Petition for Determination of Nonregulated Status for Rootworm-Resistant Event 5307 Corn

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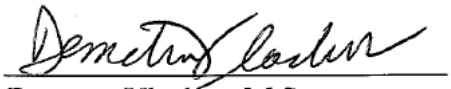
5307-USDA-1

Petition for Determination of Nonregulated Status

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

Certification

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

Petitioner: 
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Release of Information

Syngenta Biotechnology, Incorporated (Syngenta) is submitting the information in this petition for review by the United States Department of Agriculture (USDA) as part of a federal regulatory process. Except in accordance with the following, Syngenta does not authorize the release, publication, or other distribution of this information (including internet posting) to any third party without prior notice and Syngenta's consent. In responding to a request under the Freedom of Information Act (FOIA), 5 U.S.C. §552 and 7 CFR § 1, relating to all or some of the information in this petition, Syngenta expects that, in advance of the release of the information, USDA will provide Syngenta with a copy of the FOIA request and the material proposed to be released, and the opportunity to object to the release of information based on appropriate legal grounds (e.g., responsiveness, trade secret, and/or commercial concerns). Syngenta expects that no information submitted with this petition and identified as Confidential Business Information (CBI) will be disclosed to any third party.

Petition for Determination of Nonregulated Status for Rootworm-Resistant Event 5307 Corn

Summary of the Petition

Using the techniques of modern molecular biology, Syngenta has transformed corn (maize, *Zea mays* L.), to produce Event 5307 corn (also referred to hereafter to as "5307 corn"), a new cultivar that is highly resistant to larval feeding damage by western corn rootworm (*Diabrotica virgifera virgifera* Le Conte), northern corn rootworm (*D. longicornis barberi* Smith and Lawrence) and Mexican corn rootworm (*D. virgifera zeae* Krysan and Smith). These important coleopteran pests cause significant damage to the U.S. corn crop annually.

Event 5307 corn plants contain the transgene *ecry3.1Ab* encoding a novel rootworm-control protein, eCry3.1Ab, and the transgene *pmi* (also known as *manA*) encoding the enzyme phosphomannose isomerase (PMI). The eCry3.1Ab protein is an engineered chimera of the modified Cry3A (mCry3A) and Cry1Ab proteins, members of a class of insecticidal proteins derived from *Bacillus thuringiensis* (*Bt*). The gene *pmi* was obtained from *Escherichia coli* strain K-12 and the protein it encodes was utilized as a plant selectable marker during development of 5307 corn.

Event 5307 corn was produced by *Agrobacterium tumefaciens*-mediated transformation of immature corn embryos using plasmid vector pSYN12274. The region between the left and right borders of the transformation plasmid included *ecry3.1Ab* and *pmi* gene expression cassettes; this DNA was transferred into the corn genome during transformation. The *ecry3.1Ab* expression cassette consisted of the *ecry3.1Ab* coding region regulated by the CMP promoter region from the cestrum yellow leaf virus and the nopaline synthase (NOS) terminator sequence. The *pmi* expression cassette consisted of the *pmi* coding region regulated by the ZmUbiInt promoter and the NOS terminator sequence.

Southern blot analyses demonstrate that 5307 corn (1) contains, at a single locus within the corn genome, a single copy each of the gene *ecry3.1Ab*, its CMP promoter sequence, the marker gene *pmi*, its ZmUbiInt promoter sequence, and the two expected copies of the NOS terminator sequence, one NOS terminator sequence regulating *ecry3.1Ab* and one NOS terminator sequence regulating *pmi*; (2) does not contain any extraneous DNA fragments of these functional elements inserted elsewhere in the corn genome; and (3) does not contain backbone sequence from the transformation plasmid, pSYN12274.

Nucleotide sequence analysis of the transferred DNA (T-DNA) insert in 5307 corn confirms that the insert is intact and that the organization of the functional elements therein is identical to their organization within pSYN12274. One nucleotide change from the sequence of pSYN12274 was identified 48 base pairs (bp) upstream of the CMP promoter in a noncoding region of the Event 5307 insert in corn. However, this nucleotide change had no effect on the functionality of the insert. Additionally, the analysis indicates that some truncation of the nucleotide sequence occurred at the 5' and 3' ends of the T-DNA during the transformation process that resulted in Event 5307 corn; such truncation occurs commonly in transformation via *Agrobacterium*. The entire right border and three bp of noncoding

sequence at the 5′ end of the insert, and eight bp of the left border were truncated; however, these deletions had no effect on the functionality of the insert.

Sequence analysis of the T-DNA insertion site demonstrated that 33 bp of corn genomic sequence were deleted when the 5307 insert integrated into the corn genome. Bioinformatic analyses indicated that the insert does not disrupt any known endogenous corn gene. A putative 243-bp novel open reading frame (ORF) spanning the junction between corn genomic sequence and the 3′ region of the 5307 corn insert was identified. The translated 81-amino-acid sequence encoded by the putative ORF showed no biologically relevant sequence similarity to known or putative toxins or allergens. These data collectively demonstrate that there are no deleterious changes in the 5307 corn genome as a result of the T-DNA insertion.

Observations of *ecry3.1Ab* and *pmi* segregation ratios over several generations of 5307 corn plants are consistent with linkage of these transgenes at a single locus in the corn genome. These data and the results from Southern blot analyses of several generations of 5307 corn plants indicate that the T-DNA insert has integrated into the nuclear genome and that the transgenic locus is stably inherited during conventional breeding.

These genetic characterization data demonstrate that, apart from a single nucleotide change in a noncoding region of the T-DNA insert, there are no unintended changes in the 5307 corn genome as a result of the T-DNA insertion.

Laboratory, greenhouse, growth chamber, and field investigations with 5307 corn confirmed that there were no changes in seed, pollen, plant phenotypic, or composition parameters suggestive of increased plant pest risk. Compositional assessment of the grain and forage from multiple U.S. field sites demonstrate that 5307 corn is nutritionally and compositionally equivalent to, and as safe and nutritious as, its conventional counterpart. Corn does not possess weedy properties or outcross to wild relatives in the U.S.; these properties of corn have not been altered in 5307 corn.

Well-characterized modes of action, physicochemical properties, and results of safety studies demonstrate that the eCry3.1Ab and PMI proteins present in 5307 corn present no risk of harm for mammalian species.

Laboratory testing has shown no adverse effects on survival associated with exposures of eCry3.1Ab in a range of nontarget indicator species appropriate for a corn ecosystem. There was a 20% reduction in adult ground beetle weight observed after larvae of *Poecilus cupreus* were exposed to 400 µg eCry3.1Ab/g diet throughout their development, but because the larvae were tested at extreme exposure concentrations with no adverse effects on survival and only minor effects on growth, the effects of eCry3.1Ab on ground beetles are likely to be negligible under field exposure conditions. The eCry3.1Ab exposures in all studies were in excess of estimated environmental exposure levels for the nontarget organism groups represented, indicating a low probability of harm for nontarget organisms inhabiting corn ecosystems.

The narrow spectrum of insecticidal activity observed for eCry3.1Ab indicates with high certainty that no endangered or threatened species, with the potential exception of those belonging to the order Coleoptera, could be harmed by eCry3.1Ab via 5307 corn if opportunities for exposure existed. However, none of the 17 species of endangered or threatened Coleoptera listed by the US Fish and Wildlife Service is a member of the Chrysomelidae family, which is the only family of Coleoptera shown to be susceptible to eCry3.1Ab at the concentrations in 5307 plants. Moreover, due to their feeding habits and non-agricultural habitats, there is negligible opportunity for endangered or threatened Coleoptera to be exposed to eCry3.1Ab via 5307 corn cultivation.

Syngenta knows of no study results or observations associated with 5307 corn that are anticipated to result in adverse consequences to the quality of the human environment, directly, indirectly, or cumulatively. This includes a lack of anticipated adverse effects on endangered or threatened species listed by the U.S. Fish and Wildlife Service, unique geographic areas, critical habitats, public health and safety (including children and minorities), genetic diversity of corn, farmer or consumer choice, insect resistance, or the economy, either within or outside the U.S. In the near future, Syngenta intends to submit an Environmental Report containing additional information and analysis of potential environmental impacts that is not required by the regulations governing petitions for non-regulated status, but which is intended to assist APHIS in fulfilling its obligations under the National Environmental Policy Act (NEPA), 42 U.S.C. § 4321, *et seq*., as well as other applicable statutes and regulations.

Event 5307 corn will not be commercialized as a stand-alone product. Rather, it will be combined via conventional plant breeding with other insect-control traits as well as herbicide-tolerance traits in hybrid corn product offerings to growers. These hybrids will offer high efficacy, convenience, and an additional choice for protection of corn crops from feeding damage caused by important corn pests. As such, the use of 5307 corn as a component of these corn hybrids is expected to produce beneficial effects similar to previously deregulated *Bt* corn products that are commercially available. These benefits include reduced insecticide use, improved worker safety, reductions in the use of fossil fuels to apply chemical insecticides, economic benefits for growers, and increased competition in the marketplace for insect-protected seed products. Moreover, the novel mode of action of the eCry3.1Ab protein is expected to extend the useful life of *Bt* corn technology, as well as other methods, for corn rootworm control in general, by reducing the selection pressure for resistance among target pests.

The combination of eCry3.1Ab and modified Cry3A (via Syngenta's deregulated Event MIR604 corn) in the same corn hybrids will also justify a reduction in the size of the required on-farm refuge from a minimum of 20% of a grower's corn acres to 5%. This will have additional economic benefits for the grower, will further reduce insecticide use for rootworm control, and will promote grower compliance with refuge requirements. Appropriate refuge requirements will be implemented in the context of a comprehensive insect resistance management program mandated by the U.S. Environmental Protection Agency.

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Abbreviations and Acronyms

5307	Event 5307; derived from corn transformation event 5307
ADF	acid detergent fiber
AEBSF	4-(2-aminoethyl)-benzenesulfonylfluoride HCl
AOAC	Association of Analytical Communities
AOSA	Association of Seed Analysts
APHIS	Animal and Plant Health Inspection Service
ATG	translation start codon consisting of adenine, thymine, and guanine
BC	backcross
BCA	bicinchoninic acid
BCW	black cutworm (*Agrotis ipsilon*)
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
bp	base pair
BME	beta-mercaptoethanol
BSA	bovine serum albumin
Bt	*Bacillus thuringiensis*
bw	body weight
Bt11	transformation event designation for lepidopteran-resistant corn producing a Cry1Ab protein
CEW	corn earworm (*Helicoverpa zea)*
CFR	U.S. Code of Federal Regulations
cm	centimeter
CMP	cestrum yellow leaf curling virus promoter
CmYLCV	cestrum yellow leaf curling virus
CPB	Colorado potato beetle (*Leptinotarsa decemlineata*)
Cry	crystal protein from *B. thuringiensis*
CTAB	cetyltrimethyl ammonium bromide
Da	daltons
dCTP	deoxycytidine triphosphate
DDD	daily dietary dose
DNA	deoxyribonucleic acid
dsDNA	double-stranded deoxyribonucleic acid
DTT	dithiothreitol
dw	dry weight
ECB	European corn borer (*Ostrinia nubilalis*)
EDTA	ethylenediamine tetraacetic acid
EEC	estimated environmental concentration
ELISA	enzyme-linked immunosorbent assay
EPA	United States Environmental Protection Agency
F_1	first generation of progeny from a breeding cross
Fig.	figure
FARRP	Food Allergy Research and Resource Program
FAW	fall armyworm (*Spodoptera frugiperda*)

FDA	United States Food and Drug Administration
FFDCA	Federal Food, Drug and Cosmetic Act
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
fw	fresh weight
G6PDH	glucose 6-phosphate dehydrogenase
GA21	transformation event designation for glyphosate-tolerant corn producing a double mutated 5-enolpyruvyl shikimate-3-phosphate synthase enzyme
ha	hectare
IgG	immunoglobulin G
ILSI	International Life Sciences Institute
IRM	insect resistance management
kb	kilobase
kilodaltons	kDa
LB	left border
LC_{50}	median lethal concentration
LOD	limit of detection
LOQ	limit of quantification
LTR	long terminal repeat
m	meter(s)
MES	2-(N-morpholino)ethanesulfonic acid
MIR162	transformation event designation for insect resistant corn producing a Vip3Aa20 protein for lepidopteran control
MIR604	transformation event designation for insect resistant corn producing a modified Cry3A protein for corn rootworm control
MW	molecular weight
N	number of samples
N/A	not analyzed
NADP	β-nicotinamide adenine dinucleotide phosphate
NADPH	β-nicotinamide adenine dinucleotide phosphate reduced
NCBI	National Center for Biotechnology Information
NDF	neutral detergent fiber
No.	number
NOAEC	no-observable-adverse-effect concentration
NOAEL	no-observable-adverse-effect level
NOS	nopaline synthase
OD	optical density
OECD	Organisation for Economic Co-operation and Development
ORF	open reading frame
PBS	phosphate buffered saline
PBW	pink bollworm (*Pectinophora gossypiella*)
PCR	polymerase chain reaction
PMI	phosphomannose isomerase
Prob.	probability
PVDF	polyvinylidene difloride

PVP	polyvinylpyrrolidone
RB	right border
repA	pVS1 replication gene from *Pseudomonas aeruginosa*
SD	standard deviation
SCR	southern corn rootworm (*Diabrotica undecimpunctata howardi*)
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
spec	streptomycin adenylyltransferase gene from *Escherichia coli*
SSC	saline-sodium citrate buffer
TAA	translation stop codon consisting of the nucleotides thymine, adenine, and adenine
TAG	translation stop codon consisting of the nucleotides thymine, adenine, and guanine
TC1507	transformation event designation for lepidopteran-resistant corn producing a Cry1F protein
TDF	total dietary fiber
T-DNA	transfer DNA, transferred DNA
TER	toxicity exposure ratio
TGA	translation stop codon consisting of the nucleotides thymine, guanine, and adenine
TMB	tetramethylbenzidine
Tris	tris(hydroxymethyl)aminomethane
U.S.	United States
U.S.C.	United States Code
USDA	United States Department of Agriculture
vir	virulence regulon in *Agrobacterium tumefaciens*
v/v	volume per volume
WCR	western corn rootworm (*Diabrotica virgifera virgifera*)
w/v	weight per volume
w/w	weight per weight
ZmUbiInt	*Zea mays* ubiquitin promoter with intron

I. Rationale for Submission of Request for Deregulation

The introduction of crops improved through modern biotechnology has been the single most important technological innovation brought to United States (U.S.) agriculture in the past 25 years. Adoption of genetically engineered crops with insect resistance and herbicide tolerance traits has increased dramatically since the the first commercial introductions of transgenic corn, cotton and soybeans in 1996. Net economic benefits at the farm level have been substantial (Brookes and Barfoot, 2006; Hutchison et al., 2010). Improved insect and weed control have led to increased crop yields, reductions in conventional pesticide applications, and environmental benefits (Brookes and Barfoot, 2010). The continued development and introduction of transgenic crop varieties with novel traits will benefit growers, consumers and the environment.

Corn (*Zea mays* L., maize) derived from Syngenta's transformation event 5307, hereafter referred to as "Event 5307 corn" or "5307 corn," contains an engineered insecticidal protein, eCry3.1Ab, that is active on three economically important corn rootworm (*Diabrotica*) species. Event 5307 corn demonstrates excellent efficacy in controlling these damaging pests. As future product offerings to U.S. growers, the rootworm-control trait in 5307 corn will be combined via conventional corn breeding methods with other coleopteran-control, lepidopteran-control and herbicide-tolerance traits in previously deregulated corn cultivars; 5307 corn will not be deployed as a stand-alone product.

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

The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) has responsibility under the Plant Protection Act (Title IV Public Law 106-224, 114 Statute 438, 7 U.S.C. § 7701-7772) and the Plant Quarantine Act (7 U.S.C. § 151-167) to prohibit or restrict the importation, exportation, and interstate movement of plants, plant products, certain biological control organisms, noxious weeds, and plant pests. APHIS regulations under 7 CFR § 340.1 stipulate that any organism that has been altered or produced through genetic engineering is considered a regulated article if the donor organism, recipient organism, or vector or vector agent belongs to any genera or taxa designated under §340.2 and meets the definition of a plant pest, or is an unclassified organism and/or an organism whose classification is unknown, or any product that contains such an organism, or 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. The vector agent used to produce Event 5307 corn was from the genus *Agrobacterium*, an organism listed under 7 CFR § 340.2. Additionally, regulatory sequences from *Agrobacterium tumefaciens* and a plant virus, the cestrum yellow yellow leaf curling virus (CmYLCV), were introduced in the production of Event 5307 corn.

The APHIS regulation 7 CFR § 340.6 provides that any person may petition APHIS seeking a determination that an article should no longer be regulated. Syngenta herein presents data and justification for an APHIS determination of nonregulated status for Event 5307 corn based on an absence of plant pest risk.

Although the vector agent, *Agrobacterium tumefaciens*, is a plant pathogen, the transformation process that created 5307 corn used a disarmed strain. Further, no nucleotide sequences from *A. tumefaciens* imparting plant pest properties were transferred to 5307 corn. The *A. tumefaciens* sequences transferred to corn in the transformation process consisted of the right border region of T-DNA from the nopaline Ti plasmid and the NOS terminator sequence from the nopaline synthase gene.

CmYLCV is a double-stranded DNA plant pararetrovirus belonging to the Caulimoviridae family, and has an extremely narrow host range among the Solanaceae (Stavolone et al., 2003a). Although the CMP (CmYLCV) promoter (Stavolone et al., 2003b) from this virus is present in 5307 corn plants, this regulatory sequence *per se* does not impart plant pest properties.

I.B. Rationale for Development of Event 5307 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 recent 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.

Syngenta scientists (Walters et al., 2010) engineered a novel gene, *ecry3.1Ab*, by combining portions of two existing Cry genes, modified *cry3A* (*mcry3A*) and *cry1Ab*, each of which is derived from a native gene of *Bacillus thuringiensis*, a ubiquitous soil bacterium. Using the tools of modern biotechnology, the gene *ecry3.1Ab* was introduced into an elite corn cultivar, along with the gene *pmi* (also known as *manA*) from *Escherichia coli* strain K-12, which encodes a selectable marker. The phosphomannose isomerase (PMI) enzyme encoded by *pmi* allows transformed cells to survive on mannose as the primary carbon source (Negrotto et al., 2000); it serves no agronomic purpose in transformed plants (e.g., it does not confer herbicide tolerance).

The eCry3.1Ab protein produced in 5307 corn is highly 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 *ecry3.1Ab* gene at adequate levels in corn plants is a logical approach for limiting crop losses attributable to these pests, especially considering the limitations of conventional insecticides in controlling corn rootworms.

In hybrids offered for sale to U.S. growers, the gene *ecry3.1Ab* in 5307 corn will be combined through traditional breeding with the transgenes of other deregulated corn events containing insect-protection traits as well as herbicide-tolerance traits. The hybrid offerings

currently planned represent breeding "stacks" of events Bt11 × MIR604 × TC1507 × 5307 × GA21 and Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21. One of the stack components, Syngenta's MIR604 corn, also produces a rootworm-control protein, mCry3A, that is active on the same three rootworm pests targeted by eCry3.1Ab.

The combination of eCry3.1Ab and mCry3A in the same corn hybrids offers important advantages for insect resistance management. 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).

Corn is the most widely cultivated U.S. crop, in terms of acreage planted and net value. Transgenic corn hybrids delivering targeted rootworm control have been available in the U.S. since 2003. Prior to their introduction, control of *Diabrotica* rootworms accounted for the largest single use of conventional insecticides in the U.S. (Ward et al., 2005). Transgenic corn hybrids have significantly improved growers' ability to control these pests effectively and easily, while dramatically reducing their use of broad-spectrum soil-applied insecticides for rootworm control. The attendant benefits of the current transgenic hybrids on the market include higher and more consistent grain yield, economic benefits to growers, healthier plants, improved worker safety, and reduced use of fossil fuels to apply insecticide treatments. The deployment of 5307 corn (in hybrid combinations with other agronomically important traits) will offer similar benefits as well additional choices in pest control, thus promoting marketplace competition and higher adoption rates, and helping to preserve target pest susceptibility to current rootworm control methods.

I.C. RegulatoryProcess

Syngenta is actively pursuing regulatory approvals for 5307 corn cultivation in the U.S. and Canada, and may seek additional cultivation approvals in the future. In hybrid offerings to growers, 5307 corn will not be marketed as a stand-alone product, but will be combined via traditional breeding with other approved corn cultivars containing additional insect-protection traits as well as herbicide-tolerance traits, i.e., as Bt11 × MIR604 × TC1507 × 5307 × GA21 and Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 corn hybrids. Syngenta is also actively seeking regulatory approvals of products containing 5307 corn in countries that import corn from the U.S. or Canada and have functioning systems for regulating imports of genetically modified crops.

Under the Federal Coordinated Framework for the Regulation of Biotechnology, commercial biotechnology products like 5307 corn are subject to the regulatory authority of

multiple federal agencies, whose regulatory processes can operate simultaneously along parallel tracks. This section describes Syngenta's plan for obtaining regulatory approval from all of the relevant agencies. This information is intended to assist the agencies in coordinating their efforts where it is appropriate.

Syngenta will not offer hybrids containing the 5307 transgenes for sale in the U.S. or Canada until the necessary approvals in key export markets with functioning regulatory systems have been granted.

I.C.1. USDA-APHIS

Under the Plant Protection Act, APHIS has regulatory oversight over products of modern biotechnology to the extent that they could pose a plant pest risk. Under this system, any plant that has been altered or produced through genetic engineering starts out as a "regulated article." APHIS regulates the import, handling, interstate movement, and release into the environment of regulated articles, including genetically engineered plants that are undergoing confined experimental use or field trials.

APHIS regulations allow companies like Syngenta to petition the agency for a determination of non-regulated status. 7 CFR § 340.6. If APHIS grants that petition, the product of biotechnology at issue will no longer be subject to oversight as a regulated article. To determine whether non-regulated status is appropriate, APHIS evaluates a variety of issues including the potential for plant pest risk; disease and pest susceptibilities; the expression of gene products, new enzymes, or changes to plant metabolism; weediness and impact on sexually compatible plants; agricultural or cultivation practices; effects on non-target organisms; and the potential for gene transfer to other types of organisms.

This Petition is being submitted in compliance with APHIS regulations as a request for a determination of non-regulated status for 5307 corn. In the near future, Syngenta intends to submit an Environmental Report containing additional information and analysis of potential environmental impacts that is not required by the regulations governing petitions for non-regulated status, but which is intended to assist APHIS in fulfilling its obligations under the National Environmental Policy Act (NEPA), 42 U.S.C. § 4321, *et seq*., as well as other applicable statutes and regulations.

I.C.2. U.S. Environmental Protection Agency

Substances that are pesticides as defined under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) are subject to regulation by the Environmental Protection Agency (EPA). The eCry3.1Ab protein encoded by the genetic insert in 5307 corn has insecticidal properties and is, therefore, regulated by EPA. The EPA regulatory process includes issuance of Experimental Use Permits for field testing and "registration" of pesticidal products prior to their sale and distribution. As part of the registration process, EPA may establish conditions on the use of the product. EPA also sets "tolerance limits" for the presence of pesticide residues on and in food and animal feed. In some cases, EPA may establish an exemption from the tolerance limits under the Federal Food, Drug and Cosmetic Act.

Syngenta has obtained an Experimental Use Permit (67979-EUP-8) from EPA that allows for broad-scale field testing of 5307 corn and various breeding stack combinations that include 5307 corn. This permit was initially granted on June 1, 2010 with effect through February 28, 2012 (EPA, 2010f) and was extended on March 3, 2011 with effect through December 31, 2013 (EPA, 2011a). In connection with this Experimental Use Permit, EPA established and extended a temporary exemption from the requirement of a tolerance for eCry3.1Ab residues in corn commodities, pursuant to §408(d) of the Federal Food, Drug, and Cosmetic Act, 21 U.S.C. § 346a(d) (EPA, 2010a, 2011b). Phosphomannose isomerase (PMI), the selectable marker protein produced by 5307 corn plants, is exempt from food and feed tolerances (EPA, 2007).

Pursuant to FIFRA §3(c)(5), in April 2011 Syngenta submitted applications to the U.S. EPA for registration of the eCry3.1Ab plant-incorporated protectant in 5307 corn (file symbol 67979-EE) and in two breeding stacks thereof, specifically Bt11 × MIR604 × TC1507 × 5307 × GA21 and Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 corn. The registration sought for the plant-incorporated protectant in 5307 corn as a stand-alone cultivar will be for a manufacturing-use product (40 CFR § 152.3); Syngenta will not seek an end-use product registration for 5307 corn, *per se*. Rather, commercial registrations will be sought for the two breeding stack products, described above, that include 5307 corn. Concurrently, Syngenta also submitted a petition (Petition No. 1F7857) to the EPA to establish a nonexpiring exemption from the requirement of a tolerance for eCry3.1Ab residues in food and feed commodities.

I.C.3. U.S. Food and Drug Administration

The Coordinated Framework for the Regulation of Biotechnology gives FDA the responsibility to regulate the safety and proper labeling of plant-derived foods and feeds that are developed using the techniques of modern biotechnology. Under the Federal Food, Drug, and Cosmetic Act, food and feed manufacturers must ensure that the products they market are safe and properly labeled. The FDA helps sponsors of foods and feeds derived from genetically engineered crops comply with their obligations by encouraging them to participate in a voluntary consultation process.

Event 5307 corn falls within the scope of the Food and Drug Administration (FDA) policy statement concerning regulation of food products derived from new plant varieties, including those developed by recombinant DNA techniques (FDA, 1992). Syngenta initiated a voluntary pre-market consultation process with FDA and submitted a safety and nutritional assessment for 5307 corn in January 2011 (File BNF 000128).

I.C.4. Foreign Governments

Syngenta intends to commercialize 5307 corn, as part of the breeding-stack combinations Bt11 × MIR604 × TC1507 × 5307 × GA21 and Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 corn, for cultivation in Canada and will concurrently seek regulatory approvals to enable this. In April 2011, Syngenta submitted an application to the Canadian Food Inspection Agency (CFIA) for unconfined environmental release of 5307 corn.

Additional applications to Health Canada and CFIA for food and feed use approval, respectively, of 5307 corn will be submitted in May 2011.

Syngenta is also pursuing regulatory approvals for importation of corn commodities and processed goods containing 5307 corn in key export markets for U.S. and Canadian corn. Regulatory filings for 5307 corn import approvals have been made in Japan, South Korea, Taiwan, Australia/New Zealand and the European Union. Applications are currently planned for additional countries including Mexico, Colombia, China, the Philippines, Indonesia, South Africa, and Russia.

In June 2010, the Ministry of Agriculture, Forestry and Fisheries of Japan granted Syngenta approval to conduct a local field trial of 5307 corn under isolated conditions as a prerequisite for future cultivation/import approval from an environmental safety perspective. This 5307 corn was successfully harvested in October 2010.

II. The Biology of Corn

II.A. OECD Consensus Document on the Biology of *Zea mays* subsp. *mays*

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 Petition, 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. Characteristics of the Recipient Germplasm

The recipient germplasm for transformation to produce Event 5307 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* transformation and regeneration from tissue culture. NP2222 is a Stiff-Stalk family, yellow dent inbred.

III. Transformation and Development of Event 5307 Corn

III.A. Description of the Transformation System

Transformation of *Z. mays* to produce 5307 corn was accomplished via *Agrobacterium tumefaciens*-mediated transformation (as described in Negrotto et al., 2000) using immature embryos of Syngenta inbred corn line NP2222. *Agrobacterium*-mediated transformation is an efficient method for generating transformants containing simple and low-copy-number insertions with stable inheritance at a high frequency (Grimsley et al., 1987; Ishida et al., 1996). Using this method, DNA between the left border (LB) and right border (RB) of a transformation plasmid, referred to as the transferred DNA (T-DNA), is efficiently transferred integrated into the genome of the plant cell, while genetic elements beyond the plasmid borders are generally not transferred.

Event 5307 corn transformation employed a binary vector system (de Framond et al., 1983). *A. tumefaciens* strain LBA4404 carries a disarmed Ti plasmid from which the native T-DNA has been removed (Ooms et al., 1982). The Ti plasmid carries the *vir* genes which encode proteins that are required for release of the T-DNA from the transformation plasmid and its integration into the corn nuclear genome. The transformation plasmid used to create 5307 corn was p SYN12274 (see Figure III-1).

Immature embryos were excised from 8-12 day old corn ears and rinsed with fresh culture medium in preparation for transformation. Embryos were mixed with a suspension of *A. tumefaciens* strain LBA4404 harboring plasmid pSB1 (Komari et al., 1996) and the transformation plasmid pSYN12274, vortexed for 30 seconds, and allowed to incubate for an additional five minutes. Excess *A. tumefaciens* suspension was aspirated, and embryos were moved to plates containing a nonselective culture medium. Embryos were co-cultured with the remaining *A. tumefaciens* at 22°C for two to three days in the dark. 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. Embryos producing embryogenic callus were transferred to cell culture medium containing mannose. The gene *pmi* was used as a selectable marker during the transformation process (Negrotto et al., 2000). It encodes the enzyme phosphomannose isomerase (PMI), which enables transformed cells to survive on a mannose substrate (Negrotto et al., 2000).

After initial incubation with *A. tumefaciens*, transformed tissue was transferred to selective media containing 500 mg/l of the broad-spectrum antibiotic cefotaxime and grown for four months, ensuring that the *A. tumefaciens* was cleared from the transformed tissue. Cefotaxime has been shown to kill *A. tumefaciens* at this concentration (Xing et al., 2008). Regenerated plantlets were tested for the presence of both *ecry3.1Ab* and *pmi*, and for the absence of the spectinomycin (*spec*) resistance gene present on the vector backbone, by TaqMan polymerase chain reaction (PCR) analysis (Ingham et al., 2001). This screen allows for the selection of transformants that carry the T-DNA and are free of vector backbone DNA. Plants that tested positive for both *ecry3.1Ab* and *pmi* but negative for *spec* were transferred to a greenhouse for further propagation.

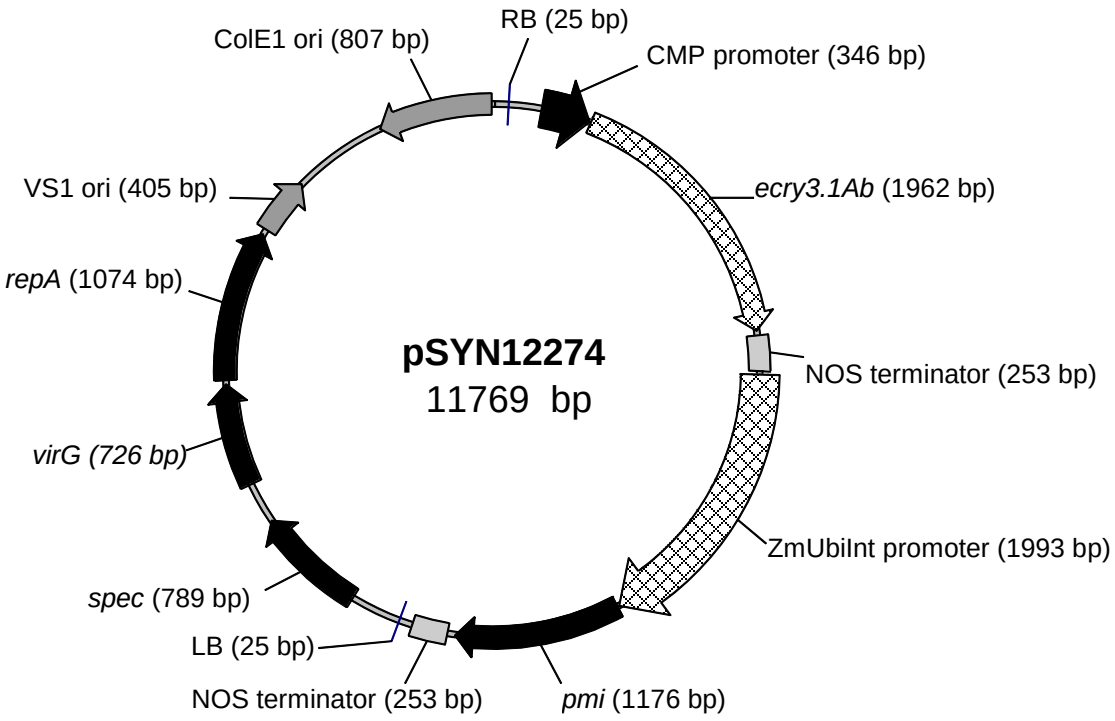


Figure III-1. Plasmid map for vector pSYN12274.

III.B. Development of Event 5307 Corn

Progeny of the original transformants (T_0 plants) were field tested for resistance to insect feeding damage and for agronomic performance after introgression of the transgenes into multiple elite lines of corn. Event 5307 corn was selected as the lead commercial candidate and underwent further field testing and development. A schematic showing the steps in development of 5307 corn is shown in Figure III-2. All interstate movements and field plantings of 5307 seed were conducted under USDA APHIS notifications; these are listed in Appendix A.

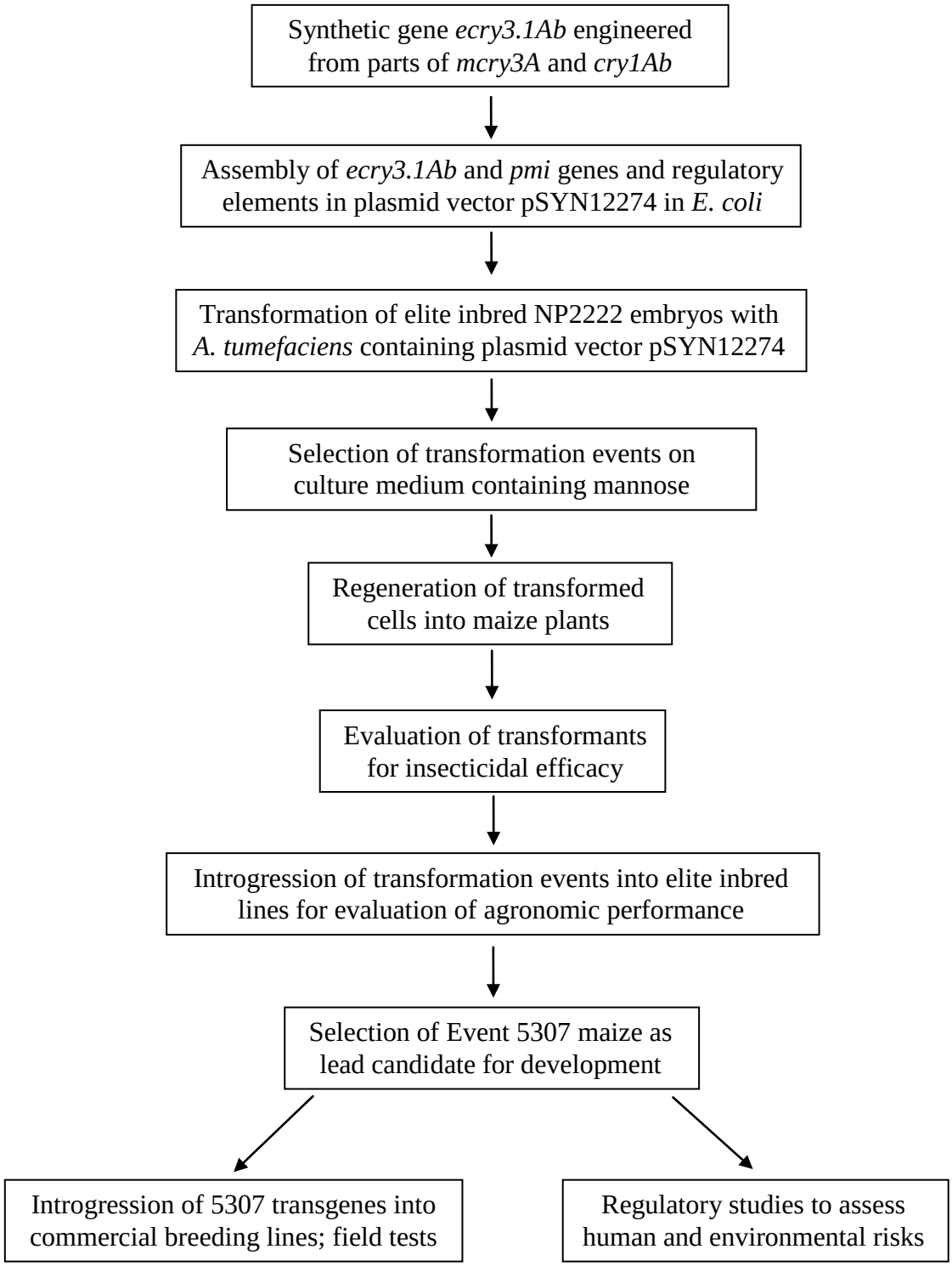


Figure III-2. Steps in the development of 5307 corn.

III.C. Production of Test and Control Materials

Table III-1 lists the genotypes and descriptions of the various 5307 corn and nontransgenic control corn seed genotypes used in the studies described within this Petition. The breeding pedigree diagram in Figure III-3 indicates how each of the 5307 genotypes was derived via conventional breeding crosses from the original Event 5307 T_0 transformant. Figure III-3 also indicates the derivation of related control seed materials of genotypes that were near-isogenic to the 5307 hybrid seed genotypes. Seed genotypes used in studies described herein are identified in the pedigree diagram by a single letter code (A through J); these correspond to seed lot identifiers in Table III-1. In Figure III-3, genotypes enclosed in ovals represent transgenic 5307 corn genotypes that were used in studies described herein; genotypes enclosed in rectangles represent nontransgenic, near-isogenic control genotypes. The goal in producing all control hybrids was to match their genetic background with that of the 5307 hybrids so that the effect of the transformation event could be assessed in an unbiased manner.

Several Syngenta studies described in this Petition were conducted using 5307 hybrid corn of genotype NP2171 x NP2460(5307), which was hemizygous for the transgenes; a hemizygous genotype is representative of future hybrids containing the 5307 transgenes that would be grown commercially. This 5307 hybrid is indicated by the letter code "E" in the breeding diagram (Figure III-3), and is alternatively referred to within this Petition as "NP2171 x $BC5F_3$." As indicated in Figure III-3, it was produced by crossing a nontransgenic inbred parent, NP2171, with the F_3 (third generation) transgenic progeny of self-pollinated plants following five successive generations of backcross (BC) breeding of the transgenes into to a recurrent NP2460 inbred. This backcross process fully introgressed the transgenes from the initial transformed germplasm (inbred line NP2222) into NP2460 germplasm. The corresponding nontransgenic control seed material was produced by crossing nontransgenic inbreds NP2171 and NP2460. Except for the presence of the transgenes, control hybrid NP2171 × NP2460 is nearly genetically identical to the corresponding transgenic hybrid; thus, this control is considered near-isogenic to the NP2171 × NP2460(5307) hybrid. The NP2171 × NP2460 seed material (indicated by the letter code "F" in Figure III-3) served as a control for two NP2171 x NP2460(5307) hybrids, one created using the 5307 $BC5F_3$ generation (letter code "E") and one created using the preceding 5307 generation, $BC5F_2$ (letter code "D").

The NP2391 × NP2222 control hybrid seed material (letter code "H" in Figure III-3), which corresponds to the transgenic hybrid seed genotype NP2391 × NP2222(5307) (letter code G), was not produced by direct crossing of the nontransgenic inbreds NP2391 × NP2222. Rather, it was derived from the null segregants of the hemizygous F_2 progeny of the initial T_0 transformant, that is, those progeny that did not inherit the transgenes from a transgenic parent as a consequence of Mendelian gene segregation. This material is also considered a suitable nontransgenic control for the corresponding 5307 hybrid material.

Two genetic characterization studies described in this Petition (genetic stability studies and Mendelian inheritance studies) required seed from multiple generations in the 5307 breeding pedigree, as indicated in Table III-1.

Table III-1. Event 5307 and control seed materials used in studies described in this Petition.

The female parent germplasm is shown first in each hybrid genotype description; the male parent germplasm is listed second. The pedigree diagram in Figure VIII-3 indicates the origin of Event 5307 seed and control seed materials of these genotypes by seed lot identifier (Lot ID).

Study Description (Relevant Part of Petition)	Event 5307 Seed Material		Nontransgenic Control Seed Material	
	Lot ID	Genotype	Lot ID	Genotype
Nucleotide sequence inserted (Part V.A.)	E	NP2171 × NP2460(5307)($BC5F_3$)*		None
Southern blots for copy no. of functional elements (Part V.B.)	E	NP2171 × NP2460(5307)($BC5F_3$)*	F	NP2171 × NP2460 + parental inbred lines NP2222, NP2460, NP2171
Southern blot analysis of genetic stability (Part V.D.)	A B C E	F_1 BC6 BC7 NP2171 × NP2460(5307)($BC5F_3$)*	 F	Parental inbred lines NP2222, NP2460, and NP2171 NP2171 × NP2460
Mendelian inheritance analysis (Part V.E.)	A B C	F_1 BC6 BC7		None
Determination of insert flanking sequences (Part V.F., Appendix B)	E	NP2171 × NP2460(5307)($BC5F_3$)*		None
Equivalence of eCry3.1Ab produced in 5307 corn and recombinant *E. coli* (Part VI.A.1.b.)	E	NP2171 × NP2460(5307)($BC5F_3$)*	F	NP2171 × NP2460
eCry3.1Ab and PMI concentrations in 5307 corn tissues (Part VI.C.)	E	NP2171 × NP2460(5307)($BC5F_3$)*	F	NP2171 × NP2460
Seed germination and dormancy evaluation (Part VII.A.1.)	E G	NP2171 × NP2460(5307)($BC5F_3$)* NP2391 × NP2222(5307)	F H	NP2171 × NP2460 NP2391 × NP2222
Field agronomic performance – 2007 (Part VII.A.2.)	D	NP2171 × NP2460(5307)($BC5F_2$)	F	NP2171 × NP2460
Field agronomic performance – 2008 (Part VII.A.2.)	E	NP2171 × NP2460(5307)($BC5F_3$)*	F	NP2171 × NP2460
Plant disease trials (Part VII.A.3)	I	5XH751 × NP2222(5307)	J	5XH751 × NP2222
Pollen viability and morphology evaluation (Part VII.A.5.)	E	NP2171 × NP2460(5307)($BC5F_3$)*	F	NP2171 × NP2460
Compositional assessment of forage and grain (Part VII.B.)	E	NP2171 × NP2460(5307)($BC5F_3$)*	F	NP2171 × NP2460
Broiler chicken and catfish feeding studies with grain (Parts VIII.D.2 & VIII.D.6.b., respectively)	E	NP2171 × NP2460(5307)($BC5F_3$)*	F	NP2171 × NP2460

* This 5307 hybrid is alternatively referred to within this Petition as "NP2171 x $BC5F_3$" or "NP2171 x NP2460(5307).

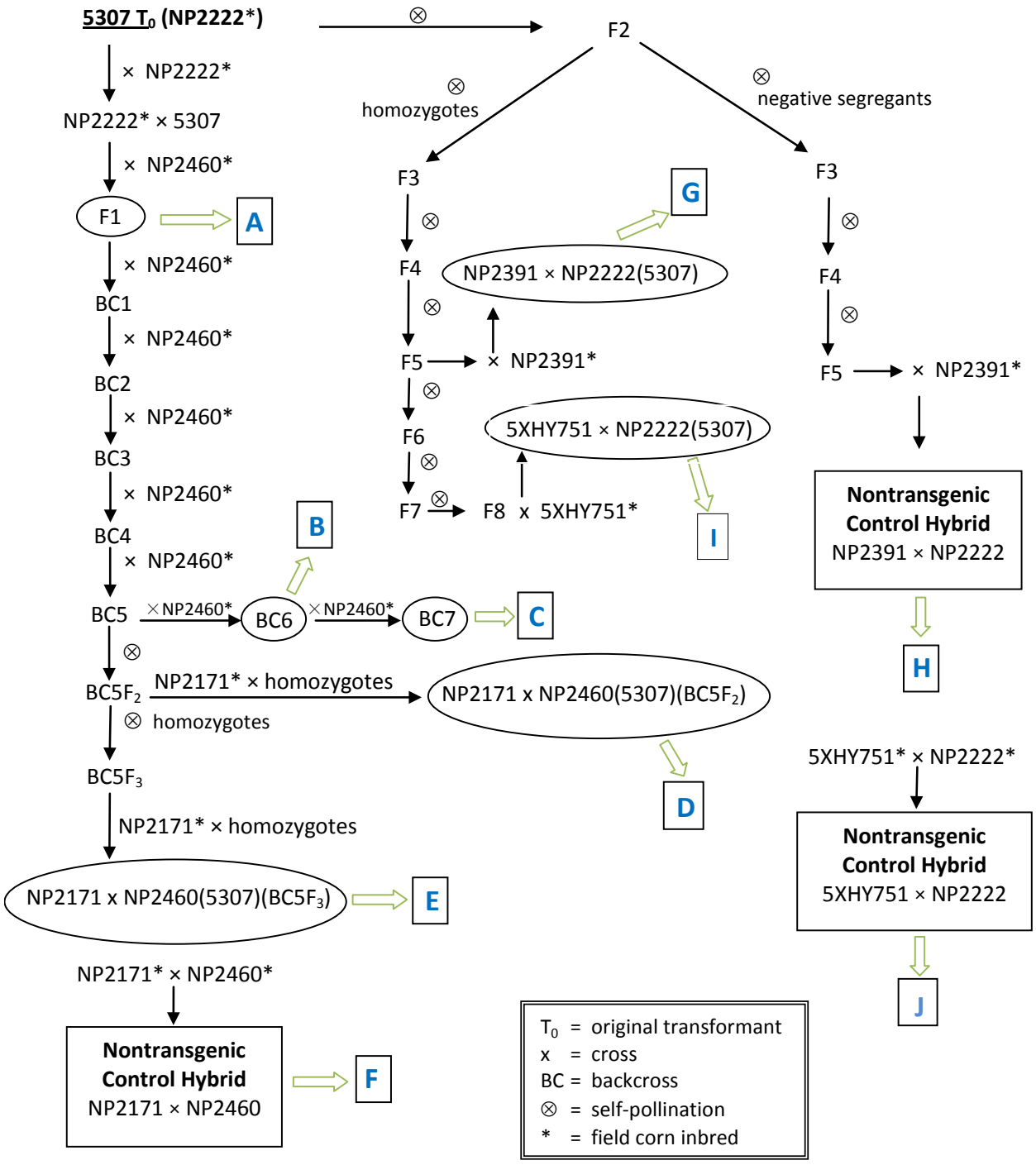


Figure III-3. Pedigree diagram of Event 5307 and control seed materials

Generations in ovals were Event 5307 materials used in studies described in this Petition. Generations in rectangles were used as controls. Boxed letter codes correspond to specific seed lots used in studies listed in Table III-1. A backcross (BC) is a cross of an individual with one of its parents. The initial Event 5307 F_1 generation was crossed with one of its inbred parents, NP2460, to create generation BC1. Likewise, BC2 originated from a cross of the BC1 with the parental line NP2460. Successive backcrosses with the recurrent parent followed similarly to introgress the transgenes into NP2460 germplasm.

III.D. Quality Control Testing of Seed Materials

All test and control seed lots were analyzed for the presence of Event 5307 DNA and adventitious DNA from other transformation events using the real-time PCR method described in Appendix B. All Event 5307 seed lots were confirmed to contain the genes *ecry3.1Ab* and *pmi* based on nucleotide sequence. Additionally, all Event 5307 seed lots were confirmed to contain Event-5307-specific DNA based on nucleotide sequences at the junction of the T-DNA insert and the corn genome. The analyses did not detect these components in control seed lots. All test and control seed lots had no detectable sequences that would be indicative of DNA from other regulated events under development at Syngenta, or deregulated events for which testing methodology is available.

IV. Donor Genes and Regulatory Sequences

Event 5307 corn was produced by *Agrobacterium tumefaciens*-mediated transformation of immature corn embryos using the transformation plasmid vector pSYN12274. The DNA region between the left and right borders of the transformation plasmid included gene expression cassettes for *ecry3.1Ab* and *pmi* (also known as *manA*); this T-DNA was transferred into the corn genome during transformation. The *ecry3.1Ab* expression cassette consisted of the *ecry3.1Ab* coding region regulated by a cestrum yellow leaf curling virus promoter (CMP) and a nopaline synthase (NOS) polyadenylation (terminator) sequence. The *pmi* expression cassette consisted of the *pmi* coding region regulated by a *Zea mays* polyubiquitin (ZmUbiInt) promoter and the NOS terminator sequence. A schematic of the plasmid vector is shown in Figure III-1. The size and description of each genetic element in the vector are shown in Table IV-1 below.

Table IV-1. Description of genetic elements in vector pSYN12274.

Genetic element	Size (bp)	Position	Description
Active ingredient cassette			
Intervening sequence	203	26 to 228	Noncoding intervening sequence with restriction sites used for cloning
CMP promoter	346	229 to 574	Cestrum Yellow Leaf Curling Virus promoter region (Hohn et al., 2007; Stavolone et al., 2003b). Provides constitutive expression in corn.
Intervening sequence	9	575 to 583	Noncoding ntervening sequence with restriction sites used for cloning
ecry3.1Ab	1962	584 to 2545	An engineered Cry gene active against certain corn rootworm (*Diabrotica*) species (Entrez® Accession No. GU327680 [NCBI, 2010a]). The gene *ecry3.1Ab* (Walters et al., 2010) 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 descriptions of *mcry3A* and *cry1Ab* and Figure IV-1 below). Upstream of the *mcry3A* domain, the gene *ecry3.1Ab* carries a 67-bp-long 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 a portion of mCry3A, followed by 172 residues that are identical to a portion of Cry1Ab. Figure IV-1 illustrates the origins of the corresponding amino acid sequences in eCry3.1Ab. Description of *mcry3A*: 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 *Bacillus 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 methionine 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 sequence alanine-alanine-proline-phenylalanine, and has replaced the amino acids valine-155, serine-156, and serine-157 in the native protein (Chen and Stacy, 2003). Description of *cry1Ab*: The gene *cry1Ab* was originally cloned from *Bacillus thuringiensis* subsp*. kurstaki* strain HD-1 (Geiser et al., 1986). Its sequence was codon-optimized (Koziel et al., 1997) to accommodate the preferred codon usage for corn (Murray et al., 1989).
Intervening sequence	30	2546 to 2575	Noncoding intervening sequence with restriction sites used for cloning
NOS	253	2576 to 2828	Terminator sequence from the nopaline synthase gene of *Agrobacterium tumefaciens* (Entrez Accession Number V00087 [NCBI, 2010a]). This sequence provides a polyadenylation site (Depicker et al., 1982).

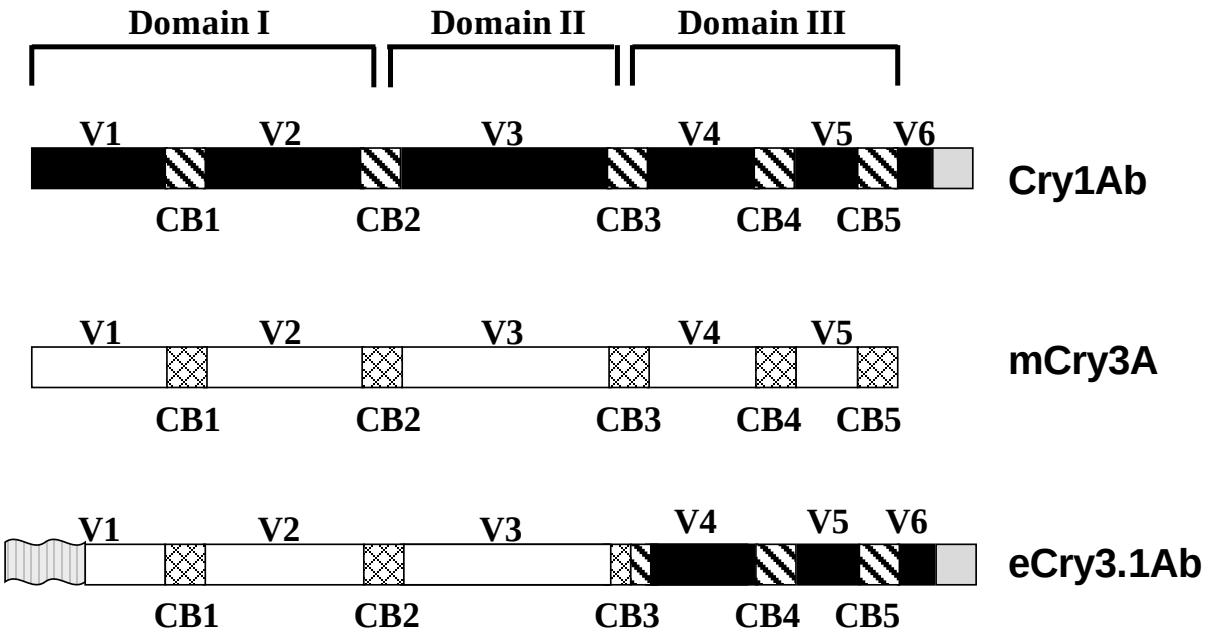
Table IV-1 (*Continued*). Description of genetic elements in vector pSYN12274

Genetic element	Size (bp)	Position	Description
Selectable marker cassette			
Intervening sequence	25	2829 to 2853	Noncoding intervening sequence with restriction sites used for cloning
ZmUbiInt promoter	1993	2854 to 4846	Promoter region from the maize polyubiquitin gene which contains the first intron (Entrez® Accession Number S94464 [NCBI, 2010a]). Provides constitutive expression in monocots (Christensen et al., 1992)
Intervening sequence	12	4847 to 4858	Noncoding intervening sequence with restriction sites used for cloning
pmi	1176	4859 to 6034	*Escherichia coli* strain K-12 gene *pmi* encoding the enzyme phosphomannose isomerase (PMI) (Entrez Accession Number M15380 [NCBI, 2010a]); this gene is also known as *manA*. Catalyzes the isomerization of mannose-6-phosphate to fructose-6-phosphate (Negrotto et al., 2000).
Intervening sequence	60	6035 to 6094	Noncoding intervening sequence with restriction sites used for cloning
NOS	253	6095 to 6347	Terminator sequence from the nopaline synthase gene of *Agrobacterium tumefaciens* (Entrez Accession Number V00087 [NCBI, 2010a]). This sequence provides a polyadenylation site (Depicker et al., 1982).
Intervening sequence	88	6348 to 6435	Noncoding intervening sequence with restriction sites used for cloning
Plasmid backbone			
Left border (LB)	25	6436 to 6460	Left border region of T-DNA from *Agrobacterium tumefaciens* nopaline Ti-plasmid (Entrez® Accession Number J01825 [NCBI, 2010a]). Short direct repeat sequence that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell (Zambryski et al., 1982)
Intervening sequence	349	6461 to 6809	Noncoding intervening sequence with restriction sites used for cloning
spec	789	6810 to 7598	Streptomycin adenylyltransferase gene, *aadA*, from *Escherichia coli* transposon Tn7 (similar to Entrez® Accession Number X03043 [NCBI, 2010a]). Confers resistance to streptomycin and spectinomycin and is used as a bacterial selectable marker (Fling et al., 1985)
Intervening sequence	299	7599 to 7897	Noncoding intervening sequence with restriction sites used for cloning
virG	726	7898 to 8623	The VirGN54D gene (*virG*) from pAD1289 (similar to Entrez® Accession Number AF242881 [NCBI, 2010a). The N54D substitution results in a constitutive virG phenotype. VirG is part of the two-component regulatory system for the virulence (*vir*) regulon in *Agrobacterium tumefaciens* (Hansen et al., 1994).
Intervening sequence	29	8624 to 8652	Noncoding intervening sequence with restriction sites used for cloning

Table IV-1 (*Continued*). Description of genetic elements in vector pSYN12274

Genetic element	Size (bp)	Position	Description
Plasmid backbone (*continued*)			
repA	1074	8653 to 9726	Gene encoding the pVS1 replication protein from *Pseudomonas aeruginosa* (similar to Entrez® Accession Number AF133831 [NCBI, 2010a]), which is a part of the minimal pVS1 replicon that is functional in Gram-negative, plant-associated bacteria (Heeb et al., 2000)
Intervening sequence	42	9727 to 9768	Noncoding intervening sequence with restriction sites used for cloning
VS1 ori	405	9769 to 10173	Consensus sequence for the origin of replication and partitioning region from plasmid pVS1 of *Pseudomonas aeruginosa* (Entrez® Accession Number U10487 [NCBI 2010a]). Serves as origin of replication in *Agrobacterium tumefaciens* host (Itoh et al., 1984)
Intervening sequence	677	10174 to 10850	Noncoding intervening sequence with restriction sites used for cloning
ColE1 ori	807	10851 to 11657	Origin of replication (similar to Entrez® Accession Number V00268 [NCBI 2010a]) that permits replication of plasmids in *Escherichia coli* (Itoh and Tomizawa, 1979)
Intervening sequence	112	11658 to 11769	Noncoding intervening sequence with restriction sites used for cloning
Right border (RB)	25	1 to 25	Right border region of T-DNA from *Agrobacterium tumefaciens* nopaline Ti-plasmid (Entrez® Accession Number J01826 [NCBI 2010a]). 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)

A.



B.

```
ecry3.1Ab   ATG ACT AGT AAC GGC CGC CAG TGT GCT GGT ATT CGC CCT TAT GAC GGC CGA
eCry3.1Ab   M   T   S   N   G   R   Q   C   A   G   I   R   P   Y   D   G   R

mcry3A      ..................................................ATG ACG GCC GAC
mCry3A                                                       M   T   A   D


ecry3.1Ab    CAA CAA CAC CGA GGC  C-TG GAC AGC AGC ACC ACC AAG GAC GTG
eCry3.1Ab    Q   Q   H   R   G    L   D   S   S   T   T   K   D   V

mcry3A       AAC AAC ACC GAG GCC  CTG GAC AGC AGC ACC ACC AAG GAC GTG
mCry3A       N   N   T   E   A    L   D   S   S   T   T   K   D   V
```

Figure IV-1. A. Schematic illustrating the origin of the amino acid residues present in eCry3.1Ab. B. Nucleotide alignment between *ecry3.1Ab* and *mcry3A* and corresponding amino acids present at the N-termini of eCry3.1Ab and mCry3A.

(A) The black rectangles represent the variable regions 1 through 6 (V1 through V6) of Cry1Ab; the rectangles with diagonal lines represent the conserved blocks 1 through 5 (CB1 through CB5) of Cry1Ab; the gray rectangles represent the Cry1Ab tail sequence. The white rectangles represent the variable regions 1 through 5 of mCry3A; the rectangles with crosshatch lines represent the conserved blocks 1 through 5 of mCry3A. The vertically striped portion of eCry3.1Ab represents the N-terminal amino-acids unique to eCry3.1Ab (see Figure IV-1B).

(B) The dotted line symbolizes sequence absent from mCry3A; the dash indicates a one-base-pair deletion in *ecry3.1Ab*. The first 40 nucleotides of *ecry3.1Ab* (and the 13 amino acids they encode) are in bold text to highlight their addition during the engineering of the gene. The 9 amino acids LDSSTTKDV shown for both proteins in bold text indicate where the amino acid sequence of eCry3.1Ab is restored to that of mCry3A.

V. Genetic Characterization of Event 5307 Corn

An extensive genetic characterization of the T-DNA insert in Event 5307 corn was conducted by Southern blot analyses and nucleotide sequencing. Further, the genetic stability of the insert was assessed both by Southern blot analyses and by examining the inheritance patterns of the transgenes over multiple generations of 5307 plants. Additionally, the corn genomic sequences flanking the Event 5307 insert were identified and characterized. Finally, it was determined that the Event 5307 T-DNA insertion into the corn genome did not disrupt the function of any known corn gene. These data collectively demonstrate that there are no deleterious changes in the 5307 corn genome as a result of the T-DNA insertion.

Descriptions, results and conclusions of each genetic characterization study are provided in Parts V.A to V.G., below. Details of the materials and methods used in these studies are provided in Appendix B. A general summary of the conclusions of these studies is provided in Part V.H., below.

V.A. Nucleotide Sequence of the T-DNA Insert

Two overlapping DNA fragments that span the 5307 corn insert were amplified from genomic DNA extracted from 5307 corn using a polymerase chain reaction method. These fragments were cloned, and sequences of the clones were aligned to create a consensus of the T-DNA sequence. The consensus nucleotide sequence data for the 5307 corn insert were compared to the sequence of the transformation plasmid pSYN12274 (Figure III-1). The data demonstrated that the insert was intact and that the organization of the functional elements within the insert, as present in plasmid pSYN12274, was maintained. The functional elements *ecry3.1Ab, pmi,* the CMP promoter, the ZmUbiInt promoter, and the NOS terminators in 5307 corn were identical to those in the transformation plasmid pSYN12274.

One nucleotide change was identified in the 5307 corn insert 48 bp upstream of the CMP promoter in a non-coding region of the T-DNA (Figure III-1). This nucleotide change has no effect on the transgenes encoded by 5307 corn.

Sequence analysis revealed that some truncation occurred at the right border (RB) and left border (LB) ends of the T-DNA during the transformation process. The entire RB, three bp of non-coding sequence at the 5′ end of the insert, and eight bp of the LB were truncated. These deletions had no effect on the functionality of the insert; similar deletions have previously been observed in transformations with *Agrobacterium tumefaciens* (Tinland and Hohn, 1995; Brunaud et al., 2002; Chilton and Que, 2003).

V.B. Copy Number of Functional Elements

Southern blot analyses demonstrated that 5307 corn contains a single copy of *ecry3.1Ab, pmi*, the CMP promoter sequence, and the ZmUbiInt promoter sequence and two copies of the NOS terminator sequence, as expected for a single insertion site. Results also indicated that there are no extraneous DNA fragments of the functional elements elsewhere in the

5307 corn genome, and that 5307 corn is free of backbone sequence from the transformation plasmid pSYN12274.

Southern blot analyses to establish the copy number of each functional element utilized five element-specific probes (the *ecry3.1Ab*-specific probe, the *pmi*-specific probe, the CMP promoter-specific probe, the ZmUbiInt promoter-specific probe, and the NOS terminator-specific probe). Southern blot analyses included genomic DNA extracted from 5307 plants of genotype 5307 NP2171 × $BC5F_3$ and the near-isogenic control plants of genotype NP2171 × NP2460 (see breeding pedigree in Figure III-3). Each Southern blot contained a positive control and a negative control. The positive control, representing one copy of a fragment of known size in the corn genome, was included to demonstrate the sensitivity of each experiment. The negative control, DNA extracted from plants grown from nontransgenic corn seed, was included to identify possible endogenous DNA sequences that hybridize with the probe. The ZmUbiInt promoter-specific probe cross-hybridizes to genomic DNA fragments of different sizes in the different corn genotypes due to restriction fragment length polymorphism of the genomic DNA that carries the endogenous maize polyubiquitin promoter. For the Southern blot analyses with the ZmUbiInt promoter-specific probe, genomic DNA extracts from control plants of inbred genotypes NP2222, NP2460, and NP2171 were also needed because the 5307 corn generation analyzed was created by crossing with these corn lines.

For these experiments, genomic DNA was analyzed using two restriction enzyme digestion strategies to assess the presence, size, intactness, and copy number of each functional element. The locations of restriction endonuclease sites are indicated in Figure V-1. In the first strategy, the corn genomic DNA was digested with an enzyme that cut once within the 5307 corn insert. The other recognition sites for this enzyme were located in the corn genome flanking the 5307 corn insert. This first strategy was used twice with two different enzymes to determine the copy number of the functional element and the presence or absence of extraneous DNA fragments of the functional elements of plasmid pSYN12274 in other regions of the 5307 corn genome. For Southern blot analyses with the *ecry3.1Ab*-specific probe, enzymes *Kpn*I and *Nco*I were used; for Southern blot analyses with the *pmi*-specific probe, enzymes *Bst*EII and *Spe*I were used; for Southern blot analyses with the CMP promoter-specific probe, enzymes *Kpn*I and *Spe*I were used; for Southern blot analyses with the ZmUbiInt promoter-specific probe, enzymes *Bst*EII and *Spe*I were used; and for Southern blot analyses with the NOS terminator-specific probe, enzymes *Kpn*I and *Nco*I were used.

For Southern blot analyses with the *ecry3.1Ab*-specific probe, the *pmi*-specific probe, the CMP promoter-specific probe, and the ZmUbiInt promoter-specific probe, these digests were expected to result in only one hybridization band specific to the corresponding functional element. More than one band with either digest would have indicated that there were multiple copies of the corresponding functional element in the plant genome. For Southern blot analyses with the NOS terminator-specific probe, the digests used were expected to result in two hybridization bands corresponding to the two NOS terminator sequences in the 5307 corn insert (the NOS terminator sequence regulating *ecry3.1Ab* and

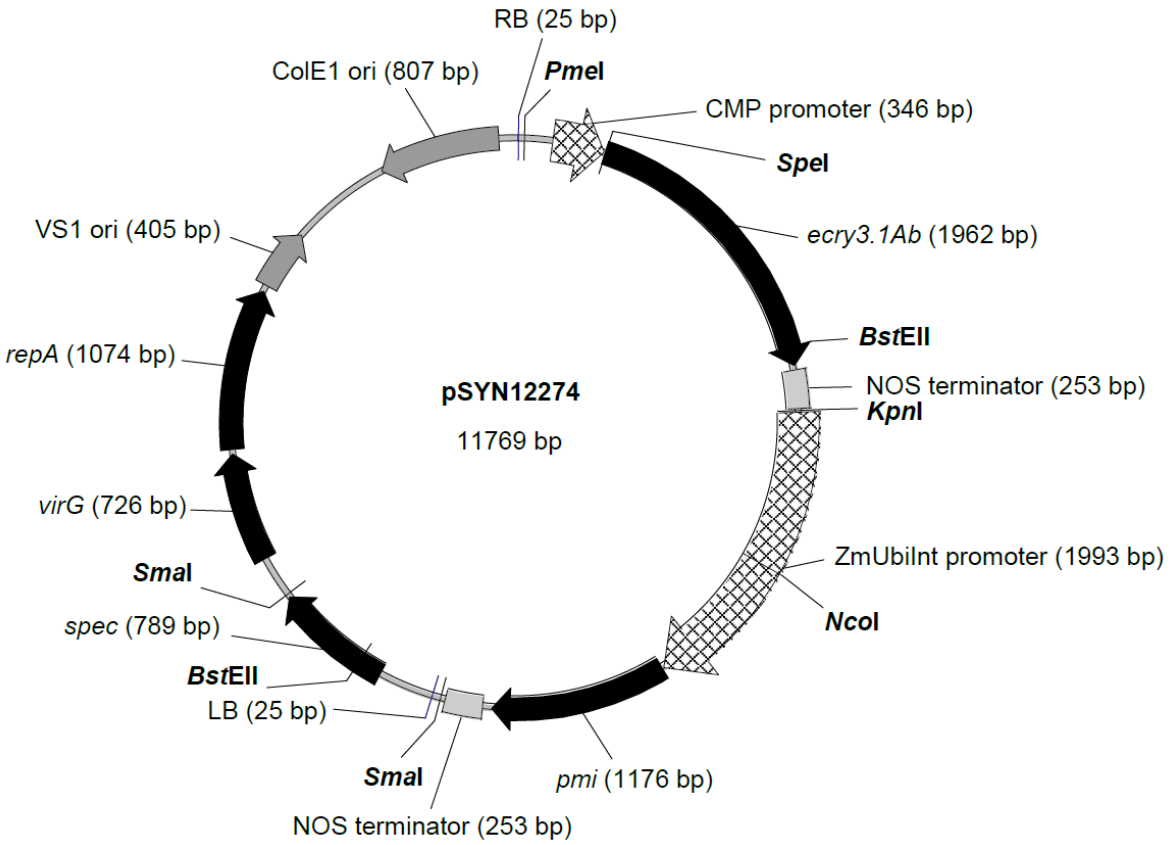


Figure V-1. Map of plasmid pSYN12274 indicating the restriction sites used in the Southern blot analyses (shown in bold type).

the NOS terminator sequence regulating *pmi*). More than two bands with either digest would have indicated additional copies of the NOS terminator sequence in the plant genome. In the second strategy, the corn genomic DNA was digested with two enzymes, which released a DNA fragment of predictable size. This strategy was used to determine the presence or absence of any closely linked extraneous DNA fragments of the functional elements in plasmid pSYN12274. For this strategy, enzymes *Sma*I + *Pme*I were used for all Southern blot analyses with element-specific probes.

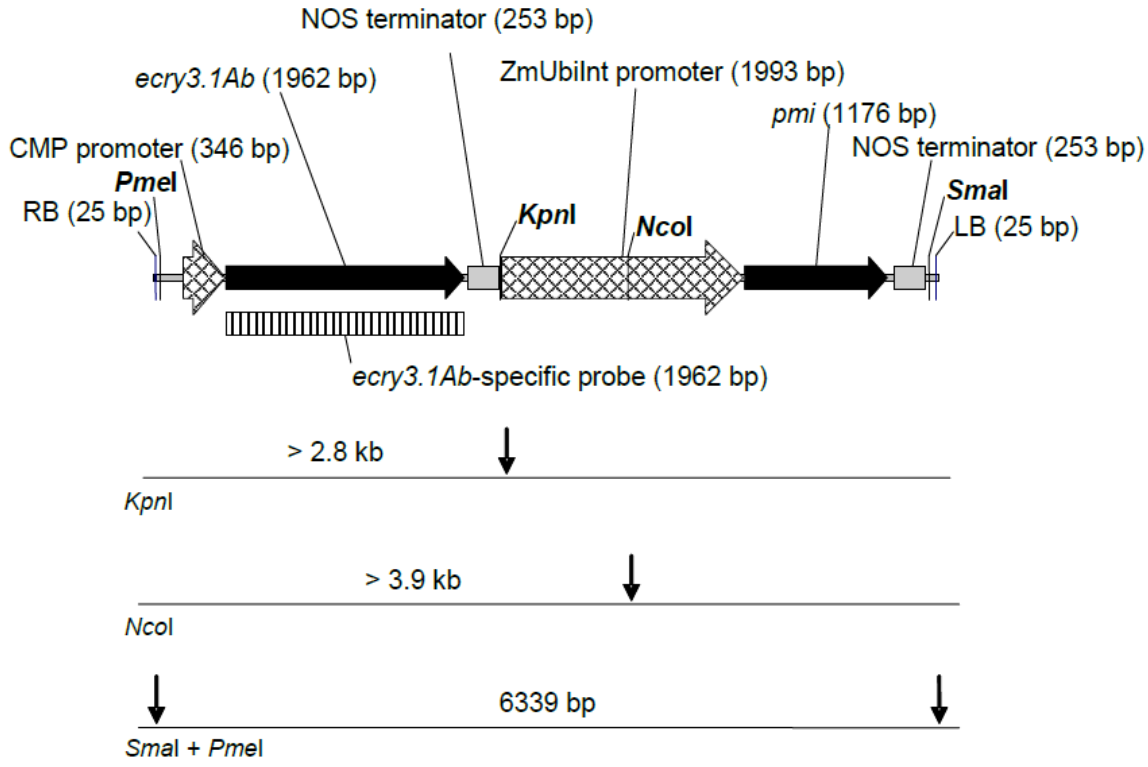
Details of each Southern blot analysis are described below. For each analysis, there is a map showing the location of the restriction endonuclease sites in relation to each element probe used.

V.B.1. Copy Number of Functional Elements: *ecry3.1Ab* Gene

A probe containing the entire sequence of *ecry3.1Ab* was used for the *ecry3.1Ab* Southern blot analyses. A map of the T-DNA region in plasmid pSYN12274 indicating locations of the *ecry3.1Ab* coding sequence and the restriction endonuclease sites targeted in these Southern blot analyses is shown in Figure V-2. The results of these analyses are displayed in Figure V-3. Table V-1 shows a comparison of expected versus observed hybridization band sizes.

For Southern blot analysis of genomic DNA digested with *Kpn*I and probed with the *ecry3.1Ab*-specific probe, one hybridization band of approximately 8.5 kb was observed in the lane containing DNA extracted from 5307 NP2171 × $BC5F_3$ plants (Figure V-3A, Lane A3) (Table V-1). This hybridization band was absent in the lane containing DNA extracted from the control plants (Figure V-3A, Lane A4) and was, therefore, specific to the 5307 corn insert. One hybridization band of approximately 6.3 kb was observed in the lane containing the positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Sma*I + *Pme*I and loaded with DNA extracted from NP2171 × NP2460 plants) (Figure V-3A, Lane A5).

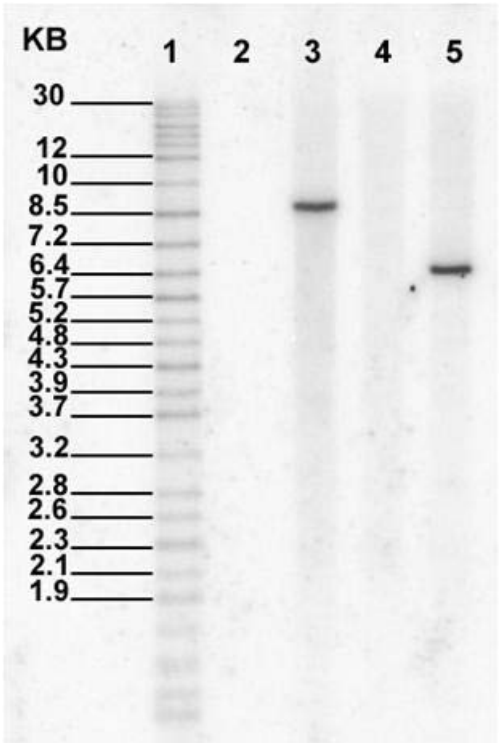
For Southern blot analysis of genomic DNA digested with *Nco*I and probed with the *ecry3.1Ab*-specific probe, one hybridization band of approximately 19 kb was observed in the lane containing DNA extracted from 5307 NP2171 × $BC5F_3$ plants (Figure V-3B, Lane B3) (Table V-1). This hybridization band was absent in the lane containing DNA extracted from the control plants (Figure V-3B, Lane B4) and was, therefore, specific to the 5307 corn insert. One hybridization band of approximately 3.9 kb was observed in the lane containing the positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Sma*I + *Pme*I + *Nco*I and loaded with DNA extracted from NP2171 × NP2460 plants) (Figure V-3B, Lane B5).



The vertical arrows indicate the site of restriction digestion.
Sizes of the expected restriction fragments are indicated.

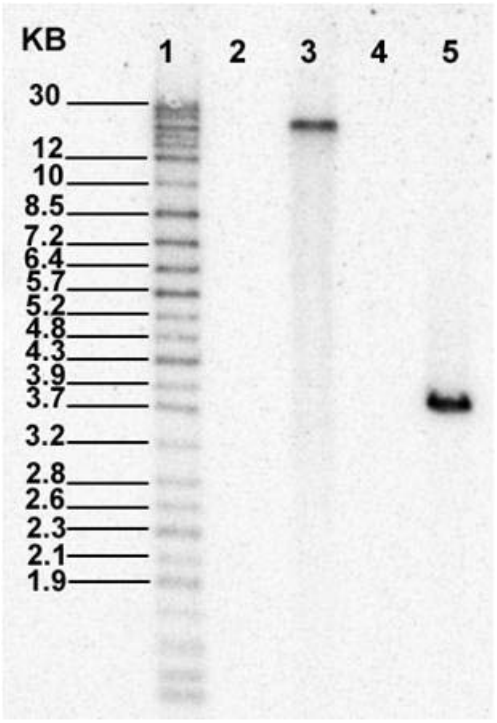
Figure V-2. Location of the *Kpn*I, *Nco*I, *Sma*I, *and Pme*I restriction sites and position of the 1962-bp *ecry3.1Ab*-specific probe in the T-DNA region of the transformation plasmid pSYN12274.

(A) *Kpn*I



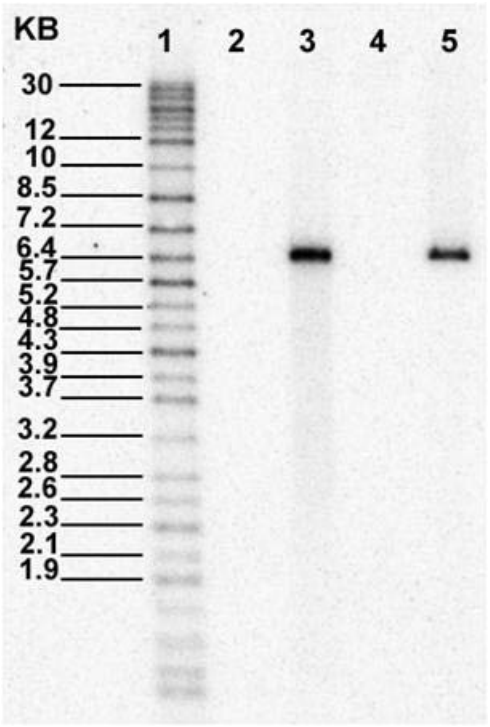
Lane A1 = molecular weight markers
Lane A2 = blank
Lane A3 = 5307 NP2171 × BC5F$_3$ digested with *Kpn*I
Lane A4 = NP2171 × NP2460 digested with *Kpn*I
Lane A5 = Positive control (NP2171 × NP2460 digested with *Kpn*I and 16.53 pg of pSYN12274 digested with *Sma*I + *Pme*I)

(B) *Nco*I



Lane B1 = molecular weight markers
Lane B2 = blank
Lane B3 = 5307 NP2171 × BC5F$_3$ digested with *Nco*I
Lane B4 = NP2171 × NP2460 digested with *Nco*I
Lane B5 = Positive control (NP2171 × NP2460 digested with *Nco*I and 16.53 pg of pSYN12274 digested with *Sma*I + *Pme*I + *Nco*I)

(C) *Sma*I + *Pme*I



Lane C1 = molecular weight markers
Lane C2 = blank
Lane C3 = 5307 NP2171 × BC5F$_3$ digested with *Sma*I + *Pme*I
Lane C4 = NP2171 × NP2460 digested with *Sma*I + *Pme*I
Lane C5 = Positive control (NP2171 × NP2460 digested with *Sma*I + *Pme*I and 16.53 pg of pSYN12274 digested with *Sma*I + *Pme*I)

Figure V-3. Southern blot analysis of 5307 corn for copy number of functional elements: 1962-bp *ecry3.1Ab*-specific probe, using restriction enzymes *Kpn*I, *Nco*I, *and Sma*I + *Pme*I

Table V-1. Expected and observed hybridization band sizes in Southern blot analysis of 5307 corn, using an *ecry3.1Ab*-specific probe and restriction enzymes *Kpn*I, *Nco*I, and *Sma*I + *Pme*I.

Figure & Lane No.	Source of DNA	Restriction enzyme	Expected number of bands	Expected band size (kb)	Observed band size (kb)
Fig. V-3A, Lane A3	5307 NP2171 × $BC5F_3$	*Kpn*I	1	> 2.8	~ 8.5
Fig. V-3A, Lane A4	NP2171 × NP2460	*Kpn*I	none	none	none
Fig. V-3A, Lane A5	Positive control (16.53 pg of pSYN12274 mixed with NP2171 × NP2460 digested with *Kpn*I)	*Sma*I + *Pme*I	1	~ 6.3	~ 6.3
Fig. V-3B, Lane B3	5307 NP2171 × $BC5F_3$	*Nco*I	1	> 3.9	~ 19
Fig. V-3B, Lane B4	NP2171 × NP2460	*Nco*I	none	none	none
Fig. V-3B, Lane B5	Positive control (16.53 pg of pSYN12274 mixed with NP2171 × NP2460 digested with *Nco*I)	*Sma*I + *Pme*I + *Nco*I[1]	1	~ 3.9	~ 3.9
Fig. V-3C, Lane C3	5307 NP2171 × $BC5F_3$	*Sma*I + *Pme*I	1	~ 6.3	~ 6.3
Fig. V-3C, Lane C4	NP2171 × NP2460	*Sma*I + *Pme*I	none	none	none
Fig. V-3C, Lane C5	Positive control (16.53 pg of pSYN12274 mixed with NP2171 × P2460 digested with *Sma*I + *Pme*I)	*Sma*I + *Pme*I	1	~ 6.3	~ 6.3

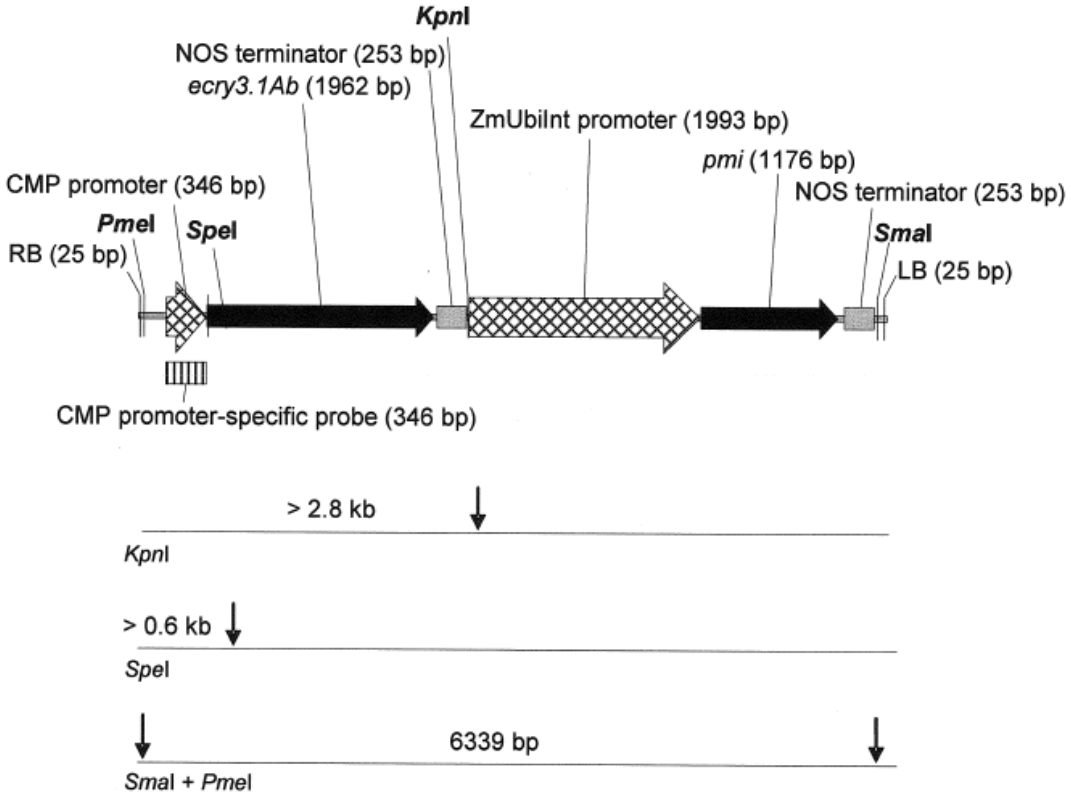
[1] Digestion of pSYN12274 with *Nco*I was the result of addition to NP2171 × NP2460 digested with *Nco*I

For Southern blot analysis of genomic DNA digested with *Sma*I + *Pme*I and probed with the *ecry3.1Ab*-specific probe, one hybridization band of approximately 6.3 kb was observed in the lane containing DNA extracted from 5307 NP2171 × $BC5F_3$ plants (Figure V-3C, Lane C3) (Table V-1). This hybridization band was absent in the lane containing DNA extracted from the control plants (Figure V-3C, Lane C4) and was, therefore, specific to the 5307 corn insert. One hybridization band of approximately 6.3 kb was observed in the lane containing the positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Sma*I + *Pme*I and loaded with DNA extracted from NP2171 × NP2460 plants) (Figure V-3C, Lane C5).

For Southern blot analyses with the *ecry3.1Ab*-specfic probe, detection of only one hybridization band of the expected size for each restriction enzyme digestion strategy demonstrated that the 5307 corn insert contains a single copy of *ecry3.1Ab* at a single locus. No unexpected bands were detected, indicating that there were no extraneous DNA fragments of *ecry3.1Ab* in the 5307 corn genome.

V.B.2. Copy Number of Functional Elements: CMP promoter

A probe containing the entire sequence of the CMP promoter was used for the CMP promoter Southern blot analyses. A map of the T-DNA region in plasmid pSYN12274 indicating locations of the CMP promoter sequence and the restriction endonuclease sites targeted in these Southern blot analyses is shown in Figure V-4. The results of these analyses are displayed in Figure V-5. Table V-2 shows a comparison of expected versus observed hybridization band sizes.



The vertical arrows indicate the site of restriction digestion.
Sizes of the expected restriction fragments are indicated.

Figure V-4. Location of the *Kpn*I, *Spe*I, *Sma*I, and *Pme*I restriction sites and position of the 346-bp CMP promoter-specific probe in the T-DNA region of the transformation plasmid pSYN12274.

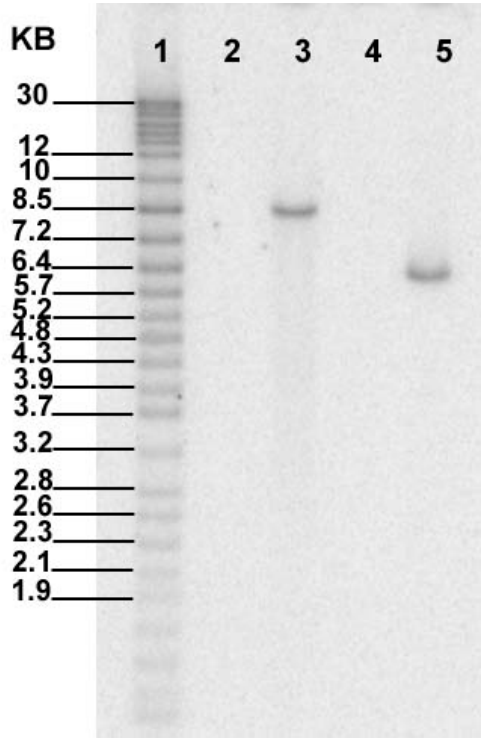
For Southern blot analysis of genomic DNA digested with *Kpn*I and probed with the CMP promoter-specific probe, one hybridization band of approximately 8.5 kb was observed in the lane containing DNA extracted from 5307 NP2171 × $BC5F_3$ plants as expected (Figure V-5A, Lane A3) (Table V-2). This hybridization band was absent in the lane containing DNA extracted from the control plants (Figure V-5A, Lane A4) and was therefore, specific to the 5307 corn insert. One hybridization band of approximately 6.3 kb was observed in the lane containing the positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Sma*I + *Pme*I and loaded with DNA extracted from NP2171 × NP2460 plants) (Figure V-5A, Lane A5).

For Southern blot analysis of genomic DNA digested with *Spe*I and probed with the CMP promoter-specific probe, one hybridization band of approximately 2.6 kb was observed in the lane containing DNA extracted from 5307 NP2171 × $BC5F_3$ plants as expected (Figure V-5B, Lane B3) (Table V-2). This hybridization band was absent in the lane containing DNA extracted from the control plants (Figure V-5B, Lane B4) and was therefore, specific to the 5307 corn insert. One hybridization band of approximately 6.3 kb was observed in the lane containing the positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Sma*I + *Pme*I and loaded with DNA extracted from NP2171 × NP2460 plants) (Figure V-5B, Lane B5).

For Southern blot analysis of genomic DNA digested with *Sma*I + *Pme*I and probed with the CMP promoter-specific probe, one hybridization band of approximately 6.3 kb was observed in the lane containing DNA extracted from 5307 NP2171 × $BC5F_3$ plants as expected (Figure V-5C, Lane C3) (Table V-2). This hybridization band was absent in the lane containing DNA extracted from the control plants (Figure V-5C, Lane C4) and was therefore, specific to the 5307 corn insert. One hybridization band of approximately 6.3 kb was observed in the lane containing the positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Sma*I + *Pme*I and loaded with DNA extracted from NP2171 × NP2460 plants) (Figure V-5C, Lane C5).

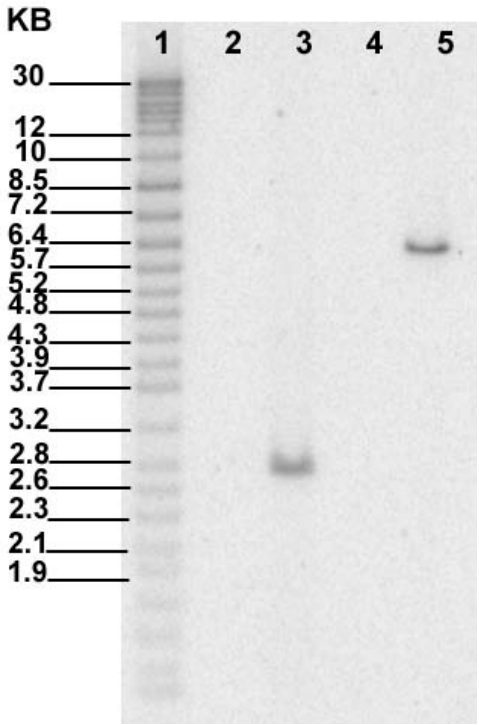
For Southern blot analyses with the CMP promoter-specfic probe, detection of only one hybridization band of the expected size for each restriction enzyme digestion strategy demonstrated that the 5307 corn insert contains a single copy of the CMP promoter sequence at a single locus. No unexpected bands were detected, indicating that there were no extraneous DNA fragments of the CMP promoter sequence in the 5307 corn genome.

(A) *Kpn*I



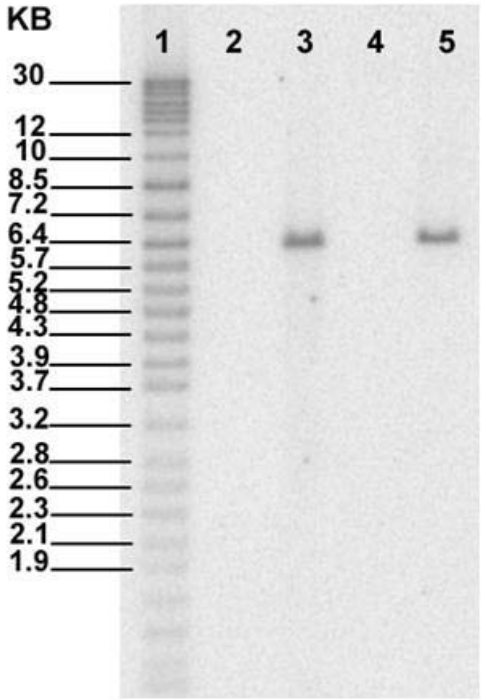
Lane A1 = molecular weight markers
Lane A2 = blank
Lane A3 = 5307 NP2171 × BC5F$_3$ digested with *Kpn*I
Lane A4 = NP2171 × NP2460 digested with *Kpn*I
Lane A5 = Positive control (NP2171 × NP2460 digested with *Kpn*I and 16.53 pg of pSYN12274 digested with *Sma*I + *Pme*I)

(B) *Spe*I



Lane B1 = molecular weight markers
Lane B2 = blank
Lane B3 = 5307 NP2171 × BC5F$_3$ digested with *Spe*I
Lane B4 = NP2171 × NP2460 digested with *Spe*I
Lane B5 = Positive control (NP2171 × NP2460 digested with *Spe*I and 16.53 pg of pSYN12274 digested with *Sma*I + *Pme*I)

(C) *Sma*I + *Pme*I



Lane C1 = molecular weight markers
Lane C2 = blank
Lane C3 = 5307 NP2171 × BC5F$_3$ digested with *Sma*I + *Pme*I
Lane C4 = NP2171 × NP2460 digested with *Sma*I + *Pme*I
Lane C5 = Positive control (NP2171 × NP2460 digested with *Sma*I + *Pme*I and 16.53 pg of pSYN12274 digested with *Sma*I + *Pme*I)

Figure V-5. Southern blot analysis of 5307 corn for copy number of functional elements: 346-bp CMP promoter-specific probe, using restriction enzymes *Kpn*I, *Spe*I, and *Sma*I + *Pme*I

Table V-2. Expected and observed hybridization band sizes in Southern blot analysis of 5307 corn, using a CMP promoter-specific probe and restriction enzymes *Kpn*I, *Spe*I, and *Sma*I + *Pme*I.

Figure & Lane No.	Source of DNA	Restriction enzyme	Expected number of bands	Expected band size (kb)	Observed band size (kb)
Fig. V-5A, Lane A3	5307 NP2171 × $BC5F_3$	*Kpn*I	1	> 2.8	~ 8.5
Fig. V-5A, Lane A4	NP2171 × NP2460	*Kpn*I	none	none	None
Fig.V-5A, Lane A5	Positive control (16.53 pg of pSYN12274 mixed with NP2171 × NP2460 digested with *Kpn*I)	*Sma*I + *Pme*I	1	~ 6.3	~ 6.3
Fig. V-5B, Lane B3	5307 NP2171 × $BC5F_3$	*Spe*I	1	> 0.6	~ 2.6
Fig. V-5B, Lane B4	NP2171 × NP2460	*Spe*I	none	none	none
Fig. V-5B, Lane B5	Positive control (16.53 pg of pSYN12274 mixed with NP2171 × NP2460 digested with *Spe*I)	*Sma*I + *Pme*I	1	~ 6.3	~ 6.3
Fig. V-5C, Lane C3	5307 NP2171 × $BC5F_3$	*Sma*I + *Pme*I	1	~ 6.3	~ 6.3
Fig. V-5C, Lane C4	NP2171 × NP2460	*Sma*I + *Pme*I	none	none	none
Fig. V-5C, Lane C5	Positive control (16.53 pg of pSYN12274 mixed with NP2171 × NP2460 digested with *Sma*I + *Pme*I)	*Sma*I + *Pme*I	1	~ 6.3	~ 6.3

V.B.3. Copy Number of Functional Elements: *pmi* Gene

A probe containing the entire sequence of *pmi* was used for the *pmi* Southern blot analyses. Figure V-6 shows a map of the T-DNA of 5307 corn transformation plasmid pSYN12274, indicating the location of the *pmi*-specific probe and restriction sites for *Bst*EII, *Spe*I, *Sma*I, and *Pme*I. Figure V-7 depicts the results of the corresponding Southern blot analyses, and Table V-3 provides the expected and observed sizes of the hybridization bands.

For Southern blot analysis of genomic DNA digested with *Bst*EII and probed with the *pmi*-specific probe, one hybridization band of approximately 7.2 kb was observed in the lane containing DNA extracted from 5307 NP2171 × $BC5F_3$ plants (Figure V-7, Lane 3) (Table V-3). This hybridization band was absent in the lane containing DNA extracted from the control plants (Figure V-7, Lane 4) and was, therefore, specific to the 5307 corn insert.

For Southern blot analysis of genomic DNA digested with *Spe*I and probed with the *pmi*-specific probe, one hybridization band of approximately 7.0 kb was observed in the lane containing DNA extracted from 5307 NP2171 × $BC5F_3$ plants (Figure V-7, Lane 5) (Table V-3). This hybridization band was absent in the lane containing DNA extracted from the control plants (Figure V-7, Lane 6) and was, therefore, specific to the 5307 corn insert.

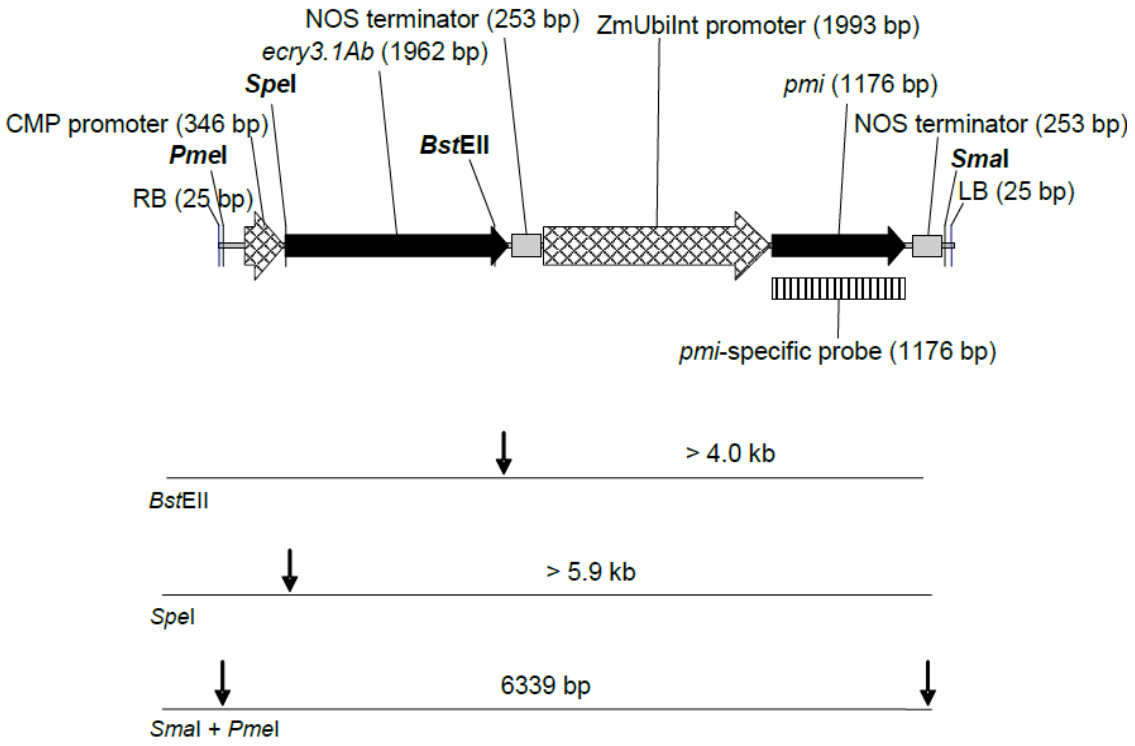
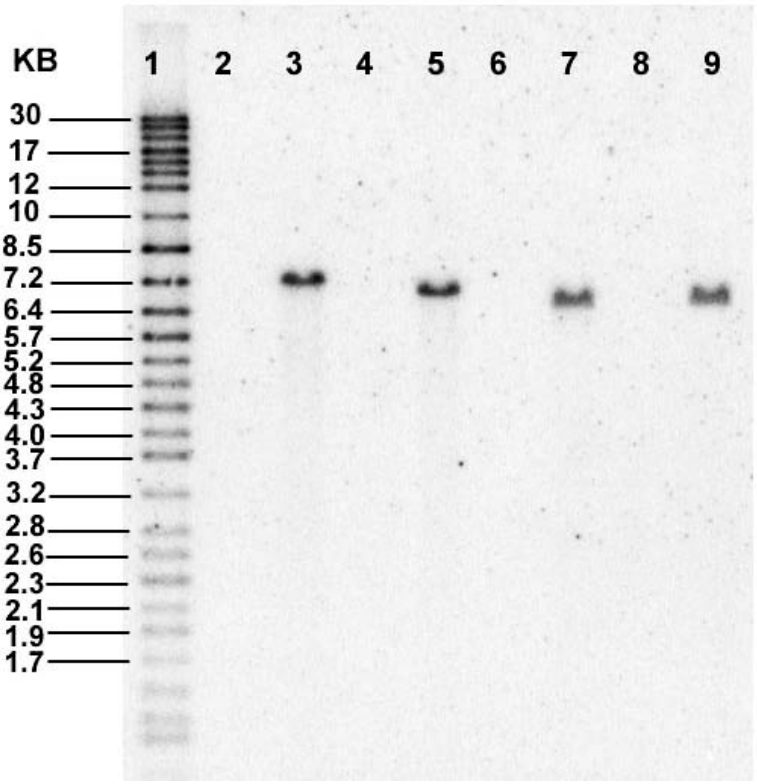


Figure V-6. Location of the *Bst*EII, *Spe*I, *Sma*I, and *Pme*I restriction sites and position of the 1176-bp *pmi*-specific probe in the T-DNA region of the transformation plasmid pSYN12274.

For Southern blot analysis of genomic DNA digested with *Sma*I + *Pme*I and probed with the *pmi*-specific probe, one hybridization band of approximately 6.3 kb was observed in the lane containing DNA extracted from 5307 NP2171 × $BC5F_3$ plants (Figure V-7, Lane 7) (Table V-3). This hybridization band was absent in the lane containing DNA extracted from the control plants (Figure V-7, Lane 8) and was, therefore, specific to the 5307 corn insert. One hybridization band of approximately 6.3 kb was observed in the lane containing the positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Sma*I + *Pme*I and loaded with DNA extracted from NP2171 × NP2460 plants) (Figure V-7, Lane 9).

For Southern blot analyses with the *pmi*-specfic probe, detection of only one hybridization band of the expected size for each restriction enzyme digestion strategy demonstrated that the 5307 corn insert contains a single copy of *pmi* at a single locus. No unexpected bands were detected, indicating that there were no extraneous DNA fragments of *pmi* in the 5307 corn genome.



Lane 1 = molecular weight markers
Lane 2 = blank
Lane 3 = 5307 NP2171 × $BC5F_3$ digested with *Bst*EII
Lane 4 = NP2171 × NP2460 digested with *Bst*EII
Lane 5 = 5307 NP2171 × $BC5F_3$ digested with *Spe*I
Lane 6 = NP2171 × NP2460 digested with *Spe*I
Lane 7 = 5307 NP2171 × $BC5F_3$ digested with *Sma*I + *Pme*I
Lane 8 = NP2171 × NP2460 digested with *Sma*I + *Pme*I
Lane 9 = Positive control (NP2171 × NP2460 digested with *Sma*I + *Pme*I and 16.53 pg of pSYN12274 digested with *Sma*I + *Pme*I)

Figure V-7. Southern blot analysis of 5307 corn for copy number of functional elements: 1176-bp *pmi*-specific probe, using restriction enzymes *Bst*EII, *Spe*I, and *Sma*I + *Pme*I.

Table V-3. Expected and observed hybridization band sizes in Southern blot analysis of 5307 corn, using a *pmi*-specific probe and restriction enzymes *Bst*EII, *Spe*I, and *Sma*I + *Pme*I.

Fig. V-7 Lane No.	Source of DNA	Restriction enzyme	Expected number of bands	Expected band size (kb)	Observed band size (kb)
3	5307 NP2171 × $BC5F_3$	*Bst*EII	1	> 4.0 kb	~ 7.2 kb
4	NP2171 × NP2460	*Bst*EII	none	none	none
5	5307 NP2171 × $BC5F_3$	*Spe*I	1	> 5.9 kb	~ 7.0 kb
6	NP2171 × NP2460	*Spe*I	none	none	none
7	5307 NP2171 × $BC5F_3$	*Sma*I + *Pme*I	1	~ 6.3 kb	~ 6.3 kb
8	NP2171 × NP2460	*Sma*I + *Pme*I	none	none	none
9	Positive control (16.53 pg of pSYN12274 mixed with NP2171 × NP2460 digested with *Sma*I + *Pme*I)	*Sma*I + *Pme*I	1	~ 6.3 kb	~ 6.3 kb

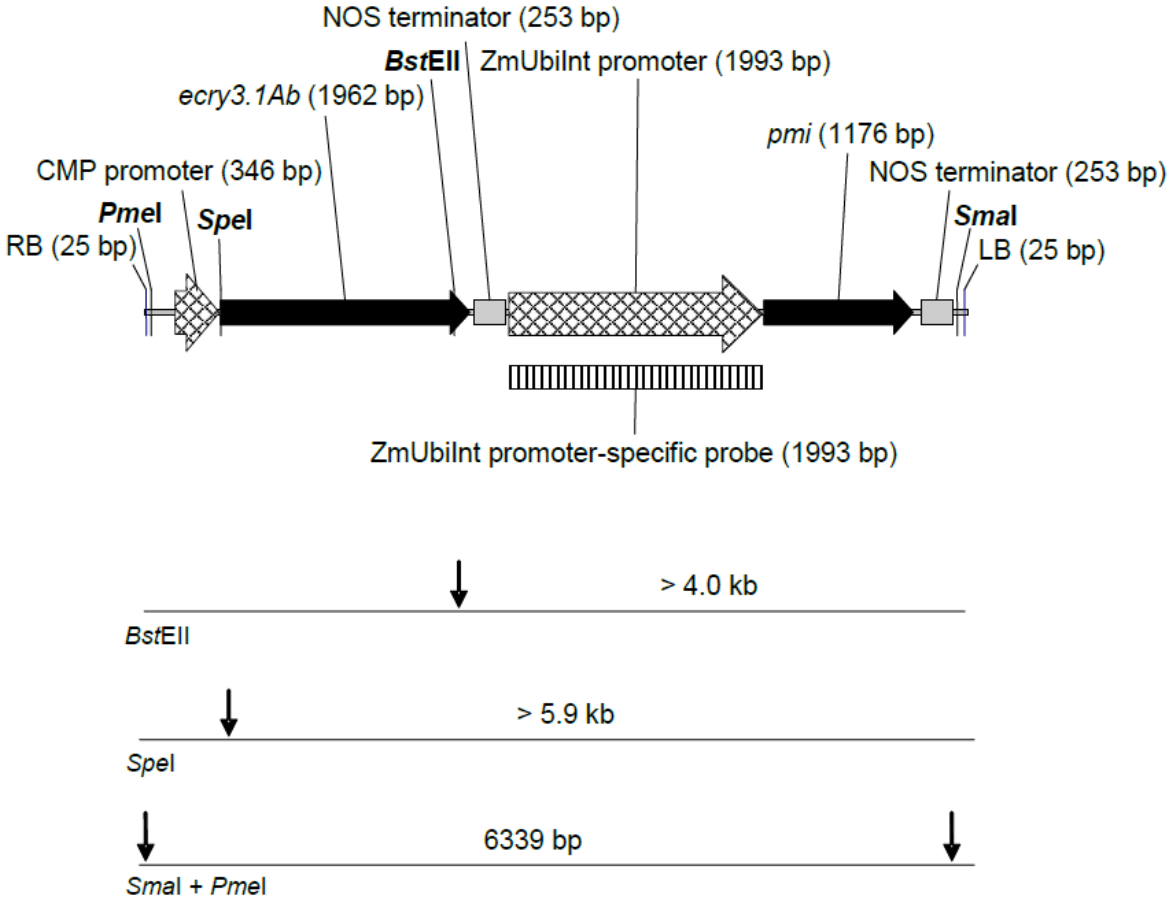
V.B.4. Copy Number of Functional Elements: ZmUbiInt promoter

A probe containing the entire sequence of the ZmUbiInt promoter was used for the ZmUbiInt promoter Southern blot analyses. Figure V-8 shows a map of the T-DNA of 5307 corn transformation plasmid pSYN12274, indicating the location of the ZmUbiInt promoter-specific probe and restriction sites for *Bst*EII, *Spe*I, *Sma*I, and *Pme*I. Figure V-9 depicts the results of the corresponding Southern blot analyses, and Table V-4 provides the expected and observed sizes of the hybridization bands.

For Southern blot analysis of genomic DNA digested with *Bst*EII and probed with the ZmUbiInt promoter-specific probe, a hybridization band of approximately 7.2 kb was observed in the lane containing DNA extracted from 5307 NP2171 × $BC5F_3$ plants as expected (Figure V-9A, Lane A3) (Table V-4). This hybridization band was absent in the lanes containing DNA extracted from the control plants NP2171 × NP2460 (Figure V-9A, Lane 4) and additional controls NP2171, NP2222 and NP2460 (Figure V-9A, Lanes A5, A6, and A7) and was, therefore, specific to the 5307 corn insert. Three hybridization bands of approximately 3.9 kb, 8.4 kb, and 18 kb were observed in the lane containing the positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Sma*I + *Pme*I + *Bst*EII and loaded with DNA extracted from NP2171 × NP2460 plants) (Figure V-9A, Lane A8).

Additional bands resulting from cross-hybridization of the ZmUbiInt promoter-specific probe with the endogenous maize polyubiquitin promoter sequence were also detected in all material analyzed. Two hybridization bands of approximately 12 kb and 18 kb corresponding to the hybridization bands observed in lanes containing DNA extracted from NP2222 plants (Figure V-9A, Lane A6) and NP2171 plants (Figure V-9A, Lane A5),

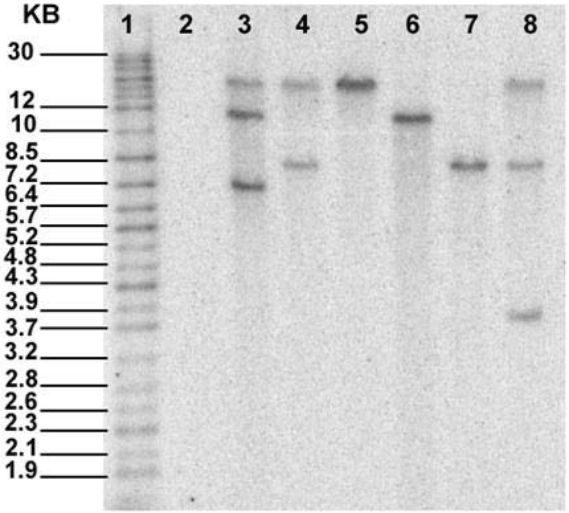
respectively, were observed in the lane containing DNA extracted from 5307 NP2171 × $BC5F_3$ plants (Figure V-9A, Lane A3). Two hybridization bands of approximately 8.4 kb and 18 kb corresponding to the hybridization bands observed in lanes containing DNA extracted from NP2460 plants (Figure V-9A, Lane A7) and NP2171 plants (Figure V-9A, Lane 5), respectively, were observed in the lane containing DNA extracted from NP2171 × NP2460 plants (Figure V-9A, Lane A4) and the lane containing the positive control (Figure V-9A, Lane A8).



The vertical arrows indicate the site of restriction digestion.
Sizes of the expected restriction fragments are indicated.

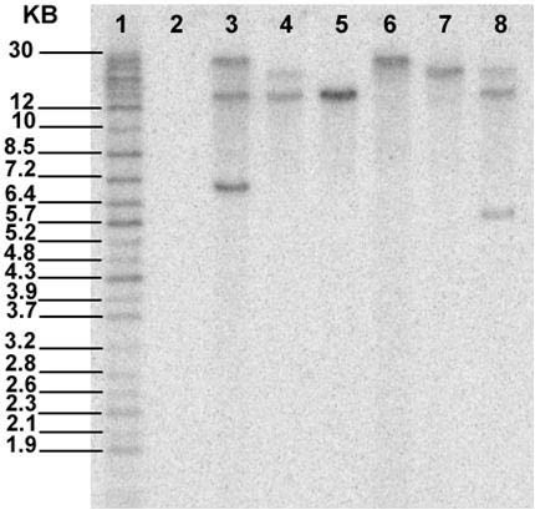
Figure V-8. Location of the *Bst*EII, *Spe*I, *Sma*I, and *Pme*I restriction sites and position of the 1993-bp ZmUbiInt promoter-specific probe in the T-DNA region of the transformation plasmid pSYN12274.

(A) *Bst*EII



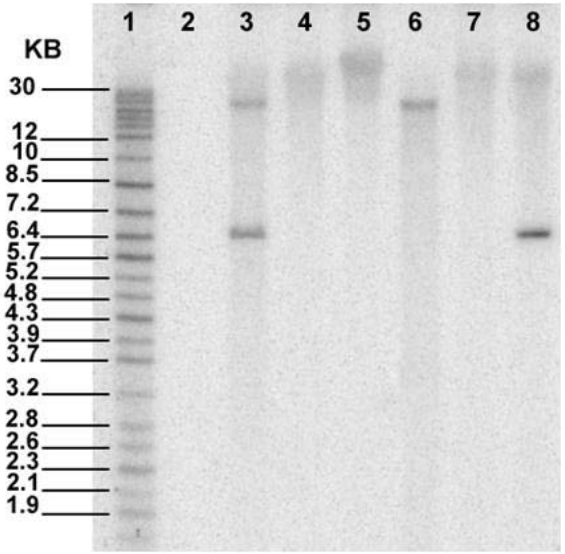
Lane A1 = molecular weight markers
Lane A2 = blank
Lane A3 = 5307 NP2171 × BC5F$_3$ digested with *Bst*EIII
Lane A4 = NP2171 × NP2460 digested with *Bst*EII
Lane A5 = NP2171 digested with *Bst*EII
Lane A6 = NP2222 digested with *Bst*EII
Lane A7 = NP2460 digested with *Bst*EII
Lane A8 = Positive control (NP2171 × NP2460 digested with *Bst*EII and 16.53 pg of pSYN12274 digested with *Sma*I + *Pme*I + *Bst*EII)

(B) *Spe*I



Lane B1 = molecular weight markers
Lane B2 = blank
Lane B3 = 5307 NP2171 × BC5F$_3$ digested with *Spe*I
Lane B4 = NP2171 × NP2460 digested with *Spe*I
Lane B5 = NP2171 digested with *Spe*I
Lane B6 = NP2222 digested with *Spe*I
Lane B7 = NP2460 digested with *Spe*I
Lane B8 = Positive control (NP2171 × NP2460 digested with *Spe*I and 16.53 pg of pSYN12274 digested with *Sma*I + *Pme*I)

(C) *SmaI + PmeI*



Lane C1 = molecular weight markers
Lane C2 = blank
Lane C3 = 5307 NP2171 × BC5F$_3$ digested with *Sma*I + *Pme*I
Lane C4 = NP2171 × NP2460 digested with *Sma*I + *Pme*I
Lane C5 = NP2171 digested with *Sma*I + *Pme*I
Lane C6 = NP2222 digested with *Sma*I + *Pme*I
Lane C7 = NP2460 digested with *Sma*I + *Pme*I
Lane C8 = Positive control (NP2171 × NP2460 digested with *Sma*I + *Pme*I and 16.53 pg of pSYN12274 digested with *Sma*I + *Pme*I)

Figure V-9. Southern blot analysis of 5307 corn for copy number of functional elements: 1993-bp ZmUbiInt promoter-specific probe, using restriction enzymes *Bst*EII, *Spe*I, and *Sma*I + *Pme*I.

Table V-4. Expected and observed hybridization band sizes in Southern blot analysis of 5307 corn, using a ZmUbiInt promoter-specific probe and restriction enzymes *Bst*EII, *Spe*I, and *Sma*I + *Pme*I.

Figure & Lane No.	Source of DNA	Restriction enzyme	Expected number of bands	Expected band size (kb)	Observed band size (kb)
Fig. V-9A, Lane A3	5307 NP2171 × $BC5F_3$	*Bst*EII	1 5307 insert x endogenous	> 4.0 unknown	~ 7.2 ~ 12 (endogenous) ~ 18 (endogenous)
Fig. V-9A, Lane A4	NP2171 × NP2460	*Bst*EII	x endogenous	unknown	~ 8.4 (endogenous) ~ 18 (endogenous)
Fig. V-9A, Lane A5	NP2171	*Bst*EII	x endogenous	unknown	~ 18 (endogenous)
Fig. V-9A, Lane A6	NP2222	*Bst*EII	x endogenous	unknown	~ 12 (endogenous)
Fig. V-9A, Lane A7	NP2460	*Bst*EII	x endogenous	unknown	~ 8.4 (endogenous)
Fig. V-9A, Lane A8	Positive control (16.53 pg of pSYN12274 mixed with NP2171 × NP2460 digested with *Bst*EII)	*Sma*I + *Pme*I + *Bst*EII[1]	1 pSYN12274 x endogenous	~ 3.9 unknown	~ 3.9 ~ 8.4 (endogenous) ~ 18 (endogenous)
Fig. V-9B, Lane B3	5307 NP2171 × $BC5F_3$	*Spe*I	1 5307 insert x endogenous	> 5.9 unknown	~ 7.0 ~ 14 (endogenous) ~ 25 (endogenous)
Fig. V-9B, Lane B4	NP2171 × NP2460	*Spe*I	x endogenous	unknown	~ 14 (endogenous) ~ 20 (endogenous)
Fig. V-9B, Lane B5	NP2171	*Spe*I	x endogenous	unknown	~ 14 (endogenous)
Fig. V-9B, Lane B6	NP2222	*Spe*I	x endogenous	unknown	~ 25 (endogenous)
Fig. V-9B, Lane B7	NP2460	*Spe*I	x endogenous	unknown	~ 20 (endogenous)
Fig. V-9B, Lane B8	Positive control (16.53 pg of pSYN12274 mixed with NP2171 × NP2460 digested with *Spe*I)	*Sma*I + *Pme*I	1 pSYN12274 x endogenous	~ 6.3 unknown	~ 6.3 ~ 14 (endogenous) ~ 20 (endogenous)
Fig. V-9C, Lane C3	5307 NP2171 × $BC5F_3$	*Sma*I + *Pme*I	1 5307 insert x endogenous	~ 6.3 unknown	~ 6.3 ~ 18 (endogenous) > 30 (endogenous)
Fig.V-9C, Lane C4	NP2171 × NP2460	*Sma*I + *Pme*I	x endogenous	unknown	> 30 (endogenous)
Fig. V-9C, Lane C5	NP2171	*Sma*I + *Pme*I	x endogenous	unknown	> 30 (endogenous)
Fig. V-9C, Lane C6	NP2222	*Sma*I + *Pme*I	x endogenous	unknown	~ 18 (endogenous)
Fig. V-9C, Lane C7	NP2460	*Sma*I + *Pme*I	x endogenous	Unknown	> 30 (endogenous)
Fig. V-9C, Lane C8	Positive control (16.53 pg of pSYN12274 mixed with NP2171 × NP2460 digested with *Sma*I + *Pme*I)	*Sma*I + *Pme*I	1 pSYN12274 x endogenous	~ 6.3 unknown	~ 6.3 > 30 (endogenous)

x = unknown number

[1] Digestion of pSYN12274 with *Bst*EII was the result of addition to NP2171 × NP2460 digested with *Bst*EII

For Southern blot analysis of genomic DNA digested with *Spe*I and probed with the ZmUbiInt promoter-specific probe, a hybridization band of approximately 7.0 kb was observed in the lane containing DNA extracted from 5307 NP2171 × $BC5F_3$ plants as expected (Figure V-9B, Lane B3) (Table V-4). This hybridization band was absent in the lane containing DNA extracted from the control plants NP2171 × NP2460 (Figure V-9B, Lane B4) and additional controls NP2171, NP2222, and NP2460 (Figure V-9B, Lanes B5, B6, and B7) and was, therefore, specific to the 5307 corn insert. Three hybridization bands of approximately 6.3 kb, 14 kb, and 20 kb were observed in the lane containing the positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Sma*I + *Pme*I and loaded with DNA extracted from NP2171 × NP2460 plants (Figure V-9B, Lane B8).

Additional bands resulting from cross-hybridization of the ZmUbiInt promoter-specific probe with the endogenous maize polyubiquitin promoter sequence were also detected in all material analyzed. Two hybridization bands of approximately 25 kb and 14 kb corresponding to the hybridization bands observed in lanes containing DNA extracted from NP2222 plants (Figure V-9B, Lane B6) and NP2171 plants (Figure V-9B, Lane B5), respectively, were observed in the lane containing DNA extracted from 5307 NP2171 × $BC5F_3$ plants (Figure V-9B, Lane B3). Finally, two hybridization bands of approximately 20 kb and 14 kb corresponding to the hybridization bands observed in lanes containing DNA extracted from NP2460 plants (Figure V-9B, Lane B7) and NP2171 plants (Figure V-9B, Lane B5), respectively, were observed in the lane containing DNA extracted from NP2171 × NP2460 plants (Figure V-9B, Lane B4) and the lane containing the positive control (Figure V-9B, Lane B8).

For Southern blot analysis of genomic DNA digested with *Sma*I + *Pme*I and probed with the ZmUbiInt promoter-specific probe, one hybridization band of approximately 6.3 kb was observed in the lane containing DNA extracted from 5307 NP2171 × $BC5F_3$ plants as expected (Figure V-9C, Lane C3) (Table V-4). This hybridization band was absent in the lane containing DNA extracted from the control plants NP2171 × NP2460 (Figure V-9C, Lane C4) and additional controls NP2171, NP2222, and NP2460 (Figure V-9C, Lanes C5, C6, and C7) and was, therefore, specific to the 5307 corn insert. A hybridization band of approximately 6.3 kb and a high molecular weight band (greater than 30 kb) was observed in the lane containing the positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Sma*I + *Pme*I and loaded with DNA extracted from NP2171 × NP2460 plants) (Figure V-9C, Lane C8).

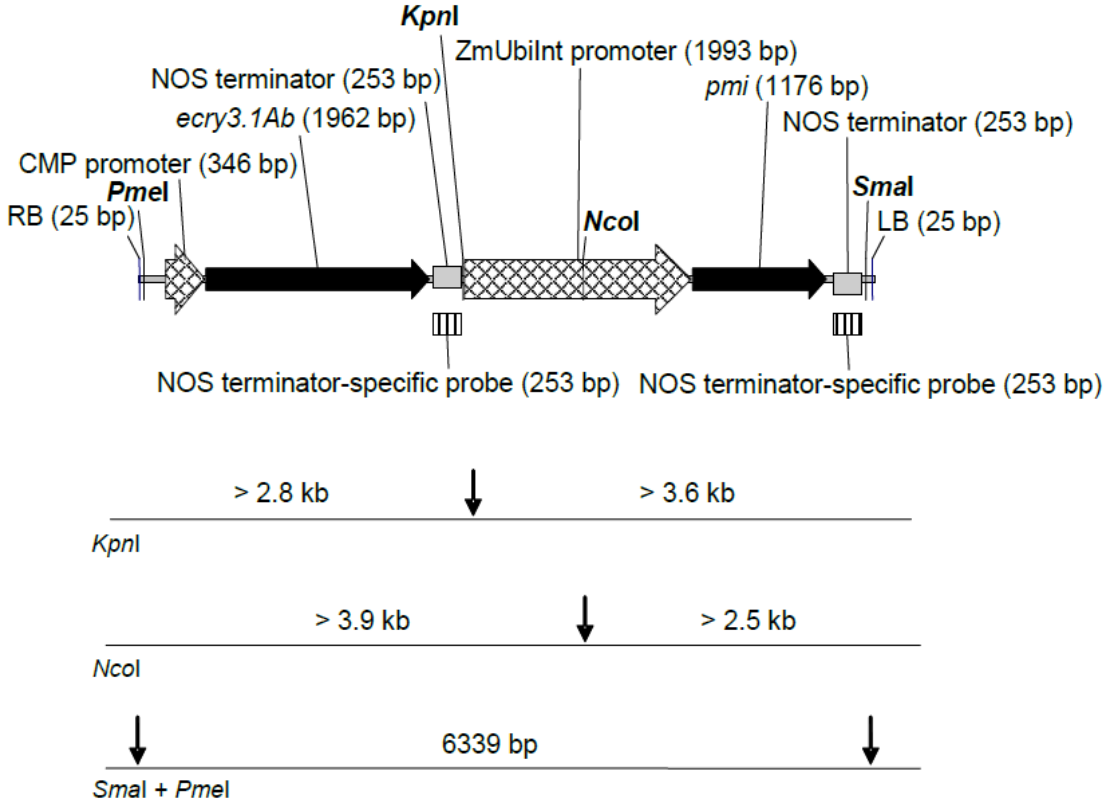
At least one additional band resulting from cross-hybridization of the ZmUbiInt promoter-specific probe with the endogenous maize polyubiquitin promoter sequence was also detected in all material analyzed. One hybridization band of approximately 18 kb corresponding to the hybridization band observed in the lane containing DNA extracted from NP2222 plants (Figure V-9C, Lane C6) was observed in the lane containing DNA extracted from 5307 NP2171 × $BC5F_3$ plants (Figure V-9C, Lane C3). An additional high molecular weight band (greater than 30 kb) was observed in lanes containing DNA extracted from NP2460 plants (Figure V-9C, Lane C7) and NP2171 plants (Figure V-9C, Lane C5). This faint high molecular weight band (greater than 30 kb) was also observed in the lane containing DNA extracted from 5307 NP2171 × $BC5F_3$ plants (Figure V-9C, Lane C3), the

lane containing DNA extracted from NP2171 × NP2460 plants (Figure V-9C, Lane C4), and the lane containing the positive control (Figure V-9C, Lane C8).

For Southern blot analyses with the ZmUbiInt promoter-specfic probe, detection of only one hybridization band specific to 5307 corn for each restriction enzyme digestion enzyme demonstrated that the 5307 corn genome contains a single copy of the ZmUbiInt promoter sequence at a single locus. No unexpected bands were detected, indicating that there were no extraneous DNA fragments of the ZmUbiInt promoter sequence in the 5307 corn genome.

V.B.5. Copy Number of Functional Elements: NOS terminator

A probe containing the entire sequence of the NOS terminator was used for the NOS terminator Southern blot analyses. Figure V-10 shows a map of the T-DNA of 5307 corn transformation plasmid pSYN12274, indicating the location of the NOS terminator-specific probe and restriction sites for *Kpn*I, *Nco*I, and *Sma*I + *Pme*I. Figure V-11 depicts the results of the corresponding Southern blot analyses, and Table V-5 provides the expected and observed sizes of the hybridization bands.



The vertical arrows indicate the site of restriction digestion.
Sizes of the expected restriction fragments are indicated.

Figure V-10. Location of the *Kpn*I, *Nco*I, *Sma*I, and *Pme*I restriction sites and position of the 253-bp NOS terminator-specific probe in the T-DNA region of the transformation plasmid pSYN12274.

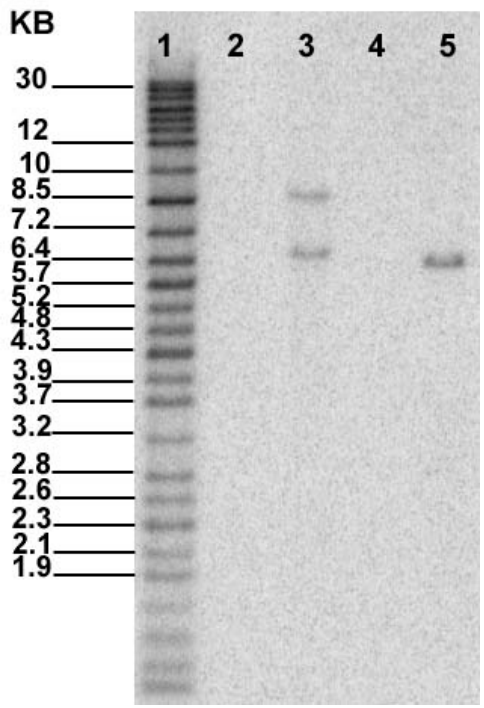
For Southern blot analysis of genomic DNA digested with *Kpn*I and probed with the NOS terminator-specific probe, two hybridization bands of approximately 6.4 kb and 8.5 kb were observed in the lane containing DNA extracted from 5307 NP2171 × $BC5F_3$ plants as expected (Figure V-11A, Lane A3) (Table V-5). These hybridization bands were absent in the lane containing DNA extracted from the control plants (Figure V-11A, Lanes A4) and were, therefore, specific to the two copies of the NOS terminator sequence in the 5307 corn insert (one NOS terminator sequence regulating *ecry3.1Ab* and one NOS terminator sequence regulating *pmi*). One hybridization band of approximately 6.3 kb was observed in the lane containing the positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Sma*I + *Pme*I and loaded with DNA extracted from NP2171 × NP2460 plants) (Figure V-11A, Lane A5).

For Southern blot analysis of genomic DNA digested with *Nco*I and probed with the NOS terminator-specific probe, two hybridization bands of approximately 16 kb and 19 kb were observed in the lane containing DNA extracted from 5307 NP2171 × $BC5F_3$ plants as expected (Figure V-11B, Lane B3) (Table V-5). These hybridization bands were absent in the lane containing DNA extracted from the control plants (Figure V-11B, Lanes B4) and were, therefore, specific to the two copies of the NOS terminator sequence in the 5307 corn insert (one NOS terminator sequence regulating *ecry3.1Ab* and one NOS terminator sequence regulating *pmi*). Two hybridization bands of approximately 2.5 kb and 3.9 kb were observed in the lane containing the positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Sma*I + *Pme*I + *Nco*I and loaded with DNA extracted from NP2171 × NP2460 plants) (Figure V-11B, Lane B5).

For Southern blot analysis of genomic DNA digested with *Sma*I + *Pme*I and probed with the NOS terminator-specific probe, one hybridization band of approximately 6.3 kb was observed in the lane containing DNA extracted from 5307 NP2171 × $BC5F_3$ plants as expected (Figure V-11C, Lane C3) (Table V-5). This hybridization band was absent in the lane containing DNA extracted from the control plants (Figure V-11C, Lanes C4) and was, therefore, specific to the 5307 corn insert. One hybridization band of approximately 6.3 kb was observed in the lane containing the positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Sma*I + *Pme*I and loaded with DNA extracted from NP2171 × NP2460 plants (Figure V-11C, Lane C9).

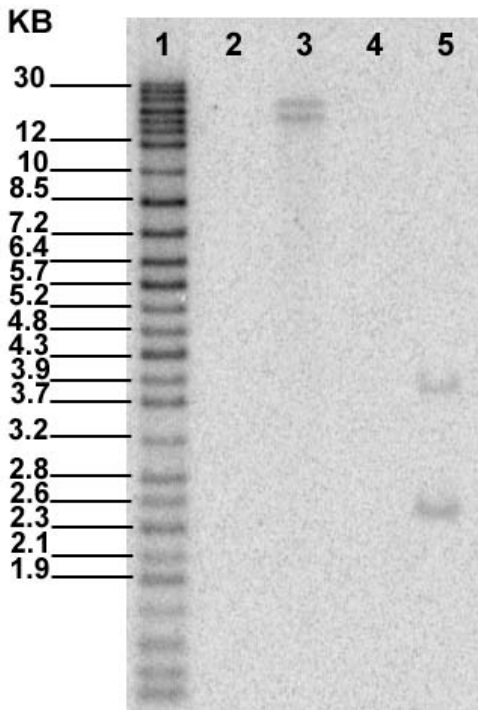
For Southern blot analyses with the NOS terminator-specific probe, detection of two hybridization bands of the expected size for each restriction enzyme digestion strategy demonstrated that 5307 corn contains two copies of the NOS terminator sequence (one NOS terminator sequence regulating *ecry3.1Ab* and one NOS terminator sequence regulating *pmi*) at a single locus. No unexpected bands were detected, indicating that there were no extraneous DNA fragments of the NOS terminator sequence in the 5307 corn genome.

(A) *Kpn*I



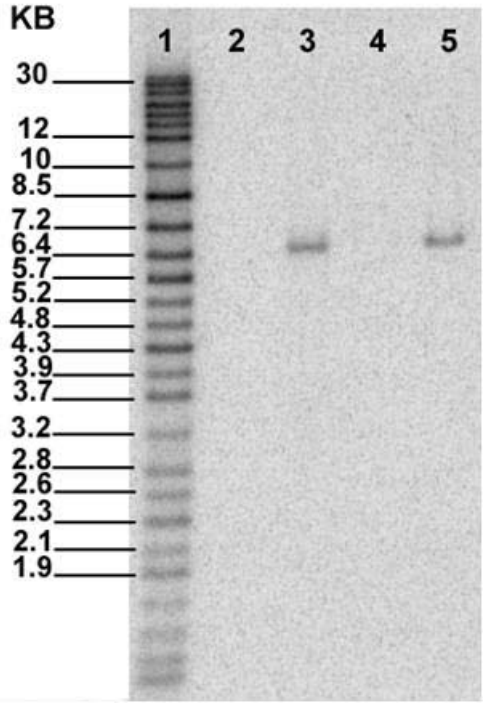
Lane A1 = molecular weight markers
Lane A2 = blank
Lane A3 = 5307 NP2171 × BC5F$_3$ digested with *Kpn*I
Lane A4 = NP2171 × NP2460 digested with *Kpn*I
Lane A5 = Positive control (NP2171 × NP2460 digested with *Kpn*I and 16.53 pg of pSYN12274 digested with *Sma*I + *Pme*I)

(B) *Nco*I



Lane B1 = molecular weight markers
Lane B2 = blank
Lane B3 = 5307 NP2171 × BC5F$_3$ digested with *Nco*I
Lane B4 = NP2171 × NP2460 digested with *Nco*I
Lane B5 = Positive control (NP2171 × NP2460 digested with *Nco*I and 16.53 pg of pSYN12274 digested with *Sma*I + *Pme*I + *Nco*I)

(C) *Sma*I + *Pme*I



Lane C1 = molecular weight markers
Lane C2 = blank
Lane C3 = 5307 NP2171 × BC5F$_3$ digested with *Sma*I + *Pme*I
Lane C4 = NP2171 × NP2460 digested with *Sma*I + *Pme*
Lane C5 = Positive control (NP2171 × NP2460 digested with *Sma*I + *Pme*I and 16.53 pg of pSYN12274 digested with *Sma*I + *Pme*I)

Figure V-11. Southern blot analysis of 5307 corn for copy number of functional elements: 253-bp NOS terminator-specific probe, using restriction enzymes *Kpn*I, *Nco*I, and *Sma*I + *Pme*I.

Table V-5. Expected and observed hybridization band sizes in Southern blot analysis of 5307 corn, using a NOS terminator-specific probe and restriction enzymes *Kpn*I, *Nco*I, and *Sma*I + *Pme*I.

Figure & Lane No.	Source of DNA	Restriction enzyme	Expected number of bands	Expected band size (kb)	Observed band size (kb)
Fig. V-11 A, Lane A3	5307 NP2171 × $BC5F_3$	*Kpn*I	2	> 2.8 > 3.6	~ 6.4 ~ 8.5
Fig. V-11A, Lane A4	NP2171/NP2460	*Kpn*I	none	none	none
Fig. V-11A, Lane A5	Positive control (16.53 pg of pSYN12274 mixed with NP2171/NP2460 digested with *Kpn*I)	*Sma*I + *Pme*I	1	~6.3	~ 6.3
Fig. V-11B, Lane B3	5307 NP2171 × $BC5F_3$	*Nco*I	2	> 2.5 > 3.9	~ 16 ~ 19
Fig. V-11B, Lane B4	NP2171/NP2460	*Nco*I	none	none	None
Fig. V-11B, Lane B5	Positive control (16.53 pg of pSYN12274 mixed with NP2171/NP2460 digested with *Nco*I)	*Sma*I + *Pme*I + *Nco*I[1]	2	~ 2.5 ~ 3.9	~ 2.5 ~ 3.9
Fig. V-11C, Lane C3	5307 NP2171 × $BC5F_3$	*Sma*I + *Pme*I	1	~ 6.3	~ 6.3
Fig. V-11C, Lane C4	NP2171/NP2460	*Sma*I + *Pme*I	none	none	none
Fig. V-11C, Lane C5	Positive control (16.53 pg of pSYN12274 mixed with NP2171/NP2460 digested with *Sma*I + *Pme*I)	*Sma*I + *Pme*I	1	~ 6.3	~ 6.3

[1] Digestion of pSYN12274 with *Nco*I was the result of addition to NP2171/NP2460 digested with *Nco*I

V.C. Absence of Plasmid Backbone Elements

Figure V-12 shows a map of the plasmid pSYN12274 indicating the location of the plasmid pSYN12274 backbone-specific probe and restriction sites for *Bst*EII, *Spe*I, and *Sma*I + *Pme*I. Figure V-13 depicts the results of the corresponding Southern blot analyses, and Table V-6 provides the expected and observed sizes of the hybridization bands.

For Southern blot analyses with genomic DNA digested with *Bst*EII and probed with the plasmid pSYN12274 backbone-specific probe, no hybridization bands were observed in the lanes containing DNA extracted from plants grown from 5307 NP2171 × $BC5F_3$ (Figure V-13, Lane 3) (Table V-6) and NP2171 × NP2460 (Figure V-13, Lane 4) (Table V-6) as expected.

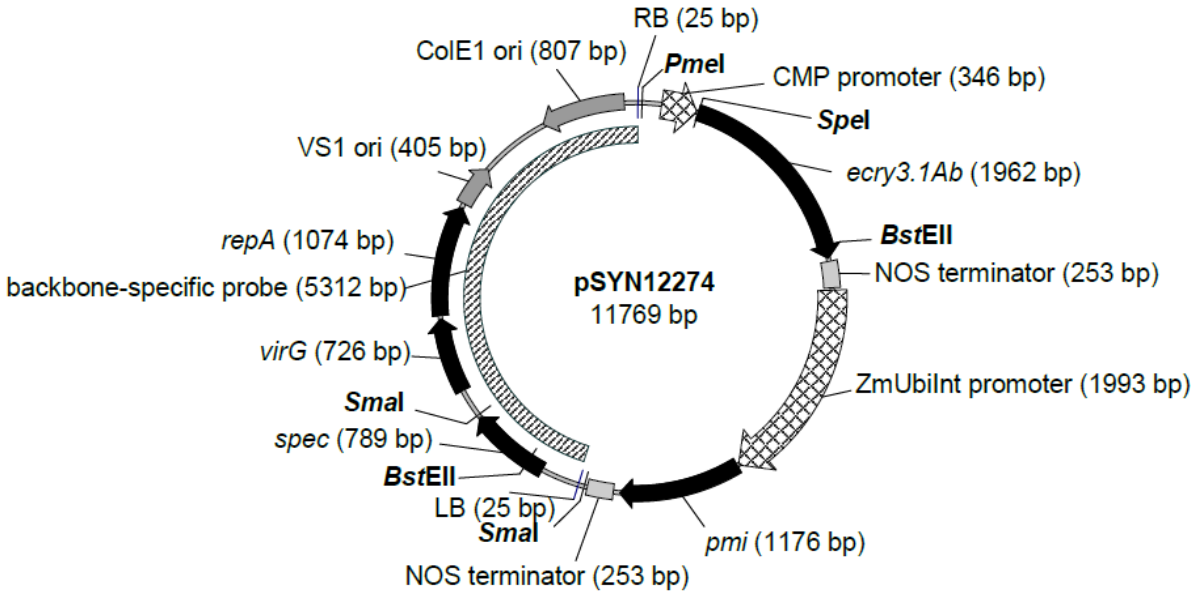
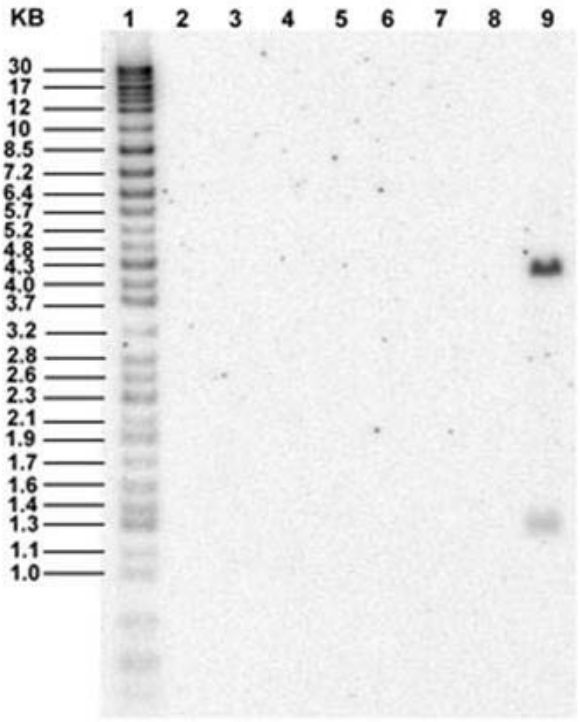


Figure V-12. Location of the *Bst*EII, *Spe*I, *Sma*I, and *Pme*I restriction sites and position of the 5312-bp plasmid backbone-specific probe in the transformation plasmid pSYN12274.

For Southern blot analyses with genomic DNA digested with *Spe*I and probed with the plasmid pSYN12274 backbone-specific probe, no hybridization bands were observed in the lanes containing DNA extracted from plants grown from 5307 NP2171 × $BC5F_3$ (Figure V-13, Lane 5) (Table V-6) and NP2171 × NP2460 (Figure V-13, Lane 6) (Table V-6) as expected.

For Southern blot analyses with genomic DNA digested with *Sma*I + *Pme*I and probed with the plasmid pSYN12274 backbone-specific probe, no hybridization bands were observed in the lanes containing DNA extracted from plants grown from 5307 NP2171 × $BC5F_3$ (Figure V-13, Lane 7) (Table V-6) and NP2171 × NP2460 (Figure V-13, Lane 8) (Table V-6) as expected. The positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Sma*I + *Pme*I and loaded with DNA extracted from NP2171 × NP2460 plants) produced the expected hybridization bands of approximately 4.2 kb and 1.2 kb band (Figure V-13, Lane 9).

No hybridization bands were detected in the 5307 plant DNA, illustrating that 5307 NP2171 × $BC5F_3$ corn does not contain any backbone sequences from the transformation plasmid pSYN12274.



Lane 1 = molecular weight markers
Lane 2 = blank
Lane 3 = 5307 NP2171 × $BC5F_3$ digested with *Bst*EII
Lane 4 = NP2171 × NP2460 digested with *Bst*EII
Lane 5 = 5307 NP2171 × $BC5F_3$ digested with *Spe*I
Lane 6 = NP2171 × NP2460 digested with *Spe*I
Lane 7 = 5307 NP2171 × $BC5F_3$ digested with *Sma*I + *Pme*I
Lane 8 = NP2171 × NP2460 digested with *Sma*I + *Pme*I
Lane 9 = Positive control (NP2171 × NP2460 digested with *Sma*I + *Pme*I and 16.53 pg of pSYN12274 digested with *Sma*I + *Pme*I)

Figure V-13. Southern blot analysis of 5307 corn for copy number of functional elements: 5312-bp plasmid backbone-specific probe, using restriction enzymes *Bst*EII, *Spe*I, and *Sma*I + *Pme*I.

Table V-6. Expected and observed hybridization band sizes in Southern blot analysis of 5307 corn, using a plasmid pSYN12274 backbone-specific probe and restriction enzymes *Bst*EII, *Spe*I, and *Sma*I + *Pme*I.

Lane No. in Figure V-13	Source of DNA	Restriction enzyme	Expected number of bands	Expected band size (kb)	Observed band size (kb)
3	5307 NP2171 × $BC5F_3$	*Bst*EII	none	none	none
4	NP2171/NP2460	*Bst*EII	none	none	none
5	5307 NP2171 × $BC5F_3$	*Spe*I	none	none	none
6	NP2171/NP2460	*Spe*I	none	none	none
7	5307 NP2171 × $BC5F_3$	*Sma*I + *Pme*I	none	none	none
8	NP2171/NP2460	*Sma*I + *Pme*I	none	none	none
9	Positive control (16.53 pg of pSYN12274 mixed with NP2171/NP2460 digested with *Sma*I + *Pme*I)	*Sma*I + *Pme*I	2	~ 1.2 ~ 4.2	~ 1.2 ~ 4.2

V.D. Genetic Stability of 5307 Corn Over Four Generations

Molecular analyses were performed to demonstrate the genetic stability of the 5307 corn insert over four generations. Southern blot analyses were performed using standard molecular biology techniques. Each Southern blot contained a positive control and a negative control. The positive control, representing one copy of a fragment of known size in the corn genome, was included to demonstrate the sensitivity of each experiment; the negative control, DNA extracted from plants grown from nontransgenic corn seed, was included in order to identify possible endogenous DNA sequences that hybridize with the probe. Two probes were used: a full-length T-DNA-specific probe containing every base of the plasmid pSYN12274 T-DNA and a plasmid pSYN12274 backbone-specific probe containing every base of plasmid pSYN12274 present outside of the T-DNA region.

These Southern blot analyses demonstrated that the 5307 corn genome contains a single, complete copy of the insert and that there are no extraneous DNA fragments of plasmid pSYN12274 T-DNA inserted elsewhere in the 5307 corn genome. Identical hybridization patterns across all generations of 5307 corn analyzed in this study indicate that the insert is stably inherited from one generation to the next. Additionally, every generation of 5307 corn examined was free of backbone sequence from the transformation plasmid pSYN12274.

Descriptions and results of these Southern blot analyses to assess genetic stability of 5307 corn are provided below. Details of the methods used are provided in Appendix B.

V.D.1. Stability of the T-DNA Using a Full-Length T-DNA-specific Probe

Genetic stability of the insert during conventional breeding of 5307 corn was determined by Southern blot analyses using a full length T-DNA-specific probe. The Southern blot

analyses included genomic DNA extracted from 5307 plants of four generations (5307 F_1, 5307 BC6, 5307 BC7, 5307 NP2171 × $BC5F_3$) and the corresponding nontransgenic, near-isogenic control genotypes (NP2171 × NP2460, NP2222, NP2460, and NP2171) (see Figure III-3 breeding pedigree). The full length T-DNA-specific probe, which contains sequence of the maize polyubiquitin promoter (ZmUbiInt), cross-hybridizes to genomic DNA fragments of different sizes in the different corn lines due to restriction fragment length polymorphism of the genomic DNA that carries the endogenous maize polyubiquitin promoter. Control genomic DNA from inbred lines NP2222, NP2460, and NP2171 was needed because the 5307 corn generations analyzed were created by crossing with these corn lines. For these experiments, genomic DNA was analyzed using two restriction enzyme digestion strategies.

In the first strategy, the corn genomic DNA was digested with an enzyme that cut once within the 5307 corn insert. The other recognition sites for this enzyme were located in the corn genome flanking the 5307 corn insert. This first strategy was used twice with two different enzymes (*Bst*EII and *Spe*I) to determine the copy number of the 5307 corn insert and the presence or absence of extraneous DNA fragments of the plasmid pSYN12274 T-DNA in other regions of the 5307 corn genome. These digests were expected to result in only two hybridization bands corresponding to the 5307 corn insert when a full length T-DNA-specific probe was used. More than two bands with either digest would have indicated that there were multiple copies of the insert in the plant genome.

In the second strategy, the corn genomic DNA was digested with *Sma*I + *Pme*I, which cut within the 5307 corn insert such that a DNA fragment of predictable size was released. This strategy was used to determine the presence of any closely linked extraneous DNA fragments of the plasmid pSYN12274 T-DNA.

Figure V-14 shows a map of the T-DNA of the 5307 corn transformation plasmid pSYN12274, indicating the location of the full-length T-DNA-specific probe and restriction sites for *Bst*EII, *Spe*I, *Sma*I, and *Pme*I. Figure V-15 depicts the results of the corresponding Southern blot analyses, and Table V-7 provides the expected and observed sizes of the hybridization bands.

For Southern blot analysis of genomic DNA digested with *Bst*EII and probed with the full length T-DNA-specific probe, two hybridization bands of approximately 2.9 kb and 7.2 kb were observed in lanes containing DNA extracted from 5307 F_1, 5307 BC6, 5307 BC7, and 5307 NP2171 × $BC5F_3$ plants as expected (Figure V-15, Lanes 3, 4, 5, and 6) (Table V-7). These hybridization bands were absent in lanes containing DNA extracted from the control plants (Figure V-15, Lanes 7, 8, 9, and 10) and were, therefore, specific to the 5307 corn insert. Three hybridization bands of approximately 11.8 kb, 14 kb, and 20 kb were observed in the lane containing the positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Spe*I and loaded with DNA extracted from NP2171 × NP2460 plants) (Figure V-15, Lane 19).

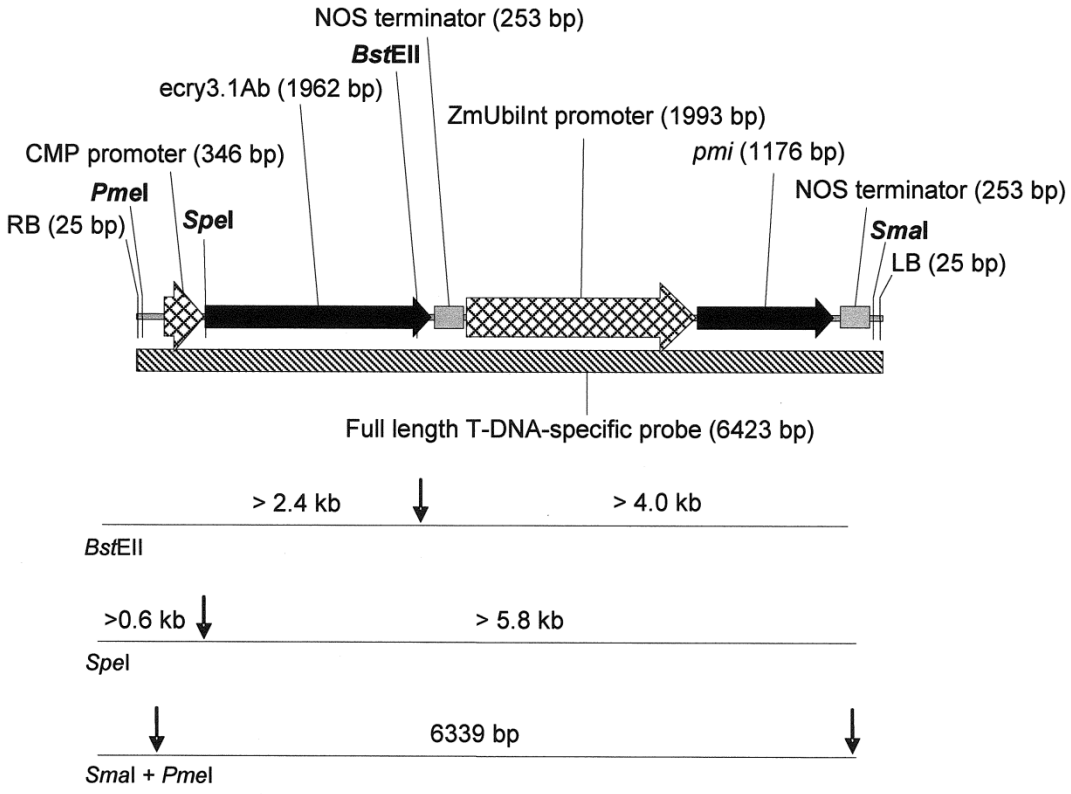
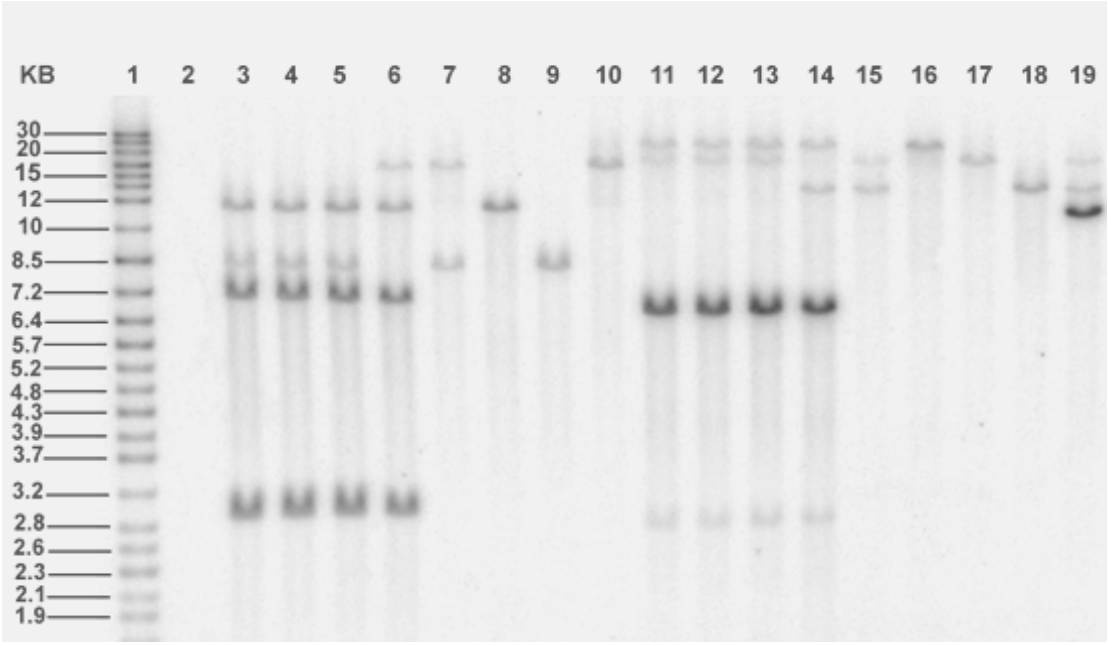


Figure V-14. Location of the *Bst*EII, *Spe*I, *Sma*I, and *Pme*I restriction sites and position of the 6423-bp full-length T-DNA-specific probe in the T-DNA region of the transformation plasmid pSYN12274.

Additional bands resulting from cross-hybridization of the ZmUbiInt promoter sequence present on the full length T-DNA-specific probe with the endogenous maize polyubiquitin promoter sequence were also detected in all material analyzed. Two hybridization bands of approximately 8.4 kb and 12 kb, corresponding to the hybridization bands observed in lanes containing DNA extracted from NP2460 plants (Figure V-15, Lane 9) and NP2222 plants (Figure V-15, Lane 8), respectively, were observed in lanes containing DNA extracted from 5307 F_1, 5307 BC6, and 5307 BC7 plants (Figure V-15, Lanes 3, 4, and 5). Two hybridization bands of approximately 12 kb and 18 kb, corresponding to the hybridization bands observed in lanes containing DNA extracted from NP2222 plants (Figure V-15, Lane 8) and NP2171 plants (Figure V-15, Lane 10), respectively, were observed in the lane containing DNA extracted from 5307 NP2171 × $BC5F_3$ plants (Figure V-15, Lane 6). Finally, two hybridization bands of approximately 8.4 kb and 18 kb, corresponding to the hybridization bands observed in lanes containing DNA extracted from NP2460 plants (Figure V-15, Lane 9) and NP2171 plants (Figure V-15, Lane 10), respectively, were observed in lanes containing DNA extracted from NP2171 × NP2460 plants (Figure V-15, Lane 7).

For Southern blot analysis of genomic DNA digested with *Spe*I and probed with the full length T-DNA-specific probe, two hybridization bands of approximately 2.6 kb and 7.0 kb were observed in lanes containing DNA extracted from 5307 F_1, 5307 BC6, 5307 BC7, and 5307 NP2171 × $BC5F_3$ plants as expected (Figure V-15A, Lanes 11, 12, 13, and 14) (Table V-7). These bands were absent in lanes containing DNA extracted from the control plants (Figure V-15A, Lanes 15, 16, 17, and 18) and were, therefore, specific to the 5307 corn insert. Three hybridization bands of approximately 11.8 kb, 14 kb, and 20 kb were observed in the lane containing the positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Spe*I and loaded with DNA extracted from NP2171 × NP2460 plants) (Figure V-15A, Lane 19).

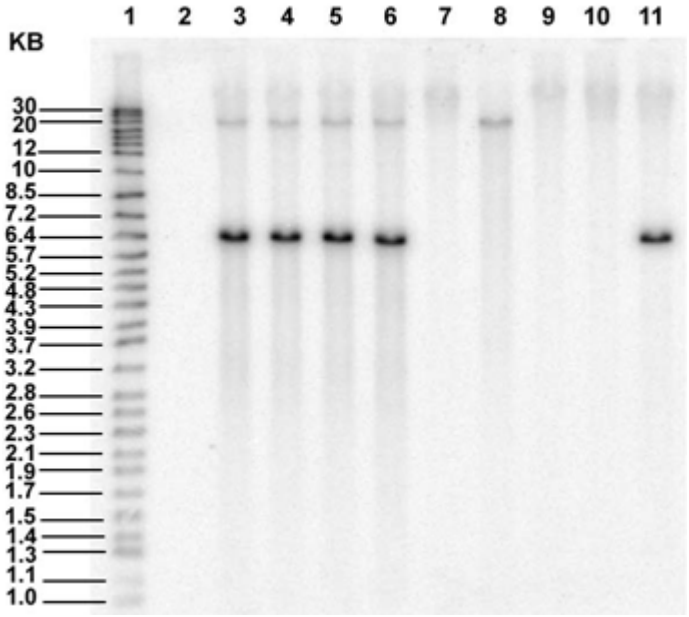
(A) *Bst*EII or *Spe*I



Lane A1 = molecular weight markers
Lane A2 = blank
Lane A3 = 5307 F_1 digested with *Bst*EII
Lane A4 = 5307 BC6 digested with *Bst*EII
Lane A5 = 5307 BC7 digested with *Bst*EII
Lane A6 = 5307 NP2171 × $BC5F_3$ digested with *Bst*EII
Lane A7 = NP2171 × NP2460 digested with *Bst*EII
Lane A8 = NP2222 digested with *Bst*EII
Lane A9 = NP2460 digested with *Bst*EII
Lane A10 = NP2171 digested with *Bst*EII
Lane A11 = 5307 F_1 digested with *Spe*I
Lane A12 = 5307 BC6 digested with *Spe*I
Lane A13 = 5307 BC7 digested with *Spe*I
Lane A14 = 5307 NP2171 × $BC5F_3$ digested with *Spe*I
Lane A15 = NP2171 × NP2460 digested with *Spe*I
Lane A16 = NP2222 digested with *Spe*I
Lane A17 = NP2460 digested with *Spe*I
Lane A18 = NP2171 digested with *Spe*I
Lane A19 = Positive control (NP2171 × NP2460 and 16.53 pg of pSYN12274 digested with *Spe*I)

Figure V-15. Genetic stability Southern blot analysis of 5307 corn with the 6423-bp full-length T-DNA-specific probe, using restriction enzymes *Bst*EII, *Spe*I, and *Sma*I + *Pme*I.

(B) *Sma*I + *Pme*I



Lane B1 = molecular weight markers
Lane B2 = blank
Lane B3 = 5307 F_1 digested with *Sma*I + *Pme*I
Lane B4 = 5307 BC6 digested with *Sma*I + *Pme*I
Lane B5 = 5307 BC7 digested with *Sma*I + *Pme*I
Lane B6 = 5307 NP2171 × $BC5F_3$ digested with *Sma*I + *Pme*I
Lane B7 = NP2171 × NP2460 digested with *Sma*I + *Pme*I
Lane B8 = NP2222 digested with *Sma*I + *Pme*I
Lane B9 = NP2460 digested with *Sma*I + *Pme*I
Lane B10 = NP2171 digested with *Sma*I + *Pme*I
Lane B11 = Positive control (NP2171 × NP2460 and 16.53 pg of pSYN12274 digested with *Sma*I + *Pme*I)

Figure V-15 (continued). Genetic stability Southern blot analysis of 5307 corn with the 6423-bp full-length T-DNA-specific probe, using restriction enzymes BstEII, SpeI, and SmaI + PmeI.

Additional bands resulting from cross-hybridization of the ZmUbiInt promoter sequence present on the full length T-DNA-specific probe with the endogenous maize polyubiquitin promoter sequence were also detected in all material analyzed. Two hybridization bands of approximately 20 kb and 25 kb, corresponding to the hybridization bands observed in lanes containing DNA extracted from NP2460 plants (Figure V-15A, Lane A17) and NP2222 plants (FigureV-15A, Lane A16), respectively, were observed in lanes containing DNA extracted from 5307 F_1, 5307 BC6, and 5307 BC7 plants (Figure V-15A, Lanes A11, A12, and A13). Two hybridization bands of approximately 14 kb and 25 kb, corresponding to the hybridization bands observed in lanes containing DNA extracted from NP2171 plants (Figure V-15A, Lane A18) and NP2222 plants (Figure V-15A, Lane A16), respectively, were observed in the lane containing DNA extracted from 5307 NP2171 × $BC5F_3$ plants (Figure V-15A, Lane A14).

Table V-7. Expected and observed hybridization band sizes in Southern blot analysis of 5307 corn, using a full length T-DNA-specific probe and restriction enzymes *Bst*EII, *Spe*I, and *Sma*I + *Pme*I.

Figure & Lane No.	Source of DNA	Restriction enzyme	Expected number of bands	Expected band size (kb)	Observed band size (kb)
Fig. V-15A, Lane A3	5307 F_1	*Bst*EII	2 5307 insert x endogenous	>2.4 >4 unknown	~2.9 ~7.2 ~8.4 (endogenous) ~12 (endogenous)
Fig. V-15A, Lane A4	5307 BC6	*Bst*EII	2 5307 insert x endogenous	>2.4 >4 unknown	~2.9 ~7.2 ~8.4 (endogenous) ~12 (endogenous)
Fig.V-15A, Lane A5	5307 BC7	*Bst*EII	2 5307 insert x endogenous	>2.4 >4 unknown	~2.9 ~7.2 ~8.4 (endogenous) ~12 (endogenous)
Fig. V-15A, Lane A6	5307 NP2171 × $BC5F_3$	*Bst*EII	2 5307 insert x endogenous	>2.4 >4 unknown	~2.9 ~7.2 ~12 (endogenous) ~18 (endogenous)
Fig. V-15A, Lane A7	NP2171 × NP2460	*Bst*EII	x endogenous	unknown	~8.4 (endogenous) ~18 (endogenous)
Fig. V-15A, Lane A8	NP2222	*Bst*EII	x endogenous	unknown	~12 (endogenous)
Fig. V-15A, Lane A9	NP2460	*Bst*EII	x endogenous	unknown	~8.4 (endogenous)
Fig. V-15A, Lane A10	NP2171	*Bst*EII	x endogenous	unknown	~18 (endogenous)
Fig. V-15A, Lane A11	5307 F_1	*Spe*I	2 5307 insert x endogenous	>0.6 >5.8 unknown	~2.6 ~7 ~20 (endogenous) ~25 (endogenous)
Fig. V-15A, Lane A12	5307 BC6	*Spe*I	2 5307 insert x endogenous	>0.6 >5.8 unknown	~2.6 ~7 ~20 (endogenous) ~25 (endogenous)
Fig. V-15A, Lane A13	5307 BC7	*Spe*I	2 5307 insert x endogenous	>0.6 >5.8 unknown	~2.6 ~7 ~20 (endogenous) ~25 (endogenous)
Fig. V-15A, Lane A14	5307 NP2171 × $BC5F_3$	*Spe*I	2 5307 insert x endogenous	>0.6 >5.8 unknown	~2.6 ~7 ~14 (endogenous) ~25 (endogenous)
Fig. V-15A, Lane A15	NP2171 × NP2460	*Spe*I	x endogenous	unknown	~14 (endogenous) ~20 (endogenous)
Fig. V-15A, Lane A16	NP2222	*Spe*I	x endogenous	unknown	~25 (endogenous)
Fig. V-15A, Lane A17	NP2460	*Spe*I	x endogenous	unknown	~20 (endogenous)
Fig. V-15A, Lane A18	NP2171	*Spe*I	x endogenous	unknown	~14 (endogenous)
Fig. V-15A, Lane A19	Positive control (NP2171 × NP2460 and 16.53 pg of pSYN12274)	*Spe*I	1 pSYN12274 x endogenous	11.8 Unknown	~11.8 ~14 (endogenous) ~20 (endogenous)

x = unknown number

Table V-7 (continued). Expected and observed hybridization band sizes in Southern blot analysis of 5307 corn, using a full length T-DNA-specific probe and restriction enzymes *Bst*EII, *Spe*I, and *Sma*I + *Pme*I.

Figure & Lane No.	Source of DNA	Restriction enzyme	Expected number of bands	Expected band size (kb)	Observed band size (kb)
Fig. V-15B, Lane B3	5307 F_1	*Sma*I + *Pme*I	1 5307 insert x endogenous	6.3 Unknown	~6.3 ~18 (endogenous) >30 (endogenous)
Fig. V-15B, Lane B4	5307 BC6	*Sma*I + *Pme*I	1 5307 insert x endogenous	6.3 unknown	~6.3 ~18 (endogenous) >30 (endogenous)
Fig. V-15B, Lane B5	5307 BC7	*Sma*I + *Pme*I	1 5307 insert x endogenous	6.3 unknown	~6.3 ~18 (endogenous) >30 (endogenous)
Fig. V-15B, Lane B6	5307 NP2171 × $BC5F_3$	*Sma*I + *Pme*I	1 5307 insert x endogenous	6.3 unknown	~6.3 ~18 (endogenous) >30 (endogenous)
Fig. V-15B, Lane B7	NP2171 × NP2460	*Sma*I + *Pme*I	x endogenous	unknown	>30 (endogenous)
Fig. V-15B, Lane B8	NP2222	*Sma*I + *Pme*I	x endogenous	unknown	~18 (endogenous)
Fig. V-15B, Lane B9	NP2460	*Sma*I + *Pme*I	x endogenous	unknown	>30 (endogenous)
Fig. V-15B, Lane B10	NP2171	*Sma*I + *Pme*I	x endogenous	unknown	>30 (endogenous)
Fig. V-15B, Lane B11	Positive control (NP2171 × NP2460 and 16.53 pg of pSYN12274)	*Sma*I + *Pme*I	1 pSYN12274 x endogenous	6.3 unknown	~6.3 >30 (endogenous)

x = unknown number

Finally, two hybridization bands of approximately 14 kb and 20 kb, corresponding to the hybridization bands observed in lanes containing DNA extracted from NP2171 plants (Figure V-15A, Lane A18) and NP246 plants (Figure V-15A, Lane A17), respectively, were observed in lanes containing DNA extracted from NP2171 × NP2460 plants (Figure V-15A, Lane A15). For Southern blot analysis of genomic DNA digested with *Sma*I + *Pme*I and probed with the full length T-DNA-specific probe, one hybridization band of approximately 6.3 kb was observed in lanes containing DNA extracted from 5307 F_1, 5307 BC6, 5307 BC7, and 5307 NP2171 × $BC5F_3$ plants as expected (Figure V-15B, Lanes B3, B4, B5, and B6) (Table V-7). This hybridization band was absent in lanes containing DNA extracted from the control plants (Figure V-15B, Lanes B7, B8, B9, and B10) and was, therefore, specific to the 5307 corn insert. One hybridization band of approximately 6.3 kb and a high molecular weight band (greater than 30 kb) was observed in the lane containing the positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Sma*I + *Pme*I and loaded with DNA extracted from NP2171 × NP2460 plants) (Figure V-15B, Lane B11). At least one additional band resulting from cross-hybridization of the ZmUbiInt promoter sequence present on the full length T-DNA-specific probe with the endogenous maize polyubiquitin promoter sequence was also detected in all material analyzed. One hybridization band of approximately 18 kb, corresponding to the hybridization band observed in the lane containing DNA extracted from NP2222 plants (Figure V-15B, Lane B8), was observed in lanes containing DNA extracted from 5307 F_1, 5307 BC6, 5307 BC7 and 5307 NP2171 ×

BC5F3 plants (Figure V-15B, Lanes B3, B4, B5, and B6). An additional high molecular weight band (greater than 30 kb) was observed in lanes containing DNA extracted from NP2460 plants (Figure V-15B, Lane B9) and NP2171 plants (Figure V-15B, Lane B10). This faint high molecular weight band (greater than 30 kb) was observed in all lanes containing DNA extracted from plants carrying either the NP2460 polyubiquitin promoter allele (5307 F1, 5307 BC6, 5307 BC7, and NP2171 × NP2460) (Figure V-15B, Lanes B3, B4, B5, and B7) and/or the NP2171 polyubiquitin promoter allele (5307 NP2171 × BC5F3 and NP2171 × NP2460) (Figure V-15B, Lanes B6 and B7).

Data from these Southern blot analyses demonstrated that the 5307 corn insert integrated into a single locus of the corn genome as only two hybridization bands specific to the 5307 corn insert were observed when genomic DNA was digested with *Bst*EII and probed with a full length T-DNA-specific probe and only two hybridization bands specific to the 5307 corn insert were observed when the genomic DNA was digested with *Spe*I and probed with a full length T-DNA-specific probe. These hybridization bands were specific to the 5307 corn insert and corresponded to each side of the restriction site of the enzyme used for Southern blot analysis. Additional hybridization bands observed resulted from cross-hybridization between the ZmUbiInt promoter sequence present on the full length T-DNA-specific probe with the endogenous maize polyubiquitin promoter sequence; these bands were consistent with the genetic make-up of the various generations analyzed. As expected, the 5307 F1, 5307 BC6, and 5307 BC7 generations carry the maize polyubiquitin promoter allelic forms present in NP2222 and NP2460, the 5307 NP2171 × BC5F3 generation carries the maize polyubiquitin promoter allelic forms present in NP2171 and NP2222, and the control NP2171 × NP2460 material carries the maize polyubiquitin promoter allelic forms present in NP2171 and NP2460.

Data from these Southern blot analyses also demonstrated that a complete copy of the 5307 corn insert integrated into the corn genome as the hybridization band specific to the 5307 corn insert observed when the genomic DNA was digested with *Sma*I + *Pme*I was the predicted size. The approximately 18 kb band observed on this Southern blot was present in lanes containing DNA extracted from NP2222 plants and all generations carrying the NP2222 polyubiquitin promoter allelic form (5307 F1, 5307 BC6, 5307 BC7, and 5307 NP2171 × BC5F3). The faint and high molecular weight band (greater than 30 kb) observed on this Southern blot was present in lanes containing DNA extracted from NP2460 plants and NP2171 plants and all generations carrying either the NP2460 polyubiquitin promoter allelic form (5307 F1, 5307 BC6, 5307 BC7, and NP2171 × NP2460) and/or the NP2171 polyubiquitin promoter allelic form (5307 NP2171 × BC5F3 and NP2171 × NP2460). Because no additional bands were observed (other than those associated with the 5307 corn insert and the corn endogenous sequence), Southern blot analyses indicated that there were no extraneous DNA fragments of plasmid pSYN12274 T-DNA in other regions of the 5307 corn genome. The data depicted in the Southern blot analyses showed that the hybridization bands specific to the insert were identical in lanes containing DNA extracted from plants grown from all generations (5307 F1, 5307 BC6, 5307 BC7, and 5307 NP2171 × BC5F3); these results indicated that the 5307 corn insert is stably inherited from one generation to the next. Southern blot analyses demonstrated that the 5307 corn genome contains a single, complete copy of the insert and that there are no extraneous DNA fragments of plasmid

pSYN12274 T-DNA inserted elsewhere in the 5307 corn genome. Identical hybridization patterns across all generations of 5307 corn analyzed in this study indicates that the insert is stably inherited from one generation to the next.

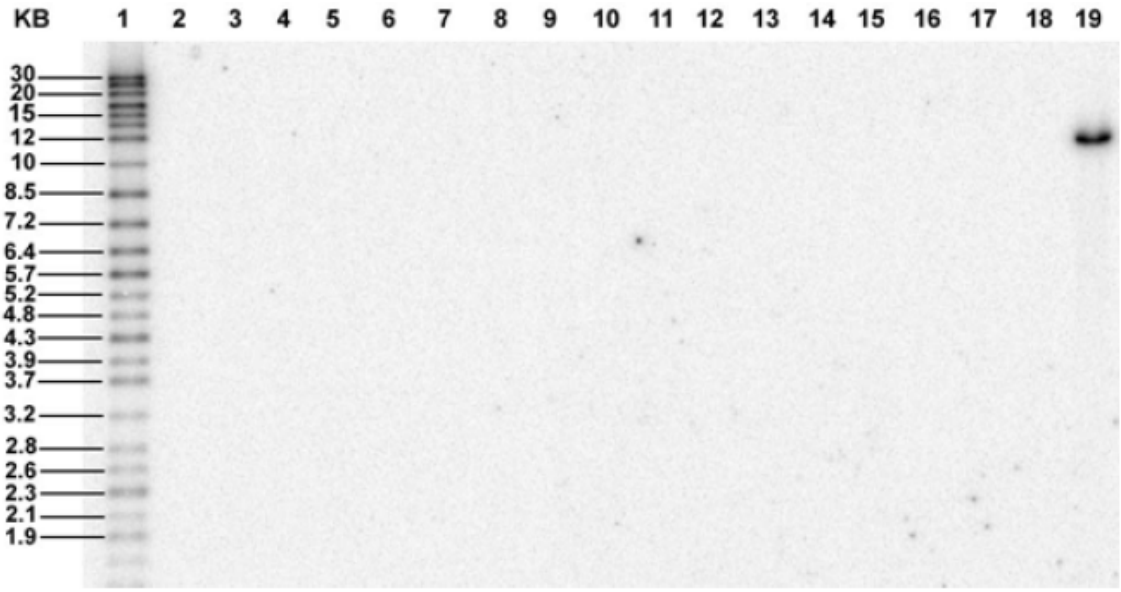
V.D.2. Confirmation of Absence of Plasmid Backbone DNA Using a Backbone-Specific Probe

The absence of plasmid backbone sequence in 5307 corn in multiple generations of 5307 plants was assessed by Southern blot analyses using plasmid pSYN12274 backbone sequence as a probe on Southern blots of DNA subjected to the two restriction enzyme digestion strategies described above. This plasmid backbone-specific probe contained every base of the plasmid pSYN12274 backbone present outside of the T-DNA region. With both restriction enzyme digestion strategies, no hybridization bands were expected.

The Southern blot analyses included genomic DNA from four generations of 5307 corn plants (5307 F_1, 5307 BC6, 5307 BC7, 5307 NP2171 × $BC5F_3$) and the corresponding nontransgenic control plants (NP2171 × NP2460, NP2222, NP2460, and NP2171) (see pedigree diagram, Figure III-3). Figure V-12 shows a map of the plasmid pSYN12274 indicating the location of the plasmid pSYN12274 backbone-specific probe and restriction sites for *Bst*EII, *Spe*I, *Sma*I, and *Pme*I. Figure V-16 depicts the results of the corresponding Southern blot analyses, and Table V-8 provides the expected and observed sizes of the hybridization bands.

For Southern blot analyses of genomic DNA digested with *Bst*EII and probed with the plasmid pSYN12274 backbone-specific probe, no hybridization bands were observed in the lanes containing DNA extracted from plants grown from the test and control substances (5307 F_1, 5307 BC6, 5307 BC7, 5307 NP2171 × $BC5F_3$, NP2171 × NP2460, NP2222, NP2460, and NP2171) (Figure V-16A, Lanes A3, A4, A5, A6, A7, A8, A9, and A10), as expected. The positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Spe*I and loaded with DNA extracted from NP2171 × NP2460 plants) produced the expected 11.8 kb band (Figure V-16A, Lane 19). For Southern blot analyses of genomic DNA digested with *Spe*I and probed with the plasmid pSYN12274 backbone-specific probe, no hybridization bands were observed in the test and control substances (5307 F_1, 5307 BC6, 5307 BC7, 5307 NP2171 × $BC5F_3$, NP2171 × NP2460, NP2222, NP2460, and NP2171) (Figure V-16A, Lanes A11, A12, A13, A14, A15, A16, A17, and A18) as expected. The positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Spe*I and loaded with DNA extracted from NP2171 × NP2460 plants) produced the expected 11.8 kb band (Figure V-16A, Lane 19). For Southern blot analyses of genomic DNA digested with *Sma*I + *Pme*I and probed with the backbone-specific probe, no hybridization bands were observed in the test and control substances (5307 F_1, 5307 BC6, 5307 BC7, 5307 NP2171 × $BC5F_3$, NP2171 × NP2460, NP2222, NP2460, and NP2171) (Figure V-16B, Lanes B3, B4, B5, B6, B7, B8, B9, and B10). The positive control (16.53 pg of plasmid pSYN12274 DNA digested with *Sma*I + *Pme*I and loaded with DNA extracted from NP2171 × NP2460 plants) produced the expected 4.2 kb and 1.2 kb bands (Figure V-16B, Lane B11). The data from the three Southern blot analyses demonstrated that all the 5307 corn generations analyzed are free of plasmid pSYN12274 backbone sequence.

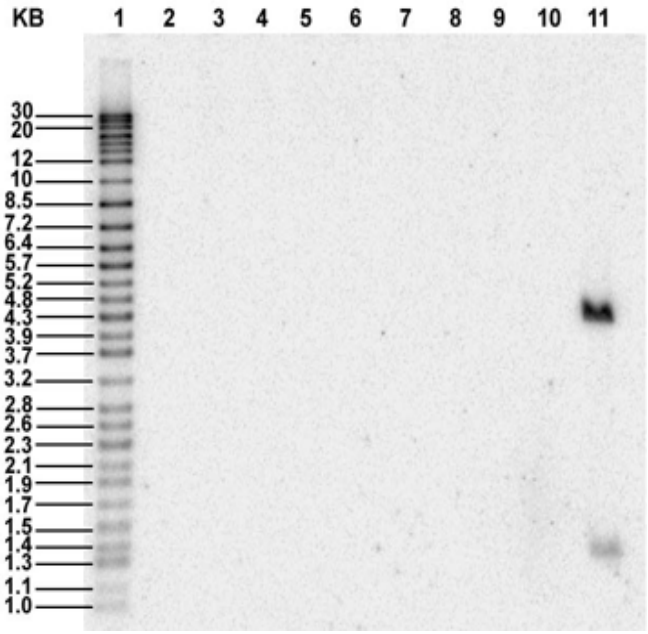
(A) *Bst*EII or *Spe*I



Lane A1 = molecular weight markers
Lane A2 = blank
Lane A3 = 5307 F_1 digested with *Bst*EII
Lane A4 = 5307 BC6 digested with *Bst*EII
Lane A5 = 5307 BC7 digested with *Bst*EII
Lane A6 = 5307 NP2171 × $BC5F_3$ digested with *Bst*EII
Lane A7 = NP2171 × NP2460 digested with *Bst*EII
Lane A8 = NP2222 digested with *Bst*EII
Lane A9 = NP2460 digested with *Bst*EII
Lane A10 = NP2171 digested with *Bst*EII
Lane A11 = 5307 F_1 digested with *Spe*I
Lane A12 = 5307 BC6 digested with *Spe*I
Lane A13 = 5307 BC7 digested with *Spe*I
Lane A14 = 5307 NP2171 × $BC5F_3$ digested with *Spe*I
Lane A15 = NP2171 × NP2460 digested with *Spe*I
Lane A16 = NP2222 digested with *Spe*I
Lane A17 = NP2460 digested with *Spe*I
Lane A18 = NP2171 digested with *Spe*I
Lane A19 = Positive control (NP2171 × NP2460 and 16.53 pg of pSYN12274 digested with *Spe*I)

Figure V-16. Genetic stability Southern blot analysis of 5307 corn with the 5312-bp plasmid backbone-specific probe, using restriction enzymes *Bst*EII, *Spe*I, and *Sma*I + *Pme*I.

(B) Sma*I* + Pme*I*



Lane B1 = molecular weight markers
Lane B2 = blank
Lane B3 = 5307 F_1 digested with *Sma*I + *Pme*I
Lane B4 = 5307 BC6 digested with *Sma*I + *Pme*I
Lane B5 = 5307 BC7 digested with *Sma*I + *Pme*I
Lane B6 = 5307 NP2171 × $BC5F_3$ digested with *Sma*I + *Pme*I
Lane B7 = NP2171 × NP2460 digested with *Sma*I + *Pme*I
Lane B8 = NP2222 digested with *Sma*I + *Pme*I
Lane B9 = NP2460 digested with *Sma*I + *Pme*I
Lane B10 = NP2171 digested with *Sma*I + *Pme*I
Lane B11 = Positive control (NP2171 × NP2460 and 16.53 pg of pSYN12274 digested with *Sma*I + *Pme*I)

Figure V-16 (continued). Genetic stability Southern blot analysis of 5307 corn with the 5312-bp plasmid backbone-specific probe, using restriction enzymes *Bst*EII, *Spe*I, and *Sma*I + *Pme*I.

Table V-8. Expected and observed hybridization band sizes in Southern blot analysis of 5307 corn, using a plasmid backbone-specific probe and restriction enzymes *Bst*EII, *Spe*I, and *Sma*I + *Pme*I.

Figure & Lane No.	Source of DNA	Restriction enzyme	Expected number of bands	Expected band size (kb)	Observed band size (kb)
Fig. V-16A, Lane A3	5307 F_1	*Bst*EII	none	none	none
Fig. V-16A, Lane A4	5307 BC6	*Bst*EII	none	none	none
Fig. V-16A, Lane A5	5307 BC7	*Bst*EII	none	none	none
Fig. V-16A, Lane A6	5307 NP2171 × $BC5F_3$	*Bst*EII	none	none	none
Fig. V-16A, Lane A7	NP2171 × NP2460	*Bst*EII	none	none	none
Fig. V-16A, Lane A8	NP2222	*Bst*EII	none	none	none
Fig. V-16A, Lane A9	NP2460	*Bst*EII	none	none	none
Fig. V-16A, Lane A10	NP2171	*Bst*EII	none	none	none
Fig. V-16A, Lane A11	5307 F_1	*Spe*I	none	none	none
Fig. V-16A, Lane A12	5307 BC6	*Spe*I	none	none	none
Fig. V-16A, Lane A13	5307 BC7	*Spe*I	none	none	none
Fig. V-16A, Lane A14	5307 NP2171 × $BC5F_3$	*Spe*I	none	none	none
Fig. V-16A, Lane A15	NP2171 × NP2460	*Spe*I	none	none	none
Fig. V-16A, Lane A16	NP2222	*Spe*I	none	none	none
Fig. V-16A, Lane A17	NP2460	*Spe*I	none	none	none
Fig. V-16A, Lane A18	NP2171	*Spe*I	none	none	none
Fig. V-16A, Lane A19	Positive control (NP2171×NP2460 and 16.53 pg of pSYN12274)	*Spe*I	1	11.8	~11.8
Fig. V-16B, Lane B3	5307 F_1	*Sma*I + *Pme*I	none	none	none
Fig. V-16B, Lane B4	5307 BC6	*Sma*I + *Pme*I	none	none	none
Fig. V-16B, Lane B5	5307 BC7	*Sma*I + *Pme*I	none	none	none
Fig. V-16B, Lane B6	5307 NP2171 × $BC5F_3$	*Sma*I + *Pme*I	none	none	none
Fig. V-16B, Lane B7	NP2171 × NP2460	*Sma*I + *Pme*I	none	none	none
Fig. V-16B, Lane B8	NP2222	*Sma*I + *Pme*I	none	none	none
Fig. V-16B, Lane B9	NP2460	*Sma*I + *Pme*I	none	none	none
Fig. V-16B, Lane B10	NP2171	*Sma*I + *Pme*I	none	none	none
Fig. V-16B, Lane B11	Positive control (NP2171×NP2460 and 16.53 pg of pSYN12274)	*Sma*I + *Pme*I	2	4.2 1.2	~4.2 ~1.2

V.E. Mendelian Inheritance of the T-DNA Insert

A chi-square (X^2) analysis of *ecry3.1Ab* and *pmi* inheritance data over three generations of Event 5307 corn was performed to test the hypothesis that the transgenes are inherited in accordance with the laws of Mendelian genetics. The chi-square analysis was based on a comparison of observed and expected gene segregation ratios from each generation. Real-time PCR analyses (Ingham et al., 2001) were conducted on DNA extracted from leaf tissue of three generations of 5307 corn plants to determine the number of plants that were positive or negative for both the transgenes *ecry3.1Ab* and *pmi* (see method in Appendix B). Ninety-two plants from 5307 F_1 seed, 91 plants from 5307 BC6 seed and 90 plants from 5307 BC7 seed were grown in a Syngenta Biotechnology, Inc. greenhouse in Research Triangle Park, North Carolina, USA (see pedigree diagram in Fig. III-3). During breeding, only progeny that tested positive for the two transgenes were selected for further crossing; thus, the expected inheritance ratio for positive to negative plants was 1:1 in each generation.

Genotypic data generated for the three 5307 corn generations were used to assess the goodness-of-fit of the observed genotypic ratios to the expected genotypic ratios using chi-square analysis with Yates' correction factor as in Armitage and Berry (1987).

$X^2 = \Sigma\ [|(\text{observed} - \text{expected})| - 0.5]^2 / \text{expected}$

All plants tested positive for the control assay targeting the endogenous corn gene *adh1*, confirming that DNA was present in all real-time PCR reactions. The expected and observed frequencies of *ecry3.1Ab* and *pmi* for each generation are presented in Tables V-9 and V-10. The critical value for rejection of the null hypothesis at the 5% level was 3.84 (Strickberger, 1976). The chi-square values for each generation tested were found to be less than 3.84. This analysis demonstrates that both the *ecry3.1Ab* and *pmi* are inherited in a predictable manner according to Mendelian principles. These results are consistent with the genetic characterization data for 5307 corn, which indicate stable integration of the T-DNA at a single locus in the genome.

Table V-9. Observed *versus* expected frequencies for *ecry3.1Ab* across generations.

Trait	F_1		BC6		BC7	
	Observed	Expected	Observed	Expected	Observed	Expected
Positive	44	46	40	45.5	43	45
Negative	48	46	51	45.5	47	45
Total	92	92	91	91	90	90
X^2 value	0.098		1.099		0.100	

Table V-10. Observed *versus* expected frequencies for *pmi* across generations.

Trait	F_1		BC6		BC7	
	Observed	Expected	Observed	Expected	Observed	Expected
Positive	44	46	40	45.5	43	45
Negative	48	46	51	45.5	47	45
Total	92	92	91	91	90	90
X^2 value	0.098		1.099		0.100	

V.F. Flanking Sequence Analysis to Determine if T-DNA Inserted into a Known Corn Gene

Nucleotide sequences flanking the 5′ and 3′ ends of the T-DNA in 5307 corn were identified by PCR, as described in Appendix B. These sequences, representing 1000 bp at the 5′ end of the T-DNA and 1000 at the 3′ end of the T-DNA, were screened for similarity with sequences found in public databases. This comparison provided an indication of whether the 5307 corn T-DNA inserted into a known plant gene. A sequence similarity analysis was performed using Basic Local Alignment Search Tool for Nucleotides (BLASTN) software, version 2.2.19 (Altschul et al., 1997) which compared the flanking sequences with nucleotide sequences in the National Center for Biotechnology Information Nucleotide Collection (nr/nt) database (NCBI, 2010a). The nr/nt database contains all sequences from the National Institutes of Health genetic sequence database (GenBank®), RefSeq Nucleotides, the European Molecular Biology Laboratory, the DNA Database of Japan, and nucleotide sequences derived from the three-dimensional structures from the Brookhaven Protein Data Bank. At the time the BLASTN analysis was run (May 7, 2010), the nr/nt database contained over 11 million sequences. Details of the parameters used in the BLASTN analysis are provided in Appendix B.

BLASTN analysis was conducted using the 1000 bp of corn genomic sequence flanking the 5' end of the 5307 T-DNA insert as the query sequence. The search identified all alignments to sequences in the nr/nt database with search results yielding an *E*-value of 10 or lower. This analysis was repeated using the 1000 bp of corn genomic sequence flanking the 3' end of the 5307 T-DNA as the query sequence. The *E*-value, or "expectation value," is a measure of the probability that matches between sequences occurred by chance. Search results involving comparisons between nucleotide sequences with highly similar sequences yield *E*-values approaching zero. The probability that sequence similarities occurred by chance increases with higher *E*-values (Ponting, 2001).

Using the results of each BLASTN query, the 20 alignments with the lowest *E*-values were examined for this analysis. The corn genomic sequences flanking the 5′ region and the 3′ region of the 5307 corn insert aligned to multiple bacterial artificial chromosome (BAC) corn sequences that were annotated as various corn chromosome numbers (i.e., corn

chromosome numbers 4, 5, 6, 8, 9, and 10). This suggests that the 5307 corn insert is located in a repetitive region of the corn genome. Both flanking sequences aligned to NCBI nucleotide database accession numbers AY530950.1, AY530951.1 and AY530952.1, which were annotated as containing gene sequences. However, the regions of AY530950.1, AY530951.1, and AY530952.1 that were similar to the corn genomic sequences flanking the 5′ region and the 3′ region of the 5307 insert were not annotated as a gene. Both flanking sequences also aligned to accession numbers AY664416.1, AY664413.1, AY664419.1, and AY664415.1. (However, alignments of corn genomic sequence flanking the 3′ region of the 5307 insert to accession numbers AY664419.1 and AY664415.1 were not among those with the 20 highest similarity scores.) These accession numbers are all annotated as containing sequences of various genes and repeat regions. However, the regions of AY664416.1, AY664413.1, AY664419.1, and AY664415.1 that were similar to the corn genomic sequences flanking the 5′ region and the 3′ region of the 5307 insert were not annotated as a gene. The corn genomic sequences flanking the 5′ region and the 3′ region of the 5307 insert aligned to a region of AY664413.1 that was annotated as repeat region Giepumx19002_LTR retrotransposon. Additionally, short regions of both flanking sequences (i.e., ~20 to 40 base pairs in length) aligned to multiple regions of multiple sequences in the database. Some of these regions were also annotated as repeat regions in accession numbers AY664416.1, AY664413.1, AY664419.1, and AY664415.1. These repeat regions were also associated with retrotransposons. Approximately 49 to 78% of the corn genome is suggested to be comprised of retrotransposons (San Miguel and Bennetzen, 1998). However, only approximately 5% of the retrotransposon sequences in the corn genome are predicted to produce proteins (Meyers et al., 2001). Because retrotranposons are repetitive, the repeat regions that align to corn genomic sequences flanking the 5′ region and the 3′ region of the 5307 insert are likely to be repeated elsewhere in the corn genome.

BLASTN analysis indicated that the corn genomic sequence flanking the 3′ region of the 5307 corn insert is also similar to accession number EU954153.1, which was annotated as a hypothetical protein mRNA sequence from *Zea mays*. The region of the flanking sequence that is similar to EU954153.1 was located 109 bp downstream of the genome-to-insert junction. BLASTN analysis also indicated that the corn genomic sequence flanking the 3′ region of the 5307 corn insert is similar to accession number EZ054274.1, annotated as mRNA sequence from *Zea mays*. The region of the flanking sequence that is similar to EZ054274.1 was located 376 bp downstream of the genome-to-insert junction. The corn genomic sequence flanking the 5′ region of the 5307 corn insert did not align to sequence EU954153.1 or EZ054274.1. None of the alignments retrieved indicates that a known endogenous corn gene was interrupted by the Event 5307 T-DNA insert.

A comparison of the sequence of the genomic insertion site from nontransgenic maize (inbred line NP2222) with the genomic sequences flanking the 5307 corn T-DNA insert determined that 33 bp of corn genomic sequence were deleted when the 5307 insert integrated into the corn genome.

V.G. Analysis of Putative Open Reading Frames Spanning the Genome to Insert Junction

The Vector NTI Advance™ program, version 10.3.0, was used to identify any putative open reading frames (ORFs) that span the junctions between the corn genomic sequence and the 5307 corn T-DNA insert. For this analysis, putative ORFs were defined as DNA sequences in any reading frame that are contained between a putative start codon (ATG) and a putative stop codon (TAG, TAA, or TGA), and have a minimum translation size of 30 amino acids. This analysis identified one putative ORF of 243 bp; it spanned the junction between the corn genomic sequence and the 3′ region of the 5307 corn insert. This putative ORF was identified solely by bioinformatic analysis; there is no evidence that it results in the production of a corresponding protein.

Bioinformatic analyses demonstrated that the translated 81-amino-acid sequence of this putative ORF had no significant sequence similarity to known or putative toxins or allergens. The methods used for these toxin and allergen similarity searches are described in Appendix B.

V.H. Summary of the Genetic Characterization of Event 5307 Corn

Southern blot analyses demonstrate that Event 5307 corn (1) contains, at a single locus within the corn genome, a single copy each of the gene *ecry3.1Ab*, its CMP promoter sequence, the marker gene *pmi*, its ZmUbiInt promoter sequence, and the two expected copies of the NOS terminator sequence, one NOS terminator sequence regulating *ecry3.1Ab* and one NOS terminator sequence regulating *pmi*; (2) does not contain any extraneous DNA fragments of these functional elements inserted elsewhere in the corn genome; (3) and does not contain plasmid backbone sequence from the transformation plasmid, pSYN12274.

Nucleotide sequence analysis of the entire Event 5307 insert confirms that the insert is intact and that the organization of the functional elements within the insert is identical to their organization within pSYN12274. One nucleotide change compared to the sequence of pSYN12274 was identified 48 base pairs (bp) upstream of the CMP promoter in a noncoding region of the insert in Event 5307 corn. However, this nucleotide change had no effect on the functionality of the insert. Additionally, the analysis indicates that some truncation of the nucleotide sequence occurred at the 5′ and 3′ ends of the T-DNA during the transformation process that resulted in Event 5307 corn; such truncation occurs commonly in transformation via *Agrobacterium*. The entire RB and three bp of noncoding sequence at the 5′ end of the insert, and eight bp of the LB were truncated; however, these deletions had no effect on the functionality of the insert.

Sequence analysis of the Event 5307 insertion site demonstrates that 33 bp of corn genomic sequence were deleted when the Event 5307 insert integrated into the corn genome. BLASTN analyses comparing the corn genomic sequence flanking the Event 5307 insert to sequences in public databases indicate that the insert does not disrupt any known endogenous corn gene.

A putative 243-bp novel open reading frame (ORF) spanning the junction between corn genomic sequence and the 3′ region of the 5307 corn insert was identified. The translated

81-amino-acid sequence encoded by the putative ORF was screened for amino acid sequence similarity to known or putative allergens or toxins. Comparisons to the FARRP AllergenOnline database indicate that the amino acid translation of the identified putative ORF shows no biologically relevant amino acid sequence similarity to any known or putative protein allergens. Additionally, the results of a comprehensive amino acid similarity search of the NCBI Entrez® Protein Database indicate that the amino acid translation of the identified putative ORF shows no biologically relevant amino acid sequence similarity to any known or putative toxins. These data collectively demonstrate that there are no deleterious changes in the 5307 corn genome as a result of the T-DNA insertion.

The *ecry3.1Ab* and *pmi* segregation ratios over several generations of Event 5307 corn plants are consistent with linkage of these transgenes at a single locus in the corn nuclear genome. These data and the results from Southern blot analyses of multiple generations of Event 5307 corn indicate that the transgenic locus is stably inherited during conventional breeding.

VI. Characterization of the eCry3.1Ab and PMI Proteins

The eCry3.1Ab and PMI proteins produced in Event 5307 corn have been characterized and tested to determine their potential for causing adverse effects in humans, livestock and wildlife. This process included an assessment of the: 1) origin and function of each protein; 2) mode of action of each protein; 3) physicochemical properties of the proteins; 4) biological activity of the proteins; and 5) potential toxicity of each protein. The concentrations of eCry3.1Ab and PMI in 5307 corn tissues were also determined for use in risk assessment.

VI.A. eCry3.1Ab – Origin, Mode of Action, and Insecticidal Specificity

The Cry (crystal) proteins produced by *Bacillus thuringiensis* share structural similarities; thus, chimeric Cry genes can be engineered via the exchange of domains that are homologous between different Cry genes. As described in Table IV-1, Figure IV-1, and Walters et al. (2010), the eCry3.1Ab[1] protein in Event 5307 represents a chimeric protein comprised of portions of two *B.-thuringiensis*-derived crystal proteins, modified Cry3A (mCry3A) and Cry1Ab. Additionally there are 22 amino acids at the N-terminus of eCry3.1Ab that are not derived from a Cry protein, *per se*, but were the result of a PCR-induced mutation that resulted in a reading-frame shift in the nucleotide sequence of a portion of the gene *mcry3A*. Following the 22 N-terminal amino acids, there are 459 consecutive amino acids from mCry3A, followed by 172 consecutive amino acids from Cry1Ab at the C-terminus, resulting in a total eCry3.1Ab polypeptide of 653 amino acids with a molecular weight of 73.7 kDa.

The mCry3A protein is a modified form of the native Cry3A produced by *B. thuringiensis* subsp. *tenebrionis*, and is present in Syngenta's coleopteran-resistant Event MIR604 corn, which was deregulated by APHIS in 2007 (in response to APHIS Petition 04-362-01p). Cry1Ab is a native protein produced by *B. thuringiensis* subsp. HD-1. A truncated version of this protein is present in Syngenta's lepidopteran-resistant Event Bt11 corn, which was deregulated by APHIS in 1996 (in response to APHIS Petition 95-195-01p). The native forms of both Cry3A and Cry1Ab are present in U.S. EPA-registered microbial *B. thuringiensis* insecticide products for coleopteran and lepidopteran control, respectively.

As an engineered chimeric protein, eCry3.1Ab has similarities to other well characterized Cry proteins. The spectrum of insecticidal activity of any individual Cry protein is quite narrow. Any given Cry protein is typically active against only a few species within a phylogenic Order. The specificity of each *B. thuringiensis* Cry protein is the result of the efficiency of the necessary steps involved in producing an active protein toxin and its subsequent interaction with the epithelial cells in the insect midgut. To exert their insecticidal activity, most known *B. thuringiensis* Cry proteins must: (1) be ingested by the

[1] The descriptor "eCry3.1Ab" was assigned by Syngenta; the "e" denotes that it was engineered, and the "Cry3" and "1Ab" descriptors relate to the respective source Cry (crystal) proteins, modified Cry3A (mCry3A) and Cry1Ab. The eCry3.1Ab protein has not been assigned an official Cry protein designation under the formal nomenclature scheme for *B. thuringiensis* Cry proteins (Crickmore et al., 2010).

insect and solubilized in the insect gut, (2) be activated by specific proteolytic cleavage by midgut enzymes, (3) bind to specific receptors on the surface of the insect midgut and (4) form ion channels in the gut membrane. The completion of all these four processes results in disruption of the normal function of the midgut leading to the death of the insect. The eCry3.1Ab protein exhibits the same behavior as other coleopteran-active *B. thuringiensis* Cry proteins, including alkaline solubility, cleavage by chymotrypsin, specificity of brush border membrane binding and ion channel formation (Walters et al., 2010). The mode of action of eCry3.1Ab, like that of most other Cry proteins, is highly specific to insects and is not operable in mammalian or other vertebrate species.

As indicated by the insect activity profile in Table VIII-1 and the results of additional tests on multiple nontarget organisms (described in Part VIII of this Petition), eCry3.1Ab is not active on species outside the Chrysomelidae family of Coleoptera. It demonstrates no lepidopteran activity, despite containing sequences from a lepidopteran-active protein (Cry1Ab).

VI.A.1. Characterization of eCry3.1Ab

A number of analytical methods were used to characterize the eCry3.1Ab protein produced in 5307 corn plants. Significant quantities of eCry3.1Ab were required to conduct mammalian and environmental safety studies with the protein. Because it was not feasible to extract the required quantities of eCry3.1Ab from 5307 plants for this safety testing program, eCry3.1Ab was produced in a recombinant *E. coli* fermentation system and purified in active form. Biochemical and bioactivity analyses were then conducted to establish the equivalence of the microbially produced eCry3.1Ab to eCry3.1Ab as produced in 5307 plants, to justify use of the microbially produced material as a surrogate for testing purposes.

VI.A.1.a. Production of eCry3.1Ab for Safety Testing

Syngenta undertook extensive efforts to produce the identical eCry3.1Ab protein in 5307 plants via microbial production systems. However, these efforts did not yield sufficient quantities of bioactive protein, and it proved necessary to produce eCry3.1Ab with an N-terminal tag of histidine residues as a purification aid. This is a common procedure for protein synthesis and purification. Except for an N-terminal tag consisting of one methionine and six histidine residues, the microbially produced eCry3.1Ab has the identical amino acid sequence as eCry3.1Ab produced in 5307 plants. The amino acid sequence of the plant-produced eCry3.1Ab can be deduced from the observation that the nucleotide sequence of *ecry3.1Ab* recovered from 5307 plants is identical to the intended sequence (see Part V.A. Nucleotide Sequence of the T-DNA Insert).

The microbially produced eCry3.1Ab is hereafter also referred to as the eCry3.1Ab test substance, or by its batch code, ECRY3.1AB-0208. The eCry3.1Ab test substance was determined by spectrophotometric analysis to contain 92.4% protein, and densitometric analysis indicated that 97.0% of the total protein in the test substance was eCry3.1Ab. Thus, the purity of test substance ECRY3.1AB-0208 was calculated to be 89.6% eCry3.1Ab by weight. The molecular weight of eCry3.1Ab in the test substance was 74,833 Da as measured by mass spectrometry; this was consistent with the predicted molecular weight.

The production and characterization of test substance ECRY3.1AB-0208 are described in Appendix C.

VI.A.1.b. Equivalence of eCry3.1Ab in Test Substance and in 5307 Plants

To justify the use of the eCry3.1Ab test substance as a suitable surrogate for eCry3.1Ab produced in 5307 plants, the proteins were compared biochemically and functionally. Both sources of eCry3.1Ab were compared with regard to their molecular weight, integrity (intactness), immunoreactivity against an antibody capable of detecting eCry3.1Ab, glycosylation status, and insecticidal activity against a sensitive species. Additionally, N-terminal sequence analysis and peptide mass mapping were done to confirm the identity of both proteins. The genotypes of the 5307 and control plants used in this study were NP2171 × NP2460($BC5F_3$)(5307) and NP2171 × NP2460, respectively. (See also Table III-1 and the breeding pedigree diagram in Figure III-3.) The plants used for this study were grown in a Syngenta greenhouse facility.

The eCry3.1Ab proteins from 5307 corn plants and microbially produced test substance ECRY3.1AB-0208 had apparent molecular weights consistent with the predicted molecular weights of 73.7 and 74.8 kDa, respectively, and immunologically cross-reacted with antibodies capable of detecting the eCry3.1Ab protein. There was no evidence of post-translational glycosylation of eCry3.1Ab from either the 5307 plants or the microbially produced test substance. N-terminal sequence analysis and peptide mass mapping confirmed the identity of eCry3.1Ab from both sources. Both the plant-produced and microbially produced eCry3.1Ab showed comparable insecticidal activity against Colorado potato beetle larvae[2] (*Leptinotarsa decemlineata*), as indicated by the 144-hour LC_{50} values in Table VI-1. These results confirm that the addition of one methionine and six histidine residues at the N-terminus in microbially produced eCry3.1Ab does not affect the function of eCry3.1Ab.

[2] Although *L. decemlineata* is not a corn rootworm, it is a member of the same family of Coleoptera (Chrysomelidae) as are the *Diabrotica* rootworms and is sensitive to eCry3.1Ab, as indicated in Table VIII-1. *L. decemlineata* larvae were used for this study because they are more amenable than rootworms to laboratory bioassays.

Table VI-1. Comparison of Colorado potato beetle larvae LC_{50} values for eCry3.1Ab from 5307 plants and test substance ECRY3.1AB-0208.

First instars were exposed to diets containing eCry3.1Ab from 5307 plants or ECRY3.1AB-0208. Leaf extract from control plants was included in the bioassays with ECRY3.1AB-0208 to control for leaf matrix effects. Details of these bioassays are provided in Appendix C. For each replicate assay, *N* = 24 larvae per treatment.

	LP5307[a]		LP-NEG[b] + ECRY3.1AB-0208	
	µg eCry3.1Ab/ml insect diet			
Replicate	LC_{50}	95% CI	LC_{50}	95% CI
#1	1.316	0.631-3.187	1.780	0.924-3.190
#2	1.669	0.875-3.279	1.113	0.113-4.001
#3	2.888	1.765-4.655	3.226	1.955-5.134

[a] Leaf protein extract of 5307 plants containing eCry3.1Ab .

[b] Leaf protein extract of control plants, tested in combination with eCry3.1Ab from test substance ECRY3.1AB-0208.

These characterization and bioactivity results support the conclusion that eCry3.1Ab produced in recombinant *E. coli* (test substance ECRY3.1AB-0208) is biochemically and functionally equivalent to the eCry3.1Ab produced in Event 5307 corn plants. These data support the use of eCry3.1Ab in test substance ECRY3.1AB-0208 as a suitable surrogate for eCry3.1Ab produced in 5307 corn for safety testing purposes. Appendix C provides further details of the methods and the results for these analyses of biochemical and functional equivalence.

VI.A.2. Safety Assessment for eCry3.1Ab

A mammalian safety data package was reviewed by the U.S. EPA in connection with the approval of the current Experimental Use Permit for 5307 corn, which was granted in June 2010 (67979-EUP-8). Effective June 16, 2010, EPA established a temporary exemption from the requirement of a tolerance for eCry3.1Ab residues in corn commodities, pursuant to §408(d) of the Federal Food, Drug, and Cosmetic Act, 21 U.S.C. §346a(d) (EPA, 2010a; 40 CFR § 174.532). Syngenta will soon petition the EPA to convert this temporary tolerance exemption to a permanent exemption, in connection with an application for FIFRA Section 3 registration of the eCry3.1Ab protein in 5307 corn. An assessment of human and animal safety of the eCry3.1Ab protein will be submitted to the U.S. FDA in December 2010 in connection with a premarket consultation.

The conclusions of the safety assessment for eCry3.1Ab are summarized below.

1. The donor organism for the source genes used to create *ecry3.1Ab* is *Bacillus thuringiensis*, a ubiquitous soil bacterium. Insecticidal Cry proteins from *B. thuringiensis*, including the source proteins from which eCry3.1Ab was engineered, 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. Additionallly, bacteria are not known to be sources of allergenic proteins (Taylor and Hefle, 2001; FAO/WHO, 2001).

2. Bioinformatic analyses showed that the eCry3.1Ab amino acid sequence (653 amino acids) has no significant sequence similarity to any toxins, other than other Cry proteins (also identified as δ-endotoxins or insecticidal crystal proteins in the search results). This comparison was conducted using the latest posting of the National Center for Biotechnology Information (NCBI) Entrez® Protein Database (NCBI, 2010b), and searching the database using the Basic Local Alignment Search Tool for Proteins (BLASTP) program[3] (Altschul et al., 1997).
3. The eCry3.1Ab protein is not acutely toxic to mice when administered orally at a very high dose, 2000 mg/kg body weight. (See study summary in Part VIII.D.2.)
4. The eCry3.1Ab protein is rapidly degraded in simulated gastric fluid containing pepsin at pH 1.2. No intact eCry3.1Ab or eCry3.1Ab-derived fragments were immunologically detectable by Western blot analysis after 30 seconds of exposure to simulated gastric fluid. In simulated intestinal fluid containing pancreatin, intact eCry3.1Ab was readily degraded within one minute, but smaller fragments of the protein remained undegraded following a 48-hour exposure. This behavior in simulated intestinal fluid is a common feature of Cry proteins. Additional intestinal proteases not present in pancreatin are involved in protein digestion *in vivo*, and would be expected to further degrade any fragments of eCry3.1Ab as part of normal digestive processes in mammals.
5. Bioinformatic analyses showed that the eCry3.1Ab amino acid sequence has no significant similarity to the sequences of known or putative allergens in the current posting of the Food Allergy Research and Resource Program (FARRP) AllergenOnline database, version 10.0 (FARRP, 2010). Sequential 80-amino-acid peptides of the eCry3.1Ab sequence were compared to the protein sequences in the Allergen Online database using the FASTA search algorithm[4] (Pearson and Lipman, 1988). Additionally, the eCry3.1Ab sequence was also examined for matches of eight contiguous amino acids (Hileman et al., 2002) with any allergen sequences, to screen for short, local regions of amino acid identity that might indicate the presence of common T-cell binding epitopes.
6. The eCry3.1Ab protein is inactivated by heating at temperatures of of 95ºC and above.
7. The eCry3.1Ab protein is not glycosylated. (See Appendix C.)
8. A standard weight-of-evidence analysis for allergenic potential (Codex, 2009) indicates that eCry3.1Ab is unlikely to be a food allergen and is unlikely to be cross-reactive to known allergens.

[3] BLASTP version 2.2.8 was used with the following parameters: no complexity filter; expectation score = 10; word size = 3; gap costs: existence = 11 and extension = 1. The similarity matrix was Blocks Substitution Matrix62 (BLOSUM62).

[4] FASTA version 3.45 was used with the following parameters: extension penalty of 2 and gap creation penalty of 12. The scoring matrix was the Blocks Substitution Matrix 50 (BLOSUM50).

VI.B. Phosphomannose Isomerase – Origin and Function

Event 5307 corn plants express the gene *pmi* (also knows as *manA*) from *E. coli* strain K-12 (Miles and Guest, 1984). This gene encodes the enzyme phosphomannose isomerase (PMI) that catalyzes the reversible conversion of mannose 6-phosphate and fructose 6-phosphate and enables many microorganisms to utilize mannose as a carbon and energy source. PMI enzymes are ubiquitous in nature, and are essential for many organisms, including humans. The purpose of PMI in 5307 corn was as a selectable marker; it does not have agronomic utility, e.g., it does not confer herbicide tolerance.

VI.B.1. Mechanism of Action

PMI has utility as a selectable marker for transformation of many plant species (Bojsen et al., 1994; Joersbo, et al., 1998; Negrotto et al., 2000). Plant cells that have been transformed with the gene *pmi* are able to survive and grow on media containing mannose as the only or primary energy source. When placed in medium containing mannose as the sole or primary sugar source, nontransformed tissue remains dormant and becomes outgrown by transformed tissue. Mannose itself is not toxic to plant cells. Inclusion of *pmi* in the T-DNA of transformation plasmid pSYN12274 allowed for selection of successfully transformed corn plantlets.

VI.B.2. Characterization of Phosphomannose Isomerase

A number of analytical methods were used to characterize the PMI protein produced in 5307 corn plants. Significant quantities of PMI were required to conduct mammalian safety studies with the protein. Because it was not feasible to extract the required quantities of PMI from 5307 plants for this safety testing program, PMI was produced in a recombinant *E. coli* fermentation system and purified in active form. Biochemical and bioactivity analyses were then conducted to establish the equivalence of the microbially produced PMI to PMI as produced in 5307 plants, to justify use of the microbially produced material as a surrogate for testing purposes.

VI.B.1.a. Production of PMI for Safety Testing

PMI test substance was produced in recombinant *E. coli* by over-expressing the same *pmi* gene that was introduced into 5307 corn. The gene *pmi* (*manA*) from *E. coli* strain K-12 was cloned into the inducible, over-expression pET-24a vector and transformed into *E. coli* strain BL21(DE3)RP. The PMI protein as encoded in this vector was identical in amino acid sequence to that encoded by the vector used to produce 5307 corn.

E. coli cell pellets were ruptured in 25 mM Tris-HCl (pH 7.5) and the cell debris was removed by centrifugation. The PMI present in the supernatant was purified with protamine sulfate precipitation followed by anion exchange chromatography using a Q Sepharose FF column. PMI was eluted from the column using a NaCl gradient, concentrated and subjected to gel filtration using a Superdex-200 column and 50 mM NH_4HCO_3 (pH 8.0) buffer. Fractions containing PMI were pooled, frozen and lyophilized. The lyophilized PMI sample preparation was designated test substance PMI-0105. This test substance was determined by spectrophotometric analysis to contain 89.9% protein, and densitometric analysis indicated that 99.6% of the total protein in the test substance was PMI. Thus the

purity of test substance PMI-0105 was calculated to be 89.5% PMI by weight. The predicted PMI molecular weight of 42.8 kDa was confirmed by mass spectrometry. Western blot analysis of test substance PMI-0105 revealed a dominant immunoreactive band corresponding to the expected PMI molecular weight. N-terminal sequencing confirmed that the first 15 amino acids of the test protein corresponded to the predicted N-terminal sequence of PMI.

VI.B.1.b. Equivalence of PMI in Test Substance and in 5307 Plants

A series of analytical methods were used to characterize the PMI protein produced in 5307 corn and to demonstrate that a PMI test substance (PMI-0105) produced in recombinant *E. coli* is a suitable surrogate for use in food and feed safety studies. The genotypes of the 5307 and control plants used in this study were NP2171 × NP2460($BC5F_3$)(5307) and NP2171 × NP2460, respectively. (See also Table III-1 and the breeding pedigree diagram in Figure III-3.) The plants used for this study were grown in a Syngenta greenhouse facility.

The identities of both the plant-produced and microbially produced PMI proteins were confirmed by apparent molecular weight, immunoreactivity, and enzymatic activity. Plant-produced and microbially produced PMI proteins were demonstrated to have the predicted molecular weights of approximately 42.8 kDa and both immunologically cross-reacted with the same anti-PMI antibodies, as determined by Western blot analysis, thus confirming the identity and integrity of the PMI proteins from both sources. It was also confirmed that both sources of PMI proteins catalyzed the same chemical reaction[5] and had similar specific activity of 455.67 U/mg PMI for the plant-produced PMI and 526.26 U/mg PMI for the microbially produced PMI.

Based on these results, the identities of the plant- and microbially produced PMI proteins were verified and it can be concluded that the PMI produced in 5307 corn and recombinant *E. coli* are biochemically and functionally equivalent. Therefore, the microbially produced PMI, as contained in test substance PMI-0105, is a suitable surrogate for PMI expressed in 5307 corn for use in safety studies. A more detailed description of the methods and results for this protein characterization work can be found in Appendix C.

VI.B.3. Safety Assessment for Phosphomannose Isomerase

Based on its safety profile, the U.S. EPA has established a permanent exemption from the requirement of food or feed tolerances for phosphomannose isomerise (PMI) in all crops (EPA, 2007; 40 CFR §174.527). PMI is also present as a selectable marker in two other Syngenta corn events that have been deregulated by APHIS, namely MIR604 corn (for rootworm control) and MIR162 corn (for lepidopteran control). An updated assessment of human and animal safety of PMI will be submitted to the U.S. FDA in December 2010 in connection with a premarket consultation for 5307 corn.

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[5] One unit (U) of PMI activity is defined as the amount of enzyme required to catalyze the conversion of 1 µmol of mannose 6-phosphate to fructose 6-phosphate per minute (equivalent to 1 µmol NADP reduced per min). Reaction: Mannose 6-P (catalyzed by PMI) → Fructose 6-P (catalyzed by phosphoglucose isomerase) → Glucose 6-P +NADP (catalyzed by glucose 6-P dehydrogenase) → 6- Gluconolactone + NADPH

The conclusions of the safety assessment for PMI are summarized below.

1. The source of the gene *pmi* is *E. coli* strain K-12, a non-pathogenic strain of this ubiquitous bacterium. The PMI protein encoded by this gene has a nontoxic mode of action. PMI enzymes serve an essential function in many organisms, including humans. Bacteria are not known to be sources of allergenic proteins (Taylor and Hefle, 2001; FAO/WHO, 2001).
2. Bioinformatic analyses showed that the PMI amino acid sequence (391 amino acids) has no significant sequence similarity to any toxins. This comparison was conducted using the latest posting of the National Center for Biotechnology Information (NCBI) Entrez® Protein Database (NCBI, 2010b), and searching the database using the Basic Local Alignment Search Tool for Proteins (BLASTP) program[6] (Altschul et al., 1997).
3. PMI (as test substance PMI-0105, containing 89.5% PMI by weight) was not acutely toxic to mice when administered orally at a very high dose, 2000 mg PMI/kg body weight. There were no mortalities and no adverse clinical signs of toxicity in mice treated with PMI and observed for 14 days. There were no adverse test substance-related effects on body weight, food consumption, clinical pathology, organ weights, macroscopic pathology or microscopic pathology. In 1998, a previous PMI acute oral toxicity study was conducted using test substance PMI-0198 (60% PMI by weight), in which the mice received 3030 mg PMI/kg and were observed for 14 days. This study evaluated a more condensed list of parameters than the acute toxicity study utilizing test substance PMI-0105, and examined organ weights of the liver, spleen, kidneys and brain. Similarly, however, there were no mortalities due to ingestion of the test substance, no adverse clinical signs of toxicity, no effects on body weight, and no effects on organ weights in mice treated with PMI-0198. This 1998 toxicity study was used to support the permanent tolerance exemption granted for PMI (EPA, 2007).
4. PMI is rapidly degraded in simulated gastric fluid containing pepsin at pH 1.2. No intact PMI or degradation products were visible on the Western blot following one minute of exposure to simulated gastric fluid. In simulated intestinal fluid containing pancreatin, the majority of intact PMI was digested within 5 minutes and no intact PMI or degradation products were detected by Western blot analysis upon sampling of the reaction mixture after 15 minutes.
5. Bioinformatic analyses showed that the PMI amino acid sequence has no biologically relevant similarity to the sequences of known or putative allergens in the current posting of FARRP AllergenOnline database, version 10.0 (FARRP, 2010). Sequential 80-amino-acid peptides of the PMI sequence were compared to the protein sequences in the Allergen Online database using the FASTA search algorithm[7] (Pearson and Lipman,

[6] BLASTP version 2.2.8 was used with the following parameters: no complexity filter; expectation score = 10; word size = 3; gap costs: existence = 11 and extension = 1. The similarity matrix was Blocks Substitution Matrix62 (BLOSUM62).

[7] FASTA version 3.45 was used with the following parameters: extension penalty of 2 and gap creation penalty of 12. The scoring matrix was the Blocks Substitution Matrix 50 (BLOSUM50).

1988). Additionally, the PMI sequence was also examined for matches of eight contiguous amino acids (Hileman et al., 2002) with any allergen sequences, to screen for short, local regions of amino acid identity that might indicate the presence of common T-cell binding epitopes. As previously identified, there was one sequence identity match of eight contiguous identical amino acids between PMI and a known allergen, α-parvalbumin from *Rana* species CH2001 (unidentified edible frog) (Hilger et al., 2002). Further investigation using IgE-specific serum screening methodology (Codex, 2009) demonstrated no cross-reactivity between PMI and the allergen α-parvalbumin, using serum from the single individual known to have demonstrated IgE-mediated allergy to this specific α-parvalbumin. The allergic patient's serum IgE did not recognize any portion of PMI as an allergenic epitope. These results support the conclusion that the eight-amino-acid sequence identity between the PMI protein and α-parvalbumin from *Rana* species CH2001 is not biologically relevant and has no implications for the potential cross-reactivity between PMI and α-parvalbumin. Therefore, the short sequence identity match with α-parvalbumin has no implications for the potential allergenicity of PMI.

6. PMI is inactivated by heating at temperatures of of 65ºC and above.
7. PMI is predicted not to be glycosylated in 5307 corn. The same PMI protein is not glycosylated in other transgenic corn cultivars.
8. A standard weight-of-evidence analysis for allergenic potential (Codex, 2009) indicates that PMI is unlikely to be a food allergen and is unlikely to be cross-reactive to known allergens.

VI.C. eCry3.1Ab and PMI Concentrations in 5307 Corn

The concentrations of the eCry3.1Ab and PMI proteins in various 5307 plant tissues were quantified using enzyme-linked immunosorbent assay (ELISA). The tissues analyzed were leaves, roots, and whole plants at four growth stages (whorl, anthesis, maturity and senescence), kernels at two stages (maturity and senescence) and pollen. Tissues were collected from a 5307 corn hybrid and a nontransgenic, near-isogenic control hybrid grown concurrently according to local agronomic practices and sampled at four US Corn Belt locations in 2008. The genotypes of the plants used in these studies were NP2171 × NP2460($BC5F_3$)(5307) and NP2171 × NP2460, respectively, as also indicated in Table III-1 and Figure III-3. These plants were grown under USDA APHIS notification 08-051-104n.

At each location, ten plants of the 5307 hybrid were collected at each sampling stage. Five of these plants were retained as whole-plant samples, and individual tissues were collected from the remaining five plants. Pollen was collected from at least 10 plants and pooled to create a single composite sample per location. Each pollen sample was air-dried and sieved to remove nonpollen debris (e.g., anthers and aphids). Tissues were also collected from nontransgenic control plants to assess the impact, if any, of the plant matrices on the ELISA. All plant tissue samples were shipped overnight on ice to Syngenta Biotechnology, Inc., where they were stored at -80°C ± 10°C until processed for analysis. Details of the tissue processing, extraction, and ELISA methods are provided in Appendix D.

The mean tissue and whole-plant concentrations of eCry3.1Ab and PMI across all four locations were determined on a fresh-weight and dry-weight basis (Tables VI-2 and VI-3). Except where noted, all values were corrected for extraction efficiency. Where the measured concentrations were below the lower limit of quantitation (< LOQ) or the limit of detection (< LOD), the respective LOQs and LODs are indicated on the data tables.

On a fresh-weight basis, the concentrations of eCry3.1Ab in individual samples across all locations and plant stages ranged from < LOQ to 71.21 µg/g in leaves, 0.40 µg/g to 9.29 µg/g in roots, 1.60 µg/g to 7.29 µg/g in kernels, < LOQ to 0.09 µg/g in pollen and 1.70 µg/g to 28.64 µg/g in whole plants (Table VI-2). On a fresh-weight basis, the concentrations of PMI in individual samples across all locations and plant stages ranged from < LOD to 1.66 µg/g in leaves, < LOQ to 1.07 µg/g in roots, 0.50 µg/g to 2.38 µg/g in kernels, 5.16 µg/g to 6.06 µg/g in pollen and 0.15 µg/g to 2.13 µg/g in whole plants (Table VI-3).

Table VI-2. Mean tissue concentrations of eCry3.1Ab in 5307 plants.

Tissue concentrations are presented on a fresh weight (FW) and dry weight (DW) basis. Low eCry3.1Ab concentrations in pollen precluded the determination of method extraction efficiency for this tissue; all other ELISA results are corrected for extraction efficiency. Control plant tissues had no detectable eCry3.1Ab.

Tissue Type	Whorl	Anthesis	Maturity	Senescence
eCry3.1Ab µg/g fresh weight (range)				
Leaves[1]	23.75 (16.81–33.80)	20.23 (13.83–27.59)	25.33 (0.89–71.21)	(< LOQ[2]–20.29)
Roots	5.54 (3.67–9.29)	3.27 (1.88–5.99)	2.98 (1.02–5.97)	2.73 (0.40–7.81)
Kernels	N/A[3]	N/A	4.56 (1.60–7.29)	3.24 (2.38–4.66)
Pollen	N/A	< LOQ–0.09	N/A	N/A
Whole Plants	15.78 (11.41–28.64)	8.11 (3.10–13.12)	8.86 (3.36–21.96)	3.60 (1.70–10.65)
eCry3.1Ab µg/g dry weight (range)				
Leaves[1]	142.96 (88.65–279.79)	84.34 (61.37–112.62)	49.04 (1.46–105.60)	(< LOQ–26.50)
Roots	42.72 (30.47–69.66)	18.20 (12.39–29.59)	11.96 (3.18–22.25)	9.13 (1.40–21.74)
Kernels	N/A	N/A	6.19 (2.37–9.64)	4.45 (2.92–6.76)
Pollen	N/A	< LOQ–0.10	N/A	N/A
Whole Plants	111.08 (75.16–178.22)	38.14 (14.18–55.67)	16.03 (6.37–38.94)	8.27 (3.41–25.46)

[1] Means were not calculated where the values for one or more plants were less than the lower limit of quantification (< LOQ); in such cases, only the range of values is shown

[2] LOQ = 0.08 µg/g FW and 0.10 µg/g DW pollen; 0.02 µg/g FW and 0.10 µg/g DW leaves at senescence

[3] N/A = not analyzed

Table VI-3. Mean tissue concentrations of PMI in 5307 plants.

Plant tissue oncentrations are presented on a fresh weight (FW) and dry weight (DW) basis. All ELISA results have been corrected for method extraction efficiency. Control plant tissues had no detectable PMI.

Tissue Type	Whorl	Anthesis	Maturity	Senescence
PMI µg/g fresh weight (range)				
Leaves[1]	0.81 (0.59–1.13)	0.70 (0.43–1.28)	(< LOQ[2]–1.66)	(< LOD[3]–0.42)
Roots[1]	0.28 (0.15–0.49)	0.31 (0.16–0.57)	0.45 (0.17–0.64)	(< LOQ–1.07)
Kernels	N/A[4]	N/A	1.36 (0.74–2.38)	0.82 (0.50–1.28)
Pollen	N/A	5.47 (5.16-6.06)	N/A	N/A
Whole Plant	0.62 (0.34–1.13)	0.93 (0.44–2.13)	0.96 (0.41–1.57)	0.43 (0.15–0.71)
PMI µg/g dry weight (range)				
Leaves[1]	4.83 (2.97–8.33)	2.91 (1.74–5.20)	(< LOQ–3.50)	(< LOD–0.54)
Roots[1]	2.11 (1.30–3.75)	1.69 (0.97–3.13)	1.85 (0.92–2.88)	(< LOQ–3.02)
Kernels	N/A	N/A	2.08 (1.04–3.82)	1.11 (0.70–1.62)
Pollen	N/A	6.51 (5.79-7.23)	N/A	N/A
Whole Plants	4.23 (2.59–7.69)	4.38 (2.00–8.83)	1.83 (0.68–2.56)	0.97 (0.39–2.02)

[1] Means were not calculated where the values for one or more plants were less than the lower limit of quantification (< LOQ) or the limit of detection (< LOD); in such cases, only the range of values are shown

[2] LOQ = 0.03 µg/g FW and 0.06 µg/g DW leaves at maturity; 0.03 µg/g FW and 0.10 µg/g DW roots at senescence

[3] LOD = 0.01 µg/g FW and DW leaves at senescence

[4] N/A = not analyzed

VII. Phenotypic and Compositional Evaluation

Laboratory, greenhouse, growth chamber, and field investigations were conducted to assess the phenotype and biochemical composition of 5307 corn. The purpose of these investigations was to determine if unintended changes occurred in 5307 corn as a result of the transformation process and to determine if any unintended changes conferred a plant pest risk potential to the new cultivar. Multiple phenotypic and compositional endpoints were evaluated and compared between 5307 corn and nontrangenic near-isogenic control corn. For several assessments, additional nontransgenic corn varieties were also used for comparison.

VII.A. Phenotypic Assessment of 5307 Plants

A range of phenotypic parameters assessing seed germination and dormancy, growth characteristics, reproductive capability, seed dispersal, and interactions with abiotic stressors and biotic stressors were examined for 5307 and control plants. Table VII-1 provides a listing of the parameters evaluated. The purpose of these evaluations was to ascertain whether the transformation that created 5307 corn imparted a phenotypic characteristic that could increase the environmental persistence of the new cultivar or result in invasive characteristics. Persistence or invasiveness could be indicative of increased weediness potential and plant pest risk. If no significant differences in these characteristics are found between 5307 corn and nontransgenic control corn, it supports the conclusion that 5307 corn does not have increased plant pest potential.

Table VII-1. Phenotypic characteristics evaluated for 5307 corn hybrids.
Evaluations were made in laboratory, greenhouse, growth chamber, or field experiments.

Phenotypic Characteristic	Variable Measured	Timing[1]	Description[1]
Dormancy/ Germination	Dormancy and germination	After 4, 7, and 12 days	Percent normal germinated, abnormal germinated, dead seed, firm swollen seed and hard seed
Emergence	Early emergence vigor	Before V3 stage	Early emergence vigor rating recorded before 14 days after planting on a scale of 1 - 9, 1 being most vigorous
	Emerged plants	Stage V3 stage	Number of emerged plants per plot
Vegetative Growth	Early growth vigor	At or after V6 stage	Early growth vigor rating recorded at or after V6 stage on a scale of 1 - 9, 1 being most vigorous
	Early root lodging	Before anthesis	Percent plants per plot leaning greater than 30 degrees from vertical at the root prior to anthesis
	Snapped plants	Before anthesis	Percent of plants per plot broken prior to anthesis due to adverse environmental conditions, such as high wind speeds

Table VII-1 (*Continued*). Phenotypic characteristics evaluated for 5307 corn hybrids.

Phenotypic Characteristic	Variable Measured	Timing[1]	Description[1]
Vegetative Growth (continued)	Ear height	After anthesis	Ear height (cm) from base of plant to node where ear connects to plant. Data collected when plant is between R2 and R6
	Plant height	After anthesis	Plant height (cm) from base of plant to collar of flag leaf. Data collected when plant is between R2 and R6
	Leaf color rating	After anthesis	Leaf color rating collected between R4 and R6 stage of corn development; 5 = same as commercial check hybrid, 1 = darker, 9 = severely chlorotic
	Late season intactness	Harvest	Rating of late-season integrity of the plant above the ear; 1 = all plant parts intact at harvest; 9 = 100% of plants in the plot are broken at the ear node prior to harvest
	Late root lodging	Harvest	Percent plants per plot leaning greater than 30° from vertical at the root after anthesis
	Push test	Harvest	Number of plants out of 10 plants tested that break the stalk or have root failure after pushing to 45 degrees from vertical
	Stalk lodging	Harvest	Number of plants per plot with broken stalks below the ear at harvest
Reproductive Growth	Heat units to 50% pollen shed	Flowering	Heat units to 50% of plants shedding pollen
	Pollen viability	Pollen shed	Percent viable pollen based on staining characteristics
	Pollen morphology	Pollen shed	Diameter (µm) of viable pollen grains
	Heat units to 50% silking	Flowering	Heat units to 50% of plants extruding silks
	Plant population at harvest	Harvest	Extrapolated estimate of plant harvest population (plants per acre)
	Barren plants	Harvest	Number of plants per plot that do not develop an ear
	Grain moisture	Harvest	Percent grain moisture at harvest
	Test weight	Harvest	Grain test weight (pounds/bushel) converted to standard 15.5% moisture
	Grain yield	Harvest	Grain yield (bushels/acre) converted to standard 15.5% grain moisture
Seed Retention	Dropped ears	Prior to harvest	Number of plants per plot that have dropped a developed ear prior to harvest
Plant-ecological Interactions	Disease susceptibility	Planting to harvest	Observations of occurrence of gray leaf spot or northern corn leaf blight disease on a 1 – 9 rating scale; 1 = no visible disease on plant, 9 = plant shows complete susceptibility to disease

[1] Plant stages described are as follows: V3 = 3-leaf stage; V6 = 6-leaf stage; R2 = kernel blister stage (10-14 days after silking); R4 = kernel dough stage (24-28 days after silking); R6 = physiologic maturity

VII.A.1. Seed Dormancy and Germination

Enhanced germination or seed dormancy are characteristics that can be indicative of plant pest potential. Dormancy mechanisms are used 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 5307 corn seed using a modification of the testing protocol established by the Association of Official Seed Analysts (AOSA). Seed samples of two 5307 hybrids, their respective nontransgenic, near-isogenic controls, and three conventional hybrids served as test, control, and reference materials, respectively, for the study. Table VII-2 shows the genotypes of the test, control, and reference hybrids. (See also Table III-1 and the breeding pedigree diagram in Figure III-3.)

Table VII-2. Event 5307, control, and reference seed varieties used in seed germination and dormancy tests.

Hybrid Designation	Variety
5307 Hybrid #1	NP2171 × NP2460(5307)
Control Hybrid #1	NP2171 × NP2460
5307 Hybrid #2	NP2391 × NP2222(5307)
Control Hybrid #2	NP2391 × NP2222
Reference Hybrid #1	N69-P9
Reference Hybrid #2	NX6176
Reference Hybrid #3	H-8991

The study design followed that described by AOSA (2005a) 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 nonoptimal temperature conditions (5°C, 10°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:

- constant temperatures: 5°C, 10°C, and 25°C
- alternating temperatures: 10°C/20°C, 10°C/30°C and 20°C/30°C

Experiments were conducted in unlighted temperature-controlled growth chambers. For the alternating temperature regimes, the lower temperature was maintained for 16 hours and the higher temperature for 8 hours, 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.

For the tests carried out at 25°C and 20°C/30°C (the standard temperatures used in the AOSA method), each germination towel was carefully unrolled and examined four and seven days after study initiation. Each seed was examined and categorized as one of the following:

- **normal germinated** (seed exhibited normal development of the root and shoot with a shoot length of at least 0.5 in (1.3 cm))
- **abnormal germinated** (seed lacked a well-developed root and shoot, or possessed a hollow coleoptile, or exhibited mechanical damage)
- **dead seed** (seed not germinated and visibly deteriorated and soft to the touch)
- **firm swollen seed** (seed visibly swollen and firm to the touch)
- **hard seed** (seed that had not imbibed water and was firm to the touch)

For the tests carried out at the nonoptimal temperature regimes of 5°C, 10°C, 10/20°C, and 10/30°C, each germination towel was carefully unrolled and examined 4, 7, and 12 days after study initiation. Each seed was examined and categorized as one of the following:

- **germinated** (seed with a radicle protruding beyond the seed coat)
- **dead seed** (seed not germinated and visibly deteriorated and soft to the touch)
- **firm swollen seed** (seed visibly swollen and firm to the touch)
- **hard seed** (seed that had not imbibed water and was firm to the touch)

At the end of each experiment, all firm swollen and hard seeds were subjected to a tetrazolium test to evaluate their viability (AOSA, 2005b). Seeds subjected to the tetrazolium test were then categorized as ‘viable firm swollen,’ ‘viable hard,’ or ‘dead’ (nonviable firm swollen or nonviable hard). Viable hard seed are indicative of dormancy potential.

The results of these seed germination and dormancy experiments are summarized in Tables VII-3 and VII-4. For each of the two 5307 hybrids and nontransgenic control hybrids, the data reported represent the combined data, averaged over four replicates, for observations at 4 and 7 days for the optimal temperature regimes and at 4, 7, and 12 days for the nonoptimal temperature regimes. For the three reference hybrids, the data reported represent the range in values across all four replicates per hybrid (12 total replicates).

As shown in Tables VII-3 and VII-4, many of the data points for seed germination and dormancy approached or equaled 100% or 0%. Such data are not well suited to statistical comparison by analysis of variance because they do not satisfy the assumptions upon which the validity of an analysis of variance depends. Instead, an alternative method of statistical analysis was applied. For each temperature regime, data for each 5307 hybrid was combined across replicates and compared to the corresponding data for its control hybrid using Fisher's Exact Test. For the nonoptimal temperature regimes (5°C, 10°C, 10/20°C, and 10/30°C), comparisons were made for the proportion of seeds classified as germinated (i.e., versus the sum of viable firm swollen seed, hard seed, and dead seed). For the AOSA

Table VII-3. Summary of 5307 corn seed germination and dormancy frequencies at nonoptimal temperatures.

Mean germination responses across four replicates from two 5307 and two control hybrids evaluated under different temperature regimes. Reference ranges across four replicates of each of three reference hybrids are provided for comparison. *P* values are provided for comparisons of the proportion of germinated seed within hybrid pairs; for values in bold text, $P < 0.05$.

Temp. (°C)	N	Genotype	Germinated		Viable Firm Swollen (%)	Viable Hard (%)	Dead (%)
			%	*p* value			
5	400	5307 Hybrid #1	0.0	1.000	91.0	0.0	9.0
	400	Control Hybrid #1	0.0		97.0	0.0	3.0
	400	5307 Hybrid #2	0.0	1.000	97.0	0.0	3.0
	400	Control Hybrid #2	0.0		97.0	0.0	3.0
	400	Range of Reference Hybrids #1, 2, and 3	0 – 0		93 – 100	0 – 0	0 – 7
10	400	5307 Hybrid #1	96.2	**0.018**	0.8	0.0	3.0
	400	Control Hybrid #1	99.0		0.8	0.0	0.2
	401[a]	5307 Hybrid #2	97.8	0.263	0.7	0.0	1.5
	400	Control Hybrid #2	99.0		0.8	0.0	0.2
	400	Range of Reference Hybrids #1, 2, and 3	67 - 100		0 – 26	0 - 0	0 – 7
10/20	400	5307 Hybrid #1	97.2	**< 0.001**	0.0	0.0	2.8
	400	Control Hybrid #1	100.0		0.0	0.0	0.0
	400	5307 Hybrid #2	98.2	0.177	0.0	0.0	1.8
	400	Control Hybrid #2	99.5		0.0	0.0	0.5
	400	Range of Reference Hybrids #1, 2, and 3	97 – 100		0 – 1	0 - 0	0 – 2
10/30	400	5307 Hybrid #1	97.0	**< 0.001**	0.0	0.0	3.0
	400	Control Hybrid #1	100.0		0.0	0.0	0.0
	400	5307 Hybrid #2	99.0	0.686	0.0	0.0	1.0
	400	Control Hybrid #2	99.5		0.0	0.0	0.5
	400	Range of Reference Hybrids #1, 2, and 3	98 – 100		0 – 0	0 – 0	0 – 2

N = Number of seeds tested

[a] One replicate inadvertently had an extra seed

Table VII-4. Summary of 5307 corn seed germination and dormancy frequencies at optimal temperatures.

Mean germination responses across four replicates from two 5307 and two control hybrids evaluated under different temperature regimes. Reference ranges across four replicates of each of three reference hybrids are provided for comparison. *P* values are provided for comparisons of the proportion of normally germinated seed within hybrid pairs; for values in bold text, $P < 0.05$.

Temp. (°C)	N	Genotype	Normally Germinated		Abnormally Germinated (%)	Viable Firm Swollen (%)	Viable Hard (%)	Dead (%)
			%	p value				
25	400	5307 Hybrid #1	97.8	0.420	0.5	0.0	0.0	1.8
	400	Control Hybrid #1	98.8		0.5	0.0	0.0	0.8
	400	5307 Hybrid #2	97.8	**0.021**	0.2	0.0	0.0	2.0
	400	Control Hybrid #2	99.8		0.2	0.0	0.0	0.0
	400	Range of Reference Hybrids #1, 2, and 3	98.0 – 100		0 – 2	0 – 1	0 – 0	0 – 1
20/30	400	5307 Hybrid #1	94.0	**< 0.001**	2.5	0.0	0.0	3.5
	400	Control Hybrid #1	99.0		0.8	0.0	0.0	0.2
	398[a]	5307 Hybrid #2	97.7	0.297	0.5	0.0	0.0	1.8
	400	Control Hybrid #2	98.8		1.2	0.0	0.0	0.0
	400	Range of Reference Hybrids #1, 2, and 3	97.0 - 100		0 – 3	0 – 0	0 – 0	0 – 2

N = Number of seeds tested

[a] One of the four replicates inadvertently contained 98 seeds instead of 100

temperature regimes (25°C and 20/30°C), statistical comparisons were made for the proportion of seeds classified as normally germinated (i.e., versus the sum of abnormally germinated seed, viable firm swollen seed, viable hard seed, and dead seed). The purpose of this comparison was to determine whether there were enhanced germination characteristics for the 5307 seeds as compared to the nontransgenic control seeds; statistically significant increases ($p < 0.05$) for the 5307 hybrid would provide evidence that such a difference exists.

At 5°C, the percentage of seeds that germinated normally was zero for all 5307, control and reference hybrids. Under all other temperature regimes, average germination ranged from 94.0% to 99.0% for the two 5307 hybrids and 98.8% to 100% for the corresponding nontransgenic controls. Under the same temperature regimes, germination ranged from 67% (at 10°C) to 100% across all replicates of the three reference hybrids. Under individual temperature regimes that supported germination, the percent germinated seed (at nonoptimal temperatures) or normally germinated seed (at optimal temperatures) was slightly lower for the 5307 hybrids when compared to the corresponding nontransgenic controls; some values for the 5307 hybrids were within the reference range and some were slightly below the reference range. Under the 10°C, 10°C/20°C, and 10°C/30°C temperature regimes (Table VII-3), germination was statistically significantly lower for 5307 Hybrid #1 than Control Hybrid #1, however, the differences between 5307 Hybrid #2 and Control Hybrid #2 were not significant. Similarly, under the 20°C/30°C regime (Table VII-4), normal germination

was statistically significantly lower for 5307 Hybrid #1 than Control Hybrid #1, and the differences between 5307 Hybrid #2 and Control Hybrid #2 were not significant. Under the 25°C regime, however, 5307 Hybrid #2 had statistically significantly lower normally germinated seed than Control Hybrid #2, while the differences between 5307 Hybrid #1 and Control Hybrid #1 were not significant. At no single temperature regime did both 5307 hybrids display significantly different germination than the corresponding control hybrids. The observation that 5307 corn hybrids did not show increased seed germination compared to control hybrids supports the conclusion that 5307 corn does not have plant pest characteristics or increased weediness potential.

Corresponding to the small decreases in germinated seed, small increases in the percent dead seed at all temperature regimes were observed for the 5307 hybrids compared to their corresponding controls. The observed values were within the corresponding reference range for six comparisons and slightly above the reference range for the remaining six comparisons. Under each temperature regime, the percent of viable firm swollen seed was identical among the 5307 and control hybrids for all comparisons except for 5307 Hybrid #1 at 5°C, for which the value was lower (91%) and slightly below the reference range (93 to 100%).

Most important from the perspective of plant pest risk is the consideration of whether the seed of the transgenic hybrids has increased dormancy potential, as measured by the presence of viable hard seeds. No viable hard seeds were found for any of the hybrids tested under any temperature regime, thus confirming that dormancy is not a normal characteristic of corn seeds and that 5307 corn demonstrates no increase in seed dormancy potential.

VII.A.2. Field Agronomic Performance

Field trials were conducted in the U.S. during the 2007 and 2008 growing seasons to compare a range of agronomic characteristics of a 5307 corn hybrid to a near-isogenic, nontransgenic hybrid. Table VII-5 shows the genotypes of the 5307 and control hybrids planted in these trials (see also Table III-1 and Figure III-3). The near-isogenic hybrid pairs were grown in five locations in 2007 and 12 locations in 2008. These agronomic trials were conducted under USDA APHIS notifications 07-043-109n and 08-051-104n. The locations of the trial sites are representative of major corn growing regions of the U.S. and are listed in Table VII-6.

The trials were planted in a randomized complete block design. In 2007, three replicate plots were planted per location. In 2008, four replicate plots were planted per location. Plot size was 0.002 acres (8 m^2), using two-row plots that were 17.5 feet long (5.3 meters), with 30-inch (0.8 m) spacing between the rows. Each plot contained approximately 68 plants. At each trial location, normal agricultural practices for pest control were followed, and the same products and application rates were used for both the 5307 plots and control plots.

Insecticides for rootworm control were applied to the control plots as well as the 5307 plots to minimize any rootworm-control advantage of the 5307 plants in these trials; soil and foliar applications were made for larval and adult rootworm control, respectively. No attempts were made to enhance natural rootworm infestation levels. The intent of these trials was to compare the underlying agronomic characteristics of 5307 and control corn in the absence of significant rootworm pressure.

Table VII-5. Event 5307 and control hybrids evaluated for agronomic performance.

Year	Hybrid Designation	Lineage
2007	5307 hybrid	NP2171 × NP2460($BC5F_2$)(5307)
	Control hybrid	NP2171 × NP2460
2008	5307 hybrid	NP2171 × NP2460(5307)($BC5F_3$)(5307)
	Control hybrid	NP2171 × NP2460

Table VII-6. Locations and dates of agronomic performance trials (2007 – 2008).

Year	City	State	Planting Date	Harvest Date
2007	Brookings	South Dakota	May 12	October 22
	Waldorf	Minnesota	May 15	October 25
	Corwith[1]	Iowa	June 7	October 13
	Green Valley	Illinois	May 10	September 21
	El Paso	Illinois	May 11	October 4
2008	Brookings	South Dakota	May 22	November 5
	Minnesota Lake	Minnesota	May 16	October 21
	Northfield[2]	Minnesota	May 15	October 27
	Janesville	Wisconsin	May 21	October 31
	New Haven	Indiana	May 25	October 31
	Beaver Crossing	Nebraska	June 2	October 5
	El Paso	Illinois	May 21	October 14
	Bloomington	Illinois	June 1	October 22
	Shirley	Illinois	June 18	November 1
	St. Joseph	Illinois	May 29	October 30
	La Salle	Illinois	May 20	October 28
	Marshall	Missouri	May 21	October 1

[1] Data were recorded for two replicate plots of the 5307 hybrid and control hybrid at this location. For all other locations in 2007, data for three replicate plots per genotype were recorded.

[2] Data were recorded for three replicate plots of the control hybrid at this location. For the 5307 hybrid at this location and both genotypes at all other locations in 2008, data for four replicate plots per genotype were recorded.

The agronomic characteristics assessed and the timing of each assessment are listed in Table VII-1. The agronomic characteristics chosen for comparison were those typically observed by professional corn breeders and agronomists, and represent a broad range of characteristics throughout the development of the corn plant. Not all characteristics were assessed at every trial location; the numbers of replicate plots (N) assessed for each variate are provided in the tables of results, described below. Natural disease infections were measured in trials where infections were sufficiently high to warrant assessment.

Data for several variates (characteristics) were subjected to an analysis of variance across locations using the model (Obert et al., 2004)

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

where Y_{ijk} is the observed response for genotype i at location j block k, U is the overall mean, T_i is the genotype effect, L_j is the location effect, $B(L)_{jk}$ is the effect of block within location, LT_{ij} is the location x genotype interaction effect and e_{ijk} is the residual error. For grain yield, results from analogous within-location analyses are also presented.

For each variate, the statistical significance of genotype (5307 versus near-isogenic control) effect was determined using a standard F-test. An F-test probability of < 5% indicates that the difference between the two genotypes was statistically significant at the customary 5% level. The JMP statistics program, version 7.0, was used for these analyses (SAS Institute; Cary, NC).

The data for other variates did not lend themselves to formal statistical analysis because they did not conform to the assumptions upon which the validity of the analysis depends. In some cases, the issue was that the data were too discrete, with values taking one of a very limited range of options. In other cases, the data set contained too few non-zero data points on which to base a reasonable estimate of residual error. Consequently, results for such variates are presented as means.

The standard error estimates of the means (SEM) were computed for the variates subjected to analysis of variance by dividing the appropriate error mean square by the sample size and then taking the square root of the resulting value. For variates not subjected to analysis of variance, the range of data values are presented because it is not possible to calculate a good estimate of error variance that takes into account the design structure. The sample sizes (N) for each of the traits were computed by summing the number of replications within or over locations.

Seventeen key agronomic traits were evaluated in the 2007 trials. Additionally, ratings for the severity of gray leaf spot and northern corn leaf blight diseases were recorded at a limited number of locations where ratings were possible. The data for these assessments are summarized in Tables VII-7 and VII-8. In these trials, the 5307 hybrid performed similarly to the corresponding nontransgenic, near-isogenic control hybrid. For seven out of the ten the traits subjected to statistical comparisons (Table VII-7), there were no significant differences between the 5307 hybrid and the control hybrid. For grain moisture, plant

height, and the number of heat units to 50% pollen shed, there were small but statistically significant differences between the 5307 and control hybrids. Approximately 20 to25 heat units were accumulated each day during pollination. Therefore, the observed average difference of 17 heat units between the 5307 hybrid and the control hybrid corresponds to less than one day, and would not have affected pollination. The absence of significant effects on grain yield suggest that the observed statistical differences in these three variates are not biologically significant.

Among the nine variates not subjected to statistical comparisons in the 2007 trials (Table VII-8), identical mean values were observed for the 5307 and control plants for four variates, and similar means were observed for three other variates. The 5307 plants did not show increased susceptibility to gray leaf spot or northern corn leaf blight diseases. The largest percent differences observed between the 5307 plots and control plots were in late root lodging and the number of barren plants. There were fewer 5307 plants with late root lodging (mean of 0.9% of plants per plot), compared to control plants (mean of 2.3% of plants per plot). (In the 2008 trials summarized below, there were not fewer 5307 plants with late root lodging.) There were a mean of 6.3 barren plants per plot for the 5307 hybrid and 2.7 barren plants per plot for the control hybrid. However, it is not possible to assign biological significance to the differences in the number of barren plants, because these observations were based on evaluation of three replicate plots at one location; as noted above, there were no significant differences in grain yield across the 2007 trial locations. Moreover, the mean number of barren plants per plot was identical between the 5307 and control plots in the 2008 trials, in which this variate was assessed in multiple locations (see below).

Twenty key agronomic traits were evaluated in the 2008 trials; the 5307 hybrid again performed similarly to the corresponding control hybrid. For the 10 the traits compared statistically (Table VII-9), there were no significant differences between the 5307 and control hybrids, except for grain yield, which was higher in the 5307 hybrid. Among the 10 other variates not compared statistically (Table VII-10), identical mean values were observed for the 5307 and control plants for four traits, and similar means were observed for the other variates. The largest percentage differences between mean values occurred for snapped plants, for which fewer were recorded in the 5307 plots (15%) than in the control plots (24%), and for late root lodging, which was higher in the 5307 plots (0.6%) versus the control plots (0.1%). This difference, however, was the result of higher numbers of root-lodged 5307 plants at a single trial location among the eight locations at which this trait was evaluated and, as such, does not suggest an agronomic deficit.

The results of these phenotypic assessments indicate that 5307 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 5307 corn were observed.

Table VII-7. 2007 agronomic performance trials: Comparisons for variates subjected to statistical comparisons.

Means across all locations, sample sizes (N = number of plots), standard errors, and statistical inferences for variates subjected to analysis of variance and F-test; significance assigned at $p < 0.05$ level.

Trait	N	5307 Mean	Control Mean	SEM	% Difference in Means	Prob. > F-value	Signifi-cance
Emerged plants/plot	12	59	58	0.57	1.7	15%	NS[1]
Early growth vigor (1 – 9 rating)	6	1.8	1.8	0.24	0.0	100%	NS
Ear height (cm)	14	94	94	1.13	0.0	84%	NS
Plant height (cm)	14	216	210	1.39	2.9	3%	*
Heat units to 50% pollen shed	12	1230	1247	0.89	− 1.4	0%	*
Heat units to 50% silking	12	1251	1265	7.92	− 1.1	29%	NS
Plant population at harvest (plants/acre)	14	27927	28524	870.14	− 2.1	65%	NS
Grain moisture (%)	14	17.9	16.9	0.18	5.9	3%	*
Test weight (pounds/bushel)	14	53.8	53.4	0.22	0.7	31.1%	NS
Yield (bushels/acre)	14	149.5	154.0	2.35	− 2.9	26.1%	NS

NS = F-test was not significant at the customary 5% level
* = F-test was significant

Table VII-8. 2007 agronomic performance trials: Comparisons for variates not subjected to formal statistical comparisons.

Means across locations, sample sizes (N = number of plots), and ranges of values for variates.

Trait	N	5307 Hybrid		Control Hybrid		% Difference in Means
		Mean	Range of values	Mean	Range of values	
Early emergence vigor (1 – 9 rating)	9	2.6	1 - 4	3.0	1 - 5	− 13.3
Leaf color rating (1 – 9)	9	5.0	5	5.0	5	0.0
Late-season intactness (1 – 9 rating)	14	6.4	4 – 8	6.2	4 - 8	3.2
Late root lodging (% plants/plot)	11	0.9	0 - 10	2.3	0 - 25	− 60.9
Stalk lodging (plants/plot)	11	0.2	0 – 1	0.2	0 - 1	0.0
Barren Plants (plants/plot)	3	6.3	5 – 9	2.7	2 - 4	133.3
Plants with dropped ears (plants/ plot)	2	0.0	0	0.0	0	0.0
Gray leaf spot disease rating (1 – 9 scale)	9	2.3	1 - 4	2.6	0 - 5	− 11.5
Northern corn leaf blight disease rating (1 – 9 scale)	2	1.0	1	1.0	1	0.0

Table VII-9. 2008 agronomic performance trials: Comparisons for variates subjected to statistical comparisons.

Means across all locations, sample sizes (N = number of plots), standard errors, and statistical inferences for variates subjected to analysis of variance and F-test; significance assigned at $p < 0.05$ level.

Trait	5307 Hybrid			Control Hybrid			% Difference in Means	Prob. > F-value	Signifi-cance
	N	Mean	SEM	N	Mean	SEM			
Emerged plants/plot	44	62	0.71	43	61	0.73	1.6	70%	NS
Early growth vigor (1-9 rating)	32	2.5	0.17	31	2.5	0.18	0.0	74%	NS
Ear height (cm)	40	113	1.81	39	117	1.87	− 3.4	16%	NS
Plant height (cm)	40	247	2.25	39	239	2.32	3.3	10%	NS
Heat units to 50% pollen shed	29	1330	2.28	28	1325	2.35	0.4	88%	NS
Heat units to 50% silking	29	1327	3.24	28	1325	3.34	0.2	61%	NS
Plant population at harvest (plants/acre)	48	29040	192.29	47	29092	197.56	− 0.2	75%	NS
Grain moisture (%)	48	20.8	0.14	47	20.3	0.14	2.5	9%	NS
Test weight (pounds/bushel)	48	57.0	0.12	47	57.0	0.12	0.0	35%	NS
Yield (bushels/ac)	48	151.6	2.34	47	138.6	2.36	9.4	0.47%	*

* = F-test was significant. NS = F-test was not significant at the customary 5% level

Table VII-10. 2008 agronomic performance trials: Comparisons for variates not subjected to formal statistical analysis.

Means across locations, sample sizes (N = number of plots), and ranges of values for variates.

Trait	5307 Hybrid			Control Hybrid			% Difference in Means
	N	Mean	Range of values	N	Mean	Range of values	
Early emergence vigor (1-9)	12	3.9	3 - 5	11	4.0	3 - 5	− 2.5
Early root lodging (% plants)	4	0.0	0	3	0.0	0	0.0
Snapped plants (%)	32	15	0 - 87	31	24	0 - 81	− 37.5
Leaf color rating (1 – 9)	32	5	5	31	5	5	0.0
Late-season intactness (1-9)	41	5.2	1 - 9	40	5.5	1 - 9	− 5.4
Late root lodging (% plants/plot)	32	0.6	0 - 5	30	0.1	0 - 2	500
Push test (no./10 plants)	24	5.9	1 - 10	24	6.9	2 - 10	− 14.5
Stalk lodging (plants/plot)	40	0.7	0 - 2	39	0.8	0 - 5	− 12.5
Barren plants (plants/plot)	28	1	0 - 4	27	1	0 - 4	0.0
Plants with dropped ears (plants/plot)	22	0	0 - 1	22	0	0	0.0

VII.A.3. Plant Disease Trials

Targeted field studies were conducted to examine whether 5307 hybrid corn plants were more susceptible to common plant fungal diseases as compared to their nontransgenic, near-isogenic counterparts grown concurrently under the same conditions. These trials were conducted in 2009 under USDA APHIS notification 09-063-103n. At one Minnesota location, susceptibility to eyespot disease (*Kabatiella zeae*) was assessed. At one Illinois location, susceptibility to southern corn leaf blight (*Helminthosporium maydis*) and gray leaf spot disease (*Cercospora zeae-maydis*) were assessed. The genotypes of the plants tested were 5XH751 × NP2222(5307) and 5XH751 × NP2222, as also described in Table III-1 and Figure III-3. Standard agronomic practices were used for all plots, except that foliar fungicides were not applied. All plots were planted in a randomized complete block design, using three replicate plots per genotype, and were artificially inoculated. Disease ratings were conducted on a 1 to 9 rating scale (ranging from no symptoms to severe symptoms). The diseases assessed and the test locations are shown in Table VII-11. The data were compared by analysis of variance. (Additional entries for other research-stage corn products in these trials are not shown.) No significant differences were observed in disease ratings between the 5307 hybrid and the control hybrid in these trials, indicating that 5307 corn is no more susceptible to these plant diseases than nontransgenic corn of the same genetic background.

Table VII-11. Plant Disease Ratings in Field Trials

Hybrid Genotype	Eyespot Disease Rating (Least Squares Mean)	Southern Corn Leaf Blight Disease Rating (Least Squares Mean)	Gray Leaf Spot Disease Rating (Least Squares Mean)
	Stanton, MN	Shirley, IL	Shirley, IL
5307 corn	5.67	2.33	5.33
Control corn	5.33	2.67	5.00
p value	0.714	0.356	0.457

VII.A.4. General Observations from Field Trials

In connection with its field trial compliance activities, Syngenta required information from the managers of each of the field sites at which 5307 corn was grown (under various USDA APHIS notifications, see Appendix A) in 2007, 2008 and 2009. (Information collection from the 2010 field trials is in progress.) This information included any observations of whether 5307 corn plants differed from control corn plants in the following characteristics:

- Plant health (e.g., susceptibility to disease, pests)
- Plant morphology (e.g., leaf color, seed set)
- Agronomic characteristics (e.g., weediness, response to drought)
- Impact on beneficial insects (e.g., ladybeetles)
- Impact on nontarget pest insects (e.g., mites)

Excluding any obvious differences in rootworm damage, no observations of differences in the above characteristics were recorded.

VII.A.5. Pollen Viability and Morphology

Pollen viability and morphology were evaluated to assess whether 5307 corn differed from near-isogenic, nontransgenic control corn in these characteristics. The 5307 and control hybrid genotypes used in these studies were NP2171 × NP2460($BC5F_3$)(5307) and NP2171 × NP2460, respectively. These genotypes are also indicated in Table III-1, and the breeding pedigree is shown in Figure III-3. Pollen grains were examined microscopically after fixing and staining according to the method described by Pedersen et al. (2004).

Twenty 5307 hybrid plants and 20 near-isogenic, nontransgenic control hybrid plants were grown in an environmentally controlled greenhouse. The greenhouse operated on a 16 hour/8 hour light/dark cycle with daytime temperatures ranging from 26°C - 29°C and nighttime temperatures ranging from 21°C - 23°C. Pollen was collected from the plants 67 - 69 days after planting. Pollen samples were immediately fixed in a 70% (v/v) ethanol solution and refrigerated. Samples were stained with a small volume of a KI/I_2 (iodine potassium iodide) solution and examined by light microscopy. Viability determinations were made at 50X magnification. Starch contained in the viable pollen cells readily bound to the iodine stain. Nonviable cells with little or no starch content were weakly stained and readily identifiable. Percent viability was determined by examining a minimum of 100 pollen cells per sample. Mean percent viable pollen was determined for the Event 5307 and control corn samples. These means were compared by a t-test with significance assigned at the standard $p < 0.05$ level. There was no significant difference in percent viable pollen detected between 5307 and control. The results of this analysis are presented in Table VII-12. Pollen viability for both hybrids was 99%.

The morphology and diameter of pollen grains in stained pollen samples from five 5307 and five control plants were examined. Morphology was assessed by a microscopic examination of all cells in the field of view. There were no discernible differences between Event 5307 and control corn pollen morphology. Pollen diameter was measured for ten pollen grains per sample. Mean diameter was calculated for the 5307 and control corn samples. The means were compared by a t-test with significance assigned at $p < 0.05$. Pollen cell diameter statistics are presented in Table VII-12. Photographs of representative Event 5307 and control corn pollen samples are shown in Figure VII-1. No significant differences in average cell diameter were detected between 5307 and control pollen samples.

Therefore, there were no discernible differences in pollen morphology, diameter, or viability between 5307 corn and nontransgenic control corn.

Table VII-12. Pollen viability and diameter measurements.
Mean percent viability and mean diameter for 5307 and control corn pollen are presented with their standard deviations (SD); means were compared by a t-test.

Variable	N	5307 Mean ± SD	Control Mean ± SD	*p* value
Pollen viability (%)	20	99.1 ± 0.76	98.8 ± 1.16	0.486
Pollen diameter (µm)	5	94.0 ± 0.001	93.9 ± 0.005	0.698

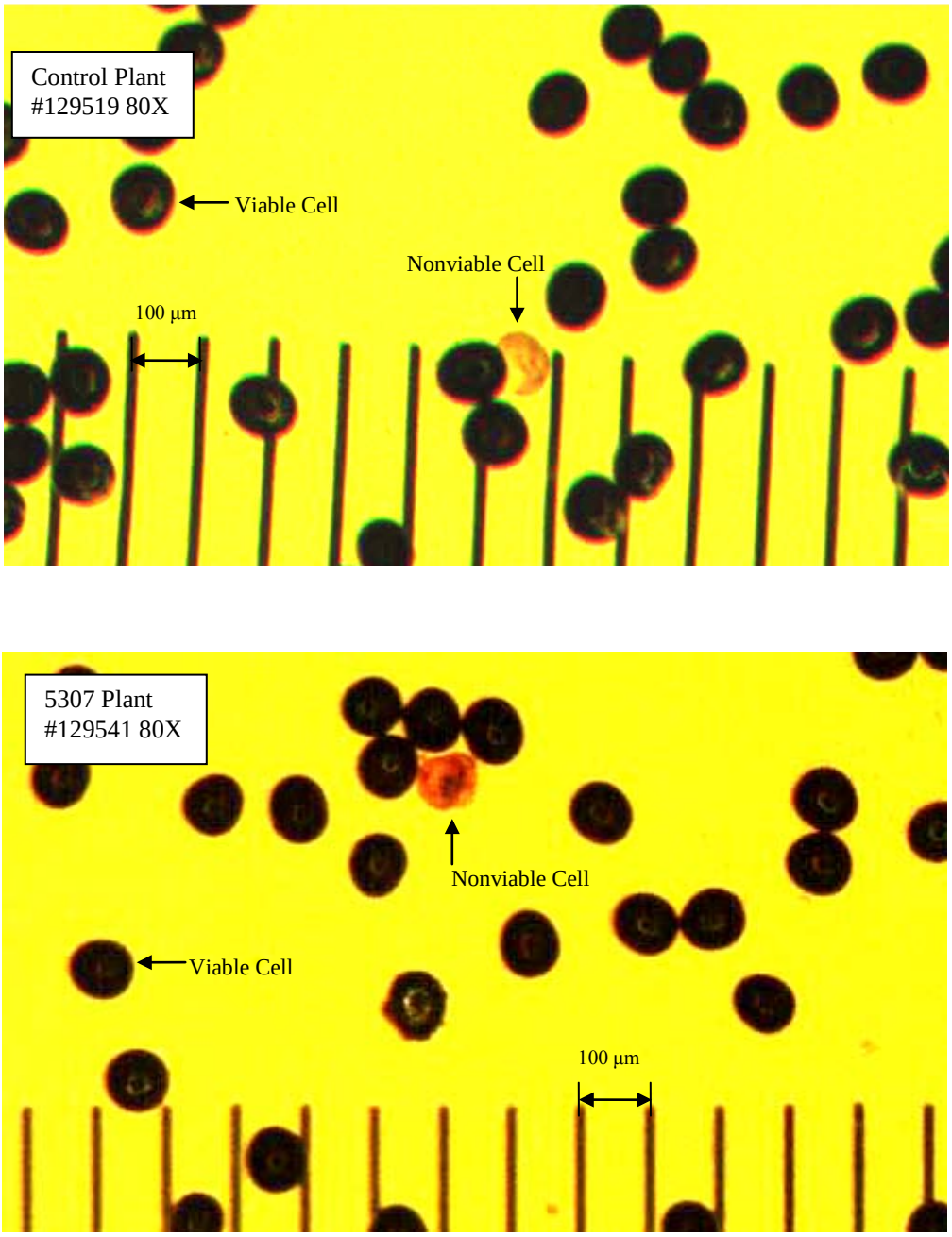

Figure VII-1. Photographs of stained pollen collected from control and 5307 plants. Magnification was 80X.

VII.A.6. Seed Dispersal

Dispersal of individual corn seeds does not occur naturally. The kernels are held inside the husks of the cob and are too heavy to be wind-blown. There was no significant difference in the propensity of 5307 corn to drop ears than that of conventional corn (see Tables VII-8 and VII-10). Dispersal of individual kernels does, however, take place as a result of mechanical harvesting and transportation. In this regard, 5307 corn kernels would be no different than conventional corn kernels. Because corn seeds lack dormancy, those that are dispersed outside of cultivated fields could germinate if environmental conditions were favorable. However, corn does not have weedy or invasive characteristics. As a highly domesticated crop, corn has lost the ability to reproduce without human intervention.

VII.B. Compositional Assessment of 5307 Forage and Grain

Compositional analyses of 5307 corn were performed to identify any changes in nutrient or anti-nutrient content of the new crop in the context of its use as food or feed and to assess its biochemical equivalence and familiarity to conventional corn. This assessment was undertaken by performing quantitative analyses of 59 biochemical components of 5307 hybrid corn forage and grain including key food and feed nutrients, antinutrients, and secondary plant metabolites. An identical set of analyses was performed on nontransgenic, near-isogenic control hybrid corn.

VII.B.1. Design and methods used in compositional analysis study

The 5307 hybrid plants were genotype NP2171 × NP2460(5307) and the control plants were genotype NP2171 × NP2460. The seed materials used to plant these composition trials are also identified in Table III-1 and in the pedigree diagram in Figure III-3. The trials were conducted under USDA APHIS notification 08-051-104n.

Forage and grain from the 5307 and control hybrids were harvested from six locations in the U.S. during 2008:

Location Code	City and State
L1	Stanton, MN
L2	Janesville, WI
L4	New Haven, IN
L6	Shirley, IL
L7	Marshall, MO
L8	Bloomington, IL

These locations are representative of major corn growing regions of the U.S. At each location, the hybrids were planted in a randomized complete block design, with three replicates for each genotype. All plots were managed according to local agronomic practices for the respective regions. Plants were self-pollinated by hand and the developing ears were bagged to avoid cross-pollination.

The components measured in this study were selected based on recommendations of the Organisation for Economic Co-operation and Development (OECD, 2002) for comparative

assessment of composition of new varieties of corn. The components analyzed are listed in Table VII-13 below.

All analyses were conducted using methods published and approved by the Association of Analytical Communities (AOAC) International or other industry-standard analytical methods. Based on the moisture content of each sample, analyte levels were converted to equivalent units of dry weight. A detailed description of the study design and methodology for the compositional analyses is provided in Appendix E.

VII.B.1.a. Statistical analysis for across-location comparisons

The data for each component were subjected to analysis of variance 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 genotype i at location j block k, U is the overall mean, T_i is the genotype effect, L_j is the location effect, $B(L)_{jk}$ is the effect of block within a location, LT_{ij} is the location-by-genotype interaction effect, and e_{ijk} is the residual error. Genotype was regarded as a fixed effect, while the effects of location, block within location, and location-by-genotype were regarded as random.

For each quantifiable component, an F test was used to assess the statistical significance of the genotype effect with an alpha level of 0.05 and with the denominator degrees of freedom determined using the Kenward-Roger method (Kenward and Roger, 1997). Moisture content of grain was not statistically analyzed because the samples had been mechanically dried.

VII.B.1.b. Statistical analysis for individual-location comparisons

The data for each component at each location were subjected to an analysis of variance with genotype and block included in the statistical model. Significance was based on an alpha level of 0.05.

Statistical analyses were performed using SAS v. 9.2 (SAS Institute, Inc.; Cary, NC).

VII.B.1.c. Comparison with ILSI Crop Composition Database

The mean levels of each component for each location and across locations were calculated and compared nonstatistically with means and ranges for forage and grain composition published in the ILSI Crop Composition Database (2008). The ILSI database is the most comprehensive and current source of crop composition data for most nutritional components.

Table VII-13. Forage and grain components measured in 5307 and conventional corn.

Forage	Grain			
Minerals	**Minerals**	**Amino acids**	**Secondary Metabolites**	**Fatty acids**
Calcium	Calcium	Alanine (Ala)	ρ-Coumaric acid	16:0 palmitic
Phosphorus	Copper	Arginine (Arg)	Ferulic acid	18:0 stearic
Proximates	Iron	Aspartic acid (Asp)	Furfural	18:1 oleic
Acid detergent fiber (ADF)	Magnesium	Cystine (Cys)	Inositol	18:2 linoleic
Ash	Manganese	Glutamic acid (Glu)	**Vitamins**	18:3 linolenic
Carbohydrates	Phosphorus	Glycine (Gly)	Vitamin A (β-carotene)	20:0 arachidic
Fat	Potassium	Histidine (His)	Vitamin B_1 (thiamine)	20:1 eicosenoic
Moisture	Selenium	Isoleucine (Ile)	Vitamin B_2 (riboflavin)	22:0 behenic
Neutral detergent fiber (NDF)	Sodium	Leucine (Leu)	Vitamin B_3 (niacin)	**Anti-nutrients**
Protein	Zinc	Lysine (Lys)	Vitamin B_6 (pyridoxine)	Phytic acid
	Proximates	Methionine (Met)	Vitamin B_9 (folic acid)	Raffinose
	ADF	Phenylalanine (Phe)	Vitamin E (α-tocopherol)	Trypsin inhibitor
	Ash	Proline (Pro)		
	Carbohydrates	Serine (Ser)		
	Fat	Threonine (Thr)		
	Moisture	Tryptophan (Trp)		
	NDF	Tyrosine (Tyr)		
	Protein	Valine (Val)		
	Starch			
	Total Dietary Fiber (TDF)			

Tables VII-14 through VII-21 report the statistical comparisons of nutritional component levels in forage and grain between 5307 corn and nontransgenic corn, the mean levels for each genotype across locations and at each location, a range of individual replicate values, and the levels for conventional hybrid corn reported in the ILSI Crop Composition Database (2008).

All data were compared with the ranges reported in the ILSI database to establish whether the results were within the range of natural variation and to provide an indication of whether the results were likely to be of biological significance.

VII.B.2. Results of statistical analysis for forage

When analyzed across all six locations, there were no statistically significant differences in any of the measured forage components (proximates, calcium, and phosphorus) between genotypes (Tables VII-14 and VII-15).

When analyzing the results at each individual location, statistically significant differences were observed in moisture, protein, calcium, and phosphorus values, but all of these components were only different at one location (out of six) and all means fell within the range of natural variation of corn reported in the ILSI database (ILSI 2008).

VII.B.3. Results of statistical analysis for grain

When analyzed across all six locations, there were no statistically significant differences in 52 of the 59 components including: proximates, starch, and fiber components (Table VII-16), minerals (Table VII-17), vitamins B_1, B_2, B_3 and E (Table VII-18), amino acids (Table VII-19), oleic, linoleic, arachidic, and behenic fatty acids (Table VII-20), antinutrients, or secondary metabolites (Table VII-21).

There were a few statistically significant differences observed in vitamins A, B_6, and B_9 (Table VII-18), as well as 16:0 palmitic, 18:0 stearic, 18:3 linolenic, and 20:1 eicosenoic acids (Table VII-20). However, the differences observed were small and the mean values observed for these vitamins and all but one fatty acid (18:3 linolenic) were all within the ranges of values observed for the nontransgenic grain, and all means fell within the natural variation of corn reported in the ILSI database (ILSI 2008).

Some statistically significant differences were observed in values for individual locations but all of these components were only different at one or two locations and all per-location means fell within the range of natural variation for corn reported in the ILSI database (ILSI 2008), with the exception of starch and Vitamin B_2 in the nontransgenic grain, for which the values exceeded the reported range.

VII.B.4. Conclusion of compositional analyses

For all 59 chemical components that were measured in 5307 forage and grain, including those for which statistically significant differences were observed, the average values (when quantifiable) were within the ranges of natural variation reported in the ILSI database (2008). No biologically significant changes in composition were found to have occurred as an unintended result of the transformation process or expression of the transgenes in 5307 corn. In conclusion, forage and grain from 5307 corn hybrids are considered similar in composition to forage and grain from both the nontransgenic comparator and conventional corn hybrids.

These data support the conclusion that 5307 corn will be as safe and nutritious as conventional corn.

Table VII-14. Proximate composition of forage from 5307 corn and nontransgenic corn.

Proximate levels are shown in % DW, except for moisture (% FW). Results significantly different at $P < 0.05$ are shown in bold italic type. For across-location analyses, $N = 18$; the range of values among these replicates is provided.

Location	Data source	Statistic	Moisture	Protein	Fat	Ash	Carbo-hydrates	ADF	NDF
Across all	Event 5307	mean	73.0	7.72	1.90	4.12	86.3	29.1	44.9
		range	66.5–79.5	5.91–10.3	0.893–2.81	2.89–5.35	82.9–88.9	22.3–40.1	35.5–56.1
	Nontransgenic	mean	72.3	7.57	1.89	4.34	86.2	28.6	45.4
		range	66.7–78.0	6.27–10.0	0.843–2.63	3.43–6.18	82.3–89.0	19.0–41.5	32.3–57.4
	ANOVA (*F* test)								
	Genotype effect	*P*	0.126	0.525	0.893	0.076	0.895	0.696	0.785
		SEM	1.49	0.449	0.118	0.295	0.64	1.33	1.91
	ILSI (2008)	mean	70.2	7.78	2.039	4.628	85.6	27.00	41.51
		range	49.1–81.3	3.14–11.57	<LOQ–4.570	1.527–9.638	76.4–92.1	16.13–47.39	20.29–63.71
		N[a]	945	945	921	945	945	945	945

[a]*N* is the number of ILSI values used to calculate the mean and excludes values <LOQ

Table VII-14 (*Continued*). Proximate composition of forage from 5307 corn and nontransgenic corn.
Proximate levels are shown in % DW, except for moisture (% FW). Results significantly different at $P < 0.05$ are shown in bold italic type.
For individual location means, $N = 3$.

Location	Data source	Statistic	Moisture	Protein	Fat	Ash	Carbo-hydrates	ADF	NDF
L1	Event 5307	mean	70.2	7.98	2.23	3.50	86.3	26.9	36.4
	Nontransgenic	mean	68.8	7.28	1.73	3.67	87.3	26.4	40.1
		P	0.208	0.286	0.415	0.162	0.162	0.887	0.480
		SEM	0.53	0.340	0.346	0.055	0.35	2.48	3.04
L2	Event 5307	mean	72.1	6.96	2.01	4.59	86.4	29.7	44.1
	Nontransgenic	mean	71.4	6.77	2.11	4.82	86.3	33.8	48.9
		P	0.630	0.731	0.772	0.363	0.961	0.433	0.563
		SEM	0.96	0.340	0.207	0.139	0.43	2.98	4.97
L4	Event 5307	mean	72.5	7.46	2.15	3.64	86.8	28.1	46.7
	Nontransgenic	mean	71.3	6.96	2.30	4.01	86.7	26.4	41.1
		P	0.105	***0.033***	0.795	0.417	0.771	0.738	0.295
		SEM	0.31	0.066	0.350	0.258	0.21	3.13	2.82
L6	Event 5307	mean	76.0	8.40	1.41	4.64	85.5	29.1	47.7
	Nontransgenic	mean	76.7	9.07	1.77	4.41	84.8	27.0	45.5
		P	0.654	0.521	0.192	0.646	0.349	0.092	0.077
		SEM	0.99	0.610	0.131	0.300	0.41	0.49	0.46
L7	Event 5307	mean	78.5	9.35	1.73	5.05	84.0	33.8	48.6
	Nontransgenic	mean	76.7	8.80	1.83	5.47	83.9	32.4	52.9
		P	***0.007***	0.141	0.695	0.377	0.804	0.672	0.541
		SEM	0.10	0.162	0.162	0.265	0.42	2.06	4.19
L8	Event 5307	mean	68.6	6.19	1.89	3.33	88.6	26.7	45.7
	Nontransgenic	mean	68.7	6.54	1.57	3.65	88.3	25.5	43.7
		P	0.225	0.057	0.323	0.162	0.625	0.688	0.594
		SEM	0.04	0.061	0.174	0.104	0.33	1.88	2.33

Table VII-15. Calcium and phosphorus composition of forage from 5307 cornand nontransgenic corn.

Calcium and phosphorus levels shown in mg/kg DW. Results significantly different at $P < 0.05$ are shown in bold italic type.
For across-location analyses, $N = 18$; the range of values among these replicates is provided.

Location	Data source	Statistic	Ca	P
Across all	Event 5307	mean	2346	1906
		range	1450–3470	1420–2870
	Nontransgenic	mean	2354	1953
		range	1660–3350	1390–2890
	ANOVA (*F* test)			
	Genotype effect	*P*	0.886	0.491
		SEM	209.3	163.9
	ILSI (2008)	mean	2028.6	2066.1
		range	713.9–5767.9	936.2–3704.1
		N	481	481

Table VII-15 *(Continued)*. Calcium and phosphorus composition of forage from 5307 corn and nontransgenic corn.

Calcium and phosphorus levels shown in mg/kg DW. Results significantly different at $P < 0.05$ are shown in bold italic type. For individual location means, $N = 3$.

Location	Data source	Statistic	Ca	P
L1	Event 5307	mean	2370	1723
	Nontransgenic	mean	2210	1523
		P	0.208	***0.003***
		SEM	61.6	8.2
L2	Event 5307	mean	2130	1500
	Nontransgenic	mean	2223	1457
		P	0.711	0.694
		SEM	154.5	67.4
L4	Event 5307	mean	2217	1783
	Nontransgenic	mean	2303	1813
		P	0.087	0.869
		SEM	19.3	113.1
L6	Event 5307	mean	2497	2493
	Nontransgenic	mean	2407	2677
		P	0.771	0.662
		SEM	191.4	255.6
L7	Event 5307	mean	3287	1947
	Nontransgenic	mean	3190	2043
		P	***0.032***	0.585
		SEM	12.5	105.9
L8	Event 5307	mean	1573	1990
	Nontransgenic	mean	1793	2203
		P	0.138	0.076
		SEM	64.8	44.0

Table VII-16. Proximate and starch composition of grain from 5307 corn and nontransgenic corn.

Proximate and starch levels shown in % DW, except moisture (% FW). Results significantly different at $P < 0.05$ are shown in bold italic type.
For across-location analyses, $N = 18$; the range of values among these replicates is provided.

Location	Data source	Statistic	Moisture[a]	Protein	Fat	Ash	Carbo-hydrates	ADF	NDF	TDF	Starch
Across all	Event 5307	mean	10.13	10.86	4.54	1.46	83.1	2.74	8.85	11.8	69.4
		range	9.54–11.4	9.12–12.6	3.85–4.93	1.22–1.60	81.0–85.3	2.23–3.34	7.68–9.52	10.8–13.4	62.0–73.7
	Nontransgenic	mean	10.18	10.92	4.72	1.40	83.0	2.85	8.83	11.7	70.3
		range	9.21–12.2	9.20–13.0	4.43–5.09	1.09–1.67	80.7–84.7	2.47–3.48	7.79–10.2	10.6–13.5	63.1–77.3
	ANOVA (*F* test)										
	Genotype effect	*P*	–	0.737	0.053	0.138	0.515	0.281	0.930	0.700	0.589
		SEM	–	0.375	0.067	0.044	0.44	0.069	0.128	0.19	1.21
	ILSI (2008)	mean	11.3	10.30	3.555	1.439	84.6	4.05	11.23	16.43	57.7
		range	6.1–40.5	6.15–17.26	1.742–5.823	0.616–6.282	77.4–89.5	1.82–11.34	5.59–22.64	8.85–35.31	26.5–73.8
		N	1434	1434	1174	1410	1410	1350	1349	397	168

– = not applicable

[a]Grain was mechanically dried after harvest; moisture levels were not subject to ANOVA

Table VII-16 *(Continued).* Proximate and starch composition of grain from 5307 corn and nontransgenic corn.

Proximate and starch levels shown in % DW, except moisture (% FW). Results significantly different at $P < 0.05$ are shown in bold italic type.
For individual location means, $N = 3$.

Location	Data source	Statistic	Moisturea	Protein	Fat	Ash	Carbo-hydrates	ADF	NDF	TDF	Starch
L1	Event 5307	mean	10.54	10.93	4.38	1.43	83.2	2.63	8.66	11.2	69.4
	Nontransgenic	mean	11.13	10.60	4.79	1.29	83.3	2.89	9.27	11.9	71.7
		P	–	0.405	0.068	***0.038***	0.578	0.390	0.623	0.456	0.401
		SEM	–	0.225	0.080	0.019	0.14	0.169	0.754	0.54	1.54
L2	Event 5307	mean	10.73	9.38	4.53	1.34	84.8	2.89	9.01	11.7	71.9
	Nontransgenic	mean	11.00	9.97	4.68	1.25	84.1	2.67	8.73	11.2	74.7
		P	–	***0.011***	0.358	0.594	0.070	0.152	0.407	0.431	0.090
		SEM	–	0.044	0.090	0.094	0.13	0.069	0.188	0.41	0.64
L4	Event 5307	mean	9.77	10.97	4.84	1.42	82.8	2.84	9.20	11.6	72.5
	Nontransgenic	mean	9.48	10.50	4.84	1.39	83.3	2.73	8.65	11.9	66.0
		P	–	0.630	0.974	0.628	0.621	0.391	0.406	0.710	***0.019***
		SEM	–	0.586	0.126	0.037	0.65	0.076	0.372	0.50	0.65
L6	Event 5307	mean	9.79	12.37	4.59	1.54	81.5	2.85	9.10	12.5	67.3
	Nontransgenic	mean	9.56	12.50	4.77	1.62	81.1	2.87	8.89	12.4	67.8
		P	–	0.801	0.257	0.159	0.593	0.962	0.442	0.978	0.940
		SEM	–	0.327	0.080	0.025	0.41	0.217	0.154	0.75	3.87
L7	Event 5307	mean	10.20	10.60	4.25	1.52	83.6	2.54	8.55	12.0	65.9
	Nontransgenic	mean	10.27	10.67	4.56	1.38	83.4	2.97	8.45	11.4	68.9
		P	–	0.868	0.384	0.112	0.805	0.188	0.842	0.135	0.222
		SEM	–	0.249	0.198	0.037	0.42	0.156	0.322	0.19	1.23
L8	Event 5307	mean	9.74	10.90	4.68	1.50	82.9	2.67	8.57	11.7	69.7
	Nontransgenic	mean	9.64	11.27	4.66	1.47	82.6	2.95	8.99	11.4	72.9
		P	–	0.053	0.792	0.159	0.057	0.479	0.519	0.189	0.283
		SEM	–	0.062	0.047	0.012	0.05	0.229	0.389	0.11	1.59

– = not applicable. [a]Grain was mechanically dried after harvest; moisture levels were not subject to ANOVA

Table VII-17. Mineral composition of grain from 5307 corn and nontransgenic corn.

Mineral levels shown in mg/kg DW. Results significant at $P < 0.05$ are shown in bold italic type.
For across-location analyses $N = 18$; the range of values among these replicates is provided.

Location	Data source	Statistic	Ca	Cu	Fe	Mg	Mn	P	K	Se[a,c]	Na[b,c]	Zn
Across all	Event 5307	mean	43.9	1.52	23.7	1323	5.65	3228	3758	–	–	23.0
		range	38.6–49.3	0.89–4.20	21.2–28.0	1150–1430	4.69–6.61	2620–3520	3400–4010	<LOQ–0.363	< LOQ	19.5–26.9
	Nontransgenic	mean	44.0	1.89	23.3	1336	5.43	3307	3776	–	–	23.4
		range	40.3–50.1	1.02–4.36	20.3–28.1	1220–1450	4.43–6.38	2650–3600	3240–4150	<LOQ–0.400	< LOQ	20.5–27.9
	ANOVA (*F* test)											
	Genotype effect	*P*	0.891	0.058	0.308	0.401	0.131	0.110	0.707	–	–	0.355
		SEM	1.28	0.253	0.85	21.2	0.249	94.7	81.0	–	–	0.78
	ILSI (2008)	mean	46.4	1.75	21.81	1193.8	6.18	3273.5	3842	0.20	31.75	21.6
		range	12.7–208.4	<LOQ–18.50	10.42–49.07	594.0–1940.0	1.69–14.30	1470.0–5330.0	1810.0–6030.0	<LOQ–0.75	<LOQ–731.54	6.5–37.2
		N[d]	1344	1249	1255	1257	1256	1349	1257	89	223	1257

– = not applicable
[a]The LOQ for selenium was 0.055–0.056 mg/kg DW
[b]The LOQ for sodium was 110–114 mg/kg DW
[c]Where some or all values were <LOQ, calculation of the mean and statistical comparison were not possible, thus only the range is shown
[d]*N* is the number of ILSI values used to calculate the mean and excludes values <LOQ

Table VII-17 *(Continued)*. Mineral composition of grain from 5307 corn and nontransgenic corn.
Mineral levels shown in mg/kg DW. Results significant at $P < 0.05$ are shown in bold italic type. For individual location means, $N = 3$.

Location	Data source	Statistic	Ca	Cu	Fe	Mg	Mn	P	K	Se[a]	Na[a]	Zn
L1	Event 5307	mean	47.6	2.66	23.6	1347	5.66	3163	3803	<LOQ–0.086	<LOQ	22.3
	Nontransgenic	mean	45.4	2.89	22.0	1330	5.03	3103	3680	0.079	<LOQ	22.1
		P	0.198	0.556	0.187	0.755	0.096	0.712	0.381	–	–	0.915
		SEM	0.81	0.232	0.55	33.0	0.149	99.7	78.1	–	–	0.78
L2	Event 5307	mean	41.6	1.35	22.4	1307	5.16	2857	3473	0.113	<LOQ	20.4
	Nontransgenic	mean	41.0	1.88	23.1	1337	5.31	2903	3403	0.125	<LOQ	22.1
		P	0.657	0.200	0.482	0.644	0.669	0.866	0.724	0.211	–	0.138
		SEM	0.87	0.198	0.63	39.4	0.219	172.4	121.7	0.0046	–	0.49
L4	Event 5307	mean	39.7	1.45	27.2	1297	5.09	3293	3657	0.348	<LOQ	25.7
	Nontransgenic	mean	41.2	1.28	27.5	1283	5.11	3413	3760	0.364	<LOQ	26.8
		P	0.200	0.453	0.701	0.732	0.910	0.230	0.446	0.569	–	***0.045***
		SEM	0.59	0.133	0.42	23.9	0.128	49.7	77.7	0.0160	–	0.16
L6	Event 5307	mean	46.7	0.95	22.5	1347	6.44	3383	3920	<LOQ–0.058	<LOQ	22.1
	Nontransgenic	mean	47.7	1.09	22.5	1353	6.25	3487	3930	<LOQ–0.063	<LOQ	21.4
		P	0.118	0.271	1.000	0.900	0.369	0.335	0.946	–	–	0.559
		SEM	0.27	0.066	0.27	33.2	0.117	58.1	92.7	–	–	0.78
L7	Event 5307	mean	46.8	1.36	24.3	1243	6.32	3227	3750	<LOQ–0.063	<LOQ	24.1
	Nontransgenic	mean	45.5	2.09	23.4	1297	6.14	3417	3883	<LOQ–0.107	<LOQ	23.9
		P	0.156	0.302	0.459	***0.047***	0.366	***0.033***	0.231	–	–	0.841
		SEM	0.40	0.372	0.73	8.5	0.108	24.8	55.4	–	–	0.83
L8	Event 5307	mean	40.8	1.34	22.1	1400	5.25	3443	3947	<LOQ–0.061	<LOQ	23.5
	Nontransgenic	mean	42.9	2.14	21.0	1417	4.73	3520	3997	<LOQ–0.062	<LOQ	24.2
		P	0.152	0.156	0.200	0.588	***0.018***	0.323	0.286	–	–	0.417
		SEM	0.66	0.252	0.39	18.4	0.049	41.7	24.5	–	–	0.51

– = not applicable
[a]Where some or all values were <LOQ, calculation of the mean and statistical comparison were not possible, thus only the range is shown

Table VII-18. Vitamin composition of grain from 5307 corn and nontransgenic corn.

Vitamin levels shown in mg/100 g DW except as indicated for vitamin E (mg/g). Results significant at $P < 0.05$ are shown in bold italic type.
For across-location analyses, $N = 18$; the range of values among these replicates is provided.

Location	Data source	Statistic	Vitamin A β-carotene	Vitamin B1 Thiamine	Vitamin B2 Riboflavin	Vitamin B3 Niacin	Vitamin B6 Pyridoxine	Vitamin B9 Folic Acid	Vitamin E[a] α-tocopherol
Across all	Event 5307	mean	0.155	0.449	0.198	3.13	0.692	0.0397	0.0093
		range	0.133–0.185	0.399–0.511	0.156–0.264	2.53–4.11	0.587–0.769	0.0305–0.0460	0.00719–0.0111
	Nontransgenic	mean	0.176	0.458	0.198	3.18	0.737	0.0382	0.0090
		range	0.155–0.216	0.408–0.518	0.152–0.318	2.51–3.70	0.621–0.815	0.0289–0.0463	0.00607–0.0110
	ANOVA (*F* test)								
	Genotype effect	*P*	***<0.001***	0.146	0.941	0.674	***0.005***	***0.031***	0.074
		SEM	0.0049	0.0126	0.0096	0.104	0.0167	0.00199	0.00055
	ILSI (2008)	mean	0.684	0.530	0.125	2.376	0.644	0.0651	0.0103
		range	0.019–4.681	0.126–4.000	0.050–0.236	1.037–4.694	0.368–1.132	0.0147–0.1464	0.0015–0.0687
		N	276	894	704	415	415	895	863

[a]Original units of mg/100 g reported by the testing laboratory were converted to mg/g

Table VII-18 *(Continued)*. Vitamin composition of grain from 5307 corn and nontransgenic corn.

Vitamin levels shown in mg/100 g DW except as indicated for vitamin E (mg/g). Results significant at $P < 0.05$ are shown in bold italic type.
For individual location means, $N = 3$.

Location	Data source	Statistic	Vitamin A β-carotene	Vitamin B1 Thiamine	Vitamin B2 Riboflavin	Vitamin B3 Niacin	Vitamin B6 Pyridoxine	Vitamin B9 Folic Acid	Vitamin Ea α-tocopherol
L1	Event 5307	mean	0.152	0.473	0.219	3.27	0.611	0.0448	0.0075
	Nontransgenic	mean	0.171	0.476	0.205	3.29	0.716	0.0434	0.0069
		P	0.297	0.939	0.612	0.963	0.105	0.399	0.176
		SEM	0.0096	0.0220	0.0162	0.225	0.0261	0.00091	0.00022
L2	Event 5307	mean	0.151	0.456	0.175	3.53	0.660	0.0422	0.0082
	Nontransgenic	mean	0.167	0.476	0.202	3.33	0.684	0.0424	0.0078
		P	0.082	0.232	0.587	0.657	0.645	0.807	0.313
		SEM	0.0035	0.0083	0.0294	0.279	0.0321	0.00059	0.00018
L4	Event 5307	mean	0.141	0.488	0.197	3.16	0.739	0.0393	0.0093
	Nontransgenic	mean	0.159	0.504	0.169	3.20	0.748	0.0360	0.0090
		P	***0.016***	0.548	0.222	0.903	0.609	0.093	0.289
		SEM	0.0017	0.0164	0.0112	0.239	0.0098	0.00078	0.00011
L6	Event 5307	mean	0.172	0.432	0.213	2.89	0.699	0.0427	0.0095
	Nontransgenic	mean	0.188	0.424	0.246	2.85	0.749	0.0407	0.0094
		P	0.061	0.218	0.403	0.826	0.484	0.506	0.743
		SEM	0.0029	0.0033	0.0222	0.104	0.0421	0.00176	0.00028
L7	Event 5307	mean	0.157	0.434	0.183	3.16	0.721	0.0317	0.0107
	Nontransgenic	mean	0.176	0.435	0.172	3.25	0.759	0.0299	0.0109
		P	0.218	0.808	0.519	0.709	0.076	0.346	0.733
		SEM	0.0076	0.0009	0.0100	0.153	0.0079	0.00104	0.00030
L8	Event 5307	mean	0.160	0.410	0.203	2.77	0.723	0.0377	0.0105
	Nontransgenic	mean	0.195	0.432	0.191	3.17	0.767	0.0368	0.0098
		P	***0.025***	0.077	0.560	0.498	***0.003***	0.744	0.454
		SEM	0.0040	0.0045	0.0123	0.345	0.0018	0.00176	0.00056

[a]Original units of mg/100 g reported by testing laboratory were converted to mg/g

Table VII-19. Amino acid composition of grain from corn 5307 corn and nontransgenic corn.

Amino acid levels shown in mg/g DW. Results significant at *P* <0.05 are shown in bold italic type.
For across-location analyses, *N* = 18; the range of values among these replicates is provided.

Location	Data source	Statistic	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Cys	Val
Across all	Event 5307	mean	6.93	3.80	5.14	20.4	9.23	3.95	8.21	2.33	5.12
		range	5.82–8.15	3.19–4.39	4.10–6.10	16.4–24.9	7.55–11.0	3.52–4.39	6.64–9.97	2.07–2.50	4.33–6.08
	Nontransgenic	mean	6.88	3.79	5.17	20.6	9.24	3.97	8.24	2.36	5.13
		range	6.00–8.20	3.36–4.47	4.44–6.28	17.4–25.3	7.84–10.9	3.61–4.35	7.06–10.0	2.14–2.59	4.28–6.01
	ANOVA (*F* test)										
	Genotype effect	*P*	0.625	0.908	0.736	0.715	0.973	0.761	0.846	0.284	0.877
		SEM	0.236	0.123	0.203	0.90	0.375	0.087	0.345	0.043	0.179
	ILSI (2008)	mean	6.88	3.75	5.12	20.09	9.51	3.85	7.90	2.21	4.90
		range	3.35–12.08	2.24–6.66	2.35–7.69	9.65–35.36	4.62–16.32	1.84–5.39	4.39–13.93	1.25–5.14	2.66–8.55
		N	1350	1350	1350	1350	1350	1350	1350	1350	1350

Table VII-19 *(Continued)*. Amino acid composition of grain from 5307 corn and nontransgenic corn.

Amino acid levels shown in mg/g DW. Results significant at $P < 0.05$ are shown in bold italic type. For individual location means, $N = 3$.

Location	Data source	Statistic	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Cys	Val
L1	Event 5307	mean	7.07	3.82	5.09	21.1	9.25	3.86	8.42	2.33	5.25
	Nontransgenic	mean	6.74	3.71	4.98	19.9	8.51	3.80	7.97	2.34	5.00
		P	0.133	0.197	0.431	0.239	0.408	0.059	0.212	0.707	0.179
		SEM	0.096	0.043	0.082	0.51	0.499	0.011	0.176	0.016	0.088
L2	Event 5307	mean	5.94	3.26	4.28	16.8	7.78	3.56	6.78	2.12	4.40
	Nontransgenic	mean	6.27	3.48	4.63	18.3	8.18	3.75	7.30	2.26	4.66
		P	***0.030***	***0.018***	0.118	***0.010***	0.380	***0.028***	***0.010***	0.148	***0.012***
		SEM	0.041	0.021	0.092	0.10	0.255	0.024	0.037	0.045	0.020
L4	Event 5307	mean	6.96	3.81	5.25	20.5	9.08	4.04	8.23	2.36	5.17
	Nontransgenic	mean	6.97	3.84	5.32	20.9	9.78	4.13	8.40	2.36	5.30
		P	0.961	0.833	0.761	0.591	0.247	0.485	0.591	0.940	0.367
		SEM	0.128	0.088	0.149	0.48	0.307	0.075	0.186	0.028	0.079
L6	Event 5307	mean	7.89	4.31	5.94	24.0	10.90	4.28	9.60	2.47	5.82
	Nontransgenic	mean	7.73	4.22	5.95	23.9	10.14	4.18	9.48	2.54	5.72
		P	0.747	0.668	0.976	0.967	0.301	0.606	0.849	0.277	0.820
		SEM	0.306	0.123	0.206	1.01	0.390	0.124	0.393	0.033	0.272
L7	Event 5307	mean	6.69	3.68	4.98	19.3	8.82	3.95	7.85	2.32	4.91
	Nontransgenic	mean	6.48	3.61	4.93	19.0	8.92	3.86	7.68	2.32	4.74
		P	0.514	0.680	0.885	0.772	0.763	0.554	0.725	0.840	0.533
		SEM	0.192	0.109	0.186	0.78	0.219	0.084	0.292	0.021	0.167
L8	Event 5307	mean	7.03	3.89	5.30	20.9	9.55	4.02	8.36	2.39	5.16
	Nontransgenic	mean	7.09	3.88	5.19	21.5	9.89	4.08	8.59	2.36	5.38
		P	0.835	0.933	0.519	0.661	0.584	0.594	0.648	0.311	0.508
		SEM	0.189	0.075	0.097	0.83	0.368	0.064	0.306	0.019	0.198

Table VII-19 (*Continued*). Amino acid composition of grain from 5307 corn and nontransgenic corn.

Amino acid levels shown in mg/g DW. Results significant at $P < 0.05$ are shown in bold italic type.
For across-location analyses, $N = 18$; the range of values among these replicates is provided.

Location	Data source	Statistic	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Trp
Across all	Event 5307	mean	2.29	3.92	13.8	3.18	5.50	3.10	2.99	4.81	0.570
		range	1.97–2.51	3.19–4.77	10.8–17.1	1.57–4.18	4.34–6.68	2.76–3.36	2.57–3.44	3.72–5.56	0.381–0.704
	Nontransgenic	mean	2.36	3.91	13.8	3.26	5.52	3.09	3.01	4.82	0.557
		range	2.08–2.56	3.23–4.71	11.5–17.3	1.67–3.98	4.73–6.70	2.74–3.38	2.57–3.43	4.20–5.32	0.380–0.700
	ANOVA (*F* test)										
	Genotype effect	*P*	0.102	0.947	0.789	0.711	0.883	0.902	0.684	0.892	0.722
		SEM	0.049	0.163	0.66	0.153	0.239	0.059	0.088	0.144	0.0298
	ILSI (2008)	mean	2.09	3.68	13.41	3.36	5.25	3.15	2.96	4.33	0.627
		range	1.24–4.68	1.79–6.92	6.42–24.92	1.03–6.42	2.44–9.30	1.72–6.68	1.37–4.34	1.19–6.39	0.271–2.150
		N	1350	1350	1350	1350	1350	1350	1350	1350	1350

Table VII-19 (*Continued*). Amino acid composition of grain from 5307 corn and nontransgenic corn.

Amino acid levels shown in mg/g DW. Results significant at $P < 0.05$ are shown in bold italic type. For individual location means, $N = 3$.

Location	Data source	Statistic	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Trp
L1	Event 5307	mean	2.32	4.03	14.3	2.88	5.63	3.08	3.01	4.43	0.497
	Nontransgenic	mean	2.32	3.74	13.3	3.47	5.26	3.06	2.92	4.61	0.601
		P	0.915	0.150	0.231	0.334	0.283	0.319	0.175	0.429	0.552
		SEM	0.039	0.092	0.39	0.330	0.177	0.014	0.031	0.132	0.1039
L2	Event 5307	mean	2.02	3.26	11.1	2.57	4.49	2.81	2.62	4.19	0.494
	Nontransgenic	mean	2.23	3.49	12.2	3.02	4.86	2.95	2.80	4.44	0.539
		P	0.144	0.066	***0.007***	0.228	0.079	0.072	***0.009***	0.161	0.656
		SEM	0.061	0.043	0.06	0.187	0.077	0.029	0.012	0.083	0.0605
L4	Event 5307	mean	2.28	3.95	13.8	3.16	5.50	3.19	3.03	5.02	0.680
	Nontransgenic	mean	2.36	4.01	14.1	3.52	5.63	3.22	3.11	5.29	0.588
		P	0.279	0.535	0.589	0.622	0.515	0.762	0.402	0.343	0.169
		SEM	0.037	0.057	0.30	0.441	0.114	0.068	0.058	0.157	0.0307
L6	Event 5307	mean	2.42	4.56	16.4	3.54	6.46	3.31	3.34	5.23	0.624
	Nontransgenic	mean	2.52	4.46	16.3	3.06	6.31	3.21	3.28	4.92	0.658
		P	0.115	0.793	0.931	0.524	0.724	0.578	0.721	0.523	0.427
		SEM	0.027	0.229	0.72	0.446	0.255	0.100	0.109	0.279	0.0238
L7	Event 5307	mean	2.37	3.74	13.0	3.43	5.27	3.08	2.91	5.02	0.554
	Nontransgenic	mean	2.39	3.58	12.7	3.39	5.18	2.97	2.82	4.84	0.502
		P	0.823	0.538	0.761	0.895	0.781	0.333	0.589	0.555	0.403
		SEM	0.037	0.154	0.61	0.189	0.193	0.063	0.100	0.178	0.0350
L8	Event 5307	mean	2.32	3.98	14.0	3.48	5.68	3.12	3.04	4.95	0.573
	Nontransgenic	mean	2.32	4.21	14.5	3.08	5.88	3.15	3.13	4.80	0.454
		P	0.919	0.477	0.644	0.665	0.592	0.607	0.509	0.703	0.134
		SEM	0.021	0.187	0.61	0.562	0.231	0.031	0.086	0.236	0.0342

Table VII-20. Fatty acid composition[a] of grain from 5307 corn and nontransgenic corn.

Fatty acids shown as % of total fatty acids. Results significant at $P < 0.05$ are shown in bold italic type.
For across-location analyses, $N = 18$; the range of values among these replicates is provided.

Location	Data source	Statistic	16:0 Palmitic	16:1 Palmitoleic[b]	18:0 Stearic	18:1 Oleic	18:2 Linoleic	18:3 Linolenic	20:0 Arachidic	20:1 Eicosenoic	22:0 Behenic
Across all	Event 5307	mean	15.7	–	1.74	24.5	55.6	1.60	0.392	0.250	0.220
		range	15.1–16.1	<LOQ–0.137	1.50–2.04	22.0–27.0	53.2–58.1	1.48–1.71	0.353–0.453	0.238–0.265	0.186–0.252
	Nontransgenic	mean	15.2	–	1.81	24.9	55.7	1.50	0.387	0.242	0.213
		range	14.6–15.9	<LOQ–0.450	1.54–2.17	22.6–26.4	53.8–58.4	1.40–1.57	0.361–0.437	0.232–0.261	0.194–0.247
	ANOVA (*F* test)										
	Genotype effect	*P*	***<0.001***	–	***0.038***	0.108	0.599	***<0.001***	0.186	***<0.001***	0.243
		SEM	0.07	–	0.059	0.54	0.60	0.017	0.0098	0.0029	0.0056
	ILSI (2008)	mean	11.50	0.154	1.82	25.8	57.6	1.20	0.412	0.297	0.176
		range	7.94–20.71	<LOQ–0.447	1.02–3.40	17.4–40.2	36.2–66.5	0.57–2.25	0.279–0.965	0.170–1.917	<LOQ–0.349
		N	1344	596	1344	1344	1344	1344	988	987	924

– = not applicable

[a] Where some or all values were <LOQ, % of total fatty acids could not be calculated and statistical analysis could not be performed. Levels <LOQ were observed for all replicates at all locations for 8:0 caprylic, 10:0 capric, 12:0 lauric, 14:0 myristic, 14:1 myristoleic, 15:0 pentadecanoic, 15:1 pentadecenoic, 17:0 heptadecanoic, 17:1 heptadecenoic, 20:2 eicosadienoic, 20:3 eicosatrienoic, and 20:4 arachidonic fatty acids

[b] Some values were <LOQ, therefore, only the range is shown

Table VII-20 (*Continued*). Fatty acid composition of grain from 5307 corn and nontransgenic corn.

Fatty acids shown as % of total fatty acids. Results significant at $P < 0.05$ are shown in bold italic type. For individual location means, $N = 3$.

Location	Data source	Statistic	16:0 Palmitic	16:1 Palmitoleic[a]	18:0 Stearic	18:1 Oleic	18:2 Linoleic	18:3 Linolenic	20:0 Arachidic	20:1 Eicosenoic	22:0 Behenic
L1	Event 5307	mean	15.5	<LOQ	1.53	22.4	58.1	1.63	0.374	0.247	0.210
	Nontransgenic	mean	15.1	<LOQ	1.58	22.7	58.2	1.51	0.371	0.237	0.204
		P	0.225	–	***0.013***	0.286	0.199	0.122	0.641	0.061	0.730
		SEM	0.16	–	0.004	0.15	0.06	0.033	0.0043	0.0019	0.0107
L2	Event 5307	mean	15.8	<LOQ	1.77	23.5	56.4	1.67	0.397	0.242	0.216
	Nontransgenic	mean	15.5	<LOQ–0.450	1.93	24.3	55.8	1.54	0.388	0.238	0.202
		P	0.149	–	0.202	0.120	0.406	***0.017***	0.467	0.476	0.100
		SEM	0.10	–	0.060	0.22	0.43	0.012	0.0077	0.0038	0.0035
L4	Event 5307	mean	15.7	0.134	1.76	25.3	54.8	1.57	0.391	0.245	0.200
	Nontransgenic	mean	15.2	0.132	1.86	25.5	55.0	1.47	0.391	0.240	0.212
		P	***0.013***	0.372	***0.023***	0.319	0.478	***0.019***	0.960	***0.047***	0.373
		SEM	0.04	0.0017	0.011	0.14	0.16	0.010	0.0042	0.0009	0.0075
L6	Event 5307	mean	15.8	<LOQ–0.132	1.68	24.5	55.5	1.60	0.368	0.246	0.228
	Nontransgenic	mean	15.1	<LOQ–0.134	1.72	24.7	56.1	1.54	0.370	0.239	0.206
		P	0.069	–	0.270	0.560	0.210	0.203	0.606	0.242	0.072
		SEM	0.14	–	0.019	0.17	0.25	0.023	0.0027	0.0029	0.0045
L7	Event 5307	mean	15.5	<LOQ	1.98	26.4	53.6	1.55	0.441	0.263	0.242
	Nontransgenic	mean	15.4	<LOQ–0.143	1.96	26.1	54.1	1.47	0.427	0.253	0.235
		P	***0.038***	–	0.701	0.493	0.291	0.086	0.336	0.132	0.678
		SEM	0.02	–	0.032	0.26	0.23	0.017	0.0077	0.0030	0.0098
L8	Event 5307	mean	15.7	<LOQ–0.137	1.71	24.9	55.1	1.59	0.380	0.254	0.221
	Nontransgenic	mean	15.0	<LOQ–0.124	1.80	25.9	54.9	1.45	0.378	0.247	0.220
		P	0.053	–	0.082	0.211	0.678	0.093	0.878	0.294	0.946
		SEM	0.13	–	0.020	0.39	0.29	0.032	0.0054	0.0037	0.0061

– = not applicable

[a]Where some or all values were <LOQ, % of total fatty acids could not be calculated, statistical analysis could not be performed, and only the range is shown

Table VII-21. Secondary metabolite and antinutrient composition of grain from 5307 corn and nontransgenic corn.

Analyte units as in column headings. Results significant at $P < 0.05$ are shown in bold italic type.
For across-location analyses, $N = 18$; the range of values among these replicates is provided.

Location	Data source	Statistic	Ferulic acid (mg/kg DW)	ρ-Coumaric acid (mg/kg DW)	Inositol (ppm DW)	Phytic acid (% DW)	Trypsin inhibitor (TIU/mg DW)	Furfural[a,b] (mg/kg DW)	Raffinose (% DW)
Across all	Event 5307	mean	1906	186	2510	0.910	3.34	–	0.156
		range	1670–2190	153–229	2120–3160	0.671–1.03	2.39–4.42	< LOQ	0.115–0.199
	Nontransgenic	mean	1889	186	2504	0.942	3.46	–	0.163
		range	1620–2090	148–226	1980–3060	0.729–1.06	2.22–3.94	< LOQ	0.119–0.188
	ANOVA (*F* test)								
	Genotype effect	*P*	0.691	0.926	0.951	0.216	0.393	–	0.066
		SEM	52.4	9.1	86.1	0.0261	0.118	–	0.0087
	ILSI (2008)	mean	2201.1	218.4	1331.5	0.745	2.73	3.697	0.312
		range	291.9–3885.8	53.4–576.2	89.0–3765.4	0.111–1.570	<LOQ–7.18	<LOQ–6.340	<LOQ–0.320
		N[c]	817	817	504	1196	696	14	701

– = not applicable
[a]The LOQ for furfural was 0.55–0.57 mg/kg DW
[b]All values were <LOQ and therefore statistical comparison was not possible
[c]*N* is the number of ILSI values used to calculate the mean and excludes values <LOQ

Table VII-21 (Continued). Secondary metabolite and antinutrient composition of grain from 5307 corn and nontransgenic corn.

Analyte units as in column headings. Results significant at $P < 0.05$ are shown in bold italic type. For individual location means, $N = 3$.

Location	Data source	Statistic	Ferulic acid (mg/kg DW)	ρ-Coumaric acid (mg/kg DW)	Inositol (ppm DW)	Phytic acid (% DW)	Trypsin inhibitor (TIU/mg DW)	Furfural[a] (mg/kg DW)	Raffinose (% DW)
L1	Event 5307	mean	1770	169	2323	0.903	3.31	<LOQ	0.179
	Nontransgenic	mean	1883	187	2160	0.878	3.58	<LOQ	0.174
		P	0.579	0.494	0.062	0.577	0.124	–	0.707
		SEM	122.2	15.9	30.1	0.0268	0.073	–	0.0082
L2	Event 5307	mean	1747	197	2517	0.840	2.90	<LOQ	0.166
	Nontransgenic	mean	1827	207	2230	0.848	3.48	<LOQ	0.178
		P	0.463	0.375	0.323	0.938	0.111	–	0.607
		SEM	62.8	6.5	155.8	0.0592	0.149	–	0.0133
L4	Event 5307	mean	2137	226	2790	1.000	3.58	<LOQ	0.164
	Nontransgenic	mean	1977	209	2760	1.006	3.73	<LOQ	0.175
		P	0.186	0.193	0.896	0.818	0.702	–	0.093
		SEM	57.2	6.3	143.5	0.0171	0.241	–	0.0026
L6	Event 5307	mean	2017	177	2527	0.842	3.44	<LOQ	0.167
	Nontransgenic	mean	1990	185	2523	0.965	3.64	<LOQ	0.173
		P	0.829	0.702	0.959	0.156	0.412	–	0.234
		SEM	76.6	12.8	40.9	0.0390	0.140	–	0.0027
L7	Event 5307	mean	1800	159	2470	0.912	3.40	<LOQ	0.139
	Nontransgenic	mean	1753	154	2627	0.984	3.28	<LOQ	0.156
		P	0.340	0.225	0.691	0.053	0.861	–	0.091
		SEM	26.6	2.0	241.1	0.0123	0.403	–	0.0039
L8	Event 5307	mean	1967	186	2433	0.964	3.41	<LOQ	0.122
	Nontransgenic	mean	1903	175	2727	0.971	3.02	<LOQ	0.123
		P	0.604	0.601	0.081	0.864	0.311	–	0.893
		SEM	73.5	13.0	62.8	0.0230	0.205	–	0.0031

– = not applicable

[a] All values were <LOQ and therefore statistical comparison was not possible

VII.C. Summary of Phenotypic and Compositional Evaluations

The results of laboratory, greenhouse, and field studies indicate that 5307 corn is no different than conventional corn with regard to phenotypic and compositional properties that bear on weediness potential for the new cultivar. Corn as a crop has lost the ability to survive outside of cultivation (OECD, 2003). It can overwinter and germinate in a subsequent crop as a volunteer weed; for example, corn is a common volunteer in soybeans. Nevertheless, several features of corn make it unlikely to form troublesome or self-sustaining weedy populations in agricultural systems: 1) it is easily controlled in subsequent crops by selective herbicides; 2) seed dispersal is limited because seeds are held inside the husks of the cob; and 3) the seeds lack dormancy characteristics. 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.

VIII. Environmental Consequences of Introduction

Event 5307 corn contains a novel protein, eCry3.1Ab, that has insecticidal activity against a number of coleopteran species that are pests of U.S. agriculture. Studies have been conducted to define the spectrum of insecticidal activity for eCry3.1Ab and to assess the efficacy of 5307 corn hybrids in resisting corn rootworm larval feeding damage. An assessment of risk for nontarget organisms and endangered species that might be exposed to eCry3.1Ab in 5307 corn has been performed. This risk assessment is comprised of multiple parts:

- A determination of estimated environmental concentrations (EECs) for eCry3.1Ab;
- A characterization of potential hazards posed by eCry3.1Ab to nontarget indicator organisms appropriate for a corn ecosystem;
- A comparison of EECs to no-observable-effect concentrations for nontarget organisms; and
- A specific characterization of risk to endangered or threatened species.

Given the ubiquitous distribution of PMI enzymes in nature, their nontoxic mode of action, and the apparent absence of environmental risk associated with PMI enzymes, it can reasonably be assumed that the presence of PMI as a selectable marker in 5307 corn will not alter the ecological balance within corn ecosystems. PMI is also present as a selectable marker in two other Syngenta corn events that have been deregulated by APHIS, namely MIR604 corn (for rootworm control) and MIR162 corn (for lepidopteran control).

VIII.A. Spectrum of eCry3.1Ab Insecticidal Activity

Syngenta has conducted mortality bioassays using 5307 corn tissue or diets containing microbially produced eCry3.1Ab in a range of insect species to identify those that are susceptible to the toxin. (A description of the microbially produced eCry3.1Ab test substance used in several of these studies is provided in Part VI.A.1.a. Production of eCry3.1Ab for Safety Testing.) The results of these bioassays demonstrate that activity of eCry3.1Ab is limited to species within the order Coleoptera, yet all coleopteran species are not sensitive to the protein. This narrow spectrum of activity for eCry3.1Ab is a very positive attribute from an ecological perspective; corn plants containing eCry3.1Ab are unlikely to pose a risk to nontarget organisms inhabiting corn ecosystems. Table VIII-1 lists the insect species that have been found by Syngenta to be sensitive or insensitive to eCry3.1Ab. These results have been compiled from a combination of both laboratory and in-field assessments.

Table VIII-1. Insect species screened for sensitivity to eCry3.1Ab.

Activity was assessed in insects fed diets comprised of 5307 corn leaf tissue or diets containing microbially produced eCry3.1Ab, or based on field evaluations of 5307 plants.

Order:	Family	Genus and Species	Common name(s)	eCry3.1Ab Activity
Coleoptera:	Chrysomelidae	*Leptinotarsa decemlineata*	Colorado potato beetle	Active[1]
		Diabrotica virgifera virgifera	Western corn rootworm	Active[2]
		Diabrotica longicornis barberi	Northern corn rootworm[1]	Active[3]
		Diabrotica virgifera zeae	Mexican corn rootworm[1]	Active[3]
		Diabrotica undecimpunctata howardi	Southern corn rootworm Spotted cucumber beetle	Not Active[4]
Coleoptera:	Coccinellidae	*Coleomegilla maculata*	Spotted ladybird beetle	Not Active[5]
Coleoptera:	Staphylinidae	*Aleochara bilineata*	Rove beetle	Not Active[6]
Coleoptera:	Carabidae	*Poecilus cupreus*	Ground beetle	Not Active[7]
Lepidoptera:	Noctuidae	*Agrotis ipsilon*	Black cutworm	Not Active[8]
		Helicoverpa zea	Corn earworm Cotton bollworm	Not Active[8]
		Spodoptera frugiperda	Fall armyworm	Not Active[8]
		Heliothis virescens	Tobacco budworm	Not Active[8]
Lepidoptera:	Crambidae	*Ostrinia nubilalis*	European corn borer	Not Active[8]
Lepidoptera:	Gelechiidae	*Pectinophora gossypiella*	Pink bollworm	Not Active[9]
Hemiptera:	Anthocoridae	*Orius laevigatus*	Flower bug	Not Active[10]

[1] Activity against this species was determined in the laboratory bioassay described below (see Table VIII-2) and confirmed in additional laboratory assays described in Part VI.A.1.b and Appendix C.

[2] Activity against this species was determined in the laboratory bioassay described below (see Table VIII-2) and confirmed in laboratory assays described by Walters et al. (2010). Activity was also confirmed in field evaluations described in Part VIII.B.

[3] Activity against this species was determined in field evaluations described in Part VIII.B.

[4] Inactivity in this species was determined in the laboratory bioassay described below; see Table VIII-2.

[5] Inactivity in this species was determined in the laboratory bioassay described in Part VIII.D.4.a.

[6] Inactivity in this species was determined in the laboratory bioassay described in Part VIII.D.5.b.

[7] Inactivity in this species was determined in the laboratory bioassay described in Part VIII.D.5.a.

[8] Inactivity in this species was determined in the laboratory bioassays described below; see Tables VIII-2 and VIII-3.

[9] Inactivity in this species was determined in the laboratory bioassay described below; see Table VIII-2.

[10] Inactivity in this species was determined in the laboratory bioassays described in Part VIII.D.4.b.

Presented below (Table VIII-2) are the results of qualitative insect screening bioassays with three coleopteran and six lepidopteran pest species listed in Table VIII-1. Included among the test species were those known to be sensitive to one of the component Cry proteins (mCry3A or Cry1Ab) represented in eCry3.1Ab (Figure IV-1). Western corn rootworm and Colorado potato beetle are sensitive to mCry3A (EPA, 2010e), whereas corn earworm, tobacco budworm, and European corn borer are sensitive to Cry1Ab (Crickmore, 2010).

The screening bioassays were conducted using the cell lysate from an *E. coli* expression system producing eCry3.1Ab. Aliquots of the cell lysate were mixed 1:1 (v/v) with molten artificial diet appropriate for each insect and the diet was allowed to solidify. For each species tested, an aliquot of the diet mixture containing eCry3.1Ab was placed in a 50 × 9 mm culture dish, to which 20 neonate larvae were then added. Control culture dishes containing larvae and untreated diet were prepared concurrently in the same manner. Additional controls were concurrently treated with cell lysate from *E. coli* transformed with a blank version of the plasmid vector (i.e., lacking the gene *ecry3.1Ab*). Lids were placed on the culture dishes and the larvae were incubated at approximately 25°C in the dark. Mortality was assessed after 5-6 days.

Among the three coleopteran species tested in this set of assays, western corn rootworm and Colorado potato beetle appeared to be highly sensitive to eCry3.1Ab and exhibited 90% and 100% mortality, respectively (Table VIII-2). (The precise eCry3.1Ab concentration in the test diets was not determined in this screening experiment, but evidently exceeded the LC_{50}s for the sensitive species, based on the high mortality observed.) Southern corn rootworm was insensitive to eCry3.1Ab at the same concentration that was highly toxic to the other coleopteran species tested; only 10% mortality was observed, and this was within the range of control mortality seen across this series of assays (0 – 20%). All six of the lepidopteran species tested were insensitive to eCry3.1Ab. The absence of lepidopteran activity is consistent with the fact that, although eCry3.1Ab contains a portion of Cry1Ab (Figure IV-1), that portion does not include the region responsible for lepidopteran activity.

Table VIII-2. Bioassays of coleopteran and lepidopteran pest larvae with eCry3.1Ab.

Treatment	% Mortality (*N* = 20)								
	WCR	SCR	CPB	FAW	BCW	CEW	TBW	ECB	PBW
eCry3A.1Ab from *E. coli* lysate + diet	90	10	100	0	10	0	0	0	20
Control *E. coli* lysate + diet	10	NT[1]	NT	0	20	10	0	0	20
Diet only	0	NT	NT	0	0	20	0	0	0

WCR = western corn rootworm; SCR = southern corn rootworm; CPB = Colorado potato beetle; FAW = fall armyworm; BCW = black cutworm; CEW = corn earworm; TBW = tobacco budworm; ECB = European corn borer; PBW = pink bollworm,

[1] NT = Not tested

Presented below (Table VIII-3) are the results of insect bioassays conducted using leaf pieces of 5307 corn and nontransgenic, near-isogenic corn. Plants were grown in a greenhouse until they had reached the V6 (six-leaf) stage. Five lepidopteran pest species were tested in five replicate cultures each per treatment. Each replicate consisted of 10 first instars placed in a culture dish with a moistened absorbent pad and a one-inch square piece of leaf tissue. After 120 hours, the percent leaf damage by the larvae was recorded. For each species, the percent of leaf area damaged was compared between the 5307 corn leaf treatments and the control corn leaf treatments by analysis of variance.

There were no statistically significant differences between the 5307 and control leaves in the percent leaf area damaged by any pest species; all the larvae fed extensively on the leaf

tissue from both sources and continued to develop normally during the test period. This study confirmed that 5307 corn containing eCry3.1Ab does not have lepidopteran activity.

Table VIII-3. Bioassays of lepidopteran pest larvae with leaf pieces from 5307 corn.

Corn Leaf Source	Replicate[1]	Percent Leaf Area Damaged by Pest Species[2]				
		ECB	FAW	BCW	CEW	TBW
Control	1	85	100	40	35	40
Control	2	95	95	60	35	95
Control	3	65	100	50	30	45
Control	4	45	100	30	40	80
Control	5	70	100	50	35	55
5307	1	75	95	50	40	45
5307	2	75	85	45	30	75
5307	3	50	90	35	35	95
5307	4	65	100	45	35	85
5307	5	40	100	40	40	90
Control Mean ± SD		72 ± 19	99 ± 2.2	46 ± 11	35 ± 3.5	63 ± 24
5307 Mean ±SD		61 ± 16	94 ± 6.5	43 ± 5.7	36 ± 4.2	78 ± 20
F-Test Probability		0.349	0.143	0.613	0.694	0.309

[1] *N* = 10 larvae per replicate.

[2] ECB = European corn borer; FAW = fall armyworm; BCW = black cutworm; CEW = corn earworm; TBW = tobacco budworm

VIII.B. Activity of eCry3.1Ab and 5307 Corn Against Target Insects

As shown in Table VIII-1, eCry3.1Ab has activity against three of the major rootworm pests of corn in the U.S., specifically: *D. 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 an *ecry3.1Ab* gene at adequate levels in corn is a logical approach for limiting crop losses attributable to these pests, especially considering the limitations of conventional insecticides in controlling corn rootworms. Thus, 5307 corn was developed. In hybrid offerings to growers, the gene *ecry3.1Ab* in 5307 corn will be combined through traditional breeding with other deregulated corn varieties containing additional insect-protection traits, as well as herbicide-tolerance traits (i.e., as Bt11 × MIR604 × TC1507 × 5307 × GA21 and Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 corn hybrids).

Field trials to date have demonstrated excellent efficacy of 5307 corn in controlling root feeding damage by western, northern and Mexican corn rootworms. While western and northern corn rootworms are pests of corn throughout the midwestern states, the Mexican corn rootworm occurs in Texas, Oklahoma and New Mexico (Krysan, 1986).
The photograph in Figure VIII-1 illustrates how destructive western corn rootworm larval feeding can be to a control hybrid under high pest pressure and how highly effective the 5307 hybrid is in preventing damage under the same pest pressure.

Figure VIII-1. Root mass of a 5307 hybrid compared to a control hybrid under high western corn rootworm pressure.

A typical root mass of a 5307 hybrid is shown on the left (Shirley, IL, 2008). Note the loss of entire nodes of roots from the control hybrid on the right due to extensive feeding by corn rootworm larvae.

Representative efficacy results from five years of field trials (2005 – 2009) are shown in Figure VIII-2. These trials were conducted in 21 locations in Illinois and Minnesota under USDA APHIS notifications 05-062-02n, 06-055-08n, 07-043-109n, 08-051-104n, and 09-063-103n. Efficacy was assessed under natural rootworm infestation, which was facilitated by planting a cucurbit trap crop the previous season to attract corn rootworm beetles for increased egg accumulation (Branson and Sutter, 1989). The efficacy of a 5307 hybrid was compared to a control hybrid and also to a MIR604 corn hybrid, both alone and in stacked combination by conventional breeding (referred to as a "MIR604 x 5307" hybrid). MIR604 corn produces a modified Cry3A (mCry3A) protein for corn rootworm control; Syngenta currently markets MIR604 corn as Agrisure® RW rootworm control.

Root damage ratings were assigned on a scale of 0.01 (no damage) to 3.0 (heavy damage) using a modification of the Iowa State University node-injury linear (0 – 3) scale for rating damage by corn rootworm larvae (Oleson et al., 2005). At the Illinois trial locations, the predominant rootworm species was western corn rootworm, whereas the trials in Minnesota had mixed infestations of western and northern corn rootworms.

The results of these trials demonstrate the high rootworm control efficacy of the eCry3.1Ab protein in 5307 hybrids. This was evident whether 5307 corn was tested as a stand-alone event or whether it was combined with mCry3A in MIR604 corn.

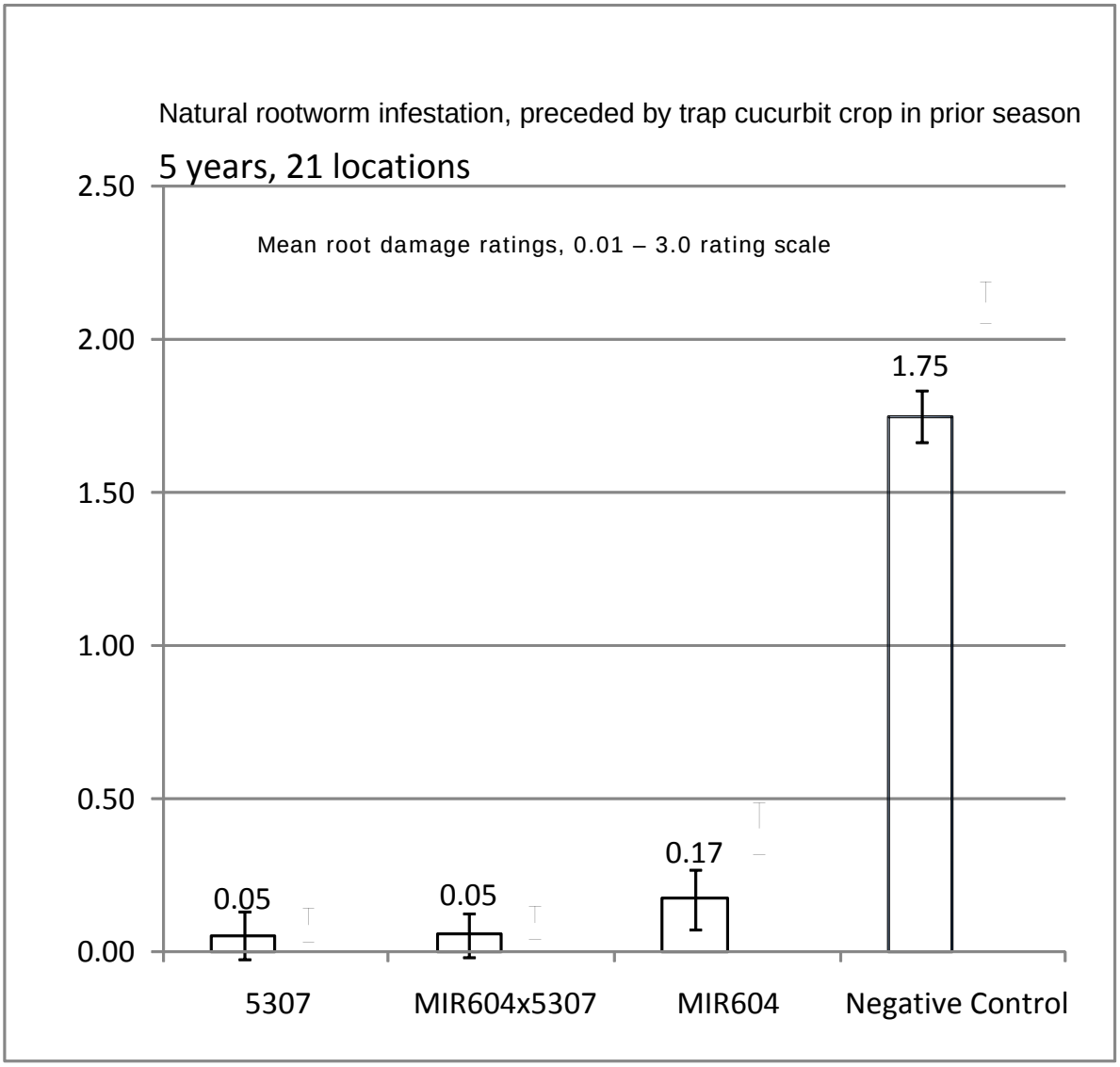

Figure VIII-2. Rootworm efficacy of 5307, MIR604 x 5307, and MIR604 corn hybrids; Illinois and Minnesota trials, 2005-2009.

Western corn rootworm was the predominant rootworm species in the Illinois trials, while mixed infestations of western and northern corn rootworms were present in the Minnesota trials.

VIII.C. Estimated Environmental Concentrations of eCry3.1Ab

Data on the concentration of eCry3.1Ab in 5307 corn were used to calculate the estimated environmental concentrations (EECs) of eCry3.1Ab for groups of organisms potentially exposed via 5307 corn. Estimates of exposure based on conservative assumptions about the dilution of eCry3.1Ab in prey and in soil have been made. Additionally, conservative assumptions about eCry3.1Ab concentrations in bodies of water near corn cultivation areas have been made. The EECs derived are suitable for protecting populations of nontarget organisms. Potential exposures and risks for endangered and threatened species are addressed separately in Part VIII.F., below.

VIII.C.1. Concentrations of eCry3.1Ab in 5307 Corn

The concentration of eCry3.1Ab was measured by enzyme-linked immunosorbent assay (ELISA) in several corn tissues and whole plants at four growth stages: whorl, anthesis, seed maturity and senescence (refer to Table VI-2). The values chosen for computation of EECs are shown in Table VIII-4. These values represent the mean[8] eCry3.1Ab concentration across four locations at the 5307 hybrid corn growth stage with the highest expression level. EECs were calculated from eCry3.1Ab tissue concentrations expressed on a fresh-weight basis.

Table VIII-4. Tissue concentrations of eCry3.1Ab used for computation of EECs.
Highest mean concentrations of eCry3.1Ab as determined by ELISA in tissues of 5307 corn hybrids grown in four field locations. All values are reported on a fresh-weight basis and are corrected for extraction efficiency.

Tissue	eCry3.1Ab (µg/g)	Growth stage
Leaf	25.33	Maturity
Kernel	4.56	Maturity
Root	5.54	Whorl
Pollen	0.14[a]	Anthesis
Whole plants	15.78	Whorl

[a] Extraction efficiency could not be derived for pollen samples, due to very low eCry3.1Ab concentrations. However, the data have been adjusted using conservative estimates of extraction efficiency for this tissue.

[8] Because these values represent the mean eCry3.1Ab concentrations at the growth stage with the *highest* expression level, they represent a conservative estimate for risk assessment purposes, and are appropriate for estimating potential exposure at a *population* level. Although a rare *individual* organism might theoretically be exposed to the highest single eCry3.1Ab concentration observed among all samples of a relevant tissue type, any such exposure is likely to be relatively brief and represent only a small portion of the total diet of the organism. This contrasts to the extended exposure to a continuous, no-choice eCry3.1Ab-treated diet in laboratory studies using nontarget indicator organisms; these factors represent additional sources of conservatism in the risk assessment, because they are unlikely to occur under natural conditions. The approach used for deriving EECs herein is similar to that used by Raybould et al. (2007) and Raybould and Vlachos (2010) for other insecticidal corn varieties.

VIII.C.2. Fate of eCry3.1Ab in Soil

Most proteins do not persist or accumulate in soil because they are inherently degradable in soils that have normal microbial populations (e.g., Burns, 1982; Marx et al., 2005). Multiple investigations have demonstrated that Cry proteins from *B. thuringiensis* are rapidly degraded in a variety of soil types and that the proteins do not accumulate (Mendelsohn et al., 2003; Head et al., 2002; Dubelman et al., 2005). There is no evidence that they accumulate in soil or are protected from the activity of proteases in soil.

A laboratory study was conducted to examine the fate of eCry3.1Ab in live soil. For this investigation, live soil representing a loam soil texture was used to examine the rate at which eCry3.1Ab biological activity declined. Microbially produced eCry3.1Ab (in test substance ECRY3.1AB-0208; see Parts VI.A.1.a and VI.A.1.b.) was added to the soil at a concentration of 50 µg/g dry weight-equivalent soil. A rapid decline in eCry3.1Ab bioactivity was observed. There was no biological activity detected above background control levels after 14 days of eCry3.1Ab incubation in live soil. The results of this study demonstrate that eCry3.1Ab biological activity is rapidly lost in healthy soils.

VIII.C.3. Expected eCry3.1Ab Environmental Concentrations for Nontarget Organisms

This section provides conservative estimates of environmental concentrations of eCry3.1Ab for multiple classes of nontarget organisms that may be exposed via cultivation of 5307 corn. Exposure through soil run-off or gene flow is unlikely for the reasons stated elsewhere in the petition (see Parts VIII.C.2. and VIII.I.) and is not considered. The approach to estimating environmental exposures for eCry3.1Ab is similar to that previously described for insect-control events MIR604 corn (Raybould et al., 2007) and MIR162 corn (Raybould and Vlachos, 2010).

VIII.C.3.a. EEC for Wild Birds

Some birds such as crows (*Corvus brachyrhynchos*), grackles (*Quiscalus quiscula*) and sandhill cranes (*Grus canadensis*) uproot sprouting corn to feed on the germinating kernels (e.g., Steffey et al., 1999; Blackwell et al., 2001; Sterner et al., 2003). Blackbirds typically slit open husks with their bills and puncture kernels in the milk stage (Steffey et al., 1999). Blackbirds are also common in corn stubble where they forage for spilled kernels and weed seeds (Linz et al., 2003). Therefore, the exposure of wild birds to eCry3.1Ab via cultivation of 5307 corn was estimated using the concentration of eCry3.1Ab in kernels.

The mean eCry3.1Ab concentration at the growth stage with the highest concentrations in 5307 corn kernels was 4.56 µg eCry3.1Ab/g fresh weight (Table VIII-4). Exposure to birds may be expressed more suitably as a daily dietary dose (DDD), which is given by a simple formula (Crocker et al., 2002):

$$DDD = \frac{FIR}{bw} \times C$$

where FIR = daily food intake rate; bw = body weight; and C = concentration of eCry3.1Ab in food.

FIR/bw ratios for cereal seed-eating birds consuming fresh food were estimated by Crocker et al. (2002). Among the seven species represented, ratios range from 0.11 (g seeds/g bw) for the pheasant (*Phasianus colchicus*) to 0.35 for the tree sparrow (*Passer montanus*); these species also represent the extreme range of body weights, i.e., 22 g for the sparrow to 953 g for the pheasant. Because smaller birds (e.g., the tree sparrow) have a higher food intake rate compared to body mass, and hence a higher DDD, the estimate of risk using the higher ratio of FIR/bw is more conservative.

Wild birds are unlikely to consume a diet of 100% corn kernels. A conservative estimate of exposure can be derived from the proportion of corn kernels in the diet of birds feeding in corn growing areas. Studies by McNichol et al. (1979) and Homan et al. (1994) of the diets of red-winged blackbirds and common grackles, respectively, showed that corn kernels comprise up to 50% of their diet. An EEC for wild birds can be calculated based on a diet of 50% 5307 corn kernels containing 4.56 µg eCry3.1Ab/g kernels. This yields a conservative EEC for wild birds of:

$$0.5 \times 4.56 \text{ µg eCry3.1Ab/g kernels} = 2.28 \text{ µg eCry3.1Ab/g kernels}$$

With a DDD for wild birds of:

$$0.5 \times 0.35 \text{ g kernels/g bw} \times 4.56 \text{ µg eCry3.1Ab/g kernels} = 0.80 \text{ µg eCry3.1Ab/g bw}$$

VIII.C.3.b. EEC for Wild Mammals

The main potential route of exposure of wild mammals to eCry3.1Ab in 5307 corn is consumption of kernels. Rodents such as thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*), deer mice (*Peromysus maniculatus*), house mice (*Mus domesticus*), prairie and meadow voles (*Microtus* spp.), and woodchucks (*Marmota monax*) feed on germinating corn seeds.

Larger mammals such as white-tailed deer (*Odocoilus virginianus*) and raccoons (*Procyon lotor*) cause injury to ripening ears. Deer typically nip off ear tips, whereas raccoons chew through husks.

The mean eCry3.1Ab concentration at the growth stage with the highest concentrations in 5307 corn kernels was 4.56 µg/g fresh weight (Table VIII-4). As with birds, exposure to wild mammals may be expressed more suitably as a daily dietary dose (DDD) calculated by the formula in Part VIII.C.3.a, above.

Crocker et al. (2002) estimated the ratio of daily food intake and body weight (FIR/bw) for several rodent species. The ratios for the harvest mouse (*Micromys minutus*) and the wood mouse (*Apodemus sylvaticus*) consuming cereal seeds are 0.33 and 0.28, respectively. Higher FIR/bw ratios give higher DDDs, and therefore more conservative estimates of risk.

Wild mammals are unlikely to consume a diet of 100% corn kernels. A conservative exposure estimate can be derived from the proportion of corn kernels in the diet of rodents

feeding in corn growing areas. The proportion of corn kernels in wild rodent diets varies greatly according to species (Houtcooper, 1978; Ellis et al., 1998), but can be up to 73%. An EEC can therefore be calculated based on a diet of 73% corn kernels containing 4.56 µg eCry3.1Ab/g kernels. These estimates give a conservative EEC for wild mammals of:

$$0.73 \times 4.56 \text{ µg eCry3.1Ab/g kernels} = 3.33 \text{ µg eCry3.1Ab/g kernels}$$

with a DDD for wild mammals of:

$$0.73 \times 0.33 \text{ g kernels/g bw} \times 4.56 \text{ µg eCry3.1Ab/g kernels} = 1.10 \text{ µg eCry3.1Ab/g bw}$$

VIII.C.3.c. EEC for Pollinators

Honey bees could potentially forage for corn pollen (Severson and Perry, 1981) and therefore could be exposed to eCry3.1Ab via 5307 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 their diet (Babendreier et al., 2004). Assuming a diet of 50% 5307 corn pollen at the mean pollen concentration of eCry3.1Ab (Table VIII-4), a conservative EEC for honey bees and other pollinators is:

$$0.5 \times 0.14 \text{ µg eCry3.1Ab/g pollen} = 0.07 \text{ µg eCry3.1Ab/g pollen}$$

VIII.C.3.d. EEC for Above-Ground Nontarget Arthropods

Nontarget arthropods rarely, if ever, eat leaves of corn plants. The more likely route of exposure to transgenic proteins is consumption of prey that have fed on corn plants (e.g., Harwood et al., 2005), or consumption of pollen if prey are scarce (Coll and Guershon, 2002).

The concentration of eCry3.1Ab in the prey of nonatarget arthropods will vary depending on the prey species, its developmental stage, and the concentration of eCry3.1Ab in plant parts on which they are feeding. Several studies have examined the concentration of Cry proteins in herbivores relative to the concentration of plants on which they are feeding; most tested the concentration of Cry1Ab in herbivores feeding on *Bt* corn (Head et al., 2001; Raps et al., 2001; Dutton et al., 2002; Obrist et al., 2005; Obrist et al., 2006a, 2006b), and other studies have been published that examine herbivores feeding on cotton and oilseed rape expressing Cry1Ac (Torres et al., 2006; Howald et al., 2003).

In general, the results show that herbivorous arthropods contain lower concentrations of *Bt* toxin than the plants on which they are feeding. Sucking insects, such as aphids, contain only trace amounts of Cry1Ab when feeding on *Bt* corn (Head et al., 2001; Raps et al., 2001; Dutton et al., 2002; Obrist et al., 2006a). Lepidopteran larvae contain between 0.1 and 0.25X the concentration of Cry1Ab in *Bt* corn on which they are feeding (Raps et al., 2001; Dutton et al., 2002; Obrist et al., 2006b), and similar results were obtained by Torres et al. (2006) with *Spodoptera exigua* feeding on cotton expressing Cry1Ac. Thrips (*Frankliniella tenuicornis*) contain up to 0.35X the concentration of Cry1Ab in *Bt* corn, although this

concentration is transitory; adults contain about half this amount and pupae less than 1/40th the concentration in larvae (Obrist et al., 2005). The herbivores with the highest concentrations of Cry protein are spider mites (*Tetranychus urticae*); they have been found to contain between 0.7 and approximately 4.6X the concentration of Cry1Ab in *Bt* corn (Dutton et al., 2002; Obrist et al., 2006a, 2006b; Alvarez-Alfageme et al., 2008).

A precise conservative EEC is difficult to set given the variety of food that nontarget arthropods are likely to consume. Setting the EEC at 0.2X the overall mean leaf concentration at the highest-expressing developmental stage seems reasonably conservative as many lepidopteran larvae contain less than this amount, and aphids and eggs of Lepidoptera contain considerably less. Spider mites may contain higher concentrations of eCry3.1Ab than leaf tissue, and serious outbreaks of spider mites can occur in corn, particularly under drought (Holtzer et al., 1988). However, most predators in corn fields are generalist feeders that do not depend on a single pest species for food (Steffey et al., 1999), and, therefore, nontarget arthropods are highly unlikely to consume a diet comprising solely spider mites.

Possible exceptions are the specialist spider mite predators *Stethorus* spp. (Coccinellidae). *S. punctillum* is found in corn (e.g., Obrist et al., 2006a), and third instars and adults of this species preferentially eat spider mite eggs (Ragkou et al., 2004), which are likely to contain low, if any, concentrations of toxin compared with adult mites. Experiments have been conducted to examine the transfer of Cry toxins to *S. punctillum* via prey consumption. Alvarez-Alfageme (2008) observed that the concentration of Cry1Ab in spider mites was 4.6 times greater than the concentration in corn leaves, while the concentration in *S. punctillum* adults was about 50% of the corn leaf concentration. Li and Romeis (2010) observed that the Cry3Bb1 concentration in spider mites was 55.9% of the concentration in *Bt* corn leaves, while larvae of *S. punctillum* that were supplied with only *Bt*-corn-reared mites for three or six days contained 10.6% or 7.5%, respectively, of the Cry3Bb1 concentration measured in leaves. After longer exposure to *Bt*-corn reared mites (up to 56 days) the concentration of Cry3Bb1 in *S. punctillum* was even lower, indicating that the Cry protein did not accumulate (Li and Romeis 2010).

Hence, 0.2X the leaf concentration of eCry3.1Ab is a reasonably conservative EEC.

The mean eCry3.1Ab concentration measured in above-ground tissue of 5307 corn at the growth stage with the highest expression was 25.33 µg/g leaf (at maturity)(Table VIII-4). The conservative EEC for above-ground nontarget arthropods is:

0.2 × 25.33 µg eCry3.1Ab/g fresh weight diet = 5.07 µg eCry3.1Ab/g fresh weight diet

VIII.C.3.e. EEC for Soil-Dwelling Nontarget Invertebrates

A conservative EEC for soil-dwelling nontarget invertebrates can be calculated as the concentration of eCry3.1Ab in soil following incorporation of corn plants into soil post-harvest. The best estimate of the eCry3.1Ab concentration in plants post-harvest is the concentration at senescence; however, a conservative EEC was calculated using the

eCry3.1Ab concentration in whole plants at the 5307 plant growth stage with the highest mean concentration.

The typical planting density of corn in the U.S. is 65,500 plants per hectare and the average fresh weight of a corn plant is 750 g. Using the mean whole-plant eCry3.1Ab concentration of 15.78 µg eCry3.1Ab/g fresh weight at the 5307 corn growth stage with the highest levels (Table VIII-4), one hectare (ha) of 5307 corn contains:

$$65{,}500 \text{ plants/ha} \times 750 \text{ g/plant} \times 15.78 \text{ µg eCry3.1Ab/g} = 7.75 \times 10^8 \text{ µg eCry3.1Ab/ha}$$

If the corn is plowed into the top 15 cm of soil, the eCry3.1Ab will be incorporated into 100 m × 100 m × 0.15 m = 1,500 m^3 of soil per hectare. The average density of soil is 1,500 kg/m^3 and, therefore, the corn will be incorporated into 1,500 m^3/ha x 1,500 kg/m^3 = 2,250,000 kg soil per hectare. Dividing the amount of eCry3.1Ab per hectare by the mass of soil per hectare gives the realistic EEC for soil organisms of:

$$\frac{7.75 \times 10^8 \text{ µg eCry3.1Ab}}{\text{ha}} \times \frac{\text{ha}}{2{,}250{,}000 \text{ kg soil}} \times \frac{1 \text{ kg soil}}{1000 \text{ g soil}} = \frac{0.34 \text{ µg eCry3.1Ab}}{\text{g soil}}$$

VIII.C.3.f. EEC for Aquatic Organisms

A potential route of exposure of aquatic organisms to eCry3.1Ab is through 5307 pollen deposited into water bodies adjacent to corn fields. However, corn pollen is heavy and wind-borne pollen densities decrease rapidly from the source (e.g., Luna et al., 2001). Corn anthesis lasts for up to 14 days, and any pollen deposited into water bodies from adjacent fields during this period is unlikely to remain in suspension (Webster et al., 1999). In addition, the bioactivity of eCry3.1Ab is likely to degrade in pollen grains suspended in water for 14 days (e.g., Pusch et al., 1998). The very low concentration of eCry3.1Ab in 5307 corn pollen (0.14 µg/g, Table VIII-4), along with the limited dispersal of pollen and likely eCry3.1Ab degradability in the environment, indicates that any exposure to eCry3.1Ab from 5307 corn pollen via water would be negligible.

The transfer of unharvested corn material to aquatic systems has been studied in water bodies adjacent to fields. Litter traps set alongside streams collected corn by-products of up to 7.9 g ash-free dry mass (AFDM)/m^2 over a year, while benthic sediments contained up to 6.4 g AFDM/m^2 (Rosi-Marshall et al., 2007). Jensen et al. (2010) observed that input of crop by-products into waterways was highest several months after field harvest.

Significant degradation of protein is likely to occur during the period between harvest and transfer to the waterways. Additionally, analysis of 5307 corn tissues by ELISA showed that the mean concentration of eCry3.1Ab was the lowest at senescence (see Table VI-2); the plant stage from which most post-harvest plant debris will originate.

Cry1Ab activity was not detectable in target organism bioassays after *Bt* corn leaves containing the protein were incubated for two weeks in a terrestrial habitat (Jensen et al.,

2010). Because many environmental parameters may affect the concentration of eCry3.1Ab present in 5307 corn crop by-products entering waterways, it is difficult to predict the concentration to which aquatic invertebrates would be exposed. However,any resulting aquatic concentrations of eCry3.1Ab are expected to be far below those at which biological activity is observed among known eCry3.1Ab-sensitive species.

Nevertheless, a conservative risk assessment was conducted based on a worst-case-exposure scenario to aquatic organisms of a diet comprised of 100% corn leaf tissue; this tissue had the highest measured concentration of eCry3.1Ab (Table VIII-4).

VIII.C.3.g. EEC for Farmed Fish

About 30% corn grain by weight is typical in commercial fish feeds used in aquaculture (National Research Council, 1983). Fish feed is heat-treated during preparation and, therefore, it is likely that eCry3.1Ab would, to at least some extent, be denatured and lose activity in feed prepared from 5307 corn grain. However, a worst-case assumption is that no activity is lost.

Corn in fish feed is unlikely to comprise 100% 5307 grain. Insect-protected corn comprised approximately 63% of corn acres planted in the United States in 2010 (USDA-NASS, 2010). In the unlikely event that 5307 corn obtains 50% market share of insect-protected corn, and using the conservative assumption of no degradation of eCry3.1Ab, the conservative EEC for farmed fish would be a diet consisting of 9.5% Event 5307 corn grain:

0.30 Event 5307 corn grain in fish feed × 0.63 US corn acres × 0.5 market share = 9.5%.

VIII.C.3.h. Summary of Environmental Exposure Estimates

Conservative EECs have been computed for groups of nontarget organisms that will be potentially exposed to eCry3.1Ab via cultivation of 5307 corn. These EEC values are compiled in Table VIII-5 and are appropriate for estimating the risks to populations of nontarget organisms. Once defined, these EEC or DDD values can be directly compared to eCry3.1Ab exposure or dose levels that cause no adverse effects in test species. This comparison results in a toxicity exposure ratio (TER).

It is unlikely that nontarget organisms will be exposed to eCry3.1Ab in environments beyond corn cultivation. Corn pollen does not drift great distances nor are corn seeds wind-borne. The transfer of plant material into waterways surrounding fields may occur; however, it is unlikely to result in ecologically relevant concentrations of eCry3.1Ab. The probability of spread of eCry3.1Ab beyond areas of corn cultivation through volunteers and self-sustaining feral populations of 5307 corn is also very low (see Part VIII.H.).

Table VIII-5. Tabulation of EEC and DDD values for nontarget organisms.

Each EEC value presented is based on the average concentration of eCry3.1Ab at the plant stage with the highest concentrations for that 5307 corn tissue and conservative estimates for consumption of these tissues by nontarget organisms. DDD values are computed where appropriate.

Nontarget Organism Group	Estimated Environmental Concentration (EEC)	Daily Dietary Dose (DDD)
Birds	2.28 µg eCry3.1Ab/g kernels	0.80 mg eCry3.1Ab/kg bw
Wild mammals	3.33 µg eCry3.1Ab/g kernels	1.10 mg eCry3.1Ab/kg bw
Pollinator	0.07 µg eCry3.1Ab/g pollen	
Above-ground arthropods	5.07 µg eCry3.1Ab/g diet	
Soil-dwelling invertebrates	0.34 µg eCry3.1Ab/g soil	
Aquatic organisms	100% 5307 corn leaf tissue	
Farmed fish	9.5% incorporation of 5307 corn grain in feed	

VIII.D. Impact on Nontarget Organisms

Eleven species representing groups of nontarget organisms potentially exposed to eCry3.1Ab via cultivation of 5307 corn were exposed to eCry3.1Ab via a microbially produced, purified protein preparation (test substance ECRY3.1AB-0208, containing 89.6% eCry3.1Ab by weight; see Part VI.A.1.a. Production of eCry3.1Ab for Safety Testing) or as 5307 corn tissue in laboratory bioassays. The purpose of these bioassays was to identify potential adverse effects and establish a no observable adverse effect concentration (NOAEC) or level (NOAEL). The source of eCry3.1Ab, test concentration or dose, and route of exposure in each study are listed in Table VIII-6.

The microbially produced eCry3.1Ab protein is biochemically and functionally equivalent to the eCry3.1Ab produced in 5307 corn (see Part VI.A.1.b. Equivalence of eCry3.1Ab in Test Substance and in 5307 Plants); therefore, it is a suitable surrogate for use in tests of the effects of eCry3.1Ab in 5307 corn.

Positive assay controls were included in the honeybee, spotted ladybird beetle, flower bug, ground beetle, earthworm, and rove beetle studies to validate the bioassay methodology. Responses for test organisms exposed to the positive control substance were observed in all studies. The presence of eCry3.1Ab was confirmed in the freshwater shrimp and catfish diets using immunostrips specific for detecting eCry3.1Ab. The intactness, concentration, and bioactivity of eCry3.1Ab were confirmed in artificial diets and soil by Western blot analysis, ELISA and bioassay of sensitive coleopteran larvae, respectively.

Table VIII-6. Source of eCry3.1Ab used to assess the hazard to NTOs.

Species Tested	Source	Route of exposure	Concentration or dose
Bobwhite quail	microbial eCry3.1Ab	gelatin capsule	900 mg eCry3.1Ab/kg bw
Chicken	5307 corn	grain incorporated in feed	0.34 - 2.13 µg eCry3.1Ab/g diet (52 – 64% Event 5307 corn grain in diet)
Mouse	microbial eCry3.1Ab	gavage	2000 mg eCry3.1Ab/kg bw
Honeybee	microbial eCry3.1Ab	sucrose solution	50 µg eCry3.1Ab/g diet
Spotted ladybird beetle	microbial eCry3.1Ab	artificial diet	353 µg eCry3.1Ab/g diet
Flower bug	microbial eCry3.1Ab	artificial diet	400 µg eCry3.1Ab/g diet
Ground beetle	microbial eCry3.1Ab	artificial diet	400 µg eCry3.1Ab/g diet
Rove beetle	microbial eCry3.1Ab	artificial diet	400 µg eCry3.1Ab/g diet
Earthworm	microbial eCry3.1Ab	artificial soil	4.06 µg eCry3.1Ab/g soil
Freshwater shrimp	5307 corn	leaf discs	100% Event 5307 corn leaf discs
Channel catfish	5307 corn	grain incorporated in feed	9.5% Event 5307 corn grain

VIII.D.1. Effect of eCry3.1Ab on Wild Birds

VIII.D.1.a Effect of eCry3.1Ab on bobwhite quail

The bobwhite quail was tested as a representative of wild birds. Five male and five female juvenile bobwhite quail (*Colinus virginianus*) were exposed to a single dose of microbial eCry3.1Ab at 900 mg/kg bw by gelatin capsule. The effects on bird mortality, body weight and feed consumption were compared with a control group for 14 days after dosing. No adverse effects were observed in the treatment group and the NOAEL was shown to be 900 mg eCry3.1Ab/kg bw, the highest dose tested. Additionally, no difference in feed consumption was detected between the study groups, and no abnormal behaviors were observed among the test animals.

VIII.D.1.b Effect of eCry3.1Ab on broiler chickens

Broiler chickens (*Gallus gallus domesticus*) were exposed to eCry3.1Ab via starter, grower and finisher diets prepared from 5307 corn grain; the corn grain concentrations in the diets ranged from 52% to 64%. Nontransgenic, near-isogenic corn grain and grain from a commercially available lot of corn were used to prepare negative control diets. The transgenic 5307 grain and near-isogenic control grain were produced from field-grown hybrid plants of genotypes NP2171 x NP2460($BC5F_3$)(5307) and NP2171 and NP2460, respectively, that were concurrently grown in the same location under USDA APHIS notification 08-051-107n.

The broilers were fed the diets for 49 days. The concentration of eCry3.1Ab in the starter, grower and finisher diets was 2.13 µg/g diet, 0.34 µg/g diet, and 0.38 µg/g diet, respectively; these concentrations were corrected for extraction efficiency. Various endpoints were measured, including mortality, body weight, carcass yield, and feed conversion ratio. There were no adverse effects of the eCry3.1Ab-containing feed on any study parameter.

The results of this study are complementary to the acute toxicity study examining the effects of eCry3.1Ab on bobwhite quail (Part VIII.D.1.a.) and support the hypothesis that cultivation of 5307 corn will pose negligible risk to wild birds.

VIII.D.2. Effect of eCry3.1Ab on Wild Mammals

Five male and five female mice (*Mus musculus*; strain Crl:CD-1) were exposed to a single dose of eCry3.1Ab, 2000 mg/kg bw by oral gavage. The effects on mortality and body weight were compared with a control group for 14 days after exposure, and multiple histological and biochemical endpoints were assessed at the end of the test. The test substance was well tolerated. All mice survived without clinical signs of distress or impairment, and anatomical pathology results did not identify any specific target organ toxicity. The necropsies included, but were not limited to, examination of the external surface, all orifices, and the cranial, thoracic, abdominal and pelvic cavities, including viscera. The organs examined included the esophagus, stomach, duodenum, jejunum, Peyer's patches, ileum, cecum, colon, rectum, selected lymph nodes, spleen, and thymus. There was no test substance-related alteration in the prevalence, severity or histologic character of any incidental tissue alterations. There were no test substance-related effects on body weight or food consumption. No adverse effects were observed in the treatment goup and the NOAEL was shown to be 2000 mg eCry3.1Ab/kg bw, the highest dose tested.

VIII.D.3. Effect of eCry3.1Ab on Pollinators

Honey bees were exposed to microbially produced eCry3.1Ab following the guideline protocol of Oomen et al. (1992). The eCry3.1Ab was provided fresh daily to the bees at 50 µg/g diet in a 50% sucrose solution (200 ml diet/day) for the first five days of the study. The negative control diet for this treatment was 50% sucrose solution without the addition of eCry3.1Ab. The hives were spatially separated (4 hives/treatment) and the diet was placed outside the hive. Worker bees collected the sucrose solutions and fed them to the brood. In each hive, 100 egg cells and 100 larval cells were marked and the development of the brood in those cells was recorded at various assessment timepoints throughout the 24-day study. Pre-imaginal mortality of brood (egg and larval cells) and the number of dead adult bees, larvae and pupae expelled from the hive were monitored and compared with the control group. Additionally, the condition of the hive was examined by measuring the relative proportions of brood and food cells within the hive.

There were no statistically significant differences in 24-day mortality between brood in hives exposed to diet containing eCry3.1Ab when compared to the negative control diet at any developmental stage. Treatment mortality, averaged over the replicates, was 38.2% and 2.3% for the egg and larval cells, respectively. Control mortality, averaged over the replicates, was 25.5% and 16.7% for egg and larval cells, respectively. The study guideline by Oomen et al. (1992) indicates that control mortality should not be, "generally above 15%."

This criterion was met for the larval cells, however it was not met for the egg cells. Both the egg- and larval-cell data provide useful information to evaluate the effects of eCry3.1Ab on brood development, however, the larval data are more robust because the control mortality meets the guideline recommendation. There was a statistically significant difference in mortality between brood in hives exposed to the toxic reference substance (diflubenzuron), 99.8% and 100% mortality for egg and larval cells, respectively, when compared to the negative control at all developmental stages.

There was no statistically significant difference in the mean number of dead adult bees expelled from the hive between the eCry3.1Ab treatment group (12.0 dead bees/hive) and the negative control (22.4 dead bees/hive). There was no statistically significant difference in the mean number of dead adult bees expelled from the hive between the toxic reference treatment group (25.3 dead bees/hive) and the negative control. Although the toxic reference substance did not have an adverse effect on the survival of adult bees this is expected because the acute oral and contact toxicity of the reference substance is reported to be greater than 100 µg active ingredient/bee (Anon., 2009); the effect on the brood was obvious as indicated by the high percent mortality in brood cells. The low number of dead adult bees indicated that the honey bees were healthy during the observation period. Exposure to eCry3.1Ab did not adversely affect the colony condition.

Because the egg cell control mortality exceeded the level that the guideline recommends, a second larval honey bee study was conducted to investigate the effects of eCry3.1Ab on honey bees. In the second study, the colony of honey bees was fed a 50 µg eCry3.1Ab/g sucrose solution fresh daily (200 g diet/day) for five days. The negative control diet for this treatment was 50% sucrose solution without the addition of eCry3.1Ab. The toxic reference substance (fenoxycarb) was also presented to the bees in a 50% sucrose solution. Prior to exposure, at least 100 egg cells were marked in three replicate colonies per treatment (at least 300 egg cells/treatment). The diet was placed inside the colony and the colony was monitored for 22 days after the initiation of exposure to the diets. The brood termination rate (percentage of egg cells that did not develop into adults) and the number of dead bees expelled from the hive were monitored and compared with the control group. The overall condition of the hive was also examined.

There was no statistically significant difference in the mean post-feeding number of dead bees/hive/day between the control (15.7 dead bees/hive/day) and eCry3.1Ab treatment (14.4 dead bees/hive/day) groups. There was a statistically significant difference between the mean number of bees expelled from the hive after exposure to the toxic reference substance (178.6 dead bees/hive/day) when compared to the control. There was no statistically significant difference in the mean post-feeding number of dead pupae/hive/day between the control group (0.6 dead pupae/hive/day) and the treatment group (0.9 dead pupae/hive/day). There was a statistically significant difference between the mean number of pupae expelled from the hive after exposure to the toxic reference substance (118.3 dead pupae/hive/day) when compared to the control.

The mean brood termination rate was 20.0% for the control group, 7.9% for the treatment group and 24.2% for the reference substance group. In this study the control mortality was

closer to the 15% level recommended by the Oomen et al. guideline (1992). The effects of the reference substance were not indicated by examining only the brood termination rate in isolation, however, effects of the toxic reference substance were demonstrated by the relatively large number of bees expelled from the hive. Fenoxycarb is a growth regulator that is known to affect honey bees late in development (pupal stage, after the cells are capped). Due to the late-acting nature of the reference substance the cells marked as empty at the end of the monitoring period were assumed to have emerged successfully as adults. However, it is possible that dead pupae affected by the toxic reference substance were instead removed from the cells by nurse bees. This is likely the case due to the large number of bees expelled from the hive with the sickle eye symptom, which is a commonly found in bees exposed to fenoxycarb. In summary, although the brood termination rate alone does not show effects of the toxic reference substance, the weight of evidence presented indicates that the honey bees were exposed to and affected by the reference substance.

Both of the honey bee studies demonstrated that exposure to eCry3.1Ab causes no adverse effects on survival, brood development, or the condition of the colonies.

VIII.D.4. Effect of eCry3.1Ab on Above-ground Arthropods

VIII.D.4.a. Ladybeetles

Three- to five-day-old spotted ladybird beetle larvae (*Coleomegilla maculata*) were exposed to a nominal eCry3.1Ab concentration of 500 µg/g diet for 21 days. The eCry3.1Ab protein was incorporated into a diet containing "bee pollen" (pollen collected by bees from various flowers) and *Ephestia* (moth) eggs. The negative control diet contained bee pollen and *Ephestia* eggs without the addition of eCry3.1Ab. Potassium arsenate (a known stomach poison to many insects) mixed with the diet of bee pollen and *Ephestia* eggs was included in the study as the positive control substance. Forty individual larvae were treated in both the test group and the positive control group; due to an error, only 30 larvae received the negative control treatment. Fresh diet was provided to individual larvae every other day, and any unconsumed diet from the previous feeding was removed. Diet analysis confirmed that the treatment group was exposed to at least 353 µg eCry3.1Ab/g diet. Western blot analysis confirmed that eCry3.1Ab remained intact in the diet matrix, and bioassays confirmed that the diet was highly bioactive against sensitive coleopteran larvae (*Leptinotarsa decemlineata*). The negative control diet was confirmed to not contain eCry3.1Ab.

The survival and development of the larvae were monitored and compared to the negative control group. Larval mortality was 13.8% in the negative control group, 2.5% in the eCry3.1Ab-treated group, and 100% in the positive control group. Larval pupation was 86.2% in the negative control group and 97.5% in the eCry3.1Ab-treated group. Percent adult emergence was 92.0% in the negative control group and 97.4% in the eCry3.1Ab-treated group. Adult beetle mortality was 14.3% in the negative control group and 10.5% in the eCry3.1Ab-treated group. The above differences between the negative control and the treated group were not statistically significant. However, the larvae in the eCry3.1Ab-treated group developed into pupae significantly more quickly (mean of 9.6 days to pupate) than the larvae in the control group (mean of 12.0 days to pupate). Additionally, the adults

that emerged from the eCry3.1Ab treatment weighed significantly more (mean of 0.0142 g) than the negative controls (mean of 0.0118). However, these are not considered adverse effects. Therefore, the NOAEC was shown to be 353 µg eCry3.1Ab/g diet, the highest concentration tested.

VIII.D.4.b. Flower bugs

Second-instar flower bugs (*Orius laevigatus*) were exposed to eCry3.1Ab via incorporation into an artificial, meat-based diet at a rate of 400 µg/g diet. A negative control group was fed an artificial, meat-based diet without the addition of eCry3.1Ab. A positive control group received the same diet treated with teflubenzuron. Each group contained 40 nymphs, which were individually provided fresh diet daily for 12 days or until they became adults. The positive control group had 100% mortality. There was no significant difference in mortality between the treatment group (5% mortality) and the negative control group (0% mortality). Therefore, the NOAEC was shown to be 400 µg eCry3.1Ab/g diet, the highest concentration tested.

Analysis of the test diet by ELISA confirmed that eCry3.1Ab was present at the nominal concentration. Western blot analysis confirmed that eCry3.1Ab remained intact in the diet matrix, and bioassays confirmed that the diet was highly bioactive against sensitive coleopteran larvae (*L. decemlineata*). The negative control diet was confirmed to not contain eCry3.1Ab.

VIII.D.5. Effect of eCry3.1Ab on Soil-dwelling Invertebrates

VIII.D.5.a. Carabid beetles

Larvae of *Poecilus cupreus*, a carabid beetle (ground beetle), were exposed to eCry3.1Ab via an artificial, meat-based diet containing 400 µg eCry3.1Ab/g diet. The negative control group was fed an artificial, meat-based diet without the addition of eCry3.1Ab. A positive control group received the same diet treated with teflubenzuron. Each group contained 40 larvae, which were individually provided fresh diet daily in their culture tubes until they ceased feeding as pre-pupae; the first pupae appeared in the culture tubes 27 days after initiation of treatment. Thereafter, the emergence of adults was monitored.

There was no significant difference between the pre-imaginal mortality of the eCry3.1Ab treatment group (8%) and the negative control group (10%). The positive control group had 100% pre-imaginal mortality. The time to adult emergence was longer (range of 41-55 days) in the eCry3.1Ab treatment group compared to the negative control (range of 41-48 days) and there was a statistically significant difference between the mean weight of the adult beetles from the treatment group (52.7 mg) and the control group (65.7 mg), representing a 20% reduction in mean beetle weight. *P. cupreus* was tested under extreme exposure concentrations, with continuous exposure to a nominal concentration of 400 µg eCry3.1Ab/g diet throughout development, with no adverse effects on survival and minor effects on growth. Therefore, effects of eCry3.1Ab on ground beetles are likely to be negligible during field exposure.

Analysis of the test diet by ELISA confirmed that eCry3.1Ab was present at a minimum of 362.2 µg eCry3.1Ab/g diet, representing 90.6% of the nominal concentration. Western blot analysis confirmed that eCry3.1Ab remained intact in the diet matrix, and bioassays confirmed that the test diet was highly bioactive against sensitive coleopteran larvae (*L. decemlineata*). Based on the results for the three endpoints evaluated in the diet analysis study, it was assumed that eCry3.1Ab was present at the nominal concentration of 400 µg/g diet. The negative control diet was confirmed to not contain eCry3.1Ab.

VIII.D.5.b. Rove beetles

Adult rove beetles (*Aleochara bilineata*) were exposed to eCry3.1Ab following the guideline protocol of Grimm et al. (2000). The treatment group was fed an artificial, meat-based diet containing 400 µg eCry3.1Ab/g diet. The negative control was the same artificial, meat-based diet without the addition of eCry3.1Ab. A positive control group received the same diet treated with teflubenzuron. Each group contained a total of 80 beetles, allocated in four replicates of 20 beetles each (10 males and 10 females). The adult beetles were fed the treated diet for 35 days after which they were removed from the test arena and the parasitic success of their offspring from fly pupae was monitored. There was no statistically significant difference between the mean number of progeny from the treatment group (824) and the control group (828), whereas no progeny were produced by the teflubenzuron-treated group. Therefore, the NOAEC was shown to be 400 µg eCry3.1Ab/g diet, the highest concentration tested.

Analysis of the test diet by ELISA confirmed that eCry3.1Ab was present at a minimum of 78.1% of the nominal concentration. Western blot analysis confirmed that eCry3.1Ab remained intact in the diet matrix, and bioassays confirmed that the test diet was highly bioactive against sensitive coleopteran larvae (*L. decemlineata*). Based on the results for the three endpoints evaluated in the diet analysis study, it was assumed that eCry3.1Ab was present at the nominal concentration of 400 µg/g diet. The negative control diet was confirmed to not contain eCry3.1Ab.

VIII.D.5.c. Earthworms

Adult earthworms (*Eisenia fetida*) were exposed to eCry3.1Ab via incorporation into an artificial soil substrate at a rate equivalent to 50 µg eCry3.1Ab/g dry wt of soil. The artificial soil was comprised of 10% w/w sphagnum peat, 20% w/w kaolinite clay, 69.8% w/w industrial quartz sand, and 0.2% w/w calcium carbonate to adjust the soil pH to 6 ± 0.5. The negative control group was placed in the same artificial soil substrate but without the addition of eCry3.1Ab. Each group contained a total of 40 worms, allocated in four replicates of 10 worms each. The treatment and control groups were monitored for 14 days. The stock earthworm culture used for this study was shown to have the expected sensitivity to chloroacetamide, a positive control substance, when mixed into artificial soil. There was 100% survival in both the eCry3.1Ab-treated and control groups and there were no statistically significant differences in the change in biomass between the groups. There were no visible lesions or effects on earthworm behavior observed in the study.

Analysis of the treated soil sampled at test initiation by ELISA confirmed that the *E. fetida* were exposed to at least 4.06 µg eCry3.1Ab/g dry weight soil. The relatively low recovery of eCry3.1Ab by ELISA likely reflects the physicochemical properties of the soil matrix, because low recovery was also seen in direct eCry3.1Ab spike and recovery experiments with the same artificial soil matrix. Western blot analysis indicated that eCry3.1Ab remained substantially intact in the artificial soil, and bioassays of *L. decemlineata* fed diets containing the treated soil indicated that the eCry3.1Ab retained bioactivity in the artificial soil matrix. Thus, it is likely that the earthworms were exposed to higher test concentrations than indicated by the ELISA results. Nevertheless, The NOAEC was shown to be 4.06 µg eCry3.1Ab/g soil, the highest concentration of eCry3.1Ab measured in the soil. The soil used in the negative control treatment was confirmed to not contain eCry3.1Ab.

VIII.D.6. Effect of eCry3.1Ab on Aquatic Organisms

VIII.D.6.a. Freshwater shrimp

Gammarid freshwater shrimp (*Gammarus fasciatus*) were selected as the test species because they are easily cultured and are sensitive to a variety of toxic substances. This species has also been observed to feed on leaves by shredding (Arsuffi and Suberkropp, 1989), which makes it suitable for testing the effects of transgenic corn leaves containing pesticidal proteins. Forty adult gammarids were exposed to a diet comprised of 100% 5307 corn leaf tissue; no other source of food was provided. The same number in a negative control group were fed a diet of nontransgenic, near-isogenic corn leaf tissue. For each treatment group, the gammarids were contained in four replicate one-liter glass vessels of fresh water, each containing ten organisms. Corn leaf discs were pre-soaked in the culture water prior to introducing fresh leaf discs to each culture vessel daily. The effects on survival in the group exposed to 5307 leaf tissue were compared with the control group. The study was terminated at day 5 because control mortality reached 20%. At study termination, mortality was 12% in the 5307 corn treatment group. Typically, mortality caused by Cry proteins to sensitive target larvae occurs within three to five days of exposure, therefore, biologically meaningful conclusions can be drawn from this study. There was no statistically significant difference in survival between *G. fasciatus* fed the 5307 corn leaf tissue and those fed the nontransgenic, near-isogenic control corn leaf tissue. Examination of the leaf discs confirmed that the gammarids had consumed leaf tissue. Analysis of the leaf discs by immunostrip assay confirmed that eCry3.1Ab was present in the 5307 corn tissue, as expected, and absent from the control corn leaf tissue.

VIII.D.6.b. Channel catfish

Juvenile channel catfish (*Ictalurus punctatus*) were exposed to 5307 corn grain via a pelleted diet. The diet was made by incorporating 41% 5307 corn grain into a fish diet formulation (prepared with typical ingredients) and using a “cold-pelleting” process, which minimized heating that could denature the eCry3.1Ab protein. (Standard, commercial fish diets are pelletted at higher temperatures to promote feed efficiency.) The negative control diet was prepared in the same manner using grain from nontransgenic, near-isogenic corn. The transgenic 5307 grain and near-isogenic control grain used to prepare these diets were produced from field-grown hybrid plants of genotypes NP2171 x NP2460($BC3F_5$)(5307)

and NP2171 and NP2460, respectively, that were concurrently grown in the same location under USDA APHIS notification 08-085-105n.

Four separate aquaria containing 20 catfish each (i.e., 80 total fish) were tested in each group, which received feed daily at the rate of approximately 8% of the fish biomass. The effects on survival and growth were compared for a 28-day observation period. There was 100% survival in both study groups. There were no statistically significant differences in mean total biomass, mean weight gain and feed conversion ratios. There were no adverse effects observed when 5307 corn grain was incorporated as 41% of the catfish diet. An immunostrip test confirmed that eCry3.1Ab was present in the treated fish diet and absent from the control diet.

VIII.E. Toxicity Exposure Ratios for Nontarget Organisms

The EEC and DDD values are compared with results of eCry3.1Ab hazard studies in order to make judgements about risk. The effects studies identified any potential for eCry3.1Ab proteins to cause adverse effects in representative nontarget organism species applicable for a corn ecosystem. In each study, a representative species was exposed to a high concentration or dose of eCry3.1Ab. No harmful effect of such exposure on survival was observed in any species tested, and the concentration of eCry3.1Ab in each study can be interpreted as the minimum value of the NOAEC for the mortality endpoint. Computing a ratio of the NOAEC to the EEC or DDD results in the toxicity exposure ratio (TER). This ratio is also known as the 'margin of exposure' or 'margin of safety.' A TER $\geq$ 1.0 provides a degree of confidence that the absence of adverse effects in these hazard studies with indicator species is predictive of the safety of eCry3.1Ab in cultivation of 5307 corn for nontarget organisms in general. The larger the TER, the higher the degree of confidence in the safety judgement and the lower the risk. Table VIII-7 contains the NOAEC and corresponding TER values for each indicator test species.

No adverse effects in mortality were observed in any study that exposed representative nontarget organisms to eCry3.1Ab. The concentration of eCry3.1Ab tested in the studies was sufficient to achieve TERs of $\geq$ 1 for all species based on conservative EEC values.

Table VIII-7 Nontarget organism TER values for eCry3.1Ab.

TER values are computed as the ratio of NOAEC: EEC (or NOAEC: DDD) and are based on estimates of exposure to eCry3.1Ab via cultivation of 5307 corn.

Test Species	NOAEC/NOAEL	EEC or DDD	TER
Bobwhite quail	900 mg/kg bw	0.80 mg/kg bw	1100
Mouse	2000 mg/kg bw	1.10 mg/kg bw	1800
Honeybee	50 µg/g diet	0.07 µg/g diet	710
Spotted ladybird beetle	353 µg/g diet	5.07 µg/g diet	70
Flower bug	400 µg/g diet	5.07 µg/g diet	79
Carabid beetle	400 µg/g diet[1]	0.34 µg/g diet	1200 (mortality) < 1200 (weight)
Rove beetle	400 µg/g diet	0.34 µg/g diet	1200
Earthworm	4.06 µg/g soil	0.34 µg/g diet	12
Freshwater shrimp	100% 5307 corn	< 100% 5307 corn	> 1[a]
Farmed fish	41% 5307 corn grain	9.5% 5307 corn grain	4.3

[1] This NOAEC is based on the mortality endpoint for this species; a 20% reduction in weight was observed at this test concentration, the only concentration tested.

[a] Limitations of the study design precluded a higher TER for freshwater shrimp. The test organism is a leaf shredder, and therefore leaves of 5307 corn were provided as the source of eCry3.1Ab in the study; higher test concentrations could not be achieved using leaves. However, the test diet consisted of 100% 5307 corn leaves for the duration of the study (in comparison to the low and intermittent exposures that might occur under more realistic exposure scenarios) and thus, represented a very conservative exposure scenario.

VIII.F. Safety Assessment for Endangered and Threatened Species

No effects of eCry3.1Ab protein on mortality were observed in any of the species tested. These studies with a wide range of taxa demonstrate that there is negligible risk associated with exposure to estimated environmental concentrations of eCry3.1Ab in 5307 corn (see Table VIII-7). There is a weight of evidence that at concentrations in 5307 corn the toxicity of eCry3.1Ab will be limited to the family Chrysomelidae in the order Coleoptera. Its receptor-mediated mechanism of action and absence of activity in bioassays with multiple species outside the order Coleoptera and outside the family Chrysomelidae support this conclusion.

As shown in Table VIII-8, currently there are 17 species of Coleoptera listed as endangered or threatened species by the US Fish and Wildlife Service (US FWS, 2010). None of these is from the same family that are potentially susceptible to eCry3.1Ab at concentrations resulting from the cultivation of 5307 corn. Moreover, due to their feeding habits and non-agricultural habitats, none of these species is expected to be exposed to eCry3.1Ab directly from 5307 corn plants, and any indirect exposure (e.g., through prey consumption) would be negligible. None of the listed species is known to feed on corn or have host plants near corn fields. Most are predatory and consume other insects; some are detritivores or consume carrion. Several are aquatic species, or inhabit saline wetlands, mud flats, stream edges, aquifers, or beaches; others inhabit caves. Therefore, due to a lack of exposure to corn as

well as likely insensitivity to eCry3.1Ab at concentrations in 5307 corn, no endangered or threatened beetles are expected to be harmed by eCry3.1Ab produced in 5307 corn hybrids.

In 2010, the U.S. EPA completed an extensive review of the data and information supporting the continued registration of several Cry protein "active ingredients" in *Bt* corn products, including multiple rootworm-control products (US EPA, 2010b; 2010c; 2010e). In all cases, EPA concluded that these products did not pose a risk to any endangered or threatened Coleoptera or other nontarget species listed by the US Fish and Wildlife Service. Additionally, EPA determined that there would be no indirect effects on endangered or threatened plant species, such as impacts on pollinators that are important and/or essential to any endangered or threatened plant.

Table VIII-8. Threatened or endangered Coleoptera listed by the U.S. Fish and Wildlife Service.

Order: Family	Genus + Species	Status
Coleoptera: Carabidae	*Elaphrus viridis*	Threatened
	Rhadine persephone	Endangered
	Rhadine exilis	Endangered
	Rhadine infernalis	Endangered
	Cicindela dorsalis dorsalis	Threatened
	Cicindela ohlone	Endangered
	Cicindela puritana	Threatened
	Cicindela nevadica lincolniana	Endangered
Coleoptera: Staphylinidae	*Batrisodes venyivi*	Endangered
	Texamaurops reddelli	Endangered
	Batrisodes texanus	Endangered
Coleoptera: Silphidae	*Nicrophorus americanus*	Endangered
Coleoptera: Dryopidae	*Stygoparnus comalensis*	Endangered
Coleoptera: Elmidae	*Heterelmis comalensis*	Endangered
Coleoptera: Haliplidae	*Brychius hungerfordi*	Endangered
Coleoptera: Scarabaeidae	*Polyphylla barbata*	Endangered
Coleoptera: Cerambycidae	*Desmocerus californicus dimorphus*	Threatened

VIII.G. Conclusion of Low Environmental Risk

No adverse effects on survival were associated with exposures of eCry3.1Ab in a range of indicator species appropriate for a corn ecosystem. There was an effect, a 20% reduction in adult beetle weight, observed after larvae of *P. cupreus* were exposed to 400 µg eCry3.1Ab/g diet throughout their development. Because the *P. cupreus* larvae were tested at extreme exposure concentrations with no adverse effects on survival and only minor

effects on growth, the effects of eCry3.1Ab on ground beetles are likely to be negligible during field exposure. The exposures in all studies were in excess of EEC or DDD levels for the nontarget organism groups represented, indicating a low probability of harm to these groups from exposure of eCry3.1Ab via cultivation of 5307 corn.

VIII.H. Gene Flow Assessment

An assessment of the environmental fate of eCry3.1Ab resulting from the cultivation of 5307 corn requires consideration not only of the production and degradation of the protein within corn fields, but also the possibility that eCry3.1Ab could persist, or spread from, areas of corn cultivation because of gene flow or the establishment of weedy populations of 5307 corn. It is highly unlikely that eCry3.1Ab will appear in environments outside of cultivated corn. Corn pollen does not drift great distances nor are corn seeds wind-borne. The probability of spread of eCry3.1Ab outside corn cultivation through volunteers and self-sustaining feral populations of 5307 corn is also very low.

As described in Part VII above, 5307 corn is not phenotypically or compositionally different from conventional corn other than in its ability to resist insect feeding damage. Thus, 5307 corn has not acquired any properties indicative of increased weediness potential. The likelihood that the *ecry3.1Ab* gene will move to other species as a result of either vertical or horizontal gene transfer is also extremely low.

VIII.I. Gene Flow to Wild Species

Z. mays L. subsp. *mays* 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 varieties despite occasional introgression (Baltazar et al., 2005). Teosinte species are not natives of the US, but isolated populations have been recorded in Florida and Texas, the former a possible remnant of the use of annual teosinte as a forage grass. These populations are apparently now extinct in both states. Teosinte species are grown in botanical gardens, but as corn pollen is heavy and relatively short-lived (e.g., EPA, 2010d; Byrne and Fromherz, 2003; Devos et al., 2005), fertilization of these plants with pollen from 5307 corn is extremely unlikely.

Species of the genus *Tripsacum* are considered close relatives of *Zea* species and some theories postulate that a *Tripsacum* species may be a progenitor of domesticated corn via hybridization and introgression with teosinte (e.g., Poggio et al., 2005). There are sixteen species of *Tripsacum* worldwide, of which three occur in the US: *T. dactyloides*, a widespread forage grass; *T. floridanum*, known from southern Florida; and *T. lanceolatum*, which is present in Arizona and possibly New Mexico (EPA, 2010d).

Corn breeders view *Tripsacum* as a potential source of useful genes for traits including apomixes, pest and disease resistance, and drought tolerance (OECD 2003). Therefore, substantial effort has been made to obtain and characterize corn-by-*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 EPA (2010d) concluded that the chance of natural introgression of genes from corn to *Tripsacum* was 'extremely remote' and that no other species in the continental US would interbreed with commercial corn.

These observations indicate a very low probability for transfer of the *ecry3.1Ab* gene from 5307 corn to wild relatives in the US. Species of *Zea* other than corn are not recorded outside botanical gardens in the US. *Tripsacum dactyloides* is widespread, but does not hybridize readily with *Z. mays*, and the probability of backcross of F_2 progeny of *Tripsacum*-by-*Zea* hybrids being produced in the field is negligible. Therefore, eCry3.1Ab is unlikely to spread from corn cultivation and persist in the environment as the result of vertical gene flow.

VIII.J. Potential for Horizontal Gene Transfer

An extensive review of information relevant to the potential risks of horizontal gene transfer for *Bt* crops to soil microbes was conducted by the US Environmental Protection Agency (EPA, 2010d). Studies reviewed by the EPA showed no evidence of horizontal gene transfer under field conditions, and only equivocal evidence for horizontal gene transfer under laboratory conditions designed to maximize the recovery of transformants. Conner et al. (2003) also reviewed the literature and found very few examples where horizontal gene transfer had been demonstrated convincingly, and these cases relied on artificially high sequence homology between the transgene and the potential recipient organism (e.g., de Vries et al., 2001). The codons in the *ecry3.1Ab* gene are optimized for expression in plants, and hence the gene is likely to have low sequence homology with genes of soil microbes. Therefore, horizontal gene transfer of *ecry3.1Ab* from 5307 corn to soil microbes is highly unlikely. The probability of spread of *ecry3.1Ab* outside corn cultivation by horizontal gene transfer is negligible.

IX. Insect Resistance Management

Following USDA APHIS deregulation of 5307 corn and EPA registration, 5307 corn will not be commercialized as a stand-alone product offering but will be sold as part of corn hybrids with multiple pyramided traits that have been combined via conventional plant breeding. The initial product offerings planned in the U.S. are Bt11 × MIR604 × TC1507 × 5307 × GA21 and Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 corn hybrids. These hybrids will combine two rootworm-active proteins (eCry3.1Ab from 5307 corn and mCry3A from MIR604 corn) that each provide control of western, northern and Mexican corn rootworms. Additionally, the hybrids will combine other *Bt*-derived proteins that will deliver broad-spectrum control of economically important lepidopteran pests. Finally, the hybrids will also contain traits that confer tolerance to glyphosate and glufosinate herbicide applications, as additional weed-control options. Apart from 5307 corn, all the other corn events in these breeding stacks have been deregulated by USDA.

An important aspect of combining eCry3.1Ab and mCry3A in a single product is that these proteins have been shown to not compete for the same binding site in western corn rootworm gut membranes (Walters et al., 2010). This means that they exert their toxicity to rootworms by different modes of action at the target site. This feature offers significant benefits from the perspective of insect resistance management, because pests are far less likely to evolve resistance to two control mechanisms deployed concurrently than they are to any single control mechanism. Not only will the combination of eCry3.1Ab and mCry3A significantly reduce the likelihood of resistance to either protein, but it can also be expected to reduce the selection pressure on other *Bt* proteins currently available in commercial rootworm-control corn hybrids as well as other established control mechanisms, including conventional insecticides and crop rotation. Some rootworm populations have evolved resistance to insecticides or have overcome traditional crop rotation methods. Hybrids containing eCry3.1Ab and mCry3A will represent an important and effective new tool in the corn grower's arsenal of rootworm control mechanisms.

Current insect resistance management strategies for *Bt* corn products are centered around the planting of a structured refuge that can provide a source of susceptible adult insects with which rare resistant insects can mate. Matings of resistant and nonresistant insects serve to dilute the frequency and establishment of resistance genes in a population. The size and configuration of the structured refuge are determined by toxin dose and insect biology. A second important aspect of combining eCry3.1Ab and mCry3A in a single product is that the minimum size of the on-farm refuge required to delay resistance can be reduced from 20% of a grower's total corn acreage (e.g., for MIR604 corn or other corn products with single rootworm-control traits) to 5% of a grower's acreage. This will (1) offer additional pest control benefits on what would previously have been refuge acres, (2) reduce the environmental impact of insecticide use on refuge acres, and (3) increase grower compliance with refuge requirements.

Syngenta is committed to implementing a product stewardship program that will help preserve the long-term efficacy of its corn hybrids. Syngenta has developed a

comprehensive insect resistance management plan, which will soon be submitted to the EPA for their review and approval. The key elements of this plan are the standard components of a robust insect resistance plan for *Bt* crop deployment, and include:

- A description of pest biology and ecology
- High-dose or effective-dose expression of insecticidal proteins
- An appropriate non-*Bt* refuge
- A extensive product stewardship program that includes grower education, programs to promote grower compliance, monitoring of pest populations, and remedial action plans

These multiple components will operate synergistically to provide long-term protection against the development of target pest resistance.

X. Adverse Consequences of Introduction

Syngenta is not aware of any unfavorable information that would have bearing on this Petition for deregulation.

Syngenta's development and testing of 5307 corn has not revealed any data or observations indicating that 5307 corn would pose a greater plant pest risk than conventional corn. Further, based on its extensive research, Syngenta does not anticipate that deregulation of 5307 corn will have any adverse environmentalconsequences. Nonetheless, Syngenta intends to submit an Environmental Report that will be submitted as a supplement to this Petition. As noted above, this Environmental Report is specifically intended to provide information that will assist APHIS in fulfilling its obligations under NEPA, as well as other applicable statutes and regulations.

The Environmental Report will discuss a range of issues related to the deregulation of 5307 corn, including any potential direct, indirect or cumulative impacts on the quality of the human environment, endangered species, unique geographic areas, critical habitats, public health and safety (including children and minorities), genetic diversity of corn, farmer or consumer choice, insect resistance, or the economy, either within or outside the U.S. Moreover, because 5307 corn offers growers an additional choice for protection of corn crops from feeding damage caused by rootworm pests, it is expected to convey benefits similar to those associated with previously deregulated *Bt* corn products that are commercially available. These benefits include reduced insecticide use, improved pest control and worker safety, better yield, and fewer environmental impacts. Additional benefits will include increased product choice for growers, increased price competition, and extended useful life of *Bt* corn as well as other approaches for rootworm control. These beneficial impacts are also expected to be part of the Environmental Report.

XI. References

Note: This list of references also includes those cited in Appendices B, C, and D.

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Appendix A. USDA Notifications for Field Trials with Event 5307 Corn

Field trials with transformation Event 5307 corn have been conducted in the U.S. under USDA-APHIS release permits or notifications since 2005. A listing of these notifications, along with a status of the field test report for each, is provided in Table A-1.

Table A-1. List of USDA-APHIS field release permits and notifications under which plantings of Event 5307 corn have occurred.

APHIS Reference Number[1]	Effective Dates (MM/DD/YY)	Release States or Puerto Rico (PR)[2]	Field Test Report Status[3]
05-062-02n	4/25/05 - 4/25/06	HI, IL, MN, NE, WI	Submitted
05-104-09n	5/12/05 - 5/12/06	HI	Submitted
06-055-08n	4/27/06 - 4/27/07	HI, IA, IL, MN, MO, NE	Submitted
07-043-109n	4/5/07 - 4/5/08	HI, IL, IN, IA, KS, MN, MO, NE, SD, WI	Submitted
07-166-101n	7/16/07 - 7/16/08	IA	Submitted
07-240-103n	11/21/07 - 11/21/08	HI, PR	Submitted
08-051-104n	3/20/08 - 3/20-09	HI, IA, IL, IN, MN, MO, PR, SD, WI	Submitted
08-051-107n	3/20/08 - 3/20/09	NE	Submitted
08-085-105n	4/25/08 - 4/25/09	WA	Submitted
08-224-102n	9/22/08 - 9/22/09	HI	Submitted
08-304-106n	11/30/08 - 11/30/09	HI, PR	Submitted
09-013-105n	3/1/09 - 3/1/10	HI, PR	Submitted
09-050-108n	3/20/09 - 3/20/10	CA, CO, NE	Submitted
09-062-103n	4/1/09 - 4/1/10	AR, IA, IL, MI, MO, NE, OH, OK, SC, WA	Submitted
09-063-103n	4/1/09 - 4/1/10	HI, IL, KY, IA, IN, MN, MO, NE, SD, WI	Submitted
09-084-109n	4/14/09 - 4/14/10	IA	Submitted
09-086-102n	4/13/09 - 4/13/10	NE	Submitted
09-114-102n	5/13/09 - 5/13/10	IA, NC, WI	Submitted
09-231-102n	9/25/09 - 9/25/10	HI, PR	Submitted
10-056-103n	3/26/10 - 3/26/11	CO, FL, PR, HI, IA, IL, IN, KY, MN, MO, NE, SD, WI	Not submitted
10-064-109n	4/6/10 - 4/6/11	AR, KY, LA, PR, CO, IA, IN, MN, HI, IL, KS, MO, NE, WI, SD	Not submitted
10-235-105n	9/23/10 - 9/23/11	HI, PR	Not submitted

[1] Only the permits and notifications under which plantings of 5307 corn actually occurred are listed.

[2] States listed are the actual release states if the Field Test Report has been submitted, or approved release states if the Field Test Report has not yet been submitted.

[3] Field Test Reports shown as "Not Submitted" are due to APHIS by six months after expiration of the permit or notification (i.e., they are due to be submitted in 2011 or 2012).

Appendix B. Materials and Methods Used in the Genetic Characterization of Event 5307 Corn

This appendix provides details of the materials and methods used in the various studies performed to genetically characterize Event 5307 corn. The design, results and conclusions of these experiments are described in **Part V** of this Petition, titled **Genetic Characterization of Event 5307 Corn**. All literature references cited in this appendix are listed in **Part IX** of this Petition, titled **References**.

B.1. Plant Material

Event 5307 corn and nontransgenic control corn seeds were planted and grown in a Syngenta Biotechnology, Inc. greenhouse in Research Triangle Park, North Carolina, USA under standard greenhouse conditions and then processed to extract genomic DNA. The specific generations of 5307 plants used and the corresponding control plants used are specified in the descriptions of the studies described in Part V of this Petition. Appropriate quality control methods were used to verify the purity and identity of the plant material used in each study. The pedigree showing the genotypes of Event 5307 seed for the various studies is provided in Figure III-1. Table III-1 provides a list of the sources of plant material used for each study.

B.2. Real-Time PCR Analysis

All plants grown for genetic characterization studies were individually analyzed for the presence of *ecry3.1Ab* and *pmi* by real-time PCR analysis (Ingham et al., 2001). A control assay targeting the endogenous corn alcohol dehydrogenase gene 1 (*adh1*) was used to confirm the presence of DNA in each reaction. Leaf discs were sampled from each individual plant. Deoxyribonucleic acid (DNA) was isolated from leaf discs of each individual plant using a method adapted from the Wizard® Magnetic 96 DNA Plant System for real-time PCR analysis.

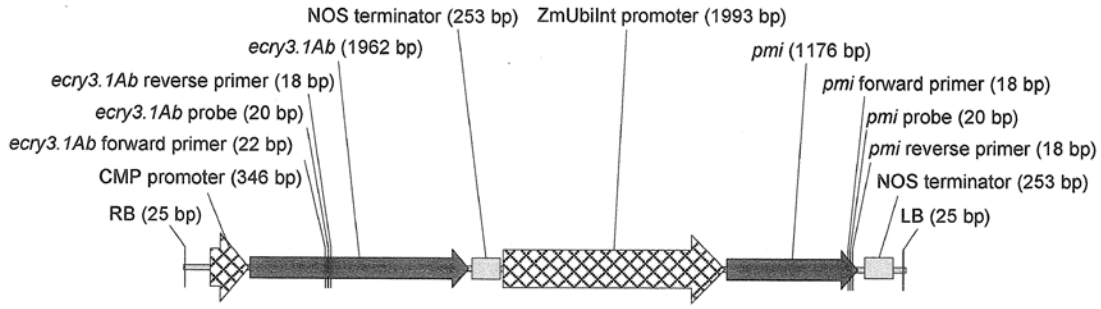
Table B-1 lists the primers and probes used to detect *ecry3.1Ab*, *pmi* and *adh1*. Figure B-1 shows the locations of the *ecry3.1Ab*-specific and *pmi*-specific primers and probes in the transferred DNA (T-DNA) of plasmid pSYN12274, the transformation plasmid used to generate 5307 corn.

The following cycling parameters were used for this reaction: 95°C for five minutes, followed by 40 cycles of 95°C for five seconds and 60°C for 30 seconds.

Table B-1. Real-time PCR primers and probes used for the detection of *ecry3.1Ab*, *pmi*, and *adh1*

Amplicon of interest	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Probe sequence (5' to 3')
ecry3.1Ab	TACGAGAGCTGGGTG AACTTCA	CGATCAGGTCCAGCA CGG	CCGCTACCGCCGCG AGATGA
pmi	CCGGGTGAATCAGCG TTT	GCCGTGGCCTTTGAC AGT	TGCCGCCAACGAATC ACCGG
adh1	GAACGGTGTTGGGTTT GCAT	TGCAGCCTAACCATG CGCAGGGTA	TCCAGCAATCCTTGC ACCTT

Figure B-1. Locations of real-time PCR primers and probes in the plasmid pSYN12274 T-DNA



bp = base pair

B.3. Genomic DNA Extraction

Following verification of the plants' identity by real-time PCR analysis (see above), leaf tissue for 10 plants of each genotype was pooled into a sampling bag and stored at -80°C ± 10°C. Genomic DNA used for nucleotide sequencing and Southern blot analyses was isolated from the pooled leaf tissue from 10 plants per genotype using a modification of the method described in Saghai-Maroof et al. (1984). Pooled leaf tissue was ground into a fine powder using a pre-chilled mortar and pestle, with liquid nitrogen, and then placed into a bottle for storage. For each DNA extraction, approximately 40 g of this tissue and 200 ml of prewarmed CTAB buffer (100 mM Tris pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 2% CTAB [w/v], 0.2% [v/v] β-mercaptoethanol) were combined in a bottle; the sample was then mixed gently and incubated for 90 minutes at 65°C ± 5°C. An equal volume of chloroform: isoamyl alcohol (24:1) was then added, followed by gentle mixing and centrifugation for 10 minutes at 7277 × *g* at room temperature. The resulting aqueous phase was transferred to a clean container, and 10 µg of ribonuclease per ml of aqueous phase was added. The sample was mixed and incubated for 30 minutes at 37°C ± 2°C. An equal volume of chloroform: isoamyl alcohol (24:1) was then added, followed by gentle mixing and centrifugation for 10 minutes at 7277 × *g* at room temperature. The aqueous phase was collected in a clean bottle, and the DNA was precipitated with a 0.8 volume of isopropanol. The DNA was then pelleted by centrifugation at 291 × *g* and washed once with 70% ethanol. The DNA pellet was air dried and dissolved in 2.5 ml of prewarmed 0.1X Tris-EDTA.

B.4. DNA Quantitation

The concentration of DNA was measured using a Quant-iT™ PicoGreen® dsDNA kit. A two-point standard curve was generated using a Lambda DNA standard. Genomic DNA was quantified by interpolation from the two point standard curve using the TBS-380 Mini-Fluorometer.

B.5. Nucleotide Sequence of the T-DNA Insert

(See Part V.A. of this Petition)

Two overlapping fragments that span the 5307 corn insert were amplified from genomic DNA using PCR analysis (Figure B-3). The 5307 plants used for this analysis were from the NP2171 × $BC5F_3$ generation, as identified in the pedigree chart of plant materials (Figure III-3). PCR amplification was carried out using the Expand™ Long Template PCR System. Table B-3 lists the primers used to amplify the insert fragments; Tables B-4 and B-5 contain the thermalcycling parameters.

Figure B-2. Map of the 5307 corn insert and location of PCR-amplified fragments from 5307 corn to determine insert sequence

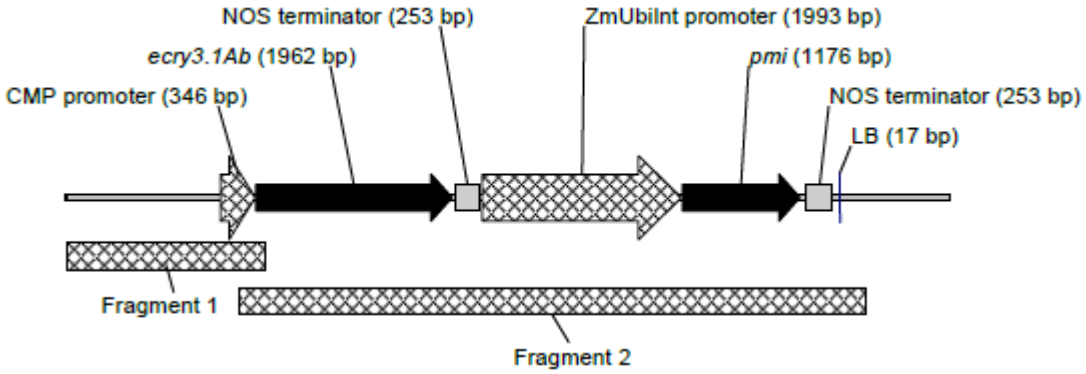


Table B-2. Primers used to amplify the insert of 5307 corn

Fragment	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')
1	GTGTAAGCCCCAAGCCATTTACTTCCTC	CGTCCTTGGTGGTGCTGCTGTCCAGGC
2	ATTCGTGGCCGACAGGTGG	AGCCGTACTATAAAGAGGGGTTGTCG

Table B-3. Cycling parameters for PCR amplification of insert Fragment 1

Cycle	Step	Temperature (°C)	Time	Number of cycles
A	1	94	2 min	1
B	1	94	15 sec	30
B	2	65	30 sec	30
B	3	72	90 sec	30
C	1	72	5 min	1
D	1	4	Hold	1

Table B-4. Cycling parameters for PCR amplification of insert Fragment 2

Cycle	Step	Temperature (°C)	Time	Number of cycles
A	1	94	2 min	1
B	1	94	10 sec	1
B	2	60	30 sec	1
B	3	68	5 min	1
C	1	94	15 sec	25
C	2	60	30 sec	25
C	3	68	5 min (+20 sec each cycle)	25
D	1	68	7 min	1
E	1	4	Hold	1

The PCR fragments were cloned into pCR®4-TOPO® vector, and three colonies for each PCR product were randomly selected and grown. The plasmid DNA was then independently extracted, and the resulting plasmid preparations, which contained the PCR amplification products, were subsequently sequenced.

Dye-terminator sequencing, a modification of the dideoxynucleotide chain-terminator sequencing method, was carried out using the ABI3730XL analyzer with ABI BigDye® 3.1 terminator chemistry. The sequence analysis was done using the Phred, Phrap, and Consed package (from the University of Washington), and was carried out to an error rate of less than 1 in 10,000 bases (Ewing and Green, 1998).

Three individual clones for each PCR product were sequenced individually, and a consensus sequence was generated for each clone. These sequences were aligned using AlignX™, a component of Vector NTI Advance™, version 10.3.0, to obtain the final consensus sequence for each segment of the insert sequence.

B.6. Southern Blot Analyses

(See Parts V.B., V.C. and V.D. of this Petition)

Southern blot analyses were performed using standard molecular biology techniques (Chomczynski, 1992). Each lane contained 7.5 µg of genomic DNA that was digested with the appropriate restriction enzyme(s) for 8 to 16 hours.

A positive control, representing one copy of a fragment of known size in the corn genome, was included on each Southern blot. The positive control for these Southern blot analyses was digested DNA from plasmid pSYN12274. This positive control was loaded in a well

together with 7.5 µg of digested DNA from NP2171 × NP2460 plants, so that the migration of this positive control DNA reflected, more accurately, the migration of the restriction fragment in the corn genome. The amount of positive control (picograms for one copy) was calculated by the following formula with a corn genome size of 2.67×10^9 bp (Arumuganathan and Earle, 1991).

$$\left\{\left(\frac{Positive\,control\,size(bp)}{Genome\,size(bp) * Ploidy}\right) * \mu g\,loaded\right\} * 1 \times 10^6 = pg\ for\ 1\ copy$$

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

maize genome size (bp)	2.67×10^9
maize ploidy	2
DNA loaded in each lane (µg)	7.5
Positive control size (bp)	11,769

The following amount of positive control was calculated:

Plasmid pSYN12274 (pg)	16.53

The molecular weight marker (serving as the reference substance), the digested genomic DNA, and the positive control were loaded onto 1% SeaKem® Gold agarose gels, and the DNA fragments were then separated by electrophoresis in 1X Tris-acetate-EDTA buffer.

Following a 10 minute depurination in 0.25 N HCl, the DNA in the gel was denatured in 0.5 M NaOH and 1.5 M NaCl for 30 minutes. The DNA was then transferred to a Zeta-Probe GT membrane, by downward alkaline transfer, for 90 minutes using a Bio-Rad Appligene Vacuum Blotter. After rinsing the membrane briefly in 2X SSC, the DNA was cross-linked to the membrane using ultraviolet light.

All PCR-generated probes and the molecular weight marker-specific probe were labeled with phosphorus-32-deoxycytidine triphosphate ([α-^{32}P]-dCTP) by random priming using the Megaprime™ DNA labeling system. Unincorporated label ([α-^{32}P]-dCTP) was removed using the Micro Bio-Spin® Chromatography Columns.

Membranes were incubated in 30 ml of PerfectHyb™ Plus Hybridization Buffer (which contained 100 µg/ml denatured Calf Thymus DNA) for at least 30 minutes at 65°C ± 5°C. Both the molecular weight marker-specific probe and either the full length T-DNAspecific probe or backbone-specific probe were added to the hybridization solution, and the membranes were incubated for 16 hours at 65°C ± 5°C. Incubation was followed by a combination of washes at 65°C ± 5°C in 2X SSC with 0.1% SDS and washes at 65°C ± 5°C in 0.1X SSC with 0.1% SDS. Finally, the membranes were subjected to imaging using a Molecular Dynamics Storm 860® phosphorimager.

B.7. Nucleotide Sequencing of T-DNA Flanking Regions in Corn Genome

(See Part V.F. of this Petition)

The 5′ and 3′ corn genomic sequences flanking the 5307 corn insert were previously recovered. This preliminary sequence was used to design primers for amplification of the flanking regions from 5307 corn. The flanking regions were amplified from genomic DNA extracted from 5307 corn using the Expand™ High-Fidelity PCR System. Table B-5 lists the primers used to amplify the flanking regions; Table B-6 contains the thermal cycling parameters.

Table B-5. Primers used to amplify the flanking regions of 5307 DNA insert

Region	Forward primer name	Forward primer sequence (5' to 3')	Reverse primer name	Reverse primer sequence (5' to 3')
5' flanking region	5307_F1	GCATTGGCATTTTCAT TAGCAAGCA	5307_R1	TGATTAAAGGCAGCCG ACCTAACCT
3' flanking region	5307_F2	CATCTCTTGCTAAGCT GGGAGCTCG	5307_R2	GACTTGTGTGGTTTCTC ACGGTCCA

Table B-6. PCR cycling parameters for the flanking regions of 5307 DNA insert

Cycle	Step	Temperature (°C)	Time	Number of cycles
A	1	95	5 min	1
B	1	95	15 sec	35
B	2	60	15 sec	35
B	3	72	2 min	35
C	1	72	10 min	1
D	1	4	Hold	1

The PCR fragments were cloned into pCR®4-TOPO® vector, and three colonies for each PCR product were randomly selected and grown. The plasmid DNA was then independently extracted, and the resulting plasmid preparations, which contained the PCR amplification products, were subsequently sequenced as described above (see Nucleotide Sequence of the T-DNA Insert).

B.8. Flanking Sequence Analysis to Determine if T-DNA Inserted into a Known Corn Gene

(See Part V.F. of this Petition)

The following parameters were used for the BLASTN analysis against the National Center for Biotechnology Information (NCBI) Nucleotide Database (NCBI, 2010a) to identify corn genomic sequences having identity or high similarity to sequences flanking the 5′ and 3′ ends of the 5307 T-DNA insert:

- Expect = 10. The expectation value (*E*-value) is a measure of the probability that matches between sequences occurred by chance. Search results involving comparisons between nucleotides with highly similar sequences yield *E*-values approaching zero; the probability that sequence similarities occurred by chance increases with higher *E*-values (Ponting, 2001). The search identified all sequences in the database with search results yielding an *E*-value of 10 or lower; this is the conservative default search setting for this parameter.
- The scoring scheme used was the default for nucleotides: +1 for a match and -3 for a mismatch.
- The following gap penalties were used for this scoring matrix: Existence = 5 and Extension = 2. A gap is a space introduced into an alignment to compensate for insertions and/or deletions in one sequence relative to another. The introduction of a gap causes the deduction of a fixed value from the alignment score to prevent the accumulation of excessive gaps in an alignment. Extension of the gap to encompass additional nucleotides is also penalized in determining the score of an alignment. The resultant score is derived from the number of identical matches between the query sequence and the database entry, with higher scores indicating more identity between the two sequences.
- A low complexity filter was used for this search.

B.9. Amino Acid Sequence Comparison of Query Peptide to Known or Putative Toxins

(See Part V.G. of this Petition)

The Basic Local Alignment Search Tool for Proteins (BLASTP) program, version 2.2.19, (Altschul et al., 1997) was used to compare the query peptide representing the translated sequence of the putative 243-bp ORF to all entries in the NCBI Entrez® Protein Database (containing over 10 million amino acid sequences) (NCBI, 2010b). Information associated with the sequences having the highest similarity to the query sequence was examined to determine if any of the sequences was a toxin or putative toxin.

The BLASTP algorithm is optimized to identify regions of local similarity between protein sequences. This approach detects more similarities than would a search that aligns two sequences over their entire length. The following default parameters were used in the BLASTP comparisons:

- Expectation value (*E*-value) = 10.
- Word size = 3
- Gap costs: existence = 11 and extension = 1
- Similarity matrix: Blocks Substitution Matrix62 (BLOSUM62)
- No complexity filter

The *E*-value is a measure of the probability that matches between sequences occurred by chance. Search results involving comparisons between proteins with highly similar sequences yield *E*-values approaching zero; the probability that sequence similarities occurred by chance increases with higher *E*-values (Ponting, 2001). The search identified all sequences in the database with search results yielding an *E*-value of 10 or lower. These sequences were evaluated for source and biological function. Any sequences described as toxins or putative toxins were identified.

B.10. Amino Acid Sequence Comparison of Query Peptide to Known or Putative Allergens

(See Part V.G. of this Petition)

The 81-amino-acid query peptide sequence representing the translated sequence of the putative 243-bp ORF was screened for biologically relevant amino acid sequence similarity to any of the known or putative protein allergens within the FARRP AllergenOnline database (FARRP, 2010). The FARRP AllergenOnline database is a curated, peer-reviewed database containing proteins identified as food allergens, respiratory allergens, allergenic venom proteins, contact allergens, gliadins, and glutenins. Entries were compiled primarily from searches of publicly available protein databases using the NCBI Entrez® search and retrieval system, most recently searched in 2009 (NCBI, 2009). The NCBI dataset was screened by searches for entries associated with allergy or celiac disease; duplicate entries were removed, and additional entries were identified from publications. The list of candidate entries was then reviewed by an international panel of allergy experts who reviewed published clinical and laboratory evidence to support the candidate sequences as allergens. Proteins are classified as known or putative allergens according to predetermined criteria set by the FARRP expert review panel. The latest version of the FARRP AllergenOnline database (2010) contains 1,471 nonredundant entries. Similarity searches were performed using an exact copy of the entire list of sequences in the current version of the FARRP AllergenOnline database (2010) (maintained at Syngenta Biotechnology, Inc., Research Triangle Park, NC, USA). Two different sequence searches were performed to compare the translated junction sequence with sequences in the FARRP AllergenOnline database. In the first search, the FASTA search algorithm, version 3.45 (Pearson and Lipman, 1988), was used to assess overall sequence similarity by comparing sequential 80-amino-acid peptides of the query sequence with the sequences in the FARRP AllergenOnline database. Each successive "window" of 80 amino acids was offset from the previous window by one residue, such that each peptide overlapped the previous peptide by 79 amino acids. The default FASTA settings used include an extension penalty of two and

gap creation penalty of 12. The scoring matrix for FASTA was the Blocks Substitution Matrix50 (BLOSUM50), the same scoring matrix used by the authors of the FARRP AllergenOnline database. The BLOSUM50 matrix is weighted to favor identical amino acids likely to impact protein structure. In the second search, the query sequence was screened for matches of eight or more contiguous amino acids (Hileman et al., 2002) using a program developed by Syngenta; this program compared every possible peptide of eight contiguous amino acids of the translated putative ORF with the sequences in the FARRP AllergenOnline database.

The FASTA search produces alignments between the 80-amino-acid peptides of the query sequence and the sequences in the allergen database. The evaluation of each query peptide sequence alignment utilizes the minimum criterion of 80 amino acids of alignment length with greater than 35% shared amino acid identity. Any alignments exceeding this criterion for shared sequence similarity indicate the potential for immunologically relevant sequence similarity (Codex, 2009). Additionally, any match of eight (or more) identical contiguous amino acids between any query sequence and any sequence in the allergen database indicates the potential for immunologically relevant sequence similarity (Codex, 2009).

Appendix C. Materials, Methods and Results of Characterization Studies on the eCry3.1Ab and PMI Proteins

This appendix presents materials, methods and detailed results of analyses of the biochemical properties and biological activity of the eCry3.1Ab and PMI proteins in 5307 corn and in the corresponding eCry3.1Ab and PMI test substances used in safety studies. A summary of the results and conclusions of these analyses is provided in **Part VI** of this Petition, titled **Characterization of the eCry3.1Ab and PMI Proteins**. All literature references cited in this appendix are listed in **Part IX** of this Petition, titled **References**.

C.1. Materials and Methods for Comparison of eCry3.1Ab Produced in 5307 Corn Plants and Recombinant *E. coli*

Three preparations containing eCry3.1Ab were evaluated in this study: (1) LP5307, extracted from leaf material of 5307 corn plants; (2) IAP5307, immunopurified eCry3.1Ab derived from leaf material of 5307 corn plants; and (3) microbial test substance ECRY3.1AB-0208, prepared from a recombinant *E. coli* overexpression system. The sample designated LP5307 was used for Western blot analysis and insecticidal activity assays. The sample designated IAP5307 was used as the source of purified plant-produced eCry3.1Ab for Western blot, glycosylation, N-terminal sequencing and peptide mass mapping analysis. Nontransgenic, near-isogenic plants were used as negative control plant material, the source of the LP-NEG material.

Event 5307 Corn Leaf Tissue and Negative Control Corn Leaf Tissue

Young leaves from greenhouse-grown 5307 plants and nontransgenic, near-isogenic plants were collected 4-6 weeks after emergence, frozen at –80°C ± 10°C, and ground into a fine powder using a Grindomix Knife Mill (Retsch).

Extracts of 5307 Corn and Control Corn Leaf Tissue for Western Blot Analysis

Leaf powder was resuspended in extraction buffer containing 100 mM sodium borate (pH 10.0), 0.2% polyvinylpyrrolidone (PVP), 7.69 mM sodium azide, 0.5% Tween 20, and supplemented with one Complete Protease Inhibitor Cocktail tablet/50 ml of buffer (Roche). The mixture was homogenized with an Omni-Prep Homogenizer (Omni International), incubated for 30 minutes on ice and centrifuged at 3,000 rpm for 15 minutes at 4°C (Sorvall Legend RT). The resulting supernatants were stored overnight at 2°C to 8°C and then stored at -20°C ± 8°C in 4X NuPage LDS Sample Buffer (Invitrogen) containing NuPage Sample Reducing Agent (Invitrogen) for subsequent Western blot analysis. The sample extracts from the 5307 and control corn leaves were designated LP5307 and LP-NEG, respectively.

Extracts of 5307 Corn and Control Corn Leaf Tissue for Insect Bioassays

Extracts for bioassays were prepared by resuspending leaf powder from 5307 and control plants as described above, in a 10 mM ammonium bicarbonate, pH 10.0 buffer. The extraction mixtures were homogenized in a Waring blender for 45 seconds, incubated on ice for 1.5 hours and centrifuged (Sorvall RC5B) at 8,000 rpm for 30 minutes at 4°C. The resulting supernatant was filtered through cheesecloth, centrifuged for an additional 30 minutes at 8,000 rpm at 4°C, and filtered through cheesecloth again. The resulting clear

supernatant was concentrated using centrifugal filter devices (Millipore). The sample was then stored overnight at 2-8°C for subsequent insect diet incorporation and ELISA analysis. The sample extracts from the 5307 and control corn leaves were designated LP5307 and LP-NEG, respectively.

Extracts of Control Leaf Tissue Fortified with Test Substance ECRY3.1AB-0208 for Western Blot Analysis

To determine whether the plant matrix affects eCry3.1Ab mobility or immunoreactivity, ECRY3.1AB-0208 was added to control leaf extract. This sample allowed for comparison of the microbially produced eCry3.1Ab and plant-produced eCry3.1Ab in the same matrix. For Western blot analysis, ECRY3.1AB-0208 was added to LP-NEG, as prepared for above Western blot analysis, such that the total protein and amount of eCry3.1Ab loaded on the gel was equivalent to that estimated for sample LP5307, as prepared for Western blot analysis. This sample was designated LP-NEG + ECRY3.1AB-0208.

Extracts of Control Leaf Tissue Fortified with Microbially Produced Test Substance ECRY3.1AB-0208 for Insect Bioassays

To determine if the plant matrix affects bioactivity, ECRY3.1AB-0208 was added to control leaf extract. This sample allowed for comparison of the microbially produced eCry3.1Ab and plant-produced eCry3.1Ab in the same matrix. For the bioassays, ECRY3.1AB-0208 was added to LP-NEG, as prepared for bioassays, such that when incorporated into the diet the concentration of eCry3.1Ab in the diet was equivalent to the eCry3.1Ab concentration in the diet containing only the ECRY3.1AB-0208 test substance. This sample was designated LP-NEG + ECRY3.1AB-0208.

Immunoaffinity-Purified Plant-Produced Protein

Leaf powder from 5307 corn, prepared as described above, was resuspended in extraction buffer (pH 7.5) containing 100 mM sodium borate, 0.2% PVP, 7.69 mM sodium azide, 1.2% concentrated hydrochloric acid, 0.5% Tween 20, supplemented with one Complete Protease Inhibitor Cocktail tablet/50 ml of buffer (Roche). The mixture was homogenized with an Omni-Prep Homogenizer and incubated for up to 2 hours on ice. The mixture was then centrifuged at approximately 2700 rpm for 10 minutes at 4°C (Sorvall Legend RT). The supernatant was filtered through cheesecloth and centrifuged at approximately 3100 rpm for 15 minutes at 4°C (Sorvall Legend RT). After a second centrifugation step (10,000 rpm for 12 minutes (Sorvall RC5B) the clarified supernatant was then loaded onto an equilibrated immunoaffinity column with mouse anti-mCry3A antibodies bound to the matrix. To remove any proteins not bound to the antibodies, the column was washed with a 50 mM sodium bicarbonate buffer pH 8.0 containing 150 mM sodium chloride. After an additional wash step with a 10 mM sodium phosphate buffer pH 6.8, eCry3.1Ab was eluted in 100 mM glycine buffer (pH 2.5), neutralized, and fractions were analyzed for eCry3.1Ab protein by ELISA. Fractions containing eCry3.1Ab protein were pooled, concentrated by ultrafiltration, and stored at 2°C to 8°C until further use. The resulting sample, designated IAP5307, was used as the source of purified plant-produced eCry3.1Ab for Western blot, glycosylation, N-terminal sequencing and peptide mass mapping analysis.

Microbially Produced Test Substance ECRY3.1AB-0208

Test substance ECRY3.1AB-0208 was prepared from an *E. coli* overexpression system. The eCry3.1Ab protein in test substance ECRY3.1AB-0208 is identical to that expressed in 5307 corn except that it contains one additional methionine and six histidine residues at the N-terminus. The intended additional seven amino acids aid in purification from the *E. coli* overexpression system. The *ecry3.1Ab* gene used for microbial expression was linked to the bacterial *tac* promoter in a vector derived from pET24a (Novagen) and transformed into *E. coli* strain DH5α (New England Biolabs). ECRY3.1AB-0208 was prepared from pooled batches of *E. coli* cell paste. *E. coli* cells were ruptured and the cell debris removed by centrifugation. The soluble material was filtered, applied to an immobilized metal affinity column (GE Healthcare Nickel Sepharose Fast Flow column), and eluted using an imidazole step gradient. Fractions containing the eCry3.1Ab protein were then further purified via anion exchange chromatography and eCry3.1Ab was eluted with a sodium chloride gradient. The eluted eCry3.1Ab-containing fractions were pooled, concentrated and the buffer was exchanged. The solution was lyophilized and designated ECRY3.1AB-0208. The test substance was stored at -20°C ± 8°C until further use. ECRY3.1AB-0208 was determined to contain 89.6% eCry3.1Ab by weight and the intact mass of the eCry3.1Ab protein, as measured by mass spectrometry, was 74.8 kDa.

eCry3.1Ab Quantification

The Beacon Analytical Systems (BAS) eCry3.1Ab ELISA kit was used as described in Appendix D., section D.4., to quantify eCry3.1Ab.

Total Protein Determination

Total protein in samples, LP5307, LP-NEG + ECRY3.1AB-0208, and LP-NEG as prepared for Western blot analysis, was quantified via the bicinchoninic acid method (Hill and Straka, 1988), using bovine serum albumin as the reference protein standard. The results were analyzed with BioMetallics DeltaSoft PC Microplate Analysis Software, v. 1.71.2 using a four-parameter fit of the standard curve.

Immunoreactivity and Molecular Weight Determination

Western blot analysis was used to investigate the integrity of eCry3.1Ab in ECRY3.1AB-0208, LP5307, LP-NEG + ECRY3.1AB-0208 and IAP5307. Aliquots containing 10 ng of eCry3.1Ab prepared in NuPage LDS Sample Buffer (Invitrogen) were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) with a NuPAGE 4-12% Bis-Tris polyacrylamide gradient gel (Invitrogen) using 3-(N-morpholino)propane-sulfonic acid (MOPS) running buffer (Bio-Rad). An aliquot of the control plant sample LP-NEG, equivalent in total protein to the amount loaded on the gel for LP5307 (29.6 µg total protein), was included in the analysis as a negative control. The molecular-weight standard was SeeBlue Plus2 pre-stained standard (Invitrogen). After electroblotting, the membrane was probed with polyclonal goat antibodies capable of detecting eCry3.1Ab protein. Alkaline phosphatase-conjugated donkey anti-goat IgG (Jackson ImmunoResearch) diluted to 1:3,000 in Tris-buffered saline with Tween 20 (Sigma-Aldrich) and 5% normal donkey serum was used to bind to the primary antibody and was visualized by development with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) alkaline

phosphatase substrate solution (Sigma-Aldrich). The Western blot was examined for the presence of intact immunoreactive eCry3.1Ab or immunoreactive eCry3.1Ab fragments.

Insecticidal Activity

The insecticidal activity of eCry3.1Ab was assessed in feeding assays with freshly hatched first-instar Colorado potato beetles *(L. decemlineata)* in three independent bioassays. The insect diet was prepared by blending a boiling mixture of 2.6 grams of agar and 169 ml of Milli-Q water with 28.1 grams of Colorado potato beetle diet powder mix and 1 gram of potassium hydroxide as per the manufacturer's instructions (Bio-Serv). The diet mixture was cooled to approximately 55°C in a water bath. Antibacterial and antifungal agents were each added to the cooled diet.

Bioassay treatments consisted of (1) test substance ECRY3.1AB-0208, (2) eCry3.1Ab extracted from 5307 corn leaves; LP5307, (3) control leaf extract fortified with test substance ECRY3.1AB-0208; LP-NEG + ECRY3.1AB-0208, and (4) control corn leaf tissue extract; LP-NEG. A a stock solution of ECRY3.1AB-0208 was prepared in 10 mM ammonium bicarbonate buffer (pH 10.0) to a concentration of 5 mg eCry3.1Ab/ml. From this, a 50 µg eCry3.1Ab/ml solution was prepared and serially diluted 1:1 (v/v) in 10 mM ammonium bicarbonate buffer (pH 10.0) to produce eight solutions with concentrations ranging from 50 to 0.390 µg eCry3.1Ab/ml. The dilution series was then mixed 1:1 (v/v) with the freshly prepared Colorado potato beetle diet to produce eight diets with eCry3.1Ab concentrations ranging from 25 to 0.195 µg/ml diet. Additional treatments containing eCry3.1Ab extracted from 5307 corn leaves, LP5307 (treatment 2), control leaf extract fortified with the microbially-produced test substance, LP-NEG + ECRY3.1AB-0208 (treatment 3) and control leaf tissue extract LP-NEG (treatment 4) as prepared for bioassays were also serially diluted 1:1 (v/v) in 10 mM ammonium bicarbonate buffer (pH 10.0) and subsequently mixed 1:1 (v/v) with freshly prepared Colorado potato beetle diet. Each of these treatments was analyzed as a series of eight dilutions. Water and buffer controls were prepared in the same manner for each bioassay. The water control was prepared by mixing 1:1 (v/v) of purified water with freshly prepared Colorado potato beetle diet. The buffer control was prepared by mixing 1:1 (v/v) of 10 mM ammonium bicarbonate buffer (pH 10.0) with freshly prepared Colorado potato beetle diet.

The bioassays were conducted in 24-well culture plates. Each well contained one freshly hatched *L. decemlineata* insect larva and 100 µl of insect diet. Larvae were transferred to each well manually using a small paint brush. The wells were covered with silicone stoppers and stored at ambient laboratory conditions. Mortality readings were taken periodically starting at 72 hours and continued until at least 144 hours.

Glycosylation Analysis

To determine whether eCry3.1Ab in ECRY3.1AB-0208 and eCry3.1Ab immuno-affinity purified from 5307 corn leaf extract (IAP5307) were glycosylated, aliquots equivalent to 1 and 2 µg of eCry3.1Ab were analyzed with the DIG Glycan Detection Kit (Roche), in accordance with the manufacturer's instructions. The positive control was transferrin (a glycosylated protein) at 100, 50, 25 and 10 ng, and the negative control was creatinase (a nonglycosylated protein) at 2 µg. Samples were separated by SDS-PAGE with a

NuPAGE4-12% Bis-Tris polyacrylamide gradient gel and NuPAGE MES SDS running buffer and electroblotted to nitrocellulose membrane (Invitrogen). While on the membrane, glycan moieties were oxidized with periodate, labeled with digoxigenin (DIG), and detected with an alkaline-phosphatase-linked anti-DIG antibody.

Peptide Mass Mapping Analysis

eCry3.1Ab purified from an extract of 5307 corn leaves (IAP5307) and from ECRY3.1AB-0208 were analyzed by peptide mass mapping. Aliquots containing 2.5 to 5 µg of eCry3.1Ab purified from 5307 corn leaf extract (IAP5307) and from test substance ECRY3.1AB-0208 were subjected to SDS-PAGE using a NuPAGE 4-12% Bis-Tris polyacrylamide gradient gel and NuPAGE MES SDS running buffer. The gel was stained with Coomassie G250 (Invitrogen), the protein band corresponding to the molecular weight of eCry3.1Ab was excised from the gel, and the protein was reduced, alkylated with iodoacetamide, and independently digested with trypsin and chymotrypsin. The mass analysis of the eCry3.1Ab-produced peptides was performed using a quadrupole time-of-flight mass spectrometer (Waters/Micromass Q-TOF Premier) connected to a Waters CapLC capillary liquid chromatography instrument. The detected peptide masses were searched using Mascot Software (Matrix Science) against a protein database containing the eCry3.1Ab protein sequence. The Mascot search parameters included likely N-terminal modifications, which have previously been reported to occur in plants. Specifically, the modifications investigated included α-N-acetylation, protein N-formylation and protein N-methylation.

N-Terminal Amino Acid Sequence Analysis

To determine the N-terminal amino acid sequence of eCry3.1Ab from test substance ECRY3.1AB-0208 and eCry3.1Ab purified from 5307 corn leaf extract (IAP5307) were both subjected to SDS-PAGE followed by electroblotting to a PVDF membrane. The blot was stained with amido black, and the band corresponding to eCry3.1Ab was excised and subjected to N-terminal amino acid sequence analysis using automated Edman-based chemistry (Brauer et al., 1984).

Statistical Methods

The LC_{50} values determined in the insecticidal activity assay were calculated using the U.S. EPA Probit Analysis Program, version 1.5.

C.2. Results of Comparison of eCry3.1Ab Produced in 5307 Corn Plants and Recombinant *E. coli*

Immunoreactivity and Molecular Weight

Western blot analysis of eCry3.1Ab in test substance ECRY3.1AB-0208, LP-NEG + ECRY3.1AB-0208, LP5307, and IAP5307 revealed immunoreactive bands consistent with the predicted molecular weight[9] of 74.8 kDa for samples containing eCry3.1Ab from ECRY3.1AB-0208 (Figure C-1, Lanes 2, 3 and 6) and 73.7 kDa for samples containing plant-produced eCry3.1Ab (Lanes 4 and 5).

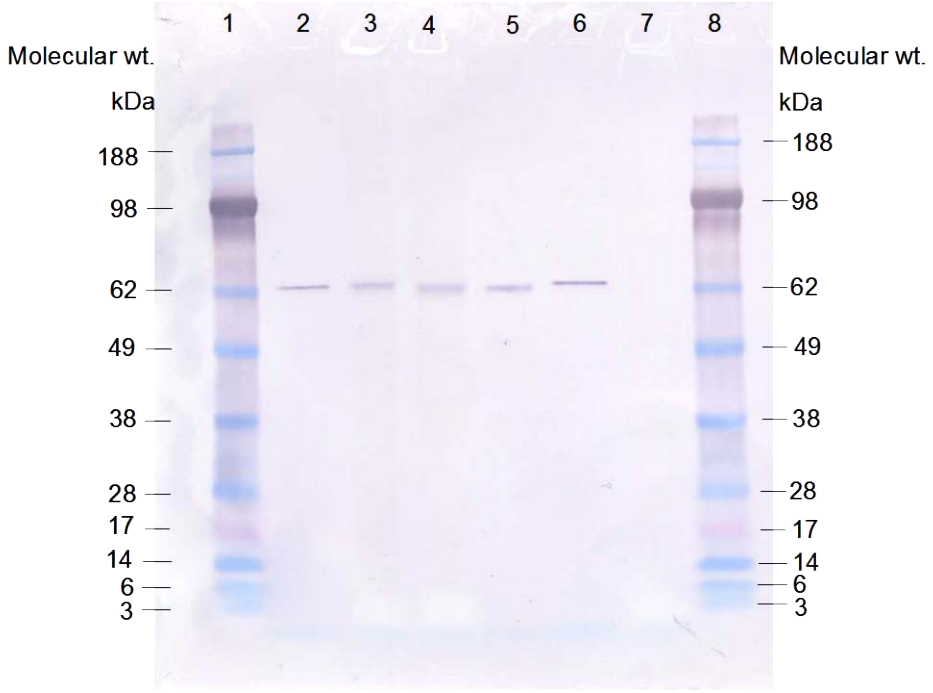


Figure C-1. Western blot analysis of eCry3.1Ab in test substance ECRY3.1AB-0208, LP-NEG + ECRY3.1AB-0208, LP5307, IAP5307 and the negative control LP-NEG.

Lane 1: Molecular-weight markers
Lane 2: Microbially produced test substance ECRY3.1AB-0208 (10 ng eCry3.1Ab)
Lane 3: Control corn leaf extract, LP-NEG (29.6 µg total protein) with the addition of test substance ECRY3.1AB-0208 (10 ng eCry3.1Ab)
Lane 4: 5307 corn leaf extract, LP5307 (10 ng eCry3.1Ab/29.6 µg total protein)
Lane 5: Immunopurified eCry3.1Ab protein from 5307 corn leaf extract, IAP5307 (10 ng eCry3.1Ab)
Lane 6: Test substance ECRY3.1AB-0208 (10 ng eCry3.1Ab)
Lane 7: Control corn leaf extract, LP-NEG (29.6 µg total protein)
Lane 8: Molecular-weight markers

[9] Although the eCry3.1Ab protein band showed slightly higher mobility (and therefore an apparent lower molecular weight) in comparison to the molecular weight standards on the Western blot (Figure C-1), the difference between the expected and observed molecular weights on the gels can be explained by the limitations of SDS-PAGE for accurate determination of molecular weight. Dube and Flynn (1988) have reviewed the reliability of SDS-PAGE for molecular weight determinations and concluded that the apparent molecular weight of a protein by this method is typically within 10% of its true molecular weight. This depends greatly on the similarity between the properties of the protein of interest and the proteins in the standard set (Sadeghi et al., 2003). Additionally, the intact mass of eCry3.1Ab in ECRY3.1AB-0208 waspreviously measured as 74.8 kDa.

The slight difference in molecular weight between the microbial and plant sources of eCry3.1Ab was consistent with the presence of the seven additional N-terminal amino acids in the microbially produced protein. As expected, no immunoreactive bands were observed in the plant-produced control substance, LP-NEG (Figure C-1, Lane 7).

Insecticidal Activity

The results of the insect bioassays were presented in Table VI-1 and discussed in Part VI.A.1.b. of this Petition: **Equivalence of eCry3.1Ab Test Substance and eCry3.1Ab in 5307 Plants**.

Glycosylation Analysis

The positive control protein, transferrin, at 10 ng generated a clearly visible band (Figure C-2, Lane 4). Transferrin has a molecular weight of approximately 80,000 Da and contains approximately 5% glycan moieties by weight. This corresponds to approximately 25 glucose equivalents per molecule (based on a calculated molecular weight of 162 Da for the glycan moiety). Of the 10 ng of transferrin loaded on the gel, 0.5 ng could be attributed to glycan moieties and was clearly detectable. The highest concentration of eCry3.1Ab from both plant and microbial sources (IAP5307 and ECRY3.1AB-0208) loaded on the blot was 2 µg (2,000 ng). If 0.5 ng of glycan were detected in eCry3.1Ab, this would correspond to 0.025% by weight (0.5/2,000 ng), or 0.115 glucose equivalents per molecule. In other words, if eCry3.1Ab bands were stained as strongly as 10 ng of transferrin in Lane 4, this would indicate glycosylation of about 1 in 8.7 of the eCry3.1Ab molecules. No bands corresponding to glycosylated eCry3.1Ab were visible for the sample prepared from the microbially-produced ECRY3.1AB-0208 test substance (Figure C-2, Lanes 9 and 10) or immunopurified plant-produced eCry3.1Ab, IAP5307 (Lanes 7 and 8). Therefore, the results indicate that neither the microbially-produced nor the plant produced eCry3.1Ab protein was glycosylated.

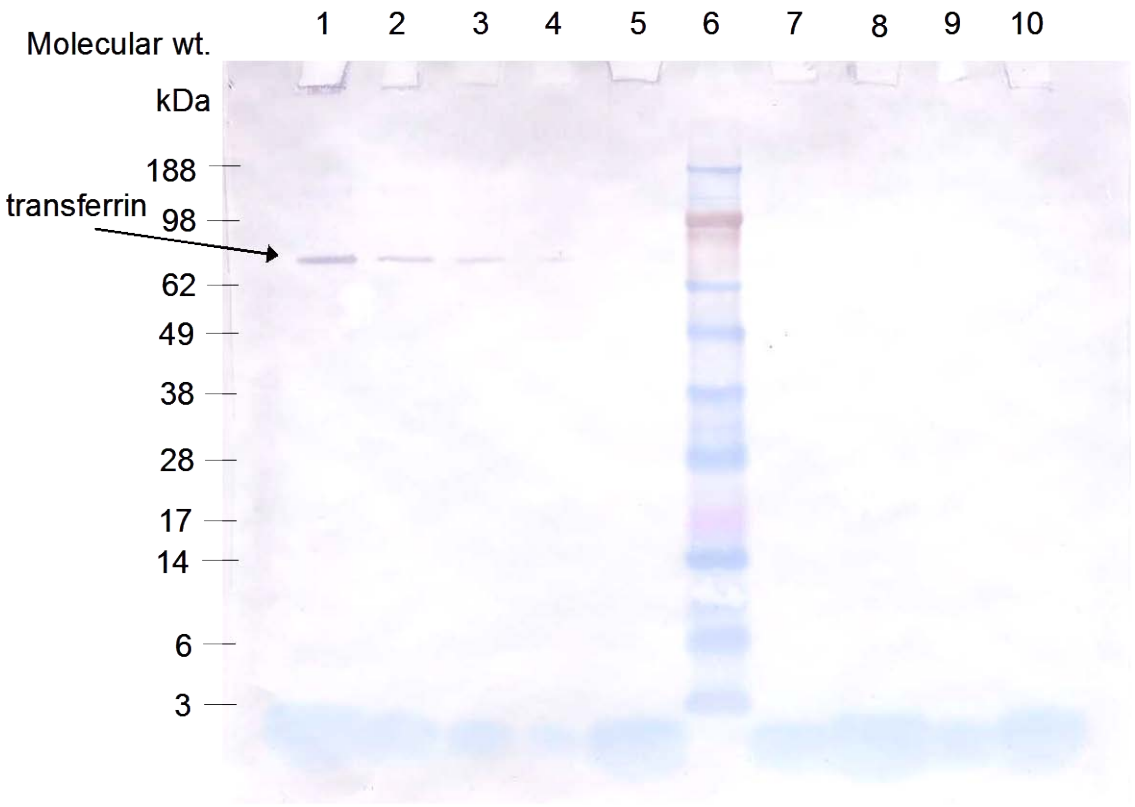


Figure C-2. Glycosylation analysis of eCry3.1Ab in IAP5307 (immunopurified plant-produced protein) and ECRY3.1AB-0208 (microbially produced test substance).

Lane 1: Transferrin (positive control), 100 ng
Lane 2: Transferrin (positive control), 50 ng
Lane 3: Transferrin (positive control), 25 ng
Lane 4: Transferrin (positive control), 10 ng
Lane 5: Creatinase (negative control), 2 µg.
Lane 6: Molecular-weight markers.
Lane 7: Immunopurified eCry3.1Ab protein from Event 5307 corn leaf extract, IAP5307; 1 µg
Lane 8: Immunopurified eCry3.1Ab protein from Event 5307 corn leaf extract, IAP5307; 2 µg
Lane 9: eCry3.1Ab from microbially produced test substance ECRY3.1AB-0208; 1 µg
Lane 10: eCry3.1Ab from microbially produced test substance ECRY3.1AB-0208; 2 µg.

Peptide Mass Mapping

Analysis of the plant-produced eCry3.1Ab yielded coverage equivalent to 76% of the total predicted eCry3.1Ab amino acid sequence, as shown in Figure C-3. Analysis of the microbially produced eCry3.1Ab yielded coverage equivalent to 87% of the total predicted eCry3.1Ab amino acid sequence, as shown in Figure C-4. The identified peptides corresponded to regions throughout the sequence of eCry3.1Ab including the N-termini of both proteins. The results of the peptide mass mapping analysis confirmed the identity of the purified proteins from both sources as eCry3.1Ab.

M**TSNGRQCAGIRPY**DGRQQHR**GLDSSTTKDVIQK**GISVVGDLLGVVGFPFGGALVSFYTNF
LNTIWPSEDPW**KAFMEQVEALMDQK**IADYAKNKALAELQGLQNNVEDYVSALSSWQK**NPAA**
PFRNPHSQGRIRELFSQAESHFRNSMPSFAISGYEVLFLTTYAQAANTHLFLLKDAQIYGE
EWGYEKEDIAEFYKRQLK**LTQEYTDHCVKWYNVGLDKLRGSSYESWVNFNR**YRR**EMTLTVL**
DLIALFPLYDVRLYPKEVKTELTRDVLTDPIVGVNNLRGYGTTFSNIENYIRKPHLFDYLH
RIQF**HTRFQPGYY**GNDSFNYWSGNY**VSTRPSIGSNDIITSPFYGNKSSEPVQNLEFNGEK**V
Y**RAVANTNLAVWPSAVYSGVTKVEFSQYNDQTDEASTQTYDSKR**NVGAVSWDSIDQLPPET
TDEPLEK**GYSHQLNYVMCFLMQGSRGTIPVLTWTHKSVDFFNMIDSKKITQLPLTKSTNLG**
SGTSVVKGPGFTGGDILRR**TSPGQISTLRVNITAPLSQR**YRVRIR**YASTTNLQFHTSIDGR**
PINQGNFSATMSSGSNLQSGSFRTVGFTTPFNFSNGSSVF**TLSAHVFNSGNEVYIDR**IEF**V**
PAEVTFEAEY**DLERAQKAVNELFTSSNQIGLKTDVTDYHID**QV

Figure C-3. Predicted amino acid sequence of eCry3.1Ab and sequence identified by peptide mass mapping analysis of eCry3.1Ab from immunopurified plant-produced sample IAP5307.

Identified eCry3.1Ab protein fragments are bold and underlined.

MHHHHHHMTSNGRQCAGIRPYDGRQQHR**GLDSSTTKDVIQK**GISVVGDLLGVVGFPFGGAL
VSFYTNFLNTIWPSEDPW**KAFMEQVEALMDQK**IADY**AKNKALAELQGLQNNVEDYVSALSS**
WQKNPAAPFRNPHSQGRIRELFSQAESHFRNSMPSFAISGYEVLFLTTYAQAANTHLFLLK
DAQIYGEEWGYEKEDIAEFYKRQLK**LTQEYTDHCVKWYNVGLDKLRGSSYESWVNFNR**YRR
EMTLTVLDLIALFPLYDVRLYPKEVKTELTRDVLTDPIVGVNNLRGYGTTFSNIENYIRKP
HLFDYLHRIQFHTRFQPGYYGNDSFNYWSGNY**VSTRPSIGSNDIITSPFYGNKSSEPVQNL**
EFNGEKVYR**AVANTNLAVWPSAVYSGVTKVEFSQYNDQTDEASTQTYDSKRNVGAVSWDSI**
DQLPPETTDEPLEKGYSHQLNYVMCFLMQGSRGTIPVLTWTHKSVDFFNMIDSKKITQLPL
TKSTNLGSGTSVVKGPGFTGGDILRRTSPGQISTLRVNITAPLSQRYRVRIR**YASTTNLQF**
HTSIDGRPINQGNFSATMSSGSNLQSGSFRTVGFTTPFNFSNGSSVFTLSAHVFNSGNEVY
IDRIEFVPAEVTFEAEYDLERAQKAVNELFTSSNQIGLKTDVTDYHIDQV

Figure C-4. Predicted amino acid sequence of eCry3.1Ab and sequence identified by peptide mass mapping analysis of eCry3.1Ab from microbially produced test substance ECRY3.1AB-0208.

Identified eCry3.1Ab protein fragments are bold and underlined.

The peptide mass analysis provided two additional results regarding the structure of the proteins. Firstly, the intact N-terminus of the plant-produced protein could be identified. The analysis showed that the N-terminal methionine was removed leaving the penultimate amino acid, threonine, at the N-terminus of the eCry3.1Ab protein. This is a common process for many proteins occurring during translation (Walling, 2006). Secondly, the nature of the N-terminal block found for the plant produced protein, as described under **N-Terminal Amino Acid Sequence Analysis**, below, was identified. The analysis of the N-terminal peptide of the plant-produced protein suggested the addition of an acetyl-residue at the primary amino group of the N-terminal threonine. This is a common modification known for plant-expressed proteins (Martinez et al., 2008).

N-Terminal Amino Acid Sequence Analysis

The N-terminal sequence results confirmed that eCry3.1Ab in the microbially produced test substance ECRY3.1AB-0208 had the predicted N-terminal amino acid sequence:

Predicted sequence:	MHHHHHHMTS
eCry3.1Ab in ECRY3.1AB-0208:	MHHHHHHMTS

N-terminal sequencing analysis of eCry3.1Ab immunoaffinity-purified from 5307 corn leaves (IAP5307) revealed that the majority of the protein was naturally blocked at the N-terminus. However, the N-terminal peptide was identified by peptide mass mapping (see **Peptide Mass Mapping**, above) and confirmed the expected sequence for the eCry3.1Ab protein, starting at threonine as described above. The analysis of the N-terminal peptide of the plant-produced protein suggested the addition of an acetyl-residue at the primary amino group of the N-terminal threonine.

C.3. Materials and Methods for Comparison of PMI Produced in 5307 Corn Plants and Recombinant *E. coli*

The purpose of these analyses was to compare PMI from 5307 corn plants with PMI from test substance PMI-0105, which was produced by overexpressing the gene *pmi* in recombinant *E. coli*. The PMI proteins from both sources were compared biochemically and functionally to justify use of PMI in PMI-0105 as a surrogate for PMI in 5307 corn plants, for safety testing purposes. Both sources of PMI were predicted to have the identical amino acid sequence, because the gene *pmi* in both the plant and *E. coli* transformation vectors encoded the same PMI enzyme.

The conclusions of these analyses are described in Part VI.B.1.b of this Petition, **Equivalence of PMI in Test Substance and in 5307 Plants.**

Event 5307 Corn Leaf Tissue and Negative Control Corn Leaf Tissue

Young leaves from greenhouse-grown 5307 plants and nontransgenic, near-isogenic plants were collected 4-6 weeks after emergence, frozen at –80°C ± 10°C, and ground into a fine powder using a Grindomix Knife Mill (Retsch). The leaf powder was then lyophilized and stored at -80 ± 10°C.

Extracts of 5307 and Control Corn Leaf Tissue

Approximately 100 mg of lyophilized leaf powder was suspended in 3 ml extraction buffer (pH 7.0) containing 50 mM Tris, 2 mM DTT), 1 mM AEBSF, and 1 µM leupeptin. The mixture was then homogenized using an Omni-Prep Homogenizer (Omni International) and centrifuged for 15 minutes at 10,000 rpm at approximately 4°C. The supernatant was desalted using PD-10 columns (GE Healthcare Life Sciences) and eluted in extraction buffer. The resulting supernatant was then concentrated using the Millipore Amicon Ultra centrifugal filter devices (Millipore) and Tween 20 was added to achieve a final concentration of 0.1%. The samples derived from the 5307 and control corn tissues were designated LP5307 and LP-NEG, respectively. They were stored at 2-8°C overnight prior to ELISA and enzymatic activity assays.

Extract of Control Leaf Tissue Fortified with Test Substance PMI-0105

PMI from test substance PMI-0105 was added to the extraction buffer with an aliquot of control leaf tissue to determine if the plant matrix or extraction procedure has an effect on PMI. For this sample preparation, 600 ng PMI from test substance PMI-0105 was added to 3 ml of extraction buffer containing 100 mg of control leaf tissue prior to homogenization. The sample was then homogenized, centrifuged, desalted and concentrated as described above (see **Extracts of 5307 and Control Corn Leaf Tissue)**. This sample was designated LP-NEG + PMI-0105.

PMI Protein Quantification

The concentration of PMI in the plant extract samples (LP5307), negative control samples (LP-NEG), and the negative control sample fortified with test substance PMI-0105 (LP-NEG + PMI-0105) was determined by ELISA (Tijssen 1985). Polyclonal rabbit antibody generated against PMI protein was diluted to 2 µg/ml in a buffer containing 35 mM sodium bicarbonate and 15 mM sodium carbonate (pH 9.5) and used to coat a Nunc MaxiSorp 96-

well plate (ThermoFisher Scientific) at a volume of 100 µl/well. The plates were incubated overnight at 5°C ± 3°C. The plate contents were then emptied and tapped on paper towels to remove residual solution. The plates were blocked with phosphate buffered saline (PBS) pH 7.4 containing 1% nonfat milk for at least 30 minutes at room temperature. The plates were washed five times with PBS containing 0.05% Tween 20 and incubated for 2 hours at 20°C ± 2°C with diluted plant extract samples and PMI-0105 standards at a volume of 100 µl/well. Dilutions were made in ELISA dilution buffer (PBS plus 1% nonfat milk and 0.05% Tween 20). The plant extract samples and standards were assayed in triplicate. After washing, the plates were incubated with 100 µl/well monoclonal mouse antibody (1 µg/ml) generated against PMI protein diluted in ELISA dilution buffer for 1 hour at 20°C ± 2°C. The plates were washed and subsequently incubated with 100 µl/well rabbit anti-mouse IgG conjugated with horseradish peroxidase (Sigma-Aldrich) diluted 1:20,000 in ELISA dilution buffer for 1 hour at 20°C ±2°C. The plates were washed again and incubated in 0.1 mg/ml TMB substrate solution (Sigma-Aldrich) in citrate buffer (24 mM citric acid monohydrate, 60 mM dibasic sodium phosphate, pH 5.0 containing 0.006% hydrogen peroxide) at a volume of 100 µl/well. Color was allowed to develop for 30 minutes in the dark at room temperature. The reaction was stopped by the addition of 50 µl 3 M sulfuric acid per well, and absorbance at 450 nm was measured with a Tecan Sunrise microplate reader (Tecan US). The results were analyzed with BioMetallics DeltaSoft PC Microplate Analysis Software, v. 1.71.2 using a four-parameter algorithm.

Total Protein Determination

Total protein of the plant extract samples (LP5307, LP-NEG + PMI-0105 and LP-NEG) were quantified via the bicinchoninic acid (BCA) method (Hill and Straka, 1988), using bovine serum albumin as the reference protein standard. The results were analyzed with BioMetallics DeltaSoft PC Microplate Analysis Software, v. 1.71.2 using a 4-parameter fit of the standard curve.

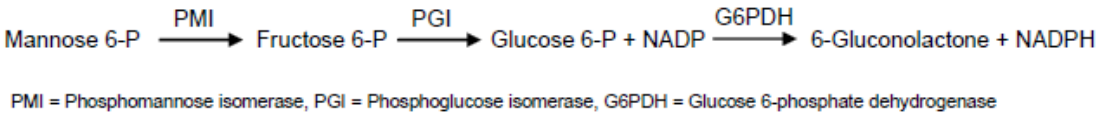
Immunoreactivity and Molecular Weight Determination

Western blot analysis was used to investigate the integrity of PMI in the plant extract sample LP5307, the control leaf extract fortified with the microbially produced test substance (LP-NEG + PMI-0105) and the microbially produced test substance PMI-0105. Aliquots equivalent to 5 ng PMI from sample LP5307, LPNEG + PMI-0105 and test substance PMI-0105 diluted in 10X Sample Buffer (as described by Laemmli (1970) containing 8% glycerol, 1% BME, 2% SDS, 65 mM Tris, and 0.01% bromophenol blue) were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) with a NuPAGE 4-12% Bis-Tris gel (Invitrogen) and NuPAGE MES SDS running buffer (Invitrogen). An aliquot of the negative control sample (LP-NEG); equivalent to 92.8 µg total protein (the total amount of protein loaded on the gel for LP5307), was included in the analysis as a negative control. Additionally, based on total protein, LP-NEG + PMI-0105 was supplemented with additional LP-NEG extract to equal 86.8 µg total protein. The molecular-weight standard was SeeBlue Plus2 pre-stained standard (Invitrogen). After electroblotting, the polyvinylidene difluoride (PVDF) membrane was probed with immunoaffinity-purified polyclonal goat antibody generated against PMI diluted to 1 µg/ml in Tris-buffered saline, pH 8.0 with 3% nonfat milk. Alkalinephosphatase-conjugated donkey anti-goat IgG (Jackson ImmunoResearch) diluted 1:3000 in Tris-buffered Saline with Tween 20, pH 8.0)

was used to bind to the primary antibody and was visualized by development with BCIP/NBT alkaline phosphatase substrate solution (Sigma-Aldrich). The Western blot was examined for the presence of intact immunoreactive PMI or immunoreactive PMI fragments.

Enzymatic Activity

The enzymatic activity of PMI was measured in triplicate using a continuous coupled spectrophotometric assay based on the method described by Gracy and Noltmann (1968) and Gill et al. (1986). PMI activity was measured by linking the formation of fructose 6-phosphate (resulting from the isomerization of mannose 6-phosphate) to the reduction of NADP via phosphoglucose isomerase and glucose 6-phosphate dehydrogenase (as shown in the diagram below). The molar reduction of NADP in this system can be directly converted into the molar isomerization of mannose 6-phosphate via PMI. The enzymatic reactions were conducted in 96-well plates. Microbially-produced and plant produced PMI were diluted in 50 mM Tris buffer, pH 7.0 containing 0.1% Tween 20. The reaction was initiated by adding 5 ng PMI from triplicate plant extract samples LP5307, LP-NEG + PMI-0105, or PMI from test substance PMI-0105 to an assay mixture containing 10 mM mannose 6-phosphate (Sigma-Aldrich), 1 mM β-nicotinamide adenine dinucleotide phosphate (NADP) sodium salt hydrate (Sigma-Aldrich), 2 U/ml phosphoglucose isomerase (Sigma-Aldrich) and 2 U/ml glucose 6-phosphate dehydrogenase (G6PDH)(Sigma-Aldrich) in 50 mM Tris buffer, pH 7.0. The total volume of the reaction mixture was 200 µl.



The assay mixture was preincubated at 25°C ± 2°C for 10 minutes prior to the addition of PMI. Following the 10 minute preincubation, PMI was added to the assay mixture and the plate was read by a SpectraMax Plus384 spectrophotometer (Molecular Devices) to determine the pathlength of the sample in each well. The reduction of NADP was monitored spectrophotometrically at 340 nm at 25ºC ± 2ºC over 10 minutes with readings taken every 15 seconds. The change in absorbance over time was monitored using SoftMax Pro version 5.2. The extinction coefficient of NADPH, 6.22 cm^{-1} mM^{-1}, was used for calculating the amount of NADPH formed. One unit (U) of PMI activity is defined as the amount of enzyme required to catalyze the conversion of 1 µmol of mannose 6-phosphate to fructose 6-phosphate per min (equivalent to 1 µmol NADP reduced per min) under the described reaction conditions. Results are reported as the mean and standard deviation of triplicate enzymatic assays.

C.4. Results of Comparison of PMI Produced in 5307 Corn and Recombinant *E. coli*

Immunoreactivity and Molecular Weight Determination

Western blot analysis of PMI in the plant extract sample LP5307, the control leaf extract fortified with PMI test substance PMI-0105 (LP-NEG + PMI-0105), and test substance PMI-0105 reveals a dominant immunoreactive band consistent with the predicted molecular weight of PMI (Figure C-5, Lanes 2, 3, and 4). The intensity of the PMI bands in the presence of the plant matrix (Lanes 2 and 3) is diminished compared to the intensity of the PMI band in the absence of the matrix (Lane 4). However, there is similar intensity between the PMI bands from test substance PMI-0105 in the presence of plant matrix (Figure C-5, Lane 3) and PMI from 5307 corn extract (Lane 2). Thus, the relative diminished intensity of the PMI bands in the presence of the plant matrix is most likely due to matrix effects. The matrix effect can be attributed to the nature of the sample. Crude plant extract preparations contain all soluble cell proteins of varying molecular weights that may interfere with the mobility of the analyzed protein and with antibody binding. The co-migration of the matrix with PMI or PMI fragments may limit access of the antibody to epitopes of the target protein and therefore diminish the signal.

The Western blot analysis also revealed some faint bands combined with a diffuse background signal in molecular weight range above 62 kDa in both LP5307 and LP-NEG + PMI-0105 (Figure C-5, Lanes 2 and 3). As this effect (with exactly the same band pattern) was also detected in the negative plant extract sample LP-NEG (Lane 5) it is not related to PMI protein and most likely represents an unspecific response on the Western blot. As expected, no immunoreactive band corresponding to the molecular weight of PMI was observed in the negative plant extract sample LP-NEG (Lane 5). The Western blot analysis also revealed a very faint protein band with a molecular weight of approximately 30 kDa in the microbially-produced test substance PMI-0105. Because this protein cross-reacted with the anti-PMI antibody it is most likely a PMI degradation product. The degradation product is not visible in the sample with the control leaf extract fortified with the microbially-produced test substance (LP-NEG + PMI-0105) due to the matrix effect described above.

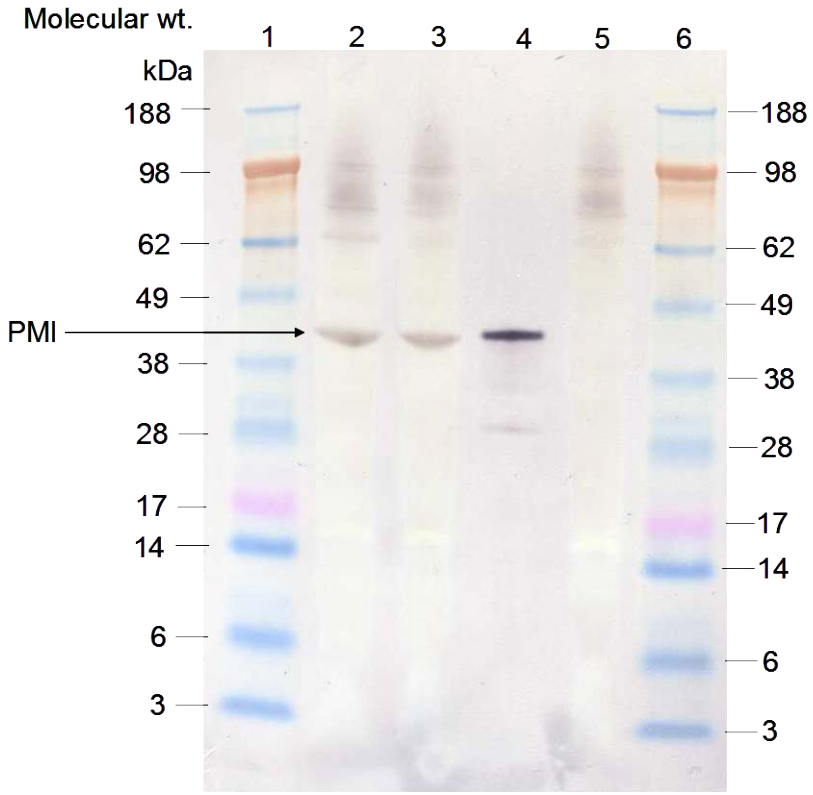


Figure C-5. Western blot analysis for PMI in LP5307, LP-NEG + PMI-0105, test substance PMI-0105, and LP-NEG.

Lane 1: Molecular weight standard SeeBlue Plus2 (Invitrogen)
Lane 2: Plant extract sample LP5307
Lane 3: Plant extract sample LP-NEG + PMI-0105
Lane 4: Microbially-produced test substance PMI-0105
Lane 5: Plant extract sample LP-NEG
Lane 6: Molecular weight standard SeeBlue Plus2

The average PMI enzymatic activity was 455.67 U/mg PMI in the triplicate plant extract samples LP5307 and 526.26 U/mg PMI in the microbially-produced test substance PMI-0105 (Table C-1). The control leaf extract fortified with the microbially-produced test substance (LP-NEG + PMI-0105) had a specific activity of 518.81 U/mg PMI (Table C-1), demonstrating that the plant matrix had little effect on the enzymatic activity of PMI. As expected, no PMI activity was detected in the negative control plant extract sample, LP-NEG (Table C-1).

Table C-1. Enzymatic activity of PMI in 5307 corn leaf extract, control corn leaf extract fortified with test substance PMI-0105, test substance PMI-0105, and control corn leaf extract.

LP5307 is leaf extract of 5307 corn plants. LP-NEG + PMI-0105 is control corn leaf extract fortified with microbially produced PMI test substance PMI-0105. LP-NEG is control corn leaf extract.

	Assay Replicate	PMI Activity[1] (U/mg PMI)	Average PMI Activity of Assay Replicates (U/mg PMI)	SD of Assay Replicate s (U/mg PMI)	CV of Assay Replicates (%)	Overall Average PMI Activity (U/mg PMI)	Overall SD (U/mg PMI)	Overall CV (%)
LP5307-1[2]	1	469.58	479.60	10.23	2.1%	455.67	30.37	6.7%
	2	490.03						
	3	479.21						
LP5307-2	1	478.77	464.39	21.67	4.7%			
	2	474.93						
	3	439.47						
LP5307-3	1	418.44	423.03	23.32	5.5%			
	2	448.30						
	3	402.34						
LPNEG + PMI-0105	1	514.24	518.81	13.82	2.7%			
	2	534.33						
	3	507.86						
PMI-0105	1	519.00	526.26	6.59	1.3%			
	2	531.87						
	3	527.90						
LPNEG	1	below LOD	below LOD	not applicable	not applicable			
	2	below LOD						
	3	below LOD						

[1] One unit of PMI activity is defined as the amount of enzyme required to catalyze the conversion of 1 µmol of mannose 6-phosphate to fructose 6-phosphate per min (equivalent to 1 µmol NADP reduced per min) under the described reaction conditions.

[2] LP5307-1, -2, and -3 represent three independent extractions.

Appendix D. Quantification of eCry3.1Ab and PMI in 5307 Corn

This appendix provides details of the materials and methods used to determine the concentrations of eCry3.1Ab and PMI in the tissues of 5307 plants at various growth stages. Refer to Part VI.C. of this Petition, eCry3.1Ab and PMI Concentrations in 5307 Corn, for a description of the study and results. All literature references cited in this appendix are listed in **Part IX** of this Petition, titled **References**.

D.1. Plant Tissue Production

Plants were grown for the collection of tissues in Bloomington, IL; Sadorus, IL; Shirley, IL; and Stanton, MN in two plots per location; one for the 5307 hybrid and one for the control hybrid.

D.2. Plant Tissue Processing

In the presence of dry ice, the leaf, root, kernel and whole-plant samples were individually processed to a fine powder. A subsample from each homogeneous, powdered sample was lyophilized and stored at –80 ºC ± 10ºC. The percent dry weight of each sample was determined from the sample weight before and after lyophilization. Frozen pollen samples were analyzed without further processing.

D.3. Tissue Extraction and Analysis

Protein extractions were performed on representative aliquots of the lyophilized leaf, root, kernel, and whole-plant samples, and fresh frozen pollen samples. The extracts were analyzed by ELISA (Tijssen, 1985) to quantify the amount of eCry3.1Ab and PMI in each sample. Sample extracts were analyzed in triplicate, and standard curves were generated with known amounts of the corresponding reference protein. Standard curves were generated for each ELISA plate. Nontransgenic plant tissue extracts were analyzed in parallel to evaluate any impact of the plant matrix on the ELISA.

D.4. eCry3.1Ab Quantification – Extraction and ELISA Procedures

Buffers

The buffers used for extraction and ELISA analysis of eCry3.1Ab are listed in the following table:

Name of buffer	Constituents
Phosphate-buffered saline (PBS)	140 mM sodium chloride, 8.24 mM sodium phosphate dibasic, 1.81mM sodium phosphate monobasic, pH 6.75
Borate buffer (pollen)	0.1 M Sodium tetraborate decahydrate, 0.2% PVP-360, 7.69 mM sodium azide, 1.2% Concentrated hydrochloric acid, 0.5% Tween 20; pH approximately 7.5. Complete Protease Inhibitor Cocktail (Roche Applied Science) added on day of extraction
Borate buffer (leaves, roots, whole plants, kernels)	0.1 M Sodium tetraborate decahydrate, 0.2% PVP-360, 7.69 mM sodium azide, 0.5% Tween 20; titrated to pH 10.0. Complete Protease Inhibitor Cocktail (Roche Applied Science) added on day of extraction
Dilution buffer	PBS, 0.05% Tween 20, 1% BSA, 0.02% sodium azide
Wash buffer	10 mM Tris, 0.05% Tween 20, 0.02% sodium azide

eCry3.1Ab Extraction

Leaves, Roots, Whole Plants, and Kernels. A ratio of 3 ml of borate buffer pH 10.0 was added to 100 mg of lyophilized tissue. The samples were vortexed, placed on wet ice for at least 30 minutes, homogenized using an Omni-Prep Homogenizer, and centrifuged at 2°C to 8°C to form a pellet. The supernatants were removed and stored at 2°C to 8°C until analysis.

Pollen. For each sample, 25 mg of fresh frozen pollen was weighed into a 2-ml Eppendorf tube containing three 4-mm glass beads and stored at -80°C ± 10°C for at least two hours. Each tube was then placed into a Four Station Titer Plate/Micro Tube Grinding Mill and set at ~3000 strokes per minute for approximately 45 seconds. The tubes were then placed on wet ice and 1.5 ml of borate buffer, pH 7.5 was added to each sample. The samples were mixed and set on wet ice for at least 30 minutes then centrifuged at 2°C to 8°C to form a pellet. The supernatants were removed and stored at 2°C to 8°C until analysis.

eCry3.1Ab Quantitation

The eCry3.1Ab ELISA kit was manufactured at Beacon Analytical Systems (BAS), Portland, ME. The assay is a double-antibody sandwich assay in which the eCry3.1Ab protein is affixed to the wells of a microtiter plate using a monoclonal, anti-mCry3A antibody that binds to the mCry3A domains of the eCry3.1Ab protein. The primary antibody was diluted and added to each well of a 96-well microtiter plate. The plate was then blocked using a proprietary method. Dilutions of each tissue extract and appropriate serial dilutions of eCry3.1Ab reference protein (ECRY3.1AB-0208), prepared in dilution buffer, were applied to the pre-coated plates at a total volume of 100 µl/well. The plates were incubated at room temperature on a titre plate shaker at 400 rpm for 1 hour. The plates were washed five times with wash buffer in a BioTek ELx405 Microplate Washer. After washing the plates, a secondary, rabbit polyclonal anti-Cry1Ab antibody (provided in the kit) was then used to bind the Cry1Ab domain of the eCry3.1Ab protein at 100 µl/well. The plates were incubated at room temperature on a titre plate shaker at 400 rpm for one hour and washed five times as described above.

After the plates were washed, a tertiary donkey anti-rabbit conjugated with alkaline phosphatase diluted in dilution buffer was added to each of the wells (100 µl/well) and incubated at room temperature on a titre plate shaker at 400 rpm for one hour. The plates were then washed five times as described above, and alkaline phosphatase substrate solution provided in the kit was added at a volume of 100 µl/well. The plates were incubated for 30 minutes at room temperature on a titre plate shaker at 400 rpm. The reaction was stopped by the addition of 3N sodium hydroxide (100 µl/well), and absorbance of the reaction was measured at a dual wavelengths (405 and 492 nm) with a Tecan Sunrise Microplate Reader. The results were analyzed with BioMetallics DeltaSoft PC Microplate Analysis Software, v. 1.71.2, using a four-parameter algorithm. The results for kernel samples were analyzed with SoftMax Pro, v. 5.2, using a four-parameter algorithm.

The lower limit of quantification (LOQ) or limit of detection (LOD) of eCry3.1Ab is shown on Table VI-2 (Part VI.C.) for any tissue type in which eCry3.1Ab could not be quantified or detected, respectively. The concentrations of eCry3.1Ab in pollen were too low to determine the efficiency of extraction from this tissue. For all other tissues, extraction efficiencies ranged from 75% to 88%; the reported tissue concentrations have been adjusted for extraction efficiency.

D.5. PMI Quantification –Extraction and ELISA Procedures

Buffers

The buffers used for extraction and ELISA analysis of PMI are listed in the following table:

Name of buffer	Constituents
PBS	138 mM sodium chloride, 2.7 mM potassium chloride, 10.1 mM disodium phosphate, 1.8 mM potassium dihydrogen phosphate, pH 7.4
Blocking buffer	PBS, 1% powdered milk
Borate buffer	0.1 M sodium tetraborate decahydrate, 0.2% PVP-360, 7.69 mM sodium azide, 1.2% concentrated hydrochloric acid, 0.5% Tween 20; pH will be approximately 7.5. Complete Protease Inhibitor Cocktail (Roche Applied Science) added on day of extraction
Carbonate-bicarbonate buffer	34.9 mM sodium bicarbonate, 15.0 mM sodium carbonate, pH 9.5
Citrate-phosphate buffer	23.8 mM citric acid, 59.9 mM disodium phosphate, pH 5.0
Dilution buffer	PBS, 0.05% Tween 20, 1% powdered milk
Wash buffer	PBS, 0.05% Tween 20

PMI Extraction

Leaves, Roots, Whole Plants, and Kernels. A ratio of 3 ml of borate buffer, pH 7.5 was added to 100 mg of lyophilized tissue. The samples were mixed, placed on wet ice for at least 30 minutes, homogenized using an Omni-Prep Homogenizer, and centrifuged at 2°C to 8°C to form a pellet. The supernatants were removed and stored at 2°C to 8°C until analysis. For root tissue, the pellet was retained and then processed through the aforementioned extraction procedure again. The supernatant removed from the second extraction was combined with that of the first extraction, mixed well and stored at 2°C to 8°C until analysis.

Pollen. For each sample, 25 mg of fresh, frozen pollen was weighed into a 2-ml Eppendorf tube containing three 4-mm glass beads and stored at -80°C ± 10°C for at least two hours. Each tube was then placed into a Four Station Titer Plate/Micro Tube Grinding Mill and set at ~3000 strokes per minute for approximately 45 seconds. The tubes were then placed on wet ice and 1.5 ml of borate buffer, pH 7.5 was added to each sample. The samples were mixed and set on wet ice for at least 20 minutes then centrifuged at 2°C to 8°C to form a pellet. The supernatants were removed and stored at 2°C to 8°C until analysis.

PMI Quantification

Rabbit polyclonal anti-PMI antibody was diluted in carbonate-bicarbonate buffer and added to each well of a 96-well microtiter plate at a volume of 100 µl/well. The plates were stored overnight in a refrigerator set at 2°C to 8°C. The antibody solution was removed and blocking buffer was added to the plate at a volume of 250 µl/well and then incubated at room temperature for at least 30 minutes. After blocking incubation, the plates were washed five times with wash buffer in a BioTek ELx405 microplate washer and dilutions of each tissue extract and appropriate serial dilutions of PMI reference protein (PMI-0105) prepared in dilution buffer were applied to the plates at a total volume of 100 µl/well. The plates were incubated at 18°C to 22°C for 2 hours. After incubation, plates were washed five times as described above and a monoclonal anti-PMI antibody diluted in dilution buffer was added to the plate at a volume of 100 µl/well and incubated at 18°C to 22°C for one hour.

The plates were washed five times after incubation and a horseradish-peroxidase-conjugated rabbit anti-mouse immunoglobin diluted in dilution buffer was added at a volume of 100 µl/well and incubated at 18°C to 22°C for one hour. After incubation, the plates were washed five times, and TMB substrate solution was added at a volume of 100 µl/well (one tablet per 10 ml of citrate-phosphate buffer) and incubated at room temperature in the dark for 30 minutes. The reaction was stopped by addition of 3 M sulfuric acid at a volume of 50 µl/well, and the absorbance of the reaction was measured at 450 nm with a Tecan Sunrise microplate reader. The results were analyzed with BioMetallics DeltaSoft PC Microplate Analysis Software, v. 1.71.2, using a four-parameter algorithm.

The lower limit of quantification (LOQ) or limit of detection (LOD) of PMI is shown on Table VI-3 (Part VI.C.) for any tissue type in which PMI could not be quantified or detected, respectively. PMI extraction efficiencies ranged from 72% to 95% across all tissue types analyzed; the reported tissue concentrations have been adjusted for extraction efficiency.

Appendix E. Compositional Analysis of 5307 Forage and Grain

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

Study Design

Forage and grain for compositional analyses were harvested from multiple locations planted in the U.S. in 2008. The locations chosen were representative of major corn producing regions in the country. For all locations, trials were planted with a 5307 hybrid and near-isogenic, nontransgenic hybrid in a randomized complete block design with three replicated 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 eight locations in an effort to ensure that grain and forage from at least six locations could be harvested in the event of loss due to adverse environmental conditions (early freeze, drought, etc.). Six locations that produced sufficient grain and forage were selected for this study (see Part VII.B.1).

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, Inc. (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.

Grain Sampling and Processing

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

Compositional Analyses

As detailed in Table VII-13 (Part VII of this Petition), forage was analyzed for proximates and the minerals calcium and phosphorus. Grain was analyzed for major constituents (proximates, including 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.

Statistical Analyses

Across-location comparisons

The data for each variate (component) were subjected to analysis of variance using the following mixed model (Obert et al., 2004):

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

In this model, Y_{ijk} is the observed response for genotype i at location j block k, U is the overall mean, T_i is the genotype effect, L_j is the location effect, $B(L)_{jk}$ is the effect of block within a location, LT_{ij} is the location-by-genotype interaction effect, and e_{ijk} is the residual error. Genotype was regarded as a fixed effect, while the effects of location, block within location, and location-by-genotype were regarded as random. For each quantifiable component, an F test was used to assess the statistical significance of the genotype effect with an alpha level of 0.05 and with the denominator degrees of freedom determined using the Kenward-Roger method (Kenward and Roger, 1997). Moisture content of grain was not statistically analyzed because the samples had been mechanically dried.

Individual-location comparisons

The data for each variate at each location were subjected to an analysis of variance with genotype and block included in the statistical model. Significance was based on an alpha level of 0.05. Statistical analysis was performed using SAS v. 9.2 (SAS Institute, Inc., Cary NC).

Comparison with ILSI Crop Composition Database

The mean levels of each component for each location and across locations were calculated and compared nonstatistically with means and ranges for forage and grain composition published in the ILSI Crop Composition Database (2008). The ILSI database is the most comprehensive and current source of crop composition data for most nutritional components.

Analytical Methods and Reference Standards for Compositional Analysis

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

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

Reference Standard: Acros 2-Furaldehyde, 99.7%, Lot Number A0219180

Acid Detergent Fiber (USDA, 1970)

The sample was washed with acetone to remove fats and pigments. It was then placed in a filter bag and positioned in an Ankom analyzer where it was washed with an acidic boiling detergent solution that dissolved the protein, carbohydrate, and ash. The lignocellulose fraction remaining was determined gravimetrically. The LOQ for this study was 0.100%, calculated on a fresh-weight basis.

Amino Acid Composition (AOAC, 2005k)

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

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

Reference Standards: ThermoScientific K18, 2.5 µmol/mL per constituent except cystine (1.25 µmol/mL), Lot Number JK126327

Sigma, L-Tryptophan, 100%, Lot Number 076K0075

Sigma/BioChemika, L-Cysteic Acid Monohydrate, >99% (used as 100%), Lot Number 1305674

Sigma, L-Methionine Sulfone, 100%, Lot Number 047K1321

Ash (AOAC, 2005b)

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

Beta-Carotene (AOAC, 2005e; Quackenbush, 1987)

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

Reference Standard: Sigma-Aldrich, Beta-carotene, Type I, 100% (stock standard concentration determined spectrophotometrically), Lot Number 068K2561

Carbohydrates (USDA, 1973)

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

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

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

Fat by Acid Hydrolysis (AOAC, 2005a)

The sample was hydrolyzed with hydrochloric acid at an elevated temperature. The fat was extracted with ether and hexane. The extract was evaporated on a steambath, redissolved in hexane and filtered through a sodium sulfate column. The hexane extract was then vaporated again on a steambath under nitrogen, dried, and weighed. The LOQfor this study was 0.1%, calculated on a fresh weight basis.

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

The lipid was extracted and saponified with 0.5N 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 0.00500%, calculated on a fresh-weight basis.

Reference Standards:

Nu Chek Prep GLC Reference Standard Hazelton No. 1, Lot Number AU18-S
Nu Chek Prep GLC Reference Standard Hazelton No. 2, Lot Number M13-O
Nu Chek Prep GLC Reference Standard Hazelton No. 3, Lot Number MA18-S
Nu Chek Prep GLC Reference Standard Hazelton No. 4, Lot Number JA16-T
Nu Chek Prep Methyl Gamma Linolenate, used as 100%, Lot Number U-63M-JY12-R
Nu Chek Prep Methyl Tridecanoate, used as 100%, Lot Number N-13M-JA16-T

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

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

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

ICP Emission Spectrometry (AOAC, 2005m)

The sample was dried, precharred, and ashed overnight in a muffle set to maintain 500°C. The ashed sample was re-ashed with nitric acid, treated with hydrochloric acid, taken to dryness, and put into a solution of 5% hydrochloric acid. The amount of each element was determined at appropriate wavelengths by comparing the emission of the unknown sample, measured on the inductively coupled plasma spectrometer, with the emission of the standard solutions. The LOQs (Table E-1) were calculated on a fresh-weight basis.

Table E-1. Inorganic Ventures Reference Standards and Limits of Quantitation

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

ICP-Mass Spectrometry (AOAC, 2005o)

The sample was wet-ashed with nitric acid using microwave digestion. Using inductively coupled plasma mass spectrometry, the amount of each element was determined by comparing the counts generated by the unknowns to those generated by standard solutions of known concentrations. The LOQ for this study was 50.0 ppb, calculated on a fresh-weight basis.

Reference Standard: SPEX, Selenium, 100 mg/L, Lot Number 6-74GS

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

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

Reference Standard: Sigma-Aldrich, Myo-Inositol, 100%, Lot Number 065K0018

Moisture (AOAC, 2005c)

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

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

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

Niacin (AOAC, 2005g)

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

Reference Standard: USP, Niacin, 99.8%, Lot Number I0E295

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

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

Reference Standards: Acros Organics, 4-Hydroxy-3-methoxycinnamic acid (ferulic acid), 99.4%, Lot Number A0248008

Acros Organics, p-Hydroxycinnamic acid (p-coumaric Acid), 99.4%, Lot Number A0236839

Phytic Acid (Lehrfeld, 1989 and 1994)

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

Reference Standard: Aldrich, Phytic Acid, Dodecasodium Salt Hydrate, 95%, Lot Number 077K0693

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

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

Pyridoxine Hydrochloride (AOAC, 2005j; Atkins et al., 1943)

The sample was hydrolyzed with dilute sulfuric acid in the autoclave and the pH was adjusted to remove interferences. The amount of pyridoxine was determined by comparing the growth response of the sample, using the yeast *Saccharomyces carlsbergensis,* with the growth response of a pyridoxine standard. The response was measured turbidimetrically. Results were reported as pyridoxine hydrochloride. The LOQ for this study was 0.00700 mg/100 g, calculated on a fresh-weight basis.

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

Raffinose (Brobst, 1972; Mason and Stover, 1971)

The sample was extracted with deionized water and the extract treated with a hydroxylamine hydrochloride solution in pyridine, containing phenyl-β-D-glucoside as an internal standard. The resulting oximes were converted to silyl derivatives by treatment with hexamethyldisilazane and trifluoracetic acid and analyzed by gas chromatography using a flame ionization detector. The acceptable LOQ for this study was 0.100%, calculated on a fresh weight basis.

Reference Standards: Sigma, D(+)-Raffinose Pentahydrate, 99% (84.0% after correction for degree of hydration), Lot Number 037K1059

Starch (AOAC, 2005p)

The sample was extracted with alcohol to remove carbohydrates other than starch, i.e. sugars. Then it was hydrolyzed into glucose with α -amylase and amyloglucosidase. Glucose was oxidized with glucose oxidase to form peroxide, which reacted with a dye in the presence of peroxidase to give a stable colored product proportional to glucose concentration. The glucose concentration was quantitated by measurement on a spectrophotometer at 540 nm. Percent starch was then calculated from the glucose concentration. The LOQ for this study was 0.05%, calculated on a fresh-weight basis.

Reference Standard: Sigma D(+)-Glucose, 99.9%, Lot Number 123K0095

Thiamine Hydrochloride (AOAC, 2005f)

The sample was autoclaved under weak acid conditions to extract the thiamine. The resulting solution was incubated with a buffered enzyme solution to release any bound thiamine. The solution was purified on a cation-exchange column. An aliquot was reacted with potassium ferricyanide to convert thiamine to thiochrome. The thiochrome was extracted into isobutyl alcohol, measured on a fluorometer, and quantitated by comparison to a known standard. The limit of quantitation was calculated and reported on a fresh weight basis. The LOQ for this study was 0.01 mg/100 g. Results were reported as thiamine hydrochloride.

Reference Standard: USP, Thiamine Hydrochloride, Purity 99.8% (used as 95.9% after correction for moisture content), Lot Number 01F236

Total Dietary Fiber (TDF)(AOAC, 2005n)

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

Trypsin Inhibitor (AOCS, 1997a)

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

Vitamin B2 (Riboflavin) (AOAC, 2005d; US Pharmacopeia, 2005)

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

Reference Standard: USP, Riboflavin, 100%, Lot Number N0C021

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

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

Reference Standard: USP, Alpha-Tocopherol, 100%, Lot Number M

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