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REQUEST FOR AN EXTENSION OF DETERMINATION OF NONREGULATED STATUS FOR HERBICIDE-TOLERANT EVENT MZHG0JG CORN

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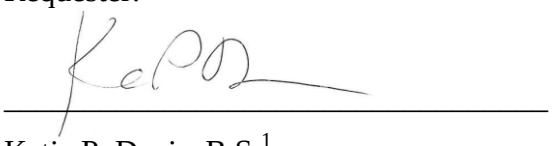
CR018-USDA-1

Certification

The undersigned submits this request under 7 CFR Part 340.6 to request that the Administrator, Animal and Plant Health Inspection Service, make a determination that the article Event MZHG0JG corn should not be regulated under 7 CFR Part 340.

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

Requester:



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Release of Information

REQUEST FOR AN EXTENSION OF DETERMINATION OF NONREGULATED STATUS FOR HERBICIDE-TOLERANT EVENT MZHG0JG CORN

Executive Summary

Syngenta requests a determination from the United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA APHIS) that herbicide-tolerant Event MZHG0JG corn (hereafter MZHG0JG corn), any progeny derived from crosses between MZHG0JG corn and conventional corn varieties, and any progeny derived from crosses of MZHG0JG corn with other biotechnology-derived corn varieties that have previously been granted nonregulated status, no longer be considered regulated articles under 7 CFR Part 340. Syngenta requests that the USDA APHIS consider this application an extension to petitions 11-342-01p and 11-244-01p based on the phenotypic similarities of MZHG0JG corn to the antecedent organisms that are the subject of petitions 11-342-01p and 11-244-01p, VCO-Ø1981-5 glyphosate-tolerant corn (hereafter VCO-Ø1981-5 corn) and DP-ØØ4114-3 insect and glufosinate-ammonium-tolerant corn (hereafter DP-ØØ4114-3 corn). Like MZHG0JG corn, VCO-Ø1981-5 corn confers tolerance to herbicides containing glyphosate and DP-ØØ4114-3 corn confers tolerance to herbicides containing glufosinate-ammonium, both of which have activity against a variety of agronomically important weed species. The antecedent organism VCO-Ø1981-5 corn received a determination of nonregulated status from the USDA APHIS on September 25, 2013 and DP-ØØ4114-3 corn received the same determination on June 20, 2013.

Syngenta has developed MZHG0JG corn (maize; *Zea mays* L.), a new cultivar that has been genetically modified to tolerate herbicides containing glyphosate and glufosinate-ammonium. Most corn currently grown in the United States (U.S.) represents transgenic herbicide-tolerant varieties. MZHG0JG corn will offer growers an additional cultivar of herbicide-tolerant corn that will allow flexibility 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 modified 5-enol pyruvylshikimate-3-phosphate synthase (mEPSPS), and the transgene *pat-09*, which encodes the enzyme phosphinothricin acetyltransferase (PAT). The native 5-enol pyruvylshikimate-3-phosphate synthase (EPSPS) from *Z. mays* is involved in the synthesis of aromatic amino acids and is inhibited by glyphosate. The enzyme mEPSPS, a variant of the native EPSPS from *Z. mays*, contains two amino acid substitutions that were introduced specifically to confer tolerance to herbicides containing 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 transgenes *mepsps-02* and *pat-09* encode protein sequences identical to those in plant varieties previously deregulated by the USDA, including GA21 corn (petition 97-099-01p) and Bt11 corn (petition 95-195-01p). Although the vector agent and the sources of some genetic elements used to create MZHG0JG corn are listed as plant pests in 7 CFR § 340.2, the introduced nucleotide sequences do not impart plant pest properties.

MZHG0JG corn was produced by *Agrobacterium tumefaciens*–mediated transformation of immature embryos of Syngenta's proprietary corn inbred NP2222. 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 (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).

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. 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 corn T-DNA insert with sequences in public databases indicated that the inserted DNA does not disrupt any known endogenous corn gene.

Southern blot analyses demonstrated that the MZHG0JG corn 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 the transgenes are inherited in a predictable manner, according to Mendelian principles.

Laboratory and field investigations confirmed that there were no changes in grain, pollen, plant phenotypic, or composition parameters suggestive of increased plant pest risk or increased susceptibility of MZHG0JG corn to plant disease or other pests. Compositional assessments of the grain and forage from multiple U.S. field sites demonstrated that MZHG0JG corn is nutritionally and compositionally equivalent to, and as safe and nutritious as, conventional corn. Corn does not possess weedy properties or outcross to wild relatives in the U.S.; these properties have not been altered in MZHG0JG 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. EPSPS and PAT proteins are exempt from the requirement for food or feed tolerances in all crops and have a history of safe use in numerous transgenic crop varieties that have been deregulated by the USDA APHIS and reviewed through the biotechnology consultation process with the U.S. Food and Drug Administration.

On the basis of the data and information described in this document and the phenotypic similarity of MZHG0JG corn to the deregulated antecedent organisms VCO-Ø1981-5 corn and DP-ØØ4114-3 corn, Syngenta requests a determination from USDA APHIS that MZHG0JG corn, and any progeny derived from crosses between MZHG0JG corn and conventional corn or deregulated corn varieties, should qualify for nonregulated status under 7 CFR Part 340.

Concurrent with its deregulation of the antecedent organisms VCO-Ø1981-5 corn and DP-ØØ4114-3 corn, USDA APHIS published an Environmental Assessment (EA) and Finding of No

Signficant Impact (FONSI) in compliance with the National Environmental Policy Act (NEPA), 42 U.S.C. §4321, *et seq*. As a supplement to Syngenta's request for a determination of nonregulated status for MZHG0JG corn, Syngenta is submitting a document that details a review of the previous EAs and associated FONSIs for VCO-Ø1981-5 corn and DP-ØØ4114-3 corn. The supplemental document is intended to assist the agency in fulfilling its obligations under NEPA, as well as other applicable statutes and regulations. Syngenta is aware of no study results or observations associated with MZHG0JG corn that are anticipated to result in adverse consequences to the quality of the human environment, directly, indirectly, or cumulatively. No adverse effects are anticipated on endangered or threatened species listed by the U.S. Fish and Wildlife Service, unique geographic areas, critical habitats, public health or safety (including children and minorities), genetic diversity of corn, farmer or consumer choice, herbicide resistance, or the economy, either within or outside of the U.S.

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35S enhancer	transcriptional enhancer region of the cauliflower mosaic virus
adh1	alcohol dehydrogenase gene 1
a.i.	active ingredient
aadA-03	spectinomycin resistance gene
ADF	acid detergent fiber
ANOVA	analysis of variance
APHIS	Animal and Plant Health Inspection Service
BC	backcross
BCS	Bayer CropScience
BLASTX	Basic Local Alignment Search Tool for Translated Nucleotides
bp	base pair
CaMV	cauliflower mosaic virus
CTAB	cetyltrimethyl ammonium bromide
CTP	chloroplast transit peptide
Dow	Dow AgroSciences, LLC
DNA	deoxyribonucleic acid
DW	dry weight
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
EPSPS	5-enol pyruvylshikimate-3-phosphate synthase
EST	expressed sequence tags
F_1	first generation of progeny from a breeding cross
FDA	Food and Drug Administration
FMV	figwort mosaic virus
FMV-05	transcriptional enhancer region of the figwort mosaic virus
FOIA	Freedom of Information Act
FW	fresh weight
ILSI	International Life Sciences Institute
kb	kilobase pairs
kDa	kilodalton
LB-01-01	left border
LOD	limit of detection
LOQ	limit of quantitation
mEPSPS	modified EPSPS enzyme
mepsps-02	modified 5-enol pyruvylshikimate-3-phosphate synthase gene
N/A	not applicable
NCBI	National Center for Biotechnology Information
NDF	neutral detergent fiber
NOS	nopaline synthase
NOS-05-01	nopaline synthase terminator sequence from *A. tumefaciens*
nr/nt	non-redundant nucleotide
OECD	Organisation for Economic Co-operation and Development
ORF	open reading frame
ori	origin of replication
OTP-02	optimized transit peptide
PAGE	polyacrylamide gel electrophoresis
PAT	phosphinothricin acetyltransferase

pat-09	phosphinothricin acetyltransferase gene
PCR	polymerase chain reaction
RB-01-01	right border
repA-03	replication gene from *Pseudomonas aeruginosa* plasmid VS1
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
T_0, T_1, T_2, T_3, etc.	T_0 is the designation used for the original transformed plant and T_1, T_2, T_3, etc., refer to successive generations produced by self-pollination
T-DNA	transferred DNA
TIU	trypsin inhibitor unit
TMV	tobacco mosaic virus
tris	2-amino-2(hydroxymethyl)-1,3-propanediol
USDA	United States Department of Agriculture
virG	regulatory gene from *Agrobacterium tumefaciens*
×	cross, cross-pollination
⊗	self-pollination

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

I. Rationale for Development of MZHG0JG Corn

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

Syngenta Crop Protection AG (Syngenta) has developed MZHG0JG corn (maize; *Zea mays* L.), a new cultivar that has been genetically modified to tolerate applications of glyphosate-based and glufosinate-ammonium-based herbicides. MZHG0JG corn was developed through *Agrobacterium*-mediated transformation to stably incorporate the transgenes *mepsps-02* and *pat-09* into the corn genome. The gene *mepsps-02* encodes the enzyme modified 5-enol pyruvylshikimate-3-phosphate synthase (mEPSPS), a variant of the native EPSPS from *Z. mays*, which contains two amino acid substitutions that were introduced specifically to confer tolerance to herbicides containing glyphosate. The gene *pat-09* encodes the enzyme phosphinothricin acetyltransferase (PAT) derived from the soil bacterium *Streptomyces viridochromogenes.* PAT acetylates glufosinate-ammonium, thus inactivating it and conferring tolerance to glufosinate-ammonium in herbicide products, and was used as the selectable marker in development of MZHG0JG corn.

I.A. Basis of the Request for a Determination of Nonregulated Status

Under the authority of the Plant Protection Act (7 U.S.C. 7701 *et seq.*) and the regulations contained in 7 CFR Part 340, the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) regulates importation, interstate movement, and environmental release of organisms and products altered or produced through genetic engineering that are plant pests or which there is reason to believe are plant pests. An organism that has been altered or produced through genetic engineering is subject to regulation if the donor organism, recipient organism, vector or vector agent belongs to any taxon designated under 7 CFR § 340.2 and meets the definition of a plant pest, or is unclassified, or its classification is unknown; any product that contains such an organism; and any other organism or product altered or produced through genetic engineering that the Administrator determines is a plant pest or has reason to believe is a plant pest.

Under 7 CFR § 340.6(e), APHIS may extend a previous determination of nonregulated status to additional regulated articles, based on an evaluation of the similarity of the regulated article to the antecedent organism(s) (i.e., an organism that has already been the subject of a determination of nonregulated status by APHIS under § 340.6, and that is used as a reference for comparison to the regulated article under consideration under the regulations). Such an extension of nonregulated status amounts to a finding that the additional regulated article does not pose a potential for plant pest risk, and should therefore not be regulated.

The vector agent used to produce MZHG0JG corn, the transgene *pat-09*, and some of the regulatory sequences used to drive expression of *mepsps-02* and *pat-09* are derived from organisms listed as plant pests under 7 CFR § 340.2. Although the vector agent, *Agrobacterium tumefaciens,* is a plant pathogen, the transformation process that created MZHG0JG corn used a disarmed strain. The gene encoding PAT, which confers tolerance to glufosinate-ammonium, was derived from *Streptomyces viridochromogenes* and codon-optimized for plant expression. In addition, regulatory sequences from figwort mosaic virus, cauliflower mosaic virus, tobacco

mosaic virus, and *A. tumefaciens* were introduced during the production of MZHG0JG corn. The transgene *pat-09* and the described regulatory sequences *per se* do not impart plant pest properties. No nucleotide sequences imparting plant pest properties from *A. tumefaciens, S. viridochromogenes,* or plant viruses were transferred to MZHG0JG corn.

Applicable regulations in 7 CFR § 340.6 provide that any person may petition APHIS to seek a determination that an article should not be regulated. USDA APHIS has reviewed and granted determinations of nonregulated status for multiple herbicide-tolerant crop varieties, including the antecedent organisms, VCO-Ø1981-5 corn and DP-ØØ4114-3 corn. These determinations were made in September 2013 and June 2013 upon finding that VCO-Ø1981-5 corn and DP-ØØ4114-3 corn did not pose a plant pest risk. Based on the similarity of the antecedent organisms VCO-Ø1981-5 corn and DP-ØØ4114-3 corn to MZHG0JG corn, Syngenta has concluded that the previous analyses of impacts completed for VCO-Ø1981-5 corn and DP-ØØ4114-3 corn are relevant to APHIS' regulatory actions associated with responding to the Syngenta extension request for MZHG0JG corn. A comparison of the antecedent organisms VCO-Ø1981-5 corn and DP-ØØ4114-3 corn to MZHG0JG corn is provided in Table I–1 below.

Table I–1. Comparison of antecedent organisms and MZHG0JG corn

Description	Antecedent VCO-Ø1981-5 Corn	Antecedent DP-ØØ4114-3 Corn	Extension MZHG0JG Corn
Organism	Corn	Corn	Corn
Phenotype	Tolerant to the broad-spectrum herbicide active ingredient glyphosate	Tolerant to the broad-spectrum herbicide active ingredient glufosinate-ammonium and insect damage from lepidopteran and coleopteran species	Tolerant to the broad-spectrum herbicide active ingredients glyphosate and glufosinate-ammonium
Proteins	EPSPS ACE5 enzyme	PAT enzyme, Cry1F, Cry34/35	mEPSPS and PAT enzymes
Method of Transformation	*Agrobacterium*-mediated	*Agrobacterium*-mediated	*Agrobacterium*-mediated
Insert Copy	Single intact insertion	Single intact insertion	Single intact insertion
Compositional Analysis	Within range of corn	Within range of corn	Within range of corn
Plant Pest Risk			
Disease and Pest Susceptibilities	Unlikely to pose plant pest risk	Unlikely to pose plant pest risk	Unlikely to pose plant pest risk
Impacts on Beneficial Non-Targets	Unlikely to pose plant pest risk	Unlikely to pose plant pest risk	Unlikely to pose plant pest risk
Enhanced Weediness	Unlikely to pose plant pest risk	Unlikely to pose plant pest risk	Unlikely to pose plant pest risk
Enhanced Weediness of Relatives	Unlikely to pose plant pest risk	Unlikely to pose plant pest risk	Unlikely to pose plant pest risk
Changes to Agriculture or Cultivation Practices	Unlikely to pose plant pest risk	Unlikely to pose plant pest risk	Unlikely to pose plant pest risk

Description	Antecedent VCO-Ø1981-5 Corn	Antecedent DP-ØØ4114-3 Corn	Extension MZHG0JG Corn
Horizontal Gene Transfer	Unlikely to pose plant pest risk	Unlikely to pose plant pest risk	Unlikely to pose plant pest risk

The data and information in the present request for an extension of deregulation demonstrate that the conclusions reached for VCO-Ø1981-5 corn and DP-ØØ4114-3 corn also apply to MZHG0JG corn, and that, likewise, MZHG0JG corn does not pose a plant pest risk.

I.B. Benefits of MZHG0JG Corn

Since 1996, genetically modified herbicide-tolerant crops have helped to revolutionize weed management and have become an important tool in crop production practices. Herbicide-tolerant crops have enabled the implementation of weed management programs that have enhanced agricultural efficiency, improving yield and profitability for growers while reducing soil erosion and better protecting the environment. Growers have recognized their benefits and have made herbicide-tolerant crops the most rapidly adopted technology in the history of agriculture (Green 2012). This technology adoption and the resulting benefits are key contributors to agricultural sustainability, which will be critical to supporting an ever-expanding global population.

Upon commercialization, MZHG0JG corn will support agricultural efficiency by facilitating the introduction of stacked-trait corn varieties to the marketplace. For example, MZHG0JG corn can be combined, through traditional breeding methods, with insecticidal traits in other deregulated corn varieties that protect against yield loss from lepidopteran and/or coleopteran pests. These next-generation stacked-trait corn products will offer the ability to improve production efficiency, enhance grower choice, and maintain pest and weed control durability. Because MZHG0JG corn is tolerant to herbicides containing glyphosate and glufosinate-ammonium, its use will avoid having to combine these herbicide-tolerance traits from separate deregulated cultivars into a single variety by traditional breeding.

MZHG0JG corn will also facilitate grower compliance with U.S. Environmental Protection Agency (EPA) mandated refuge[2] requirements for corn varieties with insecticidal traits. The U.S. EPA has authorized the use of seed blend products[3] to facilitate grower compliance with requirements to incorporate the appropriate proportion of non insect-protected refuge corn when planting corn varieties that produce EPA-registered insecticidal proteins. While many commercial stacked-trait insect-protected corn varieties are tolerant to herbicides containing glyphosate and glufosinate-ammonium, not all non-insect-protected refuge seed varieties are also tolerant to both herbicides. Consequently, growers planting seed blend products will have more limited weed control options when the seed blend is not uniformly tolerant to the same herbicides. For this reason, MZHG0JG corn is also intended for use as the non insect-protected refuge component in seed blend products, offering growers the flexibility to spray their fields

[2]The portion of a field planted with non insect-protected seed to prevent or mitigate the development of insect resistance to a particular trait or traits.

[3] Seed blend products are those that incorporate a specific blend of insect-protected and non insect-protected seed. These products offer growers the convenience of planting their fields with traited seed and the required amount of refuge seed simultaneously.

with either glyphosate-based and/or glufosinate-ammonium-based herbicides, depending on their weed management needs and recommended control practices.

I.C. Regulatory Status of MZHG0JG Corn

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

MZHG0JG corn falls within the scope of the U.S. Food and Drug Administration's (FDA's) policy statement concerning regulation of products derived from new plant varieties, including those developed through biotechnology (FDA 1992). Accordingly, Syngenta has initiated the FDA consultation process by submitting a safety and nutritional assessment for MZHG0JG corn. Additional regulatory approvals that facilitate global trade in corn commodities will be sought on an as-needed basis.

The U.S. EPA has issued permanent exemptions from food and feed tolerances for both EPSPS and PAT proteins in all crops in the United States (U.S. EPA 2007a and b). The U.S EPA has established food and feed tolerances for corn commodities containing residues of glyphosate (U.S. EPA 2015a) and glufosinate-ammonium (U.S. EPA 2015b).

II. The Biology of Corn

Corn belongs to the Poaceae family and likely originates from southern Mexico. Domestication of corn can be traced back thousands of years and corn is one of the most widely studied crops today. It is cultivated extensively around the world, with the largest production in the U.S., China, Brazil, and Argentina. In the U.S., the area planted to corn for all purposes in 2014 was estimated at 91.6 million acres, representing the fifth-largest corn acreage in the U.S. since 1944 (USDA-NASS 2015c).

II.A. Overview of Corn Biology

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 this product extension request, 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

II.B. 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. Development of MZHG0JG Corn

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

III.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, NP2222, via *Agrobacterium tumefaciens*-mediated transformation, as described by Negrotto *et al*. (2000). By this method, genetic elements between the left and right border regions of the transformation plasmid (T-DNA) 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 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 transformation events that carried the 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.

III.B. 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. MZHG0JG corn was selected as the lead commercial candidate among several transformation events and underwent further field testing and development. Figure III–1 shows the steps in the development of MZHG0JG corn.

All shipments and field releases of MZHG0JG corn in the U.S. were carried out under USDA permits and notifications, which are listed in Appendix A.

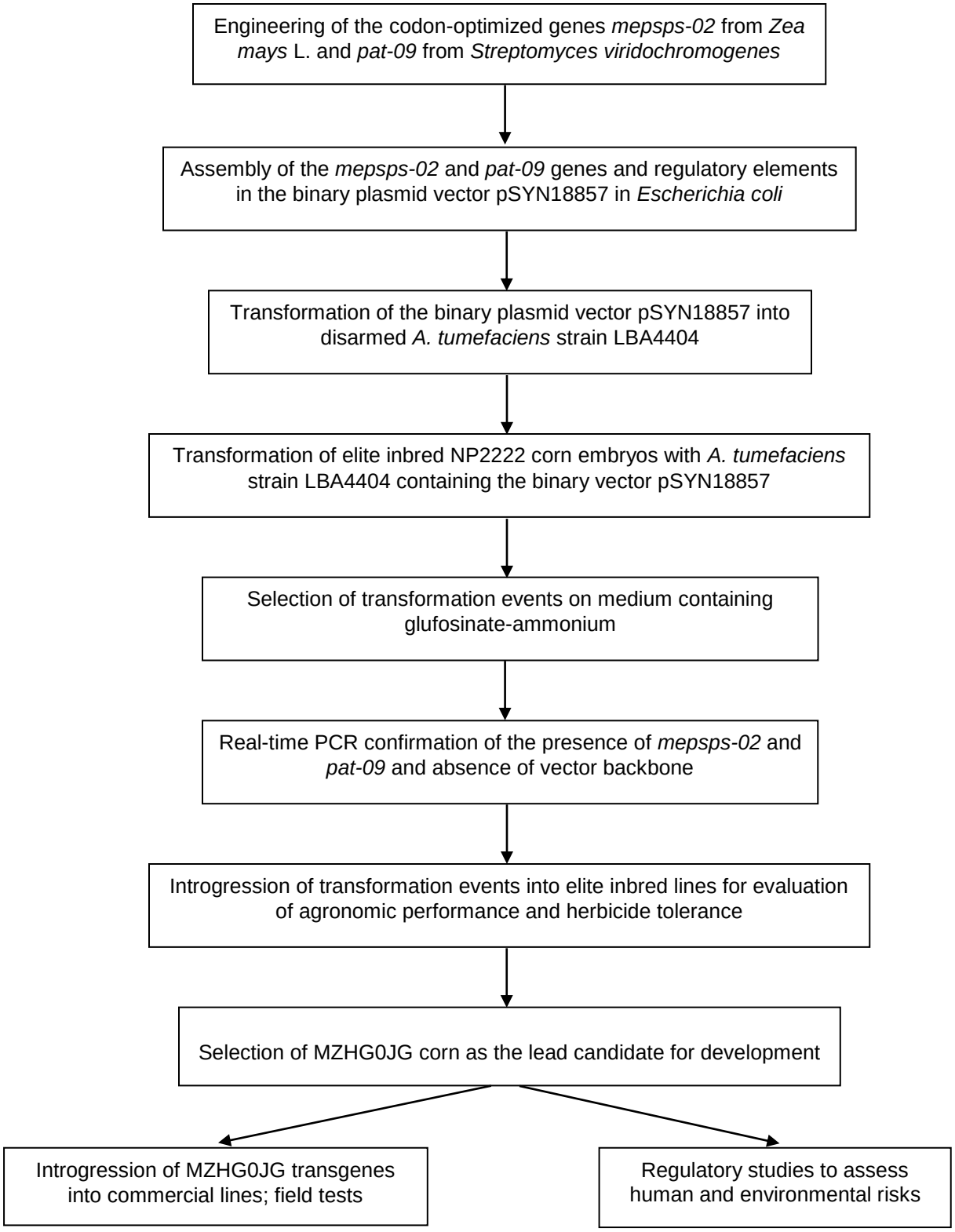


Figure III–1. Steps in the development of MZHG0JG corn

III.C. Production of Test and Control Seed

Production of all MZHG0JG corn and nontransgenic control corn seed lots used in the studies described in this extension request was carried out under controlled and isolated conditions under the direction of Syngenta breeders and field researchers. Figure III–2 shows the breeding pedigree of MZHG0JG corn seed materials. Nontransgenic, near-isogenic corn was used as a control material in all regulatory studies. Nontransgenic control corn seed lots were produced at the same time and location as the MZHG0JG corn seed lots.

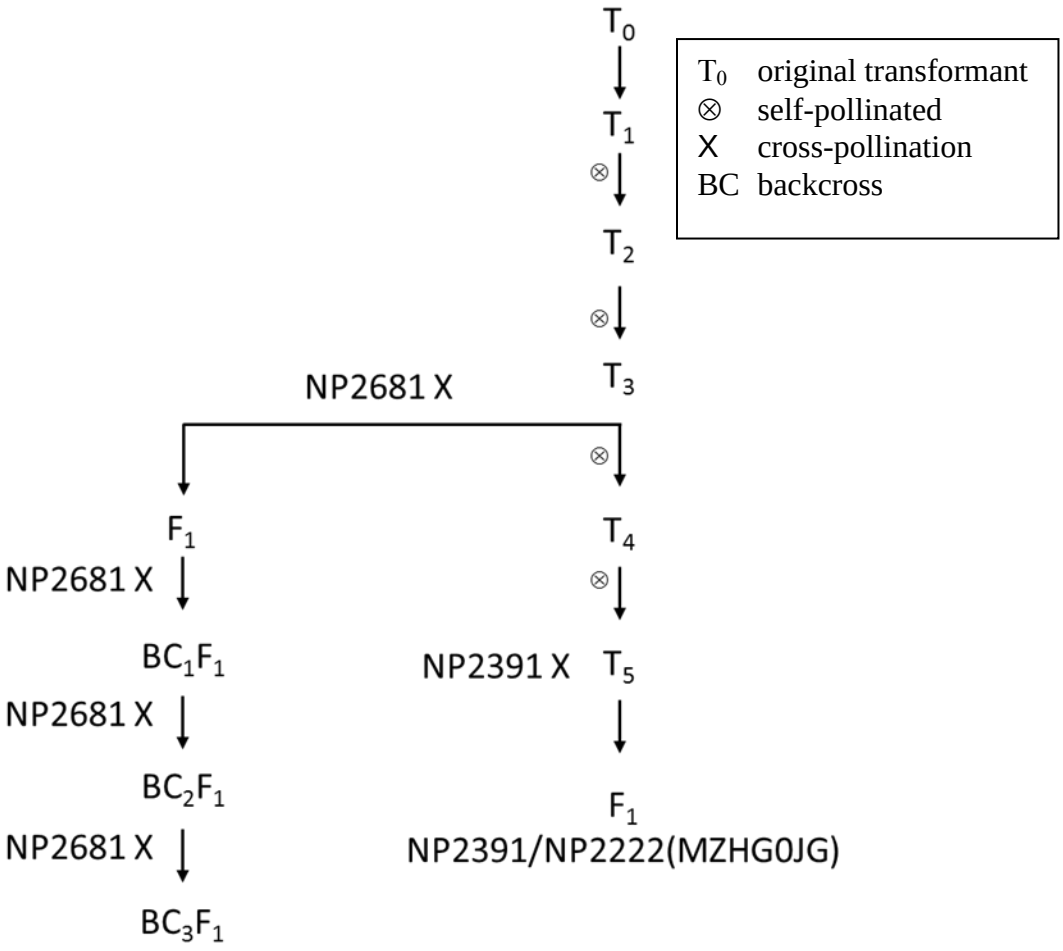


Figure III–2. Pedigree of the MZHG0JG corn plant materials used in regulatory studies

The transformation recipient line was Syngenta proprietary corn inbred NP2222. NP2681 and NP2391 are Syngenta proprietary corn inbred lines used in further breeding of MZHG0JG corn.

III.D. Quality Control of Test and Control Materials

All MZHG0JG and nontransgenic, near-isogenic control corn seed lots used in regulatory studies were analyzed by real-time polymerase chain reaction for the presence or absence of MZHG0JG deoxyribonucleic acid (DNA) and the absence of adventitious DNA from other transformation

events. All MZHG0JG corn seed lots were confirmed to contain MZHG0JG corn-specific DNA. 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 regulated transgenic corn products under development at Syngenta or from other transgenic corn products (e.g., commercial varieties) for which testing methodology is available.

IV. Donor Genes and Regulatory Sequences

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 the 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–1, and each genetic element in the transformation plasmid is described in Table IV–1.

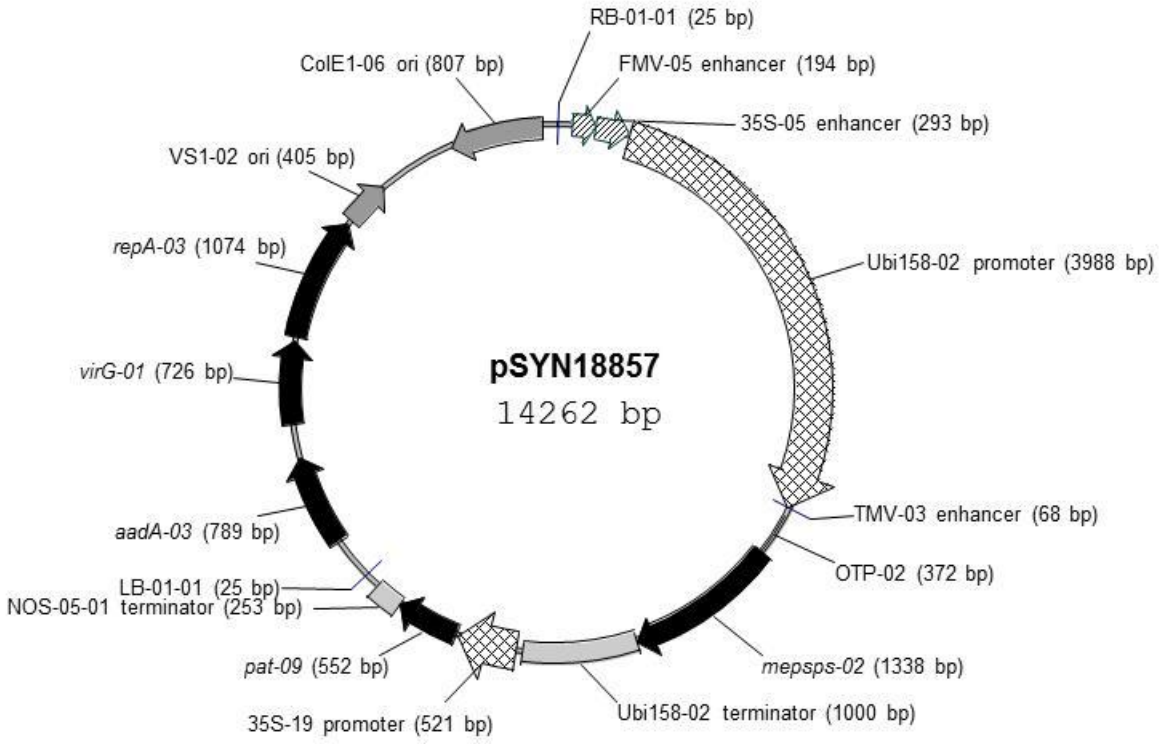


Figure IV–1. Plasmid map for the vector pSYN18857

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.
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) sequence 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.

Continued

Genetic element	Size (bp)	Position	Description
***pat-09* cassette**			
35S-19 promoter	521	7461 to 7981	Promoter region of CMV (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 gene *pat* 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).
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.

Continued

Genetic element	Size (bp)	Position	Description
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).

V. Genetic Characterization of MZHG0JG Corn

An extensive genetic characterization of the DNA insert in MZHG0JG corn was performed. 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. Nucleotide sequencing 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.

Sections V.A. through V.D., below, describe the design, results, and conclusions of each genetic characterization study. The general conclusions of the genetic characterization studies are summarized in Section V.E.

V.A. Characterization of the MZHG0JG Corn DNA Insert by Southern Blot Analysis

Southern blot analyses were performed to characterize the transgenic DNA 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 (1) the genetic integrity of the insert in the MZHG0JG T_2 generation used in further breeding to create commercial MZHG0JG corn lines and the MZHG0JG corn materials used in regulatory and safety studies and (2) the stable inheritance of the MZHG0JG insert over five generations of MZHG0JG corn.

V.A.1. 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 (Figure III–2). 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 materials 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 V–1 and Table V–1). 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 V-1 and Table V–1) were not expected to be transferred to the plant cell or integrated into the plant genome during T-DNA transfer.

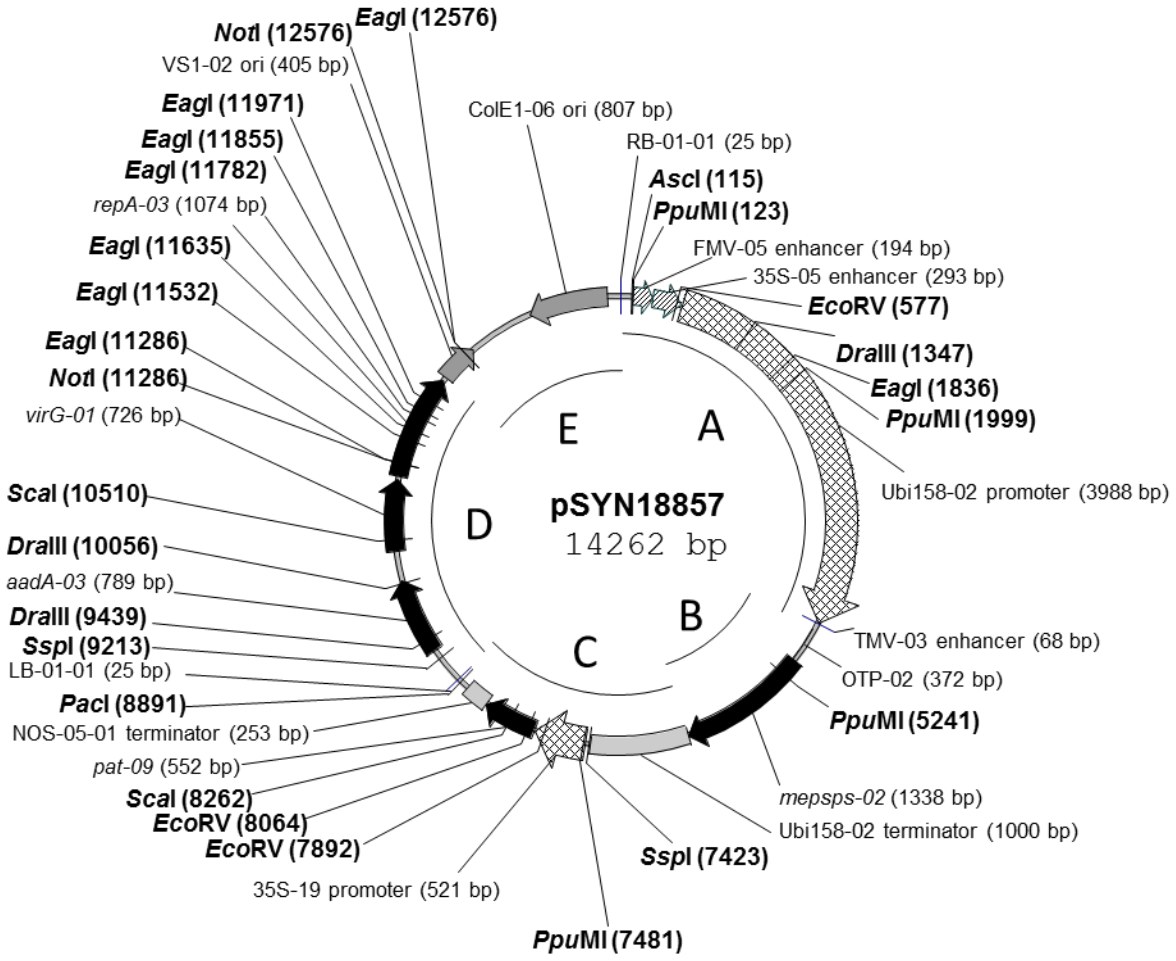


Figure V–1. Map of plasmid pSYN18857 indicating the restriction sites and probes used in the MZHG0JG corn Southern blot analyses

Table V–1. Probes used in the MZHG0JG corn 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

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 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.

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 V–2 shows the calculated amounts of the positive assay controls used in each Southern blot analysis.

Table V–2. 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 numbers 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 V–1.

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 V–3 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 V–3). 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 (the negative control). 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 were the result of cross-hybridization of the T-DNA-specific probe sequence with the endogenous corn sequence.

Table V-3. 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.

V.A.1.a. Results of Southern blot analysis with T-DNA-specific probe 1

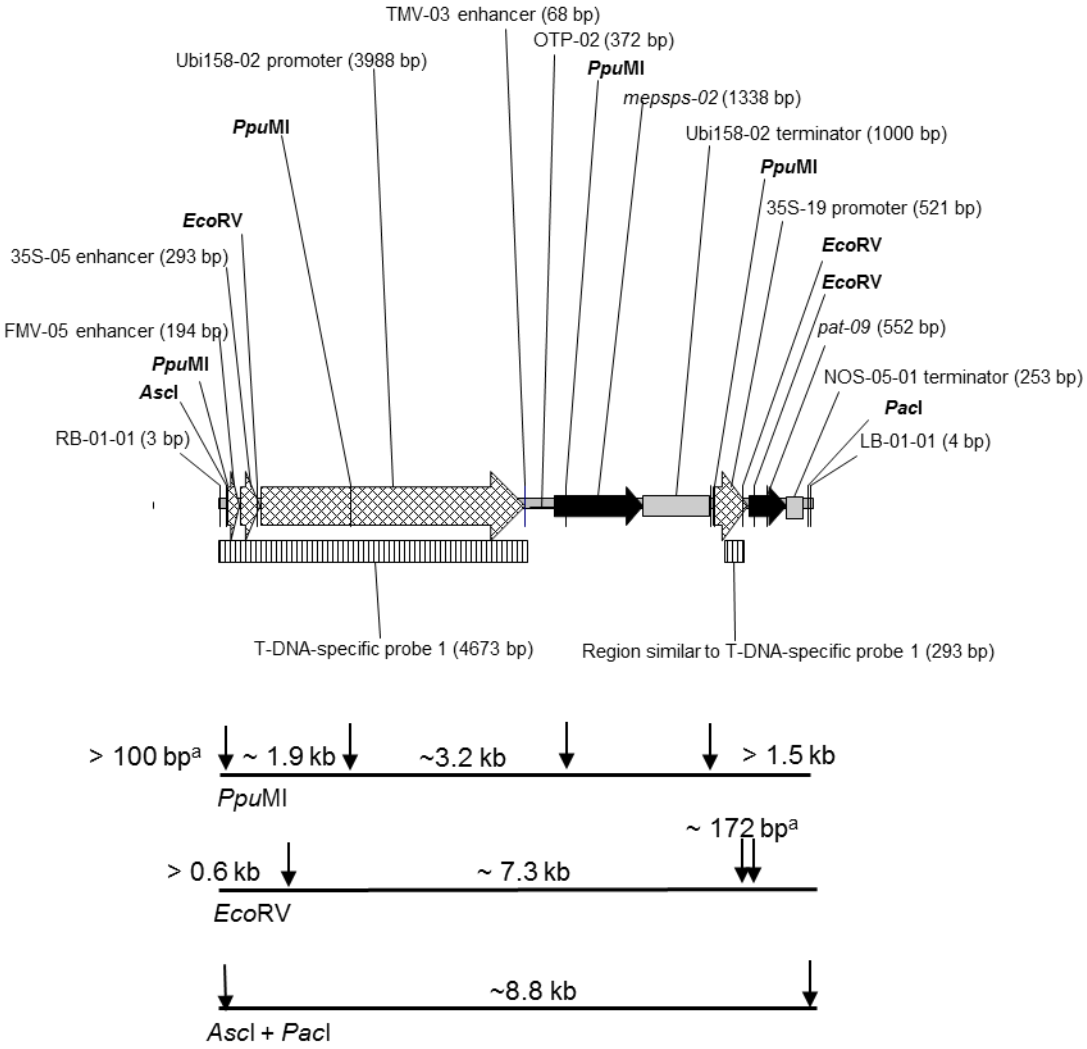
Figure V–2 shows the digestion strategy used with T-DNA-specific probe 1, Table V–4 shows the insert-specific hybridization bands expected and observed in Southern blot analyses of MZHG0JG DNA with T-DNA-specific probe 1, and Figures V–3 through V–5 show the results of the Southern blot analyses with 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 V–3, Lanes 2 through 7). These bands were absent from the lanes containing DNA from the nontransgenic NP2222, NP2391, or NP2222/NP2391 corn (Figure V–3, 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 V–3, 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 V–4, Lanes 2 through 7). These bands were absent from the lanes containing DNA from nontransgenic NP2222, NP2391, or NP2222/NP2391 corn (Figure V–4, 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 V–4, 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 V–5, Lanes 2 through 7). This band was absent from the lanes containing DNA from nontransgenic NP2222, NP2391, or NP2222/NP2391 corn (Figure V–5, 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 V–5, Lanes 11 and 12).

In the analyses with *Ppu*MI digestion (Figure V–3), 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 (Figure V–4), 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 V–2. 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 insert

Table V–4. 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

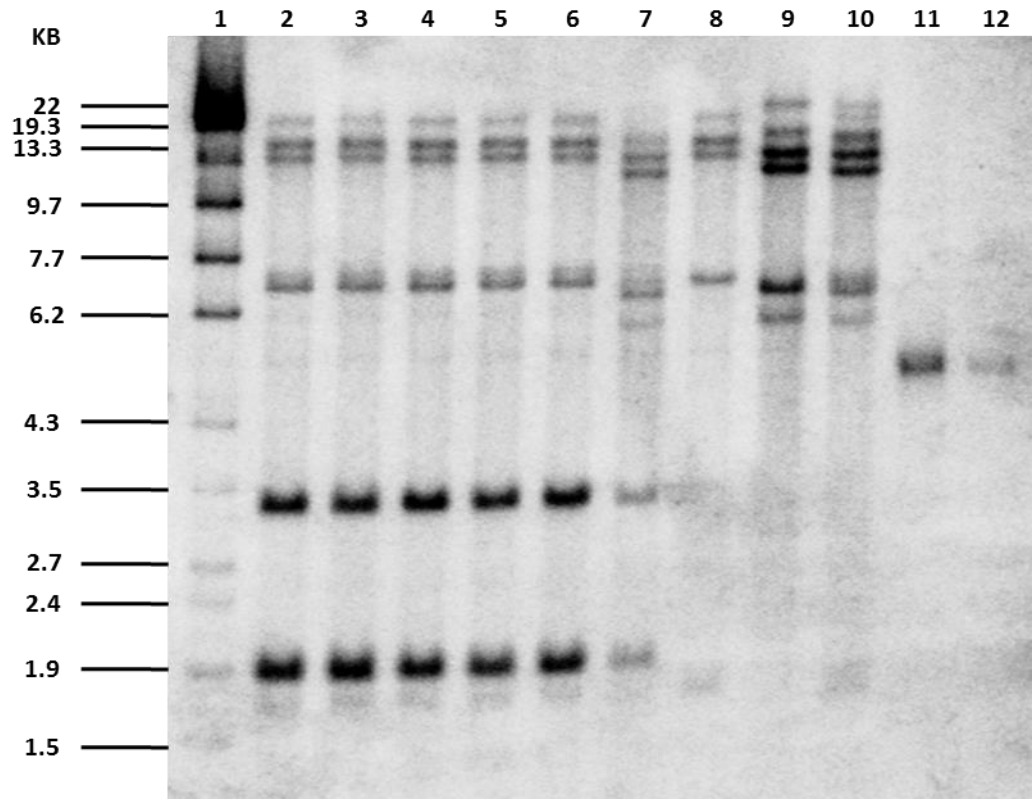
Figure & lane	Source of DNA	Restriction enzymes	Expected no. of bands[a]	Approximate band size (kb)	
				Expected	Observed[a]
V-3, 2	MZHG0JG T_2 (ear 4) corn	*Ppu*MI	3	>1.5	1.9
				1.9	3.2
				3.2	7.2
V-3, 3	MZHG0JG T_2 (ear 35) corn	*Ppu*MI	3	>1.5	1.9
				1.9	3.2
				3.2	7.2
V-3, 4	MZHG0JG T_3 corn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

Continued

Figure & lane	Source of DNA	Restriction enzymes	Expected no. of bands[a]	Approximate band size (kb)	
				Expected	Observed[a]
V-4, 2	MZHG0JG T_2 (ear 4) corn	*Eco*RV	2	>0.6	2.7
				7.3	7.3
V-4, 3	MZHG0JG T_2 (ear 35) corn	*Eco*RV	2	>0.6	2.7
				7.3	7.3
V-4, 4	MZHG0JG T_3 corn	*Eco*RV	2	>0.6	2.7
				7.3	7.3
V-4, 5	MZHG0JG T_4 corn	*Eco*RV	2	>0.6	2.7
				7.3	7.3
V-4, 6	MZHG0JG T_5 corn	*Eco*RV	2	>0.6	2.7
				7.3	7.3
V-4, 7	MZHG0JG F_1 corn	*Eco*RV	2	>0.6	2.7
				7.3	7.3
V-4, 8	NP2222 corn (negative control)	*Eco*RV	0	N/A	N/A
V-4, 9	NP2391 corn (negative control)	*Eco*RV	0	N/A	N/A
V-4, 10	NP2222/NP2391 corn (negative control)	*Eco*RV	0	N/A	N/A
V-4, 11	1-copy positive control	N/A	1	4.7	4.7
V-4, 12	1/7-copy positive control	N/A	1	4.7	4.7
V-5, 2	MZHG0JG T_2 (ear 4) corn	*Asc*I + *Pac*I	1	8.8	8.8
V-5, 3	MZHG0JG T_2 (ear 35) corn	*Asc*I + *Pac*I	1	8.8	8.8
V-5, 4	MZHG0JG T_3 corn	*Asc*I + *Pac*I	1	8.8	8.8
V-5, 5	MZHG0JG T_4 corn	*Asc*I + *Pac*I	1	8.8	8.8
V-5, 6	MZHG0JG T_5 corn	*Asc*I + *Pac*I	1	8.8	8.8
V-5, 7	MZHG0JG F_1 corn	*Asc*I + *Pac*I	1	8.8	8.8
V-5, 8	NP2222 corn (negative control)	*Asc*I + *Pac*I	0	N/A	N/A
V-5, 9	NP2391 corn (negative control)	*Asc*I + *Pac*I	0	N/A	N/A
V-5, 10	NP2222/NP2391 corn (negative control)	*Asc*I + *Pac*I	0	N/A	N/A
V-5, 11	1-copy positive control	N/A	1	4.7	4.7
V-5, 12	1/7-copy positive control	N/A	1	4.7	4.7

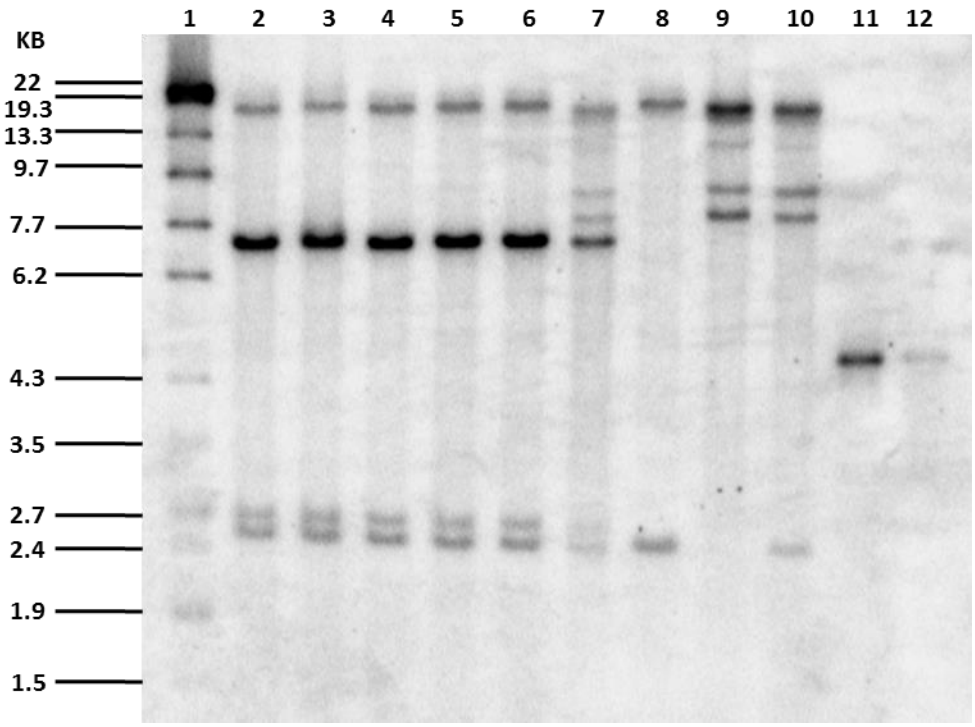
N/A = not applicable.

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



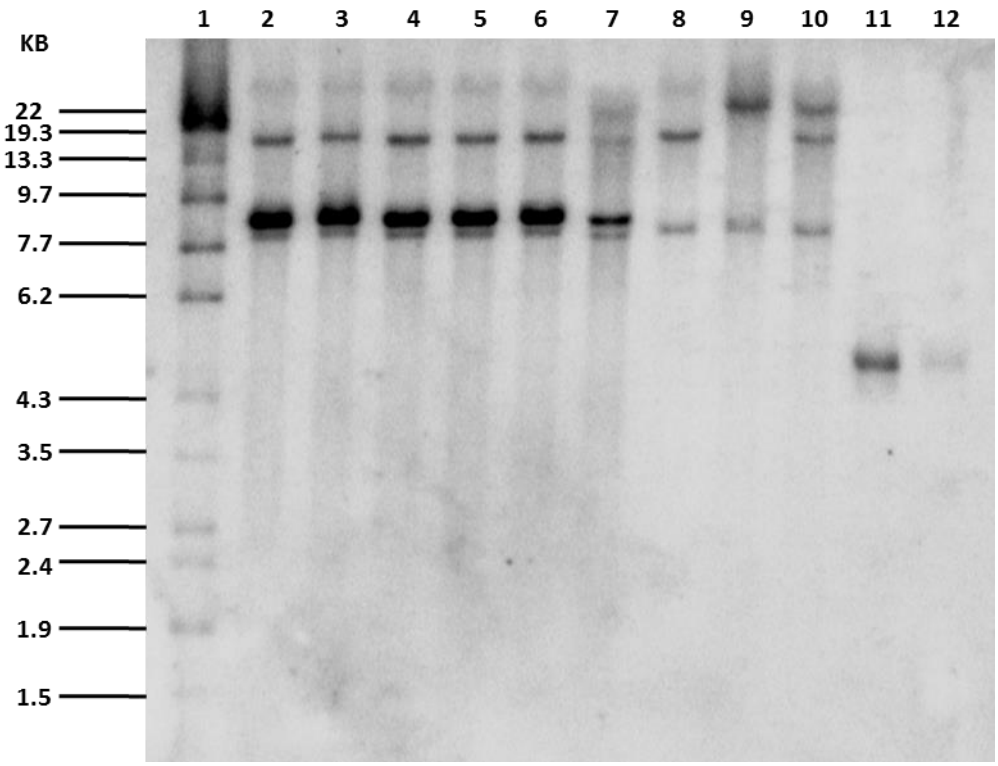
Lane 1 = molecular weight markers
Lane 2 = MZHG0JG T_2 (ear 4) corn
Lane 3 = MZHG0JG T_2 (ear 35) corn
Lane 4 = MZHG0JG T_3 corn
Lane 5 = MZHG0JG T_4 corn
Lane 6 = MZHG0JG T_5 corn
Lane 7 = MZHG0JG F_1 corn
Lane 8 = NP2222 corn (negative control)
Lane 9 = NP2391 corn (negative control)
Lane 10 = NP2222/NP2391 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 V–3. Southern blot analysis of MZHG0JG corn with the 4.7-kb T-DNA-specific probe 1 and restriction enzyme *Ppu*MI



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 V–4. 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 V–5. Southern blot analysis of MZHG0JG corn with the 4.7-kb T-DNA-specific probe 1 and restriction enzymes *Asc*I + *Pac*I

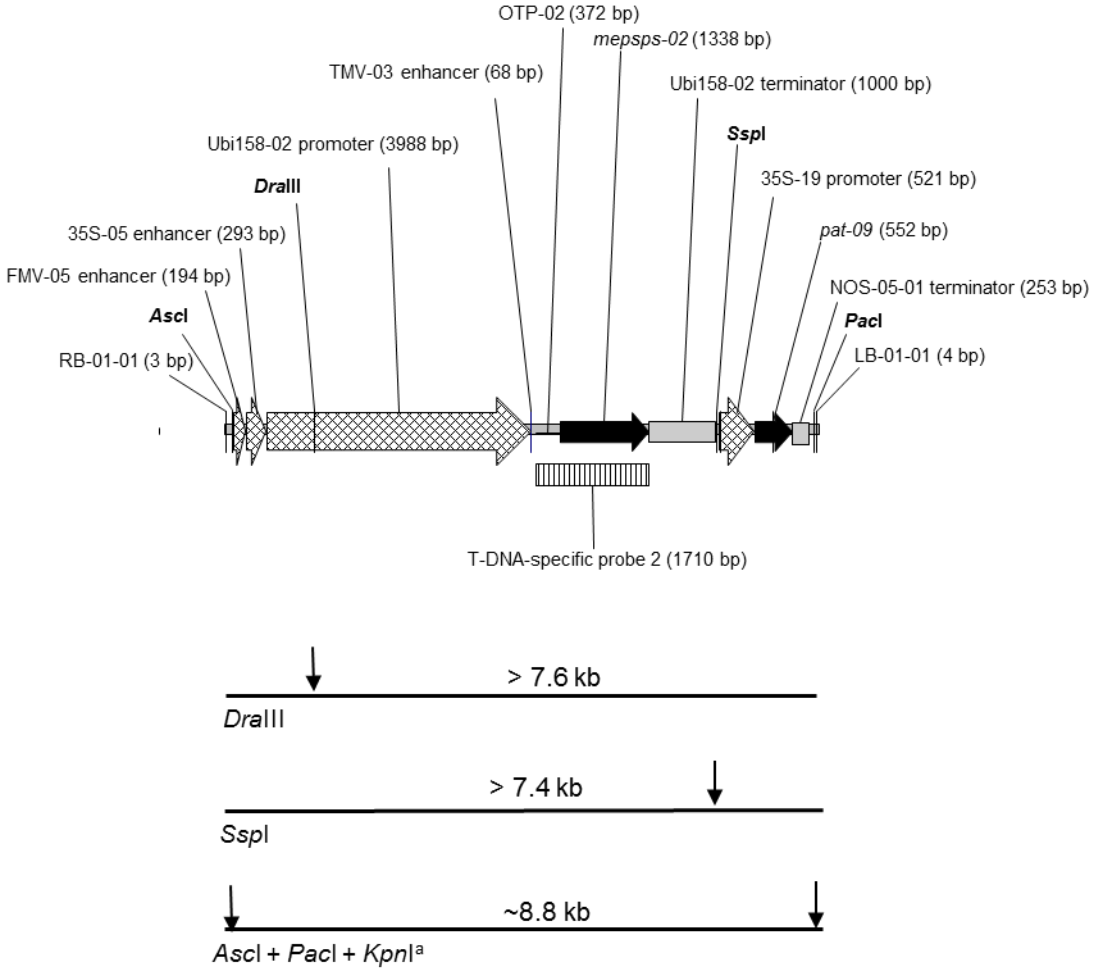
V.A.1.b. Results of Southern blot analysis with T-DNA-specific probe 2

Figure V–6 shows the digestion strategy used with T-DNA-specific probe 2, Table V–5 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 V–7 through V-9 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 V–7, Lanes 2 through 7). This band was absent from the lanes containing DNA from the nontransgenic NP2222, NP2391, or NP2222/NP2391 corn (Figure V–7, 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 V–7, 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 V–8, Lanes 2 through 7). This band was absent from the lanes containing DNA from nontransgenic NP2222, NP2391, or NP2222/NP2391 corn (Figure V–8, 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 V–8, 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 V–9, Lanes 2 through 7). This band was absent from the lanes containing DNA from nontransgenic NP2222, NP2391, or NP2222/NP2391 corn (Figure V–9, 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 V–9, 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 V–6. 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 insert

Table V–5. 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

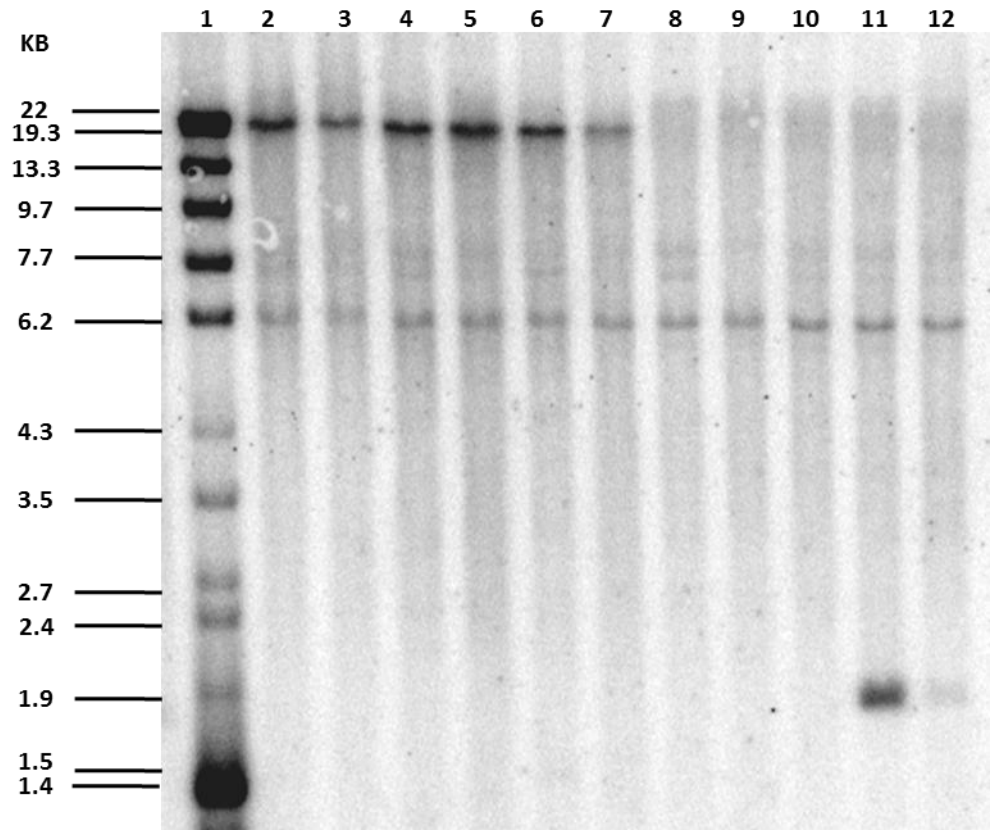
Figure & lane	Source of DNA	Restriction enzymes	Expected no. of bands[a]	Approximate band size (kb)	
				Expected	Observed[a]
V-7, 2	MZHG0JG T_2 (ear 4) corn	*Dra*III	1	>7.6	20
V-7, 3	MZHG0JG T_2 (ear 35) corn	*Dra*III	1	>7.6	20
V-7, 4	MZHG0JG T_3 corn	*Dra*III	1	>7.6	20
V-7, 5	MZHG0JG T_4 corn	*Dra*III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

Continued

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

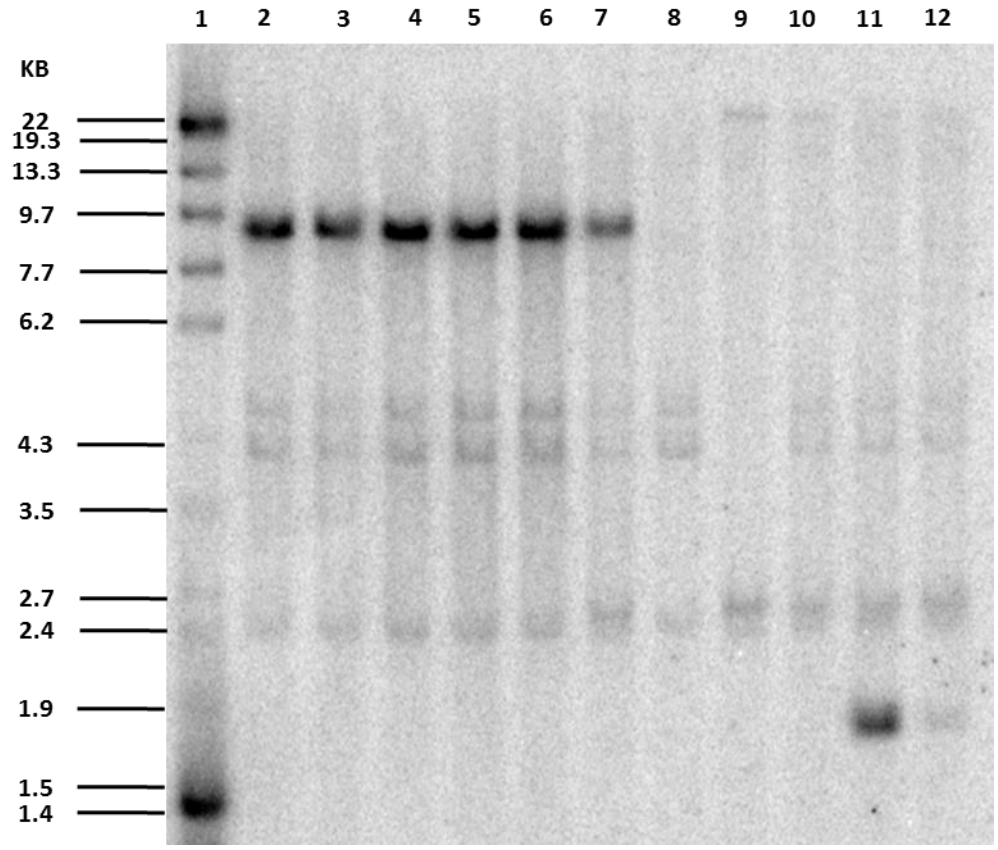
N/A = not applicable.

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



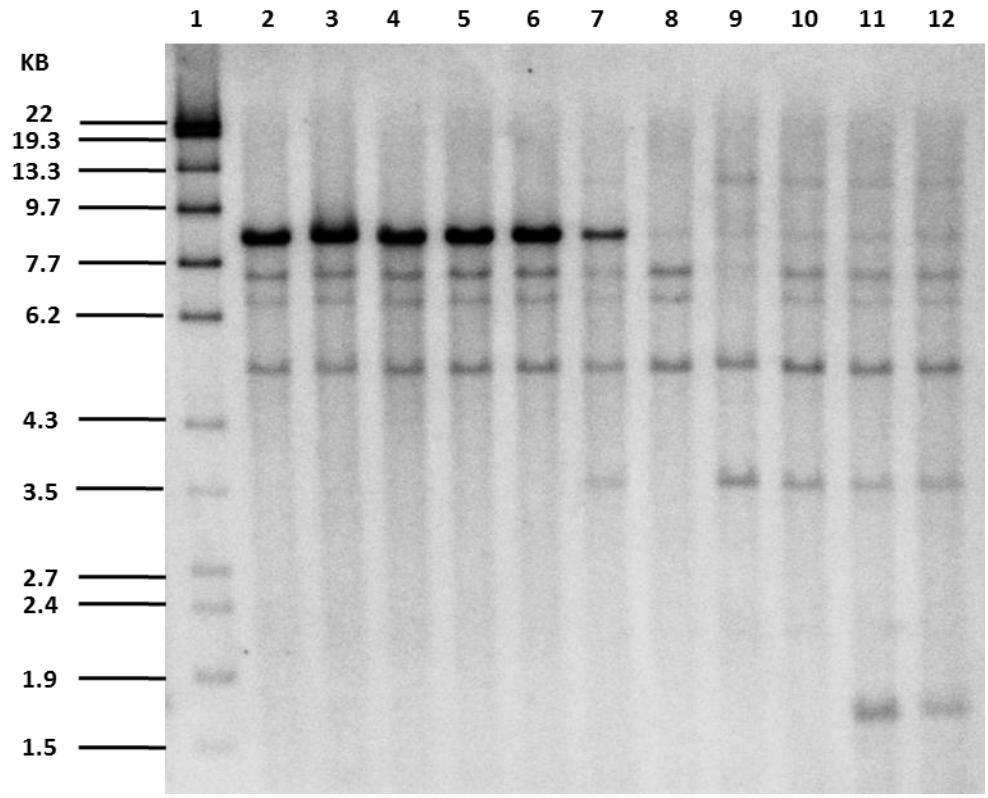
Lane 1 = molecular weight markers
Lane 2 = MZHG0JG T_2 (ear 4) corn
Lane 3 = MZHG0JG T_2 (ear 35) corn
Lane 4 = MZHG0JG T_3 corn
Lane 5 = MZHG0JG T_4 corn
Lane 6 = MZHG0JG T_5 corn
Lane 7 = MZHG0JG F_1 corn
Lane 8 = NP2222 corn (negative control)
Lane 9 = NP2391 corn (negative control)
Lane 10 = NP2222/NP2391 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 V–7. 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 V–8. 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 V–9. 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

V.A.1.c. Results of Southern blot analysis with T-DNA-specific probe 3

Figure V–10 shows the digestion strategy used with T-DNA-specific probe 3, Table V–6 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 V-11 through V–13 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 V–11, Lanes 2 through 7). These bands were absent from the lanes containing DNA from the nontransgenic NP2222, NP2391, or NP2222/NP2391 corn (Figure V–11, 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 V–11, 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 V–12, Lanes 2 through 7). These bands were absent from the lanes containing DNA from nontransgenic NP2222, NP2391, or NP2222/NP2391 corn (Figure V–12, 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 V–12, 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 V–13, Lanes 2 through 7). This band was absent in the lanes containing DNA from nontransgenic NP2222, NP2391, or NP2222/NP2391 corn (Figure V–13, 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 V–13, Lanes 11 and 12).

In the analysis with *Eag*I digestion (Figure V–11), 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 (Figure V–12), 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.

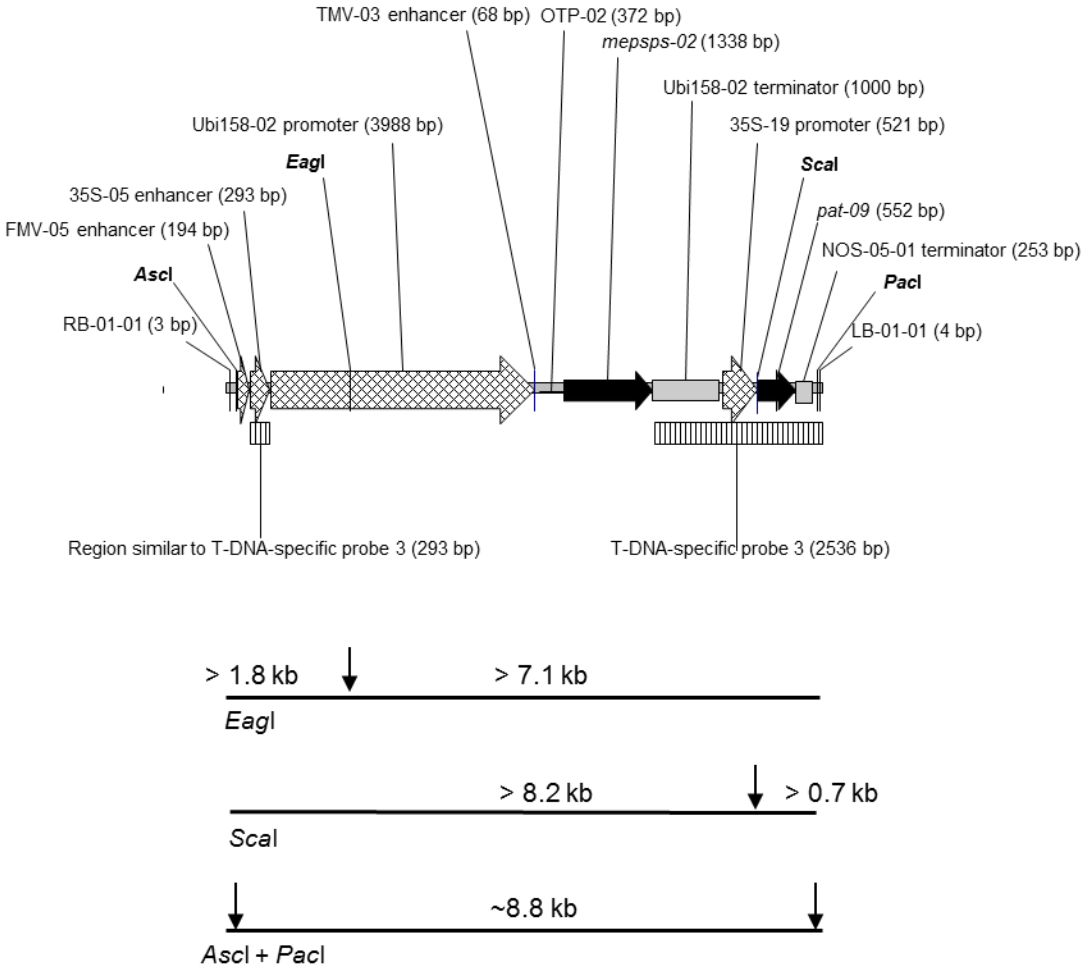


Figure V–10. Locations of the 2.6-kb T-DNA-specific probe 3 and the restriction sites *Eag*I, *Sca*I, and *Asc*I + *Pac*I in the MZHG0JG insert

Table V–6. 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

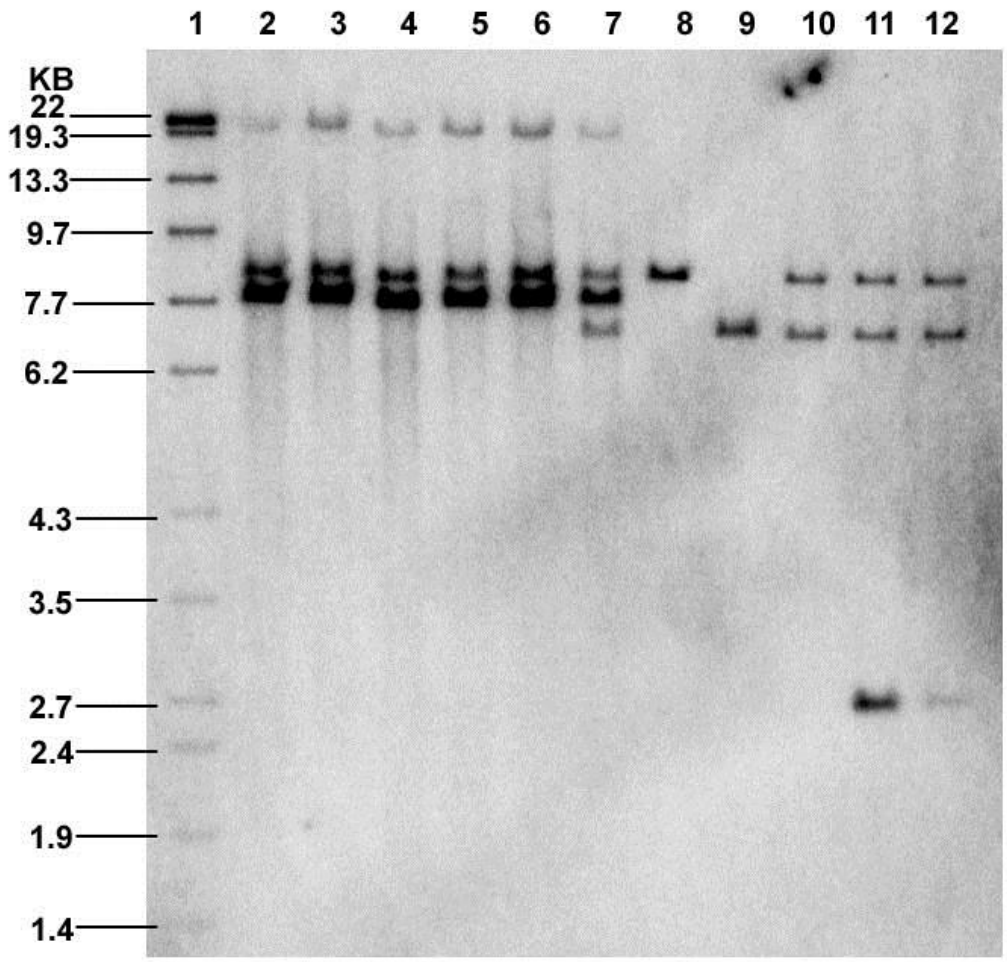
Figure & lane	Source of DNA	Restriction enzymes	Expected no. of bands[a]	Approximate band size (kb)	
				Expected	Observed[a]
V-11, 2	MZHG0JG T_2 (ear 4) corn	*Eag*I	2	>1.8	7.7
				>7.1	20
V-11, 3	MZHG0JG T_2 (ear 35) corn	*Eag*I	2	>1.8	7.7
				>7.1	20
V-11, 4	MZHG0JG T_3 corn	*Eag*I	2	>1.8	7.7
				>7.1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

Continued

Figure & lane	Source of DNA	Restriction enzymes	Expected no. of bands[a]	Approximate band size (kb)	
				Expected	Observed[a]
V-13, 2	MZHG0JG T_2 (ear 4) corn	*Asc*I + *Pac*I	1	8.8	8.8
V-13, 3	MZHG0JG T_2 (ear 35) corn	*Asc*I + *Pac*I	1	8.8	8.8
V-13, 4	MZHG0JG T_3 corn	*Asc*I + *Pac*I	1	8.8	8.8
V-13, 5	MZHG0JG T_4 corn	*Asc*I + *Pac*I	1	8.8	8.8
V-13, 6	MZHG0JG T_5 corn	*Asc*I + *Pac*I	1	8.8	8.8
V-13, 7	MZHG0JG F_1 corn	*Asc*I + *Pac*I	1	8.8	8.8
V-13, 8	NP2222 corn (negative control)	*Asc*I + *Pac*I	0	N/A	N/A
V-13, 9	NP2391 corn (negative control)	*Asc*I + *Pac*I	0	N/A	N/A
V-13, 10	NP2222/NP2391 corn (negative control)	*Asc*I + *Pac*I	0	N/A	N/A
V-13, 11	1-copy positive control	*Asc*I + *Pac*I	1	2.6	2.6
V-13, 12	1/7-copy positive control	*Asc*I + *Pac*I	1	2.6	2.6

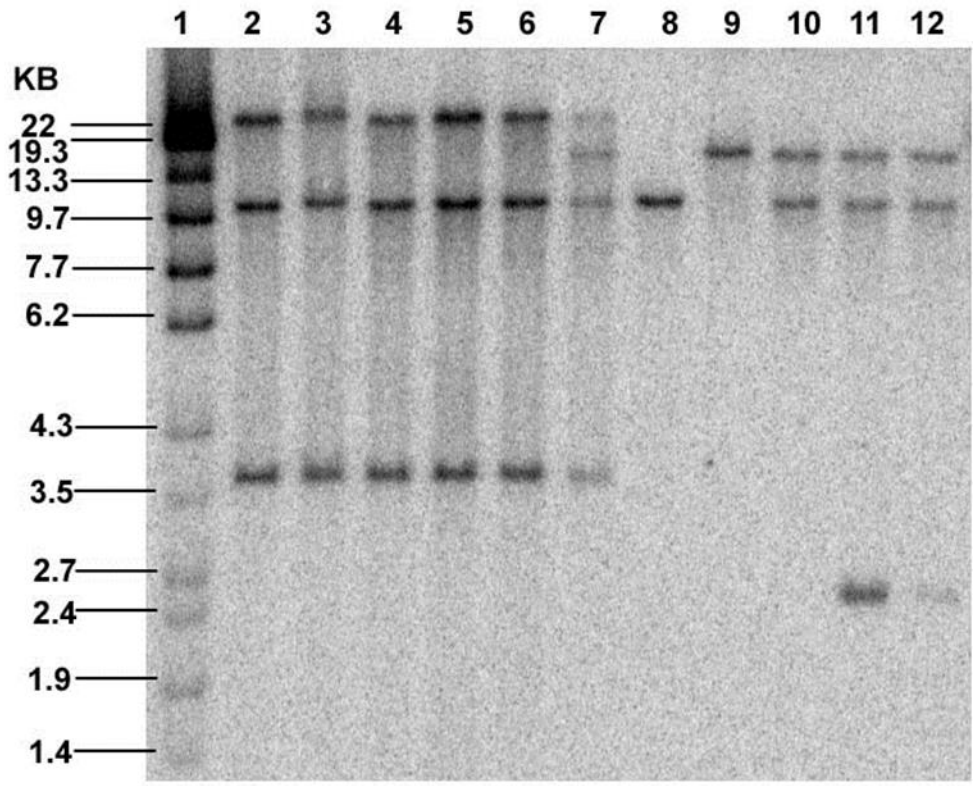
N/A = not applicable.

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



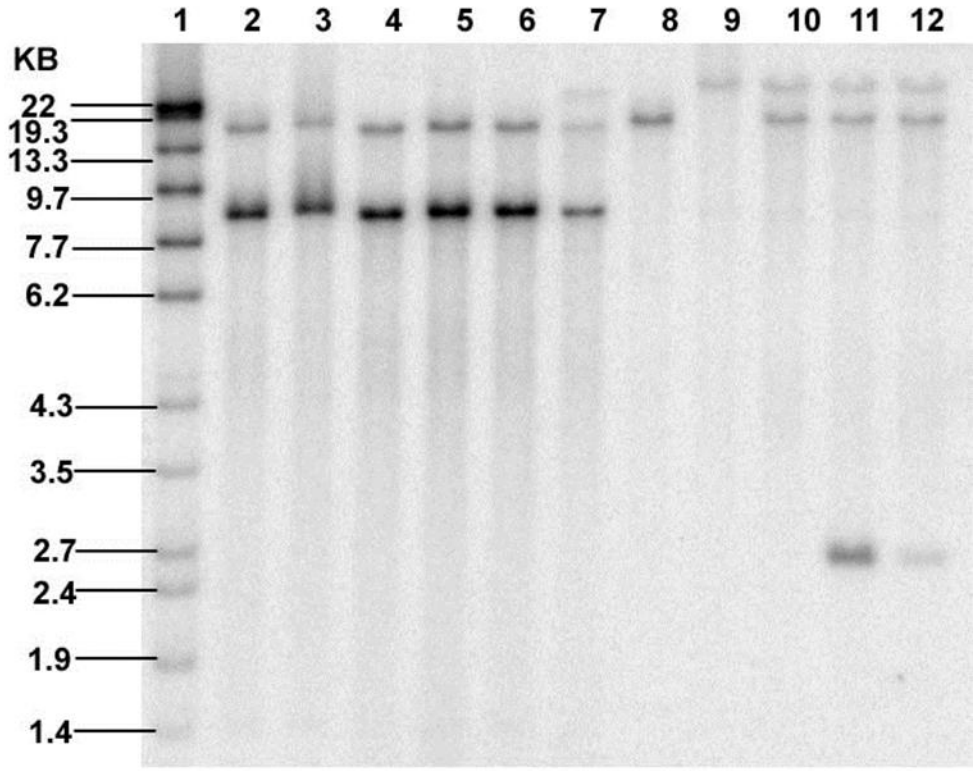
Lane 1 = molecular weight markers
Lane 2 = MZHG0JG T_2 (ear 4) corn
Lane 3 = MZHG0JG T_2 (ear 35) corn
Lane 4 = MZHG0JG T_3 corn
Lane 5 = MZHG0JG T_4 corn
Lane 6 = MZHG0JG T_5 corn
Lane 7 = MZHG0JG F_1 corn
Lane 8 = NP2222 corn (negative control)
Lane 9 = NP2391 corn (negative control)
Lane 10 = NP2222/NP2391 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 V–11. Southern blot analysis of MZHG0JG corn with the 2.6-kb T-DNA-specific probe 3 and restriction enzyme *Eag*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 V–12. 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 V–13. Southern blot analysis of MZHG0JG corn with the 2.6-kb T-DNA-specific probe 3 and restriction enzymes *Asc*I + *Pac*I

V.A.1.d. Results of Southern blot analysis with plasmid-backbone-specific probe 1

Figure V–14 shows the digestion strategy used with backbone-specific probe 1 and Figures V-15 through V–17 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 V–15 through V–17, Lanes 2 through 7) or in the lanes containing DNA from nontransgenic NP2222, NP2391, and NP2222/NP2391 corn (Figures V–15 through V–17, 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 V–15 through V–17, Lanes 11 and 12), as expected.

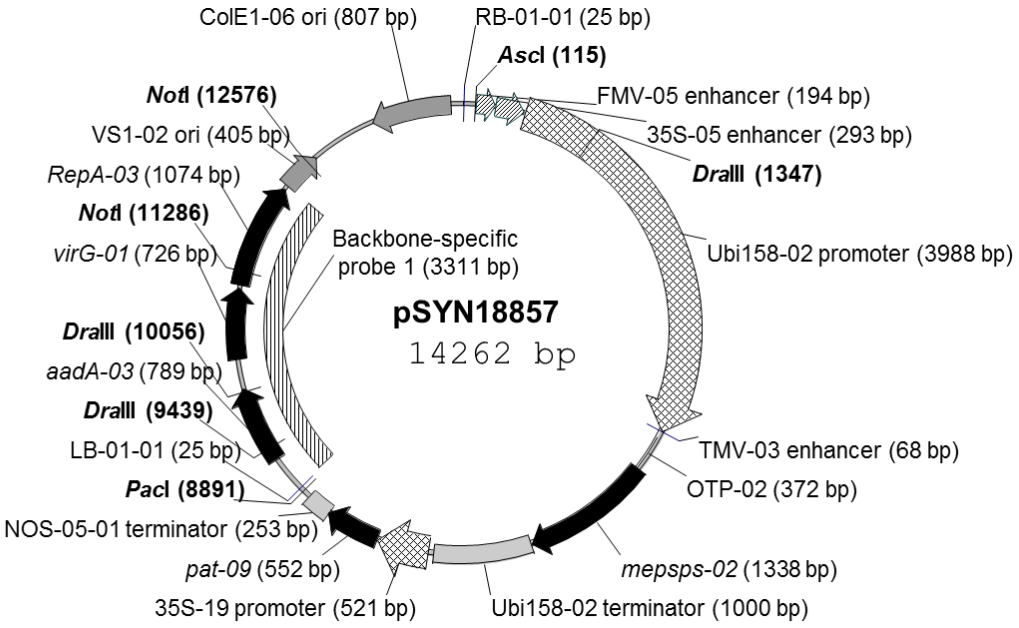
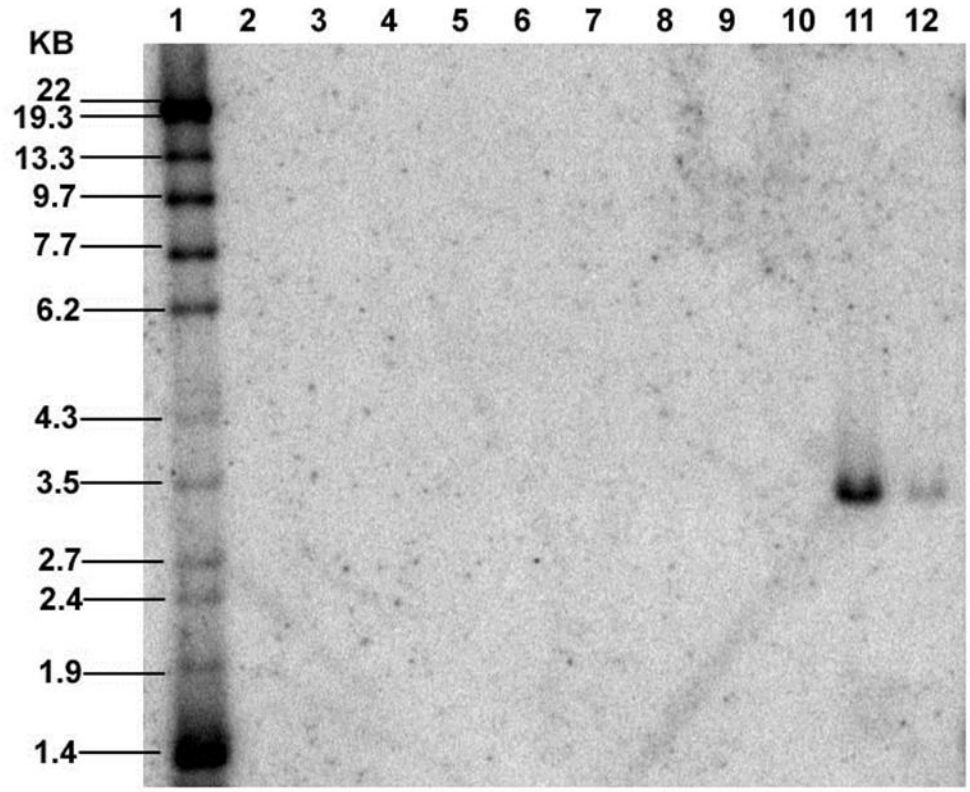
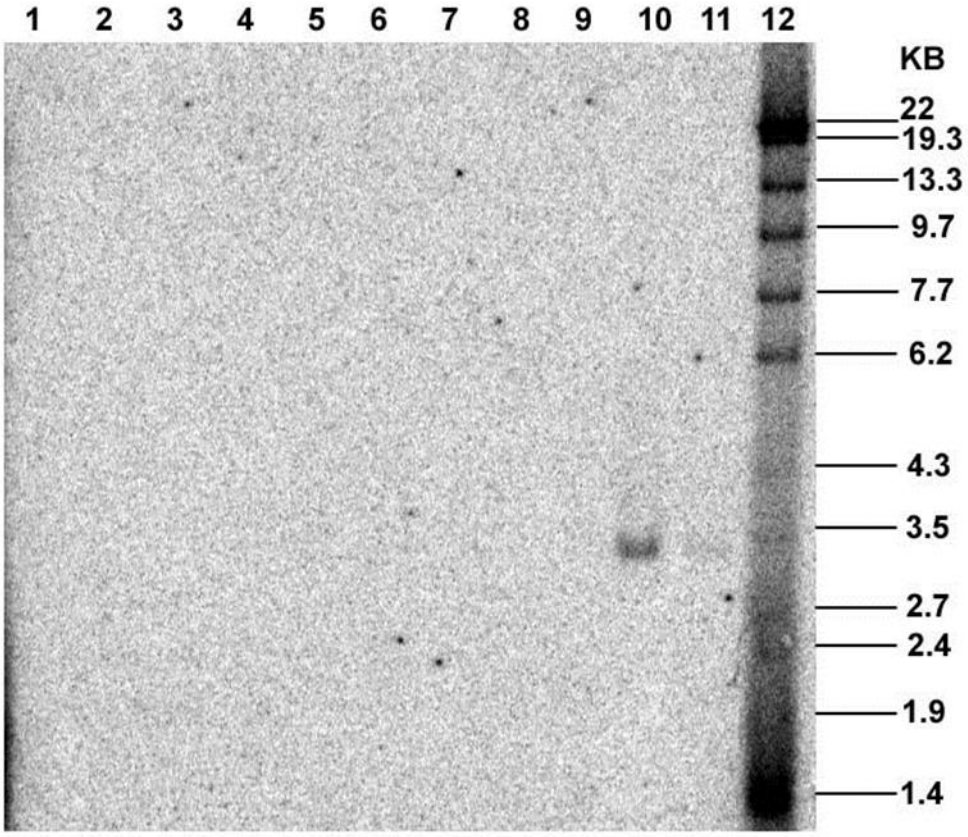


Figure V–14. Locations of the 3.3-kb backbone-specific probe 1 and the restriction sites *Dra*III, *Not*I, and *Asc*I + *Pac*I in the transformation plasmid pSYN18857



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 V–15. 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 T2 (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)

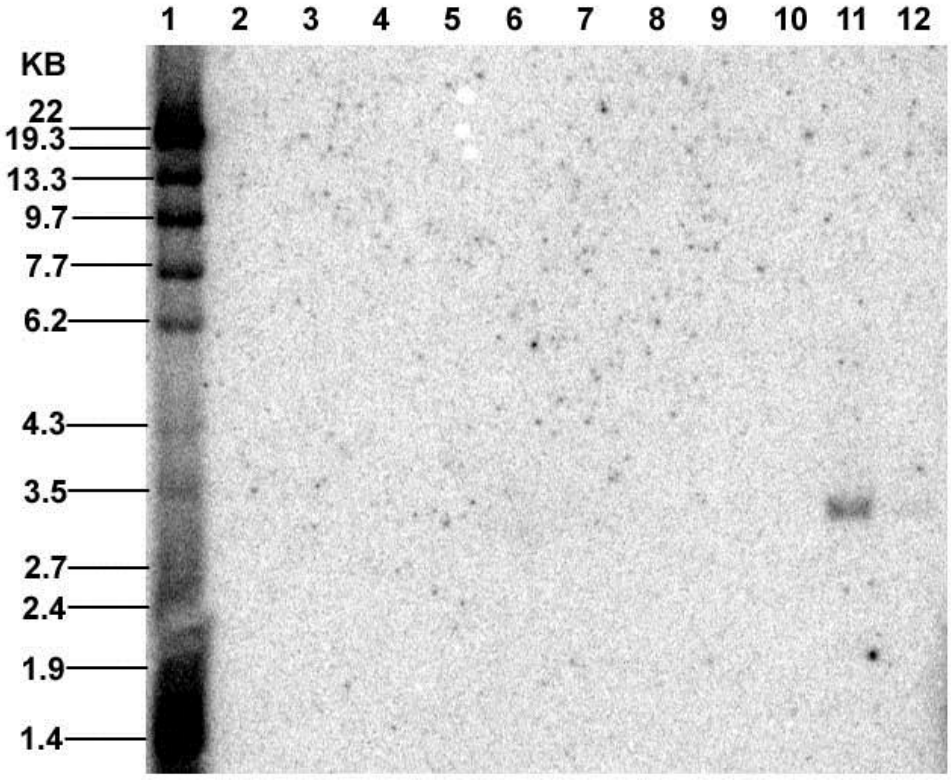
Lane 10 = 1-copy positive control (NP2222/NP2391 corn + 4.65 pg of backbone-specific fragment 1)

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 V–16. 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 11 may not be visible on the printed copy.

Figure V–17. Southern blot analysis of MZHG0JG corn with the 3.3-kb plasmid pSYN18857 backbone-specific probe 1 and restriction enzymes *Asc*I + *Pac*I

V.A.1.e. Results of Southern blot analysis with plasmid-backbone-specific probe 2

Figure V–18 shows the digestion strategy used with backbone-specific probe 2 and Figures V-19 through V–21 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 V–19 through V–21, Lanes 2 through 7) or in the lanes containing DNA from nontransgenic NP2222, NP2391, and NP2222/NP2391 corn (Figures V–19 through V–21, Lanes 8 through 10). One band of approximately 2.1 kb was observed in the lanes containing the 1-copy and 1/7-copy positive controls (Figures V–19 through V–21, Lanes 11 and 12), as expected.

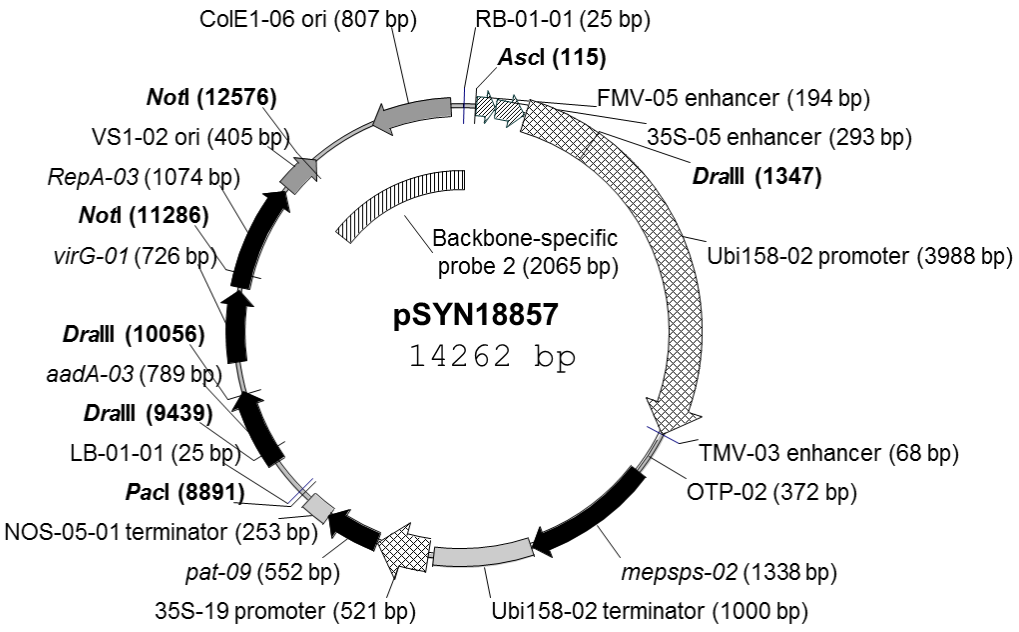
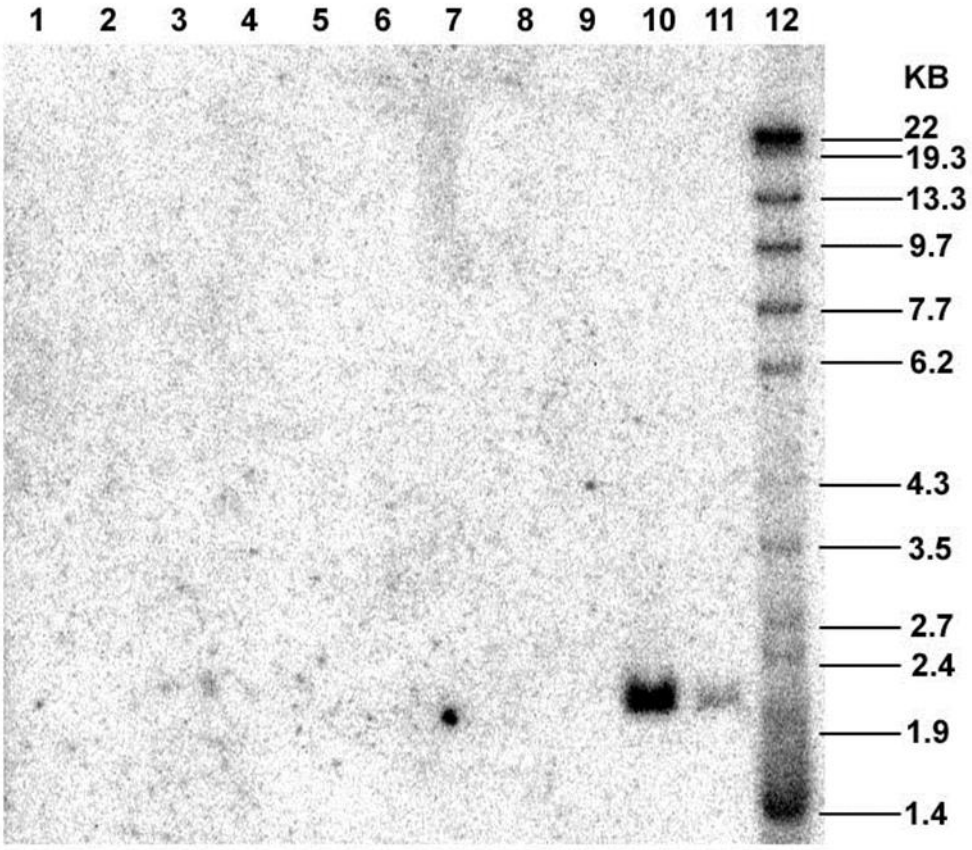
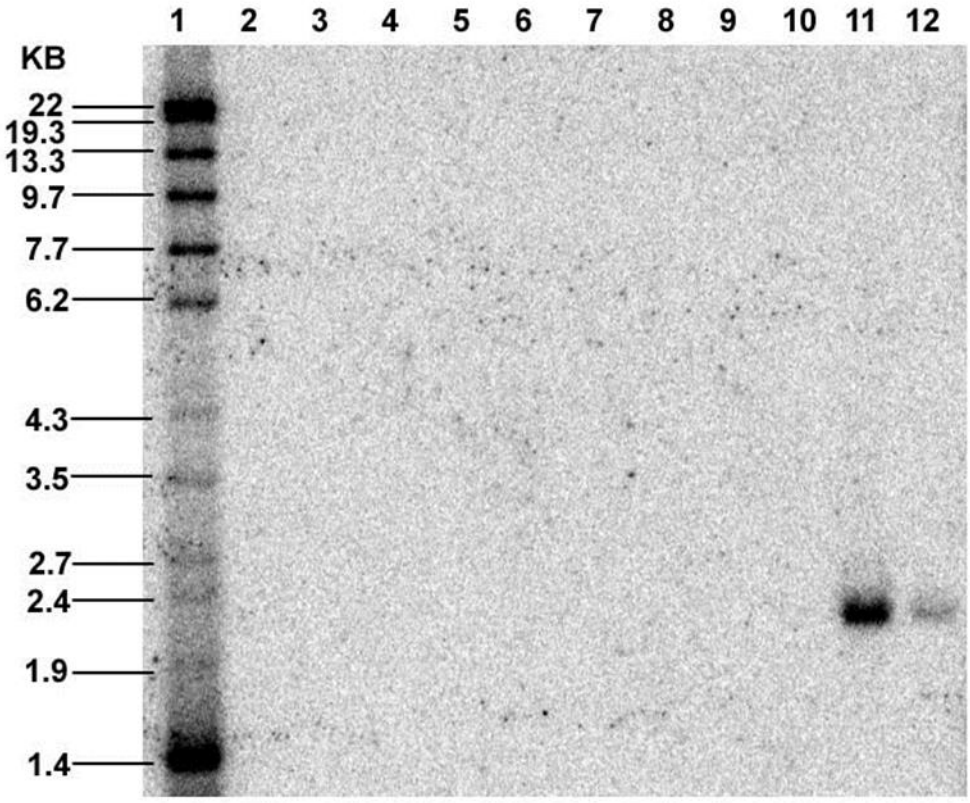


Figure V–18. Locations of the 2.1-kb backbone-specific probe 2 and the restriction sites *Dra*III, *Not*I, and *Asc*I + *Pac*I in the transformation plasmid pSYN18857



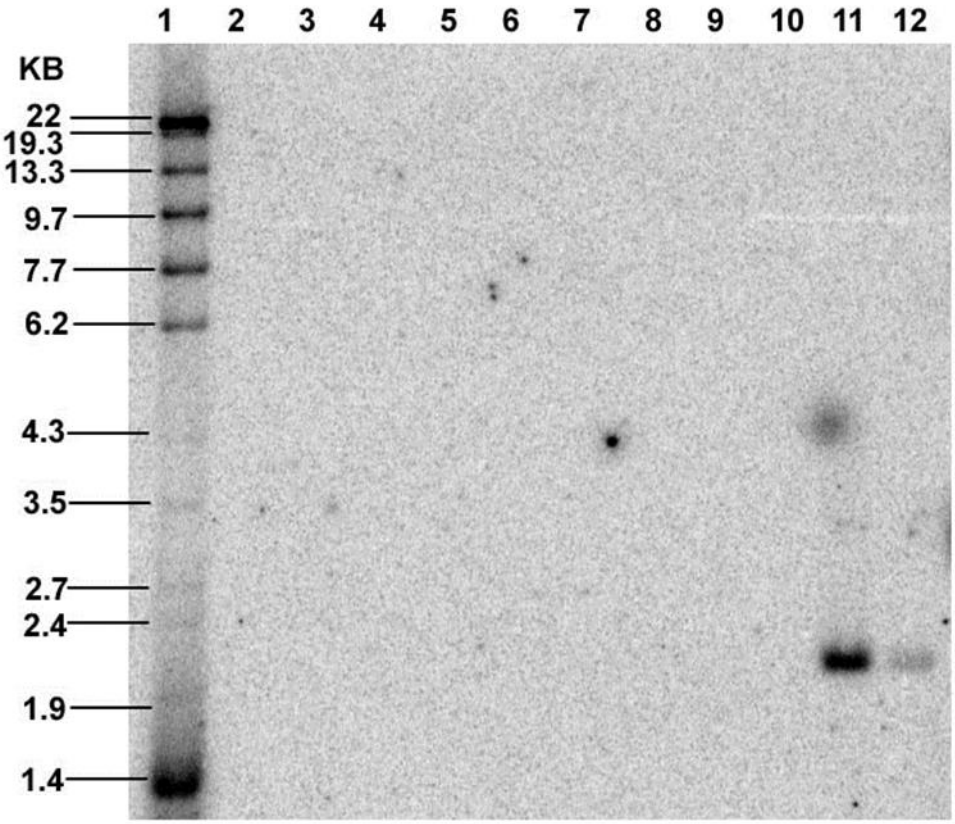
Lane 1 = MZHG0JG T2 (ear 4) corn
Lane 2 = MZHG0JG T_2 (ear 35) corn
Lane 3 = MZHG0JG T_3 corn
Lane 4 = MZHG0JG T_4 corn
Lane 5 = MZHG0JG T_5 corn
Lane 6 = MZHG0JG F_1 corn
Lane 7 = NP2222 corn (negative control)
Lane 8 = NP2391 corn (negative control)
Lane 9 = NP2222/NP2391 corn (negative control).
Lane 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 markers

Figure V–19. 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 V–20. 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 V–21. Southern blot analysis of MZHG0JG corn with the 2.1-kb plasmid pSYN18857 backbone-specific probe 2 and restriction enzyme *Asc*I + *Pac*I

V.A.2. 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.

V.B. Nucleotide Sequence of the T-DNA Insert and Copy Number of the Functional Elements

Three overlapping fragments that covered the entire MZHG0JG T-DNA insert were amplified via PCR from genomic DNA extracted from MZHG0JG T_3 corn and cloned. A consensus nucleotide sequence was generated from all of the fragments and compared with the sequence of the T-DNA in plasmid pSYN18857, the transformation plasmid used to create MZHG0JG corn.

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 ends 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. As these deletions occurred outside of the functional elements, no effect on the functionality of the transgenes is expected.

The copy number and sequence of each of the functional elements in the DNA insert of MZHG0JG corn are 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 sequences is shown in Figure V–22.

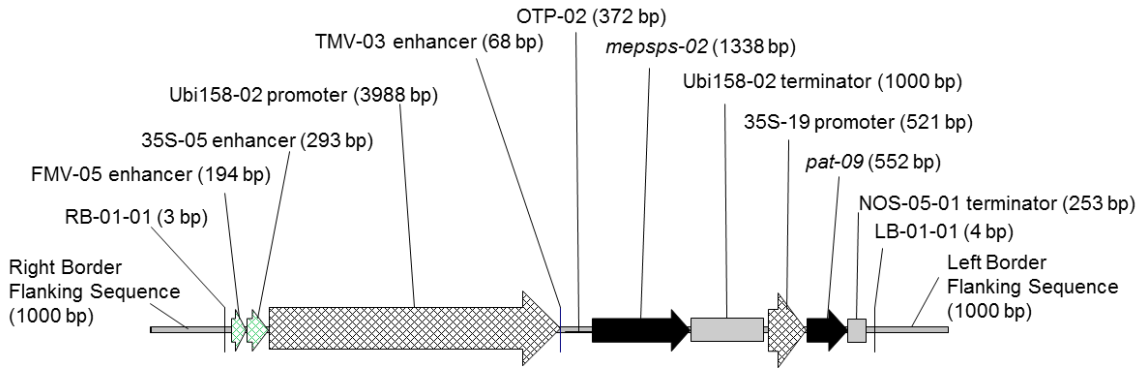


Figure V–22. Map of the MZHG0JG insert and flanking sequences

V.C. MZHG0JG Insertion Site Analysis

PCR analysis was used to determine (1) the genomic sequence in nontransgenic, near-isogenic corn at the point of integration of the MZHG0JG DNA insert and (2) the genomic sequences flanking the 5′ and 3′ ends of the MZHG0JG insert. Comparison of these two sequences showed that 22 bp of DNA from the nontransgenic 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.

The genomic sequences flanking the MZHG0JG insert were screened for similarity with DNA sequences found in a non-redundant nucleotide (nr/nt) database and an expressed sequence tags (EST) database. These comparisons provided an indication of whether the MZHG0JG insert disrupted any known endogenous corn gene. Sequence similarity analyses were performed with the Basic Local Alignment Search Tool for Nucleotides (BLASTN) program, version 2.2.28+ (Altschul *et al.* 1997). The sequences were compared with DNA sequences in the latest version of the NCBI nr/nt database (NCBI 2015) and the NCBI Viridiplantae (taxid 33090) EST database (NCBI 2014) on January 21, 2015. The results of the BLASTN analyses of the genomic sequences flanking the MZHG0JG insert indicated that the insert does not disrupt any known endogenous corn gene.

Bioinformatics analysis of the DNA sequences spanning the junctions between the corn genomic sequence and the MZHG0JG insert did not identify any nucleotide sequence contained between a start codon (ATG) and a stop codon (TAG, TAA, or TGA) for which the translated hypothetical open reading frame (ORF) is ≥30 amino acids.

V.D. 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). These results were used to determine the segregation ratios of *mepsps-02* and *pat-09*. T_3-generation MZHG0JG corn plants that were hemizygous for the transgenes were crossed with nontransgenic corn line NP2681 (Figure III-2). 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:

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

The expected and observed segregation ratios are shown in Table V-7. 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. 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 V-7. 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.
*$P < 0.05$ ($\chi^2 < 3.84$).

V.E. 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 the pSYN18857 plasmid vector. 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. BLASTN 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 the presence of start and 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.

VI. Characterization and Safety of the mEPSPS Protein

The mEPSPS protein produced in MZHG0JG corn has been well characterized and has no known toxic or allergenic properties. The enzyme mEPSPS is a variant of the native EPSPS from *Z. mays*. mEPSPS contains two amino acid substitutions that were introduced specifically to confer tolerance to herbicides containing glyphosate. International publications from ILSI (ILSI 2011a) and the OECD (OECD 1999a) have affirmed the weight of evidence regarding the human and environmental safety of EPSPS proteins and variants thereof. The mEPSPS produced in MZHG0JG corn is identical to the mEPSPS produced in Event GA21 corn (OECD Unique Identifier MON-ØØØ21-9) (hereafter GA21 corn), which was first introduced to the market in 1998 and has a history of safe use. GA21 corn was the subject of USDA APHIS Petition No. 97-099-01p for determination of nonregulated status, which was granted November 18, 1997.

To establish an expression profile for mEPSPS as expressed in MZHG0JG corn, the concentrations of mEPSPS in MZHG0JG corn tissues were determined.

VI.A. mEPSPS Protein Familiarity and History of Safe Exposure

The nucleotide sequence of *mepsps-02* in MZHG0JG corn encoding the mEPSPS protein was confirmed by nucleotide sequencing of the insert. The *mepsps* in GA21 corn encoding the mEPSPS protein was confirmed by nucleotide sequencing of the insert. The deduced amino acid sequence of the mEPSPS protein in both MZHG0JG corn and GA21 corn is identical (Figure VI–1). GA21 corn is currently approved to support cultivation activities in eight countries and is commercially available in five countries, including the United States, Canada, and Argentina. Syngenta has combined GA21 corn with other approved biotechnology-derived traits in ten novel combinations that have been reviewed and approved globally for cultivation and food/feed uses.

Because of the ubiquitous occurrence of EPSPS proteins in microorganisms and plants, it is likely that small amounts of EPSPS from various sources have always been present in the food and feed supply. Humans and animals have a long history of dietary exposure to EPSPS from the endogenous proteomes of microorganisms and of corn. Additionally, EPSPS is produced in many commercially available transgenic crop plants, including corn, cotton, and soybean. The safety of EPSPS in existing commercial transgenic crop products is supported by a permanent exemption from food and feed tolerances for EPSPS in all crops in the United States (U.S. EPA 2007a) and by regulatory approvals of numerous transgenic crops containing EPSPS encoded by genes derived from *A. tumefaciens* strain CP4, *Arthrobacter globiformis,* or corn, for U.S. cultivation (Appendix B). A complete list of commercially available U.S. corn products containing the mEPSPS protein can be found in the CropLife International BioTradeStatus Database (CLI 2015).

```
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–1. Alignment of the deduced amino acid sequence from *mepsps* in GA21 corn and *mepsps-02* in MZHG0JG corn

VI.B. Levels of mEPSPS Protein in MZHG0JG Corn Tissues

The concentrations of mEPSPS in various MZHG0JG corn tissues were quantified by enzyme-linked immunosorbent assay (ELISA) to establish an expression profile for mEPSPS 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 nontransgenic, near-isogenic control corn grown concurrently according to local agronomic practices at four U.S. locations in 2013. The corn varieties used in these studies were NP2391 × NP2222(MZHG0JG) and NP2391 × NP2222 (Figure III–2). These trials were planted under USDA permit 13-043-102rm.

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 mEPSPS protein concentrations observed in each MZHG0JG corn tissue type at several growth stages across four locations on a fresh-weight (FW) and dry-weight (DW) basis. Details of the materials and methods used to quantify levels of mEPSPS in MZHG0JG corn tissues are described in Appendix D.

Table VI–1. Concentrations of mEPSPS in MZHG0JG corn tissue samples at several growth stages, across four locations, on a dry-weight and fresh-weight basis

Tissue Type, Stage[a]	µg/g DW		µg/g FW	
	Mean ± SD[b]	Range	Mean ± SD	Range
Leaves, V6	1421 ± 410	697–2059	226 ± 70.71	95.95–327
Leaves, R1	1934 ± 678	920–3203	440 ± 182	155–808
Leaves, R6	954 ± 686	138–2760	398 ± 200	76.59–936
Leaves, Sen.[c]	–[d]	<LOD[e]–182	–	<LOD–148
Roots, V6	357 ± 149	124–707	50.53 ± 25.36	17.18–110
Roots, R1	367 ± 103	176–529	55.02 ± 21.99	18.94–85.18
Roots, R6	294 ± 87.89	147–396	43.77 ± 14.92	16.49–79.07
Roots, Sen.	153 ± 57.72	57.58–261	25.14 ± 11.79	7.02–50.91
Whole Plant, V6	1496 ± 445	734–2251	186 ± 59.16	89.41–294
Whole Plant, R1	1468 ± 398	948–2347	265 ± 90.38	167–437
Whole Plant, R6	329 ± 213	94.78–753	149 ± 88.01	51.75–315
Pollen, R1	–	–	–	–
Kernel, R6	58.23 ± 14.87	30.37–86.15	40.21 ± 8.04	24.21–58.68
Kernel, Sen.	36.89 ± 10.06	19.94–56.54	27.83 ± 5.93	16.30–35.23

[a] *N* = 20 for all tissues except pollen, where *N* = 4
[b] SD = standard deviation
[c] Sen. = senescence
[d] – = not applicable, as one or more values were below either the LOD or LOQ for the assay.
[e] LOD for mEPSPS in leaves = 2.00 µg/g DW
All concentrations were rounded to two decimal places for concentrations less than three digits and the nearest whole number for concentrations three digits and above.

VI.C. Identity and Characterization of mEPSPS Protein in MZHG0JG Corn

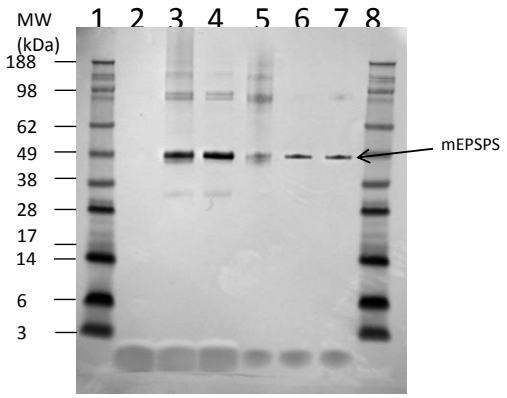
The identity of the mEPSPS protein in MZHG0JG corn was confirmed by peptide mass coverage analysis, apparent molecular weight, and immunoreactivity.

To conduct peptide mass coverage analysis of mEPSPS, the protein was extracted from MZHG0JG corn, reduced, alkylated with iodoacetamide, and enzymatically digested with trypsin, chymotrypsin, and endoproteinase Asp-N. The peptide mass coverage analysis verified 88% of the predicted amino acid sequence of mEPSPS (as shown in Figure VI–2). Western blot analysis demonstrated that the apparent molecular weight of mEPSPS was consistent with the predicted molecular weight of 47.4 kilodaltons (kDa), and the protein cross-reacted with mEPSPS-specific antibody (as 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

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

Figure VI–2. Amino acid sequence identified for mEPSPS from MZHG0JG corn by peptide mass coverage analysis



Lane 1: Molecular weight standard
Lane 2: Nontransgenic corn leaf extract (10 µg total protein)
Lane 3: Crude MZHG0JG corn leaf extract (5 ng 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 mEPSPS extract (5 ng 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–3. Western blot analysis of mEPSPS from MZHG0JG corn

VI.D. Conclusions on the Safety of mEPSPS in MZHG0JG Corn

The safety of EPSPS proteins has been previously established. This summary of safety assessment conclusions is based on existing EPSPS and mEPSPS safety data summarized by publications (ILSI 2011a, OECD 1999a) and submissions to U.S. and global regulatory authorities. Furthermore, the mEPSPS produced in MZHG0JG corn has the identical amino acid sequence as the mEPSPS produced in GA21 corn, a previously evaluated transgenic corn product in commerce. mEPSPS has a very specific and well-characterized mode of action; it is not acutely toxic, and it has no characteristics consistent with potential allergenicity. It is concluded that mEPSPS does not pose a risk to the health of humans or livestock through consumption of MZHG0JG corn.

VII. Characterization and Safety of the PAT Protein

The PAT protein produced in MZHG0JG corn has been well characterized and has no known toxic or allergenic properties. PAT is derived from the naturally occurring soil bacterium *Streptomyces viridochromogenes* and acetylates glufosinate-ammonium, thus inactivating it and conferring tolerance to glufosinate-ammonium in herbicide products. Publications from scientific literature and international organizations have detailed the characterization and affirmed the safety of the PAT protein (Hérouet *et al.* 2005, ILSI 2011b, and OECD 1999b). The PAT produced in MZHG0JG corn is identical to the PAT produced in Event Bt11 corn (OECD Unique Identifier SYN-BTØ11-1), which was first introduced to the market in 1997.

Event Bt11 corn (hereafter Bt11 corn) was the subject of USDA APHIS Petition No. 95-195-01p for the determination of nonregulated status, which was granted July 18, 1996.

To establish and expression profile for PAT as expressed in MZHG0JG corn, the concentrations of PAT in MZHG0JG corn tissues were determined.

VII.A. PAT Protein Familiarity and History of Safe Exposure

The nucleotide sequence of *pat-09* in MZHG0JG corn encoding the PAT protein was confirmed by nucleotide sequencing of the insert. The nucleotide sequence of *pat* in Bt11 corn encoding the PAT protein was confirmed by nucleotide sequencing of the insert. The deduced amino acid sequence of the PAT protein in both MZHG0JG corn and Bt11 corn is identical (Figure VII–1). Bt11 corn is currently approved to support cultivation activities in nine countries, including the United States, Canada, and Argentina. Syngenta has combined Event Bt11 with other approved biotechnology-derived traits in multiple novel combinations that have been reviewed and approved globally for cultivation and food/feed uses.

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). It is likely that small amounts of acetyltransferase enzymes from various sources have always been present in the food and feed supply, because of the ubiquitous occurrence of PAT proteins in nature. There is a long history of safe exposure to PAT proteins as part of the endogenous proteome of microorganisms that are widely distributed taxonomically. Additionally, PAT is produced in several commercially available transgenic crop plants, including corn, canola, and soybean, the products of which enter the food and feed supply. The safety of PAT in existing commercial transgenic crop products is supported by a permanent exemption from food and feed tolerances in all crops in the U.S. (U.S. EPA 2007b) and by regulatory approvals of numerous transgenic crops containing PAT (encoded by either *pat* or a similar gene, *bar*) for U.S. cultivation (Appendix B). A complete list of commercially available U.S. corn products containing the PAT protein can be found in the CropLife International BioTradeStatus Database (CLI 2015).

```
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 VII–1. Alignment of the deduced amino acid sequence from *pat* in Bt11 corn and *pat-09* in MZHG0JG corn

VII.B. Levels of PAT Protein in MZHG0JG Corn Tissues

The concentrations of PAT in various MZHG0JG corn tissues were quantified by enzyme-linked immunosorbent assay (ELISA) to establish an expression profile for PAT 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 nontransgenic, near-isogenic control corn grown concurrently according to local agronomic practices at four U.S. locations in 2013. The corn varieties used in these studies were NP2391 × NP2222(MZHG0JG) and NP2391 × NP2222 (Figure III–2). These trials were planted under USDA permit 13-043-102rm.

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 VII–1 shows the ranges of PAT protein concentrations observed in each MZHG0JG corn tissue type at several growth stages across four locations on a fresh-weight (FW) and dry-weight

(DW) basis. Details of the materials and methods used to quantify levels of PAT in MZHG0JG corn tissues are described in Appendix D.

Table VII–1. Concentrations of PAT in MZHG0JG corn tissue samples at several growth stages, across four locations, on a dry-weight and fresh-weight basis

Tissue Type, Stage[a]	µg/g DW		µg/g FW	
	Mean ± SD[b]	Range	Mean ± SD	Range
Leaves, V6	8.39 ± 1.44	6.37–11.79	1.33 ± 0.26	0.93–1.84
Leaves, R1	9.95 ± 3.02	6.21–17.03	2.18 ± 0.53	1.41–3.47
Leaves, R6	2.15 ± 1.95	0.11–6.07	0.88 ± 0.63	0.10–2.25
Leaves, Sen.[c]	–[d]	<LOD[e]–0.71	–	<LOD–0.58
Roots, V6	1.68 ± 1.08	0.77–3.91	0.24 ± 0.18	0.09–0.65
Roots, R1	1.08 ± 0.30	0.67–1.71	0.15 ± 0.05	0.08–0.22
Roots, R6	1.24 ± 0.72	0.62–2.69	0.18 ± 0.10	0.10–0.39
Roots, Sen.	0.80 ± 0.25	0.37–1.32	0.13 ± 0.05	0.05–0.25
Whole Plant, V6	6.70 ± 1.46	3.13–9.76	0.84 ± 0.22	0.39–1.34
Whole Plant, R1	4.48 ± 1.64	2.23–8.00	0.78 ± 0.25	0.43–1.45
Whole Plant, R6	–	<LOD–2.43	–	<LOD–1.20
Pollen, R1	–	–	–	–
Kernel, R6	–	<LOD	–	<LOD
Kernel, Sen.	–	<LOD	–	<LOD

[a] *N* = 20 for all tissues except pollen, where *N* = 4
[b] SD = standard deviation
[c] Sen. = senescence
[d] – = not applicable, as one or more values were below either the LOD or LOQ for the assay.
[e] LOD for PAT in leaves, whole plants, and kernels = 0.025 µg/g DW

VII.C. Identity and Characterization of PAT Protein in MZHG0JG Corn

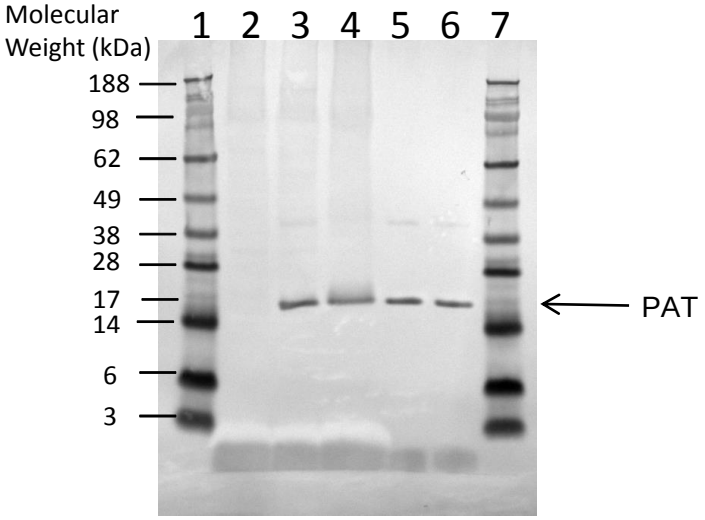
The identity of the PAT protein produced in MZHG0JG corn was confirmed by peptide mass coverage analysis, apparent molecular weight, and immunoreactivity.

To conduct peptide mass coverage of PAT, the protein was extracted from MZHG0JG plants, reduced, alkylated with iodoacetamide, and enzymatically digested with trypsin, chymotrypsin and endoproteinase Asp-N. The peptide mass coverage analysis verified 90% of the predicted amino acid sequence of PAT (as shown in Figure VII–2). 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 PAT-specific antibody (as shown in Figure VII–3).

1 *M***SPERRPVEIRPATAA***DMAAVC***DIVNHY**IETSTVNFRTEP 40
41 **QTPQ**EWI**DDLERLQDRYPWLVA**EVEGVVAGIAY*AGPW*KAR 80
81 NAYDWTVESTVYVSHRHQR*LGLGSTLY*THLLKSMEAQGFK 120
121 SVVAVIGLPN**DPSVRLH**EALGYTARGTLRAAGYKHGGWH**D** 160
161 **VGFWQRDFELPAPPRPVRPVTQI** 183

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

Figure VII–2. Amino acid sequence sequence identified for PAT from MZHG0JG corn by peptide mass coverage analysis



Lane 1: Molecular weight standard
Lane 2: Nontransgenic corn leaf extract (80 µg of total protein)
Lane 3: Nontransgenic corn leaf extract fortified with microbially produced PAT (5 ng of PAT, 80 µg of total protein)
Lane 4: Crude MZHG0JG corn leaf extract (5 ng of PAT, 80 µg of total protein)
Lane 5: PAT purified preparation from MZHG0JG extract (5 ng)
Lane 6: Microbially produced PAT (5 ng) [a]
Lane 7: Molecular weight standard
[a]Microbially produced protein was used as a positive assay control.

Figure VII–3. Western blot analysis of PAT from MZHG0JG corn

VII.D. Conclusions on the Safety of PAT in MZHG0JG Corn

The safety of PAT proteins has been previously established. This summary of safety assessment conclusions is based on existing PAT safety data, summarized by Hérouet *et al.* (2005), ILSI (2011b), OECD (1999b), and submissions to U.S. and global regulatory authorities. Furthermore, PAT as produced in MZHG0JG corn has the identical amino acid sequence as PAT

produced in Bt11 corn, a previously evaluated transgenic corn product in commerce. PAT has a very specific and well-characterized mode of action; it is not acutely toxic, and it has no characteristics consistent with potential allergenicity. It is concluded that PAT does not pose a risk to the health of humans or livestock through consumption of MZHG0JG corn.

VIII. Compositional Assessment of MZHG0JG Corn Grain and Forage

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. This Section describes a study conducted to measure and compare key nutrients and anti-nutrients in forage and grain from MZHG0JG and conventional corn.

VIII.A. 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 components of grain and nine components of 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 U.S. locations in 2013. The test material was MZHG0JG corn and the control material 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 and described in the breeding pedigree in Fig. III–2.

Table VIII–1. Plant material used in compositional analyses

Entry Identification	Seed description	Hybrid genotype
E01	Nontransgenic, near-isogenic (control)	NP2391/NP2222
E02	MZHG0JG (test)	NP2391/NP2222(MZHG0JG)
E09	Reference variety 1	H-7191
E10	Reference variety 2	H-7540
E11	Reference variety 3	SY Genoroso
E12	Reference variety 4	NK Lucius
E13	Reference variety 5	NK Cisko
E14	Reference variety 6	SY Provial

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 apart and 20 feet long, planted with approximately 40 seeds per row. The locations are listed in Table VIII–2 and shown on a satellite view 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. The soil type, previous year's crop, and planting date for each location are listed in Table VIII–2. These trials were planted under USDA permit 13-043-102rm.

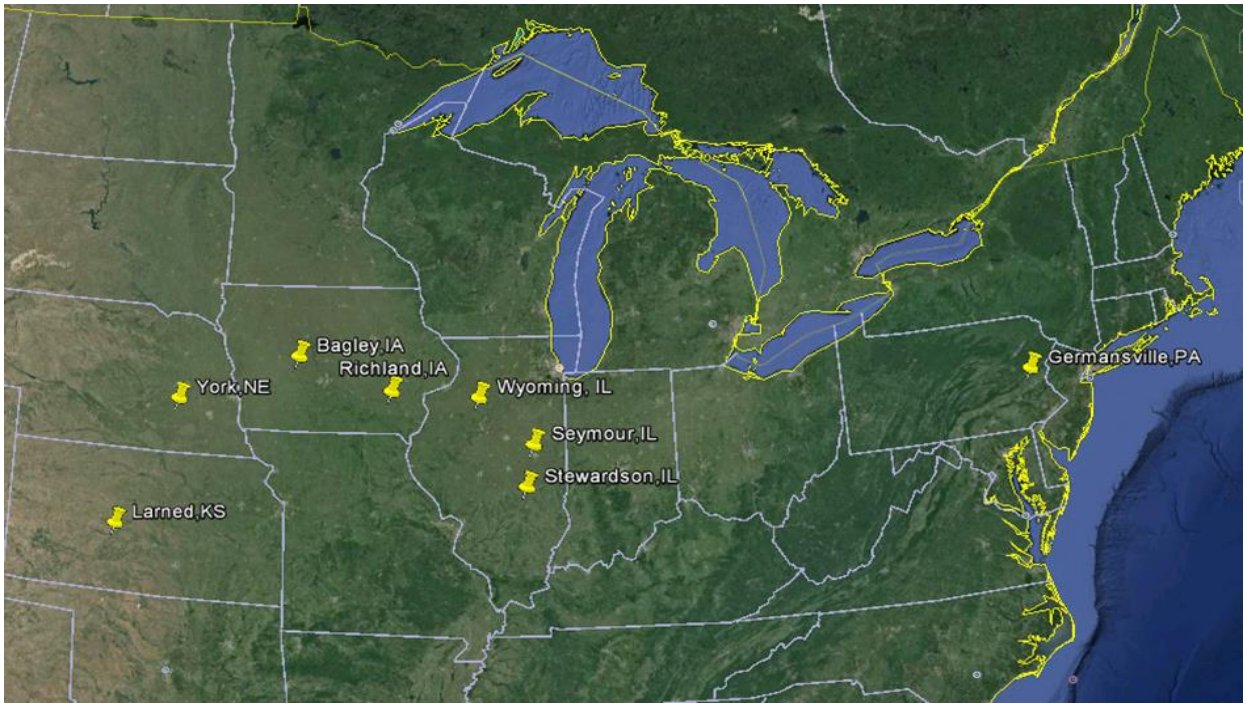


Figure VIII–1. Satellite view of composition trial locations in the United States

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

Table VIII–2. Field-trial locations for composition study

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 Organisation for Economic Co-operation and Development (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 (DW) 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 (Appendix C).

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 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 corn). 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 (<LOQ) 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.D.

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
	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
	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
	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	4316	3897	3873	4316	3897	4116	4116	3650	3650

MZHG0JG: *N* = 32.
Control: *N* = 32.
Reference: *N* = 192.
ILSI: *N* is the number of ILSI values used to calculate the mean and excludes values <LOQ.
Proximate levels shown as percent dry weight, except moisture which is shown as percent fresh weight.
Calcium and phosphorus levels shown as milligrams per kilogram dry weight.
Results significantly different ($p < 0.05$) are shown in bold italic type.

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 VIII–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
	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
	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
	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	6616	5790	5790	6190	5765	5942	5941	3763	1931

MZHG0JG: *N* = 32.
Control: *N* = 32.
Reference: *N* = 192.
Proximate and starch levels shown as percent dry weight, except moisture which is shown as percent fresh weight.
Results significantly different ($p < 0.05$) are shown in bold italic type.
[a]Grain was dried in the field, or mechanically after harvest, so moisture levels were not subjected to analysis of variance (ANOVA).

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

Data source	Statistic	Ca	Cu	Fe	Mg	Mn	P	K	Se[a]	Na[b]	Zn
MZHG0JG	Mean	34.8	1.75	18.5	1161	5.73	3012	3517	–	–	20.4
	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
	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
	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	5932	5650	5819	5823	5822	5938	5823	973	1110	5823

MZHG0JG: *N* = 32.
Control: *N* = 32. For copper, two outlying values were included in the analyses.
Reference: *N* = 192.
ILSI: *N* is the number of ILSI values used to calculate the mean and excludes values <LOQ.
Mineral levels shown as milligrams per kilogram (mg/kg) dry weight.
Results significantly different ($p < 0.05$) are shown in bold italic type. When some or all values were <LOQ, and substitution with the LOQ was not appropriate due to the number or distribution of substitutions required, calculation of the mean and analysis of variance (ANOVA) could not be performed and only the range is shown.
[a] Original units of parts per billion (ppb) were converted to mg/kg. The LOQ for selenium was 0.033–0.036 mg/kg dry weight.
[b] The LOQ for sodium was 109–121 mg/kg dry weight.

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
	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
	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
	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	4373	4981	4061	4999	4998	5460	4480

MZHG0JG: *N* = 32.
Control: *N* = 32.
Reference: *N* = 192.
ILSI: *N* is the number of ILSI values used to calculate the mean and excludes values <LOQ.
Vitamin levels shown as milligrams per 100 grams (mg/100 g) dry weight, except vitamin E which is shown as milligrams per gram (mg/g).
Results significantly different ($p < 0.05$) are shown in bold italic type.
[a] β-carotene is measured in this study vitamin A is not produced in plants.
[b] The original units of mg/100 g were converted to mg/g.

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.

Twelve fatty acids analyzed had levels below the LOQ in all replicates at all locations and could not be analyzed, including 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
	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
	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
	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
	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
	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
	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	5915	5918	5918	5918	5918	5909	5918	5918	5916

MZHG0JG: *N* = 32. Control: *N* = 32. Reference: *N* = 192.
ILSI: *N* is the number of ILSI values used to calculate the mean and excludes values <LOQ.
Amino acid levels shown as milligrams per gram dry weight.
Results significantly different ($p < 0.05$) are shown in bold italic type.

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
	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
	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
	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	4682	2119	265	4682	4682	4682	4682	4344	4322	3858

MZHG0JG: *N* = 32.
Control: *N* = 32.
Reference: *N* = 192.
ILSI: *N* is the number of ILSI values used to calculate the mean and excludes values <LOQ.
Fatty acids shown as percent of total fatty acids.
Results significantly different ($p < 0.05$) are shown in bold italic type.
When some or all values were <LOQ, and substitution with the LOQ was not appropriate due to the number or distribution of substitutions required, calculation of the mean and analysis of variance (ANOVA) could not be performed and only the range is shown. 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/mg)
MZHG0JG	mean	347	3409	–	2481	0.840	0.116	4.05
	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
	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
	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	5371	5378	14	4003	5762	4585	4089

MZHG0JG: *N* = 32.
Control: *N* = 32.
Reference: *N* = 192.
ILSI: *N* is the number of ILSI values used to calculate the mean and excludes values <LOQ.
Units for anti-nutrients are shown in column headings: milligrams per kilogram (mg/kg), parts per million (ppm), percent (%), trypsin inhibitor unit (TIU). All are expressed on a dry weight basis.
Results significantly different ($p < 0.05$) are shown in bold italic type.
When some or all values were <LOQ, and substitution with the LOQ was not appropriate due to the number or distribution of substitutions required, calculation of the mean and analysis of variance (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.

VIII.D. 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.

IX. Safety and Nutritional Assessment of MZHG0JG Corn and Derived Corn Products

The safety of MZHG0JG corn and its nutritional comparability to conventional, nontransgenic corn were assessed through consideration of the compositional assessment described in Section VIII, the safety assessments of the mEPSPS and PAT proteins described in Sections VI and VII, and the numerous publications that detail the characterization and safety of mEPSPS and PAT proteins referenced previously.

IX.A. Nutritional Assessment of MZHG0JG Corn

As discussed in Section VIII, analysis of key nutritional components of forage and grain from MZHG0JG corn identified no differences from conventional, nontransgenic corn that would affect human or animal health. No unintended, negative consequences of the transformation process or expression of the transgenes in MZHG0JG corn were evident. Grain and forage from MZHG0JG corn were found to be similar in composition to those same materials from conventional corn. Additionally, MZHG0JG corn exhibited a composition profile similar to that of reference corn varieties grown concurrently in several locations and other corn varieties represented in the historical ILSI Crop Composition Database (ILSI 2014).

Based on the data and information presented, it is concluded that MZHG0JG corn and corn products processed from raw MZHG0JG corn are nutritionally and compositionally comparable to raw and processed corn from conventional varieties, and that MZHG0JG corn is expected to provide adequate nutrition as part of human diets as well as formulated diets delivered to growing livestock.

IX.B. Safety Assessment of MZHG0JG Corn

As discussed in Sections VI and VII, both mEPSPS and PAT have specific, well-understood modes of action, and both are homologous with proteins in many species to which humans

and animals are exposed daily without concern. mEPSPS and PAT have been safely used and consumed in commercial transgenic crops and have permanent U.S. EPA tolerance exemptions in all crops. Previous evaluations of mEPSPS and PAT have shown they do not share significant amino acid similarity to known toxins and are unlikely to be human allergens. 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. 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 LOD–0.04 μg/g dry weight at maturity.

The data and information presented in this document support the conclusions that MZHG0JG corn is compositionally and nutritionally comparable to and as safe as conventional corn, and that no adverse health effects will result from exposure to either mEPSPS or PAT present in MZHG0JG corn.

X. Phenotypic and Agronomic Characteristics

Field and growth-chamber studies were conducted to determine whether reproductive, growth, or survival characteristics of MZHG0JG corn differed from those of conventional corn. Field trials were conducted to assess plant growth properties, reproductive capability, survival, seed dispersal, interactions with environmental stressors, and pollen viability and morphology. A growth-chamber study measured seed germination and dormancy characteristics. Unintended changes in these characteristics could indicate altered plant fitness and pest potential of MZHG0JG corn.

These studies employed standard designs and included a nontransgenic, near-isogenic corn variety as a control. Some studies employed additional nontransgenic commercial corn varieties as references. The phenotypic characteristics evaluated and the metrics employed are shown in Table X–1.

Table X–1. Phenotypic characteristics evaluated for MZHG0JG corn

Phenotypic Characteristic	Variable Measured[a]	Timing[b]	Description
Seed germination/ dormancy	Germination and dormancy	After 4,7, and 12 days	Percent normal germinated, abnormal germinated, dead seed, dormant seed, and hard seed
Emergence	Early stand count (pre-thinning)	14 days after planting	Number of plants emerged per plot[c]
	Early stand count (post-thinning)	14 days after planting	Number of plants emerged after thinning to a uniform stand per plot
Vegetative growth	Early growth rating	V2-V4	Rating of seedling vigor
	Ear height	R5	Distance from the soil surface at the base of the plant to the node where the ear connects to the stalk.
	Plant height	R5	Distance from the soil surface at the base of the plant to the collar of the flag leaf
	Stay green	R6	Percent stay green
	Root-lodged plants	R6	Percent of plants per plot leaning at the soil surface > 30º from vertical
	Stalk-lodged plants	R6	Percent of plants per plot with stalks broken below the ear
	Final stand count	R6	Number of plants per plot, excluding tillers
Reproductive growth	Pollen morphology	R1	Diameter (µm) of viable pollen grains
	Pollen viability	R1	Percent viable pollen based on staining characteristics
	Days to 50% pollen shed	VT-R1 (tassel)	Days from planting until 50% of plants have begun to shed pollen
	Days to 50% silking	VT-R1 (silking)	Days from planting until 50% of plants have silks exposed
	Grain moisture	R6	Moisture percentage of harvested shelled grain
	Grain test weight	R6	Harvested, shelled grain yield
	Grain yield	R6	Harvested, shelled grain yield
Seed retention	Dropped ears	R6	Number of mature ears dropped per plot
Plant-ecological interactions	Susceptibility to biotic and abiotic stressors	Every 4 weeks after V2 stage	Qualitative observations of occurrence of plot interactions with biotic and abiotic stressors

[a]Seed dormancy and germination were measured in a growth-chamber study. All other parameters were assessed in field trials.

[b]Corn vegetative and reproductive growth stages as defined by Abendroth *et al.* (2011): V2 = first two leaves collared; V4 = first four leaves collared; VT = tassel; R1 = silking; R5 = dent; R6 = physiological maturity

[c]A plot is defined as a two-row plot, 100 ft^2 (9.29 m^2).

X.A. Seed Germination and Dormancy

Enhanced germination or seed dormancy are characteristics that can be indicative of plant pest potential. Dormancy mechanisms function to distribute seed germination across multiple growing seasons. Primary dormancy is conferred by physical traits, such as hard seeds, or physiological seed traits that prevent immediate germination under conditions that would otherwise favor it. Primary dormancy is extremely rare or nonexistent in most field crops, including corn (Galinat 1988). Secondary dormancy occurs when the seed is capable of germination, but environmental conditions are unsuitable to induce germination. Overwintering of corn seed occurs via secondary dormancy.

A laboratory study was conducted to evaluate the germination and dormancy characteristics of MZHG0JG corn seed using a modification of the testing protocol established by the Association of Official Seed Analysts (AOSA 2013).

X.A.1. Test, control, and reference materials

Seed samples of MZHG0JG corn, a corresponding nontransgenic, near-isogenic control hybrid, and three conventional corn hybrids served as test, control, and reference materials, respectively, for the study. Figure III–2 shows the breeding pedigree of MZHG0JG corn seed materials. Table X–2 presents the descriptions and genotypes of the test, control, and reference materials.

Table X–2. Plant material used in seed germination and dormancy study

Entry Identification	Seed description	Hybrid genotype
E01	Nontransgenic, near-isogenic corn (control)	NP2391/NP2222
E02	MZHG0JG corn (test)	NP2391/NP2222(MZHG0JG)
E03	Corn reference variety 1	NK Octet
E04	Corn reference variety 2	NK Lucius
E05	Corn reference variety 3	NK Cisko

X.A.2. Study design

The study design followed that described by AOSA (2013) for assessment of germination and dormancy characteristics of corn seed under optimal temperature conditions for seed germination (25°C and 20°C/30°C). Additionally, similar assessments were conducted under non-optimal temperature conditions (10°C, 30°C, 10°C/20°C, and 10°C/30°C). Seed lots were divided into four replicates of 100 seeds per replicate per temperature regime. Six temperature regimes were utilized, as shown in Table X–3.

Table X–3. Temperature regimes used to test seed germination

Temperature regime
1. Constant 10°C
2. Constant 25°C[a]
3. Constant 30°C
4. Alternating 10°C for 16 hours followed by 20°C for 8 hours (10°C/20°C)
5. Alternating 10°C for 16 hours followed by 30°C for 8 hours (10°C/30°C)
6. Alternating 20°C for 16 hours followed by 30°C for 8 hours (20°C/30°C)[a]

[a] These regimes are as described in *AOSA Rules for Testing Seeds* (AOSA 2013).

Experiments were conducted in unlighted temperature-controlled growth chambers. For the alternating temperature regimes, the lower and higher temperatures were maintained as shown in Table X–3, and then the cycle was repeated. The study was initiated by rolling 100 seeds in moistened germination towels and then placing the rolled towels into the growth chambers. The day that seeds were rolled into germination towels was considered Day 0.

The seed or seedling samples subjected to the AOSA-specified temperature regimes (25°C and 20°C/30°C) were examined four and seven days after study initiation. The seed or seedling samples subjected to the additional non-AOSA-specified temperature regimes (10°C, 30°C, 10°C /20°C, and 10°C /30°C) were examined four, seven, and twelve days after study initiation. Each seed or seedling examined was categorized as described in Table X–4.

Table X–4. AOSA categories for seed and seedling evaluation[a]

Category	Description
Normal germinated	Seedlings with normal development of all essential structures including root, hypocotyl, and epicotyl.
Abnormal germinated[b]	Seedlings that lack a well-developed root, hypocotyl, or epicotyl, or possess deep lesions, or exhibit mechanical damage.
Dead	Seeds that do not germinate and are visibly deteriorated and soft to the touch.
Dormant	Viable seeds, other than hard seeds, that fail to germinate.
Hard	Seeds that do not imbibe water and are firm to the touch.

[a] AOSA (2013)
[b] Evaluated only for AOSA-specified temperature regimes (25ºC and 20°C/30ºC).

On Day 7 and, where applicable, Day 12, normal seedlings, abnormal seedlings, and dead seed were counted and removed from the towels. At each interim evaluation, seeds that were infected by bacteria or fungi were removed to reduce the chance that the remaining seed in the towel would be contaminated. On the final day of evaluation (Day 7 or Day 12, depending on temperature regime), all hard seeds were subjected to a tetrazolium test to

evaluate viability according to the method described in the Tetrazolium Testing Handbook of the AOSA (2010).

X.A.3. Statistical analyses

Statistical analyses were performed using SAS® software v. 9.4 (SAS Institute, Inc., Cary, NC). For each entry, the number of seeds or seedlings in a given category (germinated, dead, dormant, or hard seeds, or the number of abnormal seedlings), were modeled as a ratio with the number of seeds of that entry in the replicate (i.e., the proportion within a given category for each entry within a block). The ratios were subjected to analysis of variance (ANOVA) using the following generalized linear mixed model (PROC GLIMMIX of SAS® software):

$$(Y_{ij}/G_{ij}) = U + T_i + B_j + e_{ij}$$

In this model, Y_{ij} is the number of seeds that germinated for entry *i* in block *j* and G_{ij} is the number of seeds planted for entry *i* in block *j*. The overall mean is U, T_i is the entry effect, B_j is the block or container effect, and e_{ij} is the residual error. Entry was regarded as a fixed effect while block was regarded as a random effect. Because the germination of seed is fundamentally a binomial process (germinated or not germinated), GLIMMIX was used to model the effects with the binomial distribution. If the model failed to converge with blocks modeled as a random effect using residual pseudo-likelihood estimation, then the block effect was moved to the model statement as a fixed effect and maximum likelihood estimation was used. Within each temperature regime, a *t*-test was used to assess the statistical significance of the comparisons of interest (test vs. control). Significance was based on the customary alpha level of 0.05 and denominator degrees of freedom were determined by the Kenward-Roger method (Kenward and Roger 1997).

X.A.4. Results

The results of these seed germination and dormancy experiments are summarized in Table X–5. For the MZHG0JG corn and nontransgenic control corn, the data reported represent the combined data, averaged over four replicates, for each temperature regime on each day of evaluation. For the three reference hybrids, the data reported represent the range in values across all four replicates per hybrid (12 total replicates) for each temperature regime on each evaluation day.

Under all temperature regimes, average germination ranged from 97.3% to 99.5% for MZHG0JG corn and 98.8% to 99.8% for the corresponding nontransgenic control corn (Table X–5). Under the same temperature regimes, germination ranged from 73% to 100% across all replicates of the three reference varieties and the MZHG0JG corn and control germination fell within the reference range.

In comparisons between the test and control seed germination rates at the various temperature regimes, statistically significant differences were observed at the 25ºC and 20/30ºC temperature regimes. In both of these temperature regimes, the germination rate of the test was lower than that of the control and in both cases the difference was less than 3%. This small difference would not meaningfully reduce plant populations in the field. For example, corn growers in Iowa are recommended to plan on a typical loss of 4% to 7% from

seeding to surviving plants (Elmore and Abendroth 2009). The ranges of germination for both the test and the control seeds were comparable to those of the reference hybrids.

Data for abnormal germinated seedlings were only collected in the 25ºC regime and 20º/30ºC regime. Under these two conditions, there were no statistically significant differences observed between the test and control seed. Both the test and control seed had occurrences of abnormal germinated seedlings similar to those of the reference hybrids.

Comparisons of the number of dead seeds between the test and the control seed did not result in statistically significant differences for any of the temperature regimes. No entries had more than 4% dead seed, except for one replicate of one of the reference varieties in the 10°C temperature regime, which had 18% dead seed.

For most of the temperature regimes, no dormant seeds were observed among the test or control seeds. The exception was the 10°C regime where one replicate of the control had one dormant seed. However, 5.8% of the reference hybrid seeds were dormant at that temperature regime.

No seeds were classified as "hard" for the test, control, or reference hybrids.

The observation that MZHG0JG corn did not show increased seed germination compared to control corn supports the conclusion that MZHG0JG corn does not have plant pest characteristics or increased weediness potential.

Most important from the perspective of plant pest risk is the consideration of whether the seed of the test had increased dormancy potential, as measured by the presence of hard seeds. No hard seeds were found for any of the varieties tested under any temperature regime, thus confirming that dormancy is not a normal characteristic of corn seeds and that MZHG0JG corn demonstrates no increase in seed dormancy potential.

Table X–5. Summary of seed germination rates (% of total)

Temp Regime (°C)	Variety	Normal Germinated	Abnormal Germinated	Dead	Dormant	Hard
10	MZHG0JG	99.5	–	0.5	0.0	0.0
	Control	98.8	–	1.0	0.3	0.0
	ANOVA *p* value	0.301		0.516	0.978	
	Reference Range	73-–100	–	0–18	0–27	0
25	MZHG0JG	97.5	0.5	2.0	0.0	0.0
	Control	99.8	0.3	0.0	0.0	0.0
	ANOVA *p* value	***0.044***	0.580	0.976		
	Reference Range	98–100	0–2	0-1	0	0
30	MZHG0JG	99.3	–	0.8	0.0	0.0
	Control	99.3	–	0.8	0.0	0.0
	ANOVA *p* value	1.000		1.000		
	Reference Range	98–100	–	0-2	0	0
10/20	MZHG0JG	99.3	–	0.8	0.0	0.0
	Control	99.5	–	0.5	0.0	0.0
	ANOVA *p* value	0.733		0.733		
	Reference Range	98–100	–	0–2	0–1	0
10/30	MZHG0JG	97.5	–	2.5	0.0	0.0
	Control	99.8	–	0.3	0.0	0.0
	ANOVA *p* value	0.270		0.270		
	Reference Range	97-100	–	0-3	0	0
20/30	MZHG0JG	97.3	0.5	2.3	0.0	0.0
	Control	99.8	0.0	0.3	0.0	0.0
	ANOVA *p* value	***0.041***	0.985	0.063		
	Reference Range	96–100	0–1	0-3	0	0

N = 400 except for NP2391/NP2222 at Temperature Regime 10°C, where *N* = *401*
Results significantly different between test and control seed ($p < 0.05$) are shown in bold italic type.

X.B. Pollen Viability and Morphology

As a measure of potentially enhanced reproductive capability, pollen cell viability and morphology were compared between MZHG0JG corn, the nontransgenic, near-isogenic control corn, and three commercial reference varieties of hybrid corn.

X.B.1. Test, control, and reference materials

Figure III–2 shows the breeding pedigree of MZHG0JG corn seed materials. Table X–6 presents the descriptions and genotypes for the test, control, and reference corn varieties grown. The reference varieties were three commercially available, nontransgenic corn hybrids.

Table X–6. Plant material used in pollen viability and morphology study

Entry Identification	Seed Identification	Hybrid genotype
E01	Nontransgenic, near-isogenic corn (control)	NP2391/NP2222
E03	MZHG0JG corn (test)	NP2391/NP2222(MZHG0JG)
E08	Corn reference variety 1	NK Octet
E09	Corn reference variety 2	NK Lucius
E10	Corn reference variety 3	NK Cisko

X.B.2. Study design

The entries were grown in a randomized complete block design with four replicate plots in Mebane, North Carolina. This location is representative of an agricultural region suitable for the cultivation of the hybrid corn varieties shown in Table X–6. All entries were treated with conventional pesticides as needed to maintain optimal plant health.

Pollen was sampled from four representative plants per plot, for a total of 16 samples from each of the five entries. Tassels were covered with paper bags prior to collection and pollen was collected separately from each of the plants at the tasseling (VT) growth stage. The test and control plants were sampled on the same day. The reference varieties were sampled as the plants reached VT stage.

For each bagged tassel, anthers and pollen were dislodged by shaking the tassel vigorously within the tassel bag. Pollen was separated from anthers using a metal sieve, and preserved in 70% ethanol. The pollen samples were stained with 1% (w/v) Lugol's solution (iodine–potassium iodide), which readily binds to starch in viable cells (Pedersen *et al*. 2004).

The percentages of viable and nonviable pollen cells were computed after examination of at least 150 randomly selected cells by light microscopy under 48X to 64X magnification. Pollen grains that were deeply stained, spherical, and turgid (and not burst or injured) were classified as morphologically normal and viable. Pollen grains that were not stained (yellow or colorless) were classified as nonviable. The number of viable pollen grains (among a minimum of 150 evaluated per sample) were counted by use of the Object Count™ feature of the digital imaging software. The proportion of viable pollen was calculated as the number of viable pollen grains divided by the total number of pollen grains evaluated.

Morphology was determined by measuring the diameter of 10 representative stained pollen grains under 160X magnification by use of the Radius™ feature of the software.

X.B.3. Statistical analyses

Statistical analyses were performed using SAS® software v. 9.4 (SAS Institute, Inc., Cary NC).

The count of viable pollen grains of each entry was modeled as a ratio with the number of pollen grains examined for the entry in the replication (i.e., estimating the proportion viable in each

entry in each block). The ratios were subjected to analysis of variance (ANOVA) using the following generalized linear mixed model (PROC GLIMMIX of SAS® software).

$$(Y_{ij}/G_{ij}) = U + T_i + B_j + e_{ij}$$

In this model, Y_{ij} is the number of viable pollen grains for entry *i* in block *j* and G_{ij} is the total number of pollen grains examined for entry *i* in block *j*. The overall mean is U, T_i is the entry effect, B_j is the block effect, and e_{ij} is the residual error. Entry was regarded as a fixed effect while block was regarded as a random effect. Because the estimate of pollen viability is fundamentally binomial (i.e., viable or not viable), GLIMMIX was used to model the effects with the binomial distribution. For each experiment, there were five entries: MZHG0JG corn, the nontransgenic control corn, and three reference corn varieties.

A *t*-test was used to assess the statistical significance of the comparison of interest (test corn vs. control corn) for pollen viability. Significance was based on the customary alpha level of 0.05 and denominator degrees of freedom were determined by the Kenward-Roger method (Kenward and Roger 1997). There were two SEMs (standard errors of the mean) estimated (one for each entry) because variation is related to the mean in binomial data. The SEMs and range were also reported. The estimates of pollen diameter (mean of 10 estimates in each of 4 subsamples, *N* = 40 per plot) were subjected to ANOVA using the following mixed model in SAS® software.

$$Y_{ij} = U + T_i + B_j + e_{ij}$$

In this model, Y_{ij} is the observed response for entry *i* and block *j*, U is the overall mean, T_i is the entry effect, B_j is the effect of block, and e_{ij} is the residual error. Entry was regarded as a fixed effect, while the block effect was regarded as random. For each experiment, there were five entries: MZHG0JG corn, the nontransgenic control corn, and three reference corn varieties.

A *t*-test was used to assess the statistical significance of the comparison of interest (test vs. control) for pollen diameter. 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 SEM and range were also determined.

X.B.4. Results

The results of ANOVA for pollen characteristics evaluated for MZHG0JG corn (test) and the nontransgenic, near-isogenic corn (control), along with the means and ranges for the test, control, and reference varieties are shown in Table X–7. No significant differences ($p < 0.05$) were detected between the test pollen and the control pollen for viability or morphology. While not compared statistically with the reference varieties, the pollen viability and morphology means for the test and control were within the range of values for the reference varieties.

Table X–7. Corn pollen viability and diameter

	Viability (%)[a]			Diameter (µm)		
Entry	**Mean/Range**	**SEM[b]**	***P***	**Mean/Range**	**SEM**	***P***
MZHG0JG corn	99.0	0.17, 0.16	0.577	86.6	0.34	0.126
Nontransgenic control corn	99.1			87.4		
Corn reference varieties	99.0–99.5			80.9–87.4		

Test: *N* = 16
Control: *N* = 16
Reference varieties: *N* = 48
[a]Viable pollen was recorded as the ratio of stained pollen grains to total pollen grains (e.g., 0.990), and presented as a percentage of viable pollen (e.g., 99.0) for convenience.
[b]For viable pollen, there were two SEMs estimated (one for test and one for control) because variation is related to the mean in binomial data.

X.C. Field Agronomic Trials

Field trials were conducted with conventional agronomic practices to plant, maintain and harvest replicate plots at eight locations in 2013 across the U.S., comparing MZHG0JG corn to corresponding nontransgenic, near-isogenic control corn. Additionally, six nontransgenic corn reference varieties suitable for cultivation at each location were included to establish a range of natural variation in these agricultural regions, utilizing germplasm with a history of cultivation.

X.C.1. Test, control, and reference materials

Figure III–2 shows the breeding pedigree of MZHG0JG seed materials. Table X–8 presents the descriptions and genotypes for the test, control, and reference corn varieties grown. The reference varieties were six commercially-available, nontransgenic corn hybrids.

Table X–8. Plant material used in agronomic study

Entry Identification	Seed Identification	Hybrid genotype
E01	Nontransgenic, near-isogenic corn (control)	NP2391/NP2222
E02	MZHG0JG corn (test)	NP2391/NP2222(MZHG0JG)
E09	Corn reference variety 1	H-7191
E10	Corn reference variety 2	H-7540
E11	Corn reference variety 3	SY Generoso
E12	Corn reference variety 4	NK Lucius
E13	Corn reference variety 5	NK Cisko
E14	Corn reference variety 6	SY Provial

Field-grown seed lots of the test and control materials were analyzed by real-time polymerase chain reaction testing (Ingham *et al.* 2001) to confirm identity and purity.

X.C.2. Field trial locations, layout, and design

One entry of MZHG0JG corn, one entry of the nontransgenic control corn, and one entry of each of the six reference varieties were grown according to local agronomic practices at eight U.S. locations. The field trials were conducted at locations where the soil type was typical for commercial corn production, where growth and maintenance of the crop could be monitored, and that are representative of the agricultural regions suitable for the cultivation of the corn varieties shown in Table X–8.

At each location, the entries were grown in a randomized complete block design with four replicate plots. The plots consisted of six rows spaced approximately 30 inches (0.76 m) apart and 20 feet (6 m) long. The planting rate was approximately 40 seeds per row and a planting density of approximately 240 plants per plot (34,800 plants/acre; 86,000 plants/ha).

All entries were treated with conventional pesticides as needed to maintain optimal plant health. For all field observations of agronomic endpoints, data were collected for all plants in two interior rows of each plot, except plant height and ear height, which were recorded for 10 plants chosen in a nonsystematic manner from two interior rows. To monitor for naturally occurring ecological stressors, the plots were evaluated for insect damage, incidence of disease, and abiotic stress. Observations were made and visual estimates were recorded every four weeks after the plants reached the V2 vegetative growth stage. Collectively, these observations were used to identify potential differences in susceptibility between MZHG0JG corn and the nontransgenic control corn to natural environment stressors.

X.C.3. Statistical analyses

Statistical analyses were performed using SAS® software v. 9.4 (SAS Institute, Inc., Cary, NC). Some data did not lend themselves to formal statistical analysis because they did not conform to the assumptions upon which the validity of the analysis depends. Consequently, results for such variates are presented as means and ranges.

Data describing agronomic characteristics 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 the location-by-entry interaction were regarded as random. Only the control and MZHG0JG entries were included in the ANOVA. To avoid the possibility of the residual error being inflated by any interaction between location and reference varieties that may have been present, the reference varieties were not included in this analysis.

For each agronomic characteristic, *t*-tests were set up within this analysis framework to assess the statistical significance of the comparison of interest (test 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 characteristic. For characteristics for which data were limited to a very few number of values, only means were calculated because these data do not conform to the assumptions underlying the statistical analysis and consequently do not provide a valid estimate of error.

X.C.4. Comparisons with nontransgenic corn reference varieties

Agronomic characteristics of MZHG0JG corn were compared non-statistically to those in the nontransgenic corn reference varieties.

X.C.5. Results

The data for quantitative agronomic assessments of MZHG0JG corn that were suitable for statistical analysis are summarized in Table X–9. Quantitative data that were not suitable for statistical analysis are presented in Table X–10. A comparison of test weight and yield of MZHG0JG corn and nontransgenic corn are presented in Table X–11. Qualitative observations of ecological stressors are summarized in Table X–12.

No significant differences were observed between MZHG0JG corn and the nontransgenic control corn in ear height, plant height, stay green, grain moisture, and test weight (Table X–9). The mean grain yield of the test corn was statistically significantly lower than that of the control corn, but was within the range of the reference varieties. However, within individual locations, which had four replications, no statistical difference was observed. Additionally, test weight, another key agronomic characteristic, was not significantly different (Table X–9). Yield, when measured in bushels per acre, as it is in commerce, was also not statistically different between MZHG0JG corn and the control (Table X–11). The mean early stand count (before thinning to a uniform stand) of the test corn was less than the control corn. However, the difference was small (68 versus 70) and was within the range of the reference varieties.

Among the characteristics that were not suitable for statistical comparison, the mean values for early stand count post-thinning, final stand count, early growth rating, days to 50% pollen shed, days to 50% silking, and dropped ears were similar for the test corn and the nontransgenic control corn (Table X–10). Although there were higher mean numbers of root- and stalk-lodged plants in MZHG0JG corn plots than in the control plots, the observed ranges were within the observed ranges of the reference varieties. The results of these phenotypic assessments indicate that MZHG0JG corn is not phenotypically different from conventional corn with respect to characteristics that would increase its weediness potential. Additionally, no biologically relevant deficits in agronomic performance of MZHG0JG corn were observed.

Table X–9. Agronomic characteristic data: Across-location comparison (ANOVA) of MZHG0JG corn and nontransgenic (control) corn

Entry	Statistic	Early stand count (pre-thin)[a]	Ear height (cm)	Plant height (cm)	Stay green (%)	Grain moisture (%)	Test weight[b] (kg/hL)	Grain yield[c] (kg/plot)	Grain yield[c] (Mg/ha)
Test	mean	68.0	87.5	236	49.8	19.3	66.2	9.42	10.1
	range	50–75	43.6–111	155–279	5–85	14.6-–28.0	53.7–77.2	5.92–13.8	6.37–14.9
Control	mean	70.0	88.1	233	50.2	19.0	66.6	9.89	10.6
	range	53–78	54.2–104	153–268	5–75	14.4–24.7	56.5–76.7	6.24–14.0	6.72–15.0
ANOVA (*t*-test) entry effect and standard error of means (SEM)									
	p	***0.008***	0.747	0.231	0.897	0.501	0.643	***0.029***	***0.029***
	SEM	1.52	4.89	10.2	7.54	1.08	2.00	0.654	0.71
Reference varieties	mean	74.6	99.8	224	37.7	18.2	67.0	9.63	10.4
	range	59–81	53.9–133	131–277	0–80	13.5–30.3	49.7–80.2	6.01–15.5	6.47–16.7

Test: *N* = 32
Control: *N* = 32
Reference varieties: *N* = 192
Results significantly different ($p < 0.05$) are shown in bold italic type
[a]Early stand count before thinning to a uniform stand
[b]Test weight was measured in pounds per bushel, adjusted to the standard 15.5% moisture by calculation, then converted into kilograms per hectoliter (kg/hL)
[c]Yield was measured in kilograms per plot (kg/plot), adjusted to the standard 15.5% moisture by calculation; the mean, range and SEM were converted to megagrams per hectare (Mg/ha), thus the *p*-value for Mg/ha is the same as for kg/plot.

Table X–10. Agronomic characteristic data: Across-location comparison of MZHG0JG corn (test) and nontransgenic (control) corn

Entry	Statistic	Early stand count (post-thin)[a]	Final stand count	Early growth rating[b]	Days to 50% pollen shed	Days to 50% silking	Root-lodged plants (%)	Stalk-lodged plants (%)	Number of dropped ears
Test	mean	63.9	61.7	5.75	58.2	58.2	1.44	5.06	0.0625
	range	57–69	52–71	4–8	52–63	54–63	0–13	0–31	0–1
Control	mean	64.1	61.4	5.75	58.4	58.3	0.563	3.88	0.0625
	range	59–69	55–69	3–8	52–63	52–63	0–6	0–31	0–1
Reference varieties	mean	64.1	62.2	6.69	55.8	55.8	0.770	3.52	0.0890
	range	59–69	51–71	4–9	48–65	47–66	0–15	0–45	0–3

Evaluation of these characteristics resulted in a limited number of values; consequently, analysis of variance was not appropriate and only the means and ranges are shown

Test: *N* = 32

Control: *N* = 32

Reference varieties: *N* = 192

[a]Early stand count after thinning to a uniform stand.

[a]Rated on a scale of 1-9,where 1 = dead and 9 = above average vigor.

Table X–11. Across-location comparison for test weight and yield of MZHG0JG corn (test) and nontransgenic (control) corn (converted to US customary units of measure)

Entry	Statistic	Test weight (lb/bu)[a,b]	(lb/plot)[b, c]	Grain yield (tons/A)[b, c]	(bu/A)[b]
Test	mean	51.4	20.8	4.53	176
	range	41.7–60.0	13.1–30.4	2.84–6.65	121–245
Control	mean	51.7	21.8	4.74	183
	range	43.9–59.6	13.8–30.9	3.00–6.69	127–235
ANOVA (*t*-test) entry effect and standard error of means (SEM)					
	p	0.643	***0.029***	***0.029***	0.091
	SEM	1.56	1.44	0.315	10.4
Reference lines	mean	52.0	21.2	4.63	178
	range	38.6–62.3	13.2–34.2	2.89–7.45	108–271

Test and Control *N* = 32
Reference lines: *N* = 192
Results significantly different ($p < 0.05$) are shown in bold italic type.
[a] Analysis carried out for kilograms per hectoliter (kg/hL); the mean, range and SEM were converted to pounds per bushel (lb/bu); thus the *p*-value for lb/bu is the same as for kg/hL.
[b] Harvested plot size was approximately 100 ft^2 (92.9 m^2); corrected to the standard 15.5% moisture.
[c] Analysis carried out for kilograms per plot (kg/plot); the mean, range and SEM were converted to pounds per plot (lb/plot) and tons per acre (tons/A); thus the *p*-values for lb/plot and tons/A are the same as for kg/plot.

Table X–12. Qualitative observations of plot interactions with biotic and abiotic stressors

Location code	Location	Test (E02)	Control (E01)
L01	Richland, Iowa	R5-R6: mild damage from corn earworm; minimal damage from *Fusarium* ear rot; minimal stress from drought.	R5-R6: mild damage from corn earworm; minimal damage from *Fusarium* ear rot; minimal stress from drought.
L02	York, Nebraska	no insect, disease, or abiotic stressors	no insect, disease, or abiotic stressors
L03	Seymour, Ilinois	V17: minimal damage from corn rust. R3: minimal damage from corn borer, grasshopper, and root worm beetle; minimal damage from rust and gray leaf spot.	V3: minimal damage from wind. V17: minimal damage from corn rust and gray leaf spot. R3: minimal damage from grasshopper and root worm beetle.
L04	Bagley, Iowa	V2: none to minimal insect damage; minimal disease; minimal to mild abiotic stressors from wet soils. V9: minimal insect damage; mild abiotic stress from earlier wet soils. R1: minimal insect and disease damage; minimal to mild abiotic stressors from heat and low precipitation. R4: minimal insect and disease damage; minimal abiotic stressors. R5 to R6: minimal insect, disease, and abiotic stressors.	V2: none to minimal insect damage; minimal disease; minimal to mild abiotic stressors from wet soils. V9: minimal insect damage; mild abiotic stress from earlier wet soils. R1: minimal insect and disease damage; minimal to mild abiotic stressors from heat and low precipitation. R4: minimal insect and disease damage; minimal abiotic stressors. R5 to R6: minimal insect, disease, and abiotic stressors.
L05	Larned, Kansas	V2: minimal damage from Stewart's Disease and Twisted Whorl Syndrome; minimal damage from wind and nutrient deficiency. V10: minimal damage from grasshoppers, common rust, gray leaf spot, wind and heat. R5: minimal damage from grasshoppers, corn ear worm and wind. R6: minimal damage from corn ear worm; mild damage from gray leaf spot, rust, common smut and wind.	V2: minimal damage from Stewart's Disease and Twisted Whorl Syndrome; mild damage from wind and nutrient deficiency. V10: minimal damage from grasshoppers; minimal damage from common rust, gray leaf spot, wind and heat. R5: minimal damage from grasshoppers, corn ear worm and wind. R6: minimal damage from corn ear worm; mild damage from gray leaf spot and common rust; minimal damage from common smut and wind.

Continued

Location code	Location	Test (E02)	Control (E01)
L06	Stewardson, Illinois	V9–V10: minimal damage from armyworm, grasshoppers and rust. R1–R2: minimal damage from grasshoppers and rust. R5: minimal damage from ear worms and corn borers; minimal to mild gray leaf spot and rust; mild damage from wind and nutrients. R6: minimal to mild damage from ear worm and corn borer; minimal stalk rot; mild damage from wind.	V9–V10: minimal damage from grasshoppers. R1–R2: minimal damage from rust. R5: minimal to mild damage from earworm and corn borer; minimal to mild gray leaf spot and rust. R6: minimal to mild damage from corn borer; minimal stalk rot; minimal to mild wind damage.
L09	Wyoming, Illinois	V11: minimal damage from Japanese beetle; minimal damage from common rust. R1–R2: minimal damage from Japanese beetle and corn rootworm beetle; minimal rust and gray leaf spot. R4–R5: minimal corn earworm; minimal rust and gray leaf spot; minimal drought stress. R5–R6: mild rust; minimal wind damage.	V11: minimal to mild damage from Japanese beetle and armyworm; minimal damage from common rust. R1–R2: minimal damage from Japanese beetle and corn rootworm; minimal rust and gray leaf spot. R4–R5: minimal corn earworm; minimal rust and gray leaf spot; minimal drought stress. R5–R6: mild rust.
L10	Germansville, Pennsylvania	none to minimal insect and disease; no abiotic stressors.	none to minimal insect and disease; no abiotic stressors.

Corn vegetative and reproductive growth stages as defined by Abendroth *et al*. (2011).

X.D. Conclusions from Phenotypic, Agronomic, and Environmental Interactions Assessments of MZHG0JG Corn

The results of laboratory and field studies indicate that, apart from the intended phenotype of tolerance to glyphosate-based and glufosinate-ammonium-based herbicides, MZHG0JG corn is no different than conventional corn with regard to phenotypic and agronomic properties that bear on weediness potential.

While statistical differences were observed in seed germination in a laboratory setting at two temperature regimes and early stand count and yield in a field study, natural variations in seed germination and the complex multifactorial nature of yield suggest this variation is not biologically relevant. To counteract this natural variation and to ensure the highest yields, growers traditionally adjust and update their planting seeding rates to ensure optimum return on investment, factoring in field history and the background genetics of their hybrids as well as traits included in the seed.

XI. Potential Environmental Effects of MZHG0JG Corn Cultivation

The environmental impact of MZHG0JG corn cultivation is considered in the context of potential harm to wildlife, including species beneficial to agriculture, and the potential for the cultivar to become a weed.

XI.A. Potential Impact on Wildlife

EPSPS enzymes are ubiquitous in plants and microorganisms (ILSI 2011a). In addition, GA21 corn, which also produces the identical mEPSPS protein as MZHG0JG corn, was deregulated by the USDA in 1997 and is commercially available in a number of corn products. Similarly to GA21 corn, exposure to mEPSPS as expressed in MZHG0JG corn will occur mainly through the consumption of MZHG0JG corn or through contact with or consumption of soil in which MZHG0JG corn is cultivated. It is likely, therefore, that wildlife species potentially exposed to mEPSPS via MZHG0JG corn plant tissue or soil will have previously been exposed to the same enzyme or enzymes with similar function. No harmful effects of exposure to EPSPS enzymes at naturally occurring concentrations or from the cultivation of GA21 corn are known in years of field use.

Thirty-eight genetically modified cultivars expressing *pat*, including several corn cultivars, are approved for environmental release in at least one country (ILSI 2011b). PAT is normally produced in *Streptomyces* bacteria, which commonly occur in soil. Therefore, PAT or functionally similar proteins are ubiquitous in the environment. Wildlife species potentially exposed to PAT via MZHG0JG corn tissue or soil will have previously been exposed to enzymes with similar structure and function. No harmful effects of such exposure are known.

There are no material differences in crop composition or phenotype between MZHG0JG corn and conventional corn. Thus, there is no basis for concluding that cultivation of MZHG0JG corn will be more harmful to any threatened or endangered species than cultivation of conventional corn.

XI.B. Gene Flow

Corn hybridizes with a group of taxa collectively called teosinte. Several types of teosinte are classified as subspecies of *Zea mays*, whereas others are regarded as separate species of *Zea*. Teosinte species are natives of Central America and have co-existed with cultivated corn for several thousand years. They have remained genetically distinct from cultivated varieties despite occasional introgression (e.g., U.S. EPA 2010; Baltazar *et al*. 2005). Teosinte species are not natives of the US, but isolated populations have been recorded in Florida and Texas, the former a possible remnant of the use of annual teosinte as a forage grass. These populations are apparently now extinct in both states (U.S. EPA 2010). Teosinte species are grown in botanical gardens, but as corn pollen is heavy and relatively short-lived (e.g., U.S. EPA 2010; Devos *et al*. 2005), fertilization of these plants with pollen from MZHG0JG corn is extremely unlikely.

Species of the genus *Tripsacum* are considered close relatives of *Zea* species and some theories postulate that a *Tripsacum* species may be a progenitor of domesticated corn via hybridization and introgression with teosinte (e.g., Poggio *et al*. 2005). There are sixteen species of *Tripsacum* worldwide, of which three occur in the U.S.: *T. dactyloides*, a widespread forage grass; *T. floridanum*, known from southern Florida; and *T. lanceolatum*, which is present in Arizona and

possibly New Mexico (U.S. EPA 2010). Corn breeders view *Tripsacum* as a potential source of useful genes for traits including apomixis, pest and disease resistance, and drought tolerance (OECD 2003) and, therefore, substantial effort has been made to obtain and characterize corn × *Tripsacum* hybrids. Hybrids between corn and *Tripsacum* species are difficult to obtain outside the laboratory or greenhouse, and are often sterile. Only one record exists of an open-pollinated hybrid between *Zea* and *Tripsacum*, which involved species native to Guatemala. After consultation with experts on improvement of forage grasses, the U.S. EPA (2010) concluded that the chance of natural introgression of genes from corn to *Tripsacum* was 'extremely remote' and that no other species in the continental U.S. would interbreed with commercial corn.

The data reviewed above indicate the very low probability of transfer of the genes *mepsps-02* and *pat-09* from MZHG0JG corn to wild relatives in the U.S. Species of *Zea* other than corn are not recorded outside botanical gardens in the U.S. *Tripsacum dactyloides* is widespread, but does not hybridize readily with corn, and the probability of backcross or F_2 progeny of *Tripsacum* × *Zea* hybrids being produced in the field is negligible. Therefore, PAT and mEPSPS are unlikely to spread from corn cultivation and persist in the environment as the result of gene flow from MZHG0JG corn to wild relatives.

XI.C. Weediness Potential

Several characteristics make it unlikely for conventional corn to form feral populations. To evaluate whether MZHG0JG corn is potentially weedier than conventional corn, its performance in agronomic trials was compared with that of conventional, nontransgenic corn.

Corn has lost the ability to survive without cultivation (OECD 2003). It can over-winter and germinate in a subsequent crop as a volunteer weed; for example, corn is a common volunteer in soybean fields. Nevertheless, several features of corn make it unlikely to form self-sustaining weedy populations in agricultural systems: it is easily controlled in subsequent crops by selective herbicides (herbicide control of corn volunteers is not restricted only to herbicides containing glyphosate or glufosinate-ammonium); seed dispersal is limited because seeds are held inside the husks of the cob; and the seeds lack dormancy, thus young plants are exposed to harsh winter conditions. Corn does not persist in habitats outside agriculture because, in addition to the features listed above, it requires disturbed ground to germinate and it is very uncompetitive against perennial vegetation (Raybould *et al*. 2012). Expression of PAT and mEPSPS is highly unlikely to alter the dispersal or competitive ability of corn. This hypothesis was corroborated in a study comparing agronomic and phenotypic characteristics of MZHG0JG corn compared to conventional corn, as described above. The probability of spread of the transgenic proteins outside corn cultivation through volunteers and self-sustaining feral populations of MZHG0JG corn is therefore low.

A way to test whether a transgenic crop cultivar is likely to be weedier than its corresponding nontransgenic cultivar is to compare its performance in agronomic trials (White 2002, Raybould 2005). If their agronomic characteristics are similar, then it is likely that the potential to form weedy populations is no greater for the transgenic cultivar than for the nontransgenic cultivar. If the risks to endpoints potentially affected by weediness are acceptable for the nontransgenic crop, it follows that the risks should be acceptable for the transgenic crop (Raybould 2005). Agronomic characteristics typically used by breeders and agronomists to evaluate corn were compared between MZHG0JG corn and nontransgenic, near-isogenic corn. These characteristics

included stand count, yield, number of dropped ears, and plant height. Germination rate and frequency of dormant seeds were compared in a separate laboratory study.

The number of dropped ears observed in MZHG0JG corn and the nontransgenic, near-isogenic corn was comparable. The early stand count and grain yield of MZHG0JG corn were statistically significantly lower when compared to the nontransgenic, near-isogenic corn. The early stand count and grain yield of MZHG0JG corn, however, fell within the range observed in the conventional reference varieties. Therefore, the significant differences are not considered biologically relevant. Reductions in stand count and grain yields are not characterisitics associated with increased weediness potential.

The germination rate of MZHG0JG corn seed was statistically significantly lower than that of the nontransgenic, near-isogenic control corn at two of the five temperature regimes tested in a laboratory study. In those cases, however, the germination rate of MZHG0JG corn seed fell within the range observed in the conventional reference varieties. Therefore, the significant differences are not considered biologically relevant. Reductions in germination rate are not associated with increased weediness potential.

No biologically relevant differences indicative of increased weediness potential were observed in plant growth habit, life span, vegetative vigor, flowering characteristics, yield, stress adaptations, or disease susceptibility. Therefore, MZHG0JG corn is highly unlikely to be associated with an increase in the abundance of corn volunteers or be more difficult to control than conventional corn volunteers with grass-specific herbicides traditionally used in crop rotation practices. Similarly, agronomic data provide no evidence that MZHG0JG corn will form persistent feral populations.

XII. Impact on Agronomic Practices

Corn is the largest crop in the United States by acres planted. For production year 2014, corn for grain production was estimated at 14.2 billion bushels, which yielded a gross value of $52.4 billion (USDA-NASS 2015a and b). Average yield in 2014 was estimated at a record high of 171.0 bu/ac acre for which growers received an average price of $3.65/bu. The ten highest-producing states in 2014 by acres planted were Iowa, Illinois, Minnesota, Missouri, Indiana, Nebraska, Ohio, South Dakota, Wisconsin, and Kansas (USDA-NASS 2015b). The USDA estimates that 93% of the U.S. corn crop was planted to genetically egineered varieties in 2014 (USDA-NASS 2015a). Of this total, an estimated 4% of the planted U.S. corn crop was insect resistant, 13% was herbicide-tolerant, and 76% was stacked gene varieties, most of which contain an herbicide-tolerant component (USDA-NASS 2015a).

Like VCO-Ø1981-5 corn and DP-ØØ4114-3 corn, MZHG0JG corn would provide an additional cultivar option for herbicide-tolerant corn that would serve as a substitute or alternative to existing options. Therefore, like VCO-Ø1981-5 corn and DP-ØØ4114-3 corn, MZHG0JG corn would not cause an expansion of corn production or change existing corn production practices. Results of efficacy studies comparing the performance of MZHG0JG corn to a nontransgenic, near-isogenic control corn hybrid demonstrate that MZHG0JG corn is highly tolerant to broad-spectrum herbicides containing glyphosate and glufosinate-ammonium and is therefore expected to have similar agronomic performance to its commercial counterparts (Appendix E).

Glyphosate and glufosinate-ammonium are both broad-spectrum, post-emergent herbicides, and the traits created in MZHG0JG corn by genetic engineering serve to confer tolerance to herbicides containing these active ingredients. The agricultural production consequences or impacts of deregulation of MZHG0JG corn would be similar to those of the antecedents, VCO-Ø1981-5 corn and DP-ØØ4114-3 corn, because the resulting phenotype of both MZHG0JG corn and the referenced antecedents is a corn plant resistant to broad-spectrum, post-emergent herbicide products. DP-ØØ4114-3 corn additionally has introduced traits conferring tolerance to certain lepidopteran and coleopteran pests, however, these insect-resistance phenotypes are not relevant to this extension request. Further, products tolerant to both glyphosate and glufosinate-ammonium have been commercially available for many years as varieties containing combinations of events that have been previously assessed by the USDA (Appendix B).

Syngenta has conducted a detailed review of the EAs published for VCO-Ø1981-5 corn and DP-ØØ4114-3 corn. This review is outlined in the Environmental Memorandum submitted along with this extension request.

XIII. Adverse Consequences of Introduction

Syngenta is not aware of any unfavorable information that would have a bearing on a decision by USDA to deregulate MZHG0JG corn. The development and testing of MZHG0JG corn has not revealed any data or observations indicating that deregulation of this new cultivar would pose a greater risk to the environment than conventional corn.

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Appendix A. USDA Permits and Notifications for MZHG0JG Corn

Field trials with MZHG0JG corn have been conducted in the U.S. under USDA APHIS permits and notifications since 2010. A complete listing of these permits and notifications and the status of the associated field test reports by Syngenta to USDA APHIS are provided in Table A–1.

Table A–1. USDA permits and notifications for field releases of MZHG0JG corn

Notification no.	States[a]	Effective dates	Status of field test report
15-062-101n	CA, CO, FL, HI, IL, IN, IA, MN, NE, NC, PR, SD, WI	4/3/15-4/3/16	pending
15-054-103n	CA, CO, HI, IL, IN, IA, MN, NE, NC, PR, SD, WI	4/1/15-4/1/16	pending
14-255-102n	HI, IL, IA,NC, PR	10/1/14-10/1/15	pending
14-107-107n	IL, WI	5/1/14-5/1/15	pending
14-101-105n	CO, FL, IL, IA, MO, NC, TX, WI	5/1/14-5/1/15	pending
14-035-109n	CA, CO, FL, HI, IL, IN, IA, MN, NE, NC, PR, SD, WI	4/1/14-4/1/15	pending
13-247-105n	HI, PR	9/19/13-9/19/14	submitted
13-043-102rm	IA, IL, KS, NE, PA, WA, WI	4/1/13-4/1/14	submitted
13-038-103rm	HI, IL, IA, MN, NE, PR, SD, WI	4/17/13-4/17/14	submitted
12-244-104n	HI, PR	10/01/12-10/01/13	submitted
12-044-102n	HI, IL, IN, IA, MN, SD, WI	4/1/12-4/1/13	submitted
11-231-106n	HI, PR	9/19/11-9/19/12	submitted
11-047-105n	HI, IA, IL, IN, MN, WI	4/1/11-4/1/12	submitted
10-235-105n	HI, PR	9/23/10-9/23/11	submitted

[a]States listed are actual release states if the field test report has been submitted or approved release states if the field test report is still pending.

Appendix B. Deregulated Transgenic Corn Events Conferring Tolerance to Broad-Spectrum Herbicides

This appendix contains a summary of transgenic corn events previously deregulated by the USDA that display tolerance to broad-spectrum herbicides.

Table B–1. Deregulated transgenic corn events conferring tolerance to broad-spectrum herbicides

Event Name(s)	Filed By / Petition Number	Effective Date of Deregulation	Gene Conferring Tolerance to Broad-Spectrum Herbicides / Protein
VCO-Ø1981-5	Bayer/Genective / petition 11-342-01p	September 25, 2013	*epsps grg23ace5*/ EPSPS ACE5
DP-ØØ4114-3	Pioneer / petition 11-244-01p	June 20, 2013	*pat* /PAT
HCEM485	Stine Seed / petition 09-063-01p	May 3, 2013	*2mepsps*/ 2mEPSPS
98140	Pioneer / petition 07-152-01p	December 9, 2009	*gat4621*/ GAT4621
MON 88017	Monsanto / petition 04-125-01p	December 14, 2005	*cp4 epsps*/ CP4 EPSPS
59122	Dow / petition 03-353-01p	September 23, 2005	*pat* /PAT
6275	Dow / petition 03-181-01p	October 20, 2004	*pat* /PAT
1507	Mycogen c/o Dow & Pioneer / petition 00-136-01p	June 14, 2001	*pat* /PAT
NK603	Monsanto / petition 00-011-01p	September 29, 2000	*cp4 epsps*/ CP4 EPSPS
MS6	AgrEvo / petition 98-349-01p	March 16, 1999	*bar*/ PAT
676, 678, 680	Pioneer / petition 97-342-01p	May 14, 1999	*pat* /PAT
CHB-351	AgrEvo / petition 97-265-01p	May 8, 1998	*bar*/ PAT
GA21	Monsanto / petition 97-099-01p	November 18, 1997	*mepsps*/ mEPSPS
MON 802	Monsanto / petition 96-291-01p	May 27, 1997	*cp4 epsps*/ CP4 EPSPS
DBT418	DeKalb / petition 96-291-01p	March 28, 1997	*bar*/ PAT
MON 809	Monsanto / petition 96-017-01p	March 11, 1996	*cp4 epsps*/ CP4 EPSPS
Bt11	Northrup King / petition 95-195-01p	July 18, 1996	*pat* /PAT

Continued

Event Name(s)	Filed By / Petition Number	Effective Date of Deregulation	Gene Conferring Tolerance to Broad-Spectrum Herbicides / Protein
B16	DeKalb / petition 95-145-01p	December 19, 1995	*bar*/ PAT
MON 80100	Monsanto / petition 95-093-01p	August 22, 1995	*cp4 epsps*/ CP4 EPSPS
T14, T25	AgrEvo / petition 94-357-01p	June 22, 1995	*pat* /PAT
Bt176	Ciba Seeds / petition 94-319-01p	May 17, 1995	*bar*/ PAT

Source: USDA APHIS 2015

Appendix C. Methods Used for Compositional Analysis of MZHG0JG Forage and Grain

This appendix describes the methods used to conduct the compositional analysis study described in Section VII., wherein the results are also provided.

C.1. Study Design

Forage and grain for compositional analyses were harvested from multiple locations planted in the U.S. in 2013. The locations chosen were representative of major corn producing regions in the country. For all locations, trials were planted with MZHG0JG corn and nontransgenic, near-isogenic corn in a randomized complete block design with four replicate plots, and were managed following local agronomic practices. The plants were self-pollinated by hand and the developing ears were bagged to avoid cross-pollination. Trials were planted in ten locations in an effort to ensure that grain and forage from at least eight locations could be harvested in the event of loss due to adverse environmental conditions (e.g., early freeze, drought). Eight locations that produced sufficient grain and forage were selected for this study.

C.2. Forage Sampling and Processing

For each genotype, the entire above-ground portion of five plants from each of the three replicate plots at each location was harvested at dough stage (R4), the stage at which silage typically is prepared. Plants were pooled to create a composite sample for each replicate plot, then ground using a chipper-shredder. A subsample from each well-mixed composite sample was shipped overnight on ice packs to Syngenta Crop Protection (Greensboro, NC). The samples were stored at –20°C ± 10°C, then finely ground and shipped on dry ice to a contract research laboratory, where they were stored at –20°C ± 10°C until they were analyzed.

C.3. Grain Sampling and Processing

For each genotype, ears were collected from 15 plants from each replicate plot at each location. Ears were harvested after reaching physiological maturity (R6) and then mechanically dried to approximately 9% to 12% moisture content. (Mechanical drying after harvest is standard agronomic practice for improving storage characteristics of corn grain.) Each sample consisted of grain shelled from ears collected from 15 plants from one replicate plot. A well-mixed subsample of approximately 500 g of grain from each plot was shipped at ambient temperature to Syngenta Crop Protection, where it was stored at –20°C ± 10°C, then finely ground and shipped on dry ice to the contract testing facility. The samples were stored at –20°C ± 10°C until they were analyzed.

C.4. Compositional Analyses

As detailed in Section VII, forage was analyzed for proximates and the minerals calcium and phosphorus. Grain was analyzed for major constituents (proximates and starch), minerals, amino acids, fatty acids, vitamins, and selected anti-nutrients and secondary metabolites.

All compositional analyses were conducted using methods published and approved by AOAC International, or other industry-standard analytical methods, described below. Based on the moisture content of each sample, analyte levels were converted to equivalent units of dry weight.

C.5 Analytical Methods and Reference Standards for Compositional Analyses

2-Furaldehyde (Albala-Hurtado *et al.* 1997)

The ground sample was extracted with 4% trichloroacetic acid and injected directly on a high-performance liquid chromatography system for quantitation of free furfurals by ultraviolet detection. The limit of quantitation (LOQ) for this study was 0.500 ppm, calculated on a fresh-weight basis.

Reference Standard: Acros 2-Furaldehyde, 99.5%, Lot Number A0296679

Acid Detergent Fiber (USDA 1970)

Sample aliquots were weighed into pre-weighed filter bags. The fats and pigments were then removed by an acetone wash. The filter bags were placed in an ANKOM Fiber analyzer where the protein, carbohydrate, and ash content were dissolved by boiling acidic detergent solution. After drying, the bags were reweighed and the acid detergent fiber was determined gravimetrically. The limit of quantitation was calculated as 0.100% on a fresh weight basis.

Amino Acid Composition (AOAC 2005k)

Total aspartic acid (including asparagine)
Total threonine
Total serine
Total glutamic acid (including glutamine)
Total proline
Total glycine
Total alanine
Total valine
Total isoleucine
Total leucine
Total tyrosine
Total phenylalanine
Total histidine
Total lysine
Total arginine
Total tryptophan
Sulfur-containing amino acids: Total methionine
Total cystine (including cysteine)

The sample was assayed by three methods to obtain the full profile. Tryptophan required a base hydrolysis with sodium hydroxide. The sulfur-containing amino acids required an oxidation with performic acid prior to hydrolysis with hydrochloric acid. Analysis of the samples for the remaining amino acids was accomplished through direct acid hydrolysis with hydrochloric acid. Once hydrolyzed, the individual amino acids were then quantitated using an automated amino acid analyzer. The LOQ for each amino acid assay was 0.100 mg/g, calculated on a fresh-weight basis. All reference standards were manufactured by Sigma-Aldrich.

Table C–1. Reference standards for fatty acid composition

Component	Lot No.	Purity (%)
L-Alanine	060M1776V	>99
L-Arginine Monohydrochloride	SLBF3348V	100
L-Aspartic Acid	091M0201V	100.0
L-Cystine	SLBB9524V	100
L-Glutamic Acid	060M01711	100
Glycine	059K0040V	100
L-Histidine Monohydrochloride Monohydrate	110M00481V	100
L-Isoleucine	090M00842V	100
L-Leucine	110M00492V	100
L-Lysine Monohydrochloride	051M0016V	100
L-Methionine	SLBF3077V	100
L-Phenylalanine	SLBF2036V	100
L-Proline	SLBF1872V	100
L-Serine	098K0161V	99
L-Threonine	081M01921V	99
L-Trypthophan	SLBC5462V	100
L-Tyrosine	BCBF4244V	100.0
L-Valine	SLBF7406V	100

Ash (AOAC 2005b)

The sample was placed in an electric furnace at 550°C and ignited to drive off all volatile organic matter. The nonvolatile matter remaining was quantitated gravimetrically and calculated to determine percent ash. The LOQ for this study was 0.100%, calculated on a fresh-weight basis.

Beta-Carotene (AOAC 2005e, Quackenbush 1987)

The sample was saponified and extracted with hexane. The sample was then injected on a reverse phase high-performance liquid chromatography system with ultraviolet light detection. Quantitation was achieved with a linear regression analysis. The LOQ for beta-carotene was 0.0200 mg/100 g, calculated on a fresh-weight basis.

Reference Standard: Sigma-Aldrich, Beta-carotene, 98.4%, Lot Number 091M1417V

Carbohydrates (USDA 1973)

The total carbohydrate level was calculated by difference using the fresh weight-derived data and the following equation:

% carbohydrates = 100 % - (% protein + % fat + % moisture + % ash)

The LOQ for this study was 0.100%, calculated on a fresh-weight basis.

Fat by Acid Hydrolysis (AOAC 2005a)

The sample was hydrolyzed with hydrochloric acid. The fat was extracted with ether and hexane. The extract was dried down and filtered through a sodium sulfate column. The hexane extract was then evaporated, dried, and weighed. The LOQ for this study was 0.100%, calculated on a fresh weight basis.

Fatty Acids (AOAC 2005l, AOCS 1997b and 2001)

The lipid was extracted and saponified with 0.5*N* sodium hydroxide in methanol. The saponification mixture was methylated with 14% boron trifluoride in methanol. The resulting methyl esters were extracted with heptane containing an internal standard. The methyl esters of the fatty acids were analyzed by gas chromatography using external standards for quantitation. The LOQ was 0.00300%, calculated on a fresh-weight basis. The manufacturer for all standards was Nu-Check Prep GLC, Reference Standard Covance 1 and 2.

Table C–2. Reference standards for fatty acids

Component	Lot No. JY10-W		Lot No. O1-X	
	Weight (%)	Purity (%)	Weight (%)	Purity (%)
Methyl Octanoate	3.0	99.7	1.25	99.5
Methyl Decanoate	3.25	99.6	1.25	99.4
Methyl Laurate	3.25	99.8	1.25	99.7
Methyl Myristate	3.25	99.8	1.25	99.7
Methyl Myristoleate	1.0	99.5	1.25	99.4
Methyl Pentadecanoate	1.0	99.6	1.25	99.5
Methyl Pentadecenoate	1.0	99.4	1.25	99.4
Methyl Palmitate	10.0	99.8	15.75	99.7
Methyl Palmitoleate	3.0	99.7	1.25	99.7
Methyl Heptadecanoate	1.0	99.6	1.25	99.5
Methyl 10-Heptadecenoate	1.0	99.5	1.25	99.4
Methyl Stearate	7.0	99.8	14.00	99.5
Methyl Oleate	10.0	99.8	15.75	99.5
Methyl Lineoleate	10.0	99.8	15.75	99.5
Methyl Gamma Lineolenate	1.0	99.4	1.25	99.5

Continued

Component	Lot No. JY10-W		Lot No. O1-X	
	Weight (%)	Purity (%)	Weight (%)	Purity (%)
Methyl Linolenate	3.0	99.5	1.25	99.4
Methyl Arachidate	2.0	99.8	1.25	99.5
Methyl 11-Eicosenoate	2.0	99.6	1.25	99.6
Methyl 11-14 Eicosadienoate	1.0	99.5	1.25	99.5
Methyl 11-14-17 Eicosadienoate	1.0	99.5	1.25	99.6
Methyl Arachidonate	1.0	99.4	1.25	99.5
Methyl Behenate	1.0	99.8	1.25	99.5

Folic acid (AOAC 2005i, Infant Formula Council 1985a)

The sample was hydrolyzed in a potassium phosphate buffer with the addition of ascorbic acid to protect the folic acid during autoclaving. Following hydrolysis by autoclaving, the sample was treated with a chicken-pancreas enzyme and incubated approximately 18 hours to liberate the bound folic acid. The amount of folic acid was determined by comparing the growth response of the sample, using the bacteria *Lactobacillus casei*, with the growth response of a folic acid standard. This response was measured turbidimetrically. The LOQ was 0.00600 mg/100 g, calculated on a fresh-weight basis.

Reference Standard: USP, Folic acid, 98.9%, Lot Number Q0G151

Inductively Coupled Plasma Emission Spectrometry (AOAC 2005m)

The sample was dried, precharred, and ashed overnight in a muffle set to maintain 500°C. The ashed sample was re-ashed with nitric acid, treated with hydrochloric acid, taken to dryness, and put into a solution of 5% hydrochloric acid. The amount of each element was determined at appropriate wavelengths by comparing the emission of the unknown sample, measured on the inductively coupled plasma spectrometer, with the emission of the standard solutions. The LOQs (Table C–3) were calculated on a fresh-weight basis.

Table C–3. Reference standards for inductively coupled plasma emission spectrometry

Mineral	Lot Numbers	Calibration Standard Concentration (µg/ml)	LOQ (ppm)
Calcium	G2-MEB500050MCA, G2-MEB500048 H2-MEB518023MCA, H2-MEB518025	200 1000	20.0
Copper	G2-MEB500050MCA, G2-MEB499064MCA H2-MEB518023MCA, H2-MEB518024MCA	2 10	0.500
Iron	G2-MEB500050MCA, G2-MEB500049 H2-MEB518023MCA, H2-MEB518026	10 50	2.00
Magnesium	G2-MEB500050MCA, G2-MEB499064MCA H2-MEB518023MCA, H2-MEB518024MCA	50 250	20.0
Manganese	G2-MEB500050MCA, G2-MEB499064MCA H2-MEB518023MCA, H2-MEB518024MCA	2 10	0.300
Phosphorus	G2-MEB500050MCA, G2-MEB500048 H2-MEB518023MCA, H2-MEB518025	200 1000	20.0
Potassium	G2-MEB500050MCA, G2-MEB500048 H2-MEB518023MCA, H2-MEB518025	200 1000	100
Sodium	G2-MEB500050MCA, G2-MEB500048 H2-MEB518023MCA, H2-MEB518025	200 1000	100
Zinc	G2-MEB500050MCA, G2-MEB499064MCA H2-MEB518023MCA, H2-MEB518024MCA	10 50	0.400

Inositol (Infant Formula Council 1985b, Atkins *et al.* 1943)

The inositol sample was extracted with dilute hydrochloric acid at a high temperature. The amount of inositol was determined by comparing the growth response of the sample, using the yeast *Saccharomyces cerevisiae*, with the growth response of an inositol standard. The response was measured turbidimetrically. The LOQ for this study was 40.0 µg/g, calculated on a fresh-weight basis.

Reference Standard: Sigma-Aldrich, Myo-Inositol, 100%, Lot Number 090M0142V

Moisture (AOAC 2005c)

The sample was dried in a vacuum oven at approximately 100°C to a constant weight. The moisture weight loss was determined and converted to percent moisture. The LOQ for this study was 0.100%, calculated on a fresh-weight basis.

Neutral Detergent Fiber (NDF), Enzyme Method (AACC 1998, USDA 1970)

The sample was washed with acetone to remove fats and pigments. It was then placed in a filter bag and positioned in an Ankom analyzer where it was washed with a neutral boiling detergent solution that dissolved the protein, carbohydrate, enzyme, and ash. The remaining hemicellulose, cellulose, and lignin fractions were determined gravimetrically. The LOQ for this study was 0.100%.

Niacin (AOAC 2005g)

The sample was hydrolyzed with sulfuric acid and the pH was adjusted to remove interferences. The amount of niacin was determined by comparing the growth response of the sample, using the bacteria *Lactobacillus plantarum*, with the growth response of a niacin standard. This response was measured turbidimetrically. The LOQ for this study was 0.0300 mg/100 g.

Reference Standard: USP, Niacin, 99.8%, Lot Number J0J235

p-Coumaric Acid and Ferulic Acid (Hagerman and Nicholson 1982)

The sample was extracted with methanol using ultrasonication, hydrolyzed using 4*N* sodium hydroxide, buffered using acetic acid/sodium hydroxide, acidified with 3*N* hydrochloric acid, and filtered. The levels of p-coumaric and ferulic acids in the extract were determined by reverse phase high-performance liquid chromatography with ultraviolet detection. The LOQ for p-coumaric acid and ferulic acid was 50.0 ppm, calculated on a fresh-weight basis.

Reference Standards: Acros Organics, 4-Hydroxy-3-methoxycinnamic acid (ferulic acid), 99.4%, Lot Number A0294716

Sigma-Aldrich, *p*-coumaric Acid, 99.6%, Lot Number 091M1197V

Phytic Acid (Lehrfeld 1989 and 1994)

The sample was extracted using 0.5 M HCl with ultrasonication. Purification and concentration were accomplished on a silica-based anion-exchange column. The sample was analyzed on a polymer high-performance liquid chromatography column PRP-1, 5 µm (150 x 4.1 mm) with a refractive index detector. The LOQ for this study was approximately 0.100%, calculated on a fresh-weight basis.

Reference Standard: Sigma-Aldrich, Phytic Acid Sodium Salt Hydrate, 82.0%, Lot Number BCBK8062V

Protein (AOAC 2005h, Bradstreet 1965, Kalthoff and Sandell 1948)

Nitrogenous compounds in the sample were reduced in the presence of boiling sulfuric acid and a mercury catalyst mixture to form ammonia. The acid digest was made alkaline. The ammonia was distilled and then titrated with a previously standardized acid. The percent nitrogen was calculated and converted to protein using the factor 6.25. The LOQ for this study was 0.100%, calculated on a fresh-weight basis.

Selenium by Inductively Coupled Plasma-Mass Spectrometry (AOAC 2012)

The samples were closed-vessel microwave digested with nitric acid (HNO_3) and water. After digestion, the solutions were brought to a final volume with water. To normalize the organic contribution between samples and standards, a dilution was prepared for analysis that contained methanol. The selenium concentration was determined with Se^{78} using an inductively coupled plasma-mass spectrometer with a dynamic reaction cell by comparing the counts generated by standard solutions. The limit of quantitation was 30.0 ppb on a fresh weight basis.

Reference Standard: SPEX CertiPrep, Selenium, 1003 mg/L, Lot Number 19-04SEY

Starch (AOAC 2005o)

The sample was extracted with alcohol to remove carbohydrates other than starch (i.e., sugars). Then it was hydrolyzed into glucose with α-amylase and amyloglucosidase. Glucose was oxidized with glucose oxidase to form peroxide, which reacted with a dye in the presence of peroxidase to give a stable colored product proportional to glucose concentration. The glucose concentration was quantitated by measurement on a spectrophotometer at 510 nm. Percent starch was then calculated from the glucose concentration. The LOQ for this study was 0.05%, calculated on a fresh-weight basis.

Reference Standard: Sigma D(+)-Glucose, 99.8%, Lot Number 080M0142V

Sugar Profile (Brobst 1972, Mason and Stover 1971)

The sample was extracted with deionized water and the extract treated with a hydroxylamine hydrochloride solution in pyridine, containing phenyl-β-D-glucoside as an internal standard. The resulting oximes were converted to silyl derivatives by treatment with hexamethyldisilazane and trifluoracetic acid and analyzed by gas chromatography using a flame ionization detector. The acceptable LOQ for this study was 0.0500%, calculated on a fresh weight basis.

Reference Standards: Sigma-Aldrich, D(+)-Raffinose Pentahydrate, 99.6%, Lot Number 019K1156

Total Dietary Fiber (TDF) (AOAC 2005n)

Duplicate samples were gelatinized with α-amylase and digested with enzymes to break down starch and protein. Ethanol was added to each sample to precipitate the soluble fiber. The samples were filtered, and the residue was rinsed with ethanol and acetone to remove starch and protein degradation products and moisture. Protein content was determined for one of the duplicates; ash content was determined for the other. The total dietary fiber in the sample was calculated using the protein and ash values. The LOQ for this study was 1.00%, calculated on a fresh-weight basis.

Trypsin Inhibitor (AOCS 1997a)

The sample was ground and defatted with petroleum ether. A sample of matrix was extracted with 0.01*N* sodium hydroxide. Varying aliquots of the sample suspension were exposed to a known amount of trypsin and benzoy1-DL-arginine~p~nitroanilide hydrochloride. The sample was allowed to react for 10 minutes at 37°C. After 10 minutes, the reaction was halted by the addition of acetic acid. The solution was centrifuged, then the absorbance was determined at 410 nm. Trypsin inhibitor activity was determined by photometrically measuring the inhibition of trypsin's reaction with benzoyl-DL-arginine~p~nitroanilide hydrochloride. The LOQ for this study was 1.00 Trypsin Inhibitor Units (TIU)/mg, calculated on a fresh-weight basis.

Vitamin B_1 (AOAC 2005f)

The sample was autoclaved under weak acid conditions to extract the thiamine. The resulting solution was incubated with a buffered enzyme solution to release any bound thiamine. The solution was purified on a cation-exchange column. An aliquot was reacted with potassium ferricyanide to convert thiamine to thiochrome. The thiochrome was extracted into isobutyl alcohol, measured on a fluorometer, and quantitated by comparison to a known standard. The

limit of quantitation was calculated and reported on a fresh weight basis. The LOQ for this study was 0.010 mg/100 g. Results were reported as thiamine hydrochloride.

Reference Standard: USP, Thiamine Hydrochloride, Purity 99.7%, Lot Number P0K366

Vitamin B_2 (Riboflavin) (AOAC 2005d, U.S. Pharmacopeia 2005)

The sample was hydrolyzed with dilute hydrochloric acid and the pH was adjusted to remove interferences. The amount of riboflavin was determined by comparing the growth response of the sample, using the bacteria *Lactobacillus rhamnosus*, with the growth response of multipoint riboflavin standards. The growth response was measured turbidimetrically. The LOQ for this study was 0.0200 mg/100 g, calculated on a fresh-weight basis.

Reference Standard: USP, Riboflavin, 100%, Lot Number N1J079

Vitamin B_6 (AOAC 2005j, Atkins *et al.* 1943)

The sample was hydrolyzed with dilute sulfuric acid in the autoclave and the pH was adjusted to remove interferences. The amount of pyridoxine was determined by comparing the growth response of the sample, using the yeast *Saccharomyces cerveisiae* with the growth response of a pyridoxine standard. The response was measured turbidimetrically. Results were reported as pyridoxine hydrochloride. The LOQ for this study was 0.007 mg/100 g, calculated on a fresh-weight basis.

Reference Standard: USP, Pyridoxine hydrochloride, 99.8%, Lot Number Q0G409

Vitamin E (Cort *et al.* 1983, McMurray *et al*. 1980, Speek *et al*. 1983)

The sample was saponified to break down any fat and release vitamin E. The saponified mixture was extracted with ethyl ether and then quantitated by high-performance liquid chromatography using a silica column. The LOQ for this study was 0.500 mg/100 g, calculated on a fresh weight basis.

Reference Standard: USP, Alpha-Tocopherol, 98.5%, Lot Number O0K291

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Appendix D. Methods Used to Quantify mEPSPS and PAT Proteins in Corn Tissues

D.1. Test, Control, and Reference Materials

The test material for this study was MZHG0JG corn seed and the control material was nontransgenic, near-isogenic corn seed of the same genetic background as the test substance.

D.2. Plant Tissue Production and Collection

During the 2013 growing season, corn plants were grown according to local agronomic practices at four separate field-trial locations in the U.S. that were representative of agricultural regions where corn is commercially cultivated and suitable for the maturity group of the test and control seed used. These locations included York, NE; Kimballton, IA; Richland, IA; and Germansville, PA. Table D–1 shows the plant samples collected for analysis. Two samples were collected from the control entry and five samples from the test entry. All plant samples were placed on dry ice after collection and stored frozen until shipment. All samples were shipped overnight on dry ice to Covance Laboratories, Inc., Madison, WI where they were stored at –60°C or colder until they were prepared for protein extraction and analysis.

Table D-1. Tissue samples collected for analysis

Growth stage[a]	Tissues collected	Sample description
V6	leaves	all true leaves from one plant
	roots	entire root ball excluding brace roots
	whole plants	entire plant including the root ball
R1	leaves	all true leaves from one plant
	roots	entire root ball excluding brace roots
	pollen	pooled sample; 10 to 15 tassels per plot
	whole plants	entire plant including the root ball
R6	leaves	all true leaves from one plant
	roots	entire root ball excluding brace roots
	kernels	all kernels from primary ear of a single plant
	whole plants	entire plant including the root ball
Senesence	leaves	all true leaves from one plant
	roots	entire root ball excluding the brace roots
	kernels	all kernels from primary ear of a single plant

[a] Abendroth *et al* 2011

D.3. Plant Tissue Sample Preparation

The plant tissue samples collected, except pollen, from the four locations were ground to a powder. All the samples were then lyophilized, and analyzed for transgenic protein content by ELISA at Covance Laboratories, Inc. A subsample from each homogeneous powdered sample was lyophilized for protein extraction and analysis. The percent dry weight of each sample was

determined from the fresh weight of the sample before lyophilization and the dry weight after lyophilization by the following formula:

$$\%\,DW = \left(\frac{DW\,(g)}{FW\,(g)}\right) 100$$

D.4. Protein Extraction and ELISA Analysis

Protein extractions were performed on representative aliquots of the lyophilized samples. ELISA methodology was used to quantify mEPSPS and PAT in each extract. Nontransgenic plant tissue extracts were analyzed concurrently to confirm the absence of plant-matrix effects in each ELISA. For each ELISA, a standard curve was generated with known amounts of the corresponding reference protein. The mean absorbance for each sample extract was plotted against the appropriate standard curve to obtain the amount of protein as nanograms per milliliter of extract.

Phosphate-buffered saline with 0.05% Tween 20 surfactant (PBST) buffer was added to lyophilized ground sample at a ratio of 3 ml of buffer to approximately 30 mg of tissue. The samples were homogenized using an Omni Prep Multi-Sample Homogenizer set at 30,000 revolutions per minute for two 30 second bursts. Samples were centrifuged at 2°C to 8°C to form a pellet. Unless analyzed on the same day, the supernatants were stored at –20°C (±10°C) (for PAT) and at –70°C ± 10°C (for MEPSPS) until analysis.

Table D–2. Reagents and buffers used for extraction and ELISA of mEPSPS

Item	Constituents
AgraQuant EPSPS Plate Test Kit	96-well plate precoated with anti-EPSPS antibody, EPSPS antibody/enzyme conjugate, substrate solution, and stop solution
Phosphate-buffered saline with 0.05% Tween 20 (PBST), pH approximately 7.4	138 mM sodium chloride, 2.7 mM potassium chloride, 10.14 mM sodium phosphate dibasic, 1.8 mM potassium phosphate, 0.05% Tween 20
SuperBlock T20 (PBS) Blocking buffer (SuperBlock)	A protein based blocker formulation in phosphate buffered saline containing 0.05% Tween 20
Tris wash buffer, pH approximately 8.0	10 mM tris, 0.05% Tween 20

Table D–3. Reagents and buffers used for extraction and ELISA of PAT

Item	Constituents
Phosphate-buffered saline with 0.05% Tween 20 (PBST), pH approximately 7.4	138 mM sodium chloride, 2.7 mM potassium chloride, 10.14 mM sodium phosphate dibasic, 1.8 mM potassium phosphate, 0.05% Tween 20
Envirologix QualiPlate ELISA Kit for LibertyLink PAT/*pat*	96-well plate precoated with anti-PAT antibody, PAT antibody/enzyme conjugate, substrate solution

D.5. mEPSPS Quantification

Quantification of mEPSPS was performed using the AgraQuant® EPSPS Plate Test Kit. Pre-coated 96-well plates, antibody/enzyme conjugate, substrate solution, and stop solution were all removed from storage at 2°C to 8°C and allowed to equilibrate to room temperature. The tube containing the substrate solution was protected from light. Dilutions of each sample extract, the ELISA standard, and the positive assay control sample, prepared in Superblock buffer, were added to the pre-coated plates at 100 µl/well. The ELISA plates were incubated at room temperature for 27 to 33 minutes. The plates were washed with tris wash buffer and the antibody/enzyme conjugate was added to the plates at 100 µl/well. The plates were incubated at room temperature for at least 30 minutes. The plates were washed with tris wash buffer and the substrate solution was added at 100 µl/well. The plates were incubated at room temperature in the dark for approximately 10 minutes. The colorimetricreaction was stopped by the addition of the stop solution at 100 µl/well, and absorbance was measured using a spectrophotometer at 650 nm. The results were analyzed with SoftMax® Pro GxP software version 6.3. Concentrations were interpolated from a standard curve generated using a four-parameter curve fitting algorithm.

D.6. PAT Quantification

PAT quantification was performed using the QualiPlate™ ELISA Kit for LibertyLink® PAT/pat. Pre-coated 96-well plates, antibody/enzyme conjugate, and substrate were all removed from storage at 2°C to 8°C and allowed to equilibrate to room temperature. The tube containing the substrate solution was protected from light. The PAT antibody/enzyme conjugate was added to the plate at 50 µl/well. Immediately following the addition of the antibody/enzyme conjugate, dilutions of each sample extract, the ELISA standard, and the positive assay control sample, prepared in PBST buffer, were added to the pre-coated plates at 50 µl/well. The ELISA plates were shaken for 10 seconds and then incubated at room temperature for at least one hour. The plates were washed with PBST buffer and the substrate solution was added at 100 µl/well. The plates were incubated at room temperature (in the dark) for approximately 15 minutes. The colorimetric reaction was stopped by the addition of 1N hydrochloric acid at 100 µl/well, and absorbance was measured using a dual wavelength spectrophotometer at 450 nm and 650 nm. The results were analyzed with SoftMax® Pro GxP software version 6.3. The 650 nm reference measurement was subtractedfrom the 450 nm measurement prior to further analysis. Concentrations were interpolated from a standard curve generated using a quadratic curve-fitting algorithm.

D.7. Adjustments for Extraction Efficiency

Predetermined extraction efficiencies were used to adjust the mEPSPS and PAT concentrations to the estimated total mEPSPS and PAT concentration in the corresponding tissue sample. Extraction efficiency and method sensitivity data, determined during validation of the mEPSPS and PAT quantitation methods (prior to this study), are summarized in Tables D–4 and D-5.

Table D-4. Minimum dilution factors, LODs, LOQs, and extraction efficiencies for the mEPSPS ELISA

Sample Type	Minimum Dilution Factor	Extraction Efficiency	LOD (µg/g DW)	LOQ (µg/g DW)
Corn leaf	16	87%	2.00	12.8
Corn root	16	78%	2.00	12.8
Corn kernel	8	71%	2.00	4.0
Corn pollen	150	85%	37.50	75.0

Table D-5. Minimum dilution factors, LODs, LOQs, and extraction efficiencies for the PAT ELISA

Sample Type	Minimum Dilution Factor	Extraction Efficiency	LOD (µg/g DW)	LOQ (µg/g DW)
Corn leaf	1	87%	0.025	0.031
Corn root	1	87%	0.025	0.063
Corn kernel	1	97%	0.025	0.031
Corn pollen	1	79%	0.025	0.031

Appendix E. Trait Efficacy of MZHG0JG Corn

Under greenhouse conditions, the tolerance of MZHG0JG corn to glyphosate-based and glufosinate-ammonium-based herbicides applied individually or sequentially was compared with that of corresponding nontransgenic, near-isogenic control corn. Glyphosate was applied at the recommended field rate of 880 grams acid equivalent per hectare (g ae/ha) and, in a separate trial, glufosinate-ammonium was applied at the recommended field rate of 450 grams active ingredient per hectare (g ai/ha) to MZHG0JG corn and nontransgenic, near-isogenic hybrid corn at the V2 to V3 growth stage. The plants were rated for percent injury 7, 13, and 29 days after treatment. In a third trial, the plants were sprayed first with glufosinate-ammonium at the V2 to V3 growth stage and then seven days later with glyphosate at the same rates as in the preceding trials. The plants were rated for percent injury 7, 13, and 29 days after treatment. In the sequential application trial, the plants were rated for percent injury 7, 13, and 29 days after treatment with glufosinate-ammonium and 0, 6, and 22 days after treatment with glyphosate.

The results are shown in Table E–1. Glyphosate caused no or minimal injury to MZHG0JG corn, whereas the nontransgenic corn was nearly completely killed by day 13 after treatment. Similarly, glufosinate-ammonium caused no or minimal injury to MZHG0JG corn but severely injured the nontransgenic corn. In plants treated sequentially with glufosinate-ammonium and glyphosate, little or no injury to MZHG0JG corn was observed, whereas the nontransgenic corn was nearly completely killed. These results support the conclusion that MZHG0JG corn is highly tolerant to glyphosate-based and glufosinate-ammonium-based herbicides.

Table E–1. Percent injury to MZHG0JG corn and nontransgenic corn 7, 13, and 29 days after application of herbicides containing glyphosate, glufosinate-ammonium, or both herbicides sequentially

	Mean % Injury (SD)		
Herbicide and Genotype	**Day 7**	**Day 13**	**Day 29**
Glyphosate			
MZHG0JG corn	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Nontransgenic corn	63.8 (4.3)	100.0 (0.0)	100.0 (0.0)
P value	***<0.01***	N/A	N/A
Glufosinate-ammonium			
MZHG0JG corn	0.1 (1.5)	0.0 (0.0)	0.0 (0.0)
Nontransgenic corn	71.4 (2.4)	81.6 (6.3)	66.4 (13.0)
P value	***<0.01***	***<0.01***	***<0.01***
Glufosinate-ammonium followed by glyphosate one week later			
MZHG0JG corn	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Nontransgenic corn	70.9 (2.6)	88.1 (5.1)	98.9 (6.9)
P value	***<0.01***	***<0.01***	***<0.01***

The data for untreated MZHG0JG and nontransgenic corn were not included in the analyses of variance (ANOVAs).
N/A indicates that ANOVA could not be conducted because all values for both treatment groups were 0 or 100.
Results significantly different from the nontransgenic control at $P < 0.01$ are shown in bold italic type.