CP4	Australia, Brazil, Canada, Colombia, Japan, Korea, Mexico, New Zealand, Philippines, South Africa, United States
Cotton	Monsanto	Genuity® Roundup Ready® Flex Cotton	MON88913	MON-88913-8	*A. tumefaciens* strain CP4	Australia, Brazil, Canada, China, Colombia, Japan, Korea, Mexico, New Zealand, Philippines, Singapore, South Africa, United States
Cotton	Monsanto	Roundup Ready® Cotton	MON1445	MON-Ø1445-2	*A. tumefaciens* strain CP4	Argentina, Australia, Brazil, Canada, China, Colombia, European Union, Japan, Mexico, New Zealand, Philippines, Singapore, South Africa, United States

Crop	Company	Product name	Event/ stacked event	OECD unique identifier	Source of EPSPS gene	Countries with approvals
Cotton	Monsanto	Roundup Ready® with Bollgard® Cotton	MON531 × MON1445	MON-ØØ531-6 × MON-Ø1445-2	*A. tumefaciens* strain CP4	Argentina, Australia, Brazil, Colombia, Colombia, European Union, Japan, Korea, Mexico, New Zealand, Philippines, South Africa, United States
Soybean	Monsanto	Dicamba × Genuity® Roundup Ready 2 Yield® Soybeans	MON87708 × MON89788	MON-877Ø8-9 × MON-89788-1	*A. tumefaciens* strain CP4	Australia, Canada, Japan, Korea, Mexico, New Zealand, Taiwan, United States
Soybean	Monsanto	Genuity® Roundup Ready 2 Yield® Soybeans	MON89788	MON-89788-1	*A. tumefaciens* strain CP4	Australia, Canada, China, Colombia, European Union, India, Indonesia, Japan, Korea, Malaysia, Mexico, New Zealand, Philippines, Russian Federation, Singapore, South Africa, Taiwan, United States
Soybean	Monsanto	High Oleic × Genuity® Roundup Ready 2 Yield® Soybeans	MON87705 × MON89788	MON-877Ø5-6 × MON-89788-1	*A. tumefaciens* strain CP4	Canada, Japan, Korea, Mexico, Taiwan
Soybean	Monsanto	Intacta RR2 PRO® Soybeans	MON87701 × MON89788	MON-877Ø1-2 × MON-89788-1	*A. tumefaciens* strain CP4	Argentina, Australia, Brazil, Canada, China, Colombia, European Union, Japan, Korea, Mexico, New Zealand, Paraguay, South Africa, Taiwan, United States, Uruguay
Soybean	Monsanto	Omega-3 × Genuity® Roundup Ready 2 Yield® Soybeans	MON87769 × MON89788	MON-87769-7 × MON-89788-1	*A. tumefaciens* strain CP4	Australia, Canada, Mexico, New Zealand, United States
Sugar beet	Monsanto	Roundup Ready® Sugar Beet	H7-1	KM-ØØØH71-4	*A. tumefaciens* strain CP4	Australia, Canada, China, Colombia, European Union, Japan, Mexico, New Zealand, Philippines, Russian Federation, Singapore, United States
Canola	Monsanto	Roundup Ready® Canola	RT73	MON-ØØØ73-7	*A. tumefaciens* strain CP4	Australia, Canada, China, European Union, Japan, Korea, Mexico, New Zealand, Philippines, Singapore, United States

Sources: CropLife International (2015), ILSI (2011a), OECD (2015)

Table A-2. Transgenic crops approved for use globally that contain PAT proteins

Crop	Company	Product name	Event/ stacked event	OECD unique identifier	Source of PAT gene	Countries with approvals
Corn	BCS	LibertyLink® Corn	T25	ACS-ZMØØ3 -2	*S. viridochromogenes*	Argentina, Australia, Brazil, Canada, China, Colombia, European Union, Japan, Korea, Malaysia, Mexico, New Zealand, Philippines, Singapore, South Africa, Taiwan, United States
Corn	Dow	HERCULEX® I	TC1507	DAS-Ø15Ø7 -1	*S. viridochromogenes*	Argentina, Australia, Brazil, Canada, China, Colombia, European Union, Japan, Korea, Mexico, New Zealand, Paraguay, Philippines, Singapore, South Africa, Taiwan, United States, Uruguay
Corn	Dow	HERCULEX® I × Roundup Ready® Corn 2	TC1507 × NK603	DAS-Ø15Ø7-1 × MON-ØØ6Ø3-6	*S. viridochromogenes*	Argentina, Australia, Brazil, Canada, China, Colombia, European Union, Japan, Korea, Mexico, New Zealand, Philippines, Taiwan, United States, Uruguay
Corn	Dow	HERCULEX® RW Rootworm Protection	DAS-59122-7	DAS-59122-7		Australia, Canada, China, Colombia, European Union, Japan, Korea, Mexico, New Zealand, Philippines, Singapore, Taiwan, United States
Corn	Dow	HERCULEX® XTRA Insect Protection	TC1507 × DAS-59122-7	DAS-Ø15Ø7-1 × DAS-59122-7	*S. viridochromogenes*	Australia, Canada, China, European Union, Japan, Korea, Mexico, New Zealand, Philippines, South Africa, Taiwan, United States
Corn	Dow	HERCULEX® XTRA × Roundup Ready® Corn 2	TC1507 × DAS-59122-7 × NK603	DAS-Ø15Ø7-1 × DAS-59122-7 × MON-ØØ6Ø3-6	*S. viridochromogenes*	Australia, Canada, China, European Union, Japan, Korea, Mexico, New Zealand, Philippines, South Africa, Taiwan, United States
Corn	Dow	POWERCORE™	MON 89034-3 × TC1507 × NK603	MON-89Ø34-3 × DAS-Ø15Ø7-1 × MON-ØØ6Ø3-6	*S. viridochromogenes*	Argentina, Australia, Brazil, Canada, China, Colombia, European Union, Japan, Korea, Mexico, New Zealand, Paraguay, Philippines, South Africa, Taiwan, United States, Uruguay
Corn	Dow	SmartStax® Corn	MON 89034 × TC1507 × MON 88017 × DAS-59122-7	MON-89Ø34-3 × DAS-Ø15Ø7-1 × MON-88Ø17-3 × DAS-59122-7	*S. viridochromogenes*	Australia, Canada, Colombia, European Union, Japan, Korea, Mexico, New Zealand, Philippines, South Africa, Taiwan, United States
Corn	DuPont Pioneer	HERCULEX® I Insect Protection	TC1507	DAS-Ø15Ø7 -1	*S. viridochromogenes*	Argentina, Australia, Brazil, Canada, China, Colombia, European Union, Honduras, Japan, Korea, Malaysia, Mexico, New Zealand, Paraguay, Philippines, Singapore, south Africa, Taiwan, United States, Uruguay

Crop	Company	Product name	Event/ stacked event	OECD unique identifier	Source of PAT gene	Countries with approvals
Corn	DuPont Pioneer	HERCULEX® I Insect Protection × Roundup Ready® Corn 2	TC1507 × NK603	DAS-Ø15Ø7-1 × MON-ØØ6Ø3-6	*S. viridochromogenes*	Argentina, Australia, Brazil, Canada, China, Colombia, European Union, Honduras, Japan, Korea, Mexico, New Zealand, Philippines, Taiwan, United States
Corn	DuPont Pioneer	HERCULEX® RW Rootworm Protection	DAS-59122-7	DAS-59122-7	*S. viridochromogenes*	Australia, Canada, China, European Union, Japan, Korea, Mexico, New Zealand, Philippines, Taiwan, United States
Corn	DuPont Pioneer	HERCULEX® RW Rootworm Protection × Roundup Ready® Corn 2	DAS-59122-7 × NK603	DAS-59122-7 × MON-ØØ6Ø3-6	*S. viridochromogenes*	Australia, Canada, China, European Union, Japan, Korea, Mexico, New Zealand, Philippines, Taiwan, United States
Corn	DuPont Pioneer	HERCULEX® XTRA Insect Protection	TC1507 × DAS-59122-7	DAS-Ø15Ø7-1 × DAS-59122-7	*S. viridochromogenes*	Australia, Canada, China, European Union, Japan, Korea, Mexico, New Zealand, Philippines, Taiwan, United States
Corn	DuPont Pioneer	HERCULEX® XTRA × Roundup Ready® Corn 2	TC1507 × DAS-59122-7 × NK603	DAS-Ø15Ø7-1 × DAS-59122-7 × MON-ØØ6Ø3-6	*S. viridochromogenes*	Australia, Canada, China, Colombia, European Union, Japan, Korea, Mexico, New Zealand, Philippines, Taiwan, United States
Corn	DuPont Pioneer	Optimum® Intrasect®	TC1507 × MON810 × NK603	DAS-Ø15Ø7-1 × MON-ØØ81Ø-6 × MON-ØØ6Ø3-6	*S. viridochromogenes*	Australia, Brazil, Canada, China, Colombia, Japan, Korea, Mexico, New Zealand, Philippines, Taiwan, United States
Corn	DuPont Pioneer	Optimum® Intrasect® Xtra	TC1507 × DAS-59122-7 × MON810 × NK603	DAS-Ø15Ø7-1 × DAS-59122-7 × MON-ØØ81Ø-6 × MON-ØØ6Ø3-6	*S. viridochromogenes*	Australia, Canada, China, Japan, Korea, Mexico, New Zealand, Taiwan, United States
Corn	DuPont Pioneer	Optimum® Intrasect® Xtra × Xtreme	TC1507 × DAS-59122-7 × MON810 × MIR604 × NK603	DAS-Ø15Ø7-1 × DAS-59122-7 × MON-ØØ81Ø-6 × SYN-IR6Ø4-5 × MON-ØØ6Ø3-6	*S. viridochromogenes*	Australia, Canada, China, Japan, Korea, Mexico, New Zealand, Taiwan, United States
Corn	DuPont Pioneer	Optimum® Leptra®	TC1507 × MON810 × MIR162 × NK603	DAS-Ø15Ø7-1 × MON-ØØ81Ø-6 × SYN-IR162-4 × MON-ØØ6Ø3-6	*S. viridochromogenes*	Australia, Canada, Japan, Korea, Mexico, New Zealand, Taiwan, United States
Corn	DuPont Pioneer	Optimum® Trisect®	TC1507 × MIR604 × NK603	DAS-Ø15Ø7-1 × SYN-IR6Ø4-5 × MON-ØØ6Ø3-6	*S. viridochromogenes*	Australia, Canada, China, Japan, Korea, Mexico, New Zealand, Taiwan, United States

Crop	Company	Product name	Event/ stacked event	OECD unique identifier	Source of PAT gene	Countries with approvals
Corn	Monsanto	Genuity® SmartStax®	MON 89034 × TC1507 × MON 88017 × DAS-59122-7	MON-89Ø34-3 × DAS-Ø15Ø7 × MON-88Ø17-3 × DAS-59122-7	*S. viridochromogenes*	Australia, Canada, Colombia, European Union, Japan, Korea, Mexico, New Zealand, Philippines, Taiwan, United States
Corn	Monsanto	POWERCORE™	MON89034 × TC1507 × NK603	MON-89Ø34-3 × DAS-Ø15Ø7-1 × MON-ØØ6Ø3-6	*S. viridochromogenes*	Argentina, Brazil, Canada, Japan, Korea, Philippines, South Africa
Corn	Monsanto	Roundup Ready® Corn 2/Glufosinate Tolerant Corn	NK603 × T25	MON-ØØ6Ø3-6 × ACS-ZMØØ3-2	*S. viridochromogenes*	Canada, Colombia, Japan, Korea, Mexico, Philippines, Taiwan, United States
Corn	Syngenta	KnockOut®	176	SYN-EV176-9	*S. hygroscopicus*	Argentina, Australia, Canada, China, European Union, Japan, Korea, New Zealand, South Africa, Taiwan, United States
Corn	Syngenta	Agrisure® 3000GT	Bt11 × MIR604 × GA21	SYN-BTØ11-1 × SYN-IR6Ø4-5 X MON-ØØØ21-9	*S. viridochromogenes*	Argentina, Australia, Canada, Colombia, European Union, Japan, Korea, Mexico, New Zealand, Philippines, Russian Federation, South Africa, Taiwan, United States
Corn	Syngenta	Agrisure® 3122	Bt11 × DAS-59122-7 × MIR604 × TC1507 × GA21	SYN-BTØ11-1 × DAS-59122-7 × SYN-IR6Ø4-5 × DAS-Ø15Ø7-1 × MON-ØØØ21-9	*S. viridochromogenes*	Canada, Japan, Korea, Mexico, Philippines, South Africa, Taiwan, United States
Corn	Syngenta	Agrisure® CB/LL	Bt11	SYN-BTØ11-1	*S. viridochromogenes*	Argentina, Australia, Brazil, Canada, China, Colombia, European Union, Indonesia, Japan, Korea, Malaysia, Mexico, New Zealand, Paraguay, Philippines, Russia, South Africa, Switzerland, Taiwan, Turkey, United States, Uruguay, Vietnam
Corn	Syngenta	Agrisure® CB/LL/RW	Bt11 × MIR604	SYN-BTØ11-1 × SYN-IR6Ø4-5	*S. viridochromogenes*	Argentina, Australia, Canada, Colombia, European Union, Japan, Korea, Mexico, New Zealand, Philippines, Russian Federation, South Africa, Taiwan, United States
Corn	Syngenta	Agrisure Duracade™ E-Z Refuge™ 5122	Bt11 × MIR604 × TC1507 × 5307 × GA21	SYN-BTØ11-1 × SYN-IR6Ø4-5 × DAS-Ø15Ø7-1 × SYN-Ø53Ø7-1 × MON-ØØØ21-9	*S. viridochromogenes*	Australia, Canada, Japan, Korea, Mexico, New Zealand, Russian Federation, South Africa, Taiwan, United States

Crop	Company	Product name	Event/ stacked event	OECD unique identifier	Source of PAT gene	Countries with approvals
Corn	Syngenta	Agrisure Duracade™ E-Z Refuge™ 5222	Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21	SYN-BTØ11-1 × SYN-IR162-4 × SYN-IR6Ø4-5 × DAS-Ø15Ø7-1 × SYN-Ø53Ø7-1 × MON-ØØØ21-9	*S. viridochromogenes*	Australia, Canada, Japan, Korea, Mexico, New Zealand, Russian Federation, South Africa, Taiwan, United States
Corn	Syngenta	Agrisure® GT/CB/LL	Bt11 × GA21	SYN-BTØ11-1 × MON-ØØØ21-9	*S. viridochromogenes*	Argentina, Australia, Brazil, Canada, Colombia, European Union, Japan, Korea, Mexico, New Zealand, Philippines, South Africa, Taiwan, Turkey, United States, Uruguay
Corn	Syngenta	Agrisure Viptera® 3110	Bt11 × MIR162 × GA21	SYN-BTØ11-1 × SYN-IR162-4 × MON-ØØ×21-9	*S. viridochromogenes*	Argentina, Australia, Brazil, Canada, Colombia, Japan, Korea, Mexico, New Zealand, Philippines, South Africa, Taiwan, United States, Uruguay
Corn	Syngenta	Agrisure Viptera® 3111	Bt11 × MIR162 × MIR604 × GA21	SYN-BTØ11-1 × SYN-IR162-4 × SYN-IR6Ø4-5 × MON-ØØØ21-9	*S. viridochromogenes*	Argentina, Australia, Brazil, Canada, Colombia, Japan, Korea, Mexico, New Zealand, Philippines, Russian Federation, South Africa, Taiwan, United States
Corn	Syngenta	Agrisure Viptera® 3220	Bt11 × MIR162 × TC1507 × GA21	SYN-BTØ11-1 × SYN-IR162-4 × DAS-Ø15Ø7-1 × MON-ØØØ21-9	*S. viridochromogenes*	Argentina, Australia, Canada, Japan, Korea, Mexico, New Zealand, Philippines, Russian Federation, South Africa, Taiwan, United States
Corn	Syngenta	Enogen® Agrisure® 3000GT	3272 × Bt11 × MIR604 × GA21	SYN-E3272-5 × SYN-BTØ11-1 × SYN-IR6Ø4-5 × MON-ØØØ21-9	*S. viridochromogenes*	Australia, Canada, Japan, Korea, Mexico, New Zealand, Philippines, Taiwan, United States
Canola	BCS	LibertyLink® Canola	T45	ACS-BNØØ8-2	*S. viridochromogenes*	Australia, Canada, China, European Union, Japan, Korea, Mexico, Russian Federation, South Africa, Taiwan, United States
Canola	BCS	SeedLink®	MS1/RF1	ACS-BNØØ4-7 × ACS-BNØØ1-4	*S. hygroscopicus*	Australia, Canada, China, Japan, Korea, Mexico, New Zealand, South Africa, United States
Canola	BCS	SeedLink®	MS1/RF2	ACS-BNØØ4-7 × ACS-BNØØ2-5	*S. hygroscopicus*	Australia, Canada, China, Japan, Korea, Mexico, New Zealand, South Africa, United States
Canola	BCS	SeedLink®/ InVigor®	MS8/RF3	ACS-BNØØ5-8 × ACS-BNØØ3-6	*S. hygroscopicus*	Australia, Canada, China, European Union, Japan, Korea, Mexico, New Zealand, South Africa, United States
Cotton	BCS	GlyTol® × LibertyLink® Cotton	GHB614 × LL25Cotton	BCS-GHØØ2-5 × ACS-GHØØ1-3	*S. hygroscopicus*	Australia, Brazil, Canada, Colombia, Japan, Korea, Mexico, New Zealand, United States

Crop	Company	Product name	Event/ stacked event	OECD unique identifier	Source of PAT gene	Countries with approvals
Cotton	BCS	GlyTol® × TwinLink®	GHB614 × T304-40 × GHB119	BCS-GHØØ2-5 × BCS- GHØØ4-7 × BCS- GHØØ5-8	*S. hygroscopicus*	Australia, Brazil, Canada, Japan, Korea, Mexico, New Zealand, United States
Cotton	BCS	LibertyLink® Cotton	LLCotton25	ACS-GHØØ1-3	*S. hygroscopicus*	Australia, Brazil, Canada, China, Colombia, European Union, Japan, Korea, Mexico, South Africa, New Zealand, United States
Cotton	BCS	LibertyLink® × Bollgard II® Cotton	LLCotton25 × MON15985	ACS-GHØØ1-3 × MON 15985-7	*S. hygroscopicus*	Australia, Canada, Japan, Korea, Mexico, New Zealand, United States
Cotton	BCS	TwinLink®	T304-40 × GHB119	BCS- GHØØ4-7 × BCS- GHØØ5-8	*S. hygroscopicus*	Australia, Brazil, Canada, Korea, Mexico, New Zealand, United States
Cotton	Dow	WideStrike®	281-24-236 × 3006-210-23	DAS-21Ø23-5 × DAS-24236-5	*S. viridochromogenes*	Australia, Brazil, Canada, European Union, Japan, Korea, Mexico, New Zealand, United States
Cotton	Dow	WideStrike® × Roundup Ready® Flex Cotton	281-24-236 × 3006-210-23 × Mon 88913	DAS-24236-5 × DAS-21Ø23-5 × MON-88913-8	*S. viridochromogenes*	Australia, Canada, Japan, Korea, Mexico, New Zealand, United States
Cotton	Dow	WideStrike® × RR1445 Cotton	281-24-236 × 3006-210-23 × Mon 1445	DAS-24236-5 × DAS-21Ø23-5 × MON-Ø1445-2	*S. viridochromogenes*	Australia, Canada, Japan, Korea, Mexico, New Zealand, United States
Cotton	Monsanto	Bollgard II® XtendFlex™	MON 88701 × MON 88913 × MON 15985	MON-887Ø1-3 × MON-88913-8 × MON-15985-7	*S. hygroscopicus*	Australia, Canada, Japan, Mexico, New Zealand, United States
Rice	BCS	LibertyLink® Rice	LLRICE06,LLRICE 62, LLRICE601	ACS-OSØØ2-5	*S. hygroscopicus*	Australia, Canada, Colombia, Honduras, Mexico, New Zealand, Philippines, Russian Federation, South Africa, United States
Soybean	BCS	LibertyLink® Soybean	A2704-12	ACS-GMØØ5-3	*S. viridochromogenes*	Argentina, Australia, Brazil, Canada, China, European Union, Japan, Korea, Malaysia, Mexico, New Zealand, Philippines, Russian Federation, Singapore, South Africa, Taiwan, United States, Uruguay
Soybean	BCS	LibertyLink® Soybean	A5547-127	ACS-GMØØ6-4	*S. viridochromogenes*	Argentina, Australia, Brazil, Canada, China, European Union, Japan, Korea, Malaysia, Mexico, New Zealand, Philippines, Russian Federation, Singapore, Taiwan, United States, Uruguay

Sources: CropLife International (2015), ILSI (2011a), OECD (2015)

Appendix B. Summary of Safety Studies for mEPSPS and PAT

Because of the long history of safe use of food products containing EPSPS and PAT and the extensive evaluations already conducted on these proteins, additional safety assessments of these proteins is not warranted. Nonetheless, the following additional information on the protein safety, as well as corresponding study reports, are provided as supplemental data.

B.1. Equivalence of Microbially Produced and Plant-Produced Proteins

A series of analytical methods were used to characterize the mEPSPS and PAT proteins produced in MZHG0JG corn and to demonstrate that mEPSPS and PAT test substances produced from recombinant *Escherichia coli* were suitable surrogates for use in food and feed safety studies. The use of microbially produced proteins was necessary because protein safety studies require large amounts of protein, and it was infeasible to extract the plant-produced protein in quantities sufficient for all studies.

The identities of the plant-produced and microbially produced mEPSPS and PAT proteins were confirmed by apparent molecular weight, immunoreactivity, peptide mass mapping or intact mass analysis, *N*-terminal and C-terminal amino acid sequence analyses, enzymatic activity, and glycosylation status. The mEPSPS present in the microbially produced test substance (production batches GA21-0104 and MEPSPS-0113) was identical to that produced in MZHG0JG corn. The production method for the two microbial mEPSPS production batches was identical. The PAT present in the microbially produced test substance (production batch PAT-0109) was identical to that produced in MZHG0JG maize.

The results verified the identities of the plant-produced and microbially produced mEPSPS and PAT proteins, supporting the conclusion that the mEPSPS and PAT proteins produced in MZHG0JG maize and in recombinant *E. coli* were biochemically and functionally equivalent. Therefore, the microbially produced test substances containing mEPSPS or PAT were suitable surrogates for mEPSPS or PAT in MZHG0JG corn, respectively, and were appropriate for use in studies supporting the safety of MZHG0JG corn.

B.2. Assessment of Allergenic Potential of mEPSPS and PAT

Although virtually all food allergens are proteins, only a few of the many proteins found in foods are allergenic, and the probability that a novel protein will become a food allergen is small. Because there is no single definitive test to predict food allergenicity in humans, a weight-of-evidence approach was used to assess the potential allergenicity of mEPSPS and PAT. This approach is consistent with the recommendations of the Codex Alimentarius Commission (2009). The following types of characterization data were considered for mEPSPS and PAT in the weight-of-evidence assessment:

- source organism
- amino acid sequence similarity to known allergenic proteins
- susceptibility to digestive enzymes
- susceptibility to heat inactivation
- glycosylation status

- relative abundance in the commodity crop
- history of safe use

Together, this evidence indicates that mEPSPS and PAT are unlikely to be allergenic. The source organism, glycosylation status, expression, and history of safe use of these proteins are discussed elsewhere in this document. Numerous studies on the digestive fate of both mEPSPS and PAT all show rapid degradation in simulated digestion models, and exposure of mEPSPS and PAT to high temperatures, such as those reached during cooking or food processing, has been shown to destroy the function and/or structure of these proteins. Studies conducted by Syngenta confirm these conclusions and are provided below for reference.

B.2.a. Digestive Fate of mEPSPS and PAT Produced in MZHG0JG

The susceptibility of microbially produced mEPSPS and PAT to proteolytic degradation was evaluated in simulated mammalian gastric fluid (SGF) containing pepsin and in simulated mammalian intenstinal fluid (SIF) containing pancreatin. The products of the digestion were analyzed via sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and western blot analysis. The SGF digestibility studies were performed at 37ºC over a 60-minute time course, and the SIF digestibility studies were performed at 37ºC over a 48-hour time course.

The mEPSPS protein degraded rapidly upon exposure to SGF. Intact mEPSPS was readily digested in SGF in less than one minute, as assessed by SDS-PAGE and western blot analyses. No immunoreactive mEPSPS-derived fragments were observed by western blot analysis after incubation in SGF for 5 minutes. The mEPSPS protein was also readily digested in SIF, as assessed by SDS-PAGE. No intact mEPSPS protein (with a molecular weight of 47.5 kD) or mEPSPS-derived fragments were detected by western blot analysis after incubation in SIF for 10 minutes.

PAT also degraded rapidly upon exposure to SGF. Intact PAT was readily digested in SGF in less than one minute, as assessed by SDS-PAGE and western blot analyses. No immunoreactive PAT-derived fragments were observed by western blot analysis after incubation in SGF for one minute. Intact PAT (with a molecular weight of approximately 20.5 kDa) was degraded in less than 5 minutes of incubation in SIF. A protein band with a molecular weight of approximately 16 kDa, most likely representing a degradation product of PAT, was observed by western blot analysis after incubation in SIF for 1 and 2 minutes. However, after 5 minutes of incubation in SIF, this band was no longer visible.

The results of these experiments support the conclusion that mEPSPS and PAT will be readily degraded in the mammalian digestive tract.

B.2.b. Heat Stability of mEPSPS and PAT

The immunoreactivity (or ability to bind specific antibodies) of microbially produced mEPSPS and PAT proteins exposed to various temperatures (4°C, 25°C, 37°C, 65°C, and 95°C) for 30 minutes was measured via enzyme-linked immunosorbent assay (ELISA).

Temperatures of 25ºC and 37ºC had little effect on mEPSPS immunoreactivity. Following temperature treatment of mEPSPS at 65ºC and 95°C, its immunoreactivity was below the LOD

for the ELISA. The immunoreactivity of PAT decreased with increasing temperature from 25°C to 37°C to 65°C. After incubation at 95°C, the immunoreactivity of PAT was below the LOD.

The results of these experiments support the conclusion that both mEPSPS and PAT are stable for at least 30 minutes upon heating to 37°C. mEPSPS is unstable upon heating at temperature 65°C and above, whereas PAT is completely unstable at 95°C.

B.2.c. Amino Acid Similarity to Known or Putative Allergens

To determine whether mEPSPS or PAT showed biologically relevant amino acid sequence similarity to known or putative allergens, two different searches were performed against the Food Allergy Research and Resource Program Protein Allergen Protein Database (FARRP 2015), which contained 1897 nonredundant sequences of known and putative allergens. A full-length sequence search with the FASTA algorithm (Pearson and Lipman 1988) and a separate search for exact matches of eight or more contiguous amino acids were used to compare the mEPSPS and PAT amino acid sequences with each of the known or putative allergen sequences. In the FASTA search, no sequence similarity greater than 35% shared identity over 80 or more amino acids was observed between the mEPSPS or PAT amino acid sequence and any sequence in the FARRP database. In the exact match search, no alignments of eight or more contiguous amino acids were found between the mEPSPS or PAT amino acid sequence and any sequence in the FARRP database. Together, these results support the conclusion that mEPSPS and PAT share no biologically relevant amino acid sequence similarity to known or putative protein allergens.

B.2.d. Conclusions on the Allergenic Potential of mEPSPS and PAT

The weight of evidence indicates that mEPSPS and PAT are unlikely to be food allergens, because they are derived from source organisms that contains no known allergens, they are not significantly similar in amino acid sequence to known allergens, and they are not glycosylated (as discussed in Sections VI.A.4 and VI.B.4). Furthermore, exposure to mEPSPS and PAT from MZHG0JG corn grain will be low (as discussed in Section VI.C). Both mEPSPS and PAT are rapidly degraded in simulated mammalian gastric and intestinal fluids and are heat labile. Together, this evidence indicates that mEPSPS and PAT are unlikely to be allergenic and that no significant dietary exposure to mEPSPS and PAT enzymatic activity would occur in humans or other mammals via consumption of MZHG0JG corn.

B.3 Assessment of the Toxicity of mEPSPS and PAT

The potential toxicity of mEPSPS and PAT in MZHG0JG corn were evaluated through an extensive bioinformatic search to determine whether mEPSPS or PAT had significant sequence similarity to proteins identified as known or putative toxins.

The Basic Local Alignment Search Tool for Proteins (BLASTP) program (Altschul *et al.* 1997) was used to compare the mEPSPS and PAT amino acid sequences with all entries in the NCBI Entrez Protein Database (NCBI 2015). This analysis addressed two questions: (1) whether any protein(s) in the database had a high degree of sequence similarity to the mEPSPS or PAT amino acid sequence and (2) whether any proteins demonstrating a high degree of sequence similarity to the mEPSPS or PAT amino acid sequence were known or putative toxins.

The BLASTP searches were performed with the default parameters, and the threshold for statistical significance of *E*-values (a measure of the probability that matches between sequences occurred by chance) was established by analysis of searches using randomly shuffled versions of the mEPSPS and PAT amino acid sequences. The threshold *E*-values were $<1 \times 10^{-05}$ for mEPSPS and $<1x10^{-5}$ for PAT.

For both proteins, the 1000 most similar alignments with proteins in the NCBI database all had *E*-values below the threshold *E*-values and were evaluated for biological relevance. For mEPSPS, 986 of these proteins were categorized as "EPSPS/EPSPS-like," belonging to the same class of protein as mEPSPS. For PAT, approximately 90% of the proteins were other members of the acetyltransferase class of enzymes. None of the matching proteins for either mEPSPS or PAT corresponded to known or putative toxins.

BLASTP analyses were also conducted with the Syngenta Toxin Database, a separate database of known toxin sequences. No significant alignments (to indicate the potential to act as a toxin) were observed when mEPSPS or PAT were compared with any entries in the Syngenta Toxin Database.

The results of both database comparisons confirm that mEPSPS and PAT do not share significant sequence similarity with other known or putative protein toxins.

B.4. Conclusions

The weight of evidence from these additional Syngenta studies supports the conclusion that the mEPSPS and PAT proteins are unlikely to be toxins or allergens.

B.5. References Cited in Appendix B

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