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Petition for Determination of Nonregulated Status for Corn Rootworm Protected and Glyphosate Tolerant MON 87411 Maize

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 part 340

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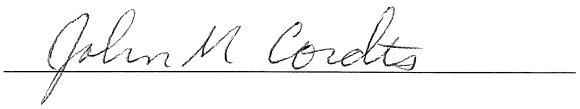
RELEASE OF INFORMATION

Monsanto is submitting the information in this petition for review by the USDA as part of the regulatory process. Monsanto understands that the USDA complies with the provisions of the Freedom of Information Act (FOIA). In the event the USDA receives a FOIA request, pursuant to 5 U.S.C., § 552, and 7 CFR Part 1, covering all or some of the information in this petition, Monsanto expects that, in advance of the release of the document(s), USDA will provide Monsanto with a copy of the material proposed to be released and the opportunity to object to the release of any information based on appropriate legal grounds, e.g., responsiveness, confidentiality, and/or competitive concerns. Monsanto understands that this information may be made available to the public in a reading room and upon individual request as part of a public comment period. Monsanto also understands that when deemed complete, a copy of the petition may be posted to the USDA-APHIS BRS website or other U.S. government websites (e.g., www.regulations.gov). Except in accordance with the foregoing, Monsanto does not authorize the release, publication or other distribution of this information without Monsanto's prior notice and consent.

CERTIFICATION

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



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EXECUTIVE SUMMARY

The Animal and Plant Health Inspection Service (APHIS) of the United States (U.S.) Department of Agriculture (USDA) has responsibility under the Plant Protection Act (Title IV Pub. L. 106-224, 114 Stat. 438, 7 U.S.C. § 7701-7772) to prevent the introduction and dissemination of plant pests into the U.S. APHIS regulation 7 CFR § 340.6 provides that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and no longer should be regulated. If APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article.

Monsanto Company is submitting this request to APHIS for a determination of nonregulated status for the new biotechnology-derived maize product, MON 87411, any progeny derived from crosses between MON 87411 and conventional maize, and any progeny derived from crosses of MON 87411 with biotechnology-derived maize that have previously been granted nonregulated status under 7 CFR part 340.

Product Description

Monsanto Company has developed biotechnology-derived maize, MON 87411, that confers protection against corn rootworm (CRW) (*Diabrotica spp*.) and tolerance to the herbicide glyphosate. MON 87411 contains a suppression cassette that expresses an inverted repeat sequence designed to match the sequence of western corn rootworm (WCR; *Diabrotica virgifera virgifera*). The expression of the suppression cassette results in the formation of a double-stranded RNA (dsRNA) transcript containing a 240 bp fragment of the WCR *Snf7* gene (DvSnf7). Upon consumption, the plant-produced dsRNA in MON 87411 is recognized by the CRW's RNA interference (RNAi) machinery resulting in down-regulation of the targeted DvSnf7 gene leading to CRW mortality. MON 87411 also contains a *cry3Bb1* gene that produces a modified *Bacillus thuringiensis* (subsp. *kumamotoensis*) Cry3Bb1 protein to protect against CRW larval feeding. In addition, MON 87411 contains the *cp4 epsps* gene from *Agrobacterium* sp. strain CP4 that encodes for the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) protein, which confers tolerance to glyphosate, the active ingredient in Roundup® agricultural herbicides.

MON 87411 builds upon the current *Bt* protein-based mode-of-action (MOA) for CRW control by the addition of a new RNA-mediated MOA that offers enhanced control of target insect pests and prolonged durability of existing *Bt* technologies designed to control CRW. MON 87411 will provide benefits to growers similar to those obtained by use of existing CRW-protected maize hybrids, which include reduced need for insecticides and associated improvements in worker safety, increased yield protection, and water conservation. MON 87411 is also glyphosate tolerant and will continue to

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provide benefits associated with conservation tillage methods, including reduced soil erosion, reduced fuel and labor costs, improved air quality and conservation of soil moisture.

MON 87411 will not be offered for commercial use as a stand-alone product, but will be combined, through traditional breeding methods, with other deregulated biotechnology-derived traits to provide protection against both above-ground and below-ground maize pests as well as tolerance to multiple herbicides. These next generation combined-trait maize products will offer broader grower choice, improved production efficiency, increased pest control durability, and enhanced grower profit potentials.

Data and Information Presented Confirms the Lack of Plant Pest Potential and the Food and Feed Safety of MON 87411 Compared to Conventional Maize

The data and information presented in this petition demonstrate that MON 87411 is agronomically, phenotypically, and compositionally comparable to commercially cultivated maize. Moreover, the data and information presented herein demonstrate that MON 87411 is not expected to pose an increased plant pest risk, including weediness, compared to commercially cultivated maize. The food, feed, and environmental safety of MON 87411 was confirmed based on multiple, well-established lines of evidence:

- The CP4 EPSPS protein in MON 87411 is identical to the CP4 EPSPS protein present in several other commercially available crops that have been reviewed by USDA and previously deregulated (*e.g.*, Roundup Ready varieties of soybean, maize, cotton, sugarbeet, canola, and alfalfa). The safety and mode-of-action of CP4 EPSPS proteins is well documented and is the subject of numerous publications. Similarly, the safety of the Cry3Bb1 protein has been previously assessed in two other corn rootworm-protected products (MON 863 and MON 88017) that have been grown on tens of millions of acres in the U.S. since their introduction. The mode-of-action of *Bt* proteins has also been extensively studied and is well-documented in numerous publications.

- The RNA-based suppression of the *Snf7* gene in western corn rootworm that results from expression of the DvSnf7 suppression cassette in MON 87411 is mediated by dsRNA molecules. Double-stranded RNAs are commonly used by eukaryotes, including plants, for endogenous gene suppression and as described in this petition, pose no novel risks from a feed/food and environment perspective. Nucleic acids, as the components of RNA, have a long history of safe consumption and are considered GRAS by the U.S. FDA.

- A compositional assessment supports the conclusion that MON 87411 grain and forage are equivalent to grain and forage of conventional maize.

- Evaluation of the agronomic and phenotypic characteristics of MON 87411, using current maize cultivation and management practices, leads to the conclusion that deregulation of MON 87411 would not have an effect on maize agronomic practices.

Maize is a Familiar Crop Lacking Weedy Characteristics

Maize is grown extensively throughout the world, and is the largest cultivated crop followed by wheat (*Triticum* sp.) and rice (*Oryza sativa* L.) in total global production. In the U.S., maize is grown in almost all states and is the largest crop grown in terms of acreage planted and net value. Maize has been studied extensively, and the domestication of maize can be traced back to approximately 10,000 years ago in southern Mexico.

Maize is not listed as a weed in the major literature references on weeds, nor is it present on the lists of noxious weed species published by the federal government (7 CFR Part 360). In addition, maize has been grown throughout the world without any report that it is a serious weed. Maize is poorly suited to survive without human assistance and is not capable of surviving as a weed due to past selection in the domestication of maize. During domestication of maize, traits often associated with weediness, such as seed dormancy, a dispersal mechanism, or the ability to establish reproducing populations outside of cultivation, have not been selected. Similarly, the history of hybrid breeding in the U.S. does not indicate there are any changes in the characteristics of maize that would change the weediness profile of the crop. Although maize seed can overwinter into a rotation with soybeans and other crops, mechanical and chemical measures are routinely used to control maize volunteers. Some populations of wild annual and perennial species that could hybridize with MON 87411 are known to exist in the U.S., however key differences in several factors such as flowering time, geographical separation, and development timings make natural crosses in the U.S. highly unlikely.

Conventional Maize MPA640B and NL6169 are Appropriate Comparators for MON 87411

Based on seed availability and the appropriate fit for various studies, conventional control materials were developed for use as comparators in safety assessment studies. The conventional control materials included the original transformation line (LH244) and two hybrid conventional control lines (hybrids MPA640B and/or NL6169), both of which have similar genetic backgrounds to the hybrid MON 87411 test material (LH244 is one parent of each of the control hybrids). Both MPA640B (LH244 × LH287) and NL6169 (LH244 × HCL645) were used as controls in molecular characterization studies. NL6169 was used as the conventional control in compositional analysis studies while MPA640B was used as the conventional control in phenotypic, agronomic and environmental interactions assessments. Where appropriate, commercial reference maize hybrids were used to establish a range of variability or responses representative of commercial maize (reference hybrids) in the U.S.

Molecular Characterization Verified the Integrity and Stability of the Inserted DNA in MON 87411

MON 87411 was developed through *Agrobacterium*-mediated transformation of maize immature embryos from line LH244 utilizing plasmid vector PV-ZMIR10871. PV-ZMIR10871 contains one transfer DNA (T-DNA) that is delineated by Left and Right

Border regions. The T-DNA contains the DvSnf7 suppression cassette, the *cry3Bb1* expression cassette, and the *cp4 epsps* expression cassette. The DvSnf7 suppression cassette is regulated by the *e35S* promoter from the 35S RNA of cauliflower mosaic virus (CaMV), the heat shock protein 70 (*Hsp70*) intron from *Zea mays*, and the 3' untranslated sequence of the *E9* gene from *Pisum sativum*. The *cry3Bb1* expression cassette is regulated by the *pIIG* promoter from *Zea mays*, the chlorophyll a/b binding protein (CAB) leader from *Triticum aestivum*, the *Ract1* intron from *Oryza sativa*, and the heat shock protein 17 (*Hsp17*) 3′ untranslated region from *Triticum aestivum*. The *cp4 epsps* expression cassette is regulated by the *TubA* promoter from *Oryza sativa*, the *TubA* leader from *Oryza sativa*, the *TubA* intron from *Oryza sativa*, the *CTP2* chloroplast-targeting sequence from *Arabidopsis thaliana*, and the *TubA* 3′ untranslated region from *Oryza sativa*.

Characterization of the DNA insert in MON 87411 was conducted using a combination of sequencing, PCR, and bioinformatics. The results of this characterization demonstrate that MON 87411 contains one copy of the intended T-DNA containing the DvSnf7 suppression cassette and the *cry3Bb1* and *cp4 epsps* expression cassettes that is stably integrated at a single locus and is inherited according to Mendelian principles over multiple generations. These conclusions are based on several lines of evidence:

- Molecular characterization of MON 87411 by Next Generation Sequencing and Junction Sequence Analysis (NGS/JSA) demonstrated that DNA from PV-ZMIR10871 DNA was integrated at a single locus in MON 87411.

- Directed sequencing (locus-specific PCR, DNA sequencing and analyses) was performed on MON 87411, which determined the complete sequence of the single PV-ZMIR10871 T-DNA insert, the adjacent flanking DNA, and the 5' and 3' insert-to-flank junctions. This confirmed that the sequence and organization of the T-DNA insert is identical to the corresponding region in PV-ZMIR10871. The sequencing analysis, along with the NGS/JSA result showing that MON 87411 contains only a single DNA insert with no unintended fragments, also confirms that no vector backbone or other unintended plasmid sequences are present in MON 87411. Furthermore, the genomic organization at the insertion site was assessed by comparing the sequences flanking the T-DNA insert in MON 87411 to the sequence of the insertion site in conventional maize. This analysis also assessed potential rearrangements at the insertion site in MON 87411 upon T-DNA integration.

- Generational stability analysis by NGS/JSA demonstrated that the single T-DNA insert in MON 87411 has been maintained through five breeding generations, thereby confirming the stability of the intended T-DNA in MON 87411.

- Segregation analyses showed expected heritability that, along with NGS/JSA, demonstrated stability of the T-DNA insert across multiple generations.

Taken together, the characterization of the genetic modification in MON 87411 demonstrates that a single copy of the intended T-DNA was stably integrated at a single

locus of the maize genome and that no plasmid backbone sequences are present in MON 87411.

Data Confirms CP4 EPSPS and Cry3Bb1 Protein Safety

A multistep approach was used to characterize and assess the safety of the CP4 EPSPS and Cry3Bb1 proteins expressed in MON 87411. The expression levels of the CP4 EPSPS and Cry3Bb1 proteins in selected tissues of MON 87411 were determined and exposure to humans and animals through diet was evaluated. In addition, the donor organisms for the CP4 EPSPS and Cry3Bb1 protein coding sequences, *Agrobacterium* sp. strain CP4 and *Bacillus thuringiensis* ssp *kumamotoensis*, are ubiquitous in the environment and are not commonly known for human or animal pathogenicity or allergenicity. Bioinformatics analysis determined that the CP4 EPSPS and Cry3Bb1 proteins lack structural similarity to known allergens or protein toxins. As has been previously shown in safety assessments of other Roundup Ready and Cry3Bb1-containing crops, the CP4 EPSPS and Cry3Bb1 proteins are rapidly digested in simulated digestive fluids and demonstrate no acute oral toxicity in mice at the levels tested. Hence, the consumption of the CP4 EPSPS and Cry3Bb1 proteins from MON 87411 or its progeny poses no meaningful risk to human and animal health or an increased plant pest risk.

Data Confirms DvSnf7 RNA Safety

DvSnf7 RNA from MON 87411 is a dsRNA that upon consumption by western corn rootworm causes gene suppression of the targeted DvSnf7 gene. In MON 87411, the predominant RNA transcript produced from the suppression cassette was identified as being 968 nucleotides (nt) in length. Because of the extremely low expression of DvSnf7 RNA in MON 87411, it was necessary to produce RNA through *in vitro* transcription methods in order to obtain sufficient quantities of DvSnf7_968 RNA for subsequent safety studies. The molecular characteristics of the MON 87411 DvSnf7 RNA were determined and equivalence between MON 87411 DvSnf7 RNA and *in vitro*-produced DvSnf7_968 RNA was demonstrated. This equivalence justifies the use of the *in vitro*-produced DvSnf7_968 RNA as a test substance in studies assessing the specificity and potential impact of DvSnf7 RNA on non-target organisms. Tissue specific expression studies demonstrated that MON 87411 DvSnf7 RNA was expressed at mean levels ranging from 0.091×10^{-3} µg/g fw (in grain) to 14.4×10^{-3} µg/g fw (in over season leaf at growth stage V14-R1). Anticipated human dietary exposure to DvSnf7 RNA is also very low (≤ 0.4 ng/kg body weight per day) relative to estimated total daily RNA intake. Based on the ubiquitous nature of RNAi suppression utilizing dsRNA in a wide variety of consumed plant species, demonstration of the specificity of DvSnf7 suppression in CRW, the long history of safe consumption of RNA from a range of sources, and the apparent lack of toxicity or allergenicity of dietary RNA; the DvSnf7 RNAi suppression sequence used in MON 87411 poses no observed or theoretical risks to humans or animals. Therefore, the consumption of the DvSnf7 RNA from MON 87411 or its progeny is considered safe for humans and animals and poses no increased plant pest risk.

MON 87411 is Compositionally Equivalent to Conventional Maize

Compositional analysis was conducted on grain and forage of MON 87411, a conventional control and 20 different commercial reference hybrids grown at eight representative sites in a 2011/2012 field production in Argentina. Production in the U.S. corn belt and Argentina maize-growing regions occurs at relatively similar latitudes with an approximate 6 month offset. The average growing season temperatures and precipitation are comparable and as a result, maize hybrids developed in the U.S. are often used directly by farmers in the southern growing regions of Argentina. As such, compositional analyses from maize grown in Argentina are appropriate for a comparative safety assessment and study results are relevant to the use of this maize grown in the U.S.

The compositional analysis, based on the OECD consensus document for maize, included measurement of nutrients, anti-nutrients and secondary metabolites in conventional commercial reference hybrids to provide data on the natural variability of each compositional component analyzed. A total of 78 components were assayed (nine in forage and 69 in grain). Of the 78 components assayed, 18 had more than 50% of observations that were below the assay limit of quantitation and were therefore excluded from statistical analysis. Of the 60 remaining components statistically assessed, only 12 components (protein, histidine, tyrosine, oleic acid, neutral detergent fiber, copper, iron, manganese, zinc, niacin, vitamin B1 in grain, and ash in forage) showed a statistically significant difference between MON 87411 and the conventional control. For these 12 components, the mean difference in component values between MON 87411 and the conventional control, however, was less than the natural variation found within the conventional control and reference hybrid values. Additionally, MON 87411 mean component values were within the tolerance intervals of the reference hybrids, the values for maize observed in the literature, and/or the International Life Sciences Institute Crop Composition Database (ILSI-CCDB) values. These data indicated that the compositional components with statistically significant differences were not meaningful from a food and feed safety or nutritional perspective.

These results support the overall conclusion that MON 87411 was not a major contributor to variation in component levels in maize grain and forage, and confirmed the compositional equivalence of grain and forage from MON 87411 to conventional maize. These results support the overall food and feed safety and lack of plant pest risk of MON 87411.

MON 87411 Does Not Change Maize Plant Pest Potential or Environmental Interactions

Plant pest potential of a biotechnology-derived crop is assessed from the basis of familiarity that the USDA recognizes as an important underlying concept in risk assessment. The concept of familiarity is based on the fact that the biotechnology-derived plant is developed from a conventional plant hybrid or variety whose biological properties and plant pest potential are well known. Familiarity considers the biology of the plant, the introduced trait, the receiving environment, and the interactions among these factors. This provides a basis for comparative risk assessment between a

biotechnology-derived plant and the conventional control. Thus, the phenotypic, agronomic, and environmental interaction assessment of MON 87411 included a genetically similar conventional control as a comparator. This evaluation used a weight of evidence approach and considered statistical differences between MON 87411 and the conventional control with respect to reproducibility, magnitude, and directionality. Comparison to a range of commercial reference hybrids grown concurrently established the range of natural variability for maize, and provided a context from which to further evaluate any observed statistical differences. Characteristics assessed included: seed dormancy and germination, pollen morphology, and plant phenotypic observations and environmental interaction evaluations conducted in the field. The phenotypic, agronomic, and environmental interaction assessment demonstrated that MON 87411 is comparable to the conventional control. Thus, MON 87411 is not expected to have increased weediness or plant pest risk compared to conventional maize.

Seed dormancy and germination characterization indicated that MON 87411 seed had dormancy and germination characteristics similar to seed of the conventional control. In particular, the lack of hard seed, a well recognized seed characteristic associated with weediness, supports a conclusion of no increased weediness of MON 87411 compared to the conventional control. For pollen characteristic assessments, there were no statistically significant differences (α=0.05) detected between MON 87411 and the conventional control for pollen viability and diameter, and no visual differences in general pollen morphology were observed.

The field evaluation of phenotypic, agronomic, and environmental characteristics also supports the conclusion that MON 87411 is not likely to have increased weediness or plant pest potential compared to conventional maize. Evaluations were conducted at nine replicated field sites across the U.S. corn belt. These assessments included 13 plant growth and development characteristics, as well as observations for plant responses to abiotic stressors and plant-disease and plant-arthropod interactions. The observed phenotypic characteristics were comparable between MON 87411 and the conventional control. Across sites, data show no statistically significant differences between MON 87411 and the conventional control for any of the assessed characteristics, including early stand count, days to 50% pollen shed and silking, stay green, ear height, plant height, dropped ears, stalk and root lodging, final stand count, grain moisture, test weight, and yield. Thus, the phenotypic characteristics of MON 87411 were not altered in terms of pest/weed potential compared to the conventional control.

In an assessment of abiotic stress response and disease damage, no differences were observed between MON 87411 and the conventional control for any of the 100 comparisons for the assessed abiotic stressors or for any of the 119 comparisons for the assessed diseases among all observations across the sites. In an assessment of arthropod-related damage, no differences were detected between MON 87411and the conventional control for any of the 102 comparisons for the assessed arthropods. Additionally, no statistically significant differences were detected across sites between MON 87411 and the conventional control for quantitative evaluations of corn earworm or European corn borer damage. The lack of differences in plant response to abiotic stress, disease damage, and arthropod-related damage support the conclusion that the introduced traits in

MON 87411 are not expected to pose an increased plant pest/weed potential compared to the conventional control.

In an assessment of arthropod abundance collected using sticky traps, no statistically significant differences were detected between MON 87411 and the conventional control plots for 104 out of 108 comparisons among the collections at the four sites where these evaluations were made. The mean arthropod abundance values from MON 87411 were within the respective range of reference hybrids for one of the four detected differences. For the remaining three differences, the mean abundance values for MON 87411 were outside of the reference range; however, these differences were not consistent across collection times or sites. These results are not indicative of a consistent response associated with the traits and are not considered biologically meaningful in terms of increased pest/weed potential of MON 87411 compared to the conventional control.

In an assessment of arthropod abundance from visual counts, no statistically significant differences were detected between MON 87411 and the conventional control for 60 out of 61 comparisons among the collections at the four sites where these evaluations were made. For the single detected difference, the mean abundance value for MON 87411 was outside of the reference range; however, this difference was not consistent across collections. Thus, this difference was not indicative of a consistent response associated with the traits and is not considered biologically meaningful in terms of increased pest/weed potential of MON 87411 compared to the conventional control.

Separate studies demonstrated the efficacy of MON 87411 against two different CRW species and low root feeding damage ratings to MON 87411 hybrids in CRW-infested fields.

In summary, the phenotypic, agronomic, and environmental interaction data were evaluated to characterize MON 87411, and to assess whether the traits introduced in MON 87411 alter the plant pest potential compared to conventional maize. The evaluation, using a weight of evidence approach, considered the reproducibility, magnitude, and direction of detected differences between MON 87411 and the conventional control, and comparison to the range of the commercial reference hybrids. Results from the phenotypic, agronomic, and environmental interactions assessment indicate that MON 87411 does not possess enhanced weediness characteristics, increased susceptibility or tolerance to specific abiotic stressors, diseases, or arthropods, or characteristics that would confer a plant pest risk compared to conventional maize.

MON 87411 Will Not Negatively Affect Non-target Organisms Including Those Beneficial to Agriculture

An evaluation of the impacts of MON 87411 on non-target organisms (NTOs) is a component of the plant pest risk assessment. The NTO assessment has taken into consideration a number of characteristics of the expressed products in MON 87411 to evaluate potential hazards to NTOs, including threatened and endangered species and organisms beneficial to agriculture. Characteristics evaluated included MOA, spectrum of insecticidal activity and exposure levels to the CP4 EPSPS and Cry3Bb1 proteins and

DvSnf7 RNA. Both the CP4 EPSPS and Cry3Bb1 proteins have been assessed in multiple products by USDA-APHIS, U.S. FDA, and U.S. EPA in past years. Cry3Bb1 protein is produced in both MON 863 and MON 88017 that were granted non-regulated status and comparable levels of this protein are produced in MON 87411. Additionally, starting in 1994 with Monsanto's 40-3-2 soybean, a number of Roundup Ready crops (canola, maize, sugarbeet, cotton, alfalfa) containing CP4 EPSPS proteins have been granted non-regulated status by USDA-APHIS. After both extensive testing and widescale commercial cultivation, in no instance have adverse impacts to NTOs been associated with exposure to Cry3Bb1 or CP4 EPSPS proteins from these biotechnology-derived crops.

As noted previously, the suppression cassette in MON 87411 contains the CaMV *e35S* promoter, maize *hsp70* intron, the two DvSnf7^{p} sequences (240 nt each) separated by a 150 bp intervening sequence, and a pea *E9* 3' untranslated region. When the suppression cassette is transcribed, the predominant RNA transcript expressed is 968 nt in length and forms a hairpin loop, thereby allowing the formation of the 240 bp DvSnf7 dsRNA. When consumed by CRW, this 240 bp DvSnf7 dsRNA activates the RNAi process leading to suppression of the targeted CRW *Snf7* gene.

To address potential impacts to NTOs, specific laboratory bioassay studies using DvSnf7_240 dsRNA, the active insecticidal product in MON 87411, were conducted on a variety of NTOs including several Coleopteran, two Hymenopteran, one Hemipteran and four Lepidopteran species. No impacts to survival, growth, or development of these species were noted when fed extremely high doses (relative to levels present in MON 87411) of DvSnf7_240 dsRNA over multi-day bioassay periods.

Additional NTO assessments were conducted on a battery of organisms based on recommendations published by the U.S. EPA. Organisms tested included earthworm, honeybee, parasitic wasp, ladybird beetle, carabid beetle and the insidious flower bug. In these studies, test concentrations were based on the measured DvSnf7 RNA expression in the tissue types that the NTO would most likely be exposed to in the environment. Based on U.S. EPA recommendations, a targeted margin of exposure (MOE) of greater than 10-times the maximum expected environmental concentration (MEEC) was used to establish test concentrations. MOEs that exceed 10 are considered as indicative of minimal risk in worst-case laboratory assays by U.S. EPA. In all cases where MOEs could be calculated, they were >10-fold the predicted exposure level for these species, indicating that DvSnf7 RNA is not likely to have effects on terrestrial beneficial invertebrate species at field exposure levels.

Additional assessments for potential exposure of aquatic organisms and threatened and endangered species to DvSnf7 RNA contained in MON 87411 conclude that due to the lack of proximity of these organisms to maize cultivation, lack of relevant exposure because of feeding ecology and the restricted activity of the DvSnf7 RNA, that cultivation of MON 87411 will have no effect on these species or their habitats.

Deregulation of MON 87411 is Not Expected to Have Effects on Maize Agronomic Practices

An assessment of current maize agronomic practices was conducted to determine whether the cultivation of MON 87411 has the potential to impact current maize agronomic practices. Maize fields are typically highly managed areas that are dedicated to grain and/or forage production.

MON 87411 was developed to provide two effective MOAs against the target corn rootworm pests of maize in the U.S. corn belt as well as glyphosate tolerance in a single product. As tolerance to glyphosate and protection from corn rootworm complex pests are present in many currently available maize hybrids that have been widely grown in the U.S. since 2003, the introduction of MON 87411 is expected to have no impact on current agronomic or management practices for maize. As phenotypic evaluations, evaluations of stress responses, and pest/disease susceptibility showed no difference between MON 87411 and reference hybrids (other than protection from CRW larval feeding), no changes are anticipated in crop rotations, tillage practices, planting practices, fertility management, weed and disease management, and volunteer management from the introduction of MON 87411.

MON 87411 is similar to conventional maize in its agronomic, phenotypic, environmental, and compositional characteristics and has naturally occurring levels of protection against pests (other than CRW) and diseases comparable to and typical of conventional commercial maize hybrids. Based on this assessment, the introduction of MON 87411 is not expected to result in changes or impacts to current maize agronomic practices.

Conclusion

Based on the data and information presented in this petition, it is concluded that MON 87411 is not expected to be a plant pest. Therefore, Monsanto Company requests a determination from USDA-APHIS that MON 87411 and any progeny derived from crosses between MON 87411 and conventional maize or deregulated biotechnology-derived maize be granted nonregulated status under 7 CFR part 340.

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ABBREVIATIONS AND DEFINITIONS[1]

~	approximately
ADF	acid detergent fiber
ANOVA	analysis of variance
AOSA	Association of Official Seed Analysts
APHIS	Animal and Plant Health Inspection Service
APS	analytical protein standards
bp	base pairs
BSA	bovine serum albumin
Bt	*Bacillus thuringiensis*
bu/A	bushels per acre
bw	body weight
cDNA	complementary deoxyribonucleic acid
CEW	corn earworm
CFR	Code of Federal Regulations
CHT	ceramic hydroxyapatite
CP4 EPSPS	5-enolpyruvylshikimate-3-phosphate synthase protein from *Agrobacterium tumefaciens* strain CP4
CRW	corn rootworm
CTAB	hexadecyltrimethylammonium bromide
CV	coefficient of variation
DAP	days after planting
dATP	deoxyadenosine triphosphate
DDI	daily dietary intake
DEEM-FCID	Dietary Exposure Evaluation Model-Food Commodity Intake Database
DHB	2,5-dihydroxybenzoic acid
dNTP	deoxyribonucleotide
dsRNA	double stranded RNA
DTT	dithiothreitol
DvSnf7	*Snf7* gene from *Diabrotica virgifera virgifera* encoding the SNF7 subunit of the ESCRT-III complex
DvSnf7 RNA	RNA expressed from the suppression cassette that contains an inverted repeat sequence designed to match the western corn rootworm (WCR; *Diabrotica virgifera virgifera*) DvSnf7 gene
DvSnf7_240	the active insecticidal RNA in MON 87411
DvSnf7_968	an *in vitro* transcribed DvSnf7 single stranded RNA
DvSnf7^{p}	partial coding sequence of the *Snf7* gene from *Diabrotica virgifera virgifera* encoding the Snf7 subunit of the ESCRT-III complex
dw	dry weight
DWCF	dry weight conversion factor
ECB	European corn borer

[1] Alred, G.J., C.T. Brusaw, and W.E. Oliu. 2003. Handbook of Technical Writing, 7th edn., pp. 2-7. Bedford/St. Martin's, Boston, MA.

EDV	extended diapause variant
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
ESCRT	Endosomal Sorting Complex Required for Transport
EUP	experimental use permit
ETS	Excellence Through Stewardship
FA	fatty acid
FDA	U.S. Food and Drug Administration
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
FMOC	fluorenylmethyl chloroformate
FSE	farm scale evaluation
fw	fresh weight
GC	gas chromatography
Gb	gigabases
ha	hectare
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
HT	herbicide tolerance
ILSI CCDB	International Life Sciences Institute-Crop Composition Database
IPM	integrated pest management
IRM	insect resistance management
JSC	junction sequence class
kDa	kilodalton
kg/hl	kilograms per hectoliter
LOD	limit of detection
LOQ	limit of quantitation
MEEC	maximum expected environmental concentration
MESA	4-Morpholinepranesulfonic acid - ethylenediaminetetraacetic acid - sodium acetate
MFI	median fluorescence intensity
Mg/ha	megagrams/hectare
miRNA	micro RNA
MMT	million metric tons
MOA	mode-of-action
MOE	margin of exposure
MVB	multi-vesicular bodies
n	number of samples
NCR	northern corn rootworm
NDF	neutral detergent fiber
NFDM	nonfat dry milk
NGS/JSA	Next Generation Sequencing/Junction Sequence Analysis
NHANES	National Health and Nutrition Examination Survey
NOAEL	no observable adverse effect level
NOEC	no observable effect concentration
nt	nucleotide
NTO	non-target organism

OECD	Organisation for Economic Co-operation and Development
OM	organic matter
OPA	o-phthalaldehyde
OSL	over season leaf
OSR	over season root
OSWP	over season whole plant
PBST	phosphate buffered saline containing 0.05% (v/v) Tween
PCR	polymerase chain reaction
PIP	plant incorporated protectant
Poly(A)	multiple adenosine monophosphates
PPA	Plant Protection Act
PTH-AA	phenylthiohydantoin-amino acid
QC-	negative quality control
QC+	positive quality control
RDR	root damage rating
RH	relative humidity
RISC	RNA-induced silencing complexes
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
RT	room temperature
SAP	Scientific Advisory Panel
SBV	soybean variant
SCR	southern corn rootworm
SD	standard deviation
SDS	sodium dodecyl sulfate
S.E.	standard error
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
siRNA	small interfering RNA
sp.	species
TDF	total dietary fiber
T-DNA	transfer DNA
TFA	trifluoroacetic acid
TSSP	tissue-specific site pool
TTC	threshold of toxicological concern
Tz	tetrazolium
USDA	United States Department of Agriculture
UTR	untranslated region
UV	ultraviolet
v/v	volume to volume
WCR	western corn rootworm

I. RATIONALE FOR THE DEVELOPMENT OF MON 87411

I.A. Basis for the Request for a Determination of Nonregulated Status under 7 CFR § 340.6

The Animal and Plant Health Inspection Service (APHIS) of the United States (U.S.) Department of Agriculture (USDA) has responsibility, under the Plant Protection Act (Title IV Pub. L. 106-224, 114 Stat. 438, 7 U.S.C. § 7701-7772), to prevent the introduction and dissemination of plant pests into the U.S. APHIS regulation 7 CFR § 340.6 provides that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and no longer should be regulated. If APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article.

Monsanto Company is submitting this request to APHIS for a determination of nonregulated status for the new biotechnology-derived maize product, MON 87411, any progeny derived from crosses between MON 87411 and conventional maize, and any progeny derived from crosses of MON 87411 with biotechnology-derived maize that have previously been granted nonregulated status under 7 CFR Part 340.

I.B. Rationale for the Development of Insect-Protected and Glyphosate Tolerant Maize MON 87411

Maize (*Zea mays* L.) is the largest crop grown in the U.S. in terms of acreage planted and net value. In 2012, maize was planted on over 97 million acres and grain harvested from 87.4 million acres (USDA-NASS 2013a). Average yields in the previous five years ranged from 147 bushels per acre (bu/A) (2011) to 165 bu/A (2009) and were valued between $46.7 billion (2009) and $76.9 billion (2011) (USDA-NASS 2013d); however, in 2012, a widespread drought in the U.S. resulted in an average yield of only 123 bu/A, with a total production of about 10.8 billion bushels (USDA-NASS 2013a), valued at approximately $77 billion (USDA-NASS 2013c).

In 2012, approximately 85 million acres in the U.S. (or 88% of the total U.S. maize acreage) were planted with biotechnology-derived maize hybrids, and approximately 64 million acres (or 67% of the total maize acreage) were planted with maize hybrids containing insecticidal crystal (Cry) proteins derived from *Bacillus thuringiensis* (*Bt*) (USDA-ERS 2013). Of those 64 million acres, over 50 million acres were planted with combined-trait hybrids containing *Bt* and herbicide tolerance (HT) traits (USDA-ERS 2013). Since the early to mid-2000's, many of these combined-trait hybrids have contained multiple *Bt* genes with multiple modes-of-action (MOA) for robust and durable efficacy against a broad range of above-ground lepidopteran and below-ground coleopteran maize pests. Estimates are that approximately 50 million acres of corn rootworm (CRW)-protected hybrids were planted in the U.S. in 2011 (Marra, et al. 2012).

I.B.1 Benefits of Insect-Protection and Herbicide Tolerance Traits

The introduction of insect-protected and herbicide tolerant (HT) biotechnology-derived maize hybrids has been valuable to growers for two primary reasons: 1) they permit the in-crop application of broad-spectrum agricultural herbicides for effective weed control, which promotes the adoption of conservation tillage practices; and 2) they provide highly effective targeted pest control to manage some of a grower's most damaging maize pests.

The value of HT maize hybrids to growers has been demonstrated by the significant growth in acres planted to HT maize. In 2000, just 7% of maize acres were planted with hybrids containing a trait conferring herbicide tolerance, while that percentage had increased to 73% by 2012 (USDA-ERS 2013). Competition for light, nutrients, and moisture resources by weeds can lead to proportional and significant reductions in crop yield (Knake, et al. 1990). Numerous studies have shown that weed control early in the growing season is necessary to reduce yield losses in corn.

Insect-protected maize hybrids have also delivered significant value to growers as demonstrated by significant growth in insect-protected maize acres planted. In 2000, 19% of planted maize acres had insect-protection traits, while that percentage had increased to 67% by 2012 (USDA-ERS 2013). Included in that total are insect-protected products for above-ground lepidopteran control, below-ground coleopteran control and hybrids containing traits for control of both types of pests.

Of the several insect species that can cause damage to maize plants, the most damaging in major U.S. maize growing regions are larvae of the CRW complex[2] (*Diabrotica* spp., Coleoptera: Chrysomelidae) (Chandler, et al. 2008). The corn rootworm complex includes *Diabrotica* species that are significant pests of maize including western CRW (*D. virgifera virgifera*), northern CRW (*D. barberi*), and southern CRW (*D. undecimpunctata howardi*). These insect larvae damage maize by feeding on the roots, reducing the ability of the plant to absorb water and nutrients from the soil, and causing harvesting difficulties because of plant lodging (Riedell 1990; Spike and Tollefson 1991). CRW has long been described as the "billion-dollar pest" complex, based on costs associated with the application of soil insecticides and crop losses from pest damage (Mitchell, et al. 2004). As the adoption of CRW-protection traits has increased from its first plantings in 2003 to approximately 50 million acres in 2011 (Marra et al. 2012), the use of these traits has led to the decreased use of conventional insecticides for CRW control by more than 75% (U.S. EPA 2011).

[2] The corn rootworm complex includes *Diabrotica* species that are significant pests of maize including western CRW (*D. virgifera virgifera*), northern CRW (*D. barberi*), and southern CRW (*D. undecimpunctata howardi*).

I.B.2. Introduction of Insect-Protection Traits in the U.S.

Biotechnology traits providing protection against CRW were first introduced in the United States in 2003 when YieldGard® Rootworm (MON 863 that expresses Cry3Bb1 protein) maize hybrids were commercialized. This trait provided a highly effective solution for CRW control. Shortly after YieldGard® Rootworm was launched, other products providing CRW protection from Dow AgroSciences (Herculex®/DAS 59122 that expresses Cry34/35Ab1 proteins) and Syngenta (Agrisure®/MIR 604 that expresses a modified Cry3A protein (mCry3A)) were introduced (Marra et al. 2012). Following the development and introduction of YieldGard® Rootworm maize hybrids, Monsanto introduced YieldGard VT Rootworm/RR2® (MON 88017 that expresses Cry3Bb1 and CP4 EPSPS proteins) with genes for both CRW protection and Roundup® herbicide tolerance in a single product. That introduction was followed shortly thereafter by combined-trait hybrids containing *Bt* proteins for both above-ground lepidopteran and below-ground coleopteran (CRW) pest control, as well as herbicide tolerance (YieldGard VT Triple®, Genuity® VT Triple PRO®, and Genuity® Smartstax®). Monsanto's YieldGard VT Triple (MON 88017 × MON 810) contains single *Bt* proteins to control targeted lepidopteran (Cry1Ab) and CRW (Cry3Bb1) maize pests while Monsanto's Genuity VT Triple PRO (MON 88017 × MON 89034) provides two *Bt* proteins with two MOAs (Cry1A.105 and Cry2Ab2) for lepidopteran pests and a single *Bt* protein (Cry3Bb1) for CRW control. The joint introduction of SmartStax maize hybrids by Monsanto and Dow AgroSciences introduced the first maize hybrids with six *Bt* proteins (Cry1A.105, Cry2Ab2, Cry1F, Cry34/35Ab1, and Cry3Bb1), three effective MOA against the primary lepidopteran pests of maize in the U.S. corn belt, and two effective MOA against the primary CRW pests of maize in the U.S. corn belt. These two MOA provided in MON 87411 (from Cry3Bb1 protein and DvSnf7 RNA) can be expected to improve the durability of CRW-protection traits and extend the useful lifetime of these products (Bates, et al. 2005; Roush 1998). All of these noted CRW-protected products have provided highly effective control of CRW across wide growing regions in the U.S.

I.B.3. Development of CRW-Protected and Glyphosate Tolerant Maize MON 87411

In its continuing efforts to provide highly effective, durable control of CRW for its customers, Monsanto Company has developed biotechnology-derived maize MON 87411 that confers protection against CRW (*Diabrotica* spp.) and tolerance to the herbicide glyphosate. MON 87411 builds upon current *Bt* protein-based CRW control technology by introducing a new MOA based on RNA-mediated gene suppression (RNAi) that offers increased control of target insect pests and will prolong the durability of existing CRW-controlling *Bt* technologies.

® YieldGard and YieldGard VT Rootworm/RR2 are registered trademarks of Monsanto Technology LLC. Herculex is a registered trademark of Dow AgroSciences LLC. Agrisure is a registered trademark of Syngenta Participations AG.

® YieldGard VT Triple, Genuity VT Triple Pro, Genuity SmartStax, and Roundup are registered trademarks of Monsanto Technology LLC.

MON 87411 contains a suppression cassette that expresses an inverted repeat sequence designed to match the sequence in western corn rootworm (WCR; *Diabrotica virgifera virgifera*). The expression of the suppression cassette results in the formation of a double-stranded RNA (dsRNA) transcript containing a 240 bp fragment of the WCR *Snf7* gene (DvSnf7). Upon consumption, the plant-produced dsRNA in MON 87411 is recognized by the CRW's RNA interference (RNAi) machinery (Hammond 2005; Ketting and Plasterk 2004; Tomari and Zamore 2005) resulting in the down-regulation of the targeted DvSnf7 gene leading to CRW mortality (Bolognesi, et al. 2012). MON 87411 also produces a modified *Bacillus thuringiensis* (subsp. *kumamotoensis*) Cry3Bb1 protein to protect against CRW larval feeding. In addition, MON 87411 contains the *cp4 epsps* gene from *Agrobacterium* sp. strain CP4 that encodes for the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) protein, which confers tolerance to glyphosate, the active ingredient in Roundup agricultural herbicides.

MON 87411 will provide benefits to growers similar to those obtained by use of existing CRW-protected maize hybrids, which includes reduced use of insecticides, increased yield protection, water conservation, and increased worker safety (Rice 2004). MON 87411 is also glyphosate tolerant and will continue to provide benefits associated with conservation tillage methods, including reduced soil erosion, reduced fuel and labor costs, improved air quality and conservation of soil moisture (CTIC 2011; Hurley, et al. 2009; Towery and Werblow 2010).

MON 87411 will not be offered for commercial use as a stand-alone product, but will be combined, through traditional breeding methods, with other deregulated biotechnology-derived traits to provide protection against both above-ground and below-ground maize pests as well as tolerance to multiple herbicides. These next generation combined-trait maize products will offer the ability to maximize grower choice, improve production efficiency, increase pest control durability, and improve grower profit potentials.

I.C. RNA Interference (RNAi)

I.C.1. Applications of RNAi in Plants

Naturally occurring RNA-mediated gene suppression (RNAi) in plants has been previously documented and includes selection for soybean seed coat color (Tuteja, et al. 2004) and maize stalk color (Della Vedova, et al. 2005). In both of these instances, production of chalcone synthase was suppressed leading to significantly decreased pigmentation in soybean seed coats and maize stalks, respectively. In addition, a low glutelin rice variety has been studied and has been determined to result from production of a dsRNA and concomitant suppression of glutelin genes (Kusaba, et al. 2003). RNA-mediated gene suppression has also been used in a number of biotechnology-derived food crops that have previously been deregulated by USDA or other regulatory authorities including virus resistant papaya, squash, potato, common bean, and plum as well as a delayed ripening tomato and a soybean with altered oil composition (Parrott, et al. 2010). Safety assessments have been conducted (Parrott et al. 2010; Petrick, et al. 2013) and global regulatory approvals have been obtained for products employing RNAi gene suppression.

I.C.2. Applications of RNAi in Insects

RNAi can also achieve gene silencing in susceptible insects following ingestion of dsRNAs (Baum, et al. 2007a; Terenius, et al. 2011; Whyard, et al. 2009). Insect control products can be developed utilizing RNAi sequence-specific gene silencing to suppress genes critical for insect survival. Because of this sequence-specific gene silencing, these products have the potential to selectively target a narrow group of closely related pest species and greatly reduce the likelihood of adverse effects on non-target organisms (NTOs), including those beneficial to agriculture. The spectrum of activity for DvSnf7 dsRNA has been shown to be narrow and activity is only evident in a subset of beetles within the Galerucinae subfamily of Chrysomelidae within the Order Coleoptera (Bachman, et al. 2013), as described in more detail in Section VI.E below.

I.D. Modes-of-Action of the Inserted Genetic Components

I.D.1. Mode-of-Action of the RNAi Component of MON 87411

MON 87411 contains a DvSnf7 suppression cassette that expresses an inverted repeat sequence designed to match the sequence in WCR and thereby utilizes the RNAi pathway to control CRW (*Diabrotica* spp.). The expression of the suppression cassette results in the formation of a dsRNA transcript containing a 240 bp fragment of the WCR *Snf7* gene (DvSnf7). Upon consumption of MON 87411 by WCR, DvSnf7 dsRNA is recognized by the pest's RNAi machinery, resulting in the down-regulation of the targeted DvSnf7 gene leading to WCR mortality (Bolognesi et al. 2012) (Figure I-1).

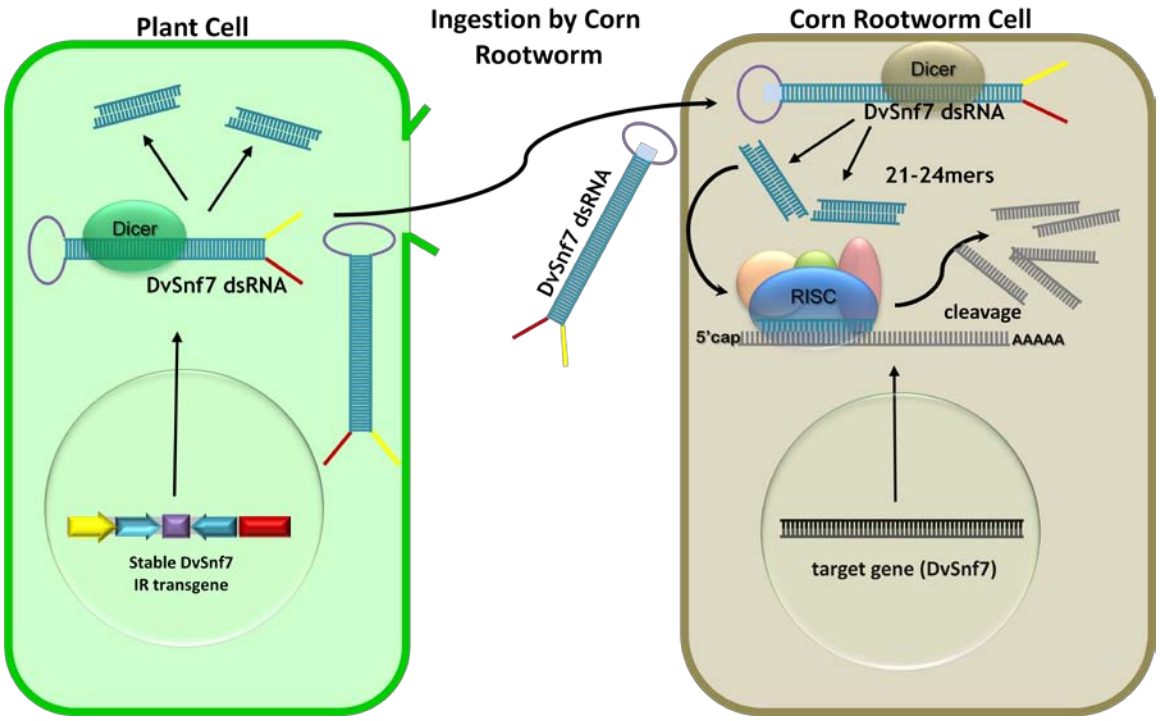

Figure I-1. Diagram of MON 87411 dsRNA Oral Delivery to Suppress DvSnf7 Expression via the RNAi Pathway in CRW

The RNAi pathway is a natural process in eukaryotic organisms for the regulation of endogenous gene expression (Dykxhoorn, et al. 2003; Parrott et al. 2010). The dsRNA molecule that activates the mechanism is first processed by a class of RNase III enzymes called Dicers into small interfering RNAs (siRNAs, ~21-25 nucleotides) (Hammond 2005; Siomi and Siomi 2009; Zamore, et al. 2000). The resulting siRNA molecules are then incorporated into multiprotein RNA-induced silencing complexes (RISC), which facilitate complementary sequence recognition and mRNA cleavage that leads to specific suppression of the target mRNA (Hammond 2005; Tomari and Zamore 2005). In the case of CRW that consume MON 87411, the DvSnf7 gene in CRW is suppressed.

DvSnf7 was selected as the target mRNA in CRW due to its vital cellular function that can be suppressed at relatively low concentrations when targeted by dsRNA (Baum et al. 2007a). Snf7 is a class E vacuolar sorting protein and belongs to the Endosomal Sorting Complex Required for Transport (ESCRT)–III complex, which has been shown to be involved in sorting of transmembrane proteins enroute to lysosomal degradation through the endosomal-autophagic pathway in a number of organisms (Kim, et al. 2011; Lee and Gao 2008; Rusten, et al. 2008; Teis, et al. 2008; Vaccari, et al. 2009) (Figure I-2). ESCRT-III components play critical roles in distinct steps of this pathway (Henne, et al. 2011; Roxrud, et al. 2010). Data have shown that suppression of DvSnf7 in WCR leads

to accumulation of ubiquitinated proteins[3] destined for lysosomal degradation (Ramaseshadri, et al. 2013). Sorting of transmembrane proteins is critical to regulate signal transduction in cells and as such, suppression of this sorting through the ESCRT-III complex impairs cell homeostasis and functioning, leading to cellular death and CRW mortality. We have also shown systemic spread of the RNAi effect to tissues distal to the WCR midgut (Bolognesi et al. 2012). Similar lethal phenotypes resulting from knockdown of *Snf7* have been shown in *Drosophila* (Sweeney, et al. 2006) and *C. elegans* (Michelet, et al. 2010).

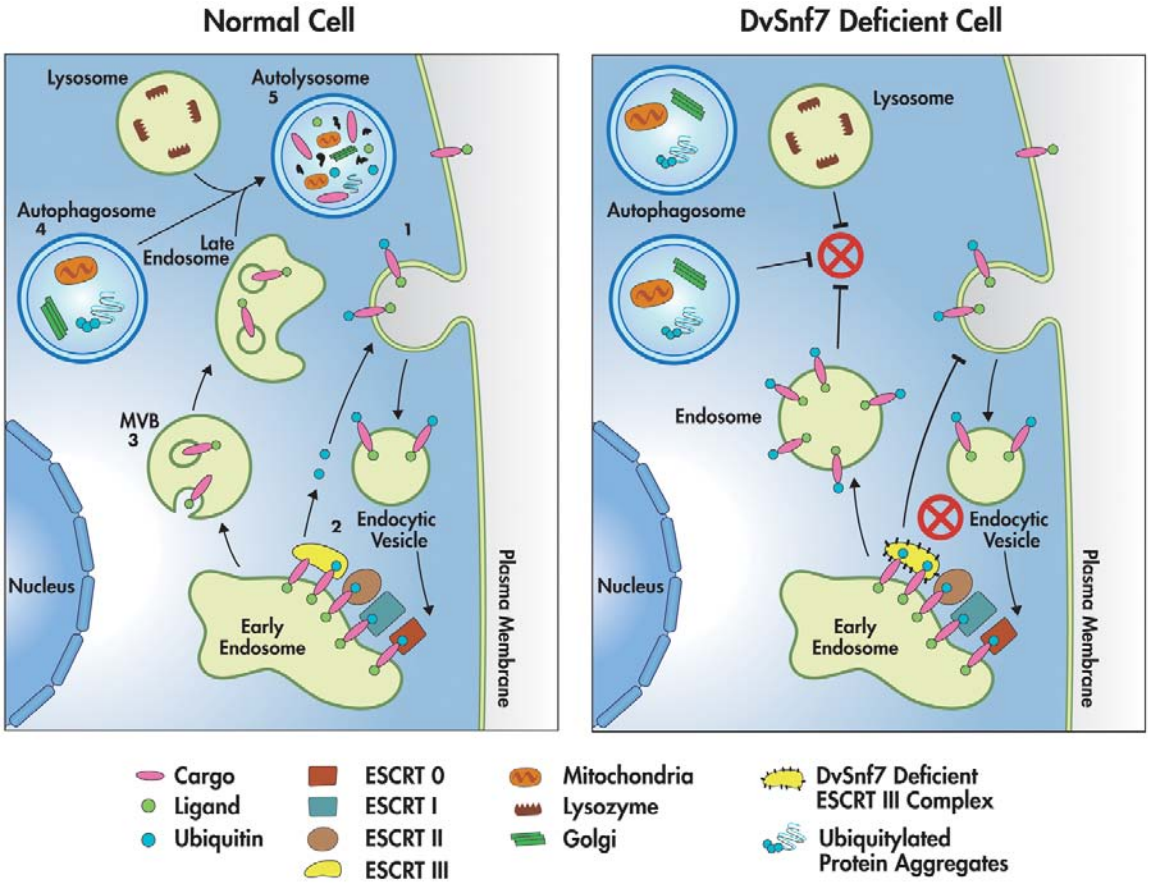

Figure I-2. Model depicting endosomal-autophagy pathway involved in intracellular sorting and degradation of receptors along with other macromolecules in a normal cell (left) and a DvSnf7 deficient cell (right).

In the normal cell, internalization and ubiquitination of cargo proteins (1) de-ubiquitination of cargo proteins (2), biogenesis of multi-vesicular bodies (MVB) (3), formation of autophagosomes engulfing macromolecules (4), and fusion of late endosomes, autophagosome and lysosomes into autolysosomes for degradation of cargo proteins and macromolecules (5) are depicted. In the

[3] Ubiquitination is a post-translational modification of a protein in which one or more ubiquitin molecules are added to the protein (Pickart 2001). Ubiquitins are small regulatory proteins found in all eukaryotic cells and their addition to a protein often leads to degradation of that protein. This process of ubiquitination and protein degradation allows the cell to modulate the concentration of essential proteins within that cell.

DvSnf7 deficient cell, the impairment of de-ubiquitination, accumulation of autophagosomes, and failure of fusion of endosomes, autophagosomes and lysosomes and autolysosomal activity are highlighted (Ramaseshadri et al. 2013).

Induction of RNAi-mediated gene suppression in insects via an oral route of exposure requires efficient uptake of dsRNAs by midgut cells followed by suppression of the target mRNA leading to significant effects on growth, development and survival. In the case of WCR, only the relatively long dsRNA (*e.g.*, DvSnf7 240-mer) is taken up by the insect and significant biological activity was only observed with dsRNA sequences ≥ 60 bp (Bolognesi et al. 2012). Finally, several key points have been identified in demonstrating efficacy of MON 87411 against WCR: 1) oral delivery/uptake of dsRNA into WCR gut cells, 2) suppression of the targeted DvSnf7 mRNA expression followed by suppression of the production of the DvSnf7 protein, 3) systemic spread of suppression of DvSnf7 expression beyond WCR midgut tissues, and 4) growth inhibition and WCR mortality (Bolognesi et al. 2012).

I.D.2. Modes-of-Action of the CP4 EPSPS and Cry3Bb1 Proteins

MON 87411 contains the identical CP4 EPSPS protein that is expressed in MON 88017 maize (USDA-APHIS Petition No. 04-125-01p) and numerous other Roundup Ready crops (maize, cotton, soybean, canola, alfalfa, sugar beet). The CP4 EPSPS protein is structurally similar and functionally identical to endogenous plant EPSPS enzymes, but has a much reduced affinity for glyphosate relative to endogenous plant EPSPS (Padgette, et al. 1996). In MON 87411, as in other Roundup Ready plants, aromatic amino acids and other metabolites necessary for plant growth and development are produced by the continued action of the CP4 EPSPS enzyme in the presence of glyphosate (Padgette et al. 1996).

MON 87411 also contains an expression cassette that codes for the same Cry3Bb1 protein as the expression cassette that is present in MON 88017 maize (USDA-APHIS Petition No. 04-125-01p) that was granted non-regulated status by USDA-APHIS in 2006 (USDA-APHIS 2013). The amino acid sequence deduced from the Cry3Bb1 expression cassettes of MON 87411 and MON 88017 is also 99.8% identical to the deduced amino acid sequence for Cry3Bb1 protein in MON 863 (USDA-APHIS Petition No. 01-137-01p) that was granted non-regulated status by USDA-APHIS in 2002 (USDA-APHIS 2013). The use of *Bt*-containing crops in U.S. agriculture has been widespread and the mode-of-action (*i.e.*, solubilization of Cry protein, processing to the active form, binding to midgut receptors and insertion of the toxin into cellular membranes) and specificity of *Bt* proteins has been studied extensively and is well understood (Gill, et al. 1992; Whalon and Wingerd 2003).

I.E. Product Efficacy

Monsanto conducted field trials in 2011 and 2012 to assess the efficacy of MON 87411 in reducing root damage caused by CRW larvae. In both years, MON 87411 was compared to genetically similar control hybrids (one parent of each hybrid was LH244) which also contained biotechnology-derived MON 89034 expressing two Cry proteins

(Cry1A.105 and Cry2Ab2) for protection against above-ground lepidopteran pests. Cry1A.105 and Cry2Ab2 are not active against Coleopteran insects and therefore do not impact root feeding by CRW larvae. Data were collected from replicated blocks at nine trial locations in 2011 and five locations in 2012 from maize production regions in Iowa, Illinois, and Indiana.

In both years, when plants reached the V2 growth stage, five plants per plot were infested with corn rootworm eggs at a rate of over 3200 eggs per plant. At the V10 growth stage, these five plants were dug and the roots were washed and evaluated for feeding damage. The evaluations were based on a root damage rating (RDR) (Oleson, et al. 2005) scale of 0 to 3, where 0 is no root damage detected and a 3 is where all three below-ground nodes are completely missing or totally damaged.

In 2011 trials, the average RDR across all 9 locations for control hybrids not containing Cry3Bb1 or DvSnf7 dsRNA was 1.5. Damage ratings across these locations ranged from 0.9 to 2.4. These ratings are indicative of the relatively high rootworm pressure overall. The average RDR for MON 87411 hybrids was 0.13, demonstrating significant efficacy against larval CRW feeding. In 2012 trials, the average RDR across the 5 locations for control hybrids was 1.06, confirming significant rootworm pressure. Averaged RDR's across these sites for MON 87411 hybrids in these trials was 0.07, again demonstrating significant efficacy against larval CRW feeding.

I.F. Submissions to Other Regulatory Agencies

Under the Coordinated Framework for Regulation of Biotechnology (CFR) (USDA-APHIS 1986), the responsibility for regulatory oversight of biotechnology-derived crops falls primarily on three U.S. agencies: U.S. Food and Drug Administration (FDA), the United States Department of Agriculture (USDA), and in the case of plant incorporated protectants (PIPs), the Environmental Protection Agency (EPA). Deregulation of MON 87411 by USDA constitutes only one component of the overall regulatory oversight and review of this product. As a practical matter, MON 87411 cannot be released and marketed until FDA, EPA, and USDA have completed their reviews and assessments under their respective jurisdictions.

I.F.1. Submission to FDA

MON 87411 falls within the scope of the 1992 FDA policy statement concerning regulation of products derived from new plant varieties, including those developed through biotechnology (U.S. FDA 1992). In compliance with this policy, Monsanto submitted a food/feed safety and nutritional assessment summary document to FDA in November 2013.

I.F.2. Submission to EPA

Substances that are pesticides, as defined under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) [7 U.S.C. §136(u)], are subject to regulation by the EPA. Pesticides produced *in planta*, referred to as PIPs, are also subject to regulation by the EPA under FIFRA.

Pursuant to §408(d) of the Federal Food Drug and Cosmetic Act [21 U.S.C. 346 a(d)], Monsanto Company petitioned EPA for an exemption from the requirement of a tolerance for the PIP *Bacillus thuringiensis* Cry3Bb1 protein in or on all food and feed commodities of field corn, sweet corn, and popcorn and the genetic material necessary for its production in these products in 1997. On March 31, 2004, the EPA established a permanent exemption from the requirement of a tolerance for the PIP *Bacillus thuringiensis* Cry3Bb1 protein and the genetic material necessary for its production in food and feed commodities of field corn, sweet corn and popcorn (40 CFR § 180.1214). Additionally, and applicable to MON 87411, is EPA's establishment of an exemption from the requirement of a tolerance for residues of nucleic acids that are part of a plant-incorporated protectant (40 CFR § 174.475).

On May 4, 2012, Monsanto filed an experimental use permit (EUP) application for MON 87411 and the genetic material necessary for its production with the U.S. EPA to facilitate MON 87411 field testing and safety evaluations. EPA granted the EUP (524-EUP-104) on March 1, 2013. Monsanto will make an application to the EPA for a Breeding Registration for MON 87411 and the genetic material (PV-ZMIR10871) necessary for its production in maize in the near future. Additionally, Monsanto will make the appropriate Section 3 registration application(s) when final decisions about specific stacked maize products (breeding stacks) are made.

I.F.3. Submissions to Foreign Government Agencies

Consistent with our commitments to the Biotechnology Industry Organization's Excellence Through Stewardship® (ETS) Program[4], Monsanto intends to obtain the appropriate approvals from all key maize import markets with functioning regulatory systems prior to commercial planting of MON 87411. As appropriate, notifications will be made to countries that import significant quantities of maize and maize products and do not have formal regulatory review processes for biotechnology-derived crops.

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[4] http://www.excellencethroughstewardship.org/.

II. THE BIOLOGY OF MAIZE

The Organisation for Economic Co-Operation and Development (OECD) Consensus Document on the biology of maize (OECD 2003) provides key information on:

- general description of maize biology, including taxonomy and morphology and use of maize as a crop plant
- agronomic practices in maize cultivation
- geographic centers of origin
- reproductive biology
- cultivated maize as a volunteer weed
- inter-species/genus introgression into relatives and interactions with other organisms
- a summary of the ecology of maize

Additional information on the biology and uses of maize can also be found on the Australian Government Department of Health and Ageing (Office of the Gene Technology Regulator) web site (OGTR 2008), and in the USDA-ARS GRIN database (USDA-ARS 2013).

To support the evaluation of the plant pest potential of MON 87411 relative to conventional maize, additional information regarding several aspects of maize biology can be found elsewhere in this petition. This includes: agronomic practices for maize in Section IX; volunteer management of maize in Section IX.H; and inter-species/genus introgression potential in Section X.I.

II.A. Maize as a Crop

Maize is grown in nearly all areas of the world and is the largest cultivated crop in the world followed by wheat (*Triticum* sp.) and rice (*Oryza sativa* L.) in total global metric ton production. In 2012, maize was planted globally on ~174 million hectares (ha) with a total grain production of an estimated 854 million metric tons (MMT) (USDA-FAS 2013). The top five production regions in 2012 were: USA (274 MMT), China (208 MMT), Brazil (73 MMT), EU-27 (55 MMT), and Argentina (27 MMT) (USDA-FAS 2013). In the U.S., maize is grown in almost every state and in 2012, its production value of over $77 billion was the highest of any crop (USDA-NASS 2013c).

In industrialized countries maize has two major uses: (1) as animal feed in the form of grain, forage or silage; and (2) as a raw material for wet- or dry-milled processed products such as high fructose maize syrup, oil, starch, glucose, dextrose and ethanol. By-products of the wet- and dry- mill processes are also used as animal feed. These processed products are used as ingredients in many industrial applications and in human food products. Most maize produced in industrialized countries is used as animal feed or for industrial purposes, but maize remains an important food staple in many developing regions, especially sub-Saharan Africa and Central America, where it is frequently the mainstay of human diets (Morris 1998).

Maize is a very familiar plant that has been rigorously studied due to its use as a staple food/feed and the economic opportunity it brings to growers. The domestication of maize

likely occurred in southern Mexico between 7,000 and 10,000 years ago (Goodman 1988). While the putative progenitor species of maize have not been recovered, it is likely that teosinte played an important role in contributing to the genetic background of maize. Although grown extensively throughout the world, maize is not considered a persistent weed or a plant that is difficult to control. Maize, as we know it today, cannot survive in the wild because the female inflorescence (the ear) is covered by a husk thereby restricting seed dispersal, it has no seed dormancy, and is a poor competitor in an unmanaged ecosystem. The transformation from a wild, weedy species to one dependent on humans for its survival most likely evolved over a long period of time through plant breeding by the indigenous inhabitants of the Western Hemisphere. Today, virtually all the maize grown in the U.S. is a hybrid, a production practice that started in the 1930's (Wych 1988). Maize hybrids are developed and used based on the positive yield increases and plant vigor associated with heterosis, also known as hybrid vigor.

Conventional plant breeding results in desirable characteristics in a plant through the unique combination of genes already present in the plant. However, there is a limit to genetic diversity with conventional plant breeding. Biotechnology, as an additional tool to conventional breeding, offers access to greater genetic diversity than conventional breeding alone, resulting in expression of highly desirable traits that are profitable to growers.

II.B. Characteristics of the Recipient Plant

The transformation for MON 87411 was conducted with inbred maize line LH244, a patented maize line assigned to Holden's Foundation Seeds LLC in 2001 (U.S. patent #6,252,148). LH244 is a medium season yellow dent maize line with a Stiff Stalk background that is best adapted to the central regions of the U.S. corn belt. LH244 was initiated from a single cross of LH197 × LH199 followed by a backcross to LH197. The F_2 combination ((LH197 × LH197) × LH199) was then selfed and used in the development of LH244.

Following transformation of immature LH244 embryos, a single transformed plant was selected and self-crossed to increase seed supplies. A homozygous inbred line was developed though further self-crossing and selection and was then used to produce other lines which were used for product testing, safety assessment studies, and commercial production.

II.C. Maize as a Test System in Product Safety Assessment

Based on seed availability and appropriate fit for various studies, hybrid maize lines MPA640B (LH244 × LH287) and NL6169 (LH244 × HCL645) were used as near isogenic, conventional controls for this submission (hereafter referred to as conventional controls). As noted, one parent of each of these control maize lines is LH244, the inbred from which MON 87411 is derived, while the other parents (LH287 and HCL645) are other maize inbreds. As such, both of these maize lines constitute relevant comparators for MON 87411. In addition, other commercial maize hybrids (hereafter referred to as reference hybrids) were used to establish ranges of natural variability representative of commercial maize hybrids. Reference hybrids used at each field trial location were selected based on their availability and agronomic fit for the respective geographic

regions. Both MPA640B and NL6169 were used in molecular characterization studies. NL6169 was used as the conventional control in compositional analysis while MPA640B was used in phenotypic, agronomic and environmental interactions assessments. Where appropriate, reference hybrids were used to establish a range of variability or responses representative of commercial maize in the U.S.

In developing the data to support this petition, appropriate MON 87411 test materials were generated for the molecular characterization (Sections III and IV), protein characterization and expression analysis (Section V), RNA characterization and expression (Section VI), compositional analysis (Section VII), and phenotypic, agronomic and environmental interactions assessment (Section VIII). The full molecular characterization studies (NGS/JSA) were conducted with the R_4 generation (Figure IV-4). Initiation of commercial breeding efforts was conducted with the R_5 generation (Figure IV-4). Protein and RNA characterization and expression analysis, composition analysis, and phenotypic, agronomic and environmental interactions assessment were conducted with various MON 87411 breeding generations as noted in the Breeding Tree (Figure IV-4).

III. DESCRIPTION OF THE GENETIC MODIFICATION

MON 87411 was developed through *Agrobacterium tumefaciens*-mediated transformation of maize immature embryos from line LH244 utilizing PV-ZMIR10871. This section describes the plasmid vector, the donor gene, and the regulatory elements used in the development of MON 87411 as well as the deduced amino acid sequence of the Cry3Bb1 protein and CP4 EPSPS protein produced in MON 87411. In this section, transfer DNA (T-DNA) refers to DNA that is transferred to the plant during transformation. An expression (or suppression) cassette is comprised of sequences to be transcribed and the regulatory elements necessary for the expression of those sequences.

III.A. The Plasmid Vector PV-ZMIR10871

PV-ZMIR10871 was used in the transformation of maize to produce MON 87411 and its plasmid map is shown in Figure III-1. The elements included in this plasmid vector are described in Table III-1. PV-ZMIR10871 is approximately 16.5 kb and contains one T-DNA that is delineated by Left and Right Border regions. The T-DNA contains the DvSnf7 suppression cassette, the *cry3Bb1* expression cassette, and the *cp4 epsps* expression cassette. The DvSnf7 suppression cassette is regulated by the *e35S* promoter from the 35S RNA of cauliflower mosaic virus (CaMV), the heat shock protein 70 (*Hsp70*) intron from *Zea mays*, and the 3' untranslated sequence of the *E9* gene from *Pisum sativum*. The *cry3Bb1* expression cassette is regulated by the *pIIG* promoter from *Zea mays*, the chlorophyll a/b binding protein (CAB) leader from *Triticum aestivum*, the *Ract1* intron from *Oryza sativa*, and the heat shock protein 17 (*Hsp17*) 3′ untranslated region from *Triticum aestivum*. The *cp4 epsps* expression cassette is regulated by the *TubA* promoter from *Oryza sativa*, the *TubA* leader from *Oryza sativa*, the *TubA* intron from *Oryza sativa*, the *CTP2* targeting sequence from *Arabidopsis thaliana*, and the *TubA* 3′ untranslated region from *Oryza sativa*.

The backbone region of PV-ZMIR10871, located outside of the T-DNA, contains two origins of replication for maintenance of the plasmid vector in bacteria (*ori V*, *ori-pBR322*), a bacterial selectable marker gene (*aadA*), and a coding sequence for repressor of primer (ROP) protein for maintenance of plasmid vector copy number in *Escherichia coli* (*E. coli*). A description of the genetic elements and their prefixes (*e.g.*, B-, P-, L-, I-, TS-, CS-, T-, and OR-) in PV-ZMIR10871 is provided in Table III-1.

III.B. Description of the Transformation System

MON 87411 was developed through *Agrobacterium*-mediated transformation of immature maize embryos based on the method described by Sidorov and Duncan (2009), utilizing PV-ZMIR10871. Immature embryos were excised from a post-pollinated maize ear of LH244. After co-culturing the excised immature embryos with *Agrobacterium* carrying the plasmid vector, the immature embryos were placed on selection medium containing glyphosate and carbenicillin disodium salt in order to inhibit the growth of untransformed plant cells and excess *Agrobacterium*. Once transformed callus developed, the callus was placed on media conducive to shoot and root development. The rooted plants (R_0) with normal phenotypic characteristics were selected and transferred to soil for growth and further assessment. As demonstrated in this petition,

the use of disarmed *Agrobacterium tumefaciens* strain ABI, a designated plant pest, as the transformation vector has not imparted plant pest characteristics to MON 87411.

The R_0 plants generated through the transformation process described above had already been exposed to glyphosate in the selection medium and demonstrated glyphosate tolerance. The R_0 plants self-pollinated to produce R_1 seed and R_1 plants were evaluated for the presence of the T-DNA via quantitative polymerase chain reaction (PCR) analysis. The R_1 plants homozygous for the T-DNA were selected for further development and their progenies were subjected to further molecular and phenotypic assessments. As is typical of a commercial event production and selection process, hundreds of different transformation events (regenerants) were generated in the laboratory using PV-ZMIR10871. After many months of careful selection and evaluation of these hundreds of events in the laboratory, greenhouse and field, MON 87411 was selected as the lead event based on superior agronomic, phenotypic, and molecular characteristics. Studies on MON 87411 were initiated to further characterize the genetic insertion and the expressed products, and to establish the food, feed, and environmental safety relative to commercial maize. The major steps involved in the development of MON 87411 are depicted in Figure III-2.

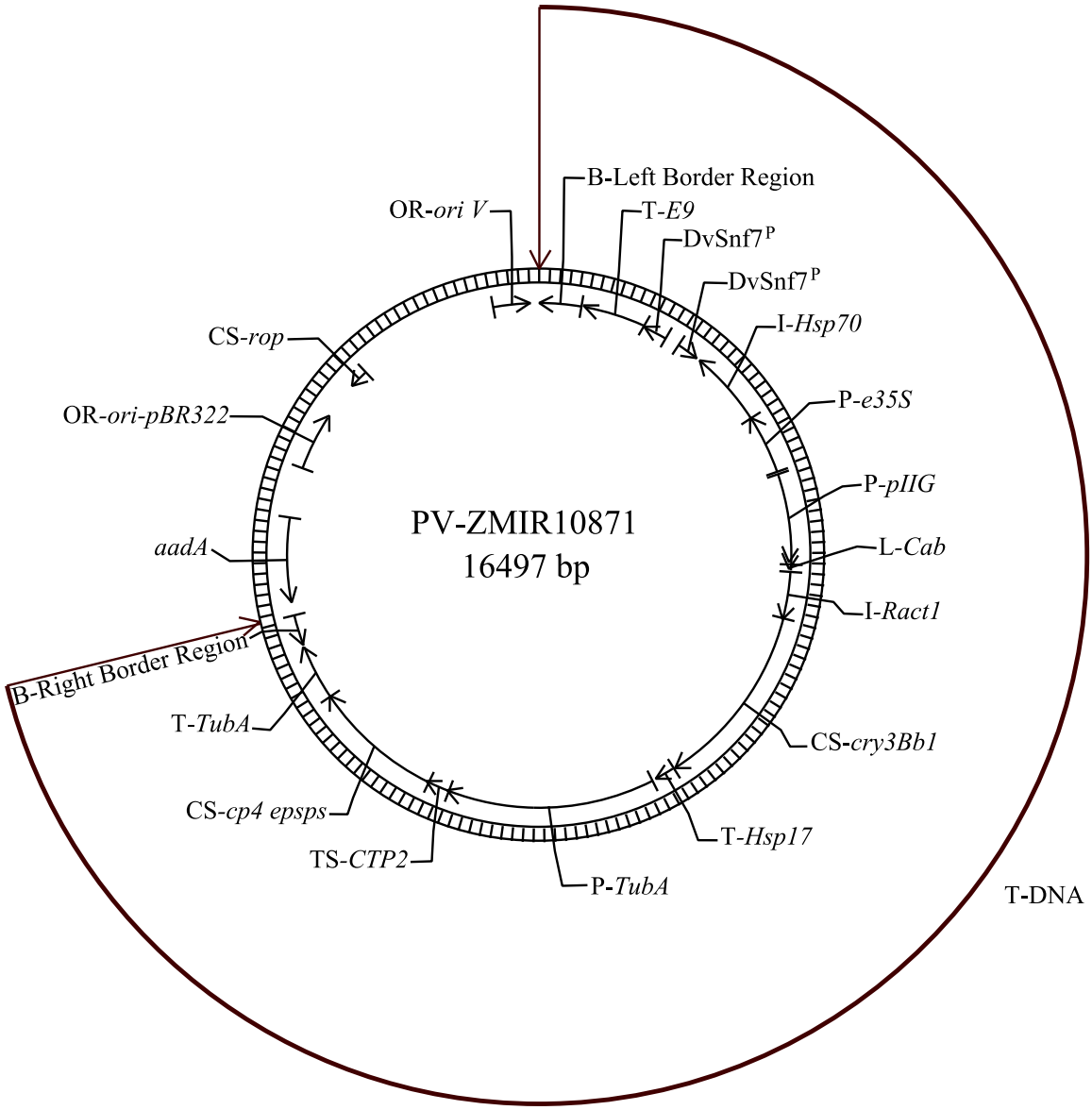


Figure III-1. Circular Map of PV- ZMIR10871
A circular map of the plasmid vector PV-ZMIR10871 used to develop MON 87411 is shown. PV-ZMIR10871 contains a single T-DNA. Genetic elements are shown on the exterior of the map. [P] Superscript in DvSnf7 indicates partial sequence.

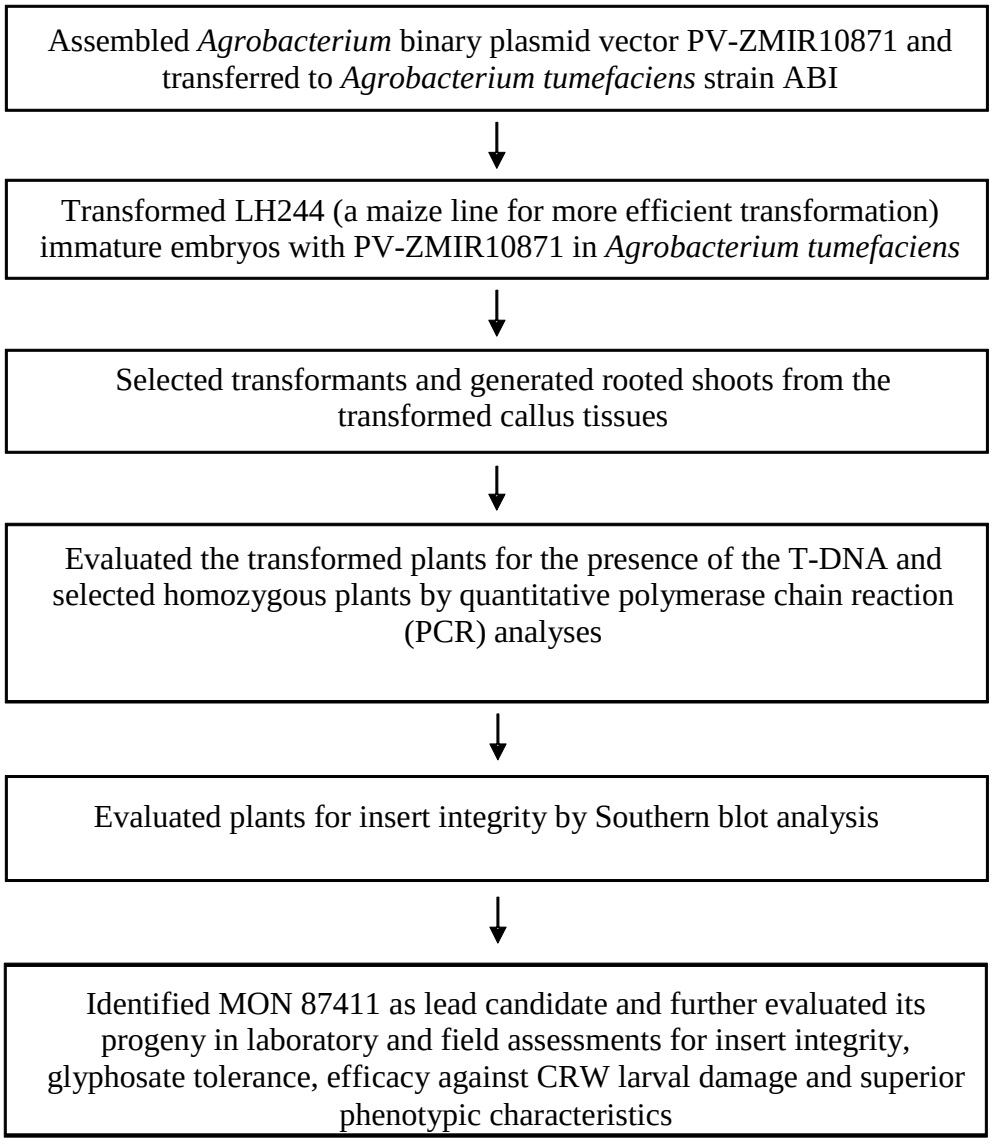


Figure III-2. Schematic of the Development of MON 87411

III.C. The *cry3Bb1* Coding Sequence and the Cry3Bb1 Protein

The *cry3Bb1* expression cassette encodes a 74.5 kDa Cry3Bb1 protein consisting of a single polypeptide of 653 amino acids (Figure III-3). The *cry3Bb1* coding sequence is the codon optimized coding sequence from *Bacillus thuringiensis* that encodes the Cry3Bb1 protein (English, et al. 2000). The presence of Cry3Bb1 protein provides protection from corn rootworm feeding.

III.D. The *cp4 epsps* Coding Sequence and the CP4 EPSPS Protein

The *cp4 epsps* expression cassette, encodes a 47.6 kDa CP4 EPSPS protein consisting of a single polypeptide of 455 amino acids (Figure III-4) (Padgette et al. 1996). The *cp4 epsps* coding sequence is the codon optimized coding sequence of the *aroA* gene from *Agrobacterium* sp. strain CP4 encoding CP4 EPSPS (Barry, et al. 2001; Padgette et al. 1996). The CP4 EPSPS protein is similar and functionally identical to endogenous plant EPSPS enzymes, but has a much reduced affinity for glyphosate, the active ingredient in Roundup agricultural herbicides, relative to endogenous plant EPSPS (Barry et al. 2001; Padgette et al. 1996). The presence of this protein renders the plant tolerant to Roundup.

III.E. DvSnf7^{p} sequence

The DvSnf7^{p} sequence is the partial coding sequence of the *Snf7* gene from *Diabrotica virgifera virgifera* (Baum et al. 2007a; Baum, et al. 2007b) encoding the SNF7 subunit of the ESCRT-III complex (Babst, et al. 2002). The DvSnf7 suppression cassette contains two 240 bp DvSnf7^{p} sequences in an inverted orientation. There is an intervening sequence of 150 nucleotides between the two DvSnf7^{p} sequences (noted on Tables III-1 and IV-1). When the suppression cassette is transcribed, the RNA expressed forms a hairpin loop thereby allowing the formation of double stranded DvSnf7 RNA. The DvSnf7^{p} sequences in the suppression cassette produce a 240 bp dsRNA that upon transcription triggers the RNAi mechanism.

III.F. Regulatory Sequences

The *cry3Bb1* coding sequence in MON 87411 is under the regulation of the *pIIG* promoter, the chlorophyll a/b binding protein (CAB) leader, the *Ract1* intron, and the heat shock protein 17 (*Hsp17)* 3′ untranslated region. The *pIIG* promoter, which directs transcription in plant cells, is from the *pIIG* gene family encoding the physical impedance induced protein from *Zea mays* (Huang, et al. 1998). The *CAB* leader is the 5' untranslated region from the chlorophyll a/b-binding (CAB) protein of *Triticum aestivum* and is involved in regulating gene expression (Lamppa, et al. 1985). The *Ract1* intron is the intron from the *act1* gene from *Oryza sativa* (McElroy, et al. 1990). The *Hsp17* 3′ non-translated region is the 3′ untranslated region from the heat shock protein, Hsp17, of *Triticum aestivum* (McElwain and Spiker 1989) that directs polyadenylation of the mRNA.

The *cp4 epsps* coding sequence in MON 87411 is under the regulation of the *TubA* promoter, the *TubA* leader, the *TubA* intron, the *CTP2* targeting sequence, and the *TubA* 3′ untranslated region. The *TubA* promoter, which directs transcription in plant cells, is

from the *OsTubA* gene family from *Oryza sativa* (rice) encoding α-tubulin (Jeon, et al. 2000). The *TubA* intron is the intron from the *OsTubA* gene family from *Oryza sativa* (rice) encoding α-tubulin (Jeon et al. 2000). The chloroplast transit peptide CTP2 directs transport of the CP4 EPSPS protein to the chloroplast in MON 87411 and is derived from *CTP2* target sequence of the *Arabidopsis thaliana ShkG* gene (Herrmann 1995; Klee, et al. 1987). The *TubA* 3′ non-translated region is the 3′ untranslated region from the *OsTubA* gene family from *Oryza sativa* (rice) encoding α-tubulin (Jeon et al. 2000) that directs polyadenylation of mRNA.

The DvSnf7^{p} sequence in MON 87411 is under the regulation of the *e35S* promoter, the heat shock protein 70 (*Hsp70)* intron, and the *E9* 3′ untranslated region. The *e35S* promoter, which directs transcription in plant cells, contains the duplicated enhancer region (Kay, et al. 1987) from the cauliflower mosaic virus (CaMV) *35S* RNA promoter (Odell, et al. 1985). As demonstrated in this petition, the use of the CaMV *35S* promoter containing the duplicated enhancer region, derived from a designated plant pest, has not imparted plant pest characteristics to MON 87411. The *hsp70* intron is the first intron from the maize heat shock protein 70 gene (Brown and Santino 1997; Rochester, et al. 1986). The *E9* 3′ non-translated region is the 3′ untranslated region from the *rbcS* gene of *Pisum sativum* (pea) encoding the small subunit of ribulose bisphosphate carboxylase protein (Coruzzi, et al. 1984) that directs polyadenylation of the mRNA.

III.G. T-DNA Borders

PV-ZMIR10871 contains Right Border and Left Border regions (Figure III-1 and Table III-1) that were derived from *Agrobacterium tumefaciens* plasmids. The border regions each contain a nick site that is the site of DNA exchange during transformation (Barker, et al. 1983; Depicker, et al. 1982; Zambryski, et al. 1982). The border regions separate the T-DNA from the plasmid backbone region and are involved in the efficient transfer of T-DNA into the maize genome. As demonstrated in this petition, the use of Right Border and Left Border regions derived from *Agrobacterium tumefaciens*, a designated plant pest, has not imparted plant pest characteristics to MON 87411.

III.H. Genetic Elements Outside of the T-DNA Borders

Genetic elements that exist outside of the T-DNA borders are those that are essential for the maintenance or selection of PV-ZMIR10871 in bacteria. The origin of replication *ori V* is required for the maintenance of the plasmid in *Agrobacterium* and is derived from the broad host plasmid RK2 (Stalker, et al. 1981). The origin of replication *ori-pBR322* is required for the maintenance of the plasmid in *E. coli* and is derived from the plasmid vector pBR322 (Sutcliffe 1979). Coding sequence *rop* is the coding sequence of the repressor of primer (ROP) protein and is necessary for the maintenance of plasmid copy number in *E. coli* (Giza and Huang 1989). The selectable marker *aadA* is a bacterial promoter and coding sequence for an enzyme from transposon Tn*7* that confers spectinomycin and streptomycin resistance (Fling, et al. 1985) in *E. coli* and *Agrobacterium* during molecular cloning. Because these elements are outside the border regions, they are not expected to be transferred into the maize genome. The absence of the backbone and other unintended plasmid sequence in MON 87411 has been confirmed by sequencing and bioinformatic analyses (see Section IV.A).

Table III-1. Summary of Genetic Elements in PV-ZMIR10871

Genetic Element	Location in Plasmid Vector	Function (Reference)
T-DNA		
B[1]-Left Border Region	1-442	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker et al. 1983)
Intervening Sequence	443-485	Sequence used in DNA cloning
T[2]-*E9*	486-1118	3′ UTR of the *rbcS* gene family from *Pisum sativum* (pea) encoding the small subunit of ribulose bisphosphate carboxylase protein (Coruzzi et al. 1984) that directs polyadenylation of the mRNA
Intervening Sequence	1119-1147	Sequence used in DNA cloning
DvSnf7[p]	1148-1387	Partial coding sequence of the *Snf7* gene designed to match that from *Diabrotica virgifera virgifera* (Baum et al. 2007a; Baum et al. 2007b) encoding the SNF7 subunit of the ESCRT-III complex (Babst et al. 2002) that forms part of the suppression cassette
Intervening Sequence	1388-1537	Sequence used in DNA cloning
DvSnf7[p]	1538-1777	Partial coding sequence of the *Snf7* gene designed to match that from *Diabrotica virgifera virgifera* (Baum et al. 2007a; Baum et al. 2007b) encoding the SNF7 subunit of the ESCRT-III complex (Babst et al. 2002) that forms part of the suppression cassette
Intervening Sequence	1778-1813	Sequence used in DNA cloning
I[3]-*Hsp70*	1814-2617	Intron and flanking exon sequence of the *hsp70* gene from *Zea mays* (maize) encoding the heat shock protein 70 (HSP70) (Rochester et al. 1986) that is involved in regulating gene expression (Brown and Santino 1997)
P[4]-*e35S*	2618-3238	Promoter from the 35S RNA of cauliflower mosaic virus (CaMV) (Odell et al. 1985) containing the duplicated enhancer region (Kay et al. 1987) that directs transcription in plant cells
Intervening Sequence	3239-3264	Sequence used in DNA cloning

Table III-1 (continued). Summary of Genetic Elements in PV-ZMIR10871

Genetic Element	Location in Plasmid Vector	Function (Reference)
P-*pIIG*	3265-4213	Promoter sequence of the *pIIG* gene encoding the physical impedance induced protein from *Zea mays* (Huang et al. 1998) (maize) that directs transcription in plant cells
Intervening Sequence	4214-4219	Sequence used in DNA cloning
L[5]-*Cab*	4220-4280	5' UTR leader sequence from chlorophyll a/b-binding (CAB) protein of *Triticum aestivum* (wheat) that is involved in regulating gene expression (Lamppa et al. 1985)
Intervening Sequence	4281-4296	Sequence used in DNA cloning
I-*Ract1*	4297-4776	Intron and flanking UTR sequence of the *act1* gene from *Oryza sativa* (rice) encoding rice Actin 1 protein is involved in regulating gene expression (McElroy et al. 1990)
Intervening Sequence	4777-4785	Sequence used in DNA cloning
CS[6]-*cry3Bb1*	4786-6747	Codon optimized coding sequence from Cry3Bb1 protein of *Bacillus thuringiensis* that provides insect resistance (English et al. 2000)
Intervening Sequence	6748-6766	Sequence used in DNA cloning
T-*Hsp17*	6767-6976	3' UTR sequence from a heat shock protein, Hsp17, of *Triticum aestivum* (wheat) (McElwain and Spiker 1989) that directs polyadenylation of the mRNA
Intervening Sequence	6977-7024	Sequence used in DNA cloning
P-*TubA*	7025-9205	Promoter, 5′UTR leader and intron sequences of the *OsTubA* gene family from *Oryza sativa* (rice) encoding α-tubulin (Jeon et al. 2000) that directs transcription in plant cells
Intervening Sequence	9206-9209	Sequence used in DNA cloning

Table III-1 (continued). Summary of Genetic Elements in PV-ZMIR10871

Genetic Element	Location in Plasmid Vector	Function (Reference)
TS[7]-*CTP2*	9210-9437	Targeting sequence of the *ShkG* gene from *Arabidopsis thaliana* encoding the EPSPS transit peptide region that directs transport of the protein to the chloroplast (Herrmann 1995; Klee et al. 1987)
CS-*cp4 epsps*	9438-10805	Codon optimized coding sequence of the *aroA* gene from *Agrobacterium* sp. strain CP4 encoding the native CP4 EPSPS protein that provides herbicide tolerance (Barry et al. 2001; Padgette et al. 1996)
Intervening Sequence	10806-10812	Sequence used in DNA cloning
T-*TubA*	10813-11394	3' UTR sequence of the *OsTubA* gene family from *Oryza sativa* (rice) encoding α-tubulin (Jeon et al. 2000) that directs polyadenylation of mRNA
Intervening Sequence	11395-11412	Sequence used in DNA cloning
B-Right Border Region	11413-11743	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker et al. 1982; Zambryski et al. 1982)
Vector Backbone		
Intervening Sequence	11744-11879	Sequence used in DNA cloning
aadA	11880-12768	Bacterial promoter, coding sequence, and 3' UTR for an aminoglycoside-modifying enzyme, 3"(9)-*O*-nucleotidyltransferase from the transposon Tn*7* (Fling et al. 1985) that confers spectinomycin and streptomycin resistance
Intervening Sequence	12769-13298	Sequence used in DNA cloning
OR[8]-*ori-pBR322*	13299-13887	Origin of replication from plasmid pBR322 for maintenance of plasmid in *E. coli* (Sutcliffe 1979)
Intervening Sequence	13888-14314	Sequence used in DNA cloning
CS-*rop*	14315-14506	Coding sequence for repressor of primer protein from the ColE1 plasmid for maintenance of plasmid copy number in *E. coli* (Giza and Huang 1989)
Intervening Sequence	14507-16014	Sequence used in DNA cloning

Table III-1 (continued). Summary of Genetic Elements in PV-ZMIR10871

Genetic Element	Location in Plasmid Vector	Function (Reference)
OR-*ori V*	16015-16411	Origin of replication from the broad host range plasmid RK2 for maintenance of plasmid in *Agrobacterium* (Stalker et al. 1981)
Intervening Sequence	16412-16497	Sequence used in DNA cloning

[1] B, Border
[2] T, Transcription Termination Sequence
[3] I, Intron
[4] P, Promoter
[5] L, Leader
[6] CS, Coding Sequence
[7] TS, Targeting Sequence
[8] OR, Origin of Replication
[p] Superscript in DvSnf7 indicates the partial sequence. Within the DvSnf7 cassette, bases 1148-1387 are reverse complement to bases 1538-1777.

```
  1 MANPNNRSEH DTIKVTPNSE LQTNHNQYPL ADNPNSTLEE LNYKEFLRMT EDSSTEVLDN
 61 STVKDAVGTG ISVVGQILGV VGVPFAGALT SFYQSFLNTI WPSDADPWKA FMAQVEVLID
121 KKIEEYAKSK ALAELQGLQN NFEDYVNALN SWKKTPLSLR SKRSQDRIRE LFSQAESHFR
181 NSMPSFAVSK FEVLFLPTYA QAANTHLLLL KDAQVFGEEW GYSSEDVAEF YRRQLKLTQQ
241 YTDHCVNWYN VGLNGLRGST YDAWVKFNRF RREMTLTVLD LIVLFPFYDI RLYSKGVKTE
301 LTRDIFTDPI FLLTTLQKYG PTFLSIENSI RKPHLFDYLQ GIEFHTRLRP GYFGKDSFNY
361 WSGNYVETRP SIGSSKTITS PFYGDKSTEP VQKLSFDGQK VYRTIANTDV AAWPNGKVYL
421 GVTKVDFSQY DDQKNETSTQ TYDSKRNNGH VSAQDSIDQL PPETTDEPLE KAYSHQLNYA
481 ECFLMQDRRG TIPFFTWTHR SVDFFNTIDA EKITQLPVVK AYALSSGASI IEGPGFTGGN
541 LLFLKESSNS IAKFKVTLNS AALLQRYRVR IRYASTTNLR LFVQNSNNDF LVIYINKTMN
601 KDDDLTYQTF DLATTNSNMG FSGDKNELII GAESFVSNEK IYIDKIEFIP VQL
```

Figure III-3. Deduced Amino Acid Sequence of the Cry3Bb1 Protein

The amino acid sequence of the Cry3Bb1 protein was deduced from the full-length coding nucleotide sequence present in PV-ZMIR10871.

```
  1 MAQVSRICNG VQNPSLISNL SKSSQRKSPL SVSLKTQQHP RAYPISSSWG LKKSGMTLIG
 61 SELRPLKVMS SVSTACMLHG ASSRPATARK SSGLSGTVRI PGDKSISHRS FMFGGLASGE
121 TRITGLLEGE DVINTGKAMQ AMGARIRKEG DTWIIDGVGN GGLLAPEAPL DFGNAATGCR
181 LTMGLVGVYD FDSTFIGDAS LTKRPMGRVL NPLREMGVQV KSEDGDRLPV TLRGPKTPTP
241 ITYRVPMASA QVKSAVLLAG LNTPGITTVI EPIMTRDHTE KMLQGFGANL TVETDADGVR
301 TIRLEGRGKL TGQVIDVPGD PSSTAFPLVA ALLVPGSDVT ILNVLMNPTR TGLILTLQEM
361 GADIEVINPR LAGGEDVADL RVRSSTLKGV TVPEDRAPSM IDEYPILAVA AAFAEGATVM
421 NGLEELRVKE SDRLSAVANG LKLNGVDCDE GETSLVVRGR PDGKGLGNAS GAAVATHLDH
481 RIAMSFLVMG LVSENPVTVD DATMIATSFP EFMDLMAGLG AKIELSDTKA A
```

Figure III-4. Deduced Amino Acid Sequence of the CTP2 Targeting Sequence and CP4 EPSPS Protein

The transit peptide CTP2 for the CP4 EPSPS protein is underlined. Accumulation of the CP4 EPSPS protein is targeted to the chloroplasts using cleavable CTP2, the transit peptide of the *Arabidopsis thaliana* EPSPS protein. The amino acid sequence of the CP4 EPSPS protein was deduced from the full-length coding nucleotide sequence present in PV-ZMIR10871.

IV. CHARACTERIZATION OF THE GENETIC MODIFICATION

Characterization of the DNA insert in MON 87411 was conducted using a combination of sequencing, PCR, and bioinformatics. The results of this characterization demonstrate that MON 87411 contains one copy of the intended transfer DNA (T-DNA) containing the DvSnf7 suppression cassette and the *cry3Bb1* and *cp4 epsps* expression cassettes that is stably integrated at a single locus and is inherited according to Mendelian principles over multiple generations. These conclusions are based on several lines of evidence:

- Molecular characterization of MON 87411 by Next Generation Sequencing and Junction Sequence Analysis (NGS/JSA) demonstrated that MON 87411 contained a single DNA insert. These whole-genome sequence analyses provided a comprehensive assessment of MON 87411 to determine the presence of sequences derived from PV-ZMIR10871 (DuBose, et al. 2013; Kovalic, et al. 2012), demonstrated that MON 87411 contained a single DNA insert.

- Directed sequencing (locus-specific PCR, DNA sequencing and analyses) of MON 87411 was used to determine the complete sequence of the single DNA insert from PV-ZMIR10871, the adjacent flanking DNA, and the 5' and 3' insert-to-flank junctions. This analysis confirmed that the sequence and organization of the DNA is identical to the corresponding region in the PV-ZMIR10871 T-DNA. The sequencing analysis, along with the NGS/JSA result showing that MON 87411 contains only a single DNA insert with no unintended fragments, also confirms that no vector backbone or other unintended plasmid sequences are present in MON 87411. Furthermore, the genomic organization at the insertion site was assessed by comparing the sequences flanking the T-DNA insert in MON 87411 to the sequence of the insertion site in conventional maize. This analysis determined that no major DNA rearrangement occurred at the insertion site in MON 87411 upon DNA integration.

- Generational stability analysis by NGS/JSA demonstrated that the single PV-ZMIR10871 T-DNA insert in MON 87411 has been maintained through five breeding generations, thereby confirming the stability of the T-DNA in MON 87411.

- Segregation analysis corroborates the insert stability demonstrated by NGS/JSA and independently establishes the nature of the T-DNA as a single chromosomal locus.

Taken together, the characterization of the genetic modification in MON 87411 demonstrates that a single copy of the intended T-DNA was stably integrated at a single locus of the maize genome and that no plasmid backbone sequences are present in MON 87411.

A schematic representation of the NGS/JSA methodology and the basis of the characterization using NGS/JSA and PCR sequencing are illustrated in Figure IV-1 below. These techniques and their value in DNA characterization in crop plants are further described in Appendices B and L.

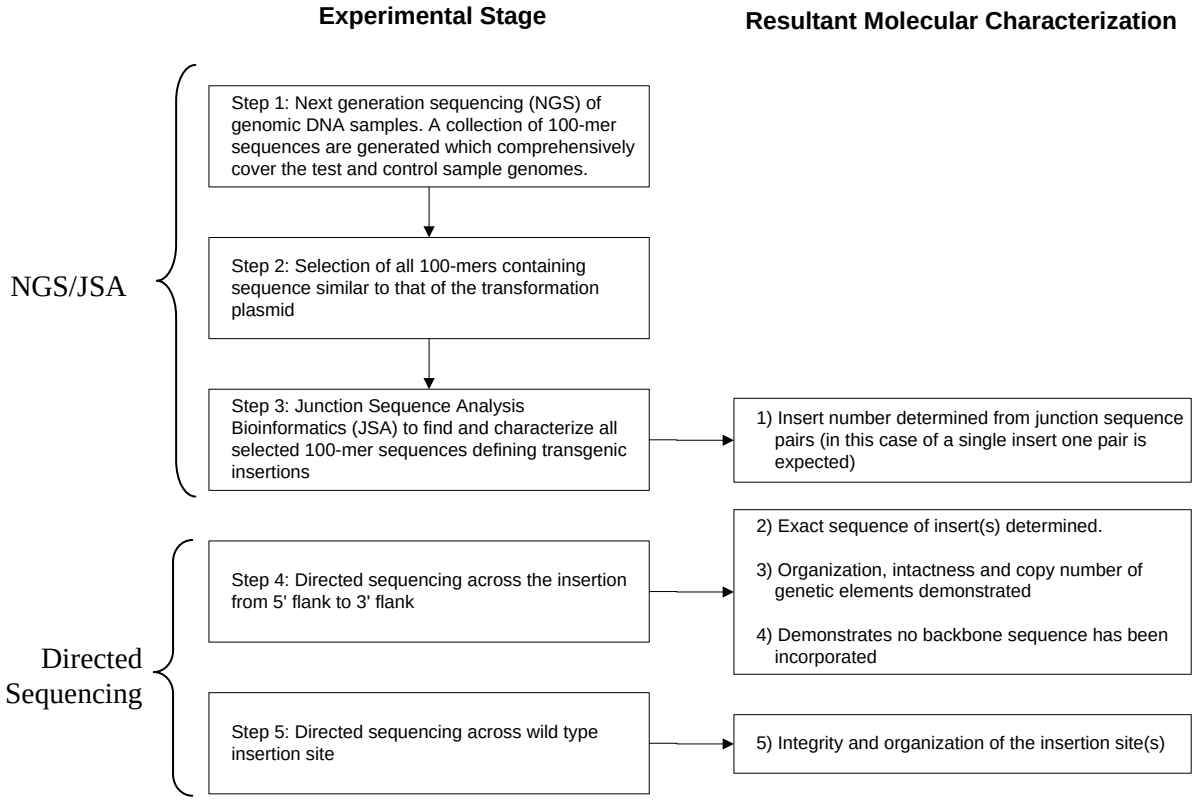


Figure IV-1. Molecular Characterization using Sequencing and Bioinformatics
Genomic DNA from MON 87411 and the conventional control was sequenced using NGS technology that produces a set of short, randomly distributed sequence reads (each approximately 100 bp long) that comprehensively cover the genomes (Step 1). Utilizing these genomic sequences, bioinformatics search tools were used to select all sequence reads (100-mers) that were significantly similar to the transformation plasmid (Step 2) and Junction Sequence Analysis (JSA) bioinformatics was used to determine the insert number (Step 3). Overlapping PCR products are produced which span any insert(s) and their wild type loci (Step 4 and Step 5, respectively). These PCR products are sequenced to provide a detailed characterization of the insertion site(s).

The NGS/JSA method characterized the genomic DNA from MON 87411 and the conventional control using short (~100 bp) randomly distributed sequence fragments (sequencing reads) generated in sufficient number to ensure comprehensive coverage of the sample genomes. Bioinformatics analysis was then used to select sequencing reads that contained sequences similar to the transformation plasmid, and these were analysed to determine the number of DNA inserts. NGS/JSA was run on all MON 87411 samples and the conventional controls; results of NGS/JSA are shown in Section IV.A and IV.D below.

The number of DNA inserts was determined by analyzing sequences for novel junctions. The junctions of the DNA insert and the flanking DNA are unique for each insertion; an example is shown in Figure IV-2 below (Kovalic et al. 2012). Therefore, insertion sites can be recognized by analyzing for sequence reads containing such junctions.

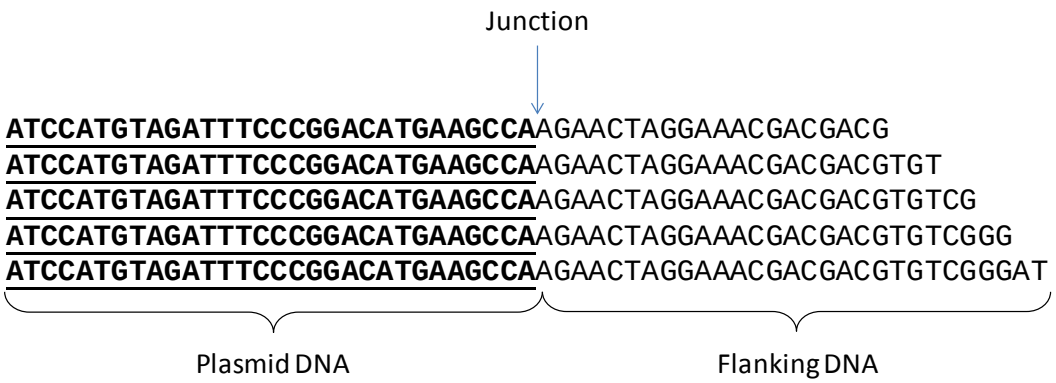


Figure IV-2. Junctions and Junction Sequences
Depicted above are five example junction sequences formatted and labeled to indicate the plasmid/flanking DNA portions of the sequences and with the junction point indicated (plasmid DNA is shown in bold, underlined text and flank DNA is shown in plain text). Junctions are detected by examining the NGS data for sequences having portions of plasmid sequences that span less than the full read. Detected junctions are typically characteristic of plasmid insertions in the genome.

Each insertion will produce two unique junction sequence classes characteristic of the genomic locus, one at the 5' end of the insert (illustrated in see Figure IV-3 below, and named junction sequence class A, or JSC-A, in this case) and one at the 3' end of the insert, named junction sequence class B, or JSC-B, in this case (Kovalic et al. 2012).

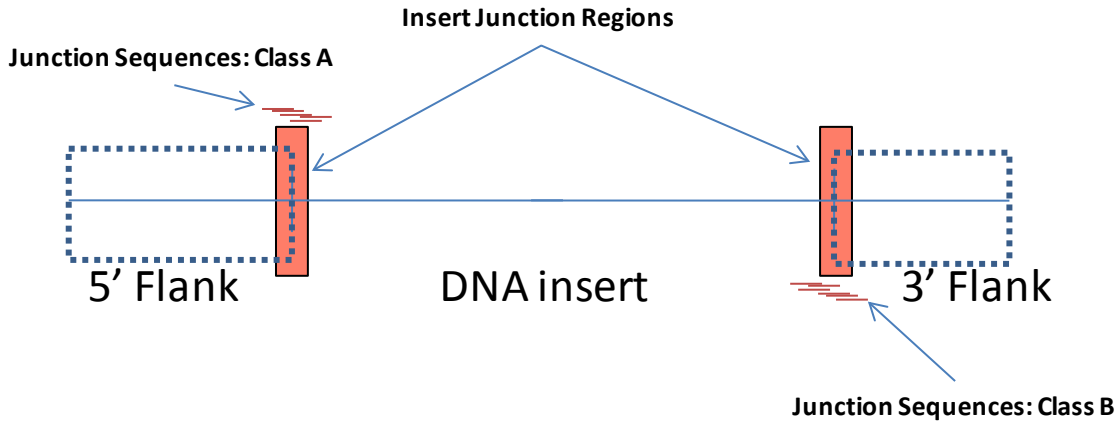


Figure IV-3. Two Unique Junction Sequence Classes are Produced by the Insertion of a Single Plasmid Region
A schematic representation of a single DNA insertion within the genome showing the inserted DNA, the 5' and 3' flanks (depicted as areas bounded by dotted lines), and the two distinct regions spanning the junctions between inserted DNA and flanking DNA (shaded boxes). The group of ~100-mer sequences in which each read contains sequences from both the DNA insert and the adjacent flanking DNA at a given junction is called a Junction Sequence Class. In this example, two distinct junction sequence classes (in this case: Class A at the 5' end and Class B at the 3' end) are represented.

By evaluating the number of unique junction classes detected, the number of insertion sites of the plasmid sequence can be determined. A longer description of the molecular methods used to characterize MON 87411 can be found in Appendices B and L. For a single insert two junction sequence classes are expected, one each originating from either end of the insert, both containing portions of plasmid DNA insert and flanking sequence. In the case of MON 87411, two unique junction sequence classes, both containing portions of T-DNA and flanking sequence, were detected indicating MON 87411 contains a single DNA insert (results are described in Section IV.A, methods and supplementary data are presented in Appendix B). The identity of the DNA insert (*i.e.*, T-DNA or backbone) is determined by direct sequencing described below.

The NGS/JSA strategy to determine insert number of the integrated plasmid DNA was designed to ensure that all transgenic segments would be identified. The depth of coverage (the average number of times each base of the genome is independently sequenced) was ≥75× for each sample genome. It has previously been demonstrated that ≥75× coverage of the soybean genome is adequate to provide comprehensive coverage and ensure detection of inserted DNA (Kovalic et al. 2012) and similarly ≥75× coverage provides comprehensive coverage of the maize genome (Clarke and Carbon 1976). The level of sensitivity of this method was demonstrated by detection of a positive control spiked at $1/10^{th}$ copy-per-genome equivalent.

Directed sequencing (locus-specific PCR and DNA sequencing analyses, Figure IV-1, step 4) complements the NGS/JSA analyses. As indicated above, NGS/JSA results determined that MON 87411 contains a single DNA insertion site. Sequencing of the insert and flanking genomic DNA determined the complete sequence of the insert and flanks; it determined that the sequence and organization of the DNA insert is identical to those in the T-DNA of PV-ZMIR10871, and that each genetic element (except for the border regions) in the insert is intact, and also that no vector backbone, or other unintended plasmid sequences were inserted in MON 87411. Furthermore, the genomic organization at the insertion site was assessed by comparing the insert and MON 87411 flanking sequence to the sequence of the insertion site in conventional maize. This assessment indicated that the integration site in the MON 87411 genome included a 118 bp deletion of genomic DNA but is otherwise identical to the native sequence. Results are described in Section IV.B and Section IV.C; methods are presented in Appendix B.

The stability of the T-DNA present in MON 87411 across multiple generations was evaluated by NGS/JSA analyses. Genomic DNA from five generations of MON 87411 (Figure IV-4) was assayed for all unique junction sequence classes as described above. This information was used to determine the number and identity of insertion sites. For a single insert, two junction sequence classes are expected; each one originates from either end of the insert, both containing portions of DNA insert and flanking sequence. In the case of MON 87411, two identical junction sequence classes were detected in all the generations tested, confirming that the single insert is stably inherited over multiple generations.

Segregation analysis of the T-DNA was conducted to determine the inheritance and stability of the insert in MON 87411. Results from this analysis demonstrate inheritance according to Mendelian principles and the stability of the insert is as expected across

multiple generations (Figure IV-11, Table IV-4, and Table IV-5). The segregation analysis corroborates the insert stability demonstrated by NGS/JSA and independently establishes the genetic behavior of the T-DNA as a single chromosomal locus.

The results of these analyses of MON 87411 demonstrate that a single copy of the intended T-DNA derived from PV-ZMIR10871 was inserted at a single locus of the MON 87411 genome, that the sequence and organization of the T-DNA insert is identical to the corresponding region in PV-ZMIR10871 and that no additional genetic elements, including backbone sequences, were detected in MON 87411. Generational stability analysis demonstrated that the single insert in MON 87411 was maintained through five generations of the breeding history, thereby confirming the stability of T-DNA in MON 87411. In addition, results from segregation analyses confirmed the genetic behavior of the T-DNA as a single chromosomal locus.

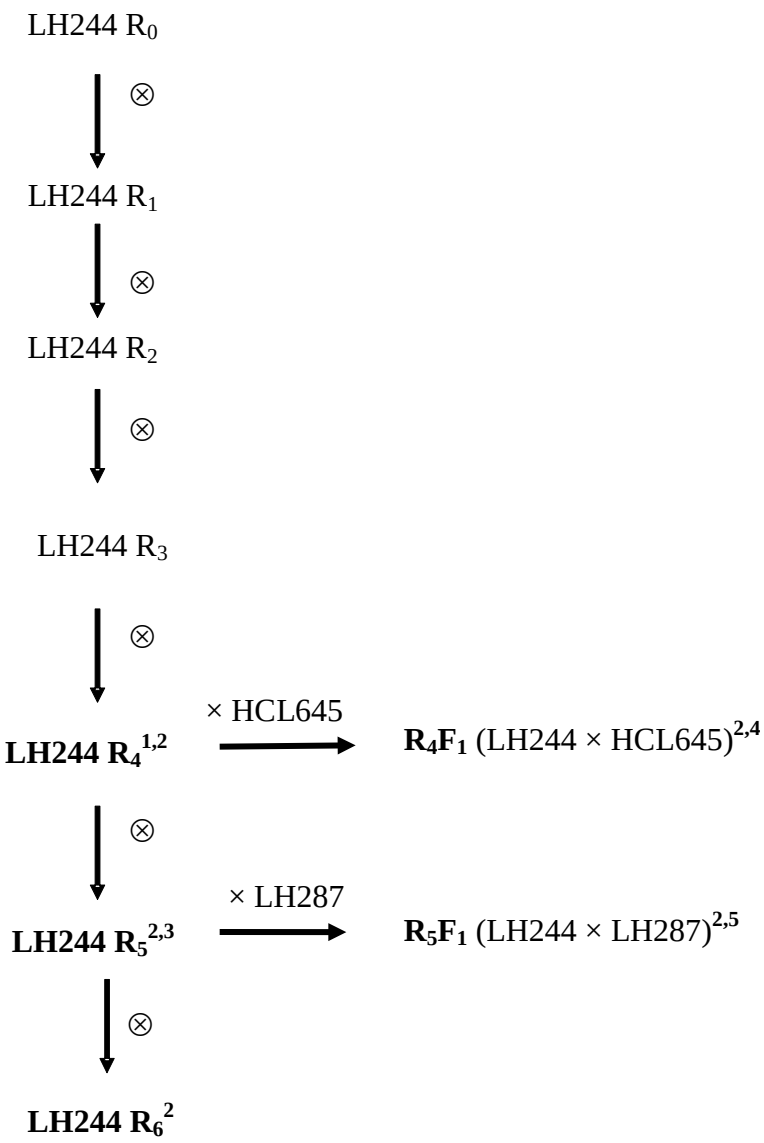


Figure IV-4. Breeding History of MON 87411

R_0 corresponds to the transformed plant, $F_{\#}$ is the filial generation, $R_{\#}$ is the regenerant and subsequent generations, ⊗ designates self-pollination.

[1]Generation used for full molecular characterization.

[2] Generations used to confirm insert stability.

[3] Generation used for commercial development of MON 87411.

[4] Generation used for compositional analysis and RNA expression studies.

[5] Generation used for agronomic/phenotypic studies

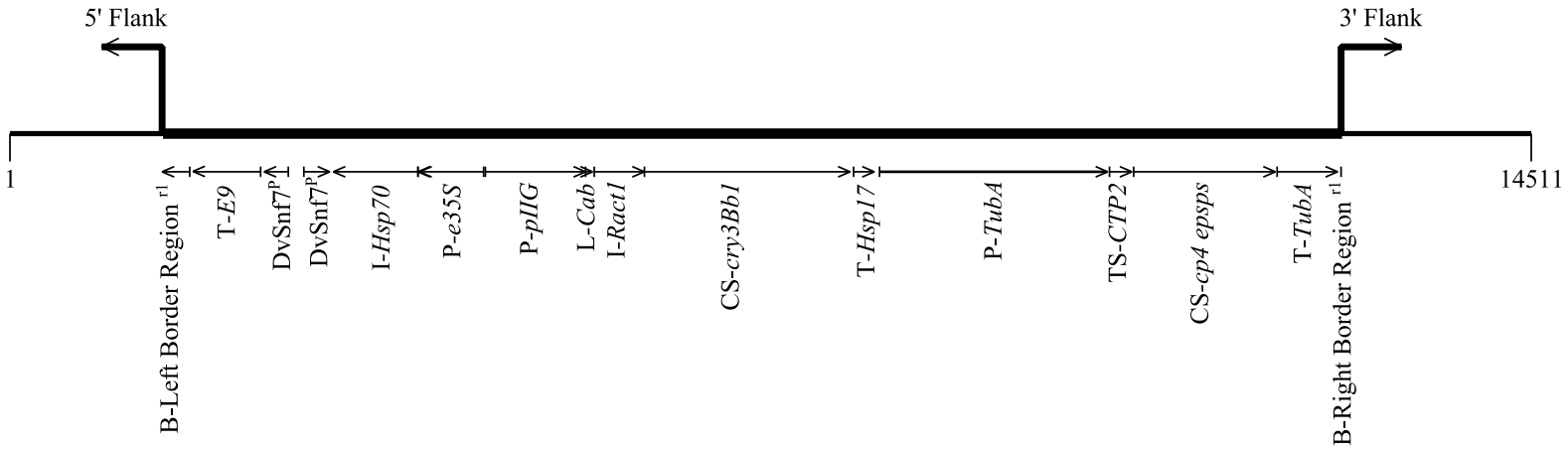


Figure IV-5. Schematic Representation of the Insert and Flanking Sequences in MON 87411

Linear map showing DNA derived from the T-DNA of PV-ZMIR10871 integrated into MON 87411. Right-angled arrows indicate the ends of the integrated T-DNA and the beginning of the flanking sequence. Identified on the map are genetic elements within the insert. This schematic diagram is drawn to scale; the exact coordinates of every element is shown in Table IV-1.

[r1] Superscript in Left and Right Border Regions indicate that the sequence in MON 87411 was truncated compared to the sequences in PV-ZMIR10871.

[P] Superscript in DvSnf7 indicates the partial sequence.

Table IV-1. Summary of Genetic Elements in MON 87411

Genetic Element[1]	Location in Sequence[2]	Function (Reference)
5′ Flank	1-1460	Sequence flanking the 5′ end of the insert
B[3]-Left Border Region[r1]	1461-1723	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker et al. 1983)
Intervening Sequence	1724-1766	Sequence used in DNA cloning
T[4]-*E9*	1767-2399	3' UTR sequence from *Pisum sativum rbcS* gene family encoding the small subunit of ribulose bisphosphate carboxylase protein (Coruzzi et al. 1984) that directs polyadenylation of the mRNA
Intervening Sequence	2400-2428	Sequence used in DNA cloning
DvSnf7[P]	2429-2668	Partial coding sequence of the *Snf7* gene designed to match that from *Diabrotica virgifera virgifera* (Baum et al. 2007a; Baum et al. 2007b) encoding the Snf7 subunit of the ESCRT-III complex (Babst et al. 2002) that forms part of the suppression cassette
Intervening Sequence	2669-2818	Sequence used in DNA cloning
DvSnf7[P]	2819-3058	Partial coding sequence of the *Snf7* gene designed to match that from *Diabrotica virgifera virgifera* (Baum et al. 2007a; Baum et al. 2007b) encoding the Snf7 subunit of the ESCRT-III complex (Babst et al. 2002) that forms part of the suppression cassette
Intervening Sequence	3059-3094	Sequence used in DNA cloning
I[5]-*Hsp70*	3095-3898	Intron and flanking exon sequence of the *hsp70* gene from *Zea mays* (maize) encoding the heat shock protein 70 (HSP70) (Rochester et al. 1986)is involved in regulating gene expression (Brown and Santino 1997)
P[6]-*e35S*	3899-4519	Promoter from the 35S RNA of cauliflower mosaic virus (CaMV) (Odell et al. 1985) containing the duplicated enhancer region (Kay et al. 1987) that directs transcription in plant cells
Intervening Sequence	4520-4545	Sequence used in DNA cloning
P-*pIIG*	4546-5494	Promoter sequence from the physical impedance induced protein of *Zea mays* (maize) (Huang et al. 1998) that directs transcription in plant cells

Table IV-1 (continued). Summary of Genetic Elements in MON 87411

Genetic Element[1]	Location in Sequence[2]	Function (Reference)
Intervening Sequence	5495-5500	Sequence used in DNA cloning
L[7]-*Cab*	5501-5561	5' UTR leader sequence from chlorophyll a/b-binding (CAB) protein of *Triticum aestivum* (wheat) that is involved in regulating gene expression (Lamppa et al. 1985)
Intervening Sequence	5562-5577	Sequence used in DNA cloning
I-*Ract1*	5578-6057	Intron and flanking UTR sequence of the *act1* gene from *Oryza sativa* (rice) encoding rice Actin 1 protein is involved in regulating gene expression (McElroy et al. 1990)
Intervening Sequence	6058-6066	Sequence used in DNA cloning
CS[8]-*cry3Bb1*	6067-8028	Codon optimized coding sequence for Cry3Bb1 protein of *Bacillus thuringiensis* that provides insect resistance (English et al. 2000)
Intervening Sequence	8029-8047	Sequence used in DNA cloning
T-*Hsp17*	8048-8257	3' UTR sequence from a heat shock protein, HSP17, of *Triticum aestivum* (wheat) (McElwain and Spiker 1989) that directs polyadenylation of the mRNA
Intervening Sequence	8258-8305	Sequence used in DNA cloning
P-*TubA*	8306-10486	Promoter, 5′ UTR leader and intron sequences of the *OsTubA* gene family from *Oryza sativa* (rice) encoding α-tubulin (Jeon et al. 2000) that directs transcription in plant cells
Intervening Sequence	10487-10490	Sequence used in DNA cloning
TS[9]-*CTP2*	10491-10718	Targeting sequence of the *ShkG* gene from *Arabidopsis thaliana* encoding the EPSPS transit peptide region that directs transport of the protein to the chloroplast (Herrmann 1995; Klee et al. 1987)
CS-*cp4 epsps*	10719-12086	Coding sequence of the *aroA* gene from *Agrobacterium* sp. strain CP4 encoding the native CP4 EPSPS protein that provides herbicide tolerance (Barry et al. 2001; Padgette et al. 1996)
Intervening Sequence	12087-12093	Sequence used in DNA cloning
T-*TubA*	12094-12675	3' UTR sequence of the *OsTubA* gene from *Oryza sativa* (rice) encoding α-tubulin (Jeon et al. 2000) that directs polyadenylation of mRNA

Table IV-1 (continued). Summary of Genetic Elements in MON 87411

Genetic Element[1]	Location in Sequence[2]	Function (Reference)
Intervening Sequence	12676-12693	Sequence used in DNA cloning
B-Right Border Region[r1]	12694-12708	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker et al. 1982; Zambryski et al. 1982)
3′ Flank	12709-14511	Sequence flanking the 3′ end of the insert

[1] Although flanking sequences and intervening sequence are not functional genetic elements, they comprise a portion of the sequence.
[2] Numbering refers to the sequence of the insert in MON 87411 and adjacent DNA.
[3] B, Border
[4] T, Transcription Termination Sequence
[5] I, Intron
[6] P, Promoter
[7] L, Leader
[8] CS, Coding Sequence
[9] TS, Targeting Sequence
[r1] Superscript in Left and Right Border Regions indicate that the sequence in MON 87411 was truncated compared to the sequences in PV-ZMIR10871
[P] Superscript in DvSnf7 indicates the partial sequence. Within the DvSnf7 cassette, bases 2429-2668 are reverse complement to bases 2819-3058.

IV.A. Determining the Number of DNA inserts in MON 87411

The number of insertion sites of PV-ZMIR10871 DNA in the MON 87411 was assessed by performing NGS/JSA on MON 87411 genomic DNA. A single genomic DNA insertion is expected to produce two junction sequence classes and any additional integration sites would produce additional junction sequence classes. A plasmid map of PV-ZMIR10871 is shown in Figure III-1. A schematic representation of the insert and flanking sequences in MON 87411 is shown in Figure IV-5. The generations studied are depicted in the breeding history diagram shown in Figure IV-4. The NGS conducted for all samples and its adequate depth of coverage in each case is summarized in Section IV.A.1, Section IV.A.2 and Table IV-2. The sensitivity of the method is demonstrated in Section IV.A.1 with data shown in Table IV-3. The JSA analysis of the R_4 generation is shown in Section IV.A.2 with data presented in Figure IV-6 and supplemental data shown in Appendix B; the other generations that were used in the generational stability analysis are shown in Figure IV-4 with the results of JSA analysis described in Section IV.D, with JSA results shown in Figure IV-9, Figure IV-10 and supplemental data included in Appendix B. For full details on materials and methods see Appendix B.

IV.A.1. Next Generation Sequencing (NGS) for MON 87411 and Conventional Control Genomic DNA

Genomic DNA from five generations of MON 87411 and the conventional controls (inbred LH244 and hybrids NL6169 and MPA640B) were isolated and prepared for sequencing according to the manufacturer's protocol (Illumina, TruSeq library protocol. For material and method details see Appendix B). These genomic DNA libraries were used to generate short (~100 bp) randomly distributed sequence fragments (sequencing reads) in sufficient numbers to ensure comprehensive coverage of the maize genome (see Figure IV-1, Step 1).

To confirm sufficient sequence coverage in all generations of MON 87411 and the conventional controls, the 100-mer sequence reads from all samples were analyzed to determine the effective depth of coverage (*i.e.*, the average number of times any base of the genome is expected to be independently sequenced) by mapping all reads to a known single-copy endogenous gene (Pyruvate decarboxylase (*pdc3*), GenBank accession.version: AF370006.2). The analysis showed that *pdc3* was covered by 100-mers at >107× for each sample (Table IV-2). It has previously been demonstrated that ≥75× coverage of the soybean genome is adequate to provide comprehensive coverage and ensure detection of inserted DNA (Kovalic et al. 2012) and similarly ≥75× coverage provides comprehensive coverage of the maize genome (Clarke and Carbon 1976). A summary of NGS and effective depth of coverage are shown in Table IV-2.

In order to confirm the method's ability to detect any sequences derived from the PV-ZMIR10871 transformation plasmid, a sample of conventional control maize DNA spiked with PV-ZMIR10871 DNA at 1 and 1/10th genome equivalent was analyzed by NGS and bioinformatics. At 1 genome equivalent, 100% nucleotide identity was observed over 100% of PV-ZMIR10871 (Table IV-3). This result demonstrates that all nucleotides of the transformation plasmid are observed by the sequencing and bioinformatic assessments performed. Also, observed coverage was adequate (Clarke and Carbon 1976) at a level of at most 1/10th genomic equivalent (99.64% coverage at 100% identity for the 1/10th genome equivalent spiked control sample, Table IV-3) and, hence, a detection level of at least 1/10th genome equivalent was achieved for the plasmid DNA sequence assessment.

Table IV-2. Sequencing (NGS) Conducted for MON 87411 and Control Genomic DNA

Sample	Total Nucleotides (Gb)	Effective Median Depth of Coverage (x-fold)
LH244	346.9	126x
LH244 × HCL645	309.5	107x
LH244 × LH287	342.3	118x
R_4	334.9	125x
R_4F_1	338.9	113x
R_5F_1	346.6	126x
R_5	352.3	140x
R_6	363.9	134x

For each sample the raw data produced are presented in terms of total nucleotide number. Effective depth of coverage is determined by mapping and alignment of all raw data to a well known single copy locus (*pdc3*: pyruvate decarboxylase) within the maize genome. The median effective depths of coverage are shown for all samples.

Table IV-3. Summary of NGS Data for the Conventional Control DNA Sample Spiked with PV-ZMIR10871 DNA

	$1/10^{th}$ copy Spike	1 copy Spike
Extent of coverage[1] of PV-ZMIR10871	99.64%	100%
Percent identity of coverage[2] of PV-ZMIR10871	100%	100%

[1] Extent of coverage is calculated as the percent of all PV-ZMIR10871 bases observed in the sequencing of the spike-in samples:

$$\text{extent of coverage} = \frac{\text{number of spike in bases detected}}{\text{total length (bp) of spike in plasmid}} \times 100$$

[2] Percent identity of coverage is calculated as the percent of all PV-ZMIR10871 bases observed in the sequencing of the spike-in samples:

$$\text{Percent identity of coverage} = \frac{\text{number of identical bases (spike in vs. plasmid sequence) detected}}{\text{total length (bp) of spike in plasmid detected}} \times 100$$

IV.A.2 Characterization of insert number in MON 87411 using Bioinformatic Analysis

The number of insertion sites of DNA from PV-ZMIR10871 in the MON 87411 was assessed by performing NGS/JSA on MON 87411 genomic DNA using the R_4 generation. A single genomic DNA insertion is expected to produce two junction sequence classes and any additional integration sites would produce additional junction sequence classes.

IV.A.2.1 Selection of Sequence Reads Containing Sequence of the PV-ZMIR10871

PV-ZMIR10871 was transformed into the parental line LH244 to produce MON 87411. Consequently, any DNA inserted into MON 87411 will consist of sequences that are similar to the PV-ZMIR10871 DNA sequence. To fully characterize the DNA from PV-ZMIR10871 inserted in MON 87411, it is sufficient to completely analyze only the sequence reads that have similarity to the transformation plasmid (Figure IV-1, Step 2). In order to analyze the sequence data for insert number, all sequences that have significant sequence similarity to PV-ZMIR10871 were selected from the full sequencing datasets (Kovalic et al. 2012). Due to the depth of sequence coverage demonstrated with this methodology (see Section IV.A.1), on average, any area of the genome will be covered by more than 107 of the 100-mer sequences; this ensures that sequences from PV-ZMIR10871 inserted into the genome will be detected by the analysis.

Using established criteria (which are described in the materials and methods, Appendix B), reads similar to the transformation plasmid were selected from MON 87411 and the conventional control sequence datasets and were then used as input data for bioinformatic junction sequence analysis.

IV.A.2.2 Determination of the Insert Number

The NGS/JSA method described above used the entire PV-ZMIR10871 plasmid as a query to determine the DNA insertion site number. Any DNA inserts, regardless of whether the sequence was from backbone or T-DNA, can be detected by junction sequences. Therefore, unlike the traditional Southern blot analysis that separately hybridizes T-DNA or backbone probes, in NGA/JSA the determination of the T-DNA insert number and of the absence of backbone or unintended sequences are simply represented by the determination of the overall insert number in the genome followed by determination of the exact identity of any DNA insert using directed sequencing and sequence analysis.

By evaluating the number of unique junction classes, the number of DNA insertion sites can be determined (Figure IV-1, Step 3). For a single insert, at a single genomic locus, a single pair of junction sequences classes, each one originating from either end of the insert, is expected. If MON 87411 contains a single T-DNA insert two junction sequence classes (JSCs) each containing portions of T-DNA sequence and flanking sequence will be detected.

To determine the insert number in MON 87411, the selected sequence reads described above were analyzed using JSA (Kovalic et al. 2012). JSA uses bioinformatic analysis to find and classify partially matched reads characteristic of the ends of insertions. The number of resultant unique JSCs were determined by this analysis and are shown in the table below.

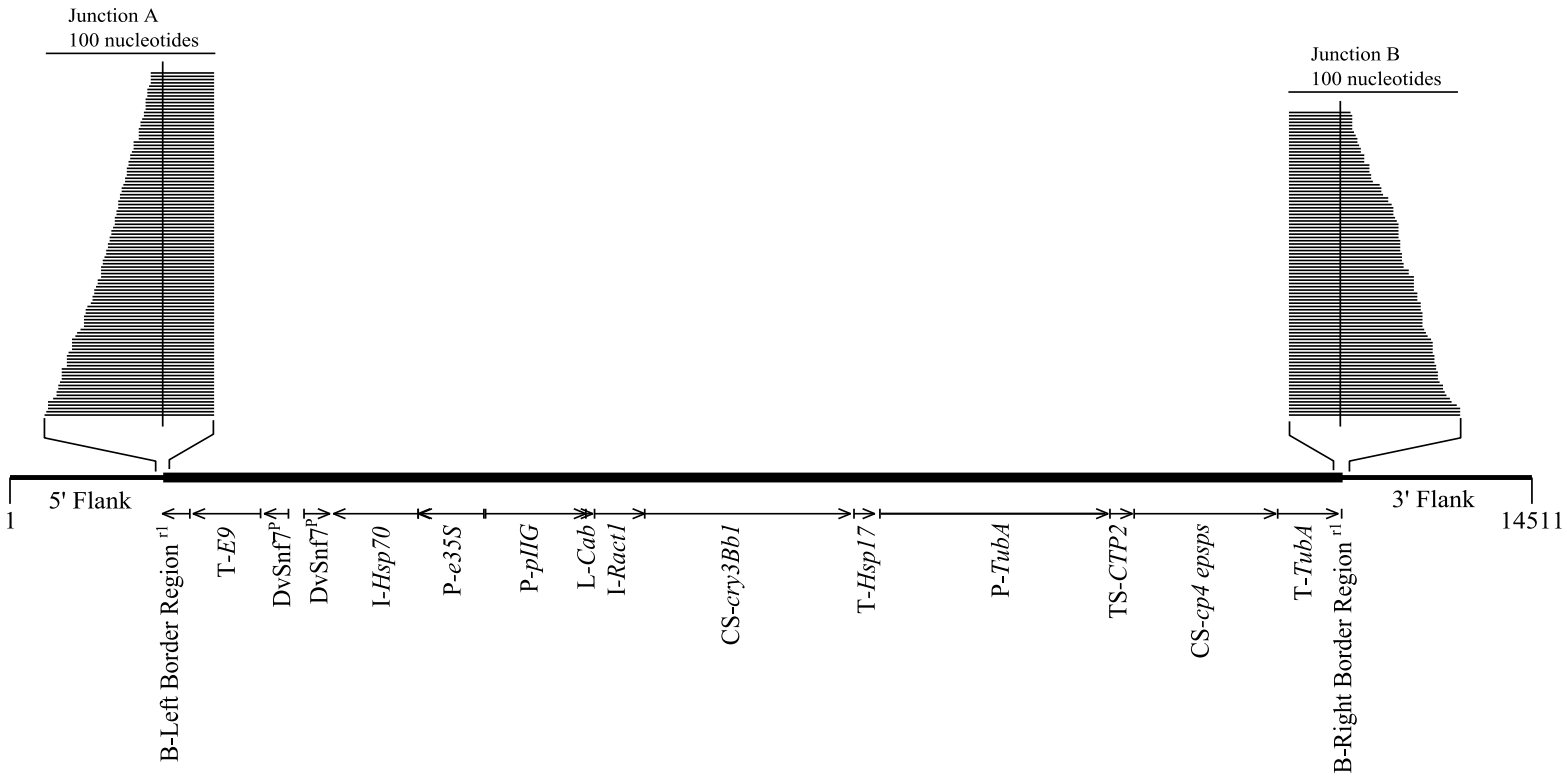
Table IV-4. Unique Junction Sequence Class Results

Sample	JSCs Detected
MON 87411	2
LH244	0

Detailed sequence information of the junction sequences detected by JSA is shown in Figure IV-6 (Panel B) and Figure B-1 in Appendix B. The location and orientation of the junction sequences relative to the T-DNA insert determined for MON 87411 (as described in Section IV.B) is shown in Figure IV-6, Panel A. As shown in the figure, there are two junction sequence classes identified in MON 87411. Junction Sequence Class A and Class B (JSC-A and JSC-B) both contain the T-DNA border sequence joined to flanking sequence, indicating that they represent the sequences at the junctions of the intended T-DNA insert and flanking sequence.

The presence of two, and only two, junction sequence classes (joining T-DNA border and flanking sequences) indicate this single pair of JSCs likely arises from the insertion of the intended PV-ZMIR10871 T-DNA at a single locus in the genome of MON 87411. JSC-A represents the junction of the T-DNA Left Border sequence to the 5' flank and JSC-B represents the junction of the T-DNA Right Border sequence to the 3' flank. As shown by exact and complete alignment of the JSCs to the full flank/insert sequence (described in Section IV.B and shown in Figure IV-6, Panel B) both of these JSCs originate from the same locus of the MON 87411 genome and are linked by contiguous, known and expected DNA that makes up the single insert.

Based on this comprehensive NGS/JSA study it is concluded that MON 87411 contains one DNA inserted into a single locus, as shown in Figure IV-5. The identity of the DNA insert was determined by the sequencing and analysis of overlapping PCR products from this locus as described below in Section IV.B. Additionally, the lack of detectable junction sequences attributable to plasmid backbone sequences leads to the conclusion that no backbone sequences from PV-ZMIR10871 are present in MON 87411.



Panel A.

Figure IV-6. Junction Sequences Detected by NGS/JSA

Panel A: Linear map of MON 87411 illustrating the relationship of the detected junction sequences to the insert locus. The individual junction sequences detected by JSA are illustrated as stacked bars; each detected junction sequence read is shown trimmed to include only 30 bases of plasmid sequence. The scale of the identified junction sequences relative to the insert map is depicted by the braces.

[r1] Superscript in Left and Right Border Regions indicate that the sequence in MON 87411 was truncated compared to the sequences in PV-ZMIR10871.

[P] Superscript in DvSnf7 indicates the partial sequence.

```
JSC-A Alignment:
JSC_A:            1   AATTGAAAAAAAATTGGTAATTACTCTTTCTTTTTCTCCATATTGACCATCATACTCATT   60
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Directed Seq.: 1554   AATTGAAAAAAAATTGGTAATTACTCTTTCTTTTTCTCCATATTGACCATCATACTCATT   1495


JSC_A:           61   GCTGATCCATGTAGATTTCCCGGACATGAAGCCA^CTTAACTATTCATTAGTGTTTTGCCT   120
                      |||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Directed Seq.: 1494   GCTGATCCATGTAGATTTCCCGGACATGAAGCCA^CTTAACTATTCATTAGTGTTTTGCCT   1435


JSC_A:          121   TTTTATTTTCCTTTTAATAAATAATCCATCACTTTAAATGAACC    164
                      |||||||||||||||||||||||||||||||||||||||||||
Directed Seq.: 1434   TTTTATTTTCCTTTTAATAAATAATCCATCACTTTAAATGAACC    1391


JSC-B Alignment:
JSC-B:            1   GGCTAGAGCCACACCCAAGTTCCTAACTATGATAAAGTTGCTCTGTAACAGAAAACACCA   60
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Directed Seq.: 12616  GGCTAGAGCCACACCCAAGTTCCTAACTATGATAAAGTTGCTCTGTAACAGAAAACACCA   12675


JSC-B:           61   TCTAGAGCGGCCGCGTTTAAACTATCAGTGTTT^AGAGAATCACAAACCTCTAGATGTATT   120
                      |||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Directed Seq.: 12676  TCTAGAGCGGCCGCGTTTAAACTATCAGTGTTT^AGAGAATCACAAACCTCTAGATGTATT   12735


JSC-B:          121   AATCTACCCTAGAACTAGTTCACTTTTGTGTGCATACTTTTCT    163
                      |||||||||||||||||||||||||||||||||||||||||||
Directed Seq.: 12736  AATCTACCCTAGAACTAGTTCACTTTTGTGTGCATACTTTTCT    12778
```

Panel B.

Figure IV-6 (continued). Junction Sequences Detected by NGS/JSA

Panel B: Full consensus sequence for junction sequence Classes A and B (JSC-A and JSC-B) showing exact alignment to the independently determined *in planta* sequence at the insert locus (labeled "Directed Seq."). The numbers flanking the sequence text represent the base pair numbering of the JSA consensus sequence or the insert sequence, respectively. Double underlined text indicates plasmid DNA sequence, single underlined text indicates plant genome sequence, and the carat character "^" indicates the junction point between the MON 87411 insert and the flank.

IV.B. Organization and Sequence of the Insert and Adjacent Flanking DNA in MON 87411

The organization of the elements within the DNA insert and the adjacent genomic DNA was assessed using directed DNA sequence analysis (refer to Figure IV-1, Step 4). PCR primers were designed to amplify eight overlapping regions of the MON 87411 genomic DNA that span the entire length of the insert (Figure IV-7). The amplified PCR products were subjected to DNA sequencing analyses. The results of this analysis confirm that the MON 87411 insert is 11,248 bp and that each genetic element in the insert is intact, with the exception of the Right and Left border regions. The border regions both contain small terminal deletions with the remainder of the inserted border regions being identical to the sequence in PV-ZMIR10871. The sequence and organization of the insert was also shown to be identical to the corresponding T-DNA of PV-ZMIR10871, confirming that a single copy of T-DNA was inserted as intended. This analysis also shows that only T-DNA elements (described in Table IV-1) were present and no PV-ZMIR10871 backbone sequences were present in MON 87411.

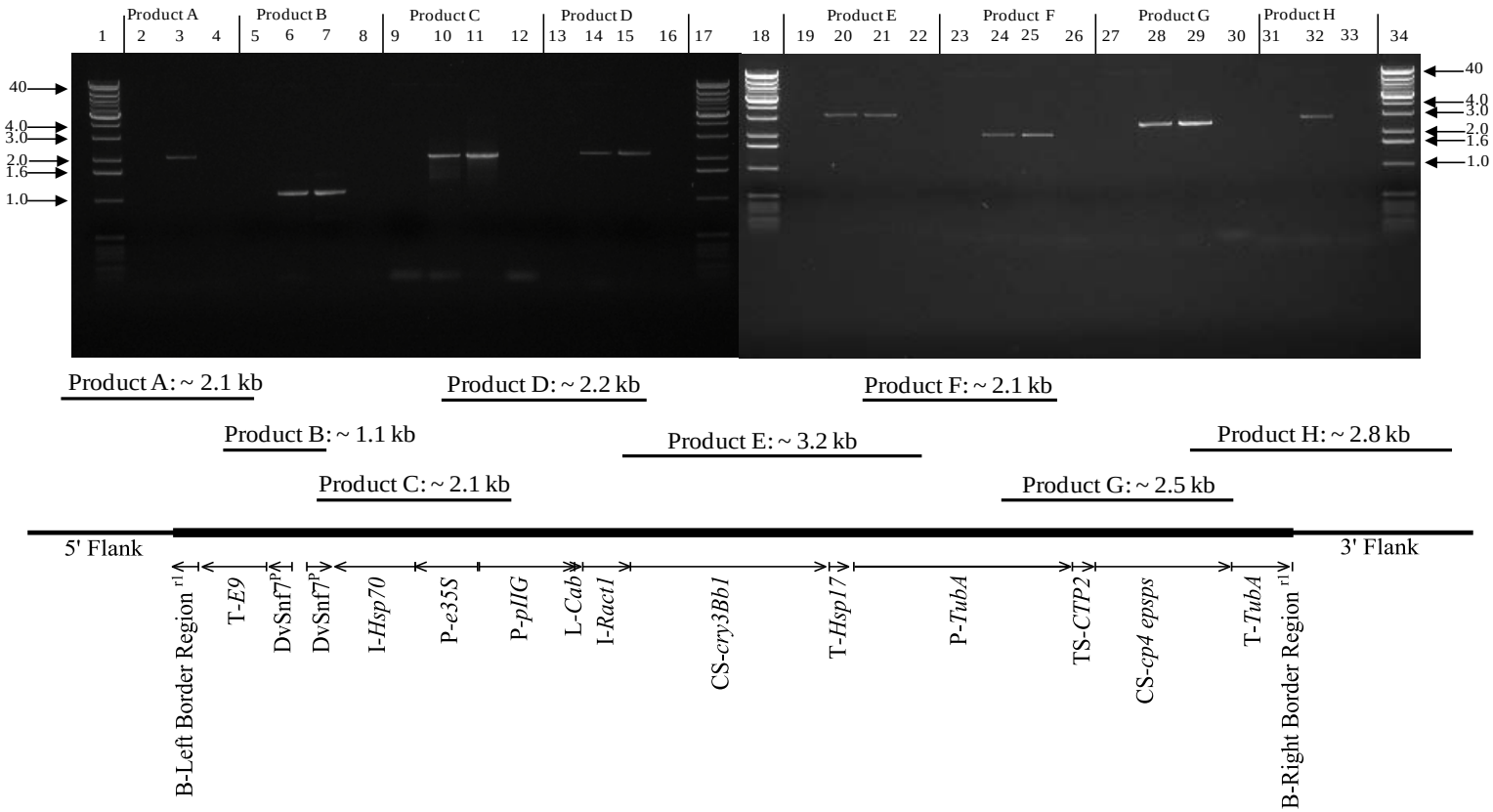


Figure IV-7. Analysis of Overlapping PCR Products Across the MON 87411 Insert

PCR was performed on both conventional control genomic DNA and genomic DNA of the R_4 generation of MON 87411 using eight pairs of primers to generate overlapping PCR fragments from MON 87411 for sequencing analysis. To verify the production of PCR products, 5 µl of each of the PCR reactions was loaded on the gel, except where noted below. The expected product size for each amplicon is provided in the illustration.

[r1] Superscript in Left and Right Border Regions indicate that the sequence in MON 87411 was truncated compared to the sequences in PV-ZMIR10871.

[P] Superscript in DvSnf7 indicates the partial sequence.

Lane designations are as follows:

Lane	Sample	Lane	Sample
1	1 kb DNA Extension Ladder	18	1 kb DNA Extension Ladder
2	Conventional Control LH244	19	Conventional Control LH244
3	MON 87411	20	MON 87411
4	No template control	21	PV-ZMIR10871
5	Conventional Control LH244	22	No template control
6	MON 87411	23	Conventional Control LH244
7	PV-ZMIR10871 (2 µl)	24	MON 87411
8	No template control	25	PV-ZMIR10871
9	Conventional Control LH244	26	No template control
10	MON 87411	27	Conventional Control LH244
11	PV-ZMIR10871 (2 µl)	28	MON 87411
12	No template control	29	PV-ZMIR10871
13	Conventional Control LH244	30	No template control
14	MON 87411 (10 µl)	31	Conventional Control LH244
15	PV-ZMIR10871	32	MON 87411
16	No template control	33	No template control
17	1 kb DNA Extension Ladder	34	1 kb DNA Extension Ladder

Figure IV-7 (continued). Analysis of Overlapping PCR Products Across the MON 87411 Insert

Arrows next to the agarose gel photograph denote the size of the DNA, in kilobase pairs, obtained from the 1 kb DNA Extension Ladder (Invitrogen, Grand Island, NY) on the ethidium bromide stained gel.

IV.C. Sequencing of the MON 87411 Insertion Site

PCR and sequence analysis were performed on genomic DNA extracted from the conventional control to examine the insertion site in conventional maize (refer to Figure IV-1, Step 5). The PCR was performed with one primer specific to the genomic DNA sequence flanking the 5' end of the MON 87411 insert paired with a second primer specific to the genomic DNA sequence flanking the 3' end of the insert (Figure IV-8). A sequence comparison between the PCR product generated from the conventional control and the sequence generated from the 5' and 3' flanking sequences of MON 87411 indicates there was a 118 base pair deletion that occurred during integration of the T-DNA, with the remainder of the flanks in MON 87411 being identical to the conventional control. Such changes are common during plant transformation and these changes presumably resulted from double-stranded break repair mechanisms in the plant during the *Agrobacterium*-mediated transformation process (Salomon and Puchta 1998).

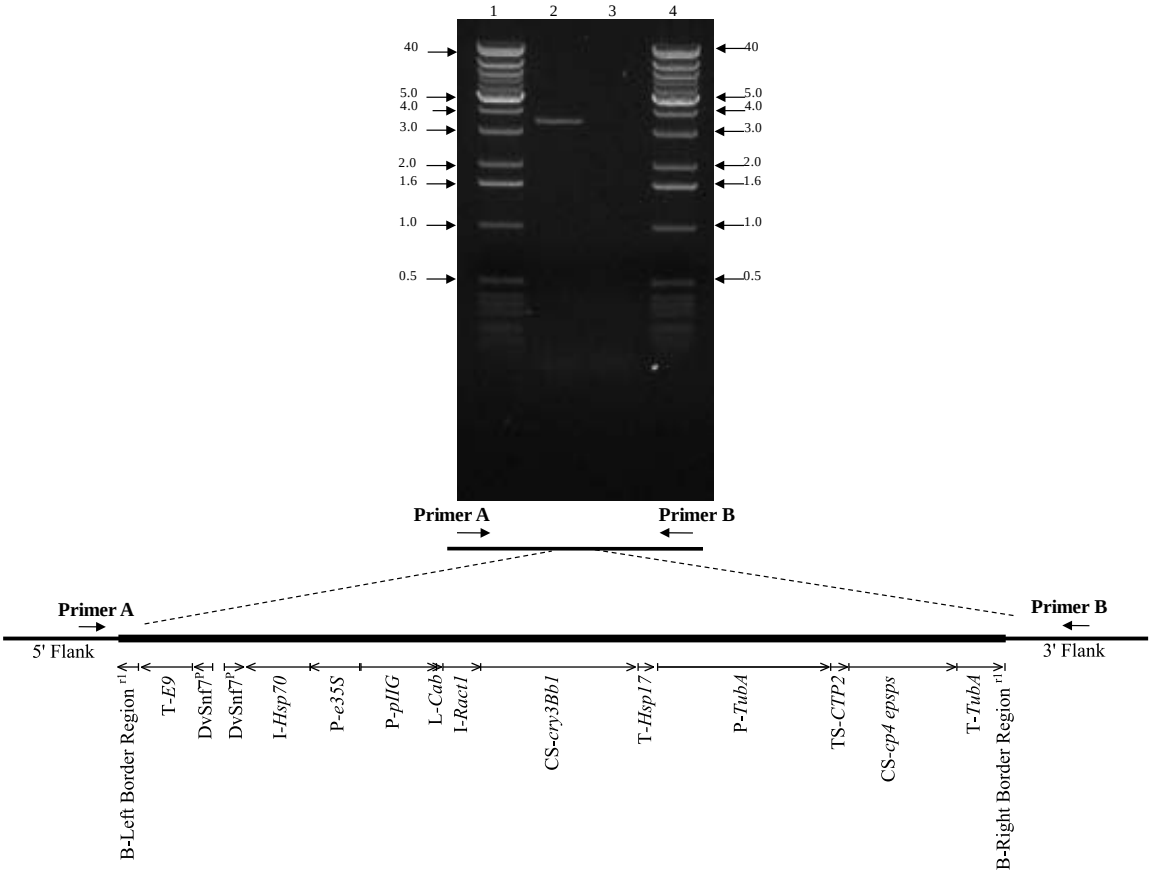


Figure IV-8. PCR Amplification of the MON 87411 Insertion Site

PCR Analysis was performed to evaluate the MON 87411 insertion site. PCR was performed on conventional control DNA using Primer A, specific to the 5′ flanking sequence, and Primer B, specific to the 3 flanking sequence of the MON 87411 insert. The DNA generated from the conventional control PCR was used for sequencing analysis. This illustration depicts the MON 87411 insertion site in the conventional control (upper panel) and a schematic of the MON 87411 insert (lower panel). Approximately 5 µl of each of the PCR reactions was loaded on the gel. Lane designations are as follows:

Lane	Sample
1	1 kb DNA Extension Ladder
2	Conventional Control LH244
3	No template DNA control
4	1 kb DNA Extension Ladder

Arrows on the agarose gel photograph denote the size of the DNA, in kilobase pairs, obtained from the 1 kb DNA Extension Ladder (Invitrogen, Grand Island, NY) on the ethidium bromide stained gel.

[r1] Superscript in Left and Right Border Regions indicate that the sequence in MON 87411 was truncated compared to the sequences in PV-ZMIR10871.
[P] Superscript in DvSnf7 indicates the partial sequence.

IV.D. Determination of Insert Stability over Multiple Generations of MON 87411

In order to demonstrate the stability of the T-DNA present in MON 87411 through multiple generations, NGS/JSA analysis was performed using DNA obtained from five breeding generations of MON 87411. The breeding history of MON 87411 is presented in Figure IV-4, and the specific generations tested are indicated in the figure legend. The MON 87411 (R_4) generation was used for the molecular characterization analyses discussed in Sections IV.A-IV.C and shown in Figure IV-6 and Figure B-1 in Appendix B. To assess stability, four additional generations were evaluated by NGS/JSA analysis as previously described in Section IV.A, and compared to the fully characterized MON 87411 (R_4) generation. The conventional controls used for the generational stability analysis included LH244, which has a genetic background similar to the MON 87411 (R_4), MON 87411 (R_5) and MON 87411 (R_6) generations and represents the original transformation line. The conventional control hybrid LH244 × HCL645 has a genetic background similar to the MON 87411 R_4F_1 hybrid. In addition, the conventional control hybrid LH244 × LH287, has a genetic background similar to the MON 87411 R_5F_1 hybrid. Genomic DNA isolated from each of the selected generations of MON 87411 and conventional controls were used for NGS/JSA analysis. The results are shown in Figure IV-9, Figure IV-10 and Figure B-1 in Appendix B.

IV.D.1 Determination of the Insert Number

To determine the insert number in the MON 87411 generations, the sequences selected as described in Section IV.A.2.1 were analyzed using JSA (Kovalic et al. 2012), where the number of resultant JSCs containing PV-ZMIR10871 DNA sequence determined by this analysis is shown in the table below.

Table IV-5. Junction Sequence Classes Detected

Sample	JSCs Detected
MON 87411 (R_4)	2
MON 87411 (R_4F_1)	2
MON 87411 (R_5)	2
MON 87411 (R_5F_1)	2
MON 87411 (R_6)	2
LH244	0
LH244 × HCL645	0
LH244 × LH287	0

Figure IV-9 and Figure IV-10, below, identify the presence of two, and only two, identical junction sequence classes in each of the five assessed MON 87411 generations (R_4, R_5, R_6, R_4F_1 and R_5F_1) as expected for a stably maintained single insert. This single identical pair of JSCs is observed due to the insertion of PV-ZMIR10871 T-DNA at a single locus in the genome of MON 87411. The consistency of these JSC data across all generations tested demonstrates that this single locus is stably maintained throughout the MON 87411 breeding process.

These results, therefore, demonstrate that the MON 87411 single locus of integration has been maintained through several generations of the breeding of MON 87411; thereby confirming the stability of the insert. Based on this comprehensive sequence data and bioinformatic analysis (NGS/JSA), it is concluded that MON 87411 contains a single and stable T-DNA insertion.

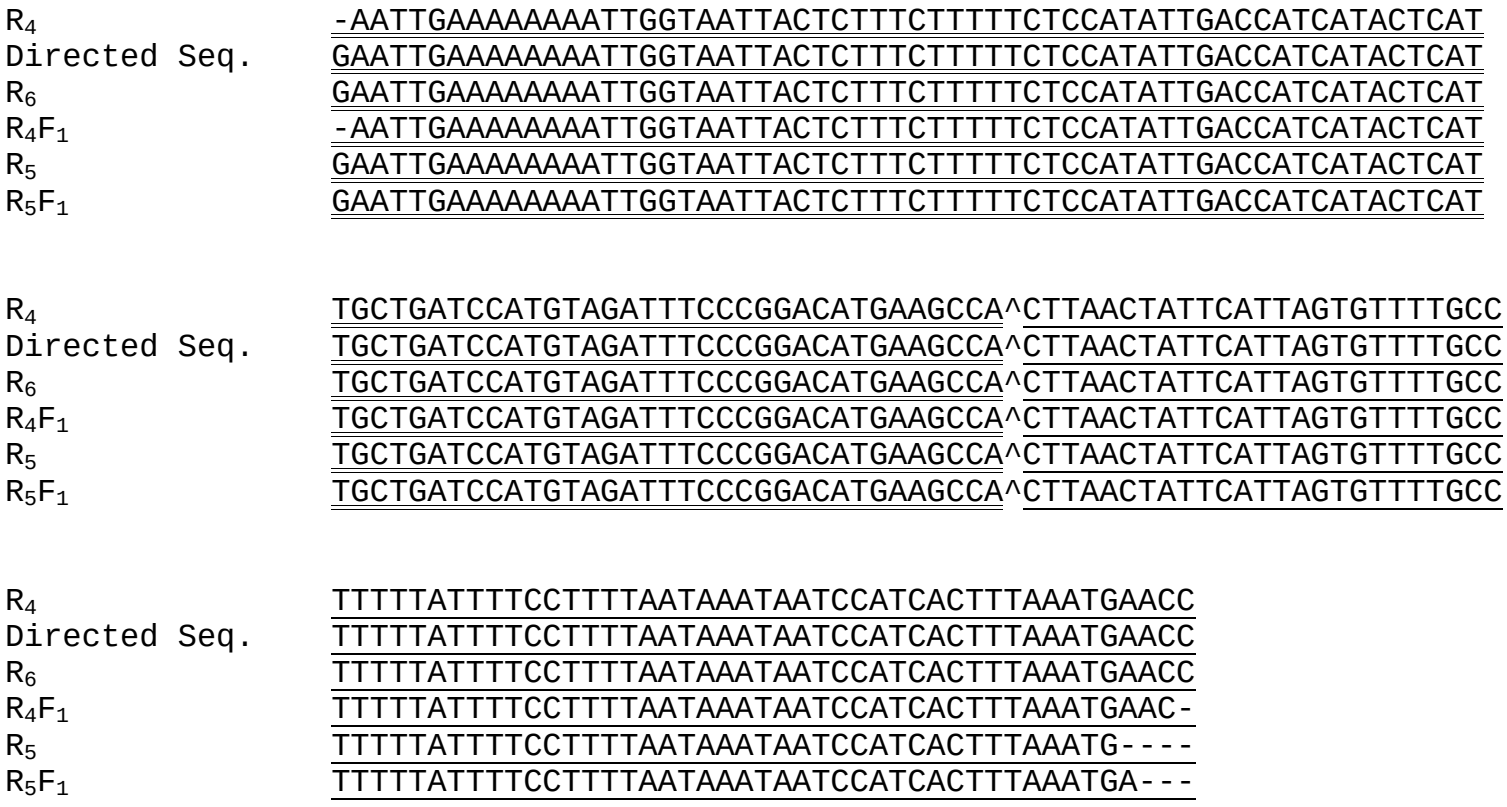


Figure IV-9. Junction sequences detected by JSA. Junction Sequence Class A Alignment (All Generations Tested)
Full consensus sequence for JSC-A showing exact alignment to the independently determined *in planta* locus specific sequence (labeled "Directed Seq." in the figure); individual consensus sequences for each of the five generations are labeled according to their generation (R_4, R_5, R_6, R_4F_1 and R_5F_1). Double underlined text indicates plasmid DNA sequence, single underlined text indicates plant genome sequence, and the carat character "^" indicates the junction point between the MON 87411 insert and the flank. The asterisk character "*" indicates identical nucleotide in every sequence at that position in the alignment. Dash characters indicate positions past the end of the consensus sequence for a particular generation.

```
R5F1              AGCGGCTAGAGCCACACCCAAGTTCCTAACTATGATAAAGTTGCTCTGTAACAGAAAACA
Directed Seq.     AGCGGCTAGAGCCACACCCAAGTTCCTAACTATGATAAAGTTGCTCTGTAACAGAAAACA
R5                --CGGCTAGAGCCACACCCAAGTTCCTAACTATGATAAAGTTGCTCTGTAACAGAAAACA
R4F1              -GCGGCTAGAGCCACACCCAAGTTCCTAACTATGATAAAGTTGCTCTGTAACAGAAAACA
R4                ---GGCTAGAGCCACACCCAAGTTCCTAACTATGATAAAGTTGCTCTGTAACAGAAAACA
R6                AGCGGCTAGAGCCACACCCAAGTTCCTAACTATGATAAAGTTGCTCTGTAACAGAAAACA

R5F1              CCATCTAGAGCGGCCGCGTTTAAACTATCAGTGTTT^AGAGAATCACAAACCTCTAGATGT
Directed Seq.     CCATCTAGAGCGGCCGCGTTTAAACTATCAGTGTTT^AGAGAATCACAAACCTCTAGATGT
R5                CCATCTAGAGCGGCCGCGTTTAAACTATCAGTGTTT^AGAGAATCACAAACCTCTAGATGT
R4F1              CCATCTAGAGCGGCCGCGTTTAAACTATCAGTGTTT^AGAGAATCACAAACCTCTAGATGT
R4                CCATCTAGAGCGGCCGCGTTTAAACTATCAGTGTTT^AGAGAATCACAAACCTCTAGATGT
R6                CCATCTAGAGCGGCCGCGTTTAAACTATCAGTGTTT^AGAGAATCACAAACCTCTAGATGT

R5F1              ATTAATCTACCCTAGAACTAGTTCACTTTTGTGTGCATACTT----—
Directed Seq.     ATTAATCTACCCTAGAACTAGTTCACTTTTGTGTGCATACTTTTCT
R5                ATTAATCTACCCTAGAACTAGTTCACTTTTGTGTGCATACTTTTCT
R4F1              ATTAATCTACCCTAGAACTAGTTCACTTTTGTGTGCATACTTTTCT
R4                ATTAATCTACCCTAGAACTAGTTCACTTTTGTGTGCATACTTTTCT
R6                ATTAATCTACCCTAGAACTAGTTCACTTTTGTGTGCATACTTTTCT
```

Figure IV-10. Junction sequences detected by JSA. Junction Sequence Class B Alignment (All Generations Tested)
Full consensus sequence for JSC-B showing exact alignment to the independently determined *in planta* locus specific sequence (labeled "Directed Seq." in the figure); individual consensus sequences for each of the five generations are labeled according to their generation (R_4, R_5, R_6, R_4F_1 and R_5F_1). Double underlined text indicates plasmid DNA sequence, single underlined text indicates plant genome sequence, and the carat character "^" indicates the junction point between the MON 87411 insert and the flank. The asterisk character "*" indicates identical nucleotide in every sequence at that position in the alignment. Dash characters indicate positions past the end of the consensus sequence for a particular generation.

IV.E. Inheritance of the Genetic Insert in MON 87411

The MON 87411 T-DNA resides at a single locus within the maize genome and therefore should be inherited according to Mendelian principles of inheritance. During development of MON 87411, phenotypic and genotypic segregation data were recorded to assess the inheritance and stability of the MON 87411 T-DNA using Chi-square (χ^2) analysis over several generations. The χ^2 analysis is based on comparing the observed segregation ratio to the expected segregation ratio according to Mendelian principles.

The MON 87411 breeding path for generating segregation data is described in Figure IV-11. The transformed R_0 plant was self-pollinated to generate R_1 seed. An individual homozygous positive plant was identified in the R1 segregating population via a Real-Time TaqMan® PCR assay.

The homozygous positive R_1 plant was self-pollinated to give rise to R_2 seed. The R_2 plants were self-pollinated to produce R_3 seed. The R_3 plants were self-pollinated to produce R_4 seed. Homozygous positive R_4 plants were crossed via traditional breeding techniques to a recurrent parent (HCL645) that does not contain the DvSnf7^p, *cp4 epsps*, or *cry3Bb1* coding sequences to produce hemizygous R_4F_1 seed. The R_4F_1 plants were crossed with the recurrent parent to produce BC_1F_1 seed. The BC_1F_1 generation was tested for the presence of the T-DNA by End-Point TaqMan PCR to select for hemizygous MON 87411 plants. BC_1F_1 plants hemizygous for MON 87411 T-DNA were crossed with the recurrent parent to produce the BC_2F_1 plants. BC_2F_1 plants hemizygous for MON 87411 T-DNA were self-pollinated to produce the BC_2F_2 plants. BC_2F_1 plants hemizygous for MON 87411 T-DNA were crossed with the recurrent parent to produce the BC_3F_1 plants.

The inheritance of the MON 87411 T-DNA was assessed in the BC_2F_1, BC_2F_2, and $BC3F_1$ generations. At the BC_2F_1 and BC_3F_1 generations, the MON 87411 T-DNA was predicted to segregate at a 1:1 ratio (hemizygous positive: homozygous negative) according to Mendelian inheritance principles. At the BC_2F_2 generation, the MON 87411 T-DNA was predicted to segregate at a 1:2:1 ratio (homozygous positive: hemizygous positive: homozygous negative) according to Mendelian inheritance principles.

A Pearson's Chi-square (χ^2) analysis was used to compare the observed segregation ratios of the MON 87411 T-DNA to the expected ratios. The Chi-square (χ^2) analysis used the statistical program R Version 2.15.2 (2012-10-26).

The Chi-square was calculated as:

$$\chi^2 = \sum [(| o - e |)^2 / e]$$

® TaqMan is a registered trademark of Roche Molecular Systems, Inc.

where o = observed frequency of the genotype or phenotype and e = expected frequency of the genotype or phenotype. The level of statistical significance was predetermined to be 5% ($\alpha = 0.05$).

The results of the χ^2 analysis of the MON 87411 segregating progeny are presented in Table IV-6 and Table IV-7. The χ^2 values in the BC_2F_1 and BC_3F_1 generations indicated no statistically significant difference between the observed and expected 1:1 segregation ratio (hemizygous positive: homozygous negative) of the MON 87411 T-DNA. The χ^2 value for the BC_2F_2 generation indicated no statistically significant difference between the observed and expected 1:2:1 segregation ratio (homozygous positive: hemizygous positive: homozygous negative) of MON 87411 T-DNA. These results support the conclusion that the MON 87411 T-DNA resides at a single locus within the maize genome and is inherited according to Mendelian principles. These results are also consistent with the molecular characterization data indicating that MON 87411 contains a single intact copy of the T-DNA inserted at a single locus in the maize genome.

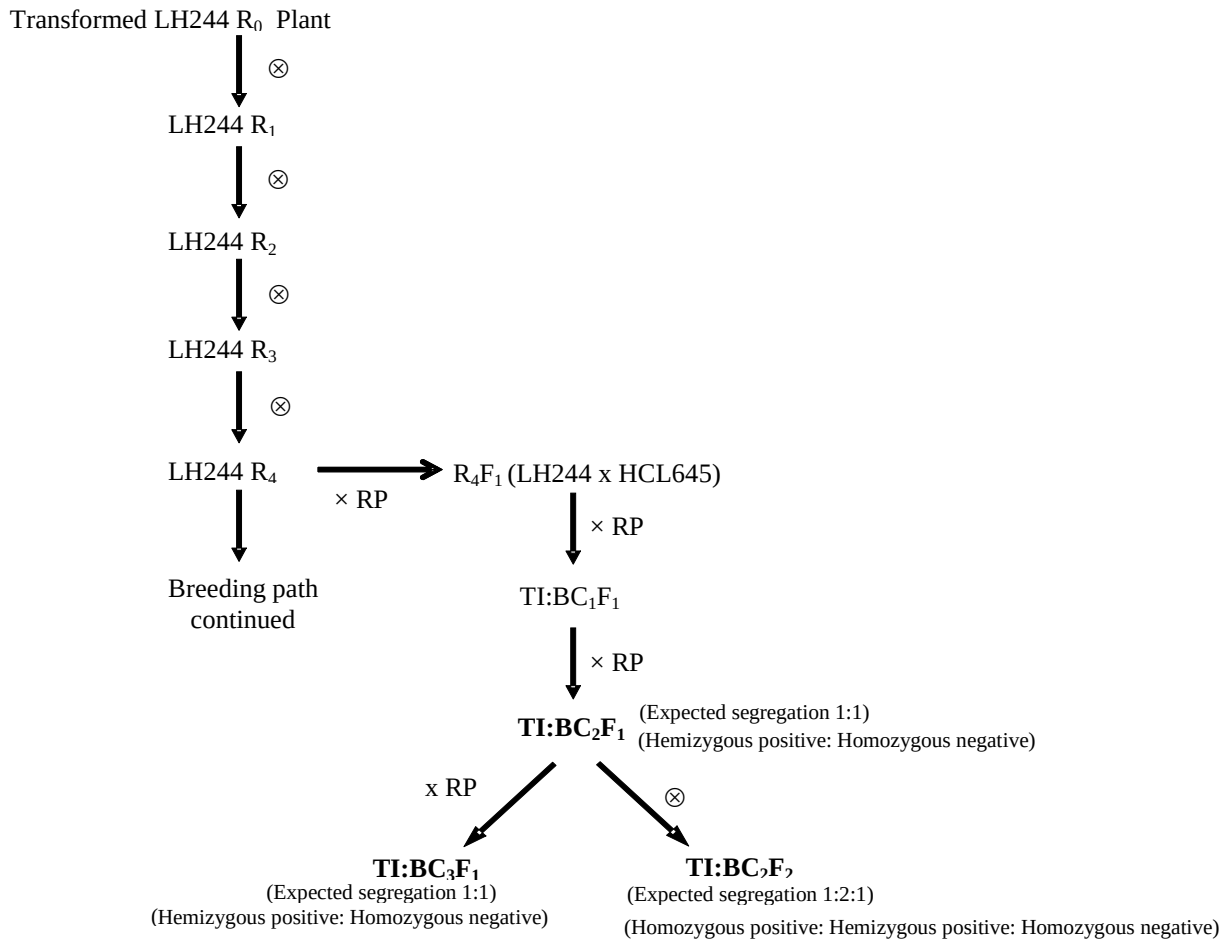


Figure IV-11. Breeding Path for Generating Segregation Data for MON 87411

*Chi-square analysis was conducted on segregation data from the BC_2F_1, BC_2F_2, and BC_3F_1 generations (bolded text).
TI: Trait Integration: Replacement of genetic background of MON 87411 by recurrent background except inserted gene.
RP: Recurring parent.
⊗=Self-Pollinated

Table IV-6. Segregation of the T-DNA During the Development of MON 87411 1:1 Segregation

				1:1 Segregation			
Generation	Total Plants	Observed # Plants Positive	Observed # Plants Negative	Expected # Plants Hemizygous (Positive)	Expected # Plants Homozygous Negative	χ^2	Probability[2]
BC_2F_1[1]	351	172	179	175.50	175.50	0.14	0.709
BC_3F_1[1]	223	104	119	111.50	111.50	1.01	0.315

[1] Segregation was evaluated using an End-Point TaqMan analysis for the MON 87411 insert.
[2] Chi-square analysis was performed to analyze the segregation ratios ($p \leq 0.05$).

Table IV-7. Segregation of the T-DNA During the Development of MON 87411 1:2:1 Segregation

					1:2:1 Segregation				
Generation	Total Plants	Observed # Plants Homozygous Positive	Observed # Plants Hemizygous	Observed # Plants Homozygous Negative	Expected # Plants Homozygous Positive	Expected # Plants Hemizygous	Expected # Plants Homozygous Negative	χ^2	Probability[2]
BC_2F_2[1]	623	152	314	157	155.75	311.50	155.75	0.12	0.942

[1] Segregation was evaluated using Real-Time TaqMan analysis for the MON 87411 insert.
[2] Chi-square analysis was performed to analyze the segregation ratios ($p \leq 0.05$).

IV.F. Characterization of the Genetic Modification Summary and Conclusion

Molecular characterization of MON 87411 by NGS/JSA and directed sequencing demonstrated that a single copy of the intended transfer DNA (T-DNA) containing the DvSnf7 suppression cassette and the *cry3Bb1* and *cp4 epsps* expression cassettes from PV-ZMIR10871 was integrated into the maize genome at a single locus. These analyses also showed no PV-ZMIR10871 backbone DNA had been inserted.

Directed sequence analyses performed on MON 87411 confirmed the organization and intactness of the full T-DNA and all expected elements within the insert, with the exception of incomplete Right and Left Border sequences that do not affect the functionality of the DvSnf7 suppression or *cry3Bb1* and *cp4 epsps* expression cassettes. Analysis of the T-DNA insertion site in maize shows the flanks in MON 87411 are identical to the conventional control, excepting a 118 bp deletion of genomic DNA at the insertion site in MON 87411. This deletion is not expected to affect food or feed safety.

Generational stability analysis by NGS/JSA demonstrated that the T-DNA in MON 87411 was maintained through five breeding generations, thereby confirming the stability of the insert. Results from segregation analyses show heritability and stability of the insert occurred as expected across multiple generations, which corroborates the molecular insert stability analysis and establishes the presence of the T-DNA in MON 87411 at a single chromosomal locus.

V. CHARACTERIZATION AND SAFETY ASSESSMENT OF THE Cry3Bb1 and CP4 EPSPS PROTEINS PRODUCED IN MON 87411

Characterization of the introduced protein(s) in a biotechnology-derived crop is important to establishing food, feed, and environmental safety. As described in Section IV, MON 87411 contains *cry3Bb1* and *cp4 epsps* expression cassettes that, when transcribed and translated, result in the expression of the Cry3Bb1 and CP4 EPSPS proteins. The characterization and safety assessment for the DvSnf7 suppression cassette is described in Section VI.

This section summarizes: 1) the identity and function of the Cry3Bb1 and CP4 EPSPS proteins produced in MON 87411; 2) assessment of equivalence between the plant-produced and *E. coli*-produced proteins; 3) the level of the Cry3Bb1 and CP4 EPSPS proteins in plant tissues from MON 87411; 4) assessment of the potential allergenicity of the Cry3Bb1 and CP4 EPSPS proteins produced in MON 87411; and 5) the food and feed safety assessment of the Cry3Bb1 and CP4 EPSPS proteins produced in MON 87411. The data are consistent with prior safety assessments of these two proteins and support a conclusion that the proteins produced in MON 87411 are safe for human or animal consumption and safe for the environment based on several lines of evidence summarized below.

V.A. Identity and Function of the Cry3Bb1 and CP4 EPSPS Proteins from MON 87411

V.A.1. Identity and Function of the Cry3Bb1 Protein from MON 87411

Cry3Bb1 protein originates from *Bacillus thuringiensis* (*Bt*), a ubiquitous gram-positive soil bacterium that accumulates crystal proteins during sporulation. Cry3Bb1 protein is a member of the 3D-Cry family of insecticidal proteins (Crickmore 2012). Proteins within the 3D-Cry proteins are subdivided into different groups based on the high specificity they have for their target category of insects. Because of their narrow spectrum of activity, they lack an impact on broader insect populations or other organisms. For example, Cry3 proteins have insecticidal activity specifically against coleopteran insects, while Cry1A proteins have insecticidal activity specifically against lepidopteran insects (Höfte and Whiteley 1989).

The generalized MOA for Cry proteins was described by English and Slatin (1992). It includes ingestion of the crystals by insects and solubilization of the crystals in the insect midgut, followed by activation through proteolytic processing of the soluble Cry protein by digestive enzymes in the midguts. The activated protein then binds to specific receptors on the surface of the midgut epithelium of target insects and inserts into the membrane, leading to pore formation and generalized disruption of the transmembrane gradients and, therefore, cell integrity. While alternate mechanisms have also been proposed, a review of the available data has recently been published and the authors concluded that the original model, pore formation, is the most valid model for Cry protein mode of action (Vachon, et al. 2012).

Cry3Bb1 protein in MON 87411 is a protein consisting of a single polypeptide of 652 amino acids. Like other Cry proteins, it is synthesized as a prototoxin and is likely cleaved by digestive enzymes in the midgut of target organisms to an approximately 60 kDa activated protein (Bravo, et al. 2007). Cry3Bb1 is also expressed in commercially available YieldGard VT Rootworm/RR2 (MON 88017) maize and SmartStax® maize. The amino acid sequence deduced from the Cry3Bb1 expression cassette present in YieldGard VT Rootworm/RR2 is identical to that deduced from the Cry3Bb1 expression cassette present in MON 87411. A related Cry3Bb1 protein, which has over 99% amino acid identity to the Cry3Bb1 in YieldGard VT Rootworm/RR2 and MON 87411, is expressed in YieldGard Rootworm maize (MON 863). Each of these products were previously reviewed by USDA-APHIS and found to not have any unique plant pest risks relative to conventional maize and were subsequently deregulated.

V.A.2. Identity and Function of the CP4 EPSPS Protein from MON 87411

The enzyme, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), catalyzes one of the enzymatic steps of the shikimic acid pathway, and is the target for the broad spectrum herbicide glyphosate (Haslam 1993; Herrmann and Weaver 1999; Kishore, et al. 1988; Steinrücken and Amrhein 1980). The shikimic acid pathway and EPSPS enzymes are ubiquitous to plants and microorganisms, but absent in mammals, fish, birds, reptiles, and insects (Alibhai and Stallings 2001). EPSPS proteins have been isolated from both plant and microbial sources and their properties have been extensively studied (Harrison, et al. 1996; Haslam 1993; Schönbrunn, et al. 2001; Steinrücken and Amrhein 1984). The plant and microbial enzymes are mono-functional with a molecular weight of 44-51 kDa (Franz, et al. 1997; Kishore et al. 1988). EPSPS enzymes catalyze the transfer of the enolpyruvyl group from phosphoenolpyruvate (PEP) to the 5-hydroxyl of shikimate-3-phosphate (S3P), thereby yielding inorganic phosphate and 5-enolpyruvylshikimate-3-phosphate (EPSP) (Alibhai and Stallings 2001). Shikimic acid is a substrate for the biosynthesis of the aromatic amino acids (phenylalanine, tryptophan and tyrosine) and other aromatic molecules necessary for plant growth.

The EPSPS transgene in MON 87411 is derived from *Agrobacterium* sp. strain CP4 (*cp4 epsps*). The *cp4 epsps* coding sequence encodes an EPSPS protein consisting of a single polypeptide of 455 amino acids (Padgette et al. 1996). The CP4 EPSPS protein is similar and functionally identical to endogenous plant EPSPS enzymes, but has a much reduced affinity for glyphosate, the active ingredient in Roundup agricultural herbicides, relative to endogenous plant EPSPS (Padgette et al. 1996). In conventional plants, including weeds, glyphosate blocks the biosynthesis of EPSP, thereby depriving plants of essential amino acids (Haslam 1993; Steinrücken and Amrhein 1980). In Roundup Ready plants, which are tolerant to Roundup agricultural herbicides, requirements for aromatic amino acids and other metabolites are met by the continued action of the CP4 EPSPS enzyme in the presence of glyphosate (Padgette et al. 1996). The CP4 EPSPS protein expressed in MON 87411 is identical to the CP4 EPSPS protein expressed in Roundup Ready products across several crops, including soybeans, corn, canola, cotton, sugar beet, and alfalfa.

V.B. Characterization and Equivalence of Cry3Bb1 and CP4 EPSPS Proteins from MON 87411

The safety assessment of crops derived through biotechnology includes characterization of the physicochemical and functional properties of the protein(s) produced from the inserted DNA, and confirmation of the safety of the protein(s). For safety data generated using proteins produced from a heterologous source (*e.g.*, *E. coli*-produced protein) to be applied to plant-produced protein(s), the equivalence of the plant and *E. coli*-produced proteins must be assessed. The physicochemical and functional characteristics of the MON 87411-produced CP4 EPSPS and MON 87411-produced Cry3Bb1 proteins were determined and each was shown to be equivalent to its respective *E. coli*-produced protein. A summary of the analytical results for each protein are shown below and the details of the materials, methods, and results are described in Appendix C.

The Cry3Bb1 and CP4 EPSPS proteins purified from grain of MON 87411 were characterized and the equivalence of the physicochemical characteristics between the MON 87411-produced and the *E. coli*-produced proteins was established using a panel of analytical tests: 1) N-terminal sequence analysis of the MON 87411-produced proteins established identity; 2) MALDI-TOF MS analysis yielded peptide masses consistent with the expected peptide masses from the theoretical trypsin digest of the MON 87411-produced sequences; 3) MON 87411-produced Cry3Bb1 and CP4 EPSPS proteins were detected on western blots probed with their respective protein-specific antibodies and the immunoreactive properties of the MON 87411-produced and *E. coli*-produced proteins were shown to be equivalent; 4) the apparent molecular weights of the MON 87411-produced and *E. coli*-produced proteins, assessed by SDS-PAGE, were shown to be equivalent; 5) MON 87411-produced and *E. coli*-produced MON 87411 proteins were both determined to be non-glycosylated; and 6) functional (biological) activity of the MON 87411-produced and *E. coli*-produced proteins were demonstrated to be equivalent for both Cry3Bb1 and CP4 EPSPS.

Taken together, these data provide a detailed characterization of the MON 87411-produced Cry3Bb1 and CP4 EPSPS proteins and establish their respective equivalence to *E. coli*-produced Cry3Bb1 and CP4 EPSPS proteins. This equivalence justifies the use of previously conducted protein studies using *E. coli*-produced Cry3Bb1 and CP4 EPSPS proteins to establish the safety of the Cry3Bb1 and CP4 EPSPS proteins expressed in MON 87411, summarized in section V.E.

V.C. Expression Levels of Cry3Bb1 and CP4 EPSPS Proteins in MON 87411

Cry3Bb1 and CP4 EPSPS protein levels in various tissues of MON 87411 relevant to the risk assessment were determined by a validated enzyme-linked immunosorbent assay (ELISA). Tissues of MON 87411 were collected from four replicate plots planted in a randomized complete block field design during the 2011 - 2012 growing season from the following five field sites in Argentina: Pergamino, Buenos Aires (Site Code BAFO); Hunter, Buenos Aires (Site Code BAHT); Pergamino, Buenos Aires (Site Code BAPE); Sarasa, Buenos Aires (Site Code BASS) and Salto, Buenos Aires (Site Code BATC). The field sites were representative of maize-producing regions suitable for commercial maize production. Maize production in the U.S. corn belt and Argentina growing regions

occurs at relatively similar latitudes with an approximate 6 month offset (Schnepf, et al. 2001). The average growing season temperatures and precipitation are comparable (Schnepf et al. 2001) and, as a result, maize hybrids developed in the U.S. are often used directly by farmers in the southern growing regions of Argentina. As such, protein expression analyses from maize grown at these sites are appropriate for a comparative assessment. Nineteen total tissue samples were collected from each replicated plot at each site, many over several time points/growth stages, throughout the season. Samples included over season leaf (OSL1 through OSL4), over season root (OSR1 through OSR4), over season whole plant (OSWP1 through OSWP4), stover, senescent root, forage root, forage, grain, pollen and silk. MON 87411 plots were treated with glyphosate to generate samples under conditions of the intended use (0.95 lbs active ingredient/ hectare) of the product.

V.C.1. Expression Levels of Cry3Bb1 Protein

Cry3Bb1 protein levels were determined in 19 tissue types. The ELISA results obtained for each sample were averaged across the five sites and are summarized in Table V-1. The details of the materials and methods are described in Appendix D. The individual Cry3Bb1 protein levels in MON 87411 across all samples analyzed from all sites ranged from 3.0 to 460 µg/g dw. The mean Cry3Bb1 protein level among all tissue types was highest in OSWP1 at 340 µg/g dw and lowest in grain at 4.0 µg/g dw.

Table V-1. Summary of Cry3Bb1 Protein Levels in Tissues from MON 87411 Grown in 2011 – 2012 Argentina Field Trials

Tissue[1]	Development Stage[2]	Days After Planting (DAP)[3]	Cry3Bb1 Mean (SD) Range (µg/g fw)[4]	Cry3Bb1 Mean (SD) Range (µg/g dw)[5]	LOQ/LOD (µg/g fw)[6]
OSL1	V3-V4	21-22	45 (9.0) 31 – 64	270 (65) 160 - 390	0.035/0.006
OSL2	V6-V8	35-44	40 (7.8) 26 – 56	210 (40) 120 – 270	0.035/0.006
OSL3	V10-V13	50-55	40 (7.9) 21 – 52	170 (35) 92 – 220	0.035/0.006
OSL4	V14-R1	59-78	56 (19) 31 – 89	220 (63) 130 – 340	0.035/0.006
OSR1	V3-V4	21-22	25 (4.6) 16 – 32	180 (43) 130 – 280	0.035/0.028
OSR2	V6-V8	35-44	16 (4.0) 9.4 – 25	120 (24) 67 – 170	0.035/0.028
OSR3	V10-V13	50-55	15 (4.0) 9.6 – 24	84 (21) 54 – 130	0.035/0.028
OSR4	V14-R1	59-78	14 (3.3) 9.0 – 21	75 (19) 43 – 120	0.035/0.028
OSWP1	V3-V4	21-22	44 (4.9) 33 – 53	340 (49) 250 – 460	0.035/0.008
OSWP2	V6-V8	35-44	30 (5.3) 21 – 40	190 (30) 130 – 270	0.035/0.008
OSWP3	V10-V13	50-55	20 (6.8) 9.2 – 33	140 (39) 59 – 210	0.035/0.008
OSWP4	V14-R1	59-78	20 (4.8) 12 – 29	120 (28) 71 – 170	0.035/0.008
Stover	R6	136-155	10 (6.2) 1.9 – 19	21 (13) 4.7 – 44	0.035/0.008
Senescent Root	R6	136-155	4.8 (3.1) 0.76 – 12	19 (13) 3.0 – 50	0.035/0.028

Table V-1. (continued) Summary of Cry3Bb1 Protein Levels in Tissues from MON 87411 Grown in 2011 – 2012 Argentina Field Trials

Tissue[1]	Development Stage[2]	Days After Planting (DAP)[3]	Cry3Bb1 Mean (SD) Range (µg/g fw)[4]	Cry3Bb1 Mean (SD) Range (µg/g dw)[5]	LOQ/LOD (µg/g fw)[6]
Forage Root	R5	101-111	7.9 (3.5) 2.6 – 15	36 (16) 13 – 66	0.035/0.028
Forage	R5	101-111	12 (4.9) 5.5 – 23	39 (17) 18 – 75	0.035/0.008
Grain	R6	139-154	3.5 (0.45) 2.7 – 4.4	4.0 (0.56) 3.1 – 5.1	0.035/0.007
Pollen	VT-R1	65-80	29 (3.0) 23 – 34	36 (4.0) 30 – 42	0.035/0.018
Silk	R1	65-81	16 (3.8) 8.5 – 23	160 (37) 89 – 220	0.035/0.010

[1] OSL= over season leaf; OSR= over season root; OSWP= over season whole plant
[2] The crop development stage each tissue was collected.
[3] The number of days after planting that each tissue was collected.
[4] Protein levels are expressed as the arithmetic mean and standard deviation (SD) as microgram (µg) of protein per gram (g) of tissue on a fresh weight basis (fw). The means, SD, and ranges (minimum and maximum values) were calculated for each tissue across all sites (n=20).
[5] Protein levels are expressed as the arithmetic mean and standard deviation (SD) as microgram (µg) of protein per gram (g) of tissue on a dry weight basis (dw). The dry weight values were calculated by dividing the µg/g fw by the dry weight conversion factor obtained from moisture analysis data.
[6] LOQ=limit of quantitation; LOD=limit of detection.

V.C.2. Expression Levels of CP4 EPSPS Protein

CP4 EPSPS protein levels were determined in all 19 tissue types. The ELISA results obtained for each sample were averaged across the five sites and are summarized in Table V-2. The details of the materials and methods are described in Appendix D. The individual CP4 EPSPS protein levels in MON 87411 across all samples analyzed from all sites ranged from less than the limit of quantitation (<LOQ) to 76 µg/g dw. The mean CP4 EPSPS protein level among all tissue types was highest in OSWP1 at 63 µg/g dw and lowest in grain at 1.9 µg/g dw.

Table V-2. Summary of CP4 EPSPS Protein Levels in Tissues from MON 87411 Grown in 2011 – 2012 Argentina Field Trials

Tissue[1]	Development Stage[2]	Days After Planting (DAP)[3]	CP4 EPSPS Mean (SD) Range (µg/g fw)[4]	CP4 EPSPS Mean (SD) Range (µg/g dw)[5]	LOQ/LOD (µg/g fw)[6]
OSL1	V3-V4	21-22	7.1 (0.83) 5.8 – 9.0	42 (5.9) 33 – 55	0.137/0.071
OSL2	V6-V8	35-44	7.0 (0.64) 6.0 – 7.9	36 (3.1) 29 – 39	0.137/0.071
OSL3	V10-V13	50-55	7.4 (0.78) 6.4 – 8.9	32 (3.8) 27 – 42	0.137/0.071
OSL4	V14-R1	59-78	7.8 (0.85) 6.6 – 9.5	31 (3.5) 24 – 37	0.137/0.071
OSR1	V3-V4	21-22	6.5 (0.86) 4.4 – 8.0	48 (6.6) 38 – 63	0.068/0.033
OSR2	V6-V8	35-44	5.2 (1.0) 3.8 – 7.1	37 (7.0) 23 – 48	0.068/0.033
OSR3	V10-V13	50-55	5.6 (0.84) 4.0 – 7.1	31 (4.7) 24 – 37	0.068/0.033
OSR4	V14-R1	59-78	5.7 (0.80) 4.2 – 7.1	30 (4.8) 20 – 38	0.068/0.033
OSWP1	V3-V4	21-22	8.1 (0.90) 6.6 – 9.8	63 (6.7) 54 – 76	0.137/0.070
OSWP2	V6-V8	35-44	5.6 (0.94) 3.4 – 7.4	36 (5.8) 21 – 46	0.137/0.070
OSWP3	V10-V13	50-55	4.6 (1.1) 2.3 – 6.6	33 (6.2) 21 – 45	0.137/0.070
OSWP4	V14-R1	59-78	4.3 (0.87) 2.9 – 5.5	25 (5.0) 17 – 32	0.137/0.070
Stover	R6	136-155	1.0 (0.60) 0.30 – 2.1	2.2 (1.2) 0.59 – 4.9	0.137/0.070
Senescent Root	R6	136-155	1.4 (0.69) 0.49 – 2.6	5.4 (2.9) 1.8 – 11	0.068/0.033

Table V-2. (continued) Summary of CP4 EPSPS Protein Levels in Tissues from MON 87411 Grown in 2011 – 2012 Argentina Field Trials

Tissue[1]	Development Stage[2]	Days After Planting (DAP)[3]	CP4 EPSPS Mean (SD) Range (µg/g fw)[4]	CP4 EPSPS Mean (SD) Range (µg/g dw)[5]	LOQ/LOD (µg/g fw)[6]
Forage Root	R5	101-111	2.2 (0.81) 1.1 – 4.1	10 (3.7) 5.1 – 19	0.068/0.033
Forage	R5	101-111	2.4 (0.71) 1.5 – 3.8	8.0 (2.3) 5.2 – 13	0.137/0.070
Grain	R6	139-154	1.7 (0.27) 1.4 – 2.7	1.9 (0.31) 1.6 – 3.1	0.228/0.152
Pollen	VT-R1	65-80	15 (1.9) 12 – 19	19 (2.8) 16 – 24	0.137/0.099
Silk	R1	65-81	4.0 (0.69) 3.1 – 5.1	40 (5.0) 32 – 49	0.137/0.121

[1] OSL= over season leaf; OSR= over season root; OSWP= over season whole plant

[2] The crop development stage each tissue was collected.

[3] The number of days after planting that each tissue was collected.

[4] Protein levels are expressed as the arithmetic mean and standard deviation (SD) as microgram (µg) of protein per gram (g) of tissue on a fresh weight basis (fw). The means, SD, and ranges (minimum and maximum values) were calculated for each tissue across all sites (n=20, except for stover where n=19 due to one sample expressing <LOQ).

[5] Protein levels are expressed as the arithmetic mean and standard deviation (SD) as microgram (µg) of protein per gram (g) of tissue on a dry weight basis (dw). The dry weight values were calculated by dividing the µg/g fw by the dry weight conversion factor obtained from moisture analysis data.

[6] LOQ=limit of quantitation; LOD=limit of detection.

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V.D. Assessment of Potential Allergenicity of the Cry3Bb1 and CP4 EPSPS Proteins

The allergenic potential of an introduced protein is assessed by comparing the physiochemical characteristics of the introduced protein to physiochemical characteristics of known allergens (Codex Alimentarius 2009). Using a weight of evidence approach, a protein is not likely to be associated with allergenicity if: 1) the protein is from a non-allergenic source; 2) the protein represents a small portion of the total plant protein; 3) the protein does not share structural similarities to known allergens based on the amino acid sequence; and 4) the protein does not show resistance to pepsin digestion. The Cry3Bb1 and CP4 EPSPS proteins have been assessed for their potential allergenicity according to these safety assessment guidelines.

V.D.1. Assessment of Potential Allergenicity of the Cry3Bb1 Protein

The Cry3Bb1 protein has been assessed for its potential allergenicity according to the Codex safety assessment guidelines described above, and conclusions were as follows.

1) The Cry3Bb1 protein originates from *Bt*, an organism that has not been reported to be a source of known allergens.

2) The Cry3Bb1 protein represents no more than 0.004% of the total protein in the grain of MON 87411.

3) Bioinformatics analyses demonstrated that the Cry3Bb1 protein does not share amino acid sequence similarities with known allergens and, therefore, is highly unlikely to contain immunologically cross-reactive allergenic epitopes.

4) Finally, *in vitro* digestive fate experiments conducted with the Cry3Bb1 protein demonstrate that the protein is rapidly digested by pepsin in a simulated gastric fluid (SGF) assay.

Taken together, these data support the conclusion that the Cry3Bb1protein does not pose a significant allergenic risk to humans or animals.

V.D.2. Assessment of Potential Allergenicity of the CP4 EPSPS Protein

The CP4 EPSPS protein has been assessed for its potential allergenicity according to the Codex safety assessment guidelines described above, and conclusions are as follows.

1) The CP4 EPSPS protein originates from *Agrobacterium* sp. strain CP4, an organism that has not been reported to be a source of known allergens.

2) The CP4 EPSPS protein represents no more than 0.002% of the total protein in the seed of MON 87411.

3) Bioinformatics analyses demonstrated that the CP4 EPSPS protein does not share amino acid sequence similarities with known allergens and, therefore, is highly unlikely to contain immunologically cross-reactive allergenic epitopes.

4) Finally, *in vitro* digestive fate experiments conducted with the CP4 EPSPS protein demonstrate that the protein is rapidly digested by pepsin in a SGF assay.

Taken together, these data support the conclusion that the CP4 EPSPS protein does not pose a significant allergenic risk to humans or animals.

V.E. Safety Assessment Summary of Cry3Bb1 and CP4 EPSPS Proteins in MON 87411

A comprehensive set of factors have been considered and assessed in the safety assessment of the Cry3Bb1 and CP4 EPSPS proteins in food and feed or the environment. The results are summarized below along with the conclusions reached from each assessment.

V.E.1. Cry3Bb1 Donor Organism, History of Safe Use, and Specificity

V.E.1.1 The *cry3Bb1* Donor Organism is Safe

The donor organism for *cry3Bb1, Bacillus thuringiensis,* has been used commercially in the United States since 1958 to produce microbial-derived products with insecticidal activity. The extremely low mammalian toxicity of *Bt*-based insecticide products has been demonstrated in numerous safety studies, and there are no confirmed cases of allergic reactions to Cry proteins in applicators of microbial-derived *Bt* products during 50 years of use.

Applications of sporulated *Bt* have a long history of safe use for pest control in agriculture, especially in organic farming (Cannon 1993; IPCS 1999; U.S. EPA 1988). Microbial pesticides containing *Bt* Cry3 proteins have been used for more than 30 years and subjected to extensive toxicity testing showing no adverse effects to human health (Baum, et al. 1999; Betz, et al. 2000; McClintock, et al. 1995; Mendelsohn, et al. 2003; U.S. EPA 2001b; 2005).

V.E.1.2. Cry3Bb1 Protein has a History of Safe Use

A history of safe use of Cry3Bb1 protein has been established (Cannon 1993; IPCS 1999; U.S. EPA 1988). Microbial pesticides containing *Bt* Cry proteins have been used for more than 50 years and subjected to extensive toxicity testing showing no adverse effects to human health (Baum et al. 1999; Betz et al. 2000; McClintock et al. 1995; Mendelsohn et al. 2003; U.S. EPA 2001b; 2005). Cry3Bb1, specifically, is one of the active ingredients in the microbial pesticide mixture Raven® Oil Flowable Bioinsecticide that was commercialized in the United States to control Colorado Potato Beetle (Baum et al. 1999). Cry3Bb1 is also expressed in commercially available YieldGard VT Rootworm/RR2 (MON 88017) maize and SmartStax® maize. The amino acid sequence deduced from the Cry3Bb1 expression cassette present in YieldGard VT Rootworm/RR2 is identical to that deduced from the Cry3Bb1 expression cassette present in MON 87411. A related Cry3Bb1 protein, which has over 99% amino acid identity to the Cry3Bb1 in YieldGard VT Rootworm/RR2 and MON 87411, is expressed in YieldGard Rootworm maize (MON 863). The United States EPA has approved commercial use of the

Cry3Bb1 as expressed in maize and has established an exemption from the requirement of a tolerance for residues of the Cry3Bb1 protein and the genetic material for its production in maize (U.S. EPA 2004a). The history of large scale cultivation of these Cry3Bb1-expressing crops without any indication of harmful impact on the environment, non-target organisms, or mammals provides additional evidence for the safety of the Cry3Bb1 protein. Taken together, these data demonstrate that the Cry3Bb1 protein has a history of safe use and does not pose any unexpected effects to human and animal health or the environment.

V.E.1.3. Cry3Bb1 Protein Demonstrates Specificity

Cry3Bb1 protein, like other Cry3 proteins, has insecticidal activity specifically against coleopteran insects (Höfte and Whiteley 1989) and is covered in more detail in Section X.C.1.

V.E.2 CP4 EPSPS Donor Organism, History of Safe Use, and Specificity

V.E.2.1 The *cp4 epsps* Donor Organism is Safe

The donor organism for *cp4 epsps*, *Agrobacterium* sp. strain CP4, is not known for human or animal pathogenicity, and is not commonly allergenic (FAO-WHO 1991). The history of safe use of *Agrobacterium* sp. strain CP4 has been previously reviewed as a part of the safety assessment of this donor organism for USDA-APHIS deregulations, as well as completed Monsanto consultations with the FDA regarding Roundup Ready varieties of soybean (1995 and 2007), canola (1995, 2002, and 2012), maize (1998 and 2000), sugar beet (1998 and 2004), alfalfa (2004), and cotton (1995 and 2005).

V.E.2.2. CP4 EPSPS Protein has a History of Safe Use

The CP4 EPSPS protein present in MON 87411 is similar to EPSPS proteins consumed in a variety of food and feed sources. CP4 EPSPS protein is homologous to EPSPS proteins naturally present in plants, including food and feed crops (*e.g.*, soybean and maize) and fungal and microbial food sources such as baker's yeast (*Saccharomyces cerevisiae*), all of which have a history of safe consumption (Harrison et al. 1996; Padgette et al. 1996). The similarity of the CP4 EPSPS protein to EPSPS proteins in a variety of foods and feeds supports extensive safe consumption of the family of EPSPS proteins and the lack of human or animal health concerns. The ubiquitous presence of homologous EPSPS enzymes in crops and common microorganisms establishes that EPSPS proteins, and their enzymatic activity, pose no hazards to humans, animals, or the environment. In addition, the CP4 EPSPS protein in MON 87411 is identical to the CP4 EPSPS protein in numerous other Roundup Ready varieties of soybean, maize, canola, sugar beet, cotton and alfalfa. Further, the U.S. EPA has established an exemption from the requirement of a tolerance for residues of CP4 EPSPS protein and the genetic material necessary for its production in all plants (U.S. EPA 1996).

V.E.2.3. CP4 EPSPS Catalyzes a Specific Enzyme Reaction

CP4 EPSPS, like other EPSPS enzymes, functions in the shikimate pathway that is integral to aromatic amino acid biosynthesis in plants and microorganisms (Levin and Sprinson 1964; Steinrücken and Amrhein 1980). Therefore, EPSPS and its activity are found widely in food and feed derived from plant and microbial sources. Genes for numerous EPSPS proteins have been cloned (Padgette et al. 1996) and the catalytic domains of this group of proteins are conserved. Bacterial EPSPS proteins have been well characterized with respect to their three dimensional X-ray crystal structures (Stallings, et al. 1991) and detailed kinetic and chemical mechanisms (Anderson and Johnson 1990).

V.E.3. Cry3Bb1 and CP4 EPSPS Proteins in MON 87411 are Not Homologous to Known Allergens or Toxins

Bioinformatics analyses were performed to assess the potential for allergenicity, toxicity, or biological activity of Cry3Bb1 and CP4 EPSPS. The analyses demonstrated that neither protein shares amino acid sequence similarities with known allergens, gliadins, glutenins, or protein toxins that could have adverse effects to human or animal health.

V.E.4. Cry3Bb1 and CP4 EPSPS Proteins in MON 87411 are Labile in *in vitro* Digestion Assays

As has been described in previous regulatory submissions for a number of Roundup Ready crops and MON 88017 (APHIS Petition No. 04-125-01), both the Cry3Bb1 and CP4 EPSPS proteins are readily digestible in SGF (USDA-APHIS 2013). Digestion in simulated intestinal fluid (SIF) was also assessed. CP4 EPSPS was readily digestible in SIF. As expected, Cry3Bb1 subjected to SIF alone was processed to a trypsin-resistant core of ~66 kDa which was stable throughout the SIF digestion period. This result is consistent with observations for this and other Cry proteins subjected to SIF digestion. Rapid degradation of the Cry3Bb1 and CP4 EPSPS proteins in SGF makes it highly unlikely that either protein would be absorbed in a form other than as component nutritional amino acids in the small intestine or have any adverse effects on human or animal health.

V.E.5. Cry3Bb1 and CP4 EPSPS Proteins in MON 87411 are Not Acutely Toxic

Acute oral toxicology studies were conducted with Cry3Bb1 and CP4 EPSPS proteins individually. Results indicate that neither Cry3Bb1 nor CP4 EPSPS caused any adverse effects in mice, with No Observable Adverse Effect Levels (NOAELs) for CP4 EPSPS at 572 mg/kg (Harrison et al. 1996) and for Cry3Bb1 at 1930 mg/kg bw (FSANZ 2006), the highest doses tested. As described below, the highest dose levels tested are many fold higher than levels of Cry3Bb1 or CP4 EPSPS that are present in tissues of MON 87411.

V.E.6. Human and Animal Exposure to the Cry3Bb1 and CP4 EPSPS Proteins

A common approach used to assess potential health risks for potentially toxic materials is to calculate a Margin of Exposure (MOE) between the lowest NOAEL from an

appropriate animal toxicity study and an estimate of human exposure. Since no evidence of mammalian toxicity has been reported for Cry3Bb1 or CP4 EPSPS, dietary risk assessments would normally not be considered necessary. Nevertheless, a dietary risk assessment was still conducted for these proteins in order to provide further assurances of safety by calculating a MOE between the NOAELs for the Cry3Bb1 and CP4 EPSPS proteins in mouse acute oral toxicity studies (Section V.E.5) and 95th percentile consumption estimates of acute dietary exposure determined using the Dietary Exposure Evaluation Model - Food Commodity Intake Database (DEEM-FCID) (U.S. EPA 2013). DEEM-FCID utilizes food consumption data from the National Health and Nutrition Examination Survey (NHANES) conducted in 2003-2008 (Included: vegetable maize, popcorn, and field maize (flour, meal, bran and starch)). Based on levels of expressed protein on a fresh weight basis (μg/g) discussed above (Sections V.C.1 and V.C.2), 95th percentile exposures to Cry3Bb1 and CP4 EPSPS for the general U.S. population were estimated to be 7.2 and 3.5 μg/kg body weight (bw), respectively. For non-nursing infants in the U.S., the most highly exposed sub-population, 95th percentile exposures to Cry3Bb1 and CP4 EPSPS were estimated to be 17.1 and 8.3 μg/kg, respectively. For the Cry3Bb1 protein, MOEs for acute dietary intake were estimated to be 270,000 (2.7×10^5) and 110,000 (1.1×10^5) for the general population and non-nursing infants, respectively. For the CP4 EPSPS protein, MOEs for acute dietary intake were estimated to be 160,000 (1.6×10^5) and 69,000 (6.9×10^4) for the general population and non-nursing infants, respectively. Actual MOEs will likely be much higher because: 1) the exposure estimates utilized are conservative (95th percentile, assume 100% MON 87411) and 2) as described in section V.E.4, Cry3Bb1 and CP4 EPSPS are rapidly digested, further minimizing exposures. These very large MOEs[5] indicate that there is no meaningful risk to human health from dietary exposure to the Cry3Bb1 or CP4 EPSPS proteins produced by MON 87411.

There are a number of steps in the processing of maize to make food ingredients, including high temperature treatments, hydrolyses, soaking in slightly acidic water, and drying that can denature a protein. Changes in temperature, pH, and physical disruptions associated with food processing and cooking/preparation generally lead to loss of protein structure and functionality (Hammond and Jez 2011). Like other proteins, the Cry3Bb1 and CP4 EPSPS proteins in MON 87411 are expected to be similarly susceptible to denaturation when exposed to high temperatures, pH extremes, and digestive environments encountered during processing and cooking of foods containing MON 87411. Thus, there are likely to be significantly lower exposures to the functionally active forms of these proteins through consumption of MON 87411 than the levels estimated above.

The potential Cry3Bb1 and CP4 EPSPS protein exposure to animals from consumption of MON 87411 in feeds was evaluated by calculating an estimate of daily dietary intake (DDI). The highest percentage of Cry3Bb1 and CP4 EPSPS proteins (g/kg bw) per total

[5] These MOEs reflect that a human would have to eat thousands of kilograms of maize in a short time period to achieve exposures to the expressed proteins in MON 87411 that were not toxic to mice, which would be a physical impossibility.

protein consumed was in the lactating dairy cow, 0.014% (g/g) and 0.0034% (g/g) of the total dietary protein intake (0.000876 g Cry3Bb1/kg bw divided by 6.2 g dietary protein, and 0.000210 g CP4 EPSPS/kg bw divided by 6.2 g dietary protein which is the total dietary protein intake for the cow), respectively. The chicken and pig percentages of the Cry3Bb1 and CP4 EPSPS proteins consumed as part of the daily protein intake are much less than for the lactating dairy cow. In the worst case scenario, per kg body weight, poultry, swine and lactating dairy cattle would be consuming 0.02% (g/g) and 0.004% (g/g) or less of their total protein as Cry3Bb1 and CP4 EPSPS proteins, respectively, from MON 87411 maize.

In summary, there is no significant risk to human and animal health associated with dietary exposure to the Cry3Bb1 and CP4 EPSPS proteins in food and feed products derived from MON 87411.

V.E.7. CP4 EPSPS Activity

The CP4 EPSPS protein produced in MON 87411 is similar to native EPSPS proteins that are ubiquitous in plant and microbial tissues in the environment and is not known to be toxic to other organisms (ILSI-CERA 2010; 2011; USDA-APHIS 2013).

V.E.8. Cry3Bb1 Activity

The spectrum of activity of the modified Cry3Bb1 protein has previously been reviewed by USDA-APHIS in submissions for both MON 863 and MON 88017 (USDA-APHIS 2013) and U.S. EPA (U.S. EPA 2010). Significant insecticidal activity has only been seen in the family Chrysomelidae within the Order Coleoptera (U.S. EPA 2010). The *Cry3Bb1* genes present in MON 863 and MON 88017 code for Cry3Bb1 proteins that are >99.8% identical and which have been shown to be functionally equivalent in insect bioassays with two species sensitive to the Cry3Bb1 protein (U.S. EPA 2010). The *Cry3Bb1* gene present in MON 87411 codes for Cry3Bb1 protein that is identical to that in MON 88017. Consequently, the Cry3Bb1 in MON 87411 is predicted to have equivalent functional activity and spectrum of activity as in those two previously registered and deregulated maize products.

V.E.9. Non-Target Assessment for CP4 EPSPS Protein

The USDA-APHIS has previously determined that the gene imparting glyphosate tolerance, *cp4 epsps*, and the CP4 EPSPS protein that it encodes, poses no significant risk to non-target organisms due to its long history of use and no known toxicity to non-target organisms (Harrison et al. 1996; ILSI-CERA 2010; USDA-APHIS 2013).

V.E.10. Non-Target Assessment for Cry3Bb1 Protein

USDA-APHIS and EPA have conducted plant pest and environmental assessments of MON 863 and MON 88017 that produce the Cry3Bb1 protein. The *Cry3Bb1* gene present in MON 87411 has the same coding sequence as the *Cry3Bb1* gene present in MON 88017 and the tissue expression of the Cry3Bb1 protein is comparable to the previously registered maize products MON 88017 and MON 863. For MON 863, a

series of NTOs were evaluated to assess for potential negative impacts of the Cry3Bb1 protein (U.S. EPA 2010). Exposure concentrations for the MON 863 studies was based upon ≥10X the maximum observed Cry3Bb1 expression in tissue and no unacceptable negative impacts were observed in the NTO testing battery. Because the maximum Cry3Bb1 protein levels are higher in MON 863 compared to MON 87411 previous studies performed for MON 863 are sufficient to inform and provide a protective NTO assessment for MON 87411. In the environmental assessment for MON 863 (USDA-APHIS 2013)(USDA-APHIS Petition No. 01-137-01p), USDA-APHIS considered the potential impact of the Cry3Bb1 protein on NTOs, including beneficial organisms and threatened or endangered species concluding:

> *"It [MON 863] has no potential to have a greater damaging, harmful, or toxic effect on organisms beneficial to agriculture than does other cultivated corn. In addition to our finding of no plant pest risk, there will be no effect on threatened or endangered species resulting from a determination of non-regulated status for MON 863 corn and its progeny."* (USDA 2002)

Additionally, USDA-APHIS concluded in their assessment of MON 88017 that;

> *"The Cry3Bb1 protein expressed in MON 88017 corn has activity only against select beetle (Order Coleoptera) species within the family Chrysomelidae, namely CRW [corn rootworm] and Colorado potato beetle. Field observations, compositional analyses, and data on the safety of the engineered EPSPS and Cry3Bb1 proteins all indicate that MON 88017 should not have greater potential than other cultivated corn to damage or harm organisms beneficial to agriculture. In addition to our finding of no plant pest risk, there will be no effect on the threatened or endangered species resulting from a determination of non-regulated status for MON 88017 and its progeny."* (USDA 2005)

The EPA also conducted an extensive environmental hazard assessment of Cry3Bb1-producing maize based on studies conducted with similar Cry3Bb1 proteins on representative species of bird, fish, and terrestrial non-target insects, including endangered species, to support the registration of MON 863 and MON 88017 concluding:

> "*Due to a demonstrated lack of toxicity and/or exposure, no effects from Cry3Bb1 protein are anticipated for any non-target species, including federally listed threatened and endangered ("listed") lepidopteran and coleopteran species and their designated critical habitats."* (U.S. EPA 2010)

V.F. Cry3Bb1 and CP4 EPSPS Proteins Characterization and Safety Conclusion

The data and information provided in this section address the questions important to the food, feed and environmental safety of the Cry3Bb1 and CP4 EPSPS proteins in MON 87411, including their potential allergenicity and toxicity. To summarize, the physicochemical characteristics of the Cry3Bb1 and the CP4 EPSPS proteins from MON 87411 were determined and shown to be equivalent to those of their *E. coli*-produced counterparts. Given the very large MOEs for Cry3Bb1 and CP4 EPSPS proteins for humans and the minimal exposure to animals, both support a conclusion of no significant risk to human or animal health associated with dietary exposure to these proteins in food and feed products derived from MON 87411. An assessment of the allergenic potential of the proteins supports the conclusion that the Cry3Bb1 and CP4 EPSPS protein do not pose a significant allergenic risk to humans or animals. The donor organisms for the Cry3Bb1 and CP4 EPSPS coding sequences, *Bacillus thuringiensis* and *Agrobacterium* sp. strain CP4, respectively, are ubiquitous in the environment and not commonly known for human or animal pathogenicity, or allergenicity. The Cry3Bb1 and CP4 EPSPS proteins lack structural similarity to allergens, toxins or other proteins known to have adverse effects on mammals. The Cry3Bb1 and CP4 EPSPS proteins are rapidly digested in simulated digestive fluid and demonstrate no oral toxicity in mice at the level tested. Based on the above information, the consumption of the Cry3Bb1 and CP4 EPSPS proteins from MON 87411 or its progeny is considered safe for humans and animals.

The protein safety data presented herein support the conclusion that food and feed products containing MON 87411 or derived from MON 87411 are as safe as maize currently on the market for human and animal consumption. Given the identical nature of these proteins to proteins contained in other products that have been registered by EPA and deregulated by USDA-APHIS, as well as previous safety assessments, the proteins contained in MON 87411 are also considered as safe for the environment as other maize products.

VI. CHARACTERIZATION AND SAFETY ASSESSMENT OF THE DvSnf7 RNA PRODUCED IN MON 87411

VI.A. History of Safe Use of RNA-mediated Gene Suppression in Plants

RNA-mediated gene suppression (RNAi) is a naturally occurring, ubiquitous process in eukaryotes, including plants and animals consumed as food and feed. Endogenous RNA-mediated gene modulation is responsible for certain characteristics of conventional crops (Della Vedova et al. 2005; Kusaba et al. 2003; Tuteja et al. 2004) and has also been utilized in some biotechnology-derived crops approved for cultivation and use as food and feed (Ivashuta, et al. 2009; Parrott et al. 2010; Petrick et al. 2013). Therefore, there is a history of safe consumption of the RNA molecules mediating gene suppression in plants, including those with homology to genes in humans and other animals (Ivashuta et al. 2009; Jensen, et al. 2013). Additionally, there is no evidence to suggest that dietary consumption of nucleic acids is associated with toxicity (Petrick et al. 2013; U.S. FDA 1992) and U.S. EPA has an established tolerance exemption for nucleic acids that are part of PIP products (U.S. EPA 2001a). FDA recognizes that all food allergens are proteins (U.S. FDA 1992; 2001) and there is also no evidence of allergenicity of dietary RNA in the peer reviewed scientific literature. This lack of toxicity or allergenicity for ingested RNA also extends to RNA molecules associated with dsRNA-mediated gene regulation. Therefore an extensive history of safe consumption for dietary RNAs, including dsRNAs, has been established, as reviewed (Petrick et al. 2013). The reason for this history of safe consumption of dietary RNAs is that extensive sequence-independent physiological and biochemical barriers are known to exist in humans and other animals that limit the potential for uptake or activity of ingested nucleic acids (Juliano, et al. 2009; O'Neill, et al. 2011; Petrick et al. 2013).

A recent publication reported that ingestion of large doses of a particular small (micro) RNA (miRNA) from rice led to some absorption of the miRNA, detection of the miRNA in serum and liver, and an apparent impact on a target protein and plasma LDL in mice (Zhang, et al. 2012a). The authors suggest that a “cross-kingdom” effect – a plant gene product (miR168a) regulating animal gene expression – may be a common phenomenon; and that miRNAs in food may regulate specific genes in animals based upon sequence identity between plant miRNAs and mammalian genes. A second publication (Heinemann, et al. 2013), a review article relying almost exclusively on the Zhang (2012a) study, suggests that the current safety/risk assessment approach is not sufficient for RNA-based biotechnology-derived products. As stated in the preceding paragraph, there are no safety concerns related to the consumption of RNA and RNAi in plants and therefore the current safety assessment approach for dsRNA-containing products, including MON 87411, is appropriate for assessing product safety. The following evidence supports this conclusion:

- Humans regularly consume plants that contain small RNAs. Recent research demonstrates that many existing plant RNAs share sequences with human genes (Ivashuta et al. 2009). This work has also been followed up by studies demonstrating that not only small RNAs but also long dsRNAs in plants share sequence identity to human transcripts (Jensen et al. 2013). Further, humans regularly consume animal-derived foods that are likely to contain more animal

miRNAs that have 100% identity to human genes than plant miRNAs. Despite this routine ingestion of plant and animal small RNAs, no impacts on human health have been reported

- A follow up study to Zhang et al. (2012a) involving Monsanto scientists (Zhang, et al. 2012b) revealed confounding factors which likely explain, in part, the unexpected findings in the 2012 study (Zhang et al. 2012a). In this follow up study, plant miRNAs (including miR168a) were shown to be over-represented relative to their dietary abundance in some public animal small RNA datasets. This indicates that their apparent presence in mouse tissues likely resulted, at least in part, from cross-contamination during the sequencing procedure, thus calling into question the potential for significant uptake of ingested plant miRNAs (Zhang et al. 2012b).
- Differences in diet composition, rather than cross kingdom gene regulation by plant miRNAs, were likely responsible for alterations in plasma LDL cholesterol when Zhang et al., (2012a) fed an all-rice diet to mice (Petrick et al. 2013). These results are consistent with the known challenges to oral delivery of nucleic acids (O'Neill et al. 2011).
- In a recent study, Monsanto and miRagen Therapeutics scientists collaborated on a rice feeding study in mice to evaluate claims of dietary miRNA uptake and physiological impact (Dickinson, et al. 2013). In this study, miR168a uptake was not reproduced, LDLRAP1 protein levels were unaffected, and LDL was only modulated with a high rice diet and not with a nutritionally balanced rice diet. These results support the conclusion that previously reported observations (Zhang et al. 2012a) were due to variability in gene expression and protein expression data and nutritional differences in animal diets, rather than dietary exposure to miR168a.
- A recent report (Snow, et al. 2013) notes the presence of endogenous miRNAs at substantial levels in diets of humans, mice and honey bees. The authors provide empirical data to demonstrate that despite consumption of miRNAs, horizontal delivery via oral ingestion from a typical diet is neither frequent nor prevalent across these consuming organisms.
- A recent report (Witwer, et al. 2013) conducted a feeding study in nonhuman primates with an miRNA rich food source and based on their results, concluded that, “there is little evidence for presence of these plant miRNA in nonhuman primate blood prior to or following dietary intake of a plant miRNA-rich substance.”
- Numerous barriers to the systemic and cellular uptake of exogenous nucleic acids exist (salivary enzymes, stomach and intestinal acids and enzymes, nucleases in serum, etc.) (Akhtar 2009; Haupenthal, et al. 2006; Jain 2008; O'Neill et al. 2011; Petrick et al. 2013).
- Other authors, organizations, and regulatory bodies have looked specifically at biotechnology-derived RNA-based products and support current risk assessment approaches (FSANZ 2013; ILSI-CERA 2011; Parrott et al. 2010).
- Data presented below in this petition regarding specificity and lack of adverse impacts on NTOs (Section VI.E.) supports the safety of the consumption of MON 87411 and the expressed construct-derived RNA molecules contained within it.

Finally, these numerous points regarding the ubiquitous nature of RNA in foods, the known barriers to systemic and cellular uptake of exogenous nucleic acids, the known sequence similarity between some plant and human RNAs, and empirical data demonstrating the lack of adverse impacts on non-target organisms lead us to conclude that unique risk issues related to expression of DvSnf7 RNA in MON 87411 do not exist and the current risk/safety assessment approach used by USDA-APHIS is appropriate for assessing this product.

VI.B. Characterization and Equivalence of DvSnf7 RNA from MON 87411

Despite the long history of exposure to and consumption of nucleic acids, a characterization and safety assessment of the DvSnf7 RNA produced in MON 87411 was conducted. The safety assessment of the DvSnf7 transcript required large amounts of DvSnf7 RNA as the test substance. Because of the extremely low expression of DvSnf7 RNA in MON 87411 (Table VI-1), it was necessary to produce RNA through *in vitro* transcription methods in order to obtain sufficient quantities of DvSnf7 RNA (DvSnf7_968 RNA[6]) for subsequent safety studies. In order to demonstrate that the *in vitro*-produced RNA was the appropriate test material for the safety assessment studies, the molecular characteristics of the RNA produced in MON 87411 (MON 87411 DvSnf7 RNA) were determined and were shown to be equivalent to those of *in vitro*-produced RNA, DvSnf7_968 RNA. A summary of the results is shown below and the details of the materials, methods, and results are described in Appendix E.

Regarding the overall potential for toxicity and allergenicity, RNA is different from proteins. While certain rare proteins are capable of demonstrating toxicity (usually occurring acutely) (Pariza and Johnson 2001; Sjoblad, et al. 1992), there is no evidence that ingested dsRNA is capable of eliciting toxicity in humans regardless of sequence (Petrick et al. 2013). Additionally, all known food allergens are proteins (U.S. FDA 1992; 2001) and there is no evidence to suggest that ingested RNA can be allergenic. Therefore, experimental endpoints intended to address toxicity and/or allergenicity of proteins are not necessary or useful for evaluating the food and feed safety of ingested RNA.

The characterization and equivalence between MON 87411 DvSnf7 RNA and DvSnf7_968 RNA was established by two molecular analyses: 1) DNA sequencing of the reverse transcribed RNA determined that the sequence of the MON 87411 DvSnf7 and DvSnf7_968 RNAs was identical, including the sequence within the 240 bp inverted repeat regions; and 2) RNase I_f[7] digestion followed by northern blot analysis detected the 240 bp dsRNA in MON 87411 DvSnf7 RNA and demonstrated its equivalence to the *in vitro* produced DvSnf7_968 RNA.

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[6] DvSnf7_968 is the predominant RNA transcript produced from the DvSnf7 suppression cassette and, in addition to the $DvSnf7^p$ sequences, contains portions of the CaMV 35S promoter, maize *hsp70* intron, and pea *E9* 3' untranslated region from PV-ZMIR10871.

[7] Endonuclease RNase I_f specifically digests single stranded RNA, leaving dsRNA intact

Taken together, these data provide a detailed characterization of MON 87411 DvSnf7 RNA and establish its equivalence to the *in vitro*-produced DvSnf7_968 RNA. The equivalence justifies the use of the DvSnf7_968 RNA as a test subtance in the safety assessment studies (Section VI.E.4.) (*e.g.*, studies on earthworms, honey bees, Lady beetle, environmental fate of DvSnf7 RNA, etc.) of MON 87411.

VI.C. Expression Levels of DvSnf7 RNA in MON 87411

DvSnf7 RNA levels in various tissues of MON 87411 were determined by a validated QuantiGene® Plex 2.0 Assay. Plant material from MON 87411 was collected in 2011-2012 from the same replicated field sites in Argentina (BAFO, BAHT, BAPE, BASS, and BATC) using the same 19 tissue types as described for protein expression analysis (Section V.C.).

DvSnf7 RNA levels were determined in all 19 tissue types. The results obtained for each sample by the QuantiGene® Plex 2.0 Assay were averaged across the five sites and are summarized in Table VI-1. The details of the materials and methods are described in Appendix F. The individual DvSnf7 RNA levels in MON 87411 across all samples analyzed from all sites ranged from lower than the limit of detection (<LOD) to 213×10^{-3} µg/g dw. The mean DvSnf7 RNA level for each tissue type was highest in OSWP1 at 84.8×10^{-3} µg/g dw and lowest in grain at 0.104×10^{-3} µg/g dw. Samples with an expression level <LOD or lower than the limit of quantitation (<LOQ) were not included in the mean determinations.

Table VI-1. Summary of DvSnf7 RNA Levels in Maize Tissues Collected from MON 87411 Produced in Argentina Field Trials during 2011-2012

Tissue Type[1]	Development Stage[2]	Mean (SD) Range (µg/g fw)[3]	Mean (SD) Range (µg/g dw)[4]	LOD/ LOQ[5] (µg/g fw)
OSL1	V3-V4	12.6×10^{-3} (2.11×10^{-3}) 8.53×10^{-3} - 16.6×10^{-3}	73.9×10^{-3} (14.5×10^{-3}) 43.3×10^{-3} - 103×10^{-3}	0.129×10^{-3}/ 0.566×10^{-3}
OSL2	V6-V8	13.2×10^{-3} (4.08×10^{-3}) 7.77×10^{-3} - 20.1×10^{-3}	67.3×10^{-3} (19.4×10^{-3}) 37.1×10^{-3}- 98.9×10^{-3}	0.114×10^{-3}/ 0.502×10^{-3}
OSL3	V10-V13	10.3×10^{-3} (1.89×10^{-3}) 6.19×10^{-3} - 12.8×10^{-3}	44.6×10^{-3} (8.51×10^{-3}) 27.5×10^{-3} - 58.8×10^{-3}	0.106×10^{-3}/ 0.468×10^{-3}
OSL4	V14-R1	14.4×10^{-3} (6.71×10^{-3}) 5.40×10^{-3} - 33.8×10^{-3}	56.9×10^{-3} (28.5×10^{-3}) 22.1×10^{-3} - 153×10^{-3}	0.110×10^{-3}/ 0.482×10^{-3}
OSR1	V3-V4	3.15×10^{-3} (1.79×10^{-3}) 1.74×10^{-3} - 8.00×10^{-3}	23.9×10^{-3} (15.1×10^{-3}) 12.5×10^{-3} - 67.0×10^{-3}	0.029×10^{-3}/ 0.128×10^{-3}
OSR2	V6-V8	2.32×10^{-3} (0.758×10^{-3}) 0.928×10^{-3} - 3.76×10^{-3}	16.3×10^{-3} (4.84×10^{-3}) 6.62×10^{-3} - 25.7×10^{-3}	0.021×10^{-3}/ 0.093×10^{-3}
OSR3	V10-V13	1.81×10^{-3} (0.749×10^{-3}) 0.942×10^{-3} - 4.00×10^{-3}	10.2×10^{-3} (4.77×10^{-3}) 5.13×10^{-3} - 24.3×10^{-3}	0.020×10^{-3}/ 0.088×10^{-3}
OSR4	V14-R1	1.28×10^{-3} (0.471×10^{-3}) 0.530×10^{-3} - 2.40×10^{-3}	6.84×10^{-3} (2.67×10^{-3}) 2.66×10^{-3} - 13.0×10^{-3}	0.015×10^{-3}/ 0.067×10^{-3}
OSWP1	V3-V4	10.5×10^{-3} (4.25×10^{-3}) 6.78×10^{-3} - 23.1×10^{-3}	84.8×10^{-3} (43.8×10^{-3}) 51.1×10^{-3} - 213×10^{-3}	0.078×10^{-3}/ 0.345×10^{-3}
OSWP2	V6-V8	8.54×10^{-3} (3.54×10^{-3}) 5.01×10^{-3} - 16.0×10^{-3}	55.1×10^{-3} (23.1×10^{-3}) 33.0×10^{-3} - 106×10^{-3}	0.054×10^{-3}/ 0.239×10^{-3}
OSWP3	V10-V13	3.53×10^{-3} (1.17×10^{-3}) 2.03×10^{-3} - 5.89×10^{-3}	25.5×10^{-3} (9.53×10^{-3}) 13.0×10^{-3} - 45.9×10^{-3}	0.027×10^{-3}/ 0.119×10^{-3}

Table VI-1. (continued). Summary of DvSnf7 RNA Levels in Maize Tissues Collected from MON 87411 Produced in Argentina Field Trials during 2011-2012

Tissue Type[1]	Development Stage[2]	Mean (SD) Range (µg/g fw)[3,6]	Mean (SD) Range (µg/g dw)[4]	LOD/ LOQ[5] (µg/g fw)
OSWP4	V14-R1	3.16×10^{-3} (1.03×10^{-3}) 1.89×10^{-3} - 5.37×10^{-3}	18.5×10^{-3} (6.27×10^{-3}) 10.3×10^{-3} - 32.2×10^{-3}	0.028×10^{-3}/ 0.123×10^{-3}
Forage Root	R5	0.536×10^{-3} (0.295×10^{-3}) 0.086×10^{-3} - 1.07×10^{-3}	2.37×10^{-3} (1.29×10^{-3}) 0.425×10^{-3} - 4.61×10^{-3}	0.013×10^{-3}/ 0.059×10^{-3}
Forage	R5	1.28×10^{-3} (0.361×10^{-3}) 0.601×10^{-3} - 2.31×10^{-3}	4.26×10^{-3} (1.26×10^{-3}) 2.00×10^{-3} - 7.72×10^{-3}	0.036×10^{-3}/ 0.157×10^{-3}
Senescent Root	R6	0.353×10^{-3} (0.203×10^{-3}) 0.127×10^{-3} - 0.947×10^{-3}	1.39×10^{-3} (0.815×10^{-3}) 0.478×10^{-3} - 3.68×10^{-3}	0.015×10^{-3}/ 0.065×10^{-3}
Stover	R6	0.310×10^{-3} (0.077×10^{-3}) 0.190×10^{-3} - 0.449×10^{-3}	0.677×10^{-3} (0.201×10^{-3}) 0.401×10^{-3} - 1.04×10^{-3}	0.047×10^{-3}/ 0.207×10^{-3}
Pollen	VT-R1	0.103×10^{-3} (0.069×10^{-3}) 0.056×10^{-3} - 0.224×10^{-3}	0.134×10^{-3} (0.090×10^{-3}) 0.073×10^{-3} - 0.292×10^{-3}	0.013×10^{-3}/ 0.057×10^{-3}
Silk	R1	0.530×10^{-3} (0.190×10^{-3}) 0.215×10^{-3} - 0.893×10^{-3}	5.42×10^{-3} (2.05×10^{-3}) 1.99×10^{-3} - 9.03×10^{-3}	0.004×10^{-3}/ 0.019×10^{-3}
Grain	R6	0.091×10^{-3} (0.028×10^{-3}) 0.049×10^{-3} - 0.153×10^{-3}	0.104×10^{-3} (0.033×10^{-3}) 0.056×10^{-3} - 0.175×10^{-3}	0.008×10^{-3}/ 0.036×10^{-3}

[1] OSL=over season leaf, OSR= over season root, and OSWP= over season whole plant

[2] The crop development stages at which each tissue was collected.

[3] The DvSnf7 RNA levels are calculated as microgram (µg) of DvSnf7 RNA (≥ 200 nt) per gram (g) of tissue on a fresh weight (fw) basis. The sample means, SDs, and ranges (minimum and maximum values) were calculated for each tissue type across all 5 sites (n=20, except for senescent root n=19, stover n=16, pollen n=5, and grain n=18 due to expressions from two pollen samples <LOD and from the rest of the samples for senescent root, stover, pollen, and grain <LOQ)

[4] The DvSnf7 RNA levels are calculated as microgram (µg) of DvSnf7 RNA per gram of tissue on a dry weight (dw) basis. The sample means, SDs, and ranges (minimum and maximum values) were calculated for each tissue type across all 5 sites (n=20, except for senescent root n=19, stover n=16, pollen n=5, and grain n=18 due to expressions from two pollen samples <LOD and from the rest of the samples for senescent root, stover, pollen, and grain <LOQ).

[5] LOD=limit of detection; LOQ=limit of quantitation.

[6] Converting µg/g to parts per billion (ppb) shows DvSnf7 RNA expression ranging from 0.091 ppb in grain to 14.4 ppb in over season leaf at these growth stages.

VI.D. Human and Animal Exposure to the **DvSnf7 RNA**

The potential dietary intake of DvSnf7 RNA from the consumption of food derived from MON 87411 can be estimated by multiplying maize consumption (total of all maize fractions consumed) by the level of DvSnf7 RNA in MON 87411. For the purposes of this assessment, the concentration of the DvSnf7 RNA in the included maize fractions (Determined using DEEM-FCID[8] as in Section V.E.6.; flour, meal, bran, starch, sweet corn and popcorn) was assumed to be equal to the mean expression level in whole MON 87411 grain. Based on DEEM-FCID exposure estimates and levels of expressed DvSnf7 RNA in grain from MON 87411 on a fresh weight basis (μg/g) discussed above (Table VI-1), 95th percentile exposure estimates for the general U.S. population and for non-nursing infants (the most highly exposed sub-population) were estimated to be 0.2 and 0.4 ng/kg body weight per day, respectively. These exposure estimates are likely to be overestimates because they assume that there is no loss of DvSnf7 RNA during storage, processing and/or cooking of the grain or food.

Total nucleic acid consumption in the diet for DNA and RNA (based on purine and pyrimidine intake) is estimated to be 1-2 grams per day (Suchner, et al. 2000) which would equate to a maximal intake of 0.2 g/kg/day (2 g in a 10 kg non-nursing infant; assuming all of the intake is RNA). In comparison, anticipated exposures to DvSnf7 RNA from consumption of MON 87411 are extremely low ($\leq$ 0 .4 ng/kg/day) relative to this estimate of total daily RNA intake (a 500 million fold difference between daily consumption of DvSnf7 RNA from MON 87411 and total RNA). Estimated DvSnf7 exposure values are more than 3,300 fold lower than the 1.5 μg/kg/day intake value defined as the threshold of toxicological concern (TTC) for compounds not triggering structural alerts for toxicity (Kroes, et al. 2005). The TTC concept establishes an exposure level for chemicals (with or without toxicity data) below which there would be negligible risk to human health. Furthermore, it is noteworthy to point out that the TTC value reflects chronic (mean per capita) intake values and the exposures herein are conservative, as they reflect acute (short-term, 95th percentile) intake values. Based on this information, the exposure assessment described above, rather than a dietary risk assessment is considered most appropriate for evaluation of the DvSnf7 RNA and the determination of safety is based on the weight-of-the-evidence for RNA safety (*e.g.*, general RNA history of safe use, very low exposure to the specific DvSnf7 RNA, and the lack of oral toxicity to nucleic acids).

The potential DvSnf7 RNA exposure to animals from consumption of MON 87411 in feeds was evaluated by calculating an estimate of daily dietary intake. The highest dietary exposure of DvSnf7 RNA is 81.0 ng/kg bw/day in the lactating dairy cow. The dietary exposure of DvSnf7 RNA for broiler chicken and finishing pig is

[8] Dietary Exposure Evaluation Model– Food Commodity Intake Database. This is a software-based exposure analysis system for performing chronic and acute dietary exposure assessments developed by the U.S. EPA. DEEM-FCID can be used to estimate dietary intake of any component of food or water.

10.5 ng/kg bw/day and 4.6 ng/kg bw/day, respectively. Therefore, there is minimal dietary exposure of animals to DvSnf7 RNA from MON 87411.

Negligible exposures and lack of oral toxicity of RNA in higher organisms indicates that there would be no significant risk to human and animal health associated with consumption of DvSnf7 RNA in food and feed products derived from MON 87411.

VI.E. Laboratory Tests to Characterize the Spectrum of Activity of DvSnf7 RNA

VI.E.1. RNAi in Insects

In plants and nematodes, exogenous dsRNAs that enter the cell are amplified via RNA-dependent RNA polymerases (RdRPs) to produce endogenous dsRNAs that supplement the RNAi pathway and prolong the RNAi effect (Miller, et al. 2012). However, insects have been shown to lack RdRPs (Miller et al. 2012). The ability of WCR and *Tribolium castaneum* to produce dose-dependent responses with RNAi is consistent with the absence of an endogenous amplification mechanism (Bolognesi et al. 2012; Miller et al. 2012). The lack of an endogenous amplification mechanism in insects suggests that exposure to dsRNA in higher trophic levels, via ingested prey species, will be limited because a mechanism for bioamplification is not evident. Other factors can also influence the efficiency of RNAi in insects, including concentration, potency and efficacy against the target, sequence and length, persistence of gene silencing and the insect life-stage (Baum et al. 2007a; Huvenne and Smagghe 2010; Whyard et al. 2009). In general, long dsRNAs that incorporate a high degree of sequence match to mRNAs in the target insect have greater potential for efficacy as a result of the number of siRNAs that can be produced (Baum et al. 2007a). Another mechanism that can affect RNAi efficiency in insects, and potentially limit environmental exposure, is the length of the dsRNA. Bolognesi et al. (2012) and Miller et al., (2012) recently demonstrated that a dsRNA must be of sufficient length (e.g.≥ 60 bp) to result in efficacy against WCR and *T. castaneum*, respectively.

VI.E.2. Test Substances Used to Assess the Activity Spectrum of DvSnf7 RNA

As noted earlier, MON 87411 maize, expresses a 968 nucleotide (nt) DvSnf7 RNA primary transcript. The DvSnf7 target sequence or "active" region is a 240 bp stretch of dsRNA included within the larger 968 nt RNA molecule. The DvSnf7 240 bp dsRNA was confirmed to be equipotent (in terms of mortality to WCR and southern corn rootworm (SCR, *Diabrotica undecimpunctata howardi*)) to the DvSnf7 968 nt RNA in diet bioassays with both the WCR and SCR, justifying the use of the 240 bp dsRNA. The DvSnf7 240 bp dsRNA, herein referred to as DvSnf7_240 dsRNA, was synthesized *in vitro* for use in initial insect diet bioassays to characterize the spectrum of activity (Table VI-2). Definitive studies used for the quantitative ecological risk assessment utilized an *in vitro* synthesized 968 nt DvSnf7 (known as DvSnf7_968 RNA) as expressed in MON 87411 maize and are described below (Table VI-3).

VI.E.3. Results from Activity Spectrum Bioassays for DvSnf7 RNA

An assessment of the spectrum of activity for a pesticide is designed to characterize activity against a range of insect taxa that includes the target organism as well as non-target organisms (Raybould 2006; Romeis, et al. 2013; U.S. EPA 2007). Efficacy of the DvSnf7 dsRNA against the target organism WCR, and the closely related SCR, was characterized with larvae in 12-day continuous-feeding diet-incorporation bioassays to characterize the concentration-effect relationship and to estimate the LC_{50}[9] value for DvSnf7_240 dsRNA (Table VI-2) (Bachman et al. 2013; Bolognesi et al. 2012). Both WCR and SCR demonstrated comparable concentration-dependent effects with mean 12-day LC_{50} values of 4.4 ng DvSnf7_240 dsRNA/ml diet and 1.2 ng DvSnf7_240 dsRNA/ml diet, respectively (Table VI-2). The relatively small difference in sensitivities between SCR and WCR species to DvSnf7_240 dsRNA may be related to each species' feeding physiology, how well they perform on an artificial diet in the laboratory bioassays and SCR typically having higher growth rates in laboratory settings than WCR. Results from feeding DvSnf7_240 dsRNA to these two species demonstrates efficacy against target pests in these assays.

Characterization of the spectrum of insecticidal activity, MOA, as well as an understanding of environmental exposure levels and pathways provides important information that can narrow the scope of NTO testing for an ecological risk assessment (Romeis, et al. 2008; Romeis et al. 2013). NTO testing is typically performed in a sequential scheme (*e.g.* Tier 1, Tier 2, etc.) and builds upon characterization of the activity spectrum. Results from the NTO battery complements specificity data by evaluating organisms that may be phylogenetically related and/or provide important functional roles (*e.g.*, detritivores, predators, parasitoids, pollinators) in relevant and reliable laboratory studies. In 2011, a tripartite group (government, industry and academia) evaluated the environmental risk assessment approach for genetically engineered plants (*e.g. Bt*-expressing plants) and concluded that the current ecological risk assessment framework and effects tests for NTOs are applicable to plants expressing an RNAi trait (ILSI-CERA 2011).

The spectrum of activity for DvSnf7 RNA was characterized by selecting and testing insects based upon their taxonomic relatedness to the WCR (Table VI-2) (Bachman et al. 2013). In total, 14 insect species were tested, representing 10 families and 4 orders. Representative insects from the following orders were tested: Hemiptera (*Orius insidiosus*), Hymenoptera (*Nasonia vitripennis* and *Pediobius foveolatus*), Lepidoptera (*Spodoptera frugiperda*, *Helicoverpa zea*, *Ostrinia nubilalis*, and *Bombyx mori*) and Coleoptera (WCR, SCR, *Leptinotarsa decemlineata, Tribolium castaneum, Coleomegilla maculata, Epilachna varivestis,* and *Poecilus chalcites).* A representative insect from the Order Diptera was not examined because a previous study on a Dipteran species (*Drosophila* spp.) had shown it to be insensitive to dietary dsRNA without the use of a

[9] The LC_{50} value is the measure of toxicity of a substance (Lethal Concentration), that when fed to a test organism over a given time period, results in death of 50% of those organisms.

transfection agent (Whyard et al. 2009). Given that the target species are in the Order Coleoptera, a strong focus was placed on this order and in total, 7 species across four families in this Order were tested with the goal to more fully characterize the range of activity of DvSnf7 dsRNA within the Order Coleoptera.

Diet bioassays were conducted utilizing *in vitro* synthesized DvSnf7_240 dsRNA, at test concentrations ranging from 500 to 5000 ng DvSnf7_240 dsRNA/ml diet and exceed DvSnf7 dsRNA expression values *in planta* (Section VI.B.). Bioassays for activity spectrum studies were designed to: 1) provide continuous exposure of the DvSnf7_240 dsRNA to each test species, and 2) provide a sufficient duration of exposure to evaluate the potential effects of DvSnf7_240 dsRNA on growth, development and survival. At exposure concentrations that will greatly exceed field exposure levels, no negative impacts attributable to DvSnf7_240 were detected against the Chrysomelidae species, *Leptinotarsa decemlineata* (phylogenetically the closest to the target species), or in representative species that are even less phylogenetically related from the other three families (Coccinellidae, Tenebrionidae, and Carabidae) tested in the order Coleoptera. Of the Coleoptera tested, two species *L. decemlineata* and *Tribolium castaneum* have, however, been shown to be sensitive to ingested dsRNA when fed their conspecific (species-specific) dsRNA (Bachman et al. 2013; Baum et al. 2007a; Whyard et al. 2009). Additionally, no negative impacts were detected in representative species from the orders Hemiptera, Hymenoptera, or Lepidoptera. Results from these bioassays demonstrate that the spectrum of activity of DvSnf7_240 dsRNA is restricted to the subfamily Galerucinae in the family Chrysomelidae within the order Coleoptera (Table VI-2).

Table VI-2. Susceptibility to DvSnf7_240 dsRNA in laboratory bioassays

Order	Family	Subfamily	Species	Common Name	Bioassay Duration (days)	Endpoints[1]	LC_{50}[2] or No Observed Effect Concentration[3] (ng/ml or g diet)
Coleoptera	Chrysomelidae	Galerucinae	*Diabrotica virgifera virgifera*	Western corn rootworm	12	S	4.4[2]
	Chrysomelidae	Galerucinae	*Diabrotica undecimpunctata howardi*	Southern corn rootworm	12	S	1.2[2]
	Chrysomelidae	Chrysomelinae	*Leptinotarsa decemlineata*	Colorado potato beetle	12	S, G	5,000[3]
	Tenebrionidae	Tenebrioninae	*Tribolium castaneum*	Red flour beetle	30	S, G	5,000[3]
	Coccinellidae	Coccinellinae	*Coleomegilla maculata*	Pink-spotted lady beetle	24	S, G, D	3,000[3]
	Coccinellidae	Epilachninae	*Epilachna varivestis*	Mexican bean beetle	28	S, G, D	3,000[3]
	Carabidae	Harpalinae	*Poecilus chalcites*	Carabid beetle	35	S, G, D	5,000[3]
Hemiptera	Anthocoridae	Anthocorinae	*Orius insidiosus*	Insidious Flower bug	9	S, D	5,000[3]
Hymenoptera	Eulophidae	Entedoninae	*Pediobius foveolatus*	Eulophid wasp	21	S	3,000[3]
	Pteromalidae	Pteromalinae	*Nasonia vitripennis*	Jewel wasp	20	S	5,000[3]
Lepidoptera	Noctuidae	Noctuinae	*Spodoptera frugiperda*	Fall armyworm	8	S, G	500[3]
	Noctuidae	Heliothinae	*Helicoverpa zea*	Corn earworm	12	S, G	5,000[3]
	Crambidae	Pyraustinae	*Ostrinia nubilalis*	European corn borer	12	S, G	5,000[3]
	Bombycidae	Bombycinae	*Bombyx mori*	Silkworm	14	S, G	5,000[3]

[1] S: Survival; G: Growth; D: Development
[2] LC_{50} survival data reported in (Bachman et al. 2013)
[3] No observed effect concentration (NOEC) is equal to the maximum concentration tested in these bioassays.

VI.E.4. Non-Target Organism Assessment for DvSnf7

Evaluation of the potential risks to NTOs is an important component of APHIS's plant pest risk assessment of a biotechnology-derived crop. Assessment of the potential risks to NTOs associated with the introduction of a biotechnology-derived crop producing an insecticidal trait is based on the characteristics of the crop and the introduced trait. Since risk is a function of hazard and exposure, it is critical to determine the potential hazard and exposure scenarios that are most likely and that require evaluation through experimental studies. Selection of the test organisms and test material are important decisions that are based on the characteristics of the trait and the product (Romeis et al. 2008). In the U.S., regulatory guidelines for NTO testing and risk assessment of insect-protected crops have been developed by the EPA and testing is conducted according to a tier-based system (U.S. EPA 2010). Additionally, the EPA has convened several Scientific Advisory Panel (SAP) meetings to make recommendations and provide guidance for NTO testing and risk assessment for agricultural products produced by methods of biotechnology (U.S. EPA 2001c; 2002; 2004b; 2010).

Based on the results from the activity spectrum (Section VI.E.3.) and known expression of DvSnf7 RNA in MON 87411 (Section VI.B.), an evaluation of the potential toxicity to selected NTOs at field exposure levels was conducted. The hazard assessment included toxicity testing against a soil decomposer [earthworm (*Eisenia andrei*)], and five beneficial insect species [honeybee (*Apis mellifera*), parasitic wasp (*Pediobius foveolatus*), ladybird beetle (*Coleomegilla maculata*), carabid beetle (*Poecilus chalcites*), and Insidious Flower bug (*Orius insidiosus*)] (Appendix K). All studies were conducted using *in vitro* produced DvSnf7_968 RNA as the test substance. With the exception of the earthworm study, all studies utilizing the *in vitro*-produced test substance included a diet analysis using a sensitive insect (*Diabrotica undecimpunctata howardi;* Southern corn rootworm, SCR) to confirm that the DvSnf7_968 RNA contained in the test substance diet was biologically active and had the expected level of biological activity. Additionally, where appropriate based upon the diet matrix, the homogeneity of the test material and stability (of the DvSnf7_968 RNA test substance) over the period of storage was also confirmed. The no observed effect concentrations (NOECs) determined for each of the tests used in the NTO risk assessment for MON 87411 are summarized in Table VI.3.

Exposure information was developed to determine the maximum expected environmental concentration (MEEC) for the DvSnf7 RNA produced in MON 87411 to NTOs. Test concentrations were based on the measured DvSnf7 RNA expression in the tissue(s) types that the NTO would most likely be exposed to in the environment. A targeted MOE of greater than 10-times the MEEC was used to set test concentrations (U.S. EPA 2010). The NTO studies were performed at a nominal concentration of 1,000 ng DvSnf7_968 RNA /ml or g diet. In addition to this concentration providing an adequate MOE, it also was high enough to allow for the assessment of the functional activity of the RNA in 12-day dose confirmation diet bioassays with SCR. For predatory beetles that consume herbivorous prey and have an indirect exposure to maize expressed DvSnf7 RNA, the maximum expression value from the leaf development stage with the highest expression (V14-R1) was used to represent worst-case scenario to calculate the MOE for

these species. The most ecologically relevant route of exposure for soil-dwelling organisms, such earthworms, was considered to be from decomposing late season plant tissue that enters the soil environment. Consequently, for soil-dwelling NTOs, the MEECs were based on the level of estimated DvSnf7 RNA in senescent maize roots.

For the non-target organism studies, MOEs were calculated based on the ratio of the NOECs to the MEECs. MOEs calculated for each species were >10-fold of the predicted exposure level for NTOs (Table VI-3). MOEs that exceed 10 are considered as indicative of minimal risk in worst-case laboratory assays by (U.S. EPA 2010). Therefore, as with the previously assessed Cry3Bb1 protein, DvSnf7 RNA is not likely to produce adverse effects on terrestrial beneficial invertebrate species at field exposure levels. This conclusion is in agreement with prior published literature which reported that DvSnf7 activity is very specific and is restricted to the Galerucinae subfamily within the Chrysomelidae family in the Order Coleoptera (Bachman et al. 2013).

The August 2002 EPA SAP report (U.S. EPA 2002) recommended that non-target testing should be focused on species exposed to the crop being evaluated (*i.e.*, for MON 87411 beneficial organisms found in maize fields). Though aquatic habitats may be located near agricultural areas, the EPA concluded that exposure of aquatic organisms to biotech crops is limited temporally and spatially and that the potential exposure of aquatic organisms is therefore low to negligible (U.S. EPA 2010). Since there is no meaningful ecologically-relevant exposure to aquatic organisms from maize, other than through purposeful feeding of processed maize products, effects tests on aquatic species were not conducted for MON 87411. Additionally, based upon the narrow spectrum of activity for DvSnf7 RNA the likelihood of adverse effects to aquatic organisms from DvSnf7 is extremely low (Bachman et al. 2013). Furthermore, successful RNAi in organisms such as fish, reptiles, and birds has only been achieved with cell lines and/or embryos and has required the use of transfection agents, direct injection, electroporation, or other invasive RNA delivery techniques (Schyth 2008; Sifuentes-Romero, et al. 2011; Ubuka, et al. 2012). Based on minimal exposure and the narrow spectrum of activity of DvSnf7, the likelihood of negative impacts to non-target aquatic organisms from cultivation of MON 87411 is concluded to be extremely low.

Table VI-3. Maximum expected environmental concentrations (MEECs), no observed effect concentrations (NOECs) from non-target organism studies and estimated margins of exposure (MOEs) for DvSnf7 RNA

Test organism	Order	MEEC [1]	NOEC	MOE [2]
Coleomegilla maculata	Coleoptera	0.224 ng/g fw pollen	1000 ng/g	≥4464
Poecilus chalcites	Coleoptera	33.8 ng/g fw leaf [3]	1000 ng/g	≥30
Apis mellifera larvae	Hymenoptera	0.000448 ng[4]	11.3 ng/larvae [5]	≥25,223
Apis mellifera adult	Hymenoptera	0.224 ng/g fw pollen	1000 ng/g	≥4464
Pediobius foveolatus	Hymenoptera	0.224 ng/g fw pollen	1000 ng/g	≥4464
Orius insidiosus	Hemiptera	0.224 ng/g fw pollen	1000 ng/g	≥4464
Eisenia andrei	Haplotaxida	3.68 ng/g dw senescent root	5000 ng/g dry soil	≥1359

[1] Maximum expression levels determined from MON 87411.

[2] MOE values were calculated based on the ratio of the NOEC to MEEC. The MOE was determined based on the maximum expression level of the DvSnf7 RNA in the tissue from MON 87411deemed most relevant to the NTO exposure.

[3] The maximum expression value from the leaf development stage with the highest expression (V14-R1) was used to represent worst-case-scenario for a predator consuming an herbivorous prey.

[4] MEEC based upon mean amount of DvSnf7 RNA expressed in 2 mg of MON 87411 pollen (fw). The average consumption of pollen by honey bee larvae is 2 mg during development (Babendreier, et al. 2004). The MEEC was calculated as follows: (2 mg pollen × (0.224 ng DvSnf7 RNA/1000 μg)).

[5] The NOEC represents the concentration of the test solution used for dosing individual larval cells. A single dose of 10 μl of 1000 ng/g solution was added to each larval cell for a total mass of 11.3 ng DvSnf7/cell. The concentration of 1000 ng/g DvSnf7_968 RNA in the diet solution is calculated based on the density of the 30% sucrose/water (w/v) solution of 1.1270 g/ml.

VI.F. Characterization and Safety Conclusions

DvSnf7 RNA from MON 87411 is a dsRNA that upon consumption by CRW species causes gene suppression of the targeted DvSnf7 gene. The molecular characteristics of the MON 87411 DvSnf7 RNA were determined and equivalence between MON 87411 DvSnf7 RNA and *in vitro*-produced DvSnf7_968 RNAs was demonstrated. This equivalence justifies the use of the *in vitro*-produced DvSnf7_968 RNA as a test substance in the RNA safety studies (described in more detail in Section VI.E.4.). Expression studies using a Quantigene Plex 2.0 assay demonstrated that MON 87411 DvSnf7 was expressed at very low mean levels ranging from 0.104×10^{-3} µg/g dw to 84.8×10^{-3} µg/g dw, representing a low percentage of the total RNA. Anticipated dietary exposures to DvSnf7 in humans are very low (≤ 0.4 ng/kg bw per day). Anticipated dietary exposures to DvSnf7 in animals are also very low, the highest anticipated levels being in lactating dairy cows (81 ng/kg bw per day).

Additionally, activity spectrum testing on 14 different species showed that activity of DvSnf7 RNA is restricted to the subfamily Galerucinae in the family Chrysomelidae within the order Coleoptera. Specific testing on a battery of NTOs (honeybee, earthworm, ladybird beetle, and others) further supports a conclusion that negative impacts resulting from exposure to DvSnf7 RNA from MON 87411 are unlikely. Based on the ubiquitous nature of the RNA-based suppression mechanism utilizing dsRNA, demonstration of specificity of DvSnf7 suppression in CRW, lack of impact to NTOs, the history of safe consumption of RNA, and the apparent lack of toxicity or allergenicity of dietary RNA; the RNA-mediated gene suppression used in MON 87411 poses no observed or theoretical risks to humans or animals. Therefore, the consumption of the DvSnf7 RNA from MON 87411 or its progeny is considered safe for humans and animals.

The safety data presented herein support the conclusion that food and feed products containing MON 87411 or derived from MON 87411 are as safe as maize products currently on the market for human and animal consumption and the environment.

VII. COMPOSITIONAL ASSESSMENT OF MON 87411

Safety assessments of biotechnology-derived crops follow the comparative safety assessment process (Codex Alimentarius 2009) in which the composition of grain and/or other raw agricultural commodities of the biotechnology-derived crop are compared to the appropriate conventional control that has a history of safe use. Compositional assessments are performed using the principles and analytes outlined in the OECD consensus document for maize composition (OECD 2002b).

A recent review of compositional assessments conducted according to OECD guidelines, which encompassed a total of seven biotechnology-derived crop varieties, nine countries and eleven growing seasons concluded that incorporation of biotechnology-derived agronomic traits has had little impact on natural variation in crop composition. Most compositional variation is attributable to growing region, agronomic practices, and genetic background (Harrigan, et al. 2010). Numerous scientific publications have further documented the extensive variability in the concentrations of crop nutrients, anti-nutrients, and secondary metabolites that reflect the influence of environmental and genetic factors as well as extensive conventional breeding efforts to improve nutrition, agronomics, and yield (Harrigan et al. 2010; Harrigan, et al. 2009; Ridley, et al. 2011; Zhou, et al. 2011).

Compositional equivalence between biotechnology-derived and conventional crops supports an "equal or increased assurance of the safety of foods derived from genetically modified plants" (OECD 2002a). OECD consensus documents on compositional considerations for new crop varieties emphasize quantitative measurements of essential nutrients and known anti-nutrients. These quantitative measurements effectively discern any compositional changes that imply potential nutritional or safety (*e.g.*, anti-nutritional) concerns. Levels of the components in grain and/or other raw agricultural commodities of the biotechnology-derived crop product are compared to: 1) corresponding levels in a conventional comparator, a genetically similar conventional line, grown concurrently under similar field conditions, and 2) natural ranges generated from an evaluation of commercial reference hybrids grown concurrently and from data published in the scientific literature. The comparison to data published in the literature places any potential differences between the assessed crop and its comparator in the context of the well-documented variation in the concentrations of crop nutrients, anti-nutrients, and secondary metabolites.

This section provides analyses of concentrations of key nutrients, anti-nutrients, and secondary metabolites of MON 87411 compared to that of a conventional control maize hybrid grown and harvested under similar conditions, as appropriate. In addition, conventional commercial reference maize hybrids (hereafter refered to as reference hybrids) were included in the composition analyses to provide additional information on the range of natural variability for each component. The production of materials for compositional analyses used a sufficient variety of field trial sites, robust field designs (randomized complete block design with four blocks), and sensitive analytical methods that allow accurate assessments of compositional characteristics over a range of environmental conditions under which MON 87411 is expected to be grown.

The information provided in this section addresses relevant factors in Codex Plant Guidelines, Section 4, paragraphs 44 and 45 for compositional analyses (Codex Alimentarius 2009).

VII.A. Compositional Equivalence of MON 87411 Grain and Forage to Conventional Maize

Grain and forage samples were collected from MON 87411, the conventional control, and a total of 20 different reference hybrids grown in Argentina during a 2011/2012 field production. The reference hybrids were included in the composition analyses to provide data on the natural variability for each component. The field production was conducted at eight sites (Appendix G). The field sites were planted in a randomized complete block design with four blocks per site. MON 87411, conventional control, and reference hybrids were grown under normal agronomic field conditions for their respective geographic regions, in areas that were typical for maize production in Argentina. Production in the U.S. corn belt and Argentina maize-growing regions occurs at relatively similar latitudes with an approximate 6 month offset (Schnepf et al. 2001). The average growing season temperatures and precipitation are comparable (Schnepf et al. 2001) and maize hybrids developed in the U.S. are often used directly by farmers in Argentina. As such, compositional analyses from maize grown in Argentina are approriate for a comparative safety assessment. MON 87411 plots were treated with glyphosate (0.95 lbs a.i./ha) to generate samples under conditions of the intended use of the product.

The evaluation of MON 87411 followed considerations relevant to the compositional quality of maize as defined by the OECD consensus document (OECD 2002b). Grain samples were analyzed for levels of nutrients including proximates, carbohydrates by calculation, fiber, amino acids, fatty acids, minerals, and vitamins. The anti-nutrients analyzed in grain included phytic acid and raffinose. Secondary metabolites analyzed in grain included furfural, ferulic acid, and p-coumaric acid. Forage samples were analyzed for levels of proximates, carbohydrates by calculation, fiber, and minerals. In total, 78 different components were assayed (nine in forage and 69 in grain).

Of those 78 components, 14 fatty acids (caprylic, capric, lauric, myristic, myristoleic, pentadecanoic, pentadecenoic, palmitoleic, heptadecanoic, heptadecenoic, gamma linolenic, eicosadienoic, eicosatrienoic, and arachidonic acids), sodium, and furfural had more than 50% of observations below the assay limit of quantitiation (LOQ) and were excluded from statistical analysis. Moisture on two components (grain and forage) was measured for conversion of components to dry weight, but was not statistically analyzed. Therefore, 60 components were statistically analyzed.

The statistical comparison of MON 87411 and the conventional control was based on compositional data combined across all field sites (the combined-site analysis). Statistically significant differences were identified at the 5% level ($\alpha = 0.05$). The compositional data from the reference hybrids were combined across all field sites to calculate a 99% tolerance interval for each component to estimate the natural variability of each component in maize.

A statistically significant difference between MON 87411 and the conventional control does not imply biological relevance from a food and feed safety perspective. Therefore, statistically significant differences observed in the combined-site analysis between MON 87411 and the conventional control were evaluated further to determine whether the detected difference indicated a biologically relevant compositional change or supported a conclusion of compositional equivalence, as follows:

1) Determination of the mean difference between MON 87411 and the conventional control that was used in steps two and three, below. For protein and amino acids only[10], the relative magnitude of the difference (percent change relative to the control) between MON 87411 and the conventional control was determined to allow an assessment of the difference in amino acids in relation to a difference in protein;

2) Assessment of the relative impact of MON 87411 compared to variation within the conventional control germplasm grown across multiple sites (*i.e.*, variation due to environmental influence). This assessment compares the mean difference between MON 87411 and the conventional control to the range of values for the conventional control (maximum value minus the minimum value) derived from the combined-site analysis. When a mean difference is less than ranges seen due to natural environmental variation within even a single, closely related germplasm, the difference is typically not a food or feed safety concern; and

3) Assessment of the relative impact of MON 87411 compared to natural variation due to multiple sources (*e.g.*, environmental and germplasm influences). This assessment compares the mean difference between MON 87411 and the conventional control to variation in conventional maize as estimated by in-study reference hybrid values and assessing whether the mean value of MON 87411 was within the 99% tolerance interval, the literature values, and/or the ILSI Crop Composition Database values (ILSI-CCDB) (ILSI 2011).

These evaluations of natural variation within the context of the conventional control and conventional maize references are important as crop composition is known to be influenced by environment and germplasm. Although used as the first step in the comparative assessment process, detection of statistically significant differences between MON 87411 and conventional control mean values does not imply a meaningful contribution by MON 87411 to compositional variability. Only if mean differences between MON 87411 and the conventional control are large relative to natural variation inherent to conventional maize would further assessments be required to establish

[10] Since total amino acids measured in a grain analysis are predominantly derived from hydrolysis of protein, changes in protein levels will likely result in corresponding changes in amino acids levels. However, the mean difference for individual amino acid levels will be less than the mean difference for protein because each amino acid constitutes only a part of the protein. For this reason, the relative magnitudes of difference (percent change relative to the control) for amino acids and protein were determined to allow an assessment of the difference in amino acids in relation to a difference in protein. When the relative magnitudes of difference for amino acids were related to the relative magnitude of difference for protein, then steps 2 and 3 are not discussed for amino acids.

whether the change in composition would have an impact from a food and feed safety perspective. The steps reviewed in this assessment therefore describe whether the differences between MON 87411 and the conventional control are meaningful from a food/feed perspective or whether they support a conclusion of compositional equivalence.

The compositional analysis provided a comprehensive comparative assessment of the levels of key nutrients, anti-nutrients, and secondary metabolites in maize grain and forage of MON 87411 and the conventional control. Of the 60 components statistically assessed there were no statistically significant differences in 48 components. Only 12 components (protein, histidine, tyrosine, oleic acid, neutral detergent fiber, copper, iron, manganese, zinc, niacin, vitamin B1 in grain and ash in forage) showed a significant difference between MON 87411 and the conventional control in the combined site analysis. For these 12 components, the mean differences in component values between MON 87411 and the conventional control were less than the observed range of the conventional control values (Step 2 above) and the reference hybrid values (Step 3 above) (Tables VII-1, -3, -4, -5, and -7 below). The MON 87411 mean component values of the 12 components that were statistically different were within the 99% tolerance intervals, the values observed in the literature, and/or the ILSI-CCDB values (Step 3 above). These results support the overall conclusion that MON 87411 was not a major contributor to variation in component levels in maize grain and forage and confirmed the equivalence of MON 87411 to the conventional control in levels of all measured components. A detailed description of the assessment of statistically significant differences observed between MON 87411 and the conventional control is provided in the following section. These data confirmed that the components with observed significant differences were not compositionally meaningful from a food and feed safety perspective.

VII.A.1. Nutrient Levels in Maize Grain

Grain samples were analyzed for levels of nutrients including ash, protein, total fat, carbohydrates by calculation, fiber (three components), amino acids (18 components), fatty acids (22 components), minerals (nine components), and vitamins (seven components). Moisture was measured for conversion of components from fresh to dry weight, but was not statistically analyzed.

VII.A.1.1. Proteins and Amino Acids

Maize grain is typically composed of approximately 10% protein and the levels of protein and associated amino acids can vary depending on local growing conditions (Harrigan et al. 2009; Ridley et al. 2011; Zhou et al. 2011). This naturally occurring variability is important in assessing the biological relevance of statistically significant differences in composition.

A statistically significant difference ($p<0.05$) between MON 87411 and the conventional control was observed for protein (Table VII-1). The mean protein value was 10.71% dw for MON 87411 and 10.28% dw for the conventional control, a mean difference of 0.43% dw. This difference was evaluated in the context of the range of the conventional control values, 4.54% dw, calculated from the minimum (8.06% dw) and maximum

(12.60% dw) protein values. The mean difference in protein values between MON 87411 and the conventional control was less than the range of the conventional control values, indicating that MON 87411 does not impact levels more than natural variation within the conventional control grown at multiple locations. The mean difference in protein values between MON 87411 and the conventional control was also less than the variation seen in the reference hybrid values (ranged 7.21 to 14.59% dw, a magnitude of 7.38% dw), and the MON 87411 mean value for protein was within the 99% tolerance interval, the values observed in the published literature and/or the ILSI-CCDB values (Table VII-8).

Since total amino acids measured in a seed analysis are predominantly derived from hydrolysis of protein, differences in amino acid levels between MON 87411 and the conventional control were assessed relative to the difference in protein levels. The relative magnitude of the difference in mean protein values for MON 87411 and the conventional control, when expressed as a percent of the conventional control, was 4.19% (Table VII-1). Correspondingly, relative magnitudes of difference for the 18 amino acids measured were≤4.77%. These differences were significant for two of the amino acids (histidine, tyrosine) (Table VII-1), and reflected small relative magnitudes of differences between MON 87411 and the conventional control, as would be expected based on the small relative magnitude of difference in protein.

The data demonstrated that MON 87411 was not a major contributor to variation in protein and amino acid levels in maize grain and confirmed the compositional equivalence of MON 87411 to the conventional control in levels of these components. Also, the mean values of these components were within the 99% tolerance intervals, the values observed in the literature, and/or the ILSI-CCDB values. These data confirmed that the significant differences ($p<0.05$) in mean values of protein and two amino acids were not compositionally meaningful from a food and feed safety perspective.

VII.A.1.2. Total Fat and Fatty Acids

Maize grain is typically composed of approximately 4% fat and the levels of total fat and fatty acid levels can vary depending on local growing conditions (Harrigan et al. 2009; Ridley et al. 2011; Zhou et al. 2011). This naturally occurring variability is important in assessing the biological relevance of statistically significant differences in composition.

No statistically significant difference was observed for total fat (Table VII-2). There were also no statistically significant differences for seven of the eight fatty acids (FA) assessed. One statistically significant difference ($p<0.05$) was observed for oleic acid (Table VII-2). The mean oleic acid value was 21.89% total FA for MON 87411 and 21.70% total FA for the conventional control, a difference of 0.19% total FA. This observed statistically significant difference was evaluated in the context of the range of the conventional control values, 2.67% total FA, calculated from the minimum (20.81% total FA) and maximum (23.49% total FA) oleic acid values. The mean difference in oleic acid values between MON 87411 and the conventional control was less than the range of the conventional control values, indicating that MON 87411 does not impact levels more than natural variation within the conventional control grown at multiple locations. The mean difference in oleic acid values between MON 87411 and the conventional control was also less than the variation seen in the reference hybrid values

(ranged from 20.52 to 42.88% total FA, a magnitude of 22.36% total FA), and the MON 87411 mean value was within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values (Table VII-8).

The data demonstrated that MON 87411 was not a major contributor to variation in total fat and fatty acid levels in maize grain and confirmed the compositional equivalence of MON 87411 to the conventional control in levels of these components. Also, the mean values of these components were within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values. These data confirmed that the significant difference in the mean value of oleic acid was not compositionally meaningful from a food and feed safety perspective.

VII.A.1.3. Carbohydrates by Calculation and Fiber

In addition to protein and fat, major biomass components assessed in maize grain included carbohydrates by calculation and fiber [acid detergent fiber (ADF), neutral detergent fiber (NDF), and total dietary fiber (TDF)]. Maize grain is typically composed of approximately 85% carbohydrates by calculation, which includes fiber (ADF, NDF, and TDF), and the levels of these components can vary widely depending on local growing conditions (Harrigan et al. 2009; Ridley et al. 2011; Zhou et al. 2011). This naturally occurring variability is important in assessing the biological relevance of statistically significant differences in composition.

No statistically significant differences were observed for carbohydrates by calculation, ADF, or TDF (Table VII-3). A statistically significant difference ($p<0.05$) was observed for NDF. The mean NDF value was 8.26% dw for MON 87411 and 8.74% dw for the conventional control, a mean difference of -0.48% dw. This difference was evaluated in the context of the range of the conventional control values, 2.94% dw, calculated from the minimum (7.36% dw) and maximum (10.30% dw) NDF values. The mean difference in NDF values between MON 87411 and the conventional control was less than the range of the conventional control values, indicating that MON 87411 does not impact levels more than natural variation within the conventional control grown at multiple locations. The mean difference in NDF values was also less than the variation seen in the reference hybrid values (ranged from 7.41 to 14.80% dw, a magnitude of 7.39% dw), and the MON 87411 mean value for NDF was within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values (Table VII-8).

The data demonstrated that MON 87411 was not a major contributor to variation in carbohydrates by calculation and fiber levels in maize grain and confirmed the compositional equivalence of MON 87411 to the conventional control in levels of these components. Also, the mean values of these components were within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values. These data confirmed that the significant difference in the mean value of NDF was not compositionally meaningful from a food and feed safety perspective.

VII.A.1.4 Ash and Minerals

Ash and minerals were also assessed in maize grain. Mineral components (calcium, copper, iron, magnesium, manganese, phosphorus, potassium and zinc), constituents of ash, are also discussed in this section. The levels of these components can vary widely depending on local growing conditions (Harrigan et al. 2009; Ridley et al. 2011; Zhou et al. 2011). This naturally occurring variability is important in assessing the biological relevance of statistically significant differences in composition.

Ash levels were not statistically significantly different between MON 87411 and the conventional control (Table VII-4). There were also no statistically significant differences observed for four of the eight minerals assessed, including calcium, magnesium, phosphorus, or potassium. Statistically significant differences ($p<0.05$) were observed between MON 87411 and the conventional control for copper, iron, manganese, and zinc (Table VII-4).

For copper, the mean value was 1.33 mg/kg dw for MON 87411 and 1.41 mg/kg dw for the conventional control, a difference of -0.077 mg/kg dw. This difference was evaluated in the context of the range of the conventional control values, 0.65 mg/kg dw, calculated from the minimum (1.13 mg/kg dw) and maximum (1.78 mg/kg dw) copper values. The mean difference in copper values between MON 87411 and the conventional control was less than the range of the conventional control values, indicating that MON 87411 does not impact levels more than natural variation within the conventional control grown at multiple locations. The mean difference in copper values was also less than the variation seen in the reference hybrid values (ranged 1.10 to 3.23 mg/kg dw, a magnitude of 2.13 mg/kg dw), and the MON 87411 mean value was within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values (Table VII-8).

For iron, the mean value was 16.84 mg/kg dw for MON 87411 and 16.33 mg/kg dw for the conventional control, a difference of 0.51 mg/kg dw. The mean difference in iron values between MON 87411 and the conventional control was less than the range of the conventional control values (4.67 mg/kg dw; 13.72 to 18.39 mg/kg dw), indicating that MON 87411 does not impact levels more than natural variation within the conventional control grown at multiple locations. The mean difference in iron values was also less than the variation seen in the reference hybrid values (ranged 13.80 to 24.48 mg/kg dw, a magnitude of 10.68 mg/kg dw), and the MON 87411 mean value was within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values (Table VII-8).

For manganese, the mean value was 6.16 mg/kg dw for MON 87411 and 5.99 mg/kg dw for the conventional control, a difference of 0.17 mg/kg dw. The mean difference in manganese values between MON 87411 and the conventional control was less than the range of the conventional control values (2.68 mg/kg dw; 4.63 to 7.32 mg/kg dw), indicating that MON 87411 does not impact levels more than natural variation within the conventional control grown at multiple locations. The mean difference in manganese values was also less than the variation seen in the reference hybrid values (ranged 4.93 to 10.42 mg/kg dw, a magnitude of 5.49 mg/kg dw), and the MON 87411 mean value was

within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values (Table VII-8).

For zinc, the mean value was 21.44 mg/kg dw for MON 87411 and 20.93 mg/kg dw for the conventional control, a difference of 0.50 mg/kg dw. The mean difference in zinc values between MON 87411 and the conventional control was less than the range of the conventional control values (6.45 mg/kg dw; 17.76 to 24.21 mg/kg dw), indicating that MON 87411 does not impact levels more than natural variation within the conventional control grown at multiple locations. The mean difference in zinc values was also less than the variation seen in the reference hybrid values (ranged 16.40 to 33.92 mg/kg dw, a magnitude of 17.52 mg/kg dw), and the MON 87411 mean value was within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values (Table VII-8).

The data demonstrated that MON 87411 was not a major contributor to variation in ash and mineral levels in maize grain and confirmed the compositional equivalence of MON 87411 to the conventional control in levels of these components. Also, the mean values of these components were within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values. These data confirmed that the significant differences in mean values of these several minerals were not compositionally meaningful from a food and feed safety perspective.

VII.A.1.5 Vitamins

Maize grain contains both water-soluble vitamins (folic acid, niacin, B1, B2, and B6) and fat-soluble vitamins [vitamins A (β-carotene) and E]. The levels of these components can vary widely depending on local growing conditions (Harrigan et al. 2009; Ridley et al. 2011; Safawo, et al. 2010). This naturally occurring variability is important in assessing the biological relevance of statistically significant differences in composition.

No statistically significant differences were observed for folic acid, vitamin A, vitamin B2, vitamin B6, and vitamin E (Table VII-5). Statistically significant differences ($p<0.05$) were observed between MON 87411 and the conventional control for niacin and vitamin B1.

For niacin, the mean value was 17.33 mg/kg dw for MON 87411 and 18.78 mg/kg dw for the conventional control, a difference of -1.45 mg/kg dw. This difference was evaluated in the context of the range of the conventional control values, 20.69 mg/kg dw, calculated from the minimum (13.73 mg/kg dw) and maximum (34.41 mg/kg dw) values. The mean difference in niacin values between MON 87411 and the conventional control was less than the range of the conventional control values, indicating that MON 87411 does not impact levels more than natural variation within the conventional control grown at multiple locations. The mean difference in niacin values was also less than the variation seen in the reference hybrid values (ranged 14.90 to 38.07 mg/kg dw, a magnitude of 23.17 mg/kg dw), and the MON 87411 mean value was within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values (Table VII-8).

For vitamin B1, the mean value was 3.44 mg/kg dw for MON 87411 and 3.56 mg/kg dw for the conventional control, a difference of -0.12 mg/kg dw. The mean difference in vitamin B1 values between MON 87411 and the conventional control was less than the range of the conventional control values (0.85 mg/kg dw; 3.10 to 3.94 mg/kg dw), indicating that MON 87411 does not impact levels more than natural variation within the conventional control grown at multiple locations. The mean difference in vitamin B1 values was also less than the variation seen in the reference hybrid values (ranged 2.79 to 4.96 mg/kg dw, a magnitude of 2.17 mg/kg dw), and the MON 87411 mean value was within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values (Table VII-8).

The data demonstrated that MON 87411 was not a major contributor to variation in vitamin levels in maize grain and confirmed the compositional equivalence of MON 87411 to the conventional control in levels of these components. Also, the mean values of these components were within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values. These data confirmed that the significant differences in mean values of these vitamins were not compositionally meaningful from a food and feed safety perspective.

VII.A.2. Anti-Nutrient Levels in Maize Grain

Anti-nutrients assessed included phytic acid and raffinose. Phytic acid, the major storage form of phosphorus in maize grain, is considered an anti-nutrient due to its mineral-chelating properties and the sequestration of phosphorus in phytic acid, reducing phosphorus bioavailabity. Raffinose is a low molecular weight non-digestible carbohydrate that is considered to be an anti-nutrient due to the enteric gas production and resulting flatulence caused by its consumption (Liener 2000). The levels of these components can vary widely depending on local growing conditions (Harrigan et al. 2009; Ridley et al. 2011). This naturally occurring variability is important in assessing the biological relevance of statistically significant differences in composition.

No statistically significant differences were observed for phytic acid and raffinose (Table VII-6). The data demonstrated that MON 87411 was not a major contributor to variation in phytic acid and raffinose levels in maize grain and confirmed the compositional equivalence of MON 87411 to the conventional control in levels of these components.

VII.A.3. Secondary Metabolites Levels in Maize Grain

Secondary metabolites measured in MON 87411 grain included furfural, ferulic acid, and p-coumaric acid according to the OECD consensus document (OECD 2002b). Furfural was not detected in the grain of MON 87411, the conventional control, or reference hybrids. Ferulic acid and p-coumaric acid are derived from phenylalanine and tyrosine (Buchanan, et al. 2000) and serve as precursors for a large group of phenylpropanoid compounds and fiber. The levels of these secondary metabolites can vary widely depending on local growing conditions (Harrigan et al. 2009; Ridley et al. 2011). This naturally occurring variability is important in assessing the biological relevance of statistically significant differences in composition.

No statistically significant differences were observed for ferulic acid or p-coumaric acid (Table VII-6). The data demonstrated that MON 87411 was not a major contributor to variation in ferulic acid and p-coumaric acid levels in maize grain and confirmed the compositional equivalence of MON 87411 to the conventional control in levels of these components.

VII.A.4. Nutrient Levels in Maize Forage

Forage samples were assessed for levels of ash, protein, total fat, carbohydrates by calculation, fiber (ADF and NDF), and minerals (calcium and phosphorus). The levels of these components can vary widely depending on local growing conditions (Harrigan et al. 2009; Ridley et al. 2011). This naturally occurring variability is important in assessing the biological relevance of statistically significant differences in composition.

With the exception of ash, there were no statistically significant differences in levels of proximates, fiber, or minerals (Table VII-7). The mean ash value was 5.57% dw for MON 87411 and 5.95% dw for the conventional control, a difference of -0.39% dw. This difference was evaluated in the context of the range of the conventional control values, 5.04% dw, calculated from the minimum (4.51% dw) and maximum (9.55% dw) values. The mean difference in ash values between MON 87411 and the conventional control was less than the range of the conventional control values, indicating that MON 87411 does not impact levels more than natural variation within the conventional control grown at multiple locations. The mean difference was also less than the variation seen in the reference values (ranged 3.53 to 8.33% dw, a magnitude of 4.80% dw), and the MON 87411 mean value was within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values (Table VII-8).

The data demonstrated that MON 87411 was not a major contributor to variation in ash levels in maize forage and confirmed the compositional equivalence of MON 87411 to the conventional control in levels of these components. Also, the mean value of this component was within the 99% tolerance interval, the values observed in the literature, and/or the ILSI-CCDB values. These data confirmed that the observed significant difference in mean value of ash was not compositionally meaningful from a food and feed safety perspective.

Table VII-1. Summary of Maize Grain Protein and Amino Acids for MON 87411, Conventional Control, and Reference Hybrids

					Difference (Test minus Control)		
Component (% dw)[1]	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Mean (S.E.)	p-Value	% Relative[6]
Protein	10.71 (0.49) 8.03 - 13.10	10.28 (0.49) 8.06 - 12.60	(7.21 - 14.59) 5.66, 15.13	4.54	0.43 (0.17)	0.023	4.19
Alanine	0.84 (0.044) 0.62 - 1.03	0.81 (0.044) 0.61 - 1.03	(0.51 - 1.20) 0.37, 1.24	0.42	0.027 (0.019)	0.166	3.34
Arginine	0.48 (0.013) 0.41 - 0.54	0.47 (0.013) 0.40 - 0.54	(0.40 - 0.66) 0.35, 0.66	0.15	0.0088 (0.0055)	0.133	1.86
Aspartic Acid	0.68 (0.028) 0.54 - 0.80	0.66 (0.028) 0.52 - 0.80	(0.49 - 0.91) 0.42, 0.91	0.28	0.022 (0.011)	0.068	3.41
Cystine/Cysteine	0.21 (0.0061) 0.17 - 0.25	0.21 (0.0061) 0.17 - 0.24	(0.16 - 0.30) 0.12, 0.32	0.07	0.0010 (0.0034)	0.768	0.50
Glutamic Acid	2.03 (0.11) 1.44 - 2.51	1.95 (0.11) 1.43 - 2.49	(1.23 - 2.87) 0.87, 3.02	1.06	0.078 (0.045)	0.108	3.98

Table VII-1 (continued). Summary of Maize Grain Protein and Amino Acids for MON 87411, Conventional Control, and Reference Hybrids

					Difference (Test minus Control)		
Component (% dw)[1]	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Mean (S.E.)	p-Value	% Relative[6]
Glycine	0.38 (0.0099) 0.34 - 0.43	0.38 (0.0099) 0.32 - 0.44	(0.31 - 0.51) 0.27, 0.52	0.12	0.0024 (0.0044)	0.591	0.64
Histidine	0.28 (0.010) 0.23 - 0.32	0.27 (0.010) 0.21 - 0.34	(0.20 - 0.46) 0.13, 0.45	0.12	0.0096 (0.0041)	0.033	3.54
Isoleucine	0.40 (0.020) 0.29 - 0.49	0.38 (0.020) 0.27 - 0.48	(0.25 - 0.54) 0.19, 0.56	0.21	0.018 (0.0084)	0.050	4.77
Leucine	1.41 (0.086) 0.96 - 1.79	1.35 (0.086) 0.95 - 1.76	(0.79 - 2.01) 0.51, 2.14	0.81	0.061 (0.034)	0.100	4.50
Lysine	0.28 (0.0057) 0.24 - 0.32	0.27 (0.0057) 0.22 - 0.31	(0.24 - 0.34) 0.22, 0.35	0.09	0.0057 (0.0042)	0.197	2.12
Methionine	0.21 (0.0074) 0.18 - 0.25	0.21 (0.0074) 0.17 - 0.25	(0.15 - 0.27) 0.13, 0.28	0.08	-0.00024 (0.0040)	0.952	-0.11

Table VII-1 (continued). Summary of Maize Grain Protein and Amino Acids for MON 87411, Conventional Control, and Reference Hybrids

Component (% dw)[1]	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Difference (Test minus Control) Mean (S.E.)	p-Value	% Relative[6]
Phenylalanine	0.56 (0.032) 0.41 - 0.70	0.54 (0.032) 0.40 - 0.70	(0.34 - 0.78) 0.24, 0.83	0.31	0.023 (0.014)	0.130	4.20
Proline	0.97 (0.045) 0.74 - 1.18	0.95 (0.045) 0.74 - 1.15	(0.64 - 1.39) 0.44, 1.47	0.41	0.019 (0.018)	0.321	1.98
Serine	0.48 (0.023) 0.35 - 0.60	0.47 (0.023) 0.33 - 0.59	(0.33 - 0.67) 0.25, 0.69	0.26	0.017 (0.0093)	0.095	3.53
Threonine	0.37 (0.015) 0.30 - 0.44	0.36 (0.015) 0.29 - 0.44	(0.27 - 0.50) 0.22, 0.51	0.14	0.010 (0.0062)	0.131	2.74
Tryptophan	0.071 (0.0016) 0.064 - 0.080	0.071 (0.0016) 0.059 - 0.082	(0.053 - 0.086) 0.053, 0.091	0.02	0.00018 (0.00070)	0.805	0.25
Tyrosine	0.42 (0.020) 0.31 - 0.52	0.40 (0.020) 0.30 - 0.52	(0.27 - 0.57) 0.22, 0.58	0.22	0.018 (0.0082)	0.046	4.42

Table VII-1 (continued). Summary of Maize Grain Protein and Amino Acids for MON 87411, Conventional Control, and Reference Hybrids

					Difference (Test minus Control)		
Component (% dw)[1]	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Mean (S.E.)	p-Value	% Relative[6]
Valine	0.49 (0.021) 0.39 - 0.58	0.48 (0.021) 0.37 - 0.59	(0.34 - 0.70) 0.27, 0.70	0.22	0.015 (0.010)	0.167	3.04

[1]dw = dry weight.
[2]Mean (S.E.) = least-square mean (standard error).
[3]Range is the minimum and maximum raw values for the conventional reference maize hybrids.
[4]With 95% confidence, the tolerance interval contains 99% of the values expressed in the population of reference hybrids. Negative limits were set to zero.
[5]Maximum value minus minimum value for the conventional control maize hybrid.
[6]The relative magnitude of the difference in mean values between MON 87411 and the conventional control, expressed as a percent of the conventional control.

Table VII-2. Summary of Maize Grain Fat and Fatty Acids for MON 87411, Conventional Control, and Reference Hybrids

Component	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Difference (Test minus Control) Mean (S.E.)	p-Value
Total Fat (% dw)[1]	3.79 (0.047) 3.42 - 4.15	3.83 (0.047) 3.43 - 4.10	(2.65 - 5.60) 1.55, 6.69	0.68	-0.036 (0.063)	0.573
16:0 Palmitic[6]	13.61 (0.045) 13.31 - 13.97	13.62 (0.045) 13.32 - 14.05	(9.98 - 12.71) 8.50, 14.14	0.73	-0.0063 (0.032)	0.842
18:0 Stearic	1.68 (0.032) 1.57 - 1.88	1.70 (0.032) 1.53 - 1.96	(1.56 - 2.58) 0.93, 2.98	0.43	-0.021 (0.018)	0.249
18:1 Oleic	21.89 (0.15) 20.86 - 22.96	21.70 (0.15) 20.81 - 23.49	(20.52 - 42.88) 7.74, 50.71	2.67	0.19 (0.091)	0.040
18:2 Linoleic	60.90 (0.22) 59.38 - 61.96	61.06 (0.22) 59.08 - 62.38	(42.82 - 64.10) 33.63, 77.43	3.30	-0.17 (0.099)	0.095
18:3 Linolenic	1.09 (0.0093) 1.04 - 1.17	1.09 (0.0093) 1.02 - 1.16	(0.85 - 1.41) 0.57, 1.65	0.14	0.0035 (0.0059)	0.552

Table VII-2 (continued). Summary of Maize Grain Fat and Fatty Acids for MON 87411, Conventional Control, and Reference Hybrids

Component	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Difference (Test minus Control) Mean (S.E.)	p-Value
20:0 Arachidic	0.41 (0.0068) 0.39 - 0.44	0.42 (0.0068) 0.37 - 0.47	(0.33 - 0.63) 0.21, 0.70	0.09	-0.0024 (0.0042)	0.571
20:1 Eicosenoic	0.26 (0.0018) 0.24 - 0.28	0.26 (0.0018) 0.24 - 0.27	(0.19 - 0.34) 0.12, 0.38	0.03	0.0038 (0.0022)	0.101
22:0 Behenic	0.16 (0.0016) 0.14 - 0.17	0.16 (0.0016) 0.15 - 0.18	(0.055 - 0.25) 0.0065, 0.31	0.03	-0.0016 (0.0020)	0.434

[1]dw = dry weight.
[2]Mean (S.E.) = least-square mean (standard error).
[3]Range is the minimum and maximum raw values for the conventional reference maize hybrids.
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional reference maize hybrids. Negative limits set to zero.
[5]Maximum value minus minimum value for the conventional control maize hybrid.
[6]Fatty acid means and ranges are expressed as a % of total fatty acid. Prefix numbers refer to number of carbon atoms and number of carbon-carbon double bonds in the fatty acid molecule; 16:0 means sixteen carbon atoms and zero double bonds.
Numbers are not included in text discussion for reasons of clarity. The following fatty acids with more than 50% of observations below the assay LOQ were excluded from statistical analysis: caprylic acid, capric acid, lauric acid, myristic acid, myristoleic acid, pentadecanoic acid, pentadecenoic acid, palmitoleic acid, heptadecanoic acid, heptadecenoic acid, gamma linolenic acid, eicosadienoic acid, eicosatrienoic acid, and arachidonic acid.

Table VII-3. Summary of Maize Grain Carbohydrates by Calculation and Fiber for MON 87411, Conventional Control, and Reference Hybrids

Component (% dw)[1]	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Difference (Test minus Control) Mean (S.E.)	p-Value
Carbohydrates by Calculation	84.13 (0.51) 81.84 - 86.85	84.53 (0.51) 82.18 - 86.95	(78.76 - 87.56) 77.72, 90.40	4.77	-0.40 (0.20)	0.068
Acid Detergent Fiber	3.06 (0.083) 2.50 - 3.62	3.26 (0.083) 2.73 - 4.00	(1.89 - 5.16) 2.18, 4.98	1.26	-0.20 (0.10)	0.074
Neutral Detergent Fiber	8.26 (0.17) 7.12 - 12.14	8.74 (0.17) 7.36 - 10.30	(7.41 - 14.80) 6.04, 13.44	2.94	-0.48 (0.20)	0.018
Total Dietary Fiber	11.50 (0.20) 10.19 - 12.90	11.82 (0.20) 10.43 - 15.03	(10.33 - 17.11) 9.83, 16.84	4.60	-0.33 (0.27)	0.247

[1]dw = dry weight.
[2]Mean (S.E.) = least-square mean (standard error).
[3]Range is the minimum and maximum raw values for the conventional reference maize hybrids.
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional reference maize hybrids. Negative limits set to zero.
[5]Maximum value minus minimum value for the conventional control maize hybrid.

Table VII-4. Summary of Maize Grain Ash and Minerals for MON 87411, Conventional Control, and Reference Hybrids

Component	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Difference (Test minus Control) Mean (S.E.)	p-Value
Ash (% dw)[1]	1.36 (0.026) 1.13 - 1.51	1.35 (0.026) 1.05 - 1.60	(1.07 - 1.75) 1.07, 1.80	0.55	0.0098 (0.030)	0.746
Calcium (% dw)	0.0031 (0.00007) 0.0024 - 0.0037	0.0030 (0.00007) 0.0026 - 0.0033	(0.0026 - 0.0056) 0.0019, 0.0062	0.00	0.00009 (0.00005)	0.063
Copper (mg/kg dw)	1.33 (0.056) 1.09 - 1.88	1.41 (0.056) 1.13 - 1.78	(1.10 - 3.23) 0.28, 3.75	0.65	-0.077 (0.030)	0.021
Iron (mg/kg dw)	16.84 (0.41) 14.59 - 19.70	16.33 (0.41) 13.72 - 18.39	(13.80 - 24.48) 10.71, 28.62	4.67	0.51 (0.18)	0.013
Magnesium (% dw)	0.11 (0.0031) 0.094 - 0.13	0.12 (0.0031) 0.094 - 0.13	(0.097 - 0.15) 0.086, 0.16	0.03	-0.00050 (0.0011)	0.657
Manganese (mg/kg dw)	6.16 (0.26) 4.64 - 7.13	5.99 (0.26) 4.63 - 7.32	(4.93 - 10.42) 2.28, 12.14	2.68	0.17 (0.070)	0.033

Table VII-4 (continued). Summary of Maize Grain Ash and Minerals for MON 87411, Conventional Control, and Reference Hybrids

Component	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Difference (Test minus Control) Mean (S.E.)	p-Value
Phosphorus (% dw)	0.31 (0.0071) 0.25 - 0.33	0.31 (0.0071) 0.23 - 0.34	(0.24 - 0.39) 0.22, 0.43	0.11	-0.0018 (0.0033)	0.591
Potassium (% dw)	0.34 (0.0061) 0.32 - 0.39	0.35 (0.0061) 0.31 - 0.40	(0.30 - 0.43) 0.28, 0.46	0.09	-0.0055 (0.0034)	0.127
Zinc (mg/kg dw)	21.44 (0.72) 18.54 - 26.54	20.93 (0.72) 17.76 - 24.21	(16.40 - 33.92) 11.63, 36.32	6.45	0.50 (0.22)	0.038

[1]dw = dry weight.
[2]Mean (S.E.) = least-square mean (standard error).
[3]Range is the minimum and maximum raw values for the conventional reference hybrids.
[4]With 95% confidence, the tolerance interval contains 99% of the values expressed in the population of conventional reference hybrids. Negative limits were set to zero.
[5]Maximum value minus minimum value for the conventional control maize hybrid.

Table VII-5. Summary of Maize Grain Vitamins for MON 87411, Conventional Control, and Reference Hybrids

Component (mg/kg dw)[1]	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Difference (Test minus Control) Mean (S.E.)	p-Value
Folic Acid	0.28 (0.0071) 0.23 - 0.39	0.28 (0.0071) 0.21 - 0.37	(0.19 - 0.52) 0.084, 0.56	0.17	0.0041 (0.0074)	0.580
Niacin	17.33 (0.75) 14.86 - 20.70	18.78 (0.75) 13.73 - 34.41	(14.90 - 38.07) 4.69, 42.03	20.69	-1.45 (0.61)	0.021
Vitamin A	1.29 (0.058) 0.75 - 1.57	1.38 (0.058) 0.65 - 3.59	(0.32 - 4.76) 0, 4.91	2.94	-0.095 (0.064)	0.145
Vitamin B1	3.44 (0.065) 3.04 - 3.85	3.56 (0.065) 3.10 - 3.94	(2.79 - 4.96) 1.86, 5.07	0.85	-0.12 (0.047)	0.021
Vitamin B2	1.53 (0.048) 1.22 - 2.03	1.64 (0.048) 1.28 - 2.30	(1.15 - 2.54) 0.94, 2.37	1.01	-0.11 (0.052)	0.058
Vitamin B6	6.16 (0.11) 5.15 - 7.44	6.10 (0.11) 5.07 - 8.56	(5.09 - 12.13) 3.84, 10.03	3.49	0.060 (0.13)	0.648

Table VII-5 (continued). Summary of Maize Grain Vitamins for MON 87411, Conventional Control, and Reference Hybrids

Component (mg/kg dw)[1]	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Difference (Test minus Control) Mean (S.E.)	Difference (Test minus Control) p-Value
Vitamin E	10.53 (0.24)	10.28 (0.24)	(6.37 - 31.91)	3.78	0.25 (0.29)	0.400
	6.99 - 15.33	8.94 - 12.72	0, 30.69			

[1]dw = dry weight.
[2]Mean (S.E.) = least-square mean (standard error).
[3]Range is the minimum and maximum raw values for the conventional reference maize hybrids.
[4]With 95% confidence, the tolerance interval contains 99% of the values expressed in the population of conventional reference hybrids. Negative limits were set to zero.
[5]Maximum value minus minimum value for the conventional control maize hybrid.

Table VII-6. Summary of Maize Grain Anti-nutrients and Secondary Metabolites for MON 87411, Conventional Control, and Reference Hybrids

Component	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Difference (Test minus Control) Mean (S.E.)	Difference (Test minus Control) p-Value
Anti-nutrient (% dw[1])						
Phytic Acid	0.99 (0.029) 0.75 - 1.27	0.98 (0.029) 0.67 - 1.19	(0.60 - 1.25) 0.56, 1.41	0.52	0.012 (0.022)	0.584
Raffinose	0.25 (0.0081) 0.14 - 0.31	0.24 (0.0081) 0.10 - 0.29	(0.093 - 0.40) 0, 0.45	0.19	0.0066 (0.0058)	0.256
Secondary Metabolite (µg/g dw)						
Ferulic Acid	1846.74 (28.24) 1528.09 - 2031.96	1896.61 (28.24) 1700.68 - 2093.02	(1337.21 - 3286.55) 749.39, 3421.84	392.34	-49.87 (29.30)	0.110
p-Coumaric Acid	148.56 (2.91) 132.88 - 197.75	148.27 (2.91) 131.03 - 164.37	(62.24 - 387.51) 0, 461.05	33.33	0.28 (3.31)	0.932

[1]dw = dry weight.
[2]Mean (S.E.) = least-square mean (standard error).
[3]Range is the minimum and maximum raw values for the conventional reference hybrids.
[4]With 95% confidence, the tolerance interval contains 99% of the values expressed in the population of conventional reference hybrids. Negative limits were set to zero.
[5]Maximum value minus minimum value for the conventional control maize hybrid.

Table VII-7. Summary of Maize Forage Proximates, Fiber and Minerals for MON 87411, Conventional Control, and Reference Hybrids

Component (% dw)[1]	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Difference (Test minus Control) Mean (S.E.)	p-Value
Ash	5.57 (0.22) 3.95 - 7.01	5.95 (0.22) 4.51 - 9.55	(3.53 - 8.33) 3.38, 7.59	5.04	-0.39 (0.16)	0.018
Carbohydrates by Calculation	84.96 (0.56) 81.65 - 88.55	84.57 (0.56) 80.91 - 88.00	(79.74 - 90.37) 80.32, 89.79	7.09	0.38 (0.25)	0.128
Protein	7.58 (0.46) 5.27 - 10.25	7.42 (0.46) 4.63 - 9.66	(4.19 - 11.38) 3.12, 11.55	5.03	0.17 (0.16)	0.321
Total Fat	1.91 (0.17) 0.49 - 2.93	2.07 (0.17) 0.99 - 3.39	(0.66 - 3.60) 0.67, 3.58	2.40	-0.16 (0.15)	0.296
Acid Detergent Fiber	25.45 (0.94) 16.58 - 34.25	25.07 (0.94) 17.89 - 30.55	(18.64 - 37.68) 14.75, 38.41	12.65	0.39 (0.66)	0.558
Neutral Detergent Fiber	44.68 (1.49) 28.32 - 55.35	42.84 (1.49) 32.16 - 51.33	(34.97 - 67.39) 28.74, 62.39	19.17	1.84 (1.19)	0.145

Table VII-7 (continued). Summary of Maize Forage Proximates, Fiber and Minerals for MON 87411, Conventional Control, and Reference Hybrids

Component (% dw)[1]	MON 87411 Mean (S.E.)[2] Range	Conventional Control Mean (S.E.) Range	Reference Hybrids (Range)[3] Tolerance Interval[4]	Conventional Control Range Value[5]	Difference (Test minus Control) Mean (S.E.)	p-Value
Calcium	0.17 (0.016)	0.18 (0.016)	(0.074 - 0.37)	0.34	-0.013 (0.0078)	0.112
	0.093 - 0.26	0.084 - 0.42	0.015, 0.32			
Phosphorus	0.19 (0.011)	0.19 (0.011)	(0.11 - 0.26)	0.13	-0.00002 (0.0058)	0.997
	0.095 - 0.24	0.13 - 0.25	0.082, 0.26			

[1]dw = dry weight.
[2]Mean (S.E.) = least-square mean (standard error).
[3]Range is the minimum and maximum raw values for the conventional reference maize hybrids.
[4]With 95% confidence, the tolerance interval contains 99% of the values expressed in the population of conventional reference hybrids. Negative limits were set to zero.
[5]Maximum value minus minimum value for the conventional control maize hybrid.

Table VII-8. Literature and ILSI Database Ranges for Components in Maize Forage and Grain

Grain Tissue Components[1]	Literature Range[2]	ILSI Range[3]
Grain Nutrients		
Proximates (% dw)		
Ash	1.17 – 2.01[a]; 1.27 – 1.63[b]	0.616 – 6.282
Carbohydrates by calculation	81.31 – 87.06[a]; 82.10 – 85.98[b]	77.4 – 89.5
Fat, total	2.95 – 4.40[a]; 3.18 – 4.23[b]	1.742 – 5.900
Protein	8.27 – 13.33[a]; 9.17 – 12.19[b]	6.15 – 17.26
Fiber (% dw)		
Acid detergent fiber	1.82 – 4.48[a]; 1.83 – 3.39[b]	1.82 – 11.34
Neutral detergent fiber	6.51 –12.28[a]; 6.08 – 10.36[b]	5.59 – 22.64
Total dietary fiber	10.65 – 16.26[a]; 10.57 – 14.56[b]	9.01 – 35.31
Amino Acids (% dw)		
Alanine	0.60 – 1.04[a]; 0.68 – 0.96[b]	0.44 - 1.39
Arginine	0.34 – 0.52[a]; 0.34 – 0.50[b]	0.12 - 0.64
Aspartic acid	0.52 – 0.78[a]; 0.59 – 0.76[b]	0.33 – 1.21
Cystine	0.19 – 0.26[a]; 0.20 – 0.26[b]	0.13 – 0.51
Glutamic acid	1.54 – 2.67[a]; 1.71 – 2.44[b]	0.97 – 3.54
Glycine	0.33 – 0.43[a]; 0.33 – 0.42[b]	0.18 – 0.54
Histidine	0.25 – 0.37[a]; 0.27 – 0.34[b]	0.14 – 0.43
Isoleucine	0.30 – 0.48[a]; 0.32 – 0.44[b]	0.18 – 0.69
Leucine	1.02 – 1.87[a]; 1.13 – 1.65[b]	0.64 – 2.49
Lysine	0.26 – 0.33[a]; 0.28 – 0.31[b]	0.17 – 0.67
Methionine	0.17 – 0.26[a]; 0.16 – 0.30[b]	0.12 – 0.47
Phenylalanine	0.43 – 0.72[a]; 0.45 – 0.63[b]	0.24 – 0.93
Proline	0.74 – 1.21[a]; 0.78 – 1.11[b]	0.46 – 1.63
Serine	0.39 – 0.67[a]; 0.43 – 0.60[b]	0.24 – 0.77
Threonine	0.29 – 0.45[a]; 0.31 – 0.39[b]	0.22 – 0.67
Tryptophan	0.047 – 0.085[a]; 0.042 – 0.070[b]	0.027 – 0.215
Tyrosine	0.13 – 0.43[a]; 0.12 – 0.41[b]	0.10 – 0.64
Valine	0.42 – 0.62[a]; 0.45 – 0.58[b]	0.27 – 0.86
Fatty Acids (% Total FA)		
16:0 Palmitic	8.80 – 13.33[a]; 9.84 – 12.33[b]	7.94 – 20.71
18:0 Stearic	1.36 – 2.14 [a]; 1.30 – 2.10[b]	1.02 – 3.40
18:1 Oleic	19.50 – 33.71[a]; 19.59 – 29.13[b]	17.4 – 40.2
18:2 Linoleic	49.31 – 64.70[a]; 56.51 – 65.65[b]	36.2 – 66.5
18:3 Linolenic	0.89 – 1.56[a]; 1.03 – 1.38[b]	0.57 – 2.25
20:0 Arachidic	0.30 – 0.49[a]; 0.30 – 0.41[b]	0.279 – 0.965
20:1 Eicosenoic	0.17 – 0.29[a]; 0.17 – 0.27[b]	0.170 – 1.917
22:0 Behenic	0.069 – 0.28[a]; 0.059 – 0.18[b]	0.110 – 0.349
Minerals		
Calcium (% dw)	0.0036 – 0.0068[a]; 0.0035 – 0.0070[b]	0.00127 – 0.02084
Copper (mg/kg dw)	1.14 – 3.43[a]; 1.39 – 2.76[b]	0.73 – 18.50
Iron (mg/kg dw)	14.17 – 23.40[a]; 15.90 – 24.66[b]	10.42 – 49.07
Magnesium (% dw)	0.091 – 0.14[a]; 0.10 – 0.14[b]	0.0594 – 0.194
Manganese (mg/kg dw)	4.83 – 8.34[a]; 4.78 – 9.35[b]	1.69 – 14.30
Phosphorous (% dw)	0.24 – 0.37[a]; 0.27 – 0.38[b]	0.147 – 0.533
Potassium (% dw)	0.29 – 0.39[a]; 0.36 – 0.43[b]	0.181 – 0.603
Zinc (mg/kg dw)	16.78 – 28.17[a]; 18.25 – 30.44[b]	6.5 – 37.2

Table VII-8 (continued). Literature and ILSI Database Ranges for Components in Maize Forage and Grain

Grain Tissue Components[1]	Literature Range[2]	ILSI Range[3]
Vitamins (mg/kg dw)		
Folic acid	0.19 – 0.35[a]; 0.23 – 0.42[b]	0.147 – 1.464
Vitamin A [β–Carotene]	122 – 4740[c]	0.19 – 46.81
Vitamin B_1 [Thiamine]	2.33 – 4.17[a]; 2.71 – 4.33[b]	1.26 – 40.00
Vitamin B_2 [Riboflavin]	0.94 – 2.42[a]; 1.64 – 2.81[b]	0.50 – 2.36
Vitamin B_3 [Niacin]	15.07 – 32.38[a]; 13.64 – 42.06[b]	10.37 – 46.94
Vitamin B_6 [Pyridoxine]	4.93 – 7.53[a]; 4.97 – 8.27[b]	3.68 – 11.32
Vitamin E [α–Tocopherol]	5.96 – 18.44[a]; 2.84 – 15.53[b]	1.537 – 68.672
Grain Anti–Nutrients (% dw)		
Phytic acid	0.69 – 1.09[a]; 0.60 – 0.94[b]	0.111 – 1.570
Raffinose	0.079 – 0.22[a]; 0.061 – 0.15[b]	0.020 – 0.320
Grain Secondary Metabolites (µg/g dw)		
Ferulic acid	1205.75 – 2873.05[a]; 1011.40 – 2539.86[b]	291.9 – 3885.8
p–Coumaric acid	94.77 – 327.39[a]; 66.48 – 259.68[b]	53.4 – 576.2
Forage Tissue Components[1]	**Literature Range[2]**	**ILSI Range[3]**
Forage Nutrients		
Proximates (% dw)		
Ash	2.67 – 8.01[a]; 4.59 – 6.90[b]	1.527 – 9.638
Carbohydrates by calculation	81.88 – 89.26[a]; 84.11 – 87.54[b]	76.4 – 92.1
Fat, total	1.28 – 3.62[a]; 0.20 – 1.76[b]	0.296 – 4.570
Protein	5.80 – 10.24[a]; 5.56 – 9.14[b]	3.14 – 11.57
Fiber (% dw)		
Acid detergent fiber	19.11 – 30.49[a]; 20.73 – 33.39[b]	16.13 – 47.39
Neutral detergent fiber	27.73 – 49.62[a]; 31.81 – 50.61[b]	20.29 – 63.71
Minerals (% dw)		
Calcium	0.12 – 0.33[a]; 0.21 – 0.41[b]	0.07139 – 0.57679
Phosphorous	0.090 – 0.26[a]; 0.13 – 0.21[b]	0.09362 – 0.37041

[1]dw=dry weight; FA = fatty acids.
[2]Literature range references: [a]US and [b]Chile (Harrigan et al. 2009), [c](Safawo et al. 2010).
[3]ILSI range is from ILSI Crop Composition Database, 2011 [Accessed 9 January 2013] (ILSI 2011).

VII.B. Compositional Assessment of MON 87411 Conclusion

Compositional analysis was conducted on grain and forage of MON 87411 treated with glyphosate grown at eight sites in a 2011/2012 field production in Argentina that are representative of commercial agricultural regions for maize production. The compositional analysis, based on the OECD consensus document for maize, included measurement of nutrients, anti-nutrients and secondary metabolites in conventional commercial reference hybrids to provide data on the natural variability of each compositional component analyzed.

Of the 60 components statistically assessed for MON 87411, only 12 components (protein, histidine, tyrosine, oleic acid, neutral detergent fiber, copper, iron, manganese, zinc, niacin, and vitamin B1 in grain and ash in forage) showed a significant difference between MON 87411 and the conventional control in the combined-site analysis. For these 12 components, the mean difference in component values between MON 87411 and the conventional control was less than the range of the conventional control values and the reference hybrid values. For all components analyzed, the MON 87411 mean component values were within the tolerance intervals, the values observed in the literature, and/or the ILSI-CCDB values.

These results support the overall conclusion that MON 87411 is not a major contributor to variation in component levels in maize grain and forage and confirmed the compositional equivalence of MON 87411 to the conventional control in levels of these components. These data confirmed that the components with a statistically significant differences ($p<0.05$) between MON 87411 and the conventional control were not compositionally meaningful from a food and feed safety perspective.

VIII. PHENOTYPIC, AGRONOMIC, AND ENVIRONMENTAL INTERACTIONS ASSESSMENT

This section provides a comparative assessment of the phenotypic, agronomic, and environmental interaction characteristics of MON 87411 compared to the conventional control. The data support a conclusion that MON 87411 is not meaningfully different from the conventional control with the exception of the insect-protected and glyphosate-tolerant traits, and therefore, is not expected to pose a plant pest risk compared to conventional maize. These conclusions are based on the results of multiple evaluations from laboratory and field assessments.

Phenotypic, agronomic, and environmental interaction characteristics of MON 87411 were evaluated in a comparative manner to assess plant pest potential. These assessments included evaluation of seed germination characteristics, plant growth and development characteristics, observations of plant responses to abiotic stressors, plant-disease and plant-arthropod interactions, and pollen characteristics. Results from these assessments demonstrate that MON 87411 does not possess a) increased weediness characteristics; b) increased susceptibility or tolerance to specific abiotic stressors, diseases, or arthropods; or c) characteristics that would confer a plant pest risk compared to the conventional control.

VIII.A. Characteristics Measured for Assessment

In the phenotypic, agronomic, and environmental interactions assessment of MON 87411, data were collected to evaluate altered plant pest potential. A detailed description of the regulated article phenotype is requested as part of the petition for determination of nonregulated status in 7 CFR § 340.6 including differences from the unmodified recipient organism that would "substantiate that the regulated article is unlikely to pose a greater plant pest risk than the unmodified organism from which it was derived". As part of the characterization of MON 87411, data were collected to provide a detailed description of the phenotypic, agronomic, and environmental interaction characteristics of MON 87411. A subset of these data were included in an evaluation of specific characteristics related to altered plant pest potential (*e.g.* seed dormancy, lodging, ear drop, and environmental interactions data).

The plant characterization of MON 87411 encompassed five general data categories: 1) seed germination, dormancy, and emergence; 2) vegetative growth; 3) reproductive development (including pollen characteristics); 4) lodging and seed retention on the plant; 5) plant response to abiotic stress and interactions with diseases and arthropods. An overview of the characteristics assessed is presented in Table VIII-1.

The phenotypic, agronomic, and environmental interactions data were evaluated from a basis of familiarity (OECD 1993) and were comprised of a combination of field and laboratory studies conducted by scientists who are familiar with the production and evaluation of maize. In each of these assessments, MON 87411 was compared to an appropriate conventional control (*i.e.*, MPA640B) that had a genetic background similar to MON 87411 but did not possess insect-protected or glyphosate-tolerance traits. In

addition, multiple commercial conventional maize reference hybrids developed through conventional breeding and selection (see Appendix H, I, and J, and Tables H-1, I-1, and J-1) were included to provide a range of comparative values for each characteristic that are representative of the variability in existing commercial maize hybrids. Data collected for the various characteristics from the commercial reference hybrids provide context for interpreting experimental results.

Table VIII-1. Phenotypic, Agronomic, and Environmental Interaction Characteristics Evaluated in U.S. Field Trials and Laboratory Studies

Data category	Characteristics measured (associated section where discussed)	Evaluation timing (Setting of evaluation)[1]	Evaluation description (measurement endpoints)
Germination, dormancy, and emergence	Normal germinated (VIII.C.1)	Day 4 and 7: 20/30°C (Laboratory)	Percentage of seed producing seedlings exhibiting normal developmental characteristics
	Abnormal germinated (VIII.C.1)	Day 7: 20/30°C (Laboratory)	Percentage of seed producing seedlings that could not be classified as normal germinated
	Germinated (VIII.C.1)	Day 4, Day 7, and Day 12: 5, 10, 20, 30, 10/20 and 10/30°C (Laboratory)	Percentage of seed that had germinated (both normally or abnormally)
	Dead (VIII.C.1)	Day 4 and 7: 5, 10, 20, 30, 10/20, 10/30, and 20/30°C. Day 12: 5, 10, 20, 30, 10/20 and 10/30°C (Laboratory)	Percentage of seed that had visibly deteriorated and become soft to the touch (also included non-viable hard and non-viable firm-swollen seed)
	Viable hard (VIII.C.1)	Day 7: 20/30°C Day 12: 5, 10, 20, 30, 10/20 and 10/30°C (Laboratory)	Percentage of seed that did not imbibe water and remained hard to the touch (viability determined by a tetrazolium test[2])
	Viable firm-swollen (VIII.C.1)	Day 7: 20/30°C Day 12: 5, 10, 20, 30, 10/20 and 10/30°C (Laboratory)	Percentage of seed that imbibed water and were firm to the touch but did not germinate (viability determined by a tetrazolium test[2])
	Early stand count (VIII.C.2.1)	V2 – V5 growth stage (Field)	Number of emerged plants in two rows
	Final stand count (VIII.C.2.1)	Pre-harvest (Field)	Number of plants in two rows
Vegetative growth	Plant vigor (VIII.C.2.1)	V2 – V4 growth stage (Field)	Rated on a 1-9 scale, where 1 = excellent vigor and 9 = poor vigor
	Stay green (VIII.C.2.1)	Maturity (Field)	Rated on a 1-9 scale, 1 = 90 to 100%; 2 = 80-89%; 3 = 70-79%; 4 = 60-69%; 5 = 50-59%; 6 = 40-49%; 7 = 30-39%; 8 = 20-29%; and 9 = 0 – 19% green tissue
	Ear height (VIII.C.2.1)	R1 – Maturity (Field)	Distance from the soil surface at the base of the plant to the ear attachment node on five plants per plot
	Plant height (VIII.C.2.1)	R1 – Maturity (Field)	Distance from the soil surface at the base of the plant to the flag leaf collar on five plants per plot

Table VIII-1 (continued). Phenotypic, Agronomic and Environmental Interaction Characteristics Evaluated in U.S. Field Trials and Laboratory Studies

Data category	Characteristics measured	Evaluation timing (Setting of evaluation)[1]	Evaluation description
Reproductive growth	Days to 50% pollen shed (VIII.C.2.1)	Pollen shed (Field)	Days from planting until 50% of the plants have begun to shed pollen
	Days to 50% silking (VIII.C.2.1)	Silking (Field)	Days from planting until 50% of the plants have multiple silks exposed
	Pollen viability (VIII.C.3)	Tasseling (Laboratory)	Percentage of viable pollen based on pollen grain staining characteristics
	Pollen morphology (VIII.C.3)	Tasseling (Laboratory)	Diameter of viable pollen grains and observations
	Grain moisture (VIII.C.2.1)	Harvest (Field)	Percentage moisture of harvested shelled grain
	Test weight (VIII.C.2.1)	Harvest (Field)	Test weight (kilogram/hecto liter) of harvested shelled grain
	Yield (VIII.C.2.1)	Harvest (Field)	Calculated in Mg/ha, adjusted to 15.5% grain moisture content
Lodging and Seed retention	Stalk lodged plants (VIII.C.2.1)	Pre-harvest (Field)	Number of plants per plot broken below the ear
	Root lodged plants (VIII.C.2.1)	Pre-harvest (Field)	Number of plants per plot leaning at the soil surface at >30° from the vertical
	Dropped ears (VIII.C.2.1)	Pre-harvest (Field)	Number of mature ears dropped from plants
Environmental interactions	Abiotic stress response (VIII.C.2.2.1)	Four times during growing season (Field)	Qualitative assessment of each plot, with categorical scale of increasing severity (none, slight, moderate, severe)
	Disease damage (VIII.C.2.2.1)	Four times during growing season (Field)	Qualitative assessment of each plot, with categorical scale of increasing severity (none, slight, moderate, severe)
	Arthropod damage (VIII.C.2.2.1)	Four times during growing season (Field)	Qualitative assessment of each plot, with categorical scale of increasing severity (none, slight, moderate, severe)
	Stalk rot disease (VIII.C.2.2.1)	Harvest (Field)	Qualitative assessment of each plot, with categorical scale of increasing severity (none, slight, moderate, severe)
	Ear/kernel rot disease (VIII.C.2.2.1)	Harvest (Field)	Qualitative assessment of each plot, with categorical scale of increasing severity (none, slight, moderate, severe)

Table VIII-1 (continued). Phenotypic, Agronomic and Environmental Interaction Characteristics Evaluated in U.S. Field Trials and Laboratory Studies

Data category	Characteristics measured	Evaluation timing (Setting of evaluation)[1]	Evaluation description
Environmental interactions	Corn earworm damage (VIII.C.2.2.2)	R5 growth stage (Field)	Quantitative assessment on 10 plants per plot by examining ears for damaged area using a plastic film grid (each grid cell = 0.5 cm^2)
	European corn borer damage (VIII.C.2.2.2)	R6 growth stage (Field)	Quantitative assessment on 10 plants per plot by counting number of feeding galleries and total length of feeding galleries in each stalk
	Arthropod abundance (VIII.C.2.2.2)	Five collection times during growing season (Field)	Quantitative assessment of arthropod abundance via sticky traps collections and visual counts

[1] Plant growth stages were determined using descriptions and guidelines outlined in Corn Growth and Development (Ritchie, et al. 1997).
[2] Viability of hard and firm-swollen seed were determined by a tetrazolium test (AOSA/SCST 2010).

VIII.B. Interpretation of Phenotypic and Environmental Interaction Data

Plant pest risk assessments for biotechnology-derived crops are comparative assessments, and are considered from a basis of familiarity. The concept of familiarity is based on the fact that the biotechnology-derived plant is developed from a well-characterized conventional crop whose biological properties and plant pest potential are well-known. Familiarity considers the biology of the crop, the introduced traits, the receiving environment and the interaction of these factors, and provides a basis for comparative environmental risk assessment between a biotechnology-derived plant and its conventional counterpart.

Expert knowledge and experience with conventionally bred maize was the basis for selecting appropriate endpoints and estimating the range of responses that would be considered typical for maize. As such, MON 87411 was compared to the conventional control in the assessment of phenotypic, agronomic, and environmental interaction characteristics. An overview of the characteristics assessed is presented in Table- VIII-1. A subset of the data relating to well-understood weedy characteristics (*e.g.*, seed dormancy, ear drop, and lodging) was used to assess whether there was an increase in weediness potential of MON 87411 compared to a conventional maize. Evaluation of environmental interaction characteristics (*e.g.*, plant abiotic stress, plant-disease, and plant-arthropod interactions) was also considered in the plant pest assessment. Prior to analysis, the overall dataset was evaluated for possible evidence of biologically-relevant changes and unexpected plant responses. No unexpected observations or issues were identified. Based on all of the data collected, an assessment was made to determine if MON 87411 could be expected to pose an increased plant pest risk compared to conventional maize.

VIII.B.1. Interpretation of Detected Differences Criteria

Comparative plant characterization data between a biotechnology-derived crop and the conventional control are interpreted in the context of contributions to increased plant pest/weed potential. Under the framework of familiarity, characteristics for which no differences are detected support a conclusion of no increased plant pest/weed potential. Characteristics for which differences are detected are considered in a step-wise method (Figure VIII-1) or in a similar fashion. All detected differences for a characteristic are considered in the context of whether or not the difference would increase the crop's plant pest/weed potential. Ultimately, a weight of evidence approach considering all characteristics and data is used for the overall risk assessment of differences and their significance. In detail, Figure VIII-1 illustrates the stepwise assessment process employed:

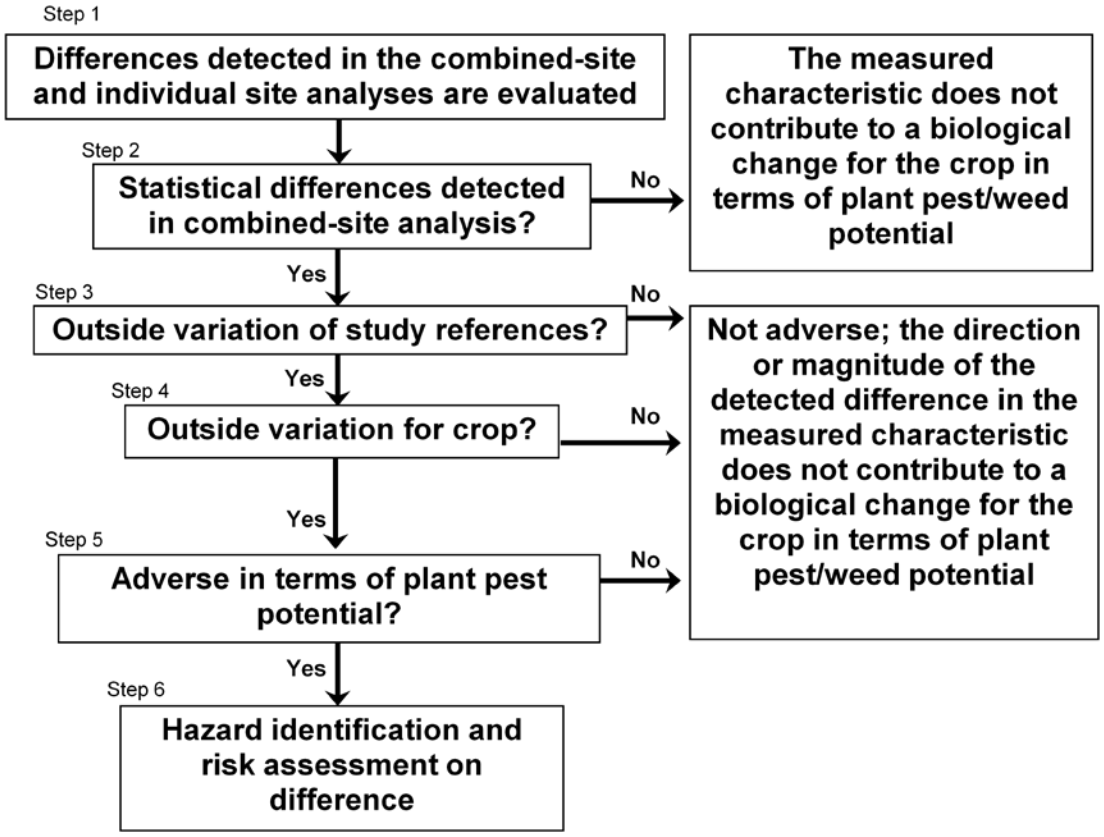

Figure VIII-1. Schematic Diagram of Agronomic and Phenotypic Data Interpretation Methods

Note: A "no" answer at any step indicates that the characteristic does not contribute to a biological change for the crop in terms of plant pest/weed potential and subsequent steps are not considered. If the answer is "yes" or "uncertain", the subsequent step is considered.

Steps 1 and 2 – Evaluate Detected Statistically Significant Differences

Data on each measured characteristic are statistically analyzed, where appropriate, within each individual site and in a combined-site analysis, in which the data are pooled among sites. All statistically significant differences are evaluated and considered in the context of a change in plant pest/weed potential. Differences detected in individual-site analyses that are not detected when data across multiple environments are pooled in the combined-site analysis are considered not biologically meaningful in terms of plant pest/weed potential and, therefore, are not further considered in subsequent steps. Any difference detected in the combined-site analysis is further assessed.

Step 3 – Evaluate differences in the context of commercial reference materials included in the Study

If a difference for a characteristic is detected in the combined-site analysis across multiple environments, then the mean value of the biotechnology-derived crop for the characteristic is assessed relative to the range of variation of the commercial reference materials included in the study (*e.g.*, reference range).

Step 4 – Evaluate Differences in the Context of the Crop

If the mean value of the characteristics for a biotechnology-derived crop is outside the variation of the commercial reference materials included in the study, the mean value of the biotechnology-derived crop is assessed relative to known values common for the crop (*e.g.*, published values).

Step 5 – Relevance of Difference to Plant Pest/Weed Potential

If the mean value of the characteristics for a biotechnology-derived crop is outside the range of values common for the crop, the difference is then assessed for whether or not it is meaningful in terms of plant pest/weed potential.

Step 6 – Conduct Risk Assessment on Identified Hazard

If an adverse effect (hazard) is identified, risk assessment on the difference is conducted. The risk assessment considers contributions to enhanced plant pest/weed potential of the crop itself, the impact of differences detected in other measured characteristics, and potential for and effects of trait introgression into any populations growing outside of cultivated environments or into a sexually-compatible species.

VIII.B.2. Interpretation of Environmental Interactions Data

For the qualitative assessments of abiotic stress response, disease damage, and arthropod damage, the biotechnology-derived crop and conventional control are considered different in susceptibility or tolerance if the range of injury symptoms of each did not overlap across all four replications. Any observed differences are assessed for biological significance in the context of the range of the commercial reference materials, and for consistency in other observation times and sites. Differences that are not consistently observed in multiple environments are considered not biologically meaningful in terms of plant pest potential.

Quantitative assessments of corn earworm and European corn borer damage are analyzed within individual sites and pooled across sites in a combined site analysis. Statistically significant differences detected between the biotechnology-derived crop and conventional controls are evaluated using the method outlined in Figure VIII-1.

Quantitative assessments of arthropod abundance are only analyzed within each individual site. Statistically significant differences between the biotechnology-derived crop and conventional control are assessed for biological significance in the context of the range of the commercial reference hybrids, and for consistency in other collection times and collection sites and in the context of pest potential. Differences that are not consistently detected in multiple environments are considered not biologically meaningful in terms of plant pest potential.

VIII.C. Comparative Assessments of the Phenotypic, Agronomic, and Environmental Interaction Characteristics of MON 87411

This section provides the results of comparative assessments conducted in replicated laboratory and/or multi-site field experiments to provide a detailed phenotypic, agronomic, and environmental interactions description of MON 87411. The characteristics for MON 87411 evaluated in these assessments included: seed germination and dormancy characteristics (Section VIII.C.1), plant phenotypic, agronomic, and environmental interaction observations under field conditions (Section VIII.C.2), and pollen characteristics (Section VIII.C.3). Additional details for each assessment are provided in Appendices G, H, and I.

VIII.C.1. Seed Dormancy and Germination Characteristics

USDA-APHIS considers the potential for weediness to constitute a plant pest factor (7 CFR § 340.6). Seed germination and dormancy mechanisms vary with species and their genetic basis tends to be complex. Seed dormancy (*e.g.*, hard seed) is an important characteristic that is often associated with plants that are considered weeds (Anderson 1996; Lingenfelter and Hartwig 2007). Information on germination and dormancy characteristics is therefore useful when assessing a plant for increased weediness potential. To assess germination characteristics, standardized germination assays are available and routinely used. The Association of Official Seed Analysts (AOSA), an internationally recognized seed testing organization, recommends a temperature range of 20/30 °C as optimal for testing the germination and dormancy characteristics of maize seed (AOSA 2012b; a). Additional temperature regimes were also evaluated.

A comparative assessment of seed germination and dormancy characteristics was conducted on MON 87411 and the conventional control. The seed lots to be used for the germination testing (selfed F_2 grain) of MON 87411, the conventional control, and the reference hybrids (4 per site, 11 unique across all locations) were produced in replicated field trials during 2012 in Iowa (IA), Illinois (IL), and Kansas (KS). These geographic areas represent a broad range of environmental conditions for maize production for this product. The plots were not treated with glyphosate. The germination and dormancy characteristics (as noted in Table VIII-1) were assessed in seven separate split-plot experiments with four replications, one for each temperature regime.

Descriptions of the evaluated germination and dormancy characteristics and the timing of the evaluations for all temperature regimes are listed in Table VIII-1. Additional details on the materials and experimental methods used in this evaluation are presented in Appendix H.

In the combined-site analysis, in which data were pooled across the three seed production sites, no statistically significant differences (α=0.05) were detected between MON 87411 and the conventional control for any characteristic at the AOSA temperature regime (20/30 °C), or at the temperature regimes of 5, 10, 20, 30, 10/20, 10/30 °C (Table VIII-2). In addition, no hard seed were observed at any temperature.

The germination and dormancy characteristics evaluated were used to assess MON 87411 in the context of plant pest risk. The results of this assessment, particularly the lack of increased hard seed, and no changes in other germination and dormancy characteristics, support the conclusion that the introduction of insect-protected and glyphosate-tolerant traits is not expected to result in increased plant pest/weed potential compared to conventional maize.

Table VIII-2. Germination Characteristics of MON 87411 and the Conventional Control

Temperature (°C)	Assessment Category	Mean % (S.E.)[1] MON 87411	Mean % (S.E.)[1] Control	Reference Range[2]
5	Germinated	0.3 (0.14)	0.2 (0.11)	0.0 - 0.3
	Viable Hard†	0.0 (0.00)	0.0 (0.00)	0.0 - 0.0
	Dead	3.0 (0.62)	2.2 (0.47)	1.3 - 5.3
	Viable Firm-Swollen	96.7 (0.68)	97.7 (0.47)	94.5 - 98.6
10	Germinated	82.0 (2.40)	83.1 (2.30)	86.3 - 97.5
	Viable Hard†	0.0 (0.00)	0.0 (0.00)	0.0 - 0.0
	Dead	0.9 (0.36)	1.0 (0.28)	0.5 - 3.8
	Viable Firm-Swollen	17.1 (2.14)	15.9 (2.14)	0.0 - 11.3
20	Germinated	98.6 (0.38)	99.0 (0.25)	95.5 - 99.5
	Viable Hard†	0.0 (0.00)	0.0 (0.00)	0.0 - 0.0
	Dead	1.3 (0.36)	1.0 (0.25)	0.5 - 4.5
	Viable Firm- Swollen	0.1 (0.08)	0.0 (0.00)	0.0 - 0.0
30	Germinated	99.1 (0.23)	98.6 (0.42)	92.5 - 99.3
	Viable Hard†	0.0 (0.00)	0.0 (0.00)	0.0 - 0.0
	Dead	0.9 (0.23)	1.4 (0.42)	0.8 - 7.5
	Viable Firm- Swollen†	0.0 (0.00)	0.0 (0.00)	0.0 - 0.0
10/20	Germinated	98.7 (0.26)	98.3 (0.47)	94.8 - 99.3
	Viable Hard†	0.0 (0.00)	0.0 (0.00)	0.0 - 0.0
	Dead	0.7 (0.22)	0.9 (0.29)	0.8 - 4.5
	Viable Firm- Swollen	0.7 (0.28)	0.8 (0.28)	0.0 - 0.8
10/30	Germinated	98.8 (0.37)	99.3 (0.25)	96.3 - 99.5
	Viable Hard†	0.0 (0.00)	0.0 (0.00)	0.0 - 0.0
	Dead	1.1 (0.31)	0.8 (0.25)	0.5 - 3.8
	Viable Firm- Swollen	0.1 (0.08)	0.0 (0.00)	0.0 - 0.3
20/30 (AOSA)	Normal Germinated	98.2 (0.37)	98.3 (0.33)	93.8 - 99.8
	Abnormal Germinated	0.8 (0.24)	0.3 (0.14)	0.0 - 2.8
	Viable Hard†	0.0 (0.00)	0.0 (0.00)	0.0 - 0.0
	Dead	1.0 (0.28)	1.3 (0.36)	0.3 - 4.3
	Viable Firm- Swollen	0.0 (0.00)	0.0 (0.00)	0.0 - 0.1

Note: The experimental design was a split-plot with four replications.
No statistically significant difference between MON 87411 and the conventional control were detected (α=0.05) using ANOVA.
†No statistical comparison could be made due to lack of variability in the data.
[1]MON 87411 and the conventional control values represent means with standard error (S.E.) in parentheses. n = 12. In some instances, the total percentage of both MON 87411 and the conventional control did not equal 100% due to numerical rounding of the means.
[2]Reference range is the minimum and maximum mean values observed among the 11 unique reference hybrids.

VIII.C.2. Field Phenotypic, Agronomic, and Environmental Interactions Characteristics

Phenotypic and agronomic characteristics, and environmental interactions were evaluated under field conditions as part of the plant characterization assessment of MON 87411. These data were developed to provide USDA-APHIS with a detailed description of MON 87411 relative to the conventional control and reference hybrids. According to 7 CFR § 340.6, as part of the petition to seek deregulation, a petitioner must submit "a detailed description of the phenotype of the regulated article." This information is being provided to assess whether there are phenotypic differences between MON 87411 and the conventional control that may impact its plant pest/weed potential. Specific characteristics that are related to weediness (*e.g.*, lodging and ear drop) were used to assess whether there is a potential increase in weediness of MON 87411 compared to conventional maize. Environmental interactions including plant response to abiotic stress, disease damage, specific arthropod-related damage, and arthropod abundance were also assessed as an indicator of changes to MON 87411 and are also considered in the plant pest assessment.

The results of the assessments of agronomic and phenotypic characteristics demonstrated that the introduction of the glyphosate tolerant and insect protected traits did not meaningfully alter the plant pest/weed potential of MON 87411 compared to conventional maize. Furthermore, the lack of meaningful differences in plant response to abiotic stress, disease damage, arthropod-related damage, and arthropod abundance also support the conclusion that the introduction of the glyphosate tolerant and insect protected traits is not expected to result in increased plant pest/weed potential for MON 87411 compared to conventional maize.

VIII.C.2.1. Field Phenotypic and Agronomic Characteristics

Data were collected at nine sites in the U.S. during 2012 to evaluate phenotypic and agronomic characteristics of MON 87411 compared to the conventional control (Table VIII-3). These nine sites provided a diverse range of environmental and agronomic conditions representative of commercial maize production areas in North America. The experimental design at each site was a randomized complete block with four replications. At all sites, MON 87411, the conventional control, and four reference hybrids were evaluated. A total of 22 unique reference hybrids were evaluated among the nine sites (Appendix I, Table I-1). The planted plot dimensions varied between sites, due to variability in available planting equipment (Appendix I, Table I-2). All plots of MON 87411, the conventional control, and the reference hybrids at each site were uniformly managed in order to assess whether the introduction of insect-protected and glyphosate-tolerant traits altered the phenotypic and agronomic characteristics of MON 87411 compared to the conventional control.

Descriptions of the evaluated phenotypic characteristics and the timing of the evaluations are listed in Table VIII-1. The materials, methods, details concerning the timing of

phenotypic assessments, and detailed results of the individual-site data comparisons are presented and discussed in Appendix I (Table I-4). The results of the combined-site analyses are summarized below.

In a combined-site analysis in which the data were pooled among the sites, no statistically significant differences were detected (α=0.05) between MON 87411 and the conventional control for any of the assessed characteristics, including early stand count, 50% pollen shed, days to 50% silking, stay green rating, ear height, plant height, dropped ear count, stalk lodged plants, root lodged plants, final stand count, grain moisture, test weight, and yield (Table VIII-4). Thus, the phenotypic and agronomic characteristics of MON 87411 were not altered in terms of pest/weed potential compared to conventional maize (Figure VIII-1, step 2, answer "no").

Plant vigor data were summarized as ranges within individual sites. MON 87411 and the conventional control were considered different if the range of vigor values did not overlap across all four replications. There were no differences observed between MON 87411 and the conventional control in plant vigor across sites (Appendix I, Table I-4).

The phenotypic and agronomic characteristics evaluated in this study were used to provide a detailed description of MON 87411 compared to the conventional control. A subset of these characteristics was used to assess the weediness of MON 87411. The results of the agronomic and phenotypic assessment demonstrate that there were no unexpected changes in the phenotype of MON 87411 compared to the conventional control when managed under the agronomic practices for maize production. Thus, the introduction of insect-protected and glyphosate-tolerant traits is not expected to result in increased plant pest/weed potential from MON 87411 compared to conventional maize.

Table VIII-3. Field Phenotypic Evaluation Sites for MON 87411 during 2012

Site Code	County, State
IABG	Greene, Iowa
IARL	Jefferson, Iowa
ILMN	Warren, Illinois
INSH	Boone, Indiana
KSLA	Pawnee, Kansas
NCBD	Perquimans, North Carolina
NEDC	Butler, Nebraska
NEYO	York, Nebraska
PAHM	Berks, Pennsylvania

Table VIII-4. Combined-Site Comparison of MON 87411 to Conventional Control for Phenotypic and Agronomic Characteristics During 2012

Phenotypic Characteristic (units)	Mean (S.E.)[1]		Reference Range[2]
	MON 87411	Control	
Early stand count (#/plot)	82.1 (1.59)	83.4 (1.20)	72.0 – 92.5
Days to 50% pollen shed	63.3 (0.64)	63.1 (0.59)	60.0 – 67.0
Days to 50% silking	63.4 (0.62)	63.3 (0.54)	59.3 – 66.5
Stay green rating (1-9 scale)	5.6 (0.42)	5.6 (0.40)	2.8 – 9.0
Ear height (cm)	104.1 (1.99)	103.6 (2.07)	87.4 – 132.2
Plant height (cm)	238.3 (4.13)	239.1 (4.32)	204.4 – 262.6
Dropped ears (#/plot)	0.7 (0.29)	1.0 (0.42)	0.0 – 10.9
Stalk lodged plants (#/plot)	1.2 (0.29)	1.1 (0.39)	0.0 – 5.7
Root lodged plants (#/plot)	0.1 (0.06)	0.2 (0.13)	0.0 – 1.3
Final stand count (#/plot)[3]	65.0 (0.96)	64.3 (0.81)	60.5 – 70.8
Grain moisture (%)[4]	17.6 (0.38)	17.9 (0.42)	12.6 – 22.4
Test Weight (kg/hl)[5]	72.8 (0.57)	73.2 (0.64)	68.2 – 77.7
Yield (Mg/ha)[6]	11.1 (0.44)	10.9 (0.44)	7.4 – 15.8

Note: The experimental design was a randomized complete block with four replicates per site. No statistically significant differences were detected between MON 87411 and the conventional control (α=0.05) using ANOVA.

[1]MON 87411 and the conventional control values represent means with standard error (S.E.) in parentheses. n = 36, except where noted.

[2]Reference range is calculated from the minimum and maximum mean values from among the 22 unique reference hybrids.

[3]Final stand count was excluded from a single replication of MON 87411 at the NEDC site because it was identified as an outlier. n = 35 for MON 87411; n = 36 for the conventional control.

[4]Grain moisture was excluded from a single replication of the conventional control at the IARL site because it was collected incorrectly. n = 36 for MON 87411; n = 35 for the conventional control.

[5]Test weight (kg/hl = kilogram/hectoliter) was excluded from a single replication of the conventional control at two sites (IARL and PAHM). n = 36 for MON 87411; n = 34 for the conventional control.

[6]Yield (Mg/ha = megagram/hectare) data was dropped from a single replication of the conventional control at the IARL site because the shelled plot weight was collected incorrectly. n = 36 for MON 87411; n = 35 for the conventional control.

VIII.C.2.2. Environmental Interaction Characteristics

USDA-APHIS considers the environmental interaction of the biotechnology-derived crop compared to its conventional control to determine the potential for increased plant pest characteristics. Evaluations of environmental interactions were conducted as part of the plant characterization for MON 87411. In the 2012 U.S. field trials conducted to evaluate the phenotypic and agronomic characteristics of MON 87411, data were also collected on plant response to abiotic stress (*e.g.*, drought, wind, nutrient deficiency, etc.), disease damage, arthropod-related damage, and arthropod abundance (Tables VIII-5 and VIII-6; Appendix I; Tables I-5, I-6, I-7, I-8, I-9, and I-10, respectively). These data were used as part of the plant pest/environmental analysis (Section X) to assess plant pest potential compared to the conventional control and provide confimatory data for the conclusion of laboratory NTO testing data (Section VI.E.). The results of the field evaluations showed that insect-protected and glyphosate-tolerant traits did not unexpectedly alter the assessed environmental interactions of MON 87411 compared to the conventional control. The lack of significant biological differences in plant responses to abiotic stress, disease damage, arthropod-related damage, and pest- and beneficial-arthropod abundance support the conclusion that the introduction of the insect-protected and glyphosate-tolerant traits is not expected to result in increased plant pest potential from MON 87411 compared to commercial maize.

VIII.C.2.2.1. Qualitative Environmental Interactions Assessment

Plant responses to abiotic stressors, disease damage, and arthropod damage were assessed at natural levels, *i.e.*, no artificial infestation or imposed abiotic stress; therefore these levels typically varied between observations at a site and among sites. Plant responses to abiotic stress, disease damage, and arthropod damage data were collected from each plot using a categorical scale (none, slight, moderate, and severe) of increasing severity of observed damage for each stressor. This scale was utilized to allow for the evaluation of the wide variety of potential abiotic stressor, disease damage, and arthropod damage symptoms potentially occurring across the season and across sites. These data were categorical and therefore were summarized and not subjected to ANOVA. For a particular stressor, all comparisons of the range of responses for MON 87411 to the range of responses for the conventional control across all observation times and sites are reported.

Descriptions of the evaluated environmental interactions characteristics and the timing of the evaluations are listed in Table VIII-1. The materials, methods, additional details concerning the qualitative environmental interactions assessments, and detailed results of the qualitative data comparisons are presented and discussed in Appendix I (Tables I-5 through I-7).

In the qualitative assessment, no differences in the range of responses were observed between MON 87411 and the conventional control for any of the 100 comparisons of plant response to abiotic stressors, including cold, drought, flood, frost, hail, heat, nutrient deficiency, soil compaction, sunscald, and wind (Table VIII-5 and Appendix I; Table I-5). Additionally, no differences in the range of responses were observed between

MON 87411 and the conventional control for any of the 119 comparisons for plant damage caused by diseases, including anthracnose, bacterial leaf spot, ear rot, eyespot, *Fusarium* sp., Goss's bacterial wilt, gray leaf spot, leaf blight, maize rough dwarf virus, *Pythium* sp., *Rhizoctonia* sp., rust, seedling blight, smut, stalk rot, and Stewart's bacterial wilt (Table VIII-5 and Appendix I; Table I-6). Finally, no differences in the range of responses were observed between MON 87411 and the conventional control for any of the 102 comparisons for plant damage caused by arthropods, including aphid, armyworm, billbug, cutworm, corn earworm, corn flea beetle, rootworm beetle, European corn borer, grasshopper, Japanese beetle, sap beetle, spider mite, stink bug, and wireworm adult (Table VIII-5 and Appendix I; Table I-7).

The lack of differences observed between MON 87411 and the conventional control for plant responses to abiotic stressors, disease damage, and arthropod-related damage in multiple environments across the U.S. supports the conclusion that the introduction of the insect-protected and glyphosate-tolerant traits is not expected to cause a biologically meaningful change in terms of plant pest potential compared to the conventional control (See Section VIII.B.2.).

Table VIII-5. Summary of Qualitative Environmental Interactions Assessments during 2012

Stressor	Number of observations across all sites	Number of observations with no differences between MON 87411 and the conventional control across all sites[1]
Abiotic stressors	100	100
Disease damage	119	119
Arthropod-related damage	102	102
Total	321	321

Note: The experimental design was a randomized complete block with four replicates per site.
No differences were observed between MON 87411 and the conventional control during any observation for damage caused by any of the assessed stressors.
[1]MON 87411 and the conventional control were considered different in susceptibility or tolerance if the range of injury symptoms across four replications did not overlap between MON 87411 and the conventional control.

VIII.C.2.2.2. Quantitative Environmental Interactions Assessment

Quantitative arthropod assessments on corn earworm (CEW: *Helicoverpa zea*) damage, European corn borer (ECB: *Ostrinia nubilalis*) damage, and arthropod abundance were conducted at four sites (IABG, NEYO, NCBD, and PAHM). CEW and ECB damage was assessed once during the growing season at each site. Arthropod abundance was assessed from collections performed five times during the growing season at each site using sticky traps and visual counts.

Damage data were collected for CEW and ECB at the four sites in 2012. Both individual and combined-site (the four site pooled data) analyses were conducted. Descriptions of the evaluated environmental interactions characteristics and the timing of the evaluations are listed in Table VIII-1. The materials, methods, additional details concerning the specific arthropod damage assessments, and detailed results of the individual-site data comparisons are presented and discussed in Appendix I (Table I-8). The results of the combined-site analysis are summarized below.

In the combined-site analysis, no statistically significant differences (α=0.05) were detected between MON 87411 and the conventional control for plant damage caused by corn earworm or European corn borer (Tables VIII-6). Considering this lack of significant differences, the results indicate no changes in susceptibility or resistance of MON 87411 to these common maize pests. (See Figure VIII-1, Step 2, answer “no”).

Arthropod abundance was assessed from collections performed using sticky traps and visual counts at the four sites in 2012. Variations in temporal activity and geographical distribution of arthropod taxa occurred between sites, therefore, only individual-site

analyses were conducted for arthropod abundance data. Statistical analyses and significance testing of differences were only performed for the arthropods present in sufficient numbers to allow more robust statistical analysis. An inclusion criterion was established where a given arthropod must have an average count per plot per collection time (across all materials) ≥ of 1. Additional details of the arthropod abundance assessments and detailed results of the individual-site data comparisons are provided in Appendix I (Tables I-9 and I-10). The results of these analyses are summarized below and in Table VIII-7.

In an assessment of arthropod abundance from sticky traps, a total of 108 statistical comparisons were made between MON 87411 and the conventional control for arthropod abundance involving the following arthropods: aphid, corn flea beetle, delphacid planthopper, green lacewing, ladybird beetle, leafhopper, micro-parasitic hymenoptera, macro-parasitic hymenoptera, sap beetle, minute pirate bug, and spider. Lack of sufficient arthropod abundance precluded statistical comparisons between MON 87411 and the conventional control for 79 additional comparisons; however, descriptive statistics were provided for these comparisons (Appendix I; Table I-9).

No statistically significant differences (α=0.05) were detected between MON 87411 and the conventional control for 104 out of 108 comparisons. The four differences detected between MON 87411 and the conventional control included aphid, delphacid planthopper, minute pirate bug, and spider (Table VIII-7 and Appendix I; Table I-9). The abundance of aphids was higher in MON 87411 than the conventional control in Collection 3 (1.8 vs. 0.0 per plot) at the NEYO site. The mean value for aphid abundance on MON 87411 was within the range of the reference hybrids. The abundance of delphacid planthoppers was lower in MON 87411 than the conventional control in Collection 1 at the IABG site (0.0 vs. 1.8 per plot). MON 87411 had higher abundance of spiders than the conventional control (3.3 vs. 0.3 per plot) in Collection 5 and lower abundance than the conventional control for minute pirate bugs (1.0 vs. 3.0 per plot) in Collection 1 at the PAHM site. The mean values for delphacid planthopper, minute pirate bug, and spider abundance on MON 87411 were outside the respective ranges of reference hybrids. However, these differences were not consistently detected across collections or sites (Table VIII-7 and Appendix I; Table I-9). Thus, these differences in aphid, delphacid planthopper, minute pirate bug, and spider abundance were not indicative of a consistent response associated with the trait and are not considered biologically meaningful in terms of increased pest potential of MON 87411 compared to the conventional control (See Section VIII.B.2.).

In an assessment of arthropod abundance from visual counts, a total of 61 statistical comparisons were made between MON 87411 and the conventional control for arthropod abundance involving the following pest and beneficial arthropods: ant-like flower beetle, click beetle, corn flea beetle, ladybird beetle adult, ladybird beetle larvae, minute pirate bug, sap beetle, shining flower beetle, and spider. Lack of sufficient arthropod abundance precluded statistical comparisons between MON 87411 and the conventional control for 152 additional comparisons; however, the descriptive statistics were provided for these comparisons (Appendix I, Table I-10).

No statistically significant differences (α=0.05) were detected between MON 87411 and the conventional control for 60 out of 61 comparisons. One difference was detected between MON 87411 and the conventional control for ant-like flower beetle (Table VIII-7 and Appendix I; Table I-10). The abundance of ant-like flower beetles was lower on MON 87411 than the conventional control in Collection 2 at the IABG site (0.8 vs. 4.3 per plot). The mean value for ant-like flower beetle abundance on MON 87411 was lower than the range of reference hybrids (1.8 – 3.3 per plot). However, the difference for ant-like flower beetle was not consistently detected across collections at the IABG site (Table VIII-7 and Appendix I; Table I-10). Thus, this difference in ant-like flower beetle abundance was not indicative of a consistent response associated with the trait and is not considered biologically meaningful in terms of increased pest potential of MON 87411 compared to the conventional control (See Section VIII.B.2.).

Table VIII-6 Combined-Site Comparison of Pest Damage to MON 87411 Compared to the Conventional Control during 2012

Pest Arthropod	Damage Assessment	Mean (S.E.)[1]		Reference range[2]
		MON 87411	Control	
Corn earworm (*H. zea*)	Damage area (cm^2) of 10 plants per plot	1.9 (0.47)	1.3 (0.30)	0.2 – 3.2
European corn borer (*O. nubilalis*)	Number of stalk galleries of 10 plants per plot	0.4 (0.16)	0.5 (0.21)	0.0 – 1.8
European corn borer (*O. nubilalis*)	Stalk gallery length (cm) of 10 plants per plot	1.6 (0.72)	2.0 (0.94)	0.0 – 8.3

Note: The experimental design was a randomized complete block with four replicates per site.
No statistically significant differences were detected between MON 87411 and the conventional control (α=0.05) using ANOVA.
[1]MON 87411 and the conventional control values represent means with standard error (S.E.) in parentheses. n = 16.
[2] Reference range is calculated from the minimum and maximum mean values from among 15 unique reference hybrids across four sites (IABG, NCBD, NEYO, and PAHM).

Table VIII-7. Summary of Arthropod Abundance Assessments and Detected Differences during 2012

Summary of Statistical Comparisons[1]

Arthropod Abundance Assessment	Number of sites	Number of comparisons across sites	Number of comparisons where no differences were detected
Sticky Traps	4	108	104
Visual Counts	4	61	60

Summary of Detected Differences[2]

Arthropod	Site	Collection Number	Within reference range?	Consistently detected across collections or sites?
Aphids	NEYO	3	Yes	No
Delphacid planthopper	IABG	1	No	No
Minute pirate bug	PAHM	1	No	No
Spiders	PAHM	5	No	No
Ant-like flower beetle	IABG	2	No	No

[1]Quantitative arthropod abundance assessments were statistically analyzed at α=0.05 using ANOVA. Lack of sufficient arthropod abundance precluded statistical comparisons between MON 87411 and the conventional control for additional 79 comparisons (sticky traps) and 152 comparisons (visual counts); however, descriptive statistics were provided for these comparisons in Appendix I (Tables I-9 and I-10).

[2]Five statistically significant differences were detected. These differences are further discussed in Section VIII.C.2.2.2 using the approach outlined in Section VIII.B.2 .

VIII.C.3. Pollen Characteristics

USDA-APHIS considers the potential for gene flow and introgression of the biotechnology-derived trait(s) into sexually compatible plants and wild relatives to determine the potential for increased weedy or invasive characteristics of the receiving species. Pollen morphology and viability information are pertinent to this assessment and, therefore, were assessed for MON 87411. In addition, morphological characterization of pollen produced by MON 87411 and the conventional control is relevant to the plant pest risk assessment because it adds to the detailed description of the phenotype of MON 87411 compared to the conventional control.

The viability and morphology of pollen collected from MON 87411 compared to that of the conventional control was also assessed. Pollen was collected from MON 87411, the conventional control, and four commercial references grown under similar agronomic conditions in a field trial in Iowa, a geographic area that represents environmentally relevant conditions for maize production for this product. The trial was arranged in a randomized complete block design with four replications. Once all plants across the replications reached the flowering stage, pollen was collected from three non-systematically selected plants per plot and stained for assessment. Descriptions of the evaluated pollen viability and morphology characteristics and the timing of the evaluations are listed in Table VIII-1. The details of the materials and experimental methods used in this evaluation are presented in Appendix J.

No statistically significant differences (α=0.05) were detected between MON 87411 and the conventional control for percent viable pollen or pollen grain diameter (Table VIII-8). Furthermore, no visual differences in general pollen morphology were observed between MON 87411 and the conventional control (Appendix J, Figure J-1).

The pollen characterization data contribute to the detailed phenotypic description of MON 87411 compared to the conventional control. Based on the assessed characteristics, the results support a conclusion that neither pollen viability nor morphology of MON 87411 were altered compared to conventional maize.

Table VIII-8. Pollen Characteristics of MON 87411 Compared to the Conventional Control during 2012

Pollen Characteristic (unit)	Mean (S.E.)[1]		Reference Range[2]
	MON 87411	Control	
Viability[3] (%)	98.7 (0.43)	98.9 (0.21)	99.0 – 99.2
Diameter[4] (µm)	87.0 (1.02)	88.9 (1.29)	80.4 – 88.9

Note: The experimental design was a randomized complete block design with four replications. No significant differences were detected between the MON 87411 and the conventional control (α=0.05) using ANOVA.
[1]MON 87411 and the conventional control values represent means with standard error (S.E.) in parentheses. n=4
[2]Reference range is the minimum and maximum mean value observed among the four reference hybrids.
[3]Evaluated from three sub-samples per replication at 100X magnification.
[4]Evaluated from 10 representative viable pollen grains per replication at 200X magnification.

VIII.D. Conclusions for Phenotypic, Agronomic, and Environmental Interactions Evaluation

Comparative plant characterization data between a biotechnology-derived crop and the conventional control are interpreted in the context of contributions to increased plant pest potential as assessed by USDA-APHIS. Under the framework of familiarity, characteristics for which no differences are detected support a conclusion of no increased plant pest potential of the biotechnology-derived crop compared to the conventional crop. Ultimately, a weight-of-evidence approach that considers all characteristics and data is used for the overall risk assessment of differences and their significance.

An extensive and robust set of agronomic, phenotypic, and environmental interactions data, including specific weedy and plant pest potential characteristics, were used to assess whether the introduction of insect-protected and glyphosate-tolerant traits altered the plant pest potential of MON 87411 compared to the conventional control, considered within the context of the variation among commercial reference hybrids. These assessments included five general data categories: 1) seed germination, dormancy, and emergence; 2) vegetative growth; 3) reproductive development (including pollen characteristics); 4) lodging and seed retention on the plant; 5) plant response to abiotic stress and interactions with diseases and arthropods. Within these data categories, data relevant to understanding specific characteristics associated with weediness were also assessed to determine whether there was a potential increase in weediness of MON 87411 compared to conventional maize.

Results from these assessments comparing MON 87411 and the conventional control demonstrate that MON 87411 does not possess: 1) increased weediness characteristics; 2) increased susceptibility or tolerance to specific abiotic stress, diseases, or arthropods; or 3) characteristics that would confer a plant pest risk compared to conventional maize. Therefore, based on the results of multiple assessments discussed above and presented in the appendices, the weight-of-evidence indicates that MON 87411 is not meaningfully different from conventional maize with the exception of insect-protected and glyphosate-tolerant traits and is not expected to pose a plant pest risk compared to conventional maize.

IX. U.S. AGRONOMIC PRACTICES

IX.A. Introduction

As part of the plant pest assessment required by 7 CFR § 340.6(c)(4), impacts of deregulation on agricultural and cultivation practices must be considered. This section provides a summary of current agronomic practices in the U.S. and North America for producing maize, and is included in this petition as a baseline to assess possible impacts to agricultural practices due to the cultivation of MON 87411. Discussions include maize production, plant growth and development, general management practices during the season, management of weeds, insects and diseases, maize rotational crops, and volunteer management. Information presented in the previous section demonstrated that MON 87411 is no more susceptible to diseases or pests than commercially cultivated maize. Additionally, data presented in Section VIII show that MON 87411 is not expected to pose a plant pest risk compared to conventional maize. Current CRW-control products available in the U.S. were grown on over half of the maize acres in 2011 (Marra et al. 2012). MON 87411 is protected from CRW feeding using two different modes of action derived from Cry3Bb1 protein and DvSnf7 dsRNA. Thus, there are no expected changes to the inputs needed for MON 87411, and no expected impacts to agronomic practices employed for production of maize compared to the current situation.

IX.B. Overview of U.S. Maize Production

IX.B.1. Maize Production

The U.S., China, Brazil, Argentina, and Mexico are the top five countries producing maize globally (USDA-FAS 2013). As noted in Section I.B., maize (*Zea mays* L.) is the largest crop grown in the U.S. in terms of acreage planted (97.2 million acres in 2012) (USDA-NASS 2013d), exceeding soybean and wheat with acreages of 77.2 and 55.7 million acres, respectively (USDA-NASS 2013a). The value of maize reached $77.4 billion in the United States in 2012, exceeding soybeans and wheat with values of $43.2 and $17.9 billion, respectively (USDA-NASS 2013c). The principal uses of maize are feed and residual, ethanol fuel, export, and high-fructose corn syrup (Capehart, et al. 2012).

The planting of 97.2 million acres of maize in 2012 was the largest acreage since 1936 (Table IX-1) (USDA-NASS 2013d) and was up over 5.2 million acres from 2011. Much of that production occurs in upper Midwest states (Figure IX-1). Of the 2012 acreage, approximately 87.4 million acres were harvested for grain and 7.4 milli(Gassmann, et al. 2012)on acres were harvested for silage (USDA-NASS 2013d). Total production was approximately 10.8 billion bushels with an average yield of 123 bushels per acre (Table IX-1). The value of maize grain production in the U.S. has ranged from $46.7 to $77.4 billion in the past 6 years (Table IX-1) (USDA-NASS 2013d).

Table IX-1. Maize Production in the U.S., 2007-2012

Year	Acres Planted (×1000)	Acres Harvested (×1000)	Average Yield (bushels/acre)	Total Production (×1000 bushels)	Value (billions $)
2012	97,155	87,375	123.4	10,780,296	77.35
2011	91,936	83,989	147.2	12,359,612	76.94
2010	88,192	81,446	152.8	12,446,865	64.64
2009	86,382	79,490	164.7	13,091,862	46.73
2008	85,982	78,570	153.9	12,091,648	49.31
2007	93,527	86,520	150.7	13,037,875	54.67

Source: (USDA-NASS 2013d)

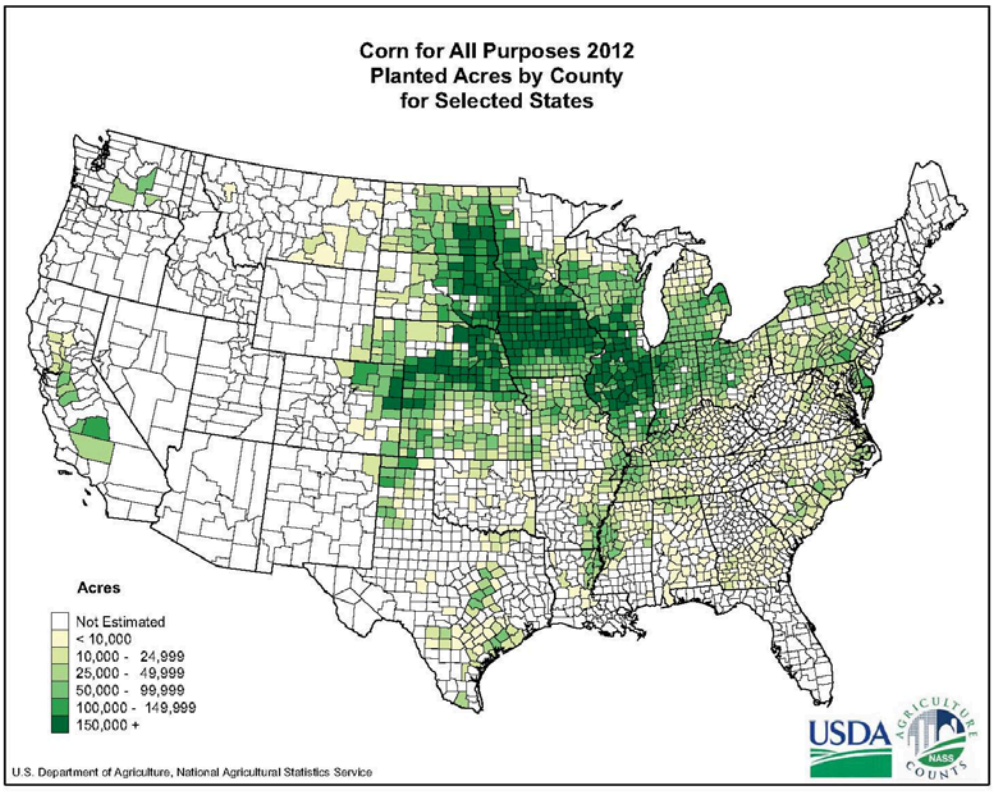

Figure IX-1. Planted Maize Acres by County in the U.S. in 2012
Source: (USDA-NASS 2012)

IX.C. Production Management Considerations

Other than the specific insertion of a partial coding sequence of the DvSnf7 gene that provides another MOA for protection against CRW, MON 87411 is similar to several other events present in CRW-protected maize hybrids being grown in the U.S. (*e.g.*, Genuity SmartStax, Genuity VT Triple PRO, etc). Herbicide tolerant maize has been in use commercially in the U.S. since 1997 and constituted 73% of the total maize crop in 2012 (USDA-ERS 2013). CRW-protected maize has been planted commercially since 2003 and in 2010 constituted 53% of the total U.S. crop (Brookes and Barfoot 2012). Given the widespread use of these HT and insect-protected maize hybrids, Monsanto anticipates no specific production management changes from introduction of MON 87411 above and beyond those in current use on a majority of U.S. maize acres.

IX.D. Management of Insect Pests

Maize is subject to attack by numerous insects and global production losses from arthropod pests are minimally estimated at 12%, with potential losses being much higher (Culy 2000; Vaadia 1985). Approximately 100 species of insects cause most of the pest damage to maize production in the U.S. (O'Day, et al. 1998). Certain insect pests are abundant almost every year and tend to be chronic problems while other pest infestations are sporadic and occur only every 5 to 10 years with a severity great enough to reach economic thresholds (O'Day et al. 1998). For migratory pests, weather events must favor the movement into maize-producing regions. In addition, maize or other suitable host plants must be available and in the preferred growth stage when the insects arrive and local weather conditions must also be favorable for the insects to survive and reproduce. Some of the most economically important migratory maize pests in the U.S. include black cutworm, fall armyworm and corn earworm (O'Day et al. 1998). Other economically important pests such as European corn borer and corn rootworm overwinter in the field (O'Day et al. 1998). Table IX-2 lists the most important insect pests in maize in the North Central states of the U.S.

Table IX-2. Insect Pests in Maize in the North Central States and Typical Time of Damage

Typical Time of Damage	Growth Stage	Common Name of Insect Pest
Planting to Full Emergence	Planting to V2	Seed corn maggot Seed corn beetles Wireworms
Emergence to Knee-High	VE to V8	Wireworms White grubs Grape colaspis larva Cinch bugs Black cutworm Stalk borer Thrips Corn leaf beetle Sod webworms Southern corn leaf beetle Billbugs Armyworms
Knee-High to Tasseling	V8 to VT	Fall armyworm Grasshoppers European corn borer Southwestern corn borer Corn rootworm
Tasseling to Maturity	VT to R6	Corn leaf aphid Corn earworm

Source: (Dicke and Guthrie 1988; O'Day et al. 1998)

Insect pests are best managed through the use of integrated pest management (IPM) programs where all viable control strategies are considered and appropriate strategies are selected for use against specific insect pests (Bradley, et al. 2013). Control strategies employed in IPM programs may include chemical, cultural, mechanical, biological and genetic options (*i.e.*, use of biotechnology-derived or naturally occurring resistant or tolerant crop varieties). Almost all maize seed includes a treatment of an insecticide such as thiamethoxam or clothianidin for control or suppression of several seedling pests including white grubs, wireworms, seed corn maggots, cinch bugs, black cutworm, and billbugs (Bradley et al. 2013; University of Tennessee 2013). Applications of insecticide for control of various maize insect pests has ranged from 12% to 31% of the planted acreage over the past 20 years (USDA-NASS 2013b) In 2010, however, only 12% of planted acres were treated with an insecticide (USDA-NASS 2013b). Numerous insecticidal active ingredients are registered for use in maize for the control of insect pests. However, only seven insecticide active ingredients (bifenthrin (2%), chlorpyrifos (1%), cyfluthrin (2%), lambda-cyhalothrin (2%), permethrin (1%), tebupirimphos (2), and tefluthrin (3%)) represent almost all of the total treated maize acres in the U.S. (USDA-NASS 2013b). These insecticides are effective in managing insect pests that may cause damage to maize from emergence through maturity (Table IX-3) (Bradley et al. 2013). Other IPM practices that are used include rotation to non-host crops to break pest life cycles, soil tillage to manage weed, arthropod and disease pests, and removal of crop residues that can harbor both arthropod and disease organisms (Vasileiadis, et al.

2011). Additionally, some researchers have evaluated and found effective the use of specific beneficial nematodes for biological control of specific maize pests (Kurtz, et al. 2007).

Table IX-3. Insecticide Applications in Maize in 2010 in the U.S.

Herbicide	Chemical Family	Mode of Action (MOA)	Percent of Maize Acres Treated	Quantity Applied (1000 lbs ai)
Bifenthrin	Pyrethroids	Sodium channel modulators	2.0	68
β-Cyfluthrin	Pyrethroids		2.0	15
Lambda-Cyhalothrin	Pyrethroids		2.0	24
Permethrin	Pyrethroids		1.0	72
Tefluthrin	Pyrethroids		3.0	242
Z-Cypermethrin	Pyrethroids			2
Chlorpyrifos	Organophosphates	Acetylcholin-esterase inhibitors	1.0	478
Tebupirimphos	Organophosphates		2.0	195
Tebufos	Organophosphates			137
Dimethoate	Organophosphates			52
Propargite	Propargite	Inhibitors of mitochondrial ATP synthase		109
Spiromesifen	Tetronic & tetramic acid derivatives	Inhibitors of acetyl CoA carboxylase		59
Total			12	1631

Source: (USDA-NASS 2013b).

The most damaging root-feeding pests of maize in the major U.S. maize growing regions are larvae of the corn rootworm complex (CRW: *Diabrotica* ssp., Coleoptera; Chrysomelidae) (Chandler et al. 2008). There are three primary species of CRW in the U.S., namely western corn rootworm (WCR: *Diabrotica virgifera virgifera*), northern corn rootworm (NCR: *Diabrotica barberi*) and southern corn rootworm (SCR: *Diabrotica undecimpunctata howardi*) and all are considered major insect pests of maize (Culy 2000). In the Midwest region, the WCR is the dominant species while the SCR is not considered an economic pest there (O'Day et al. 1998). Corn rootworms can cause serious injury and yield losses in the following ways: 1) root pruning and tunneling

disrupting the transport of nutrients and water from the root system, 2) lack of root support causing gooseneck lodging, which can complicate harvesting, and 3) root feeding promotes invasion by secondary pathogens such as bacteria and fungi, increasing the incidence of root rots (O'Day et al. 1998). Yield losses depend on the number of larvae per plant and on plant maturity, soil fertility, and amount of moisture following peak injury, as well as the ability of the plant to regenerate root tissue (O'Day et al. 1998). Historically, populations of the NCR and WCR had been estimated to result in annual yield losses and control costs that exceeded $1 billion (Metcalf 1986). Researchers have reported yield losses of 0.8 to 2.5% per larva per plant (Petty, et al. 1969; Smith 1979). In addition, adult rootworm beetles can cause stripping of the upper layer of tissue from the leaves and silk clipping, although this rarely has an impact on maize yields (O'Day et al. 1998).

In past years, various management practices have been employed to manage CRW pests including crop rotation, insecticide application at planting to manage larvae, and foliar insecticide application to manage adult beetles. In 2003, maize hybrids containing *Bt* proteins to protect against CRW feeding became available and have been widely adopted on broad acreages in the U.S. since then (Figure IX-2). Prior to introduction of these corn rootworm-protected *Bt* hybrids, two variants of WCR and NCR were identified that made crop rotation as a means to manage rootworm populations less effective. The soybean variant (SBV) of the WCR adapted its egg-laying behavior to lay eggs in crops other than maize, namely soybean (Levine and Oloumi-Sadeghi 1996). The SBV has been reported to occur in Illinois, Indiana, Michigan, Ohio, Minnesota, Iowa, and Missouri (Gray, et al. 2009; Onstad, et al. 1999). The extended diapause variant (EDV) of the NCR has adapted to crop rotations as well. Although most NCR eggs hatch the following spring, some of the eggs of EDV hatch after two winters and the larval stages are able to feed on maize roots even in rotated maize (Krysan, et al. 1984). The EDV variant is most prevalent in portions of South Dakota, Nebraska, Iowa, and Minnesota (Alston, et al. 2002). While these variant CRW populations do exist, corn rootworm-protected *Bt* maize products (both single and dual MOA) available to growers have provided and continue to provide excellent control of corn rootworm-complex pests on a vast majority of acres.

The introduction of biotechnology-derived insect-protected maize has offered growers alternative and highly effective solutions for control of major insect pests in maize. As noted in Section I (Product Rationale), in 2012, approximately 85 million acres in the U.S. (or 88% of the total U.S. maize acreage) were planted with biotechnology-derived maize hybrids, and approximately 64 million acres (or 67% of the total maize acreage) were planted with maize hybrids containing insecticidal crystal (Cry) proteins derived from *Bt* (USDA-ERS 2013). Of the 97.2 million acres of maize planted in the U.S. in 2012, approximately 50 million contained *Bt* proteins for protection against corn rootworm (Figure IX-2).

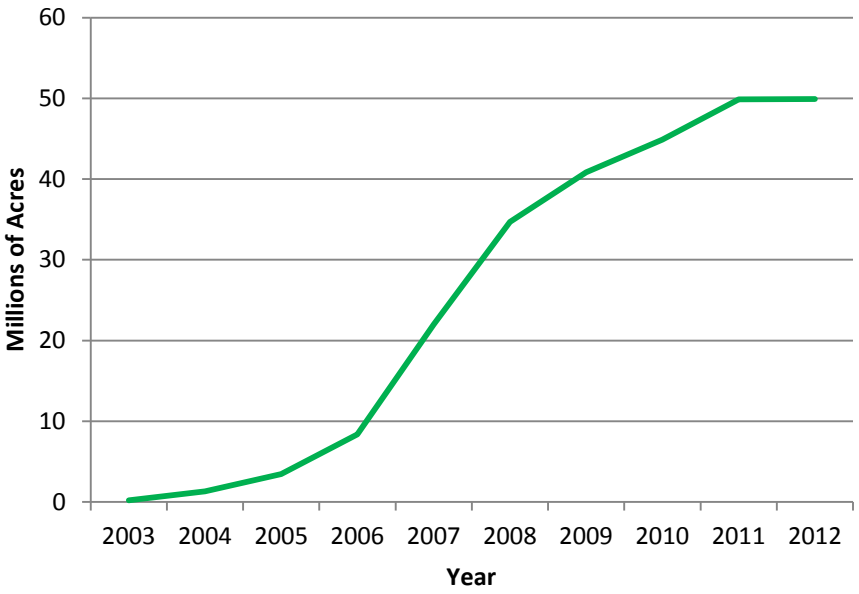

Figure IX-2. Maize acres in the U.S. planted to hybrids containing *Bt* proteins for corn rootworm protection
Source: (Marra et al. 2012) (updated with 2012 internal Monsanto data)

MON 87411 was developed to build upon current *Bt* protein-based CRW control technology by introducing a new MOA based on RNAi that will continue to effectively control corn rootworm-complex pests in maize fields and is expected to prolong the durability of existing *Bt* technologies. By incorporating two distinct MOAs, MON 87411 is expected to offer increased control of target insect pests and will continue to provide benefits to growers similar to those obtained with the use of existing CRW-protected maize hybrids. Thus, no changes to agronomic practices are expected related to grower use of MON 87411.

IX.D.1. Insect Resistance Management

MON 87411 will not be offered for commercial use as a stand-alone single-event product. Thus, no specific insect resistance management (IRM) program is being developed for MON 87411 alone. Instead MON 87411 is intended to be commercialized as combined-trait breeding stacks with previously deregulated biotechnology-derived traits. As these selected insect-protected combined-trait products are identified and developed, Monsanto (or other developers) will develop appropriate IRM program(s) for these products and submit them to U.S. EPA as part of its registration package(s). EPA has required IRM proposals for each of the insect-protected corn products it has previously registered. IRM plans, which are included as conditions of U.S. EPA registration, are designed to delay the development of insect resistance to specific PIPs and prolong the useful life of these products.

IX.E. Management of Diseases and Other Pests

Management of diseases during maize growth and development is important for protecting the yield of harvested grain. Diseases can cause stand loss, reduce photosynthesis, increase lodging, and lower yields and grain quality (Mueller and Pope 2009). The incidence of diseases varies from year to year because they are highly influenced by climate and other environmental factors. Estimates for annual yield losses because of disease have ranged from 7 to 17% (Shurtleff 1980). More than 60 diseases occur in maize in the U.S., but many occur infrequently or are not prevalent enough to cause measurable loss (Stuckey, et al. 1993). Maize diseases can be grouped into six categories: 1) seed and seedling diseases, 2) leaf diseases, 3) stalk rots, 4) ear rots, 5) viral diseases, and 6) nematode diseases. Most maize seed is treated with fungicide for the prevention of seed and seedling diseases (Stuckey et al. 1993). Other diseases can be managed to varying degrees with use of resistant hybrids, crop rotation, tillage, foliar fungicides, and various cultural practices (Mueller and Pope 2009; Stuckey et al. 1993). In addition, several nematode species have been known to cause disease in maize (Smith and White 1988). The best methods for management of pest nematodes are primarily crop rotation and stress protection (Stuckey et al. 1993). Other than use as part of a seed treatments, fungicides were applied on just 8% of U.S. maize acreage in 2010 (USDA-NASS 2013b). Fungicides currently used on maize plants in the U.S. include azoxystrobin, propiconazole, pyraclostrobin, and trifloxystrobin (USDA-NASS 2013b).

Environmental observations in field studies have demonstrated no apparent impact of MON 87411 on diseases of maize (Section VIII). Therefore, no changes in current disease management practices are anticipated from the introduction of MON 87411.

IX.F. Weed Management

Weed control in maize is essential to optimizing yield because weeds compete with maize for light, nutrients, and moisture and can lead to reductions in yield (Knake et al. 1990). Weed control the first several weeks after maize emergence is the most critical period to avoid yield losses in maize (Bosnic and Swanton 1997; Carey and Kells 1995; Hall, et al. 1992). Some weeds can tolerate cold, wet conditions better than maize, and can gain an advantage prior to planting. Annual weed species such as giant foxtail (*Setaria* spp.), barnyardgrass (*Echinochloa crus-galli* (L.) *Beauv*.) and Palmer pigweed (*Amaranthus* spp.) can reduce maize yields by up to 13, 35 and 74%, respectively (Bosnic and Swanton 1997; Fausey, et al. 1997; Gianessi, et al. 2002; Knake and Slife 1965). In a study of mixed weed populations competing with maize, yields were reduced by up to 20% when the weeds reached a height of eight inches (Carey and Kells 1995; Gianessi et al. 2002).

A survey of Extension Service weed scientists solicited estimates of the percent of maize acreage infested with individual weed species by state or region, as well as the potential impact on maize yields if the species were left uncontrolled. In this survey, twelve annual broadleaf, nine annual grass, and seven perennial species were identified as troublesome weeds (Table IX-4) (Gianessi et al. 2002). Estimates of yield loss ranged from a low of 15% due to wirestem muhly and sandburs to a high of 48% from burcucumber.

Table IX-4. Troublesome Weeds in Maize

Weed Species	Infestation Area[11]	Acreage Infested (%)	Potential Yield Loss (%)
Annuals			
Broadleaves			
Burcucumber	PA/OH/TN/SE	5-10	48
Cocklebur	MW/NP/SE	20-60	33
Jimsonweed	MW/CO	5-20	17
Kochia	NP/NW	10-70	33
Lambsquarters	MW/SE/NE/CA	15-80	33
Morningglory	MW/SE/SP	20-75	33
Nightshade	MW/NP/CA	25-50	26
Pigweeds/Waterhemp	US	30-90	36
Ragweed, Common	MW/SE/NE	20-70	30
Ragweed, Giant	MW/NP	10-45	28
Smartweeds	MW/SD/NE/SE	30-70	22
Velvetleaf	MW/NE/NP	25-70	28
Grasses			
Barnyardgrass	SP/NW/CA	80-90	23
Bermudagrass	MD/SE/UT/CA	10-20	47
Crabgrass spp	MW/SE/NE	20-80	29
Cupgrass, Woolly	IA/WI	15-20	29
Foxtail spp	MW/NE/NP	50-90	31
Millet, Wild-Proso	UT/WY/CO/ID	15-40	31
Panicum, Fall	MW/SE/NE/NP	15-80	30
Sandburs	NP/UT/WY	5-30	15
Shattercane	MW/SP	5-40	33
Perennials			
Bindweed, Field	ND/SW/CA	40-80	18
Dogbane, Hemp	IL/MO	2-20	21
Johnsongrass	MW/SE/SW/CA	20-60	45
Muhly, Wire stem	PA	2	15
Nutsedge, Yellow	MW/SE/NE/NP/CA	10-70	21
Quackgrass	MW/NE/UT	10-70	27
Thistle, Canada	NE/MW/NP/CO	5-25	26

Adapted from (Gianessi et al. 2002)

[11] Area Abbreviations:
PA: Pennsylvania
MD: Maryland
TN: Tennessee
MW: Midwest
SP: Southern Plains
NP: Northern Plains
WI: Wisconsin
ID: Idaho
OH: Ohio
CA: California
SE: Southeast
NW: Northwest
SD: South Dakota
NE: Northeast
MO: Missouri
US: United States
UT: Utah
WY: Wyoming
SW: Southwest
ND: North Dakota
IA: Iowa
CO: Colorado

Until the early 1950s, tillage and cultivation practices were primarily used for weed control in maize, but they have been largely replaced by the use of herbicides. Herbicide use in maize became widespread by the end of the 1970s. In 2010, herbicides were applied to 98% of the planted maize acreage (USDA-NASS 2013b). Currently, glyphosate is the most widely applied herbicide in maize followed by atrazine at 78% and 61%, respectively, of planted acreage. The chloracetamide herbicides (long chain fatty acid inhibitors such as acetochlor, s-metolachlor, dimethenamid, and flufenacet) were applied to 56% of the planted maize acreage in 2010 (USDA-NASS 2013b).

The introduction of biotechnology-derived HT maize (like MON 87411) continues to offer growers an effective in-crop solution for control of weeds. In 2012, approximately 73% of the total maize acreage in the U.S. was planted with hybrids possessing HT traits. Although glyphosate provides effective broad-spectrum control of numerous annual and perennial weed species, pre-emergence residual herbicides are also recommended components of weed control programs for Roundup Ready maize (Monsanto Company 2013). Pre-emergence residual herbicides provide early season control to reduce early weed competition, improve control of certain hard to control broadleaf weed species (*e.g.*, morning glory spp.), and can provide control of glyphosate-resistant weeds.

IX.F.1. Weed Resistance Management

As MON 87411 uses the same gene (*cp4 epsps*) as other Roundup Ready maize products, no separate or additional recommendations for managing glyphosate-resistant weeds have been developed for MON 87411. As described in its Technology Use Guide (Monsanto Company 2013), Monsanto continues to recommend that growers implement diversified weed management programs. Recommendations include starting with clean weed-free fields, use of a diverse set of weed control tools, including residual herbicides and multiple MOA, use of other cultural practices, as needed, and control/removal of weed escapes. Guidelines have been developed by a wide variety of stakeholders to help facilitate the effective management of herbicide resistance (HRAC 2011).

IX.G. Crop Rotation Practices in Maize

Crop rotation is a well-established farming practice and a useful management tool for maize production. Crop rotations are used to diversify farm income, spread labor requirements throughout the year, and spread the crop loss risk associated with weather and pest damage across two or more crops. In terms of soil and pest management, rotations are used to 1) manage weed, insect, and disease pests, 2) reduce soil erosion by wind and water, 3) maintain or increase soil organic matter, 4) provide biologically fixed nitrogen when legumes are used in the rotation, and 5) manage excess nutrients (Singer and Bauer 2009). Studies in U.S. corn belt states indicate maize yield is about 10-15% higher in maize grown following soybean than maize grown following maize (Singer and Bauer 2009). Despite the many benefits of crop rotations, crop price fluctuations, input costs, rental agreements, government price supports, weather, choice of farming system and on-farm resources, and other factors all contribute to decisions regarding crop rotations. Market conditions such as U.S. government-mandated ethanol use and record high commodity maize prices have increased the demand for maize grain and resulted in increases in maize acreage in recent years (Singer and Bauer 2009; USDA-NASS 2013d).

Introduction of MON 87411 is not, however, expected to impact crop rotation practices any more so than current CRW-protected and HT products available to growers.

IX.H. Maize Volunteer Management

Volunteer maize commonly occurs in rotational crops in the season following cultivation of conventional or biotechnology-derived maize. Viable grain is not produced on the approximately 8% of U.S. maize acres that are cultivated for the production of silage, and volunteer maize plants typically do not occur in the rotational crops that follow maize harvested as silage. In the warmer climates of the Southeast and Southwest, volunteer maize is rare because maize grain remaining after harvest is likely to germinate in the fall and the resulting plants can usually be controlled by tillage or by freezing temperatures in the winter. In northern maize-growing regions, volunteer maize does not always occur in rotational crops because of seed decomposition over the winter, efficient harvest procedures, and tillage prior to planting rotational crops. None of the agronomic or phenotypic data collected for MON 87411 (Section VIII.C.), including data regarding germination and seed dormancy, indicates that MON 87411 is any more likely to volunteer than existing commercial maize hybrids.

Management of volunteer maize in rotational crops involves minimizing or reducing the potential for volunteers through practices that include: 1) adjusting harvest equipment to minimize the amount of grain lost in the field; 2) planting hybrids that reduce the extent of ear drop; 3) choosing hybrids with superior stalk strength and reduced lodging; and 4) practicing no-till production to significantly reduce the potential for volunteer growth in rotational crops. If volunteer maize does occur in subsequent crops, pre-plant tillage, in-crop cultivation and the use of selective herbicides are very effective management tools. Introduction of MON 87411 is not expected to impact maize volunteer management practices any more so than current CRW-protected and HT products available to growers.

IX.I. Stewardship of MON 87411

Monsanto develops effective products and technologies that deliver value to growers and conserve resources that agriculture depends on, and is committed to assuring that its products and technologies are safe and environmentally responsible. Monsanto demonstrates this commitment by implementing product stewardship processes throughout the lifecycle of a product and by participation in the Excellence Through Stewardship® (ETS) Program (BIO 2010). ETS policies and practices include rigorous field compliance and quality management systems and verification through auditing. Monsanto's Stewardship Principles are also articulated in Technology Use Guides (Monsanto Company 2013) and Monsanto Technology Stewardship Agreements that are signed by growers who utilize Monsanto branded traits, to ensure stewardship compliance.

As an integral action of fulfilling this stewardship commitment, Monsanto will seek biotechnology regulatory approvals for MON 87411 in all key maize importing countries with functioning regulatory systems to assure global compliance and support the flow of international trade. These actions will be consistent with the Biotechnology Industry Organization Policy on Product Launch (BIO 2010). Monsanto continues to monitor

other countries that are key importers of maize from the U.S., for the development of formal biotechnology approval processes. If new functioning regulatory processes are developed, Monsanto will make appropriate and timely regulatory submissions.

Monsanto also commits to industry best practices on seed quality assurance and control to ensure the purity and integrity of MON 87411 seed. As with all of Monsanto's products, before commercializing MON 87411 products in any country, the appropriate detection methods will be made available to maize producers, processors, and buyers.

IX.J. Impact of the Introduction of MON 87411 on Agricultural Practices

MON 87411 has been developed to provide two MOA against the primary CRW-complex pests of maize in the corn belt as well as glyphosate tolerance in a single product. As tolerance to glyphosate and protection from corn rootworm complex pests are present in many currently available maize hybrids and are widely grown in the U.S., the introduction of MON 87411 is not expected to have major impacts on current agronomic, cultivation and management practices for maize. No changes are anticipated in crop rotations, tillage practices, planting practices, fertility management, weed and disease management, and volunteer management from the introduction of MON 87411.

MON 87411 has been shown to be comparable to conventional maize in its compositional, phenotypic, and agronomic characteristics (Sections VII and VIII). CRW-protected and glyphosate tolerant maize products have been widely grown in the U.S. since 2003 and the introduction of MON 87411, with its new MOA, will improve the durability of CRW-protection traits and extend the useful lifetime of these products.

Biotechnology-derived CRW-protected maize has been cultivated and consumed in the U.S. since 2003 and has reduced insecticide applications directed at this pest (U.S. EPA 2011). MON 87411 introduces a new MOA based on RNAi that offers increased control of CRW pests and is expected to prolong the durability of existing CRW-control technologies. As such, it will continue to provide benefits to growers similar to those obtained by use of existing CRW-protected maize hybrids, including reduced use of insecticides, increased yield protection, water conservation, and increased worker safety.

X. PLANT PEST ASSESSMENT

This section provides a brief review and assessment of the plant pest potential of MON 87411 and its impact on agronomic practices and the environment. USDA-APHIS has responsibility, under the Plant Protection Act (PPA) (7 U.S.C. § 7701-7772), to prevent the introduction and dissemination of plant pests into the U.S. Regulation 7 CFR § 340.6 provides that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and should no longer be regulated. If APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article.

According to the PPA, the definition of "plant pest" includes the living stage of any of the following that can directly or indirectly injure, cause damage to, or cause disease in any plant or plant product: (A) a protozoan; (B) a nonhuman animal; (C) a parasitic plant; (D) a bacterium; (E) a fungus; (F) a virus or viroid; or (G) an infectious agent or other pathogen; (H) Any article similar to or allied with any of the articles specified in the preceding subparagraphs (7 U.S.C. § 7702[14]).

The regulatory endpoint under the PPA for biotechnology-derived crop products is not zero risk, but rather a determination that deregulation of the article in question is not expected to pose a greater plant pest risk than its unmodified comparator. Information in this petition related to plant pest risk characteristics includes: 1) mode-of-action and changes to plant metabolism; 2) composition; 3) expression and characteristics of the gene product; 4) potential for weediness of the regulated article; 5) impacts to NTOs; 6) disease and pest susceptibilities; 7) impacts on agronomic practices; and 8) impacts on the weediness of any other plant with which it can interbreed, as well as the potential for gene flow. Using the assessment above, the data and analysis presented in this petition lead to a conclusion that MON 87411 is not expected to be a plant pest, and therefore should no longer be subject to regulation under 7 CFR § 340.

X.A. Characteristics of the Genetic Insert and Expressed Products

X.A.1. Genetic Insert

As described in Section III, MON 87411 was developed by *Agrobacterium tumefaciens*-mediated transformation of maize embryos using plasmid vector PV-ZMIR 10871. Characterization of the DNA insert in MON 87411 was conducted using a combination of sequencing, PCR, and bioinformatics methods. The results of this characterization demonstrate that MON 87411 contains one copy of the intended T-DNA containing the DvSnf7 suppression cassette and the *cry3Bb1* and *cp4 epsps* expression cassettes that is stably integrated at a single locus and is inherited according to Mendelian principles over multiple generations. These methods also confirmed that no vector backbone or other unintended plasmid sequences are present in MON 87411. Additionally, the genomic organization at the insertion site was assessed by comparing the sequences flanking the T-DNA insert in MON 87411 to the sequence of the insertion site in conventional maize. This analysis determined that no major DNA rearrangement occurred at the insertion site in MON 87411 upon DNA integration.

X.B. Mode-of-Action

As noted in Section I.D.2., MON 87411 contains the same CP4 EPSPS and Cry3Bb1 proteins as those found in MON 88017 maize (APHIS petition No. 04-125-01p) that was granted non-regulated status by USDA-APHIS in 2006 (USDA-APHIS 2013). The MOA of the CP4 EPSPS protein, which is found in numerous other approved Roundup Ready crops, is well understood (Padgette et al. 1996). Similarly, the MOA of *Bt* proteins such as Cry3Bb1 has been extensively studied and is well understood (Gill et al. 1992; Whalon and Wingerd 2003).

As previously described in Section I.C.1., the RNAi mechanism is a natural process in eukaryotic organisms for the regulation of gene expression. Double-stranded RNA molecules that activate the mechanism are first processed into siRNAs which are then incorporated into multi-protein RNA-induced silencing complexes (RISC). These RISCs facilitate target sequence recognition and mRNA cleavage, leading to specific suppression of the targeted mRNA. The targeted DvSnf7 is a vacuolar sorting protein in the ESCRT-III complex and is involved in sorting transmembrane proteins enroute to lysomsomal degradation. *Snf7* is an essential gene in CRW with significant roles in intracellular trafficking of proteins and because of its key cellular function, its suppression resulting from the expression of specific DvSnf7 dsRNAs, results in CRW mortality.

Characterization of the insecticidal mode of action of DvSnf7 dsRNA has demonstrated that *Snf7* is an essential gene in WCR that plays a significant role in intracellular protein trafficking and its function is consistent with its known function in other eukaryotes (Bolognesi et al. 2012; Ramaseshadri et al. 2013). The spectrum of activity for DvSnf7 dsRNA has been shown to be narrow and activity is only evident in a subset of beetles within the Galerucinae subfamily of Chrysomelidae within the Order Coleoptera (Section VI.E.) (Bachman et al. 2013). For the NTO assessment of MON 87411, in addition to the activity spectrum testing (Sections VI.E.2. and VI.E.3.), an additional battery of beneficial organisms was tested that included representative pollinators (honeybee), a detritivore (earthworm) and insect predators/parasitoids (ladybird beetle, carabid beetle, *Orius*, parasitic wasp). The testing and assessment for DvSnf7 RNA lead to the conclusion that no negative impacts to NTOs, including those beneficial to agriculture, will result from the cultivation of MON 87411.

X.C. Expression and Characterization of Gene Products

X.C.1. Protein Safety and Expression Levels

The safety and expression of the Cry3Bb1 and CP4 EPSPS proteins are detailed in Section V. Expression levels were determined from 19 tissue types from trials conducted in Argentina and are presented in Section V.C. The expression in the various tissues ranged from 3.0 to 460 µg/g dw for Cry3Bb1 and <LOQ to 76 µg/g dw for CP4 EPSPS. These proteins are present at a very small percentage of the total protein in maize seed (no more than 0.004% and 0.002% for Cry3Bb1 and CP4 EPSPS, respectively). Both proteins also have established histories of safe use, having been assessed by USDA, FDA and U.S. EPA on multiple occasions (Section V.E.). Neither protein originates from an

organism known to be a source of allergens, a bioinformatic assessment of each shows no shared amino acid sequence similarities to known allergens, and each protein is rapidly digested in a simulated gastric fluid assay (Sections V.D.1 and V.D.2). Taken together, the results of these analyses support a determination that MON 87411 is no more likely to pose a plant pest risk than conventional maize.

X.C.2. RNA Safety and Expression Levels

The safety and expression of the DvSnf7 RNA expressed in MON 87411 is detailed in Section VI. Expression levels were determined from 19 tissue types from trials conducted in Argentina and are presented in Section VI.C. The expression in the various tissue types ranged from <LOQ to 213×10^{-3} µg/g dw. As RNAi is a naturally occurring, ubituitous process in eukaryotes, there is a long history of safe consumption of the RNA molecules mediating this process. Further, there is no evidence suggesting that dietary consumption of nucleic acids, like RNA, is associated with toxicity or allergenicity (Petrick et al. 2013; U.S. FDA 1992). Total nucleic acid consumption in the human diet for DNA and RNA is estimated to be 1-2 grams per day (Suchner et al. 2000) which equates to an approximate maximal intake of 0.2 g/kg/day for a non-nursing infant (the highest consumer per body weight). Given these parameters, consumption of DvSnf7 RNA from MON 87411 would be extremely low (≤ 0.4 ng/kg/day) relative to the total estimated dietary RNA intake. Taken together, the results of these assessments, along with the demonstrated specificity of DvSnf7 RNA to CRW and the history of safe consumption of RNA, support a determination that MON 87411 is no more likely to pose a plant pest risk than conventional maize.

X.D. Compositional Characteristics

Compositional comparisons based on OECD guidance were presented in Section VII to assess whether levels of nutrients, anti-nutrients, and secondary metabolites in grain and forage derived from MON 87411 are comparable to levels in the conventional control and several reference hybrids for which there is an established history of safe consumption. Nutrients assessed in this analysis included proximates, carbohydrates by calculation, acid detergent fiber, neutral detergent fiber, total dietary fiber, minerals, amino acids, and vitamins. Anti-nutrients assessed in grain included phytic acid and raffinose. Secondary metabolites assessed in grain included furfural, ferulic acid, and p-coumaric acid. Forage samples were assessed for levels of proximates, fiber, minerals (calcium and phosphorus), and carbohydrates by calculation. In all, 78 different components were assayed (9 in forage and 69 in grain). While 78 components were assayed, 16 of these had more than 50% of observations below the assay LOQ and were excluded from statistical analysis.

A combined-site analysis of the data was conducted to determine statistically significant differences (at 5%) between MON 87411 and the conventional control. The biological significance of differences from the data was reviewed using considerations relevant to food and feed safety and nutritional quality. These considerations included: 1) a determination of the mean differences of nutrient and anti-nutrient components of MON 87411 and the conventional control; 2) consideration of the mean differences between MON 87411 and the conventional control in the context of the range of values

for the conventional control, 3) comparison of the mean differences between MON 87411 and the conventional control to variation in conventional maize as estimated by in-study reference hybrid values and assessing whether the mean values for MON 87411 were within 99% tolerance intervals, literature values, and/or ILSI Crop Composition Database values. These evaluations of variation within the conventional control and conventional maize, including reference hybrids, are important as crop composition is known to be influenced by environment and germplasm.

The compositional analysis provided a comprehensive comparative assessment of the levels of key nutrients, anti-nutrients, and secondary metabolites in maize grain and forage of MON 87411 and the conventional control. Of the 60 components statistically assessed there were no statistically significant differences in 48 components. Only 12 components (protein, histidine, tyrosine, oleic acid, neutral detergent fiber, copper, iron, manganese, zinc, niacin, vitamin B1 in grain and ash in forage) showed significant differences between MON 87411 and conventional control. For these 12 components, the mean difference in component values between MON 87411 and the conventional control was less than the range of the conventional control values and the reference hybrid values. Overall, MON 87411 mean component values were within 99% tolerance intervals, values observed in the literature, and/or ILSI-CCDB values. These data indicate that the components with significant differences were not compositionally meaningful from a food and feed safety or nutritional perspective. These results further support the overall conclusion that MON 87411 was not a major contributor to variation in component levels in maize grain and forage and confirmed the equivalence of MON 87411 to the conventional control in levels of these components.

X.E. Phenotypic, Agronomic, and Environmental Interaction Characteristics

An extensive set of comparative plant characterization data were used to assess whether the introduction of the insect-protection and glyphosate tolerance traits altered the plant pest potential of MON 87411 compared to the conventional control (Section VIII). Phenotypic, agronomic, and environmental interaction characteristics of MON 87411 were evaluated and compared to those of the conventional control. As described below, these assessments included: seed dormancy and germination characteristics; agronomic and plant phenotypic characteristics; observations for abiotic stress response, disease damage, arthropod-related damage, arthropod abundance, and pollen characteristics. Results from all phenotypic, agronomic, and environmental interaction assessments demonstrated that MON 87411 does not possess weedy characteristics, or increased susceptibility or tolerance to specific diseases, insects, or abiotic stressors compared to the conventional control. Taken together, the results of the analysis support a determination that MON 87411 is no more likely to pose a plant pest risk than conventional maize.

X.F. Weediness Potential of MON 87411

Maize is not listed as a weed in the major weed references (Crockett 1977; University of Montana 2011), nor is it present on the lists of noxious weed species distributed by the federal government (7 CFR § 360). In addition, maize has been grown throughout the world without any report that it is a serious weed. During domestication of maize, traits

often associated with weediness, such as, seed dormancy, a seed dispersal mechanism, or the ability to form reproducing populations outside of cultivation, have not been selected. Even if individual kernels of maize were distributed within a field or along transportation routes from the fields to storage or processing facilities, sustainable volunteer maize populations are not found growing in fence rows, ditches, or road sides. Maize is poorly suited to survive without human assistance and is not capable of surviving as a weed (Galinat 1988; Keeler 1989).

In comparative studies between MON 87411 and a conventional control, phenotypic, agronomic and environmental interaction data were evaluated (Section VIII) for changes that would impact the plant pest potential, in particular, plant weediness potential. Results of these evaluations show that there is no fundamental difference between MON 87411 and the conventional control for traits potentially associated with weediness. Furthermore, comparative field observations between MON 87411 and its conventional control and their response to abiotic stressors indicated no differences and, therefore, no increased weediness potential. Collectively, these findings support the conclusion that MON 87411 has no increased weed potential compared to conventional maize and it is no more likely to become a weed than conventional maize.

X.F.1. Seed Dormancy and Germination

A comparative assessment of seed germination and dormancy characteristics was conducted on MON 87411 and the conventional control. The results of this assessment, particularly the lack of increased hard seed, and no changes in other germination and dormancy characteristics, support the conclusion that the introduction of MON 87411 is not expected to result in increased plant pest/weed potential compared to conventional maize.

X.F.2. Plant Growth and Development

Evaluations of plant growth and development characteristics in the field are useful for assessing potential weediness characteristics such as stalk and root lodging. Phenotypic characteristics such as early stand count, days to 50% pollen shed and silking, stay green, ear height, plant height, dropped ears, stalk lodged plants, root lodged plants, final stand count, grain moisture, test weight, and yield were assessed. In the combined-site analysis in which the data were pooled among the sites, no statistically significant differences were detected between MON 87411 and the conventional control for any of the assessed characteristics. Thus, the phenotypic and agronomic characteristics of MON 87411 were not altered in terms of pest/weed potential compared to conventional maize.

X.F.3. Pollen Morphology and Viability

Evaluations of pollen morphology and viability from field-grown plants provide useful information in a plant pest assessment as it relates to the potential for gene flow to, and possible introgression of a biotechnology-derived trait into sexually-compatible plants and wild relatives. No statistically significant differences were detected between MON 87411 and the conventional control for percent viable pollen or pollen grain diameter. Furthermore, no visual differences in general pollen morphology were observed between MON 87411 and the conventional control. Based on the assessed characteristics, the results support a conclusion that neither pollen viability nor morphology of MON 87411 were altered compared to conventional maize.

X.G. Impact to Non-Target Organisms, Including Those Beneficial to Agriculture

Information presented previously in Sections V.E. and VI.E. is relevant to the plant pest risk assessment for MON 87411 because it describes the spectrum of activity for the CP4 EPSPS protein, the Cry3Bb1 protein, and the DvSnf7 RNA. According to 7 CFR Part 340.6, this information is part of the required information needed for evaluation of plant pest potential. Given that the safety of CP4 EPSPS and Cry3Bb1 proteins as well as the CRW-resistance and glyphosate- tolerance traits have previously been evaluated by both USDA-APHIS in regulatory submissions for MON 863 and MON 88017 (USDA-APHIS 2013) and EPA (U.S. EPA 2010), the primary focus of the NTO section (Section VI.E.) was on DvSnf7 RNA.

The NTO assessment for MON 87411 has taken into consideration the MOAs, the spectrum of insecticidal activity, and exposure levels to the CP4 EPSPS and Cry3Bb1 proteins and the DvSnf7 RNA produced by MON 87411. The data, analyses and assessments presented support a conclusion that MON 87411 will not negatively impact NTOs, including those beneficial to agriculture, and does not present an increased plant pest risk compared to conventional maize.

X.G.1. Impact on Threatened and Endangered Species

Threatened and endangered species risk assessments were previously conducted by USDA (USDA-APHIS 2013) and EPA (U.S. EPA 2010) for Cry3Bb1-containing maize products MON 863 and MON 88017, and indicated no direct or indirect effects to threatened or endangered species.

Because of the specificity of the DvSnf7 RNA for coleopteran species within the family Chrysomelidae (Bachman et al. 2013), endangered species concerns for this trait are properly focused on the order Coleoptera. Currently, there are 18 listed Coleoptera on the threatened and endangered species list, however none of these species are members of the family Chrysomelidae (USFWS 2013a). Additionally, the listed threatened or endangered coleopteran species are not expected to be in or near maize fields as many of the endangered and threatened coleopteran species occur in caves or aquatic habitats (U.S. EPA 2010).

Threatened and endangered coleopteran species in the U.S. have restrictive habitat ranges, which generally do not include maize fields (U.S. EPA 2010). However, some endangered and threatened coleopteran habitats may occur near agricultural production areas. An analysis of the county level distributions of threatened or endangered terrestrial coleopteran species indicates that the potential concern regarding range overlap with maize production was restricted to the American burying beetle (*Nicrophorus americanus).* The American burying beetle is the largest carrion beetle in North America (USFWS 1991) and is only found in limited areas encompassing parts of 9[12] states, including Arkansas, Kansas, Massachusetts, Nebraska, Ohio, Oklahoma, Rhode Island, South Dakota, and Texas (USFWS 2013b). Adults feed on carrion and occasionally other insects (USFWS 1991), while larvae feed exclusively on buried carrion or carrion regurgitations provided by their parents (U.S. EPA 2010; USFWS 2008)). The American burying beetle's habitat is variable and includes mature forests, shrub-covered areas, and some grassland habitats and the beetle's preferred habitat has been correlated with an abundance of small vertebrate biomass (USFWS 2008). Considering that both larvae and adult beetles are carrion feeders, exposure to the DvSnf7 RNA in MON 87411 is highly unlikely due to their feeding ecology.

There are several threatened and endangered coleopteran species that occur in aquatic habitats. As discussed above, however, exposure of aquatic organisms to biotechnology-derived crops has been shown to be limited temporally and spatially and the U.S. EPA has previously concluded that potential exposure of aquatic organisms is low to negligible (U.S. EPA 2010).

Due to the lack of proximity to maize cultivation, lack of relevant exposure because of feeding ecology and the restricted activity of the DvSnf7 RNA, it is concluded that cultivation of MON 87411 will have no effect on threatened and endangered species, including coleopteran species.

X.H. Environmental Fate of CRW Products Expressing DvSnf7_968

The two components of the insecticidal activity of MON 87411 are the Cry3Bb1 protein and the DvSnf7_968 RNA. The environmental safety of the Cry3Bb1 protein has been demonstrated in multiple environmental studies and assessed extensively as part of many previous EPA product registrations, *e.g.*, MON 863, EPA Reg. # 524-528; MON 863 x MON 810, EPA Reg. # 524-545; MON 88017, EPA Reg. # 524-551, MON 88017 x MON 810, EPA Reg. # 524-552 and MON 89034 x MON 88017, EPA Reg. # 524-576 among others (U.S. EPA 2010). Thus, the current environmental assessment pertains exclusively to the RNA component of the insecticidal activity, DvSnf7_968.

Soil organisms may be exposed to DvSnf7_968 by contact with roots and with above-ground plant residues or pollen reaching the soil. Soil microorganisms may be exposed to DvSnf7_968 by degrading living or decaying maize biomass or by ingestion or absorption of the dsRNA after its release into the soil. Assessment of the environmental

[12] An experimental population that is listed as non-essential exists in Missouri (USFWS 2013a).

fate of DvSnf7_968 and any other nucleic acid (DNA or RNA) from biotechnology-derived crops should consider the amount of nucleic acids already in the environment from conventional sources. Thousands of tons of nucleic acids are released into the environment every year from conventional plant biomass (roots, leaves, pollen, etc.), in addition to the nucleic acid contribution of other decaying animal and microbial matter (Dale, et al. 2002). It is unlikely that nucleic acids originating from biotechnology-derived plants will persist in soil or interact differently than nucleic acids from non-engineered organisms, or persist by being incorporated into microbes via soil uptake (Dale, et al. 2001).

There are currently no published laboratory or field monitoring studies that directly address the persistence or accumulation of RNA in maize fields. However, a laboratory soil study of DNA that encodes recombinant neomycin phosphotransferase II (rNPT-II), a marker used in early plant genetic engineering, showed rapid dissipation of the target DNA (Widmer, et al. 1996). The study showed that after the rNPT-II DNA was added to soil by incorporating ground tissue of transgenic tobacco containing the rNPT-II sequence and by addition to soil of the purified plasmid DNA, both the tissue-derived rNPT-II sequence and the plasmid DNA degraded rapidly. Only a small proportion of the DNA was detectable for longer periods. Up to 0.08% of the plasmid DNA was detectable at 40 days, and up to 0.14% of tissue-derived DNA sequence was detectable for 120 days.

X.I. Potential for Pollen Mediated Gene Flow and Introgression

Pollen mediated gene flow is a process whereby one or more genes successfully integrate into the genome of a recipient plant. Introgression is affected by both biotic and abiotic factors such as plant biology, pollen biology/volume, plant phenology, overlap of flowering times, proximity of the pollen source and sink, ambient conditions such as temperature and relative humidity, and field architecture. Because gene introgression is a natural biological process, it does not constitute an environmental risk in and of itself. Gene introgression must be considered in the context of the transgenes inserted into the biotechnology derived plant, and the likelihood that the presence of the transgenes and their subsequent transfer to recipient plants will result in increased plant pest potential. The potential for gene introgression from MON 87411 is discussed below.

X.I.1. Hybridization with Cultivated Maize

Maize morphology fosters cross pollination, therefore, high levels of pollen mediated gene flow can occur in this species. In addition, researchers recognize that (1) the amount of gene flow that occurs can be high because of open pollination; (2) the percent gene flow can vary by population, hybrid or inbred; (3) the level of gene flow decreases with greater distance between the source and recipient plants; (4) environmental factors affect the level of gene flow; (5) maize pollen is viable for a short period of time under field conditions; (6) maize produces ample pollen over an extended period of time; and, (7) maize is almost exclusively wind pollinated.

Based on several studies conducted on the extent of pollen mediated gene flow between maize fields, results were found to vary depending on the experimental design,

environmental conditions, and detection method, as expected. In general, the percent of gene flow diminished with increasing distance from the source field, generally falling below 1% at distances >200 m (~660 feet) (Table X-1). This information is useful for managing gene flow during maize breeding, seed production, identity preservation or other applications; in addition, it forms the basis for the USDA-APHIS performance standards for maize. All testing and production of regulated MON 87411 seed or grain have been conducted under USDA notification according to these standards. Gene flow from fields planted with MON 87411 to other maize would not be of concern because of the lack of potential to cause harm to humans and to the environment.

Table X-1. Summary of Published Literature on Maize Cross Pollination

Pollinator Distance (m)	Reported Outcrossing (%)	Comments	Country	Reference
0	28.6	Frequencies by distance investigated. Three year study. Single male and female. Pollen source was a yellow dent and the female was a white sweet corn..	USA	(Jones and Brooks 1950)
25	14.2			
75	5.8			
125	2.3			
200	1.2			
300	0.5			
400	0.2			
500	0.2			
1	-	Frequencies by distance investigated. Single yellow sweet corn hybrid was used as a pollen source and as pollen recipient. To measure levels of outcrossing, pollen recipient plants were detasseled.	UK	(Haskell and Dow 1951)
3.6	95			
4.8	-			
6	-			
7.3	-			
8.5	-			
9.8	-			
11	-			
12	-			
13.4	-			
18	10			

Table X-1 (continued). Summary of published literature on maize outcrossing

Pollinator Distance (m)	Reported Outcrossing (%)	Comments	Country	Reference
1 10 20 30 34	2.25 0.02 0.008 0.005 0.003	Dispersal of maize pollen investigated. Single hybrid. Gene flow decreased with greater distance from the source. Closer correlation of number of plants with gene flow than physical distance. Data reported in this table represent means from two of four fields	Brazil	(Paterniani and Stort 1974)
2-4	0.01	Gene flow in isolated and crossing blocks was evaluated. Two year study. Single male and female. *Bt* female hybrid was detasseled.	Mexico	(Garcia, et al. 1998)
30 40 350	1.04 0.03 0	Frequencies by distance investigated. Two year study. Single RR male and non-RR female. Data reported in this table represent one of two years.	USA	(Jemison and Vayda 2001)

Table X-1 (continued). Summary of published literature on maize outcrossing

Pollinator Distance (m)	Reported Outcrossing (%)	Comments	Country	Reference
100 150 200 300 400	0.01 - 0.01 - -	Frequencies by distance and pollen viability investigated. Two year study. Single male and female. A purple gene marker was utilized to measure pollen mobility. Pollen viability lasted one hour in the driest-hottest year and two hours in the most humid, less hot year.	Mexico	(Luna, et al. 2001)
1 3 8 16 24 32	30-40 18-22 9-12 3-5 0-2 2-4	Frequencies by distance investigated. Two sites/one year. Six hybrid pairs. Six *Bt* and six near isogenic non-*Bt* hybrids. Hybridization was assessed by measuring the expression of *Bt* gene in kernels collected from neighboring plants. Alternatively, sampled kernels were grown and seedlings tested for expression of *Bt* gene. Data reported in this table represent estimates from a graph.	USA	(Chilcutt and Tabashnik 2004)
1 5 10 14 19 24 28 33 36	9.7-19.0 1.3-2.6 0.7-2.0 0.3-0.6 0.4 0-0.3 0.1-0.5 0-0.3 0-0.1	Frequency by distance investigated. Three year, three sites. Single male and female/location.	Canada	(Ma, et al. 2004)

Table X-1 (continued). Summary of published literature on maize outcrossing

Pollinator Distance (m)	Reported Outcrossing (%)	Comments	Country	Reference
200 300	0.03 0.02	Detasseling efficiency on pollen containment investigated. Four inbreds were used as a source of pollen; yellow inbred, two GM inbreds (*Bt* and RR) and an IT (imidazolinone tolerant) inbred. The recipient pollen traps were two white inbreds and a male-sterile hybrid. Two year/three locations study.	USA	(Stevens, et al. 2004)
24-32 60-62 123-125 244-254 486-500 743-745	0.01-0.7 0.01-0.2 0.001-0.08 0-0.02 0-0.005 0-0.002	Frequencies by distance investigated. Single male and 7 females with different RM used. The male parent source of pollen contains the genetic markers P1-rr and R1-nj. When male pollen pollinated female yellow plants a purple coloration occurred in the fertilized yellow kernels. Two year/two site study. Data reported in this table represent results from one site.	USA	(Halsey, et al. 2005)
1.8 9.4 20.6 35.8 200	1.0-2.5 1.2-2.5 1.0-2.2 0.5-2.3 0.6-1.4	Isolation distance investigated. The objectives were (i) to evaluate current industry isolation practices to produce hybrid seed that meets higher levels of genetic purity and (ii) to identify practices that will improve reproductive isolation in hybrid seed fields. Three year/315 fields. Multiple hybrids from 24 seed companies tested. Data reported in this table represent estimates from a graph.	USA	(Ireland, et al. 2006)

Table X-1 (continued). Summary of published literature on maize outcrossing

Pollinator Distance (m)	Reported Outcrossing (%)	Comments	Country	Reference
1 10 35 100 150 200 250	17.0-29.9 1.5-2.5 0.4 0.03-0.05 0.01-0.03 0.007-0.03 0.002-0.03	Frequencies of cross-pollination by distance investigated. Pollination was quantified by measuring out-crossing from a transgenic hybrid plot into a conventional grain production field. A combination of three marker genes was utilized to detect outcrosses: y1 (seed color gene), *Bt* and RR. Two years/two sites. Single male and female.	USA	(Goggi, et al. 2006)
0 4.6 18.3	< 0.9% at distances ≤ 20 m	Efficiency of border rows and isolation distance on cross-pollination investigated. Available datasets were utilized to make predictions for reducing out-crossing to levels below 0.9%.	USA	(Gustafson, et al. 2006)
0 2 5 10 20 40 80	3-13 0.2-10 0.1-2.3 0.2-3.7 0.1-0.8 0-0.7 0.1-0.2	Frequencies of cross-pollination with a PCR based method investigated. The main objective of the study was to compare a PCR based method to real cross-fertilization rates as determined by phenotypic analysis. Four *Bt* hybrids and a single non-*Bt* hybrid were used as a male and female respectively. One year/one site.	Spain	(Pla, et al. 2006)

Table X-1 (continued). Summary of published literature on maize outcrossing

Pollinator Distance (m)	Reported Outcrossing (%)	Comments	Country	Reference
0	10.5	Frequency of cross pollination (expressed as %GM DNA) by distance investigated. The study was conducted in large farm scale evaluation (FSE) across the UK. Data reported here are maximum raw values	UK	(Weekes, et al. 2007)
2	34.9			
5	9.9			
10	12.2			
15	0.5			
20	8.2			
25	4			
40	3.7			
50	5.9			
70	0.13			
75	0.28			
80	0.12			
100	2.3			
120	0.16			
142	0.06			
147	0			
150	5.4			
160	0			
200	0.24			

Table X-1 (continued). Summary of published literature on maize outcrossing

Pollinator Distance (m)	Reported Outcrossing (%)	Comments	Country	Reference
52 85 105 125 149 150 200 287 371 402 458 4125 4440	0.009 0.015 0.003 0.01 0.016 0.007 0.009 0.005 0.008 0.005 0.0002 0.006 0.0005	Cross-pollination investigated using occurrence of yellow kernels in 13 white maize fields. In no case, the cross-pollination of the whole field was > than 0.02%. In every field some cross-pollination with a low rate, on an average of 1.8% of the sampled ears, could be found. These pollinations were mostly single cross-pollinations on the ear.	Switzerland	(Bannert and Stamp 2007)
1 10 35 100	42.2 6.3 1.3 0.1	Frequencies of cross-pollination by distance investigated. The pollen source was a stacked RR/*Bt* yellow hybrid. The recipient was a nontransgenic white hybrid. Higher outcrossing detected when white hybrid used detasseled.	USA	(Goggi, et al. 2007)
12 12 12	4.2 11.7 3.8	Frequency of cross pollination and coexistence by distance investigated. Two crops were used as barriers to determine their usefulness as buffer crops in maize. Three genetic markers to measure outcrossing were used: GM *Bt* maize, a kernel color maize and a molecular marker test	EU	(Langhof, et al. 2008)

X.I.2. Hybridization with Wild Annual Species of *Zea mays* subsp. *mexicana*

For gene flow to occur by normal sexual transmission, the following conditions must exist: (1) the two parents must be sexually compatible; (2) there must be overlapping flowering times; and (3) a suitable factor (such as wind or insects) must be present and capable of transferring pollen between the two parents.

Maize and annual teosinte (*Zea mays* subsp. *mexicana*), are genetically compatible, wind-pollinated and teosinte pollen can pollinate maize silks when in close proximity to each other, *e.g.* in areas of Mexico and Guatemala (Wilkes 1972). Maize crosses with teosinte; however, teosinte is not present in the U.S. other than as an occasional botanical garden specimen or small feral populations in Florida, Alabama and Maryland. In experimental studies where maize and teosinte species were planted together, very low hybridization rates were observed for maize and *Zea mays* subsp. *mexicana* (Baltazar, et al. 2005; Ellstrand, et al. 2007). Differences in factors such as flowering time, geographical distribution, and development factors make natural crosses in the U.S. highly unlikely.

X.I.3. Hybridization with the Wild Perennial Species of Subgenus *Tripsacum*

In contrast with maize and teosinte, which hybridize under certain conditions, it is only with extreme difficulty and special techniques that maize and the related perennial species, *Tripsacum dactyloides* (gamma grass) hybridize. Furthermore, hybrids of the cross are male sterile, even after several backcrosses to maize (Russell and Hallauer 1980).

A single species, *Tripsacum floridanum* (Florida gamma grass), found in the extreme southern Florida counties of Miami-Dade, Collier and Monroe has been categorized as a threatened species by the state of Florida and listed on the USDA Natural Resources Conservation Service (USDA-NRCS) database (USDA-NRCS 2012). Another species, *Tripsacum dactyloides* (Eastern gamma grass), found primarily throughout the eastern U.S., has been categorized as endangered in Massachusetts and Pennsylvania, and as threatened in New York (USDA-NRCS 2013). However, given the level of difficulty for natural hybridization between species of *Tripsacum* and *Zea* as mentioned above, the occurrence of *T. floridanum* primarily in both highly urbanized and non-agricultural, swampy areas of the state where commercial maize is not typically grown, as well as the preference of *T. dactyloides* for wet habitats where hybrid maize production would not occur, it is very unlikely there would be any impact on this species due to the introduction of MON 87411.

X.J. Transfer of Genetic Information to Species with which Maize Cannot Interbreed (Horizontal Gene Flow)

Monsanto is aware of no reports confirming the transfer of genetic material from maize to other species with which maize cannot interbreed. The probability for horizontal gene flow to occur is judged to be exceedingly small. Even if it were to occur, the consequences would be negligible since the CP4 EPSPS and Cry3Bb1 proteins produced

in MON 87411 are the same proteins as in commercial MON 863 and MON 88017 maize products and have been shown to have no meaningful toxicity to humans and NTOs. Similar to the situation with transfer of the protein genes, the likelihood of horizontal transfer of the partial DvSnf7 gene from MON 87411 is also exceedingly small. The consequence of such transfer, given the known lack of toxicity to all but a very small subset of organisms (CRW species), would also be expected to be inconsequential. In either case, the presence of these genes would not be expected to increase the pest potential of the recipient species.

X.K. Potential Impact on Maize Agronomic Practices

An assessment of current maize agronomic practices was conducted to determine whether the cultivation of MON 87411 has the potential to impact current maize management practices (Section IX). Maize fields are typically highly managed agricultural areas that are dedicated to crop production. Other than the specific insertion of the partial DvSnf7 gene that provides another MOA for protection against CRW, MON 87411 is similar to many other maize hybrids being grown in the U.S. (*e.g.*, Genuity SmartStax, Genuity VT Triple PRO, etc). Herbicide tolerant maize has been in use commercially in the U.S. since 1997 and constituted 73% of the total maize crop in 2012 (USDA-ERS 2013). CRW-protected maize has been planted commercially since 2003 and in 2010 constituted 53% of the total U.S. crop (Brookes and Barfoot 2012). CRW-protected maize hybrids have been grown on tens of millions of acres in the U.S. since their introduction. Given the widespread use of these HT and insect-protected maize hybrids, Monsanto anticipates no specific changes to agronomic practices from introduction of MON 87411 above and beyond those in current use.

MON 87411 is similar to commercially cultivated maize in its agronomic, phenotypic, ecological, and compositional characteristics, and has levels of resistance to insect pests and diseases comparable to other commercially cultivated maize. Based on this assessment, the introduction of MON 87411 is not likely to impact current U.S. maize agronomic or cultivation practices or lead to an increased plant pest potential compared to other maize hybrids widely available to growers.

X.L. Conventional Breeding with Other Biotechnology-derived or Conventional Maize

Numerous biotechnology-derived maize products have been deregulated or are under consideration for deregulation by APHIS. Once deregulated, MON 87411 may be bred with these deregulated maize products, as well as with conventional maize, creating new improved hybrids. APHIS has determined that none of the individual biotechnology-derived maize products it has previously deregulated displays increased plant pest characteristics. APHIS has also concluded that progeny derived from crosses of these deregulated maize products with conventional or previously deregulated maize are unlikely to exhibit new plant pest properties. This presumption, that combined-trait biotechnology products are unlikely to exhibit new characteristics that would pose new plant pest risks not observed in the single event biotech product, is based upon several facts. Namely: 1) stability of the genetic inserts is confirmed in each approved biotech-

derived maize product across multiple generations; 2) stability of each of the introduced traits is continually and repeatedly assessed as new combined-trait hybrids are created by plant breeders and tested over multiple seasons prior to commercialization; 3) combined-trait products are developed using conventional breeding that has been safely used for thousands of years to generate new varieties (Steiner, et al. 2013; WHO 1995); 4) worldwide organizations, such as World Health Organization, Food and Agriculture Organization/ World Health Organization, International Seed Federation, CropLife International and U.S. FDA, conclude that the safety of the combined-trait product can be based on the safety of the parental GE events (CLI 2005; FAO-WHO 1996; ISF 2005; WHO 1995); and 5) practical applications in the field have shown that two unrelated biotechnology traits combined together by conventional breeding do not display new characteristics or properties distinct from those present in the single event biotech products (Pilacinski, et al. 2011).

Therefore, based on the considerations above and the conclusion that MON 87411 is no more likely to pose a plant pest risk than commercially cultivated maize, it can be concluded that any progeny derived from crosses between MON 87411 and conventional maize or deregulated biotechnology-derived maize are no more likely to pose a plant risk than commercially cultivated maize.

X.M. Summary of Plant Pest Assessments

A plant pest, as defined in the PPA, is the living stage of any of the following, or a similar article, that can directly or indirectly injure, damage, or cause disease in any plant or plant product: (A) a protozoan; (B) a nonhuman animal; (C) a parasitic plant; (D) a bacterium; (E) a fungus; (F) a virus or viroid; (G) an infectious agent or other pathogen, or (H) any article similar to or allied with any of the articles specified in the preceding subparagraphs (7 U.S.C. § 7702[14]). Characterization data presented in Sections IV through VIII and Section X of this petition confirm that MON 87411, with the exception of protection from CRW larval feeding and glyphosate tolerance, is not fundamentally different from conventional maize, in terms of plant pest potential. Monsanto is not aware of any study results or observations associated with MON 87411 that would suggest an increased plant pest risk would result from its introduction.

The plant pest assessment was based on multiple lines of evidence developed from a detailed characterization of MON 87411 compared to conventional maize, followed by a risk assessment on detected differences. The plant pest risk assessment in this petition was based on the following lines of evidence: 1) insertion of a single functional copy of the Cry3Bb1 and CP4 EPSPS genes and DvSnf7 suppression cassette; 2) characterization and safety of the expressed products; 3) compositional equivalence of MON 87411 forage and grain compared to a conventional control; 4) phenotypic, agronomic, and environmental characteristics demonstrating no increased plant pest potential compared to conventional maize; 5) negligible risk to NTOs, including organisms beneficial to agriculture; 6) familiarity with maize as a cultivated crop and 7) no greater likelihood to impact agronomic practices, cultivation practices, or the management of weeds, diseases and insects, than conventional maize.

Based on the data and information presented in this petition, it is concluded that, like conventional maize and previously deregulated biotechnology-derived maize, MON 87411 is not expected to be a plant pest. Results also support a conclusion of no increased weediness potential of MON 87411 compared to conventional maize. Therefore, Monsanto Company requests a determination from USDA-APHIS that MON 87411 and any progeny derived from crosses between MON 87411 and other commercial maize be granted nonregulated status under 7 CFR part 340.

XI. ADVERSE CONSEQUENCES OF INTRODUCTION

Monsanto knows of no study results or observations associated with MON 87411 indicating that there would be adverse consequences from its introduction. MON 87411 produces the Cry3Bb1 and CP4 EPSPS proteins and the DvSnf7 RNA. The Cry3Bb1 and CP4 EPSPS proteins produced in MON 87411 are identical to the same proteins present in MON 88017 that was previously granted a determination of nonregulated status by USDA-APHIS. The unique DvSnf7 RNA in MON 87411 has been thoroughly characterized and its safety has been thoroughly assessed in this submission. As demonstrated by field results and laboratory tests, the only phenotypic differences between MON 87411 and conventional maize are protection from CRW larval feeding and glyphosate tolerance.

The data and information presented in this petition demonstrate that MON 87411 is unlikely to pose an increased plant pest risk compared to conventional maize. This conclusion is reached based on multiple lines of evidence developed from a detailed characterization of the product compared to conventional maize, followed by risk assessment on detected differences. The characterization evaluations included molecular analyses, which confirmed the insertion of one copy of the intended DNA containing the DvSnf7 suppression cassette and the *cry3Bb1* and *cp4 epsps* expression cassettes that is stably integrated at a single locus and is inherited according to Mendelian principles over multiple generations.

Analysis of key nutrients, anti-nutrients, and secondary metabolites of MON 87411 demonstrate that MON 87411 is compositionally equivalent to conventional maize. The phenotypic evaluations, including an assessment of seed germination and dormancy characteristics, plant growth and development characteristics, pollen characteristics, ecological interaction characteristics, and environmental interactions also indicated MON 87411 is unchanged compared to conventional maize. There is no indication that MON 87411 would have an adverse impact on beneficial or non-target organisms, including threatened or endangered species. Therefore, based on the lack of increased pest potential compared to conventional maize, the risks for humans, animals, and other NTOs from MON 87411 are negligible.

The introduction of MON 87411 will not adversely impact cultivation practices or the management of weeds, diseases, and insects in maize production systems. Farmers familiar with CRW-protected maize products (*e.g.*, Genuity VT Triple Pro or Genuity SmartStax) currently available will be advised to continue to employ the same crop rotational practices, weed control practices and/or volunteer control measures currently in place for these products.

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APPENDICES

Appendix A: USDA Notifications and Permits

Field trials of MON 87411 have been conducted in the U.S. since 2010. The protocols for these trials include field performance, breeding and observation, agronomics, and generation of field materials and data necessary for this petition. In addition to the MON 87411 phenotypic assessment data, observational data on pest and disease stressors were collected from these product development trials. The majority of the final reports have been submitted to the USDA. However, some final reports, mainly from the 2012-2013 seasons, are still in preparation. A list of trials conducted under USDA notifications or permits and the status of the final reports for these trials are provided in Table A-1.

Table A-1. USDA Notifications and Permits Approved for MON 87411 and Status of Trials Planted under These Notifications

USDA No.	Effective date	Release State (# of sites)	Trial Status
2010 Field Trials			
10-050-101n	21-Mar-2010	IL (4)	Submitted
10-050-105n	23-Mar-2010	IA (4)	Submitted
10-054-141n	25-Mar-2010	OH (1)	Submitted
10-081-101n	16-Apr-2010	HI (1)	Submitted
2011 Field Trials			
11-014-104n	13-Feb-2011	HI (2)	Submitted
10-351-113rm	15-Feb-2011	HI (1)	Submitted
11-034-104n	02-Mar-2011	IA (10)	Submitted
11-034-102n	02-Mar-2011	IL (12)	Submitted
11-034-107n	02-Mar-2011	OH (6)	Submitted
11-034-105n	02-Mar-2011	KS (6)	Submitted
11-039-101n	09-Mar-2011	IA (2), IL (4), NE (1)	Submitted
11-041-107n	11-Mar-2011	KS (5)	Submitted
11-045-106n	13-Mar-2011	IA (7)	Submitted
10-351-117rm	15-Mar-2011	IL (1), IN (1)	Submitted
11-045-103n	16-Mar-2011	IL (8)	Submitted
11-045-109n	16-Mar-2011	NE (1)	Submitted
11-047-102n	17-Mar-2011	OH (4)	Submitted
11-056-102n	27-Mar-2011	IA (2), IL (2), NE (1)	Submitted
11-062-101n	01-Apr-2011	IN (1)	Submitted
11-152-104n	01-Jul-2011	PR (1)	Submitted
11-123-104rm	01-Sep-2011	HI (2)	Submitted
11-265-104n	24-Oct-2011	HI (2)	Submitted
11-292-101n	18-Nov-2011	HI (1)	Submitted
11-322-102n	18-Dec-2011	HI (1)	Submitted
11-322-101n	18-Dec-2011	HI (1)	Submitted
2012 Field Trials			
12-006-101n	05-Feb-2012	GA (1)	Submitted
11-291-111rm	15-Feb-2012	IA (4), IL (1), IN (1), KS (3)	Submitted
11-305-105rm	01-Mar-2012	HI (3)	Submitted
12-038-103n	08-Mar-2012	IA (1)	Submitted
11-320-105rm	15-Mar-2012	IA (5), IL (3), NE (4), SD (1)	Submitted
12-051-101n	20-Mar-2012	IA (2), NE (3)	Submitted
12-051-102n	20-Mar-2012	IA (1), IL (9)	Submitted
12-051-103n	20-Mar-2012	IA (4), IL (4)	Submitted

Table A-1 (continued). USDA Notifications and Permits Approved for MON 87411 and Status of Trials Planted under These Notifications

USDA No.	Effective date	Release State (# of sites)	Trial Status
12-051-104n	20-Mar-2012	IA (4), KS (2)	Submitted
11-326-108rm	22-Mar-2012	IA (11), IL (20)	Submitted
12-059-120n	28-Mar-2012	IL (1), NE (1)	Submitted
12-059-109n	28-Mar-2012	HI (1)	Submitted
12-060-110n	30-Mar-2012	IA (1), IL (1), IN (1)	Submitted
12-061-105n	31-Mar-2012	IA (1), IL (1), NC (1), NE (1), PA (1)	Submitted
12-062-111n	01-Apr-2012	IA (1), IL (1), IN (1), KS (1)	Submitted
12-065-109n	04-Apr-2012	IL (2), NE (1)	Submitted
12-074-110n	13-Apr-2012	IA (1)	Submitted
12-075-114n	14-Apr-2012	IA (1), IL (1), NE (1), SD (1)	Submitted
12-116-105n	07-May-2012	CO (2), IA (2), IL (1), KS (5), NE (1), SD (1)	Submitted
12-143-104n	20-Jun-2012	HI (4), PR (1)	Submitted
12-125-106rm	01-Sep-2012	HI (3)	Submitted
12-251-101n	07-Oct-2012	AR (1), IL (1), KS (1), NE (1)	Submitted
12-312-103n	07-Dec-2012	HI (2)	In Progress
2013 Field Trials			
12-320-125rm	07-Mar-2013	KS (32), NE (20)	In Progress
13-044-101rm	07-Mar-2013	HI (2), PR (2)	In Progress
12-320-109rm	08-Mar-2013	IA (32), NE (7), SD (10), TN (3)	In Progress
12-312-109rm	15-Mar-2013	HI (8), PR(2)	In Progress
12-320-114rm	15-Mar-2013	IA (1), IL (37), IN (3), KS (10), MS (1)	In Progress
13-039-102n	15-Mar-2013	HI (11), PR (3)	In Progress
13-051-101n	22-Mar-2013	IA (8), IL (23), KS (12), NE (7)	In Progress
13-052-105n	23-Mar-2013	IA (2), IL (1), MN (12), SD (1)	In Progress
13-053-109n	24-Mar-2013	IA (8), IL (8), MS (1), NE (4), SD (4)	In Progress
13-053-110n	24-Mar-2013	IA (8), IL (8), MS (1), NE (4), SD (4)	In Progress
13-059-103n	30-Mar-2013	IA (5), IL (5), NE (4)	In Progress
13-064-123n	04-Apr-2013	IA (3)	In Progress
13-066-105n	06-Apr-2013	AR (1), CA (1), IA (5), IL (10), IN (3), KS (3), LA (1), MI (1), MO (3), MS (3), NC (2), NE (5), OH (1), PA (2), TX (2), WI (1)	In Progress

Table A-1 (continued). USDA Notifications and Permits Approved for MON 87411 and Status of Trials Planted under These Notifications

USDA No.	Effective date	Release State (# of sites)	Trial Status
13-066-109n	06-Apr-2013	IA (11), IL (6), NE (4), SD (5)	In Progress
13-119-103n	29-May-2013	HI (11), PR (3)	In Progress
13-191-102n	09-Aug-2013	GA (1)	In Progress
13-301-101n	04-Dec-2013	HI (8), PR (3)	In Progress

Appendix B: Materials, Methods, and Supplementary Results for Molecular Analyses of MON 87411

B.1. Materials and Methods

B.1.1 Test Substance

The test substance in this study was MON 87411. Genomic DNA for use in this study was extracted from seed tissue listed in the table below.

Generation	Seed Lot Number
R_4	11308817
R_4F_1	11320173
R_5	11326313
R_5F_1	11333176
R_6	11338858

B.1.2 Control Substance

The control substances were the conventional maize lines which have similar genetic background as the generations as shown in the table below. Genomic DNA for use in this study was extracted from seed tissue listed in the table below.

Control Substance	Generations	Seed Lot Number
LH244	R_4, R_5, R_6	11264747
LH244 × HCL645	R_4F_1 (hybrid)	11320031
LH244 × LH287	R_5F_1 (hybrid)	11333170

B.1.3 Reference Substance

The reference substance was plasmid vector PV-ZMIR10871, which was used to develop MON 87411. Whole plasmid served as a positive control for sequencing and bioinformatic analyses. The identity of the reference plasmid was confirmed by restriction enzyme digestion prior to the study. Documentation of the confirmation of the plasmid vector identity was archived with the raw data. Appropriate molecular size markers from commercial sources were used for size estimations on agarose gels. The unique identity of the molecular weight markers was documented in the raw data.

B.1.4 Characterization of Test, Control, and Reference Substances

The seed for the test and control substances used in this study were obtained from Monsanto Trait Development. The synthesis records for these materials are located in the ORION system. The identities of the test substance and the conventional control substance were confirmed by the sequencing in the study. No certificates of analysis (COA) or verification of identity (VOI) certificates were generated for these materials.

The Study Director reviewed the chain of custody documentation to confirm the identity of the test and control substances prior to the use of these materials in the study.

Test, control and reference DNA substance were considered stable during storage if they yielded interpretable signals in sequencing experiments and/or did not appear visibly degraded on the stained gels.

B.1.5 Genomic DNA Isolation

For sequencing library construction and PCR reactions, genomic DNA was isolated from seed tissues of the test and control substances. First the seeds were decontaminated by vigorously agitating them by hand for 30 seconds with 0.05% (v/v) Tween-20, followed by a tap water rinse. The seeds were then vigorously agitated with 0.5% (w/v) NaOCl, allowed to stand for one minute at room temperature, and rinsed with tap water. The seeds were then vigorously agitated with 1% (v/v) HCl, allowed to stand for one minute at room temperature, and rinsed with tap water. The 1% (v/v) HCl rinse was repeated one time, and then the seeds were rinsed with distilled water and placed in a drying oven at 75°C-80°C to dry. The dried seeds were ground to a fine powder in a Harbil paint shaker. Genomic DNA was extracted using a hexadecyltrimethylammonium bromide (CTAB) extraction protocol. Briefly, 16 ml CTAB buffer (1.5% (w/v) CTAB, 75 mM Tris HCl (pH 8.0), 100 mM EDTA (pH 8.0), 1.05 M NaCl, and 0.75% (w/v) PVP) and RNase A was added to ground seed tissue. The samples were incubated at ~65° for 60 minutes with intermittent mixing. The samples were cooled to room temperature and subjected to three rounds of chloroform:isoamyl alcohol (24:1) extraction. Approximately 1.6 ml of 10% CTAB solution (10% (w/v) CTAB and 0.7 M NaCl) was added to the samples, mixed by inversion and extracted once with chloroform:isoamyl alcohol (24:1). Approximately 15 ml of CTAB precipitation buffer (1% (w/v) CTAB, 50 mM Tris HCl (pH 8.0), 10 mM EDTA (pH 8.0)) was added to the samples, mixed by inversion, and allowed to stand at room temperature for 50-70 minutes. Following centrifugation to precipitate the DNA, the samples were dissolved in high salt TE buffer (10 mM Tris HCl (pH 8.0), 1 mM EDTA (pH 8.0), 1 M NaCl). The DNA was precipitated with 3 M sodium acetate (pH 5.2) and 100% (v/v) ethanol. The DNA pellets were washed with 70% ethanol, air dried and resuspended in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0).All extracted DNA was stored in a 4°C refrigerator.

B.1.6 DNA Quantification

PV-ZMIR10871 DNA and extracted genomic DNA were quantified using a Qubit™ Fluorometer (Invitrogen, Carlsbad, CA). For directed sequencing, genomic DNA was quantified using a Nanodrop™ Spectrophotometer (Thermo Scientific, Wilmington, DE) according to manufacturer's instructions.

B.1.7 Agarose Gel Electrophoresis

After quantification, approximately 0.5-1 microgram (µg) of the extracted DNA for NGS/JSA sequencing library construction was run on a 1% (w/v) agarose gel to check the quality.

B.1.8 Shearing of DNA

Approximately 1 µg of DNA from the test, control and reference substances were sheared using a Covaris S-220 ultrasonicator. The DNA was diluted to ~ 18-20 ng/µl in Buffer EB (Qiagen Inc., Valencia, CA) and fragmented using the following settings to create approximately 325 bp fragments with 3' or 5' overhangs: duty cycle of 10; peak incident power of 175; intensity of 5.0, 200 bursts per cycle, in the frequency sweeping mode at ~ 6°C for 80 seconds for test and control DNA or 60 seconds for reference DNA.

B.1.9 Bioanalyzer Analysis

One microliter of sheared genomic DNA was diluted 1:10 in Buffer EB and run on a DNA High Sensitivity chip on an Agilent 2100 Bioanalyzer to check the quality of the shearing. After preparing the chip according to the manufacturer's instructions, 1 µl of each diluted DNA sample or water was added to individual wells and the chip was run on the Bioanalyzer using the dsDNA, High Sensitivity Assay reagents.

B.1.10 Paired End Library Preparation

Paired end genomic DNA libraries were prepared for the test, control, and reference substances using the Illumina TruSeq DNA Sample Preparation Kit (Illumina, San Diego, CA) according to the manufacturer's instructions for the low-throughput procedure with the following exception: a Sage Science Pippin Prep DNA Size Selection system (Sage Science Inc., Beverly, MA) was used to size select the DNA fragments instead of agarose gel electrophoresis.

First, the 3' and 5' overhangs of the DNA fragments generated by the shearing process were converted into blunt ends by adding 10 µl of Illumina Resuspension Buffer and 40 µl of Illumina End Repair mix to each sample and mixing thoroughly by pipette.

Then the libraries were incubated for 30 minutes at 30°C. The end-repaired samples were purified using AMPure XP beads (Beckman Coulter, Inc., Brea, CA) and resuspended in 17.5 µl of Illumina Resuspension Buffer.

Fifteen microliters of each library was transferred to a new tube for adenylation, which adds a single adenosine nucleotide to the 3' ends of the blunt fragments. Then 2.5 µl of Illumina Resuspension Buffer and 12.5 µl of Illumina A-Tailing Mix were added to each library and mixed thoroughly by pipetting. The libraries were incubated for 30 minutes at 37°C. After incubation, 2.5 µl each of individual adapter index, Illumina Resuspension Buffer, and Illumina DNA Ligase Mix was immediately added to each tube, and mixed thoroughly by pipetting to begin ligation of each library. The libraries were incubated for 10 minutes at 30°C. Then 5 µl of Illumina Stop Ligase Buffer was added to each tube and mixed thoroughly by pipetting to stop the ligation reaction. Next, another AMPure XP bead cleanup was performed on the libraries which were then resuspended in 32.5 µl of Illumina Resuspension Buffer prior to size selection.

The libraries were run on the Sage Science Pippin Prep Size Selection system using 2% gel cassettes according to the manufacturer's instructions. Ten microliters of loading solution were added to 30 µl of each of the purified libraries and mixed thoroughly by pipetting. Forty microliters of Marker B was loaded in the cassette well designated for the reference sample, and 40 µl of each DNA library was loaded in the remaining wells for analysis. After elution of the desired size range (~445 bp) of DNA fragments, the DNA sample in the elution chamber of the cassette was removed from the cassette by pipette and transferred into PCR strip tubes.

After removal from the Pippin Prep, the libraries were again put through the AMPure XP bead cleanup procedure and resuspended in 22.5 µl of Illumina Resuspension Buffer. Twenty µl of the resuspended library was added to five microliters of Illumina PCR Primer Cocktail and 25 µl of Illumina PCR Master Mix and mixed thoroughly by pipetting. The DNA fragments were enriched through PCR using the following cycling conditions: 1 cycle at 98°C for 30 seconds; 10 cycles at 98°C for 10 seconds, 60°C for 30 seconds, 72°C for 30 seconds; 1 cycle at 72°C for 5 minutes. Following PCR amplification, a final AMPure XP bead cleanup was performed on the libraries which were resuspended in 32.5 µl of Illumina Resuspension Buffer. Finally, 1 µl of each DNA library was diluted 1:10 in Buffer EB for running in a DNA High Sensitivity chip on an Agilent 2100 Bioanalyzer as described above. All purified library DNA was stored in a -20°C freezer.

B.1.11 Next-Generation Sequencing

The library samples described above were sequenced by The Genome Analysis Center (TGAC, Monsanto) using Illumina HiSeq technology that produces short sequence reads (~100 bp long). Sufficient numbers of these sequence fragments were obtained (>75x genome coverage) to comprehensively cover the genomes of the test event and the conventional control (Kovalic et al., 2012). Sequencing runs performed by the TGAC were assessed versus standard QC criteria.

B.1.12 Junction Sequence Analysis Bioinformatics

High-throughput sequence reads were enriched by mapping to the PV-ZMIR10871 transformation plasmid sequence using the local alignment software BlastAll (V2.2.21) in

order to collect all reads that were sourced from the plasmid as well as reads with sequences representing integration point. All collected reads were further refined by removing sequencing artefacts of sequencing adapters, redundant reads and low quality read ends. All quality refined reads were then used to identify junction points with custom developed bioinformatics tools as detailed below. All significant junctions are reported for both the test and the control samples. All software versions were documented in the archived data package and the software versions which were used in this study have been archived.

B.1.12.1 Sequencing Read Enrichment

The transformation plasmid PV-ZMIR10871 sequence was used as reference to find all reads that were either fully matched to the insert plasmid fragments or partially matched with junction sequences. A junction sequence is characterized by a combination of transformation plasmid sequence and flanking sequence that is likely to be host genome flanking sequence or any other co-inserted sequence. Local alignment with BlastAll (V2.2.21) was performed to collect all sequencing reads with an e-Score of less than 1e-5 and at least 30 bases match of greater than 96.7% identity to the transformation plasmid (Kovalic et al., 2012). Both reads of the paired-end sequences were collected in all cases.

B.1.12.2 Read Quality Refinement

In order to identify all duplicate read pairs, a high quality segment (bases 3-42) of all collected pairs was compared to all others with short sequence alignment software (Bowtie v.0.12.3) allowing up to 1 mismatch. If multiple read pairs were matched at both paired reads, such read pairs were deemed redundant and only the best quality pair of reads was kept for further analysis.

Computer software Novoalign (v.2.06.09) was used to remove any adapter sequences at either end of the sequencing reads. Low quality read ends (with phred scores of 12 or lower) were trimmed. Only reads of 30 bases or longer after adapter and quality trimming were collected. A custom developed Perl script "farm_gen_sm_bucket.pl" was used to perform read enrichment and read quality refinement as described above.

B.1.12.3 Junction Detection

Enriched and quality refined reads of both test and control samples were aligned against the whole PV-ZMIR10871 transformation plasmid sequence in order to detect junction sequences using custom developed Perl script "farm_blast_map.pl". Reads with partial match to the transformation plasmid of at least 30 bases match and 96.7% identity were collected as potential junction sequences (Kovalic et al., 2012). The collected reads were also aligned against the genomic sequence collection of the host genome in order to remove junction reads sourced from the plant endogenous homologues. Custom developed Perl script “junctions_by_bn.pl” was used to identify the junction position on the transformation plasmid and their supporting junction reads. For each junction position, all supporting junction reads were aligned at the 30 plasmid bases proximal to

the junction position. The remaining bases of these reads were sorted to show the alignment and the consensus of the flanking junction sequences past the junction point.

B.1.12.4 Effective Sequencing Depth Determination

A single copy locus from the native plant genome (Pyruvate decarboxylase (*pdc3*), GenBank accession.version: AF370006.2) was selected from the *Zea mays* genome and used to determine the effective sequence depth coverage. All reads with at least 30 bases match and 96.7% identity were considered as reads sourced from this locus. A custom developed Perl script "farm_match_reads.pl" was used to perform such alignment and calculate the actual depth distribution at this locus.

B.1.12.5 Positive Spike-in Controls

To produce "spike-in" positive control samples for sequencing, plasmid DNA libraries were created as described above (B.1.8 – B.1.10) and then diluted to 1 and 1/10 maize genome equivalents (representation of the plasmid DNA at concentrations equivalent to single copy or 1/10 copy per genome) before pooling with samples produced from the control materials (as described above).

B.1.13 PCR and DNA Sequence Analyses to Examine the Insert and Flanking Sequences in MON 87411

Overlapping PCR products, denoted as Product A, Product B, Product C, Product D, Product E, Product F, Product G and Product H were generated that span the insert and adjacent 5′and 3′ flanking DNA sequences in MON 87411. For each fragment generation experimental conditions were chosen to successfully produce on-target amplifications. These products were analyzed to determine the nucleotide sequence of the insert in MON 87411, as well as that of the DNA flanking the 5′ and 3′ ends of the insert.

The PCR analyses for Product A, Product D, Product E and Product H were each conducted using 50 ng of genomic DNA template in a 50 µl reaction volume. The reaction contained a final concentration of 0.5 µM of each primer and 1x concentration of Phusion High Fidelity PCR Master Mix with HF Buffer (NEB, Ipswich, MA).

The PCR analyses for Product B, Product C and Product F were each conducted using 50 ng of genomic DNA template in a 50 µl reaction volume. The reaction contained a final concentration of 0.2 µM of each primer and 1.25 units/reaction of Ex Taq Polymerase (Takara Bio Inc., Shiga, Japan).

The PCR analysis for Product G was each conducted using 50 ng of genomic DNA template in a 20 µl reaction volume. The reaction contained a final concentration of 0.5 µM of each primer and 1x concentration of Phusion High Fidelity PCR Master Mix with HF Buffer (NEB, Ipswich, MA).

The amplification of Product A, Product D, Product F and Product H were performed under the following cycling conditions: 1 cycle at 98°C for 30 seconds; 25 cycles at 98°C for 10 seconds, 72°C for 2 minutes 20 seconds; 1 cycle at 72°C for 5 minutes.

The amplification of Product B and Product C were performed under the following cycling conditions: 1 cycle at 98°C for 10 seconds; 10 cycles at 70°C, decreasing 1 °C per cycle for 30 seconds, 72°C for 2 minutes; 25 cycles at 98°C for 10 seconds, 60°C for 30 seconds, and 72°C for 2 minutes.

The amplification of Product E was performed under the following cycling conditions: 1 cycle at 98°C for 30 seconds; 25 cycles at 98°C for 10 seconds, 65 °C for 20 seconds, 72°C for 2 minutes; 1 cycle at 72°C for 5 minutes.

The amplification of Product G was performed under the following cycling conditions: 1 cycle at 98°C for 30 seconds; 25 cycles at 98°C for 10 seconds, 71 °C for 20 seconds, 72°C for 2 minutes; 1 cycle at 72°C for 5 minutes.

Aliquots of each PCR product were separated on a 1.0% (w/v) agarose gel and visualized by ethidium bromide staining to verify that the products were the expected size. Prior to sequencing, each verified PCR product was either purified using the QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions and quantified using the Nanodrop spectrophotometer according to the manufacturer's instructions or purified using Exo-SAP IT (Affymetrix, Santa Clara, CA). The purified PCR products were sequenced using multiple primers, including primers used for PCR amplification. All sequencing was performed by Monsanto TGAC (The Genome Analysis Center) using BigDye terminator chemistry (Applied Biosystems, Foster City, CA).

A consensus sequence was generated by compiling sequences from multiple sequencing reactions performed on the overlapping PCR products. This consensus sequence was aligned to the PV-ZMIR10871 sequence to determine the integrity and organization of the integrated DNA and the 5′ and 3′ insert-to-flank DNA junctions in MON 87411.

B.1.14 PCR and DNA Sequence Analyses to Examine the Integrity of the DNA Insertion Site in MON 87411.

To examine the MON 87411 T-DNA insertion site in control maize, PCR and sequence analyses were performed on genomic DNA from the conventional control maize. The primers used in this analysis were designed from the DNA sequences flanking the insert in MON 87411. A forward primer specific to the DNA sequence flanking the 5′ end of the insert was paired with a reverse primer specific to the DNA sequence flanking the 3′ end of the insert.

The PCR reactions were conducted using 50 ng of genomic DNA template in a 50 µl reaction volume. The reaction contained a final concentration of 0.5 µM of each primer and 1x concentration of Phusion High Fidelity PCR Master Mix with HF Buffer (NEB). The amplification was performed under the following cycling conditions: 1 cycle at 98°C

for 30 seconds; 25 cycles at 98°C for 10 seconds, 72°C for 2 minutes 20 seconds; 1 cycle at 72°C for 5 minutes.

A small aliquot of each PCR product was separated on a 1.0% (w/v) agarose gel and visualized by ethidium bromide staining to verify that the PCR products were the expected size prior to sequencing. Only the verified PCR product from the conventional control LH244 was either purified using the QIAquick PCR Purification Kit (Qiagen, Inc.) according to the manufacturer's instructions and quantified using the Nanodrop spectrophotometer according to the manufacturer's instructions or purified using Exo-SAP IT (Affymetrix, Santa Clara, CA). The purified PCR product was sequenced using multiple primers, including primers used for PCR amplification. All sequencing was performed by TGAC using BigDye terminator chemistry (Applied Biosystems).

A consensus sequence was generated by compiling sequences from multiple sequencing reactions performed on the verified PCR product. This consensus sequence was aligned to the 5′ and 3′ sequences flanking the MON 87411 insert to determine the integrity and organization of the insertion site.

B.2 Supplementary Results

Junction Sequence Class A:

R_4

Plasmid DNA sequence ^Flank

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R_4F_1

Plasmid DNA sequence ^Flank

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R_5

Plasmid DNA sequence ^Flank

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R_5F_1

Plasmid DNA sequence ^Flank

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R_6
Plasmid DNA sequence   ^Flank

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Junction Sequence Class B:

R_4

Plasmid DNA sequence ^Flank

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Plasmid DNA sequence ^Flank

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R_5

Plasmid DNA sequence ^Flank

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R_5F_1
Plasmid DNA sequence   ^Flank

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R_6

Plasmid DNA sequence ^Flank

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Figure B-1. Junction sequences of MON 87411 detected by NGS/JSA

Trimmed nucleotide alignment of the detected junction sequences. The detected junction sequences are ordered by junction sequence class (Kovalic et a., 2012) and then by length. The junction point between the T-DNA border and flanking sequence is indicated by the "^" character. Both alignments are trimmed to include only the 30 plasmid bases proximal to the junction point, as well as all sequence of the flank. Both alignments are shown 5′->3′ beginning with the detected plasmid sequence.

References for Appendix B

Kovalic, D., C. Garnaat, L. Guo, Y. Yan, J. Groat, A. Silvanovich, L. Ralston, M. Huang, Q. Tian, A. Christian, N. Cheikh, J. Hjelle, S. Padgette and G. Bannon. 2012. The use of next generation sequencing and junction sequence analysis bioinformatics to achieve molecular characterization of crops improved through modern biotechnology. The Plant Genome Journal 5:149-163.

Appendix C: Materials, Methods and Results for Characterization of Cry3Bb1 and CP4 EPSPS Proteins Produced in MON 87411

C.1 Characterization of Cry3Bb1 Protein in MON 87411

C.1.1 Materials for Cry3Bb1 Characterization

The MON 87411-produced Cry3Bb1protein (lot 11355394) was purified from the grain of MON 87411 (lot 11330153). The MON 87411-produced Cry3Bb1 protein was stored in a -80 ºC freezer in a buffer solution containing 10 mM sodium carbonate/bicarbonate, 0.1 mM EDTA, pH 10.0.

The E. Coli-produced Cry3Bb1 protein (lot 11309151) was used as the reference substance. The Cry3Bb1 protein reference substance was generated from cell paste produced by large-scale fermentation of E. coli containing the pMON70855 expression plasmid. The coding sequence for cry3Bb1 contained on the expression plasmid (pMON70855) was confirmed prior to and after fermentation. The E. coli-produced Cry3Bb1 protein was characterized previously.

C.1.2 Cry3Bb1 Protein Purification

The plant-produced Cry3Bb1 protein was purified from grain of MON 87411. The purification procedures were not performed under a GLP plan; however, all procedures were documented on worksheets and, where applicable, SOPs were followed. Cry3Bbl protein was extracted from seven batches (~ 3 kg each) of MON 87411 grain with 50 mM sodium carbonate/bicarbonate, pH 10.8, 1 mM EDTA, 1mM Benzamidine HCl, 0.5 mM PMSF, 0.1% Triton X-100 (1: 5 (w/v) grain to buffer ratio). The extracts were clarified by centrifugation. The clarified extract was brought to 40% saturation by the addition of ammonium sulfate and clarified by centrifugation. The resulting supernatant was brought to 50% saturation by the addition of ammonium sulfate, clarified by centrifugation, and the pellet resuspended in one-tenth the original volume in Capto Q Buffer A (50 mM sodium carbonate/bicarbonate, pH 10.8, 1 mM EDTA, 1mM Benzamidine HCl, 0.5 mM PMSF). The dissolved pellets were then diluted 1:1 with CaptoQ buffer A and loaded onto a Capto Q (GE Healthcare, Piscataway, NJ) anion exchange column that was pre-equilibrated with CaptoQ buffer A. The column was washed with 20% buffer B (50 mM sodium carbonate/bicarbonate, pH 10.8, 1 mM EDTA, 1 mM Benzamidine HCl, 0.5 mM PMSF, 500 mM NaCl). The Cry3Bbl protein was eluted with 40% buffer B. The eluted protein was diluted 1:1 with 100mM Tris- HCl pH 8, 1M ammonium sulfate, 1 mM EDTA, 1 mM Benzamidine HCl, 0.5 mM PMSF. The diluted sample was loaded onto a Phenyl Sepharose HP (GE Healthcare) column pre-equilibrated with Phenyl Sepharose buffer A (50 mM Tris-HCl pH 8, 0.5 M ammonium sulfate, 1 mM EDTA, 1 mM Benzamidine HCl, 0.5 mM PMSF). The column was washed with Phenyl Sepharose buffer A and then eluted with 38% Phenyl Sepharose buffer B (50 mM Tris-HCl pH8, 1 mM EDTA, 1 mM Benzamidine HCl, 0.5 mM PMSF). As a final purification step, the Phenyl Sepharose 38% buffer B pool containing the Cry3Bb1 passed at a slow flow rate

through a PBS equilibrated immunoaffinity column. The column consisted of Protein L Agarose cross-linked to an anti-Cry3Bb1 monoclonal antibody. The bound Cry3Bb1 protein was eluted from the column using 100 mM glycine/150 mM NaCl, pH 2.5. Fractions were collected, immediately neutralized to pH 8.0 using 1 M Tris-HCl, pH 8.0. Elution fractions were pooled and concentrated using Spin X spin concentrators (Corning, Corning NY) with a 10K MWCO pretreated with 5% PEG (average molecular weight 3350). This MON 87411 Cry3Bb1 purified from the grain of MON 87411 was aliquoted and stored in a -80 °C freezer.

The final buffer composition of the sample was 10 mM sodium carbonate/bicarbonate, 0.1 mM EDTA, pH 10.0. The purified MON 87411-produced Cry3Bb1 protein was aliquoted, assigned lot 11355394, and stored at in a -80°C freezer.

C.1.3 N-Terminal Sequencing of Cry3Bb1

C.1.3.1 Methods

N-terminal sequencing by automated Edman degradation chemistry was carried out in an attempt to confirm the identity of MON 87411-produced Cry3Bb1

MON 87411-produced Cry3Bb1 was separated by SDS-PAGE and transferred to PVDF membrane. The blot was stained using Coomassie Blue R-250. The major bands at 77 and ~65 kDa containing MON 87411-produced Cry3Bb1 were excised from the blot and used for N-terminal sequence analysis. The analysis was performed for 15 cycles using automated Edman degradation chemistry (Hunkapiller et al., 1983) using an Applied Biosystems 494 Procise Sequencing System equipped with 140C Microgradient system a Perkin Elmer Series 200 UV/VIS Absorbance Detector with Procise™ Control Software (version 2.1). Chromatographic data were collected using SequencePro (Applied Biosystems, Foster City CA; version 2.1). A phenylthiohydantoin-amino acid (PTH-AA) standard solution (Applied Biosystems) was used to chromatographically calibrate the instrument for the analysis. A control protein, β-lactoglobulin, (Applied Biosystems) was analyzed before and after the sequence analysis of the MON 87411-produced Cry3Bb1 protein to verify that the sequencer met performance criteria for repetitive yield and sequence identity.

C.1.3.2 Results of the N-terminal Sequence Analysis

N-terminal sequencing analysis was performed on the MON 87411-produced Cry3Bb1 protein. The reaction did not yield any observable sequence for the ~77.0 kDa band, presumably because the N-terminus was blocked. It is well documented that the N-terminal residue of many proteins is blocked due to post-translational modification *in vivo* (Polevoda and Sherman, 2000). Blocked N-terminal amino acids cannot be directly sequenced by Edman degradation.

N-terminal sequencing of the first 15 amino acids was performed on the ~65 kDa band of MON 87411-produced Cry3Bb1. A sequence for the Cry3Bb1 protein deduced from the *cry3Bb1* gene present in grain of MON 87411 was observed. The data obtained

correspond to the deduced Cry3Bb1 protein beginning at amino acid position- 50 (Table C-1). Hence, the sequence information confirms the identity of the ~65 kDa Cry3Bb1 band isolated from the grain of MON 87411.

Table C-1. N-Terminal Sequence of the MON 87411-produced Cry3Bb1

MW	Expected Sequence	Experimental Results	Position
77 kDa	MANPNNRSEHDTIKV	No Sequence Observed	
65 kDa	TEDSSTEVLDNSTVK	TEDSSTEVLDNSTVK	50-64

The expected amino acid sequence of the N-terminus of the ~65 kDa Cry3Bb1 protein was deduced from the *cry3Bb1* gene present in MON 87411. The experimental sequences obtained from the MON 87411-produced Cry3Bb1 were compared to the expected sequence. The single letter IUPAC-IUB amino acid code is A, alanine; D, aspatic acid; E, glutamic acid; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; R, arginine; S, serine; T, threonine; V, valine.

C.1.4 MALDI-TOF Tryptic Mass Map Analysis of Cry3Bb1

C.1.4.1 Methods

MALDI-TOF MS mass fingerprint analysis was used to confirm the identity of the MON 87411-produced Cry3Bb1 protein. A MON 87411-produced Cry3Bb1 protein sample was separated by SDS-PAGE and the gel was stained using Brilliant Blue G-Colloidal stain (Sigma-Aldrich, St. Louis, MO). Each ~77 kDa band was excised and destained with 40% (v/v) methanol / 10% (v/v) acetic acid. The excised bands were washed in 100 mM ammonium bicarbonate and treated with 10 mM dithiothreitol (DTT) at 37°C for 2 h followed by incubation for 20 minutes with 10 mM iodoacetic acid in the dark. The excised bands were then washed with 25 mM ammonium bicarbonate, dried using vacuum centrifugation and rehydrated with 20 µl 20 µg/ml trypsin (Promega, Madison, WI). After 1 h, excess liquid was removed and the excised bands were incubated at 38°C overnight in 40 µl of 10% (v/v) acetonitrile in 25 mM ammonium bicarbonate. The excised bands were sonicated for 5 min and the resulting extract was transferred to new microcentrifuge tube labeled Extract 1 and dried using vacuum centrifugation. The excised bands were then extracted two more times, each with 30 µl of a 60% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid, 0.1% (v/w) β-octyl-glucopyranoside solution and sonicated for 5 min. These two extracts were pooled into a new tube labeled Extract 2 and dried using vacuum centrifugation. Extract 1 and 2 were then treated with 0.1% trifluoroacetic acid (TFA) and dried. The extracts were solubilized in 50% (v/v) acetonitrile, 0.1% TFA and sonicated for 5 min. Extracts were spotted to wells on an analysis plate and mixed with either 2,5-dihydroxybenzoic acid (DHB, Thermo Fisher Scientific Inc. Waltham, MA), α-cyano-4-hydroxycinnamic acid (α-Cyano, Thermo Fisher Scientific Inc.), or 3,5-dimethoxy-4-hydroxycinnamic acid (Sinapinic acid, Thermo Fisher Scientific Inc.). The samples in DHB, α-Cyano, and Sinapinic acid matrix were analyzed in the 300 to 5000 Da, the 500 to 5000 Da, and 500 to 7000 Da range, respectively. CalMix 2™ was used as the external calibrant (Sequazyme™ Peptide Mass Standards kit, AB SciEx, Foster City, CA). The analysis was performed on a Voyager™ DE Pro Biospectrometry™ workstation (Applied

Biosystems) using Voyager Instrument Control Panel software (version 5.10.2) and Data Explorer data analysis software (version 4.0.0.0). Protonated peptide masses were isotopically resolved in reflector mode (Aebersold 1993; Billeci and Stults 1993). GPMAW32 software (Lighthouse Data, Odense M, Denmark) was used to generate an in silico digest of the Cry3Bb1 protein sequence. Masses within 1 Da of the monoisotopic mass were matched against the in silico digest of the MON 87411-produced Cry3Bb1 sequence. All matching masses were tallied and a coverage map was generated for the mass fingerprint.

C.1.4.2 Results of MALDI-TOF Tryptic Mass Map Analysis

The identity of the MON 87411-produced Cry3Bb1 protein was confirmed by MALDI-TOF MS analysis of peptide fragments produced by the trypsin digestion of the MON 87411-produced Cry3Bb1 protein. There were 41 unique peptides identified that corresponded to the expected masses (Table C-2). The identified masses were used to assemble a peptide map of the Cry3Bb1 protein (Figure C-1). The experimentally determined coverage of the Cry3Bb1 protein was 73% (478 out of 652 amino acids). This analysis further confirms the identity of MON 87411-produced Cry3Bb1 protein.

As part of the mass fingerprint analysis, a mass was identified that corresponded to the predicted mass of an acetylated N-terminal tryptic fragment. The N-terminus of the ~77.0 kDa MON 87411-produced Cry3Bb1 protein begins with alanine at position two.

Table C-2. Summary of the Tryptic Masses Identified for the MON 87411-Produced Cry3Bb1 Using MALDI-TOF MS

Observed Mass[1]	Expected Mass	Diff.[2]	Fragment	Sequence[3]
564.33	564.31	0.02	45-48	EFLR
619.35	619.34	0.01	299-303	TELTR
686.45	686.42	0.03	155-160	TPLSLR
727.37	727.35	0.02	2-7	[4]Ac-ANPNNR
794.44	794.40	0.04	394-400	LSFDGQK*
	794.48	0.04	292-298	LYSKGVK*
877.13	876.47	0.66	161-167	SKRSQDR
897.61	897.58	0.03	513-520	ITQLPVVK
925.51	925.47	0.04	573-580	YASTTNLR
937.56	937.53	0.03	348-355	LRPGYFGK
958.58	958.56	0.02	646-653	IEFIPVQL
1026.52	1026.49	0.03	258-266	GSTYDAWVK
1067.58	1067.52	0.06	181-190	NSMP…AVSK
1128.55	1128.56	0.01	377-386	TITS…YGDK
1185.67	1185.70	0.03	556-566	VTLN...LLQR
1350.67	1350.64	0.03	170-180	ELFS...SHFR
1362.72	1362.70	0.02	490-500	GTIP...WTHR
1385.68	1385.66	0.02	501-512	SDVF.…DAEK
1457.84	1457.74	0.10	404-417	TIAN….PNGK
1460.93	1460.86	0.07	554-566	FKVT….LLQR
1496.82	1496.77	0.05	319-331	YGPT….NSIR
1590.97	1590.91	0.06	641-653	IYID…..PVQL
1619.90	1619.83	0.07	168-180	IREL….SHFR
1649.96	1649.84	0.12	626-640	NELI….SNEK
1765.05	1764.98	0.07	304-318	DIFT….TLQK
2001.13	2001.03	0.10	332-347	KPHL….FHTR
2041.22	2041.08	0.14	581-597	LFVQ….YINK
2147.03	2146.93	0.10	472-488	AYSH….MQDR
2298.38	2298.25	0.13	273-291	EMTL….YDIR
2394.23	2394.10	0.13	356-376	DSFN….GSSK
2402.48	2402.35	0.13	191-211	FEVL….LLLK
2483.41	2483.32	0.09	521-545	AYAL…LFLK
2484.26	2484.06	0.20	212-232	DAQV….EFYR
2499.18	2499.08	0.10	425-445	VDFS….YDSK
2552.36	2552.20	0.16	237-257	LTQQ….NGLR
2637.54	2637.29	0.25	131-153	ALAE….NSWK
2655.39	2655.18	0.21	425-446	VDFS…..DSKR
2734.57	2734.28	0.29	447-471	NNGH….PLEK
3444.04	3443.64	0.40	15-44	VTPN…..LNYK*
	3443.71	0.33	404-434	TIAN…...DDQK*
3988.68	3988.93	0.25	15-48	VTPN…..EFLR

[1]The observed mass was collected from at least one of three matrices including α-cyano, DHB and sinapinic acid. The observed mass shown is the mass closest to the expected mass.

[2]The data represent the calculated difference between the expected mass and the observed mass

[3]For peptide matches greater than nine amino acids in length the first 4 residues and last 4 residues are shown separated by three dots (…)

[4] AC is the abbreviation for acetylation

*The expected peptide masses are nearly identical (< 1 dalton). Because this analysis could not determine with certainty which expected peptide was actually observed, the peptides with an asterisk (*) were not included in determining sequence coverage (Figure C-1).

001 ~~M~~ANPNNRSEH DTIKVTPNSE LQTNHNQYPL ADNPNSTLEE LNYKEFLRMT

051 EDSSTEVLDN STVKDAVGTG ISVVGQILGV VGVPFAGALT SFYQSFLNTI

101 WPSDADPWKA FMAQVEVLID KKIEEYAKSK ALAELQGLQN NFEDYVNALN

151 SWKKTPLSLR SKRSQDRIRE LFSQAESHFR NSMPSFAVSK FEVLFLPTYA

201 QAANTHLLLL KDAQVFGEEW GYSSEDVAEF YRRQLKLTQQ YTDHCVNWYN

251 VGLNGLRGST YDAWVKFNRF RREMTLTVLD LIVLFPFYDI RLYSKGVKTE

301 LTRDIFTDPI FLLTTLQKYG PTFLSIENSI RKPHLFDYLQ GIEFHTRLRP

351 GYFGKDSFNY WSGNYVETRP SIGSSKTITS PFYGDKSTEP VQKLSFDGQK

401 VYRTIANTDV AAWPNGKVYL GVTKVDFSQY DDQKNETSTQ TYDSKRNNGH

451 VSAQDSIDQL PPETTDEPLE KAYSHQLNYA ECFLMQDRRG TIPFFTWTHR

501 SVDFFNTIDA EKITQLPVVK AYALSSGASI IEGPGFTGGN LLFLKESSNS

551 IAKFKVTLNS AALLQRYRVR IRYASTTNLR LFVQNSNNDF LVIYINKTMN

601 KDDDLTYQTF DLATTNSNMG FSGDKNELII GAESFVSNEK IYIDKIEFIP

651 VQL

Figure C-1. MALDI-TOF MS Coverage Map of the MON 87411-produced Cry3Bb1

The amino acid sequence of the MON 87411-produced Cry3Bb1 protein was deduced from the *cry3Bb1* gene present in MON 87411. Boxed regions correspond to tryptic peptides that were identified from the MON 87411-produced Cry3Bb1 protein sample using MALDI-TOF MS. In total, 73% (478 out of 652 amino acids) of the expected protein sequence was identified.

C.1.5 Western Blot Analysis-Immunoreactivity of Cry3Bb1

C.1.5.1 Methods

Western blot analysis was performed as follows to confirm the identity of the MON 87411-produced Cry3Bb1 protein and to compare the immunoreactivity of the MON 87411-produced and *E. coli*-produced Cry3Bb1 protein. MON 87411-produced and *E. coli*-produced Cry3Bb1 protein were diluted in 1× LB (62 mM Tris-HCl, 5% (v/v) 2-mercaptoethanol, 2% (w/v) sodium dodecyl sulfate (SDS), 0.005% (w/v) bromophenol blue, 10% (v/v) glycerol, pH 6.8) and heated to 100°C for 3 minutes. Each protein sample was loaded in duplicate at ~1, 2, and 3 ng purity corrected Cry3Bb1 protein per lane onto a pre-cast Tris-glycine (4-20 %) polyacrylamide gradient mini-gel (Invitrogen). Pre-stained molecular weight standards (Precision Plus Protein Standards, Bio-Rad) were loaded on the gel for molecular weight reference and to verify electrotransfer of the proteins to the membrane. Following electrophoresis at a constant voltage, proteins were electrotransferred to a PVDF membrane (Invitrogen).

The membrane was blocked with 5% nonfat dry milk (NFDM) in 1× phosphate buffered saline containing 0.05% (v/v) Tween-20 (PBST) and incubated with a goat anti-Cry3Bb1

antibody (lot G839074) at a dilution of 1:2500 in 1 % NFDM in PBST. After washing with PBST, the membrane was incubated with horseradish peroxidase (HRP) -conjugated horse anti-goat IgG (H+L) IgG (Vector Labs, Burlingame CA) at a dilution of 1:5000 in 1 % NFDM in PBST and washed again, with PBST. Immunoreactive bands were detected using the ECL™ detection system (GE Healthcare) and Amersham Hyperfilm (GE Healthcare). The film was developed using a Konica SRX-101A automated film processor (Konica Minolta Medical & Graphic, Inc., Tokyo, Japan).

Quantification of the bands on the blot was performed on a GS-800 densitometer with the supplied Quantity One® software (version 4.6.7, Bio-Rad) using the using the lane selection and contour tool. The signal intensities of the immunoreactive bands migrating at the expected position for the Cry3Bb1 protein were quantified as "contour quantity" values. The immunoreactivity was reported in OD × mm^2.

C.1.5.2 Results of Cry3Bb1 Protein Immunoreactivity Equivalence

Western blot analysis was conducted using goat anti-Cry3Bb1 polyclonal antibody as additional means to confirm the identity of the Cry3Bb1 protein isolated from the grain of MON 87411 and to assess the equivalence of the immunoreactivity of the MON 87411-produced and *E. coli*-produced Cry3Bb1 proteins.

The results showed that immunoreactive bands migrating at the expected apparent MW were present in all lanes loaded with the MON 87411-produced (Figure C-2, lanes 10-15) or *E. coli*-produced (Figure C-2, lanes 3-8) Cry3Bb1 proteins. For each amount loaded, comparable signal intensity was observed between the MON 87411- and *E. coli*-produced Cry3Bb1 protein bands. As expected, the signal intensity increased with increasing load amounts of the MON 87411-produced and *E. coli*-produced Cry3Bb1 proteins, thus supporting identification of MON 87411-produced Cry3Bb1 protein.

To compare the immunoreactivity of the MON 87411- and the *E. coli*-produced Cry3Bb1 proteins, densitometric analysis was conducted on bands that migrated to the expected apparent MW for Cry3Bb1 proteins (~77-55 kDa). The signal intensity (reported in OD × mm^2) of the immunoreactive bands identified by anti-Cry3Bb1 antibodies and migrating between the full-length protein at 77.0 kDa and the insecticidal tryptic core (at ~55 kDa) were included in the mean signal intensities (Table C-3). Because the mean signal intensity of the MON 87411-produced Cry3Bb1 protein band was within 35% of the mean signal intensity of the *E. coli*-produced Cry3Bb1 protein, the MON 87411-produced and *E. coli*-produced Cry3Bb1 proteins were determined to have equivalent immunoreactivity.

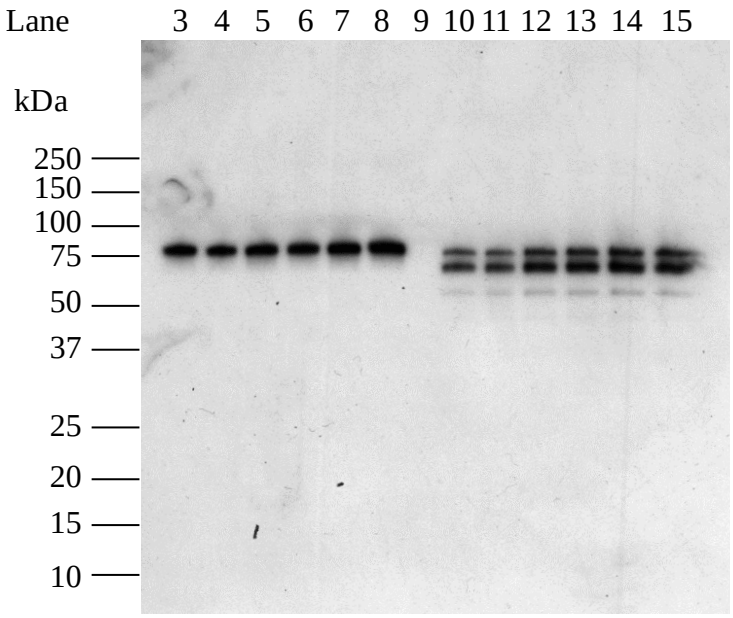

Figure C-2. Western Blot Analysis of MON 87411- and *E. coli* -produced Cry3Bb1 Proteins

Aliquots of the MON 87411-produced Cry3Bb1 protein and the *E. coli*-produced Cry3Bb1 protein were subjected to SDS-PAGE and electrotransferred to a PVDF membrane. Proteins were detected using anti-Cry3Bb1 antibodies as the primary antibodies. Immunoreactive bands were visualized using HRP-conjugated secondary antibodies and an ECL system. The MWs (kDa) of the standards are shown on the left. Lane 1 and 2 were cropped from the image. The 15 second exposure is shown. Lane designations are as follows:

Lane	Sample	Amount (ng)
1	Precision Plus Protein Standards Dual color	
2	Blank	
3	*E. coli*-Produced Cry3Bb1 protein	1
4	*E. coli*-Produced Cry3Bb1 protein	1
5	*E. coli*-Produced Cry3Bb1 protein	2
6	*E. coli*-Produced Cry3Bb1 protein	2
7	*E. coli*-Produced Cry3Bb1 protein	3
8	*E. coli*-Produced Cry3Bb1 protein	3
9	Blank	
10	MON 87411-produced Cry3Bb1 protein	1
11	MON 87411-produced Cry3Bb1 protein	1
12	MON 87411-produced Cry3Bb1 protein	2
13	MON 87411-produced Cry3Bb1 protein	2
14	MON 87411-produced Cry3Bb1 protein	3
15	MON 87411-produced Cry3Bb1 protein	3

Table C-3. Comparison of Immunoreactive Signal Between MON 87411- and *E coli*-produced Cry3Bb1Proteins

Mean Signal intensity from MON 87411 -produced Cry3Bb1[1] (OD x mm^2)	Mean Signal intensity from *E. coli*-produced Cry3Bb1[1] (OD x mm^2)	Acceptance limits[2]for MON 87411-produced Cry3Bb1(OD x mm^2)
7.35	7.02	4.56-9.48

[1]Each value represents the mean of six values (n=6)

[2] The acceptance limits are for the MON 87411-produced Cry3Bb1 protein and are based on the interval between +35% (7.02 × 1.35) and −35% (7.02 × 0.65) of the mean of the *E. Coli*-produced Cry3Bb1 signal intensity across all loads.

C.1.6 Molecular Weight Estimation of Cry3Bb1 using SDS-PAGE

C.1.6.1 Methods

MON 87411-produced and *E. coli*-produced Cry3Bb1 protein were diluted in 1× LB and heated to 95-105 °C for 3-5 min. The MON 87411-produced Cry3Bb1 protein was loaded in duplicate at 1, 2, and 3 µg onto a pre-cast Tris-glycine (4-20 %) polyacrylamide gradient mini-gel (Invitrogen). The *E. coli*-produced Cry3Bb1 protein was loaded at 1 µg total protein in a single lane. Broad Range Molecular Weight Standards (Bio-Rad) were prepared and loaded on the gel in parallel. Following electrophoresis at a constant voltage, proteins were briefly fixed in 40% (v/v) methanol, 7% (v/v) acetic acid and stained for 17 hours with Brilliant Blue G-Colloidal stain (Sigma-Aldrich, St. Louis, MO). Gels were briefly destained in 10% (v/v) acetic acid, 25% (v/v) methanol followed by 6.5 hours in 25% (v/v) methanol. Analysis of the gel was performed using a Bio-Rad GS-800 densitometer supplied with Quantity One software (4.6.7). Apparent MW was reported as an average of all six lanes containing the MON 87411-produced Cry3Bb1 protein.

C.1.6.2 Results of Cry3Bb1 Protein Molecular Weight Equivalence

The intact MON 87411-produced Cry3Bb1 protein (Figure C-3, lanes 3-8) migrated to the same position on the gel as the *E. coli*-produced Cry3Bb1 protein (Figure C-3, lane 2) and the apparent MW was calculated to be 77.0 kDa (Table C-4). Because the experimentally determined apparent MW of the MON 87411-produced Cry3Bb1 protein was within the acceptance limits for equivalence, the MON 87411- and *E. coli*-produced Cry3Bb1 proteins were determined to have equivalent apparent molecular weights.

Table C-4. Molecular Weight Comparison Between the MON 87411-and *E. coli*-produced Cry3Bb1 Proteins

Apparent MW of MON 87411-Produced Cry3Bb1Protein (kDa)	Apparent MW of *E. coli*-Produced Cry3Bb1 Protein[1] (kDa)	Preset Acceptance Limits for the MON 87411-Produced Cry3Bb1Protein(kDa)
77.0	74.5	72.9 - 81.7

[1]As reported on the Certificate of Analysis for lot 11309151

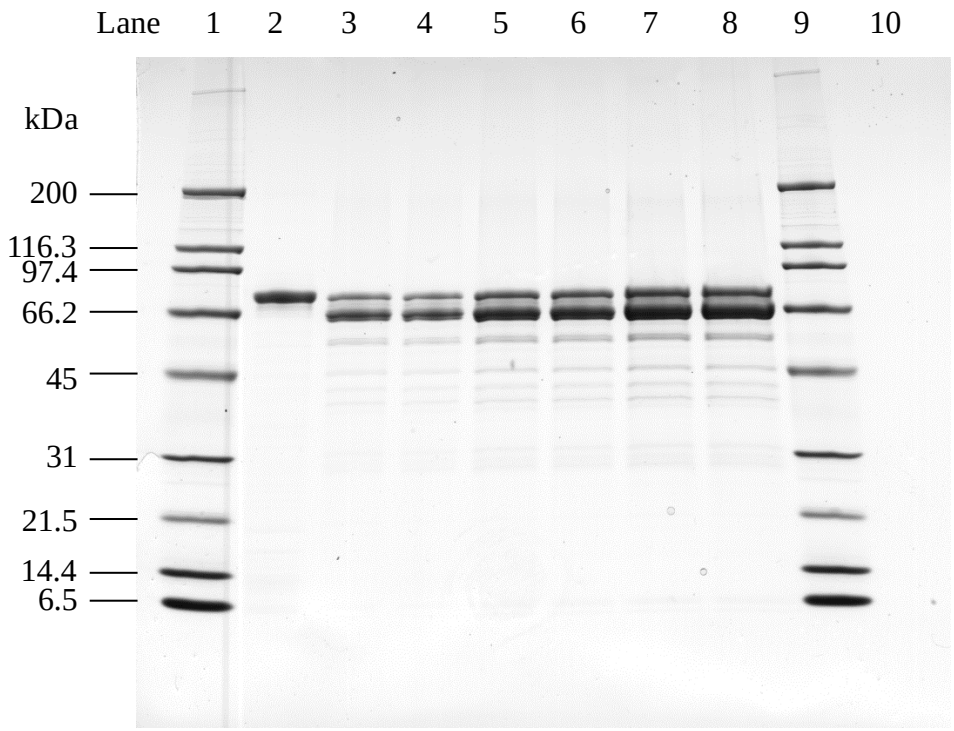

Figure C-3. Molecular Weight and Purity Analysis of the MON 87411-produced Cry3Bb1 Protein

Aliquots of the MON 87411-produced Cry3Bb1 and the *E. coli*-produced Cry3Bb1 proteins were subjected to SDS-PAGE and gel was stained with Brilliant Blue G-Colloidal stain. The MWs (kDa) are shown on the left and correspond to the standards loaded in Lanes 1 and 9. Lane designations are as follows:

Lane	Sample	Amount (µg)
1	Broad Range MW Standards	4.5
2	*E. coli*-produced Cry3Bb1 protein	1
3	MON 87411-produced Cry3Bb1 protein	1
4	MON 87411-produced Cry3Bb1 protein	1
5	MON 87411-produced Cry3Bb1 protein	2
6	MON 87411-produced Cry3Bb1 protein	2
7	MON 87411-produced Cry3Bb1 protein	3
8	MON 87411-produced Cry3Bb1 protein	3
9	Broad Range MW Standards	4.5
10	Blank	

C.1.7 Glycosylation Analysis of Cry3Bb1

C.1.7.1 Methods

ECL Glycoprotein Detection Kit (GE Healthcare) was used for glycoprotein detection. The MON 87411-produced Cry3Bb1 protein, the *E. coli*-produced Cry3Bb1 protein, and a positive control, transferrin (Sigma-Aldrich), were diluted in 1× LB and heated to 100 °C for 4 min. Two amounts (~100 and ~200 ng) of the intact the MON 87411-produced Cry3Bb1 protein (purity corrected) and the *E. coli*-produced Cry3Bb1 protein (purity corrected) were loaded onto a pre-cast Tris-glycine 4 - 20% polyacrylamide gradient mini-gel (Invitrogen). Four amounts (~50, ~100, ~150, and ~200 ng) of the positive control were loaded on the gel. Protein MW Standards (Precision Plus Protein™ Standards Dual color, Bio-Rad) were also loaded for molecular weight reference and to verify electrotransfer of the proteins to the membrane. Following electrophoresis at a constant voltage, proteins were electrotransferred to a PVDF membrane (Invitrogen).

Glycosylation analysis was performed on the PVDF membrane at room temperature using the Amersham ECL™ glycoprotein Detection Module (GE Healthcare) as directed by the manufacturer. Glycosylated proteins were detected using ECL™ reagents (GE Healthcare) and Amersham Hyperfilm (GE Healthcare). The film was developed using a Konica SRX-101A automated film processor (Konica Minolta). An identical gel was run and electrotransferred to a PVDF membrane in parallel. Proteins were stained with Coomassie Brilliant Blue R-250 staining solution (Bio-Rad) and then destained with 1× Coomassie Brilliant Blue R-250 Destaining Solution (Bio-Rad). After washing with water, the blot was scanned using Bio-Rad GS-800 densitometer.

C.1.7.2 Results of Glycosylation Analysis

Eukaryotic proteins can be post-translationally modified with carbohydrate moieties (Rademacher et al., 1988). To test whether Cry3Bb1 protein was glycosylated when expressed in the grain of MON 87411, the MON 87411-produced Cry3Bb1 protein was analyzed using an ECL ™ Glycoprotein Detection Module (GE Healthcare). To assess equivalence of the MON 87411- and *E. coli*-produced Cry3Bb1 proteins, the *E. coli*-produced Cry3Bb1 protein was also analyzed.

A clear glycosylation signal was observed at the expected in the lanes containing the positive control (transferrin) and the band intensity increased with increasing concentration (Figure C-4 panel A, lanes 1-4). In contrast, no glycosylation signals were observed at the expected molecular weight for Cry3Bb1 in the lanes containing the MON 87411-produced Cry3Bb1 protein (Figure C-4 panel A, lanes 7 and 8) or *E. coli*-produced Cry3Bb1 protein (Figure C-4 panel A, lanes 9 and 10). The band observed in the *E. coli*-produced Cry3Bb1 sample was at a lower molecular weight than the Cry3Bb1 protein. This band likely represents a naturally biotinylated protein that co-purified with the *E. coli*-produced Cry3Bb1 protein (Choi-Rhee and Cronan 2003), because binds to the streptavidin-HRP conjugate and therefore is detectable by the ECL system.

To confirm that MON 87411- and *E. coli*-produced Cry3Bb1 proteins were appropriately loaded for glycosylation analysis, a second membrane with identical loadings and transfer time was stained with Coomassie Blue R250 for protein detection (Figure C-4 Panel B). Both the MON 87411-produced Cry3Bb1 (Figure C-4 panel B, lanes 7 and 8) and *E. coli*-produced Cry3Bb1 (Figure C-4 panel B, lanes 9, and 10) proteins were detected. These data indicate that the glycosylation status of MON 87411-produced Cry3Bb1 protein is equivalent to that of the *E. coli*-produced Cry3Bb1 protein and that neither is glycosylated.

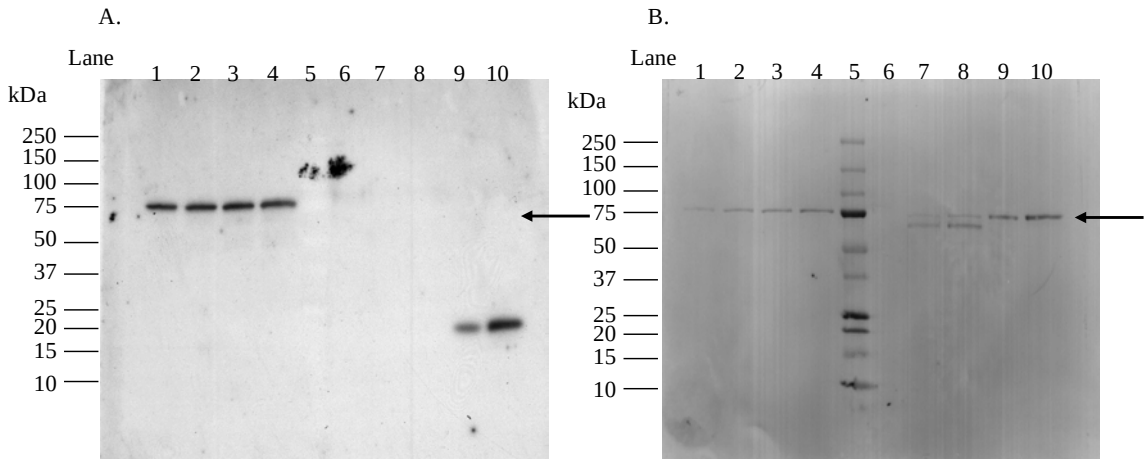

Figure C-4. Glycosylation Analysis of the MON 87411-produced Cry3Bb1 Protein
Aliquots of the transferrin (positive control), *E. coli*-produced Cry3Bb1 and MON 87411-produced Cry3Bb1 were subjected to SDS-PAGE and electrotransferred to PVDF membranes. The MWs (kDa) correspond to the Precision Plus Protein™ Dual Color Standards. The arrows show the expected migration of the MON 87411- and *E. coli*-produced Cry3Bb1 protein. (A) Where present, labeled carbohydrate moieties were detected by addition of streptavidin conjugated to HRP followed by a luminol-based the detection using ECL reagents and exposure to Hyperfilm®. (B) An equivalent blot was stained with Coomassie Blue R250 to confirm the presence of proteins. Lane designations and the approximate mass loaded are as follows:

Lane	Sample	Amount (ng)
1	Transferrin (positive control)	50
2	Transferrin (positive control)	100
3	Transferrin (positive control)	150
4	Transferrin (positive control)	200
5	Precision Plus Protein™ Standards	
6	Blank	
7	MON 87411-produced Cry3Bb1 protein	100
8	MON 87411-produced Cry3Bb1 protein	200
9	*E. coli*-produced Cry3Bb1 protein	100
10	*E. coli*-produced Cry3Bb1 protein	200

C.1.8 Cry3Bb1 Functional Activity Analysis

C.1.8.1 Methods

Insects. CPB eggs were obtained from French Agricultural Research, Inc. (Lamberton, MN). The eggs were incubated at temperatures ranging from 15 °C to 27 °C, to achieve the desired hatch time.

Bioassays. CPB larvae (≤ 30 hours old) were used to measure biological activity of the MON 87411-produced and *E. coli*-produced Cry3Bb1 protein samples in accordance with the current version of Monsanto SOP BR-ME-1048. The bioassay was replicated three times on separate days, each with a separate batch of insects. The MON 87411-produced and *E. coli*-produced substances were run in parallel during each bioassay. Each bioassay replicate consisted of a series of six dilutions yielding a dose series with a two-fold separation factor ranging from 0.078 – 2.5 µg Cry3Bb1 protein/ml diet for the *E. coli*-produced and MON 87411-produced Cry3Bb1 and a single buffer control. The Cry3Bb1 protein dosing solutions were prepared by diluting the respective protein with purified water and incorporating the dilution into a Bio-Serv CPB agar-based insect diet (pre-mix #F9380B; Frenchtown, NJ). This dose series in diet was chosen to adequately characterize the dose-effect relationship on CPB mortality for the *E. coli*-produced and MON 87411-produced Cry3Bb1 proteins. The diet mixture was then dispensed in 0.5 ml aliquots into a 128 well tray (# BAW128, Bio-Serv, Frenchtown, NJ). Individual insect larvae were placed on these diets using a fine paintbrush, with a target number of 24 insects per treatment. The infested wells were covered by a ventilated adhesive cover (# BACV16, Bio-Serv, Frenchtown, NJ) and the insects were allowed to feed for a period of approximately 7 days in an environmental chamber programmed at 27 °C, 60% relative humidity and a lighting regime of 14 light : 10 dark. The number of insects infested and the number of surviving insects at each dose level was recorded at the end of the 7 day incubation period. For a bioassay to be accepted, control mortality cannot be > 20%.

C.1.8.2 Results of Functional Activity

The functional activity of the MON 87411-produced and *E. coli*-produced Cry3Bb1 protein was determined Colorado potato beetle diet-incorporation bioassays. In this assay, activity is expressed as $LC_{50,}$ µg/ml diet**.** The MON 87411- and *E. coli*-produced Cry3Bb1 proteins were considered functionally equivalent if the $LC_{50,}$ of both were within acceptance limits of 0.37 µg/ml of diet to 0.97 µg/ml; the prediction interval calculated from data obtained for the *E. coli*-produced Cry3Bb1 protein activity. The LC_{50} of the MON 87411- and *E. coli*-produced Cry3Bb1 proteins were determined to be 0.77 µg/ml diet and 0.67 µg/ml diet respectively (Table C-5). Because the LC_{50} of MON 87411-produced and *E. coli*-produced Cry3Bb1 proteins were within the acceptance limits (Table C-5), the proteins were determined to have equivalent functional activity.

Table C-5. Cry3Bb1 Functional Activity Assay

MON 87411-produced Cry3Bb1 Protein[1] ($LC_{50,}$ µg/ml diet)	*E. coli*-produced Cry3Bb1 Protein[1] ($LC_{50,}$ µg/ml diet)	Preset Acceptance Limits for MON 87411-produced Cry3Bb1 Protein[2] ($LC_{50,}$ µg/ml diet)
0.77	0.67	0.37 – 0.97

[1]Value refers to mean based on n = 3
[2] Data obtained for the *E. coli*-produced protein was used to generate the acceptance limits

C.2 Characterization of CP4 EPSPS Protein in MON 87411

C.2.1 Materials for CP4 EPSPS Characterization

The MON 87411-produced CP4 EPSPS protein (lot 11351676) was purified from the grain of MON 87411 (lot 11330153). The MON 87411-produced CP4 EPSPS protein was stored in a -80 ºC freezer in a buffer solution containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM dithiothreitol (DTT), 1 mM benzamidine-HCl, and 25% glycerol.

The *E. coli*-produced CP4 EPSPS protein (lot 10000739) was used as the reference substance. The CP4 EPSPS protein reference substance was generated from cell paste produced by large-scale fermentation of *E. coli* containing the pMON21104 expression plasmid. The coding sequence for *cp4 epsps* contained on the expression plasmid (pMON21104) was confirmed prior to and after fermentation. The *E. coli*-produced CP4 EPSPS protein was characterized previously.

C.2.2. CP4 EPSPS Protein Purification

The plant-produced CP4 EPSPS protein was purified from grain of MON 87411. The purification procedures were not performed under a GLP plan; however, all procedures were documented on worksheets and, where applicable, SOPs were followed. Three CP4 EPSPS protein purification batches were prepared to generate a sufficient amount of MON 87411 produced CP4 EPSPS protein. All purification steps were performed at ~4 °C. CP4 EPSPS was purified from an extract of ground grain using a combination of purification methods. The purification process for the first two batches used ammonium sulfate fractionation, hydrophobic interaction chromatography, anion-exchange chromatography, and a second hydrophobic interaction chromatography. The purification process for the third batch used ammonium sulfate fractionation, hydrophobic interaction chromatography, anion-exchange chromatography, cellulose phosphate affinity chromatography, and hydroxyapatite chromatography. Chromatography fractions containing CP4 EPSPS protein were identified by western blot analysis or SDS-PAGE stained gels. The final purified CP4 EPSPS protein product from all three batches was combined, concentrated and buffer-exchanged to prepare the CP4 EPSPS protein lot used in the equivalence study. Each of the three purification procedures are briefly described below and a detailed description of the purification procedures are filed under Lot 11351676 in the Monsanto Regulatory Archives.

CP4 EPSPS protein Batch 1. Grain of MON 87411 was ground in the presence of dry ice and stored at -80 °C until use. A total of 920 g of ground powder was mixed with EX buffer (100 mM Tris-HCl pH 7.5, 2 mM EDTA, 2 mM benzamidine-HCl, 4 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 % polyvinyl-polypyrrolidone and 10 % glycerol) at a sample weight (g) to buffer volume (ml) of approximately 1:10 for ~1.5 hr. The slurry was centrifuged to clarify, and the supernatant was collected, filtered, and brought to 40 % ammonium sulfate saturation by addition of solid ammonium sulfate. Following equilibration, the 40 % saturated solution was centrifuged to pellet precipitated proteins. The supernatant was brought to 70 % ammonium sulfate saturation by addition of the solid salt. Following equilibration, the precipitated protein containing the majority of CP4 EPSPS protein was collected by centrifugation. The 40-70 % ammonium sulfate pellet was re-suspended in PS-A buffer (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 10 % glycerol (v/v), and 1.5 M ammonium sulfate). The dissolved suspension was centrifuged, filtered, and loaded onto a Phenyl Sepharose Fast Flow (GE Healthcare, Piscataway, NJ) column equilibrated with PS-A buffer. Proteins were eluted with a linear salt gradient that decreased from 1.5 M to 0 M ammonium sulfate over 6 column volumes. Fractions containing the CP4 EPSPS protein were pooled and desalted by dialysis against S30Q-A buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM benzamidine-HCl, and 4 mM DTT). The desalted sample was loaded onto a Source 30Q (GE Healthcare) column equilibrated with S30Q-A buffer. The bound CP4 EPSPS was eluted with a linear salt gradient that increased from 0 M to 0.4 M KCl over 15 column volumes. Fractions containing CP4 EPSPS were pooled and dialyzed against PS-A buffer. The dialyzed sample was then loaded onto a Source 15PHE (GE Healthcare) column equilibrated with PS-A buffer. Proteins were eluted with a linear salt gradient that decreased from 1.5 M to 0 M ammonium sulfate over 20 column volumes. Fractions containing the CP4 EPSPS protein were pooled and buffer exchanged by dialysis against 50 mM Tris-HCl pH 7.5, 50 mM KCl, 1 mM benzamidine-HCl, 2 mM DTT, and 10 % glycerol. The buffer exchanged sample was initially concentrated within the dialysis tubing by packing in Aquacide II (EMD Chemicals, San Diego, CA), then further concentrated by centrifugation in an Amicon Ultra spin concentrator (ULTRACEL-10K, 10,000 MWCO, regenerated cellulose membrane). The sample was then buffer exchanged into 1.2X formulation buffer (60 mM Tris-HCl pH 7.5, 60 mM KCl, 1.2 mM benzamidine-HCl, 2.4 mM DTT, and 10 % glycerol), yielding a final volume of ~500 µl. This Batch 1 purified CP4 EPSPS sample was frozen and stored in a -80 °C freezer.

CP4 EPSPS protein Batch 2. The purification procedure used for the Batch 2 isolation of CP4 EPSPS from the grain of MON 87411 followed the same sequence of fractionation steps as used for Batch 1, except that the initial amount of grain extracted for Batch 2 was ~3 kg. Because a larger amount of grain was extracted for Batch 2, the bed size of chromatography columns, the column gradient volumes and the dialysis volumes used were increased, as appropriate, to accommodate the larger amounts of sample handled at each step. The final volume of Batch 2 protein sample was ~350 µl. This Batch 2 purified CP4 EPSPS sample was frozen and stored in a -80 °C freezer.

CP4 EPSPS protein Batch 3. The initial purification steps used for the Batch 3 isolation of CP4 EPSPS from the grain of MON 87411 followed the same sequence of fractionation steps as used for Batch 1 through the Source 30Q step, starting with ~2 kg

of ground grain. Following Source 30Q fractionation, fractions containing CP4 EPSPS were pooled, brought to 25 % glycerol (v/v) and stored frozen and-20 °C until the next step. The pooled sample was thawed, concentrated and buffer exchanged into CPE buffer (10 mM MES pH 5.9, 1 mM benzamidine-HCl, 1 mM DTT, 10 µM Na-tungstate, and 10 % glycerol) using 15 ml Amicon Ultracell-10K centrifugal concentrators. The buffer exchanged and concentrated sample was diluted with CPE buffer and loaded on a column of P11 Cellulose Phosphate (Whatman/GE Healthcare, Maidstone, UK) equilibrated in CPE buffer. The column was washed with CPE buffer followed by CPE buffer with 0.5 mM phosphoenolpyruvate (PEP). CP4 EPSPS was then eluted with CPE buffer with 0.5 mM PEP and 0.5 mM shikimate-3-phosphate (S-3-P). Fractions containing CP4 EPSPS were pooled and concentrated using a Vivaspin 15R centrifugal concentrator equipped with a 10,000 MWCO membrane (Sartorius Stedim Biotech, Goettingen, Germany). The glycerol concentration was increased to 25 % and the sample frozen and held at -20 °C until the next step. The sample was thawed, diluted with CHT-A buffer (50 mM HEPES pH 7.0, 1 mM benzamidine-HCl, 1 mM DTT, and 10 % glycerol), and loaded on a ceramic hydroxyapatite (CHT Type 1, Bio-Rad Laboratories, Hercules, CA) column. Protein was eluted with linear phosphate gradient from 0 to 50 mM sodium phosphate in CHT-A buffer over 20 column volumes. Fractions containing CP4 EPSPS were pooled and concentrated using an Amicon Ultracel -10K centrifugal concentration device. The pooled sample was buffer exchanged into 1.2X formulation buffer (60 mM Tris-HCl, pH 7.5, 60 mM KCl, 2.4 mM DTT, 1.2 mM benzamidine-HCl, 10 % glycerol), yielding a final volume of ~0.5 ml of buffer exchanged protein concentrate.

Preparation of the final pooled protein product. The Batch 1 and 2 samples were thawed and pooled with purified protein from Batch 3. The pooled protein sample was concentrated to ~0.5 ml by centrifugation in a 4 ml Amicon Ultracel-10K centrifugal concentration device, equipped with a 10,000 MWCO membrane. Buffer exchange was conducted in the same unit using 1.2X formulation buffer (60 mM Tris-HCl, pH 7.5, 60 mM KCl, 2.4 mM DTT, 1.2 mM benzamidine-HCl, 10 % glycerol), to a final volume of 300 µl, then brought to 500 µl volume with a 200 µl buffer rinse of the spin concentrator. Glycerol was added bringing the final protein volume to 600 µl in 1X formulation buffer and 25 % glycerol. Final buffer composition of the sample was: 50 mM Tris-HCl pH 7.5, 50 mM KCl, 2 mM DTT, 1 mM benzamidine-HCl and 25 % glycerol. This CP4 EPSPS protein purified from the grain of MON 87411 was aliquoted, assigned lot 11351676, and stored in a -80 °C freezer.

C.2.3 N-Terminal Sequencing of CP4 EPSPS

C.2.3.1 Methods

N-terminal sequencing, carried out by automated Edman degradation chemistry, was used to confirm the identity of the MON 87411-produced CP4 EPSPS.

A MON 87411-produced CP4 EPSPS protein sample was separated by SDS-PAGE and transferred to a PVDF membrane. The blot was stained using Coomassie Blue R-250 and destained with 1× Coomassie Brilliant blue R-250 Destaining Solution (Bio-Rad). The

major band at ~44 kDa containing the test protein was excised from the blot and was used for N-terminal sequence analysis. The analysis was performed for 15 cycles using automated Edman degradation chemistry (Hunkapiller et al., 1983). An Applied Biosystems 494 Procise Sequencing System with a 140C Microgradient system and a Perkin Elmer Series 200 UV/VIS Absorbance Detector and Procise™ Control Software (version 2.1) were used. Chromatographic data were collected using SequencePro software (Applied Biosystems, Foster City CA; version 2.1). A PTH-AA standard solution (Applied Biosystems) was used to chromatographically calibrate the instrument for the analysis. A control protein, β-lactoglobulin, (Applied Biosystems) was analyzed before and after analysis of the MON 87411-produced CP4 EPSPS protein.

C.2.3.2 Results of the N-terminal Sequence Analysis

Fifteen cycles of N-terminal sequencing was performed on the MON 87411-produced CP4 EPSPS protein. The expected sequence for the CP4 EPSPS protein deduced from the *cp4 epsps* gene present in MON 87411 was observed. The data obtained correspond to the deduced CP4 EPSPS protein beginning at amino acid position 2 (Table C-6). Hence, the sequence information confirms the identity of the CP4 EPSPS protein isolated from the seed of MON 87411.

Table C-6. N-Terminal Sequence of the MON 87411-produced CP4EPSPS

Amino acid residue # from the N-terminus →	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Expected Sequence →	M	L	H	G	A	S	S	R	P	A	T	A	R	K	S	S
		|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Experimental Sequence →	–	L	H	G	A	S	S	R	P	A	T	A	R	K	X	X

The experimental sequences obtained from the MON 87411-produced CP4 EPSPS protein were compared to the expected sequence. The single letter IUPAC-IUB amino acid code is M, methionine; L, leucine; H, histidine; G, glycine; A, alanine, S, serine; R, arginine; P, proline; T, threonine; K, lysine; (X) indicates that the residue was not identifiable.

C2.4 MALDI-TOF Tryptic Mass Map Analysis of CP4 EPSPS

C2.4.1 Methods

MALDI-TOF MS tryptic mass fingerprint analysis was used to confirm the identity of the MON 87411-produced CP4 EPSPS protein. A MON 87411-produced CP4 EPSPS protein sample was separated by SDS-PAGE and the gel was stained using Brilliant Blue G-Colloidal stain (Sigma-Aldrich, St. Louis, MO). The major band at ~44 kDa band was excised, transferred to a microcentrifuge tube, and destained. The gel band was washed in 200 µl of 100 mM ammonium bicarbonate, and then incubated in 100 µl of 10 mM DTT at 37 °C for 2 h to reduce protein disulfide bonds. The protein was then alkylated in

the dark for 20 min with 100 µl of 20 mM iodoacetic acid and washed with 200 µl of 25 mM ammonium bicarbonate for 3 × 15 min washes. The gel band was dried with a Speed-Vac® concentrator (Thermo Fisher Scientific, Waltham, MA) and then rehydrated with 20 µl of trypsin solution (20 µg/ml). After 1 h, excess liquid was removed and the gel was incubated overnight at 37 °C in 40 µl of 10 % acetonitrile in 25 mM ammonium bicarbonate. The gel band was sonicated for 5 min to further elute proteolytic fragments. The resulting extract was transferred to a new tube labeled Extract 1 and dried using a Speed-Vac concentrator. The gel band was re-extracted twice with 30 µl of a 60 % acetonitrile, 0.1 % trifluoroacetic acid, 0.1 % β-octyl-glucopyranoside solution and sonicated for 5 min. These samples were pooled into a new tube labeled Extract 2 and dried with a Speed-Vac concentrator. A solution of 0.1 % trifluoroacetic acid (TFA) was added to all Extract 1 and 2 tubes and they were dried as before. A solution of 50 % acetonitrile, 0.1 % TFA was added to each tube and all were sonicated for 5 min. Each extract (0.3 µl) was spotted to three wells on an analysis plate. For each extract 0.75 µl of 2, 5-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (α Cyano), or 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) (Thermo Fisher Scientific) was added to one of the spots. The samples in DHB matrix were analyzed in the 300 to 5000 Da range. Samples in α-Cyano and Sinapinic acid were analyzed in the 500 to 5000 and 500 to 7000 Da range, respectively. CalMix 2™ was used as the external calibrant (Sequazyme™ Peptide Mass Standards kit, AB SciEx). The analysis was performed on a Voyager™ DE Pro Biospectrometry™ workstation (Applied Biosystems) using Voyager Instrument Control Panel software (version 5.10.2) and Data Explorer data analysis software (version 4.0.0.0). Protonated peptide masses were isotopically resolved in reflector mode (Aebersold 1993; Billeci and Stults 1993). GPMAW32 software (Lighthouse Data) was used to generate an *in silico* digest of the CP4 EPSPS protein sequence. Masses within 1 Da of the monoisotopic mass were matched against the *in silico* digest of the MON 87411-produced Cry3Bb1 sequence. All matching masses were tallied and a coverage map was generated for the mass fingerprint.

C2.4.2 Results of MALDI-TOF Tryptic Mass Map Analysis

The identity of the MON 87411-produced CP4 EPSPS protein was confirmed by MALDI-TOF MS analysis of peptide fragments produced by the trypsin digestion of the MON 87411-produced CP4 EPSPS protein. There were 23 unique peptides identified that corresponded to the masses expected to be produced by trypsin digestion of the CP4 EPSPS protein (Table C-7). The identified masses were used to assemble a coverage map of the entire CP4 EPSPS protein (Figure C-5). The experimentally determined mass coverage of the CP4 EPSPS protein was 67.3% (306 out of 455 amino acids).

Table C-7. Summary of the Tryptic Masses Identified for the MON 87411-produced CP4 EPSPS Using MALDI-TOF MS

Observed Mass[1]	Expected Mass	Difference[2]	Fragment	Sequence[3]
389.20	389.25	0.05	225-227	TIR
474.23	474.27	0.04	228-231	LEGR
599.33	599.33	0.00	29-33	SISHR
616.33	616.34	0.01	128-132	RPMGR
629.34	629.29	0.05	201-205	DHTEK
711.45	711.45	0.00	133-138	VLNPLR
733.44	733.38	0.06	352-357	VKESDR
835.39	835.39	0.00	62-69	AMQAMGAR
863.47	863.46	0.01	15-23	SSGLSGTVR
872.45	872.45	0.00	313-320	GVTVPEDR*
872.46	872.52	0.06	358-366	LSAVANGLK*
930.49	930.51	0.02	169-177	VPMASAQVK
948.51	948.52	0.01	161-168	TPTPITYR
991.56	991.55	0.01	14-23	KSSG…GTVR
1115.57	1115.57	0.00	295-305	LAGG...ADLR
1357.72	1357.71	0.01	146-157	SEDG...VTLR
1359.67	1359.72	0.05	354-366	ESDR...NGLK*
1359.63	1359.64	0.01	34-46	SFMF...GETR*
1558.82	1558.83	0.01	47-61	ITGL...NTGK
1646.85	1646.84	0.01	389-405	GLGN...LDHR
1763.83	1763.81	0.02	367-382	LNGV...LVVR
1993.97	1993.97	0.00	206-224	MLQG...DGVR
2183.19	2183.17	0.02	275-294	TGLI...INPR
2367.33	2367.33	0.00	178-200	SAVL...IMTR
2450.24	2450.23	0.01	24-46	IPGD...GETR*
	2450.22	0.02	105-127	LTMG...SLTK*
3244.52	3244.52	0.00	73-104	EGDT...TGCR
3249.59	3249.62	0.03	321-351	APSM...EELR
4188.47	4188.26	0.21	234-274	LTGQ...NPTR

[1]The observed mass was obtained from at least one of three matrices including α-cyano, DHB, and sinapinic acid. The observed mass shown is the mass closest to the expected mass.

[2]The data represents the calculated difference between the expected mass and the observed mass.

[3]For peptide matches greater than nine amino acids in length the first 4 residues and last 4 residues are show separated by three dots (…)

*The expected peptide masses are nearly identical (<1 dalton). Because this analysis did not determine with certainty which expected peptide was actually observed, the peptides with an asterisk (*) were not included in determining sequence coverage (Figure C-5).

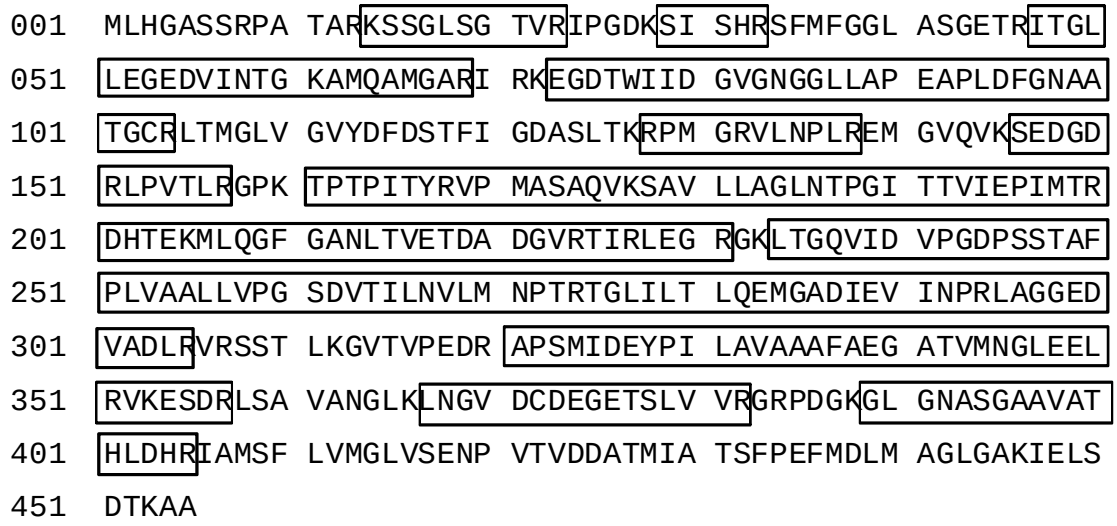

Figure C-5. MALDI-TOF MS Coverage Map of the MON 87411-Produced CP4 EPSPS Protein

The amino acid sequence of the CP4 EPSPS protein was deduced from the *cp4 epsps* gene present in MON 87411. Boxed regions correspond to regions covered by tryptic peptides that were unambiguously identified from the MON 87411-produced CP4 EPSPS protein sample using MALDI-TOF MS. In total, 67.3 % (306 of 455 total amino acids) of the expected protein sequence was covered by the identified peptides.

C.2.5 Western Blot Analysis-Immunoreactivity of CP4 EPSPS

C.2.5.1 Methods

Western blot analysis was performed to confirm the identity of the MON 87411-produced CP4 EPSPS protein and to compare the immunoreactivity of the MON 87411- and *E. coli*-produced proteins.

The MON 87411- and *E. coli*-produced CP4 EPSPS proteins were analyzed concurrently on the same gel using three protein loadings of 1, 2 and 3 ng. Loadings of the three concentrations were made in duplicate on the gel. In preparation for analysis the *E. coli*-produced CP4 EPSPS was diluted with storage buffer to the same purity corrected concentration as the MON 87411-produced protein (0.263 mg CP4 EPSPS/ml). Aliquots of each protein were subsequently diluted in water and 1× (LB), and heated at 95 °C for 4 min. Samples were further diluted in 5× LB to a final concentration of 0.25 ng/µl, and applied to a pre-cast Tris-glycine 4-20 % polyacrylamide gradient gel (Invitrogen). Pre-stained molecular weight markers (Precision Plus Protein Standards Dual Color; Bio-Rad) were loaded to verify electrotransfer of the proteins to the membrane and to estimate the size of the immunoreactive bands observed. Following electrophoresis, electrotransfer to a PVDF membrane (Invitrogen) was performed.

After electrotransfer, the membrane was blocked for 1 h with 5 % (w/v) NFDM in PBST) at room temperature. The membrane was then probed with a 1:10,000 dilution of goat anti-CP4 EPSPS antibody (lot G-848018) in 1 % NFDM in PBST for 60 min at room

temperature. Excess antibody was removed using washes with PBST. Finally, the membrane was probed with HRP-conjugated rabbit anti-goat IgG (Thermo, Rockford, IL) at a dilution of 1:5,000 in 1 % NFDM in PBST for 1 h at room temperature. Excess HRP-conjugate was removed using washes with PBST. All washes were performed at room temperature. Immunoreactive bands were visualized using the ECL detection system (GE, Healthcare) with exposure to Amersham Hyperfilm ECL (GE, Healthcare). The film was developed using a Konica SRX-101A automated film processor.

Quantification of the bands on the blot was performed using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0) using the band volume tool. The signal intensities of the immunoreactive bands observed for the MON 87411- and *E. coli*-produced proteins migrating at the expected position on the blot film were quantified as "adjusted volume" values (OD × mm^2).

C.2.5.2 Results of CP4EPSPS Protein Immunoreactivity Equivalence

Western blot analysis was conducted using goat anti-CP4 EPSPS polyclonal antibody as an additional means to confirm the identity of the CP4 EPSPS protein isolated from the grain of MON 87411 and to assess the equivalence of the immunoreactivity of the MON 87411- and the *E. coli*-produced CP4 EPSPS proteins.

The results demonstrated that the anti-CP4 EPSPS antibody recognized the MON 87411-produced CP4 EPSPS protein that migrated to an identical position as the *E. coli*-produced CP4 EPSPS protein (Figure C-6). Furthermore, the immunoreactive signal increased with increasing amounts of CP4 EPSPS protein loaded.

To compare the immunoreactivity of the MON 87411-produced CP4 EPSPS protein and the *E. coli*-produced CP4 EPSPS proteins, densitometric analysis was conducted on bands that migrated to the expected apparent MW for CP4 EPSPS proteins (~44 kDa). The signal intensity (reported in OD × mm^2) of the band of interest in lanes loaded with the MON 87411-produced and the *E. coli*-produced CP4 EPSPS protein was measured (Table C-8). Because signal intensity of the MON 87411-produced CP4 EPSPS protein band was within 35% of the *E. coli*-produced CP4 EPSPS protein, the MON 87411-produced and *E. coli*-produced CP4 EPSPS proteins were determined to have equivalent immunoreactivity.

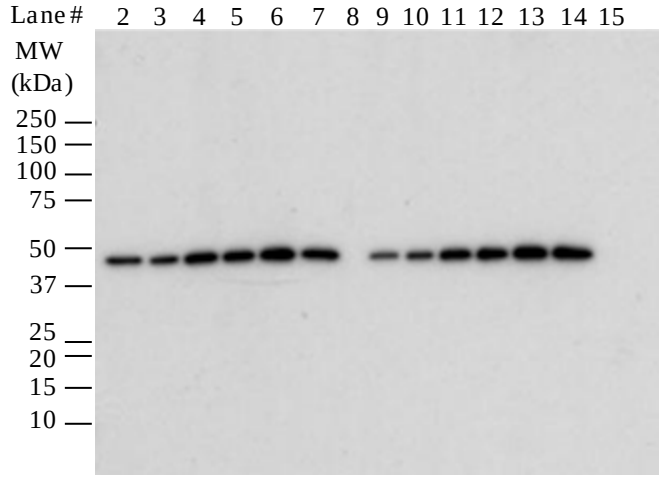

Figure C-6. Western Blot Analysis of MON 87411- and *E. coli*-Produced CP4 EPSPS Proteins

Aliquots of the MON 87411-produced CP4 EPSPS protein and the *E. coli*-produced CP4 EPSPS protein were separated by SDS-PAGE and electrotransferred to a PVDF membrane. The membrane was incubated with anti-CP4 EPSPS antibodies as the primary antibodies and immunoreactive bands were visualized using HRP-conjugated secondary antibodies and an ECL system (GE Healthcare). Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in lane 1. Lane 1 was cropped from the image. The 3 min exposure is shown. Lane designations are as follows:

Lane	Sample	Amount (ng)
1	Precision Plus Protein Standards Dual color (cropped from the image)	-
2	*E. coli*-produced CP4 EPSPS protein	1
3	*E. coli*-produced CP4 EPSPS protein	1
4	*E. coli*-produced CP4 EPSPS protein	2
5	*E. coli*-produced CP4 EPSPS protein	2
6	*E. coli*-produced CP4 EPSPS protein	3
7	*E. coli*-produced CP4 EPSPS protein	3
8	Blank	-
9	MON 87411-produced CP4 EPSPS protein	1
10	MON 87411-produced CP4 EPSPS protein	1
11	MON 87411-produced CP4 EPSPS protein	2
12	MON 87411-produced CP4 EPSPS protein	2
13	MON 87411-produced CP4 EPSPS protein	3
14	MON 87411-produced CP4 EPSPS protein	3
15	Blank	-

Table C-8. Comparison of Immunoreactive Signal Between MON 87411- and *E coli*-produced CP4 EPSPS Protein

Mean Signal Intensity from MON 87411-Produced CP4 EPSPS[1] (OD × mm^2)	Mean Signal Intensity from *E. coli*-Produced CP4 EPSPS[1] (OD × mm^2)	Acceptance Limits[2] for MON 87411-Produced CP4 EPSPS (OD × mm^2)
6.0	6.0	3.9–8.1

[1]Each value represents the mean of six values (n=6).
[2]The acceptance limits are for the MON 87411-produced CP4 EPSPS protein and are based on the interval between +35% (6.0 × 1.35) and –35% (6.0 × 0.65) of the mean of the *E. coli*-produced CP4 EPSPS signal intensity across all loads.

C.2.6 Molecular Weight Estimation of CP4 EPSPS using SDS-PAGE

C.2.6.1 Methods

MON 87411-produced CP4 EPSPS protein and *E. coli*-produced CP4 EPSPS protein, as well as Bio-Rad Broad Range molecular weight standards, were diluted in water and 5× LB. The diluted samples were heated to 95 °C for 3-5 min. The MON 87411-produced CP4 EPSPS protein was analyzed in duplicate at 1, 2, and 3 µg protein per lane. The *E. coli*-produced CP4 EPSPS reference standard was analyzed at 1 µg total protein in a single lane. The samples were loaded onto a pre-cast Tris glycine 4-20 % polyacrylamide gradient mini-gel (Invitrogen) and electrophoresis was performed at a constant voltage. Proteins were fixed by placing the gel in a solution of 40 % (v/v) methanol and 7 % (v/v) acetic acid for 30 min, and stained for 18 h with Brilliant Blue G-Colloidal stain. Gels were destained for 30 to 45 sec with a solution containing 10 % (v/v) acetic acid and 25 % (v/v) methanol, and for 8 h with 25 % (v/v) methanol. Analysis of the gel was performed using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0). Apparent MW was reported as an average of all six lanes containing the MON 87411-produced Cry3Bb1 protein.

C.2.6.2 Results of CP4EPSPS Protein Molecular Weight Equivalence

For molecular weight and purity analysis, the MON 87411-produced CP4 EPSPS protein was separated using SDS-PAGE. Following electrophoresis, the gel was stained with Brilliant Blue G-Colloidal stain and analyzed by densitometry (Figure C-7). The MON 87411-produced CP4 EPSPS protein (Figure C-7, lanes 3-8) migrated to the same position on the gel as the *E. coli*-produced CP4 EPSPS protein (Figure C-7, lane 2) and the apparent molecular weight was calculated to be 42.9 kDa (Table C-9). Because the experimentally determined apparent MW of MON 87411-produced CP4 EPSPS protein was within the pre-set acceptance limits for equivalence (Table C-9; 42.6 – 45.1 kDa), the MON 87411-produced and *E. coli*-produced CP4 EPSPS proteins were determined to have equivalent apparent molecular weights.

Table C-9. Molecular Weight Comparison Between the MON 87411-and *E. coli*-produced CP4 EPSPS Proteins

Apparent MW of MON 87411-Produced CP4 EPSPS Protein (kDa)	Apparent MW of *E. coli*-Produced CP4 EPSPS Protein[1] (kDa)	Preset Acceptance Limits for MON 87411-Produced CP4 EPSPS (kDa)
42.9	43.8	42.6-45.1

[1]As reported on the Certificate of Analysis for lot 10000739.

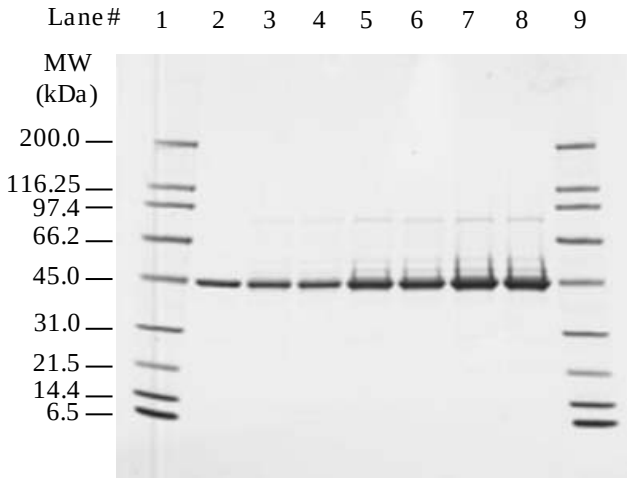

Figure C-7. Molecular Weight and Purity Analysis of the MON 87411-produced CP4EPSPS Protein

Aliquots of the MON 87411-produced and the *E. coli*-produced CP4 EPSPS proteins were subjected to SDS-PAGE and the gel was stained with Brilliant Blue G-colloidal stain. The MWs (kDa) are shown on the left and correspond to the standards loaded in Lanes 1 and 9. Lane 10 (blank lane) was cropped from the image. Lane designations are as follows:

Lane	Sample	Amount (µg)
1	Broad Range MW Standards	4.5
2	*E. coli*-produced CP4 EPSPS protein	1
3	MON 87411-produced CP4 EPSPS protein	1
4	MON 87411-produced CP4 EPSPS protein	1
5	MON 87411-produced CP4 EPSPS protein	2
6	MON 87411-produced CP4 EPSPS protein	2
7	MON 87411-produced CP4 EPSPS protein	3
8	MON 87411-produced CP4 EPSPS protein	3
9	Broad Range Molecular Weight Markers	4.5
10	Blank (cropped from the image)	--

C.2.7 Glycosylation Analysis of CP4 EPEPS

C.2.7.1 Methods

ECL Glycoprotein Detection Kit (GE Healthcare) was used for glycoprotein detection. The MON 87411-produced CP4 EPSPS protein, the *E. coli*-produced CP4 EPSPS protein, and a positive control, transferrin (Sigma-Aldrich), were diluted in 1× LB and heated to 100 °C for 4 min. Two amounts (~100 and ~200 ng) of the intact the MON 87411-produced CP4 EPSPS protein (purity corrected) and the *E. coli*-produced CP4 EPSPS protein (purity corrected) were loaded onto a pre-cast Tris-glycine 4 – 20 % polyacrylamide gradient mini-gel (Invitrogen). Four amounts (~50, ~100, ~150, and ~200 ng) of the positive control were loaded on the gel. Protein MW Standards (Precision Plus Protein™ Standards Dual color, Bio-Rad,) were also loaded for MW reference and to verify electrotransfer of the proteins to the membrane. Following electrophoresis at a constant voltage, proteins were electrotransferred to a PVDF membrane (Invitrogen).

Glycosylation analysis was performed on the PVDF membrane at room temperature using the Amersham ECL™ glycoprotein Detection Module (GE Healthcare) as directed by the manufacturer. Glycosylated proteins were detected using ECL™ reagents (GE Healthcare) and Amersham Hyperfilm (GE Healthcare). The film was developed using a Konica SRX 101A automated film processor (Konica Minolta). An identical gel was run and electrotransferred to a PVDF membrane in parallel. Proteins were stained with Coomassie Brilliant Blue R 250 staining solution (Bio-Rad) and then destained with 1× Coomassie Brilliant Blue R 250 Destaining Solution (Bio-Rad). After washing with water, the blot was scanned using Bio Rad GS-800 densitometer.

C.2.7.2 Results of Glycosylation Analysis

To test whether the CP4 EPSPS protein was glycosylated when expressed in the seed of MON 87411, the MON 87411-produced CP4 EPSPS protein was analyzed using an ECL™ Glycoprotein Detection Module (GE Healthcare, Piscataway, NJ). To assess equivalence of the MON 87411- and *E. coli*-produced CP4 EPSPS proteins, the *E. coli*-produced CP4 EPSPS protein, previously been shown to be free of glycosylation (Harrison et al. 1996) was also analyzed.

A clear glycosylation signal was observed at the expected molecular weight in the lanes containing the positive control (transferrin) and the band intensity increased with increasing concentration (Figure C-8, Panel A, lanes 2-5). In contrast, signals were not observed in the lanes containing the MON 87411- or *E. coli*-produced protein at the expected molecular weight for the CP4 EPSPS protein (Figure C-8 panel A, lanes 6-9).

To confirm that sufficient MON 87411- and *E. coli*-produced CP4 EPSPS proteins were present for glycosylation analysis, a second membrane (with identical loadings and transfer times) was stained with Coomassie Blue R250 for protein detection (Figure C-8 Panel B). Both the MON 87411- and *E. coli*-produced CP4 EPSPS proteins were clearly detected (Figure C-8, Panel B, Lanes 6-9).

These data indicate that the glycosylation status of MON 87411-produced CP4 EPSPS protein is equivalent to that of the *E. coli*-produced CP4 EPSPS protein and that neither is glycosylated.

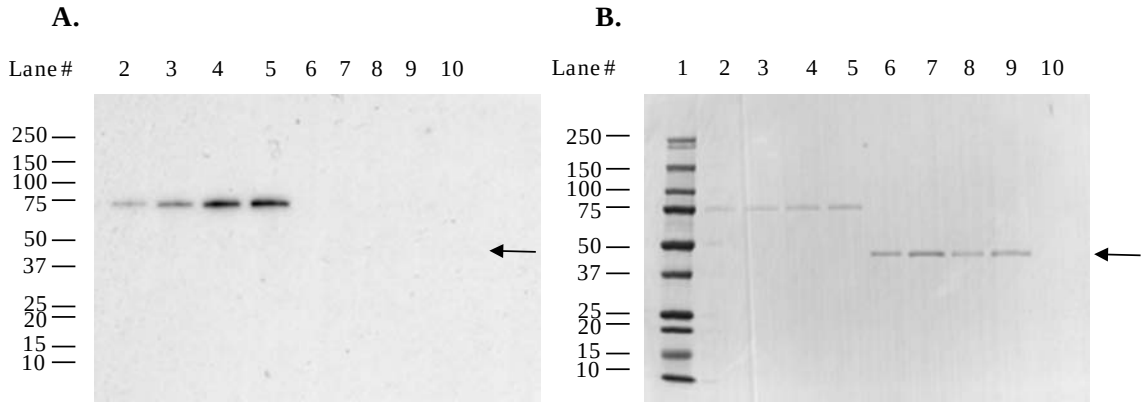

Figure C-8. Glycosylation Analysis of the MON 87411-produced CP4 EPSPS Protein

Aliquots of transferrin (positive control), *E. coli*-produced CP4 EPSPS protein and MON 87411-produced CP4 EPSPS protein were subjected to SDS-PAGE and electrotransferred to PVDF membranes. The MWs (kDa) correspond to the Precision Plus Protein™ Standards. The arrows show the expected migration of the MON 87411-produced and *E. coli*-produced CP4 EPSPS proteins. (A) Where present, labeled carbohydrate moieties were detected by addition of streptavidin conjugated to HRP followed by a luminol-based detection using ECL reagents and exposure to Hyperfilm®. (B) An equivalent blot was stained with Coomassie Blue R250 to confirm the presence of proteins. Lane designations and the approximate mass loaded are as follows:

Lane	Sample	Amount (ng)
1	Precision Plus Protein™ Standards	-
2	Transferrin (positive control)	50
3	Transferrin (positive control)	100
4	Transferrin (positive control)	150
5	Transferrin (positive control)	200
6	*E. coli*-produced CP4 EPSPS protein (negative control)	100
7	*E. coli*-produced CP4 EPSPS protein (negative control)	200
8	MON 87411-produced CP4 EPSPS protein	100
9	MON 87411-produced CP4 EPSPS protein	200
10	Blank	-

C.2.8 CP4 EPSPS Functional Activity Analysis

C.2.8.1 Methods

In preparation for analysis the *E. coli*-produced CP4 EPSPS was diluted with storage buffer to the same purity corrected concentration as the MON 87411-produced protein (0.263 mg CP4 EPSPS/ml). Prior to functional activity analysis, both MON 87411- and *E. coli*-produced proteins were diluted with 50 mM HEPES, pH 7.0 buffer to a purity corrected concentration of ~50 µg/ml. Assays for both proteins were conducted in triplicate. The reactions were performed in 50 mM HEPES, pH 7.0, 0.1 mM ammonium molybdate, 1 mM PEP and 5 mM potassium fluoride with or without 2 mM S-3-P for 2 min at ~25 °C. The reactions were initiated by the addition of PEP. After 2 min, the reactions were quenched with phosphate assay reagent (0.033% malachite green, 1.1% ammonium molybdate) and then fixed with 33% (w/v) sodium citrate. A standard curve was prepared using 0 to 10 nmoles of inorganic phosphate in water treated with the phosphate assay reagent and 33% (w/v) sodium citrate. The absorbance of each reaction and each standard was measured in duplicate at 660 nm using a PowerWave Xi™ microplate reader. The amount of inorganic phosphate released from PEP in each reaction was determined using the standard curve. For CP4 EPSPS, the specific activity was defined in unit per mg of protein (U/mg), where a unit (U) is defined as 1 µmole of inorganic phosphate released from PEP per min at 25 °C. Calculations of the specific activities were performed using Microsoft Excel (2007).

C.2.8.2 Results of Functional Activity

The functional activities of the MON 87411- and *E. coli*-produced CP4 EPSPS proteins were determined using a colorimetric assay that measures formation of inorganic phosphate (Pi) from the EPSPS-catalyzed reaction between shikimate-3-phosphate (S-3-P) and PEP. In this assay, protein-specific activity is expressed as units per milligram of protein (U/mg), where a unit is defined as one µmole of inorganic phosphate released from PEP per minute at 25 °C. The MON 87411- and *E. coli*-produced CP4 EPSPS proteins were considered to have equivalent functional activity if the specific activities of both were within the acceptance limits of 1.96 to 7.90 U/mg.

The experimentally determined specific activities for the MON 87411- and *E. coli*-produced CP4 EPSPS proteins are presented in Table C-10. The specific activities of MON 87411- and *E. coli*-produced CP4 EPSPS proteins were 5.78 U/mg and 5.00 U/mg of CP4 EPSPS protein, respectively. Because the specific activity of the MON 87411-produced CP4 EPSPS protein falls within the preset acceptance criterion (Table C-10), the MON 87411-produced CP4 EPSPS protein was considered to have equivalent functional activity to that of the *E. coli*-produced CP4 EPSPS protein.

Table C-10. CP4 EPSPS Functional Activity Assay

MON 87411-Produced CP4 EPSPS Protein[1] (U/mg)	*E. coli*-Produced CP4 EPSPS Protein[1] (U/mg)	Preset Acceptance Limits for MON 87411-Produced CP4 EPSPS (U/mg)
5.78 ± 0.19	5.00 ± 0.38	1.96 – 7.90

[1]Value refers to mean and standard deviation calculated based on n=6 which includes three replicate assays spectrophometrically read in duplicate plate wells.

References for Appendix C

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Appendix D: Materials and Methods Used for the Analysis of the Levels of Cry3Bb1 and CP4 EPSPS Proteins in MON 87411

D.1. Materials

Over season leaf (OSL1-4), over season root (OSR1-4), over season whole plant (OSWP1-4), stover, senescent root, forage root, forage, grain, pollen and silk tissue samples from glyphosate-treated MON 87411 were harvested from five field sites in Argentina during the 2011 – 2012 growing season from starting seed lot 11320173. *E coli*-produced Cry3Bb1 (lot 10000775) and CP4 EPSPS protein (lot 10000739) were used as the analytical reference standards.

D.2. Characterization of the Materials

The identity of MON 87411 was confirmed by conducting MON 87411 event-specific polymerase chain reaction (PCR) analyses on the harvested grain from each site.

D.3. Field Design and Tissue Collection

Field trials were initiated during the 2011 – 2012 planting season to generate MON 87411 at various maize growing locations in Argentina. OSL1-4, OSR1-4, OSWP1-4, stover, senescent root, forage root, forage, grain, pollen and silk tissue samples from the following field sites were analyzed: Pergamino, Buenos Aires (Site Code BAFO); Hunter, Buenos Aires (Site Code BAHT); Pergamino, Buenos Aires (Site Code BAPE); Sarasa, Buenos Aires (Site Code BASS) and Salto, Buenos Aires (Site Code BATC). At each site, four replicated plots of plants containing MON 87411 were planted using a randomized complete-block field design. Tissue samples were collected from each replicated plot at all field sites. See Tables V-1 and V-2 for detailed descriptions of when the samples were collected.

D.4. Tissue Processing and Protein Extraction

All tissue samples harvested were shipped to Monsanto's processing facility, and were ground, except pollen, by the Monsanto Sample Management Team to facilitate protein extraction. The ground tissue samples, and pollen, were stored in a -80 °C freezer until transferred on dry ice to the analytical facility.

D.4.1. Cry3Bb1 Protein

The Cry3Bb1 protein was extracted from maize tissues as described in Table D-1. The protein extracts were aliquoted and stored frozen in a -80 °C freezer until analysis.

Table D-1. Cry3Bb1 Protein Extraction Methods[1] for Tissue Samples

Sample Type	Tissue-to-Buffer Ratio	Extraction Buffer
Leaf[2]/Grain/Pollen/Silk/Root[3]/Forage[4]	1:100	50 mM sodium carbonate-bicarbonate + 0.1% (w/v) BSA[5]

[1]Cry3Bb1 protein was extracted from each tissue by adding the appropriate volume of extraction buffer and chrome steel beads, and shaking in a Harbil mixer (Harbil Industries Inc., Compton, CA). The extracted samples were clarified using a serum filter.
[2]Over season leaf (OSL1, OSL2, OSL3, and OSL4).
[3]Over season root (OSR1, OSR2, OSR3, and OSR4), forage root and senescent root.
[4]Forage, stover and over season whole plant (OSWP1, OSWP2, OSWP3, and OSWP4).
[5]50 mM sodium carbonate-bicarbonate with 0.1 % bovine serum albumin (BSA) [0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, and 0.1% (w/v) BSA].

D.4.2. CP4 EPSPS Protein

CP4 EPSPS protein was extracted from maize tissues samples as described in Table D-2. The protein extracts were aliquoted and stored frozen in a -80 °C freezer until analysis.

Table D-2. CP4 EPSPS Protein Extraction Methods[1] for Tissue Samples

Sample Type	Tissue-to-Buffer Ratio	Extraction Buffer
Leaf[2]/Pollen/Silk/Forage[3]	1:100	1× PBST + 0.1% (w/v) BSA[5]
Grain	1:100	1× TBA + 10 mM DCA[6]
Root[4]	1:50	1× PBST + 0.1% (w/v) BSA

[1]CP4 EPSPS protein was extracted from each tissue by adding the appropriate volume of extraction buffer and chrome steel beads, and shaking in a Harbil mixer (Harbil Industries Inc., Compton, CA). The extracted samples were clarified using a serum filter.
[2]Over season leaf (OSL1, OSL2, OSL3, and OSL4).
[3]Forage, stover and over season whole plant (OSWP1, OSWP2, OSWP3, and OSWP4).
[4]Over season root (OSR1, OSR2, OSR3, and OSR4), forage root and senescent root.
[5]Phosphate buffered saline buffer with Tween-20 and 0.1% bovine serum albumin [0.001 M KH_2PO_4, 0.01 M Na_2HPO_4, 0.137 M NaCl, and 0.0027 M KCl with 0.05% (v/v) Tween-20 and 0.1% (w/v) BSA].
[6]Tris-borate buffer (pH 7.8) with L-ascorbic acid and 10 mM deoxycholic acid [0.1 M Tris, 0.1 M $Na_2B_4O_7$, 0.005 M $MgCl_2$, 0.05% (v/v) Tween-20, 0.2% (w/v) L-ascorbic acid and 0.01 M DCA].

D.5. Cry3Bb1 and CP4 EPSPS Antibodies

D.5.1. Cry3Bb1 Antibodies

Goat polyclonal antibodies specific for the Cry3Bb1 protein were purified using Protein G affinity chromatography. The concentration of the purified IgG was determined to be 4.5 mg/ml by spectrophotometric methods. The purified antibody was stored in (1×PBS) (0.001 M KH_2PO_4, 0.01 M Na_2HPO_4, 0.137 M NaCl, and 0.0027 M KCl).

Purified Cry3Bb1 antibodies were coupled with biotin (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. The detection reagent was NeutrAvidin (Thermo Fisher Scientific) conjugated to horseradish peroxidase.

D.5.2. CP4 EPSPS Antibodies

Mouse monoclonal antibody clone 39B6.1 (IgG2a isotype, kappa light chain) specific for the CP4 EPSPS protein was purified from mouse ascites fluid using Protein A affinity chromatography. The concentration of the purified IgG was determined to be 2.3 mg/ml by spectrophotometric methods. Production of the 39B6.1 monoclonal antibody was performed by Strategic Biosolutions (Newark, DE). The purified antibody was stored in a buffer (0.02 M NaH_2PO_4, 0.15 M NaCl, and 15 ppm Proclin 300). The detection reagent was goat anti-CP4 EPSPS antibody, otherwise known as anti-protein 4 (Sigma-Aldrich, Saint Louis, MO) conjugated to horseradish peroxidase.

D.6. Cry3Bb1 and CP4 EPSPS ELISA Methods

D.6.1. Cry3Bb1 Protein

Goat anti-Cry3Bb1 capture antibodies were diluted in a coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$,) and immobilized onto 96-well microtiter plates at 5 µg/ml followed by incubation in a 4 °C refrigerator for ≥12 hours. Prior to each step in the assay, plates were washed with 1× PBS containing 0.05 % (v/v) Tween 20 (1× PBST) . Plates were blocked with the addition of 150 µl per well of 1× PBS with 0.25% (w/v) casein for 60 to 70 minutes at 37 °C. Cry3Bb1 protein standard or sample extract was added at 100 µl per well and incubated for 60 to 70 minutes at 37 °C. Biotinylated goat anti-Cry3Bb1 antibodies were added at 100 µl per well and incubated for 60 to 70 minutes at 37 °C. NeutraAvidin-horseradish peroxidase conjugate was added at 100 µl per well and incubated for 30 to 35 minutes at 37 °C. Plates were developed by adding 100 µl per well of horseradish peroxidase substrate, 3,3',5,5'- tetramethylbenzidine (TMB, Kirkegaard & Perry, Gaithersburg, MD). The enzymatic reaction was terminated by the addition of 100 µl per well of 6 M H_3PO_4. Quantification of the Cry3Bb1 protein was accomplished by interpolation from a Cry3Bb1 protein standard curve that ranged from 0.35 - 11.2 ng/ml.

D.6.2. CP4 EPSPS Protein

The CP4 EPSPS ELISA was performed using plates commercially coated at 2 µg/ml (Agri Diagnostic Manufacturing, Rickreall, OR). CP4 EPSPS protein standard or sample extract was added at 100 µl per well and incubated for 60 to 70 minutes at 37 °C. Prior to each step in the assay, plates were washed with 1× PBST. Goat anti-CP4 EPSPS conjugated to horseradish peroxidase was added at 100 µl per well and incubated for 60 to 70 minutes at 37 °C. Plates were developed by adding 100 µl per well of horseradish peroxidase substrate, TMB. The enzymatic reaction was terminated by the addition of 100 µl per well of 6 M H_3PO_4. Quantification of the CP4 EPSPS protein was accomplished by interpolation from a CP4 EPSPS protein standard curve that ranged from 0.456 - 14.6 ng/ml.

D.7. Moisture Analysis

Tissue moisture content was determined using a Mettler Toledo HR83 Moisture Analyzer System (Mettler-Toledo, Inc. Columbus, OH). A homogeneous tissue-specific site pool (TSSP) was prepared consisting of samples of a given tissue type grown at a specific site. The mean percent moisture for each TSSP was calculated from triplicate analyses. A TSSP dry weight conversion factor (DWCF) was calculated using MoistureDirect version 4.0 software as follows:

$$\text{DWCF} = 1 - \left(\frac{\text{Mean\% TSSP Moisture}}{100}\right)$$

The DWCF was used to convert protein levels assessed on a µg/g fresh weight (fw) basis into levels reported on a µg/g dry weight (dw) basis using the following calculation:

$$\text{Protein Level in Dry Weight} = \frac{\text{Protein Level Fresh Weight}}{\text{DWCF}}$$

The protein levels (ng/ml) that were reported to be less than or equal to the limit of detection (LOD) or less than the limit of quantitation (LOQ) on a fresh weight basis were not reported on a dry weight basis.

D.8. Data Analyses

ELISA plates were analyzed on a SPECTRAmax Plus 384 (Molecular Devices, Sunnyvale, CA) microplate spectrophotometer, using a dual wavelength detection method. Protein concentrations were determined by optical absorbance at a wavelength of 450 nm with a simultaneous reference reading of 620 nm. Data reduction analyses were performed using Molecular Devices SOFTmax PRO GxP version 5.4 software. Absorbance readings and protein standard concentrations were fitted with a four-parameter logistic curve fit. Following the interpolation from the standard curve, the amount of protein (ng/ml) in the tissue was reported on a "µg/g fw" basis for data that were greater than or equal to the LOQ. This conversion utilized a sample dilution factor, and tissue-to-buffer ratio. The protein values in "µg/g fw" were also converted to

"µg/g dw" by applying the DWCF. Microsoft Excel 2007 (Microsoft, Redmond, WA) was used to calculate the protein levels in all maize tissues. The sample means, standard deviations (SDs), and ranges were also calculated using Microsoft Excel 2007. All protein expression levels were rounded to two significant figures.

A test substance extract that resulted in unexpectedly negative result by ELISA analysis was re-extracted twice for the protein of interest and re-analyzed by ELISA to confirm the result. The sample with the confirmed unexpected result was omitted from all calculations.

Appendix E: Materials, Methods and Results for Characterization of the DvSnf7 RNA Produced in MON 87411

E.1 Materials

The MON 87411 RNA was extracted from ~V6 maize MON 87411 leaf tissue. The MON 87411 RNA was resuspended in water and stored in a -80 °C freezer. The *in vitro*-produced DvSnf7 RNA (Dvsnf7_968 RNA; lot 11331164) was used as the reference substance. The DvSnf7_968 RNA was *in vitro* transcribed from the plasmid pMON149601 and purified using acid phenol:chloroform, precipitated with isopropanol and resuspended in UltraPure Water (Invitrogen). The *in vitro* transcribed DvSnf7_968 RNA was previously characterized.

E.2. RNA extraction and Poly(A) enrichment

The MON 87411 RNA was extracted and enriched from maize MON 87411 leaf tissue. The extraction and enrichment procedure was not performed under a GLP plan; however, all procedures were documented on worksheets and, where applicable, SOPs were followed.

MON 87411 RNA was extracted from MON 87411 leaf tissue. Prior to extraction, leaf tissue was processed into a fine powder using a Harbil paint shaker. Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. All extracted RNA was eluted by water and stored in a -80°C freezer.

MON 87411 poly(A) RNA was enriched from MON 87411 RNA using the Ambion MicroPoly (A) Purist MAG kit (Life Technologies, Carlsbad, CA) as per manufacturer's specifications. All MON 87411 poly(A) RNA was resuspended in nuclease-free water and stored in a -80 °C freezer.

E.3. Sequence analyses

E.3.1. Methods

DNA sequence analysis was performed to confirm the identity of MON 87411 DvSnf7 RNA and to compare its sequence to that of DvSnf7_968 RNA.

MON 87411 DvSnf7 RNA was reverse transcribed using a sequence specific primer to produce cDNA (complementary DNA) utilizing an Omniscript Reverse Transcription kit from Qiagen (Valencia, CA). The manufacturer's specifications were followed with the exception of the incubation time, which was run for 30 min. The resulting cDNA was stored in a -20 °C freezer.

Two overlapping PCR products (Product A and Product B) were generated that span the expected primary transcript sequence of MON 87411 DvSnf7 RNA (Figure E-1). These products were analyzed to determine the nucleotide sequences of MON 87411 DvSnf7 RNA.

The PCR amplification of Product A was performed on 1 µl of diluted MON 87411 DvSnf7 cDNA in a 20 µl reaction volume. Each reaction contained a final concentration of 0.2 µM of each primer, 1 mM $MgCl_2$, 0.2 mM dNTP mix, and 0.5 units of PrimeSTAR® GXL Polymerase (Takara Bio Inc., Shiga, Japan). The following cycling conditions were followed: 38 cycles at 98 °C for 10 seconds; 60 °C for 15 seconds; 68 °C for 5 seconds with an initial denaturation step at 98 °C for 2 min and a final extension cycle for 5 min at 68 °C.

The PCR amplification of Product B was performed on 1 µl of diluted MON 87411 DvSnf7 cDNA in a 20 µl reaction volume. Each reaction contained a final concentration of 0.2 µM of each primer, 1 mM $MgCl_2$, 0.2 mM dNTP mix, and 0.5 units of PrimeSTAR® GXL Polymerase (Takara Bio Inc., Shiga, Japan). The following cycling conditions were followed: 35 cycles at 98 °C for 10 seconds; 60 °C for 15 seconds; 68 °C for 30 seconds with an initial denaturation step at 98 °C for 2 min and a final extension cycle for 5 min at 68 °C.

Aliquots of each PCR product were separated on a 1.2% (w/v) agarose gel and visualized by ethidium bromide staining to verify that the products were the expected size. Prior to sequencing, each verified PCR product was purified using the QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions and quantified using a Nanodrop spectrophotometer according to the manufacturer's instructions. The purified PCR products were sequenced using multiple primers, including primers used for PCR amplification.

All sequencing was performed by Monsanto TGAC (The Genome Analysis Center) using BigDye™ terminator chemistry (Applied Biosystems, Foster City, CA). A consensus sequence was generated by compiling sequences from multiple sequencing reactions performed on the overlapping PCR products. This consensus sequence was obtained to determine the identity of MON 87411 DvSnf7 RNA. The consensus sequence was also aligned to the sequence of DvSnf7_968 cDNA which was established during its characterization (lot 11331164). This alignment established the equivalence between MON 87411 DvSnf7 RNA and DvSnf7_968 RNA.

E.3.2. Results of Sequence Analyses

In order to characterize the MON 87411 DvSnf7 RNA, and to demonstrate molecular equivalence between it and DvSnf7_968 RNA, the sequence of MON 87411 DvSnf7 cDNA (complementary DNA) was determined. MON 87411 DvSnf7 RNA was reverse transcribed to cDNA and amplified by PCR. The amplified MON 87411 DvSnf7 cDNA was sequenced to obtain the primary transcript sequence which contains the predicted transcription start and polyadenylation sites (Coruzzi et al. 1984), and most importantly, the 240 bp dsRNA functional region (inverted repeat) (Bolognesi et al. 2012). The consensus sequence of MON 87411 DvSnf7 cDNA was aligned to the sequence of cDNA from DvSnf7_968 RNA (DvSnf7_968 cDNA) which was previously characterized under APS characterization plan 11331164. In this APS characterization, DvSnf7_968 RNA was shown to consist of 968 nucleotides encompassing the predicted transcription start,

the polyadenylation site, and the 240 bp dsRNA. This analysis demonstrated that the sequenced regions between MON 87411 DvSnf7 cDNA and DvSnf7_968 cDNA are identical. More importantly, it was shown that there is a 100% sequence identity in the inverted repeat regions between the MON 87411 DvSnf7 cDNA consensus sequence and the DvSnf7_968 cDNA reference sequence (Figure E-2). This analysis serves as identity confirmation of the MON 87411 DvSnf7 RNA as well as demonstrating the molecular equivalence between MON 87411 DvSnf7 RNA and DvSnf7_968 RNA.

E.4. Northern Blot Analysis

E.4.1. Methods

Northern blot analysis was carried out to characterize MON 87411 DvSnf7 RNA and to demonstrate the molecular equivalence between MON 87411 DvSnf7 RNA and DvSnf7_968 RNA. The dsRNA regions of MON 87411 DvSnf7 RNA and DvSnf7_968 RNA were specifically targeted through RNase I_f treatment of the RNAs. RNase I_f preferentially digests single stranded RNA leaving behind double stranded regions such as the active region of DvSnf7. It is this 240 bp dsRNA, that is efficiently taken up by WCR and is processed by the RNAi machinery, ultimately leading to mRNA degradation. Therfore, RNase I_f digestion facilitates a direct comparison of the size equivalence between the active dsRNA regions of MON 87411 DvSnf7 RNA and DvSnf7_968 RNA.

DvSnf7_968 RNA and poly (A) RNA from MON 87411 plant tissues were digested with RNase I_f (New England Biolabs, Ipswich, MA) using the manufacturer's suggested reaction conditions for 5 minutes. Following digestion, the samples were heat inactivated at 70 °C for 20 minutes, precipitated, and resuspended in nuclease-free water.

RNase I_f digested MON 87411 poly (A) RNA and DvSnf7_968 RNA were resolved on a ~6.6% (w/v) formaldehyde/2.5% agarose gel buffered by 1x MESA. The gel was run at 100V for ~2.5 hours. An appropriate molecular size marker was included on the gel. After running, the gel was stained with SYBR Gold (Life Technologies, Carlsbad, CA) and the RNA visualized and photographed.

The RNA resolved on the formaldehyde/agarose gel was transferred to a neutral nylon membrane. The ladder and wells were marked before UV light cross-linking the RNA to the membrane.

The northern blot was hybridized with a [α-^{32}P]-dATP-labeled probe specific to the 240 bp dsRNA region of DvSnf7. Prior to hybridization, the DvSnf7 probe template was generated by PCR using plasmid PV-ZMIR10871 as the template and primers specific to the DvSnf7 240 bp dsRNA. The PCR products were separated by agarose gel electrophoresis and purified from the gel using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) according to the manufacturer's specifications. After purification, the probe template was quantified using a Qubit Fluorometer (Life Technologies, Carlsbad, CA). Approximately 25 ng of probe template was labeled with [α-^{32}P]-dATP

(~6000 Ci/mmol) using the RadPrime DNA Labeling System (Life Technologies, Carlsbad, CA).

The hybridization was carried out at 55 °C and the membrane was washed at the same temperature. Two exposures of the blot were generated using Kodak Biomax MS film (Eastman Kodak, Rochester, NY) in conjunction with two Kodak Biomax MS intensifying screen in a -80 °C freezer. The banding in the MON 87411 DvSnf7 RNA and DvSnf7_968 RNA lanes was visually compared, after a one hour exposure, to determine their size similarity and the identity of MON 87411 DvSnf7 RNA.

E.4.2. Results of the Northern Blot Analysis

Poly (A) RNA from MON 87411 plant tissues (MON 87411 poly (A) RNA) and DvSnf7_968 RNA were digested by RNase I_f which preferentially digests single stranded RNA leaving dsRNA regions intact. Each RNase I_f digested sample was loaded in triplicate and subjected to northern blot analyses. The northern blot was hybridized with a probe specific to the 240 bp dsRNA region of DvSnf7 in order to characterize MON 87411 DvSnf7 RNA by identifying the presence of the functionally active double stranded region of DvSnf7 RNA. Additionally, a comparison of the dsRNA regions of MON 87411 DvSnf7 RNA and DvSnf7_968 RNA was conducted using this northern blot analysis to determine equivalence. The results are shown in Figure E-3. MON 87411 DvSnf7 RNA contains the expected ~240 bp dsRNA region of DvSnf7 as indicated by the ~240 base band on the blot (Lanes 4-6). This is the expected size of the double stranded functionally active region of DvSnf7 (Bolognesi et al. 2012). Furthermore, DvSnf7_968 RNA (lanes 1-3) exhibits a single band at approximately 240 bases, similar to MON 87411 DvSnf7 RNA (lanes 4-6), indicating that MON 87411 DvSnf7 RNA and DvSnf7_968 RNA are equivalent with respects to their dsRNA functionally active regions.

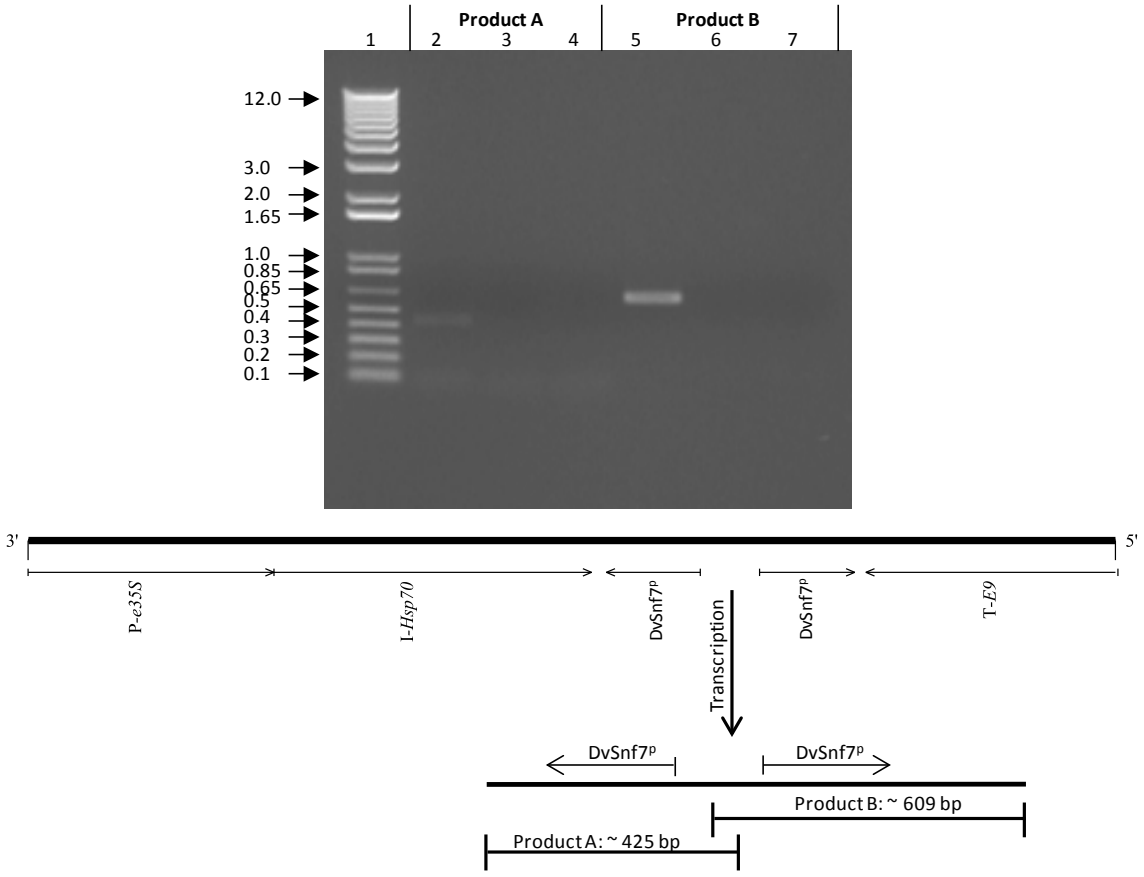

Figure E-1. PCR Amplification of MON 87411 DvSnf7 cDNA

PCR was performed on MON 87411 DvSnf7 cDNA using two pairs of primers to generate overlapping PCR fragments for sequencing analysis. To verify the PCR products, the PCR reactions were run on a gel. The expected product size for each amplicon is provided in the illustration of the MON 87411 DvSnf7 transcript (A region of I-Hsp70 is spliced out.) The MON 87411 DvSnf7 suppression cassette is depicted above the DvSnf7 transcript. This figure is a representative of the data generated in the study and the illustrations are not to scale. Lane designations are as follows:

Lane	Sample
1	1 Kb DNA Plus Ladder
2	MON 87411 DvSnf7 cDNA
3	Reverse transcriptase reaction minus reverse transcriptase
4	No template control
5	MON 87411 DvSnf7 cDNA
6	Reverse transcriptase reaction minus reverse transcriptase
7	No template control

Arrows next to the agarose gel image denote the size of the DNA, in kilobase pairs, obtained from the 1kb DNA Plus Ladder (Invitrogen) on the ethidium bromide stained gel.

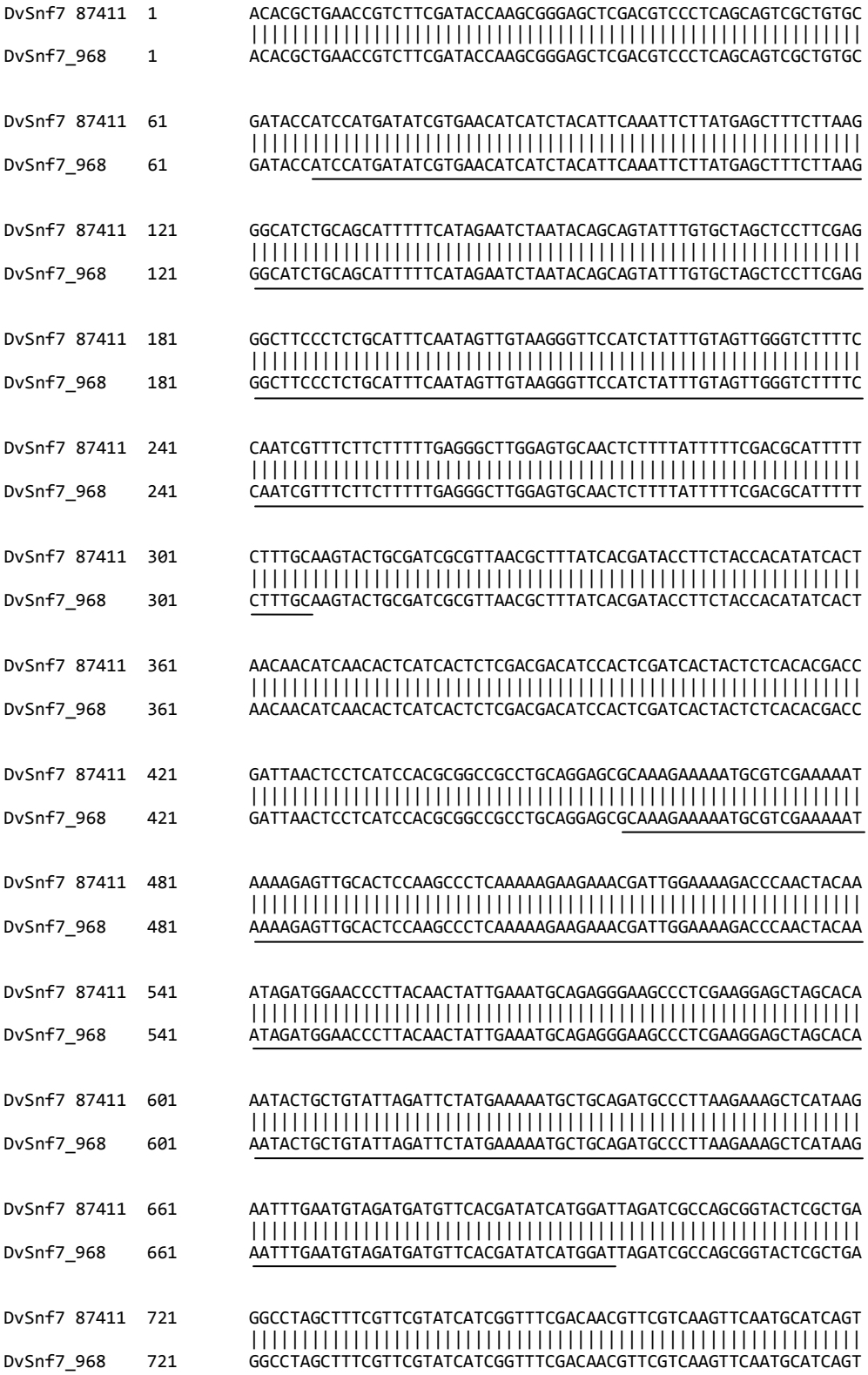

Figure E-2. Sequence Alignment between MON 87411 DvSnf7 cDNA and DvSnf7_968 cDNA

```
DvSnf7 87411  781       TTCATTGCGCACACACCAGAATCCTACTGAGTTTGAGTATTATGGCATTGGGAAAACTGT
                        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
DvSnf7_968    781       TTCATTGCGCACACACCAGAATCCTACTGAGTTTGAGTATTATGGCATTGGGAAAACTGT


DvSnf7 87411  841       TTTTCTTGTACCATTTGTTGTGCTTGTAATTTACTGTGTTTTTTATTCGGTTTTCGCTAT
                        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
DvSnf7_968    841       TTTTCTTGTACCATTTGTTGTGCTTGTAATTTACTGTGTTTTTTATTCGGTTTTCGCTAT


DvSnf7 87411  901       CGAACTGTGAAATGGAAATGGATGGAGAAGAGTTAATGAATGATATGGTCCTTTTGTTCA
                        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
DvSnf7_968    901       CGAACTGTGAAATGGAAATGGATGGAGAAGAGTTAATGAATGATATGGTCCTTTTGTTCA


DvSnf7 87411  961       TTCTCAAA   968
                        ||||||||
DvSnf7_968    961       TTCTCAAA   968
```

Figure E-2 (continued). Sequence Alignment between MON 87411 DvSnf7 cDNA and DvSnf7_968 cDNA

The consensus sequence of MON 87411 DvSnf7 cDNA(top sequence labeled DvSnf7 87411) aligned to that of the previously characterized DvSnf7_968 cDNA (bottom sequence labeled DvSnf7_968) sequence. The inverted repeat regions are underlined. The numbering indicates base position.

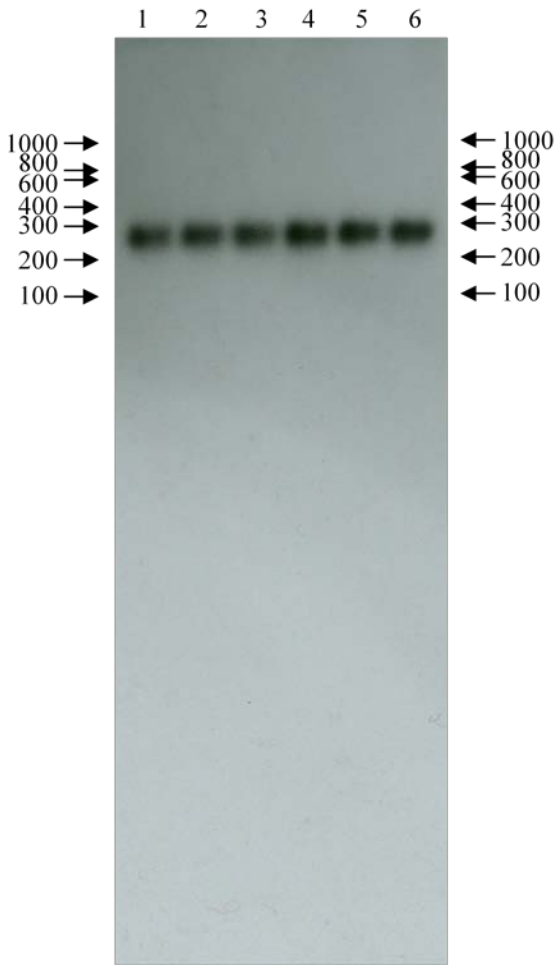

Figure E-3. Northern Blot Analysis to Confirm the Equivalence between the dsRNA in DvSnf7_968 RNA and MON 87411 DvSnf7 RNA

The blot was hybridized with a 32P-labled, DvSnf7 probe specific to the 240 bp dsRNA. Lanes 1-3 contain 75 pg of DvSnf7_968 RNA that was RNase I_f digested and lanes 4-6 contain 200 ng of MON 87411 poly (A) RNA also subjected to RNase I_f digestion. Lane designations are as follows:

Lane	Sample
1	DvSnf7_968 RNA (RNase I_f digested)
2	DvSnf7_968 RNA (RNase I_f digested)
3	DvSnf7_968 RNA (RNase I_f digested)
4	MON 87411 poly (A) RNA (RNase I_f digested)
5	MON 87411 poly (A) RNA (RNase I_f digested)
6	MON 87411 poly (A) RNA (RNase I_f digested)

Arrows denote the size of the RNA, in bases, obtained from the Low Range Ribo Ruler ladder on the SYBR Gold stained gel.

References for Appendix E

Bolognesi, R., P. Ramaseshadri, J. Anderson, P. Bachman, W. Clinton, R. Flannagan, O. Ilagan, C. Lawrence, S. Levine, W. Moar, G. Mueller, J. Tan, J. Uffman, E. Wiggins, G. Heck and G. Segers. 2012. Characterizing the mechanism of action of double-stranded RNA activity against Western corn rootworm (*Diabrotica virgifera virgifera* LeConte). PLoS ONE 7:e47534.

Coruzzi, G., R. Broglie, C. Edwards and N.-H. Chua. 1984. Tissue-specific and light-regulated expression of a pea nuclear gene encoding the small subunit of ribulose-1, 5-bisphosphate carboxylase. EMBO Journal 3:1671-1679.

Appendix F: Materials and Methods Used for the Analysis of Expression Levels of DvSnf7 RNA in MON 87411

F.1 Materials

Over season leaf (OSL1, OSL2, OSL3, and OSL4), over season root (OSR1, OSR2, OSR3, and OSR4), over season whole plant (OSWP1, OSWP2, OSWP3, and OSWP4), forage root, forage, senescent root, stover, pollen, silk, and grain tissue samples from glyphosate treated plants grown from starting seed lot 11320173 were collected at five field sites in Argentina during 2011-2012 growing season. MON 87411 plots were treated at the 2-4 leaf stage with glyphosate herbicide at the label rate (0.95 kg active ingredient [a.i.]/ha). An *in vitro* transcribed DvSnf7 RNA, DvSnf7_968 (lot: 11331164), was used as an analytical reference standard for the assessment of DvSnf7 RNA levels from MON 87411.

F.2 Characterization of the Materials

The identity of MON 87411 was confirmed by analysis of the harvested grain DNA by an event-specific polymerase chain reaction (PCR) method.

F.3. Field Design and Tissue Collection

Field trials were initiated during the 2011 planting season to generate MON 87411 over season leaf (OSL1 through OSL4), over season root (OSR1 through OSR4), over season whole plant (OSWP1 through OSWP4), forage root, forage, senescent root, stover, pollen, silk, and grain at various maize growing locations in Argentina. The tissue samples from the following field sites were analyzed: Pergamino, Buenos Aires (site code BAFO); Hunter, Buenos Aires (site code BAHT); Pergamino, Buenos Aires (site code BAPE); Sarasa, Buenos Aires (BASS); and Salto, Buenos Aires (site code BATC). These field sites were representative of maize producing regions suitable for commercial production. At each site, four replicated plots of plants containing MON 87411 were planted using a randomized complete-block field design. Over season leaf (OSL1 through OSL4), over season root (OSR1 through OSR4), over season whole plant (OSWP1 through OSWP4), forage root, forage, senescent root, stover, pollen, silk, and grain samples were collected from each replicated plot at all field sites. See Table VI-1for detailed descriptions of when the samples were collected.

F.4. Tissue Processing and Total RNA Extraction

Tissue samples were shipped on dry ice to Monsanto Company (Saint Louis, Missouri) and stored in -80° C freezers. All tissue samples except for pollen, were roughly ground with dry ice by the Monsanto Sample Management Team. The roughly ground samples and pollen samples were further processed to fine powder using liquid nitrogen. The prepared tissue samples were stored in -80° C freezers before and after transferred on dry ice to the analytical facility.

Total RNA from leaf (OSL1 through OSL4), root (OSR1 through OSR4), whole plant (OSWP1 through OSWP4), forage root, senescent root, and silk tissues was extracted using a Trizol method (Chomczynski and Sacchi 1987) and is briefly described below. On dry ice, approximately 0.1 grams of frozen processed tissue were transferred into a tube and weighed. Trizol reagent (Ambion Biotechnology, Austin, TX) was added to the tube and mixed by vortexing. Following an incubation at RT, chloroform (Fisher Scientific, Pittsburgh, PA) was added and mixed. Following another incubation at RT, the tube was centrifuged at 12,000 *x g* and ~4°C for 10 minutes. The aqueous phase was transferred to a new tube, and the RNA was precipitated with an equal volume of isopropyl alcohol (Sigma, Saint Louis, MO). To pellet the RNA, the tube was centrifuged at 12,000 *x g* and ~4°C for 20 minutes. The RNA pellet was washed using 70% (v/v) ethanol and resuspended in nuclease-free water (Gibco by Life Technologies, Grand Island, NY). All extracted RNA samples were stored in a -80°C freezer.

Total RNA from forage, stover, pollen, and grain tissues was extracted as follows. On dry ice, approximately 0.1 grams of frozen processed tissue were transferred to a tube and weighed. RNA extraction buffer (25 mM Tris-HCl pH 8.0, 25 mM EDTA, 75 mM NaCl, 1% SDS, and 1% β-mercaptolethanol) was added to the tube and mixed by vortexing. An equal volume of acid phenol (pH 4.3): chloroform [2:1 (v/v)] (Sigma, Saint Louis, MO; Fisher Scientific, Pittsburgh, PA, respectively) was added to the tube and mixed. The tube was centrifuged at 12,000 *x g* and 2-6 °C for 15 minutes to separate the phases. The aqueous phase was transferred to a new tube and an equal volume of acid phenol (pH 4.3): chloroform [1:1 (v/v)] was added to the tube and mixed. The tube was centrifuged at 12,000 *x g* and 2-6 °C for 10 minutes to separate the phases. The aqueous phase was transferred to a new tube and ½ volume of 15 M LiCl was added to the tube while gently mixing. The tube was incubated on ice for ~ 2 hours to precipitate RNA. To pellet the RNA, the tube was centrifuged at 20,000 *x g* and 2-6 °C for 30 minutes. The RNA pellet was washed with 75% (v/v) ethanol and resuspended in nuclease-free water (Gibco by Life Technologies). All extracted RNA samples were stored in a -80°C freezer.

All extracted RNA samples were quantified using a NanoDrop-8000 (Thermo Scientific, Wilmington, DE) according to the manufacturer's instructions. Prior to the analysis, each RNA sample was normalized in water to an appropriate concentration manually and/or using a Biomek FX^P Laboratory Automatic Workstation (Beckman Coulter Inc., Brea, CA) according to the manufacturer's instructions. All normalized RNA samples were stored in a -80 °C freezer before analysis.

F.5. Analytical Reference Standard DvSnf7_968

The Analytical Reference Standard DvSnf7_968 was produced by *in vitro* transcription. This *in vitro* transcribed DvSnf7 RNA corresponds to the expected *in planta* DvSnf7 RNA transcribed from the DvSnf7 suppression cassette in MON 87411. The sequence of the DvSnf7_968 was confirmed by sequencing analysis. The DvSnf7_968 concentration was 2.21 mg/ml as determined by spectrophotometric analysis.

F.6. QuantiGene® Plex 2.0 Assay

The normalized total RNA (Section D.2.4) was analyzed using a validated QuantiGene® Plex 2.0 Assay (Affymetrix Inc., Santa Clara, CA). This bead-based assay relies on a series of oligonucleotide probes that recognize and hybridize specifically to a portion of the DvSnf7 RNA. Capture probes conjugated to a magnetic polystyrene microbead are designed to capture the DvSnf7 RNA, while blocking probes act to prevent non-specific hybridization to unrelated RNAs, thereby increasing assay specificity. The assay also includes oligonucleotide probes which hybridize to the DvSnf7 RNA and the branched DNA molecules that mediate the signal amplification. Detection of the DvSnf7 RNA is by fluorescence detection of the hybridization complex while it passes through a signal detector. The fluorescence intensity is proportional to the number of the DvSnf7 RNA molecules present in the sample allowing the DvSnf7 RNA to be quantified by including a reference standard in the assay. The DvSnf7_968 (Section F.5) was used as an analytical reference standard. To ensure quality, each assay also includes a background control, a negative control (QC-), and a positive control (QC+).

F.6.1. Target Specific Probe Set Design and Generation

The probe set used in the QuantiGene® Plex 2.0 Assay (Affymetrix Inc.) was designed to detect transcripts containing DvSnf7 RNA (Affymetrix Inc. 2010). The probe set was generated and supplied by Affymetrix and stored in a -20 °C freezer upon receipt.

Three RNA targets, DvSnf7, actin, and EF-1α, were included in this assay. The DvSnf7 RNA was to be measured and reported. Actin and EF-1α were included in the assay to meet Affymetrix's requirements for the QuantiGene® Plex 2.0 Assay design, which has to be at least three targets. However, actin and EF-1α will be neither analyzed nor reported since they are not relevant to the DvSnf7 RNA quantification.

F.6.2. Background Control, Negative Quality Control (QC-), and Positive Quality Control (QC+)

Nuclease-free water was used as a background control for each assay since all RNA samples, including the DvSnf7_968 and the total RNA extracted from the plant tissues, were dissolved and normalized in nuclease-free water.

Total RNA extracted from conventional maize NL6169 leaf tissue was used as a QC- for each assay. The leaf tissue sample was collected from NL6169 plants grown from seed lot 11320471. The absence of the transgene containing the DvSnf7 expression cassette was confirmed by analysis of the leaf DNA extracted from individual plant leaf punches using an endpoint Taqman® PCR analysis method.

The DvSnf7_968 was spiked to an appropriate concentration in a separate aliquot of the same conventional maize RNA to serve as a QC+ for each assay.

F.6.3. Reference Standard Curve Generation

The DvSnf7_968 was spiked in separate aliquots of the same conventional maize NL6169 RNA at eight different concentrations to serve as reference standards for each assay. A standard curve was generated for each assay plate.

F.6.4. Execution of the QuantiGene® Plex 2.0 Assay

The QuantiGene® Plex 2.0 Assay (Affymetrix Inc.) was performed according to the manufacturer's instructions (Affymetrix Inc. 2010) with a modification to introduce a denaturation step. Briefly, each sample, including the nuclease-free water background control, QC-, QC+, DvSnf7_968 reference standards, and the test samples, was assayed in triplicate wells in a volume of 20 microliters (µl). For the test samples, the total RNA was normalized to a concentration such that an equal amount of RNA for each tissue type was added to the assay. To ensure the capture of the DvSnf7 RNA during hybridization, a denaturation step was introduced: the sample was mixed with the target-specific oligonucleotide probe set in a well of a 96-well PCR microplate (Axygen Scientific, Union City, CA) and heated for 5 minutes at 95 °C using a thermocycler (Applied Biosystems, Foster, CA). After heating, the samples were kept in the thermocycler at 46°C until use. Before removing the plate from the thermocycler, the hybridization buffer containing the rest of the components for the capture of the DvSnf7 RNA were added to each sample well. The PCR microplate was then removed from the thermocycler and the content of each well (~100 µl) was transferred to the corresponding well of a Hybridization Plate (Affymetrix Inc.) followed by overnight hybridization (Affymetrix Inc. 2010).

After signal amplification following the overnight hybridization, all QuantiGene® Plex 2.0 Assay plates were read by a Luminex LX200 analyzer (Luminex Corp., Austin, TX) according to the manufacturer's instructions. The net median fluorescence intensity (MFI) from each assay well, which is the MFI value subtracted by the MFI from the nuclease-free water background control, was reported digitally.

F.7. Moisture Analysis

Tissue moisture content was determined using an IR-200 Moisture Analyzer (Denver Instrument Company, Arvada, CO). A homogeneous tissue-specific site pool (TSSP) was prepared consisting of samples of a given tissue type grown at a given site. The average percent moisture for each TSSP was calculated from triplicate analyses. A TSSP Dry Weight Conversion Factor (DWCF) was calculated as follows:

$$\mathrm{DWCF} = 1 - \left(\frac{\mathrm{Mean\%\ TSSP\ Moisture}}{100}\right)$$

The DWCF was used to convert DvSnf7 levels assessed on a µg/g fresh weight (fw) basis into levels reported on a µg/g dry weight (dw) basis using the following calculation:

$$\text{DvSnf7 Level in Dry Weight} = \frac{\text{DvSnf7 Level in Fresh Weight}}{\text{DWCF}}$$

The DWCFs were only applied to test samples with DvSnf7 RNA levels greater than or equal to the assay's limit of quantitation (LOQ).

F.8. Data Analyses

Each net MFI value was converted to a log2 value. The sample mean (mean), standard deviation (SD), and coefficient of variation (CV) from the three replicate wells of each sample were calculated and retained in the raw data. For each analysis, a standard curve was generated based on the log2 values of the theoretical concentrations (ng/ml) of the eight DvSnf7_968 reference standards and the log2 values of their net MFI, which established the correlation between the DvSnf7_968 concentration in the assay and the log2 value of the net MFI obtained from the assay. Using this standard curve, the net MFI of the test samples was converted to a DvSnf7 concentration (ng/ml). Each test sample with a DvSnf7 RNA level greater than or equal to the LOQ was converted to a µg/g fw value using the total RNA/tissue ratio and dilution factor. All the µg/g fw values were converted to µg/g dw values by applying the DWCFs. The test samples with the DvSnf7 RNA level lower than or equal to the assay's LOD or less than the assay's LOQ were reported as ≤ LOD or < LOQ. The mean, SD, and range of the DvSnf7 RNA levels in MON 87411 were determined on both fw and dw basis for each tissue type across all five sites.

References for Appendix F

Affymetrix Inc. 2010. QuantiGene® Plex 2.0 Assay: Using a magnetic plate washer. Affymetrix, Inc., Santa Clara, California.

Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry 162:156-159.

Appendix G: Materials, Methods, and Individual Site Results for Compositional Analysis of MON 87411 Maize Grain and Forage

Compositional comparisons between MON 87411 and the conventional control maize hybrid were performed using the principles and analytes outlined in the OECD consensus documents for maize composition (OECD, 2002). These principles are accepted globally and have been employed previously in assessments of maize products derived through biotechnology. The compositional assessment was conducted on grain and forage samples harvested from a single growing season conducted in Argentina during 2011/2012 under normal agronomic practices.

G.1. Materials

Harvested grain and forage from MON 87411, a conventional control that has similar genetic background to that of MON 87411, and conventional, commercial reference maize hybrids were compositionally assessed. The reference hybrids are listed in Table G-1.

Table G-1. Conventional Commercial Reference Maize Hybrids

Material Name	Seed Lot #	Field Site Codes
Dow AgroSciences Mill 527	11319757	BABE, BATC
Phillips 717	11300073	BABE, BAFO, BATC
Stewart S518	11226920	BABE
Syngenta NK 880	11319758	BABE, BAGH, BASS
ACA 2002	11319751	BAFO
NC+ 4443	11226700	BAFO, BALN
Nidera AX 878	11319754	BAFO, BAHT
ACA 430	11319753	BAGH
Gateway 6116	11227211	BAGH
Midland Phillips 7B15P	11226702	BAGH
Stewart S602	11226919	BAHT
Stine 9724	11298951	BAHT
La Tijereta LT 625	11319756	BALN, BATC
Legacy L7671	11226598	BALN, BATC
Syngenta NK 940	11319760	BALN
H-9180	11226704	BAPE
LG2597	11226862	BAPE
Syngenta NK 910	11319759	BAPE
Dekalb DK747	11319755	BASS
Mycogen 2M746	11226705	BASS

G.2. Characterization of the Materials

The identities of MON 87411, the conventional control, and reference hybrids were confirmed prior to use in the compositional assessment.

G.3. Field Production of the Samples

Grain and forage samples from MON 87411, the conventional control, and the reference hybrids were collected from eight replicated sites in Argentina during the 2011/2012 growing season. The field sites were located in: Berdier, Buenos Aires (BABE); three sites in Pergamino, Buenos Aires (BAFO, BAPE, and BATC); Gahan, Buenos Aires (BAGH); Hunter, Buenos Aires (BAHT); Los Indios, Buenos Aires (BALN); and Sarasa, Buenos Aires (BASS). Starting seeds were planted in a randomized complete block design with four plots for each of MON 87411, the conventional control, and the reference hybrids. The production was conducted under normal agronomic field conditions for their respective geographic regions that are typical areas for maize production in Argentina. MON 87411 plots were treated with glyphosate at approximately 0.95 kg a.i./hectare at approximately the V2 growth stage to generate samples under conditions of the intended use of the product.

Forage was collected at early dent (R5) and grain was collected at physiological maturity. Forage samples were shipped on dry ice and grain was shipped at ambient temperature from the field sites to Monsanto Company (Saint Louis, Missouri). Subsamples were ground to a powder, stored in a freezer set to maintain -20°C located at Monsanto Company (Saint Louis, Missouri). Subsamples were shipped on dry ice to Covance Laboratories Inc. (Madison, Wisconsin) for compositional analysis.

G.4. Summary of Analytical Methods

Nutrients analyzed in this study included moisture, ash, protein, total fat, carbohydrates by calculation, acid detergent fiber (ADF), neutral detergent fiber (NDF), total dietary fiber (TDF), amino acids (18 components), fatty acids (22 components), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc) and vitamins [β-carotene (referred to as vitamin A), B1, B2, B6, E (α-tocopherol), niacin, and folic acid], in the grain, and moisture, ash, protein, total fat, carbohydrates by calculation, ADF, NDF, calcium and phosphorus in the forage. The anti-nutrients assessed in grain included phytic acid and raffinose. Secondary metabolites assessed in grain included furfural, ferulic acid, and p-coumaric acid.

All compositional analyses were performed at Covance Laboratories, Inc. (Madison, Wisconsin). Methods for analysis were based on internationally-recognized procedures and literature publications. Brief descriptions of the methods utilized for the analyses are described below.

G.4.1. 2-Furaldehyde (Furfural)

The ground samples were extracted with 4% trichloroacetic acid and injected directly on a high-performance liquid chromatography (HPLC) system for quantitation of free

furfurals by ultraviolet detection (Albala-Hurtado, et al., 1997). The limit of quantitation was 0.500 ppm.

Reference Standard:

- ACROS Organics, 2-Furaldehyde, 99.5%, Lot Number A0296679

G.4.2. Acid Detergent Fiber

The ANKOM2000 Fiber Analyzer automated the process of removal of proteins, carbohydrates, and ash. Fats and pigments were removed with an acetone wash prior to analysis. The fibrous residue that was primarily cellulose and lignin and insoluble protein complexes remained in the Ankom filter bag, and was determined gravimetrically (Goering and Van Soest, 1970; Komarek, et al., 1993). The limit of quantitation was 0.100%.

G.4.3. Amino Acid Composition

The following 18 amino acids were analyzed:

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

The samples were hydrolyzed in 6N hydrochloric acid for approximately 24 hours at approximately 106-118ºC. Phenol was added to the 6N hydrochloric acid to prevent halogenation of tyrosine. Cystine and cysteine were converted to S-2-carboxyethylthiocysteine by the addition of dithiodipropionic acid. Tryptophan was hydrolyzed from proteins by heating at approximately 110ºC in 4.2N sodium hydroxide for approximately 20 hours.

The samples were analyzed by HPLC after pre-injection derivatization. The primary amino acids were derivatized with o-phthalaldehyde (OPA) and the secondary amino acids were derivatized with fluorenylmethyl chloroformate (FMOC) before injection (AOAC, 2012f; Barkholt and Jensen, 1989; Henderson and Brooks, 2010; Henderson, et al., 2000; Schuster, 1988). The limit of quantitation for this study was 0.100 mg/g.

Reference Standards:

Component	Manufacturer	Lot No.	Purity (%)
L-Alanine	Sigma-Aldrich	BCBC5470	99.8
L-Arginine Monohydrochloride	Sigma-Aldrich	1361811	100
L-Aspartic Acid	Sigma-Aldrich	BCBB9274	100.6
L-Cystine	Sigma-Aldrich	1451329	100
L-Glutamic Acid	Sigma-Aldrich	1423805	100.2
Glycine	Sigma-Aldrich	1119375	100
L-Histidine Monohydrochloride Monohydrate	Sigma-Aldrich	BCBB1348	99.9
L-Isoleucine	Sigma-Aldrich	1423806	100
L-Leucine	Sigma-Aldrich	BCBC6907	99.9
L-Lysine Monohydrochloride	Sigma-Aldrich	1362380	100.2
L-Methionine	Sigma-Aldrich	1423807	99.9
L-Phenylalanine	Sigma-Aldrich	BCBC5774	100
L-Proline	Sigma-Aldrich	1414414	99.7
L-Serine	Sigma-Aldrich	1336081	99.9
L-Threonine	Sigma-Aldrich	1402329	100
L-Tyrosine	Sigma-Aldrich	BCBC2417	100
L-Valine	Sigma-Aldrich	1352709	100
L-Tryptophan	Sigma-Aldrich	BCBB1284	99.8

G.4.4. Ash

All organic matter was driven off when the samples were ignited at approximately 550ºC in a muffle furnace for at least 5 hours. The remaining inorganic material was determined gravimetrically and referred to as ash (AOAC, 2012p). The limit of quantitation was 0.100%.

G.4.5. Beta Carotene (Vitamin A)

Samples were saponified and extracted with hexane. The samples were then injected on a reverse-phase HPLC system with ultraviolet light detection. Quantitation was achieved with a linear regression analysis (AOAC, 2012q; Quackenbush, 1987). The limit of quantitation was 0.0200 mg/100g.

Reference Standard:

- Sigma-Aldrich, Beta-Carotene Type I, 98.7% (based on E1% = 2280 for Lambda Maximum of 478 nm to 479 nm in Hexane), Lot Number 021M1304V

G.4.6. Carbohydrate

The total carbohydrate level was calculated by difference using the fresh weight-derived data and the following equation (USDA, 1973):

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

The limit of quantitation was calculated as 0.100%.

G.4.7. Fat by Acid Hydrolysis

The samples were hydrolyzed with hydrochloric acid. The fat was extracted using ether and hexane. The extracts were dried down and filtered through a sodium sulfate column. The remaining extracts were then evaporated, dried, and weighed (AOAC, 2012t; u). The limit of quantitation was 0.100%.

G.4.8. Fat by Soxhlet Extraction

The samples were weighed into a cellulose thimble containing sodium sulfate and dried to remove excess moisture. Pentane was dripped through the samples to remove the fat. The extract was then evaporated, dried, and weighed (AOAC, 2012v; a). The limit of quantitation was 0.100%.

G.4.9. Fatty Acids

The following 22 fatty acids were analyzed:

- 8:0 Caprylic
- 10:0 Capric
- 12:0 Lauric
- 14:0 Myristic
- 14:1 Myristoleic
- 15:0 Pentadecanoic
- 15:1 Pentadecenoic
- 16:0 Palmitic
- 16:1 Palmitoleic
- 17:0 Heptadecanoic
- 17:1 Heptadecenoic
- 18:0 Stearic
- 18:1 Oleic
- 18:2 Linoleic
- 18:3 gamma-Linolenic
- 18:3 Linolenic
- 20:0 Arachidic
- 20:1 Eicosenoic
- 20:2 Eicosadienoic
- 20:3 Eicosatrienoic
- 20:4 Arachidonic
- 22:0 Behenic

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 (GC) using external standards for quantitation (AOCS 2009). The limit of quantitation was 0.00400%.

References:

Manufacturer	Lot No.	Component	Weight (%)		Purity (%)
			JY10-W	MA7-W	
		Methyl Octanoate	3.0	1.25	99.7
		Methyl Decanoate	3.25	1.25	99.6
		Methyl Laurate	3.25	1.25	99.8
		Methyl Myristate	3.25	1.25	99.8
		Methyl Myristoleate	1.0	1.25	99.5
		Methyl Pentadecanoate	1.0	1.25	99.6
		Methyl Pentadecenoate	1.0	1.25	99.4
		Methyl Palmitate	10.0	15.75	99.8
		Methyl Palmitoleate	3.0	1.25	99.7
		Methyl Heptadecanoate	1.0	1.25	99.6
Nu-Chek Prep GLC Reference Standard		Methyl 10-Heptadecenoate	1.0	1.25	99.5
		Methyl Stearate	7.0	14.00	99.8
Covance 1	JY10-W	Methyl Oleate	10.0	15.75	99.8
Covance 2	MA7-W	Methyl Linoleate	10.0	15.75	99.8
		Methyl Gamma Linolenate	1.0	1.25	99.4
		Methyl Linolenate	3.0	1.25	99.5
		Methyl Arachidate	2.0	1.25	99.8
		Methyl 11-Eicosenoate	2.0	1.25	99.6
		Methyl 11-14 Eicosadienoate	1.0	1.25	99.5
		Methyl 11-14-17 Eicosatrienoate	1.0	1.25	99.5
		Methyl Arachidonate	1.0	1.25	99.4
		Methyl Behenate	1.0	1.25	99.8

G.4.10. Folic Acid

The samples were hydrolyzed in a potassium phosphate buffer with the addition of ascorbic acid to protect the folic acid during autoclaving. Following hydrolysis by autoclaving, the samples were treated with a chicken-pancreas enzyme and incubated approximately 18 hours to liberate the bound folic acid. The amount of folic acid was determined by comparing the growth response of the samples, using the bacteria *Lactobacillus casei*, with the growth response of a folic acid standard. This response was measured turbidimetrically (AOAC, 2012c; d; Infant Formula Council, 1985). The limit of quantitation was 0.0600 µg/g.

Reference Standard:

- USP, Folic acid, 98.9%, Lot Number Q0G151

G.4.11. Minerals/ ICP Emission Spectrometry

The following nine minerals were analyzed:

- Calcium
- Copper
- Iron
- Magnesium
- Manganese
- Phosphorus
- Potassium
- Sodium
- Zinc

The samples were dried, precharred, and ashed overnight in a muffle furnace set to maintain 500ºC. The ashed samples were re-ashed with nitric acid, treated with hydrochloric acid, taken to dryness, and put into a solution of 5% hydrochloric acid. The amount of each element was determined at appropriate wavelengths by comparing the emission of the unknown samples, measured on the inductively coupled plasma spectrometer, with the emission of the standard solutions (AOAC, 2012e; g).

Reference Standard:

Inorganic Ventures Reference Standards and Limits of Quantitation:

Mineral	Lot Numbers	Concentration (µg/mL)	LOQ (ppm)
Calcium	F2-MEB417082MCA, F2-MEB417084	200, 1000	20.0
Copper	F2-MEB417082MCA, F2-MEB417083MCA	2.00, 10.0	0.500
Iron	F2-MEB417082MCA, F2-MEB417085	10.0, 50.0	2.00
Magnesium	F2-MEB417082MCA, F2-MEB417083MCA	50.0, 250	20.0
Manganese	F2-MEB417082MCA, F2-MEB417083MCA	2.00, 10.0	0.300
Phosphorus	F2-MEB417082MCA, F2-MEB417084	200, 1000	20.0
Potassium	F2-MEB417082MCA, F2-MEB417084	200, 1000	100
Sodium	F2-MEB417082MCA, F2-MEB417084	200, 1000	100
Zinc	F2-MEB417082MCA, F2-MEB417083MCA	10.0, 50.0	0.400

G.4.12. Moisture

The samples were dried in a vacuum oven at approximately 100ºC. The moisture weight loss was determined and converted to percent moisture (AOAC, 2012h; i). The limit of quantitation was 0.100%.

G.4.13. Neutral Detergent Fiber

The ANKOM2000 Fiber Analyzer automated the process of the removal of protein, carbohydrate, and ash. Fats and pigments were removed with an acetone wash prior to analysis. Hemicellulose, cellulose, lignin and insoluble protein fraction were left in the

filter bag and determined gravimetrically (AACC, 1998; Goering and Van Soest, 1970; Komarek, et al., 1994). The limit of quantitation was 0.100%.

G.4.14. Niacin

The samples were hydrolyzed with sulfuric acid and the pH was adjusted to remove interferences. The amount of niacin was determined by comparing the growth response of the samples, using the bacteria *Lactobacillus plantarum*, with the growth response of a niacin standard. This response was measured turbidimetrically (AOAC, 2012j; c). The limit of quantitation was 0.300 µg/g.

Reference Standard:

- USP, Niacin, 99.8%, Lot Number J0J235

G.4.15. p-Coumaric Acid and Ferulic Acid

The ground samples were extracted with methanol followed by alkaline hydrolysis and buffering prior to injection on an analytical HPLC system for quantification of p-coumaric acid and ferulic acid by ultra violet (UV) detection (Hagerman and Nicholson, 1982). The limit of quantitation for the p coumaric acid and ferulic acid was 33.3 ppm.

Reference Standards:

Component	Manufacturer	Lot No.	Purity (%)
p-Hydroxycinnamic Acid (p-Coumaric Acid)	Sigma-Aldrich	091M1197V	99.6
4-Hydroxy-3-methoxycinnamic Acid (Ferulic Acid)	ACROS Organics	A0261354	99.4
4-Hydroxy-3-methoxycinnamic Acid (Ferulic Acid)	ACROS Organics	A0294716	99.4

G.4.16. Phytic Acid

The samples were extracted using hydrochloric acid and sonication, purified using a silica based anion exchange column, concentrated and injected onto a HPLC system with a refractive index detector (Lehrfeld, 1989; Lehrfeld, 1994). The limit of quantitation was 0.100%.

Reference Standard:

- Sigma-Aldrich, Phytic Acid Sodium Salt Hydrate, 97.9%, Lot Number BCBH8701V

G.4.17. Protein

The protein and other organic nitrogen in the samples were converted to ammonia by digesting the samples with sulfuric acid containing a catalyst mixture. The acid digest was made alkaline. The ammonia was distilled and then titrated with a previously standardized acid. Instrumentation was used to automate the digestion, distillation and titration processes. The percent nitrogen was calculated and converted to equivalent protein using the factor 6.25 (AOAC, 2012k; l). The limit of quantitation was 0.100%.

G.4.18. Raffinose

Sugars in the samples were extracted with a 50:50 water:methanol solution. Aliquots were taken, dried under inert gas, and then reconstituted with a hydroxylamine hydrochloride solution in pyridine containing phenyl-β-D-glucopyranoside as the internal standard. The resulting oximes were converted to silyl derivatives by treatment with hexamethyldisilazane and trifluoracetic acid treatment, and then analyzed by GC using a flame ionization detector (Brobst, 1972; Mason and Slover, 1971). The limit of quantitation was 0.0500%.

Reference Standard:

- Sigma-Aldrich, D-(+)-Raffinose pentahydrate, 99.6%, Lot Number 019K1156

G.4.19. Total Dietary Fiber

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 sample was 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 protein and ash values (AOAC, 2012m). The limit of quantitation was 1.00%.

G.4.20. Vitamin B1 (Thiamine Hydrochloride)

The samples were autoclaved under weak acid conditions to extract the thiamine. The resulting solutions were incubated with a buffered enzyme solution to release any bound thiamine. The solutions were purified on a cation-exchange column. Aliquots were reacted with potassium ferricyanide to convert thiamine to thiochrome. The thiochrome was extracted into isobutyl alcohol, measured on a fluorometer, and quantitated by comparison to a known standard (AOAC, 2012n; o; r). The limit of quantitation was 0.010 mg/100g.

Reference Standard:

- USP, Thiamine Hydrochloride, 99.7%, Lot Number P0K366

G.4.21. Vitamin B2 (Riboflavin)

The samples were hydrolyzed with dilute hydrochloric acid and the pH was adjusted to remove interferences. The amount of riboflavin was determined by comparing the growth response of the samples, using the bacteria *Lactobacillus rhamnosus*, with the growth response of multipoint riboflavin standards. The growth response was measured turbidimetrically (AOAC, 2012s; c). The limit of quantitation was 0.200 µg/g.

Reference Standard:

- USP, Riboflavin, 99.7%, Lot Number N1J079

G.4.22. Vitamin B6 (Pyridoxine Hydrochloride)

The samples were hydrolyzed with dilute sulfuric acid in the autoclave and the pH was adjusted to remove interferences. The amount of pyridoxine was determined by comparing the growth response of the samples, using the yeast *Saccharomyces cerevisiae*, with the growth response of a pyridoxine standard. The response was measured turbidimetrically. Results were reported as pyridoxine hydrochloride (AOAC, 2012b; Atkins, et al., 1943). The limit of quantitation was 0.0700 µg/g.

Reference Standard:

- USP, Pyridoxine hydrochloride, 99.8%, Lot Number Q0G409

G.4.23. Vitamin E (α-Tocopherol)

The samples were saponified to break down any fat and release vitamin E. The saponified mixtures were extracted with ethyl ether and then quantitated by high-performance liquid chromatography using a silica column (Cort, et al., 1983; McMurray, et al., 1980; Speek, et al., 1985). The limit of quantitation was 0.00500 mg/g.

Reference Standard:

- USP, Alpha Tocopherol, 98.9%, Lot Number N0F068

G.5. Data Processing and Statistical Analysis

After compositional analyses were performed, data spreadsheets containing individual values for each analysis were sent to Monsanto Company for review. Data were then transferred to Certus International, Inc., where they were converted into the appropriate units and statistically analyzed. The following formulas were used for re-expression of composition data for statistical analysis (Table G-2):

Table G-2. Re-expression Formulas for Statistical Analysis of Composition Data

Component	From (X)	To	Formula[1]
Proximates (excluding Moisture), Fiber, Anti-nutrients	% fwt	% dw	X/d
Amino Acids (AA)	mg/g fwt	% dw	X/(10d)
Secondary Metabolites	ppm fwt	µg/g dw	X/d
Copper, Iron, Manganese, Zinc	ppm fwt	mg/kg dw	X/d
Calcium, Magnesium, Phosphorus, Potassium	ppm fwt	% dw	$X/(10^4 d)$
Folic Acid, Niacin, Vitamin B2, Vitamin B6	µg/g fwt	mg/kg dw	X/d
Vitamin A, Vitamin B1	mg/100g fwt	mg/kg dw	10X/d
Vitamin E	mg/g fwt	mg/kg dw	$10^3 X/d$
Fatty Acids (FA)	% fwt	% Total FA	$(100)X_j/\Sigma X$, for each FA_j where ΣX is over all the FA

[1]'X' is the individual sample value; d is the fraction of the sample that is dry matter.

In order to complete a statistical analysis for a compositional constituent in this compositional assessment, at least 50% of all the values for an analyte in grain or forage had to be greater than the assay limit of quantitation (LOQ). Analytes with more than 50% of observations below the assay LOQ were excluded from summaries and analysis. The following 16 analytes in grain with more than 50% of observations below the assay LOQ were excluded from statistical analysis: 8:0 caprylic acid, 10:0 capric acid, 12:0 lauric acid, 14:0 myristic acid, 14:1 myristoleic acid, 15:0 pentadecanoic acid, 15:1 pentadecenoic acid, 16:1 palmitoleic acid, 17:0 heptadecanoic acid, 17:1 heptadecenoic acid, 18:3 gamma linolenic acid, 20:2 eicosadienoic acid, 20:3 eicosatrienoic acid, 20:4 arachidonic acid, sodium, and furfural.

Otherwise, individual results below the LOQ were assigned a value equal to one-half the quantitation limit. Fourteen observations for 22:0 Behenic were assigned a value equal to one-half of the LOQ (0.002% fwt).

The data were assessed for potential outliers using a studentized PRESS residuals calculation. A PRESS residual is the difference between any value and its value predicted from a statistical model that excludes the data point. The studentized version scales these residuals so that the values tend to have a standard normal distribution when outliers are absent. Thus, most values are expected to be between ± 3. Extreme data points that are also outside of the ± 6 studentized PRESS residual ranges are considered for exclusion, as outliers, from the final analyses. Seven components had PRESS residual values outside of the ± 6 range.

Of the seven flagged values, only the vitamin E value from a conventional reference was removed from further analysis as an outlier. The remaining values were not removed

because they were not extreme values or they were deemed sufficiently close to neighboring values to lack sufficient evidence for removal.

The outlier test procedure was reapplied to the remaining vitamin E data to detect potential outliers that were masked in the first analysis. One vitamin E value from a commercial reference was identified as an outlier, but the value was not an extreme value and was not removed as an outlier.

Maize compositional components were statistically analyzed using a mixed-model analysis of variance with the SAS MIXED procedure.

Analyses of the combined replicated sites were performed using model (1).

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

where Y_{ijk} = unique individual observation, U = overall mean, T_i = substance effect, L_j = random site effect, $B(L)_{jk}$ = random block within site effect, LT_{ij} = random site by substance interaction effect, and e_{ijk} = residual error.

For each component analysis, individual mean comparison tests of MON 87411 vs. conventional control were conducted.

A range of observed values from the reference hybrids was determined for each analytical component. Additionally, data from the reference hybrids were used to develop 99% tolerance intervals. A tolerance interval is an interval that one can claim, with a specified degree of confidence, contains at least a specified proportion, p, of an entire sampled population for the parameter measured.

For each compositional component analyzed, two-sided 99% tolerance intervals were calculated that are expected to contain, with 95% confidence, 99% of the quantities expressed in the population of reference hybrids. Each estimate was based upon the average of all observations per unique reference hybrid. Because negative quantities are not possible, negative calculated lower tolerance bounds were set to zero.

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Appendix H: Materials, Methods, and Individual Site Results for Seed Dormancy and Germination Assessment of MON 87411

H.1. Materials

Seed germination and dormancy characteristics were assessed on seed from MON 87411, the conventional control, and reference hybrids produced in replicated field trials during 2012 at the following sites: Jefferson County, IA (IARL), Warren County, IL (ILMN), and Pawnee County, KS (KSLA).

H.2. Characterization of the Materials

The identities of the MON 87411 and the conventional control starting seed were verified by event-specific polymerase chain reaction (PCR) analyses. During the growing season, the field planting order of MON 87411 and the conventional control plots was confirmed by event-specific PCR analyses. Chain-of-custody documentation for all starting seed for dormancy and germination study was maintained from harvest through shipment to the performing laboratory with the use of packaging labels and plant sample transfer forms.

H.3. Germination Testing Facility and Experimental Methods

Germination and dormancy evaluations were conducted at BioDiagnostics, Inc. in River Falls, WI. The principal investigator was qualified to conduct seed germination and dormancy testing consistent with the standards established by the Association of Official Seed Analysts (AOSA), a seed trade association (AOSA/SCST 2010; AOSA, 2012a; 2012b).

The seed lots (Selfed F_2 grain) of MON 87411, the conventional control, and four reference hybrids from each location were tested under seven different temperature regimes. Seven germination chambers were used in the study and each chamber was maintained dark under one of the following seven temperature regimes: constant temperature of approximately 5, 10, 20, or 30 °C or alternating temperatures of approximately 10/20, 10/30, or 20/30° C. The alternating temperature regimes were maintained at the lower temperature for 16 hours and the higher temperature for 8 hours. The temperature inside each germination chamber was monitored and recorded throughout the duration of the study.

Approximately 100 seeds each of MON 87411, the conventional control, and the reference hybrids were placed on pre-moistened germination towels using a vacuum planting system. Additional pre-moistened germination towels were placed on top of the seed. The germination towels were then rolled up in a wax cover. All rolled germination towels were labeled and placed into an appropriately labeled bucket. Each bucket within a temperature regime represented a replicate per site. There were 4 reps per site for a total of 12 buckets for each temperature regime. Each bucket contained 6 towels or 1 towel per entry. Buckets were then placed in the appropriate germination chambers.

Each temperature regime constituted a separate split-plot experiment with four replications where the whole-plot was the location where starting seed (Selfed F_2 grain) was produced and the sub-plot was the material type (*i.e.*, MON 87411, the conventional control or reference material). Whole-plots and sub-plots were randomized within each temperature regime.

A description of each germination characteristic evaluated and the timing of evaluations are presented in Table VIII-1. The types of data collected depended on the temperature regime. Each rolled germination towel in the AOSA-recommended temperature regime (*i.e.*, alternating 20/30 °C) was assessed periodically during the study for normally germinated, abnormally germinated, hard (viable and nonviable), dead, and firm swollen (viable and nonviable) seed as defined by AOSA guidelines (AOSA, 2012a; 2012b). AOSA only provides guidelines for testing seed under optimal temperatures, whereas additional temperature regimes were included to test diverse environmental conditions. Therefore, each rolled germination towel in the additional temperature regimes (*i.e.*, 5, 10, 20, 30, alternating 10/20, and 10/30 °C) was assessed periodically during the study for germinated, hard (viable and nonviable), dead, and firm swollen (viable and nonviable) seed. Because temperature extremes could affect the development of seedlings, AOSA standards were not applied and no distinction was made between normal or abnormal germinated seed. Therefore, any seedling with a radical of 1 mm or more was classified as germinated.

The calculation of percent seed in each assessment category was based on the actual number of seeds evaluated (*e.g.*, 99 or 101). Across temperature regimes, the total number of seeds evaluated from each germination towel was approximately 100.

Within both AOSA and the additional temperature regimes, hard and firm-swollen seeds remaining at the final evaluation date were subjected to a tetrazolium (Tz) test for evaluation of viability according to AOSA standards (AOSA/SCST 2010). The number of nonviable hard and nonviable firm-swollen seed was added to the number of dead seed counted on all collection dates to determine the total percent dead seed. Total counts for percent viable hard and viable firm-swollen seed were determined from the Tz test.

H.4. Statistical Analysis

An analysis of variance was conducted using SAS® (SAS 2008) according to a split-plot design (production site as the whole plot and starting seed material as the sub-plot) with four replications. MON 87411 was compared to the conventional control for germination and dormancy characteristics of seed produced within each site (*i.e.*, individual site analysis) and in a combined-site analysis in which the data were pooled across all three sites. The seed germination and dormancy characteristics analyzed included percent germinated seed, percent viable hard seed, percent dead seed, and percent viable firm-swollen seed. The percent germinated seed were categorized as either normal germinated

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or abnormal germinated for the AOSA temperature regime. The level of statistical significance was predetermined to be 5% (α=0.05). MON 87411 was not statistically compared to the reference hybrids, nor were comparisons made across temperature regimes. The minimum and maximum mean values were determined from the reference materials across all sites to provide a range of values (*i.e.*, reference range) representative of commercial maize hybrids. Results from the combined-site analysis are presented in Table VIII-2.

H.5. Individual-Site Seed Dormancy and Germination Analysis

In the individual site analyses, five statistically significant differences in total were detected between MON 87411 and the conventional control for the measured characteristics (*i.e.*, percent germinated, viable hard, dead, or viable firm-swollen seed) of the seed (Selfed F_2 grain) produced at the IARL, ILMN, and KSLA sites (Table H-2). MON 87411 had fewer dead seed than the conventional control at 10/20°C (0.7% vs. 2.0%) and more viable firm swollen seed than the conventional control at 20°C (0.3% vs. 0.0%) for the seed produced at the IARL site. MON 87411 had more germinated seed than the conventional control at 20/30°C (99.3% vs. 97.8%) and fewer dead seed than the conventional control at 20/30°C (0.3% vs. 1.8%) for the seed produced at the ILMN site. MON 87411 had more germinated seed than the conventional control at 5°C (0.5% vs. 0.0%) for the seed produced at the KSLA site. Statistically significant differences between MON 87411 and the conventional control for germination amd dormancy characteristics in the individual site analyses were not consistently detected across temperature regimes or the individual sites. These differences were not detected in the combined site analysis (Section VIII-1, Table VIII-2) and are unlikely to be biologically meaningful in terms of increased pest/weed potential (See Figure VIII-1, Step 2, answer “no”).

Table H-1. Starting Seed of MON 87411, Conventional Control and Commercial Maize Reference Hybrids Used in Dormancy Assessment

Site Code	Material Name	Phenotype	Monsanto Lot Number
IARL	MON 87411	Glyphosate-Tolerant and Insect-Protected	11354899
IARL	MPA640B[1]	Conventional Control	11354894
IARL	Legacy L7671	Conventional Reference	11354895
IARL	Lewis 7007	Conventional Reference	11354896
IARL	Gateway 6116	Conventional Reference	11354897
IARL	Phillips 717	Conventional Reference	11354898
KSLA	MON 87411	Glyphosate-Tolerant and Insect-Protected	11354905
KSLA	MPA640B	Conventional Control	11354900
KSLA	Lewis 7007	Conventional Reference	11354901
KSLA	Midland Phillips 799	Conventional Reference	11354902
KSLA	NC+ 5220	Conventional Reference	11354903
KSLA	LG2540	Conventional Reference	11354904
ILMN	MON 87411	Glyphosate-Tolerant and Insect-Protected	11354911
ILMN	MPA640B	Conventional Control	11354906
ILMN	Gateway 4148	Conventional Reference	11354907
ILMN	H-9180	Conventional Reference	11354908
ILMN	Stewart S588	Conventional Reference	11354909
ILMN	LG2548	Conventional Reference	11354910

[1]MPA640B = LH244 × LH287

Table H-2. Germination and Dormancy Characteristics of MON 87411 and the Conventional Control Seed (Selfed F2 Grain) Produced at each of the Three Field Sites

Temperature Regime	Assessment Category	IARL[1] Mean % (S.E.)[2]		ILMN[1] Mean % (S.E.)[2]		KSLA[1] Mean % (S.E.)[2]	
		MON 87411	Control	MON 87411	Control[3]	MON 87411	Control
5 °C	Germinated	0.0 (0.00)	0.3 (0.25)	0.5 (0.29)	0.3 (0.25)	0.5 (0.29)*	0.0 (0.00)
	Viable Hard	0.0 (0.00)†	0.0 (0.00)	0.0 (0.00)†	0.0 (0.00)	0.0 (0.00)†	0.0 (0.00)
	Dead	2.0 (0.91)	1.0 (0.41)	4.3 (1.38)	2.3 (0.48)	2.8 (0.75)	3.3 (1.11)
	Viable Firm Swollen	98.0 (0.91)	98.8 (0.25)	95.3 (1.49)	97.5 (0.65)	96.8 (0.85)	96.8 (1.11)
10 °C	Germinated	73.5 (2.60)	75.9 (1.34)	90.5 (1.55)	91.0 (1.22)	82.0 (2.48)	82.5 (4.09)
	Viable Hard	0.0 (0.00)†	0.0 (0.00)	0.0 (0.00)†	0.0 (0.00)	0.0 (0.00)†	0.0 (0.00)
	Dead	2.0 (0.82)	2.0 (0.41)	0.0 (0.00)	0.8 (0.25)	0.8 (0.25)	0.3 (0.25)
	Viable Firm Swollen	24.5 (2.10)	22.1 (1.28)	9.5 (1.55)	8.3 (1.03)	17.3 (2.43)	17.3 (3.88)
20 °C	Germinated	98.0 (0.71)	99.0 (0.41)	99.3 (0.25)	99.3 (0.48)	98.5 (0.87)	98.8 (0.48)
	Viable Hard	0.0 (0.00)†	0.0 (0.00)	0.0 (0.00)†	0.0 (0.00)	0.0 (0.00)†	0.0 (0.00)
	Dead	1.8 (0.63)	1.0 (0.41)	0.8 (0.25)	0.8 (0.48)	1.5 (0.87)	1.3 (0.48)
	Viable Firm Swollen	0.3 (0.25)*	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)	0.0(0.00)	0.0 (0.00)
30 °C	Germinated	98.8 (0.48)	97.8 (0.95)	99.3 (0.48)	99.8 (0.25)	99.3 (0.25)	98.3 (0.48)
	Viable Hard	0.0 (0.00)†	0.0 (0.00)	0.0 (0.00)†	0.0 (0.00)	0.0 (0.00)†	0.0 (0.00)
	Dead	1.3 (0.48)	2.3 (0.95)	0.8 (0.48)	0.3 (0.25)	0.8 (0.25)	1.8 (0.48)
	Viable Firm Swollen	0.0 (0.00)†	0.0 (0.00)	0.0 (0.00)†	0.0 (0.00)	0.0 (0.00)†	0.0 (0.00)

Table H-2 (continued). Germination and Dormancy Characteristics of MON 87411 and the Conventional Control Seed (Selfed F_2 Grain) Produced at each of the Three Field Sites

Temperature Regime	Assessment Category	IARL[1] Mean % (S.E.)[2]		ILMN[1] Mean % (S.E.)[2]		KSLA[1] Mean % (S.E.)[2]	
		MON 87411	Control	MON 87411	Control[3]	MON 87411	Control
10/20 °C	Germinated	98.0 (0.41)	97.0 (0.58)	99.3 (0.25)	100.0 (0.00)	98.8 (0.48)	98.0 (0.71)
	Viable Hard	0.0 (0.00)†	0.0 (0.00)	0.0 (0.00)†	0.0 (0.00)	0.0 (0.00)†	0.0 (0.00)
	Dead	0.7 (0.47)*	2.0 (0.00)	0.5 (0.29)	0.0 (0.00)	0.8 (0.48)	0.8 (0.48)
	Viable Firm Swollen	1.3 (0.63)	1.0 (0.58)	0.3 (0.25)	0.0 (0.00)	0.5 (0.50)	1.3 (0.48)
10/30 °C	Germinated	98.5 (0.29)	98.3 (0.25)	99.0 (1.00)	99.5 (0.29)	99.0 (0.58)	100.0 (0.00)
	Viable Hard	0.0 (0.00)†	0.0 (0.00)	0.0 (0.00)†	0.0 (0.00)	0.0 (0.00)†	0.0 (0.00)
	Dead	1.5 (0.29)	1.8 (0.25)	0.8 (0.75)	0.5 (0.29)	1.0 (0.58)	0.0 (0.00)
	Viable Firm Swollen	0.0 (0.00)	0.0 (0.00)	0.3 (0.25)	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)
20/30 °C (AOSA)	Normal Germinated	97.5 (0.29)	98.5 (0.65)	99.3 (0.48)*	97.8 (0.63)	97.8 (0.75)	98.8 (0.48)
	Abnormal Germinated	0.8 (0.48)	0.0 (0.00)	0.5 (0.29)	0.5 (0.29)	1.3 (0.48)	0.5 (0.29)
	Viable Hard	0.0 (0.00)†	0.0 (0.00)	0.0 (0.00)†	0.0 (0.00)	0.0 (0.00)†	0.0 (0.00)
	Dead	1.8 (0.48)	1.5 (0.65)	0.3 (0.25)*	1.8 (0.85)	1.0 (0.41)	0.8 (0.25)
	Viable Firm Swollen	0.0 (0.00)†	0.0 (0.00)	0.0 (0.00)†	0.0 (0.00)	0.0 (0.00)†	0.0 (0.00)

Note: The experimental design was a split-plot with four replications.
*Indicates a statistically significant difference between MON 87411 and the conventional control (α=0.05) using ANOVA.
†No statistical comparison could be made due to lack of variability in the data.
[1]Site codes are as follows: IARL = Jefferson County, Iowa, KSLA = Pawnee County, Kansas, and ILMN = Warren County Illinois.
[2]MON 87411 and the conventional control values represent means with standard error (S.E.) in parentheses. N = 4. In some instances, the total percentage of both MON 87411 and the conventional control did not equal 100% due to numerical rounding of the means.

References for Appendix H

AOSA/SCST. 2010. Tetrazolium testing handbook. Association of Official Seed Analysts and the Society of Commercial Seed Technologists, Ithaca, New York.

SAS. 2008. SAS/STAT software version 9.2. SAS Institute, Inc., Cary, North Carolina.

Appendix I: Materials, Methods, and Individual Site Results from Phenotypic, Agronomic, and Environmental Interaction Assessment of MON 87411 under Field Conditions

I.1. Materials

Agronomic, phenotypic, and environmental interaction characteristics were assessed for MON 87411, the conventional control, and 22 reference hybrids grown under similar agronomic conditions. Four reference hybrids were planted per site (Table I-1).

I.2. Characterization of the Materials

The presence or absence of the MON 87411 event in the starting seed of MON 87411 and the conventional control was verified by event-specific polymerase chain reaction (PCR) analyses. No molecular analyses were performed on the reference starting seed.

I.3. Field Sites and Plot Design

Field trials were established in 2012 at nine sites that provided a range of environmental and agronomic conditions representative of U.S. maize growing regions (Section VIII, Table VIII-3). The Principal Investigator at each site was familiar with the growth, production, and evaluation of maize characteristics.

At all sites, seed of MON 87411, the conventional control, and four conventional reference hybrids were planted in a randomized complete block design with four replications. The planted plot dimensions varied between sites, due to variability in available planting equipment and data collection (Table I-2). At IABG (Iowa), NEYO (Nebraska), NCBD (North Carolina), and PAHM (Pennsylvania) sites, each replicated plot consisted of 16 rows of maize spaced approximately 0.76 - 0.96 m apart and approximately 6 m long. Phenotypic and qualitative environmental interactions data were collected from rows 2 and 3 (rows 14 and 15 at NCBD site). Rows six and eight were used to collect arthropod samples using sticky traps. Rows 9, 10, 11, and 12 were designated for visual counts of arthropod abundance. Rows 14 and 15 (rows 2 and 3 at NCBD site) were used to assess plant damage caused by corn earworm (*Helicoverpa zea*) and European corn borer (*Ostrinia nubilalis*).

At IARL (Iowa), ILMN (Illinois), INSH (Indiana), KSLA (Kansas), and NEDC (Nebraska) sites, each replicated plot consisted of six rows of maize spaced approximately 0.76 m apart and approximately 6 m long. The plots were separated by two rows of conventional maize along their length. Phenotypic and qualitative environmental interactions data were collected from rows 4 and 5 except one plot at ILMN and two plots at NEDC.

I.4. Planting and Field Operations

Planting information, soil description, and cropping history of the trial area are listed in Table I-2. Prior to planting, the Principal Investigator at each site prepared the plot area with a proper seed bed according to local agronomic practices, including tillage,

fertilization, and pest management. During the growing season, all plots were assessed for agronomic conditions and pest populations, including pest arthropods, diseases and weeds. Fertilizer, irrigation, agricultural chemicals, and other management practices were applied as necessary. Maintenance operations were performed uniformly across all plots.

Table I-1. Starting Seed for Phenotypic, Agronomic, and Environmental Interaction Assessment

Site Code[1]	Material Name	Monsanto Lot Number	Phenotype
All	MON 87411	11333176	Glyphosate-Tolerant and Insect-Protected
All	MPA640B[2]	11333170	Conventional Control
IABG	Jacobsen Seed JS4431	11267096	Coventional Reference
	Phillips 713	11300072	Coventional Reference
	LG2615CL	11226863	Coventional Reference
	Pioneer 32B81	11226578	Coventional Reference
IARL	Legacy L7671	11226598	Coventional Reference
	Lewis 7007	11226559	Coventional Reference
	Gateway 6116	11227211	Coventional Reference
	Phillips 717	11300073	Coventional Reference
ILMN	Gateway 4148	11273005	Coventional Reference
	H-9180	11226704	Coventional Reference
	Stewart S588	11226918	Coventional Reference
	LG2548	11266731	Coventional Reference
INSH	Gateway 6158	11273006	Coventional Reference
	LG2620	11226861	Coventional Reference
	Stewart S518	11226920	Coventional Reference
	Legacy L7671	11226598	Coventional Reference
KSLA	Lewis 7007	11226559	Coventional Reference
	Midland Phillips 799	11226703	Coventional Reference
	NC+ 5220	11226701	Coventional Reference
	LG2540	11266730	Coventional Reference
NCBD	LG2548	11266731	Coventional Reference
	Stewart S602	11226919	Coventional Reference
	Midland Phillips 799	11226703	Coventional Reference
	Gateway 6158	11273006	Coventional Reference
NEDC	Gateway 4148	11273005	Coventional Reference
	Midland Phillips 799	11226703	Coventional Reference
	NC+ 4443	11226700	Coventional Reference
	LG2540	11266730	Coventional Reference
NEYO	LG2540	11266730	Coventional Reference
	NC+ 5220	11266701	Coventional Reference
	Mycogen 2M746	11226705	Coventional Reference
	Gateway 6158	11273006	Coventional Reference
PAHM	Phillips 717	11300073	Coventional Reference
	Stewart S588	11226918	Coventional Reference
	Pioneer 33T56	11226580	Coventional Reference
	Gateway 4148	11273005	Coventional Reference

[1]Site code: IABG = Greene County, IA; IARL = Jefferson County, IA; ILMN = Warren County, IL; INSH = Boone County, IN; KSLA = Pawnee County, KS; NCBD = Perquimans County, NC; NEDC = Butler County, NE; NEYO = York County, NE; PAHM = Berks County, PA.
[2]MPA640B = LH244 × LH287.

Table I-2. Field and Planting Information

Site[1]	Planting Date[2]	Harvest Date[2]	Approximate Planting Rate (seeds/m)	Approximate Plot Size (m × m)	Rows per Plot	Soil Type	% OM[3]	Previous Crop 2011
IABG	05/09/2012	10/05/2012	7.2	6.1 × 12.2	16	Loam	4.0	Soybean
IARL	05/11/2012	10/12/2012	7.2	6.1 × 4.6	6	Silty Clay Loam	3.4	Soybean
ILMN	05/10/2012	10/01/2012	6.9	6.2 × 4.6	6	Silty Clay Loam	4.5	Soybean
INSH	05/11/2012	11/02/2012	7.2	6.1 × 4.6	6	Silt Loam	2.5	Maize
KSLA	05/11/2012	09/21/2012	7.2	6.1 × 4.6	6	Silt Loam	2.6	Sorghum
NCBD	05/11/2012	09/20/2012	6.6	6.1 × 15.5	16	Sandy Loam	2.6	Cotton
NEDC	05/05/2012	09/11/2012	7.2	6.2 × 4.6	6	Silt Loam	2.6	Soybean
NEYO	05/08/2012	10/09/2012	7.2	6.1 × 12.2	16	Silt Loam	3.0	Soybean
PAHM	05/19/2012	10/19/2012	8.2	6.1 × 12.2	16	Loam	1.6	Vegetables[4]

Note: All plots were thinned to a uniform density of approximately 31 plants (IABG, INSH, KSLA, NCBD, NEYO, and PAHM) and 38 plants (IARL, ILMN, and NEDC) per 6.0 m row.

[1]Site code: IABG = Greene County, IA; IARL = Jefferson County, IA; ILMN = Warren County, IL; INSH = Boone County, IN; KSLA = Pawnee County, KS; NCBD = Perquimans County, NC; NEDC = Butler County, NE; NEYO = York County, NE; PAHM = Berks County, PA.

[2]Planting and Harvest Date = mm/dd/yyyy.

[3]% OM = Percent Organic Matter.

[4]Vegetables = peppers, tomatoes, potatoes, cabbage, maize.

I.5. Phenotypic Observations

The description of the characteristics measured and the designated developmental stages where observations occurred are listed in Section VIII, Table VIII-1.

I.6. Environmental Observations

Environmental interactions (*i.e.*, interactions between the crop plants and their receiving environment) were used to characterize MON 87411 by evaluating plant response to abiotic stressors, disease damage, and arthropod-related damage using qualitative methods described in section I.7. In addition, specific arthropod damage (corn earworm damage and European corn borer) and arthropod abundance were evaluated using the quantitative methods described in Section I.8.

I.7. Plant Response to Abiotic Stress, Disease Damage, and Arthropod-Related Damage

MON 87411 and the conventional control were evaluated at all sites for plant response to abiotic stressors, disease damage, and arthropod damage. A target of three abiotic stressors, three diseases, and three arthropod pests were evaluated four times during the following four crop developmental stages: V6–V8; V12-VT; R1-R3; and R5-R6.

Abiotic stressor, disease damage and arthropod damage observations were collected from each plot using the categorical scale of increasing severity listed below:

Category	Severity of plant damage
None	No symptoms observed
Slight	Symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); mitigation likely not required
Moderate	Intermediate between slight and severe; likely requires mitigation
Severe	Symptoms damaging to plant development (*e.g.*, stunting or death); mitigation unlikely to be effective

Method used for selecting stressors at each field site:

1. Prior to each data collection, maize was surveyed in proximity to the study area or the border rows of the study for abiotic stressors (*e.g.*, drought), diseases (*e.g.*, gray leaf spot), and arthropod damage (*e.g.*, corn flea beetle).
2. The Principal Investigator chose three abiotic stressors, three diseases, and three arthropod species that are actively causing damage for subsequent evaluation in the study plots. The Principal Investigators were requested to select additional stressors if present.
3. If fewer than three abiotic stressors, diseases, or arthropod species were present, the cooperator chose additional abiotic stressors, diseases, and arthropod species that are known to commonly occur in that geographical region and cause damage at the study site at that time.

4. All plots at a site were rated for the same abiotic stressors, diseases, and arthropod pests at a given observation, even if that selected stressor was not present in some or all of the plots.
5. If a selected stressor was not present, the cooperator recorded the rating as "none".

As indicated above, the Principal Investigator at each field site chose abiotic stressors, diseases, and arthropod pests that were either actively causing plant injury in the study area or were likely to occur in maize during the given observation period. Therefore, the type of abiotic stressors, diseases, and arthropod pests assessed varied between observations at a site and between sites.

In addition, ear and kernel rot disease and stalk rot disease were evaluated at harvest (R6 growth stage) using the above categorical scale. Ear and kernel rot disease data were collected by evaluating five non-systematically selected ears (one per plant) from each plot. The husks were pulled back and each ear was examined for disease. To evaluate stalk rot, five non-systematically selected stalks in each plot were cut longitudinally. The stalks were then examined for disease.

I.8. Arthropod Abundance

Specific arthropod (corn earworm and European corn borer) damage and arthropod abundance were assessed quantitatively from observations/collections performed at IABG, NEYO, NCBD, and PAHM sites.

Corn earworm damage was evaluated at R5 growth stage by examining ears from ten plants (5 consecutive plants per row) in each plot. The husks were pulled back and each ear was examined for corn earworm damage using a plastic film grid (size of each grid 0.5 cm^2). Damage (cm^2) per plant was calculated as the total number of grid cells matching the damage area multiplied by 0.5 (each grid cell = 0.5 cm^2).

European corn borer damage was evaluated at R6 growth stage by examining ten plants (5 consecutive plants per row) in each plot. Damage was assessed by splitting each of ten plants and counting the number of feeding galleries per plant and length of feeding gallery (cm.) in each stalk.

Arthropods were collected using yellow sticky traps five times during the growing season at the following intervals: late vegetative – VT, R1, R2, R3, and R4 growth stage. Sticky traps (two per plot) were deployed in rows 6 and 8 of each plot at the approximate midpoint between the ground level and the top of the plant canopy for all arthropod collections. At each specified collection, traps were deployed for approximately 7 days. Sticky traps were then sent to the University of Arkansas, Fayetteville, AR for arthropod identification and enumeration. A maximum of twelve arthropods were enumerated for each collection. Seven preselected arthropods (or arthropod groups), namely aphid, corn flea beetle, leafhopper, spider, micro parasitic hymenoptera, ladybird beetle, and minute pirate bug were enumerated at all sites for each collection time. Additionally, for each individual collection (*e.g.*, Collection 1, IABG site), four non-systematically selected samples were examined to determine the most abundant arthropods to obtain a total of up

to twelve arthropods to be enumerated for that particular collection and site. Thus, the suite of arthropods assessed often varied between collections from a site and between sites due to differences in temporal activity and geographical distribution of arthropod taxa.

Five visual counts were conducted during the growing season at approximately VT-R1, R1, R2, R3, and R4-R5 from five non-systematically selected plants per plot to collect abundance data per plot. Visual counts were made by examining the stalk, the leaf blade, the leaf collar, the ear tip, the silk, and the tassel of each plant.

I.9. Data Assessment

Experienced scientists familiar with the experimental design and evaluation criteria were involved in all components of data collection, summarization, and analysis. Study personnel assessed that measurements were taken properly, data were consistent with expectations based on experience with the crop, and the experiment was carefully monitored. Prior to analysis, the overall dataset was evaluated for evidence of biologically relevant changes and for possible evidence of an unexpected plant response. Any unexpected observations or issues during the trials that would impact the trial objectives were noted. Data were then subjected to data summarization or statistical analysis as indicated I.10.

I.10. Environmental Interactions Evaluation Criteria for Qualitative Data

I.10.1 Agronomic and Phenotypic Data

Plant vigor data were summarized but not subjected to an analysis of variance (ANOVA). MON 87411 was considered different from the conventional control in vigor if the ranges of vigor of MON 87411 did not overlap with the range of vigor of the conventional control across all replications. Any observed differences between the MON 87411 and conventional control were further assessed in the context of the range of the reference materials, and for consistency at other sites.

An ANOVA was conducted according to a randomized complete block design using SAS® to compare MON 87411 and the conventional control for the 13 phenotypic characteristics listed in Table VIII-1, with the exception of plant vigor. The level of statistical significance was predetermined to be 5 % ($\alpha = 0.05$). Comparisons of MON 87411 and the conventional control were conducted within site (individual site analysis) and in a combined-site analysis, in which the data were pooled across sites. MON 87411 and the conventional control materials were not statistically compared to the reference materials. The reference range for each measured phenotypic characteristic was determined from the minimum and maximum mean values from the 22 conventional reference hybrids that were included across all sites.

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Data excluded from analysis and the reasons for exclusion are listed in Table I-3.

I.10.2 Environmental Interaction Data

The environmental interaction data (*i.e.*, plant response to abiotic stressors, disease damage, and arthropod damage) are categorical and were not subjected to ANOVA. MON 87411 and conventional control were considered different in susceptibility or tolerance if the range of injury symptoms of each did not overlap across all four replications. Any observed differences were further assessed in the context of the range of the reference materials, and for consistency at other sites.

An ANOVA was conducted according to a randomized complete block design SAS® (SAS 2008; 2012) for corn earworm (CEW) damage, European corn borer (ECB) damage, and arthropod abundance. The level of statistical significance was predetermined to be 5 % ($\alpha = 0.05$). MON 87411 was compared to the conventional control at each site (individual-site analysis) for CEW damage, ECB damage, and the arthropod abundance. Additionally, corn earworm damage and European corn borer damage data were pooled across sites (combined-site analysis) for statistical comparison between MON 87411 and the conventional control. Minimum and maximum mean values were calculated for CEW damage and ECB damage from 15 reference hybrids that were included at IABG, NEYO, NCBD, and PAHM sites. The reference range for arthropod abundance evaluated from a given collection and site was determined from the minimum and maximum mean values collected from the reference maize at the site.

For the arthropod abundance data, statistical analyses and significance testing of differences between MON 87411 and the conventional control materials were only performed for the arthropods present in sufficient numbers to estimate the material mean arthropod counts and the variation of the means. An inclusion criterion was established where a given arthropod must have an average count per plot per collection time (across all materials) of ≥ 1.

Data excluded from analysis and the reasons for exclusion are listed in Table I-3.

I.11. Statistical Analysis

In individual site assessments of plant vigor, MON 87411 and the conventional control were considered different if the range of vigor values did not overlap across all four replications. There were no differences observed between MON 87411 and the conventional control in plant vigor at all sites (Table I-4).

In the individual-site analysis, a total of 11 statistically significant differences were detected out of 107 comparisons between MON 87411 and the conventional control (Table I-4). These differences were distributed among eight of the 13 phenotypic characteristics. MON 87411 had a lower early stand count than the conventional control at the IABG (82.3 vs. 86.3 plants per plot) and PAHM (72.5 vs. 78.8 plants per plot) sites but a higher early stand count at the NEDC site (95.3 vs. 89.8 plants per plot). MON 87411 had fewer days to 50% pollen shed than the conventional control at the NCBD site (59.3 vs. 60.5 days) and more days to 50% silking than the conventional

control at the NEYO site (66.5 vs. 65.5 days). MON 87411 exhibited a higher stay green rating (less green tissue) than the conventional control at the KSLA site (1.8 vs. 1.0 rating). MON 87411 had a higher final stand count than the conventional control at the KSLA site (77.3 vs.74.0 plants/plot). Grain moisture percentage was lower for MON 87411 than the conventional control at the IARL (16.9 vs. 17.7 %) and PAHM (21.1 vs. 22.3 %) sites. The test weight was higher for MON 87411 than the conventional control at the PAHM site (68.5 vs. 65.8 kg/hl). MON 87411 had a higher yield than the conventional control at the INSH site (11.5 vs. 9.2 Mg/ha). The statistical differences between MON 87411 and the conventional control detected in the individual-site analysis for early stand count, days to 50% pollen shed, days to 50% silking, stay green, final stand count, grain moisture percentage, test weight, and yield were not detected in the combined-site analysis. Thus, the differences detected for these phenotypic characteristics do not indicate a consistent response associated with the trait and are unlikely to be biologically meaningful, in terms of increased pest/weed potential of MON 87411 compared to the conventional maize (See Figure VIII-1, Step 2, answer "no").

I.12. Individual Field Site Plant Growth, Development, and Environmental Interactions Results and Discussion

Plant Response to Abiotic Stressor, Disease Damage, and Arthropod-related Damage:

In the individual-site assessment, no differences were observed between MON 87411 and the conventional control for any of the 100 comparisons for the assessed abiotic stressors, including cold, drought, flood, frost, hail, heat, nutrient deficiency, soil compaction, sunscald, and wind (Table I-5).

In the individual-site assessment, no differences were observed between MON 87411 and the conventional control for any of the 119 comparisons for the assessed diseases, including anthracnose, bacterial leaf spot, ear rot, eyespot, Fusarium sp., Goss's bacterial wilt, gray leaf spot, leaf blight, maize rough dwarf virus, Pythium sp., Rhizoctonia sp., rust, seedling blight, smut, stalk rot, and Stewart's bacterial wilt (Table I-6).

In the individual-site assessment, no differences were observed between MON 87411 and the conventional control for any of the 102 comparisons for the assessed arthropods, including aphid, armyworm, billbug, cutworm, corn earworm, corn flea beetle, rootworm beetle, European corn borer, grasshopper, Japanese beetle, sap beetle, spider mite, stink bug, and wireworm adult (Table I-7).

Corn Earworm and European Corn Borer Damage:

In the individual-site analysis, one statistically significant difference was detected out of 12 comparisons between MON 87411 and the conventional control for CEW and ECB among all observations at all four sites (Table I-8). CEW damage was higher for MON 87411 than the conventional control at the NCBD site (3.3 vs. 1.5 cm^2 damaged area per plot). This statistical difference in the individual-site analysis was not detected in the combined-site analysis. Thus, this difference was not indicative of a consistent plant

response associated with the trait and is unlikely to be biologically meaningful in terms of increased pest potential of MON 87411 compared to the conventional control (See Figure VIII-1, Step 2, answer "no").

Sticky Trap:

A total of 108 statistical comparisons were made between MON 87411 and the conventional control for arthropod abundance involving the following arthropods: aphid, corn flea beetle, delphacid planthopper, green lacewing, ladybird beetle, leafhopper, micro-parasitic hymenoptera, macro-parasitic hymenoptera, sap beetle, minute pirate bug, and spider (Table I-9). Lack of sufficient arthropod abundance precluded statistical comparisons between MON 87411 and the conventional control for 79 additional comparisons; however, descriptive statistics were provided for these comparisons (Table I-9).

No statistically significant differences were detected between MON 87411 and the conventional control for 104 out of 108 comparisons (Table I-9). The abundance of aphids was higher in MON 87411 than the conventional control in Collection 3 (1.8 vs. 0.0 per plot) at the NEYO site. The mean value for aphid abundance on MON 87411 was within the range of the reference hybrids. The abundance of delphacid planthoppers was lower in MON 87411 than the conventional control in Collection 1 at the IABG site (0.0 vs. 1.8 per plot). MON 87411 had higher abundance than the conventional control for spiders (3.3 vs. 0.3 per plot) in Collection 5 and lower abundance than the conventional control for minute pirate bugs (1.0 vs. 3.0 per plot) in Collection 1 at the PAHM site. The mean values for delphacid planthopper, minute pirate bug, and spider abundance on MON 87411 were outside the respective ranges of reference hybrids. However, these differences were not consistently detected across collections or sites (Table I-9). Thus, these differences in aphid, delphacid planthopper, minute pirate bug, and spider abundance were not indicative of a consistent response associated with the trait and are not considered biologically meaningful in terms of increased pest potential of MON 87411 compared to conventional maize (See Section VIII.B.2.).

Visual counts:

A total of 61 statistical comparisons were made between MON 87411 and the conventional control for arthropod abundance involving the following pest and beneficial arthropods: ant-like flower beetle, click beetle, corn flea beetle, ladybird beetle adult, ladybird beetle larvae, minute pirate bug, sap beetle, shining flower beetle, and spider (Table I-10). Lack of sufficient arthropod abundance precluded statistical comparisons between MON 87411 and the conventional control for 152 additional comparisons; however, the descriptive statistics were provided for these comparisons (Table I-10).

No statistically significant differences were detected between MON 87411 and the conventional control for 60 out of 61 comparisons (Table I-10). The abundance of ant-like flower beetles was lower in MON 87411 than the conventional control in Collection 2 at the IABG site (0.8 vs. 4.3 per plot). The mean value for ant-like flower beetle abundance on MON 87411 was lower than the range of reference hybrids (1.8 – 3.3 per

plot). However, the difference for ant-like flower beetle was not consistently detected across collections at the IABG site (Table I-10). Thus, this difference in ant-like flower beetle abundance was not indicative of a consistent response associated with the trait and is not considered biologically meaningful in terms of increased pest potential of MON 87411 compared to conventional maize (See Section VIII.B.2.).

Table I-3. Data Missing or Excluded from Analysis

Site Code[1]	Material Name	Material Type	Plots	Characteristics	Reason for Exclusion
IALL, ILCY, ILPH, and ILWY	All	All	All	Phenotypic and environmental interactions data	Extreme weather conditions (strong wind or drought) that caused extensive damage across the plots
IARL	MON 87411	Test	406	Root lodged plants	Wind damage
IARL	MPA640B Legacy L7671	Control Reference	106 107	Moisture (%), shelled plot weight, and test weight	Incorrect data collection; Mechanical errors
INSH	Legacy L7671	Reference	205	Days to 50% silking and days to 50% pollen shed	Identified as an outlier
NEDC	MON 87411 Midland Phillips 799	Test Reference	104 106	Final stand count	Identified as an outlier
PAHM	MON 87411 Phillips 717 Pioneer 33T56 MPA640B	Test Reference Reference Control	106 206 306 406	Sticky trap data and visual count	Missing data
PAHM	MPA640B	Control	406	Test weight	Missing data
NEDC	All	All	All	Environmental interactions evaluation # 4 (Animal damage)	Improper selection of stressor
NEYO	All	All	All	Environmental interaction evaluation # 4 (Black stem disease)	Improper selection of stressor
NEYO	All	All	All	Environmental interaction evaluation # 3 (abiotic stressor)	Missing data for two abiotic stressors
IARL	All	All	All	Environmental interaction evaluation # 2	Incorrect data collection
IARL	All	All	All	Environmental interaction evaluation # 3 and 4 (Bean leaf beetle)	Improper selection of stressor
KSLA	All	All	All	Environmental interaction evaluation # 2 (Velvetbean caterpillar)	Improper selection of stressor

Table I-3 (continued). Data Missing or Excluded from Analysis

Site Code[1]	Material Name	Material Type	Plots	Characteristics	Reason for Exclusion
NCBD	All	All	All	Environmental interaction evaluation # 1 (Other arthropod)	Improper selection of stressor
IABG	All	All	All	Environmental interaction evaluation # 4 (Other arthropod)	Improper selection of stressor
IABG	All	All	All	Visual count – Collection # 1	Incorrect data collection
INSH	LG2620	Reference	101	Environmental interaction evaluation # 1 (Bacterial blight - Pseudomonas)	Incorrect data collection; not rated for all plots

[1]Site code: IABG = Greene County, IA; IALL = Story, IA; IARL = Jefferson County, IA; ILCY = Clinton, IL; ILPH = Champaign, IL; ILWY = Stark, IL; ILMN = Warren County, IL; INSH = Boone County, IN; KSLA = Pawnee County, KS; NCBD = Perquimans County, NC; NEDC = Butler County, NE; NEYO = York County, NE; PAHM = Berks County, PA.

Table I-4. Individual Site Phenotypic Comparison of MON 87411 Compared to the Conventional Control

	Phenotypic Characteristics					
	Plant Vigor (1-9 rating)[2]		Early stand count (#/plot)		Days to 50% pollen shed	
	Range		Mean (S.E.)[3]		Mean (S.E.)[3]	
Site Code[1]	MON 87411	Control	MON 87411	Control	MON 87411	Control
IABG	2 – 3	1 – 3	82.3 (1.11)*	86.3 (1.44)	65.3 (0.25)	65.3 (0.48)
IARL	7 – 8	5 – 7	84.8 (2.25)	82.3 (3.12)	62.8 (0.63)	62.0 (0.41)
ILMN	2 – 3	1 – 3	83.0 (0.41)	82.8 (1.18)	62.0 (0.00)†	62.0 (0.00)
INSH	4 – 6	3 – 4	76.5 (3.52)	83.5 (1.44)	66.5 (1.66)	64.8 (0.75)
KSLA	2	2	97.3 (0.75)	96.0 (0.82)	56.0 (0.00)	56.0 (0.00)
NCBD	3 – 4	3 – 4	71.3 (3.04)	72.3 (1.65)	59.3 (0.25)*	60.5 (0.50)
NEDC	2	2	95.3 (1.49)*	89.8 (1.70)	68.0 (0.41)	68.8 (0.25)
NEYO	1	1	76.0 (0.82)	78.8 (1.44)	66.3 (0.48)	65.5 (0.29)
PAHM	3 – 4	3 – 5	72.5 (1.89)*	78.8 (1.55)	63.5 (0.50)	63.0 (0.41)

Table I-4 (continued). Individual Site Phenotypic Comparison of MON 87411 Compared to the Conventional Control

	Phenotypic Characteristics					
	Days to 50% silking		Stay-green rating (1-9 scale)		Ear height (cm)	
	Mean (S.E.)[3]		Mean (S.E.)[3]		Mean (S.E.)[3]	
Site Code[1]	MON 87411	Control	MON 87411	Control	MON 87411	Control
IABG	64.3 (0.25)	65.3 (0.63)	7.3 (1.03)	7.0 (0.00)	96.8 (2.19)	101.4 (1.22)
IARL	61.0 (0.58)	61.0 (0.00)	3.0 (0.00)	3.5 (0.29)	97.7 (3.95)	90.8 (2.40)
ILMN	62.0 (0.00)†	62.0 (0.00)	4.0 (0.00)	4.0 (0.00)	107.9 (1.03)	109.5 (0.81)
INSH	67.3 (1.49)	66.0 (0.82)	4.5 (0.29)	5.5 (0.87)	97.1 (2.21)	93.6 (2.51)
KSLA	58.0 (0.00)	58.0 (0.00)	1.8 (0.25)*	1.0 (0.00)	103.2 (1.45)	102.2 (1.13)
NCBD	58.8 (0.63)	59.8 (0.25)	5.5 (0.50)	6.0 (0.00)	126.9 (2.69)	130.1 (1.73)
NEDC	68.3 (0.25)	68.3 (0.48)	9.0 (0.00)	9.0 (0.00)	113.5 (0.83)	107.8 (4.33)
NEYO	66.5 (0.50)*	65.5 (0.29)	6.3 (0.25)	6.0 (0.00)	107.7 (1.05)	104.3 (1.20)
PAHM	64.8 (0.63)	64.3 (0.25)	8.8 (0.25)	8.0 (0.41)	85.9 (3.05)	93.3 (5.68)

Table I-4 (continued). Individual Site Phenotypic Comparison of MON 87411 Compared to the Conventional Control

	Phenotypic Characteristics					
	Plant height (cm)		Dropped ears (#/plot)		Stalk lodged plants (#/plot)	
	Mean (S.E.)[3]		Mean (S.E.)[3]		Mean (S.E.)[3]	
Site Code[1]	MON 87411	Control	MON 87411	Control	MON 87411	Control
IABG	259.8 (2.51)	266.0 (6.25)	0.0 (0.00)†	0.0 (0.00)	0.3 (0.25)	0.0 (0.00)
IARL	200.6 (9.72)	202.7 (3.79)	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)†	0.0 (0.00)
ILMN	257.5 (1.56)	263.7 (3.37)	0.0 (0.00)	0.0 (0.00)	0.3 (0.25)	0.0 (0.00)
INSH	214.0 (9.56)	204.2 (7.99)	0.5 (0.29)	1.3 (0.48)	3.3 (0.63)	3.3 (0.85)
KSLA	223.7 (3.50)	221.9 (1.02)	0.0 (0.00)†	0.0 (0.00)	0.0 (0.00)†	0.0 (0.00)
NCBD	259.2 (0.67)	262.2 (1.09)	5.3 (0.95)	7.8 (0.95)	0.3 (0.25)	0.0 (0.00)
NEDC	252.4 (4.75)	247.3 (5.50)	0.0 (0.00)	0.0 (0.00)	1.8 (0.85)	1.0 (0.41)
NEYO	260.4 (1.63)	257.1 (2.47)	0.3 (0.25)	0.0 (0.00)	1.0 (0.71)	0.3 (0.25)
PAHM	217.2 (3.54)	227.2 (7.10)	0.0 (0.00)	0.3 (0.25)	4.3 (0.85)	5.8 (2.06)

Table I-4 (continued). Individual Site Phenotypic Comparison of MON 87411 Compared to the Conventional Control

	Phenotypic Characteristics					
	Root lodged plants (#/plot)		Final stand count (#/plot)		Grain moisture (%)	
	Mean (S.E.)[3]		Mean (S.E.)[3]		Mean (S.E.)[3]	
Site Code[1]	MON 87411	Control	MON 87411	Control	MON 87411	Control
IABG	0.3 (0.25)	1.0 (1.00)	60.3 (0.25)	60.8 (0.48)	17.0 (0.79)	16.9 (0.59)
IARL	0.0 (0.00)	0.8 (0.48)	61.5 (0.50)	61.8 (0.25)	16.9 (0.15)*	17.7 (0.33)
ILMN	0.0 (0.00)†	0.0 (0.00)	62.0 (0.71)	61.5 (0.50)	19.7 (0.28)	19.5 (0.17)
INSH	0.5 (0.50)	0.0 (0.00)	70.0 (1.47)	69.3 (2.95)	18.2 (0.26)	18.3 (0.14)
KSLA	0.0 (0.00)†	0.0 (0.00)	77.3 (0.48)*	74.0 (0.00)	15.1 (0.19)	15.4 (0.43)
NCBD	0.0 (0.00)	0.3 (0.25)	67.8 (1.03)	67.0 (0.58)	20.0 (0.19)	20.0 (0.19)
NEDC	0.0 (0.00)	0.0 (0.00)	62.0 (0.00)	61.3 (0.25)	14.8 (0.33)	15.6 (0.35)
NEYO	0.0 (0.00)†	0.0 (0.00)	61.0 (1.35)	60.8 (0.75)	15.4 (0.22)	15.2 (0.20)
PAHM	0.0 (0.00)†	0.0 (0.00)	62.3 (0.63)	62.5 (0.29)	21.1 (0.27)*	22.3 (1.01)

Table I-4 (continued). Individual Site Phenotypic Comparison of MON 87411 Compared to the Conventional Control

Phenotypic Characteristics				
	Test weight (kg/hl)		Yield (Mg/ha)	
	Mean (S.E.)[3]		Mean (S.E.)[3]	
Site Code[1]	MON 87411	Control	MON 87411	Control
IABG	76.9 (0.80)	77.8 (0.33)	11.7 (1.18)	9.5 (0.42)
IARL	76.6 (0.64)	75.8 (0.64)	8.2 (1.00)	8.3 (0.43)
ILMN	69.6 (0.18)	69.9 (0.18)	14.4 (0.29)	14.9 (0.04)
INSH	71.6 (1.01)	73.2 (1.18)	11.5 (0.66)*	9.2 (0.77)
KSLA	74.8 (0.36)	75.5 (0.40)	14.1 (0.48)	14.0 (0.51)
NCBD	69.0 (0.41)	70.1 (0.78)	9.5 (0.28)	9.3 (0.59)
NEDC	72.1 (1.00)	73.5 (1.07)	7.5 (1.16)	8.0 (0.52)
NEYO	76.2 (0.28)	76.3 (0.18)	12.5 (0.07)	13.0 (0.17)
PAHM	68.5 (0.46)*	65.8 (0.88)	10.4 (0.27)	11.1 (0.48)

Note: The experimental design was a randomized complete block with four replicates per site.
*Indicates statistically significant difference between MON 87411 and the conventional control (α =0.05) using ANONA.
†Indicates p-values could not be generated due to lack of variability in the data.
[1]Site code: IABG = Greene County, IA; IARL = Jefferson County, IA; ILMN = Warren County, IL; INSH = Boone County, IN; KSLA = Pawnee County, KS; NCBD = Perquimans County, NC; NEDC = Butler County, NE; NEYO = York County, NE; PAHM = Berks County, PA.
[2]Data were not subjected to statistical analysis. Plant vigor rating range (minimum - maximum); the range of plant vigor ratings for the references is as follows: IABG 1 – 3; IARL 5 – 8; ILMN 1 – 3; INSH 3 – 5; KSLA 2; NCBD 3 – 4; NEDC 2; NEYO 1; PAHM 2 – 4
[3]MON 87411 and the conventional control values represent means with standard error in parentheses. N = 4 except where noted in Table I-3.

Table I-5. Qualitative Assessment: Abiotic Stressor Evaluations Using a Categorical Scale for MON 87411 and the Conventional Control

Abiotic Stressor	Number of Observations across Sites[1]	Number of Observations where No Differences were Observed between MON 87411 and the Conventional Control
Total	**100**	**100**
Cold	1	1
Drought[2]	22	22
Flood[3]	5	5
Frost	2	2
Hail	8	8
Heat	20	20
Nutrient deficiency	12	12
Soil compaction	3	3
Sunscald	5	5
Wind	22	22

Note: The experimental design was a randomized complete block with four replicates per site.
No differences were observed between MON 87411 and the conventional control during any observation for damage caused by any of the assessed abiotic stressors. Data were not subjected to statistical analysis.
Observational data collected at four crop development stages: V6–V8; V12-VT; R1-R3; and R5-R6.

[1]Site code: IABG = Greene County, IA; IARL = Jefferson County, IA; ILMN = Warren County, IL; INSH = Boone County, IN; KSLA = Pawnee County, KS; NCBD = Perquimans County, NC; NEDC = Butler County, NE; NEYO = York County, NE; PAHM = Berks County, PA.
[2] Includes dryness, dry/heat.
[3] Includes wet soil.

Table I-6. Qualitative Assessment: Disease Damage Evaluations Using a Categorical Scale for MON 87411 and the Conventional Control

Disease	Number of Observations across Sites[1]	Number of Observations where No Differences were Observed between MON 87411 and the Conventional Control
Total	**119**	**119**
Anthracnose	6	6
Bacterial leaf spot	1	1
Ear rot[2]	12	12
Eyespot	5	5
Fusarium sp.	8	8
Goss's bacterial wilt	10	10
Gray leaf spot	20	20
Leaf blight[3]	16	16
Maize rough dwarf virus	2	2
Pythium sp.	2	2
Rhizoctonia sp.	2	2
Rust[4]	7	7
Seedling blight	2	2
Smut	8	8
Stalk rot[5]	10	10
Stewart's bacterial wilt	8	8

Note: The experimental design was a randomized complete block with four replicates per site.
No differences were observed between MON 87411 and the conventional control during any observation for damage caused by any of the assessed diseases. Data were not subjected to statistical analysis.
Observational data collected at four crop development stages: V6–V8; V12-VT; R1-R3; and R5-R6.
Additional assessments of ear rot disease and stalk rot disease were made on 5 plant/plots at harvest.
[1]Site code: IABG = Greene County, IA; IARL = Jefferson County, IA; ILMN = Warren County, IL; INSH = Boone County, IN; KSLA = Pawnee County, KS; NCBD = Perquimans County, NC; NEDC = Butler County, NE; NEYO = York County, NE; PAHM = Berks County, PA.
[2]Assessed on 5 non-systematically selected plants. At ILMN and INSH, stalk rot data were collected both on a per plot basis and on 5 plant/plots.
[3]Includes northern and southern.
[4]Includes common and southern.
[5]Assessed on 5 non-systematically selected plants. At NEDC, stalk rot data were collected both on a per plot basis and on 5 plant/plots.

Table I-7. Qualitative Assessment: Arthropod Damage Evaluations Using a Categorical Scale for MON 87411 and the Conventional Control

Arthropod	Number of Observations across Sites[1]	Number of Observations where No Differences were Observed between MON 87411 and the Conventional Control
Total	102	102
Aphid (Aphididae)	3	3
Armyworm (Noctuidae)[2]	18	18
Billbugs (*Sphenophorus parvulus*)	2	2
Cutworm (Noctuidae)[3]	6	6
Corn earworm (*Helicoverpa zea*)	5	5
Corn flea beetle (*Chaetocnema pulicaria*)	5	5
Rootworm beetle (*Diabrotica* spp.*)*	16	16
European corn borer (*Ostrinia nubilalis*)	9	9
Grasshopper (*Melanoplus* spp.)	14	14
Japanese beetle (*Popillia japonica*)	9	9
Sap beetle (Nitidulidae)	3	3
Spider mites (*Tetranychus* spp.)	3	3
Stink bugs (Pentatomidae)	8	8
Wireworm beetle (Elateridae)	1	1

Note: The experimental design was a randomized complete block with four replicates per site.
No differences were observed between MON 87411 and the conventional control during any observation for damage caused by any of the assessed arthropods. Data were not subjected to statistical analysis.
Observational data collected at four crop development stages: V6–V8; V12-VT; R1-R3; and R5-R6.
[1]Site code: IABG = Greene County, IA; IARL = Jefferson County, IA; ILMN = Warren County, IL; INSH = Boone County, IN; KSLA = Pawnee County, KS; NCBD = Perquimans County, NC; NEDC = Butler County, NE; NEYO = York County, NE; PAHM = Berks County, PA.
[2]Includes beet armyworm and fall armyworm.
[3]Includes black cutworm.

Table I-8. Individual-Site Analysis: Quantitative Assessment of Corn Earworm and European Corn Borer Damage to MON 87411 Compared to the Conventional Control

Pest	Damage assessment[1]	Site[2]	MON 87411	Control
Corn earworm (*H. zea*)[3]	Mean (S.E.) damage area of 10 plants per plot (cm^2)	IABG	0.7 (0.38)	0.5 (0.28)
		NCBD	3.3 (1.25)*	1.5 (0.39)
		NEYO	3.2 (0.13)	3.0 (0.22)
		PAHM	0.3 (0.23)	0.2 (0.11)
European corn borer (*O. nubilalis*)[4]	Mean (S.E.) number of stalk galleries of 10 plants per plot	IABG	0.0 (0.03)	0.0 (0.00)
		NCBD	0.1 (0.03)	0.1 (0.04)
		NEYO	0.0 (0.00)	0.0 (0.00)
		PAHM	1.4 (0.24)	1.8 (0.33)
European corn borer (*O. nubilalis*)[4]	Mean (S.E.) stalk gallery length (cm) of 10 plants per plot	IABG	0.1 (0.08)	0.0 (0.00)
		NCBD	0.5 (0.19)	0.2 (0.14)
		NEYO	0.0 (0.00)	0.0 (0.00)
		PAHM	5.9 (1.46)	7.9 (1.46)

Note: The experimental design was a randomized complete block with four replicates per site.
*Indicates a statistically significant difference between MON 87411 and the conventional control (α=0.05) using ANOVA.
[1] MON 87411and the conventional control values represent means with standard error in parentheses. N = 4.
[2] Site code: IABG = Greene, IA; NCBD = Perquimans, NC; NEYO = York, NE; PAHM = Berks, PA.
[3] Damage assessment for *H. zea* was conducted at R5 growth stage.
[4] Damage assessments for *O. nubilalis* were conducted at R6 growth stage.

Table I-9. Individual-Site Analysis: Arthropod Abundance in Sticky Trap Samples Collected from MON 87411 Compared to the Conventional Control

		Aphid (Aphididae)			Brown Lacewing (Hemerobiidae)			Corn Flea Beetle (Chrysomelidae)		
		Pest Arthropod			Beneficial Arthropod			Pest Arthropod		
		Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference
Coll.[1]	Site[2]	MON 87411	Control	range[4]	MON 87411	Control	range[4]	MON 87411	Control	range[4]
1	IABG	1.5 (0.87)	1.0 (0.41)	0.5 – 3.3	—	—	—	0.3 (0.25)†	0.5 (0.50)	0.0 – 0.8
	NCBD	0.8 (0.75)†	0.3 (0.25)	0.3 – 1.8	0.5 (0.29)†	0.3 (0.25)	0.0 – 0.5	0.5 (0.29)†	0.0 (0.00)	0.0 – 4.3
	NEYO	0.5 (0.29)†	0.0 (0.00)	0.0 – 2.0	—	—	—	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0
	PAHM	0.7 (0.33)†	0.7 (0.33)	0.3 – 1.0	0.7 (0.33)†	0.3 (0.33)	0.0 – 0.3	3.7 (1.45)	2.3 (1.33)	3.7 – 11.3
2	IABG	1.3 (0.75)	1.0 (0.41)	1.3 – 3.5	0.3 (0.25)†	0.3 (0.25)	0.3 – 1.0	0.5 (0.50)†	0.5 (0.50)	0.0 – 0.3
	NCBD	0.8 (0.25)	0.3 (0.25)	0.3 – 4.3	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3	0.0 (0.00)	2.0 (1.08)	0.5 – 5.3
	NEYO	0.5 (0.29)†	0.3 (0.25)	0.0 – 2.3	—	—	—	0.5 (0.29)†	0.0 (0.00)	0.0 – 0.8
	PAHM	1.0 (1.00)†	0.7 (0.67)	0.0 – 0.8	—	—	—	3.0 (1.53)	6.7 (0.88)	5.7 – 12.5
3	IABG	1.5 (0.29)	0.5 (0.29)	1.0 – 3.8	—	—	—	0.3 (0.25)†	0.0 (0.00)	0.5 – 1.0
	NCBD	1.5 (0.65)	1.0 (0.71)	0.0 – 2.3	—	—	—	0.8 (0.48)	1.3 (0.95)	0.3 – 16.0
	NEYO	1.8 (0.48)*	0.0 (0.00)	1.0 – 2.5	—	—	—	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0
	PAHM	0.3 (0.33)†	0.0 (0.00)	0.0 – 0.3	—	—	—	9.3 (4.48)	5.0 (1.73)	7.7 – 20.8
4	IABG	4.0 (1.73)	0.8 (0.25)	1.5 – 5.5	0.0 (0.00)†	0.0 (0.00)	0.3 – 0.8	0.5 (0.29)	0.3 (0.25)	1.3 – 4.8
	NCBD	1.3 (0.25)†	0.0 (0.00)	0.0 – 1.8	—	—	—	1.0 (0.71)	3.3 (1.93)	0.3 – 19.8
	NEYO	1.8 (0.48)	0.8 (0.25)	2.5 – 7.0	—	—	—	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3
	PAHM	0.3 (0.33)†	1.0 (0.58)	0.0 – 1.7	—	—	—	7.7 (3.53)	6.7 (3.28)	12.3 – 23.3
5	IABG	7.5 (4.25)	2.8 (1.11)	6.3 – 12.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.8	0.3 (0.25)†	0.5 (0.29)	0.5 – 1.5
	NCBD	1.0 (0.71)	1.0 (0.41)	0.5 – 1.8	—	—	—	3.3 (1.11)	6.8 (1.03)	4.3 – 27.8
	NEYO	21.0 (5.58)	19.3 (7.06)	15.8 – 30.0	—	—	—	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3
	PAHM	1.7 (0.88)†	0.7 (0.33)	0.0 – 1.0	—	—	—	5.0 (1.73)	3.0 (1.53)	3.0 – 12.0

Table I-9. (continued). Individual-Site Analysis: Arthropod Abundance in Sticky Trap Samples Collected from MON 87411 Compared to the Conventional Control

		Delphacid Planthopper (Delphacidae)			Grasshopper (Acrididae)			Green Lacewings (Chrysopidae)		
		Pest Arthropod			Pest Arthropod			Beneficial Arthropod		
		Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference
Coll.[1]	Site[2]	MON 87411	Control	range[4]	MON 87411	Control	range[4]	MON 87411	Control	range[4]
1	IABG	0.0 (0.00)*	1.8 (0.85)	1.5 – 3.5	—	—	—	3.8 (1.25)	2.8 (1.31)	1.5 – 4.8
	NCBD	51.0 (5.58)	64.0 (6.92)	63.8 – 106.0	—	—	—	0.0 (0.00)†	0.8 (0.25)	0.0 – 0.3
	NEYO	—	—	—	—	—	—	0.0 (0.00)†	0.3 (0.25)	0.0 – 0.3
	PAHM	6.7 (1.45)	3.0 (1.53)	3.8 – 10.0	—	—	—	—	—	—
2	IABG	0.8 (0.48)	0.5 (0.29)	1.3 – 3.0	—	—	—	1.8 (0.85)	2.5 (1.19)	0.0 – 3.0
	NCBD	10.0 (4.02)	11.8 (2.29)	15.3 – 23.0	—	—	—	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3
	NEYO	0.3 (0.25)†	0.5 (0.29)	0.3 – 0.5	—	—	—	0.8 (0.75)†	0.3 (0.25)	0.5 – 1.5
	PAHM	4.7 (2.73)	5.0 (1.73)	2.3 – 10.5	—	—	—	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.5
3	IABG	—	—	—	—	—	—	—	—	—
	NCBD	8.0 (1.08)	5.0 (2.52)	9.3 – 12.8	—	—	—	—	—	—
	NEYO	0.8 (0.25)†	0.3 (0.25)	0.3 – 0.5	—	—	—	4.0 (1.68)	2.5 (0.29)	1.5 – 4.8
	PAHM	6.0 (2.00)	4.7 (1.20)	2.7 – 8.8	—	—	—	—	—	—
4	IABG	1.3 (0.75)†	0.3 (0.25)	0.5 – 0.8	—	—	—	4.3 (1.11)	7.0 (1.22)	5.8 – 6.5
	NCBD	4.8 (1.11)	6.0 (0.41)	7.5 – 8.3	—	—	—	—	—	—
	NEYO	—	—	—	—	—	—	0.5 (0.29)	0.8 (0.25)	0.8 – 7.3
	PAHM	4.7 (1.20)	2.3 (0.33)	0.7 – 10.3	—	—	—	—	—	—
5	IABG	—	—	—	—	—	—	6.3 (2.75)	5.3 (1.11)	4.5 – 9.8
	NCBD	3.3 (1.25)	4.3 (1.44)	3.8 – 8.3	—	—	—	—	—	—
	NEYO	—	—	—	—	—	—	3.0 (0.71)	1.5 (1.19)	0.3 – 2.0
	PAHM	1.0 (0.58)†	0.7 (0.33)	0.0 – 2.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	—	—	—

Table I-9. (continued). Individual-Site Analysis: Arthropod Abundance in Sticky Trap Samples Collected from MON 87411 Compared to the Conventional Control

		Ladybird Beetle (Coccinellidae)			Leafhopper (Cicadellidae)			Micro-Parasitic Hymenoptera		
		Beneficial Arthropod			Pest Arthropod			Beneficial Arthropod		
		Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference
Coll.[1]	Site[2]	MON 87411	Control	range[4]	MON 87411	Control	range[4]	MON 87411	Control	range[4]
1	IABG	1.3 (0.75)	0.5 (0.50)	1.0 – 3.3	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3	33.8 (2.66)	45.5 (3.40)	34.3 – 67.3
	NCBD	3.5 (0.87)	5.3 (3.20)	2.5 – 3.5	37.8 (4.53)	40.0 (5.43)	37.3 – 53.0	129.8 (8.43)	141.5 (20.97)	214.5 – 278.0
	NEYO	0.8 (0.25)	0.3 (0.25)	0.0 – 7.5	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	7.5 (1.94)	9.0 (1.47)	8.8 – 13.0
	PAHM	15.3 (0.88)	14.3 (1.33)	21.5 – 32.3	4.3 (1.20)	4.0 (2.08)	3.5 – 7.3	128.0 (18.0)	104.0 (18.36)	145.7 – 189.3
2	IABG	0.8 (0.25)	1.3 (0.25)	1.0 – 5.8	0.3 (0.25)†	0.0 (0.00)	0.0 – 0.0	36.3 (7.72)	44.8 (10.46)	48.0 – 88.3
	NCBD	7.5 (1.85)	10.0 (2.58)	9.3 – 16.5	40.5 (8.91)	42.3 (15.10)	33.5 – 79.8	92.3 (15.37)	97.8 (12.25)	153.3 – 187.5
	NEYO	0.0 (0.00)†	0.8 (0.48)	0.5 – 1.3	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	14.3 (3.28)	17.3 (4.35)	14.3 – 17.5
	PAHM	39.0 (11.14)	33.7 (6.36)	26.3 – 36.0	3.0 (1.53)	5.0 (1.15)	3.0 – 10.3	102.7 (12.81)	119.0 (8.96)	114.0 – 141.8
3	IABG	0.0 (0.00)†	0.8 (0.25)	0.5 – 1.5	0.0 (0.00)†	0.3 (0.25)	0.0 – 0.0	30.0 (4.78)	28.8 (3.88)	38.3 – 102.3
	NCBD	5.8 (1.44)	6.5 (0.50)	4.0 – 7.0	40.8 (9.00)	43.3 (10.66)	48.0 – 69.5	66.8 (7.96)	62.5 (13.12)	61.3 – 87.3
	NEYO	0.3 (0.25)†	0.8 (0.48)	0.3 – 1.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	17.8 (1.03)	12.8 (1.65)	14.3 – 33.0
	PAHM	20.7 (5.90)	13.0 (2.08)	10.3 – 14.5	2.3 (0.33)	3.0 (1.00)	1.5 – 4.5	78.7 (22.51)	61.3 (2.96)	59.3 – 104.0
4	IABG	1.3 (0.48)	0.5 (0.50)	1.3 – 4.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3	47.0 (5.15)	47.5 (3.30)	44.5 – 89.8
	NCBD	8.5 (1.85)	7.0 (1.68)	5.5 – 8.0	27.5 (0.87)	33.5 (7.60)	28.3 – 45.0	65.8 (11.53)	56.5 (15.54)	61.5 – 104.3
	NEYO	0.5 (0.29)†	0.8 (0.75)	0.3 – 1.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	12.5 (1.50)	9.3 (2.29)	13.8 – 69.0
	PAHM	7.3 (0.67)	5.7 (1.20)	2.7 – 5.0	3.7 (0.88)	4.3 (1.33)	4.0 – 11.0	118.3 (24.44)	85.0 (20.43)	100.0 – 177.0
5	IABG	2.0 (0.82)	2.0 (2.00)	1.3 – 4.5	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3	17.5 (4.33)	13.8 (2.39)	20.0 – 58.5
	NCBD	1.5 (0.29)	3.8 (0.48)	1.8 – 5.0	17.3 (2.25)	20.3 (4.71)	19.5 – 27.3	92.0 (25.01)	65.8 (18.86)	86.8 – 107.5
	NEYO	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	11.3 (0.75)	13.5 (2.10)	9.8 – 20.0
	PAHM	5.7 (0.67)	4.3 (2.33)	2.5 – 3.7	5.0 (1.00)	3.3 (2.03)	4.0 – 9.8	182.7 (21.84)	152.0 (33.56)	136.7 – 229.8

Table I-9. (continued). Individual-Site Analysis: Arthropod Abundance in Sticky Trap Samples Collected from MON 87411 Compared to the Conventional Control

		Macro-parasitic hymenoptera			Sap Beetle (Nitidulidae)			Minute Pirate Bug (Anthocoridae)		
		Beneficial Arthropod			Pest Arthropod			Beneficial Arthropod		
		Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference
Coll.[1]	Site[2]	MON 87411	Control	range[4]	MON 87411	Control	range[4]	MON 87411	Control	range[4]
1	IABG	—	—	—	—	—	—	1.0 (0.71)	0.8 (0.48)	0.8 – 3.8
	NCBD	—	—	—	—	—	—	1.3 (0.63)†	0.3 (0.25)	0.5 – 0.8
	NEYO	—	—	—	—	—	—	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0
	PAHM	—	—	—	—	—	—	1.0 (0.58)*	3.0 (0.58)	2.0 – 7.5
2	IABG	—	—	—	—	—	—	1.5 (0.96)	3.3 (0.25)	3.3 – 9.5
	NCBD	—	—	—	1.3 (0.48)†	1.0 (0.71)	0.0 – 1.0	1.3 (0.95)	1.3 (0.48)	0.0 – 1.8
	NEYO	—	—	—	—	—	—	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3
	PAHM	0.7 (0.33)	1.0 (0.58)	0.7 – 2.5	0.7 (0.33)†	1.3 (0.33)	0.3 – 1.8	2.7 (1.20)	4.7 (2.19)	5.0 – 10.0
3	IABG	—	—	—	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3	1.3 (0.48)	1.0 (0.41)	0.8 – 3.5
	NCBD	0.5 (0.50)†	0.3 (0.25)	0.0 – 0.3	0.8 (0.25)†	0.3 (0.25)	0.3 – 1.0	0.3 (0.25)	0.5 (0.29)	1.0 – 1.8
	NEYO	0.0 (0.00)†	0.0 (0.00)	0.3 – 0.8	—	—	—	0.3 (0.25)†	0.0 (0.00)	0.0 – 0.3
	PAHM	0.3 (0.33)	1.7 (0.33)	0.5 – 1.7	0.3 (0.33)†	0.0 (0.00)	0.7 – 0.8	4.0 (1.53)	2.0 (0.00)	2.3 – 10.0
4	IABG	—	—	—	—	—	—	0.3 (0.25)	1.0 (0.41)	2.3 – 6.3
	NCBD	—	—	—	—	—	—	1.0 (0.58)	0.8 (0.25)	1.5 – 4.0
	NEYO	—	—	—	—	—	—	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3
	PAHM	—	—	—	—	—	—	0.3 (0.33)	0.3 (0.33)	2.3 – 4.0
5	IABG	—	—	—	0.0 (0.00)†	0.3 (0.25)	0.0 – 1.0	0.5 (0.29)	1.8 (1.44)	1.3 – 3.5
	NCBD	—	—	—	1.5 (0.65)	1.0 (0.71)	0.3 – 1.5	1.3 (0.75)†	0.5 (0.29)	0.5 – 1.5
	NEYO	0.0 (0.00)†	0.3 (0.25)	0.0 – 0.3	—	—	—	1.0 (0.41)†	0.0 (0.00)	0.5 – 0.8
	PAHM	0.3 (0.33)†	0.0 (0.00)	0.0 – 0.5	—	—	—	2.0 (0.58)	1.0 (1.00)	4.0 – 6.8

Table I-9. (continued). Individual-Site Analysis: Arthropod Abundance in Sticky Trap Samples Collected from MON 87411 Compared to the Conventional Control

		Spider (Araneae)		
		Beneficial Arthropod		
		Mean (S.E.)[3]		Reference Range[4]
Coll.[1]	Site[2]	MON 87411	Control	
1	IABG	0.5 (0.29)†	0.3 (0.25)	0.5 – 0.8
	NCBD	3.3 (0.48)	1.0 (0.41)	1.5 – 2.3
	NEYO	0.5 (0.29)†	0.3 (0.25)	0.0 – 1.3
	PAHM	0.7 (0.33)†	1.7 (0.33)	0.5 – 1.3
2	IABG	0.0 (0.00)†	0.3 (0.25)	0.0 – 1.0
	NCBD	1.5 (0.29)	2.5 (1.50)	1.5 – 3.3
	NEYO	0.3 (0.25)†	0.0 (0.00)	0.0 – 0.5
	PAHM	1.3 (0.33)†	0.0 (0.00)	1.0 – 1.5
3	IABG	0.3 (0.25)†	0.0 (0.00)	0.0 – 0.3
	NCBD	1.8 (0.75)	1.3 (0.25)	1.3 – 3.0
	NEYO	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3
	PAHM	2.3 (1.45)	0.7 (0.33)	1.3 – 2.5
4	IABG	0.8 (0.75)†	0.3 (0.25)	0.0 – 1.0
	NCBD	1.0 (0.58)	2.0 (1.00)	1.5 – 4.0
	NEYO	0.0 (0.00)†	0.3 (0.25)	0.3 – 0.3
	PAHM	2.0 (2.00)	2.0 (0.00)	1.0 – 1.8
5	IABG	0.3 (0.25)†	0.3 (0.25)	0.3 – 0.5
	NCBD	1.5 (0.29)	0.8 (0.48)	1.5 – 2.8
	NEYO	0.3 (0.25)†	0.0 (0.00)	0.0 – 0.5
	PAHM	3.3 (0.88)*	0.3 (0.33)	0.3 – 1.3

Note: The experimental design was a randomized complete block with four replicates per site.
*Indicates a statistically significant difference between MON 87411 and the conventional control ($\alpha = 0.05$) using ANOVA.

[†]Indicates p-values could not be generated where the taxa did not meet inclusion criteria (see appendix I.10.2).
[1]Arthropods were enumerated at five crop development stages: Collection 1 = late vegetative – VT growth stage; Collection 2 = R1 growth stage; Collection 3 = R2 growth stage; Collection 4 = R3 growth stage; Collection 5 = R4 growth stage.
[2]Site code: IABG = Greene, IA; NCBD = Perquimans, NC; NEYO = York, NE; PAHM = Berks, PA.
[3]MON 87411 and the conventional control values represent means with standard error in parentheses. N = 4 (IABG, NCBD, and NEYO sites); N= 3 (PAHM site).
[4]Reference range is calculated from the minimum and maximum mean values from among reference hybrids at each site.
A dash (—) indicates data not available.

Table I-10. Individual-Site Analysis: Arthropod Abundance in Visual Counts from MON 87411 Compared to the Conventional Control

		Ant-like Flower Beetle (Anthicidae)			Click Beetle			Corn Flea Beetle		
		Pollen Feeder			Pest Arthropod			Pest Arthropod		
		Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference
Coll.[1]	Site[2]	MON 87411	Control	range[4]	MON 87411	Control	range[4]	MON 87411	Control	range[4]
1	IABG	—	—	—	—	—	—	—	—	—
	NCBD	—	—	—	1.0 (0.71)	1.8 (0.85)	0.8 – 1.5	2.5 (1.26)	3.3 (1.97)	1.0 – 5.0
	NEYO	—	—	—	—	—	—	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0
	PAHM	—	—	—	—	—	—	6.3 (4.37)	4.7 (1.45)	7.8 – 10.3
2	IABG	0.8 (0.48)*	4.3 (2.29)	1.8 – 3.3	—	—	—	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0
	NCBD	—	—	—	1.3 (0.75)	1.3 (0.75)	1.0 – 1.8	0.0 (0.00)†	0.3 (0.25)	0.0 – 0.8
	NEYO	—	—	—	—	—	—	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0
	PAHM	—	—	—	—	—	—	8.0 (5.13)	7.3 (0.88)	7.5 – 11.0
3	IABG	0.5 (0.50)	1.0 (0.71)	0.8 – 2.0	—	—	—	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0
	NCBD	—	—	—	0.0 (0.00)†	0.8 (0.48)	0.5 – 0.8	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3
	NEYO	—	—	—	—	—	—	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0
	PAHM	—	—	—	—	—	—	12.0 (3.51)	7.7 (3.84)	7.8 – 9.8
4	IABG	1.3 (0.95)	2.8 (1.49)	1.5 – 3.0	—	—	—	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0
	NCBD	—	—	—	0.5 (0.50)†	0.0 (0.00)	0.0 – 0.5	0.3 (0.25)†	0.3 (0.25)	0.0 – 0.8
	NEYO	—	—	—	—	—	—	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0
	PAHM	—	—	—	—	—	—	3.7 (1.76)	2.0 (0.58)	4.0 – 6.0
5	IABG	1.8 (1.11)	1.8 (1.44)	0.5 –1.8	—	—	—	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0
	NCBD	—	—	—	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	1.3 (1.25)	3.3 (1.89)	0.8 – 3.0
	NEYO	—	—	—	—	—	—	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0
	PAHM	—	—	—	—	—	—	2.3 (0.67)	7.3 (3.93)	5.5 – 7.3

Table I-10. (continued). Individual-Site Analysis: Arthropod Abundance in Visual Counts from MON 87411 Compared to the Conventional Control

		Japanese Beetle (**Scarabaeidae)**			Lacewing Adult (Chrysopidae)			Lacewing Larvae (Chrysopidae)		
		Pest Arthropod			Beneficial Arthropod			Beneficial Arthropod		
		Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference
Coll.[1]	Site[2]	MON 87411	Control	range[4]	MON 87411	Control	range[4]	MON 87411	Control	range[4]
1	IABG	—	—	—	—	—	—	—	—	—
	NCBD	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3	0.5 (0.50)†	0.5 (0.50)	0.3 – 0.5	0.3 (0.25)†	0.0 (0.00)	0.0 – 0.5
	NEYO	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0
	PAHM	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.3 (0.33)	0.0 – 0.5	0.0 (0.00)†	0.0 (0.00)	0.0 – 1.0
2	IABG	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.3 (0.25)†	0.0 (0.00)	0.0 – 0.8	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0
	NCBD	0.3 (0.25)†	0.0 (0.00)	0.0 – 0.0	0.3 (0.25)†	0.0 (0.00)	0.0 – 0.0	0.8 (0.48)†	0.5 (0.50)	0.0 – 0.5
	NEYO	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0
	PAHM	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.7	0.3 (0.33)†	0.0 (0.00)	0.0 – 0.8
3	IABG	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3
	NCBD	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.3 (0.25)†	1.3 (0.48)	0.0 – 0.3
	NEYO	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.3 (0.25)†	0.0 (0.00)	0.0 – 0.3	0.5 (0.29)†	0.3 (0.25)	0.3 – 0.5
	PAHM	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.3 (0.33)	0.0 – 0.7	0.3 (0.33)†	0.0 (0.00)	0.0 – 0.7
4	IABG	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.5
	NCBD	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0
	NEYO	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3	0.0 (0.00)†	0.3 (0.25)	0.0 – 0.0
	PAHM	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3	0.7 (0.67)†	0.0 (0.00)	0.0 – 0.0
5	IABG	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.3 – 0.5	0.3 (0.25)†	0.0 (0.00)	0.0 – 0.3
	NCBD	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.5	0.0 (0.00)†	0.5 (0.50)	0.0 – 0.0
	NEYO	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.5	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0
	PAHM	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0

Table I-10. (continued). Individual-Site Analysis: Arthropod Abundance in Visual Counts from MON 87411 Compared to the Conventional Control

		Ladybird Beetle Adult (Coccinellidae)			Ladybird Beetle Larvae (coccinellidae)			Lygus Bug (Miridae)		
		Beneficial Arthropod			Beneficial Arthropod			Pest Arthropod		
		Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference
Coll.[1]	Site[2]	MON 87411	Control	range[4]	MON 87411	Control	range[4]	MON 87411	Control	range[4]
1	IABG	—	—	—	—	—	—	—	—	—
	NCBD	0.5 (0.29)†	0.5 (0.29)	0.3 – 1.0	0.5 (0.50)	0.5 (0.50)	0.3 – 1.8	—	—	—
	NEYO	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	—	—	—
	PAHM	3.7 (1.45)	7.0 (2.08)	2.5 – 3.3	0.7 (0.67)†	0.0 (0.00)	0.0 – 0.5	—	—	—
2	IABG	0.3 (0.25)†	0.0 (0.00)	0.0 – 0.3	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3	—	—	—
	NCBD	0.5 (0.50)†	0.5 (0.50)	0.3 – 1.0	2.8 (0.95)	3.5 (0.50)	1.5 – 2.8	—	—	—
	NEYO	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3	—	—	—
	PAHM	3.0 (2.52)	5.0 (4.04)	1.5 – 2.5	0.3 (0.33)†	0.7 (0.67)	0.0 – 1.3	0.0 (0.00)†	1.0 (1.00)	0.0 – 0.3
3	IABG	0.3 (0.25)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	—	—	—
	NCBD	1.0 (0.41)†	0.3 (0.25)	0.0 – 1.5	1.3 (0.63)†	0.3 (0.25)	0.8 –1.3	—	—	—
	NEYO	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	—	—	—
	PAHM	1.3 (0.33)†	0.3 (0.33)	0.0 – 0.8	1.3 (0.67)†	0.0 (0.00)	0.0 – 1.7	0.0 (0.00)†	0.7 (0.67)	0.0 – 0.3
4	IABG	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3	—	—	—
	NCBD	0.8 (0.48)†	0.0 (0.00)	0.0 – 0.8	0.5 (0.50)†	0.0 (0.00)	0.0 – 0.3	—	—	—
	NEYO	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	—	—	—
	PAHM	0.0 (0.00)†	0.3 (0.33)	0.0 – 0.7	0.7 (0.67)†	0.0 (0.00)	0.0 – 0.3	0.7 (0.67)†	1.0 (0.00)	0.0 – 1.3
5	IABG	0.0 (0.00)†	0.3 (0.25)	0.0 – 0.3	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3	—	—	—
	NCBD	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3	0.5 (0.29)†	0.3 (0.25)	0.3 – 0.5	—	—	—
	NEYO	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	—	—	—
	PAHM	0.0 (0.00)†	0.7 (0.33)	0.0 – 0.8	0.7 (0.33)†	0.7 (0.67)	0.0 – 1.0	0.7 (0.33)†	0.3 (0.33)	0.0 – 0.5

Table I-10. (continued). Individual-Site Analysis: Arthropod Abundance in Visual Counts from MON 87411 Compared to the Conventional Control

		Minute Pirate Bug (Anthocoridae)			Sap Beetle (Nitidulidae)			Shining Flower Beetle (Phalacridae)		
		Beneficial Arthropod			Pest Arthropod			Pollen Feeder		
		Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference
Coll.[1]	Site[2]	MON 87411	Control	range[4]	MON 87411	Control	range[4]	MON 87411	Control	range[4]
1	IABG	—	—	—	—	—	—	—	—	—
	NCBD	1.5 (0.65)	0.3 (0.25)	1.0 – 3.5	4.5 (2.63)	5.5 (2.96)	3.8 – 7.5	6.3 (1.49)	4.3 (1.84)	5.8 – 10.5
	NEYO	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	—	—	—
	PAHM	6.3 (2.33)	10.3 (5.49)	8.3 - 13.3	6.0 (2.08)	4.3 (1.45)	5.3 – 12.8	2.0 (0.58)	5.3 (3.18)	1.5 – 5.3
2	IABG	5.0 (0.41)	4.3 (1.11)	1.5 - 4.5	6.3 (1.44)	10.8 (4.77)	4.0 – 7.5	—	—	—
	NCBD	2.0 (0.71)	2.3 (0.63)	0.5 - 3.3	4.8 (4.09)	3.5 (1.55)	2.8 – 7.8	9.0 (2.74)	15.3 (3.09)	7.8 – 17.8
	NEYO	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.3 (0.25)	0.0 – 0.3	—	—	—
	PAHM	5.3 (1.45)	6.0 (0.00)	4.0 – 10.0	8.0 (1.53)	5.0 (2.52)	1.3 – 7.3	1.7 (0.88)	4.0 (1.53)	2.0 – 3.5
3	IABG	1.5 (0.65)	0.8 (0.48)	0.5 – 1.8	2.0 (0.91)	2.0 (0.58)	0.3 – 4.3	—	—	—
	NCBD	1.3 (0.48)	1.5 (1.19)	0.5 – 1.0	2.8 (1.80)	1.8 (1.18)	1.3 – 3.8	5.3 (0.85)	3.8 (1.11)	2.0 – 2.5
	NEYO	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	4.8 (0.75)	4.8 (0.85)	1.8 – 3.0	—	—	—
	PAHM	3.3 (1.20)	9.3 (2.73)	4.0 – 9.0	3.3 (1.45)	2.3 (0.88)	1.3 – 5.3	1.3 (0.88)	1.3 (0.67)	0.0 – 2.8
4	IABG	0.8 (0.48)	1.3 (0.75)	1.3 – 1.8	4.0 (1.47)	6.8 (3.61)	2.3 – 6.0	—	—	—
	NCBD	0.3 (0.25)†	0.3 (0.25)	0.0 – 0.8	0.8 (0.75)†	0.3 (0.25)	0.0 – 1.3	1.0 (0.58)†	1.3 (0.63)	0.0 – 1.0
	NEYO	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	1.5 (0.87)	2.5 (0.96)	0.8 – 2.8	—	—	—
	PAHM	5.0 (1.53)	7.3 (0.67)	1.7 – 6.5	2.7 (2.19)	5.0 (2.31)	0.3 – 2.3	1.3 (0.67)†	0.7 (0.67)	0.0 – 0.7
5	IABG	1.3 (0.48)	1.0 (0.58)	0.8 – 1.8	14.0 (7.39)	8.8 (4.85)	2.5 – 4.0	—	—	—
	NCBD	0.8 (0.75)†	0.8 (0.48)	0.3 – 1.0	3.5 (0.96)	4.8 (1.11)	1.8 – 5.0	0.5 (0.29)†	0.5 (0.29)	0.3 – 0.8
	NEYO	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	1.8 (0.85)†	1.8 (1.11)	0.0 – 0.5	—	—	—
	PAHM	2.0 (1.00)	4.7 (1.45)	1.3 – 3.5	0.3 (0.33)	1.7 (1.20)	1.7 – 2.3	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3

Table I-10. (continued). Individual-Site Analysis: Arthropod Abundance in Visual Counts from MON 87411 Compared to the Conventional Control

		Spider (Araneae)			Stink bug (Pentatomidae)		
		Beneficial Arthropod			Pest Arthropod		
		Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference
Coll.[1]	Site[2]	MON 87411	Control	range[4]	MON 87411	Control	range[4]
1	IABG	—	—	—	—	—	—
	NCBD	7.3 (1.60)	4.0 (1.08)	3.0 - 5.8	1.0 (0.00)†	0.3 (0.25)	0.3 – 1.8
	NEYO	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0
	PAHM	1.0 (0.58)	1.7 (0.88)	0.8 – 3.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 5.7
2	IABG	1.8 (0.85)	1.5 (0.96)	0.5 – 2.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3
	NCBD	6.8 (2.69)	4.8 (1.25)	3.5 – 5.0	1.0 (0.71)†	1.5 (0.87)	0.3 – 1.0
	NEYO	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0
	PAHM	1.7 (1.20)	0.3 (0.33)	1.0 – 2.3	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3
3	IABG	0.3 (0.25)†	0.3 (0.25)	0.0 – 0.5	0.0 (0.00)†	0.3 (0.25)	0.0 – 0.5
	NCBD	4.5 (1.32)	5.0 (1.47)	3.3 – 5.8	0.5 (0.29)†	0.3 (0.25)	0.0 – 1.8
	NEYO	0.5 (0.29)†	0.8 (0.48)	0.3 – 1.8	0.0 (0.00)†	0.3 (0.25)	0.0 – 0.5
	PAHM	1.7 (0.33)	1.0 (0.58)	1.5 – 2.7	0.0 (0.00)†	0.0 (0.00)	0.0 – 2.3
4	IABG	0.3 (0.25)†	0.3 (0.25)	0.0 – 0.8	0.5 (0.29)†	0.5 (0.50)	0.3 – 0.8
	NCBD	2.3 (1.44)	3.3 (2.29)	3.0 – 4.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.5
	NEYO	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.3	0.0 (0.00)†	0.3 (0.25)	0.0 – 0.3
	PAHM	2.0 (1.15)	0.3 (0.33)	1.3 – 2.3	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0
5	IABG	0.3 (0.25)†	1.0 (0.71)	0.3 – 0.8	0.5 (0.29)†	0.5 (0.29)	0.3 – 0.5
	NCBD	3.8 (1.18)	2.3 (0.48)	1.5 – 5.3	0.3 (0.25)†	0.0 (0.00)	0.0 – 0.3
	NEYO	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0	0.0 (0.00)†	0.0 (0.00)	0.0 – 0.0
	PAHM	1.0 (0.00)	2.7 (1.45)	1.5 - 3.3	0.0 (0.00)†	0.3 (0.33)	0.0 – 0.0

Note: The experimental design was a randomized complete block with four replicates per site.

[*]Indicates a statistically significant difference between MON 87411 and the conventional control ($\alpha = 0.05$) using ANOVA.
[†]Indicates p-values could not be generated where the taxa did not meet inclusion criteria (see appendix I.10.2).
[1]Arthropods were enumerated at five crop development stages: Collection 1 = VT-R1 growth stage; Collection 2 = R1 growth stage; Collection 3 = R2 growth stage; Collection 4 = R3 growth stage; Collection 5 = R4-R5 growth stage.
[2]Site code: IABG = Greene, IA; NCBD = Perquimans, NC; NEYO = York, NE; PAHM = Berks, PA.
[3]MON 87411 and the conventional control values represent means with standard error in parentheses. N = 4.
[4]Reference range is calculated from the minimum and maximum mean values from among reference hybrids at each site.
A dash (—) indicates data not available.

References for Appendix I

SAS. 2008. SAS/STAT software version 9.2. SAS Institute, Inc., Cary, North Carolina.

SAS. 2012. SAS/STAT software version 9.3. SAS Institute, Inc., Cary, North Carolina.

Appendix J: Materials and Methods for Pollen Morphology and Viability Assessment

J.1. Plant Production

MON 87411, the conventional control, and four reference hybrids were grown under similar agronomic conditions in a field trial in Story County, Iowa. The trial was arranged in a randomized complete block design with four replications. Each plot consisted of six rows approximately 6 m in length.

J.2. Flower Collection and Pollen Sample Preparation

Tassel bags were placed on three non-systematically selected plants during pollen shed. The following morning, pollen was collected from three plants per plot and transferred to a uniquely labeled tube. Pollen collected from each plant in a plot represented a subsample. Within approximately 30 minutes of collection, Alexander's stain solution (Alexander 1980), in a 1:5 dilution with distilled water, was added to each tube (at least 2:1 (v/v) stain to pollen) to fix and stain the pollen, rendering the pollen non-viable. The tubes were closed and the contents shaken until thoroughly mixed. Subsamples were placed on wet ice within 30 minutes of pollen collection and maintained under those conditions until receipt at the performing laboratory.

J.3. Data Collection

Slides were prepared by aliquoting suspended pollen/stain solution onto a slide. Pollen characteristics were assessed under an Olympus© BX53 light microscope equipped with an Olympus© DP72 digital color camera. The microscope and camera were connected to a computer running Microsoft Windows XP® and installed with an Olympus© cellSens (version 1.4.1) software.

J.3.1. Pollen Viability

When pollen grains were exposed to the staining solution, viable pollen grains stained red to purple due to the presence of living cytoplasmic content. Non-viable pollen grains stained light blue to green or colorless, and the shape appeared round to collapsed depending on the degree of hydration. For each pollen sample, the number of viable and non-viable pollen grains was counted from a random field of view under the microscope. A minimum of 100 pollen grains were counted for each of the three subsamples per plot. Mean pollen viability for each replicate was calculated from the subsamples as shown in Table VIII-8.

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J.3.2. Pollen Diameter

For a single predetermined subsample per plot, pollen grain diameter was measured along two perpendicular axes for 10 representative pollen grains per replication. Mean pollen diameter for each replicate was calculated from the total of 20 diameter measurements as shown in Table VIII-8.

J.3.3. General Pollen Morphology

General pollen morphology of MON 87411, the conventional control, and the reference hybrids was observed as shown in Figure J-1.

J.4. Statistical Analysis

An analysis of variance was conducted according to a randomized complete block design SAS® (SAS 2012). The level of statistical significance was predetermined to be 5% (α=0.05). MON 87411 was compared to the conventional control for percent viable pollen and pollen grain diameter. MON 87411 and the conventional control were not statistically compared to the reference hybrids. Minimum and maximum mean values were calculated for each characteristic from the four reference hybrids. General pollen morphology was qualitative; therefore, no statistical analysis was conducted on these observations.

Table J-1. Starting Seed for Pollen Morphology and Viability Assessment

Material	Phenotype	Monsanto Lot Number
MPA640B[1]	Conventional Control	11333170
MON 87411	Glyphosate -Tolerant and Insect-Protected	11333176
Gateway 6158	Conventional Reference	11273006
Mycogen 2M746	Conventional Reference	11226705
LG2597	Conventional Reference	11226862
Phillips 713	Conventional Reference	11300072

[1]MPA640B = LH244 × LH287.

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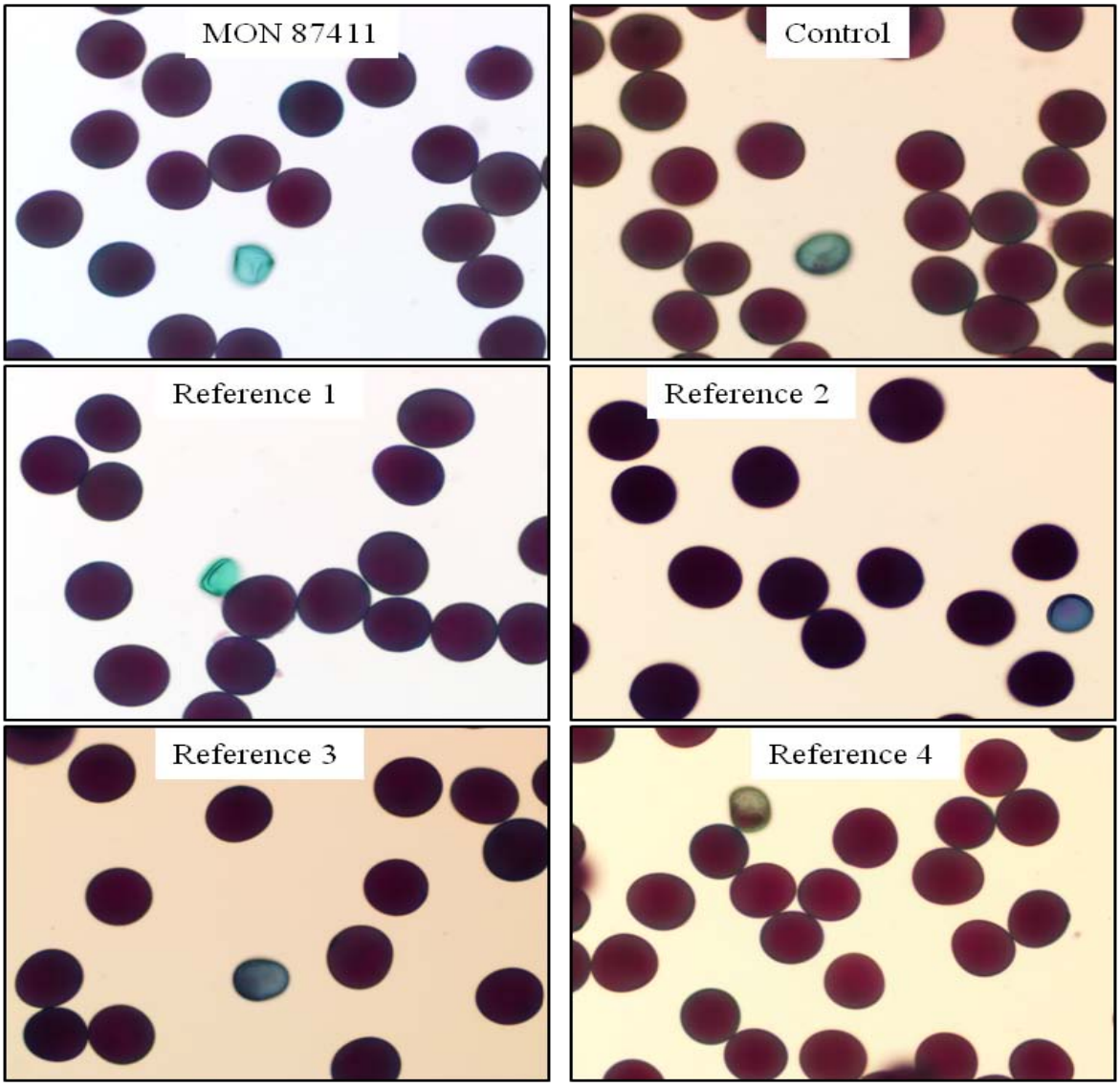

Figure J-1. General Morphology of Pollen from MON 87411, the Conventional Control, and the Reference Hybrids under 200X Magnification

The maize pollen samples were stained with Alexander's stain diluted 1:5 with distilled water. Viable pollen grains stained red to purple, while non-viable pollen grains stained blue to green and the shape appeared round to collapsed depending on the degree of hydration.

References for Appendix J

Alexander, M.P. 1980. A versatile stain for pollen fungi, yeast and bacteria. Stain Technology 55:13-18.

SAS. 2012. SAS/STAT software version 9.3. SAS Institute, Inc., Cary, North Carolina.

Appendix K: Summary of Non-target Organism Studies

This appendix provides a summary of the studies conducted to assess the potential effects of DvSnf7 RNA on non-target organisms. All studies were conducted using *in vitro*-produced DvSnf7 RNA, referred to as DvSnf7_968 RNA. With the exception of the earthworm study, all studies utilizing the *in vitro*-produced test substance included a diet analysis using a sensitive insect (*Diabrotica undecimpunctata howardi;* Southern corn rootworm) to confirm that the DvSnf7_968 RNA was biologically active and had the expected level of biological activity in diet. Additionally, where appropriate based upon the diet matrix, the homogeneity of the test material and stability over the period of storage was also confirmed.

K.1. Evaluation of Potential Effects of DvSnf7_968 RNA to the Earthworm *Eisenia andrei* in an Acute Exposure Study in an Artificial Soil Substrate.

The purpose of this study was to evaluate the potential effects of dietary and contact exposure of DvSnf7_968 RNA on adults of the earthworm *Eisenia andrei* (Oligochaeta: Lumbricidae) over a 14 day exposure period. For this study, a dilution of DvSnf7_968 RNA was prepared in purified water and incorporated into a standard artificial soil medium following the standard methodology described in OECD guideline 207. The test substance was evaluated at a single test concentration and compared with a water-treated assay control. Treatments were mixed into an artificial soil in replicated 1 L glass jars (test arenas/chambers). At initiation of the test, ten adult *E. andrei* were placed on the soil surface of each jar. Each treatment was replicated four times for a total of 40 earthworms exposed per treatment. All jars were maintained in an incubator under a 24L: 0D photoperiod at 20° C. The survival, biomass and behavior of the worms were assessed at 7 and 14 days after commencement of the bioassay. In lieu of a positive control, the sensitivity of the *E. andrei* worm culture used for this study was previously confirmed in a separate testing at the Test Facility, in accordance with the guideline, and conducted within 12 months from the start of the study in-life phase. After 14 days, no effect on survival (100% survival in both treatments), was observed with *E. andrei* treated with DvSnf7_968 RNA at a concentration equivalent to 5000 ng RNA/g soil dry weight. Additionally, the biomass of worms exposed to DvSnf7_968 RNA (-8.4 ± 1.4% change) was not significantly reduced compared to the assay control (-9.4 ± 2.4% change) ($p > 0.05$) and there were no adverse observed effects on worm behavior. Based on the results of assessments for mortality, behavior and change in biomass, the NOEC for DvSnf7_968 RNA was concluded to be 5000 ng RNA/g soil dry weight.

K.2. Evaluation of the Potential Dietary Effects of DvSnf7_968 RNA on Honeybee Larvae (*Apis mellifera* L.).

The purpose of this study was to evaluate the potential dietary effects of DvSnf7_968 RNA on the survival and development of honey bee (*Apis mellifera* L.) larvae. Larvae of *A. mellifera*, 2 to 3 days old, were exposed to DvSnf7_968 RNA in a single dose

administered to the brood cell. A single dose 1000 ng/g solution, in a 10 µl aliquot of 30% (w/v) sucrose/purified water, was added to each larval cell at test initiation for a total mass of 11.3 ng DvSnf7/cell[13]. In addition, an assay control treated with 10 µL of 30% (w/v) sucrose/purified water was included as well as a positive control (2000 µg potassium arsenate/ml) in a 30% (w/v) sucrose/purified water solution to confirm that the test honey bee larvae were feeding. For each treatment there were four replicate frames with a target number of 20 larvae assessed per frame. Treatments were administered to the cells and frames were returned to the hives for incubation. Treated brood cells were mapped in each treated frame and identified on acetate overlay maps to indicate the study, hive, replicate, and treatment group numbers. Post-capping and prior to emergence, all treated frames were removed from the hives, placed into screened hive boxes in a growth chamber and maintained under a 0L: 24D photoperiod at approximately 27 to 30ºC and 47.3 to 72.6% relative humidity (RH). The endpoints measured were survival at two different life stages: dosing to cell-capping (larval stage) and cell-capping to test termination (pupal stage). There was 100% survival in both the DvSnf7_968 RNA and assay control treatments. Additionally, emergence was initiated in the test and control on the same day for the DvSnf7_968 RNA treatment and the assay control (day 14), approximately 50% emergence occurred on the same day (day 15) for the DvSnf7_968 RNA treatment and the assay control and 100% emergence was achieved on the same day (day 17) for the DvSnf7_968 RNA treatment and the assay control. Behavioral observations at emergence indicated no adverse behavior or morphological effects. Survival for the positive control treatment was 0%, confirming the validity of the test system. Based on no differences in survival and development between the test and control treatments, the NOEC of the DvSnf7_968 RNA for honeybee larvae was ≥11.3 ng/larvae.

K.3. Evaluation of the Potential Dietary Effects of DvSnf7_968 RNA on Honeybee Adults (*Apis mellifera* L.).

The purpose of this study was to evaluate the potential dietary effects of DvSnf7_968 RNA on the survival of honey bee (*Apis mellifera* L.) adults in 14-day continuous feeding study. Honey bee adults, <1 day post-emergence, were exposed to three treatments that included: 1000 ng/g DvSnf7_968 RNA in a 50% (w/v) sucrose/purified water solution, an assay control with 50% (w/v) sucrose/purified water solution and a positive control (200 µg potassium arsenate/ml) in a 50% (w/v) sucrose/purified water solution to confirm that the test bees are feeding. For each of the three treatments there were four cages (replicates) containing a target number 20 adult bees per replicate. Each cage was provided with approximately 10 ml of appropriate treatment diet solution in a vial inserted on the top of each cage. The vial was replaced every two days with fresh treatment diets over the study duration of 14 days. Prior to test initiation, adult bees were starved for a maximum of two hours. Test bees for each treatment group were observed

[13] A single dose of 10 µl of 1000 ng/g solution was added to each larval cell for a total mass of 11.3 ng DvSnf7/cell. The concentration of 1000 ng/g DvSnf7_968 RNA in the diet solution is calculated based on the density of the 30% sucrose/water (w/v) solution of 1.1270 g/ml.

daily for mortality, abnormal behavior, and appearance. During the test period, adult bees were maintained under a 0L: 24D photoperiod, with the exception of the time during which mortality and behavior assessments were conducted. Environmental conditions were maintained at approximately 26 to 32ºC and 41 to 80% relative humidity (RH). The positive control produced 100% mortality, confirming the validity of the test system. Though the study was designed to be a 14-day exposure, the mortality in the assay control exceeded 20% on day 10 and continued to increase until test termination at day 14. Therefore, day 10 survival data from the DvSnf7_968 RNA treatment and assay control were analyzed. A 10-day exposure is considered to be appropriate exposure duration for a chronic feeding study for adult honey bees (EFSA, 2013). There were no significant differences ($p > 0.05$) in mean survival between the DvSnf7_968 RNA (79%) and the assay control (76%) treatments after 10 days of continuous dietary exposure. Mean survival on day 14 was not analyzed and survival in the DvSnf7_968 RNA and the assay control was both 30%. The NOEC of the DvSnf7_968 RNA for adult honeybees was ≥1000 ng/g.

K.4. Evaluation of Potential Dietary Effects of DvSnf7_968 RNA on the Lady Beetle, *Coleomegilla maculata* (DeGeer) (Coleoptera: Coccinellidae).

The purpose of this study was to evaluate potential dietary effects of the DvSnf7_968 RNA on the survival, development and growth of the lady beetle, *Coleomegilla maculata*. The test substance dissolved in water was incorporated into an agar based artificial *C. maculata* diet at a single concentration of 1000 ng DvSnf7_968 RNA/g of diet. In addition, appropriate assay control (purified water) and a positive control (120 µg potassium arsenate/g diet) were included in the study. Each treatment was replicated three times and with a target of 20 insects per diet treatment. Larvae were exposed for a maximum of 18 days. *C. maculata* larvae originating from the same egg batch were used for each replicate. All diet treatments were maintained in an incubator with a temperature of 27° C, a relative humidity (RH) of 70 % and a 14L: 10D photoperiod. There were no significant differences ($p > 0.05$) for survival between the DvSnf7_968 RNA (92%) and the assay control (90%) treatments. Additionally, there were no significant differences ($p > 0.05$) for the mean percent that developed to adults between the DvSnf7_968 RNA (92%) and the assay control (90%) treatments. There was no significant difference for development days to adult emergence between the larvae fed the test or control diet ($p > 0.05$). For the test and the assay control diet treatment groups the development time to adult emergence was 15 days. There was no significant difference with adult biomass between the test and control diet ($p > 0.05$). Adults that developed from larvae fed test or control diet averaged 10.2 mg for both treatments. Because of significant differences in body mass between males and females in *C. maculata* populations, separate analyses were performed by gender. There was no significant difference between the biomass of females averaging 10.7 mg and 10.9 mg ($p > 0.05$) and males averaging 9.7 mg and 9.5 mg ($p > 0.05$) for test and control treatments respectively. The positive control group had 17% survival and none of the insects in the positive control group developed to the pupa stage, confirming the validity of the test system. The NOEC of the DvSnf7_968 RNA for *C. maculata* was concluded to be ≥1000 ng/g of diet.

K.5. Evaluation of Potential Dietary Effects of DvSnf7_968 RNA on the Carabid ground beetle, *Poecilus chalcites* (Say) (Coleoptera: Carabidae)

The purpose of this study was to evaluate potential dietary toxicity of the DvSnf7_968 RNA against larvae of the carabid beetle, *Poecilus chalcites*. The test substance dissolved in water was incorporated into an agar-based artificial diet at a single concentration of 1000 ng DvSnf7_968 RNA/g of diet. In addition, appropriate an assay controls (purified water) and a positive control (220 µg potassium arsenate/g diet) were included in the study. Each treatment was replicated three times and each replicate was initiated with a target of 20 insects per diet treatment. Larvae were exposed for a maximum of 35 days. All dietary exposures were initiated with first instar larvae and larvae were individually housed throughout the study. All diet treatments were maintained in an incubator with a temperature of 27° C, a RH of 70 % and a 14L: 10D photoperiod. The endpoints measured were survival, development to the adult stage, and development time (days) to adult emergence, and adult biomass. There was no significant difference for survival between the DvSnf7_968 RNA (93%) and the assay control (92%) treatments and all of the sublethal endpoints ($p > 0.05$). For the test or control diets, the percentage that developed to the adult stage averaged 70% and 75%, respectively, adult biomass averaged 31.9 mg and 32.3 mg, respectively, and the development time to adult emergence averaged 33 days for both treatments. The positive control group had 65%, survival and none of the insects in the positive control group developed to the pupal or adult stage, confirming the validity of the test system. The NOEC of the DvSnf7_968 RNA for *P. chalcites* was ≥1000 ng/g of diet.

K.6. Evaluation of Potential Dietary Effects of DvSnf7_968 RNA on the Insidious Flower Bug, O*rius insidiosus* (Say) (Heteroptera: Anthocoridae)

The purpose of this study was to evaluate potential dietary effects of DvSnf7_968 RNA on the survival and development of *O. insidiosus* nymphs over 10 days of continuous exposure following the methodology described in Tan et al., (2011).Three treatment diets were prepared for separate feeding exposures to *O. insidiosus* nymphs, including an assay control diet, a DvSnf7_968 RNA treatment diet at 1000 ng /g diet, and a positive control diet (100 µg potassium arsenate/g diet). All dietary exposures were initiated with 5-day old nymphs and each treatment consisted of a total of 40 nymphs. All test nymphs were individually housed in test arenas and supplied with appropriate treatment diet in two domes. The dome diets were replaced every two days. All test nymphs were allowed to feed *ad libitum* on the treated diet for 10 days. All diet treatments were maintained in an incubator with a temperature of 25 ± 5° C, a RH of 70 ± 10 % and a 16L: 8D photoperiod. The test nymphs were observed every day to record mortality and development. Survival of the nymphs was 93% in both the assay control and DvSnf7_968 RNA treatment diets after 10 days of continuous feeding. Nymph development to the adult stage was not significantly different ($p > 0.05$) at 95% and 98% in the assay control and DvSnf7_968 RNA treatment, respectively. The average development time of the test nymphs was not significantly different ($p > 0.05$) between the control and test groups, with mean development times of 11.11 ± 0.15 days in the assay control treatment and 10.87 ± 0.13 days in DvSnf7_968 RNA treatment. In contrast, the *O. insidiosus* nymphs fed the positive control treatment diet had 100% mortality by day 10, indicating that the

test system was effective in detecting toxic effects through the dietary exposure. The NOEC of the DvSnf7 RNA for the insidious flower bug, *O. insidiosus,* was ≥1000 ng/g diet.

K.7. Evaluation of Potential Dietary Effects of DvSnf7_968 RNA on the Parasitic Wasp, *Pediobius foveolatus* (Hymenoptera: Eulophidae)

The purpose of this study was to evaluate potential dietary effects of DvSnf7_968 RNA on the survival of adult parasitic wasp, *Pediobius foveolatus* Crawford over 20 days of continuous exposure. Three treatment diets were prepared for separate feeding exposures to *P. foveolatus* adults. An assay control diet was 30% honey/water (v/v) solution, the DvSnf7_968 RNA treatment diet prepared at 1000 ng/g in a 30% honey/water (v/v) solution and a positive control (200 µg potassium arsenate/g) in a 30% honey/water (v/v) solution. Exposure of *P. foveolatus* adults to the three treatment diets was replicated four times with 10 adult wasps per replicate for a total of 40 wasps per treatment. All dietary exposures were initiated with newly emerged adults after approximately 24 hours of acclimation. The adult wasps in each replicate were housed together in a single arena and allowed to feed *ad libitum* on the treated diet for 20 days. All diet treatments were maintained in an incubator at a target temperature of 25° C, a target RH of 70 % and a 16L: 8D photoperiod. Mortality was observed every two days at diet replacement and the dead wasps, if any, were removed from the test arenas at the time of observation. There was no mortality of *P. foveolatus* adults fed the DvSnf7_968 RNA treatment diet or the assay control diet after 20 days. In contrast, the *P. foveolatus* adults fed the positive control treatment diet showed 100% mortality at day 12, indicating that the test system was effective in detecting toxic effects through the dietary exposure. The NOEC of the DvSnf7 RNA for the parasitic wasp, *P. foveolatus*, was ≥1000 ng/g diet.

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Appendix L: Next-Generation Sequencing and Junction Sequence Analysis for the Characterization of DNA Inserted into Crop Plants

Background

Safety assessments of biotechnology-derived crops include a detailed molecular characterization of the inserted DNA sequence and its location within the genome (Codex Alimentarius 2009). Typically, molecular characterization has relied on Southern blot analysis to establish locus and copy number along with targeted sequencing of polymerase chain reaction products spanning any inserted DNA to complete the characterization process. With the advent of next-generation sequencing (Shendure and Ji 2008; Zhang et al. 2011), improvements in sequencing technologies have enabled alternative methods for molecular characterizations which do not require Southern blot analysis. Next-Generation Sequencing and Junction Sequence Analysis bioinformatics (NGS/JSA) utilizes sequencing (both next-generation technologies and traditional methods) and bioinformatics to produce characterizations equivalent to those achieved by current Southern blot based methods.

There are multiple advantages to using next-generation sequencing and bioinformatics, most notably the robustness, simplicity and consistency of the method compared with Southern blot studies, which require customized experimental design for every transformation event. The new sequencing-based method overcomes many technical challenges inherent in Southern blot analyses (*e.g.*, false positive hybridization bands resulting from incomplete digestion or star activity (Wei et al. 2008)) and the need for radioactive ^{32}P-labeled probes. This new method provides higher reproducibility, because it is less dependent on complex lab based procedures. The method described here is essentially identical for all transformation events and it robustly establishes molecular characteristics of genetically engineered crops (Kovalic et al. 2012). Additionally, similar techniques are being used to characterize transgene integration sites and insert molecular anatomy in mammalian systems (DuBose et al. 2013; Zhang et al. 2012).

Method Synopsis

Molecular characterization of the inserted DNA and associated native flanking sequences consists of a multistep approach to determine:

1. the number of insertion sites;
2. the presence/absence plasmid backbone;
3. insert copy number at each insertion site;
4. DNA sequence of each inserted DNA;
5. sequence of the native locus at each insertion site.

Additionally, current methods also establish a description of any genetic rearrangements that may have occurred at the insertion site as a consequence of transformation. Generational stability analysis, which demonstrates the stable heritability of inserted DNA sequences over a number of breeding generations, is also routinely conducted.

The first step of the molecular characterization, determination of number of insert sites, is conducted using a combination of next-generation sequencing technologies (NGS) and Junction Sequence Analysis (JSA) bioinformatics (DuBose et al. 2013; Kovalic et al. 2012). A schematic representation of the basis of the characterization, including the NGS/JSA methodology and the directed sequencing, is presented in Figure 1 (Kovalic et al. 2012).

Genomic DNA from the transformation event and the conventional control are used to generate short (~100 bp) randomly distributed sequence fragments (sequencing reads) in sufficient numbers to ensure comprehensive coverage of the genomes (Shendure and Ji 2008) (Figure 1 box 1). Sufficient numbers of sequence fragments are obtained (≥75x effective genome coverage) to comprehensively cover the genomes of the sequenced samples (Ajay et al. 2011; Clarke and Carbon 1976; Wang et al. 2008). Previous studies with a variety of transformation events demonstrate that 75x coverage of the genome is adequate to provide comprehensive coverage and ensure detection of inserted DNA, producing results equivalent to Southern blot analysis (Kovalic et al. 2012). The 75x coverage used in this method is predicted, based on established and accepted methods (Clarke and Carbon 1976; Lander and Waterman 1988), to provide genome coverage that would be expected to not miss a single basepair in complex genomes (Kovalic et al. 2012). Furthermore, even with know biases in next-generation sequencing techniques, including the Illumina sequencing by synthesis method employed here (Minoche et al. 2011), it has previously been established experimentally that given deep next-generation sequencing, it is possible to achieve comprehensive coverage of complex genomes that form the foundation for accurate whole genome studies (Ajay et al. 2011; Wang et al. 2008).

To confirm sufficient sequence coverage in both the transformation event and the control, the 100 bp sequence reads are analyzed to determine the coverage of a known single-copy endogenous gene, this analysis demonstrates coverage at ≥75x median depth in each sample. Furthermore, in order to confirm the method's ability to detect any sequences derived from the transformation plasmid, plasmid DNA is spiked into conventional control DNA at a single copy genome equivalent ratio and 1/10 copy genome equivalent ratio. This analysis demonstrates that any portion of the plasmid may be detected at a single copy per genome level and 1/10 copy genome equivalent level, which is adequate sensitivity to observe any inserted fragment.

Also of note is that although the method presented here provides ≥75x coverage of the genomes under study, accurate assembly of complete genome sequences for the transformation event and conventional control is not technically possible using currently available sequence assembly tools. This is due to the nature of the sequences generated in this study, short reads of a single short insert length (Miller et al. 2010), in addition to limitation on available sequence assembly algorithms (Zhang et al. 2011). The sequences generated with this method represent datasets sufficient for achieving precise molecular characterization of transformed DNA in transformation events where reference to a template sequence (plasmid DNA) is utilized for comparison (Kovalic et al. 2012).

Using bioinformatics tools, the sequence reads that are derived from the plasmid vector are selected for further analysis out of the comprehensive genomic sequence dataset produced from the transformation event. To determine the insert number, the known sequence of the transformation vector plasmid is used as an "e-probe" in the bioinformatics analysis to search for and select the sequences that contain any portion of sequence of the plasmid. The DNA sequencing reads with a match to the query sequence having an e-value of 1×10^{-5} or less and having a match length of at least 30 bases with at least 96.7% sequence identity are collected. The results of a parameter optimization study that systematically evaluated many different potential parameter sets established these selection criteria as providing the best possible combination of sensitivity and specificity.

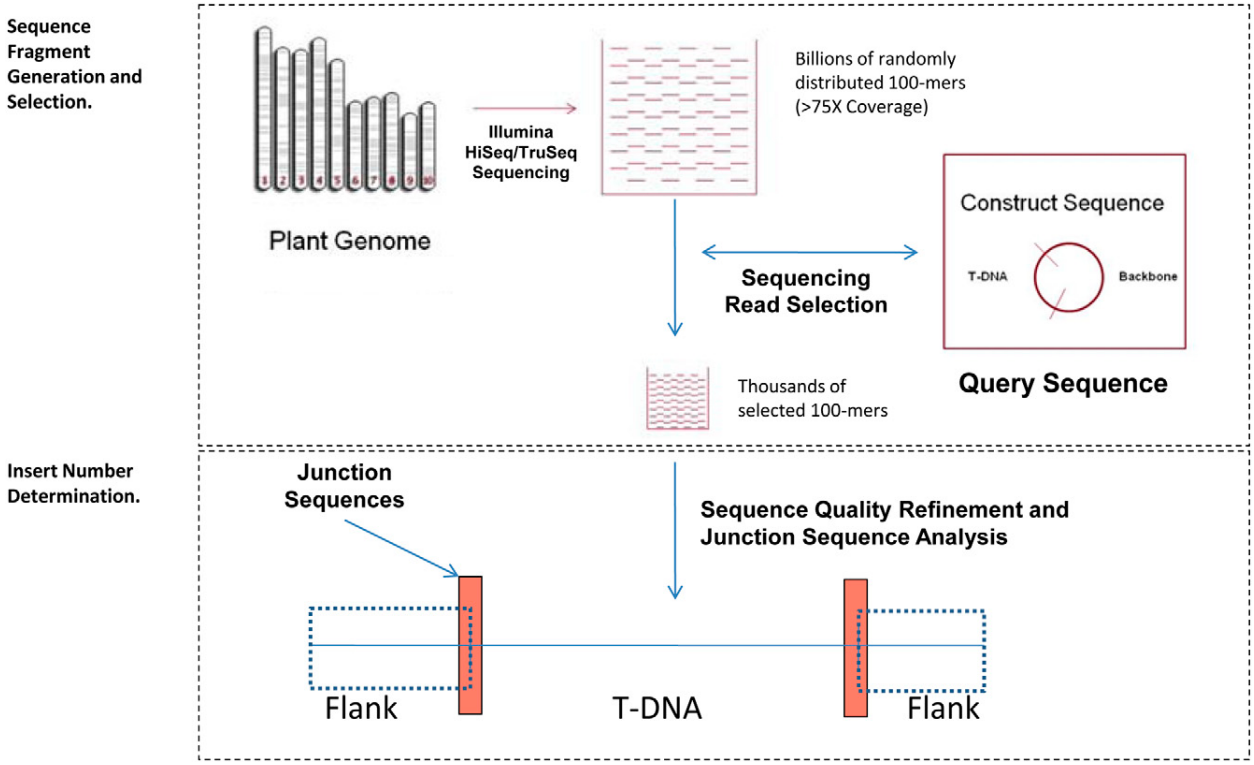

Figure L-1. Sequencing and Sequence Selection

Genomic DNA from the test and control material were sequenced using Illumina HiSeq/TruSeq technology (Illumina, Inc.) that produces large numbers of short sequence reads approximately 100 bp in length. Sufficient numbers of these sequence fragments were obtained to comprehensively cover the genomes of each sample at ≥75x average coverage. Using these genome sequence reads, bioinformatics search tools were used to select all sequence reads that are significantly similar (as defined in the text) to the transformation plasmid. Only the selected sequence reads were used in further bioinformatics analysis to determine the insert number by detecting and characterizing all junction sequences and the presence or absence of the plasmid backbone sequences by

lack of detectable sequences, including the use of suitable controls for experimental comprehensiveness and sensitivity.

The number of DNA inserts is determined by analyzing the selected sequences for novel junctions. The junctions of the DNA insert and flanking DNA are unique for each insertion and an example is shown in Figure 2 below (Kovalic et al. 2012). Therefore, insertion sites can be recognized by analyzing for sequence reads containing such junctions. Each insertion will produce two unique junction sequence classes characteristic of the genomic locus, with one at the 5' end of the insert, in this case named Junction Sequence Class A (JSC-A), and similarly one at the 3' end of the insert, JSC-B (as illustrated in Figure 3 from (Kovalic et al. 2012)). By evaluating the number and the sequences of all unique junction classes detected, the number of insertion sites of the plasmid sequence can be determined. For a single insert, two junction sequence classes are expected, one each originating from either end of the insert, both containing portions of T-DNA and flanking sequence.

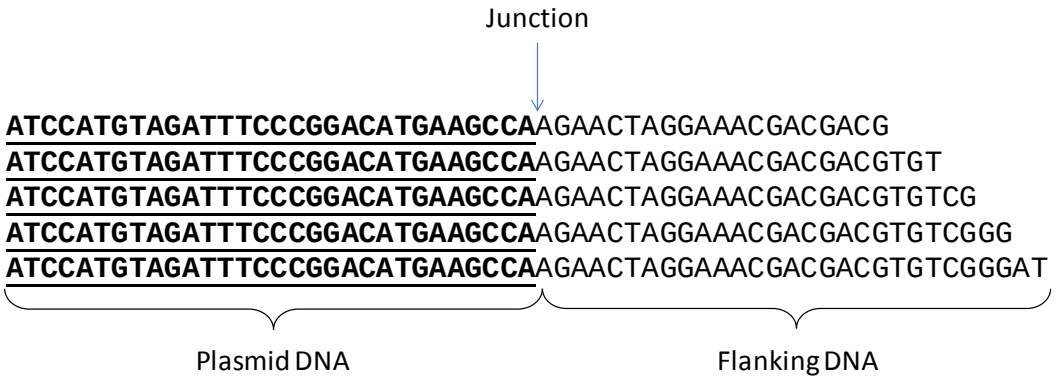

Figure L-2. Junctions and Junction Sequences
Depicted above are five example junction sequences formatted and labeled to indicate the plasmid/flanking DNA portions of the sequences and with the junction point indicated (plasmid DNA is shown in bold, underlined text and flank DNA is shown in plain text). Junctions are detected by examining the NGS data for sequences having portions of plasmid sequences that span less than the full read. Detected junctions are typically characteristic of plasmid insertions in the genome. A group of junction sequences which share the same junction point and common flanking sequence (as shown above) is called a Junction Sequence Class (or JSC).

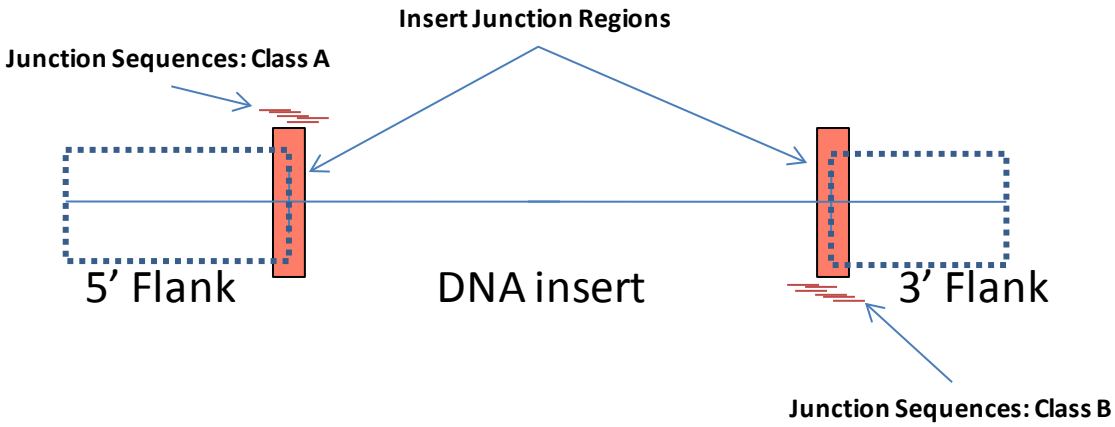

Figure L-3. Two Unique Junction Sequence Classes are Produced by the Insertion of a Single Plasmid Region
A schematic representation of a single DNA insertion within the genome showing the inserted DNA, the 5' and 3' flanks (depicted as areas bounded by dotted lines), and the two distinct regions spanning the junctions between inserted DNA and flanking DNA (shaded boxes). The group of ~100-mer sequences in which each read contains sequences from both the DNA insert and the adjacent flanking DNA at a given junction is called a Junction Sequence Class. In this example, two distinct junction sequence classes (in this case: Class A at the 5’ end and Class B at the 3’ end) are represented.

The next step in the molecular characterization is determination of the insert copy number, integrity of the insert, lack of backbone or other unintended plasmid sequences, and flanking sequence of the native locus at the insertion site. This analysis is conducted using directed sequencing, locus-specific PCR and DNA sequencing analyses, which complements the NGS/JSA analyses, and is common to both the Southern-based and the NGS/JSA characterization methods. Directed sequencing (locus-specific PCR and DNA sequencing analyses) of the transformation event determines the complete sequence of the insert and flanks. This determines if the sequence of the insert is identical to the corresponding sequence in plasmid vector, if each genetic element in the insert is intact, if the plasmid vector sequence is inserted as a single copy, and establishes no vector backbone or other unintended plasmid sequences were inserted in the event. This comparison allows a determination of whether the T-DNA elements are present in the intended order. Furthermore, the genomic organization at the insertion site is assessed by comparing the insert and flanking sequence to the sequence of the insertion site in conventional control genome.

Finally, the stability of the T-DNA across multiple generations is evaluated by NGS/JSA analyses. Genomic DNA from multiple generations of the transformation event is assayed for the number and sequences of all unique junction classes, as described above. This information is used to determine the number and identity of insertion sites. For a single insert, two junction sequence classes are expected, both containing portions of T-DNA and flanking sequence (Figure 2), with one each originating from either end of the insert (Figure 3). In the case of an event where a single locus is stably inherited over multiple generations, two identical junction sequence classes are expected in all the generations tested.

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