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REQUEST FOR AN EXTENSION OF DETERMINATION OF NONREGULATED STATUS FOR INSECT-RESISTANT AND HERBICIDE-TOLERANT EVENT MZIR098 CORN

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AUTHORS

Katie P. Davis, B.S.

Sydney G. Jarrett, M.S.

Melissa L. Yanek-Eichner, M.S.

SUBMITTED BY

Syngenta Seeds, Inc.
P.O. Box 12257
3054 E. Cornwallis Road
Research Triangle Park, NC 27709 USA

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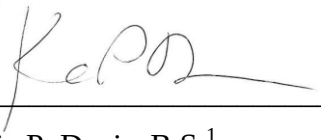
Certification

The undersigned submits this request under 7 CFR §340.6 to request that the Administrator, Animal and Plant Health Inspection Service, make a determination that the article Event MZIR098 corn should not be regulated under 7 CFR §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:



Katie P. Davis, B.S.[1]
Regulatory Affairs Manager, U.S.
Syngenta Seeds, Inc.
P.O. Box 12257
3054 E. Cornwallis Rd.
Research Triangle Park, NC 27709 USA
Tel. 919.226.7374
Email katie.davis@syngenta.com

[1] Person to whom correspondence should be addressed.

CONTRIBUTING SCIENTISTS

Katie Bailey, Andrea Burns, Rebecca Cade, Karrie Christenson, Lisa Darmo, Anic de Framond, Rakesh Jain, Karen Launis, Tae-Jin Lee, Beth Matthews, James Merchant, Nicki Vanderpool, Andy Noe, David Roberts, Shuang Song, Frederick Walters, and Fasica Woldeyes

EDITORS

Demetra Vlachos and Nicole Juba

Release of Information

REQUEST FOR AN EXTENSION OF DETERMINATION OF NONREGULATED STATUS FOR INSECT-RESISTANT AND HERBICIDE-TOLERANT EVENT MZIR098 CORN

Executive Summary

Syngenta requests a determination from the United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA APHIS), that insect-resistant and herbicide-tolerant Event MZIR098 corn (hereafter MZIR098 corn), any progeny derived from crosses between MZIR098 corn and conventional corn varieties, and any progeny derived from crosses of MZIR098 corn with other biotechnology-derived corn varieties that have previously been granted nonregulated status, no longer be considered regulated articles under 7 CFR §340. Syngenta requests that the USDA APHIS consider this application an extension to petition 11-244-01p based on the phenotypic similarities of MZIR098 corn to the antecedent organism that is the subject of petition 11-244-01p, DP-ØØ4114-3 insect-resistant and glufosinate-ammonium-tolerant corn (hereafter DP-ØØ4114-3 corn). Like MZIR098 corn, DP-ØØ4114-3 corn confers resistance to corn rootworm and tolerance to herbicides containing glufosinate-ammonium, which has activity against a variety of agronomically important weed species. The antecedent organism DP-ØØ4114-3 corn received a determination of nonregulated status from the USDA APHIS on June 20, 2013.

Syngenta has developed MZIR098 corn (maize; *Zea mays* L.), a new cultivar that has been genetically modified to provide dual modes of action for control of corn rootworm (*Diabrotica* spp.) and tolerate herbicides containing glufosinate-ammonium. Most corn currently grown in the United States (U.S.) represents transgenic insect-resistant herbicide-tolerant varieties. MZIR098 corn will offer growers two traits for corn rootworm control in the same corn hybrid, and an option for weed management programs.

MZIR098 corn plants contain the transgenes *ecry3.1Ab* and *mcry3A*, which encode the insecticidal proteins eCry3.1Ab and mCry3A, and the transgene *pat-08*, which encodes the enzyme phosphinothricin acetyltransferase (PAT). The native Cry3A from the soil bacterium *Bacillus thuringiensis* subsp. *tenebrionis* is active against certain coleopteran pests. The modified protein mCry3A produced by MZIR098 corn has enhanced activity against western corn rootworm *(Diabrotica virgifera virgifera)* and other related coleopteran pests of corn. The engineered protein eCry3.1Ab is a chimera of mCry3A and Cry1Ab that is also active against *D. virgifera virgifera* and other related pests of corn. The native Cry1Ab from *B. thuringiensis* subsp. *kurstaki* is active against certain lepidopteran pests; however, the portion of Cry1Ab included in eCry3.1Ab has not preserved the activity of Cry1Ab against lepidopterans. The transgene *pat-08* 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 MZIR098 corn. The transgenes *ecry3.1Ab*, *mcry3A*, and *pat-08* encode protein sequences identical to those in plant varieties previously deregulated by the USDA, including 5307 corn (petition 10-336-01p), MIR604 corn (petition 04-362-01p), and Bt11 corn (petition 95-195-01p), respectively. Although the vector agent and the sources of some genetic elements used to create MZIR098 corn are listed as plant pests in 7 CFR §340.2, the introduced nucleotide sequences do not impart plant pest properties.

MZIR098 corn was produced by transformation of immature embryos of proprietary line NP2222 via *Agrobacterium tumefaciens*-mediated transformation. The DNA region between the left and right borders of the transformation plasmid included gene-expression cassettes for *ecry3.1Ab*, *mcry3A*, and *pat-08*. The *ecry3.1Ab* expression cassette consisted of the *ecry3.1Ab* coding region regulated by a CMP promoter from cestrum yellow leaf curling virus (CMP-04) and the nopaline synthase (NOS) terminator sequence from *A. tumefaciens* (NOS-05-01), as well as the NOS enhancer sequence (NOS-02). The *mcry3A* expression cassette consisted of the *mcry3A* coding region regulated by a corn ubiquitin promoter (Ubi1-18) and NOS terminator (NOS-20). The *pat-08* expression cassette consisted of the *pat-08* coding region regulated by the 35S promoter from cauliflower mosaic virus (35S-04) and the NOS terminator (NOS-05-01).

Genetic characterization studies demonstrated that MZIR098 corn contains, at a single locus within the corn genome, a single copy of each of the following functional elements: *ecry3.1Ab*, *mcry3A*, *pat-08*, NOS-02 enhancer, CMP-04 promoter, Ubi1-18 promoter, NOS-20 terminator, 35S-04 promoter and two copies of the NOS-05-01 terminator, as expected. No extraneous DNA fragments of these functional elements occur elsewhere in the MZIR098 corn genome. Similarly, plasmid backbone sequence from transformation plasmid pSYN17629 is not present in the MZIR098 corn genome. Analyses comparing the corn genomic sequence flanking the MZIR098 insert with sequences in public databases indicate that the inserted DNA does not disrupt any known endogenous corn gene.

Southern blot analyses demonstrated that the MZIR098 T-DNA insert is stably inherited from one generation to the next and that the MZIR098 corn genome contains a single T-DNA insert. The observed segregation ratios for *ecry3.1Ab*, *mcry3A*, and *pat-08* in three generations of MZIR098 corn plants indicated that they 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 compositional parameters suggestive of increased plant pest risk or increased susceptibility of MZIR098 corn to plant disease or other pests. Compositional assessments of grain and forage from multiple U.S. field sites demonstrated that MZIR098 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 MZIR098 corn.

Well-characterized modes of action, physicochemical properties, and a history of safe use demonstrate that the eCry3.1Ab, mCry3A, and PAT proteins present in MZIR098 corn present no risk of harm to humans or livestock that consume corn products. The eCry3.1Ab and mCry3A proteins are exempt from the requirement for food and feed tolerances in corn and PAT is exempt from food and feed tolerances in all crops. All three proteins have a history of safe use in transgenic crop varieties that have been deregulated by the USDA APHIS and reviewed by the U.S. Food and Drug Administration through the biotechnology consultation process.

On the basis of the data and information described in this document and the phenotypic similarity of MZIR098 corn to the deregulated antecedent organism DP-ØØ4114-3 corn, Syngenta requests a determination from USDA APHIS that MZIR098 corn, and any progeny derived from crosses between MZIR098 corn and conventional corn or deregulated corn varieties, should qualify for nonregulated status under 7 CFR §340.

Concurrent with its deregulation of the antecedent organism 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 MZIR098 corn, Syngenta is submitting a document that details a review of the previous EA and associated FONSI for 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 MZIR098 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 the U.S.

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35S-04 promoter	promoter region of the cauliflower mosaic virus
aadA-03	spectinomycin resistance gene
ADF	acid detergent fiber
a.i.	active ingredient
ANOVA	analysis of variance
APHIS	Animal and Plant Health Inspection Service
BC	backcross
BLASTX	Basic Local Alignment Search Tool for Translated Nucleotides
bp	base pair
CaMV	cauliflower mosaic virus
CMP-04 promoter	promoter from cestrum yellow leaf curling virus
Dow	Dow AgroSciences, LLC
DNA	deoxyribonucleic acid
DW	dry weight
eCry3.1Ab	*Bacillus thuringiensis* eCry3.1Ab protein
ecry3.1Ab	*Bacillus thuringiensis* eCry3.1Ab gene
ELISA	enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
F_1	first generation of progeny from a breeding cross
FDA	Food and Drug Administration
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
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
mCry3A	*Bacillus thuringiensis* modified Cry3A protein
mcry3A	*Bacillus thuringiensis* modified cry3A gene
N/A	not applicable
NCBI	National Center for Biotechnology Information
NDF	neutral detergent fiber
NOS	nopaline synthase
NOS-02 enhancer	nopaline synthase sequence from *Agrobacterium tumefaciens* that increases gene expression
NOS-05-01 terminator	nopaline synthase terminator sequence from *A. tumefaciens*
NOS-20 terminator	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
PAT	phosphinothricin acetyltransferase
pat-08	phosphinothricin acetyltransferase gene
PCR	polymerase chain reaction
RB-01-01	right border
repA-03	replication gene from *Pseudomonas aeruginosa* plasmid VS1

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
TDF	total dietary fiber
TIU	trypsin inhibitor unit
tris	2-amino-2(hydroxymethyl)-1,3-propanediol
Ubi1-18 promoter	corn ubiquitin promoter
U.S.	United States
USDA	United States Department of Agriculture
virG-01	virulence regulon in *A.faciens*
×	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 MZIR098 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 MZIR098 corn (maize; *Zea mays* L.), a new cultivar that has been genetically modified to provide dual modes of action for control of corn rootworm (*Diabrotica* spp.) and tolerate herbicides containing glufosinate-ammonium. MZIR098 corn was developed through *Agrobacterium*-mediated transformation to stably incorporate the transgenes *ecry3.1Ab*, *mcry3A*, and *pat-08* into the corn genome. The native Cry3A from the soil bacterium *Bacillus thuringiensis* subsp. *tenebrionis* is active against certain coleopteran pests. The modified protein mCry3A produced by MZIR098 corn has enhanced activity against western corn rootworm *(Diabrotica virgifera virgifera)* and other related coleopteran pests of corn. The engineered protein eCry3.1Ab is a chimera of mCry3A and Cry1Ab that is also active against *D. virgifera virgifera* and other related pests of corn. The native Cry1Ab from *B. thuringiensis* subsp. *kurstaki* is active against certain lepidopteran pests; however, the portion of Cry1Ab included in eCry3.1Ab has not preserved the activity of Cry1Ab against lepidopterans. The transgene *pat-08* was 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 MZIR098 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 §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 MZIR098 corn, the transgene *pat-08*, and some of the regulatory sequences used to drive expression of *ecry3.1Ab*, *mcry3A*, and *pat-08* 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 MZIR098 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 cauliflower mosaic virus, yellow leaf curling virus, and *A. tumefaciens* were introduced during the production of MZIR098 corn. The transgene *pat-08* 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 MZIR098 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 corn-rootworm-resistant and/or glufosinate-tolerant crop varieties, including the antecedent organism, DP-ØØ4114-3 corn. This determination was made in June 2013 upon finding that DP-ØØ4114-3 corn did not pose a plant pest risk. Based on the similarity of the antecedent organism DP-ØØ4114-3 corn to MZIR098 corn, Syngenta has concluded that the previous analyses of impacts completed for DP-ØØ4114-3 corn are relevant to APHIS' regulatory actions associated with responding to the Syngenta extension request for MZIR098 corn. A comparison of the antecedent organism DP-ØØ4114-3 corn to MZIR098 corn is provided in Table I–1 below.

Table I–1. Comparison of antecedent organism and MZIR098 corn

Description	**Antecedent DP-ØØ4114-3 Corn**	**MZIR098 Corn**
Organism	Corn	Corn
Phenotype	Resistant to insect damage from coleopteran and lepidopteran species and tolerant to the broad-spectrum herbicide active ingredient glufosinate-ammonium	Resistant to insect damage from coleopteran species and tolerant to the broad-spectrum herbicide active ingredient glufosinate-ammonium
Proteins	Cry34/35 and Cry1F proteins and PAT enzyme	eCry3.1Ab and mCry3A proteins and PAT enzyme
Method of transformation	*Agrobacterium*-mediated	*Agrobacterium*-mediated
Insert copy	Single intact insertion	Single intact insertion
Compositional analysis	Within range of corn	Within range of corn
Disease and pest susceptibilities	Unlikely to pose plant pest risk	Unlikely to pose plant pest risk
Impacts on beneficial non-target organisms	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
Enhanced weediness of relatives	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
Potential for horizontal gene transfer	Unlikely to pose plant pest risk	Unlikely to pose plant pest risk

In addition to DP-ØØ4114-3 corn, USDA APHIS has evaluated and subsequently reached a determination of nonregulated status for other corn varieties expressing transgenic traits both identical and/or similar to those in MZIR098 corn. The transgenes *ecry3.1Ab*, *mcry3A*, and *pat-08* encode protein sequences identical to those in 5307 corn (petition 10-336-01p), MIR604 corn (petition 04-362-01p), and Bt11 corn (petition 95-195-01p), respectively. Like DP-ØØ4114-3 corn, the analysis of impacts for the relevant traits in these deregulated corn varieties would also be similar to that of MZIR098 corn. As such, Table I–2 below contains a list of additional deregulated corn cultivars that USDA APHIS may choose to consider in connection with the evaluation of this MZIR098 corn extension request.

Table I–2. Additional deregulated corn cultivars for consideration

Event Name(s)	Filed By/ Petition Number	Effective Date of Deregulation	Relevant Transgene / Protein
5307	Syngenta/ 10-336-01p	February 27, 2013	*ecry3.1Ab* / eCry3.1Ab
MIR604	Syngenta/ 00-136-01p	March 16, 2007	*mcry3A* / mCry3A
MON 88017	Monsanto/ 04-125-01p	December 14, 2005	*cry3Bb1* / Cry3Bb1
59122	Dow/ 03-353-01p	September 23, 2005	*cry34Ab1, cry35Ab1* / Cry34Ab1, Cry35Ab1, *pat* / PAT
MON 863	Monsanto/ 01-137-01p	October 8, 2002	*cry3Bb1* / Cry3Bb1
59122	Dow/ 03-353-01p	September 23, 2005	*pat* / PAT
6275	Dow/ 03-181-01p	October 20, 2004	*pat* / PAT
1507	Mycogen c/o Dow & Pioneer / 00-136-01p	June 14, 2001	*pat* / PAT
MS6	AgrEvo/ 98-349-01p	March 16, 1999	*bar* / PAT
676, 678, 680	Pioneer/ 97-342-01p	May 14, 1999	*pat* / PAT
CHB-351	AgrEvo/ 97-265-01p	May 8, 1998	*bar* / PAT
DBT418	DeKalb/ 96-291-01p	March 28, 1997	*bar* / PAT
Bt11	Northrup King / 95-195-01p	July 18, 1996	*pat* / PAT
B16	DeKalb/ 95-145-01p	December 19, 1995	*bar* / PAT
T14, T25	AgrEvo/ 94-357-01p	June 22, 1995	*pat* / PAT

Source: USDA APHIS 2015

The data and information in the present request for an extension of nonregulated status demonstrate that the conclusions reached for DP-ØØ4114-3 corn also apply to MZIR098 corn and that, likewise, MZIR098 corn does not pose a plant pest risk.

I.B. Benefits of MZIR098 Corn

Corn rootworm larvae feed on the roots of growing corn plants and are widespread and major pests of U.S. corn. Prior to the introduction of transgenic corn cultivars for rootworm control, the average economic costs of rootworm damage to the U.S. corn crop were estimated at $1 billion annually (Metcalf 1986; Chandler 2003). The damage inflicted by rootworm larvae can significantly reduce grain yield by interfering with photosynthetic rates, limiting the uptake of water and nutrients, and by increasing the plant's susceptibility to lodging (Oleson *et al.* 2005). Lodging (leaning) further reduces the effective grain yield by making the plants more susceptible to breaking, reducing their access to sunlight, and increasing the difficulty with which the grain can be harvested efficiently.

The eCry3.1Ab and mCry3A proteins produced in MZIR098 corn are effective in controlling three of the major rootworm pests of corn in the U.S., specifically: *Diabrotica virgifera virgifera* LeConte (western corn rootworm), *D. longicornis barberi* Smith and Lawrence (northern corn rootworm), and *D. virgifera zeae* Krysan and Smith (Mexican corn rootworm). Expressing the transgenes *ecry3.1Ab* and *mcry3A* at adequate levels in corn plants is a logical approach for limiting crop losses attributable to these pests.

The combination of eCry3.1Ab and mCry3A in the same corn variety will offer the same advantages to growers that both 5307 corn and MIR604 corn have provided for pest control and insect resistance management since their introduction to the marketplace. It has been demonstrated that eCry3.1Ab and mCry3A have unique properties that, when combined, will serve to prevent, delay, or mitigate the evolution of target pest resistance to either protein. Although the proteins act by the same general mechanism, i.e., pore formation in the target pest gut, the evidence indicates that they have unique gut binding sites in the target pest, thus effectively representing different modes of action (Walters *et al.* 2010). The concurrent deployment of both eCry3.1Ab and mCry3A in the same hybrid offerings to growers is expected to help preserve pest susceptibility to both proteins. Additionally, by reducing the selection pressure on target pest populations to evolve resistance to any single method of rootworm control, this strategy is predicted to help prolong pest susceptibility to other *B. thuringiensis*-derived proteins in transgenic corn cultivars used for rootworm control, as well as to other traditional control methods (e.g., insecticides and crop rotation).

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 and productivity, which will be critical to supporting an ever-expanding global population.

Upon commercialization, MZIR098 corn will support agricultural efficiency by facilitating the introduction of corn varieties containing stacked agronomically important traits to the marketplace. For example, the traits in MZIR098 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 as well as traits that provide alternative modes of action for herbicide tolerance. 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. MZIR098 corn increases breeding efficiency by combining two traits for resistance to coleopteran pest damage with tolerance to herbicides containing glufosinate-ammonium in a single variety, rather than having to combine these traits from separate deregulated cultivars into a single variety by traditional breeding.

I.C. Regulatory Status of MZIR098 Corn

Syngenta is pursuing regulatory approvals for MZIR098 corn cultivation in the U.S. and Canada, and may seek cultivation approvals in other countries in the future.

MZIR098 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 (U.S. FDA 1992). Accordingly, Syngenta has initiated the FDA consultation process by submitting a safety and nutritional assessment for MZIR098 corn. Additional regulatory approvals that will facilitate global trade in corn commodities will be sought on an as-needed basis.

Substances that are pesticides as defined under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) are subject to regulation by the U.S. Environmental Protection Agency (EPA). The eCry3.1Ab and mCry3A proteins encoded by the genetic insert in MZIR098 corn have insecticidal properties and are, therefore, regulated by U.S. EPA. Pursuant to FIFRA §3(c)(5), Syngenta is preparing an application for the registration of MZIR098 corn.

The U.S. EPA has granted permanent exemptions from food and feed tolerances for eCry3.1Ab and mCry3A in field corn, sweet corn, and popcorn when used as plant-incorporated protectants in the U.S. (U.S. EPA 2007a and 2012b). The U.S. EPA has granted a permanent exemption from food and feed tolerances for the PAT protein in all crops in the U. S. (U.S. EPA 2007b). Although PAT does not have pesticidal activity, U.S. tolerance exemptions were granted for this protein because it accompanies the use of some plant-incorporated protectants. Additionally, the U.S EPA has established food and feed tolerances for corn commodities containing residues of glufosinate-ammonium (U.S. EPA 2015).

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 MZIR098 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*-mediated transformation and regeneration from tissue culture. NP2222 is a Stiff-Stalk family, yellow dent inbred.

III. Development of MZIR098 Corn

This section describes the method by which corn was transformed to produce insect-resistant and herbicide-tolerant corn plants, the development of MZIR098 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

Agrobacterium-mediated transformation of immature embryos of a proprietary corn line (NP2222), as described by Negrotto *et al*. (2000), was used to produce MZIR098 corn. By this method, genetic elements between the left and right border regions of the transformation plasmid are efficiently transferred and integrated into the genome of the target plant cell, while genetic elements outside these border regions are 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 pSYN17629. The embryos in suspension were mixed by vortex 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 transgene *pat-08* 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 *ecry3.1Ab, mcry3A,* and *pat-08* 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 transferred deoxyribonucleic acid (T-DNA) and were free of plasmid backbone deoxyribonucleic acid (DNA). Plants that tested positive for *ecry3.1Ab, mcry3A,* and *pat-08* and negative for *aadA-03* were transferred to the greenhouse for further propagation.

III.B. Development of MZIR098 Corn

Progeny of the original transformants (T_0 plants) were field tested for resistance to corn rootworm feeding damage, tolerance to glufosinate-ammonium, and agronomic performance in multiple elite lines of corn and in test hybrids. MZIR098 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 MZIR098 corn.

All shipments and field releases of MZIR098 corn in the U.S. were carried out under USDA APHIS notifications, which are listed in Appendix A.

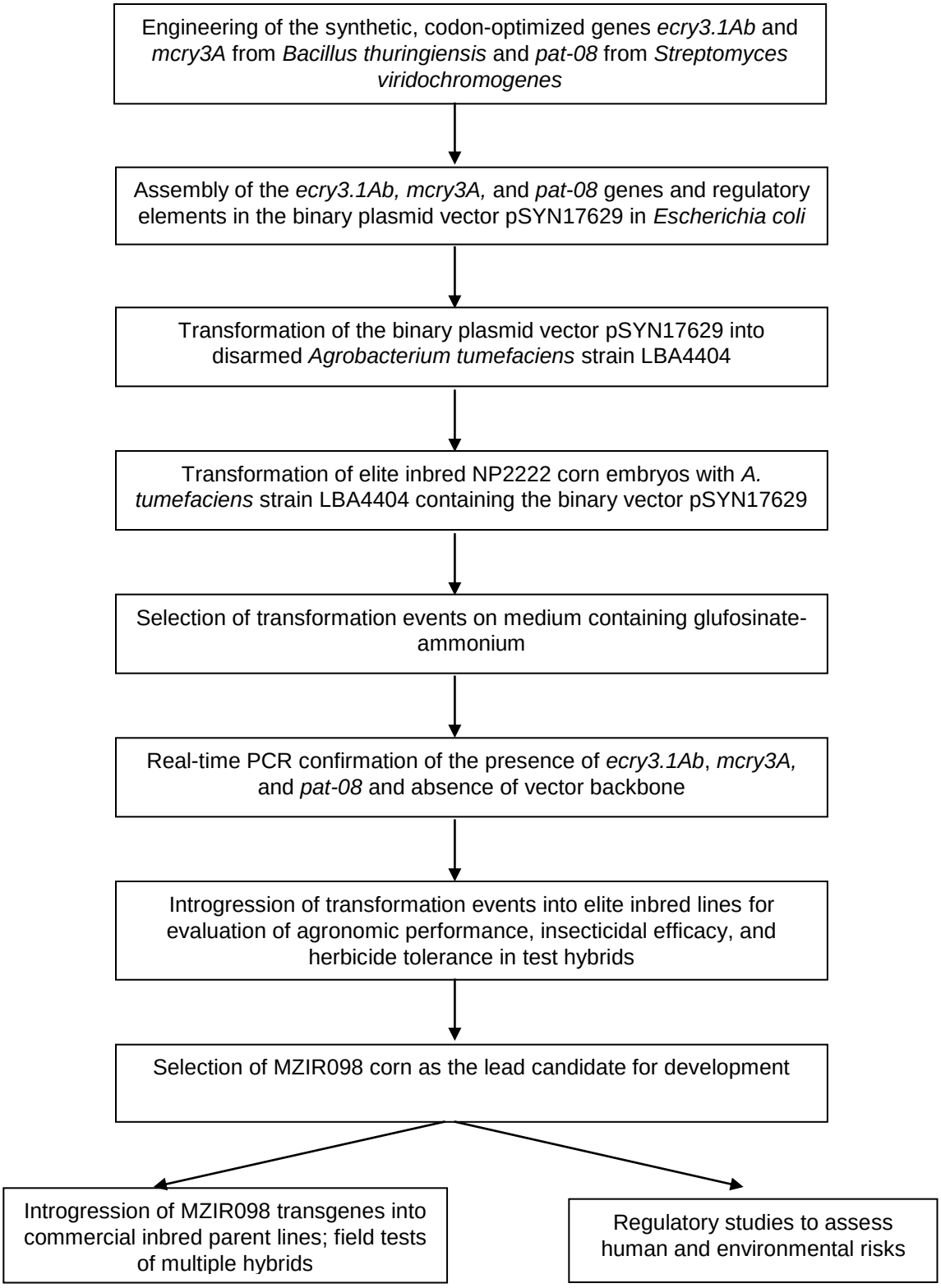


Figure III–1. Steps in the development of MZIR098 corn

III.C. Production of Test and Control Seed

Production of all MZIR098 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 MZIR098 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 MZIR098 corn seed lots. Both test and control materials were analyzed by real-time PCR testing (Ingham *et al.* 2001) to confirm identity and purity.

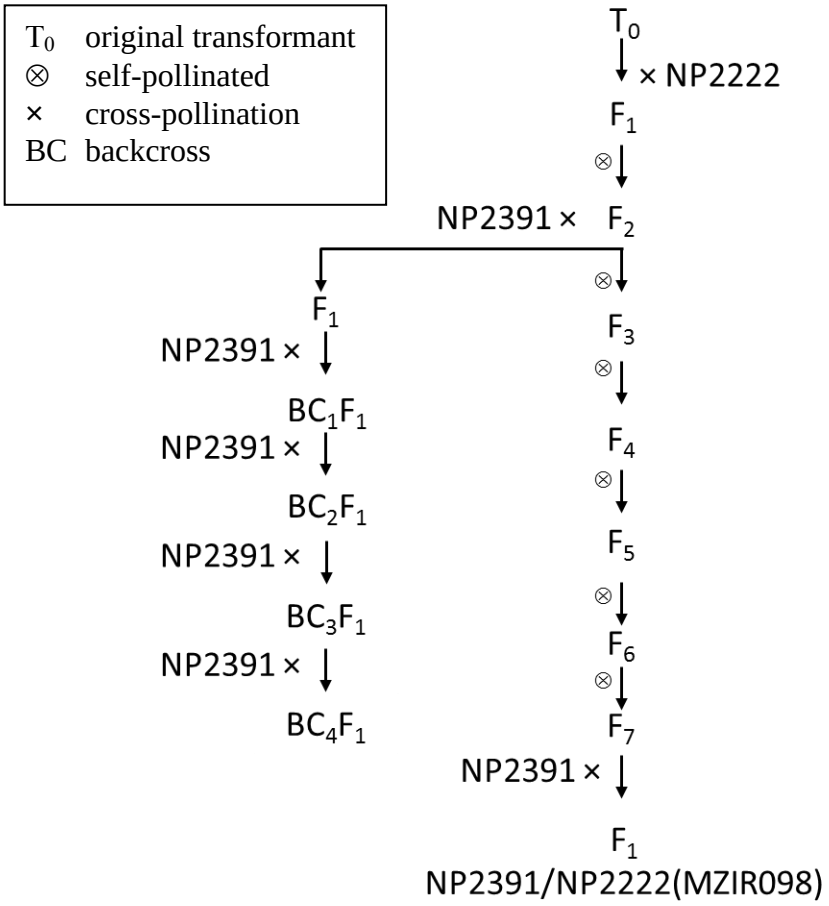


Figure III–2. Pedigree of the MZIR098 corn plant materials used in regulatory studies

The transformation recipient line was Syngenta proprietary corn inbred NP2222. NP2391 is a Syngenta proprietary corn inbred line used in further breeding of MZIR098 corn.

III.D. Quality Control of Test and Control Materials

All MZIR098 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 MZIR098 DNA and the absence of adventitious DNA from other transformation events. All MZIR098 corn seed lots were confirmed to contain MZIR098-corn-specific DNA. MZIR098 DNA was not

detected in any nontransgenic control corn seed lots. None of the MZIR098 or nontransgenic 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 pSYN17629 was used to produce MZIR098 corn by *Agrobacterium*-mediated transformation of immature corn embryos. The DNA region between the left and right borders of the transformation plasmid included gene-expression cassettes for *ecry3.1Ab*, *mcry3A*, and *pat-08*. The *ecry3.1Ab* expression cassette consisted of the *ecry3.1Ab* coding region regulated by a CMP promoter from cestrum yellow leaf curling virus (CMP-04) and the nopaline synthase (NOS) terminator sequence from *A. tumefaciens* (NOS-05-01), as well as the NOS enhancer sequence (NOS-02). The *mcry3A* expression cassette consisted of the *mcry3A* coding region regulated by a corn ubiquitin promoter (Ubi1-18) and NOS terminator (NOS-20). The *pat-08* expression cassette consisted of the *pat-08* coding region regulated by the 35S promoter from cauliflower mosaic virus (35S-04) and the NOS terminator (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.

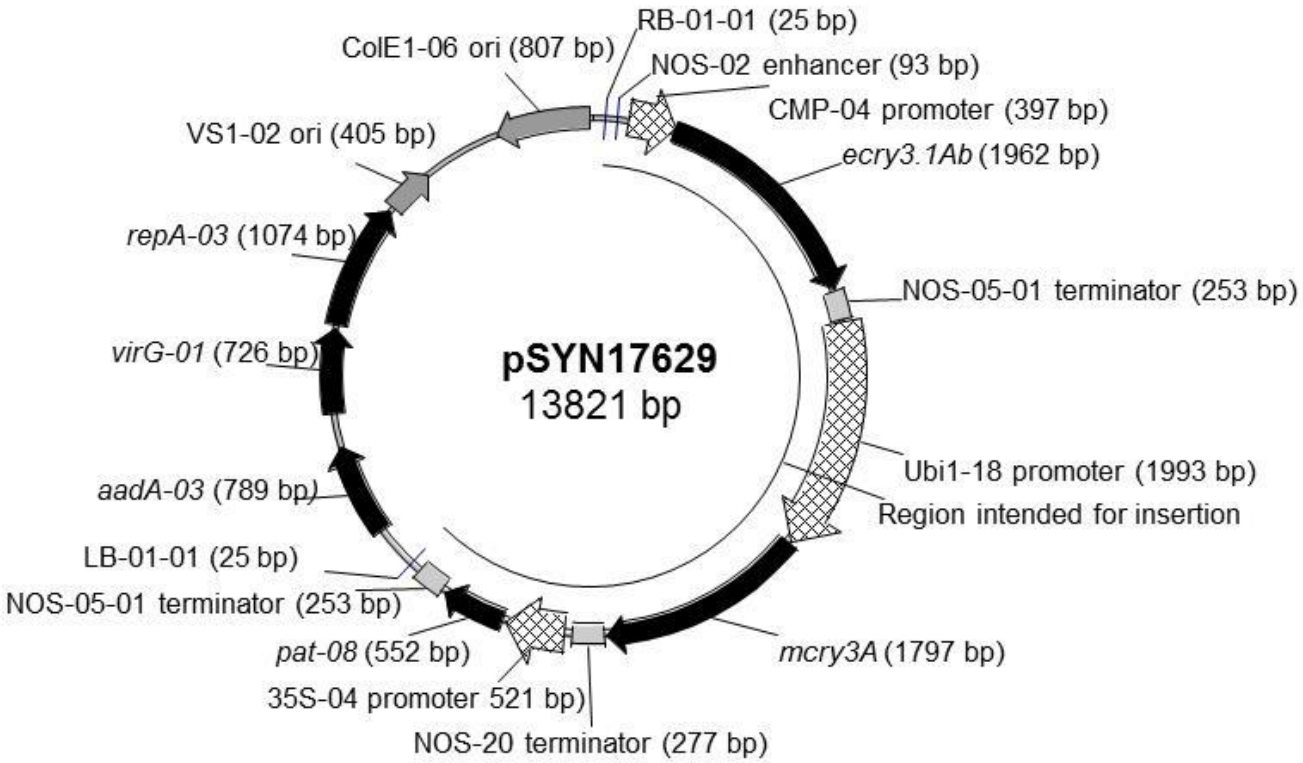


Figure IV–1. Plasmid map for the vector pSYN17629

Table IV–1. Description of the genetic elements in vector pSYN17629

Genetic element	Size (bp)	Position	Description
***ecry3.1Ab* cassette**			
Region-1	80	26 to 105	Region used for cloning.
NOS-02 enhancer	93	106 to 198	Enhancer sequence from the NOS gene of *A. tumefaciens* which increases gene expression (NCBI accession number V00087.1) (Bevan *et al.* 1983).
Region-2	5	199 to 203	Region used for cloning.
CMP-04 promoter	397	204 to 600	Cestrum yellow leaf curling virus promoter region (Hohn *et al*. 2007). Provides constitutive expression in corn.
Region-3	9	601 to 609	Region used for cloning.
ecry3.1Ab	1962	610 to 2571	An engineered Cry gene active against certain corn rootworm *(Diabrotica)* species (NCBI accession number GU327680.1). As an engineered chimeric protein, eCry3.1Ab has similarities to other well-characterized Cry proteins. Because Cry proteins share structural similarities, chimeric Cry genes can be engineered via the exchange of domains that are homologous between different Cry genes. The gene *ecry3.1Ab* consists of a fusion between the 5′ end (domain I, domain II, and 15 amino acids of domain III) of a modified Cry3A gene (*mcry3A*) and the 3′ end (domain III and variable region 6 [Höfte and Whiteley 1989]) of a synthetic Cry1Ab gene (see description of *mcry3A* and *cry1Ab* below). Upstream of the *mcry3A* domain, the gene *ecry3.1Ab* carries a 67-bp oligomer extension at its 5′ end, which was introduced during the engineering of the variable regions and is translated into the following 22 amino acid residues: MTSNGRQCAGIRPYDGRQQHRG. The next 459 amino acid residues are identical to those of mCry3A, followed by 172 amino acid residues of Cry1Ab. Description of *cry1Ab*: The gene *cry1Ab* was originally cloned from *B. thuringiensis* subsp. *kurstaki* strain HD-1 (Geiser *et al*. 1986). Its amino acid sequence has been codon-optimized (Koziel *et al*. 1997) to accommodate the preferred codon usage for corn (Murray *et al*. 1989).
Region-4	21	2572 to 2592	Region used for cloning.
NOS-05-01 terminator	253	2593 to 2845	Terminator sequence from the NOS gene of *A. tumefaciens* (NCBI accession number V00087.1). Provides a polyadenylation site (Bevan *et al.* 1983).
***mcry3A* cassette**			
Region-5	20	2846 to 2865	Region used for cloning.

Continued

Genetic element	Size (bp)	Position	Description
Ubi1-18 promoter	1993	2866 to 4858	Promoter region from *Z. mays* polyubiquitin gene which contains the first intron (NCBI accession number S94464.1), in which three basepairs have been altered to remove restriction sites. Provides constitutive expression in monocots (Christensen *et al*. 1992).
Region-6	9	4859 to 4867	Region used for cloning.
mcry3A	1797	4868 to 6664	A corn-optimized *cry3A* was synthesized to accommodate the preferred codon usage for corn (Murray *et al*. 1989). The synthetic sequence was based on the native Cry3A protein sequence from *B. thuringiensis* subsp. *tenebrionis* (Sekar *et al*. 1987). The corn-optimized gene was then modified to incorporate a consensus cathepsin G protease recognition site within the expressed protein. The amino acid sequence of the encoded mCry3A corresponds to that of the native Cry3A, except that (1) its N-terminus corresponds to M-48 of the native protein and (2) a cathepsin G protease recognition site has been introduced, beginning at amino acid residue 155 of the native protein. This cathepsin G recognition site has the amino acid sequence AAPF, and has replaced the amino acids V-155, S-156, and S-157 in the native protein (Chen and Stacy 2007).
Region-7	19	6665 to 6683	Region used for cloning.
NOS-20 terminator	277	6684 to 6960	Terminator sequence from the NOS gene of *A. tumefaciens* (NCBI accession number V00087.1). Variation of the NOS-05-01 terminator with nucleotide changes to eliminate cross-border unintended open reading frames (ORFs). Provides a polyadenylation site (Bevan *et al*. 1983).
Region-8	59	6961 to 7019	Region used for cloning.
***pat-08* cassette**			
35S-04 promoter	521	7020 to 7540	Promoter region of cauliflower mosaic virus (Odell *et al.* 1985). Provides constitutive expression in plants.
Region-9	24	7541 to 7564	Region used for cloning.
pat-08	552	7565 to 8116	*S. viridochromogenes* strain Tü494 gene encoding the selectable marker PAT. The native coding sequence (Wohlleben *et al*. 1988) was codon-optimized for enhanced expression (NCBI accession number DQ156557.1). The synthetic *pat* was obtained from AgrEvo, Germany. PAT confers resistance to herbicides containing glufosinate-ammonium (phosphinothricin). The gene *pat*-08 was altered to remove an *Xma*I site.
Region-10	31	8117 to 8147	Region used for cloning.

Continued

Genetic element	Size (bp)	Position	Description
NOS-05-01 terminator	253	8148 to 8400	Terminator sequence from the NOS gene of *A. tumefaciens* (NCBI accession number V00087.1). Provides a polyadenylation site (Bevan *et al*. 1983).
Region-11	87	8401 to 8487	Region used for cloning.
Border Region			
LB-01-01	25	8488 to 8512	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-12	349	8513 to 8861	Region used for cloning.
aadA-03	789	8862 to 9650	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-13	299	9651 to 9949	Region used for cloning.
virG-01	726	9950 to 10675	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-14	29	10676 to 10704	Region used for cloning.
repA-03	1074	10705 to 11778	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-15	42	11779 to 11820	Region used for cloning.
VS1-02 ori	405	11821 to 12225	Consensus sequence for the origin of replication and partitioning region from pVS1 of *P. aeruginosa* (NCBI accession number U10487.1). Serves as the origin of replication in the *A. tumefaciens* host (Itoh *et al*. 1984).
Region-16	677	12226 to 12902	Region used for cloning.
ColE1-06 ori	807	12903 to 13709	Origin of replication (similar to NCBI accession number V00268.1) that permits replication of plasmids in *E. coli* (Itoh and Tomizawa 1979).
Region-17	112	13710 to 13821	Region used for cloning.

Continued

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 MZIR098 Corn

An extensive genetic characterization of the T-DNA insert in MZIR098 corn was performed by means of nucleotide sequencing and Southern blot analyses. Sequencing results confirmed the expected copy number of each of the functional elements in the T-DNA. In addition, the corn genomic sequences flanking the MZIR098 insert were identified and characterized and it was determined that the MZIR098 insert did not disrupt the function of any known corn gene. The genetic stability of the insert and absence of plasmid backbone sequence in the MZIR098 corn genome were assessed by Southern blot analyses over at least three generations of MZIR098 corn. Further, stability of the insert was assessed by examining the inheritance patterns of the transgenes over three generations of MZIR098 corn. These data collectively demonstrate that no deleterious changes occurred in the MZIR098 corn genome as a result of the T-DNA insertion.

Sections V.A. through V.B., describe the design, results, and conclusions of each genetic characterization study, and the general conclusions of the genetic characterization studies are summarized in Section V.C.

V.A. Nucleotide Sequence of the DNA Insert

Five overlapping fragments that covered the entire MZIR098 insert were amplified via PCR from genomic DNA extracted from MZIR098 F_3 corn. Four of the fragments were cloned. A fifth fragment was not cloned but was directly sequenced. A consensus sequence was generated from all of the fragments and compared with the sequence of the T-DNA in plasmid pSYN17629, the transformation plasmid used to create MZIR098 corn.

Comparison of the MZIR098 insert sequence with the transformation plasmid pSYN17629 showed that the 8467-base-pair (bp) MZIR098 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. The right border, along with 10 bp of non-coding sequence, was truncated, and 10 bp from the left border were truncated. These deletions have no effect on the functionality of the T-DNA insert. Sequence analysis of the MZIR098 insertion site demonstrated that 24 bp from the corn genomic sequence were deleted during the integration of the MZIR098 insert.

The copy number and sequence of each of the functional elements in MZIR098 corn are as expected based on the pSYN17629 T-DNA sequence. The MZIR098 insert contains a single copy of the functional elements *ecry3.1Ab*, *mcry3A*, *pat-08*, NOS-02 enhancer, CMP-04 promoter, Ubi1-18 promoter, NOS-20 terminator, and 35S-04 promoter and two copies of the NOS-05-01 terminator, as expected.

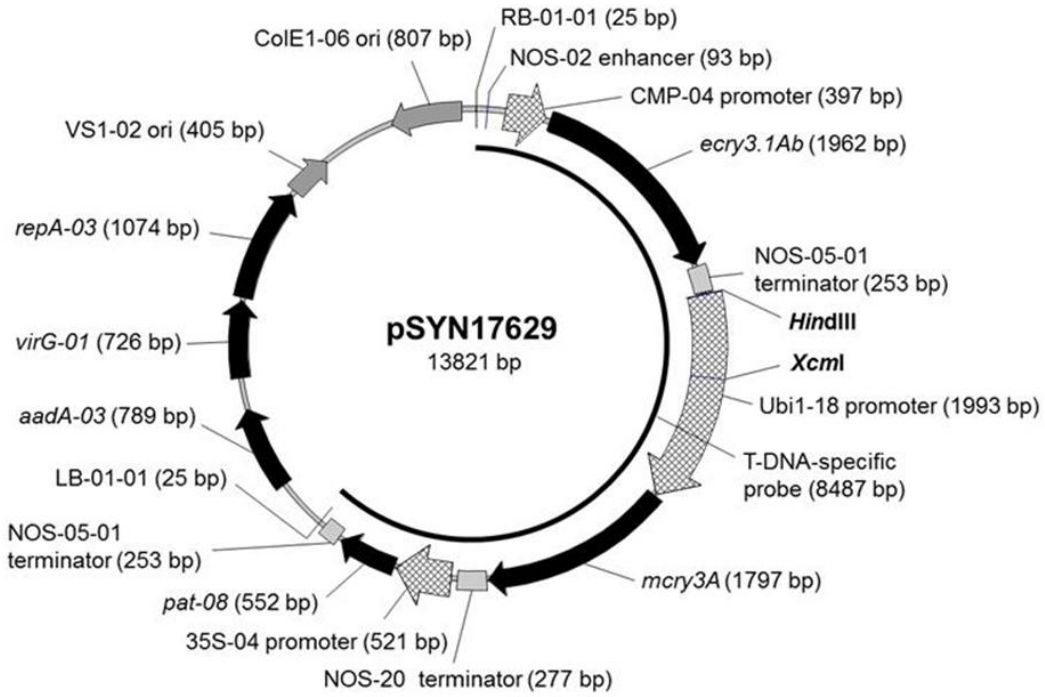
The genomic sequences flanking the MZIR098 insert were screened for similarity with translated DNA sequences found in the latest version of the National Center for Biotechnology Information (NCBI) non-redundant (nr) protein database (NCBI 2014). Sequence similarity analyses were performed with the BLASTX program, version 2.2.28+ (Altschul *et al.*1997), which translates the query DNA sequence in all six reading frames into corresponding amino acid sequences. The results of these analyses indicate that the MZIR098 insert does not disrupt any known endogenous corn gene.

V.B. Characterization of the MZIR098 Corn Insert by Southern Blot Analyses

Southern blot analyses were performed to characterize the transgenic insert of MZIR098 corn by determining the number of plasmid pSYN17629 T-DNA integration sites and the presence or absence of pSYN17629 backbone sequence or additional extraneous fragments of T-DNA.

V.B.1. T-DNA integration sites in MZIR098 corn and stability across multiple generations

The number of T-DNA integration sites within the MZIR098 corn genome and the number of copies of the T-DNA at each integration site within the MZIR098 corn genome were determined through the use of a single T-DNA-specific probe that covered every base pair of the pSYN17629 T-DNA expected to be transferred and integrated into the corn genome. The template for the probe was a segment of the pSYN17629 T-DNA corresponding with the NOS-02 enhancer through the NOS-05-01 terminator near the left border region (Figure V–1).



The restriction enzyme *Bmt*I was used during the Southern blot analyses but is not present on this map because pSYN17629 does not contain the recognition sequence for this enzyme.

Figure V–1. Location of the 8.5-kb full-length T-DNA-specific probe and the restriction sites *Hind*III and *Xcm*I in the transformation plasmid pSYN17629

The MZIR098 corn generations used in Southern blot analyses were the F_2, F_3, F_4, F_5, and F_1 generations. The F_2 through F_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. Five generations of MZIR098 corn were included to demonstrate stability of the T-DNA insert over multiple generations. The control substances were nontransgenic, near-isogenic NP2222, NP2391, and NP2222/NP2391 corn. DNA from nontransgenic, near-isogenic control corn was used as a negative control to identify any endogenous corn DNA sequences that hybridize to the probe. For all samples, leaf tissue from seven plants were sampled, pooled, and subjected to DNA extraction. Genomic DNA was isolated from leaf tissue by a method modified from that described by Murray and Thompson (1980).

To demonstrate the sensitivity of the analyses, each Southern blot analysis 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 the full-length T-DNA-specific fragment and 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 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:

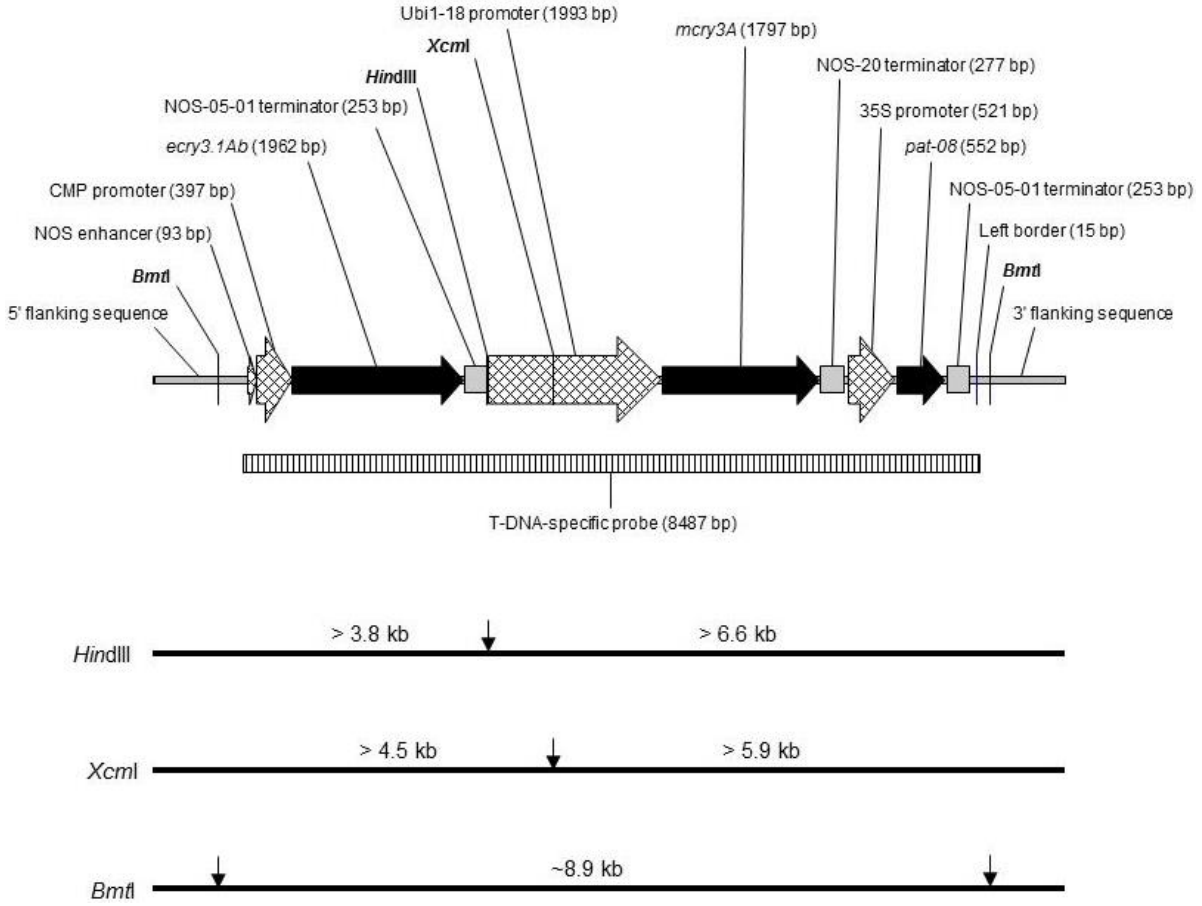
corn genome size (bp)	2.67×10^9
corn ploidy	2
DNA loaded in each lane (µg)	7.5
Full-length T-DNA-specific DNA fragment (bp)	8487

A total of 11.9 pg of the full-length T-DNA-specific DNA fragment was added to the 1-copy Southern blot lanes and 1.7 pg was added to the 1/7-copy lanes.

Corn genomic DNA was analyzed via two restriction enzyme digestion strategies. The first strategy was used to determine the number of T-DNA integration sites in the MZIR098 genome, while the second strategy was used to determine the integrity of the insert and the presence of any extraneous DNA fragments of plasmid pSYN17629 T-DNA closely linked to the MZIR098 insert. Data from both strategies were used to infer the number of copies of the insert at the MZIR098 locus.

In the first strategy, the genomic DNA was digested with an enzyme that cut within the MZIR098 insert and in the corn genome flanking the MZIR098 insert. This first strategy was used twice, with two different enzymes, *Hind*III and *Xcm*I. The locations of the restriction sites are shown in Figure V–1, above.

In the second strategy, the genomic DNA was digested with a restriction enzyme that cut within the insertion site 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 MZIR098 corn genome, the intactness of the insert, and the presence or absence of any closely linked extraneous T-DNA fragments. The restriction enzyme *Bmt*I was used for Southern blot analysis but is not present on the plasmid map because pSYN17629 does not contain the recognition sequence for this enzyme. The *Bmt*I recognition sequences were verified from the known flanking sequences of the MZIR098 insert. Figure V–2 shows the digestion strategy used with the full-length T-DNA-specific probe.



The vertical arrows indicate the site of restriction digestion.
Sizes of the expected restriction fragments are indicated.
The restriction enzyme *Bmt*I was used during the Southern blot analysis but is not present on pSYN17629. The *Bmt*I recognition sequences were verified from the known flanking sequences of the MZIR098 insert.

Figure V–2. Locations of the 8.5-kb T-DNA-specific probe and the restriction sites *Hind*III, *Xcm*I, and *Bmt*I in the MZIR098 corn insert

Table V–1 shows the insert-specific hybridization bands expected and observed in Southern blot analyses of MZIR098 corn with the full-length T-DNA-specific probe. 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 MZIR098 corn genome. No hybridization bands were expected in the analyses of genomic DNA from nontransgenic, near-isogenic corn (the negative control). In the analyses of NP2222, NP2391, or NP2391/NP2222 corn genomic DNA, the observation of bands that were also present in genomic DNA from MZIR098 corn were the result of cross-hybridization of the T-DNA-specific probe sequence with the endogenous corn sequence. Figures V–3 through V–5 show the results of the Southern blot analyses with the full-length T-DNA-specific probe.

In the analysis of genomic DNA digested with *Hin*dIII, two bands of approximately 5.9 kb and 12.0 kb were observed in the lanes containing DNA from MZIR098 corn F_2, F_3, F_4, F_5, and F_1 generations (Figure V–3, Lanes 2 through 6). These bands were absent from the lanes containing DNA from the nontransgenic NP2222, NP2391, and NP2222/NP2391 corn (Figure V–3, Lanes 7 through 9) and were, therefore, specific to the MZIR098 insert. As expected, one band of approximately 8.5 kb was observed in the lanes containing the positive controls (Figure V–3, Lanes 10 and 11).

In the analysis of genomic DNA digested with *Xcm*I, two bands of approximately 8.0 kb and 11.5 kb were observed in the lanes containing DNA from MZIR098 corn F_2, F_3, F_4, F_5, and F_1 generations (Figure V–4, Lanes 2 through 6). These bands were absent from the lanes containing DNA from nontransgenic NP2222, NP2391, and NP2222/NP2391 corn (Figure V–4, Lanes 7 through 9) and were, therefore, specific to the MZIR098 insert. As expected, one band of approximately 8.5 kb was observed in the lanes containing the positive controls (Figure V–4, Lanes 10 and 11).

In the analysis of genomic DNA digested with *Bmt*I, one band of approximately 8.9 kb was observed in the lanes containing DNA from MZIR098 corn F_2, F_3, F_4, F_5, and F_1 generations (Figure V–5, Lanes 2 through 6). This band was absent in the lanes containing DNA from nontransgenic NP2222, NP2391, and NP2222/NP2391 corn (Figure V–5, Lanes 7 through 9) and was, therefore, specific to the MZIR098 insert. As expected, one band of approximately 8.5 kb was observed in the lanes containing the positive controls (Figure V–5, Lanes 10 and 11).

Table V–1. Expected and observed insert-specific hybridization bands in Southern blot analyses of multiple generations of MZIR098 corn DNA with a full-length T-DNA-specific probe and restriction enzymes *Hind*III, *Xcm*I, and *Bmt*I

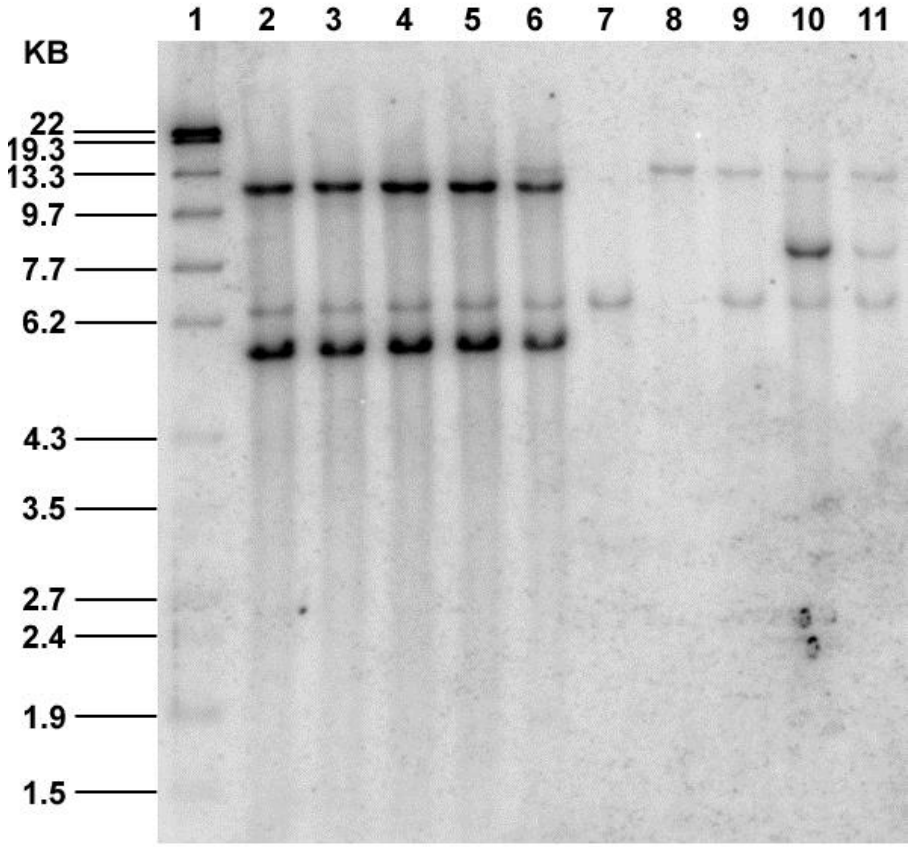
Figure & lane	Source of DNA	Restriction enzyme	Expected no. of bands[a]	Approximate band size (kb)	
				Expected	Observed[a]
V-3, 2	MZIR098 F_2 corn	*Hin*dIII	2	>3.8 >6.6	5.9 12.0
V-3, 3	MZIR098 F_3 corn	*Hin*dIII	2	>3.8 >6.6	5.9 12.0
V-3, 4	MZIR098 F_4 corn	*Hin*dIII	2	>3.8 >6.6	5.9 12.0
V-3, 5	MZIR098 F_5 corn	*Hin*dIII	2	>3.8 >6.6	5.9 12.0
V-3, 6	MZIR098 F_1 corn	*Hin*dIII	2	>3.8 >6.6	5.9 12.0
V-3, 7	NP2222 corn (negative control)	*Hin*dIII	0	N/A	N/A
V-3, 8	NP2391 corn (negative control)	*Hin*dIII	0	N/A	N/A
V-3, 9	NP2222/NP2391 corn (negative control)	*Hin*dIII	0	N/A	N/A
V-3, 10	1-copy positive control	*Hin*dIII	1	8.5	8.5
V-3, 11	1/7-copy positive control	*Hin*dIII	1	8.5	8.5
V-4, 2	MZIR098 F_2 corn	*Xcm*I	2	>4.5 >5.9	8.0 11.5
V-4, 3	MZIR098 F_3 corn	*Xcm*I	2	>4.5 >5.9	8.0 11.5
V-4, 4	MZIR098 F_4 corn	*Xcm*I	2	>4.5 >5.9	8.0 11.5
V-4, 5	MZIR098 F_5 corn	*Xcm*I	2	>4.5 >5.9	8.0 11.5
V-4, 6	MZIR098 F_1 corn	*Xcm*I	2	>4.5 >5.9	8.0 11.5
V-4, 7	NP2222 corn (negative control)	*Xcm*I	0	N/A	N/A
V-4, 8	NP2391 corn (negative control)	*Xcm*I	0	N/A	N/A
V-4, 9	NP2222/NP2391 corn (negative control)	*Xcm*I	0	N/A	N/A
V-4, 10	1-copy positive control	*Xcm*I	1	8.5	8.5
V-4, 11	1/7-copy positive control	*Xcm*I	1	8.5	8.5
V-5, 2	MZIR098 F_2 corn	*Bmt*I	1	8.9	8.9
V-5, 3	MZIR098 F_3 corn	*Bmt*I	1	8.9	8.9
V-5, 4	MZIR098 F_4 corn	*Bmt*I	1	8.9	8.9
V-5, 5	MZIR098 F_5 corn	*Bmt*I	1	8.9	8.9
V-5, 6	MZIR098 F_1 corn	*Bmt*I	1	8.9	8.9

Continued

Figure & lane	Source of DNA	Restriction enzyme	Expected no. of bands[a]	Approximate band size (kb)	
				Expected	Observed[a]
V-5, 7	NP2222 corn (negative control)	*Bmt*I	0	N/A	N/A
V-5, 8	NP2391 corn (negative control)	*Bmt*I	0	N/A	N/A
V-5, 9	NP2222/NP2391 corn (negative control)	*Bmt*I	0	N/A	N/A
V-5, 10	1-copy positive control	*Bmt*I	1	8.5	8.5
V-5, 11	1/7-copy positive control	*Bmt*I	1	8.5	8.5

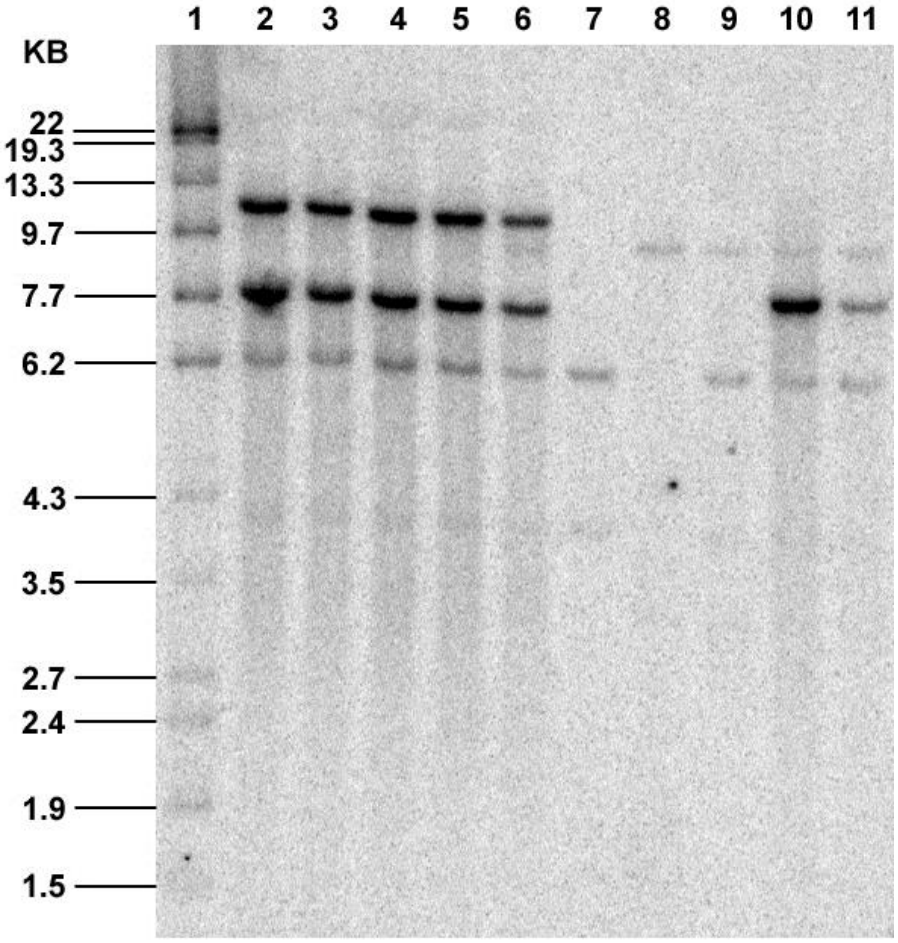
N/A = not applicable.

[a]Bands resulting from cross-hybridization to endogenous corn elements that are not specific to the MZIR098 insert are not included.



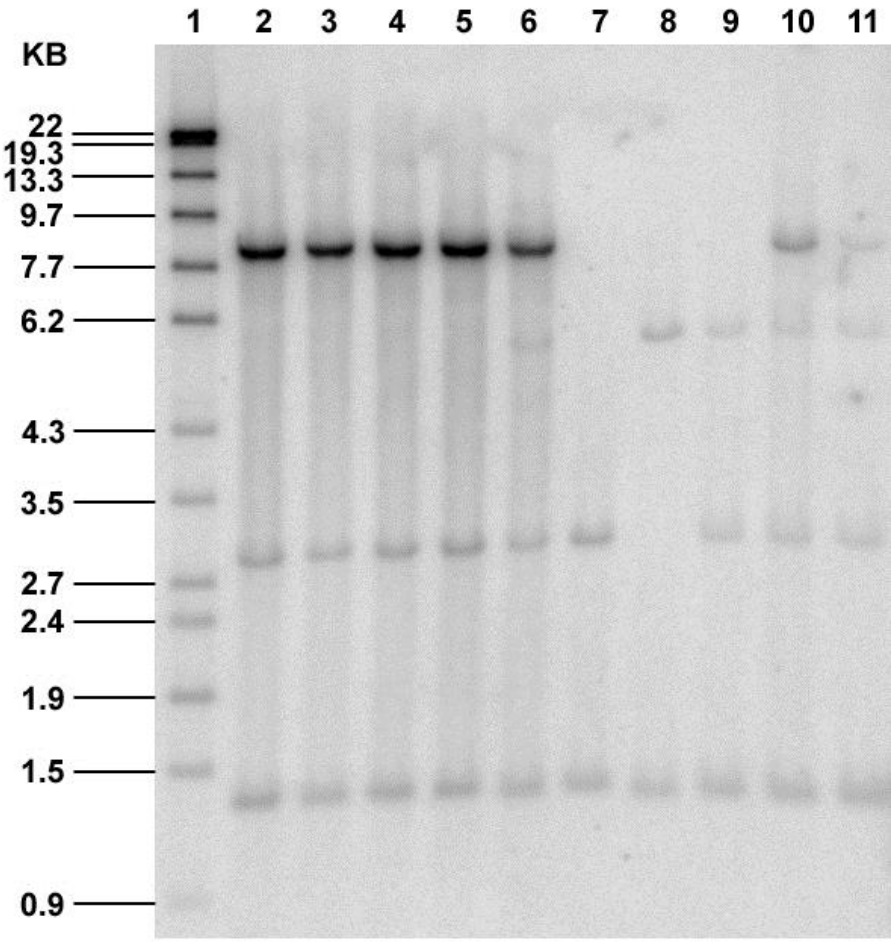
Lane 1 = molecular weight markers
Lane 2 = MZIR098 F_2 corn
Lane 3 = MZIR098 F_3 corn
Lane 4 = MZIR098 F_4 corn
Lane 5 = MZIR098 F_5 corn
Lane 6 = MZIR098 F_1 corn
Lane 7 = NP2222 corn
Lane 8 = NP2391 corn
Lane 9 = NP2222/NP2391 corn
Lane 10 = 1-copy positive control (NP2222/NP2391 corn + 11.9 pg of T-DNA-specific fragment)
Lane 11 = 1/7-copy positive control (NP2222/NP2391 corn + 1.7 pg of T-DNA-specific fragment)

Figure V–3. Southern blot analysis of MZIR098 corn with the a 8.5-kb full-length T-DNA-specific probe and restriction enzyme *Hind*III



Lane 1 = molecular weight markers
Lane 2 = MZIR098 F_2 corn
Lane 3 = MZIR098 F_3 corn
Lane 4 = MZIR098 F_4 corn
Lane 5 = MZIR098 F_5 corn
Lane 6 = MZIR098 F_1 corn
Lane 7 = NP2222 corn
Lane 8 = NP2391 corn
Lane 9 = NP2222/NP2391 corn
Lane 10 = 1-copy positive control (NP2222/NP2391 corn + 11.9 pg of T-DNA-specific fragment)
Lane 11 = 1/7-copy positive control (NP2222/NP2391 corn + 1.7 pg of T-DNA-specific fragment)

Figure V–4. Southern blot analysis of MZIR098 corn with the a 8.5-kb full-length T-DNA-specific probe and restriction enzyme *Xcm*I



Lane 1 = molecular weight markers
Lane 2 = MZIR098 F_2 corn
Lane 3 = MZIR098 F_3 corn
Lane 4 = MZIR098 F_4 corn
Lane 5 = MZIR098 F_5 corn
Lane 6 = MZIR098 F_1 corn
Lane 7 = NP2222 corn
Lane 8 = NP2391 corn
Lane 9 = NP2222/NP2391 corn
Lane 10 = 1-copy positive control (NP2222/NP2391 corn + 11.9 pg of T-DNA-specific fragment)
Lane 11 = 1/7-copy positive control (NP2222/NP2391 corn + 1.7 pg of T-DNA-specific fragment)

Figure V–5. Southern blot analysis of MZIR098 corn with the a 8.5-kb full-length T-DNA-specific probe and restriction enzyme *Bmt*I

V.B.2. Confirmation of absence of plasmid backbone through Southern blot analyses

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 pSYN17629 outside of the T-DNA. These elements were not expected to be transferred to the plant cell or integrated into the plant genome during T-DNA transfer.

The MZIR098 corn generations used in Southern blot analyses were the F_1 and F_2 generations in the genetic background NP2222. These represent the earliest generations of MZIR098 corn (Figure III–2). The control substance was nontransgenic, near-isogenic NP2222 corn. DNA from nontransgenic, near-isogenic control corn was used as a negative control to identify any endogenous corn DNA sequences that hybridize to the probe. For the MZIR098 F_1 generation, leaf tissue from 14 plants was sampled and pooled in groups of seven and used for DNA extraction. For the MZIR098 F_2 generation and the nontransgenic, near-isogenic NP2222 corn, leaf tissue from seven plants was sampled, pooled, and used for DNA extraction. Genomic DNA was isolated from leaf tissue by a method modified from that described by Murray and Thompson (1980).

To demonstrate the sensitivity of the method, each Southern blot analysis 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 the two backbone-specific probes and were loaded in a well together with 7.5 µg of digested DNA from nontransgenic, near-isogenic NP2222 corn, in order to more accurately reflect their migration speeds in the corn genome matrix.

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

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

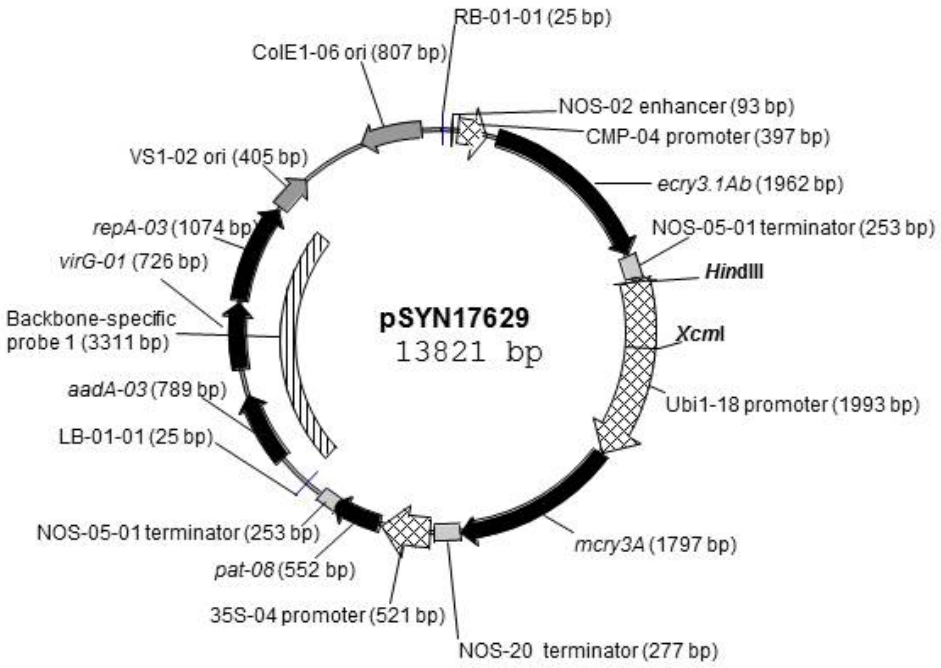
The following factors were used to calculate the amounts of the positive assay controls:

corn genome size (bp)	2.67×10^9
corn ploidy	2
DNA loaded in each lane (µg)	7.5
backbone-specific DNA fragment 1 (bp)	3311
backbone-specific DNA fragment 2 (bp)	2065

A total of 4.65 and 0.66 pg of backbone-specific DNA fragment 1 was added to the Southern blot for the 1-copy and 1/7-copy lanes, respectively. A total of 2.90 and 0.41 pg of backbone-specific DNA fragment 2 was added to the Southern blot for the 1-copy and 1/7-copy lanes, respectively.

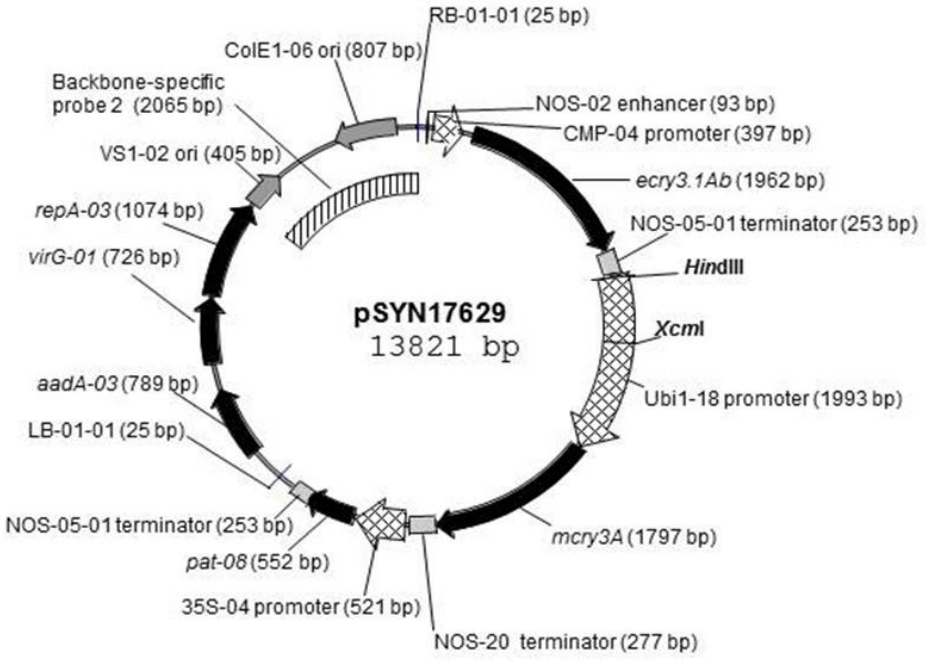
Corn genomic DNA was analyzed via two restriction enzyme digestion strategies. In the first strategy, the genomic DNA was digested with an enzyme that cut within the MZIR098 insert and

in the corn genome flanking the MZIR098 insert. This first strategy was used twice, with two different enzymes. The enzymes used were *Hind*III and *Xcm*I (Figures V–6 and V–7). In the second strategy the genomic DNA was digested with restriction enzymes that cut within the insert to release DNA fragments of predictable size. The enzyme used was *Bmt*I.



The restriction enzyme *Bmt*I was used during the Southern blot analysis but is not present on this map because pSYN17629 does not contain the recognition sequence for this enzyme.

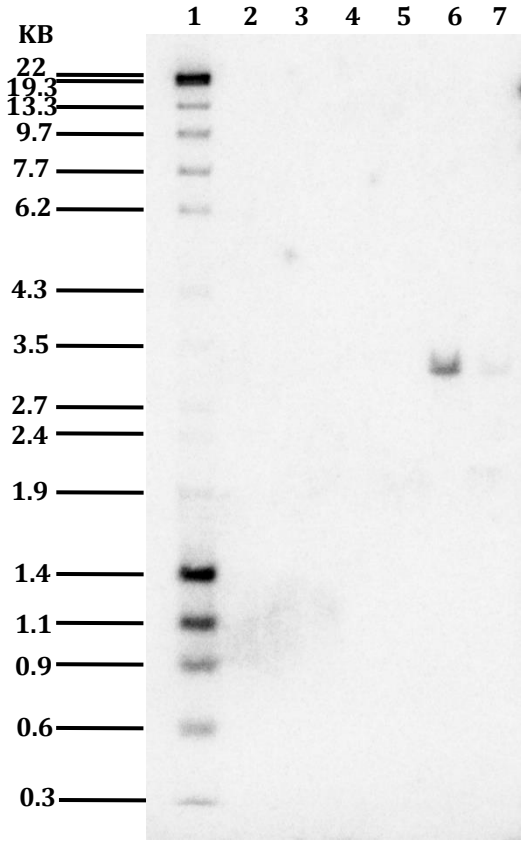
Figure V–6. Locations of the 3.3-kb backbone-specific probe 1 and the restriction sites *Hind*III and *Xcm*I in the transformation plasmid pSYN17629



The restriction enzyme *Bmt*I was used during the Southern blot analysis but is not present on this map because pSYN17629 does not contain the recognition sequence for this enzyme.

Figure V–7. Locations of the 2.1-kb backbone-specific probe 2 and the restriction sites *Hind*III and *Xcm*I in the transformation plasmid pSYN17629

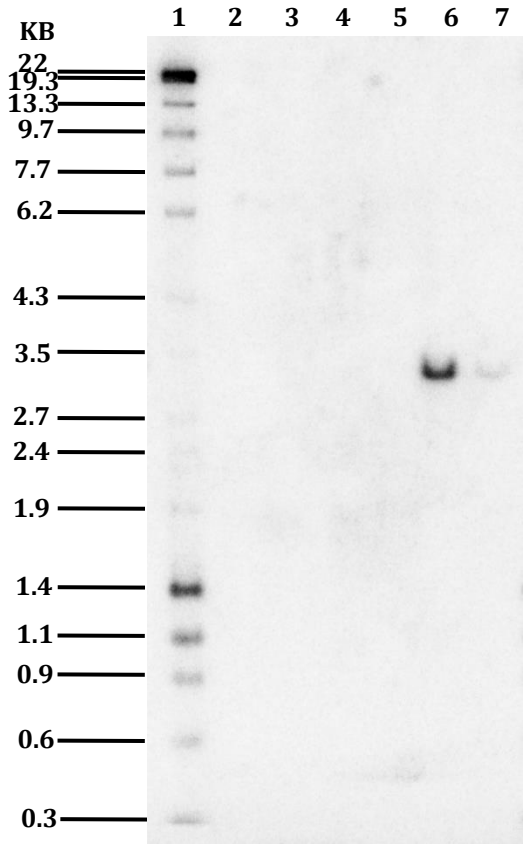
Figures V–8 through V–10 show the results of the Southern blot analyses with backbone-specific probe 1. In the analyses of genomic DNA digested with *Hin*dIII, *Xcm*I, and *Bmt*I, no bands were observed in any of the lanes containing DNA from MZIR098 corn F_1 and F_2 generations (Figures V–8 through V–10, Lanes 2 through 4) or in the lanes containing DNA from nontransgenic NP2222 corn (Figures V–8 through V–10, Lanes 5 through 7). One band of approximately 3.3 kb was observed in the lanes containing the 1-copy and 1/7-copy positive controls (Figures V–8 through V–10, Lanes 6 and 7), as expected.



Lane 1 = molecular weight markers
Lane 2 = MZIR098 F_1 (pool 1) corn
Lane 3 = MZIR098 F_1 (pool 2) corn
Lane 4 = MZIR098 F_2 corn
Lane 5 = NP2222 corn
Lane 6 = 1-copy positive control (NP2222 corn + 4.65 pg of backbone-specific fragment 1)
Lane 7 = 1/7-copy positive control (NP2222 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 7 may not be visible on the printed copy.

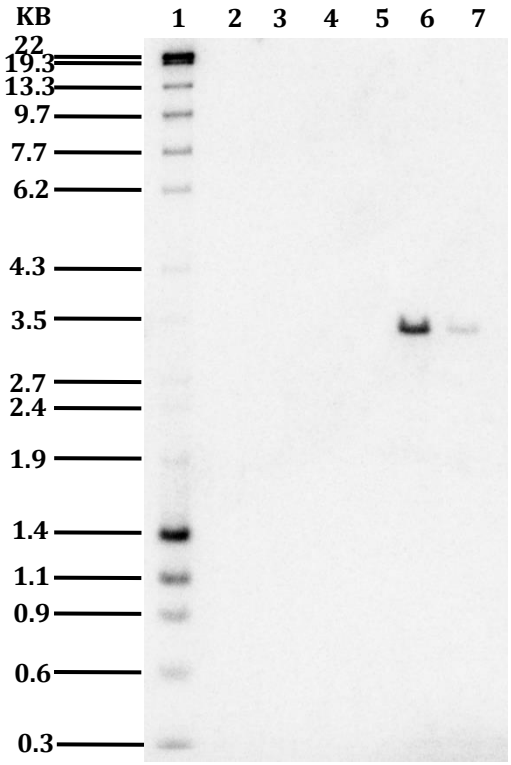
Figure V–8. Southern blot analysis of MZIR098 corn with the 3.3-kb plasmid pSYN17629 backbone-specific probe 1 and restriction enzyme *Hind*III



Lane 1 = molecular weight markers
Lane 2 = MZIR098 F_1 (pool 1) corn
Lane 3 = MZIR098 F_1 (pool 2) corn
Lane 4 = MZIR098 F_2 corn
Lane 5 = NP2222 corn
Lane 6 = 1-copy positive control (NP2222 corn + 4.65 pg of backbone-specific fragment 1)
Lane 7 = 1/7-copy positive control (NP2222 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 7 may not be visible on the printed copy.

Figure V–9. Southern blot analysis of MZIR098 corn with the 3.3-kb plasmid pSYN17629 backbone-specific probe 1 and restriction enzyme *Xcm*I

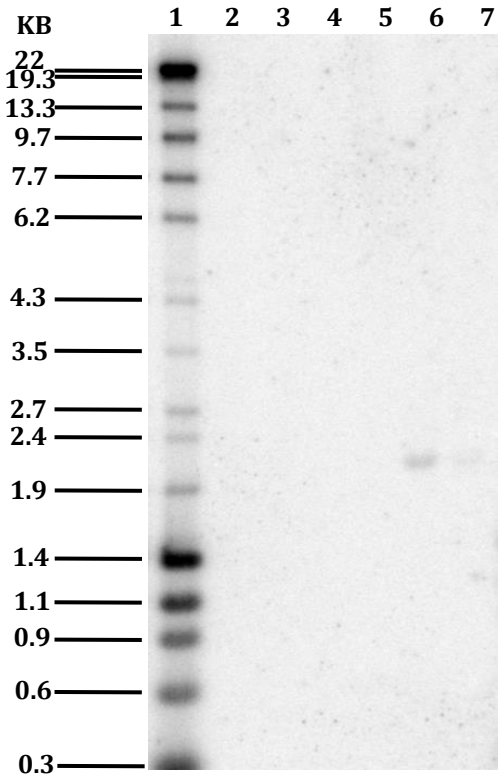


Lane 1 = molecular weight markers
Lane 2 = MZIR098 F_1 (pool 1) corn
Lane 3 = MZIR098 F_1 (pool 2) corn
Lane 4 = MZIR098 F_2 corn
Lane 5 = NP2222 corn
Lane 6 = 1-copy positive control (NP2222 corn + 4.65 pg of backbone-specific fragment 1)
Lane 7 = 1/7-copy positive control (NP2222 corn + 0.66 pg of backbone-specific fragment 1)[a]

[a]Because of limitations in printer resolution, the faint band visible at approximately 3.3 kb in lane 7 may not be visible on the printed copy.

Figure V–10. Southern blot analysis of MZIR098 corn with the 3.3-kb plasmid pSYN17629 backbone-specific probe 1 and restriction enzyme *Bmt*I

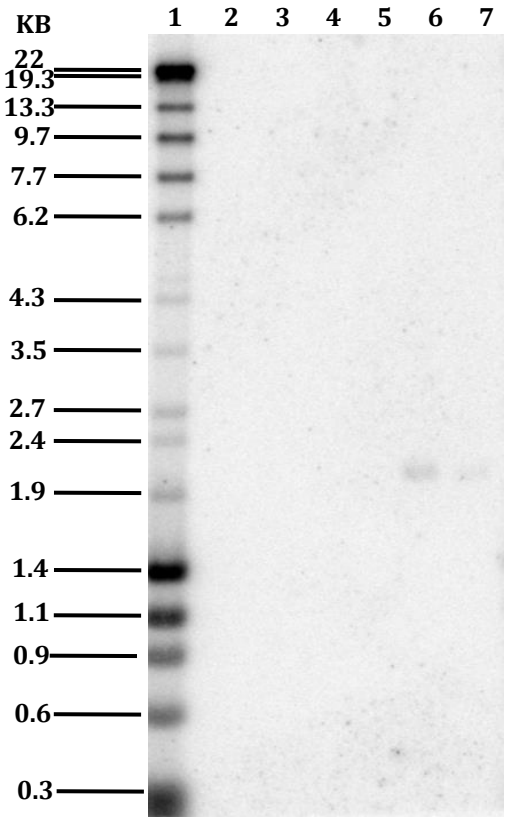
Figures V–11 through V–13 show the results of the Southern blot analyses with backbone-specific probe 2. In the analyses of genomic DNA digested with *Hin*dIII, *Xcm*I, and *Bmt*I, no bands were observed in any of the lanes containing DNA from MZIR098 corn F_1 and F_2 generations (Figures V–11 through V–13, Lanes 2 through 4) or in the lanes containing DNA from nontransgenic NP2222 corn (Figures V–11 through V–13, Lanes 5 through 7). One band of approximately 2.1 kb was observed in the lanes containing the 1-copy and 1/7-copy positive controls (Figures V–11 through V–13, Lanes 6 and 7), as expected.



Lane 1 = molecular weight markers
Lane 2 = MZIR098 F_1 (pool 1) corn
Lane 3 = MZIR098 F_1 (pool 2) corn
Lane 4 = MZIR098 F_2 corn
Lane 5 = NP2222 corn
Lane 6 = 1-copy positive control (NP2222 corn + 2.90 pg of backbone-specific fragment 2)
Lane 7 = 1/7-copy positive control (NP2222 corn + 0.41 pg of backbone-specific fragment 2)[a]

[a]Because of limitations in printer resolution, the faint band visible at approximately 2.1 kb in lane 7 may not be visible on the printed copy.

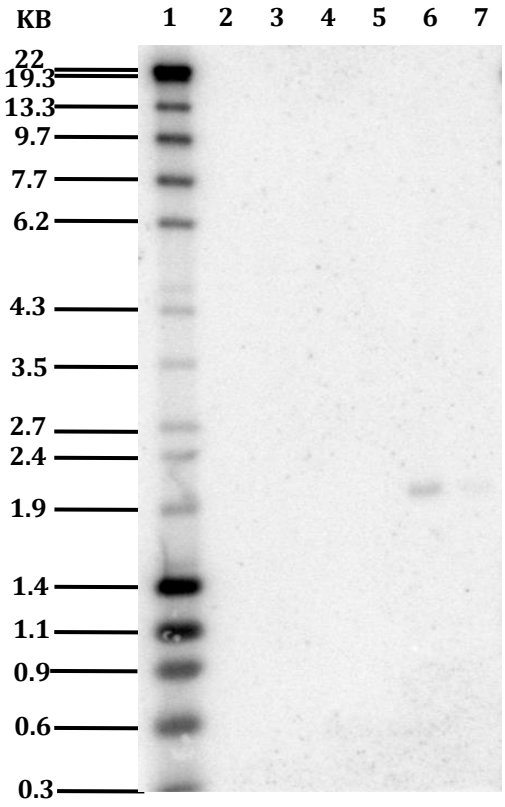
Figure V–11. Southern blot analysis of MZIR098 corn with the 2.1-kb plasmid pSYN17629 backbone-specific probe 2 and restriction enzyme *Hind*III



Lane 1 = molecular weight markers
Lane 2 = MZIR098 F_1 (pool 1) corn
Lane 3 = MZIR098 F_1 (pool 2) corn
Lane 4 = MZIR098 F_2 corn
Lane 5 = NP2222 corn
Lane 6 = 1-copy positive control (NP2222 corn + 2.90 pg of backbone-specific fragment 2)
Lane 7 = 1/7-copy positive control (NP2222 corn + 0.41 pg of backbone-specific fragment 2)[a]

[a]Because of limitations in printer resolution, the faint band visible at approximately 2.1 kb in lane 7 may not be visible on the printed copy.

Figure V–12. Southern blot analysis of MZIR098 corn with the 2.1-kb plasmid pSYN17629 backbone-specific probe 2 and restriction enzyme *Xcm*I



Lane 1 = molecular weight markers
Lane 2 = MZIR098 F_1 (pool 1) corn
Lane 3 = MZIR098 F_1 (pool 2) corn
Lane 4 = MZIR098 F_2 corn
Lane 5 = NP2222 corn
Lane 6 = 1-copy positive control (NP2222 corn + 2.90 pg of backbone-specific fragment 2)
Lane 7 = 1/7-copy positive control (NP2222 corn + 0.41 pg of backbone-specific -specific fragment 2)[a]
[a]Because of limitations in printer resolution, the faint band visible at approximately 2.1 kb in lane 7 may not be visible on the printed copy.

Figure V–13. Southern blot analysis of MZIR098 corn with the 2.1-kb plasmid pSYN17629 backbone-specific probe 2 and restriction enzyme *Bmt*I

V.B.3. Conclusions from the results of the Southern blot analyses

The Southern blot analyses demonstrated that the hybridization bands specific to the MZIR098 insert were identical in all lanes containing genomic DNA extracted from MZIR098 corn plants of the generation tested. These results support the conclusion that the MZIR098 insert is stably inherited from one generation to the next and that MZIR098 corn contains a single T-DNA insert. No unexpected bands were detected, indicating that the MZIR098 corn genome contains no extraneous fragments of T-DNA. The Southern blot analyses also demonstrated that MZIR098 corn does not contain any backbone sequence from the transformation plasmid pSYN17629.

V.B.4. Mendelian Inheritance of the T-DNA Insert

Three generations of MZIR098 corn were individually analyzed for the presence of *ecry3.1Ab*, *mcry3A*, and *pat-08* by real-time PCR analysis (Ingham *et al.* 2001). The results were used to determine the segregation ratios of *ecry3.1Ab*, *mcry3A*, and *pat-08*. MZIR098 F_2 generation plants that were hemizygous for the transgenes were crossed with nontransgenic corn line NP2391. The resulting F_1 generation was backcrossed with the nontransgenic recurrent parent (NP2391) to yield the BC_1F_1 generation (Figure III–2). MZIR098 corn plants from the BC_1F_1 generation were backcrossed three more times with the nontransgenic recurrent parent (NP2391) to yield the BC_2F_1, BC_3F_1, and BC_4F_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 genes). The segregation data were examined to test the hypothesis that the MZIR098 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–2. The genes *ecry3.1Ab*, *mcry3A*, and *pat-08* co-segregated (i.e., when one gene was present, the other genes were 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 *ecry3.1Ab*, *mcry3A*, and *pat-08* were inherited in a predictable manner, according to Mendelian principles. These results support the conclusion that the MZIR098 corn insert integrated into a chromosome within the corn nuclear genome.

Table V–2. Observed and expected frequencies of *ecry3.1Ab*, *mcry3A*, and *pat-08* in three generations of MZIR098 corn

	BC_2F_1		BC_3F_1		BC_4F_1	
Trait[a]	**Observed**	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**
Positive	85	93	69	70	75	74.5
Negative	101	93	71	70	74	74.5
Total	186	186	140	140	149	149
χ^2	1.38[b]		0.03[b]		0.01[b]	

[a]The observed frequencies of *ecry3.1Ab*, *mcry3A*, and *pat-08* were identical; the three genes segregated as one locus.
[b]$P < 0.05$ ($\chi^2 < 3.84$).

V.C. Summary of the Genetic Characterization of Event MZIR098 Corn

Genetic characterization studies demonstrated that MZIR098 corn contains, at a single locus within the corn genome, a single copy of each of the following functional elements: *ecry3.1Ab*, *mcry3A*, *pat-08*, NOS-02 enhancer, CMP-04 promoter, Ubi1-18 promoter, NOS-20 terminator, and 35S-04 promoter and two copies of the NOS-05-01 terminator, as expected. It does not contain any extraneous DNA fragments of these functional elements elsewhere in the MZIR098

corn genome, and it does not contain backbone sequence from transformation plasmid pSYN17629.

Nucleotide sequence analysis determined that the MZIR098 insert consists of the intact T-DNA region of the pSYN17629. The results of the Southern blot analyses are consistent with the results of the nucleotide sequence analysis. Sequence analysis of the MZIR098 insertion site demonstrated that 24 bp from the corn genomic sequence and 10 bp of non-coding sequence from each border of the insert were deleted during the integration of the MZIR098 insert. Analyses comparing the corn genomic sequence flanking the MZIR098 insert with sequences in public databases indicate that the inserted DNA does not disrupt any known endogenous corn gene.

The observed segregation ratios for *ecry3.1Ab*, *mcry3A*, and *pat-08* in three generations of MZIR098 corn plants were as expected for genes 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 MZIR098 corn indicate that the transgenic locus is stably inherited during conventional breeding.

VI. Characterization and Safety of the eCry3.1Ab Protein

The eCry3.1Ab protein produced in MZIR098 corn has been well characterized and no safety concerns have been identified. Syngenta scientists (Walters *et al.* 2010) engineered the gene, *ecry3.1Ab*, by combining portions of two existing Cry genes, *cry1Ab* and modified *cry3A (mcry3A)*, each of which is derived from a native gene of *Bacillus thuringiensis*, a ubiquitous soil bacterium. The safety of eCry3.1Ab in existing commercial transgenic crop products is supported by a permanent exemption from food and feed tolerances in corn in the U.S. (U.S. EPA 2012b). Insecticidal Cry proteins from *B. thuringiensis* have a long history of safe use in food crops. Their modes of action are highly specific within narrow ranges of related insect species and are not relevant to mammals or other vertebrates. The eCry3.1Ab protein produced in MZIR098 corn is identical to the eCry3.1Ab protein produced in 5307 corn (OECD Unique Identifier SYN-Ø53Ø7-1). Event 5307 corn was the subject of an FDA consultation, as summarized in Biotechnology Consultation Note to File BNF No. 000128, dated February 29, 2012. Event 5307 corn was the subject of USDA APHIS Petition No. 10-336-01p for the determination of nonregulated status, which was granted February 27, 2013 and subsequently introduced to the marketplace in 2014.

To establish an expression profile for eCry3.1Ab as expressed in MZIR098 corn, the concentrations of eCry3.1Ab in MZIR098 corn tissues were determined, Section VI.B.

VI.A. eCry3.1Ab Protein Familiarity and History of Safe Exposure

The nucleotide sequence of the *ecry3.1Ab* gene in MZIR098 corn encoding the eCry3.1Ab protein was confirmed by nucleotide sequencing of the T-DNA insert. Similarly, the nucleotide sequence of the *ecry3.1Ab* gene in 5307 corn encoding the eCry3.1Ab protein was confirmed by nucleotide sequencing of the insert. The deduced amino acid sequences of the eCry3.1Ab

protein in both MZIR098 corn and 5307 corn are identical (Figure VI–1). There are no scientific reports of concern about eCry3.1Ab as it exists in commercially available transgenic food crops.

5307 corn is currently approved to support cultivation activities in the U.S. and Canada. Syngenta has combined the traits in 5307 corn with other deregulated biotechnology-derived traits in two novel combinations that have been reviewed and approved globally in a variety of countries for cultivation and food/feed uses. In addition to the permanent exemption from food and feed tolerances, the safety of eCry3.1Ab in existing commercial transgenic crop products is supported by regulatory approvals of numerous transgenic crops containing insecticidal Cry proteins encoded by genes derived from from *B. thuringiensis*. A complete list of previously evaluated traits containing *B. thuringiensis* dervied corn rootworm active proteins can be found in Appendix B. A complete list of commercially available U.S. corn products containing 5307 corn can be found in the CropLife International BioTradeStatus Database (CLI 2015).

```
Translation of Event 5307 ecry3.1Ab       (1) MTSNGRQCAGIRPYDGRQQHRGLDS
Translation of Event MZIR098 ecry3.1Ab    (1) MTSNGRQCAGIRPYDGRQQHRGLDS

Translation of Event 5307 ecry3.1Ab      (26) STTKDVIQKGISVVGDLLGVVGFPF
Translation of Event MZIR098 ecry3.1Ab   (26) STTKDVIQKGISVVGDLLGVVGFPF

Translation of Event 5307 ecry3.1Ab      (51) GGALVSFYTNFLNTIWPSEDPWKAF
Translation of Event MZIR098 ecry3.1Ab   (51) GGALVSFYTNFLNTIWPSEDPWKAF

Translation of Event 5307 ecry3.1Ab      (76) MEQVEALMDQKIADYAKNKALAELQ
Translation of Event MZIR098 ecry3.1Ab   (76) MEQVEALMDQKIADYAKNKALAELQ

Translation of Event 5307 ecry3.1Ab     (101) GLQNNVEDYVSALSSWQKNPAAPFR
Translation of Event MZIR098 ecry3.1Ab  (101) GLQNNVEDYVSALSSWQKNPAAPFR

Translation of Event 5307 ecry3.1Ab     (126) NPHSQGRIRELFSQAESHFRNSMPS
Translation of Event MZIR098 ecry3.1Ab  (126) NPHSQGRIRELFSQAESHFRNSMPS

Translation of Event 5307 ecry3.1Ab     (151) FAISGYEVLFLTTYAQAANTHLFLL
Translation of Event MZIR098 ecry3.1Ab  (151) FAISGYEVLFLTTYAQAANTHLFLL

Translation of Event 5307 ecry3.1Ab     (176) KDAQIYGEEWGYEKEDIAEFYKRQL
Translation of Event MZIR098 ecry3.1Ab  (176) KDAQIYGEEWGYEKEDIAEFYKRQL

Translation of Event 5307 ecry3.1Ab     (201) KLTQEYTDHCVKWYNVGLDKLRGSS
Translation of Event MZIR098 ecry3.1Ab  (201) KLTQEYTDHCVKWYNVGLDKLRGSS

Translation of Event 5307 ecry3.1Ab     (226) YESWVNFNRYRREMTLTVLDLIALF
Translation of Event MZIR098 ecry3.1Ab  (226) YESWVNFNRYRREMTLTVLDLIALF

Translation of Event 5307 ecry3.1Ab     (251) PLYDVRLYPKEVKTELTRDVLTDPI
Translation of Event MZIR098 ecry3.1Ab  (251) PLYDVRLYPKEVKTELTRDVLTDPI

Translation of Event 5307 ecry3.1Ab     (276) VGVNNLRGYGTTFSNIENYIRKPHL
Translation of Event MZIR098 ecry3.1Ab  (276) VGVNNLRGYGTTFSNIENYIRKPHL

Translation of Event 5307 ecry3.1Ab     (301) FDYLHRIQFHTRFQPGYYGNDSFNY
Translation of Event MZIR098 ecry3.1Ab  (301) FDYLHRIQFHTRFQPGYYGNDSFNY

Translation of Event 5307 ecry3.1Ab     (326) WSGNYVSTRPSIGSNDIITSPFYGN
Translation of Event MZIR098 ecry3.1Ab  (326) WSGNYVSTRPSIGSNDIITSPFYGN

Translation of Event 5307 ecry3.1Ab     (351) KSSEPVQNLEFNGEKVYRAVANTNL
Translation of Event MZIR098 ecry3.1Ab  (351) KSSEPVQNLEFNGEKVYRAVANTNL

Translation of Event 5307 ecry3.1Ab     (376) AVWPSAVYSGVTKVEFSQYNDQTDE
Translation of Event MZIR098 ecry3.1Ab  (376) AVWPSAVYSGVTKVEFSQYNDQTDE

Translation of Event 5307 ecry3.1Ab     (401) ASTQTYDSKRNVGAVSWDSIDQLPP
Translation of Event MZIR098 ecry3.1Ab  (401) ASTQTYDSKRNVGAVSWDSIDQLPP

Translation of Event 5307 ecry3.1Ab     (426) ETTDEPLEKGYSHQLNYVMCFLMQG
Translation of Event MZIR098 ecry3.1Ab  (426) ETTDEPLEKGYSHQLNYVMCFLMQG

Translation of Event 5307 ecry3.1Ab     (451) SRGTIPVLTWTHKSVDFFNMIDSKK
Translation of Event MZIR098 ecry3.1Ab  (451) SRGTIPVLTWTHKSVDFFNMIDSKK

Translation of Event 5307 ecry3.1Ab     (476) ITQLPLTKSTNLGSGTSVVKGPGFT
Translation of Event MZIR098 ecry3.1Ab  (476) ITQLPLTKSTNLGSGTSVVKGPGFT

Translation of Event 5307 ecry3.1Ab     (501) GGDILRRTSPGQISTLRVNITAPLS
Translation of Event MZIR098 ecry3.1Ab  (501) GGDILRRTSPGQISTLRVNITAPLS

Translation of Event 5307 ecry3.1Ab     (526) QRYRVRIRYASTTNLQFHTSIDGRP
Translation of Event MZIR098 ecry3.1Ab  (526) QRYRVRIRYASTTNLQFHTSIDGRP

Translation of Event 5307 ecry3.1Ab     (551) INQGNFSATMSSGSNLQSGSFRTVG
Translation of Event MZIR098 ecry3.1Ab  (551) INQGNFSATMSSGSNLQSGSFRTVG

Translation of Event 5307 ecry3.1Ab     (576) FTTPFNFSNGSSVFTLSAHVFNSGN
Translation of Event MZIR098 ecry3.1Ab  (576) FTTPFNFSNGSSVFTLSAHVFNSGN

Translation of Event 5307 ecry3.1Ab     (601) EVYIDRIEFVPAEVTFEAEYDLERA
Translation of Event MZIR098 ecry3.1Ab  (601) EVYIDRIEFVPAEVTFEAEYDLERA

Translation of Event 5307 ecry3.1Ab     (626) QKAVNELFTSSNQIGLKTDVTDYHI
Translation of Event MZIR098 ecry3.1Ab  (626) QKAVNELFTSSNQIGLKTDVTDYHI

Translation of Event 5307 ecry3.1Ab     (651) DQV-
Translation of Event MZIR098 ecry3.1Ab  (651) DQV-
```

Figure VI–1. Alignment of the deduced amino acid sequence from *ecry3.1Ab* in 5307 corn and *ecry3.1Ab* in MZIR098 corn

VI.B. Levels of eCry3.1Ab Protein in MZIR098 Corn Tissues

The concentrations of eCry3.1Ab in various MZIR098 corn tissues were quantified by enzyme-linked immunosorbent assay (ELISA) to establish an expression profile for eCry3.1Ab as produced in MZIR098 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 MZIR098 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(MZIR098) and NP2391/NP2222 (Figure III–2). These trials were planted under USDA notification 13-044-12n.

At each location, one plot was planted with MZIR098 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 eCry3.1Ab protein concentrations observed in each MZIR098 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 eCry3.1Ab in MZIR098 corn tissues are described in Appendix C.

Table VI–1. Concentrations of eCry3.1Ab in MZIR098 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	216.07 ± 72.13	129.62–333.92	32.70 ± 10.01	20.72–46.68
Leaves, R1	107.35 ± 32.74	61.05–157.55	24.98 ± 8.80	13.50–41.36
Leaves, R6	49.70 ± 19.72	9.68–75.20	21.11 ± 6.58	5.82–31.98
Leaves, Sen.[c]	9.58 ± 4.88	2.09–20.12	7.37 ± 3.38	1.68–14.81
Roots, V6	73.07 ± 13.68	37.63–96.44	9.42 ± 3.00	4.90–13.84
Roots, R1	21.76 ± 11.21	7.82–47.09	3.05 ± 1.57	1.35–6.21
Roots, R6	14.37 ± 5.74	5.06–23.19	2.26 ± 0.77	1.16–3.72
Roots, Sen.	7.87 ± 4.60	1.84–18.76	1.14 ± 0.63	0.34–2.56
Whole Plant, V6	168.14 ± 40.40	99.40–221.05	20.75 ± 5.16	12.22–29.39
Whole Plant, R1	52.72 ± 19.31	26.20–98.55	9.95 ± 4.09	4.59–19.97
Whole Plant, R6	7.72 ± 3.48	2.70–18.38	3.47 ± 1.09	1.57–6.47
Pollen, R1	<LOD[d]	<LOD	<LOD	<LOD
Kernel, R6	2.42 ± 1.15	1.28–5.90	1.58 ± 0.62	1.02–3.44
Kernel, Sen.	2.08 ± 1.29	0.82–4.52	1.50 ± 0.79	0.67–3.03

[a] *N* = 20 for all tissues except pollen, where *N* = 4
[b] SD = standard deviation
[c] Sen. = senescence
[d] LOD for eCry3.1Ab in pollen = 0.08 µg/g DW

VI.C. Identity and Characterization of the eCry3.1Ab Protein in MZIR098 Corn

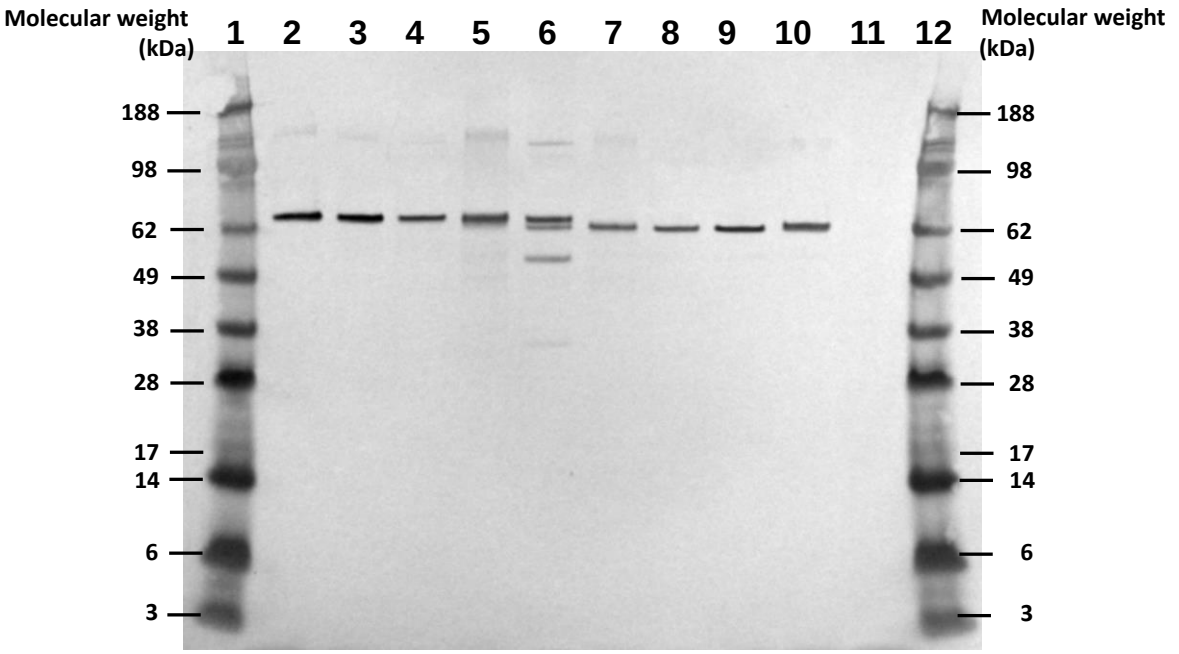
The identity of the eCry3.1Ab protein in MZIR098 corn was confirmed by peptide mass coverage analysis, apparent molecular weight, and immunoreactivity.

To conduct peptide mass coverage analysis of eCry3.1Ab, the protein was extracted from MZIR098 corn, reduced, alkylated with iodoacetamide, and enzymatically digested separately with trypsin, chymotrypsin and endoproteinase Asp-N. The peptide mass coverage analysis verified 85.9% of the predicted amino acid sequence of eCry3.1Ab (Figure VI–2). Western blot analysis (Figure VI–3) demonstrated that eCry3.1Ab from MZIR098 corn cross-reacted with an antibody capable of detecting eCry3.1Ab, displaying an apparent molecular weight consistent with the predicted molecular weight of 73.7 kilodalton (kDa). The same figure also demonstrated the predicted molecular weight and cross-reactivity of mCry3A, the other insect resistance protein produced in MZIR098 corn.

1 *M***TSNGRQCAGIRPYDGRQQHRGLDSSTTKDVIQKGISVVG** 40
41 *DLLGVVGFPFGGALVSFY*TNFLNTIWPSEDPWKAF*MEQVE* 80
81 *ALMDQKIA***DYAKNKALAELQGLQNNVE**DYVSALSSWQKNP 120
121 AAPFRNPHSQGRIRELFSQAESHFRNSMPSFAISGYEVL*F* 160
161 *LTTY*AQAANTHLFLLKDAQIYGEEWGYEKE**DIAEFYKRQL** 200
201 **KLTQEYTDHCVKWYNVGL**DKLRGSSYESWVNFNRYRR*EMT* 240
241 *LTVL***DLIALFPLYDVRLYPKEVKTELTR**DVLT**DPIVGVNN** 280
281 **LRGYGTTFSNIENYIRKPHLFDYLHRIQFHTRFQPGYYGN** 320
321 **DSFNYWSGNYVSTRPSIGSNDIITSPFYGNKSSEPVQNL**E 360
361 FNGEKVYRAVANTNLAVWPSAVYSGVTKVEFSQYNDQT**DE** 400
401 **ASTQTYDSKRNVGAVSWDSIDQLPPETT**DEPLEKGY*SHQL* 440
441 *NYVMCF*LMQGSRGTIPVLTWTHKSVDFFNMI**DSKKITQLP** 480
481 **LTKSTNLGSGTSVVKGPGFTGG**DILRRTSPGQISTLRVNI 520
521 TAPLSQRYR*VRIRY*ASTTNLQFHTSIDGRPINQGNF*SATM* 560
561 *SSGSNLQSGSFRTVGFTTPFNFSNGSSVF*TLSAHVFNSGN 600
601 EVYI**DRIEFVPAEVTF**EAEY**DLERAQKAVNELFTSSNQIG** 640
641 **LKTDVTDYHIDQV** 653

Legend: Trypsin-detected
Chymotrypsin-detected
Endoproteinase Asp-N-detected
Italic type indicates amino acids not identified

Figure VI–2. Amino acid sequence identified for eCry3.1Ab from MZIR098 corn by peptide mass coverage analysis



Lane 1: Molecular weight standard
Lane 2: Microbially produced eCry3.1Ab (7.44 ng of eCry3.1Ab)[a]
Lane 3: Microbially produced eCry3.1Ab (7.44 ng of eCry3.1Ab)[a]
Lane 4: Nontransgenic corn extract, (12.2 µg of total protein) fortified with microbially produced eCry3.1Ab (7.44 ng of eCry3.1Ab)[a]
Lane 5: eCry3.1Ab purified preparation from MZIR098 corn extract (7.44 ng of eCry3.1Ab)
Lane 6: MZIR098 corn extract (12.2 µg of total protein, 7.44 ng of eCry3.1Ab, 3.94 ng of mCry3A)
Lane 7: mCry3A purified preparation from MZIR098 corn extract (3.94 ng of mCry3A)
Lane 8: Nontransgenic corn extract, (12.2 µg of total protein) fortified with microbially produced mCry3A (3.94 ng of mCry3A)[a]
Lane 9: Microbially produced mCry3A (3.94 ng of mCry3A)[a]
Lane 10: Microbially produced mCry3A (3.94 ng of mCry3A)[a]
Lane 11: Nontransgenic corn extract, (12.2 µg of total protein)
Lane 12: Molecular weight standard

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

Figure VI–3. Western blot analysis of eCry3.1Ab and mCry3A as expressed in MZIR098 corn

VI.D. Conclusions on the Safety of eCry3.1Ab in MZIR098 Corn

The safety of the eCry3.1Ab protein has been previously established. These safety conclusions are based on existing safety data and the fact that the eCry3.1Ab produced in MZIR098 corn has the identical amino acid sequence as the eCry3.1Ab produced in 5307 corn, a previously evaluated transgenic corn event in commerce. Previous assessments have demonstrated that eCry3.1Ab has a very specific and well-characterized mode of action, it is not acutely toxic, and it has no characteristics consistent with potential allergenicity (U.S. EPA 2012a). It is concluded that eCry3.1Ab does not pose a risk to the health of humans or livestock through consumption of MZIR098 corn.

VII. Characterization and Safety of the mCry3A Protein

The mCry3A protein produced in MZIR098 corn has been well characterized and no safety concerns have been identified. The gene *mcry3A* is a modified version of a native *cry3A* gene from *Bacillus thuringiensis* subsp. *tenebrionis* (Sekar *et al.* 1987). The safety of mCry3A in existing commercial transgenic crop products is supported by a permanent exemption from food

and feed tolerances in corn in the U.S. (U.S. EPA 2007a). The mCry3A protein produced in MZIR098 corn is identifical to the mCry3A protein produced in MIR604 corn (OECD Unique Identifier SYN-IR6Ø4-5). MIR604 corn was the subject of an FDA consultation, as summarized in Biotechnology Consultation Note to File BNF No. 000099, dated January 30, 2007. MIR604 corn was the subject of USDA APHIS Petition No. 04-362-01p for the determination of nonregulated status, which was granted March 16, 2007 and subsequently introduced to the marketplace in 2007.

To establish an expression profile for mCry3A as expressed in MZIR098 corn, the concentrations of mCry3A in MZIR098 corn tissues were determined, Section VII.B.

VII.A. mCry3A Protein Familiarity and History of Safe Exposure

The nucleotide sequence of the gene *mcry3A* in MZIR098 corn encoding the mCry3A protein was confirmed by nucleotide sequencing of the T-DNA insert. Similarly, the nucleotide sequence of the gene *mcry3A* in MIR604 corn encoding the mCry3A protein was confirmed by nucleotide sequencing of the insert. The deduced amino acid sequences of the mCry3A protein in both MZIR098 corn and MIR604 corn are identical (Figure VII–1). There are no scientific reports of concern about mCry3A as it exists in commercially available MIR604 corn and breeding stacks thereof.

MIR604 corn is currently approved to support cultivation activities in the U. S. and Canada. Syngenta has combined the traits in MIR604 corn with other deregulated biotechnology-derived traits in multiple novel combinations that have been reviewed and approved globally in a variety of countries for cultivation and food/feed uses. In addition to the mCry3A permanent exemption from food and feed tolerances in corn, the safety of mCry3A in existing commercial transgenic corn products is supported by regulatory approvals of numerous transgenic crops containing insecticidal Cry proteins encoded by genes derived from from *B. thuringiensis*. A complete list of commercially available U.S. corn products containing MIR604 corn can be found in the CropLife International BioTradeStatus Database (CLI 2015).

```
Translation of Event MIR604 mcry3A        (1) MTADNNTEALDSSTTKDVIQKGISV
Translation of Event MZIR098 mcry3A       (1) MTADNNTEALDSSTTKDVIQKGISV

Translation of Event MIR604 mcry3A       (26) VGDLLGVVGFPFGGALVSFYTNFLN
Translation of Event MZIR098 mcry3A      (26) VGDLLGVVGFPFGGALVSFYTNFLN

Translation of Event MIR604  mcry3A      (51) TIWPSEDPWKAFMEQVEALMDQKIA
Translation of Event MZIR098 mcry3A      (51) TIWPSEDPWKAFMEQVEALMDQKIA

Translation of Event MIR604  mcry3A      (76) DYAKNKALAELQGLQNNVEDYVSAL
Translation of Event MZIR098 mcry3A      (76) DYAKNKALAELQGLQNNVEDYVSAL

Translation of Event MIR604 mcry3A      (101) SSWQKNPAAPFRNPHSQGRIRELFS
Translation of Event MZIR098 mcry3A     (101) SSWQKNPAAPFRNPHSQGRIRELFS

Translation of Event MIR604 mcry3A      (126) QAESHFRNSMPSFAISGYEVLFLTT
Translation of Event MZIR098 mcry3A     (126) QAESHFRNSMPSFAISGYEVLFLTT

Translation of Event MIR604 mcry3A      (151) YAQAANTHLFLLKDAQIYGEEWGYE
Translation of Event MZIR098 mcry3A     (151) YAQAANTHLFLLKDAQIYGEEWGYE

Translation of Event MIR604 mcry3A      (176) KEDIAEFYKRQLKLTQEYTDHCVKW
Translation of Event MZIR098 mcry3A     (176) KEDIAEFYKRQLKLTQEYTDHCVKW

Translation of Event MIR604 mcry3A      (201) YNVGLDKLRGSSYESWVNFNRYRRE
Translation of Event MZIR098 mcry3A     (201) YNVGLDKLRGSSYESWVNFNRYRRE

Translation of Event MIR604 mcry3A      (226) MTLTVLDLIALFPLYDVRLYPKEVK
Translation of Event MZIR098 mcry3A     (226) MTLTVLDLIALFPLYDVRLYPKEVK

Translation of Event MIR604 mcry3A      (251) TELTRDVLTDPIVGVNNLRGYGTTF
Translation of Event MZIR098 mcry3A     (251) TELTRDVLTDPIVGVNNLRGYGTTF

Translation of Event MIR604 mcry3A      (276) SNIENYIRKPHLFDYLHRIQFHTRF
Translation of Event MZIR098 mcry3A     (276) SNIENYIRKPHLFDYLHRIQFHTRF

Translation of Event MIR604 mcry3A      (301) QPGYYGNDSFNYWSGNYVSTRPSIG
Translation of Event MZIR098 mcry3A     (301) QPGYYGNDSFNYWSGNYVSTRPSIG

Translation of Event MIR604 mcry3A      (326) SNDIITSPFYGNKSSEPVQNLEFNG
Translation of Event MZIR098 mcry3A     (326) SNDIITSPFYGNKSSEPVQNLEFNG

Translation of Event MIR604 mcry3A      (351) EKVYRAVANTNLAVWPSAVYSGVTK
Translation of Event MZIR098 mcry3A     (351) EKVYRAVANTNLAVWPSAVYSGVTK

Translation of Event MIR604 mcry3A      (376) VEFSQYNDQTDEASTQTYDSKRNVG
Translation of Event MZIR098 mcry3A     (376) VEFSQYNDQTDEASTQTYDSKRNVG

Translation of Event MIR604 mcry3A      (401) AVSWDSIDQLPPETTDEPLEKGYSH
Translation of Event MZIR098 mcry3A     (401) AVSWDSIDQLPPETTDEPLEKGYSH

Translation of Event MIR604 mcry3A      (426) QLNYVMCFLMQGSRGTIPVLTWTHK
Translation of Event MZIR098 mcry3A     (426) QLNYVMCFLMQGSRGTIPVLTWTHK

Translation of Event MIR604 mcry3A      (451) SVDFFNMIDSKKITQLPLVKAYKLQ
Translation of Event MZIR098 mcry3A     (451) SVDFFNMIDSKKITQLPLVKAYKLQ

Translation of Event MIR604 mcry3A      (476) SGASVVAGPRFTGGDIIQCTENGSA
Translation of Event MZIR098 mcry3A     (476) SGASVVAGPRFTGGDIIQCTENGSA

Translation of Event MIR604 mcry3A      (501) ATIYVTPDVSYSQKYRARIHYASTS
Translation of Event MZIR098 mcry3A     (501) ATIYVTPDVSYSQKYRARIHYASTS

Translation of Event MIR604 mcry3A      (526) QITFTLSLDGAPFNQYYFDKTINKG
Translation of Event MZIR098 mcry3A     (526) QITFTLSLDGAPFNQYYFDKTINKG

Translation of Event MIR604 mcry3A      (551) DTLTYNSFNLASFSTPFELSGNNLQ
Translation of Event MZIR098 mcry3A     (551) DTLTYNSFNLASFSTPFELSGNNLQ

Translation of Event MIR604 mcry3A      (576) IGVTGLSAGDKVYIDKIEFIPVN-
Translation of Event MZIR098 mcry3A     (576) IGVTGLSAGDKVYIDKIEFIPVN-
```

Figure VII–1. Alignment of the deduced amino acid sequence from *mcry3A* in MIR604 corn and *mcry3A* in MZIR098 corn

VII.B. Levels of mCry3A Protein in MZIR098 Corn Tissues

The concentrations of mCry3A in various MZIR098 corn tissues were quantified by ELISA to establish an expression profile for mCry3A as produced in MZIR098 corn. Plant tissue collection and analysis were as previously described in Section VI.B. for eCry3.1Ab.

Table VII–2 shows the ranges of mCry3A protein concentrations observed in each MZIR098 corn tissue type at several growth stages across four locations on a FW and DW basis. Details of the materials and methods used to quantify levels of mCry3A in MZIR098 corn tissues are described in Appendix C.

Table VII–1. Concentrations of mCry3A in MZIR098 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	79.51 ± 14.24	60.65–114.42	12.08 ± 1.87	9.63–15.87
Leaves, R1	49.57 ± 6.74	32.62–61.07	11.44 ± 1.97	7.34–15.60
Leaves, R6	35.00 ± 13.67	7.71–71.74	15.09 ± 4.63	4.63–26.90
Leaves, Sen.[c]	11.91 ± 5.07	3.50–22.81	9.23 ± 3.68	2.81–20.01
Roots, V6	61.63 ± 18.73	27.11–110.86	8.06 ± 3.65	2.74–18.23
Roots, R1	24.58 ± 6.67	13.36–39.51	3.39 ± 0.69	2.05–5.01
Roots, R6	15.53 ± 5.78	5.47–22.89	2.42 ± 0.77	1.32–4.63
Roots, Sen.	11.86 ± 4.31	5.07–19.06	1.77 ± 0.71	0.53–2.87
Whole Plant, V6	71.48 ± 11.48	49.38–95.09	8.08 ± 1.33	6.12–10.41
Whole Plant, R1	35.43 ± 8.42	24.48–52.35	6.63 ± 1.81	4.26–10.61
Whole Plant, R6	7.72 ± 3.48	2.70–18.38	3.47 ± 1.09	1.57–6.47
Pollen, R1	302.93 ± 6.37	293.87–308.71	187.75 ± 41.14	152.64–246.96
Kernel, R6	14.59 ± 3.76	8.91–22.83	9.76 ± 2.36	6.05–15.38
Kernel, Sen.	11.21 ± 3.41	6.69–19.65	8.30 ± 2.02	5.38–14.14

[a] *N* = 20 for all tissues except pollen, where *N* = 4
[b] SD = standard deviation
[c] Sen. = senescence

VII.C. Identity and Characterization of the mCry3A Protein in MZIR098 Corn

The identity of the mCry3A protein produced in MZIR098 corn was confirmed by peptide mass coverage analysis, apparent molecular weight, and immunoreactivity.

To conduct peptide mass coverage of mCry3A, the protein was extracted from MZIR098 plants, reduced, alkylated with iodoacetamide, and enzymatically digested separately with trypsin, chymotrypsin and endoproteinase Asp-N. The peptide mass coverage analysis verified 91.6% of the predicted amino acid sequence of mCry3A (Figure VII–2). Western blot analysis demonstrated that mCry3A form MZIR098 corn cross-reacted with an antibody capable of detecting mCry3A, displaying an apparent molecular weight consistent with the predicted molecular weight of 67.7kDa (Figure VI–3).

1 MTADNNTEALDSSTTKDVIQKGISVVG*DLLGVVGFPF*GGA 40
41 LVSF*Y*TNFLNTIWPSEDPWKAFMEQVEALMDQKIA**DYAKN** 80
81 **KALAELQGLQNNVEDYVSALSSWQKNPAAPFRNPHSQGRI** 120
121 **R**ELFSQAESHFRNSMPSFAISGY*EVLFLTTY*AQAANTHLF 160
161 LLKDAQIYGEEWGYEKE**DIAEFYKRQLKLTQEYTDHCVKW** 200
201 **YNVGLDKLRGSSYESWVNFNRYRR**EMTLTVL**DLIALFPLY** 240
241 **DVRLYPKEVKTELTR**DVLT**DPIVGVNNLRGYGTTFSNIEN** 280
281 **YIRKPHLFDYLHRIQFHTRFQPGYYGNDSFNYWSGNYVST** 320
321 **RPSIGSNDIITSPFYGNKSSEPVQNL**EFNGEKVYRAVANT 360
361 NLAVWPSAVYSGVTKVEFSQYNDQTDEASTQTY**DSKRNVG** 400
401 **AVSWDSIDQLPPETT**DEPLEKGYSHQL*NYVMCFLMQGSR*G 440
441 TIPVLTWTHKSVDFFNMI**DSKKITQLPLVKAYKLQSGASV** 480
481 **VAGPRFTGGDIIQCTENGSAATIYVTP**DVSYSQKYRAR*IH* 520
521 *YASTSQITFTLSL***DGAPFNQYYF**DKTINKGDTLTYNSFNL 560
561 ASF*STPF***ELSGNNLQIGVTGLSAGDKVYIDKIEFIPVN** 598

Legend: Trypsin-detected
Chymotrypsin-detected
Endoproteinase Asp-N-detected
Italic type indicates amino acids not identified

Figure VII–2. Amino acid sequence identified for mCry3A from MZIR098 corn by peptide mass coverage analysis

VII.D. Conclusions on the Safety of mCry3A in MZIR098 Corn

The safety of the mCry3A protein has been previously established. These safety conclusions are based on existing safety data and the fact that mCry3A as produced in MZIR098 corn has the identical amino acid sequence as mCry3A produced in MIR604 corn, a previously evaluated transgenic corn event in commerce. Previous assessments have demonstrated that mCry3A has a very specific and well-characterized mode of action, it is not acutely toxic, and it has no characteristics consistent with potential allergenicity (U.S. EPA 2010b). It is concluded that mCry3A does not pose a risk to the health of humans or livestock through consumption of MZIR098 corn.

VIII. Characterization and Safety of the PAT Protein

The PAT protein produced in MZIR098 corn has been well characterized and no safety concerns have been identified. 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 PAT (Hérouet *et al.* 2005, ILSI 2011, and OECD 1999). The PAT produced in MZIR098 corn is identical to the PAT produced in Event Bt11 corn (OECD Unique Identifier SYN-BTØ11-1). Event Bt11 corn (hereafter Bt11 corn) was the subject of an FDA consultation, as summarized in Biotechnology Consultation Note to File BNF No. 000017, dated May 22, 1996. 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 and subsequently introduced to the market in 1997.

To establish an expression profile for PAT as expressed in MZIR098 corn, the concentrations of PAT in MZIR098 corn tissues were determined, Section VIII.B.

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

The nucleotide sequence of *pat-08* in MZIR098 corn encoding the PAT protein was confirmed by nucleotide sequencing of the T-DNA insert. Similarly, 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 MZIR098 corn and Bt11 corn is identical (Figure VIII–1). Bt11 corn is currently approved to support cultivation activities in nine countries, including the U.S., Canada, and Argentina. Syngenta has combined the traits in Bt11 corn 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 PAT 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 enzymes 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 Bt11 corn can be found in the CropLife International BioTradeStatus Database (CLI 2015).

```
Translation of Event Bt11 pat            (1) MSPERRPVEIRPATAADMAAVCDIV
Translation of Event MZIR098 pat-08      (1) MSPERRPVEIRPATAADMAAVCDIV

Translation of Event Bt11 pat           (26) NHYIETSTVNFRTEPQTPQEWIDDL
Translation of Event MZIR098 pat-08     (26) NHYIETSTVNFRTEPQTPQEWIDDL

Translation of Event Bt11 pat           (51) ERLQDRYPWLVAEVEGVVAGIAYAG
Translation of Event MZIR098 pat-08     (51) ERLQDRYPWLVAEVEGVVAGIAYAG

Translation of Event Bt11 pat           (76) PWKARNAYDWTVESTVYVSHRHQRL
Translation of Event MZIR098 pat-08     (76) PWKARNAYDWTVESTVYVSHRHQRL

Translation of Event Bt11 pat          (101) GLGSTLYTHLLKSMEAQGFKSVVAV
Translation of Event MZIR098 pat-08    (101) GLGSTLYTHLLKSMEAQGFKSVVAV

Translation of Event Bt11 pat          (126) IGLPNDPSVRLHEALGYTARGTLRA
Translation of Event MZIR098 pat-08    (126) IGLPNDPSVRLHEALGYTARGTLRA

Translation of Event Bt11 pat          (151) AGYKHGGWHDVGFWQRDFELPAPPR
Translation of Event MZIR098 pat-08    (151) AGYKHGGWHDVGFWQRDFELPAPPR

Translation of Event Bt11 pat          (176) PVRPVTQI-
Translation of Event MZIR098 pat-08    (176) PVRPVTQI-
```

Figure VIII–1. Alignment of the deduced amino acid sequence from *pat* in Bt11 corn and *pat-08* in MZIR098 corn

VIII.B. Levels of PAT Protein in MZIR098 Corn Tissues

The concentrations of PAT in various MZIR098 corn tissues were quantified by enzyme-linked immunosorbent assay (ELISA) to establish an expression profile for PAT as produced in MZIR098 corn. Plant tissue collection and analysis were as previously described in Section VIII.B. for PAT.

Table VIII–1 shows the ranges of PAT protein concentrations observed in each MZIR098 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 MZIR098 corn tissues are described in Appendix C.

Table VIII–1. Concentrations of PAT in MZIR098 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	7.62 ± 2.77	4.16–13.05	1.14 ± 0.34	0.68–1.85
Leaves, R1	3.57 ± 1.31	1.58–5.94	0.82 ± 0.29	0.38–1.36
Leaves, R6	– [c]	<LOD[d] –1.93	–	<LOD –0.74
Leaves, Sen.[e]	–	<LOD–0.13	–	<LOD–0.10
Roots, V6	1.47 ± 0.81	0.52–3.27	0.20 ± 0.13	0.05–0.50
Roots, R1	0.69 ± 0.31	0.08–1.24	0.09 ± 0.04	0.02–0.15
Roots, R6	–	<LOD–0.61	–	<LOD–0.07
Roots, Sen.	–	<LOD–0.55	–	<LOD–0.07
Whole Plant, V6	4.60 ± 1.73	1.81–6.82	0.57 ± 0.20	0.19–0.80
Whole Plant, R1	1.69 ± 0.92	0.63–4.60	0.32 ± 0.18	0.11–0.91
Whole Plant, R6	–	<LOD–0.36	–	<LOD–0.14
Pollen, R1	–	–	–	–
Kernel, R6	–	<LOD–<LOQ[f]	–	<LOD–<LOQ
Kernel, Sen.	–	<LOD	–	<LOD

[a] *N* = 20 for all tissues except pollen, where *N* = 4
[b] SD = standard deviation
[c] not applicable, as one or more values were below either the LOD or LOQ for the
[d] – = assay LOD for PAT in leaves, whole plants, and kernels = 0.025 µg/g DW.
[e] Sen. = senescence
[f] LOQ for PAT in kernels = 0.031 µg/g DW

VIII.C. Identity and Characterization of PAT Protein in MZIR098 Corn

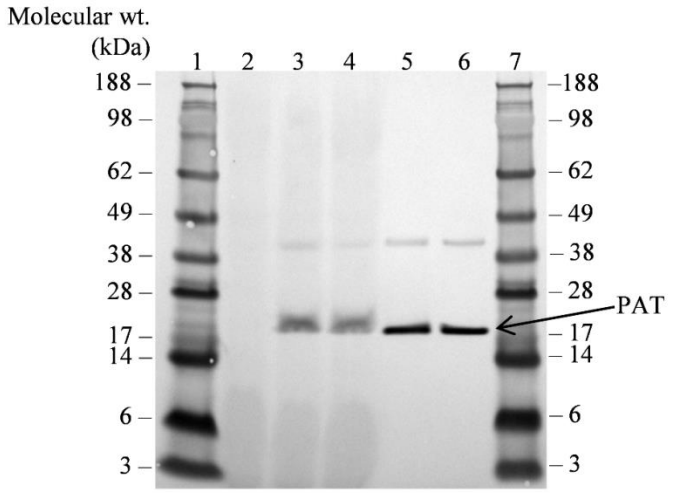
The identity of the PAT protein produced in MZIR098 corn was confirmed by peptide mass coverage analysis, apparent molecular weight, and immunoreactivity.

To conduct peptide mass coverage of PAT, the protein was extracted from MZIR098 corn, reduced, alkylated with iodoacetamide, and enzymatically digested separately with trypsin, chymotrypsin and endoproteinase Asp-N. The peptide mass coverage analysis verified 96% of the predicted amino acid sequence of PAT (Figure VIII–2). Western blot analysis demonstrated that PAT from MZIR098 corn cross-reacted with PAT-specific antibody, displaying an apparent molecular weight consistent with the predicted molecular weight of 20.5 kDa (Figure VIII–3).

1 *M***SPERRPVEIRPATAA***DMAAVC***DIVNHYIETSTVNFRTEP** 40
41 **QTPQEWIDDLERLQDRYPWLVAEVEGVVAGIAYAGPWKAR** 80
81 **NAY**DWTVESTVYVSHRHQRLGLGSTLYTHLLKSM**EAQGFK** 120
121 **SVVAVIGLPNDPSVRLHEALGYTARGTLRAAGYKHGGWHD** 160
161 **VGFWQRDFELPAPPRPVRPVTQI** 183

Legend: Trypsin-detected
Chymotrypsin-detected
Endoproteinase Asp-N-detected
Italic type indicates amino acids not identified

Figure VIII–2. Amino acid sequence sequence identified for PAT from MZIR098 corn by peptide mass coverage analysis



Lane 1: Molecular weight standard
Lane 2: Nontransgenic corn extract (165 µg of total protein)
Lane 3: Nontransgenic corn extract fortified with microbially produced PAT (10 ng of PAT, 165 µg of total protein)
Lane 4: MZIR098 corn extract (10 ng of PAT, 165 µg of total protein)
Lane 5: Purified PAT preparation from MZIR098 corn extract (10 ng of PAT)
Lane 6: Microbially produced PAT (10 ng of PAT)[a]
Lane 7: Molecular weight standard
[a]Microbially produced protein was used as a positive assay control.

Figure VIII–3. Western blot analysis of PAT from MZIR098 corn

VIII.D. Conclusions on the Safety of PAT in MZIR098 Corn

The safety of PAT proteins has been previously established. These safety conclusions are based on existing PAT safety data, summarized by Hérouet *et al.* (2005), ILSI (2011), OECD (1999), and submissions to U.S. and global regulatory authorities. Furthermore, PAT as produced in MZIR098 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 MZIR098 corn.

IX. Compositional Assessment of MZIR098 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 MZIR098 and conventional corn.

IX.A. Study Design and Methods

Compositional analyses of MZIR098 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 9 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 MZIR098 corn and the control material was nontransgenic, near-isogenic corn. 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. The test, control, and reference entries are listed in Table IX–1 and described in the breeding pedigree in Figure III–2.

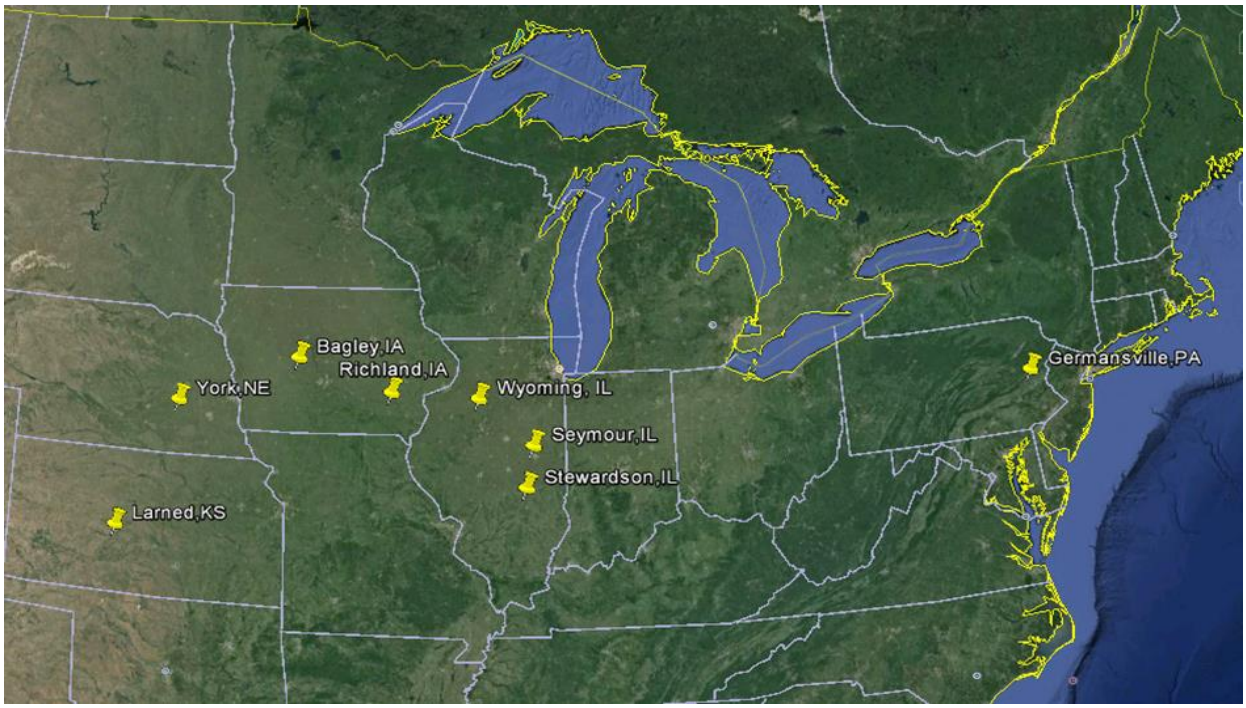
Table IX–1. Identification of test, control, and reference corn varieties

Seed description	Hybrid genotype
Nontransgenic, near-isogenic (control)	NP2391/NP2222
MZIR098 (test)	NP2391/NP2222(MZIR098)
Reference variety 1	H-7191
Reference variety 2	H-7540
Reference variety 3	SY Sincero
Reference variety 4	NK Lucius
Reference variety 5	NK Cisko
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 IX–2 and shown on a satellite view map in Figure IX–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 IX–2. These trials were planted under USDA APHIS notification 13-044-123n.



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

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

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

Location	Soil type	Previous crop	Planting date (2013)
Richland, Iowa	silty clay loam	soybean	June 4
York, Nebraska	silty clay loam	soybean	June 3
Seymour, Illinois	silty clay loam	corn	June 20
Bagley, Iowa	clay loam	field corn	June 13
Larned, Kansas	loam	sorghum	June 12
Stewardson, Illinois	silt loam	corn	June 10
Wyoming, Illinois	silt loam	corn	June 8
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), 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 IX–3 and IX–4.

Table IX–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 IX–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 D).

IX.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 (MZIR098 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 MZIR098 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).

IX.C. Compositional Analysis Results

Sections IX.C.1 and IX.C.2 describe the compositional analysis results for MZIR098 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 IX.D.

IX.C.1. Forage

Across-location statistics for proximate and mineral composition of corn forage are shown in Table IX–5. In statistical comparisons between MZIR098 corn and the nontransgenic control corn varieties, no significant differences were observed in the levels of moisture, protein, fat, ash, carbohydrates, ADF, NDF, calcium, or phosphorus.

In both MZIR098 corn and the nontransgenic, near-isogenic 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 IX–5. Proximate and mineral composition of forage from MZIR098 corn and nontransgenic corn

Data source	Statistic	Moisture	Protein	Fat	Ash	Carbohydrates	ADF	NDF	Calcium	Phosphorus
MZIR098	mean	68.7	7.17	1.90	4.00	86.9	25.7	43.8	1971	1731
	range	62.2-75.9	5.24-9.50	1.05-2.84	2.73-5.70	84.0-90.2	17.2-32.2	35.0-52.9	1240-2800	1290-2570
Control	mean	68.4	7.36	1.95	3.94	86.8	24.9	42.5	1861	1832
	range	65.5-74.8	5.61-9.10	1.14-2.81	3.03-5.26	84.1-89.8	20.0-32.2	33.9-49-.8	1300-2570	1200-2450
ANOVA (*t*-test) entry effect and SEM										
	P	0.649	0.206	0.626	0.608	0.386	0.264	0.311	0.176	0.052
	SEM	0.99	0.211	0.099	0.239	0.37	0.72	0.98	76	101
Reference varieties	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

MZIR098: *N* = 32.
Control: *N* = 32.
Reference varieties: *N* = 192.
ILSI: *N* is the number of ILSI values used to calculate the mean and excludes values < LOQ.
Proximate levels are shown as percent dry weight, except moisture, which is shown as percent fresh weight.
Calcium and phosphorus levels are shown as milligrams per kilogram dry weight.

IX.C.2. Grain

IX.C.2.a. Proximates, starch, minerals, and vitamins

Across-location statistics for proximate and starch components of corn grain are shown in Table IX–6. In statistical comparisons between MZIR098 corn and the nontransgenic, near-isogenic control corn, no significant differences were observed in the levels of any proximates or starch. Grain was dried in the field or mechanically after harvest, therefore, moisture levels were not subjected to ANOVA.

Across-location statistics for mineral components of corn grain are shown in Table IX–7. In statistical comparisons between MZIR098 corn and the nontransgenic, near-isogenic control corn, no significant differences were observed in the levels of iron, magnesium, manganese, phosphorus, or zinc. The levels of calcium, copper, and potassium were significantly higher in MZIR098 corn than in the control corn. For selenium and sodium, levels below the LOQ precluded calculation of the means and statistical comparisons across locations.

Across-location statistics for vitamin components of corn grain are shown in Table IX–8. In statistical comparisons between MZIR098 corn and the nontransgenic, near-isogenic control corn, no significant differences were observed in the levels of vitamins B_1, B_2, B_3, B_6, B_9, or vitamin E. The level of vitamin A was significantly higher in MZIR098 corn than in the control corn.

In both MZIR098 corn and the nontransgenic, near-isogenic control corn, the mean levels of all proximates, starch, quantifiable minerals, and vitamins were within the ranges for the reference varieties and the ranges reported in the ILSI database.

Table IX–6. Proximate and starch composition of grain from MZIR098 corn and nontransgenic corn

Data source	Statistic	Moisture[a]	Protein	Fat	Ash	Carbohydrates	ADF	NDF	TDF	Starch
MZIR098	mean	12.9	10.3	3.96	1.43	84.3	3.98	11.3	16.4	65.5
	range	8.59-18.8	8.58-13.1	3.33-4.69	1.16-1.73	81.8-86.2	3.20-4.72	9.91-12.4	14.3-19.9	56.0-76.6
Control	mean	12.8	10.4	3.93	1.42	84.3	4.06	11.1	16.3	65.8
	range	9.22-17.6	8.46-13.9	3.22-4.76	1.25-1.69	81.3-86.6	3.12–4.88	9.58-12.8	14.0-20.1	58.1-75.0
ANOVA (*t*-test) entry effect and SEM										
	P	–	0.416	0.654	0.974	0.618	0.294	0.223	0.726	0.657
	SEM	–	0.32	0.098	0.035	0.34	0.100	0.17	0.36	0.75
Reference varieties	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

MZIR098: *N* = 32.
Control: *N* = 32.
Reference varieties: *N* = 192.
Proximate and starch levels are shown as percent dry weight, except moisture, which is shown as percent fresh weight.
[a]Grain was dried in the field or mechanically after harvest, therefore, moisture levels were not subjected to analysis of variance (ANOVA).

Table IX–7. Mineral composition of grain from MZIR098 corn and nontransgenic corn

Data source	Statistic	Ca	Cu	Fe	Mg	Mn	P	K	Se[a]	Na[b]	Zn
MZIR098	Mean	37.8	2.02	19.9	1178	5.98	3030	3680	–	–	22.2
	Range	26.1-51.3	1.47-3.49	16.2-23.2	1030-1350	3.86-9.29	2450-3600	3190-4040	<LOQ–0.667	<LOQ	17.3-53.2
Control	Mean	35.5	1.90	19.7	1176	5.93	2989	3549	–	–	20.4
	Range	23.6-50.1	1.32-2.57	16.9-28.0	994–1360	3.34-10.4	2540-3620	3220-3930	<LOQ–0.582	<LOQ	15.7-24.4
ANOVA (*t*-test) entry effect and SEM											
	P	***0.023***	***0.013***	0.655	0.926	0.717	0.314	***<0.001***	–	–	0.105
	SEM	2.28	0.131	0.35	25	0.586	82	59	–	–	0.92
Reference varieties	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

MZIR098: *N* = 32.
Control: *N* = 32.
Reference varieties: *N* = 192.
ILSI: *N* is the number of ILSI values used to calculate the mean and excludes values < LOQ.
Mineral levels are 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 IX–8. Vitamin composition of grain from MZIR098 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)
MZIR098	Mean	0.154	0.380	0.206	2.11	0.557	0.0440	0.0124
	Range	0.125-0.175	0.275-0.504	0.131-0.336	1.74-2.53	0.372-0.642	0.0322-0.0584	0.00887-0.0156
Control	Mean	0.145	0.374	0.217	2.09	0.563	0.0441	0.0121
	Range	0.105-0.190	0.305-0.458	0.132-0.346	1.76-2.42	0.410-0.698	0.0355-0.0602	0.00814-0.0155
ANOVA (*t*-test) entry effect and SEM								
	P	***0.037***	0.359	0.437	0.453	0.580	0.940	0.286
	SEM	0.0045	0.0130	0.0115	0.044	0.0161	0.00233	0.00066
Reference varieties	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

MZIR098: *N* = 32.
Control: *N* = 32.
Reference varieties: *N* = 192.
ILSI: *N* is the number of ILSI values used to calculate the mean and excludes values < LOQ.
Vitamin levels are 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]Original units of mg/100 g were converted to mg/g.

IX.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 IX–9. In statistical comparisons between MZIR098 corn and the nontransgenic, near-isogenic control corn, no significant differences were observed in the levels of 17 amino acids. The levels of lysine were significantly lower in MZIR098 corn than in the control corn.

The across-location statistics for 10 quantifiable fatty acids in corn grain are shown in Table IX–10. In statistical comparisons between MZIR098 corn and the nontransgenic, near-isogenic control corn, no significant differences were observed in the proportions of 16:0 palmitic, 16:1 palmitoleic, 18:3 linolenic, 20:1 eicosenoic, or 22:0 behenic acid. The proportions of 17:0 heptadecanoic and 18:2 linoleic acid were significantly higher in MZIR098 corn than in the control corn, and the proportions of 18:0 stearic, 18:1 oleic, and 20:0 arachidic were significantly lower.

Twelve fatty acids analyzed had levels below the LOQ in all replicates at all locations 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 γ-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 IX–11. In statistical comparisons between MZIR098 corn and the nontransgenic, near-isogenic control corn, no significant differences were observed in the levels of *p*-coumaric acid, ferulic acid, inositol, phytic acid, raffinose or trypsin inhibitor. For furfural, levels below the LOQ precluded calculation of the means and statistical comparisons across locations.

In both MZIR098 corn and the nontransgenic control corn, the mean levels of all amino acids, quantifiable fatty acids, quantifiable secondary metabolites, and anti-nutrients, except ferulic acid, were within the ranges for the reference varieties and the ranges reported in the ILSI database. In both MZIR098 corn and the nontransgenic control corn, the mean levels of ferulic acid were within the ranges reported in the ILSI database.

Table IX–9. Amino acid composition of grain from MZIR098 corn and nontransgenic corn

Data source	Statistic	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Cys	Val
MZIR098	mean	6.57	3.54	4.65	19.0	8.91	3.76	7.85	2.03	4.61
	range	5.70-8.30	3.02-4.37	3.87-6.16	15.8-25.7	7.75-11.2	3.17-4.35	6.50-10.2	1.71–2.44	3.84-5.81
Control	mean	6.66	3.57	4.70	19.1	8.97	3.78	7.91	2.03	4.63
	range	5.52-9.19	2.99-4.93	3.72-6.58	15.1-29.4	7.16-12.6	3.14-4.55	6.17-11.8	1.59-2.46	3.67-6.42
ANOVA (*t*-test) entry effect and SEM										
	P	0.277	0.370	0.470	0.689	0.615	0.637	0.619	0.981	0.857
	SEM	0.195	0.107	0.155	0.75	0.277	0.085	0.297	0.048	0.140
Reference varieties	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**
MZIR098	mean	2.14	3.55	12.8	4.01	5.13	2.89	2.58	4.90	0.843
	range	1.45-2.55	3.08–4.66	10.6-17.4	3.518-5.02	4.38-6.73	2.19-3.26	2.04-3.08	3.84-5.87	0.716-0.930
Control	mean	2.16	3.55	12.9	4.02	5.17	2.97	2.56	4.91	0.839
	range	1.73–2.84	2.81-5.31	10.1–20.9	3.29-5.90	4.09-8.01	2.43-3.41	2.16-3.27	3.83-6.09	0.690-.965
ANOVA (*t*-test) entry effect and SEM										
	P	0.602	0.982	0.670	0.829	0.595	***0.044***	0.596	0.767	0.704
	SEM	0.066	0.131	0.56	0.148	0.206	0.051	0.067	0.138	0.0202
Reference varieties	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

MZIR098: *N* = 32.
Control: *N* = 32.
Reference varieties: *N* = 192.
ILSI: *N* is the number of ILSI values used to calculate the mean and excludes values <LOQ.
Amino acid levels are shown as milligrams per gram dry weight.
Results significantly different ($p < 0.05$) are shown in bold italic type.

Table IX–10. Fatty acid composition of grain from MZIR098 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
MZIR098	mean	14.2	0.131	0.0842	2.10	26.3	54.6	1.78	0.421	0.227	0.174
	range	13.7-15.0	0.104-0.149	0.0745-0.0947	1.75–2.39	22.6-28.7	52.3-58.6	1.67-1.92	0.358-0.468	0.201–0.244	0.134–0.202
Control	mean	14.2	0.131	0.0821	2.13	27.1	53.8	1.76	0.427	0.228	0.174
	range	13.8-14.7	0.106-0.157	0.0730-0.0913	1.73-2.44	23.5-29.2	51.7-58.0	1.65-1.85	0.353-0.477	0.203–0.253	0.134–0.206
ANOVA (*t*-test) entry effect and SEM											
	P	0.493	0.686	***0.010***	***0.038***	***<0.001***	***<0.001***	0.219	***0.029***	0.626	0.921
	SEM	0.08	0.0034	0.00199	0.058	0.63	0.63	0.017	0.0107	0.0039	0.0058
Reference varieties	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

MZIR098: *N* = 32.
Control: *N* = 32.
Reference lines: *N* = 192.
ILSI: *N* is the number of ILSI values used to calculate the mean and excludes values <LOQ.
Fatty acids are 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 γ-linolenic, 20:2 eicosadienoic, 20:3 eicosatrienoic, and 20:4 arachidonic fatty acids.

Table IX–11. Secondary metabolite and anti-nutrient composition of grain from MZIR098 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)
MZIR098	mean	304	3357	–	2505	0.859	0.111	3.94
	range	220-346	2930-3820	<LOQ	1850-3540	0.643-1.13	<LOQ–0.196	2.49-5.38
Control	mean	300	3357	–	2520	0.885	0.105	4.03
	range	220-364	3030-3970	<LOQ	1900-3470	0.707-1.12	<LOQ–0.169	2.90-4.90
ANOVA (*t*-test) entry effect and SEM								
	P	0.445	0.987	–	0.852	0.282	0.076	0.580
	SEM	9.2	62	–	74	0.0358	0.0130	0.123
Reference varieties	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

MZIR098: *N* = 32.
Control: *N* = 32.
Reference varieties: *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 units (TIU). All are expressed on a dry weight basis.
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.

IX.D. Conclusions from Compositional Analyses

In the compositional assessment of MZIR098 corn forage and grain, 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. However, the levels of ferulic acid did not differ significantly between the MZIR098 and nontransgenic control corn. The across-location mean levels of all components of MZIR098 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 MZIR098 and near-isogenic, nontransgenic control corn, and that those levels that did differ fell within ranges considered to be normal for conventional corn.

X. Safety and Nutritional Assessment of MZIR098 Corn and Derived Corn Products

The safety of MZIR098 corn and its nutritional comparability to conventional, nontransgenic corn were assessed through consideration of the compositional assessment described in Section IX, the safety assessments of the eCry3.1Ab, mCry3A, and PAT proteins described in Sections VI through VIII, and the history of safe use of the eCry3.1Ab, mCry3A, and PAT proteins referenced previously.

X.A. Nutritional Assessment of MZIR098 Corn

As discussed in Section IX, analyses of key nutritional components of forage and grain from MZIR098 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 MZIR098 corn were evident. Grain and forage from MZIR098 corn were found to be similar in composition to those same materials from conventional corn. Additionally, MZIR098 corn exhibited a compositional 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 MZIR098 corn and corn products processed from raw MZIR098 corn are nutritionally and compositionally comparable to raw and processed corn from conventional varieties, and that MZIR098 corn is expected to provide adequate nutrition as part of human diets as well as formulated diets delivered to growing livestock.

X.B. Safety Assessment of MZIR098 Corn

As discussed in Sections VI through VIII, the eCry3.1Ab, mCry3A, and PAT proteins have specific, well-understood modes of action, and are identical to proteins in commercially available crop products to which humans and animals are exposed daily without concern. eCry3.1Ab, mCry3A, and PAT have been safely used and consumed in commercial transgenic crops and have permanent U.S. EPA tolerance exemptions established in the U.S. Previous evaluations of eCry3.1Ab, mCry3A, and PAT have shown they do not share

significant amino acid similarity to known toxins and are unlikely to be human allergens. Kernels from MZIR098 corn are the most likely tissue to enter the food supply, either as grain or grain by-products. Humans would potentially consume corn kernels at the senescence stage of development, whereas livestock would be more likely to consume the kernels at maturity (R6 stage). The average eCry3.1Ab concentration measured in kernels from MZIR098 corn was 2.08 µg/g dry weight at senescence and 2.42 µg/g dry weight at maturity (Table VI–1). The mCry3A concentration measured in kernels from MZIR098 corn was 11.21 µg/g dry weight at senescence and 14.59 µg/g dry weight at maturity (Table VII–1). The PAT concentration measured in kernels from MZIR098 corn was less than the LOD for the assay at senescence and ranged from less than the LOD (0.025 µg/g dry weight) to less than the LOQ (0.031 µg/g dry weight) for the assay at maturity on a dry weight basis (Table VIII–1). The above-cited concentrations do not account for protein degradation that would occur during processing of corn grain or storage under ambient conditions.

The data and information presented in this document support the conclusions that MZIR098 corn is compositionally and nutritionally comparable to and as safe as conventional corn, and that no adverse health effects will result from exposure to the eCry3.1Ab, mCry3A, and PAT proteins present in MZIR098 corn.

XI. Phenotypic and Agronomic Characteristics

Field and growth-chamber studies were conducted to determine whether reproductive, growth, or survival characteristics of MZIR098 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 MZIR098 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 reference varieties. The phenotypic characteristics evaluated and the metrics employed are shown in Table XI–1.

Table XI–1. Phenotypic characteristics evaluated for MZIR098 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 are 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).

XI.A. Seed Germination and Dormancy

Enhanced germination or seed dormancy are characteristics that can be indicative of plant pest potential. Dormancy mechanisms serve to distribute seed germination across multiple growing seasons. Primary dormancy is conferred by physical traits, such as hard seeds, or physiological traits of the seed 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 MZIR098 corn seed using a modification of the testing protocol established by the Association of Official Seed Analysts (AOSA).

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

Seed samples of MZIR098 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 MZIR098 corn seed materials. Table XI–2 presents the descriptions and genotypes of the test, control, and reference materials.

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

Seed description	Hybrid genotype
Nontransgenic, near-isogenic corn (control)	NP2391/NP2222
MZIR098 corn (test)	NP2391/NP2222(MZIR098)
Corn reference variety 1	NK Octet
Corn reference variety 2	NK Lucius
Corn reference variety 3	NK Cisko

XI.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 XI–3.

Table XI–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 XI–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.

Table XI–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).

The seed or seedling samples subjected to the AOSA-specified temperature regimes (25°C and 20°C/30°C) were examined at Days 4 and 7. The seeds subjected to the additional non-AOSA-specified temperature regimes (10°C, 30°C, 10°C/20°C, and 10°C/30°C) were examined at Days 4, 7, and 12. At each 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). Each seed or seedling examined was categorized as described in Table XI–4.

XI.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).

XI.A.4. Results

The results of these seed germination and dormancy experiments are summarized in Table XI–5. For the MZIR098 corn and nontransgenic control corn seed, 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 98.3% to 99.5% for MZIR098 corn seed and 98.0% to 99.8% for the corresponding nontransgenic control corn seed (Table XI–5). Under the same temperature regimes, germination ranged from 58% to 100% across all replicates of the three reference varieties. In comparisons between the test and control seed germination rates at the various temperature regimes, no statistically significant differences were observed.

Data for abnormal germinated seedlings were only collected in the 25ºC regime and 20ºC/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 MZIR098 and the control did not result in statistically significant differences for any of the temperature regimes and the mean percent

dead seed was 1.5% or less; however, one replicate of the reference hybrid had 11% dead seed.

Among the test and control seed, only one test seed in one replicate at the 10ºC temperature regime was classified as dormant. No seeds were classified as "hard" for the test, control, or reference varieties.

The observation that MZIR098 corn seed did not show increased seed germination compared to control corn seed supports the conclusion that MZIR098 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 MZIR098 corn seed 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 MZIR098 corn demonstrates no increase in seed dormancy potential.

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

Temperature Regime (°C)	Variety	Normal Germinated	Abnormal Germinated	Dead	Dormant	Hard
10	MZIR098	99.0	–	1.0	0.3	0.0
	Control	99.5	–	0.5	0.0	0.0
	ANOVA *p* value	0.303		0.481	0.979	
	Reference Range	58–99	–	0–11	0–40	0
25	MZIR098	99.5	0	0.5	0.0	0.0
	Control	99.3	0	0.8	0.0	0.0
	ANOVA *p* value	0.644	–	0.664		
	Reference Range	97–100	0–2	0–2	0	0
30	MZIR098	98.5	–	1.5	0.0	0.0
	Control	99.8	–	0.3	0.0	0.0
	ANOVA *p* value	0.121		0.121		
	Reference Range	99–100	–	0–1	0	0
10/20	MZIR098	99.0	–	1.0	0.0	0.0
	Control	99.0	–	1.0	0.0	0.0
	ANOVA *p* value	1.000		1.000		
	Reference Range	96–100	–	0–4	0–1	0
10/30	MZIR098	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
20/30	MZIR098	98.3	0.3	1.5	0.0	0.0
	Control	98.0	0.5	1.5	0.0	0.0
	ANOVA *p* value	0.811	0.672	1.000		
	Reference Range	96–100	0–2	0–2	0–1	0

N = 400 except for MZIR098 seed at the 10°C temperature regime, for which *N* = 401. *N* = 1200 for combined reference variety data.

XI.B. Pollen Viability and Morphology

As a measure of potentially enhanced reproductive capability, pollen cell viability and morphology were compared between MZIR098 corn, the nontransgenic, near-isogenic control corn, and three commercial reference varieties of hybrid corn.

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

Table XI–6 presents the descriptions and genotypes for the test, control, and reference corn varieties grown. Figure III–2 shows the breeding pedigree of MZIR098 corn seed material used in this study. The reference varieties were three commercially available, nontransgenic corn hybrids.

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

Seed Identification	Hybrid genotype
Nontransgenic, near-isogenic corn (control)	NP2391/NP2222
MZIR098 corn (test)	NP2391/NP2222(MZIR098)
Corn reference variety 1	NK Octet
Corn reference variety 2	NK Lucius
Corn reference variety 3	NK Cisko

XI.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 XI–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 pollen collection. Pollen was collected separately from each of the plants. The test and control plants were sampled on the same day, when the plants reached the tasseling (VT) growth stage. 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 80X 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, Nikon NIS-Elements, BR ver. 3.01 program. 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.

XI.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: MZIR098 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: MZIR098 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.

XI.B.4. Results

The results of ANOVA for pollen characteristics evaluated for MZIR098 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 XI–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 XI–7. Corn pollen viability and diameter

	Viability (%)[a]			Diameter (µm)		
Entry	**Mean/Range**	**SEM[b]**	***P***	**Mean/Range**	**SEM**	***P***
MZIR098	99.2	0.16, 0.15	0.848	87.4	0.34	0.866
Control	99.1			87.4		
Reference varieties	99.0–99.5			80.9–87.4		

MZIR098: *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.

XI.C. Field Agronomic Trials

Field trials were conducted using conventional agronomic practices to plant, maintain and harvest replicate plots at eight locations in 2013 across the U.S., comparing MZIR098 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.

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

Table XI–8 presents the descriptions and genotypes for the test, control, and reference corn varieties grown. Figure III–2 shows the breeding pedigree of the MZIR098 seed material used in this study. The reference varieties were six commercially available, nontransgenic corn hybrids.

Table XI–8. Plant material used in field agronomic trials

Seed Identification	Hybrid genotype
Nontransgenic, near-isogenic corn (control)	NP2391/NP2222
MZIR098 corn (test)	NP2391/NP2222(MZIR098)
Corn reference variety 1	H-7191
Corn reference variety 2	H-7540
Corn reference variety 3	SY Generoso
Corn reference variety 4	NK Lucius
Corn reference variety 5	NK Cisko
Corn reference variety 6	SY Provial

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

One entry of MZIR098 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 XI–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 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. Collectively, these observations were used to identify potential differences in susceptibility between MZIR098 corn and the nontransgenic control corn to natural environment stressors.

XI.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 MZIR098 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.

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

Agronomic characteristics of MZIR098 corn were compared non-statistically to those in the nontransgenic corn reference varieties.

XI.C.5. Results

The data for quantitative agronomic assessments of MZIR098 corn that were suitable for statistical analysis are summarized in Table XI–9. Quantitative data that were not suitable for statistical analysis are presented in Table XI–10.

No significant differences were observed between MZIR098 corn and the nontransgenic control corn for any of the agronomic characteristics analyzed statistically (Table XI–9). Among the characteristics that were not suitable for statistical comparison, the mean values were similar for MZIR098 corn and the control corn (Table XI–10).

Qualitative observations of naturally occurring ecological stressors on the MZIR098 corn and control plots at each location are summarized in Table XI–11. The occurrence and severity of insects, diseases, and damage from abiotic stressors (e.g., wind) were similar between and the MZIR098 corn and control corn plots. Thus, MZIR098 corn did not appear to be more susceptible to ecological stressors than control corn.

The results of these phenotypic assessments indicate that MZIR098 corn is not phenotypically different from conventional corn with respect to characteristics that would increase its weediness potential. Additionally, no deficits in agronomic performance of MZIR098 corn were observed.

Table XI–9. Agronomic characteristics: Across-location comparison (ANOVA) of MZIR098 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)
MZIR098	mean	74.9	89.2	233	46.7	18.7	67.3	10.1	10.9
	range	65–81	52.7–108	152–273	5–75	14.3-–27.0	54.0–82.1	6.28–13.7	6.76–14.8
Control	mean	73.8	90.0	234	49.5	19.0	66.8	9.92	10.7
	range	58–80	54.4–110	145–272	5–85	14.8–27.3	56.4–78.7	6.55–14.3	7.05–15.4
ANOVA (*t*-test) entry effect and standard error of means (SEM)									
	p	0.15	0.477	0.778	0.330	0.54	0.498	0.555	0.555
	SEM	1.39	4.83	10.45	7.31	1.04	2.13	0.696	0.75
Reference varieties	mean	74.6	99.7	224	37.5	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

MZIR098: *N* = 32.

Control: *N* = 32.

Reference varieties: *N* = 192.

[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 XI–10. Agronomic characteristics: Across-location comparison of MZIR098 (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
MZIR098	mean	64.1	61.6	5.75	58.3	58.3	1.06	7.03	0.0625
	range	60–69	49–69	4–8	52–64	52–63	0–16	0–70	0–1
Control	mean	64.1	61.7	5.97	58.3	58.3	0.688	4.66	0.0313
	range	60–69	51–69	4–9	52–64	52–63	0–8	0–34	0–1
Reference varieties	mean	64.1	62.2	6.68	55.8	55.8	0.776	3.58	0.0885
	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 being appropriate for statistical comparison; consequently, analysis of variance was not applied and only the means and ranges are shown.

MZIR098: *N* = 32.

Control: *N* = 32.

Reference varieties: *N* = 192.

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

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

Table XI–11. Qualitative observations of plot interactions with biotic and abiotic stressors

Location	Test	Control
Richland, Iowa	R2: minimal common rust. 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.
York, Nebraska	no insect, disease or abiotic stressors	no insect, disease or abiotic stressors
Seymour, Ilinois	V17: minimal damage from corn borer and root worm beetle; minimal damage from corn rust and gray leaf spot. R3: minimal damage from grasshopper and rust.	V3: minimal damage from wind. V17: minimal damage from corn rust and gray leaf spot. R3: minimal damage from grasshopper and root worm beetle.
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.
Larned, Kansas	V2: minimal damage from Stewart's Disease and Twisted Whorl Syndrome; minimal damage from wind. V10: minimal damage from grasshoppers; minimal common smut and gray leaf spot; minimal wind and heat damage. R5: minimal damage from grasshoppers and corn ear worm; minimal wind damage. R6: mild damage from corn ear worm; minimal damage from gray leaf spot, common rust and common smut; minimal damage from wind.	V2: minimal damage from Stewart's Disease and Twisted Whorl Syndrome; mild damage from wind and minimal damage from nutrient deficiency. V10: minimal damage from grasshoppers; minimal damage from common rust and 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	Test	Control
Stewardson, Illinois	V9–V10: mild damage from armyworm, grasshoppers and rust. R1–R2: minimal damage from grasshoppers; mild rust. R5: minimal to mild damage from earworms and corn borer; minimal to mild gray leaf spot and rust; minimal nutrient damage; moderate wind damage. R6: mild damage from earworms and corn borer; moderate stalk rot; mild wind damage.	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.
Wyoming, Illinois	V11: minimal damage from Japanese beetle and common rust. R1–R2: minimal damage from Japanese beetle and corn rootworm beetle; minimal rust and gray leaf spot. R4–R5: minimal corn earworm; minimalrust and gray leaf spot; minimal drought stress. R5–R6: minimal common 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.
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).

XI.D. Conclusions from Phenotypic, Agronomic, and Environmental Interactions Assessments of MZIR098 Corn

The results of laboratory and field studies indicate that, apart from the intended phenotype of corn rootworm resistance and tolerance to herbicides containing glufosinate-ammonium (see Section XIII and Appendix E), MZIR098 corn is no different than conventional corn with regard to phenotypic and agronomic properties that bear on weediness potential.

XII. Potential Environmental Effects of MZIR098 Corn Cultivation

The environmental impact of MZIR098 corn cultivation is considered in the context of potential harm to nontarget organisms (NTOs), including species beneficial to agriculture, and the potential for the cultivar to become a weed.

XII.A. Assessments of the Effects of eCry3.1Ab and mCry3A on Nontarget Organisms

The transgenes *ecry3.1Ab* and *mcry3A* in MZIR098 corn encode protein sequences identical to the eCry3.1Ab and mCry3A proteins produced in 5307 corn and MIR604 corn, respectively. Cultivation of 5307 corn and MIR604 corn separately and in combination has been determined to pose negligible environmental risk, because it is unlikely that the insecticidal proteins produced during the cultivation of these crops will have harmful effects on NTOs (Burns and Raybould 2014; U.S. EPA 2012a; Raybould *et al*. 2007; U.S. EPA 2010b). As assessments of the risk to nontarget species have been performed for the eCry3.1Ab and mCry3A proteins expressed in 5307 and MIR604 corn, and because the eCry3.1Ab and mCry3A proteins expressed in 5307 and MIR604 corn are the same as those expressed in MZIR098 corn, the environmental assessments for 5307 and MIR604 corn inform the assessment of MZIR098 corn. Although MZIR098 corn produces the same eCry3.1Ab and mCry3A proteins produced in 5307 corn and MIR604 corn, it is a different transformation event with different transgenic protein concentrations and expression patterns. This analysis, therefore, used the data for eCry3.1Ab and mCry3A concentrations in the tissues of MZIR098 corn plants (Tables VI–1 and VII–1) to update the environmental safety assessments of these proteins.

XII.A.1. Methodology for Determining Exposure Estimates and Margins of Exposure

The effects of the insecticidal proteins on NTOs were determined in laboratory studies that exposed representative surrogate NTOs to purified Cry protein. The highest concentration of insecticidal protein that has no observed adverse effect on a particular surrogate NTO is referred to as the no-observed-adverse-effect concentration (NOAEC) for that species.

The concentration of insecticidal protein to which the taxonomic or functional group of organisms represented by that surrogate is likely to be exposed via cultivation of the crop producing that protein is referred to as the estimated environmental concentration (EEC). EECs are usually estimated using the worst-case assumption that the organisms consume a diet comprising solely of transgenic crop tissue containing the proteins of interest. The diets of non-pest organisms are unlikely to comprise crop tissue only; therefore, refinements of the worst-case EEC may be made using conservative assumptions about the dilution of the insecticidal protein in the diets of NTOs in the field (e.g., Raybould *et al.* 2007; Raybould and Vlachos 2011).

The ratio of NOAEC/EEC is the margin of exposure, and if the margin of exposure for a certain protein and surrogate species is one or greater, the risk to the functional group represented by the surrogate from cultivation of the crop producing that protein may be deemed negligible. If a sufficiently representative set of surrogate species is determined to have margins of exposure of one or greater, the risk to all NTOs posed by cultivation of the transgenic crop may be deemed negligible with sufficient confidence for decision-making.

The comparison of the no observable adverse effects concentration or level (NOAEC or NOAEL) to the EEC or the daily dietary dose (DDD) reflects the margin of exposure (MoE) in the effect study, as discussed in Raybould *et al*. 2007 and Crocker *et al*. 2002.

$$\frac{\text{NOAEC}}{\text{EEC}} = \text{MoE}$$

$$\frac{\text{NOAEL}}{\text{DDD}} = \text{MoE}$$

The comparison of the hazard data (determined from the laboratory studies of effects) and exposure data estimates the confidence (i.e., high margin of exposure = high confidence) that the absence of observable adverse effects in the effect studies is predictive of the safety of eCry3.1Ab and mCry3A in MZIR098 corn to NTOs in general.

XII.A.2. Comparisons of Test Concentrations and Exposure Estimates

Exposure estimates for MZIR098 corn were calculated as in Raybould *et al.* 2007 and compared to the NOAECs from laboratory studies exposing NTOs to either eCry3.1Ab or mCry3A. The test concentrations for existing eCry3.1Ab and mCry3A NTO laboratory studies were selected to exceed the EEC calculated for cultivation of 5307 and MIR604 corn. Based on this comparison of the EECs for MZIR098 cultivation and the NOAECs from the laboratory studies, a single NTO effects test, an evaluation for potential effects on a lady beetle indicator species was selected to assess the risk to foliar arthropods that may consume MZIR098 corn pollen. The other eCry3.1Ab and mCry3A laboratory studies, with the exception of the effects test of mCry3A on honey bee, had test concentrations that exceeded the EECs for MZIR098 corn cultivation and therefore are a strong test of the hypothesis of no effects on NTOs. Further exposure refinements were applied to estimate honey bee exposure to mCry3A; the NOAEC exceeded the conservative EEC, resulting in a margin of exposure of greater than one, indicating that honey bees would not be impacted under the worst case exposure scenario. Aquatic exposure to eCry3.1Ab and mCry3A from the cultivation of MZIR098 corn is negligible; therefore, aquatic effects tests are not presented.

The concentrations of eCry3.1Ab and mCry3A measured in relevant MZIR098 corn tissues (Tables VI–1 and VII–1) were used to determine the EECs of eCry3.1Ab and mCry3A for groups of organisms potentially exposed via cultivation of MZIR098 corn. Two sets of EECs for NTOs were calculated: worst-case and conservative EECs. The worst-case EECs were taken as the highest mean concentration of eCry3.1Ab and mCry3A measured in the relevant tissue type from a single field trial location. Worst-case EECs were refined via conservative assumptions of the dilution of eCry3.1Ab and mCry3A in prey, in soil, or by other means to calculate conservative EECs.

All of the relevant NTO effects tests presented in Tables XII–1 and XII–2 have been previously reviewed by USDA APHIS except for the mCry3A *Coleomegilla maculata* lady beetle study. No adverse effects were observed in a previous laboratory study in which

Coccinella septempunctata, another lady beetle species, was exposed to mCry3A; however, that study did not achieve margins of exposure of greater than one when assuming exposure to the mCry3A concentration measured in MZIR098 corn pollen. A second study, with *Coleomegilla maculata,* was conducted in which the test group was exposed to a more realistic but still highly conservative EEC determined for MZIR098 corn.

XII.A.2.a. mCry3A Coleomegilla maculata study

Four- to five-day-old lady beetle larvae (*Coleomegilla maculata*) were exposed to mCry3A for 21 days. The mCry3A protein was incorporated into a diet containing bee pollen and *Ephestia* eggs at 500 µg mCry3A/g diet. The negative control diet for this treatment contained the bee pollen and *Ephestia* eggs without the addition of mCry3A. The survival and body weight of the organisms in the test group were compared with those in the negative control group. No statistically significant reductions in survival or body weight were observed. Analyses of the test diets measured the concentration of mCry3A in the diet using ELISA, examined the intactness of mCry3A by western blot analysis, and examined the biological activity of mCry3A with feeding assays using a sensitive insect. The results of the diet analyses suggest that the *Coleomegilla maculata* larvae in the test group were exposed to the nominal concentration (500 µg mCry3A/g diet).

Coleomegilla maculata was tested as a representative foliage-dwelling nontarget arthropod. Lady beetles are reported to consume pollen and EECs of mCry3A via cultivation of MZIR098 corn are highest via this route of exposure. Therefore, the worst-case estimate of exposure for above-ground nontarget arthropods that may consume pollen is 246.96 µg mCry3A/g pollen (Table XII–4), the highest mean concentration of mCry3A measured at any location (Table VII–1). *Coleomegilla maculata* larvae consume pollen more frequently than adults and they tend to consume pollen more frequently than prey during anthesis (Lundgren 2009). At certain times, maize pollen may comprise up to 50% of a ladybird beetle larva's diet (US EPA 2010b). Therefore, a conservative estimate of exposure would be 187.75 µg mCry3A/g pollen × 0.5= 93.88 µg mCry3A/g diet. Based on these data, 500 µg mCry3A/g diet represents a margin of exposure of 2.0 under worst-case conditions and 5.3 under conservative conditions. Thus, the data suggest that cultivation of MZIR098 corn will pose negligible risk to above-ground nontarget arthropods.

XII.A.2.b. mCry3A exposure refinements for honey bee

Honey bees could potentially forage for corn pollen (Severson and Perry 1981) and, therefore, could be exposed to eCry3.1Ab and mCry3A via MZIR098 pollen. Honey bees can successfully rear young on a diet of 100% corn pollen; however, it is unlikely that corn pollen regularly comprises more than 50% of the pollen in a hive (Babendreier *et al.* 2004), and that pollen comprises no more than 16.7% of a bee's diet (U.S. EPA 2014). Assuming a diet of 100% MZIR098 pollen at the highest mean concentration of mCry3A measured at any location (Table VII–1), the worst-case EEC for pollinators is 246.96 µg mCry3A/g pollen (Table XII–4). A conservative EEC for honey bees utilizing the mean concentration of mCry3A in pollen (Table VII–1) is 0.5 × 0.167 × 187.75 µg mCry3A/g fresh weight = 15.68 µg mCry3A/g diet. The NOAEC in the honey bee effects test was 50 µg mCry3A/g diet,

resulting in a margin of exposure of 3.2 under conservative conditions. Thus, the cultivation of MZIR098 corn will pose negligible risk to pollinators.

XII.A.3. Aquatic Exposure Estimates

The August 2002 EPA SAP report (U.S. EPA 2002) recommended that nontarget testing should be focused on species exposed to the crop being cultivated. Though aquatic habitats may be located near agricultural areas, the EPA concluded that exposure of aquatic organisms to transgenic crops is limited temporally and spatially and that the potential exposure of aquatic organisms is therefore low to negligible (U.S. EPA 2010c). Since there is no meaningful ecologically-relevant exposure to aquatic organisms from corn, other than through purposeful feeding of processed corn products, effects tests on aquatic species are not relevant for corn products developed via agricultural biotechnology. Additionally, based upon the narrow spectrum of activity for eCry3.1Ab and mCry3A the likelihood of adverse effects to aquatic organisms from cultivation of MZIR098 corn is extremely low.

Tables XII–1 and XII–2 show a summary of the relevant species tested, the group represented, and the NOAEC or NOAEL. All studies, with the exception of the mCry3A *Coleomegilla maculata* lady beetle study, have been previously evaluated by USDA APHIS.

Table XII–1. Summary of the results of ecotoxicology studies on eCry3.1Ab

Test species	Common name	Group represented	NOAEC or NOAEL
Coleomegilla maculata	Pink-spotted lady beetle	Foliar nontarget arthropods	353 µg eCry3.1Ab/g diet
Orius laevigatus	Flower bug	Foliar nontarget arthropods	400 µg eCry3.1Ab/g diet
Aleochara bilineata	Rove beetle	Soil-dwelling invertebrates	400 µg eCry3.1Ab/g diet
Poecilus cupreus	Carabid beetle	Soil-dwelling invertebrates	400 µg eCry3.1Ab/g diet for survival <400µg eCry3.1Ab/g diet for growth
Eisenia fetida	Earthworm	Soil-dwelling invertebrates	4.06 µg eCry3.1Ab/g soil
Apis mellifera	Honey bee	Pollinators	50 µg eCry3.1Ab/g diet
Colinus virginianus	Bobwhite quail	Wild birds	900 mg eCry3.1Ab/kg bw
Mus musculus	Mouse	Wild mammals	2000 mg eCry3.1Ab/kg bw

Table XII–2. Summary of the results of ecotoxicology studies on mCry3A

Test species	Common name	Group represented	NOAEC or NOAEL
Coccinella septempunctata	Seven-spotted lady beetle	Foliar nontarget arthropods	9 µg mCry3A/g diet
Coleomegilla maculata	Pink-spotted lady beetle	Foliar nontarget arthropods	500 µg mCry3A/g diet
Orius insidiosus	Flower bug	Foliar nontarget arthropods	50 µg mCry3A/g diet
Aleochara bilineata	Rove beetle	Soil-dwelling invertebrates	50 µg mCry3A/g diet
Poecilus cupreus	Carabid beetle	Soil-dwelling invertebrates	12 µg mCry3A/g diet
Eisenia fetida	Earthworm	Soil-dwelling invertebrates	250 µg mCry3A/g soil
Apis mellifera	Honey bee	Pollinators	50 µg mCry3A/g diet
Colinus virginianus	Bobwhite quail	Wild birds	652 mg mCry3A/kg bw
Mus musculus	Mouse	Wild mammals	2377 mg mCry3A/kg bw

Table XII–3. eCry3.1Ab margins of exposure

Test organism	Worst-case EEC or DDD	Conservative EEC or DDD	NOAEC or NOAEL	Worst-case MoE	Conservative MoE
Coleomegilla maculata	43.57 µg/g leaves	6.54 µg/g diet	353 µg/g diet	8.1	54
Orius laevigatus	43.57 µg/g leaves	6.54 µg/g diet	400 µg/g diet	9.2	61
Aleochara bilineata	15.64 µg/g roots	0.45 µg/g diet	400 µg/g diet	26	890
Poecilus cupreus	15.64 µg/g roots	0.45 µg/g soil	400 µg/g diet (survival)	26	890
			<400 µg/g diet (growth)		
Eisenia fetida	15.64 µg/g roots	0.45 µg/g soil	4.06 µg/g soil	0.26	9
			10.3 µg/g soil[1]	0.66	23
Apis mellifera	0.08 µg/g pollen	0.007 µg/g diet	50 µg/g diet	625	7100
Colinus virginianus	0.96 mg/kg bw	0.26 mg/kg bw	900 mg/kg bw	940	3500
Mus musculus	0.50 mg/kg bw	0.36 mg/kg bw	2000 mg/kg bw	4000	5600

[1] NOAEC or NOAEL were corrected for soil matrix effects observed in ELISA measurements.

Table XII–4. mCry3A margins of exposure

Test organism	Worst-case EEC or DDD	Conservative EEC or DDD	NOAEC or NOAEL	Worst-case MoE	Conservative MoE
Coccinella septempunctata	246.96 µg/g pollen	93.88 µg/g pollen	9 µg/g diet	0.04	0.1
Coleomegilla maculata	246.96 µg/g pollen	93.88 µg/g pollen	500 µg/g diet	2.0	5.3
Orius insidiosus	22.51 µg/g leaves	3.75 µg/g diet	50 µg/g diet	2.2	13
Aleochara bilineata	12.68 µg/g roots	0.20 µg/g diet	50 µg/g diet	3.9	250
Poecilus cupreus	12.68 µg/g roots	0.20 µg/g diet	12 µg/g diet	0.95	60
Eisenia fetida	12.68 µg/g roots	0.20 µg/g diet	250 µg/g soil	20	1250
Apis mellifera	246.96 µg/g pollen	15.68 µg/g diet	50 µg/g diet	0.20	3.2
Colinus virginianus	4.07 mg/kg bw	1.71 mg/kg bw	652 mg/kg bw	160	380
Mus musculus	3.22 mg/kg bw	2.35 mg/kg bw	2377 mg/kg bw	740	1000

XII.A.4. Conclusions of the Test of the Effects of eCry3.1Ab and mCry3A on Nontarget Organisms

Effects tests on a variety of relevant NTOs exposed to either eCry3.1Ab or mCry3A have been previously evaluated by the USDA APHIS. No adverse effects were observed in a laboratory study in which *Coccinella septempunctata* lady beetles were exposed to mCry3A; however, the study did not achieve margins of exposure of greater than one when assuming exposure to MZIR098 corn pollen. Therefore, a second lady beetle study, with *Coleomegilla maculata*, was conducted in which the test group was exposed to 2X the worst-case EEC and 5.3X the more realistic but still highly conservative EEC. No adverse effects were observed in this study, thus the data suggest that cultivation of MZIR098 corn poses negligible risk to above-ground nontarget arthropods that might consume pollen. Using further exposure refinements to estimate honey bee exposure to mCry3A from MZIR098 corn, the NOAEC exceeded the conservative EEC, giving a margin of exposure of greater than one. Similarly for earthworm, using further exposure refinements to estimate exposure to eCry3.1Ab from MZIR098 corn, the NOAEC exceeded the conservative EEC, giving a margin of exposure of greater than one. Aquatic exposure to eCry3.1Ab and mCry3A from the cultivation of MZIR098 corn is negligible; therefore aquatic effects tests are not relevant to this assessment.

The comparison of the hazard and exposure data corroborates the hypothesis that eCry3.1Ab and mCry3A are not harmful to NTOs at concentrations likely to result from the cultivation of MZIR098 corn, and provides a weight of evidence that eCry3.1Ab and mCry3A in MZIR098 corn will have no harmful effects on populations of potentially exposed NTOs.

XII.B. Potential Impact of the PAT Protein on Nontarget Organisms

Forty-six transgenic plant cultivars (unique transformation events), including 16 corn cultivars, that produce PAT via either the transgene *pat* or *bar* have been deregulated by USDA APHIS (CERA, 2015). PAT is normally produced in *Streptomyces* bacteria, which commonly occur in soil. Functionally similar phosphinothricin acetyl transferases also occur in other bacteria. Therefore, PAT or functionally similar proteins are ubiquitous in the environment. Wildlife species potentially exposed to PAT via MZIR098 corn tissue or soil will have previously been exposed to enzymes with similar structure and function. No harmful effects of such exposure are known.

XII.C. 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 2010a; Baltazar *et al*. 2005). Teosinte species are not natives of the U.S., 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 2010a). Teosinte species are grown in botanical gardens, but as corn pollen is heavy and relatively short-lived (e.g., U.S. EPA 2010a; Devos *et al*. 2005), fertilization of these plants with pollen from MZIR098 corn is extremely unlikely.

Species of the genus *Tripsacum* are considered close relatives of *Zea* species and some theories (e.g., Poggio *et al*. 2005) postulate that a *Tripsacum* species may be a progenitor of domesticated corn via hybridization and introgression with teosinte.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 2010a). 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 (2010a) 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 *ecry3.1Ab*, *mcry3A*, and *pat-08* from MZIR098 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, eCry3.1Ab, mCry3A, and PAT are unlikely to spread from corn cultivation and persist in the environment as the result of gene flow from MZIR098 corn to wild relatives.

XII.D. Weediness Potential

Several characteristics make it unlikely for conventional corn to form feral populations. To evaluate whether MZIR098 corn is potentially weedier than conventional corn, its seed germination and dormancy characteristics and its performance in agronomic trials were compared with those of conventional, nontransgenic as discussed in Section XI.

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; 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 eCry3.1Ab, mCry3A, and PAT are highly unlikely to alter the dispersal or competitive ability of corn. This hypothesis was corroborated in a study comparing seed germination/dormancy and agronomic and phenotypic characteristics of MZIR098 corn to conventional corn, as described Section XI.A. The probability of spread of the transgenic proteins outside corn cultivation through volunteers and self-sustaining feral populations of MZIR098 corn is therefore low.

To test whether a transgenic crop cultivar is likely to be weedier than its corresponding nontransgenic cultivar, performance in agronomic trials can be compared (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 MZIR098 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 numbers of dropped ears observed in MZIR098 corn and the nontransgenic, near-isogenic corn were comparable (Table XI–10). The early stand count and grain yield of MZIR098 corn were not statistically significantly different than the nontransgenic, near-isogenic corn (Table IX–9). The germination rate of MZIR098 corn seed was not statistically significantly different than that of the nontransgenic, near-isogenic control corn under any of the five temperature regimes tested in a laboratory study (Table XI–5).

No biologically relevant differences indicative of increased weediness potential were observed in seed germination/dormancy, plant growth habit, life span, vegetative vigor, flowering characteristics, yield, stress adaptations, or disease susceptibility. Therefore, MZIR098 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 MZIR098 corn will form persistent feral populations.

XII.E. Conclusions of the Analysis of Potential Environmental Impacts of MZIR098 Corn Cultivation

Testing with purified eCry3.1Ab and mCry3A protein at levels greatly exceeding the concentrations found in MZIR098 corn tissues resulted in no biologically relevant effects on several beneficial arthropods and other nontarget species. Ecologically significant concentrations of eCry3.1Ab and mCry3A are unlikely to occur outside of fields where MZIR098 corn is cultivated. PAT and functionally similar proteins have been identified in *Streptomyces* and other bacteria and commonly occur in soil, and are thus ubiquitous in the environment. Pollen- or seed-mediated gene flow is unlikely because there are no free-living wild relatives of corn in the U.S. and the probability that self-sustaining feral populations of MZIR098 corn will form is very low. Aside from the intended phenotypes of corn rootworm resistance and tolerance to herbicides containing glufosinate-ammonium, there are no material differences in crop agronomic characteristics between MZIR098 corn and conventional corn. Thus, there is no basis for concluding that MZIR098 corn will be associated with increased weediness potential.

XIII. Impact on Agronomic Practices

Corn is the largest crop in the U. S. 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 engineered 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-trait varieties, most of which contained traits for both insect resistance and herbicide tolerance (USDA-NASS 2015a).

Like the antecedent DP-ØØ4114-3 corn, MZIR098 corn would provide an additional cultivar option for corn rootworm-resistant, herbicide-tolerant corn that would serve as a substitute or alternative to existing options. Therefore, like DP-ØØ4114-3 corn, MZIR098 corn would not cause an expansion of corn production or change existing corn production practices. Results of efficacy studies comparing the performance of MZIR098 corn to nontransgenic, near-isogenic control corn demonstrate that MZIR098 corn is highly resistant to damage from corn rootworm feeding and tolerant to herbicide containing glufosinate-ammonium (Appendix E). As such, MZIR098 corn is expected to have similar agronomic performance to its rootworm-resistant and glufosinate-ammonium tolerant commercial counterparts.

The agricultural production impacts of deregulation of MZIR098 corn would be similar to those of the antecedent, DP-ØØ4114-3 corn, as the resulting phenotype of both MZIR098 corn and the referenced antecedent is a corn plant resistant to damage from corn rootworm species and tolerant to herbicides containing glufosinate-ammonium. DP-ØØ4114-3 corn additionally expresses the Cry1F protein conferring tolerance to certain lepidopteran pests, however, this insect-resistant phenotype is not relevant to this extension request.

Syngenta has conducted a detailed review of the Environmental Assessment published for DP-ØØ4114-3 corn. This review is outlined in the Environmental Memorandum submitted concurrently with this extension request.

XIV. Adverse Consequences of Introduction

Syngenta is not aware of any unfavorable information that would have a bearing on a decision by USDA to deregulate MZIR098 corn. The development and testing of MZIR098 corn have 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 Notifications for MZIR098 Corn

Field trials with MZIR098 corn have been conducted in the U.S. under USDA APHIS notifications since 2009. A complete listing of these 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 notifications for field releases of MZIR098 corn

Notification no.	States[a]	Effective dates	Status of field test report
09-230-106n	HI	09/24/2009 - 09/24/2010	Submitted
10-056-103n	HI, IL, IA, MN	03/26/2010 - 03/26/2011	Submitted
10-235-105n	HI	09/23/2010 - 09/23/2011	Submitted
11-046-107n	HI, IA, IL, MN, MO, NE, SD, WI	04/01/2011 - 04/01/2012	Submitted
11-231-106n	HI, PR	09/19/2011 - 09/19/2012	Submitted
12-045-107n	HI, IL, IN, IA, MN, NE, SD, WI	04/01/2012 - 04/01/2013	Submitted
12-074-103n	IA	04/01/2012 - 04/01/2013	Submitted
12-076-106n	IA	04/15/2012 - 04/15/2013	Submitted
12-248-101n	HI, PR	10/01/2012 - 10/01/2013	Submitted
13-036-103n	HI, IL, IN, IA, MN, NE, WI	04/01/2013 - 04/01/2014	Submitted
13-044-123n	IL, IA, KS, MN, NE, PA, WA, WI	04/01/2013 - 04/01/2014	Submitted
13-052-111n	IA, IL, IN, MO,	03/14/2013 - 03/14/2014	Submitted
13-248-118n	HI, PR	09/18/2013 - 09/18/2014	Submitted
14-035-108n	HI, IL, IA, MN, SD	04/01/2014 - 04/01/2015	Submitted
14-055-102n	WA	05/01/2014 - 05/01/2015	Submitted
14-063-105n	IL, IN, IA, MO	04/01/2014 - 04/01/2015	Submitted
14-090-111n	IL	04/14/2014 - 04/14/2015	Submitted
14-101-105n	IA, NC	05/01/2014 - 05/01/2015	Submitted
14-107-106n	WI	05/09/2014 - 05/09/2015	Submitted
14-255-103n	HI, PR	09/26/2014 - 09/26/2015	Pending
15-054-106n	CA, CO, HI, IL, IN, IA, MN, NE, PR, SD, WI	04/01/2015 - 04/01/2016	Pending
15-062-101n	KS, WA	04/03/2015 - 04/03/2016	Pending
09-230-106n	HI	09/24/2009 - 09/24/2010	Submitted
10-056-103n	HI, IL, IA, MN	03/26/2010 - 03/26/2011	Submitted
10-235-105n	HI	09/23/2010 - 09/23/2011	Submitted
11-046-107n	HI, IA, IL, MN, MO, NE, SD, WI	04/01/2011 - 04/01/2012	Submitted
11-231-106n	HI, PR	09/19/2011 - 09/19/2012	Submitted
12-045-107n	HI, IL, IN, IA, MN, NE, SD, WI	04/01/2012 - 04/01/2013	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 Resistance to Damage from Corn Rootworm Species or Tolerance to the Broad-Spectrum Herbicide Glufosinate-Ammonium

This appendix contains a summary of transgenic corn events previously deregulated by the USDA that display resistance to damage from corn rootworm species and tolerance to the broad-spectrum herbicide glufosinate-ammonium.

Table B–1. Deregulated transgenic corn events conferring resistance to damage from corn rootworm species

Event Name(s)	Filed By/ Petition Number	Effective Date of Deregulation	Transgene /Protein Conferring Resistance to Corn Rootworm
DP-ØØ4114-3	Pioneer/ 11-244-01p	June 20, 2013	*cry34Ab1, cry35Ab1* / Cry34Ab1, Cry35Ab1
5307	Syngenta/ 10-336-01p	February 27, 2013	*ecry3.1Ab* / eCry3.1Ab
MIR604	Syngenta/ 00-136-01p	March 16, 2007	*mcry3A* / mCry3A
MON 88017	Monsanto/ petition 04-125-01p	December 14, 2005	*cry3Bb1* / Cry3Bb1
59122	Dow/ 03-353-01p	September 23, 2005	*cry34Ab1, cry35Ab1* / Cry34Ab1, Cry35Ab1
MON 863	Monsanto/ 01-137-01p	October 8, 2002	*cry3Bb1* / Cry3Bb1

Source: USDA APHIS 2015

Table B–2. Deregulated transgenic corn events conferring tolerance to herbicides containing glufosinate-ammonium

Event Name(s)	Filed By/ Petition Number	Effective Date of Deregulation	Transgene / Protein Conferring Tolerance to Herbicides Containing Glufosinate-Ammonium
DP-ØØ4114-3	Pioneer/ 11-244-01p	June 20, 2013	*pat* / PAT
59122	Dow/ 03-353-01p	September 23, 2005	*pat* / PAT
6275	Dow/ 03-181-01p	October 20, 2004	*pat* / PAT
1507	Mycogen c/o Dow & Pioneer/ 00-136-01p	June 14, 2001	*pat* / PAT
MS6	AgrEvo/ 98-349-01p	March 16, 1999	*bar* / PAT
676, 678, 680	Pioneer/ 97-342-01p	May 14, 1999	*pat* / PAT

Continued

Event Name(s)	Filed By/ Petition Number	Effective Date of Deregulation	Transgene / Protein Conferring Tolerance to Herbicides Containing Glufosinate-Ammonium
CHB-351	AgrEvo/ 97-265-01p	May 8, 1998	*bar* / PAT
DBT418	DeKalb/ 96-291-01p	March 28, 1997	*bar* / PAT
Bt11	Northrup King / 95-195-01p	July 18, 1996	*pat* / PAT
B16	DeKalb/ 95-145-01p	December 19, 1995	*bar* / PAT
T14, T25	AgrEvo/ 94-357-01p	June 22, 1995	*pat* / PAT
Bt176	Ciba Seeds/ 94-319-01p	May 17, 1995	*bar* / PAT

Source: USDA APHIS 2015

Appendix C. Methods Used to Quantify eCry3.1Ab, mCry3A, and PAT Proteins in MZIR098 Corn Tissues

C.1. Test, Control, and Reference Materials

The test material for this study was MZIR098 corn seed and the control material was nontransgenic, near-isogenic corn seed of the same genetic background as the test substance.

C.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; Richland, IA; Germansville, PA; and Delvan, WI. Table C–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 C–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

C.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:

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

C.4. Protein Extraction and ELISA Analysis

Protein extractions were performed on representative aliquots of the lyophilized samples. ELISA methodology was used to quantify eCry3.1Ab, mCry3A, 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.

For all tissues types and proteins, with the exception of pollen analyzed for eCry3.1Ab, 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 eCry3.1Ab and mCry3A) until analysis.

For pollens samples intended for analysis to quantify eCry3.1Ab, lyophilized pollen (approximately 25 mg) was weighed into a 2 ml microfuge tube containing three 4 mm glass beads; the samples were stored at -70°C (±10°C) for at least two hours. Samples were homogenized using a KLECO homogenizer set at approximately 3000 strokes per minute for approximately 60 seconds. Borate extraction buffer, pH approximately7.5, was added to each sample (1.5 ml) and samples were incubated on ice for at least 20 minutes. Samples were centrifuged at 2°C to 8°C to form a pellet. Unless analyzed on the same day, the supernatants were stored at -70°C ± 10°C until analysis.

Tables C–2 through C–4 list the reagents, buffers, and kits used for extraction and ELISA analysis.

Table C–2. Reagents, buffers, and kits used for extraction and ELISA of eCry3.1Ab

Item	Constituents
Beacon eCry3.1Ab 10 plate kit	96-well plates pre-coated with anti-mCry3A antibody, rabbit anti-G6-Cry1Ab antibody, and substrate solution
Borate extraction buffer, pH approximately 7.5	0.1 M sodium tetraboratedecahydrate, 0.2% polyvinylpyrrolidine, 7.69 mM sodium azide, 1.2% concentrated hydrochloric acid, 0.5% Tween 20, Complete Protease Inhibitor Cocktail Tablet (added on day of extraction)
ELISA diluent, pH approximately 7.4	1.4 M sodium chloride, 18.1 mM sodium phosphate (monobasic), 82.4 mM sodium phosphate (dibasic), 1% bovine serum albumin, 0.02% sodium azide, 0.05% Tween 20
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
Tris wash buffer, pH approximately 8.0	10 mM tris, 0.05% Tween 20

Table C–3. Reagents, buffers, and kits used for extraction and ELISA of mCry3A

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 Kit for Modified Cry3A	96-well plate pre-coated with mCry3A antibody, mCry3A antibody/enzyme conjugate, and substrate solution

Table C–4. Reagents, buffers, and kits 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

C.5. eCry3.1Ab Quantification

Quantification of eCry3.1Ab was performed using the eCry3.1Ab 10 plate kit. Pre-coated 96-well plates, rabbit anti-G6-Cry1Ab antibody solution, 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. Dilutions of each tissue extract, the ELISA standard, and the positive assay control sample, prepared in PBST buffer (prepared in ELISA diluent for pollen samples), were added to the pre-coated plates at 100 µl/well. The plates were incubated at room temperature for at least one hour with shaking. The plates were washed with PBST buffer

(tris wash buffer for pollen samples) and the rabbit anti-G6-Cry1Ab antibody was added at 100 µl/well. The plates were incubated at room temperature for at least one hour with shaking. The plates were washed with PBST buffer (tris wash buffer for pollen samples) and an alkaline phosphatase-conjugated donkey anti-rabbit immunoglobin G antibody diluted in PBST buffer (in ELISA diluent for pollen samples) was added at 100 µl/well. The plates were incubated at room temperature for at least one hour with shaking. The plates were washed with PBST buffer (tris wash buffer for pollen samples) and the substrate solution was added at 100 µl/well. The plates were incubated at room temperature (in the dark) for approximately 20 minutes with shaking. The colorimetric reaction was stopped by the addition of 3N sodium hydroxide at 100 µl/well, and absorbance was measured using a dual wavelength spectrophotometer at 405 nm and 490 nm. The results were analyzed with SoftMax® Pro Enterprise software version 4.6 and SoftMax® Pro GxP software version 6.3. The 490 nm reference measurement was subtracted from the 405 nm measurement prior to further analysis. Concentrations were interpolated from a standard curve generated using a four parameter algorithm.

C.6. mCry3A Quantification

Quantification of mCry3A protein was performed using the QualiPlate™ ELISA kit for Modified Cry3A. 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. Dilutions of each tissue 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 plates were incubated at room temperature for at least 30 minutes with shaking. After incubation, the plates were washed with PBST buffer, and the mCry3A antibody/enzyme conjugate was added at 50 µl/well. The plates were incubated at room temperature for at least one hour with shaking. 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 with shaking. The colorimetric reaction was stopped by the addition of 1N hydrochloric acid at 100 µl/well, and absorbance was measured using a spectrophotometer at 450 nm. The results were analyzed with SoftMax® Pro Enterprise software version 4.6 and SoftMax® Pro GxP software version 6.3. Concentrations were interpolated from a standard curve generated using a four-parameter algorithm.

C.7. 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.

C.8. Adjustments for Extraction Efficiency

Predetermined extraction efficiencies were used to adjust the eCry3.1Ab, mCry3A, and PAT concentrations to the estimated total eCry3.1Ab, mCry3A, and PAT concentrations in the corresponding tissue sample. Extraction efficiency and method sensitivity data, determined during validation of the eCry3.1Ab, mCry3A, and PAT quantitation methods (prior to this study), are summarized in Tables C–5 through C–7.

Table C–5. Minimum dilution factors, LODs, LOQs, and extraction efficiencies for the eCry3.1Ab ELISA

Sample Type[a]	Minimum Dilution Factor	Extraction Efficiency	LOD (µg/g DW)	LOQ (µg/g DW)
Corn leaf	undiluted (1)	82%	0.063	0.15
Corn root	undiluted (1)	87%	0.125	0.25
Corn kernel	2	84%	0.250	0.30
Corn pollen	undiluted (1)	not determined[b]	0.08	0.08

[a]The eCry3.1Ab protein was below the limit of quantitation for all iterative extrations, therefore an extraction efficiency could not be determined

[b] Whole plant uses the most conservative extraction efficiency present in other samples.

Table C–6. Minimum dilution factors, LODs, LOQs, and extraction efficiencies for the mCry3A ELISA

Sample Type[a]	Minimum Dilution Factor	Extraction Efficiency	LOD (µg/g DW)	LOQ (µg/g DW)
Corn leaf	64	81%	0.203	0.407
Corn root	4	85%	0.006	0.012
Corn kernel	undiluted (1)	83%	0.002	0.0031
Corn pollen	16	96%	0.025	0.099

[a]Whole plant uses the most conservative extraction efficiency present in other samples.

Table C–7. 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	undiluted (1)	87%	0.025	0.031
Corn root	undiluted (1)	87%	0.025	0.063
Corn kernel	undiluted (1)	97%	0.025	0.031
Corn pollen	undiluted (1)	79%	0.025	0.031

Appendix D. Methods Used for Compositional Analysis of MZIR098 Corn Forage and Grain

This appendix describes the methods used to conduct the compositional analysis study described in Section IX., wherein the results are also provided. References cited for individual methods in this appendix are listed at the end of the appendix.

D.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 MZIR098 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.

D.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, LLC. (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.

D.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 dried either in the field or mechanically until the grain contained not more than 17% moisture. (Mechanical drying after harvest is standard agronomic practice for improving storage characteristics of corn grain.) A well-mixed subsample of approximately 500 g of grain from each plot was shipped at ambient temperature to Syngenta Crop Protection, LLC., 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.

D.4. Compositional Analyses

As detailed in Section IX, 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.

D.5. Analytical Methods and Reference Standards for Compositional Analyses

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

The ground samples were 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 was calculated as 0.500 ppm on a fresh weight basis.

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

Acid Detergent Fiber (Komarek *et al.* 1994, 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 2005q, Barkholt and Jensen 1989, Henderson *et al.* 2000, Henderson and Brooks 2010, and Schuster 1988)

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 samples were hydrolyzed in 6N hydrochloric acid for approximately 24 hours at approximately 106-118ºC. Phenol was added to the 6N hydrochloric acid to prevent halogenation of tyrosine. Cystine and cysteine were converted to S-2-carboxyethylthiocysteine by the addition

of dithiodipropionic acid. Tryptophan was hydrolyzed from proteins by heating at approximately 110ºC in 4.2N sodium hydroxide for approximately 20 hours.

The samples were analyzed by HPLC after pre-injection derivatization. The primary amino acids were derivatized with o-phthalaldehyde (OPA) and the secondary amino acids were derivatized with fluorenylmethyl chloroformate (FMOC) before injection The limit of quantitation for this study was 0.100 mg/g on a fresh weight basis.

All reference standards were manufactured by Sigma-Aldrich.

Table D-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)

All organic matter was driven off when the samples were ignited at approximately 550ºC in a muffle furnace for at least 5 hours. The remaining inorganic material was determined gravimetrically and referred to as ash. The limit of quantitation was calculated as 0.100% on a fresh weight basis.

Beta Carotene (AOAC 2012a, Quackenbush 1987)

The samples were saponified with enzyme and potassium hydroxide and extracted with hexane. After concentration, the samples were brought up in methanol. The samples were then injected on a reverse phase high-performance liquid chromatography system with ultraviolet light detection. Quantitation

was achieved with a linear regression analysis using an external standard curve. The limit of quantitation was calculated as 0.0200 mg/100 g 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 and i)

The samples were hydrolyzed with hydrochloric acid. The fat was extracted using ether and hexane. The extracts were dried down and filtered through a sodium sulfate column. The remaining extracts were then evaporated, dried, and weighed. The LOQ was calculated as 0.100% on a fresh weight basis.

Fatty Acids (AOCS 2009a and b)

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 D-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

Component	Lot No. JY10-W		Lot No. O1-X	
	Weight (%)	Purity (%)	Weight (%)	Purity (%)
Methyl Lineoleate	10.0	99.8	15.75	99.5
Methyl Gamma Lineolenate	1.0	99.4	1.25	99.5
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 2005k and r, Infant Formula Council 1985a)

The samples were hydrolyzed in a potassium phosphate buffer with the addition of ascorbic acid to protect the folic acid during autoclaving. Following hydrolysis by autoclaving, the samples were 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 samples, using the bacteria *Lactobacillus casei*, with the growth response of a folic acid standard. This response was measured turbidimetrically. The LOQ was calculated as 0.00600 mg/100 g on a fresh weight basis.

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

Inductively Coupled Plasma Emission Spectrometry (AOAC 2005n and o)

The samples were dried, precharred, and ashed overnight in a muffle furnace set to maintain 500ºC. The ashed samples were 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 samples, measured on the inductively coupled plasma spectrometer, with the emission of the standard solutions. The LOQs (Table D-3) were calculated on a fresh weight basis:

Table D-3. Reference standards for inductively coupled plasma emission spectrometry

Mineral	Lot Numbers	Calibration Standard Concentration (µg/ml)	LOQ (ppm)
Calcium	B2-MEB280039 B2-MEB266040	200 1000	20.0
Copper	B2-MEB280039 B2-MEB280036	2 10	0.50
Iron	B2-MEB280039 B2-MEB280035	10 50	2.00
Magnesium	B2-MEB280039 B2-MEB280036	50 250	20.0
Manganese	B2-MEB280039 B2-MEB280036	2 10	0.30
Phosphorus	B2-MEB280039 B2-MEB266040	200 1000	20.0
Potassium	B2-MEB280039 B2-MEB266040	200 1000	100
Sodium	B2-MEB280039 B2-MEB266040	200 1000	100
Zinc	B2-MEB280039 B2-MEB280036	10 50	0.40

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

The inositol samples were 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 was calculated as 40.0 µg/g on a fresh weight basis.

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

Moisture (AOAC 2005c and d)

The samples were dried in a vacuum oven at approximately 100º C. The moisture weight loss was determined and converted to percent moisture. The LOQ was calculated as 0.100% on a fresh weight basis.

Neutral Detergent Fiber (NDF) (AACC 1998, 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 a boiling detergent solution at a neutral pH. The starches were removed via an alpha amylase soak. Hemicellulose, cellulose, lignin and insoluble protein fraction were left in the filter bag and determined gravimetrically. The LOQ was calculated as 0.100% on a fresh weight basis.

Niacin (AOAC 2005g and k)

The samples were 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 samples, using the bacteria *Lactobacillus plantarum,* with the growth response of a niacin standard. This response was measured turbidimetrically. The LOQ was calculated as 0.0300 mg/100 g on a fresh weight basis.

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

p-Coumaric Acid and Ferulic Acid (Covance 2009, Hagerman and Nicholson 1982)

The ground samples were extracted with methanol followed by alkaline hydrolysis and buffering prior to injection on an analytical high-performance liquid chromatography (HPLC) system for quantification of sinapic acid, p-coumaric acid, caffeic acid, and ferulic acid by ultra violet (UV) detection. The LOQ for the p-coumaric acid and ferulic acid assays was calculated as 16.7 ppm 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 samples were extracted using hydrochloric acid and sonication, purified using a silica based anion exchange column, concentrated and injected onto a high-performance liquid chromatography (HPLC) system with a refractive index detector. The LOQ was calculated as 0.125% on a fresh weight basis.

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

Protein (AOAC 2005m, AOCS 2011)

The protein and other organic nitrogen in the samples were converted to ammonia by digesting the samples with sulfuric acid containing a catalyst mixture. The acid digest was made alkaline. The ammonia was distilled and then titrated with a previously standardized acid. Instrumentation was used to automate the digestion, distillation and titration processes. The percent nitrogen was calculated and converted to equivalent protein using the factor 6.25. The LOQ was calculated as 0.100% on a fresh weight basis.

Selenium by Inductively Coupled Plasma-Mass Spectrometry (AOAC 2012b, Sullivan *et al.* 2013)

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 (ICP-MS) with a dynamic reaction cell (DRC) 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 2005s)

The samples were 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 was calculated as 0.05% on a fresh weight basis.

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

Sugar Profile (Brobst 1972, Mason and Slover 1971)

The samples were 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 LOQ for this study was 0.0500% on a fresh weight basis.

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

Total Dietary Fiber (TDF) (AOAC 2005p)

Duplicate samples were gelatinized with α-amylase and digested with enzymes to break down starch and protein. Ethanol was added to each of the samples 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 samples was calculated using protein and ash values and the weighed residue fractions. The LOQ was calculated as 1.00% on a fresh weight basis.

Trypsin Inhibitor (AOCS 1997, Kakade *et al*. 1974)

The samples were defatted with petroleum ether and extracted with 0.01N sodium hydroxide. Varying aliquots of the sample suspensions were exposed to a known amount of trypsin and benzoy1-DL-arginine~p~nitroanilide hydrochloride. The samples were allowed to react for 10 minutes at 37°C. After 10 minutes, the reaction was halted by the addition of acetic acid. The solutions were centrifuged or filtered, and 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 was calculated as 1.00 Trypsin Inhibitor Units (TIU)/mg on a fresh weight basis.

Vitamin B_1 (AOAC 2005f, h, and j)

The samples were autoclaved under weak acid conditions to extract the thiamine. The resulting solutions were incubated with a buffered enzyme solution to release any bound thiamine. The solutions were purified on a cation-exchange column. Aliquots were 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 results are

reported as thiamine hydrochloride. The LOQ was calculated as 0.010 mg/100 g on a fresh weight basis.

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

Vitamin B_2 (Riboflavin) (AOAC 2005e and k) The samples were 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 samples, using the bacteria *Lactobacillus rhamnosus*, with the growth response of multipoint riboflavin standards. The growth response was measured turbidimetrically. LOQ was calculated as 0.0200 mg/100 g on a fresh weight basis.

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

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

The samples were 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 samples, using the yeast *Saccharomyces cerevisiae*, with the growth response of a pyridoxine standard. The response was measured turbidimetrically. Results were reported as pyridoxine hydrochloride. The LOQ was calculated as 0.00700 mg/100 g 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*. 1985)

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

Note: Alpha tocopherol is part of a mixed standard which also includes beta, delta, and gamma isomers. The reference standard material for those isomers may contain small amounts of alpha tocopherol. All reference standards that contributed to the alpha tocopherol concentration are listed below.

Table D-4. Reference standards for Vitamin E composition

Manufacturer	Component	Lot No.	Purity (%)
USP	Alpha Tocopherol	O0K291	98.5
ACROS Organics	D-gamma-Tocopherol	A0083534	99.3
Sigma-Aldrich	(+)-δ-Tocopherol	SLBG1716V	93

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Appendix E. Trait Efficacy of MZIR098 Corn

E.1. Insecticidal Efficacy of MZIR098 Corn against Western Corn Rootworm

Under field conditions, the protection MZIR098 corn provides from western corn rootworm feeding damage was compared with that of corresponding nontransgenic, near-isogenic control corn. At two test sites (Bloomington, Illinois, and Shirley, Illinois), a trap crop had been planted the previous season for increased western corn rootworm egg accumulation (Branson and Sutter 1989) and was artificially infested with western corn rootworm eggs at a rate of 442 eggs/plant when plants were at the V3 growth stage. A third test site (Napier, Iowa) was artificially infested with western corn rootworm eggs at a rate of 442 eggs/plant when the plants were at the V3 growth stage. Root masses of six plants from each plot were collected at the R1 growth stage, washed, and assessed according to the Iowa State University node-injury scale for root injury by corn rootworm (Oleson *et al.* 2005), a quantitative linear scale (shown in Table E–1).

Table E–1. Node-injury scale for rating root damage by corn rootworm

Rating	Description of rootworm damage
0.01	No damage to 1–2 light surface scars on roots
0.02	3+ light surface scars or ≤4 moderate scars (combined across all roots on a plant)
0.05	5+ heavy scars (long, deep scars), but NO root pruning (pruning ≤1.5 in. from crown)
0.10	One root pruned to ≤1.5 in. accompanied by heavy scars
0.25	2+ roots pruned to ≤1.5 in. (up to the equivalent of 0.25 nodes pruned)
0.50	Equivalent of 0.50 node of roots pruned
0.75	Equivalent of 0.75 node of roots pruned
1.00	Equivalent of 1.00 node of roots pruned
1.25	Equivalent of 1.25 nodes of roots pruned
1.50	Equivalent of 1.50 nodes of roots pruned
1.75	Equivalent of 1.75 nodes of roots pruned
2.00	Equivalent of 2.00 nodes of roots pruned
2.25	Equivalent of 2.25 nodes of roots pruned
2.50	Equivalent of 2.50 nodes of roots pruned
2.75	Equivalent of 2.75 nodes of roots pruned
3.00	Equivalent of 3.00 nodes of roots pruned

Adapted from Oleson *et al*. (2005).

The results are shown in Table E–2. MZIR098 corn showed little root injury, whereas the nontransgenic corn showed significantly higher damage ratings. These results support the conclusion that MZIR098 corn provides excellent protection against root damage from feeding by western corn rootworm.

Table E–2. Root-injury ratings for MZIR098 corn and nontransgenic corn infested with western corn rootworm in field trials at three locations

Location Entry	Mean	*p* (ANOVA entry effect)	SEM
Bloomington, IL			
MZIR098	0.02	***0.0002***	0.09
Control	2.76		
Shirley, IL			
MZIR098[a]	0.05	***0.0180***	0.21
Control	2.46		0.29
Napier, IA			
MZIR098	0.14	***0.0167***	0.14
Control	1.08		

SEM = standard error of the mean.
N = 4 except as indicated.
p-values for significantly different results ($P < 0.05$) are shown in bold italic type.
[a]*N* = 3. Replicate number 3 was lost as the result of mechanical damage in the field.

E.2. Tolerance of MZIR098 Corn to Herbicide Products Containing Glufosinate-Ammonium

Under greenhouse conditions, the tolerance of MZIR098 corn to herbicide containing glufosinate-ammonium was compared with that of corresponding nontransgenic, near-isogenic corn. Glufosinate-ammonium was applied at the recommended field rate of 450 g ai/ha to MZIR098 corn and nontransgenic, near-isogenic hybrid corn at the V2 to V3 growth stage. The plants were rated for percent injury 5, 16, and 26 days after treatment.

The results are shown in Table E–3. Glufosinate-ammonium caused no or minimal injury to MZIR098 corn, whereas the nontransgenic corn was nearly completely killed by 26 days after treatment. These results support the conclusion that MZIR098 hybrid corn is highly tolerant to glufosinate-ammonium.

Table E–3. Percent injury to MZIR098 and nontransgenic corn 5, 16, and 26 days after application of glufosinate-ammonium

	Mean % injury (SD)		
Genotype	**Day 5**	**Day 16**	**Day 26**
MZIR098	0.0 (0.0)	0.5 (1.2)	0.0 (0.0)
Control	62.0 (6.0)	99.1 (2.5)	100.0 (0.0)
p-value	***<0.01***	***<0.01***	N/A

SD = standard deviation.
p-values for significantly different results ($P < 0.05$) are shown in bold italic type.
N/A indicates that ANOVA could not be conducted, because all values for both treatment groups were 0 or 100.

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