Petition for Determination of Nonregulated Status
for Insect-Resistant MIR162 Maize

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MIR162-USDA-1

Petition for Determination of Nonregulated Status

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

Certification

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

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Release of Information

Syngenta Biotechnology, Incorporated (Syngenta) is submitting the information in this petition for review by the United States Department of Agriculture (USDA) as part of a federal regulatory process. By submitting this information to USDA, Syngenta does not authorize its release to any third party. In responding to a request made under the Freedom of Information Act (FOIA), 5 U.S.C. §552 and 7 CFR Part 1, covering all or some of the information in this petition, Syngenta expects that, in advance of the release of the information, USDA will provide Syngenta with a copy of the FOIA request and the material proposed to be released, and the opportunity to object to the release of information based on appropriate legal grounds (*e.g.* responsiveness, trade secret, and/or commercial concerns). Syngenta understands that a copy of this petition may be made available to the public as part of the public comment process. Except in accordance with the foregoing, Syngenta does not authorize the release, publication, or other distribution of this information (including internet posting) without prior notice and Syngenta consent.

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Summary of the Petition

Using the techniques of modern molecular biology, Syngenta has transformed maize (*Zea mays* L.), to produce event MIR162 maize (hereafter 'MIR162 maize'), a new cultivar that is resistant to lepidopteran insect feeding. MIR162 maize plants contain the *vip3Aa20* gene encoding the Vip3Aa20 protein and the *manA* gene encoding the enzyme phosphomannose isomerase (PMI). The Vip3Aa20 protein is a variant of the native insecticidal Vip3Aa protein from *Bacillus thuringiensis* strain AB88. The Vip3Aa20 protein is insecticidally active against a number of significant lepidopteran pests of maize. The *manA* gene was obtained from *Escherichia coli* strain K-12 and the protein it encodes was utilized as a plant selectable marker during development of MIR162.

MIR162 maize was produced by *Agrobacterium tumefaciens*-mediated transformation of immature maize embryos using plasmid vector pNOV1300. The region between the left and right borders of the transformation plasmid included *vip3Aa* and *manA* gene expression cassettes; this T-DNA was transferred into the maize genome during transformation. The *vip3Aa* expression cassette consisted of the *vip3Aa* coding region regulated by the *Z. mays* polyubiquitin promoter (ZmUbiInt) and cauliflower mosaic virus 35S 3′ polyadenylation sequences. The *manA* expression cassette consisted of the *manA* coding region regulated by the ZmUbiInt promoter and the nopaline synthase (NOS) polyadenylation sequence.

Southern blot analyses and nucleotide sequencing demonstrated that MIR162 maize contains a single intact T-DNA insert in the nuclear maize genome. Southern blot analyses further demonstrated that the T-DNA insert contains: i) single copies of a *vip3Aa* gene and a *manA* gene; ii) two copies of the ZmUbiInt promoter; iii) one copy of the NOS terminator; and iv) no backbone sequences from transformation plasmid pNOV1300. Nucleotide sequencing of the T-DNA insert in MIR162 maize revealed two codon changes within the *vip3Aa* coding sequence relative to the intended *vip3Aa* sequence; one of these was a silent mutation and the other codon change resulted in an amino acid substitution. The *vip3Aa* gene variant present in MIR162 maize has been designated *vip3Aa20.* Nucleotide sequencing additionally determined that the MIR162 maize T-DNA insert did not locate within any known *Z. mays* gene. Further, no novel open reading frames were created that spanned either the 5′ or 3′ junctions between the T-DNA and *Z. mays* genomic sequences. These genetic characterization data demonstrate that, apart from the well-characterized change that resulted in a single altered amino acid in the *vip3Aa* coding sequence, there are no unintended changes in the MIR162 maize genome as a result of the T-DNA insertion. Observations of *vip3Aa20* and *manA* segregation ratios over several generations of MIR162 maize are consistent with the genes being linked at a single locus in the maize genome, and indicate stable inheritance of the transgenes. These data also indicate that no novel proteins, other than Vip3Aa20 and PMI, will be produced in MIR162 maize.

Based on a well-characterized mode of action, physiochemical properties, and results of safety studies, it has been demonstrated that the Vip3Aa20 and PMI proteins present in

MIR162 maize pose no risk of harm for mammalian species.

Laboratory, greenhouse, growth chamber, and field investigations with MIR162 maize confirmed that there were no changes in seed, pollen, plant phenotypic, or composition parameters suggestive of increased plant pest risk.

No adverse effects were associated with exposures to Vip3Aa proteins in a range of nontarget indicator species appropriate for a maize ecosystem and the highest doses tested represented no adverse effect levels. With one exception, the levels tested were in excess of expected environmental exposure levels, indicating a low probability of harm for nontarget organisms inhabiting maize ecosystems. In one study it was not possible to exceed a theoretical expected environmental concentration; however, no adverse effects were observed on this study.

The narrow spectrum of insecticidal activity observed for Vip3Aa proteins indicates with high certainty that no endangered or threatened species other than Lepidoptera would be harmed by contact with Vip3Aa20 *via* MIR162 maize. Furthermore, there is minimal exposure of endangered Lepidoptera to maize and therefore, cultivation of MIR162 maize is unlikely to harm any endangered or threatened species in the U.S.

Syngenta knows of no study results or observations associated with MIR162 maize that are anticipated to result in adverse consequences to the quality of the human environment, directly, indirectly, or cumulatively. This includes a lack of anticipated adverse effects on endangered species, unique geographic areas, critical habitats, public health and safety (including children and minorities), genetic diversity of maize, farmer or consumer choice, insect resistance, or the economy, either within or outside the U.S. MIR162 maize offers growers high efficacy, convenience, and an additional choice for protection of maize crops from feeding damage caused by lepidopteran pests. As such, MIR162 maize is expected to produce beneficial effects similar to previously deregulated *Bt* maize products that are commercially available. These benefits include increased competition in the marketplace for insect-protected seed products. Moreover, the novel mode of action of the Vip3A20 protein is expected to extend the useful life of *Bt* maize technology for lepidopteran insect control in general by reducing the selection pressure for resistance among target pests.

The Animal and Plant Health Inspection Service of the United States Department of Agriculture has responsibility, under the Plant Protection Act (Title IV Public Law 106-224, 114 Statute 438, 7 U.S.C. §7701-7772) to prevent the introduction and dissemination of plant pests into the United States. The APHIS regulation 7 CFR Part 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. Syngenta is hereby submitting this petition for a determination of nonregulated status for MIR162 maize and provides all necessary data upon which to make this determination.

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I. Rationale for Submission of Request for Deregulation

The introduction of crops improved through modern biotechnology has been the single most important technological innovation brought to United States (U.S.) agriculture in the past 25 years. Adoption of genetically engineered crops with insect and herbicide tolerance traits has increased dramatically since their commercial introduction in 1995. Net economic benefits at the farm level have been substantial (Brookes and Barfoot, 2005). Improved insect and weed control have led to increased crop yields and reductions in conventional insecticide applications. The continued development and introduction of these traits is expected to benefit both growers and consumers. Transformation event MIR162 maize has been developed by Syngenta to provide growers with maize varieties that are resistant to feeding damage caused by a number of significant lepidopteran insect pests. This trait will be offered to growers in combination with other deregulated maize traits (*e.g.* herbicide tolerance).

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

The Animal and Plant Health Inspection Service (APHIS) of USDA has responsibility under the Plant Protection Act (Title IV Public Law 106-224, 114 Statute 438, 7 U.S.C. 7701-7772) to prohibit or restrict the importation, exportation, and interstate movement of plants, plant products, certain biological control organisms, noxious weeds, and plant pests. APHIS regulations under 7 CFR §340.1 stipulate that any organism which has been altered or produced through genetic engineering is considered a regulated article if the donor organism, recipient organism, or vector or vector agent belongs to any genera or taxa designated under §340.2 and meets the definition of a plant pest, or is an unclassified organism and/or an organism whose classification is unknown, or any product which contains such an organism, or any other organism or product altered or produced through genetic engineering which the Administrator determines is a plant pest or has reason to believe is a plant pest. The transformation system used to produce MIR162 maize was derived from the genus *Agrobacterium*, an organism listed under §340.2. APHIS regulations further state that any person may submit a petition seeking a determination that an article should not be regulated under this regulation. Syngenta herein presents data and justification for an APHIS determination of nonregulated status for MIR162 maize based on an absence of plant pest risk.

I.B. Rationale for Development of MIR162 Maize

Maize is susceptible to attack by a variety of insects from the time it is planted until it is consumed as food or feed. Insect pests can be categorized as major and consistent pests, major and sporadic pests, and moderate to minor pests based on annual destructiveness and their geographic distribution. Table 1 categorizes most of the insect pests of maize found in the U.S. The most economically significant insect pests of maize are: *Ostrinia nubilalis* (European corn borer), *Diatraea saccharalis* (sugarcane borer), *Diatraea grandiosella* (southwestern corn borer), *Diabrotica spp.* (corn rootworm complex), *Helicoverpa zea* (corn earworm/cotton bollworm), *Spodoptera frugiperda* (fall armyworm), *Agrotis ipsilon* (black cutworm), *Elasmopalpus lignosellus* (lesser cornstalk borer), *Rhopalosiphum maidis* (corn leaf aphids), and *Striacosta albicosta* (western bean cutworm). Pests of secondary economic

importance in maize include both soil-dwelling insects that feed on roots (*e.g.,* wireworms, billbugs, webworms, white grubs, corn root aphids, the seed corn maggot, grape colaspis and seedcorn beetles) and above-ground insects that attack the stalk, leaf, and ear (*e.g.,* cutworms, chinch bugs, grasshoppers, corn flea beetles and Japanese beetles). *S. albicosta* is a pest of increasing economic importance because of its movement into Iowa, Illinois, and Indiana.

Table 1. Categorization of maize insect pests.
Categorized based on their potential for causing economic losses (modified from Gray and Luckmann, 1994).

Major & Consistent Insect Pests	**Moderate to Minor Insect Pests (cont.)**
Ostrinia nubilalis (European corn borer)	*Blissus leucopterus* (cinch bug)
Helicoverpa zea (corn earworm)	*Diabrotica undecimpunctata* (southern corn rootworm)
Spodoptera frugiperda (fall armyworm)	Other cutworms, many species
Diabrotica virgifera virgifera (western corn rootworm)	*Stenolophus lecontei* (seedcorn beetle)
Diabrotica barberi (northern corn rootworm)	*Delia platura* (seedcorn maggot)
Major – Sporadic Insect Pests	*Oligonychus pratensis* (banks grass mite)
Agrotis ipsilon (black cutworm)	*Tetranychus urticae* (two-spotted spider mite)
Rhopalosiphum maidis (corn leaf aphid)	Billbugs, many species
Diatraea grandiosella (southwest corn borer)	White grubs, many species
Diatraea saccharalis (sugarcane borer)	*Papaipema nebris* (stalk borer)
Elasmopalpus lignosellus (lesser cornstalk borer)	*Scutigerella immaculate* (garden symphylan)
Striacosta albicosta (western bean cutworm)	*Popillia japonica* (Japanese beetle)
	Sod webworms, several species
Moderate to Minor Insect Pests	*Colaspis brunnea* (grape colaspis)
Wireworms, many species	Thrips, several species
Pseudaletia unipunctata (armyworm)	*Carpophilus lugubris* (dusky sap beetle)
Graphognathus spp. (white-fringed beetles)	Stink bugs, several species
Grasshoppers, many species	*Diatraea crambidoides* (southern cornstalk borer)
Chaetocnema pulicaria (corn flea beetle)	*Anuraphis maidiradicis* (corn root aphid)

In addition to direct damage caused by feeding on plant tissue, insects play an important role in the transmission and dissemination of pathogenic organisms during maize development. Soil abounds in microorganisms, particularly fungi, which may infect plant parts injured by soil-dwelling insects. In much of the Corn Belt, pathogenic fungi probably pose more problems in maize production than any other group of microorganisms. Primary roots of the seedling and the radical and seminal roots are commonly infected with *Fusarium* spp. after the roots have served their function and become senescent. Feeding by *Diabrotica* rootworms has been associated with increased frequencies of *Fusarium* infection (Dicke and Guthrie, 1988); rootworm feeding may also lead to increased incidences of stalk rots. These pathogenic infections can lead to reduced crop quality, harvestability, and yield.

Ear, kernel, and cob rots occur wherever maize is grown and can result in reduced test weight, poor grain quality, and mycotoxin contamination of food and feed. *Fusarium* kernel or ear rot is the most widespread disease of maize ears and is frequently associated with insect feeding damage.

Crop losses attributable to *O. nubilalis* and *Diabrotica* infestations have been well characterized and are significant. The introductions of transgenic cultivars which encode proteins that are toxic to these species have provided U.S. maize growers with a powerful tool for effectively protecting crop yields. There is not as much quantitative information available on the economic impacts of other major insect pests of maize, specifically *H. zea*, *S. frugiperda*, *A. ipsilon*, and *S. albicosta*. These pests are not as widespread as corn borers and rootworms; however, crop infestations by these leaf and ear-feeding pests can be very costly to growers, as they have the potential to significantly lower grain yield and quality. Conventional insecticide applications are an option for reducing feeding damage caused by these insects; however, most growers do not treat their crops to control these pests because of cost and limited effectiveness of the chemical agents. Currently available transgenic varieties are not as efficacious against these lepidopteran insects as they are against *O. nubilalis*. For example, Bt11 maize containing the Cry1Ab toxin provides only limited or no protection against feeding damage caused by *H. zea*, *S. frugiperda*, *A. ipsilon*, and *S. albicosta*.

Transformation event MIR162 maize has been developed by Syngenta to provide U.S. growers with maize hybrids that are resistant to feeding damage caused by a number of lepidopteran insect pests. MIR162 maize contains a Vip3Aa protein from *Bacillus thuringiensis* that is highly toxic to *H. zea*, *S. frugiperda*, *A. ipsilon*, and *S. albicosta* larvae. In combination with an *O. nubilalis*-protected maize trait, the Vip3Aa protein in MIR162 can provide growers the means of protecting their maize crops from damage caused by a broad range of lepidopteran pests. Commercialization of this new trait has the potential to reduce conventional insecticide use in maize, increase grower profits, and improve grain quality.

I.C. Status with Other Regulatory Agencies

Syngenta is actively pursuing regulatory approvals for MIR162 maize in countries with functioning regulatory systems for genetically modified organisms and that import maize from the U.S. or Canada. Regulatory filings for MIR162 will be made in Mexico, Colombia,

Japan, South Korea, Taiwan, China, the Philippines, Australia and New Zealand, South Africa, the European Union, Russia, and Switzerland.

I.C.1. U.S. Environmental Protection Agency

Substances that are pesticides as defined under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) are subject to regulation by the Environmental Protection Agency (EPA). The Vip3Aa20 protein encoded by the genetic insert in MIR162 maize has insecticidal properties and is, therefore, regulated by EPA. Syngenta has obtained an experimental use permit from EPA that allows for broad-scale field testing of MIR162 maize; this permit was granted on March 26, 2007 and is in effect through March 31, 2008. On April 4, 2007 EPA established a temporary exemption from the requirement of a tolerance for Vip3Aa20 residues in maize commodities, pursuant to §408(d) of the Federal Food, Drug, and Cosmetic Act, 21 U.S.C. §346a(d). Applications to extend both the experimental use permit and temporary tolerance exemption have been submitted to EPA.

On May 17, 2007 Syngenta submitted to EPA an application for registration of the plant-incorporated protectant encoded by MIR162 maize pursuant to FIFRA §3(c)(5) and a petition to establish a permanent exemption from the requirement of a tolerance for Vip3Aa20 residues in food and feed commodities of all crops.

I.C.2. U.S. Food and Drug Administration

MIR162 maize falls within the scope of the Food and Drug Administration's (FDA) policy statement concerning regulation of food products derived from new plant varieties, including those developed by recombinant DNA techniques (FDA, 1992). Syngenta has initiated a voluntary consultation process with FDA and has submitted a safety and nutritional assessment document for MIR162 maize.

I.C.3. Foreign Governments

Syngenta intends to commercialize MIR162 maize in Canada and will seek regulatory approvals to enable this. Syngenta will also seek regulatory approvals to commercialize MIR162 in other countries where armyworms, cutworms, and ear-feeding lepidopteran species are significant economic pests of maize (*e.g.,* Argentina and Brazil). Syngenta will also pursue regulatory approvals for importation of MIR162 maize commodities and processed goods in key export markets for U.S. and Canadian maize growers (*e.g.*, Japan, Taiwan, Korea, Philippines, and Mexico).

II. The Maize Family

II.A. Maize as a Crop

Zea mays L. subspecies *mays*, known as maize throughout the world, and as corn in the U.S., is one of the few major crop species indigenous to the Western Hemisphere. It has been cultivated in the Americas since early historic times. Field maize is the leading production

crop globally, with the 2005/2006 growing season yielding 695 million metric tons of grain (USDA, 2006a). The U.S. accounts for nearly 41% of global maize production. Maize is the largest crop grown in the U.S. in terms of both volume and value. Approximately 78.3 million acres were planted in 2006, yielding 10.5 billion bushels (267 million metric tons) with a gross crop value of $33.7 billion (USDA, 2007a).

Maize is grown for animal feed, human food, vegetable oil, high fructose corn syrups (HFCS), starch, fermentation into ethanol, and a multitude of industrial uses. U.S. maize usage by market segment is shown in Figure 1. Maize as a source of fuel ethanol has increased dramatically over the past two years and is expected to continue doing so as the U.S. focuses on utilizing renewable sources of energy. By 2010, U.S. ethanol production could displace the equivalent of 311,000 barrels of imported crude oil per day.

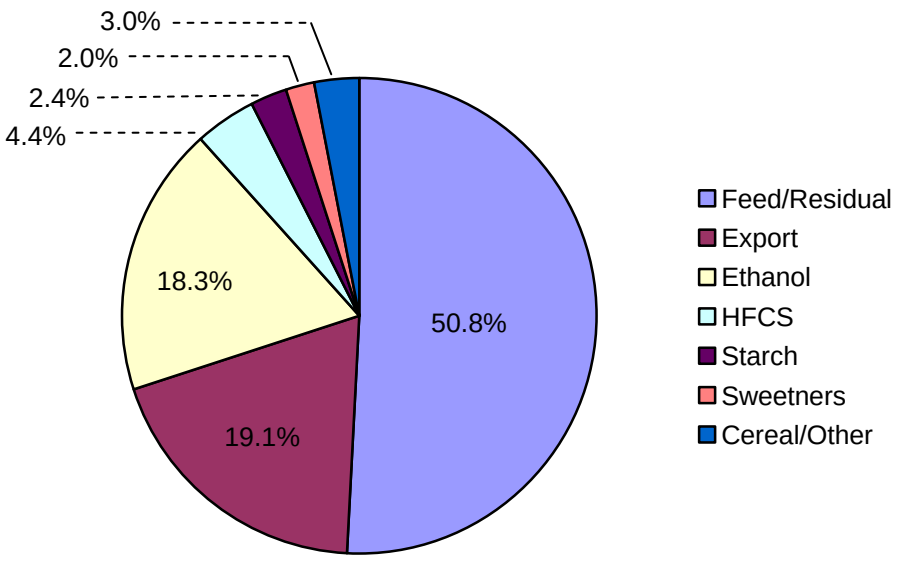

Figure 1. U.S. maize usage by segment in 2006.

The U.S. is by far the world's largest exporter of maize, accounting for 68% of global exports. Total U.S. agricultural exports in 2006 were valued at $71 billion, 10% of which was attributable to maize (Brooks, 2007). Agricultural exports generate employment, income, and purchasing power in both farm and nonfarm sectors of the economy. Production from almost one-third of U.S. cropland moved into export channels in 2005 and generated $166.1 billion in business activity. Technology advances increase agricultural productivity and keep domestic growers competitive in the global market.

II.B. Biology of Maize

Maize is probably the most studied and best characterized of the crops grown in North America. The biology of maize (*Zea mays* L. subspecies *mays*, corn) has been summarized in a consensus document prepared by the Organisation for Economic Co-operation and Development (OECD, 2003). Other authoritative works on the origin of maize (Galinat, 1988; Goodman and Brown, 1988), its genetics (Coe *et al.*, 1988), physiology (Hageman and

Lambert, 1988), and breeding (Hallauer *et al.*, 1988) are also available.

Maize is a large, annual monoecious grass; the duration of its life cycle depends on the cultivar and the environment in which the cultivar is grown. The bulk of maize is grown between latitudes 30˚ and 47˚. Practically no maize is grown where the mean midsummer temperature is < 19˚C or where the average nighttime temperature during the summer months falls much below 13˚C. The greatest production occurs where the warmest month isotherms range between 21˚ and 27˚C and the freeze-free season lasts 120 to 180 days. Maize is grown in areas where annual precipitation ranges from 25 to > 500 cm. Summer rainfall of 15 cm is approximately the lower limit for maize production without irrigation.

The upper Midwest region of the U.S. provides an ideal combination of temperature, rainfall, and soil type for the cultivation of maize. Iowa, Illinois, Nebraska, Minnesota, Indiana, Ohio, Wisconsin, Missouri, Kansas, and South Dakota are major maize growing states. Production in these ten states accounts for 84% of total annual production (USDA, 2007a). Figure 2 displays the geographic distribution of acres planted in 2006 (USDA, 2007b).

Farmers have hundreds of maize varieties from which to choose. Available varieties differ widely in agronomic characteristics, including length of growing period. Technology providers continue to develop varieties with desirable traits and increasing yield. Maize yields have increased an average of 3.5 bu/ac per year over the past decade. The average yield reported for the 2006 growing season was 149.1 bu/ac (NCGA, 2007).

The adoption of new maize varieties improved through biotechnology has added greatly to farm productivity and profits since their introduction in 1996 (Fernandez-Cornejo and Caswell, 2006). Varieties containing herbicide-tolerance and insect-protection traits have been widely adopted by maize, soybean, and cotton growers because they protect the inherent yield potential of these crops or typically reduce grower input costs. In 2007, herbicide tolerant varieties accounted for 91% of soybean acres planted, 70% of cotton acres planted, and 52% of maize acres planted (USDA, 2007c). Insect-protected varieties accounted for 59% of cotton and 49% of maize acres planted. Planting of these varieties has also benefited the environment by displacing conventional pesticide applications and reducing the production of greenhouse gases. The adoption of biotech maize varieties has been estimated to reduce the application of conventional pesticides by more than 20 million pounds annually (NCGA, 2007).

II.C. Weediness of Maize

Modern-day maize cannot survive outside of cultivation (Gould and Shaw, 1968). Volunteer maize is not found growing in fence rows, ditches, or roadsides as a weed. Although maize seed from a previous year's crop can overwinter and germinate the following year, it cannot persist as a weed. The appearance of maize in soybean fields following a maize crop is a common occurrence. Manual or chemical measures are often applied to remove these volunteers, but the plants that are not removed do not persist in following years.

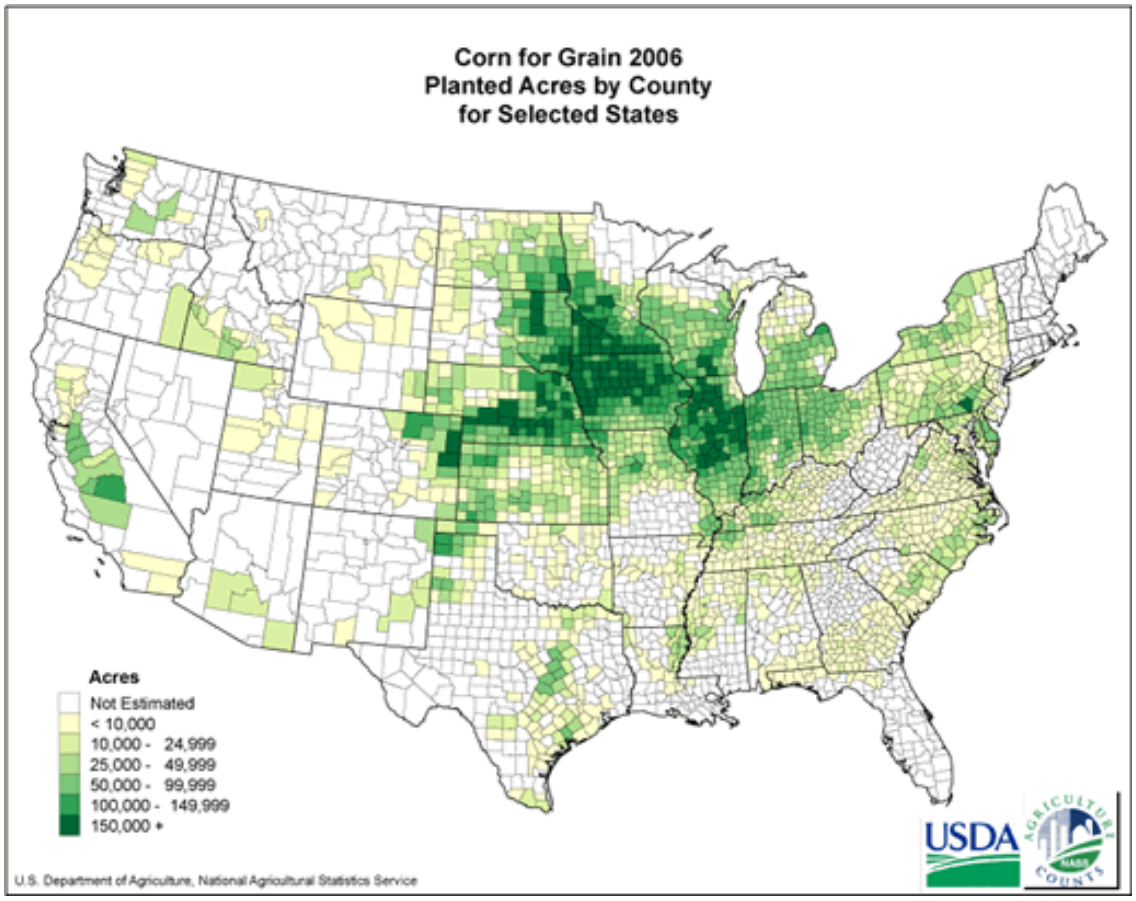

Figure 2. Geographic distribution of acres planted with maize in 2006.

It is difficult for maize to survive as a weed because of past selection in its evolution. In contrast to weedy species, maize has a polystichous female inflorescence on a stiff central spike enclosed with husks. Consequently, seed dispersal of individual seeds does not occur naturally because of the structure of the maize ears. Individual seeds, however, can be dispersed during grain harvest and transportation. In neither instance does maize become a troublesome weed. Maize is not suited to survive without human assistance and is not capable of surviving as a weed.

II.D. Characteristics of the Recipient Germplasm

The recipient germplasm for transformation to produce MIR162 was Hi-IIxA188. This germplasm is well suited for Type II cultures of maize, has a high transformation frequency, and is easily regenerated from callus. Hi-II is publicly available from the Maize Genetics Stock Center.[1] Inbred line Hi-II, created from a cross of line A188 with line B73, was specifically developed for use in embryonic tissue transformation systems (Armstrong *et al.*, 1991). A188 is a publicly available stiff-stalk inbred developed at the University of

[1] USDA/ARS. Maize Genetics Cooperation Stock Center, University of Illinois at Urbana, Champaign, Department of Crop Sciences. http://maizecoop.cropsci.uiuc.edu/

Minnesota. B73 is an elite, publicly available stiff-stalk inbred developed at Iowa State University.

III. Transformation and Development of MIR162 Maize

III.A. Description of the Transformation System

Event MIR162 was produced by *Agrobacterium tumefaciens*-mediated transformation of immature embryos of maize. *Agrobacterium*-mediated transformation is an efficient method for generating transformants containing simple and low copy number insertions with stable inheritance at a high frequency (Grimsley *et al.*, 1987; Ishida *et al.*, 1996). Using this method, DNA within a left border (LB) and right border (RB) of a transformation plasmid, referred to as the transferred DNA (T-DNA), is integrated into the genome of infected cells, while genetic elements outside of the plasmid borders are not.

MIR162 transformation employed a binary vector system (Hoekema, 1983). Plasmid vector pNOV1300 containing the *manA* gene from *E. coli* and maize-optimized coding sequence for *vip3Aa19* between the right and left borders was placed into *A. tumefaciens* strain LBA4404 (licensed from Japan Tobacco, Inc.); see Figure 3.

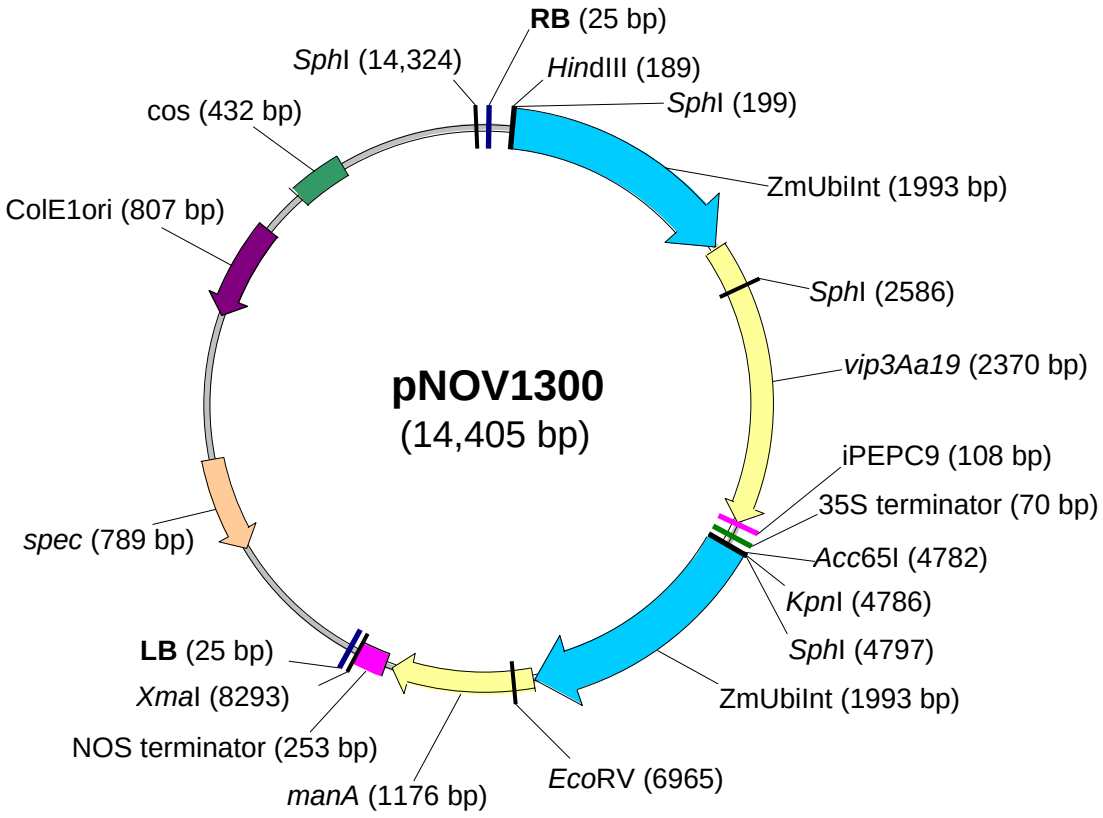

Figure 3. Plasmid map for vector pNOV1300.

Replication of pNOV1300 in *A. tumefaciens* was made possible by homologous recombination with an 'acceptor vector' carrying the broad host range replicon origin RK2, which is also known as pSB1 (Komari *et al.*, 1996). *A. tumefaciens* strain LBA4404 carries a disarmed Ti plasmid from which the native T-DNA has been removed (Ooms *et al.*, 1982). The Ti plasmid carries the *vir* genes which encode proteins that are required for release of the T-DNA from pNOV1300 and its integration into the maize nuclear genome.

Immature embryos were excised from 8- to 12-day old ears of Hi-IIxA188 maize and rinsed with fresh medium in preparation for transformation. Embryos were then mixed with a suspension of *A. tumefaciens* harboring vector pNOV1300. Following a five-minute incubation, excess solution was aspirated and embryos were transferred to plates containing culture medium. Embryos and the *Agrobacterium* were incubated at 22°C for two to three days in the dark. Embryos were transferred to culture medium containing ticarcillin and silver nitrate and incubated for ten days. Embryos producing embryogenic callus were transferred to cell culture medium containing mannose. The *manA* gene encodes the enzyme phosphomannose isomerase (PMI) that served as a selectable marker and enables transformed cells to survive on a mannose substrate (Negrotto *et al.*, 2000).

Transformed tissue was transferred to culture medium containing 500 mg/l of the broad-spectrum antibiotic cefotoxime to clear the *A. tumefaciens*; the transformed tissue was allowed to grow for four months. Regenerated plantlets were tested for the presence of both *vip3Aa19* and *manA*, as well as for the spectinomycin resistance gene (*spec*), by TaqMan polymerase chain reaction (PCR) (Ingham *et al.*, 2001). Plants that tested positive for both *vip3Aa19* and *manA*, but negative for *spec*, were transferred to a greenhouse for growth and propagation.

III.B. Development of MIR162 Maize

Progeny of the original transformants (T_0 plants) were field tested for resistance to insect feeding damage and for agronomic performance after introgression of the transgenes into multiple elite lines of maize. Event MIR162 was selected as the lead commercial candidate and placed into regulatory trials. A schematic showing the steps in development of MIR162 maize is shown in Figure 4. All interstate movements and field plantings of MIR162 were conducted under USDA permit or notification; these are listed in Appendix A.

III.C. Production of Test and Control Materials

A number of factors (*e.g.*, seed availability, trial location, hybrid maturity group) bore on the selection of plant materials for use in field and greenhouse studies with MIR162 maize. With the exception of plant materials utilized in the genetic characterization studies, finished hybrids, produced from converted MIR162 inbreds, were selected for use as test materials in regulatory studies. A converted inbred is a line in which a specific trait(s) has been introgressed and genetically stabilized through backcrossing, followed by self-pollinations. Parental control hybrids (near-isogenic hybrids) were utilized in all studies except the genetic characterization study. Control plants utilized in the study to characterize the T-DNA were derived from null segregants of a hemizygous MIR162 parent. The goal in producing all

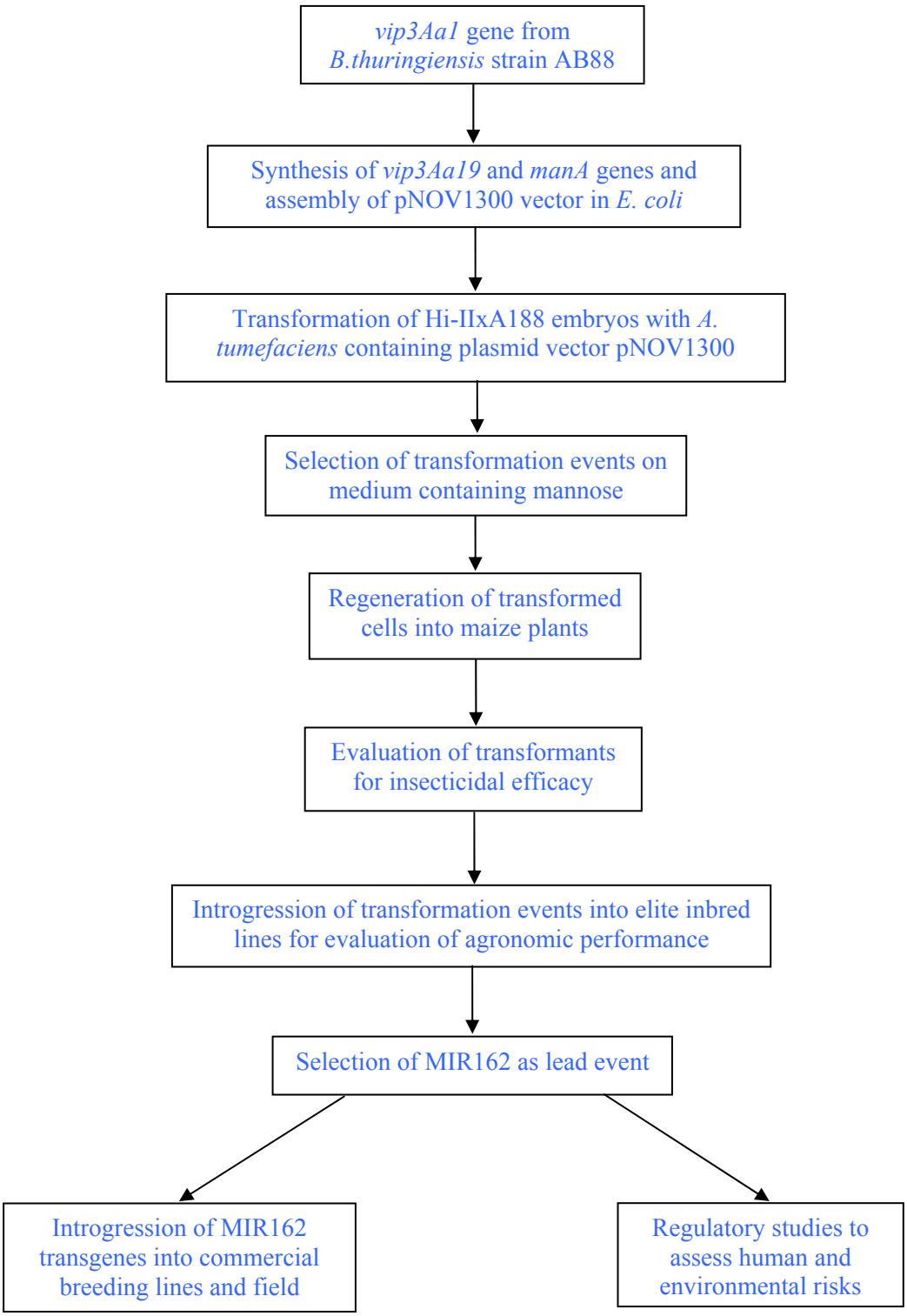

Figure 4. Steps in the development of MIR162 maize.

control hybrids was to match their genetic background with that of the MIR162 hybrids so that the effect of the transformation event could be assessed in an unbiased manner. Table 2 provides a listing of the seed lots used for planting of regulatory trials. A pedigree chart showing the origin of MIR162 seed lots is shown in Figure 5.

Table 2. Description of test and control seed materials used in regulatory studies.
The genetic identity is shown for test and control seed materials planted in regulatory trials; generation number in this table is derived from the pedigree chart displayed in Figure 4. The female parent germplasm is stated first in the pedigree for each generation. The parent genotype containing the MIR162 transgenes is accompanied by the generation number in parentheses.

Trial Type	Seed Lot Code	Generation	MIR162 Genotype	Control Genotype
Genetic characterization & Mendelian inheritance	**A**	BC_1F_1	B9620	B9620
Genetic characterization & Mendelian inheritance	**B**	BC_2F_1	B9620	B9620
Genetic characterization & Mendelian inheritance; Characterization of plant transgenic protein	**C**	BC_4F_1	B9620	B9620
Germination / dormancy	**D**	F_1	NP2391 x NP2222(BC_6F_4)	NP2391 x NP2222
Germination / dormancy	**E**	F_1	NP2222(BC_6F_5) x NP2391	NP2391 x NP2222
Pollen viability / morphology	**E**	F_1	NP2222(BC_6F_5) x NP2391	NP2222 x NP2391
2005 agronomic equivalence	**F**	F_1	NP2010 x NP2222(BC_6F_4)	NP2222 x NP2010
2006 agronomic equivalence	**I**	F_1	NP2673 x NP2171(BC_4F_5)	NP2673 x NP2171
Compositional analysis	**G & H**	F_1 & F_2	NP2276(BC_4F_4) x NP2391	NP2276 x NP2391
Tissue protein quantification – Hybrid 1	**G & H**	F_1 & F_2	NP2276(BC_4F_4) x NP2391	NP2276 x NP2391
Tissue protein quantification – Hybrid 2	**J & K**	F_1 & F_2	NP2153(BC_4F_4) x NP2391	NP2153 x NP2391

All test and control seed lots were analyzed by real time PCR methods for the presence of MIR162 and adventitious transgenic events. All MIR162 seed lots were confirmed to contain the desired gene of interest and plant selectable marker based on nucleotide sequence, and the event of interest based on the genome-insert junction nucleotide sequence. The analyses did not detect these components in control seed lots. All test and control seed lots had no detectable sequences that would be indicative of other regulated events under

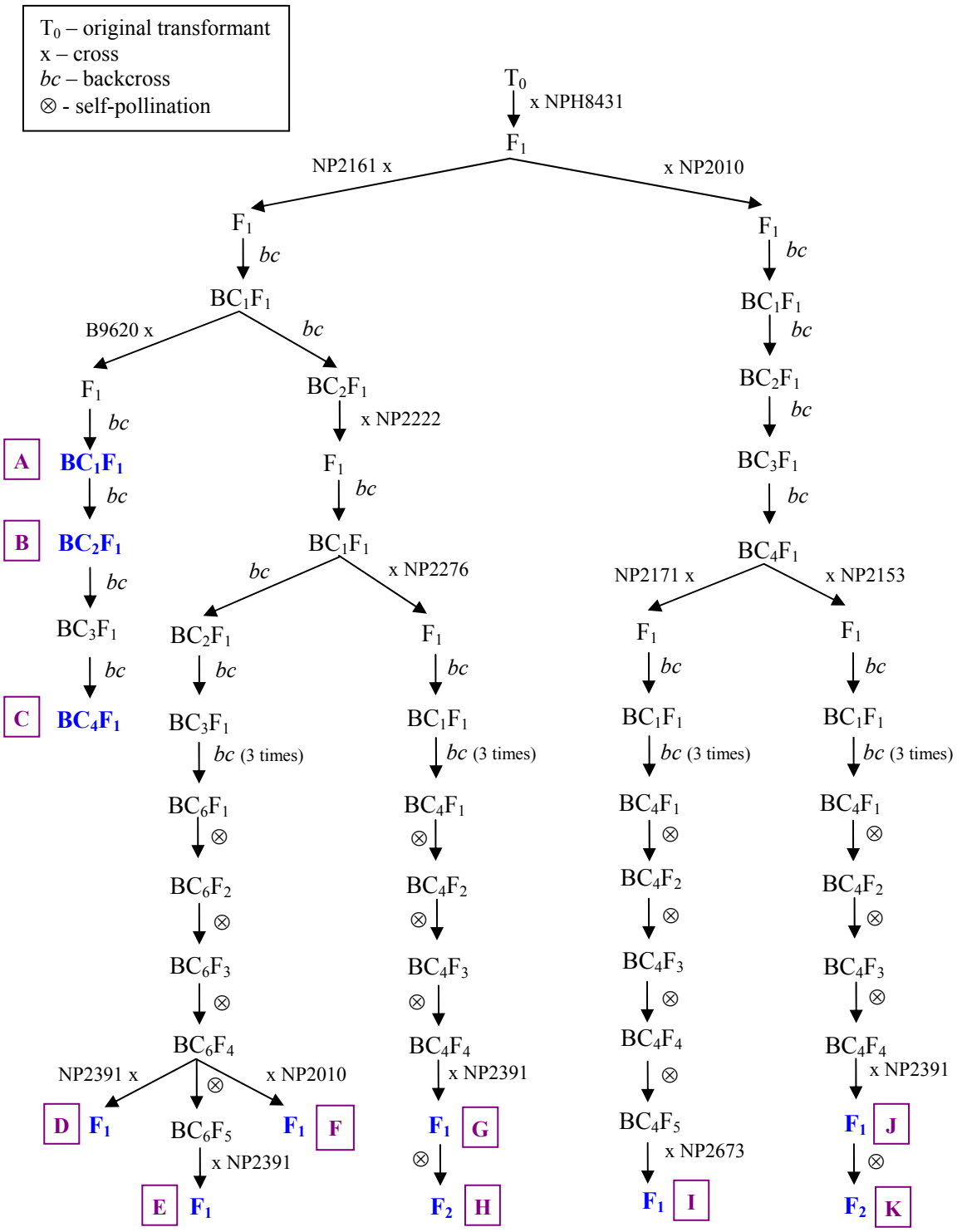

Figure 5. MIR162 pedigree chart.
Generations in blue and bold type were used in regulatory studies; boxed letter code corresponds to specific seed lots used in regulatory studies (see Table 2).

development at Syngenta, or deregulated events for which testing methodology is available.

A number of human and environmental safety studies employed purified Vip3Aa proteins as the test substances. One of three Vip3Aa variants was used: Vip3Aa1, the native protein from *B. thuringiensis* strain AB88; Vip3Aa19, the variant found in transgenic COT102 cotton and Pacha maize; or Vip3Aa20, the variant found in MIR162 maize. Vip3Aa1 was extracted from a culture of *B. thuringiensis* strain AB88. Vip3Aa19 and Vip3Aa20 were produced in recombinant cultures of *Escherichia coli*. The three proteins share >99.7% amino acid sequence homology and have been shown to be biochemically and functionally equivalent.

IV. Donor Genes and Regulatory Sequences

IV.A. Transformation Vector pNOV1300

MIR162 maize was produced by *Agrobacterium tumefaciens*-mediated transformation of immature embryos using the plasmid vector pNOV1300. The T-DNA region between the left and right borders, which included the *vip3Aa19* and *manA* gene expression cassettes, was inserted into the maize genome during transformation. The first expression cassette consists of the *vip3Aa19* coding region regulated by the *Z. mays* polyubiquitin promoter (ZmUbiInt) and 35S 3′ polyadenylation sequences. The second expression cassette consists of the *manA* coding region regulated by the ZmUbiInt promoter and the nopaline synthase (NOS) polyadenylation sequence. A schematic of the vector is shown in Figure 3. The size and location of each genetic element in the vector are shown below in Table 3 below.

Table 3. Description of genetic elements inserted in vector pNOV1300.

Genetic Element	Location in pNOV1300 (bp)	Size (bp)	Source and Function
RB	1 - 25	25	Right border region of T-DNA from *A. tumefaciens* nopaline Ti-plasmid (Entrez Accession Number J01826; NCBI, 2007). This region is a short, direct repeat that flanks the T-DNA and is required for transfer of the T-DNA into plant cells (Wang *et al*., 1984).
ZmUbiInt	200 - 2192	1993	Promoter region from *Z. mays* polyubiquitin gene which contains the first intron (Entrez Accession Number S94464; NCBI, 2007). Provides constitutive expression in monocots (Christensen *et al*., 1992).
vip3Aa19	2214 - 4583	2370	A variant of the native *vip3Aa1* gene (Estruch *et al.*, 1996) from *B. thuringiensis* strain AB88. The *vip3Aa19* gene was codon optimized for expression in maize (Murray *et al.*, 1989). The *vip3A19* gene (Entrez Accession Number DQ539887; NCBI, 2007) encodes a Vip3Aa19 protein that has insecticidal activity against many lepidopteran insect pests.

(Continued)

Table 3 (cont.). Description of genetic elements inserted in vector pNOV1300.

Genetic Element	Location in pNOV1300 (bp)	Size (bp)	Source and Function
iPEPC9	4600 - 4707	108	Intron #9 from the phosphoenolpyruvate carboxylase gene (Entrez Accession Number X15239; NCBI, 2007) from *Z. mays* (Hudspeth and Grula, 1989).
35S	4710 - 4779	70	Terminator sequence from 35S RNA of cauliflower mosaic virus genome (Similar to Entrez Accession Number AF140604; NCBI, 2007). Its function is to provide a polyadenylation sequence (Franck *et al*., 1980).
ZmUbiInt	4798 - 6790	1993	Promoter region from *Z. mays* polyubiquitin gene which contains the first intron (Entrez Accession Number S94464; NCBI, 2007). Provides constitutive expression in monocots (Christensen *et al*., 1992).
manA	6803 - 7978	1176	*manA* gene from *E. coli* strain K-12 encoding the enzyme phosphomannose isomerase (PMI; Entrez Accession Number M15380; NCBI, 2007). PMI catalyzes the interconversion of mannose-6-phosphate to fructose-6-phosphate (Negrotto *et al*., 2000). Used as a selectable marker during transformant selection.
NOS	8039 - 8291	253	Terminator sequence from the nopaline synthase gene of *A. tumefaciens* (Entrez Accession Number V00087; NCBI, 2007). Its function is to provide a polyadenylation site (Depicker *et al*., 1982).
LB	8362 - 8386	25	Left border region of T-DNA from *A. tumefaciens* nopaline Ti-plasmid (Entrez Accession Number J01825; NCBI, 2006). This region is a short, direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell (Zambryski *et al*., 1982).
spec	9562 - 10350	789	Spectinomycin adenylyltransferase *aadA* gene from *E. coli* Tn7 (Entrez Accession Number X03043; NCBI, 2007). Confers resistance to erythromycin, streptomycin, and spectinomycin; used as a bacterial selectable marker (Fling *et al*., 1985).
ColE1ori	11549 - 12355	807	Origin of replication that permits replication of plasmid in *E. coli*. (similar to Entrez Accession Number V00268: NCBI, 2007) (Itoh and Tomizawa, 1978).
cos	12736 - 13167	432	Cohesive end site that is cut to produce the cohesive, single-stranded extensions located at the ends of the linear DNA molecules of certain phages, such as lambda (Sanger *et al*., 1982).

IV.B. Characterization of the T-DNA in MIR162 Maize

A combination of Southern blot, nucleotide sequence, and Mendelian inheritance analyses were performed to characterize the MIR162 maize T-DNA. As described in detail below, data from Southern analyses demonstrated that MIR162 maize contains: i) a single intact insert in the maize genome; ii) single copies of a *vip3Aa20* gene and a *manA* gene; iii) two copies of the ZmUbiInt promoter; iv) one copy of the NOS terminator; and v) no backbone sequences from transformation plasmid pNOV1300.

The MIR162 T-DNA was found to be stable over three breeding generations. Sequence analysis of the entire T-DNA present in MIR162 maize confirmed the intactness of the T-DNA and that the contiguousness of the functional elements had been maintained. Sequence analysis also revealed two single nucleotide changes in the *vip3Aa* coding sequence contained in the MIR162 maize T-DNA, as compared with the sequence present in the transformation plasmid. One of these mutations resulted in a single codon change for the amino acid originally encoded, while the other mutation was silent. The new gene incorporated into the MIR162 maize genome has been designated *vip3Aa20*. See Section V.A for a discussion of the Vip3Aa variants. Nucleotide sequence analysis also confirmed the presence of a single, intact DNA insert in MIR162 maize. A schematic representation of the T-DNA in MIR162 maize is shown in Figure 6.

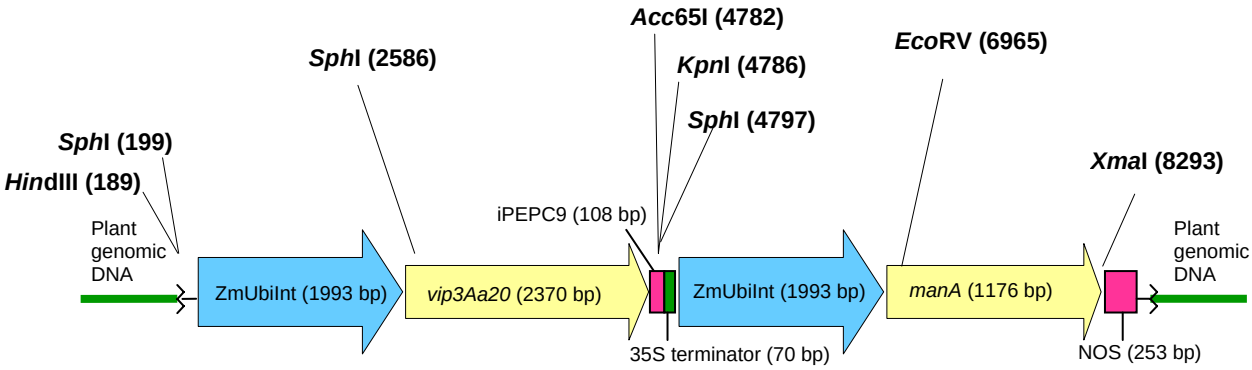

Figure 6. Schematic representation of the MIR162 T-DNA.
This schematic shows the linear orientation of the genetic elements and restriction endonuclease sites within the T-DNA.

Statistical analysis of gene segregation patterns over three generations of MIR162 maize confirmed the expected Mendelian inheritance ratio for both the *vip3Aa20* and *manA* genes; this segregation pattern was consistent with the genes being linked at a single locus. The MIR162 maize DNA insert does not disrupt any known endogenous *Z. mays* genes. There is a region of homology between the maize genomic sequence flanking the 5′ region of the MIR162 maize DNA insert and maize transposable element sequences. There are also regions of homology between the maize genomic sequence flanking the 3′ region of the MIR162 maize T-DNA and identified maize sequences. These regions of homology likely do not have any function. Additionally, no novel open reading frames were identified that spanned either the 5′ or 3′ junction between the MIR162 maize T-DNA and *Z. mays* genomic

sequence. A detailed description of the materials and methods for the molecular characterization of MIR162 maize can be found in Appendix B.

IV.B.1. Sequence of the T-DNA

Consensus nucleotide sequence data demonstrated that the MIR162 T-DNA is intact and that the contiguousness of the functional elements within the insert as intended in pNOV1300 has been maintained. Two nucleotide changes were noted within the coding sequence of the *vip3Aa* gene in MIR162 maize. The first of these nucleotide changes results in a single amino acid change: methionine at position 129 of Vip3Aa19 has been substituted by isoleucine (M129I). The second nucleotide change within the *vip3Aa* coding sequence did not result in an amino acid change. The *vip3Aa* gene in MIR162 maize was given the designation *vip3Aa20* (Entrez Accession Number DQ539888; NCBI, 2007). The coding sequences of *manA*, the ZmUbiInt promoters, the 35S terminator, and the NOS terminator in MIR162 maize were identical to those in the transformation plasmid pNOV1300. Sequence analysis revealed that some truncation occurred at the right border (RB) and left border (LB) ends of the T-DNA during the transformation process. The entire RB, along with two base pairs of noncoding sequence was truncated, and the entire LB, along with 32 base pairs of noncoding sequence, was truncated. These deletions have no apparent effect on the functionality of the DNA insert as this phenomenon has been previously observed in transformations with *A. tumefaciens* (Tinland and Hohn, 1995; Brunaud *et al*., 2002; Chilton and Que, 2003).

IV.B.2. Copy Number of Functional Elements

The hybridization data demonstrated that the T-DNA in MIR162 contains single copies of the *vip3Aa20* and *manA* genes. As expected, the MIR162 maize T-DNA contains two copies of the ZmUbiInt promoter, corresponding to the two copies of the ZmUbiInt promoter present in the transformation plasmid pNOV1300, and contains a single copy of the NOS terminator. The Southern blot analyses demonstrated that MIR162 maize contains no pNOV1300 plasmid backbone sequences.

For each Southern blot analyses, there is a map showing the location of the restriction endonuclease digestion sites in relation to each element probe used in the analysis. These are found in Figures 7, 9, 11, and 13. The Southern blots are shown in Figures 8, 10, 12, and 14, respectively. In addition, comparisons of the expected and observed hybridization bands for each Southern blot are displayed in Tables 4, 5, 6, and 7 as an aid to the reader.

IV.B.2.a. Copy Number of Functional Elements: *vip3Aa20* Gene

A *vip3Aa19*-specific probe was used for the *vip3Aa20* Southern blot analyses. The nucleotide sequences of *vip3Aa19* and *vip3Aa20* are 99.9% identical, differing by only two nucleotides. The two nucleotide changes did not interfere with the ability of the *vip3Aa19* probe to hybridize with the *vip3Aa20* coding sequence in MIR162 maize.

A map of the T-DNA region in vector pNOV1300 indicating locations of the *vip3Aa19*

coding sequence and the restriction endonuclease sites targeted in these Southern blot analyses is shown in Figure 7. Three endonuclease digestion strategies were utilized to confirm the presence, size, intactness, and copy number of the *vip3Aa20* coding sequence.

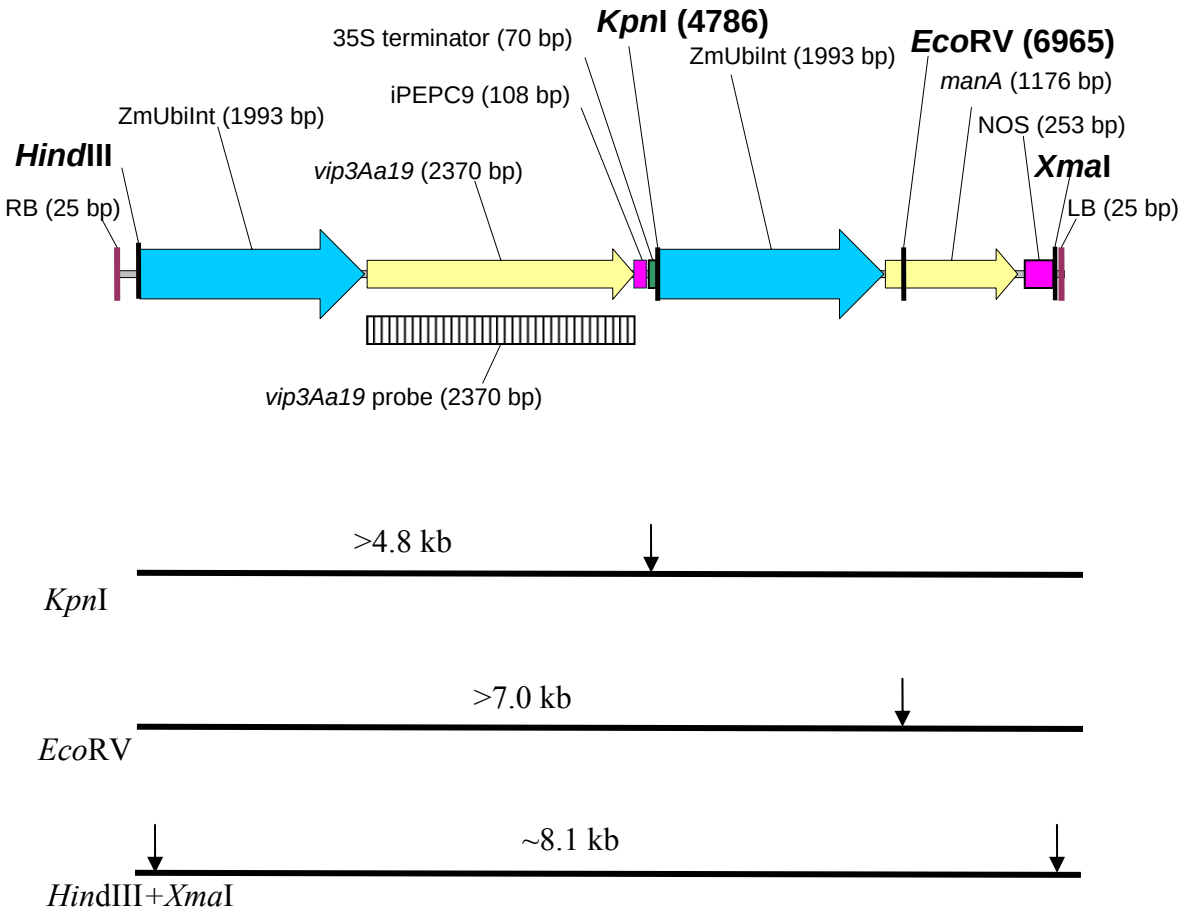

Figure 7. Restriction sites and probe for *vip3Aa20*.
A schematic of the 8.4 kb T-DNA of pNOV1300 is shown along with the location of the *Kpn*I, *Eco*RV, *Hin*dIII, and *Xma*I restriction sites and their location relative to the *vip3Aa20* coding sequence. The vertical arrows indicate the endonuclease cutting sites and the expected size of the resulting restriction fragments that should be detected when probed with *vip3Aa19*.

The results of these analyses are displayed in Figure 8. Table 4 shows a comparison of expected *versus* observed hybridization band sizes.

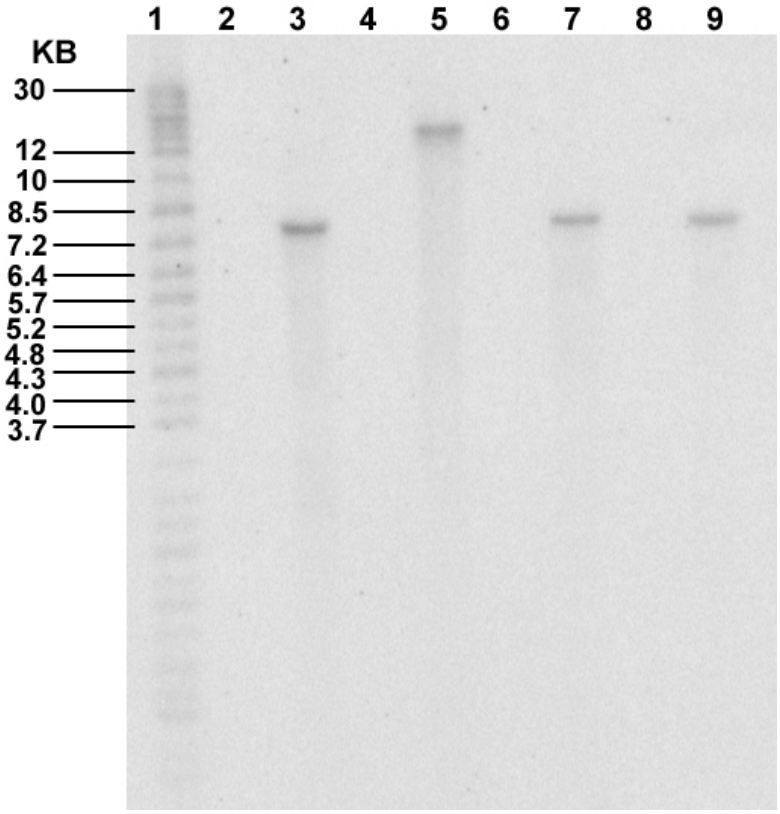

Figure 8. Southern blot analysis of MIR162 maize with a *vip3Aa19*-specific probe**.**
Maize genomic DNA (7.5 µg) was subjected to three separate digestions with *Kpn*I or *Eco*RV or *Hin*dIII plus *Xma*I restriction endonucleases and subjected to Southern blot analysis with a *vip3Aa19*-specific probe (2370 bp).

Lane 1: Molecular weight marker
Lane 2: Blank
Lane 3: BC_4F_1 generation of MIR162 maize digested with *Kpn*I
Lane 4: Control from BC_4F_1 generation maize digested with *Kpn*I
Lane 5: BC_4F_1 generation of MIR162 maize digested with *Eco*RV
Lane 6: Control from BC_4F_1 generation maize digested with *Eco*RV
Lane 7: BC_4F_1 generation of MIR162 maize digested with *Hin*dIII + *Xma*I
Lane 8: Control from BC_4F_1 generation maize digested with *Hin*dIII + *Xma*I
Lane 9: Control from BC_4F_1 generation maize digested with *Hin*dIII + *Xma*I plus 20.2 pg of *Hin*dIII plus *Xma*I digested pNOV1300 plasmid.

Table 4. Expected *versus* observed hybridization band sizes for probes with *vip3Aa19*.

Lane # (Figure 8)	DNA	Restriction Enzyme	Probe	Expected Bands	Expected Band Size (kb)	Observed Band Size (kb)
Lane 3	BC_4F_1	*Kpn*I	*vip3Aa19*	1	>4.8	8.0
Lane 4	Negative	*Kpn*I	*vip3Aa19*	0	-	-
Lane 5	BC_4F_1	*Eco*RV	*vip3Aa19*	1	>7.0	14.0
Lane 6	Negative	*Eco*RV	*vip3Aa19*	0	-	-
Lane 7	BC_4F_1	*Hin*dIII + *Xma*I	*vip3Aa19*	1	8.1	8.1
Lane 8	Negative	*Hin*dIII + *Xma*I	*vip3Aa19*	0	-	-
Positive Control						
Lane 9	pNOV1300	*Hin*dIII + *Xma*I	*vip3Aa19*	1	8.1	8.1

Genomic MIR162 maize DNA digested with *Kpn*I (Figure 8, Lane 3) produced a single hybridization band of approximately 8.0 kb, corresponding to a single copy of *vip3Aa20*. Genomic MIR162 maize DNA digested with *Eco*RV (Lane 5) produced a single hybridization band of approximately 14.0 kb, corresponding to a single copy of *vip3Aa20*. Genomic MIR162 maize DNA digested with *Hin*dIII plus *Xma*I (Lane 7) produced a single hybridization band of approximately 8.1 kb, corresponding to a single copy of *vip3Aa20* and confirming the intactness of the insert. The negative control samples for each enzymatic digestion showed no hybridization bands and none were expected (*Kpn*I Lane 4, *Eco*RV Lane 6, and *Hin*dIII plus *Xma*I Lane 8). The *Hin*dIII plus *Xma*I digestion of the pNOV1300 vector produced the expected hybridization signal at 8.1 kb (positive control, Lane 9).

For Southern blot analyses with the *vip3Aa19*-specific probe, each restriction endonuclease digestion resulted in single hybridization band of the expected size. This demonstrates that the MIR162 maize T-DNA contains an intact *vip3Aa20* coding sequence and the absence of bands other than the three expected indicates that there is only a single copy of the gene present.

IV.B.2.b. Copy Number of Functional Elements: *manA* Gene

A map of the T-DNA region in the MIR162 maize transformation vector pNOV1300 indicating locations of the *manA* coding sequence and the restriction endonuclease sites targeted in these Southern blot analyses is shown in Figure 9. Three digestion strategies were utilized to confirm the presence, size, intactness, and copy number of the *manA* coding sequence. The results of these analyses are shown in Table 5 and Figure 10.

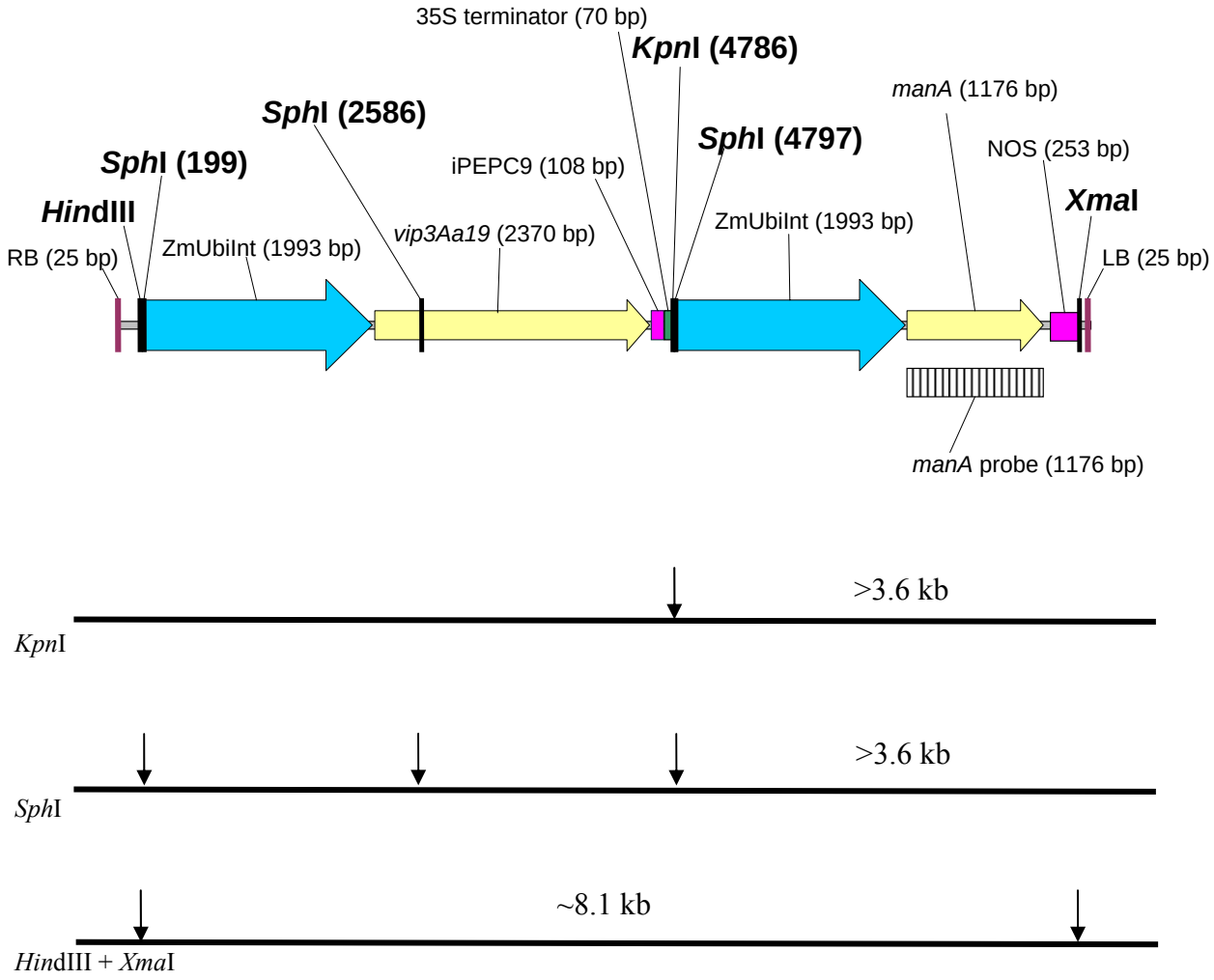

Figure 9. Restriction sites and probe for *manA*.
A schematic of the 8.4 kb T-DNA of pNOV1300 is shown along with the location of the *Kpn*I, *Sph*I, *Hin*dIII, and *Xma*I restriction sites and their location relative to the *manA* coding sequence. The vertical arrows indicate the endonuclease cutting sites and the expected size of the resulting restriction fragments that should be detected when probed with *manA*.

The results of these Southern blot analyses are displayed in Figure 10. Table 5 shows a comparison of expected *versus* observed hybridization band sizes.

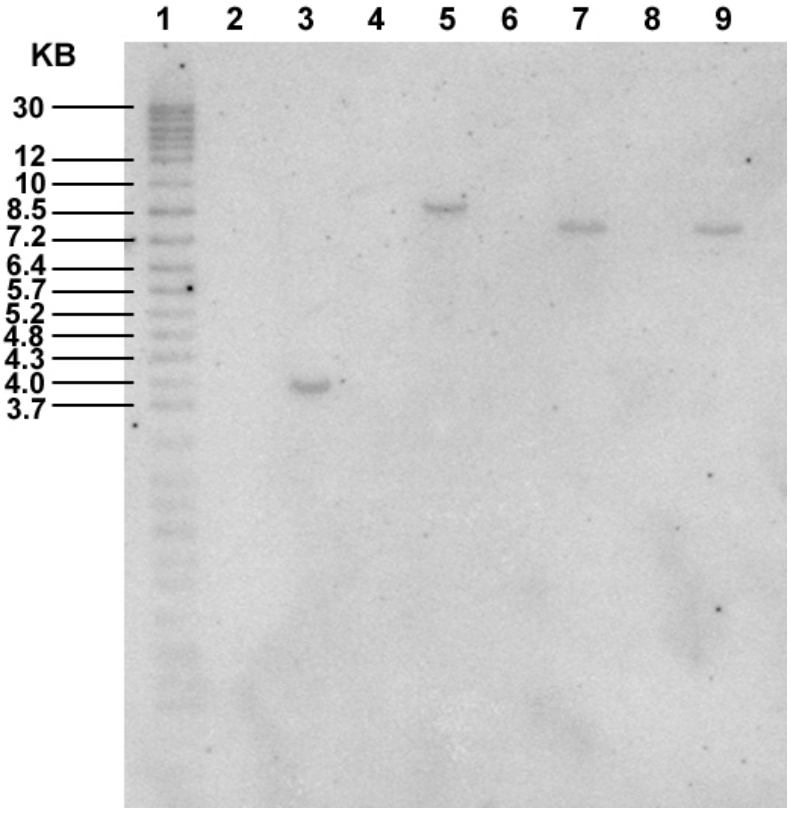

Figure 10. Southern blot analysis of MIR162 maize with a *manA*-specific probe**.**
Maize genomic DNA (7.5 µg) was subjected to three separate digestions with *Kpn*I or *Sph*I or *Hin*dIII plus *Xma*I restriction endonucleases and subjected to a Southern blot analysis with a *manA*-specific probe (1176 bp).

Lane 1: Molecular weight marker
Lane 2: Blank
Lane 3: BC_4F_1 generation of MIR162 maize digested with *Kpn*I
Lane 4: Control from BC_4F_1 generation maize digested with *Kpn*I
Lane 5: $BC4F_1$ generation of MIR162 maize digested with *Sph*I
Lane 6: Control from BC_4F_1 generation maize digested with *Sph*I
Lane 7: BC_4F_1 generation of MIR162 maize digested with *Hin*dIII + *Xma*I
Lane 8: Control from BC_4F_1 generation maize digested with *Hin*dIII + *Xma*I
Lane 9: Control from BC_4F_1 generation maize digested with *Hin*dIII + *Xma*I plus 20.2 pg of *Hin*dIII + *Xma*I digested pNOV1300 plasmid.

Table 5. Expected *versus* observed hybridization band sizes for probes with *manA*.

Lane # (Figure 10)	DNA	Restriction Enzyme	Probe	Expected Bands	Expected Band Size (kb)	Observed Band Size (kb)
Lane 3	BC_4F_1	*Kpn*I	*manA*	1	>3.6	4.0
Lane 4	Negative	*Kpn*I	*manA*	0	-	-
Lane 5	BC_4F_1	*Sph*I	*manA*	1	>3.6	9.0
Lane 6	Negative	*Sph*I	*manA*	0	-	-
Lane 7	BC_4F_1	*Hin*dIII + *Xma*I	*manA*	1	8.1	8.1
Lane 8	Negative	*Hin*dIII + *Xma*I	*manA*	0	-	-
Positive Control						
Lane 9	pNOV1300	*Hin*dIII + *Xma*I	*manA*	1	8.1	8.1

Genomic MIR162 maize DNA digested with *Kpn*I (Figure 10, Lane 3) produced a single hybridization band of approximately 4.0 kb, corresponding to a single copy of *manA*. Genomic MIR162 maize DNA digested with *Sph*I (Lane 5) produced a single hybridization band of approximately 9.0 kb, corresponding to a single copy of *manA*. Genomic MIR162 maize DNA digested with *Hin*dIII + *Xma*I (Lane 7) produced a single hybridization band of approximately 8.1 kb, corresponding to a single copy of *manA* and confirming the intactness of the insert. The negative control samples for each enzymatic digestion showed no hybridization bands and none were expected (*Kpn*I Lane 4, *Sph*I Lane 6, and *Hin*dIII + *Xma*I Lane 8). The *Hin*dIII plus *Xma*I digestion of the pNOV1300 vector produced the expected hybridization band at 8.1 kb (positive control, Lane 9).

For the *manA*-specific probe Southern blot analyses, each restriction endonuclease digestion resulted in a single hybridization band closely matching the expected size for each. This demonstrates that MIR162 maize contains an intact *manA* coding sequence and the absence of bands other than the three expected, indicates that there is only a single copy of the gene present.

IV.B.2.c. Copy Number of Functional Elements: ZmUbiInt promoter

A map of the T-DNA region in vector pNOV1300 indicating locations of the ZmUbiInt promoter and the restriction endonuclease sites targeted for use in these Southern blot analyses is shown in Figure 11. Three digestion strategies were utilized to confirm the presence, size, intactness, and copy number of the ZmUbiInt promoter. The results of this analysis are shown in Table 6 and Figure 12.

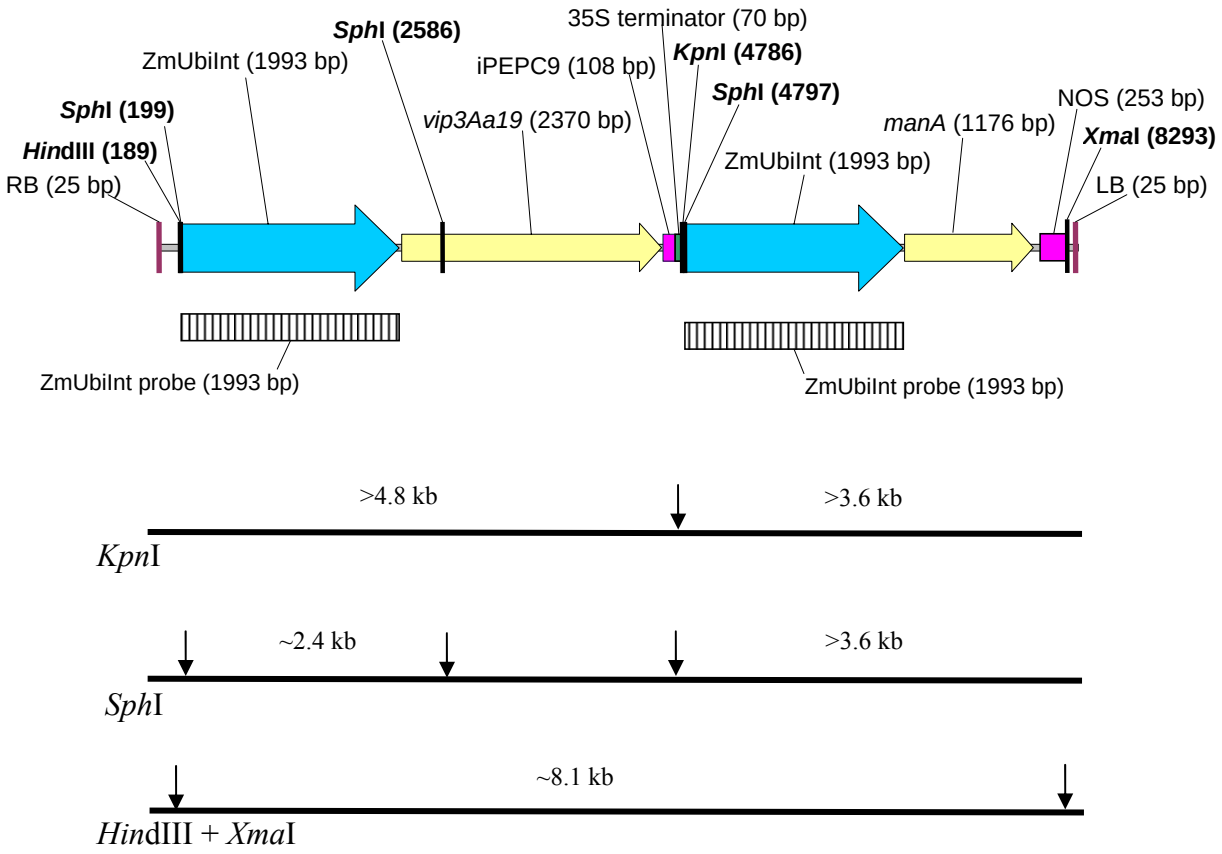

Figure 11. Restriction sites and probe for the ZmUbiInt promoter.
A schematic of the 8.4 kb T-DNA of pNOV1300 is shown along with the location of the KpnI, SphI, HindIII, and XmaI restriction sites and their location relative to the ZmUbiInt promoter. The vertical arrows indicate the endonuclease cutting sites and the expected size of the resulting restriction fragments that should be detected when probed with ZmUbiInt.

Since two copies of the ZmUbiInt promoter are present in the T-DNA region of plasmid pNOV1300 (see Figure 3), two unique bands were expected for the *Kpn*I and *Sph*I enzyme digestions, and one band for the *Hind*III and *Xma*I digestion. Additionally, ZmUbiInt is an endogenous maize promoter and, thus, the probe will hybridize to this endogenous maize sequence.

The results of these Southern blot analyses are displayed in Figure 12. Table 6 shows a comparison of expected *versus* observed hybridization band sizes.

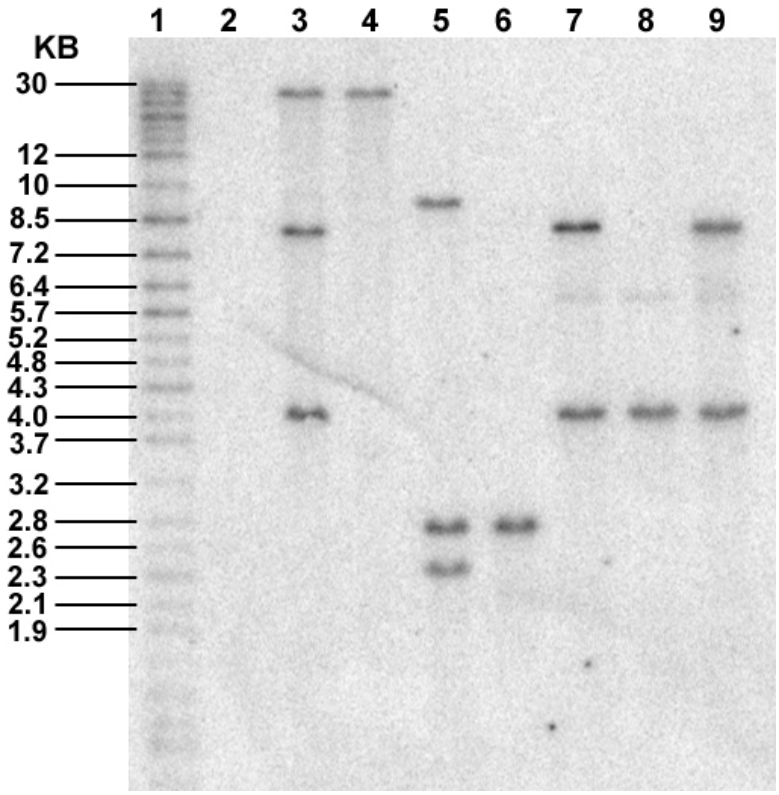

Figure 12. Southern analysis of MIR162 maize with ZmUbiInt-specific probe.
Maize genomic DNA (7.5 µg) was subjected to three separate digestions with *Kpn*I or *Sph*I or *Hin*dIII plus *Xma*I restriction endonucleases and to Southern blot analysis with a ZmUbiInt promoter-specific probe (1993 bp).

Lane 1: Molecular weight marker
Lane 2: Blank
Lane 3: BC_4F_1 generation of MIR162 maize digested with *Kpn*I
Lane 4: Control from BC_4F_1 generation maize digested with *Kpn*I
Lane 5: BC_4F_1 generation of MIR162 maize digested with *Sph*I
Lane 6: Control from BC_4F_1 generation maize digested with *Sph*I
Lane 7: BC_4F_1 generation of MIR162 maize digested with *Hin*dIII + *Xma*I
Lane 8: Control from BC_4F_1 generation maize digested with *Hin*dIII + *Xma*I
Lane 9: Control from BC_4F_1 generation maize digested with *Hin*dIII + *Xma*I plus 20.2 pg of *Hin*dIII + *Xma*I digested pNOV1300 plasmid.

Table 6. Expected *versus* observed hybridization band sizes for probes with ZmUbiInt.

Lane # (Figure 12)	DNA	Restriction Enzyme	Probe	Expected Bands	Expected Band Size (kb)	Observed Band Size (kb)
Lane 3	BC_4F_1	*Kpn*I	ZmUbiInt	3	>3.6 >4.8 Unknown Endogenous	4.0 8.0 22.0
Lane 4	Negative	*Kpn*I	ZmUbiInt	1	Unknown Endogenous	22.0
Lane 5	BC_4F_1	*Sph*I	ZmUbiInt	3	~2.4 >3.6 Unknown Endogenous	2.4 9.0 2.8
Lane 6	Negative	*Sph*I	ZmUbiInt	1	Unknown Endogenous	2.8
Lane 7	BC_4F_1	*Hin*dIII + *Xma*I	ZmUbiInt	2	8.1 Unknown Endogenous	8.1 4.0, 5.8
Lane 8	Negative	*Hin*dIII + *Xma*I	ZmUbiInt	1	Unknown Endogenous	4.0, 5.8
Lane 9	pNOV1300	*Hin*dIII + *Xma*I	ZmUbiInt	2	8.1 Unknown Endogenous	8.1 4.0, 5.8

Genomic MIR162 maize DNA digested with *Kpn*I (Figure 12, Lane 3) produced two hybridization bands of approximately 4.0 kb and 8.0 kb, corresponding to the expected size of the two copies of the ZmUbiInt promoter present in the T-DNA. There was also a hybridization band representing endogenous maize sequence at approximately 22.0 kb present in MIR162 maize (Lane 3) and the negative control (Lane 4). Genomic MIR162 DNA digested with *Sph*I (Lane 5) produced two unique hybridization signals of approximately 2.4 kb and 9.0 kb, corresponding to the expected two copies of the ZmUbiInt promoter present in the T-DNA. There was also a hybridization band observed representing endogenous maize sequence at approximately 2.8 kb present in MIR162 maize (Lane 5) and the negative control (Lane 6). Genomic MIR162 maize DNA digested with *Hin*dIII + *Xma*I (Lane 7) produced a single hybridization signal of approximately 8.1 kb, corresponding to the full-length MIR162 T-DNA which contains two copies of the ZmUbiInt promoter, thus confirming the intactness of the insert. There were also two hybridization bands of approximately 4.0 kb and 5.8 kb present in both MIR162 maize (Lane 7) and the negative control (Lane 8) that represent endogenous ZmUbiInt sequence. The negative control corresponding to each digest showed no hybridization band other than that observed with endogenous ZmUbiInt sequences (*Kpn*I Lane 4, *Sph*I Lane 6, and *Hin*dIII + *Xma*I Lane 8). The *Hin*dIII + *Xma*I digestion of the pNOV1300 vector produced the expected hybridization signal at 8.1 kb (positive control, Lane 9). Two hybridization bands of 4.0 and 5.8 were

observed which represent endogenous ZmUbiInt sequence.

For the Southern blot analyses with the ZmUbiInt promoter-specific probe, each restriction endonuclease digestion resulted in hybridization bands demonstrating that MIR162 maize contains two intact copies of the ZmUbiInt promoter within the T-DNA and confirmed the known presence of endogenouos maize ZmUbiInt promoter sequence.

IV.B.2.d. Copy Number of Functional Elements: NOS Terminator

A map of the T-DNA region in pNOV1300 indicating locations of the NOS terminator and the restriction endonuclease sites targeted for these Southern blot analyses is shown in Figure 13. Three digestion strategies were utilized to confirm the presence, size, intactness, and copy number of the NOS terminator sequence.

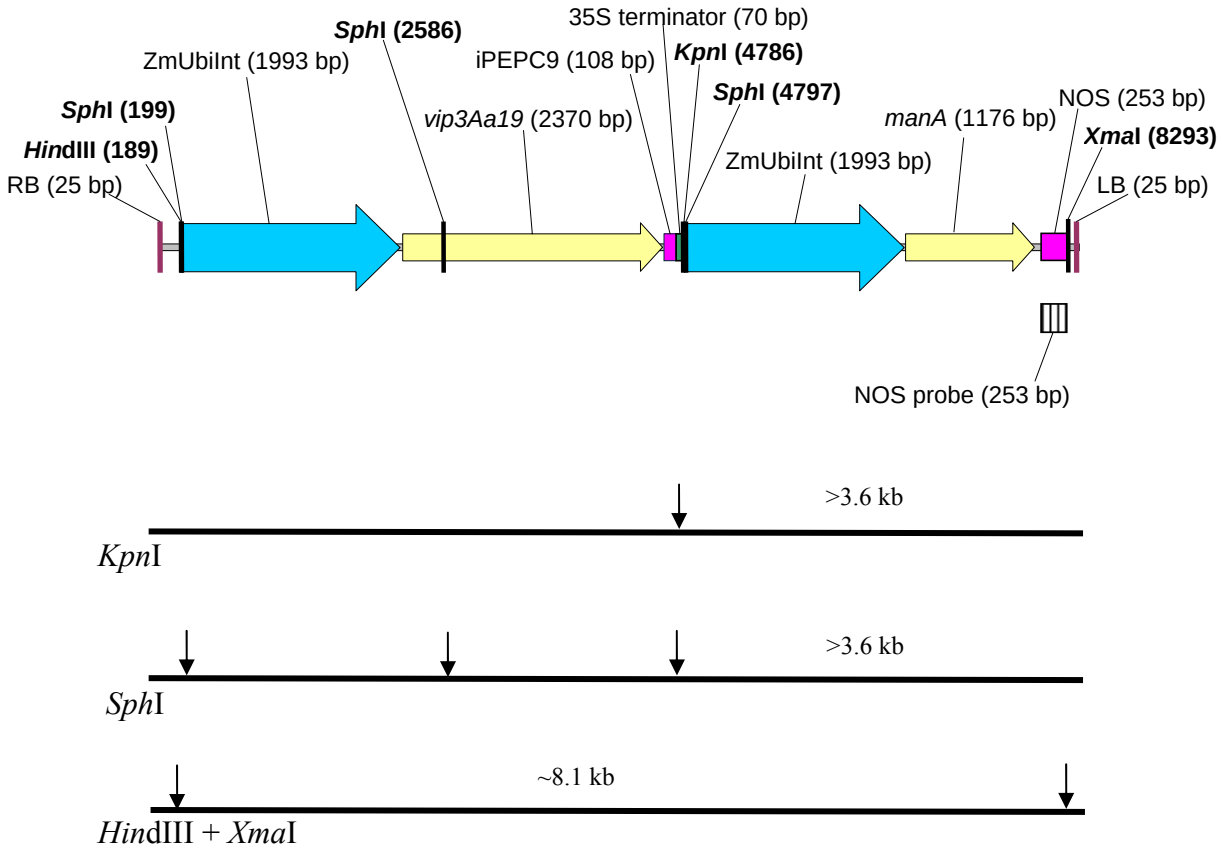

Figure 13. Restriction sites and probe for NOS terminator.
A schematic of the 8.4 kb T-DNA of pNOV1300 is shown along with the location of the *Kpn*I, *Sph*I, *Hin*dIII, and *Xma*I restriction sites and their location relative to the NOS terminator. The vertical arrows indicate the endonuclease cutting sites and the expected size of the resulting restriction that should be detected when probed with NOS.

The results of these Southern blot analyses are displayed in Figure 14. Table 7 shows a comparison of expected *versus* observed hybridization band sizes.

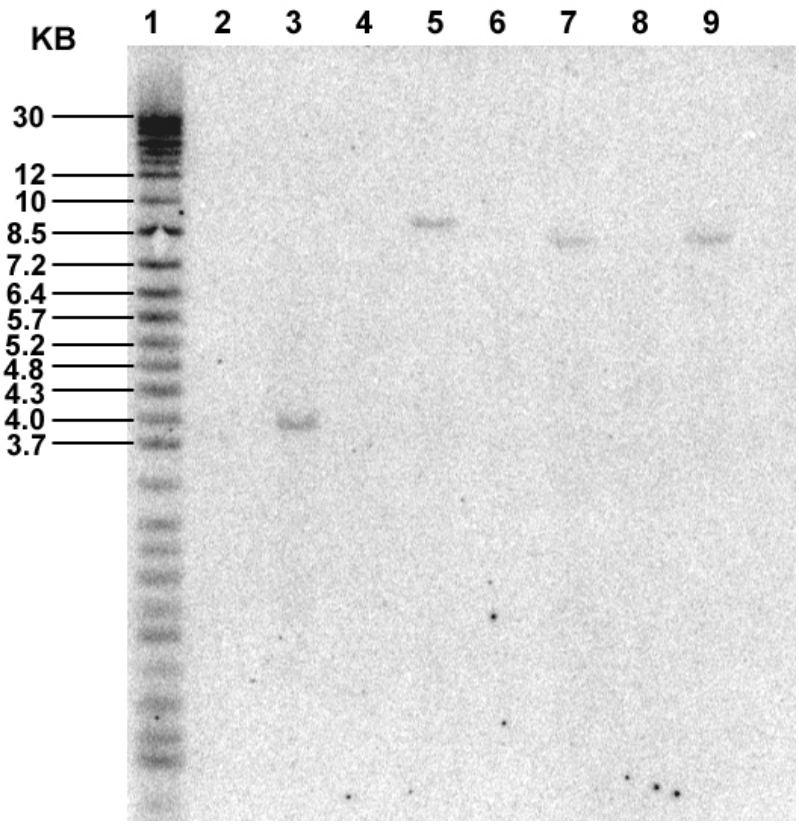

Figure 14. Southern analysis of MIR162 maize with NOS terminator-specific probe.
Maize genomic DNA (7.5 µg) was subjected to three separate digestions with *Kpn*I or *Sph*I or *Hin*dIII plus *Xma*I restriction endonucleases and subjected to Southern blot analysis with a NOS-specific probe.

Lane 1: Molecular weight marker
Lane 2: Blank
Lane 3: BC_4F_1 generation of MIR162 maize digested with *Kpn*I
Lane 4: Control from BC_4F_1 generation maize digested with *Kpn*I
Lane 5: BC_4F_1 generation of MIR162 maize digested with *Sph*I
Lane 6: Control from BC_4F_1 generation maize digested with *Sph*I
Lane 7: BC_4F_1 generation of MIR162 maize digested with *Hin*dIII + *Xma*I
Lane 8: Control from BC_4F_1 generation maize digested with *Hin*dIII + *Xma*I
Lane 9: Control from BC_4F_1 generation maize digested with *Hin*dIII + *Xma*I plus 20.2 pg of *Hin*dIII + *Xma*I digested pNOV1300 plasmid.

Table 7. Expected *versus* observed hybridization band sizes for probes with NOS.

Lane # (Figure 14)	DNA	Restriction Enzyme	Probe	Expected Bands	Expected Band Size (kb)	Observed Band Size (kb)
Lane 3	BC_4F_1	*Kpn*I	NOS	1	>3.6	4.0
Lane 4	Negative	*Kpn*I	NOS	0	-	-
Lane 5	BC_4F_1	*Sph*I	NOS	1	>3.6	9.0
Lane 6	Negative	*Sph*I	NOS	0	-	-
Lane 7	BC_4F_1	*Hin*dIII + *Xma*I	NOS	1	8.1	8.1
Lane 8	Negative	*Hin*dIII + *Xma*I	NOS	0	-	-
Lane 9	pNOV1300	*Hin*dIII + *Xma*I	NOS	1	8.1	8.1

Genomic MIR162 maize DNA digested with *Kpn*I (Figure 14, Lane 3) produced a single hybridization band of approximately 4.0 kb, corresponding to a single copy of the NOS terminator. Genomic MIR162 maize DNA digested with *Sph*I (Lane 5) produced a single hybridization band of approximately 9.0 kb, corresponding to a single copy of the NOS terminator. Genomic MIR162 maize DNA digested with *Hin*dIII + *Xma*I (Lane 7) produced a single hybridization band of approximately 8.1 kb, corresponding to a single copy of the NOS terminator and confirming the intactness of the insert. The negative control corresponding to each digest showed no hybridization bands and none were expected (*Kpn*I Lane 4, *Sph*I Lane 6 and *Hin*dIII + *Xma*I Lane 8). The *Hin*dIII + *Xma*I digestion of the pNOV1300 vector produced the expected hybridization signal at 8.1 kb (positive control, Lane 9).

For the Southern blot analyses with the NOS terminator-specific probe, each restriction endonuclease digestion resulted in a single hybridization band of the expected size. This demonstrates that the MIR162 maize T-DNA contains an intact NOS terminator sequence, and the absence of bands other than the three expected indicates that there is only a single copy of the terminator present.

IV.B.3. Absence of Plasmid Backbone Elements

Genomic DNA was digested and probed for the possible presence of plasmid backbone elements in a Southern blot analysis. None of these backbone elements should have been incorporated into the MIR162 maize genome. A map of the pNOV1300 plasmid vector

indicating the region covered by the backbone-specific probe, the location of each backbone element, and the restriction endonuclease sites targeted for this Southern blot analyses is shown in Figure 15. Three digestion strategies were utilized to test for the presence of backbone elements.

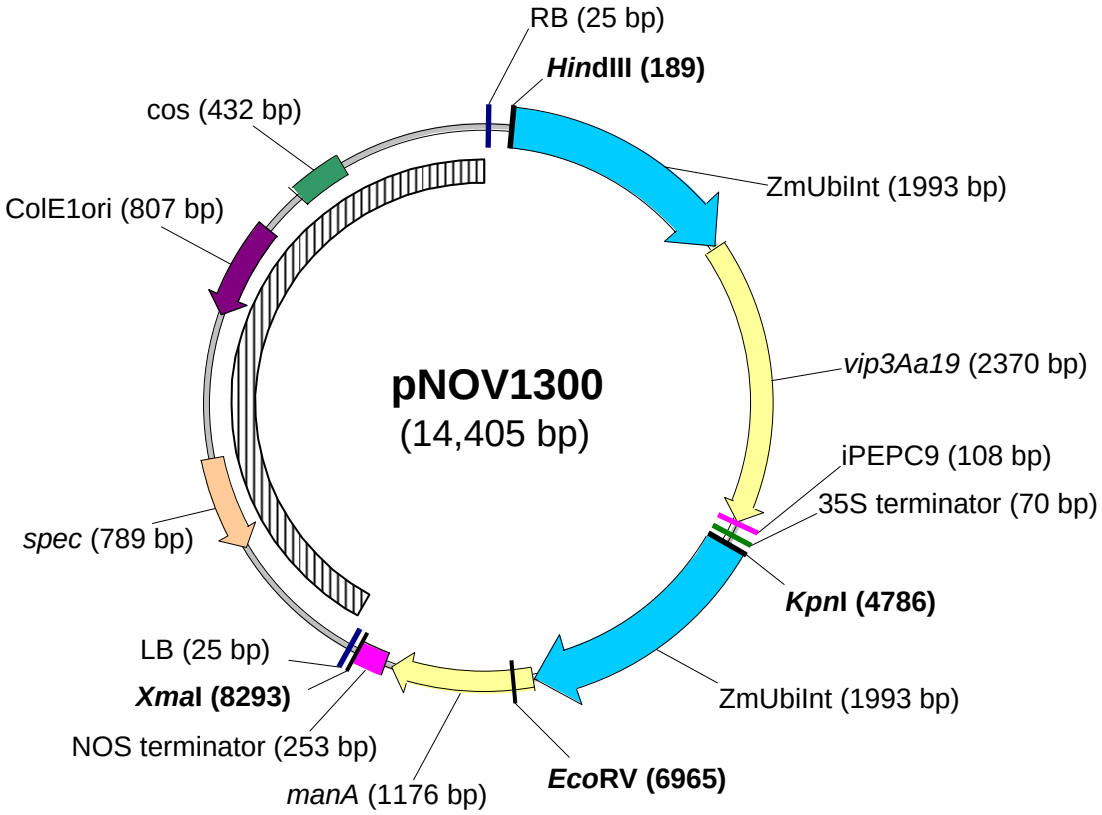

Figure 15. Restriction sites and probe for plasmid backbone elements.
A schematic of the pNOV1300 plasmid vector showing the location of the *Kpn*I, *Eco*RV, *Hin*dIII, and *Xma*I restriction sites and their location relative to the region outside of the right and left borders of the T-DNA. The backbone-specific probe spans the 6019 base pairs outside of the borders.

The results of these Southern blot analyses are displayed in Figure 16. Table 8 shows a comparison of expected *versus* observed hybridization band sizes. Genomic MIR162 maize DNA was digested with *Kpn*I (Lane 3), *Eco*RV (Lane 5), and *Hin*dIII + *Xma*I (Lane 7). The blot was hybridized with a probe covering the entire backbone region of pNOV1300. No detectable hybridization bands were observed in the MIR162 maize genomic samples (Lanes 3, 5, and 7) or in the negative control samples (Lanes 4, 6, and 8). The *Hin*dIII plus *Xma*I digestion of the pNOV1300 vector produced an expected 6.3 kb band (Lane 9). These results demonstrate that the MIR162 maize T-DNA does not contain any backbone sequences from the pNOV1300 transformation plasmid.

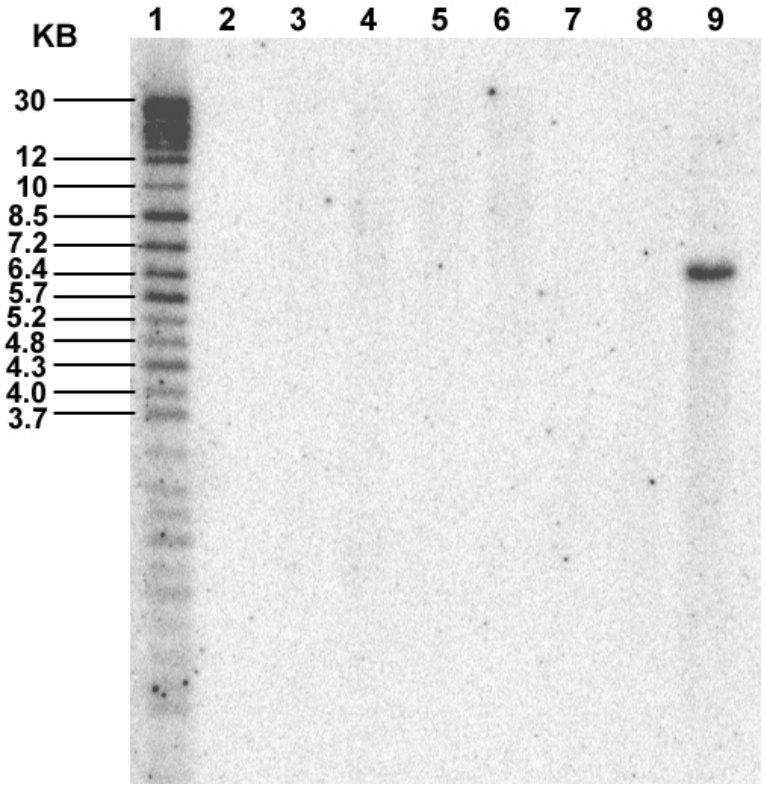

Figure 16. Southern blot analysis for plasmid backbone sequence.
Maize genomic DNA (7.5 µg) was subjected to three separate digestions with *Kpn*I or *Eco*RV or *Hin*dIII plus *Xma*I restriction endonucleases and subjected to Southern blot analysis with a pNOV1300 backbone-specific probe (6019 bp).

Lane 1: Molecular weight marker
Lane 2: Blank
Lane 3: BC_4F_1 generation of MIR162 maize digested with *Kpn*I
Lane 4: Control from BC_4F_1 generation maize digested with *Kpn*I
Lane 5: BC_4F_1 generation of MIR162 maize digested with *Eco*RV
Lane 6: Control from BC_4F_1 generation maize digested with *Eco*RV
Lane 7: BC_4F_1 generation of MIR162 maize digested with *Hin*dIII + *Xma*I
Lane 8: Control from BC_4F_1 generation maize digested with *Hin*dIII + *Xma*I
Lane 9: Control from BC_4F_1 generation maize digested with *Hin*dIII + *Xma*I plus 20.2 pg of *Hin*dIII + *Xma*I digested pNOV1300 plasmid.

Table 8. Expected *versus* observed band sizes for probes with pNOV1300 backbone.

Lane # (Figure 16)	DNA	Restriction Enzyme	Probe	Expected Bands	Expected Band Size (kb)	Observed Band Size (kb)
Lane 3	BC_4F_1	*Kpn*I	Backbone	0	None	None
Lane 4	Negative	*Kpn*I	Backbone	0	None	None
Lane 5	BC_4F_1	*Eco*RV	Backbone	0	None	None
Lane 6	Negative	*Eco*RV	Backbone	0	None	None
Lane 7	BC_4F_1	*Hin*dIII + *Xma*I	Backbone	0	None	None
Lane 8	Negative	*Hin*dIII + *Xma*I	Backbone	0	None	None
Positive Control						
Lane 9	pNOV1300	*Hin*dIII + *Xma*I	Backbone	1	6.3	6.3

IV.C. Stability of the T-DNA Across Generations

Stability of the T-DNA in MIR162 maize across multiple generations of conventional breeding was determined by Southern blot analysis, using backcross generations BC_1F_1, BC_2F_1, and BC_4F_1 (see Figure 5). For this analysis, MIR162 maize and negative control genomic DNA samples were digested with a restriction enzyme that digests once within the DNA insert but not within the functional element being probed. Southern blot analysis was performed using a *vip3Aa19*-specific probe.

A schematic of the T-DNA region in vector pNOV1300 indicating locations of the *vip3Aa19*-coding sequence and the restriction endonuclease site targeted for this Southern blot analysis is shown in Figure 17.

The results of this Southern blot analysis are displayed in Figure 18. Table 9 shows a comparison of the expected *versus* observed hybridization band sizes.

Genomic MIR162 maize DNA from BC_1F_1, BC_2F_1, and BC_4F_1 digested with *Acc*65I produced a single hybridization signal of approximately 8.0 kb corresponding to the single copy of the *vip3Aa20* gene present in MIR162 maize (Figure 18; Lanes 3, 4, and 5). The negative control showed no hybridization (negative segregant of BC_4F_1; Lane 6). The *Acc*65I digestion of pNOV1300 produced a 14.4 kb band, as expected (positive control; Lane 7). The hybridization patterns for generations BC_1F_1, BC_2F_1, and BC_4F_1 of MIR162 maize in

this Southern blot analysis were identical, demonstrating the stability of the *vip3Aa20* cassette over multiple generations.

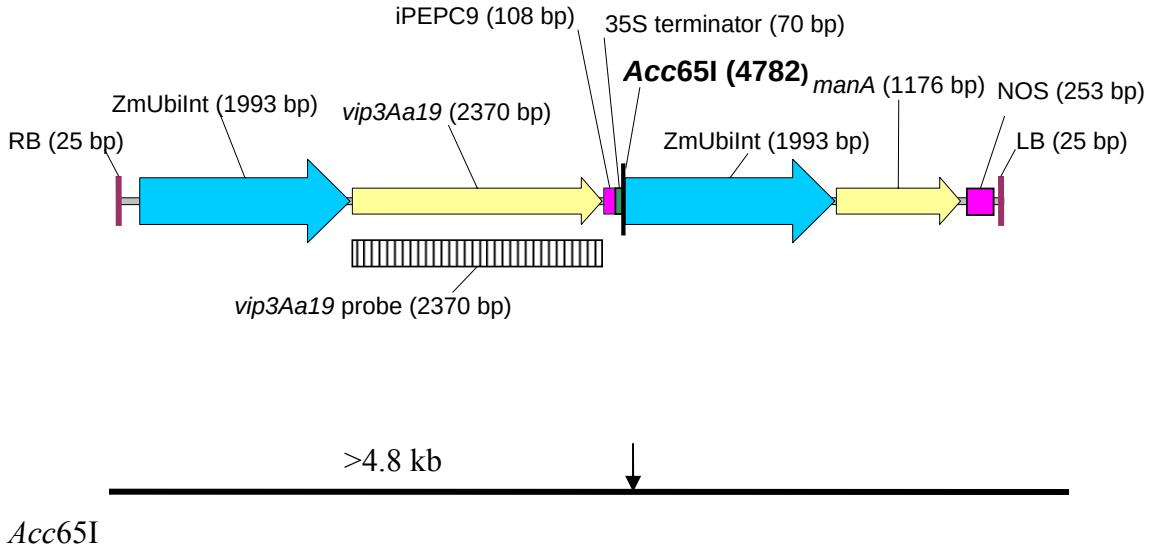

Figure 17. Restriction site and probe for *vip3Aa19*.
The vertical arrow indicates the endonuclease cutting site and the expected band size for the resulting restriction fragment that should be detected when probed with *vip3Aa19*.

These Southern blot analysis data demonstrate that the expected hybridization pattern was observed for the *vip3Aa20* gene over several generations of MIR162 maize. Therefore, the stability of the insert in MIR162 maize during conventional breeding has been established over multiple generations.

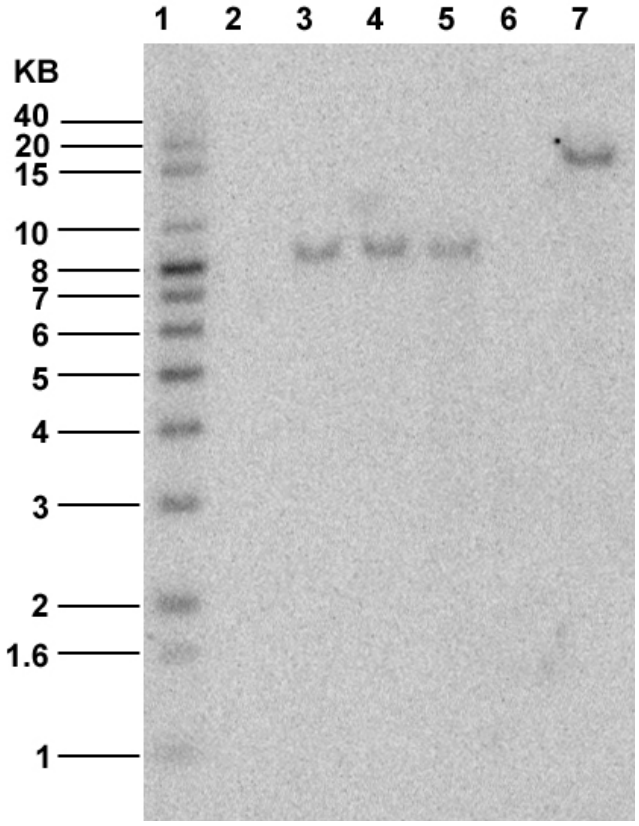

Figure 18. Southern blot of BC_1F_1, BC_2F_1, and BC_4F_1 generations from MIR162.
Maize genomic DNA (7.5µg) was digested with *Acc*65I restriction endonuclease and subjected to Southern blot analysis with a *vip3Aa19*-specific probe (2370 bp).

Lane 1: Molecular weight marker
Lane 2: Blank
Lane 3: BC_1F_1 generation of MIR162 maize digested with *Acc*65I
Lane 4: BC_2F_1 generation of MIR162 maize digested with *Acc*65I
Lane 5: BC_4F_1 generation of MIR162 maize digested with *Acc*65I
Lane 6: Control from BC_4F_1 generation maize digested with *Acc*65I
Lane 7: Control from BC_4F_1 generation maize digested with *Acc*65I plus 20.2 pg of *Acc*65I digested pNOV1300 plasmid.

Table 9. Expected *versus* observed hybridization band size with *vip3Aa19* probe.

Lane # (Figure 18)	DNA	Restriction Enzyme	Probe	Expected Bands	Expected Band Size (kb)	Observed Band Size (kb)
Lane 3	BC_1F_1	*Acc*65I	*vip3Aa19*	1	>4.8	8.0
Lane 4	BC_2F_1	*Acc*65I	*vip3Aa19*	1	>4.8	8.0
Lane 5	BC_4F_1	*Acc*65I	*vip3Aa19*	1	>4.8	8.0
Lane 6	Negative	*Acc*65I	*vip3Aa19*	0	-	-
Positive Control						
Lane 7	pNOV1300	*Acc*65I	*vip3Aa19*	1	14.4	14.4

IV.D. Mendelian Inheritance of the T-DNA

Chi-square analysis of *vip3Aa20* and *manA* inheritance data over three generations of MIR162 backcrossing was performed to test the hypothesis that the genes are inherited in accordance with the laws of Mendelian genetics. See Table 2 and Figure 5 for identification of the three generations used in this analysis. The Chi-square analysis was based on a comparison of observed and expected gene segregation ratios from each generation. TaqMan PCR analyses were conducted on plants from three generations of MIR162 maize to determine the number of plants that were positive or negative for both the *vip3Aa20* and *manA* gene. Only progeny that tested positive for the two genes were selected for backcrossing; thus, the expected inheritance ratio for positive to negative plants was 1:1 in each generation.

The expected and observed frequencies of the *vip3Aa20* and *manA* genes for each generation are presented in Tables 10 and 11. The critical value for rejection of the null hypothesis at $p<0.05$ was 3.84. The Chi-square values for each generation tested were found to be less than 3.84. This analysis demonstrates that both the *vip3Aa20* and *manA* genes are inherited in a predictable manner according to Mendelian principles. These results are consistent with the genetic characterization data, which indicate a stable integration of the T-DNA at a single locus in the genome.

Table 10. Observed *versus* expected frequencies for *vip3Aa20* across generations.
The progeny of three generations were tested for the presence of *vip3Aa20* coding sequence by PCR over three backcross generations.

Trait	BC_1F_1		BC_2F_1		BC_4F_1	
	o*	e*	o*	e*	o*	e*
Positive	21	20.5	45	48.5	148	143.5
Negative	20	20.5	52	48.5	139	143.5
Total	41	41.0	97	97.0	287	287.0
χ^2 value	0.000		0.371		0.223	

* o = observed value; e = expected value

$\chi^2 = \sum (| o - e | - 0.5)^2 / e$

Table 11. Observed *versus* expected frequencies for *manA* across generations.
The progeny of three generation were tested for the presence of *manA* coding sequence by PCR over three backcross generations.

Trait	BC_1F_1		BC_2F_1		BC_4F_1	
	o*	e*	o*	e*	o*	e*
Positive	21	20.5	45	48.5	148	143.5
Negative	20	20.5	52	48.5	139	143.5
Total	41	41.0	97	97.0	287	287.0
χ^2 value	0.000		0.371		0.223	

* o = observed value; e = expected value

$\chi^2 = \sum (| o - e | - 0.5)^2 / e$

IV.E. Flanking Sequence Analysis

Nucleotide sequences flanking the 5′ and 3′ ends of the T-DNA in MIR162 maize were screened for homology with sequences found in public databases. This comparison provides an indication of whether the MIR162 maize T-DNA inserted into a known plant functional genetic unit. A sequence similarity analysis was performed using the BLASTN software (Altschul *et al.*, 1997; version 2.2.6 Apr-9-2003) which compared the flanking sequences with nucleotide sequences in the latest version of the National Center for Biotechnology Information nonredundant database. An analysis of the nucleotide junctions between the T-DNA and genomic DNA was also conducted to determine if novel open reading frames were generated at the point of T-DNA insertion (see Appendix B for details).

The BLASTN analysis of maize genomic sequence flanking the 5′ border of the T-DNA showed significant homology with *Dissociation1 (Ds1)*-related transposable elements. *Ds1* is a nonautonomous transposable element that requires an active *Activator* (*Ac*) element to become mobile. Previous studies have shown that the excision of *Ds* elements can extend beyond the element, but the largest amount of surrounding DNA that has been shown to be deleted is 36 bp (Shen *et al*., 1992). The region of homology between the 5′ flanking sequence and the *Ds1*-related elements is located more than 500 bp from the insert sequence. BLASTN analysis of the maize genomic sequence flanking the 3′ region of the MIR162 maize DNA insert shows homology to sequence defined as a *Z. mays* cyclophilin gene, but the region of homology lies outside of the cyclophilin coding region.

Analysis of all six potential reading frames at both the 5′ and 3′ DNA insert to maize genome junctions did not detect the presence of any functional novel open reading frames.

IV.F. Summary of the Genetic Characterization of MIR162 Maize

Data from Southern blot analyses demonstrated that MIR162 maize contains: i) single copies of the *vip3Aa20* gene and the *manA* gene in the T-DNA; ii) two copies of the ZmUbiInt promoter within the T-DNA region, in addition to endogenous maize ZmUbiInt promoter elsewhere in the genome; iii) a single copy of the NOS terminator in the T-DNA; and iv) no backbone sequences from transformation plasmid pNOV1300. Additionally, Southern blot analyses demonstrated that the MIR162 maize T-DNA was stable over several breeding generations. Sequence analysis of the entire T-DNA in MIR162 maize confirmed the intactness of the insert and the contiguousness of the functional elements. Sequence analysis also revealed two transformation-induced nucleotide changes in the *vip3Aa* coding sequence contained in the T-DNA. Only one of the nucleotide changes encoded an amino acid change, whereby methionine at position 129 has been substituted by isoleucine. Sequence analysis revealed that some truncation occurred at the RB and LB ends of the T-DNA. The entire right border, along with two base pairs of noncoding sequence, was truncated, and the entire left border, along with 32 base pairs of noncoding sequence, was truncated. These deletions have no effect on the functionality of the DNA insert. A schematic diagram of the experimentally confirmed T-DNA in MIR162 maize is shown in Figure 6.

Data from Southern blot analyses and DNA sequencing demonstrated the presence of a single intact transgene insert in MIR162 maize. Observations of *vip3Aa20* and *manA* segregation ratios over several generations of MIR162 maize are consistent with the genes being linked at a single locus in the maize genome. The MIR162 maize DNA insert did not locate within any known *Z. mays* gene. There is a region of homology between the maize genomic sequence flanking the 5′ region of the MIR162 T-DNA and maize transposable element sequences, but the homologous region is more than 500 bp removed from the insert. There is a region of homology between the maize genomic sequence flanking the 3′ region of the MIR162 maize T-DNA and the *Z. mays* cyclophilin gene, but the region of homology lies outside of the cyclophilin coding sequence. Additionally, no novel open reading frames were identified that spanned either the 5′ or 3′ junctions between the T-DNA and *Z. mays* genomic sequences.

V. Characterization of the Vip3Aa20 and PMI Proteins

The two novel proteins produced in MIR162 maize have been characterized and tested to determine their potential for causing adverse effects in mammals. This process included an assessment of: i) the origin and function of each protein; ii) the mode of action of each protein; iii) physiochemical properties of the proteins; iv) biological activity of the proteins; v) results of safety studies for each protein; and vi) Vip3Aa20 and PMI concentrations in plant tissues.

V.A. Vip3Aa20 Protein – Origin and Function

Vip3Aa is a class of recently discovered 'vegetative insecticidal proteins' produced by *B. thuringiensis*, a gram positive bacterium (Estruch *et al.,* 1996). Unlike the crystal (Cry) proteins of *B. thuringiensis*, Vip protein toxins are produced during vegetative bacterial growth and are secreted as soluble proteins into the extracellular environment. *B. thuringiensis* cultures continue to produce Vip proteins during the stationary phase of growth and sporulation. In contrast to the thermostabile nonproteinaceous β-exotoxins secreted by some *B. thuringiensis* strains, Vip proteins are thermolabile. Vip3Aa1 is the toxin designation assigned to the native protein discovered in *B. thuringiensis* strain AB88. Vip3Aa19 and Vip3Aa20 are toxin designations assigned by the *Bacillus thuringiensis* Toxin Nomenclature Committee to two engineered variants of the native protein.[2] *B. thuringiensis* is a scavenger organism that does not readily proliferate in soil. Production of Cry and Vip toxins conveys upon the microorganism a means of killing insect prey that can then serve as nutrient sources.

V.A.1. Mechanism of Action and Insecticidal Specificity

The mechanism by which Vip proteins exert their insecticidal activity has been studied and found to be similar, but not identical, to that which has been previously described for the *B. thuringiensis* Cry proteins. Following ingestion, full-length Vip proteins are proteolytically processed into active fragments of approximately 66 kDa which bind to receptors in the midgut epithelium of susceptible insects. Competitive binding assays have shown that Vip proteins and Cry proteins bind to different receptors (Lee *et al.*, 2003). Receptor binding is followed by the formation of selective ion channels (pores) in epithelial membranes which leads to cell lysis and death (Yu *et al*., 1997). Each of these steps plays a role in establishing the insecticidal specificity of a given protein for different insect species. As discussed later in this petition, the insecticidal activity of Vip3Aa proteins is limited to species within selected families of the order Lepidoptera (see Table 27).

Native Vip3Aa1 is 789 amino acids in length and approximately 89 kDa molecular weight. The Vip3Aa20 variant produced in MIR162 maize is also 789 amino acids in length but differs from the native amino acid sequence by two amino acids (at positions 129 and 284). A Vip3Aa variant is also present in Syngenta cotton transformation event COT102; this

[2] Bacillus thuringiensis Toxin Nomenclature Committee (2007). http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/

variant has been assigned the designation Vip3Aa19. Vip3Aa19 differs from the native sequence by one amino acid at position 284. Vip3Aa19 and Vip3Aa20 differ by one amino acid at position 129. Table 12 compares selected sequence information for these protein variants. COT102 cotton was deregulated by USDA in 2005.

Table 12. Amino acid differences between Vip3Aa1, Vip3Aa19, and Vip3Aa20.

Vip3Aa Source	Toxin Designation	Amino Acids	Position 129 [a]	Position 284 [a]
B. thuringiensis strain AB88	Vip3Aa1	789	M	K
COT102 cotton	Vip3Aa19	789	M	Q
MIR162 maize	Vip3Aa20	789	I	Q

[a] – M = methionine; I = isoleucine; K = lysine; Q = glutamine

Based on Syngenta laboratory bioassay results, the amino acid differences between these Vip3Aa variants do not impact insecticidal activity against target insect pests. The single amino acid difference between Vip3Aa19 and Vip3Aa20 (position 129) occurs outside of the protein tryptic core. Lee *et al.* (2003) have shown that full-length Vip3Aa is proteolytically activated to form a 66 kDa active core in midgut extracts of susceptible insects. N-terminal sequencing of the 66 kDa active core indicates that cleavage occurs at amino acid residue 199 (Estruch and Yu, 2001). This finding suggests that amino acid residue 129 does not play a role in defining species specificity of the toxin since it would be lost during proteolytic processing in the insect midgut.

V.A.2. Characterization of Vip3Aa20

A number of analytical methods were used to characterize the Vip3Aa20 protein produced in MIR162 maize. In order to conduct animal and environmental toxicology studies with the protein, significant quantities of Vip3Aa20 were produced in a recombinant *E. coli* fermentation system. Production and extraction of Vip3Aa20 from MIR162 maize is not a feasible approach for obtaining the large quantities of the protein needed for these safety studies. Biochemical and bioactivity analyses were conducted to establish the equivalence of plant- and microbially produced Vip3Aa20; the latter being produced in an *E. coli* fermentation system.

The Vip3Aa20 protein produced in MIR162 maize was characterized by its molecular weight, mobility in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), immunoreactivity, glycosylation status, and amino acid sequence. The amino acid sequence of full length Vip3Aa20 was deduced from the nucleotide sequence of the *vip3Aa20* gene found in MIR162 maize. Peptide mass mapping by matrix-assisted laser desorption ionization time of flight mass spectrometry confirmed the deduced amino acid sequence of Vip3Aa20. Based on its amino acid sequence, Vip3Aa20 has a predicted molecular weight of 88,647 Da. Western blot analysis of protein extracts from MIR162 leaf tissue using anti-

Vip3Aa antibodies revealed an immunoreactive band of approximately 89 kDa molecular weight, a size that is consistent with the deduced sequence for full-length protein (see Figure 19). The plant-produced protein was found not to be glycosylated. A more detailed description of the methods and results for the protein characterization analyses can be found in Appendix C.

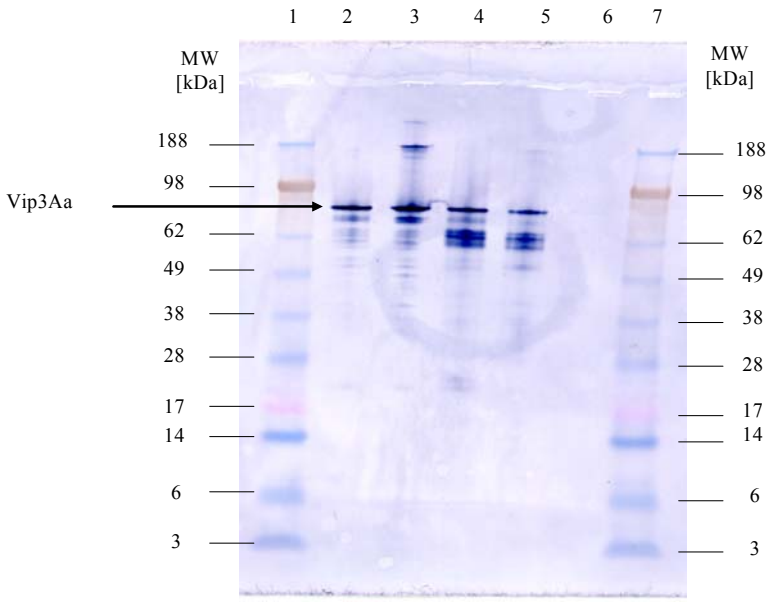

Figure 19. Western blot of plant- and microbially produced Vip3Aa proteins.
This Western blot shows the immunoreactivity of Vip3Aa19 from *E. coli*, Vip3Aa20 from *E. coli*, and Vip3Aa20 from MIR162 maize. The molecular weights of Vip3Aa19 and Vip3Aa20 correspond to approximately 89 kDa.

Lanes 1 and 7: molecular weight standard
Lane 2: 10 ng Vip3Aa19 from *E. coli*
Lane 3: 10 ng Vip3Aa20 from *E. coli*
Lane 4: 10 ng Vip3Aa20 from MIR162 maize leaf tissue extract
Lane 5: 10 ng immunoaffinity-purified Vip3Aa20 from MIR162 maize leaf tissue
Lane 6: protein extract from control maize leaf tissue.

These same analytical methods, plus an assessment of insecticidal activity, were used to compare plant-produced Vip3Aa20 to microbially produced protein. Similar analyses were performed to compare the biochemical properties and bioactivity of the Vip3Aa19 and Vip3Aa20 variants.

As shown in lanes 3, 4, and 5 of Figure 19, the electrophoretic mobility, molecular weight, and immunoreactivity of microbial Vip3Aa20 and plant Vip3Aa20 are essentially the same. N-terminal sequencing and peptide mass mapping of microbial Vip3Aa20 matched the amino acid sequence of plant Vip3Aa20. Neither microbial- nor plant-produced Vip3Aa20 was

found to be glycosylated. The efficacy and potency of the two proteins were found to be similar as measured by an assessment of lethality against first instar *S. frugiperda* larvae. A comparison of median lethal concentration (LC_{50}) values for each protein is shown in Table 13. Although a numeric difference in the computed LC_{50} values is observed, their overlapping confidence intervals indicate that the difference was not biologically significant.

Table 13. Comparative bioactivity of Vip3Aa proteins from different sources.
Results of mortality bioassays with first-instar *S. frugiperda* larvae are presented as LC_{50} values with their 95% confidence intervals. In the first set of bioassays, Vip3Aa20 from MIR162 maize leaf tissue was compared to Vip3Aa20 produced in *E. coli*. In the second set of bioassays, Vip3Aa20 from maize leaf tissue was compared to Vip3Aa19 produced in *E. coli*.

Test Substance	LC_{50} (ng protein/cm^2)	95% Confidence Intervals
Vip3Aa20 – MIR162 leaf extract	318 [a]	232 – 451
Vip3Aa20 – *E. coli*	225	155 – 289
Vip3Aa20 – MIR162 leaf extract	154 [a]	94 – 222
Vip3Aa19 – *E. coli*	137	82 – 199

[a] – no significance should be assigned to the apparent difference in LC_{50} values from the two assays with MIR162 leaf extract (318 *vs* 154). The assays were temporally separated, conducted with different leaf tissue extracts, and different batches of insect larvae.

Based on these results, the identities of plant- and microbially produced Vip3Aa20 proteins have been verified and it can be concluded that Vip3Aa20 proteins produced in recombinant *E. coli* and MIR162 maize are biochemically and functionally equivalent. Having established equivalence of the proteins, it was appropriate to utilize microbially produced Vip3Aa20 as a surrogate for the plant-produced protein in toxicology tests.

The biochemical and functional properties of Vip3Aa20 were compared to those of Vip3Aa19 in a separate set of experiments. This comparison was made because the Vip3Aa19 variant has been used as a surrogate test substance in environmental safety studies. These protein variants differ by only one amino acid. They are of comparable molecular weight, electrophoretic mobility, and immunoreactivity as determined by Western blot analysis. Neither protein is glycosylated. As shown in Table 13, a comparison of insecticidal activity results shows no biologically significant difference between the computed LC_{50} values for the Vip3Aa20 from MIR162 leaf tissue and Vip3Aa19 from *E. coli*.

V.A.3. Safety Assessment for Vip3Aa20

A detailed assessment of human and animal safety for the Vip3Aa20 protein has been provided to the FDA as part of the MIR162 premarket consultation. A toxicology data

package has also been submitted to EPA in support of a petition to exempt Vip3Aa20 residues from the requirement of a tolerance in food and feed commodities of all crops. A summary of the pertinent information and toxicology data bearing on the potential risk of Vip3Aa20 for humans is presented below:

- *B. thuringiensis* microbial insecticides that are commercially available and registered by EPA (*e.g.* Dipel® Biological Insecticide, Javelin® Biological Insecticide, and Condor® Bioinsecticide) have been found to contain Vip3Aa or Vip3Aa-like proteins.[3] Thus, there is a history of human and environmental exposure to Vip3Aa or Vip3Aa-like proteins without corresponding evidence of adverse effects.
- No adverse effects were observed in mice administered a single oral dose of Vip3Aa20 at 1250 mg/kg body weight. The no observable effect level (NOEL) was found to be ≥ 1250 mg/kg, which was the highest dose that could be physically administered to the animals. Mice have historically been a suitable surrogate species for detecting evidence of toxicity in mammals.
- The amino acid sequence of Vip3Aa20 has been systematically compared to the sequence of other proteins in the latest posting of the National Center for Biotechnology Information (NCBI) Entrez Protein Database (NCBI, 2007). This comparison determined if any proteins in the database showed significant amino acid sequence identity to Vip3Aa20, indicating they may be closely related to Vip3Aa20, and if any proteins with significant sequence identity to Vip3Aa20 are known to be toxins, indicating possible implications for the toxic potential of Vip3Aa20. The NCBI Entrez protein database was searched using the BLASTP program with Vip3Aa20 as the query sequence (Altschul *et al.,* 1997).[4] All database sequences with a conservative Expect value (E-value) of 10 or lower were identified by default by the BLASTP program. Comparisons between highly homologous proteins yield E-values approaching zero, indicating a very low probability that such matches would occur by chance. The probability that amino acid sequence similarities observed in a database search occur by chance increases with higher E-values (Ponting, 2001). The Vip3Aa20 query sequence showed no significant sequence identity to any proteins identified as, or known to be, toxins other than the vegetative insecticidal proteins from *B. thuringiensis*. Vip3Aa20 has no significant sequence homology to known mammalian protein toxins.
- The Vip3Aa20 amino acid sequence was compared to the Syngenta Allergen Database (version 4.0) to determine if it has significant sequence identity to proteins known or suspected to be allergens, indicating possible implications for the allergenic potential of Vip3Aa20. The Syngenta Allergen Database was compiled from entries identified as allergens or putative allergens in public protein databases, and was supplemented with additional amino acid sequences identified from the scientific literature as being known or putative allergens. The Syngenta Allergen Database

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[3] Vip3Aa-like indicates proteins contained in the specified products were bound by Vip3Aa antibodies in western blot analyses but differed in molecular weight from Vip3Aa.

[4] BLASTP version 2.2.6 was used with the following parameters: No complexity filter; expect score = 10; word size = 3; gap costs: existence = 11 and extension = 1. The similarity matrix was Blosum 62.

contained 1414 nonredundant entries at the time this search was performed. Sequential 80-amino acid peptides of the Vip3Aa20 protein sequence were compared to protein sequences in the database using the FASTA search algorithm (Pearson and Lipman, 1988). Any 80-amino acid peptide of the query sequence having greater than 35% amino acid identity to an allergen sequence was identified as having significant identity to the allergen sequence in accordance with the recommendations of FAO/WHO (2001). The results of this analysis revealed that there was no significant identity between any of the sequential 80-amino acid peptides of Vip3Aa20 and any entry in the Syngenta Allergen Database. Therefore, Vip3Aa20 does not share overall sequence homology with any known allergenic protein.

- The Vip3Aa20 protein sequence was also screened for matches of eight contiguous amino acids between the Vip3Aa20 sequence and the allergen sequences in the Syngenta Allergen Database to screen for short, local regions of amino acid identity that might indicate the presence of common T-cell binding epitopes (Hileman *et al*., 2002; Stadler and Stadler, 2003; Silvanovich *et al*., 2006). There were no matches of eight contiguous amino acids between Vip3Aa20 and any entry in the allergen database. No significant amino acid sequence identity of Vip3Aa20 to known or putative allergenic proteins having implications for allergenic potential was identified.
- The mode of insecticidal action for *B. thuringiensis* Cry and Vip proteins is not operative in mammalian species because they do not possess the epithelial cell receptors for binding of the proteins.
- Vip3Aa20 protein was degraded within two minutes in simulated gastric fluid containing pepsin. Thus, there will be minimal opportunity for human and domestic animal systemic exposure to Vip3Aa20.
- Vip3Aa20 protein degrades upon exposure to elevated temperatures (≥ 65°C); thus, it will degrade at temperatures used in maize cooking and processing.

Collectively, the results of these studies indicate that the Vip3Aa20 protein present in MIR162 maize presents no risk of harm for mammalian species.

V.B. Phosphomannose Isomerase – Origin and Function

MIR162 maize plants express the *manA* gene from *E. coli* strain K-12 (Miles and Guest, 1984). This gene encodes the enzyme PMI that catalyzes the reversible conversion of mannose 6-phosphate and fructose 6-phosphate and enables many microorganisms to utilize mannose as a carbon and energy source. Many plant species do not possess genes that encode PMI enzymes.

V.B.1. Mechanism of Action

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

cells. Inclusion of the *manA* gene in the T-DNA of transformation plasmid pNOV1300 allowed for selection of successfully transformed maize plantlets.

V.B.2. Characterization of Phosphomannose Isomerase

Western blot analysis was used to characterize the PMI enzyme produced in MIR162 maize. In order to conduct animal toxicology studies with the protein, significant quantities of PMI were produced in a recombinant *E. coli* fermentation system. Production and extraction of PMI from MIR162 maize is not a feasible approach for obtaining the large quantities of the protein needed for these safety studies. Biochemical and bioactivity analyses were conducted to establish the equivalence of plant- and microbially produced PMI.

PMI from MIR162 maize was found to be approximately 42.8 kDa molecular weight which matched the predicted molecular weight based on the amino acid sequence deduced from having sequenced the *manA* gene in the T-DNA. The observed molecular weight for PMI produced in the recombinant *E. coli* system was approximately 44.4 kDa. The microbial protein contained an additional 16 nonfunctional amino acids at the N-terminus which were added for purification purposes (13 amino acid T7-Tag and three additional amino acids arising from the polylinker). Both proteins reacted with the same anti-PMI antibodies. Specific activity was measured for both sources of the enzyme. PMI extracted from MIR162 maize leaf had a specific activity of 55.5 U/mg and the microbial enzyme had a specific activity of 33.2 U/mg. Both of these unit activity measurements fell within the range of historically observed values at Syngenta.

Based on these results, it can be concluded that PMI from MIR162 maize and PMI from *E. coli* are biochemically and functionally equivalent. A more detailed description of the methods and results for this protein characterization work can be found in Appendix C.

V.B.3. Safety Assessment for Phosphomannose Isomerase

An assessment of human and animal safety of the PMI protein has been provided to the FDA as part of the MIR162 premarket consultation. A toxicology data package has been submitted to EPA and supported establishment of an exemption of PMI residues from the requirement of a tolerance in food and feed commodities of all crops (40 CFR §174.527). A summary of the pertinent information and toxicology data bearing on the potential risk of PMI for humans is presented below:

- PMI enzymes are ubiquitous in nature. PMI is a functional enzyme in *E. coli*, a common contaminant of food and feed. The enzyme is also found in a wide variety of other species. Thus, there is a history of safe consumption of PMI proteins in human food and animal feed sources.
- No adverse effects were observed in mice administered a single oral dose of PMI at 3030 mg/kg body weight. The NOEL was found to be ≥ 3030 mg/kg, which was the highest dose that could be physically administered to the animals. Mice have historically been a suitable surrogate species for detecting evidence of toxicity in mammals.

- The amino acid sequence of PMI has been compared to the sequence of other proteins in a comprehensive sequence database. This sequence comparison was conducted following the methodology described above for Vip3Aa20. PMI was found to have no significant sequence homology to known protein toxins.
- PMI is derived from a donor organism (*E. coli*) that is not known to be a source of allergenic proteins and it possesses no significant amino acid sequence identity to known or putative allergenic protein sequences that are biologically relevant or that have implications for allergenic potential. This sequence comparison was conducted following the methodology described above for Vip3Aa20. There was one region of sequence homology of eight contiguous identical amino acids between PMI and a known allergen, α-parvalbumin from *Rana* species CH2001 (unidentified edible frog) (Hilger *et al.,* 2002). Further investigation using sensitive serum screening methodology (CODEX, 2003) demonstrated no cross-reactivity between PMI and the serum from the single individual known to have demonstrated IgE-mediated allergy to this specific α-parvalbumin. The patient's serum did not recognize any portion of the PMI protein as an allergenic epitope. Therefore, the sequence identity between PMI and the α-parvalbumin from *Rana* species CH2001 is not biologically meaningful and has no implications for the potential allergenicity of PMI.
- PMI protein is degraded within seconds in simulated gastric fluid containing pepsin. Thus, there will be minimal opportunity for human or domestic animal systemic exposure to PMI.
- PMI protein degrades upon exposure to elevated temperatures (≥ 65°C); thus, it will degrade at temperatures used in maize cooking and processing.

Collectively, the results of these studies indicate that the PMI protein present in MIR162 maize presents no risk of harm for mammalian species.

V.C. Vip3Aa20 and PMI Levels in MIR162 Maize

Concentrations of the Vip3Aa20 and PMI proteins in plant tissues from two MIR162 hybrids were determined using an enzyme-linked immunosorbent assay (ELISA). Plant tissues were collected from two locations at four stages of growth: V9-V12, anthesis, seed maturity, and senescence. The materials and methods for these ELISA experiments are described in Appendix D.

Vip3Aa20 and PMI concentrations on both a fresh-weight and dry-weight basis were determined and are reported in Tables 14 and 15, respectively. No meaningful differences between the two hybrids were noted in their concentrations of Vip3Aa20 and PMI in each tissue type at each growth stage sampled. Therefore, values presented in the tables are means of the two hybrids. Vip3Aa20 and PMI concentrations in the conventional maize tissues were less than the limit of detection or limit of quantification.

Vip3Aa20 fresh weight levels in leaf, pith, and whole plant were highest at seed maturity. Levels in root tissue were relatively constant throughout the plant's life cycle. These fresh-weight values are used in computing expected environmental concentrations for risk assessment purposes (see Section VII.C.3).

Table 14. Mean tissue concentrations of Vip3Aa20 in MIR162 plants.
Concentrations are presented on both a fresh tissue and dry weight basis. All ELISA results have been corrected for method extraction efficiency. Vip3Aa20 concentrations in control plant tissues were all <LOD or <LOQ. [a] Not all tissues were analyzed at all growth stages.

Tissue Type	V9-V12	Anthesis	Seed Maturity	Senescence
Vip3Aa20 µg/g fresh weight (Range)				
Leaves	17.63 (13.11 – 22.35)	24.44 (21.08 – 29.07)	50.41 (35.85 – 60.92)	13.40 (8.87 – 18.20)
Roots	5.23 (3.99 – 7.07)	4.32 (4.18 – 4.69)	4.81 (2.27 – 6.60)	5.29 (4.61 – 5.82)
Pith	N/A	3.54 (3.17 – 4.16)	11.47 (10.21 – 12.75)	N/A
Kernels	N/A	N/A	29.81 (27.78 – 34.13)	28.65 (25.06 – 32.42)
Silk	N/A	12.55 (8.05 – 18.91)	N/A	N/A
Pollen [b]	N/A	43.21 (37.42 – 49.70)	N/A	N/A
Whole Plants	11.98 (8.96 – 15.39)	12.16 (11.51 – 12.97)	20.84 (15.54 – 25.98)	17.35 (13.24 – 24.07)
Vip3Aa20 µg/g dry weight (Range)				
Leaves	97.26 (76.12 – 119.12)	107.74 (97.10 – 118.80)	121.79 (77.25 – 159.66)	21.32 (12.93 – 30.28)
Roots	31.80 (28.10 – 35.65)	28.34 (26.30 – 30.20)	20.29 (9.87 – 27.48)	21.66 (11.58 – 32.13)
Pith	N/A	31.71 (29.43 – 36.18)	58.21 (52.74 – 63.68)	N/A
Kernels	N/A	N/A	43.56 (40.47 – 50.50)	34.25 (30.90 – 37.67)
Silk	N/A	97.40 (60.54 – 149.00)	N/A	N/A
Pollen	N/A	47.13 (41.45 – 53.52)	N/A	N/A
Whole Plants	91.53 (88.68 – 96.51)	67.61 (61.68 – 72.63)	49.04 (34.84 – 63.14)	34.30 (21.12 – 55.17)

[a] - LOQ = limit of quantification; LOD = limit of detection; N/A = not analyzed
[b] - pollen was analyzed as received, air-dried overnight

Table 15. Mean tissue concentrations of PMI in MIR162 plants.
Concentrations are presented on both a fresh tissue and dry weight basis. All ELISA results have been corrected for method extraction efficiency. PMI concentrations in control plant tissues were all <LOD or <LOQ. [a] Not all tissues were analyzed at all growth stages.

Tissue Type	V9-V12	Anthesis	Seed Maturity	Senescence
PMI µg/g fresh weight (Range) [b]				
Leaves	2.02 (1.42 – 3.14)	2.18 (1.69 – 3.06)	2.44 (1.94 – 3.50)	< 0.17 (<LOD – <LOQ)
Roots	0.69 (0.43 – 0.90)	0.54 (0.36 – 0.83)	0.48 (0.25 – 0.74)	0.36 (0.19 – 0.49)
Pith	N/A	0.23 (0.17 – 0.27)	0.54 (0.47 – 0.62)	N/A
Kernels	N/A	N/A	1.32 (0.92 – 1.71)	0.63 (0.46 –0.79)
Silk	N/A	2.68 (1.68 – 3.45)	N/A	N/A
Pollen [c]	N/A	4.87 (3.45 – 7.07)	N/A	N/A
Whole Plant	1.15 (0.76 – 1.56)	1.28 (1.18 – 1.32)	1.60 (0.96 – 2.21)	1.22 (1.11 – 1.35)
PMI µg/g dry weight (Range) [b]				
Leaves	11.12 (8.26 – 16.76)	9.75 (6.92 – 14.68)	5.78 (4.57 – 7.55)	< 0.26 (<LOD – <LOQ)
Roots	4.32 (3.17 – 7.08)	3.49 (2.51 – 5.22)	2.00 (1.08 – 3.09)	1.51 (0.47 – 2.53)
Pith	N/A	2.02 (1.53 – 2.40)	2.75 (2.36 – 3.19)	N/A
Kernels	N/A	N/A	1.93 (1.33 – 2.54)	0.75 (0.54 –0.97)
Silk	N/A	20.70 (12.60 – 27.16)	N/A	N/A
Pollen	N/A	5.30 (3.82 – 7.62)	N/A	N/A
Whole Plants	8.74 (7.49 – 9.75)	7.10 (6.32 – 7.61)	3.77 (2.16 – 5.37)	2.36 (1.85 – 3.09)

[a] - LOQ = limit of quantification; LOD = limit of detection; N/A = not analyzed
[b] - mean values preceded by '<' indicate that the LOQ or LOD was used for some samples in calculating the mean
[c] - pollen was analyzed as received, air-dried overnight

VI. Phenotypic and Compositional Evaluation

Laboratory, greenhouse, growth chamber, and field investigations have been conducted to assess the phenotype and biochemical composition of MIR162 maize. The purpose of these investigations was to determine if unintended changes occurred in MIR162 maize as a result of the transformation process and to determine if any unintended changes conveyed a plant pest risk potential to the new cultivar. For each parameter evaluated in a MIR162 hybrid, a comparable set of data were collected from a genetically matched nontransgenic hybrid.

VI.A. Phenotypic Assessment of MIR162 Plants

A range of phenotypic parameters assessing seed germination and dormancy, growth characteristics, reproductive capability, seed dispersal, and interactions with biotic and abiotic stressors have been examined for MIR162 and control plants. Table 16 provides a listing of the parameters evaluated. The purpose of these evaluations was to ascertain whether or not the transformation that created MIR162 maize has imparted some phenotypic characteristic on cultivars that would make it undesirable to growers, for example, a reduction in yield potential, or that could increase the new cultivars' persistence or result in invasive characteristics. These latter characteristics could be considered indicative of increased weediness potential and plant pest risk. If no significant differences are found between MIR162 and control hybrids it can be concluded that the transformation has not increased the new cultivars' plant pest potential.

Table 16. Phenotypic characteristics evaluated for MIR162 maize hybrids. Evaluations were conducted in laboratory, greenhouse, growth chamber, or field experiments.

Phenotypic Characteristic	Variable Measured	Timing	Description
Dormancy / Germination	Dormancy and germination	After 4, 7, and 12 days	Percent normal germinated, abnormal germinated, dormant, dead, and viable firm swollen seed
Emergence	Emerged plants	14 days post-planting	Number of emerged plants per plot
Vegetative Growth	Early growth rating	Stage V6	Growth rating
	Green snapped plants	Prior to anthesis	Percent of plants per plot broken due to adverse environmental conditions
	Root lodged plants	After anthesis	Percent of plants leaning >30° from vertical
	Ear height	Stage R2-R6	Height from plant base to node where ear connects to stalk (cm)
	Plant height	Stage R2-R6	Height from plant base to collar of flag leaf (cm)
	Leaf color rating	Stage R4-R6	Color rating: 5=same as commercial check, 1=darker, 9=severely chlorotic

(continued)

Table 16 (cont.). Phenotypic characteristics evaluated for MIR162 maize.
Evaluations were conducted in laboratory, greenhouse, growth chamber, or field experiments.

Phenotypic Characteristic	Variable Measured	Timing	Description
Vegetative Growth	Late season intactness	Prior to harvest	Plant integrity above the ear: 1=all plant parts intact at harvest, 9=all plants broken at the ear node prior to harvest
	Stalk lodging	Harvest	Percent of plants per plot broken below the ear
	Stay green rating	Harvest	Percent staygreen at harvest as measure of late season plant health
	Population count	Harvest	Extrapolated estimate of plant population per acre
Reproductive Growth	Heat units to 50% pollen shed	Pollen shed	Heat units to 50% pollen shed
	Pollen viability	Pollen shed	Percent viable pollen based on staining characteristics
	Pollen morphology	Pollen shed	Diameter (μm) of viable pollen grains
	Heat units to 50% silking	Silk emergence	Heat units to 50% silk emergence
	Barren plants	Harvest	Percent of plants per plot that do not develop an ear
	Grain moisture	Harvest	Percent grain moisture
	Test weight	Harvest	Grain test weight (lb/bu) converted to 15.5% moisture
	Yield	Harvest	Grain yield (bu/ac) converted to 15.5% moisture
Seed Retention	Dropped ears	Prior to harvest	Percent of plants per plot that dropped a developed ear
Plant-ecological Interactions	Disease susceptibility	Planting to harvest	Observations of disease occurrence

VI.A.1. Seed Dormancy and Germination

Enhanced germination or seed dormancy are characteristics that can be indicative of plant pest potential. Dormancy mechanisms, including hard seed, vary with species and are generally complex processes. Seed dormancy is not a characteristic of maize. A laboratory study was conducted to evaluate the germination and dormancy characteristics of MIR162 seed using a modification of the testing protocol established by the Association of Official Seed Analysts

Seed samples of two MIR162 hybrids, their respective near-isogenic controls, and three

conventional hybrids served as test, control, and reference materials for the study. Table 17 shows the genotypes of the test, control, and reference hybrids (see also Table 2 and Figure 5). MIR162 hybrid #1 corresponds to seed lot code 'D' and MIR162 hybrid #2 corresponds to seed lot code 'E' as described in Table 2 and Figure 5.

Table 17. Test, control, and reference seeds used in germination and dormancy test.

Hybrid Designation	Seed Generation	Lineage or Variety
MIR162 hybrid #1	F_1	NP2391 x NP2222(BC_6F_4)
Control hybrid #1	F_1	NP2391 x NP2222
MIR162 hybrid #2	F_1	NP2222(BC_6F_5) x NP2391
Control hybrid #2	F_1	NP2222 x NP2391
Reference hybrid #1	F_1	N59-Q9
Reference hybrid #2	F_1	N72-G8
Reference hybrid #3	F_1	N36-J2

The study design followed that described by AOSA (2005) with the addition of four temperature regimes to assess germination and dormancy characteristics of the maize seed under nonoptimal conditions. Seed lots were divided into four replicates of 40 seeds per replicate per temperature regime. Six temperature regimes were utilized:

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

Experiments were conducted in unlighted temperature-controlled growth chambers. For the alternating temperature regimes, the lower temperature was maintained for 16 hours and the higher temperature for eight hours, and then the cycle was repeated. The study was initiated by rolling 40 seeds in moistened germination towels and then placing the rolled towels into the growth chambers.

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

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

For the tests carried out at 5°C, 10°C, 25°C, 5/20°C, and 10/20°C, each germination towel

was carefully unrolled and examined four, seven, and 12 days after study initiation. Each seed was examined and categorized as one of the following:

- normal germinated (seed with a radicle protruding beyond the seed coat)
- abnormal germinated (seed lacked a well-developed root and shoot, or possessed a hollow coleoptile, or exhibited mechanical damage)
- dead seed (seed not germinated and visibly deteriorated and soft to the touch)
- firm swollen seed (seed visibly swollen and firm to the touch)
- hard seed (seed that had not imbibed water and was firm to the touch)

At the end of each experiment, all firm swollen and hard seeds were subjected to a tetrazolium test to evaluate their viability (AOSA, 2000). Seeds subjected to the tetrazolium test were then categorized as 'viable firm swollen', 'viable hard', or 'dead' (nonviable firm swollen and nonviable hard).

The results of these germination and dormancy experiments are summarized in Table 18. Inspection of the data revealed a high frequency of zero response rates for the endpoints being measured. For example, only one of 28 replicates gave a response rate above zero for percent normal germinated at 5°C. At the higher temperatures, there was a high frequency of 100% normal germinated seeds among the replicates. These data are not considered suitable for analysis of variance because they do not satisfy the assumptions upon which the validity of an analysis of variance depends. Instead, the data for each MIR162 and control hybrid were combined across replicates and assembled into 2x2 contingency tables and subjected to Fisher's Exact Test. Significance was assigned at $p<0.05$. Only the results for 'normal germinated' seeds were statistically analyzed.

There were no statistically significant differences in the proportions of normal germinated MIR162 and control seeds. Table 19 presents the probability values for each of the contingency table analyses. No viable hard seeds were found for any of the hybrids tested under any temperature regime, thus confirming that dormancy is not a normal characteristic of maize seeds and that MIR162 maize demonstrates no increase in seed dormancy potential.

Table 18. Summary of seed germination and dormancy frequencies.
Mean germination responses of four replicates from test, control, and reference hybrids evaluated under different temperature regimes. No significant differences were observed between MIR162 and control proportions of normal germinated seeds ($p<0.05$).

Temp (°C)	Hybrid Genotype	Normal Germinated (%)	Abnormal Germinated (%)	Viable Firm Swollen (%)	Viable Hard (%)	Dead (%)
5	MIR162 #1	0.0	0.0	95.6	0.0	4.4
	Control #1	0.0	0.0	96.3	0.0	3.8
	MIR162 #2	0.0	0.0	94.4	0.0	5.6
	Control #2	0.6	0.0	96.3	0.0	3.1
	Reference Range	0.0 – 0.0	0.0 – 0.0	90.6 - 96.3	0.0	3.8 - 9.4
10	MIR162 #1	98.1	0.0	0.0	0.0	1.9
	Control #1	97.5	0.0	2.5	0.0	0
	MIR162 #2	94.4	0.0	4.4	0.0	1.3
	Control #2	98.8	0.0	0.0	0.0	1.3
	Reference Range	90.6 - 98.1	0.0 – 0.0	0.0 - 8.6	0.0 - 0.0	0.6 - 1.9
5/20	MIR162 #1	97.5	0.0	0.6	0.0	1.9
	Control #1	99.4	0.0	0.0	0.0	0.6
	MIR162 #2	95.6	0.0	1.9	0.0	2.5
	Control #2	98.6	0.0	0.6	0.0	0.6
	Reference Range	91.9 - 100	0.0 - 0.0	0 - 5.6	0.0 - 0.0	0 - 2.5
10/20	MIR162 #1	100.0	0.0	0.0	0.0	0.0
	Control #1	99.4	0.0	0.0	0.0	0.6
	MIR162 #2	97.5	0.0	1.9	0.0	0.6
	Control #2	99.4	0.0	0.0	0.0	0.6
	Reference Range	97.5 - 99.4	0.0 - 0.0	0 - 0.6	0.0 – 0.0	0.6 - 1.9
25	MIR162 #1	100.0	0.0	0.0	0.0	0.0
	Control #1	98.1	1.3	0.0	0.0	0.6
	MIR162 #2	99.4	0.6	0.0	0.0	0.0
	Control #2	96.3	1.9	0.0	0.0	1.9
	Reference Range	98.1 - 99.4	0 – 1.3	0 - 0.6	0.0 – 0.0	0.0 - 0.6
20/30	MIR162 #1	98.1	0.6	0.0	0.0	1.3
	Control #1	98.1	0.6	0.0	0.0	1.3
	MIR162 #2	96.3	1.9	0.0	0.0	1.9
	Control #2	99.4	0.6	0.0	0.0	0.0
	Reference Range	97.5 - 99.4	0.6 - 1.3	0.0 – 0.0	0.0 – 0.0	0.0 - 1.3

Table 19. Statistical analysis of proportions for normal germinated seeds.
Fisher's Exact Test was used to compare the proportion of MIR162 and control seeds classified as normally germinated versus not normally germinated. Probability values for each comparison are presented in the table.

Temperature (°C)	*p* values	
	MIR162 #1 *vs* Control #1	**MIR162 #2 *vs* Control #2**
5	1.000	1.000
10	1.000	0.061
5/20	0.371	0.174
10/20	1.000	0.371
25	0.248	0.121
20/30	1.000	0.121

VI.A.2. Field Agronomic Performance

Small field trials were conducted during the 2005 and 2006 growing seasons to compare a range of agronomic characteristics of two MIR162 maize hybrids to near-isogenic nontransgenic commercial varieties. Table 20 shows the pedigree of the MIR162 and control hybrids planted in these trials (see also Table 2 and Figure 5). The MIR162 hybrid used in 2005 trials corresponds to seed lot code 'F' and the MIR162 hybrid used in 2006 trials corresponds to seed lot code 'I' as described in Table 2 and Figure 5.

Table 20. Test and control hybrids planted in field agronomic equivalence trials.

Trial Year	Hybrid Designation	Seed Generation	Lineage or Variety
2005	MIR162 hybrid	F_1	NP2010 x NP2222(BC_6F_4)
	Control hybrid	F_1	NP2222 x NP2010
2006	MIR162 hybrid	F_1	NP2673 x NP2171(BC_4F_5)
	Control hybrid	F_1	NP2673 x NP2171

Near-isogenic hybrid pairs were grown in six locations in 2005 and 10 locations in 2006. These locations are representative of the major maize growing regions of the U.S. and are listed in Table 21.

Table 21. Locations and dates of MIR162 agronomic performance trials.

Trial Year	City	State	Planting Date	Harvest Date	APHIS Notification #
2005	Alleman	Iowa	June 20	October 26	05-062-02n
	Bloomington	Illinois	May 28	October 10	
	Hudson	Illinois	May 28	October 19	
	Mackinaw	Illinois	May 28	October 19	
	Seward	Nebraska	June 15	October 28	
	Wapella	Illinois	May 27	October 17	
2006	Bloomington	Illinois	May 28	October 10	06-055-08n
	Brookings	South Dakota	May 31	October 30	
	El Paso	Illinois	June 3	October 24	
	Gaylord	Minnesota	May 31	October 26	
	Janesville	Wisconsin	June 1	October 29	
	Mackinaw	Illinois	May 29	October 23	
	Maxwell	Iowa	June 6	October 30	
	Monroeville	Indiana	May 30	October 28	
	Sadorus	Illinois	June 5	October 22	
	Seward	Nebraska	June 1	October 7	

Trials were planted in a randomized complete block design. The trials planted in 2005 included four replicates per location and trials planted in 2006 included five replicates per location. Plot size was 0.02 acres, using two-row plots, 17.5 ft long, with 30-inch spacing between the rows. Each plot contained approximately 68 plants. The agronomic characteristics assessed and the timing of each assessment are listed in the Table 16 above.

The agronomic data sets were subjected to an analysis of variance across locations using the following model:

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

Y_{ijk} is the observed response for entry i at location j block k; U is the overall mean; T_i is the entry effect, L_j is the location effect, $B(L)_{jk}$ is the effect of block within a location; LT_{ij} is the location-by-entry interaction effect and e_{ijk} is the residual error. For each variate, the statistical significance of the genotype effect for each of the hybrids was determined using a standard F-test. An F-test probability of <0.05 indicates that the difference observed between

the two genotypes was statistically significant at the customary 5% level.

The data for several variates were not subjected to formal statistical analysis because they did not satisfy the assumptions upon which the validity of an analysis of variance depends. In some cases, the data were too discrete to be considered normally distributed, with values taking one of a very limited range of options. In other cases, the data sets contained too few nonzero entries upon which to compute a reasonable estimate of residual error.

Results of the 2005 agronomic equivalence trials are presented in Table 22. Evaluation of the 2005 trial results revealed only one statistically significant difference between a control and MIR162 variate. The average number of germinated plants per plot was slightly higher in the 2005 MIR162 plots compared to control plots; the difference was only 3.2%. This difference is considered to be of no biological significance because the effect was not repeated in the 2006 trials and there was no effect of genotype observed in the seed germination and dormancy study (see Tables 18 and 19). No other differences between MIR162 and control plots were observed for the wide range of phenotypic endpoints assessed in the 2005 trials.

Results of the 2006 agronomic equivalence trials are presented in Table 23. Evaluation of the 2006 trial results revealed two statistically significant differences between MIR162 and control mean values. Grain moisture at harvest was 3.8% lower from the MIR162 plots and grain test weight was 1.4% lower from the MIR162 plots. Both of these differences are of very small magnitude and are inconsequential. There was no difference in yield observed between the MIR162 and control plots and there was no effect observed on either variate in the 2005 trials. These differences represent random variation and are of no biological significance.

The results of these phenotypic assessments indicate that MIR162 maize is not phenotypically different from conventional maize with respect to characteristics that would increase its weediness potential.

Table 22. Results of agronomic performance trials conducted in 2005.
Trials were planted with a mid-maturity MIR162 hybrid and a near-isogenic control. Trials were conducted at multiple locations representative of the major maize-growing areas of the U.S. Mean values across locations are presented in the table for each variate measured. Not all traits were evaluated at all locations.

Trait	Locations	Control $\bar{X}$	MIR162 $\bar{X}$	Δ	*p*-value	Significance [a]
Emerged plants (#)	6	62	64	2.0	0.04	*
Early growth rating	5	4.1	4.3	0.2		NA
Green snapped plants (%)	1	0	0	0		NA
Late root lodging (%)	6	4.0	2.0	-2.0		NA
Ear height (cm)	6	97.0	96.0	-1.0	0.62	NS
Plant height (cm)	6	228.0	227.0	-1.0	0.82	NS
Leaf color rating	5	5.0	5.0	0		NA
Late season intactness rating	4	3.1	3.3	0.2		NA
Stalk lodging (%)	6	0.7	0.9	0.2		NA
Stay green rating (%)	3	67.0	66.0	-1.0	0.66	NS
Plant population at harvest (plants/ac)	6	30,617	30,887	270	0.29	NS
Heat units to 50% pollen shed	5	1,378.0	1,386.0	8.0	0.45	NS
Heat units to 50% silking	5	1,428.0	1,438.0	10.0	0.18	NS
Barren plants (%)	6	0	0	0		NA
Grain moisture at harvest (%)	6	20.0	20.0	0.0	0.38	NS
Test weight (lb/bu)	6	56.7	55.8	-0.9	0.19	NS
Yield (bu/ac)	6	175.5	184.9	9.4	0.07	NS
Dropped ears	2	0	0	0		NA
Grey leaf spot rating	4	4.9	6.3	1.4		NA

[a] * - difference is significant; NS – difference is not significant; NA – data not statistically analyzed

Table 23. Results of agronomic performance trials conducted in 2006.
Trials were planted with a mid-maturity MIR162 hybrid and a near-isogenic control. Trials were conducted at multiple locations representative of the major maize-growing areas of the U.S. Mean values across locations are presented in the table for each variate measured. Not all traits were evaluated at all locations.

Trait	Locations	Control $\bar{X}$	MIR162 $\bar{X}$	Δ	*p*-value	Significance [a]
Emerged plants (#)	8	62.1	63.5	1.4	0.13	NS
Early growth rating	8	3.1	3.0	-0.1		NA
Late root lodging (%)	7	0.4	0.2	-0.2		NA
Ear height (cm)	9	105.9	106.0	0.1	0.96	NS
Plant height (cm)	9	236.8	236.3	-0.5	0.82	NS
Late season intactness rating	9	4.9	5.4	0.5		NA
Stalk lodging (%)	10	2.4	3.5	1.2	0.21	NS
Plant population at harvest (plants/ac)	10	31,005.4	31,383.8	378.4	0.24	NS
Heat units to 50% pollen shed	8	1,242.1	1,245.9	3.8	0.32	NS
Heat units to 50% silking	8	1,254.2	1,262.8	8.6	0.07	NS
Barren plants (%)	5	0.1	0.1	0		NA
Grain moisture at harvest (%)	10	18.3	17.6	-0.7	0.0001	*
Test weight (lb/bu)	8	57.4	56.6	-0.8	0.03	*
Yield (bu/ac)	10	176.4	178.4	2.2	0.51	NS
Dropped ears	2	0.3	0.5	0.2		NA
Grey leaf spot rating	4	3.7	3.9	0.2		NA

[a] * - difference is significant; NS – difference is not significant; NA – data not statistically analyzed

VI.A.3. Pollen Viability and Morphology

Viability and morphology were evaluated to assess the potential impact of transformation on pollen characteristics of MIR162 maize. Pollen morphology and viability were investigated by microscopically examining pollen grains that had been fixed and stained according to the method described by Pedersen *et al.* (2004).

Twenty MIR162 hybrid plants and 20 near-isogenic control plants were grown in an environmentally controlled greenhouse. The greenhouse operated on a 16 hr/8 hr light/dark cycle with daytime temperatures ranging from 23°C-28°C and nighttime temperatures ranging from 18°C-22°C. Pollen was collected from each plant 69 or 70 days after planting. Pollen samples were immediately fixed in a 70% (v/v) ethanol solution and refrigerated. Samples were stained with a small volume of a KI/I_2 solution and examined by light microscopy. Viability determinations were made at 50X magnification. Starch contained in the viable pollen cells readily bound to the iodine stain. Nonviable cells with little or no starch content were weakly stained and readily identifiable. Percent viability was determined by examining a minimum of 100 pollen cells per sample. Mean percent viable pollen was determined for the MIR162 and control samples. These means were compared by a t-test with significance assigned at the standard $p<0.05$ level. The results of this analysis are presented in Table 24. There was no significant difference detected between MIR162 and control percent viable pollen. In fact, the frequency of nonviable cells averaged less than 1% in both MIR162 and control samples.

Table 24. Pollen viability and diameter measurements.
Mean ($\bar{X}$) percent pollen viability and mean diameter measurements are presented with their respective standard error of the mean (SEM). The control and MIR162 means were compared by a t-test; no significant differences were detected.

Variable	Control $\bar{X}$ (SEM)	MIR162 $\bar{X}$ (SEM)
Pollen viability (%)	99.71 (0.133)	99.74 (0.094)
Pollen diameter (μm)	96.8 (0.734)	97.2 (0.583)

Cell morphology and the dimension of stained pollen samples from five MIR162 and five control plants were examined at 80X magnification. Morphology was assessed by a visual examination of all cells in the field of view. Pollen diameter was measured on ten cells per sample. Mean diameter was computed for the MIR162 and control samples. These means were compared by a t-test with significance assigned at the $p<0.05$ level. Photographs of representative MIR162 and control pollen samples are shown in Figure 20. Cell diameter statistics are presented in Table 24. There were no readily discernible differences in MIR162 and control pollen morphology. No significant difference in average cell diameter was detected between MIR162 and control pollen samples.

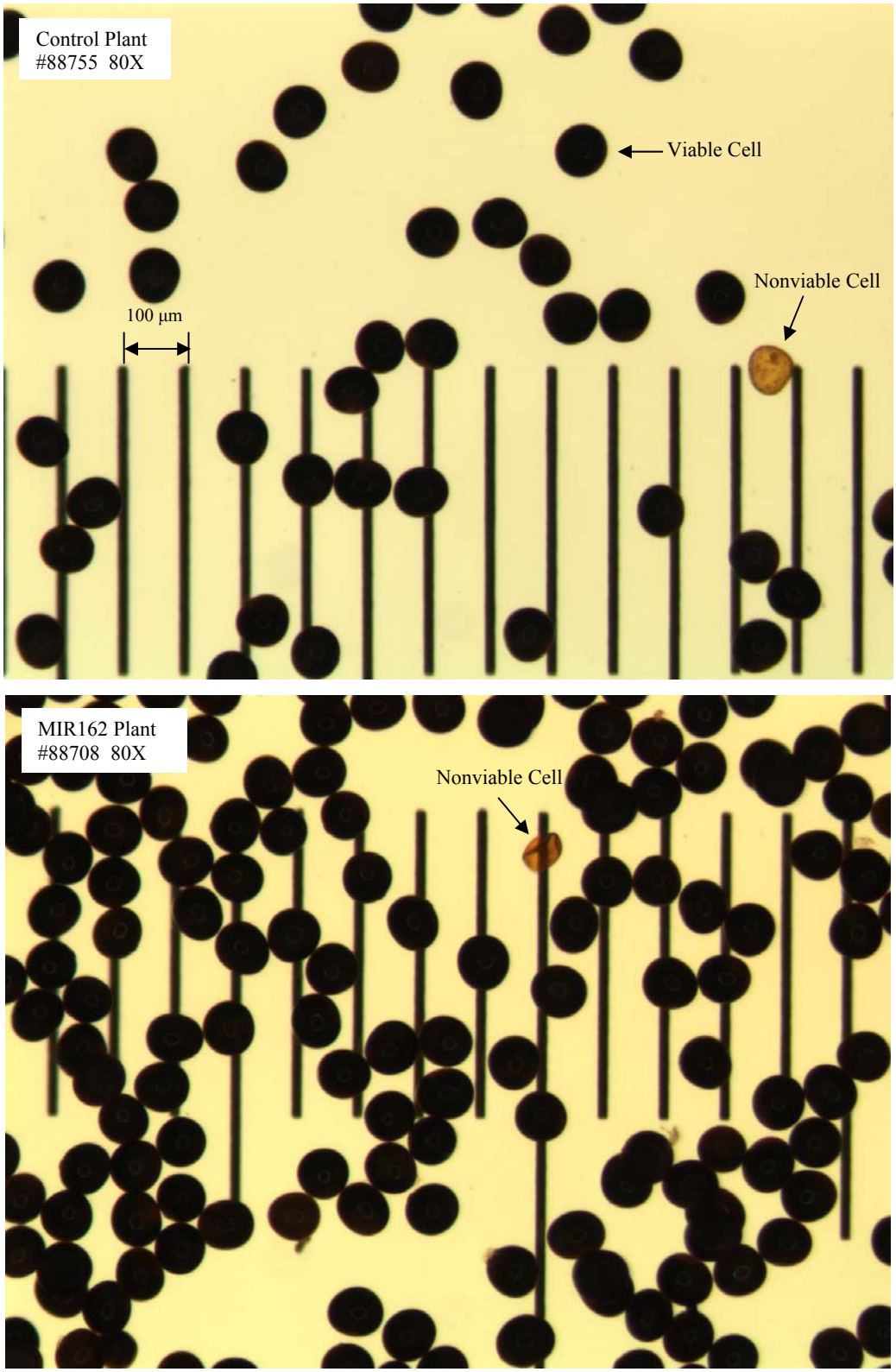

Figure 20. Photographs of stained pollen collected from control and MIR162 plants. Magnification was set at 80X.

VI.A.4. Seed Dispersal

Dispersal of individual maize seeds does not occur naturally. The kernels are held inside the husks of the cob and are too heavy to be wind-blown. There was no significant difference in the propensity of MIR162 maize to drop ears than that of conventional maize (see Tables 22 and 23). Dispersal of individual kernels does, however, take place as a result of mechanical harvesting and transportation. In this regard, MIR162 maize kernels would be no different than conventional maize kernels. Since maize seeds lack dormancy, those that are dispersed outside of cultivated fields would germinate and the young plants could be exposed to harsh winter conditions and die.

VI.B. Compositional Assessment of MIR162 Forage and Grain

Compositional analyses of MIR162 maize were performed in order to identify any changes in nutrient or anti-nutrient content of the new crop in the context of its use as food or feed and to assess its biochemical equivalence and familiarity to conventional maize. This assessment was undertaken by performing quantitative analyses of 65 components of MIR162 hybrid maize forage and grain. An identical set of analyses was performed on a nontransgenic control variety.

Forage and grain from a MIR162 transgenic hybrid and its corresponding near-isogenic control were harvested from six locations in the U.S. during 2005. These locations are representative of the major corn growing regions of the U.S. At each location, the hybrids were planted in a randomized complete block design, with three replicates for each genotype. All plots were managed according to local agronomic practices for the respective regions. Plants were self-pollinated by hand and the developing ears were bagged to avoid cross-pollination.

The analytes measured in this study were selected based on recommendations of the Organisation for Economic Co-operation and Development (OECD, 2002) for comparative assessment of composition of new varieties of maize. The components analyzed are listed in Table 25.

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

The analytical results for each component were subjected to an analysis of variance using the model:

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

where Y_{ijk} is the observed response for genotype i at location j block k, U is the overall mean, T_i is the genotype effect, L_j is the location effect, $B(L)_{jk}$ is the effect of block within a location, LT_{ij} is the location-by-genotype interaction effect and e_{ijk} is the residual error.

Table 25. Forage and grain components measured in MIR162 and conventional maize.

Forage	Grain		
Minerals	**Amino acids (18)**	**Proximates**	**Secondary Metabolites**
Calcium	**Anti-nutrients**	Acid detergent fiber	Ferulic acid
Phosphorus	Phytic acid	Ash	ρ-Coumaric acid
Proximates	Raffinose	Carbohydrates	Furfural
Acid detergent fiber (ADF)	Trypsin inhibitor	Fat	Inositol
Ash	**Minerals**	Moisture	**Vitamins**
Carbohydrates	Calcium	Neutral detergent fiber	Vitamin A (β-carotene)
Fat	Copper	Protein	Vitamin B_1 (thiamine)
Moisture	Iron	Starch	Vitamin B_2 (riboflavin)
Neutral detergent fiber (NDF)	Magnesium	Total Dietary Fiber (TDF)	Vitamin B_3 (niacin)
Protein	Manganese	**Fatty acids**	Vitamin B_6 (pyridoxine)
	Phosphorus	Palmitic acid	Vitamin B_9 (folic acid)
	Potassium	Stearic acid	Vitamin E (α-tocopherol)
	Selenium	Oleic acid	
	Sodium	Linoleic acid	
	Zinc	Linolenic acid	

For each analyte, the statistical significance of the difference between the MIR162 and control mean values (*i.e.,* the genotype effect) was determined using a standard F-test. An F-test $p<0.05$ indicates that the difference between the genotypes was statistically significant at the 5% level. An F-test was also used to detect potential location-by-genotype interactions. In this case a significant outcome ($p<0.05$) indicates that the effect of genotype was not consistent across locations, and that the comparison of genotypes averaged across locations may not be valid. In such cases, the average analyte levels measured at each location were compared to ranges of normal values reported in the literature. For all analytes measured, the average levels across locations were compared to the range of natural variation, as reported in the International Life Sciences Institute crop composition database (ILSI, 2006) and the OECD consensus document on compositional considerations for maize (OECD, 2002).

The results of these compositional analyses, expressed as mean values across locations and a range of individual replicate values, are presented in the tables found in Appendix E (Tables E-3 through E-11). Nine components of maize forage were measured; the difference between MIR162 and control mean values was found to be statistically significant for one of these analytes. Fifty-six components of grain were measured; the difference between MIR162 and control mean values was found to be statistically significant for 13 of these analytes. The results for these 14 analytes that had a statistically significant outcome for genotype effect are presented in Table 26.

The forage compositional analyses for proximates and minerals revealed a single statistically significant difference between MIR162 and control mean values. The mean value for MIR162 NDF was 11.34% higher than the corresponding control value. This difference is considered relatively small and the MIR162 mean falls well within the range of normal values reported by ILSI and OECD. No statistically significant genotype by location interactions were noted for the forage compositional analyses.

Compositional analyses of grain revealed no statistically significant differences between MIR162 and control means for 43 of the 56 analytes examined in across-location comparisons. Statistically significant differences were noted for levels of the proximates ash, NDF, and starch. These differences were small (< 8%) and the MIR162 mean values were well within the ranges of normal values for the control maize. Additionally, the average values for all proximates were within the ranges reported by ILSI and OECD.

Statistically significant differences were noted for three grain minerals: calcium, iron, and phosphorus. The differences observed were small, less than 8%, and the mean MIR162 values were each within the ranges of normal values reported by ILSI and OECD for conventional maize.

Statistically significant differences were noted between MIR162 and control mean levels of vitamin A (β-carotene), vitamin B_6 (pyridoxine), and vitamin E (α-tocopherol). These differences were small (< 7%) and the mean values observed for these vitamins in MIR162 grain were well within the range of values observed for the control grain. Additionally, the MIR162 means for all vitamins fell within the normal range of values reported for conventional maize by ILSI and OECD. For vitamin A and vitamin B_9 a statistically significant genotype-by-location interaction was noted, which suggests that the effect of genotype was not consistent across locations, hence, the comparison of genotypes averaged across locations may not be valid. Individual location means for the two analytes are provided in Table E-8 (Appendix E). The vitamin A and vitamin B_9 levels at all locations were within the ranges reported in the literature.

There were no significant differences noted for any of the 18 amino acids or anti-nutrients measured and all average values were within the ranges reported by ILSI and OECD.

Statistically significant differences were noted for linoleic and linolenic fatty acids. These differences were very small (< 4%) and the MIR162 mean values observed for these fatty acids were within the ranges of values observed for the control grain. Furthermore, the

Table 26. Statistically significant differences between MIR162 and control analytes. Summary composition results for the one forage analyte and 13 grain analytes that had statistically significant ($p<0.05$) differences between MIR162 and control maize across location mean values. All values reported are on a dry weight (dw) basis.

Analyte	MIR162 $\bar{X}$ (Range)	Control $\bar{X}$ (Range)	% Δ [a]	ILSI $\bar{X}$ (Range)	Literature Range [b]
Proximates					
NDF (%)	43.2 (35.1 – 56.1)	38.8 (32.13 – 46.9)	11.34	41.51 (20.29 – 63.71)	40 – 48.2
Grain					
Ash (%)	1.4 (1.1 - 1.6)	1.3 (1.1 – 1.5)	7.69	1.439 (0.616 - 6.282)	1.1 – 3.9
NDF (%)	11.7 (10.1 – 13.0)	11.1 (9.5 – 12.8)	5.41	11.23 (5.59 – 22.64)	8.3 – 11.9
Starch (%)	63.1 (54.8 – 68.1)	64.9 (60.6 – 69.2)	-2.77	57.7 (26.5 – 73.8)	
Calcium (mg/kg)	38.1 (29.4 – 47.2)	35.3 (25.7 – 44.0)	7.93	46.4 (12.7 – 208.4)	3 – 100 g /100 g
Iron (mg/kg)	20.2 (17.3 – 22.9)	19.2 (15.7 – 22.5)	5.21	21.81 (10.42 – 49.07)	0.1 – 10 g /100 g
Phosphorus (mg/kg)	3173 (2810 - 3550)	3073 (2710 - 3400)	3.25	3273.5 (1470.0 – 5330.0)	234 – 750 mg /100 g
Vitamin A (mg/100 g)	0.277 (0.241 – 0.316)	0.294 (0.244 – 0.358)	-5.78	0.684 (0.019 – 4.681)	
Vitamin B_6 (mg/100 g)	0.565 (0.434 – 0.694)	0.605 (0.486 – 0.738)	-6.61	0.644 (0.368 – 1.132)	4.6 – 9.6
Vitamin E (mg/g)	0.0125 (0.0097 – 0.0154)	0.0132 (0.0110 – 0.0154)	-5.44	0.0103 (0.0015 – 0.0687)	
18:2 Linoleic acid (% total FA) [c]	56.99 (55.86 – 59.74)	57.36 (56.26 – 59.47)	-0.65	57.60 (36.2 – 66.5)	0.67 – 2.81% dw
18:3 Linolenic acid (% total FA) [c]	1.81 (1.72 – 1.89)	1.75 (1.64 – 1.86)	3.43	1.20 (0.57 – 2.25)	0.03 – 0.10% dw
Ferulic acid (mg/kg)	2682 (2490 - 2980)	2453 (2010 - 2760)	9.33	2201.1 (291.9 – 3885.8)	200 – 3000
ρ-Coumaric acid (mg/kg)	179 (148 - 202)	157 (137 - 179)	14.01	218.4 (53.4 – 576.2)	3 – 300

[a] – % difference: [(MIR162 – control)/control] x 100
[b] – literature values in comparable units of measure not available for some analytes
[c] – FA = fatty acids

average values for all fatty acids were within the range of normal values reported for conventional maize by ILSI and OECD.

Statistically significant differences were noted in the secondary metabolites ferulic acid and *p*-coumaric acid. These differences were relatively small (< 15%) and the MIR162 mean values for these secondary metabolites were within the ranges of values observed for the control grain. Additionally, the mean values for all MIR162 secondary metabolites and anti-nutrients were within the normal range of values reported for conventional maize by ILSI and OECD.

Collectively, the observed differences between MIR162 and control means are considered of no biological significance and represent typical random variance. The magnitude of the differences was small, all MIR162 values fell within normal ranges for conventional maize, and the MIR162 and control data ranges significantly overlapped. MIR162 is therefore, not compositionally different than conventional maize.

VI.C. Summary of Phenotypic and Compositional Evaluations

Maize has lost the ability to survive without cultivation (OECD, 2003). It can overwinter and germinate in a subsequent crop as a volunteer weed; for example, maize is a common volunteer in soybeans. Nevertheless, several features of maize make it unlikely to form self-sustaining weedy populations in agricultural systems: i) it is easily controlled in subsequent crops by selective herbicides; ii) seed dispersal is limited because seeds are held inside the husks of the cob; and iii) the seeds lack dormancy. Maize does not persist in habitats outside agriculture because, in addition to the features listed above, it requires disturbed ground to germinate and it is very uncompetitive against perennial vegetation. The results of laboratory, greenhouse, and field studies indicate that MIR162 maize is no different than conventional maize with regard to phenotypic and compositional properties that bear on weediness potential for the new cultivar.

VII. Environmental Consequences of Introduction

MIR162 maize contains a novel protein that has toxicological activity against a number of lepidopteran species which are pests of U.S. agriculture. Studies have been conducted to define the spectrum of insecticidal activity for Vip3Aa proteins and to assess the efficacy of MIR162 maize hybrids in resisting larval feeding damage. An assessment of risk for nontarget organisms and endangered species that might be exposed to the Vip3Aa20 protein in MIR162 maize has been performed. This risk assessment is composed of multiple parts: i) a determination of expected environmental concentrations (EECs) for Vip3Aa20; ii) a characterization of potential hazards posed by the protein to nontarget indicator organisms appropriate for a maize ecosystem; iii) a comparison of EECs to no observable effect concentrations for nontarget organisms; and iv) a specific characterization of endangered species risk.

Given the ubiquitous nature of PMI enzymes in nature and its apparent absence of environmental risk, it can reasonably be assumed that the presence of PMI protein in

MIR162 will do nothing to alter the ecological balance within maize ecosystems.

VII.A. Spectrum of Insecticidal Activity for Vip3Aa Proteins

Syngenta has conducted mortality bioassays with Vip3Aa protein variants in a range of insect species to identify those that are susceptible to the toxin. The results of these bioassays and the results of assays reported in the scientific literature demonstrate that activity of Vip3Aa proteins is limited to species within the order Lepidoptera, yet all lepidopteran species are not sensitive to the protein. It is notable that Vip3Aa proteins appear to have no activity against *O. nubilalis*, probably the most significant lepidopteran pest of U.S. maize. Table 27 provides a listing of insect species that have been found by Syngenta, or reported in the literature, as being sensitive to one or more Vip3Aa protein variants. The *Bacillus thuringiensis* Toxin Nomenclature Committee currently lists 25 variants of the Vip3Aa protein.[5] This narrow spectrum of activity for Vip3Aa proteins is a very positive attribute from an ecological perspective; maize hybrids containing a Vip3Aa protein are unlikely to pose a risk to nontarget organisms inhabiting maize ecosystems.

Table 27. Insect species screened for sensitivity to Vip3Aa protein variants. Mortality was assessed in standard diet-surface or diet-incorporation bioassays. Treatment-related mortality was the criterion used for evidence of sensitivity.

Order: Family	Genus: Species	Activity
Lepidoptera: Noctuidae	*Agrotis ipsilon*	Active
	Helicoverpa zea	Active
	Helicoverpa armigera	Active
	Helicoverpa punctifera	Active
	Heliothis virescens	Active
	Spodotera exigua	Active
	Spodoptera frugiperda	Active
	Spodoptera litura	Active
	Striacosta albicosta	Active
	Trichoplusia ni	Active
Lepidoptera: Gelechidae	*Phthorimea operculella*	Active
Lepidoptera: Sphingidae	*Manduca sexta*	Active
Lepidoptera: Pyralidae	*Chilo partellus*	Active
	Ostrinia nubilalis	Not Active
Lepidoptera: Plutellidae	*Plutella xylostella*	Active

(continued)

[5] http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/vip.html (August 25, 2007).

Table 27 (cont.). Insect species screened for sensitivity to Vip3Aa protein variants. Mortality was assessed in standard diet-surface or diet-incorporation bioassays. Treatment-related mortality was the criterion used for evidence of sensitivity.

Order: Family	Genus: Species	Activity
Lepidoptera: Danaidae	*Danaus plexippus*	Not Active
Lepidoptera: Pieridae	*Pieris brassicae*	Not Active
Other Lepidoptera	*Bombyx mori*	Not Active
	Earias vittella	Active
	Ephestia kuehniella	Active
Diptera	*Culex pipiens*	Not Active
	Drosophila melanogaster	Not Active
	Culex quinquefasciatus	Not Active
Coleoptera	*Coleomegilla maculata*	Not Active
	Diabrotica virgifera virgifera	Not Active
	Leptinotarsa decemlineata	Not Active
	Tenebrio molitor	Not Active
	Anthonomus grandis grandis	Not Active
	Aleochara bilineata	Not Active
Hymenoptera	*Apis mellifera*	Not Active
Hemiptera: Anthocoridae	*Orius insidious*	Not Active
Thysanoptera	*Frankliniella occidentalis*	Not Active
Isotomidae	*Folsomia candida*	Not Active
Neuroptera	*Chrysoperla carnea*	Not Active

VII.B. Activity Against Target Insects

As shown in Table 27, Vip3Aa has activity against several of the major lepidopteran pests of maize, specifically: *A. ipsilon*, *H. zea*, *S. albicosta*, and *S. frugiperda*. Expressing a *vip3Aa* gene at adequate levels in maize is a logical approach for limiting crop losses attributable to these four pests, especially considering the deficiencies of conventional insecticides in controlling these pests (see discussion in Section VII.I). Thus, MIR162 maize was developed. In hybrid offerings to growers, the MIR162 trait will be combined through traditional breeding with other insect protection traits (*e.g.*, Bt11xMIR162 maize).

Trait efficacy trials with MIR162 maize hybrids were conducted at multiple locations in 2005 and 2006 by Syngenta entomologists, academic cooperators, and extension agents. Figure 21 provides a graphic representation of the comparative feeding damage for each of the

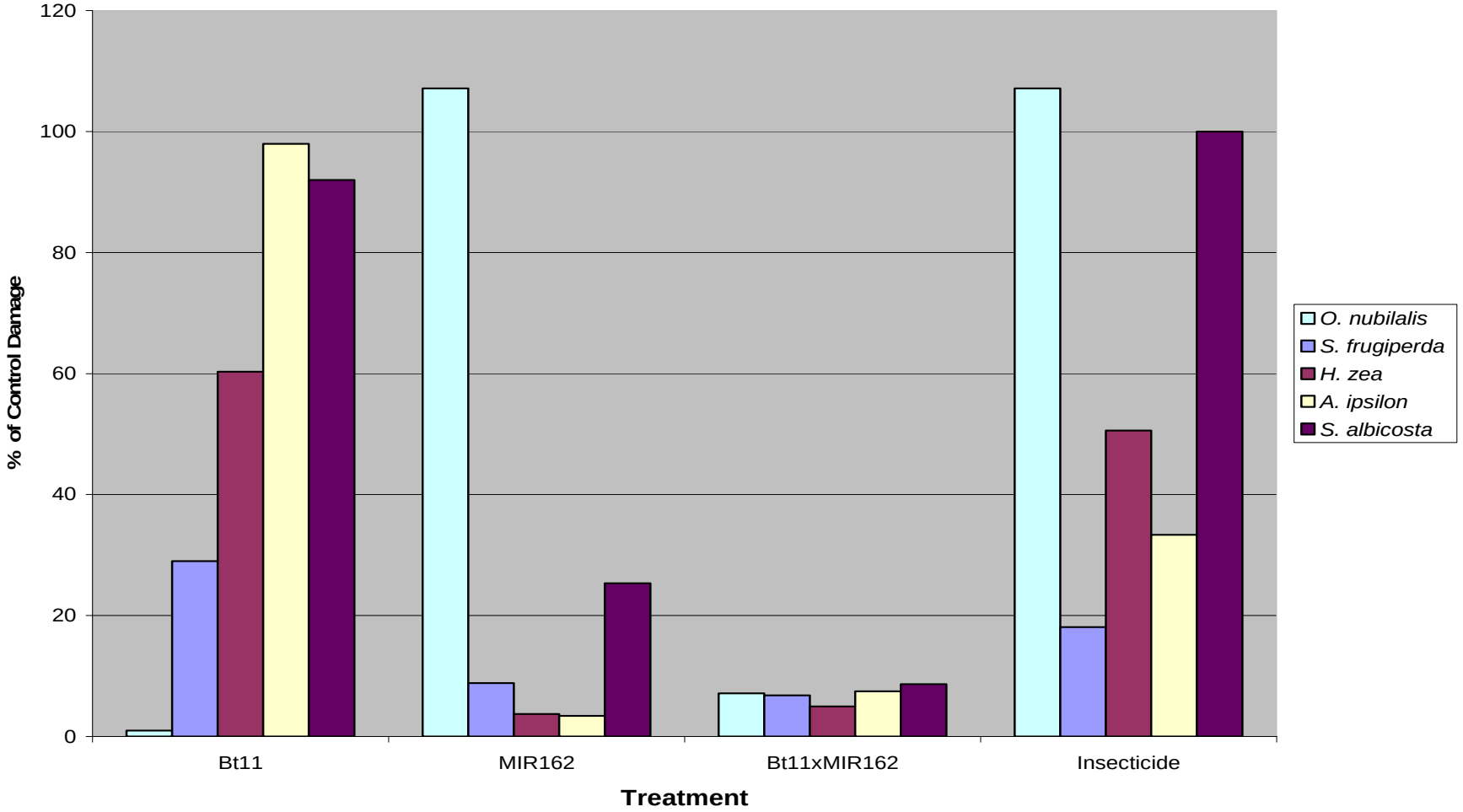

Figure 21. MIR162 field efficacy trial results.
Comparative feeding damage ratings are from replicated field efficacy trials conducted in 2005 and 2006 with Bt11, MIR162, and Bt11xMIR162 maize, and a conventional insecticide treatment (*Warrior® Insecticide*). Mean damage ratings are expressed as a percentage of the damage observed in the untreated control plots.

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treatments, expressed as a percentage of damage measured in the control plants, for five of the insects evaluated. Trials were placed in locations to take advantage of natural pest infestations. In some locations trials were artificially infested to test trait performance under high levels of insect pressure. MIR162 maize, Bt11 maize, Bt11xMIR162 maize, conventional maize, and conventional maize with an insecticide application were the treatments employed in most trials. These trials were conducted under USDA notifications 05-062-02n and 06-055-08n.

MIR162 alone has no activity against *O. nubilalis* but is efficacious in limiting feeding damage caused by the other four insect pests. Whereas Bt11 is highly efficacious against *O. nubilalis*, it has limited or no activity against the other four insects. The combined-trait Bt11xMIR162 hybrid is very efficacious against all five insects. MIR162 addresses the lepidopteran pest-control limitations of existing Cry1Ab-containing maize hybrids. In combination with the Bt11 trait, it will provide growers with excellent control of the following significant lepidopteran insect pests of maize (not all efficacy data shown): *A. ipsilon*, *D. crambidoides*, *D. grandiosella*, *D. saccharalis*, *H. zea*, *O. nubilalis*, *P. nebris*, *S. exigua*, *S. frugiperda*, and *S. albicosta*.

VII.C. Expected Environmental Concentrations for Vip3Aa20

Data on the concentration of Vip3Aa20 in MIR162 maize are used to make estimates of the EEC for Vip3Aa20 in groups of organisms potentially exposed *via* MIR162 maize. Eestimates of exposure based on conservative assumptions about the dilution of Vip3Aa20 in prey, in soil, and in water have been made. These EECs are suitable for protecting populations of nontarget organisms. Potential exposures and risks for endangered species are addressed separately in Section VII.F.

VII.C.1. Concentrations of Vip3Aa20 in MIR162 Maize

The concentrations of Vip3Aa20 were measured by ELISA in several MIR162 maize tissues and whole plants at growth stages V9-V12, anthesis, seed maturity and senescence (refer to Table 14). The values chosen for computation of EECs are shown in Table 28. These values represent the highest mean concentration across locations at any growth stage of MIR162 hybrid maize. EECs were calculated from fresh weight values.

VII.C.2. Soil Fate of Vip3Aa Proteins

Most proteins do not persist or accumulate in the soil because they are inherently degradable in soils that have healthy microbial populations (*e.g.*, Burns, 1982; Marx *et al*., 2005). Multiple investigations have demonstrated that Cry proteins from *B. thuringiensis* are rapidly degraded in a variety of soil types and that the proteins do not accumulate (EPA, 2001; Head *et al.,* 2002; Dubelman *et al.,* 2005). Vip proteins are similar to Cry proteins in that they are also found in naturally occurring soil bacteria and commercial microbial insecticides (De Maagd *et al*., 2003). There is no evidence that they accumulate in soil or are protected from the activity of proteases in soil.

Table 28. Tissue concentrations of Vip3Aa20 used for computation of EECs.
Highest mean concentrations of Vip3Aa20 as determined by ELISA in tissues of two field-grown MIR162 hybrids. All values are reported on a fresh weight basis.

Tissue	Vip3Aa20 (µg/g)	Growth stage
Leaves	50.41	Seed maturity
Kernels	29.81	Seed maturity
Roots	5.29	Senescence
Pollen	43.21	Anthesis
Whole plant	17.35	Seed maturity

A laboratory study was conducted to determine the degradability of Vip3Aa proteins in live soils. For this investigation, the Vip3Aa19 variant was used. Live soil samples from Brazil and the U.S., plus one artificial soil sample, representing four soil textures (clay, sandy clay loam, sandy loam, and silt loam) were used to examine the rate of degradation of Vip3Aa19. The source of Vip3Aa19 for this study was a concentrated protein extract from lyophilized leaf tissue of transgenic event Pacha maize. The protein was tested at two concentrations, 4 mg/g and 16 mg/g dry weight-equivalent soil (corresponding to approximately 14 and 58 µg/g dry weight equivalent soil). This concentration was expected to greatly exceed levels of Vip3Aa protein entering the soil as a result of Vip3Aa maize cultivation.[6] A rapid decline in the levels of Vip3Aa19 was observed in all soil types; degradation was measured as loss of insecticidal activity. The time to 50% dissipation (DT_{50}) was estimated to be between 6.0 and 12.6 days across soil types and test concentrations. The results of this study show that Vip3Aa19 is inherently degradable in healthy soils and indicate it is likely to be degraded rapidly in the field.

VII.C.3. Expected Environmental Concentrations for Nontarget Organisms

This section provides estimates of realistic environmental concentrations of Vip3Aa20 for multiple classes of nontarget organisms that may be exposed *via* cultivation of MIR162 maize. Exposure through soil run-off or gene flow is unlikely for the reasons stated elsewhere in the petition (see Sections VII.C.2 and VII.H) and is not considered. The approach to estimating environmental exposures for Vip3Aa20 is similar to that previously described for corn rootworm-protected maize event MIR604 (Raybould *et al.*, 2007).

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

Birds rarely feed on leaf tissue of maize; however, birds such as *Corvus brachyrhynchos* (crows), *Quiscalus quiscula* (grackles), and *Grus canadensis* (sandhill cranes) uproot sprouting corn to feed on the germinating kernels (*e.g.*, Steffey *et al.*, 1999; Blackwell *et al.*,

[6] MIR162 maize was not the lead line under development.

2001; Sterner *et al*., 2003). *Agelaius phoeniceus* (red-winged blackbirds) and common grackles destroy over 360,000 metric tons per annum of ripening field corn in the U.S. and Canada. Blackbirds typically slit open husks with their bills and puncture kernels in the milk stage (Steffey *et al*., 1999). Blackbirds are also common in corn stubble where they forage for spilled corn kernels and weed seeds (Linz *et al*., 2003). Therefore, the concentration of Vip3Aa20 in kernels was used to estimate the exposure of wild birds to Vip3Aa20 *via* cultivation of MIR162 maize.

The mean concentration of Vip3Aa20 in MIR162 maize kernels is 29.81µg/g fresh weight (Table 28). Exposure to birds may be expressed more suitably as a daily dietary dose (DDD), which is computed by a simple formula:

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

FIR = daily food intake; bw = body weight; C = concentration of Vip3Aa20 in food

FIR/bw ratios for cereal seed-eating birds consuming fresh food were estimated by Crocker *et al*. (2002). Among the seven species represented, values range from 0.11 for the pheasant (*Phasianus colchicus*) to 0.35 for the tree sparrow (*Passer montanus*). These species represent a range of mean body weights; 22 g for the sparrow to 953 g for the pheasant (heavier species have lower *FIR/bw* ratios). Higher *FIR/bw* ratios give higher DDDs and therefore, more conservative estimates of risk.

Wild birds are unlikely to consume a diet of 100% maize kernels. A more realistic estimate of exposure can be derived from the proportion of maize kernels in the diet of birds feeding in maize-growing areas. Studies by McNichol *et al*. (1979) and Homan *et al*. (1994) of the diets of red-winged blackbirds and common grackles, respectively, showed that maize kernels comprise up to 50% of their diet. An EEC can be calculated based on a diet of 50% maize kernels containing 29.81 µg Vip3Aa20/g kernels. This yields a realistic EEC for wild birds of:

0.5 x 29.81 µg Vip3Aa20/g = 14.91 µg Vip3Aa20/g kernels

with a DDD for wild birds of:

0.5 x 0.35 x 29.81 µg Vip3Aa20/g = 5.22 µg Vip3Aa20/g bw

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

The main route of exposure of wild mammals to Vip3Aa20 in MIR162 maize is consumption of kernels. Rodents such as *Spermophilus tridecemlineatus* (thirteen-lined ground squirrels), *Peromysus maniculatus* (deer mice), *Mus domesticus* (house mice), and *Microtus* spp. (prairie and meadow voles) feed on germinating corn seeds. Frequently these species remove so many seeds that the field needs to be replanted. *Marmota monax* (woodchucks) also feed on sprouting corn seed, but because they feed along the edges of fields, they usually cause less serious damage than other rodents. Larger mammals such as *Odocoilus virginianus*

(white-tailed deer) and *Procyon lotor* (raccoons) cause injury to ripening ears. Deer typically nip off ear tips, whereas raccoons chew through husks. In some areas these species are hunted specifically to reduce damage to cornfields (Steffey *et al*., 1999).

The mean concentration of Vip3Aa20 in MIR162 maize seeds is 29.81 µg/g fresh weight (Table 28). As with birds, exposure to mammals may be expressed more suitably as a DDD (calculated by the formula above). Crocker *et al.* (2002) has estimated the ratio of daily food intake and body weight (*FIR/bw*) for several rodent species. The values for the *Micromys minutus* (harvest mouse) and the *Apodemus sylvaticus* (wood mouse) consuming cereal seeds are 0.33 and 0.28, respectively. Higher *FIR/bw* ratios give higher DDDs, and therefore, more conservative estimates of risk.

Wild mammals are unlikely to consume a diet of 100% maize kernels. A more realistic estimate of exposure can be derived from the proportion of maize kernels in the diet of rodents feeding in maize growing areas. The proportion of maize kernels in wild rodent diets varies greatly according to species (Houtcooper, 1978; Ellis *et al*., 1998), but can be up to 73%. An EEC can be calculated based on a diet of 73% maize kernels containing 29.81 µg Vip3Aa20/g kernels. This yields a realistic EEC for wild mammals of:

0.73 x 29.81 µg Vip3Aa20/g = 21.76 µg Vip3Aa20/g kernels

with a DDD for wild mammals of:

0.73 x 0.33 x 29.8 µg Vip3Aa20/g = 7.18 µg Vip3Aa20/g bw

VII.C.3.c. EEC for Pollinators

Honeybees forage for maize pollen and therefore, can be exposed to Vip3Aa20 *via* MIR162 pollen (Severson and Perry, 1981). Honeybees can successfully rear young on a diet of 100% maize pollen; however, it is unlikely that maize pollen regularly comprises more than 50% of their diet (Babendreier *et al*., 2004). Assuming a diet of 50% MIR162 pollen at the mean concentration of Vip3Aa20, a realistic EEC for honeybees and other pollinators is computed to be:

0.5 x 29.81 µg Vip3Aa20/g = 14.91 µg Vip3Aa20/g pollen

VII.C.3.d. EEC for Above-ground Nontarget Arthropods

Nontarget arthropods rarely, if ever, eat leaves of maize. The more likely route of exposure to Vip3Aa20 for these species is consumption of prey that have fed on maize (*e.g.*, Harwood *et al.*, 2005), or consumption of pollen if prey are scarce (Coll and Guershon, 2002).

The concentration of Vip3Aa20 in the prey of nontarget arthropods will vary depending on the prey species, its developmental stage, and the concentration of Vip3Aa20 in plant parts on which they are feeding. Several studies have examined the concentration of Cry proteins in herbivores relative to the concentration of plants on which they are feeding; most tested

the concentration of Cry1Ab in herbivores feeding on *Bt* maize and others have examined herbivores feeding on cotton and oilseed rape containing Cry1Ac (Head *et al*., 2001; Raps *et al.*, 2001; Dutton *et al*., 2002; Obrist *et al*., 2005, 2006a, 2006b; Torres *et al*., 2006; Howald *et al*., 2003).

In general, the results of these studies show that herbivores contain lower concentrations of *B. thuringiensis* toxins than the plants on which they are feeding. Sucking insects, such as aphids, contain only trace amounts of Cry1Ab when feeding on Cry1Ab maize (Head *et al*., 2001; Raps *et al*., 2001; Dutton *et al*., 2002; Obrist *et al*., 2006a). Lepidopteran larvae contain between 0.1 and 0.25X the concentration of Cry1Ab in Cry1Ab maize on which they are feeding (Raps *et al*., 2001; Dutton *et al*., 2002; Obrist *et al*., 2006b), and similar results were obtained by Torres *et al*. (2006) with *Spodoptera exigua* feeding on cotton containing Cry1Ac. *Frankliniella tenuicornis* (thrips) contain up to 0.35X the concentration of Cry1Ab in Cry1Ab-maize, although this concentration is transitory; adults contain about half this amount and pupae less than $1/40^{th}$ the concentration in larvae (Obrist *et al*., 2005). The herbivores with the highest concentrations of Cry protein are *Tetranychus urticae* (spider mites); they have been found to contain between 0.7 and 3X the concentration of Cry1Ab in Cry1Ab maize (Dutton *et al*., 2002; Obrist *et al*., 2006a,b). All of these pests are found in maize, and therefore, nontarget arthropods may be exposed to Vip3Aa20 through consumption of these species.

A precise EEC is difficult to compute given the variety of food that nontarget arthropods are likely to consume. Setting the EEC at 0.2X the overall mean leaf concentration at the highest expressing developmental stage seems reasonably conservative as many lepidopteran larvae contain less than this amount, and aphids and lepidopteran eggs contain considerably less. Spider mites may contain higher concentrations of Vip3Aa20 than leaf tissue, and serious outbreaks of spider mites can occur in maize, particularly under drought conditions (Holtzer *et al*., 1988). However, most predators in maize fields are generalist feeders that do not depend on a single pest species as a food source (Steffey *et al*., 1999); therefore, nontarget arthropods are highly unlikely to consume a diet comprising solely spider mites. Possible exceptions are the specialist spider mite predators *Stethorus* spp. (Coccinellidae). *S. punctillum* is found in maize (*e.g.*, Obrist *et al.*, 2006a); however, this species preferentially eats spider mite eggs, which are likely to contain low concentrations of toxin compared with adult mites (Roy *et al*., 2002). Hence, 0.2X the leaf concentration of Vip3Aa20 is a reasonably conservative EEC. The highest average concentration of Vip3Aa20 in above ground tissue of MIR162 plants is 50.41 µg/g leaf fresh weight at seed maturity (Table 28). The realistic EEC for above-ground nontarget arthropods is computed to be:

$$0.2 \times 50.41 \text{ µg Vip3Aa20/g} = 10.08 \text{ µg Vip3Aa20/g diet}$$

VII.C.3.e. EEC for Soil-dwelling Nontarget Invertebrates

A realistic EEC for soil-dwelling nontarget arthropods can be calculated as the concentration of Vip3Aa20 in soil following incorporation of maize plants into soil post-harvest. The average concentration of Vip3Aa20 in MIR162 whole plants is 17.35 µg/g at seed maturity (Table 28). The average planting density of maize is 65,500 plants per hectare and the

average fresh weight of a corn plant is 750 g. If MIR162 plants contain an average of 17.35 µg Vip3Aa20/g), one hectare of MIR162 maize contains:

$$65{,}500 \text{ plants} \times 750 \text{ g/plant} \times 17.35 \text{ µg Vip3Aa20/g} = 8.52\text{x}10^{8} \text{ µg Vip3Aa20.}$$

If the maize is ploughed into the top 15 cm of soil, the Vip3Aa20 will be incorporated into 100 m x 100 m x 0.15 m = 1,500 m^3 of soil per hectare. The average density of soil is 1,500 kg/m^3. Therefore, the MIR162 maize will be incorporated into 1,500 m^3/ha x 1,500 kg/m^3 = 2,250,000 kg soil per hectare. Dividing the amount of Vip3Aa20 per hectare by the mass of soil per hectare gives the realistic EEC for soil organisms of:

$$8.52\text{x}10^{8} \text{ µg Vip3Aa20/ha} \div 2{,}250{,}000 \text{ kg soil/ha} = 379 \text{ µg Vip3Aa20/kg soil}$$
$$(\text{or } 0.38 \text{ µg Vip3Aa20/g soil})$$

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

The main route of potential exposure of aquatic organisms to Vip3Aa20 is through MIR162 pollen deposited in water bodies adjacent to maize fields. Maize produces up to $3.15\text{x}10^{11}$ pollen grains per hectare during anthesis (Westgate *et al*., 2003); 1 mg of maize pollen contains approximately 2,500 pollen grains (Depuis *et al*., 1987). Therefore, maize produces up to $1.26\text{x}10^{8}$ mg of pollen per hectare. A worst-case assumption for loading of maize pollen into a body of water is that it is deposited onto the surface of the water at the same density it is produced in the field. For a 1-hectare pond (10,000 m^2) that is 2 m deep, the pollen concentration would be:

$$1.26\text{x}10^{8} \text{ mg pollen} \div (10{,}000 \text{ m}^2 \times 2 \text{ m}) = 6300 \text{ mg pollen/m}^3 \text{ water}$$
$$(\text{or } 6.3 \text{ mg pollen/l water})$$

Deposition of maize pollen falls dramatically from the edge of a maize field; for example, Pleasants *et al*. (2001) found that pollen deposition 5 m from the edge of the field was approximately one-tenth of that at the immediate edge. A reasonably conservative extrapolation from this observation is that pollen loading into an adjacent water body is likely to be at least 0.05X the density of pollen within the maize field.[7] Estimates of settling and degradation of maize pollen in ponds are not available, but reduction of the maximum pollen density by 0.5 seems a conservative assumption. Using the mean pollen concentration of 43.21 µg Vip3Aa20/g, an EEC for aquatic organisms exposed *via* pollen is calculated by multiplying the concentration of Vip3Aa20 in MIR162 pollen, the density of pollen under worst-case conditions, 0.05 to account for realistic pollen loading, and 0.5 to account for degradation and settling:

$$43.21 \text{ µg Vip3Aa20/g MIR162 pollen} \times 0.0063 \text{ g MIR162 pollen/l} \times 0.05 \times 0.5$$
$$= 0.0068 \text{ µg Vip3Aa20/l water}$$

About 25% maize grain by weight is typical in the feed of farm-raised fish (National

[7] The assumption is that under worst-case conditions, a 100 m x 100 m field deposits pollen into a 1 ha pond; under more realistic conditions, only a 5 m x 100 m strip of maize deposits pollen into the pond.

Research Council, 1983). The mean concentration of Vip3Aa20 in MIR162 grain is 29.81 µg/g. Fish feed is heat-treated during preparation and therefore, it is likely that Vip3Aa20 will be denatured and lose activity in feed prepared from MIR162 grain. Maize in fish feed is unlikely to comprise 100% MIR162 grain. Insect-protected maize comprised approximately 40% of maize acres planted in the United States in 2006 (Doane Marketing Research, 2006a). If MIR162 maize were to achieve a 50% market share of the insect-protected maize market and assuming that no Vip3Aa20 is lost during heat treatment of the feed, a realistic EEC for farmed fish is:

29.81 µg Vip3Aa20/g x 0.25 x 0.40 x 0.5 = 1.49 µg Vip3Aa20/g feed

VII.C.3.g. Summary of Environmental Exposure Estimates

Realistic EECs have been computed for groups of nontarget organisms that will be potentially exposed to Vip3Aa20 *via* cultivation of MIR162 maize. These EEC values are compiled in Table 29 and are appropriate for estimating the risks to populations of nontarget organisms. Once defined, these EEC or DDD values can be directly compared to Vip3Aa exposure or dose levels that cause no adverse effects in test species. This comparison results in a toxicology exposure ratio (TER).

It is highly unlikely that nontarget organisms will be exposed to Vip3Aa20 in environments outside of cultivated maize. Maize pollen does not drift great distances nor are maize seeds wind-borne. The probability of spread of Vip3Aa20 outside maize cultivation through volunteers and self-sustaining feral populations of MIR162 maize is also very low.

Table 29. Tabulation of EEC and DDD values for nontarget organisms.
Each EEC value presented is based on the highest average concentration of Vip3Aa20 in applicable MIR162 plant tissues and conservative estimates for consumption of these tissues by nontarget organisms. DDD values are computed where appropriate.

Nontarget Organism Group	Expected Environmental Concentration (EEC)	Daily Dietary Dose (DDD)
Birds	14.91 µg/g kernels	5.22 µg/g bw
Wild mammals	21.76 µg/g kernels	7.18 µg/g bw
Pollinator	14.91 µg/g pollen	-
Above-ground arthropods	10.08 µg/g diet	-
Soil-dwelling invertebrates	0.38 µg/g soil	-
Aquatic organisms	0.0068 µg/l water	-
Farmed fish	1.49 µg/g feed	-

VII.D. Impact on Nontarget Organisms

Thirteen species representing groups of nontarget organisms potentially exposed to Vip3Aa20 in MIR162 maize were exposed to Vip3Aa variants in laboratory bioassays for the purpose of identifying adverse effects and establishing a no observable effect concentration (NOEC) or NOEL. Several test substances, containing one of three variants of Vip3Aa, were used in these studies. The identity and source of Vip3Aa variant used in each study are listed in Table 30. The reason that variants other than Vip3Aa20 have been used for studies supporting this MIR162 petition for deregulation is that Syngenta has had other transgenic crops under development that have incorporated the Vip3Aa19 variant. [8] Also, this petition is relying on nontarget organism bioassay results for Vip3Aa1 that are found in the scientific literature. As discussed earlier in this petition (see Section V.A.1), these variants share 99.7% sequence identity and are functionally equivalent. Therefore, Vip3Aa1 and Vip3Aa19 are suitable test substance surrogates for Vip3Aa20 in nontarget organism bioassays.

Table 30. Vip3Aa test substances used in nontarget organism hazard studies.

Test Substance	Source [a]	Concentration of Vip3Aa	Species Tested
Vip3Aa1 purified protein	Recombinant *E. coli*	200 mg/g	*Colinus virginianus*
Plant-incorporated Vip3Aa19	Pacha maize pollen	83.8 µg/g	*Daphnia magna*
Plant-incorporated Vip3Aa19	Pacha maize pollen	144.8 µg/g	*Colleomegilla maculata, Chrysoperla carnea*
Plant-incorporated Vip3Aa19	Pacha maize leaf	3640 µg/g	*Eisenia foetida*
Plant-incorporated Vip3Aa19	Pacha maize leaf	86.7 µg/g	*Folsomia candida*
Plant-incorporate Vip3Aa19	Pacha maize grain	7.10 µg/g	*Ictaluris punctatus*
Vip3Aa19 purified protein	Recombinant *E. coli*	631 mg/g	*Orius insidiosus Chrysoperla carnea*
Vip3Aa19 purified protein	Recombinant *E. coli*	897 mg/g	*Coccinella septempunctata*
Vip3Aa20 purified protein	Recombinant *E. coli*	840 mg/g	*Apis mellifera Aleochara bilineata*

[a] – Pacha is transgenic maize producing the Vip3Aa19 variant which is no longer under development by Syngenta.

Pacha maize was a predecessor Vip3Aa19 maize cultivar under development by Syngenta.

[8] COT102 is a component event of VipCot™ cotton currently under review by the EPA. COT102 cotton has been deregulated by USDA.

Its development was discontinued for agronomic performance reasons; it was replaced in commercial development by MIR162 maize. Many of the nontarget organism bioassays described below utilized Pacha maize tissues as test substances, specifically pollen, leaf, and grain. Data from these bioassays are applicable for predicting hazards and risk for MIR162 maize because Vip3Aa19 is biochemically and functionally equivalent to the Vip3Aa20 protein in the corresponding tissues of MIR162 maize.

All nontarget organism studies, except the *Colinus virginianus* feeding study, employed an appropriate positive control substance to validate the bioassay methodology. Expected responses for test organisms exposed to the positive control substance were observed in all studies (data not shown). The presence and concentration of Vip3Aa protein was confirmed in all artificial meat-based diets by Western blot analysis, ELISA, and bioassay.

VII.D.1. Effect of Vip3Aa on Wild Birds

Five male and five female juvenile *Colinus virginianus* (bobwhite quail) were exposed to a single dose of microbial Vip3Aa1 at 400 mg protein/kg bw, by oral gavage. The effects on bird mortality and growth were compared with a control group for 14 days after exposure, and histological endpoints were assessed at the end of the test. No adverse effects were observed in the treatment or control groups, and the Vip3Aa1 NOEL was shown to be ≥ 400 mg protein/kg bw.

VII.D.2. Effect of Vip3Aa on Wild Mammals

Mus musculus strain Alpk:AD$_f$CD-1 (white laboratory mouse) males and females (five per sex) were exposed to the highest feasible single dose of the microbial Vip3Aa20 test substance, corresponding to 1250 mg protein/kg bw, by oral gavage. The effects on mortality and growth were compared with a control group of male and female mice for 14 days after exposure, and many histological and biochemical endpoints were assessed at the end of the test. No adverse effects were observed in the treatment or control groups, and NOEL was shown to be ≥ 1250 mg protein/kg bw.

VII.D.3. Effect of Vip3Aa on Pollinators

Apis mellifera (honeybees) were exposed to microbial Vip3Aa20 following the protocol of Oomen *et al*. (1992). Vip3Aa20 was incorporated into a diet of 50% sucrose solution at concentrations of 50, 200 and 500 µg/g diet; these concentrations represented approximately 1, 4 and 10 times the concentration of Vip3Aa20 in MIR162 pollen, respectively. The negative control was 50% sucrose solution in buffer. Worker bees collected the sucrose solutions and fed them to brood for 24 days. There were no statistically significant differences in 24-day survival between brood in hives exposed to the Vip3Aa20-treated diet and brood in hives exposed to the negative control diet at any developmental stage. Therefore, the NOEC to bee brood exposed in-hive *via* a sugar solution diet was shown to be ≥ 500 µg protein/g diet.

VII.D.4. Effect of Vip3Aa on Above-ground Arthropods

Eight- to nine-day old adult *Coleomegilla maculata* (pink spotted ladybird beetle) were exposed to Vip3Aa19 *via* Pacha maize pollen for 21 days. The test substance was incorporated into an artificial diet at 5% weight by weight (w/w). The negative control diet comprised 5% w/w pollen from nontransgenic, near-isogenic maize. Fresh diet was supplied daily. The difference in survival of the beetles in the treatment and control groups was not statistically significant, and the NOEC was shown to be ≥ 7.24 µg protein/g diet.

Three- to seven-day old adult *Coccinella septempunctata* (seven-spot ladybird beetle) were exposed to microbial Vip3Aa19 test substance. The test substance was incorporated into a 50% sucrose solution diet at 7250 µg Vip3Aa19/g diet. The negative control diet comprised 50% sucrose solution only. The treatment and control groups were fed fresh diet daily. The difference in survival between the treatment and control groups was not statistically significant, and the NOEC was shown to be ≥ 7250 µg protein/g diet.

Second-instar *Orius insidiosus* (minute pirate bug) were exposed to microbial Vip3Aa19 test substance. The test substance was incorporated into an artificial liver-based diet at 7250 µg Vip3Aa19/g diet. The negative control liver-based diet was treated with buffer only. The treatment and control groups were fed fresh diet daily for 21 days or until they became adults. The difference in survival between the treatment and control groups was not statistically significant. The NOEC was shown to be ≥ 7250 µg protein/g diet.

Adult *Chrysoperla carnea* (green lacewings) were exposed to Vip3Aa19 *via* Pacha maize pollen for 13 days. The pollen was incorporated into an artificial diet, comprising dry milk, sugar, brewer's yeast, and de-ionized water, at 15% w/w. The corresponding negative control diet contained 15% w/w pollen from nontransgenic, near-isogenic maize. The treatment and control groups were provided with fresh diet daily. The difference in survival between the treatment and control groups was not statistically significant, and the NOEC was shown to be ≥ 21.7 µg protein/g diet.

Two- to three-day old *C. carnea* larvae were exposed to microbial Vip3Aa19 test substance. The test substance was incorporated into an artificial meat-based diet at 7250 µg Vip3Aa19/g diet. The negative control was diet treated with buffer only. The treatment and control groups were fed fresh diet daily for 30 days or until adult emergence. After 30 days, the difference between the treatment and negative control groups was not statistically significant; however, the control mortality exceeded the guideline validity criterion of not greater than 25% (Vogt *et al*., 2000); the control mortality remained within the guideline validity criterion up to day 21. The OPPTS Guideline 885.4340 (EPA, 1996) for testing of microbial pesticides states that tests should be terminated when the control mortality exceeds 20%; the control mortality remained within this guideline up to day 14. At day 14 and day 21, the differences between the treatment and negative control groups were not statistically significant. Fourteen and 21 days represent significant portions of larval development, and therefore, the data can be considered a rigorous assessment of the toxicity of Vip3Aa19 to green lacewings. The NOEC was shown to be ≥ 7250 µg protein/g diet

VII.D.5. Effect of Vip3Aa on Soil-dwelling Invertebrates

Adult *Eisenia foetida* (earthworms) were exposed to Vip3Aa19 protein extracted from Pacha maize leaf tissue. The test substance was incorporated into an artificial soil substrate at 3.60 µg Vip3Aa19/g soil. The negative control was artificial soil treated with a leaf protein test substance prepared from nontransgenic, near-isogenic maize. After 14 days, there was 100% survival in both the treatment and negative control groups, and there were no statistically significant differences in body weight between the groups. The NOEC was shown to be ≥ 3.60 µg protein/g soil.

Juvenile *Folsomia candida* (collembola) were exposed to Vip3Aa19 *via* lyophilized Pacha maize leaf tissue. The test substance was mixed with an equal weight of yeast to form the treatment diet containing 43.2 µg Vip3Aa19/g diet. A negative control diet containing equal parts yeast and lyophilized leaves of nontransgenic, near-isogenic maize was also prepared. The collembola were provided with fresh diet daily. After 28 days, there were no statistically significant differences in survival, or in the number of new juveniles, between the Pacha maize leaf treated- and the nontransgenic maize leaf-treated groups. The NOEC was shown to be ≥ 43.2 µg protein/g diet.

Adult *Aleochara bilineata* (rove beetles) were exposed to microbial Vip3Aa20 test substance following the protocol of Grimm *et al*. (2000). The treatment group was fed a meat-based diet containing 500 µg Vip3Aa20/g diet, which was intended to represent approximately 10 times the concentration of Vip3Aa20 in leaves of MIR162 maize. The negative control group was fed diet treated with buffer only. Fresh diet was supplied daily. After 35 days, beetles were allowed to parasitize pupae of *Delia antique* (onion fly). The number of adult offspring of the beetles exposed to the test diets was monitored until the average emergence fell below two beetles per day. The number of beetles that emerged from the Vip3Aa20-treated group was not statistically significantly different from that of the control group. The NOEC was shown to be ≥ 500 µg protein/g diet.

VII.D.6. Effect of Vip3Aa on Aquatic Organisms

Neonate *Daphnia magna* (water fleas) were exposed to Vip3Aa19 *via* Pacha maize pollen. The test substance was suspended in water at 120 mg pollen/l, representing 10.1 µg Vip3Aa19/l. The negative control was nontransgenic, near-isogenic maize pollen suspended in water at 120 mg/l. A water-only control of maize pollen was also included. After 48 hours, survival was 100% in each group, and there was no sign of immobilization or other sublethal effects. The NOEC was ≥ 10.1 µg protein/l water.

Juvenile *Ictalurus punctatus* (channel catfish) were exposed to Vip3Aa19 *via* fish feed prepared from Pacha maize grain. The feed was formulated to contain the maximum proportion of maize grain that provides a nutritious diet and was made using a cold-pelleting technique, which minimizes heating that could denature the protein. Control fish feed was prepared in the same manner using grain from nontransgenic, near-isogenic maize. Treatment and control groups of catfish were fed the respective feeds three times daily for 30 days. There were no statistically significant differences in mean wet body weight increase

between catfish exposed to the Vip3Aa19 diet and fish exposed to the control diet. No abnormalities were noted among the fish during the study except for one thin individual in the control group that died on the last day of the study. The NOEC was shown to be ≥ 7.1 µg protein/g feed.

VII.E. Toxicity Exposure Ratios for Nontarget Organisms

The EEC and DDD values are compared with results of Vip3Aa hazard studies in order to make judgments about risk. The hazard studies identified any potential for Vip3Aa proteins to cause adverse effects in representative nontarget organism species applicable for a maize ecosystem. In each study, a representative species was exposed to high concentrations or doses of Vip3Aa; typically this exposure was the highest attainable. No harmful effect of such exposure was observed in any species tested, and the concentration of Vip3Aa in each study can be interpreted as the minimum value of the NOEC or NOEL. Computing a ratio of the NOEC to the EEC or the NOEL to the DDD results in the toxicity exposure ratio (TER). This ratio is also known as the 'margin of exposure' or 'margin of safety'. A TER ≥ 1.0 provides a degree of confidence that the absence of adverse effects in these hazard studies with indicator species is predictive of the safety of Vip3Aa20 in MIR162 maize for nontarget organisms in general. The larger the TER the higher the degree of confidence in the safety judgment or the lower the risk. Table 31 contains the NOEC or NOEL, and corresponding TER values for each indicator test species.

No adverse effects were observed in any study that exposed representative nontarget organisms to Vip3Aa proteins. The concentration of Vip3Aa tested in the studies was sufficient to achieve margins of exposure of ≥ 1 for all but one species based on realistic EEC values. In the case of the *C. maculata* where the TER was ≥ 0.7, this lower TER value for the pink-spotted ladybird beetle is not an indication of elevated risk but rather a reflection of the fact that the test substance was pollen and there were limitations on the fraction of pollen that could comprise the diet utilized. Additionally, the study conducted with a second ladybird beetle species (*C. septempunctata*) provided a TER of ≥ 719. Collectively, these TER values indicate that plant-incorporated Vip3Aa20 will not be harmful to nontarget organisms likely to be found in a maize ecosystem.

Table 31. Nontarget organism TER values for Vip3Aa proteins.
TER values are computed as the ratio of NOEC : EEC (or NOEL : DDD) and are based on estimates of exposure to Vip3Aa20 contained in MIR162 maize. All NOEC and NOEL values are shown as greater than or equal to (≥) the specified value because no adverse effect was observed in any species tested at the maximum exposure tested.

Test Species	NOEC or NOEL	EEC or DDD	TER
C. virginianus	≥ 400 µg/g bw	5.22 µg/g bw	≥ 77
M. musculus	≥ 1250 µg/g bw	7.18 µg/g bw	≥ 174
A. mellifera	≥ 500 µg/g diet	14.91 µg/g pollen	≥ 34
C. maculata	≥ 7.24 µg/g diet	10.08 µg/g diet	≥ 0.7
C. septempunctata	≥ 7250 µg/g diet	10.08 µg/g diet	≥ 719
O. insidiosus	≥ 7250 µg/g diet	10.08 µg/g diet	≥ 719
C. carnea [a]	≥ 21.7 µg/g diet	10.08 µg/g diet	≥ 2
C. carnea [b]	≥ 7250 µg/g diet	10.08 µg/g diet	≥ 719
E. foetida	≥ 3.6 µg/g soil	0.38 µg/g soil	≥ 9
F. candida	≥ 43.2 µg/g diet	0.38 µg/g soil	≥ 114
A. bilineata	≥ 500 µg/g soil	0.38 µg/g soil	≥ 1316
D. magna	≥ 10.1 µg/l water	0.0068 µg/l water	≥ 1507
I. punctatus	≥ 7.1 µg/g diet	1.49 µg/g diet	≥ 4

a - adults
b - larvae

VII.F. Safety Assessment for Endangered and Threatened Species

There is a weight of evidence that at concentrations in MIR162 maize the toxicity of Vip3Aa20 will be limited to Lepidoptera. Its receptor-mediated mechanism of action and absence of activity in bioassays with multiple species outside of the order Lepidoptera support this conclusion. Furthermore, no harmful effects of Vip3Aa proteins have been observed in nontarget organism hazard identification studies. These studies used a wide range of taxa and at expected environmental concentrations they indicate a lack of risk associated with exposure to Vip3Aa20 in MIR162 maize (see Table 31).

The only endangered or threatened lepidopteran species with potential for exposure to insecticidal proteins in maize is the Karner blue butterfly (*Lycaeides melissa samuelis*) (EPA, 2001; USFWS, 2007). The potential route of exposure for this species is consumption of maize pollen that has settled on the leaves of its larval food plant, the wild lupine (*Lupinus*

perennis). Peterson *et al*. (2006) determined that exposure of Karner blue larvae to maize pollen was minimal because most lupine populations are separated from maize fields by at least 500 metres, and because maize anthesis usually occurs after Karner blue larvae have finished feeding.

The restriction of toxicity of Vip3Aa20 to Lepidoptera, and the minimal exposure of endangered Lepidoptera to maize, indicates that Vip3Aa20 in MIR162 maize is expected to have no harmful effects on any endangered or threatened species in the U.S.

Although not an endangered or threatened species, *Danaus plexippus* (monarch butterfly) is a species of high conservation interest, and there has been concern that it may be harmed by consuming pollen from transgenic insect-protected maize. The monarch is susceptible to Cry1Ab (Hellmich *et al*., 2001), the commonest insecticidal protein in transgenic maize. However, the distribution of the monarch's food plant (*Asclepias syriaca* - common milkweed), its pattern of migration, and the timing of maize anthesis means that very few monarchs are exposed to harmful concentrations of Cry1Ab (Sears *et al*., 2001).

The exposure assessments used to assess the risks of maize containing Cry1Ab to monarchs are also valid for MIR162 maize. In addition, it has been shown that monarchs are not susceptible to Vip3Aa1. Lee *et al*. (2003) showed that trypsinized Vip3Aa1 did not form pores in the midgut of monarchs; pore formation appears to be essential for toxicity and occurs in the guts of insects susceptible to Vip3Aa1. These investigators also found no mortality of *D. plexippus* in a surface diet bioassay limit test at 1000 ng/cm^2. MIR162 maize, therefore, poses low risk to monarchs because of minimal hazard of Vip3Aa20 and low exposure to Vip3Aa20-containing pollen.

VII.G. Conclusion on Environmental Risk

No adverse effects were associated with exposures to Vip3Aa1, Vip3Aa19, or Vip3Aa20 proteins in a range of indicator species appropriate for a maize ecosystem, and the NOEC or NOEL was the highest concentration (or dose) tested in each study. The exposures in all but one study were in excess of EEC or DDD levels for the nontarget organism groups represented, indicating a low probability of harm to these groups from Vip3Aa20 in MIR162 maize.

The very narrow spectrum of insecticidal activity observed for Vip3Aa proteins indicates with high certainty that no endangered or threatened species other than Lepidoptera would be harmed by contact with Vip3Aa20 *via* MIR162 maize. There is minimal exposure of endangered Lepidoptera to maize and therefore, cultivation of MIR162 maize is unlikely to harm any endangered or threatened species in the U.S.

VII.H. Gene Flow Assessment

An assessment of the environmental fate of Vip3Aa20 resulting from the cultivation of MIR162 maize requires consideration not only of the production and degradation of the protein within maize fields, but also the possibility that Vip3Aa20 could persist, or spread

from, areas of maize cultivation because of gene flow or the establishment of weedy populations of MIR162 maize. It is highly unlikely that Vip3Aa20 will appear in environments outside of cultivated maize. Maize pollen does not drift great distances nor are maize seeds wind-borne. The probability of spread of Vip3Aa20 outside maize cultivation through volunteers and self-sustaining feral populations of MIR162 maize is also very low.

As described in Section VI above, MIR162 is not phenotypically or compositionally different from conventional maize other than for its ability to resist insect feeding damage. Thus, MIR162 maize has not acquired any properties indicative of increased weediness potential. The likelihood that the *vip3Aa20* gene will move to other species as a result of either vertical or horizontal gene transfer is also extremely low.

VII.H.1. Gene Flow to Wild Species

Z. mays L. subsp. *mays* hybridizes with a group of taxa collectively called teosinte. Several types of teosinte are classified as subspecies of *Zea mays*, whereas others are regarded as separate species of *Zea*. Teosinte species are natives of Central America and have co-existed with cultivated maize for several thousand years. They have remained genetically distinct from cultivated varieties despite occasional introgression (Baltazar *et al*., 2005). Teosinte species are not natives of the U.S., but isolated populations have been recorded in Florida and Texas, the former a possible remnant of the use of annual teosinte as a forage grass. These populations are apparently now extinct in both states. Teosinte species are grown in botanical gardens, but as maize pollen is heavy and relatively short-lived (*e.g.*, EPA, 2001; Byrne and Fromherz, 2003; Devos *et al*., 2005), fertilization of these plants with pollen from MIR162 maize is extremely unlikely.

Species of the genus *Tripsacum* are considered close relatives of *Zea* species and some theories postulate that a *Tripsacum* species may be a progenitor of domesticated corn *via* hybridization and introgression with teosinte (*e.g*., Poggio *et al*., 2005). There are sixteen species of *Tripsacum* worldwide, of which three occur in the U.S.: *T. dactyloides*, a widespread forage grass; *T. floridanum*, known from southern Florida; and *T. lanceolatum*, which is present in Arizona and possibly New Mexico (EPA, 2001).

Maize breeders view *Tripsacum* as a potential source of useful genes for traits including apomixis, pest and disease resistance, and drought tolerance (OECD, 2003). Therefore, substantial effort has been made to obtain and characterize maize-by-*Tripsacum* hybrids. Hybrids between maize and *Tripsacum* species are difficult to obtain outside the laboratory or greenhouse, and are often sterile. Only one record exists of an open-pollinated hybrid between *Zea* and *Tripsacum*, which involved species native to Guatemala. After consultation with experts on improvement of forage grasses, the EPA (2001) concluded that the chance of natural introgression of genes from maize to *Tripsacum* was 'extremely remote' and that no other species in the continental U.S. would interbreed with commercial maize.

These observations indicate a very low probability for transfer of the *vip3Aa20* gene from MIR162 maize to wild relatives in the U.S. Species of *Zea* other than maize are not recorded outside botanical gardens in the U.S. *Tripsacum dactyloides* is widespread, but does not

hybridize readily with *Z. mays*, and the probability of backcross or F_2 progeny of *Tripsacum*-by-*Zea* hybrids being produced in the field is negligible. Therefore, Vip3Aa20 is unlikely to spread from maize cultivation and persist in the environment as the result of vertical gene flow.

VII.H.2. Potential for Horizontal Gene Transfer

An extensive review of information relevant to the potential risks of horizontal gene transfer for *Bt* crops to soil microbes was conducted as part of the 2001 reregistration of *Bt*-based plant-incorporated protectants (EPA, 2001). Studies reviewed by the EPA showed no evidence for horizontal gene transfer under field conditions, and only equivocal evidence for horizontal gene transfer under laboratory conditions designed to maximize the recovery of transformants. Conner *et al*. (2003) also reviewed the literature and found very few examples where horizontal gene transfer had been demonstrated convincingly, and these cases relied on artificially high sequence homology between the transgene and the potential recipient organism (*e.g*., de Vries *et al*., 2001). The codons in the *vip3Aa20* gene are optimized for expression in plants, and hence the gene is likely to have low sequence homology with genes of soil microbes. Therefore, horizontal gene transfer of *vip3Aa20* from MIR162 maize to soil microbes is highly unlikely. If transfer did take place, expression of *vip3Aa20* is improbable. In addition to the fact that codon use *vip3Aa20* is optimized for expression in plants, rather than microbes, the maize ubiquitin promoter is unlikely to function in microbes. The probability for spread of *vip3Aa20* outside maize cultivation by horizontal gene transfer is negligible.

VII.I. Impact of MIR162 Introduction on Current Maize Agronomic Practices

Field maize is the leading production crop globally, with the 2005/2006 growing season yielding 695 million metric tons of grain (USDA, 2006a). The U.S. accounts for nearly 41% of global maize production. Maize is the largest crop grown in the U.S. in terms of both volume and value. Approximately 78.3 million acres were planted in 2006, yielding 10.5 billion bushels (267 million metric tons) with a gross crop value of $33.7 billion (USDA, 2007a). The total plantings in 2007 are estimated at 92.9 million acres, a 19% increase over 2006 plantings (USDA, 2007c). This increase has been driven by the demand for fuel ethanol. Field corn is planted in almost every state of the U.S. with the majority concentrated in ten states of the upper Midwest: Illinois, Iowa, Indiana, Kansas, Minnesota, Missouri, Nebraska, Ohio, South Dakota, and Wisconsin. The average annual yield from 2006 was 149.1 bu/ac (USDA, 2006b).

Yield losses due to weeds, diseases, and insects were substantial until the introduction of crop protection chemicals in the 1960s. Weeds compete with crops for light, nutrients, water, and other growth factors. If weeds are left uncontrolled, maize simply cannot be grown successfully. Estimates of maize yield loss caused by pathogens have ranged from 2 to 17% (Smith and White, 1988). In addition, a maize crop is susceptible to attack by a variety of insects from the time it is planted until it is consumed as food or feed. As a result, maize crops are intensively managed.

For the 2005 crop year in the 19 program states tracked by the National Agricultural Statistics Service of USDA, nitrogen was applied to 96% of maize acres at a rate of 138 lb/ac, phosphate was applied to 81% of maize acres at a rate of 58 lb/ac, potash was applied to 84% of acres at a rate of 65 lb/ac, and sulfur was applied to 13% of acres at a rate of 12 lb/ac (USDA, 2006c). The introduction of MIR162 maize is not expected to alter fertilizer application practices.

Weeds are controlled to varying degrees by crop rotation, tillage, and herbicide applications. Herbicides were applied to 97% of the program state maize acres in 2005 (USDA, 2006c). Atrazine was applied to 66% of acres, glyphosate was applied to 31% of planted acres, *S*-metolachlor and acetochlor were each applied to 23% of planted maize acres. The introduction of MIR162 maize is not expected to alter maize weed control practices.

Insect control options available to growers include conventional insecticide applications, microbial insecticide applications, crop rotation, and planting of insect resistant cultivars. The major and moderate insect pests of maize are described earlier in this petition (see Section I.B and Table 1). The most widespread and damaging insects of maize in the U.S. Corn Belt have been *O. nubilalis* and *Diabrotica* species. Before the introduction of *Bt* maize hybrids ten years ago, growers had few practical options for controlling stalk boring insects; only about 10% of growers applied insecticides for control of corn borers. As a result, most growers incurred significant annual yield losses because most fields were not treated and chemical applications were not always effective. Timing of insecticide applications had to be nearly perfect because there was only a very short period of time (two to six days) that these insects would be physically positioned on the plant where they could be exposed to an insecticide application. The introduction of the first *Bt* maize hybrids in 1996 provided growers with an effective means of limiting damage caused by *O. nubilalis*. By plant-incorporating the insecticide, exposure of the insect to the toxin was guaranteed. In 2006, 42% of maize acres were planted with *Bt* corn borer-protected hybrids (Doane Marketing Research, 2006a). These hybrids express either a *cry1Ab* or *cry1F* gene from *B. thuringiensis*, both of which encode proteins that are highly toxic to *O. nubilalis*.

More options have been available to growers for mitigating damage caused by corn rootworms. In many maize growing regions crop rotation has been effective in limiting *Diabrotica* populations because it breaks the life cycle of the insect. There have also been many effective conventional insecticide products available to growers for control of these pests. Prior to the introduction of rootworm-protected *Bt* varieties in 2003, an estimated 14 million acres were treated annually with conventional insecticides to control corn rootworms. This equated to more than 7.7 million pounds of insecticide active ingredient being applied annually in maize fields for the control of *Diabrotica* species (Ward *et al.*, 2005). Control of *Diabrotica* rootworms accounted for the largest single use of insecticides in the U.S. In 2005, 23% of maize acres in the program states were treated with conventional insecticides. Tefluthrin, cyfluthrin, and tebupirimphos were the most widely applied active ingredients (USDA, 2006c).

Controlling above-ground insects presents a challenge for maize growers. The majority of maize fields are not treated for control of leaf-, stalk-, and ear-feeding insects. A grower

decision not to treat is generally not reversible because of the feeding location of the pests (in the soil, under the leaf, inside the stalk, or in the ear); the pests are shielded from aerial chemical applications. Timing of applications is critical for efficacy.

Data obtained from the 2005 and 2006 Doane Marketing Research AgroTrak studies indicate that growers are currently treating approximately three million acres a year with conventional insecticides for control of *H. zea*, *A. ipsilon*, *S. albicosta*, and *S. frugiperda* (see Table 32). Compared to the total number of maize acres planted annually in the U.S., this represents a relatively small use of conventional pesticides; however, three million acres treated represents a significant use compared to chemical usage in other crops.

Table 32. Conventional insecticide usage for control of selected lepidopteran pests. Cost and maize acres treated with conventional insecticides during 2005 and 2006 for the control of *S. frugiperda*, *A. ipsilon*, *S. albicosta*, and *H. zea* (Doane Marketing Research, 2005 and 2006b).

Pest	Acres Treated		Grower Cost ($)	
	2005	2006	2005	2006
S. frugiperda	0	20,441	0	86,550
A. ipsilon	2,721,543	3,064,137	19,457,090	22,328,818
S. albicosta	44,410	72,373	363,801	343,552
H. zea	99,620	161,002	825,654	848,502
Totals	2,865,573	3,317,953	$20,646,545	$23,607,422

In addition to direct damage caused by feeding on plant tissue, insects play an important role in the transmission and dissemination of pathogenic organisms during maize development. Soil abounds in microorganisms, particularly fungi, which may infect plant parts injured by soil-dwelling insects. In much of the Corn Belt, pathogenic fungi probably pose more problems in corn production than any other group of organisms. Primary roots of the seedling and the radical and seminal roots are commonly infected with *Fusarium* spp. after they have served their function and become senescent. Feeding by *Diabrotica* rootworms has been associated with increased frequencies of *Fusarium* infection (Dicke and Guthrie, 1988); rootworm feeding may also lead to increased incidences of stalk rots. Ear, kernel, and cob rots occur wherever maize is grown and result in reduced test weight, poor grain quality, and mycotoxin contamination of food and feed. *Fusarium* kernel or ear rot is the most widespread disease of maize ears and is frequently associated with insect feeding damage. Mycotoxin contamination of maize grain presents a potential threat to livestock health and it is occasionally necessary to reject or reformulate field lots because of contamination. These pathogenic infections can lead to reduced crop quality, harvestability, and yield.

MIR162 maize provides excellent protection against feeding damage caused by *A. ipsilon*, *H. zea*, *S. albicosta*, and *S. frugiperda*. For this reason, its introduction will impact in a very

positive way current maize insect control practices. This product has the potential to displace all of the conventional insecticide applications listed in Table 32. Control of these above-ground insect pests is currently challenging for growers. Conventional insecticide applications are costly and intensive scouting of fields is required to identify the appropriate timing for applications. Growers only have a very narrow time window during which insecticides can be applied because many of the above-ground feeding insects are shielded from contact with the insecticides by virtue of their feeding location on the plant.

Two years of efficacy field trials, conducted at multiple locations under varying levels of insect pressure, have demonstrated the superior leaf, stalk, and ear protection provided by MIR162 maize compared to hybrids treated with a conventional insecticide product (see Figure 21). Vip3Aa20 possesses a number of unique properties that conventional insecticides do not. The protein is efficacious *via* a mode of action that is selective to lepidopteran insects. The protein is expressed throughout all tissues of the maize plant. This ensures protection where it is needed and eliminates the risk of insecticide failures associated with timing of applications or unfavorable environmental conditions. Furthermore, the delivery of Vip3Aa20 in the maize seed and its production in plants eliminates many risks associated with conventional insecticide usage, some of which include improper calibration and maintenance of application equipment, handling of hazardous chemical insecticides, container disposal, chemical misplacement, runoff, and spray drift.

Integrated pest management (IPM) in agriculture includes insect scouting or monitoring to determine pest populations, consideration and application of compatible alternative biological, cultural, mechanical and chemical controls, and the establishment of action thresholds for agricultural inputs. The delivery of pest management interventions on target and on time is a key to successful IPM. Planting of MIR162 hybrids provides much greater accuracy of application compared to chemical treatments due to the localization of Vip3Aa20 within the plant tissues. Timing of application is not a factor with MIR162 hybrids since Vip3Aa20 is present in the plant throughout the growing season. Planting of MIR162 hybrids is compatible with current insect scouting and monitoring programs that provide data upon which to base crop management decisions. The product is also fully compatible with cultural control measures such as crop rotation. MIR162 fits seamlessly into the concept of integrated pest management for maize.

From data collected in a telephone survey of 150 maize growers in 12 states, average yield losses in 2006 attributable to *H. zea* were estimated to be 4.9 bu/ac and losses attributable to *S. albicosta* were estimated to be 4.8 bu/ac (see Appendix F). Examination of data provided by these growers for the past five seasons suggests that yield losses attributable to the two pests are increasing. This conclusion is supported by analysis of insecticide use data for 2005 and 2006 which indicate that economically significant infestations of *H. zea* and *S. albicosta* are on the rise in the Corn Belt and Great Plains. Additionally, there is evidence that populations of *S. albicosta* are spreading eastward and will have the potential to cause greater harm in critical maize-producing states. While economic losses attributable to the insects that are effectively controlled by MIR162 maize are not as large as those attributable to *O. nubilalis* and *Diabrotica* rootworms, they are significant when severe infestations occur. As the price of maize grain continues to rise, the economic threshold for growers to

respond to infestations of *A. ipsilon*, *H. zea*, *S. albicosta*, or *S. frugiperda* will fall. Even relatively small reductions in crop yield (< 5%) will result in a significant economic loss for growers.

In a combined-trait hybrid offering with Bt11 maize (Bt11xMIR162 maize),[9] the crop will be protected from the damage and yield losses attributable to all of the economically significant lepidopteran insect pests. This superior protection against insect feeding will also result in a corresponding decrease in mycotoxin levels in grain.

VIII. Insect Resistance Management

Current insect resistance management (IRM) strategies for *Bt* maize products are centered around the planting of a structured refuge that can provide a source of susceptible adult insects for rare resistant insects to mate with. Matings of resistant and nonresistant adults serve to dilute the frequency and establishment of resistance genes in a population. The size and configuration of the structured refuge is determined by toxin dose and insect biology. The existing EPA policy for resistance management of maize lepidopteran pests is focused primarily *O. nubilalis*, *D. grandiosella*, and *H. zea*. Growers planting Cry1Ab- and Cry1F-containing maize hybrids in the Corn Belt are required to plant a 20% structured refuge of nonlepidopteran-protected maize. The refuge can be configured as strips within or surrounding a field or as a block within, adjacent to, or up to ½ mile away from the *Bt* maize field. For maize planted in cotton-growing areas of the south, the refuge must be at least 50% of a grower's total planted areas because Cry1Ab- and Cry1F-containing hybrids do not deliver a high dose against *H. zea* and are present in both corn and cotton varieties. *H. zea* is a pest of both maize and cotton and has the potential to undergo selection pressure from feeding on both corn and cotton varieties that express similar Cry proteins. The refuge configuration options are the same as in the Corn Belt.

As MIR162 maize provides no protection against feeding damage caused by *O. nubilalis* it will not likely be offered to growers as a stand-alone trait. Instead, it will be commercialized as a combined-trait hybrid with Syngenta's Bt11 maize event. Together the two traits have been shown to deliver a high dose against *O. nubilalis* and *H. zea*, as well as against *S. frugiperda*. Syngenta has submitted an IRM plan for Bt11xMIR162 maize that requires growers to plant a 20% structured refuge that can be planted as strips within or surrounding the *Bt* maize field or as a block within, adjacent to, or up to ½ a mile away. The proposed refuge requirements are the same in the Corn Belt and cotton growing areas.

A unique benefit that will be offered by Bt11xMIR162 hybrids is that the Cry1Ab and Vip3Aa20 proteins are present at levels that have been demonstrated to provide high-dose control of *O. nubilalis, H. zea* and *S. frugiperda*, thus minimizing the risk of resistance developing in these species. Bt11xMIR162 hybrids offer IRM advantages over other lepidopteran-control options that do not demonstrably provide a high dose against the target pests. Moreover, Vip3Aa20 operates by a mode of action different from that of Cry1Ab or Cry1F and targets a unique binding site(s) in susceptible larvae. The available data support a

[9] An application for registration of the plant-incorporated protectants in Bt11xMIR162 maize is currently under review by the U.S. EPA.

conclusion that Vip3Aa20 shows no potential for cross-resistance with Cry proteins (Lee *et al.*, 2003). Thus, for *H. zea* and *S. frugiperda*, which are sensitive to both Cry1Ab and Vip3Aa20, Bt11xMIR162 maize is predicted to significantly extend the durability of both traits for control of these pests because local populations are very unlikely to evolve resistance to two proteins that act on independent receptor sites.

For growers of Bt11xMIR162 maize hybrids, the reduced refuge requirement in cotton-growing regions will translate into a higher proportion of insect-protected maize acres, with a proportional increase in all the attendant benefits of the product in these areas. As an added advantage, compliance with the refuge requirement for IRM can be predicted to increase because *Bt* maize growers in cotton-growing regions have heretofore not been able to fully experience the benefits enjoyed by *Bt* maize growers in other regions of the U.S. The potential for increased maize acres in cotton-growing regions can also help meet the current high demand for maize grain.

IX. Adverse Consequences of Introduction

Syngenta knows of no data or observations that indicate MIR162 maize would adversely impact the quality of the human environment, directly, indirectly, or cumulatively. This includes a lack of anticipated effects on endangered species, unique geographic areas, critical habitats, public health and safety (including children and minorities), genetic diversity of maize, farmer or consumer choice, insect resistance or the economy, either within or outside the U.S. MIR162 maize offers growers an additional choice for protection of maize crops from feeding damage caused by lepidopteran pests. As such, MIR162 is expected to convey benefits similar to those associated with previously deregulated *Bt* maize products that are commercially available. Additional benefits will include increased product choice for growers, price competition, and extended useful life of *Bt* maize technology generally.

This section of the petition addresses potential beneficial and adverse consequences associated with deregulation of MIR162 maize and demonstrates that the introduction of MIR162 maize will only affect the quality of the human environment in a positive way. The direct, indirect, and cumulative impact of deregulating of MIR162 maize is analyzed within the framework of the factors listed by the Council on Environmental Quality (CEQ).[10]

IX.A. Effects on the Quality of the Human Environment

The National Environmental Policy Act (NEPA), 42 U.S.C. §4321 *et seq*., requires that agencies undertaking a major federal action significantly affecting the quality of the human environment provide a detailed statement of the environmental impact of the proposed action, any adverse environmental effects that cannot be avoided, and alternatives to the action (42 U.S.C. §4332 (C)). Where the significance of an action is uncertain, agencies may use an environmental assessment (EA) to identify, analyze, and evaluate the impacts of the

[10] The Council on Environmental Quality coordinates federal environmental efforts and works closely with agencies and other White House offices in the development of environmental policies and initiatives. Congress established the Council on Environmental Quality within the Executive Office of the President as part of the National Environmental Policy Act of 1969.

proposed action. The EA will satisfy a NEPA obligation where it provides sufficient evidence and analysis to support a finding of no significant impact (FONSI) for the proposed federal action.

The test of whether an action 'significantly affects' the environment requires considerations of both context and intensity (40 CFR §1508.27). The term 'context' refers to the setting within which the proposed action takes place.[11] The term 'intensity' refers to the severity of the impact and includes factors such as effects on human health, cumulative impacts, and effects on endangered species.

Data and information submitted by Syngenta in support of this petition for deregulation of MIR162 maize is sufficient under NEPA for APHIS to prepare an EA in support of a FONSI. The context within which the deregulation of MIR162 maize will occur is one in which multiple lepidopteran-protected *Bt* maize cultivars are currently in use, and have been for many years. These cultivars provide a valuable tool for growers to protect their crops from feeding damage caused by lepidopteran insects that infest maize and that have developed resistance to a number of conventional insecticides. These *Bt* maize cultivars do not present plant pest risks.

Currently available lepidopteran-protected *Bt* maize cultivars produce insecticidal Cry proteins derived from *B. thuringiensis*. MIR162 maize produces a vegetative insecticidal protein that is also derived from *B. thuringiensis*. Vip and Cry proteins differ in terms of their solubility characteristics, yet they act by the same fundamental mechanism of action (*i.e.*, they are activated to insecticidal toxins in the insect midgut and form pores in the epithelial membranes). The MIR162 technology will provide benefits in the marketplace in the form of crop protection against lepidopteran pests that are poorly or only partially controlled by existing *Bt* maize cultivars. Its introduction will bring market diversification with no corresponding plant pest or environmental risks.

These same facts demonstrate the low intensity of this action. The cumulative impacts of an additional lepidopteran-protected maize product entering the market will only be beneficial. These benefits will come in the form of reduced insecticide usage, increased crop yields, and marketplace competition. Maize is a well-characterized and intensively managed crop. Its genetic diversity is carefully maintained and safeguarded by seed producers. The modification of maize to produce MIR162 maize will not present a risk of adverse effects for endangered, threatened, or other nontarget species. A comprehensive and scientifically based IRM plan will be executed to delay the development of insect resistance to the MIR162 technology. The following sections delineate the evidence supporting these conclusions.

IX.B. Context of the Proposed Action

The context or setting of the proposed deregulation is based in the production of an intensively managed row crop, maize. Maize is the largest crop grown in the U.S. in terms of both volume and value. Approximately 78.3 million acres were planted in 2006, yielding 10.5 billion bushels (267 million metric tons) with a gross crop value of $33.7 billion

[11] See Coliseum Square Association *v.* Jackson, 465 F.3d 215, 239-41 (5th Cir. 2006).

(USDA, 2007a). Maize is grown for animal feed, human food, vegetable oil, high fructose corn syrups, starch, fermentation into ethanol, and a multitude of industrial uses. Maize cultivars improved through modern biotechnology have contributed significantly to this value. Improved insect control has led to increased crop yields and reductions in conventional pesticide use (EPA, 2001; Marra *et al.*, 2002).

Lepidopteran insects are significant insect pests of field maize and more so, of sweet maize in the U.S. In addition to direct damage caused by feeding on plant tissue, these insects play an important role in the transmission and dissemination of pathogenic organisms during maize development. Syngenta plans to market the MIR162 trait in a combined-trait hybrid with the insecticidal trait in Bt11 maize. The broad efficacy of the Bt11xMIR162 product will allow maize growers across wide geographies to benefit from routinely planting Bt11xMIR162 hybrids to control all of the major lepidopteran maize pests that could be prevalent in any given year.

The improved pest-protection profile of Bt11xMIR162 maize can be expected to translate into correspondingly higher overall economic benefits to growers, consumers, and other downstream users of maize products. Some lepidopteran pests, such as *A. ipsilon*, can cause major yield losses due to cutting of an entire stand of maize in a field. If the crop is left untreated, the grower is sometimes forced to replant an entire field. Such catastrophic losses will be prevented by planting Bt11xMIR162 maize. Other, stalk-boring lepidopteran larvae cause physiologic yield loss because the stalk damage interferes with nutrient uptake and increases susceptibility to plant diseases. They can also cause severe stalk lodging, which results in a physical yield loss because the ears cannot be mechanically harvested by the combine. Ear-feeding pests reduce grain yield and quality, and occasionally result in elevated grain fumonisin levels that render the grain unsafe and unusable as food or feed.

Growers have significant experience with the management of *Bt* maize cultivars. Deregulated *Bt* maize cultivars that resist lepidopteran feeding have been commercially available for more than a decade, and more recent *Bt* maize cultivars offer corn rootworm resistance, as well. USDA statistics show that 40% of the U.S. maize crop in 2006 consisted of *Bt* varieties (including single and stacked biotech trait products) and 49% in 2007 (USDA, 2007c). The presence of *Bt* maize has not adversely affected genetic diversity.

The importance of maize as a food crop, and its dependence on human management, has produced a long history of great care to protect germplasm lines of maize. Decades prior to the introduction of transgenic maize cultivars, the maize seed industry developed effective methods for maintaining product segmentation and genetic purity standards. Specialty maizes, for example, were successfully isolated for years and continue to be grown today, even with transgenic maize being widely adopted in the U.S. Moreover, with respect to both conventional and transgenic maize, the ability to protect and maintain the genetic purity of breeding lines is critical to seed companies and developers of new varieties such as those containing event MIR162. Consequently, seed companies routinely apply rigorous breeding techniques, including physical and temporal isolation, that have proven effective in maintaining the genetic purity of breeding lines.

The EPA registrations for MIR162 and all other *Bt* maize cultivars require refugia and related measures that have been used successfully for several years, as evidenced by the fact that there have been no documented instances of confirmed insect resistance to *Bt* maize having developed in the field, and Syngenta is not aware of any studies demonstrating the development of insect resistance in the field. Monitoring target pest populations for resistance development is an ongoing stewardship requirement imposed by EPA for all *Bt* maize cultivars.

IX.C. Intensity of the Proposed Action

With regard to the intensity element of the 'significance' determination, CEQ regulations provide ten factors to guide the analysis (40 CFR §1508.27(b)). These factors "…do not constitute categorical rules such that their presence or absence means an impact is *per se* significant." The ten CEQ intensisty factors are as follows:

i. Impacts that may be both beneficial and adverse. A significant effect may exist even if the federal agency believes that, on balance, the effect will be beneficial.
ii. The degree to which the proposed action affects public health or safety.
iii. Unique characteristics of the geographic area such as proximity to historic or cultural resources, park lands, prime farmlands, wetlands, wild and scenic rivers, or ecologically critical areas.
iv. The degree to which the effects on the quality of the human environment are likely to be highly controversial.
v. The degree to which the possible effects on the human environment are highly uncertain or involve unique or unknown risks.
vi. The degree to which the action may establish a precedent for future actions with significant effects or represents a decision in principle about a future consideration.
vii. Whether the action is related to other actions with individually insignificant but cumulatively significant impacts. Significance exists if it is reasonable to anticipate a cumulatively significant impact on the environment. Significance cannot be avoided by terming an action temporary or by breaking it down into small component parts.
viii. The degree to which the action may adversely affect districts, sites, highways, structures, or objects listed in or eligible for listing in the National Register of Historic Places or may cause loss or destruction of significant scientific, cultural, or historical resources.
ix. The degree to which the action may adversely affect an endangered or threatened species or its habitat that has been determined to be critical under the Endangered Species Act of 1973.
x. Whether the action threatens a violation of federal, state, or local law or requirements imposed for the protection of the environment.

IX.C.1. Impacts That May Be Both Beneficial and Adverse

The NEPA regulations (40 CFR §1508.27(b)(1)) emphasize that agencies must take into account beneficial effects, as well as adverse ones. As set out below, MIR162 maize is expected to deliver the same beneficial effects as previously deregulated *Bt* maize cultivars

that are also registered by the EPA as plant-incorporated protectants and which are commercially available. These benefits include additional grower choice of technologies, increased price competition and extended useful life of *Bt* maize technology generally. These effects do not constitute significant impacts on the human environment because they merely provide incremental benefits over the *status quo* and do not constitute an adverse impact on the human environment.

USDA has already deregulated multiple lepidopteran-protected maize cultivars, each containing proteins derived from *B. thuringiensis*, which limit the destructive feeding damage caused by insects such as *O. nubilalis*, *D. grandiosella*, *A. ipsilon*, *H. zea, S. albicosta,* and *S. frugiperda*, the same insects targeted by the Vip3Aa20 protein contained in MIR162 maize.[12] These *Bt* maize cultivars have reduced the use of conventional pesticides since their market introduction and this has been viewed as a very favorable benefit to the environment. The commercial introduction of MIR162 maize is expected to reduce conventional insecticide use further. The superior protection of ears from insect feeding damage offered by the MIR162 trait will preserve grain yield and quality, and will limit fumonisin levels that render the grain unsafe and unusable as food or feed.

IX.C.2. Effects on Public Health or Environmental Safety

Previously deregulated *Bt* maize cultivars have resulted in reduced conventional pesticide use, as farmers find the *Bt* products more effective in mitigating insect feeding damage. It is reasonable to expect that deregulation and commercialization of MIR162 maize will result in further reductions in the use of conventional pesticides. This reduction in conventional pesticide use would diminish the environmental risks of insect control, as the chemical alternatives to MIR162 present well-characterized risks to humans and other wildlife, whereas Vip3Aa20 presents no such risk. Substantial data support a conclusion that Vip3Aa20 toxicity will be limited to sensitive lepidopteran species that are sufficiently exposed to the protein.

The toxicity of insecticidal *B. thuringiensis* proteins such as Vip3a20 depends on their binding to specific receptors present in the insect midgut. Research demonstrates that this specificity limits the proteins' toxic effect to certain lepidopteran species. A discussion on the mechanism of action for Vip3Aa20, its spectrum of activity, and its lack of toxicity for nonlepidopteran species is presented in Section VII of this petition.

Health and safety studies have been conducted with the novel proteins contained in MIR162 maize. A comprehensive assessment of the safety of the introduced proteins, Vip3Aa20 and PMI, demonstrate that both proteins are nontoxic to mammalian species and are unlikely to be food allergens. The Vip3Aa20 protein is considered nontoxic because it does not share significant amino acid homology with known protein toxins, is nontoxic to mice at a very high dose of 1250 mg Vip3Aa20/kg bw, it is rapidly degraded in simulated mammalian gastric fluid, and its insecticidal mode of action for Vip3Aa20 is not relevant to mammals.

[12] See determinations of nonregulated status: Monsanto maize line MON 810, 61 *Federal Register* 10720 (March 15, 1996); Pioneer/Dow maize line 1507, 66 *Federal Register* 42624 (August 14, 2001); Northrup King maize line Bt11, 61 *Federal Register* 2789 (January 29, 1996).

Furthermore, Vip3Aa20 is not likely to be a food allergen because it is not derived from a known source of allergenic proteins, it does not have any significant amino acid sequence identity to known allergenic proteins, it is rapidly degraded in simulated mammalian gastric fluid, and it is labile upon heating at temperatures of 65ºC and above. The PMI protein is considered nontoxic because it does not share significant amino acid homology with known protein toxins, it is nontoxic to mice at a very high dose of 3030 mg PMI/kg bw, and it is rapidly degraded in simulated mammalian gastric fluid. PMI is not likely to be a food allergen because it is not derived from a known source of allergenic proteins, it does not have any significant amino acid sequence identity to known allergenic proteins with implications for its allergenic potential, it is rapidly degraded in simulated mammalian gastric fluid, and it is labile upon heating at temperatures of 37ºC and above.

A temporary exemption from the requirement of a food tolerance currently exists under 40 CFR §174.458 for Vip3Aa20 in maize and a permanent exemption from the requirement of a tolerance exists under 40 CFR §180.1252 for PMI in all plants. A petition for establishment of a permanent tolerance exemption for Vip3Aa20 in all plants is currently under review at EPA.

IX.C.3. Unique Characteristics of the Geographic Area

There is no indication that this action would have a significant effect on the unique characteristics of any particular geographic area. As with *Bt* maize cultivars already deregulated and commercialized, MIR162 maize is expected to be used throughout maize-producing areas of the country.

IX.C.4. Effects on Human Environment are Unlikely to be Controversial

There is no 'controversy' as that term is used in the NEPA context regarding the use of transgenic *Bt* maize varieties. "The term 'controversial' refers to the existence of a substantial dispute ... as to the size, nature, or effect of the major federal action rather than to the existence of opposition to a use."[13] The experience with existing *Bt* maize and cotton cultivars that are used for limiting lepidopteran insect feeding damage demonstates that there is no controversy regarding the use of MIR162 maize. This petition contains substantial evidence demonstrating why the deregulation of MIR162 maize would have only beneficial effects on the quality of the human environment. Additionally, there is no scientific evidence that contradicts the data submitted in this petition.

IX.C.5. Potential Unique or Unknown Risks

Syngenta's petition has the advantage of having been preceded by the deregulation of multiple *Bt* maize cultivars.[12] Experience with maize cultivars producing Cry1Ab and Cry1F proteins from *B. thuringiensis* serves as a guide to the expected effects of MIR162 maize producing the Vip3Aa20 protein. Moreover, there is a history of safe use for *B. thuringiensis* proteins because microbial *Bt* pesticides have been used for decades and

[13] See Heartwood, Inc. *v.* U.S. Forest Service, 380, F.3d 428, 8th Circuit 2004.

transgenic *Bt* maize and cotton have been grown commercially for more than 10 years.[14] Therefore, the likely effects of *Bt* maize are well known.

To the extent that risks such as insect resistance may manifest themselves over time, these risks are known and there will be preventative measures implemented upon commercialize of Bt11xMIR162 maize. As a condition of the EPA registration for Bt11xMIR162 maize, Syngenta will be required to implement an IRM plan. This is not a hypothetical solution to the potential development of insect resistance nor is it a response that might be implemented at an uncertain future date. A detailed IRM plan that includes a refuge and other requirements designed to prevent the development of insect resistance has been documented and submitted to EPA as part of the application for a FIFRA Section 3 registration of Bt11xMIR162 maize.[15]

A significant and unique benefit offered by MIR162 relates to its distinct advantages in the area of resistance management when combined through conventional breeding with Bt11 maize containing the Cry1Ab protein. The Cry1Ab and Vip3Aa20 proteins are present in these hybrids at levels that have been demonstrated to provide a high-dose for control of *O. nubilalis, H. zea* and *S. frugiperda*, thus minimizing the risk of resistance developing in these species. Bt11xMIR162 hybrids will offer IRM advantages in comparison to other control options that do not demonstrably provide a 'high dose' against the target pests. Moreover, Vip3Aa20 operates by a mode of action different from that of Cry1Ab or Cry1F and targets a different binding site in susceptible larvae. The available data support a conclusion that Vip3Aa20 shows no potential for cross-resistance with Cry proteins (Lee *et al.*, 2003). Thus, for *H. zea* and *S. frugiperda*, which are sensitive to both Cry1Ab and Vip3Aa20, Bt11xMIR162 maize is predicted to significantly extend the durability of both proteins for control of these pests because insect populations are very unlikely to evolve resistance to two proteins that act by independent modes of action.

The possibility of resistance development in *H. zea* is of particular concern, as it is also a pest of cotton and has the potential to undergo selection pressure from both *Bt* maize and *Bt* cotton cultivars that express similar *cry* genes, where the two crops are grown in the same geographies. The principal reason that the EPA requires growers in cotton-growing areas to plant 50% of their maize acres to non-*Bt* maize hybrids concerns the potential for resistance evolution in *H. zea* populations. In its application for EPA registration of Bt11xMIR162, Syngenta provides data and rationale to justify reduction of the maize refuge in cotton-growing areas from 50% to 20% of maize acres planted. For growers of Bt11xMIR162 maize hybrids, the reduced refuge requirement in cotton-growing regions will translate into a higher proportion of insect-protected maize acres, with a proportional increase in all the attendant benefits of the product in these areas. If EPA grants the reduction in refuge for Bt11xMIR162 it will have the advantage that IRM compliance will likely increase because *Bt* maize growers in cotton-growing regions have heretofore not been able to fully experience the benefits enjoyed by *Bt* maize growers in other regions of the U.S. No other *Bt* product offers comparable IRM advantages of Bt11xMIR162 maize.

[14] See http://www.aphis.usda.gov/brs/not_reg.html for listing of deregulated *Bt* cultivars dating from 1994.
[15] MIR162 will be offered to growers as a combined trait hybrid with the Bt11 trait; it will not be offered for sale to growers as a stand-alone trait.

The IRM program is itself one component of an overall package of integrated crop management techniques, which include crop rotation, maintenance of refuge quality, and education as to the proper use of insecticides in refugia in order for them to serve as an effective source of susceptible adults to mate with any resistant adults surviving in the *Bt* maize field. The effectiveness of IRM plans is not hypothetical. Refugia have been required for all other commercial *Bt* maize cultivars and have been deployed over the past decade. The fact that there have been no documented instances of confirmed insect resistance in the field to *Bt* maize cultivars is evidence of the success of these IRM plans.

The IRM plan will be implemented as part of a product stewardship program. The specific refuge requirements and other stewardship practices to be used are set out in detail in a stewardship agreement. Growers will not have a choice whether to follow these procedures when choosing to grow hybrids containing the MIR162 and Bt11 traits; they will be contractually bound to follow the procedures, by means of the stewardship agreement, and their compliance will be monitored and enforced according to a fully documented compliance program, which is reviewed and evaluated by EPA. Syngenta will communicate these requirements to growers using a wide-ranging grower education campaign. The stewardship program also requires resistance monitoring, a remedial action plan to be implemented in the event of unexpected levels of lepidopteran pest damage, and an annual IRM plan review.

Thus, Syngenta is obligated to implement specific and detailed stewardship program aimed at preventing or significantly delaying insect resistance. The program requires that growers plant a structured refuge and compliance with this requirement will be monitored and enforced. Data collected to date for existing *Bt* maize cultivars indicate that this type of stewardship program has been effective in preserving the effectiveness of the *Bt* technology. Thus, the absence of significant uncertainty is demonstrated by large volumes of data and analysis indicating predicted effects, observations of the effects of similar products over previous years, and plans in place to account for any deviation from expected effects.

IX.C.6. Potential Impacts for Future Considerations

By its terms, this petition for deregulation applies only to MIR162 maize. There is no indication that APHIS intends to use this action as a decision in principle about future deregulations. Nor is there any suggestion that another federal action would be effectively decided as a result of an APHIS conclusion with regard to MIR162.

IX.C.7. Potential Cumulative Impacts

A cumulative impact is "…the impact on the environment which results from the incremental impact of the action when added to other past, present, and reasonably foreseeable future actions regardless of what agency . . . undertakes such other actions" (40 CFR §1508.7). Cumulative impact refers to the combination of the effect of the proposed action 'with the effects of other federal actions', not combinations of interactions between this event and features of the environment, such as multiple factor interactions between the *Bt*-based protein

and other chemicals in a given Bt maize variety.[16]

APHIS has previously made determinations of nonregulated status as to other *Bt* maize cultivars.[12] Data and analysis submitted in this petition demonstrate that MIR162 is not a 'tipping point' that will combine with these previous deregulations to cause a significant impact. The crucial requirement is that the overall analysis take into account the effects of other actions in assessing the predicted effects of the action at issue: "It makes sense to consider the 'incremental impact' of a project for possible cumulative effects by incorporating the effects of other projects into the background 'data base' of the project at issue, rather than by restating the results of the prior studies."[17] Studies and testing that support Syngenta's petition took place in an environment in which numerous cultivars of *Bt* maize are widely used (USDA, 2007c). Thus, 'incorporating the effects of other projects into the background' data. See Sections VI and VII of this petition for discussions of field trial results for MIR162 that showed no enhanced plant pest potential and no adverse effects for nontarget organisms. Accordingly, the data in this petition indicate that the Vip3Aa20 protein contained in MIR162 maize is not toxic to nontarget organisms and that the protein will not accumulate in the environment. To the extent that there may be other cumulative effects on the human environment, such effects could only be considered positive. These effects include the potential for reduced conventional insecticide use, reduced emissions of greenhouse gases, improved maize grain quality, and for economic gain accruing to U.S. growers.

Maintaining genetic purity has been a feature of maize cultivation for decades as part of hybrid seed and specialty maize production, and the deregulation of multiple *Bt* maize cultivars has not significantly affected these processes, even considering the effects of these transgenic cultivars cumulatively. Since the introduction of hybrid maize in the 1930s, maize production has required separation of inbred parent and hybrid seed production activities from the production of grain. This has been necessary to maintain the genetic purity of inbred lines and guarantee the quality of hybrid seed sold to growers.

Standards for genetic purity and seed quality are largely set by industry associations, state, national, and international institutions. Many of these standards were developed decades prior to the advent of transgenic maize. A basic requirement for maintaining genetic purity in seed production fields is ensuring that only intended cross-pollination occurs. Many options are available for this purpose: maintaining isolation distances to prevent pollen movement from other maize, planting border or barrier rows to intercept pollen, employing natural barriers to pollen movement such as treelines, manual, or mechanical detasseling, genetic male sterility, and staggered planting dates. In addition to these practices, a series of widely-used standard operating procedures ensure that the genetic purity and identity of seed is maintained in all phases of production, from planting to harvest, processing, storage and

[16] See TOMAC *v.* Norton, 433 F.3d 852, 864 (D.C. Circuit. 2006). "Appellant appears to misunderstand the function of a cumulative impacts analysis. TOMAC construes the requirement to mean that BIA was required to consider the 'cumulative impact of all the casino's expected impacts when added together." This is not correct. The 'cumulative' impacts to which the regulation refers are those outside of the project in question; it is a measurement of the effect of the current project along with any other past, present, or likely future actions."
[17] See Coalition on Sensible Transp., 826 F.2d at 70.

sale (Wych, 1988). The ubiquity and robustness of these procedures developed for maintaining genetic purity in seed production, and the rigor with which they are routinely applied in the maize industry, these procedures have effectively managed the introduction of transgenic maize into the marketplace and have continued to do so through the introduction of successive transgenic maize cultivars.

In addition to the specialization adopted across the industry to enable hybrid seed production for commodity field maize, about 5% of U.S. maize production has been composed of various specialty maize varieties. These include popcorn, waxy (high amylopectin) maize, high oil maize, high protein and modified protein maize, sweet corn, white corn, blue corn, Indian corn, and high amylose corn.[18] More recently, niche markets for organic maize products have also been developed, and in 2006 there were at least 18 seed companies in the U.S. specializing in organic maize seed (see Section IX.D.3). Similar to the production of conventional inbred and hybrid seed, industry quality standards for these maize products have led specialty maize seed producers and growers to employ a variety of techniques to ensure that their products are not pollinated by or commingled with other field maize varieties. In general, all the management practices used in conventional seed production to ensure quality standards are also employed in, and are sufficient to meet standards for, the production of specialty maize seed.

In contrast to commodity field maize, rigorous quality standards are also required for specialty grain production. To meet these standards, specialty grain growers employ a range of additional measures to meet purity standards. Most commonly, to prevent cross-pollination of specialty maize by commodity field maize, growers employ isolation distances, border or barrier rows, natural barriers, and in some cases, plantings staggered to ensure specialty maize is not flowering at the same time as nearby commodity field maize.

The same isolation standards that apply to seed production fields may result in much higher purity in hybrid grain production fields; a hybrid grain field produces a pollen load 10 to 100 times greater than that of a single-cross seed production field. In addition, the timing of pollen shed in a hybrid field is usually highly synchronized with silk emergence. The large pollen load and synchronous timing of pollen and silks serve to greatly limit pollen mixing from outside sources. In addition, since the 1950s a gametophytic self-incompatability system (*GaI* ^{S}) has been available, and is widely used in popcorn production, to prevent cross-pollination of specialty maize by field maize (Thomas, 1955; Ziegler, 2000).

For decades prior to the introduction of transgenic maize cultivars and since their introduction, the maize industry has had effective methods and means to maintain product segmentation and genetic purity standards. As a result, these widespread practices have served to ensure that the broad adoption of transgenic maize in the U.S. (including the sale and cultivation of multiple *Bt* maize varieties for more than a decade) has had no significant impact, even in the aggregate, on the production of maize seed and specialty maize products. There is no evidence to suggest that deregulation of MIR162 maize will act as a 'tipping

[18] See U.S. Grains Council, *Value Enhanced Corns Report 2005/2006, available at:* http://www.grains.org/galleries/technical_publications/USGC%20Value%20Enhanced%20Corn%20Report%202006%20%20(English).pdf

point' that will undermine the effectiveness of these methods.

The adoption of multiple varieties of transgenic maize has had no significant impact on the genetic diversity of cultivated maize or the availability of diverse maize germplasm resources, even considering the effects of these transgenic events cumulatively. Genetically distinct maize hybrids have always been developed for various geographies and purposes, and are continually improved by plant breeding. This has in no way been altered by the introduction of transgenic maize; transgenes are simply introgressed into these breeding programs, and have not interfered with the continuous improvement of the base genetics that underlie the performance of modern maize hybrids.

Futhermore, the adoption of transgenic maize cultivars was preceded by worldwide efforts to identify and preserve sources of maize genetic diversity, and to make these resources available for utilization by public and private maize breeders. Among these efforts are the Germplasm Enhancement of Maize program, a cooperative effort undertaken by USDA, public, and private plant sector breeders, nongovernment organizations and international public cooperators, which was established to further identify maize genetic diversity and to provide it in useful form in order to broaden the genetic base of this crop (USDA, 1999). The germplasm sources being developed through the Germplasm Enhancement of Maize program are available free of charge through the extensive national germplasm collections and germplasm repository programs for conservation of maize genetic diversity.

Maize collections sponsored by the National Plant Germplasm System are located in Ames, Iowa (North Central Regional Plant Introduction Station) and at the Maize Genetics Stock Center in Urbana, Illinois. According to the Germplasm Resources Information Network, there are four maize species in the national collection: *Zea diploperennis, Z. luxurians*, *Z. mays* and *Z. perennis*. *Z. mays*, which is the primary genetic source of cultivated maize, has 19,384 accessions. [19] At Urbana, 5101 accessions are available, primarily *Z. mays*. Other public maize germplasm resource centers include world collections at the International Maize and Wheat Improvement Center (Mexico), tropical maize materials at International Institute of Tropical Agriculture (Nigeria), and various collections residing within national programs of different countries around the world. Specific maize germplasm is also available from individual breeders working on maize at public institutions and universities. The deregulation of transgenic maize events provides yet another source of genetic diversity that can be utilized in the improvement of maize performance.

Thus, observation of the cumulative effects of numerous other transgenic maize products indicates that the genetic diversity of maize has been maintained in coexistence with conventional maize. There is no evidence on the record, nor is Syngenta aware of any, to suggest that MIR162 will act as a 'tipping point' that will undermine the effectiveness of these methods for maintaining genetic diversity, and which have been successful through the previous deregulation of *Bt* cultivars.

There have been no documented instances of confirmed insect resistance to *Bt* maize having developed in the field, and Syngenta is not aware of any studies showing insect resistance to

[19] http://www.ars-grin.gov/npgs/searchgrin.html

Bt maize products, despite the introduction of multiple previous cultivars over the past decade. All commercialized *Bt* maize cultivars are subject to mandatory refuge requirements. The fact that there have been no documented instances of confirmed insect resistance to *Bt* maize in the field indicates that the use of mandatory refugia is effective in preventing or delaying the development of insect resistance to *B. thuringiensis* insecticidal proteins, even cumulatively after multiple *Bt* maize cultivar introductions. A description of IRM program and supporting data and studies is provided in Section VIII. There is no evidence on the record, nor is Syngenta aware of any, to suggest that MIR162 will act as a 'tipping point' that will cause the previously-effective refuge system to become ineffective.

IX.C.8. Historical and Cultural Effects

There is no indication that this action would adversely affect districts, sites, highways, structures, or objects listed in or eligible for listing in the National Register of Historic Places or that it may cause loss or destruction of significant scientific, cultural, or historical resources.

IX.C.9. Effects on Endangered Species

There is a weight of evidence that at the concentrations of Vip3Aa20 in MIR162 maize adverse effects will be limited to lepidopteran species. No harmful effects of Vip3Aa proteins have been observed in any nontarget organism study. These studies used a wide range of taxa, and even at conservative estimates of expected environmental concentrations, there is an adequate margin of safety for nontarget organisms (see Section VII).

EPA determined that the only endangered lepidopteran with potential for exposure to insecticidal proteins in maize is the Karner blue butterfly (*Lycaeides melissa samuelis*) (US EPA, 2001). The potential route of exposure is consumption of maize pollen that has settled on the leaves of its food plant, the wild lupine (*Lupinus perennis*). Peterson *et al*. (2006) determined that exposure of the Karner blue to maize pollen was minimal because most lupine populations are separated from maize fields by at least 500 metres, and because maize anthesis usually occurs after the Karner blue has finished feeding.

The restriction of toxicity of Vip3Aa20 to Lepidoptera, and the minimal exposure of engendered Lepidoptera to maize, indicates that planting of MIR162 maize is expected to have no harmful effects on any endangered or threatened species in the U.S.

IX.C.10. Compliance with Law

There has been no indication that this action would violate federal, state, or local law or requirements imposed for the protection of the environment, nor is there any evidence on the record that would so indicate.

IX.D. Economic Impacts

Economic considerations are not explicitly described within the factors listed in 40 CFR

§1508.27. However, economic impacts do relate to the significance of the requested action and have been considered by some courts in reviewing NEPA compliance.

Although some maize-growing areas experience significant lepidopteran infestations in most years, it is not possible for individual growers to accurately predict whether lepidopteran pest pressure will be economically significant in any particular growing season. Growers must make seed purchase decisions prior to knowing whether the seed price premium for lepidopteran control in a given year will actually be recouped as higher crop yields when compared to the seed cost for unprotected hybrids or the costs of other control measures that might be applied. Nevertheless, the acres planted to *Bt* maize have continued to increase steadily since the first *Bt* maize hybrids were introduced in the U.S. in 1996. This is a testament to the actual yield-preserving benefits that *Bt* maize growers have experienced over the long term, and to the value of the built-in 'insurance' against a potential pest outbreak that could otherwise result in high economic losses. For many growers, the broad lepidopteran control offered by Bt11xMIR162 hybrids will represent a higher insurance value than currently available *Bt* maize cultivars. Additionally, Bt11xMIR162 hybrids will offer unsurpassed convenience to growers by reducing the need to scout fields for pest pressure or to apply other control measures for lepidopteran larvae.

The continued success of *Bt* maize hybrids in the marketplace since their initial introduction in 1996 attests to their economic benefits for growers. Although it is difficult at this time to accurately predict the magnitude of economic benefits that growers of MIR162 maize hybrids in the marketplace, the improved pest protection profile of Bt11xMIR162 maize can be expected to translate into correspondingly higher overall economic benefits to growers, consumers, and other downstream users of maize products. The magnitude of these economic benefits will necessarily depend upon the seed price premium paid for the pest-control traits, the level of local pest pressure, and the value of the crop. Commodity prices for maize grain have dramatically increased recently due to high demand for fuel ethanol, and sustained demand is predicted for the coming years. Such demand will function to increase the value of a grower's investment in any agricultural practice, technology, or product, including the MIR162 traits, that increases or preserves yield.

Another predicted economic benefit for growers and downstream consumers is increased competition in the marketplace for pest-control products, including hybrid seed from multiple providers of lepidopteran-tolerant *Bt* maize varieties. The commercial availability of MIR162 hybrid maize seed will represent a significant new pest control option and tool for growers. Increased grower choice can be expected to exert downward pressure on the cost of products that offer control of lepidopteran pests.

IX.D.1. Enhanced Productivity

Studies to quantify the expected yield advantage of Bt11xMIR162 maize across multiple geographies, environmental conditions, cropping practices and pest pressure are ongoing. The data available from a limited set of trials indicate that, in the absence of pest pressure, Bt11xMIR162 hybrids will provide the same yield as their nontransgenic counterparts. It is expected that, as a result of superior and season-long control of significant lepidopteran

pests, Bt11xMIR162 hybrids will demonstrate significant yield advantages under both low and high insect pressure.

Some lepidopteran pests, such as *A. ipsilon*, can cause major yield losses due to cutting of an entire stand of maize in a field. If the crop is left untreated, the grower is sometimes forced to replant an entire field. Such catastrophic losses will be prevented by planting Bt11xMIR162 maize. Other, stalk-boring lepidopteran larvae cause physiologic yield loss because the stalk damage interferes with nutrient uptake and increases susceptibility to plant diseases. They can also cause severe stalk lodging and dropped ears, which results in a physical yield loss because the ears cannot be mechanically harvested by the combine. Ear-feeding pests reduce grain yield and quality, and occasionally result in elevated grain fumonisin levels that render the grain unsafe and unusable as food or feed (see Sections I.B and VII.I; EPA, 2001). In the presence of pest pressure, Bt11xMIR162 maize hybrids can be expected to preserve yield potential, grain quality, and silage quality by minimizing the damage that could otherwise be caused by lepidopteran insects.

IX.D.2. Increased Competition for *Bt* Lepidopteran Control Products

Another predicted economic benefit for growers and downstream consumers is increased competition in the marketplace for pest-control technologies, including hybrid seed from multiple providers of lepidopteran-tolerant *Bt* maize varieties. The commercial availability of hybrid maize seed with the MIR162 trait will represent a significant new pest control option and tool for growers. Increased grower choice can be expected to exert downward pressure on the cost of other products that offer control of lepidopteran pests.

IX.D.3. Farmer and Consumer Choice

As described above, maize cultivation has long included effective methods of maintaining genetic purity, and mechanisms are in place to protect the genetic diversity of maize. Specialization of maize cultivation, in hybrid seed production for commodity field maize and in production of specialty maizes, has required genetic purity procedures for more than 70 years, and maize growers have utilized these methods effectively to prevent undesired gene flow. Syngenta is aware of no studies showing that these methods have been any less effective at preventing gene flow from transgenic maize varieties, or that specialty maizes, such as popcorn, have become less available since transgenic maize has come into general use.

Thus, despite the introduction and adoption of transgenic maize cultivars over the past decade, including multiple varieties of *Bt* maize, specialty and organic maize remains readily available. In 2006, there were at least 18 seed companies in the U.S. specializing in organic maize seed:[20]

Albert Lea Seed House, Albert Lea, MN
The American Organic Seed Co., Warren IL

[20] http://www.mosesorganic.org/umord/suppliers.htm#seeds and http://www.cropsci.ncsu.edu/organicgrains/production/seedsuppliers.htm

Ames Seed Farms, Kelley, IA
Brown Seed Farms, Inc., Bay City, WI
Brunner Seed, Durand, WI
Doebler's, Inc., Callaway, VA
Falk's Seed Farm, Murdock, MN
Foundation Direct Seeds/Mid State Supply, Inc., Onalaska, WI
Gold Country Seeds, Hutchinson, MN 55350
Golden Grains, Sparta, WI
Great Harvest Organics, Atlanta, IN 46031
Lakeland Organics/Bruenner Seeds, Colfax, WI
Lancaster Ag Products, Bird-in-Hand , PA
Lawler Farm Center, Lawler, IA
Merit Seeds, Berlin, OH
Prairie Gold Seeds, Mankato, MN
Prairie Hybrids Seeds, Deer Gove, IL
Welter Seed and Honey Co., Onslow, IA

See also U.S. Grains Council website for a listing of large suppliers of organic seed as of 2005.[21] There is no indication on the record that MIR162 will alter the coexistence between organic/specialty maize and the widespread use of transgenic maize varieties. Accordingly, there is no reason to believe that MIR162 will in any way limit farmer or consumer choice.

IX.D.4. Effects on the Export Market

There should be no effects on the U.S. maize export market since Syngenta is actively pursuing regulatory approvals for MIR162 maize in countries with functioning regulatory systems for genetically modified organisms and that import maize from the U.S. or Canada. Regulatory filings for MIR162 maize are in process for Colombia, Japan, South Korea, Taiwan, China, the Philippines, Australia and New Zealand, South Africa, the European Union, Russia, and Switzerland.

Syngenta's stewardship agreements with growers will include a term requiring growers to divert this product away from export markets (*i.e.* channeling) where the grain has not yet received regulatory approval for import. Syngenta will communicate these requirements to growers using a wide-ranging grower education campaign (*e.g.*, grower Stewardship Guide). As noted in the context of the IRM program, these procedures are not hypothetical.

The ability to channel particular types of maize for particular uses, such as the export market, is demonstrated by the continuing success of the specialty maize market. Use of identity preservation measures has enabled growers to maintain a wide variety of specialized maize products, including white food maize, waxy maize, hard endosperm maize, high oil maize, nutritionally enhanced maize, high extractable starch maize, nonGMO maize, and organic maize (U.S. Grains Council, 2006). Channeling programs are well established for separating each of these maize varieties. As set out above, these practices have continued successfully long after the introduction of numerous varieties of transgenic maize.

[21] http://www.grains.org/index.ww

IX.E. Conclusion

Syngenta is seeking a determination of deregulated status for MIR162 maize under APHIS regulation 7 CFR §340.6. NEPA (42 U.S.C. 4321 *et seq*.) requires agencies undertaking such actions to provide a detailed statement of the environmental impact of the proposed action, any adverse environmental effects that cannot be avoided, and alternatives to the action (42 U.S.C. 4332). Where the significance of an action is uncertain, agencies may use an EA to identify, analyze and evaluate the impacts of the proposed action. The EA will satisfy the NEPA obligation where it provides sufficient evidence and analysis to support a FONSI. Factors to assess significance of a proposed action as listed by the CEQ are addressed in this petition. Based on the analysis of data and information provided in this petition, Syngenta believes an EA is sufficient to evaluate the impact of deregulation of MIR162 and that a conclusion of no significant impact is warranted thereby satisfying the requirements of NEPA.

X. References

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Key to Abbreviations and Acronyms

ADF	Acid detergent fiber
AOAC	Association of Analytical Communities
APHIS	Animal and Plant Health Inspection Service
bc	Backcross
bp	Base pair
BSA	Bovine serum albumin
bw	Body weight
CFR	U.S. Code of Federal Regulations
Cry	Crystal protein from *B. thuringiensis*
DDD	Daily dietary dose
DF	Dilution factor
DT_{50}	Time to 50% dissipation
DTT	Dithiotheitol
dw	Dry weight
EA	Environmental assessment
EDTA	Ethylenediamine tetraacetic acid
EEC	Expected environmental concentration
ELISA	Enzyme linked immunosorbent assay
EPA	United States Environmental Protection Agency
FA	Fatty acids
FDA	United States Food and Drug Administration
FFDCA	Federal Food, Drug and Cosmetic Act
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
FONSI	Finding of no significant impact
fw	Fresh weight
HFCS	High fructose corn syrup
HPLC	High performance liquid chromatography
ILSI	International Life Sciences Institute
IPM	Integrated pest management
IRM	Insect resistance management
kb	Kilobase
LB	Left border

(Continued)

Key to Abbreviations and Acronyms (cont.)

LC_{50}	Median lethal concentration
LOD	Limit of detection
LOQ	Limit of quantification
MES	2-(N-morpholino)ethanesulfonic acid
MIR162	Maize insect resistant transformation event encoding a Vip3Aa20 protein derived from *B. thuringiensis*
MW	Molecular weight
NADP	β-nicotinamide adenine dinucleotide phosphate
NADPH	β-nicotinamide adenine dinucleotide phosphate reduced
NCBI	National Center for Biotechnology Information
NDF	Neutral detergent fiber
NEPA	National Environmental Policy Act
NOEC	No observable effect concentration
NOEL	No observable effect level
OD	Optical density
OECD	Organisation for Economic Co-operation and Development
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMI	Phosphomannose isomerase
PVDF	Polyvinylidene diflouride
RB	Right border
SDS PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
TDF	Total dietary fiber
TER	Toxicology exposure ratio
T-DNA	Transferred DNA
Tris	Tris(hydroxymethyl)aminomethane
U.S.	United States
U.S.C.	United States Code
USDA	U.S. Department of Agriculture
v/v	Volume per volume
Vip	Vegetative insecticidal protein from *B. thuringiensis*
w/v	Weight per volume
w/w	Weight per weight

Appendix A. USDA Notifications for Field Trials with MIR162 Maize

Field trials with maize transformation event MIR162 have been conducted in the U.S. under USDA release permits or notifications since 1999. A listing of these notifications, along with a status of the report for each, is provided in Table A.1.

Table A.1. List of field release permits and notifications that MIR162 maize have been planted under.

Reference #	Effective Dates	Release Sites (State) [a]	Report Status
99-032-02r	03/31/99-03/31/00	IL	Submitted
00-024-02r	4/30/00-04/30/01	HI	Submitted
01-022-07r/m	4/30/01-4/30/02	AR, FL, ID, IL, MN, PR	Submitted
02-022-01r/m	4/10/02-4/10/03	IL, MN, MS	Submitted
02-022-02r/m	4/30/02-4/30/03	HI	Submitted
03-021-01r/m	4/30/03-4/30/04	AZ, CA, FL, IA, IL, KS, MN, MO, MS, NC, NE, PR, TX, WI	Submitted
04-072-06n	4/26/04-4/26/05	FL, HI, IA, IL, MN, PR, WI	Submitted
04-203-05n	8/24/04-8/24/05	PR	Submitted
05-062-02n	4/25/05-4/25/06	AR, CA, FL, HI, IA, IL, IN, KS, KY, LA, MD, MN, MO, MS, NC, NE, NY, OH, PA, PR, TX, VA, WI	Submitted
05-104-09n	5/12/05-5/12/06	HI	Submitted
05-117-07n	5/10/05-5/10/06	OH	Submitted
05-255-03n	11/9/05-11/9/06	HI	Submitted
06-055-08n	4/27/06-4/27/07	CA, CO, FL, HI, IA, IL, IN, KS, LA, MN, MS, NE, NY, OH, PR, SD, TX, WI	Submitted
06-059-07n	4/27/06-4/27/07	IL	Submitted

(Continued)

Table A.1 (cont.). List of field release permits and notifications that MIR162 maize have been planted under.

Reference #	Effective Dates	Release Sites (State)	Report Status
06-284-101n	11/14/06-11/14/07	PR	Trials in progress
06-284-102n	11/13/06-11/13/07	HI	Trials in progress
07-032-104n	3/19/07-3/19/08	FL, GA, ID, MN	Trials in progress
07-043-109n	4/5/07-4/5/08	AR, CO, DE, FL, GA, HI, ID, IL, IN, IA, KS, KY, LA, ME, MD, MN, MO, NE, NC, OH, PR, SD, TX, VA, WI	Trials in progress
07-050-101n	4/5/07-4/5/08	HI, IA, IL, MN, NE, PR, TN, WI	Trials in progress
07-166-101n	7/16/07-7/16/08	IA	Trials in progress

[a] – Includes only states where plantings took place.

Appendix B. Genetic Characterization of MIR162 Maize

Materials and Methods

Plant Material

MIR162 maize seeds were obtained for multiple backcross (BC) generations: BC_1F_1, BC_2F_1, and BC_4F_1. Seeds from each of these generations were planted and grown under standard greenhouse conditions and then processed to extract genomic DNA. See Table 2 and Figure 5 for the pedigree of each generation.

Plant Processing: From Plants to DNA

All plants grown in the greenhouse were individually sampled and analyzed by TaqMan PCR for the presence of the *vip3Aa* and *manA* genes. Leaf tissue was collected from the plants of each generation, pooled, and DNA was extracted and quantified for use in Southern blot analyses. See schematic in Figure B-1.

TaqMan PCR

All plants were individually analyzed using TaqMan PCR (Ingham *et al.*, 2001) for the presence of the *vip3Aa* and *manA* genes. For each individual plant, DNA was isolated from leaf discs using the Wizard Genomic DNA Purification kit. MIR162 maize plants were confirmed positive for both the *vip3Aa* and *manA* genes, while absence of these genes was confirmed for the negative segregant plants. All plants were TaqMan PCR positive for the assay's internal control, the endogenous maize *adh1* gene, as expected. See Table B-1 for primers used in the detection of *vip3Aa, manA,* and *adh1*. The cycling parameters for this reaction were as follows: 95ºC for 5 minutes, 40 cycles of 95ºC for 15 seconds, and 60ºC for 1 minute.

Table B-1. Primers and probes used for detection of the *vip3Aa*, *manA*, and *adh1*.

Gene	Forward Primer 5' to 3'	Reverse Primer 5' to 3'	Probe 5' to 3'
vip3Aa	CACCTTCAGCAACCCGAACTA	GCTTAGCCTCCACGATCATCTT	GTCCTCGTCGCTGCCCTTCACCT
manA	CCGGGTGAATCAGCGTTT	GCCGTGGCCTTTGACAGT	TGCCGCCAACGAATCACCGG
adh1	GAACGTGTGTTGGGTTTGCAT	TGCAGCCTAACCATGCGCAGGGTA	TCCAGCAATCCTTGCACCTT

Plant Pooling for Genomic DNA Extraction

Based upon the TaqMan results, ten positive plants and ten negative segregant plants (hereafter negative control) were selected to be used for molecular characterization. Leaf tissue from the ten positive plants was pooled into one sample bag and stored at -80ºC. This process was repeated for the ten negative control plants.

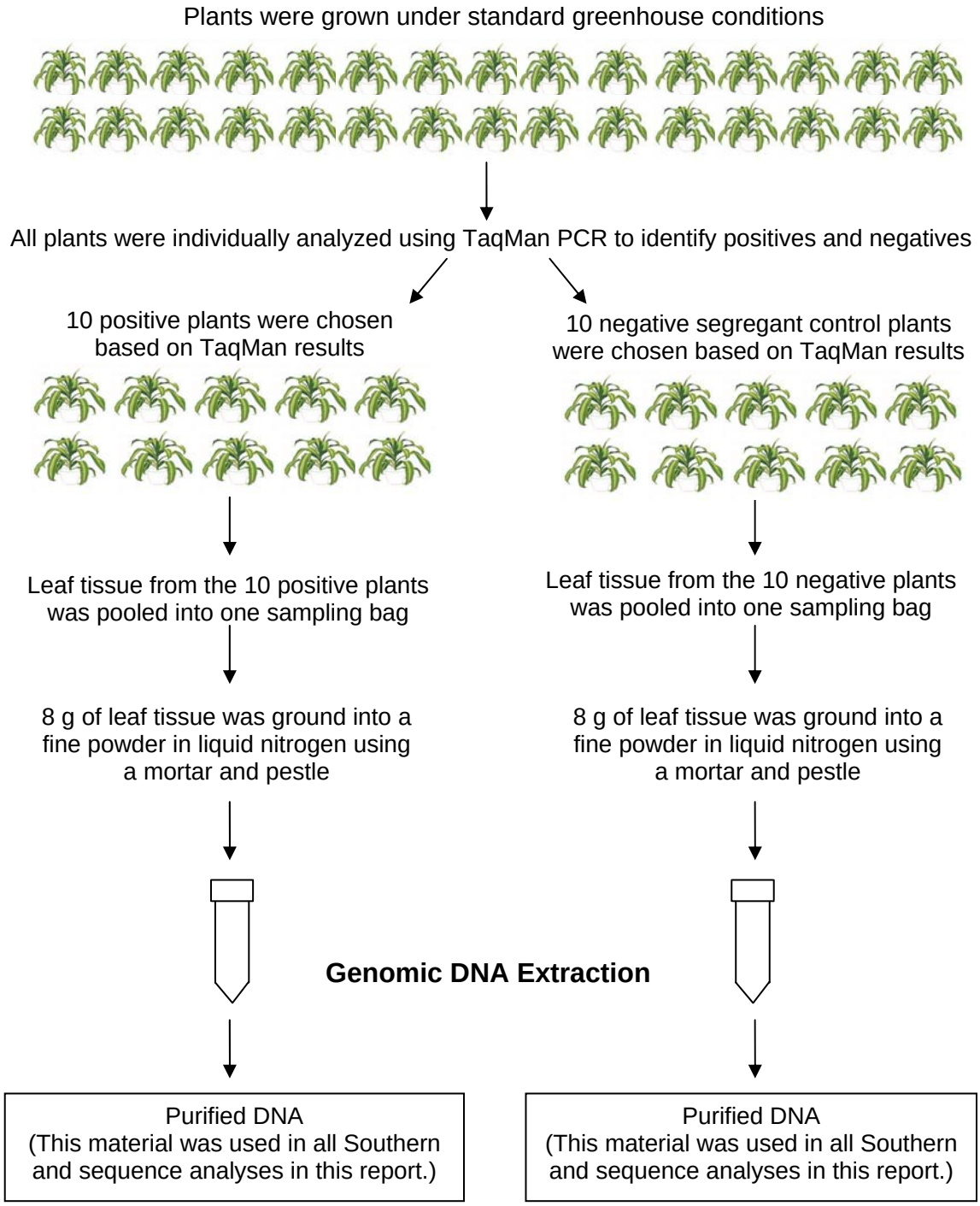


Figure B-1. Schematic representation of the procedure for selection and processing of plant samples for DNA extraction.

Genomic DNA Extraction

Genomic DNA used for blot Southern analyses was isolated from the pooled leaf tissue using a method from Thomas *et al*. (1993).

The following buffers were used for genomic DNA extraction:

Extraction Buffer A: 0.25 M NaCl, 0.2 M Tris pH 8.0, 50 mM EDTA, 0.1% v/v 2-mercaptoethanol, 2.5% w/v polyvinylpyrolidone (PVP-40)

Extraction Buffer B: 0.5 M NaCl, 0.2 M Tris pH 8.0, 50 mM EDTA, 1% v/v 2-mercaptoethanol, 2.5% PVP-40, 3% sarkosyl, 20% ethanol

DNA was extracted by first grinding the leaf tissue into a fine powder using a mortar and pestle under liquid nitrogen. Eight grams of the positive plant tissue and eight grams of the negative control plant tissue were ground and placed into separate 50 ml conical tubes. A 25 ml volume of Extraction Buffer A was added to each tube, and samples were gently mixed and centrifuged for 10 minutes at 2755 x g. The supernatant was discarded and 6 mls of Extraction Buffer B was added to each sample. The samples were mixed and incubated for 30-60 minutes at 65ºC. Using a sterile loop, the samples were mixed once during the incubation period. After the incubation, an equal volume of chloroform/isoamyl alcohol (24:1) was added to each sample. The samples were mixed gently by inversion and centrifuged for 20 minutes at 2755 x *g*. The aqueous layer was collected in a clean 50 ml conical tube, and a 0.1 volume of 3M NaOAc pH5.2 was added and mixed. Next, a 0.7 volume of 100% isopropanol was added, and the samples were mixed by inversion and centrifuged for 5 minutes at 2755 x *g*. The aqueous layer was decanted and the pellet was allowed to air dry briefly. The pellet was resuspended in 1000 µl of 1X TE (10 mM Tris, 1 mM EDTA pH 8.0) overnight at 4ºC.

After complete resuspension, samples were transferred to microcentrifuge tubes and 6 µl of Ribonuclease A (10 mg/ml) was added, and the sample was incubated at 37ºC for 30 minutes. Samples were then centrifuged at 19,600 x *g* for 10 minutes. The supernatant was removed and placed into a new tube, and a 0.5 volume of 7.5M NH_4OAc was added. The tubes were centrifuged for 10 minutes at 19,600 x *g*. The supernatant was removed and placed in a new tube. A 0.7 volume of isopropanol was added and the samples were mixed by inversion to precipitate the DNA. The samples were centrifuged for 10 minutes at 19,600 x *g* to pellet the DNA. The supernatant was decanted and the pellet was washed twice in 500 µl of 70% ethanol, spinning for 5 minutes at 19,600 x *g* after each wash. The supernatant was decanted and the pellet air dried briefly. Finally, the pellet was resuspended in approximately 500 µl of 1X TE at 65ºC. DNA was stored at 4ºC.

DNA Quantification

The concentration of each DNA sample was measured using the PicoGreen QuantiT technology with a Turner Biosystems TBS-380 Fluorometer following the manufacturer's instructions. A Lambda DNA standard was used to calibrate the instrument prior to

quantitation.

DNA Insert Sequencing

The DNA insert was amplified from MIR162 maize DNA derived from a BC_4F_1 generation (see Figure 5) as four overlapping fragments (see Figure B-2 below). PCR amplification was carried out using either an Expand High-Fidelity PCR system or *Pfu*Ultra Hotstart High-Fidelity DNA polymerase. Each PCR product was cloned into either a pCR-XL-TOPO vector or a pCR-BluntII-TOPO vector, and three clones for each PCR product were sequenced. Sequencing was carried out using an ABI3730XL analyzer with ABI BigDye 1.1 or Big Dye 3.1 dGTP (for GC-rich templates) chemistry. The sequence analysis was performed using the Phred, Phrap, and Consed package from the University of Washington and was carried out to an error rate of less than 1 in 10,000 bases (Ewing and Green, 1998). The final consensus sequence of the DNA was determined by combining sequence data from the three individual clones from each of the four PCR fragments to generate one consensus sequence. Sequence alignment was performed using the ClustalW program with the following parameters: scoring matrix BLOSUM55, gap opening penalty 15, gap extension penalty 6.66 (Thompson *et al.*, 1994).

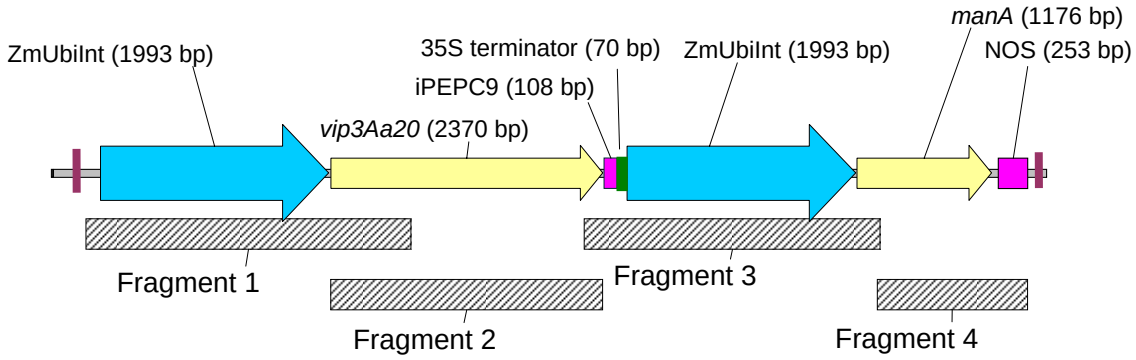


Figure B-2. Location of PCR-amplified fragments from MIR162 maize to determine insert sequence.

Southern Blot Analysis

Southern blot analyses were performed using standard molecular biology techniques (Chomczynski, 1992). Genomic DNA (7.5 µg) was digested with the appropriate restriction enzymes overnight at the optimal temperature for each enzyme. Additional enzyme was added to each reaction the following morning, and the reaction was allowed to continue for approximately four hours. Digested DNA was loaded onto 1% agarose gels and bands were separated electrophoretically in 1X TAE buffer. Following a 10 minute depurination in 0.25 N HCl, DNA was denatured in 0.5 M NaOH and 1.5 M NaCl for 30 minutes. The DNA was then transferred to a Zeta-Probe GT membrane *via* alkaline transfer for one hour using a Boekel/Appligene Vacuum Blotter. The membranes were briefly rinsed in 2X SSC. The DNA was then crosslinked to the membrane using a Stratalinker UV Crosslinker with the

'auto crosslink' setting.

Element-specific, full-length PCR-generated probes were labeled with dCTP-^{32}P *via* random priming using the Megaprime DNA labeling system. For all element-specific probes 5-25 ng was used for labeling; 5 ng was used for DNA molecular weight marker probes. Unincorporated isotope was removed using the Micro Bio-Spin Chromatography Columns. Membranes were incubated in prehybridization solution (PerfectHyb Plus Hybridization Buffer), and calf thymus DNA (100 µg/ml) for approximately one hour at 65ºC. The element-specific and molecular weight marker radiolabeled probes were added to the prehybridization solution, and the membranes were incubated for a minimum of three hours. Hybridization was carried out at 65°C, followed by multiple washes in 2X SSC, 0.1% SDS and 0.1X SSC, 0.1% SDS. The membranes were then subjected to autoradiography.

One positive control was included in each Southern blot: a pNOV1300 plasmid control representing one copy equivalent based on plasmid size was added to DNA from negative control plants. The calculation for the pNOV1300 plasmid is shown below (Table B-2).

Table B-2. Formula used to determine one copy equivalent based on plasmid size.

((Plasmid size/(Genome size * Ploidy)) * µg loaded) * 1x10^6 =	pg for 1 copy
Example:	
Z. mays genome size in bp:	2.67x10^9
Ploidy:	2
pNOV1300 size in bp:	14405
µg of digested MIR162 maize DNA loaded for Southern analysis:	7.5
Calculation for pNOV1300:	
((14405 / (2.67x10^9 * 2)) * 7.5) * 1x10^6 =	20.2 pg

The copy number of the T-DNA functional elements present in the MIR162 maize genome was determined by Southern blot analyses. For each functional element of MIR162 maize, genomic DNA was digested using three restriction enzyme strategies. In the first enzyme digestion, MIR162 maize genomic DNA was digested with an enzyme that digests once within the DNA insert but not within the functional element being probed. This digest will result in a single unique hybridization band for each copy of the functional element present in the MIR162 maize genome. In the second enzyme scheme, another enzyme that digests once within the DNA insert but not within the functional element being probed was employed. This digest will also result in a single unique hybridization band for each copy of the functional element present. Additionally, MIR162 maize genomic DNA was digested with an enzyme(s) that released a known size fragment. This restriction enzyme strategy gives further evidence of copy number and also demonstrates the intactness of the insert. A negative control was included in each Southern blot experiment to identify endogenous *Z.*

mays sequences that may hybridize with the element-specific probe. Digested pNOV1300 plasmid equal to one copy equivalent based on plasmid size was included in each Southern blot analysis to demonstrate a positive control for hybridization, as well as the sensitivity of the experiment.

Mendelian Inheritance of DNA Insert

The initial MIR162 maize plant (T_0 generation) was crossed with maize inbred line NPH8431, creating the F_1 generation. NPH8431 (MIR162 maize) plants from this F_1 generation were crossed to the inbred line NP2161 to yield the F_1 generation. NP2161 (MIR162 maize) plants from this F_1 generation were backcrossed to the inbred line NP2161 to yield the BC_1F_1 generation. MIR162 maize plants from this BC_1F_1 generation were backcrossed again to inbred line B9620 to yield BC_2F_1 generation. The F_1 generation was backcrossed two more time with inbred line B9620 to yield a BC_4F_1 generation (refer to Figure 5). Positive segregants were utilized in each backcross. Material from the BC_1F_1, BC_2F_1, and BC_4F_1 generations of B9620 was used in the Mendelian inheritance study.

Individual plants from the BC_1F_1, BC_2F_1, and BC_4F_1 generations were assayed by TaqMan PCR for the presence of the *vip3Aa* and *manA* genes. The expected Mendelian inheritance ratio of positive to negative plants for a hemizygous trait in these populations was 1:1. Genotypic data (Tables 10 and 11) were used to assess the goodness-of-fit for the ratio of observed (o) genotypes to the ratio of expected (e) genotypes using Chi-square (X^2) analysis with Yates correction factor:

$$\chi^2 = \sum (| o - e | - 0.5)^2 / e$$

Flanking Sequence

Nucleotide sequences flanking the 5' and 3' ends of the T-DNA in MIR162 maize were screened for homology with sequences found in public databases. The purpose of this comparison was to provide an indication of whether the MIR162 maize T-DNA inserted into a known plant functional genetic unit. A sequence similarity analysis was performed using the BLASTN software (Altschul *et al.*, 1997; version 2.2.6 Apr-9-2003) which compared the flanking sequences with nucleotide sequences in the latest version of the National Center for Biotechnology Information nonredundant database.

The nonredundant database contains all sequences from the National Institutes of Health genetic sequence database (GenBank), RefSeq Nucleotides, the European Molecular Biology Laboratory (EMBL), the DNA Database of Japan (DDBJ) and sequences derived from the three-dimensional structure (PDB) database. Whereas GenBank is an archival repository of all sequences, the RefSeq database is a nonredundant set of reference standards that includes chromosomes, complete genomic molecules (organelle genomes, viruses, and plasmids), intermediate assembled genomic contigs, curated genomic regions, mRNAs, RNAs, and proteins. The nonredundant database does not contain any EST, STS, GSS, or phase 0, 1, or 2 HTGS sequences. At the time of this analysis, the nonredundant database contained 4,654,881 unique sequences and was last updated on December 14, 2006. The query

sequence was not filtered for low complexity. Parameters for the BLASTN analysis were as follows:

- Expect = 10. The Expect value (E-value) is the significance threshold for reporting matches against database sequences; the default value is 10, meaning that 10 matches are expected to be found merely by chance, according to the stochastic model of Karlin and Altschul (1990). Those results for which the Expect value is close to 0 will be unlikely to have occurred at random and, therefore, will be statistically significant hits. Those hits with E-values close to 10 will have happened randomly and are, therefore, not statistically significant.

- The scoring scheme (bits) used is the default for nucleotides: +1 for a match and –3 for a mismatch. Gap penalties: Existence = 5, Extension = 2. A gap is a space introduced into an alignment to compensate for insertions and deletions in one sequence relative to another. Gap existence and extension penalties are chosen empirically. To prevent the accumulation of excessive gaps in an alignment, the introduction of a gap causes the deduction of a fixed amount from the alignment score. Extension of the gap to encompass additional nucleotides is also penalized in determining the score of an alignment. The resultant score is derived from the number of identical matches between the query sequence and the database entry, with higher scores indicating greater homology between the two sequences.

To ascertain if any potential novel open reading frames were generated at the point of insertion in the *Z. mays* genome, the junctions between genomic sequences and the MIR162 maize DNA insert were examined for the presence of open reading frames using Vector NTI (version 9.0) software. An open reading frame was defined in the bioinformatic program as a region corresponding to at least 50 amino acids in length initiating with an ATG codon and ending with any of the three stop codons: TAA, TAG, or TGA. All six possible reading frames at both the 5' and 3' regions were examined using the above criteria.

Appendix C. Characterization of the Vip3Aa20 and PMI Proteins

Vip3Aa Test, Control, and Reference Materials

Vip3Aa20 Extracted from MIR162 Maize Leaf Tissue

Maize leaf tissue was used as a source of MIR162 maize-expressed Vip3a20. Leaf tissue samples were harvested from greenhouse-grown MIR162 maize plants. Leaves were collected four to six weeks after plant emergence, frozen at -80 ± 10°C, and subsequently ground into fine powder and lyophilized. Lyophilized leaf powder was suspended in Vip3Aa extraction buffer containing 50 mM Tris-HCl (pH 9.5), 2 mM EDTA, 100 mM NaCl, 2 mM DTT and 1 µM leupeptin. The mixture was homogenized in a pre-cooled CB6 Waring industrial blender equipped with a double blade. The homogenate was then filtered through eight layers of cheesecloth and the collected solids were retained and extracted again in half of the original extraction volume. Both extracts were pooled, adjusted to a final concentration of 0.075% in polyethylenimine and centrifuged at *approximately* 7280 x g for 20 min at 2 - 8˚C. The supernatant was filtered through Miracloth filtration material and fine powdered crystalline ammonium sulfate was added to a final concentration of 40% (24.6 g ammonium sulfate/100 ml extract). The mixture was slowly stirred overnight at 2 - 8˚C and then centrifuged at *approximately* 7280 x g for 20 min at 2 - 8˚C. The supernatant was discarded and the resultant precipitates were stored at 2 - 8˚C. The precipitate was re-suspended in dialysis buffer containing 50 mM ammonium carbonate (pH 9.5), centrifuged to remove any insoluble debris and dialyzed 3 times (5 - 12 hr each) in dialysis buffer. The dialyzed extract was then lyophilized and stored at -20 ± 8˚C.

Two batches of purified MIR162 maize-derived Vip3Aa20 were prepared and designated test substances LPMIR162-0105 and LPMIR162-0106 and were used for the Western blot analysis and insect bioassays.

Immuno-affinity Purified Vip3Aa20 from MIR162 Maize Leaf Tissue

Immuno-affinity-purified Vip3Aa20 was prepared from the ammonium sulfate precipitate as described above for test substances LPMIR162-0105 and LPMIR162-0106. The precipitate was re-suspended in column equilibration buffer containing 50 mM Na_2HCO_3 and 100 mM NaCl, then centrifuged to remove insoluble debris. The supernatant was dialyzed three times (5 - 12 hr each) in column equilibration buffer, and subsequently loaded onto an equilibrated immuno-affinity column which had rabbit anti-Vip3Aa antibody bound to the matrix. Vip3Aa20 was eluted with 100 mM CAPS buffer (pH 11), neutralized, concentrated with ultra-filtration using Centricon 20-Plus centrifugal filters and stored on ice before further use. Two batches of immuno-affinity purified MIR162 maize-derived Vip3Aa20 were prepared and designated test substances IAPMIR162-0105 and IAPMIR162-0106. These test substances were used in the Western blot and peptide mapping analyses.

Vip3Aa20 from *E. coli*

Test substance MIR162VIP3A-0106, containing Vip3Aa20 protein, was prepared by

expressing a synthetic *vip3Aa20* gene in an *E. coli* over-expression system. The Vip3Aa20 in test substance MIR162VIP3A-0106 is predicted to be identical in amino acid sequence to Vip3Aa20 expressed in MIR162 maize. The synthetic *vip3Aa20* gene was cloned into the inducible, over-expression vector pET-24a in *E. coli* strain BL21DE3pLysS.

Test substance MIR162VIP3A-0106 was prepared from pooled batches of *E. coli* cell paste, was lyophilized, and stored at -20 ± 8°C. Purification was accomplished as follows: *E. coli* cell pellets were ruptured and the cell debris removed by centrifugation. The Vip3Aa20 present in the supernatant was further purified with anion exchange chromatography using a Q Sepharose FF column. Vip3Aa20 was eluted from the column with a NaCl gradient. Fractions containing Vip3Aa20 were pooled and dialyzed in 20 mM ammonium bicarbonate buffer (pH 10), frozen and lyophilized. The lyophilized Vip3Aa20 sample preparation was designated test substance MIR162VIP3A-0106.

Control Substance – Maize Leaf Extract

Control substance LPMIR162-0105C was prepared from leaf material derived from MIR162 null segregant plants in a similar manner as described above for Vip3Aa20 extracted from MIR162 maize leaf tissue. Since its preparation, this control substance has been stored at -20 ± 8˚C.

Vip3Aa19 from *E. coli* - Reference Substance

Reference substance VIP3A-0204, containing Vip3Aa19, was prepared from pooled batches of *E. coli* cell paste and was determined to contain approximately 92% Vip3Aa19 by weight, and was demonstrated to have insecticidal activity against *S. frugiperda* larvae.

A listing of the test, control, and reference substances used in these characterization studies is shown in Table C-1.

Table C-1. Identification and source of test, control, and reference substances.

Test Substance ID	Vip3Aa Variant	Source
LPMIR162-0105	Vip3Aa20	Extract of MIR162 leaf tissue
LPMIR162-0106	Vip3Aa20	Extract of MIR162 leaf tissue
IAPMIR162-0105	Vip3Aa20	Immuno-affinity purified extract of MIR162 leaf tissue
IAPMIR162-0106	Vip3Aa20	Immuno-affinity purified extract of MIR162 leaf tissue
MIR162VIP3A-0106	Vip3Aa20	*E. coli*
LPMIR162-0105C	No Vip3Aa protein	Control maize leaf extract
VIP3A-0204	Vip3Aa19	*E. coli*

Methods for Vip3Aa20 Characterization and Equivalence

Total Protein Determination

Total protein in test substance MIR162VIP3A-0106 was quantified spectrophotometrically by determining the absorption at 280 nm (Gill and von Hippel, 1989). The A280 method is based upon the absorption of the aromatic amino acids tryptophan and tyrosine at 280 nm. The extinction coefficient at 280 nm varies with the abundance of these amino acids in the target protein. A Genesys 6 spectrophotometer was used to measure absorption of MIR162VIP3A-0106 at 280 nm and the extinction coefficient of Vip3Aa20 was calculated with Vector NTI software version 9. The absorbance at 280 nm was multiplied by the correlation factor for the extinction coefficient, to give an approximate total protein concentration.

Total protein in test substances LPMIR162-0105, LPMIR162-0106 and control substance LPMIR162-0105C was quantified using the BCA method (Hill and Straka, 1988), using bovine serum albumin (BSA) as the protein standard.

Densitometry

For purity determination, aliquots of MIR162VIP3A-0106 (ranging from 1 to 20 µg Vip3Aa20 per lane) were subjected to SDS-PAGE using NuPAGE 4-12% Bis-Tris polyacrylamide gradient gel and MES running buffer. After electrophoretic separation, protein bands were visualized with Coomassie staining and the distribution of the visible protein bands was estimated by densitometric analysis using a Personal Densitometer SI and ImageQuant analysis software for Windows.

Glycosylation Analysis

Vip3Aa20 from MIR162 maize was previously determined to show no evidence of glycosylation. To determine whether Vip3Aa20 in test substance MIR162VIP3A-0106 was glycosylated, 1 and 2 µg samples of the insecticidal protein were analyzed using the DIG Glycan Detection Kit, in accordance with the manufacturer's instructions. Creatinase, a nonglycosylated protein, was included as a negative control. Transferrin, a glycosylated protein, was used as a positive control. Samples were separated by SDS-PAGE using NuPAGE 4-12% Bis-Tris polyacrylamide gradient gel and MES running buffer, and electro-blotted to PVDF membrane. While on the membrane, glycan moieties were oxidized using periodate, labeled with digoxigenin, and detected with an anti-digoxigenin antibody coupled to alkaline phosphatase.

N-Terminal Amino Acid Sequence Analysis

For N-terminal amino acid sequence analysis, test substance MIR162VIP3A-0106 was subjected to SDS-PAGE followed by electro-blotting of the protein to a PVDF membrane. After staining the membrane with Amido black, the corresponding Vip3Aa20 band was excised and sent to Proseq, Inc. Protein Sequencing Services for N-terminal amino acid

sequence analysis. ProSeq's methodology was developed specifically for proteins immobilized on PVDF membrane and optimized for automated Edman-based chemistry (Brauer *et al.,* 1984).

Vip3Aa20 Quantification

The concentration of Vip3Aa20 in test substances LPMIR162-0105, LPMIR162-0106, IAPMIR162-0105, IAPMIR162-0106 and MIR162VIP3A-0106 was determined using sandwich ELISA (Tijssen, 1985). Control substance LPMIR162-0105C was used as a negative control in the ELISA analysis.

96-well Nunc MaxiSorp plates were coated overnight and incubated at 2 - 8°C with goat anti-Vip3Aa immuno-affinity purified polyclonal antibodies in BBS containing 100 mM boric acid, 25 mM sodium borate, 75 mM sodium chloride (pH 8.5; 100 µl/well). The plates were then washed five times with ELISA wash buffer containing 10 mM Tris, 0.05% Tween 20, 0.02% sodium azide (pH 8.0), and incubated in ELISA diluent containing phosphate buffered saline plus 0.05% Tween 20, 1% BSA and 0.02% sodium azide (pH 7.4), for at least 45 min. The plates were then washed again as described above and incubated with the Vip3Aa20 sample solutions (100 µl/well) for 1.5 hr at 2 - 8°C followed by 30 min at room temperature. After washing, the plates were incubated with rabbit anti-Vip3Aa antibody diluted in ELISA diluent for 1 h at 37 ± 1°C. The plates were washed and subsequently incubated with donkey anti-rabbit IgG conjugated with alkaline phosphatase antibody, diluted to 1 µg per ml in ELISA diluent, for 1 h at 37 ± 1°C. The plates were washed again and incubated in phosphatase substrate solution at room temperature for 30 min to allow color development. The reaction was stopped by addition of 3 N sodium hydroxide and absorbance was measured at 405 nm with a Tecan Sunrise multi-well plate reader. The results were analyzed using the DeltaSoft Curve fitting software program. The four parameters algorithm was used to generate a curve.

Molecular Weight Determination and Immunoreactivity Analysis

The integrity of Vip3Aa protein in test substances LPMIR162-0105, LPMIR162-0106, MIR162VIP3A-0106, and VIP3A-0204 was investigated using Western blot analysis. Aliquots containing 10 ng Vip3Aa20 prepared in 4X LDS NuPAGE Sample Buffer from the described test substances were subjected to SDS-PAGE using NuPAGE 4-12% Bis-Tris polyacrylamide gradient gel and MES running buffer. Based on total soluble protein, an equivalent amount (containing approximately 676 µg of total soluble protein) of control substance LPMIR162-0105C was also included in the analysis as a negative control. After electroblotting, the membrane was incubated with goat anti-Vip3Aa immuno-affinity purified polyclonal antibodies. Donkey anti-goat IgG linked to alkaline phosphatase diluted 1:3,000 was used as the secondary antibody to bind to the primary antibody and visualized by development with alkaline phosphatase substrate solution. The Western blot was examined for the presence of intact immunoreactive Vip3Aa20 and other immunoreactive Vip3Aa polypeptides. SeeBlue Plus2 molecular weight standard was used to establish approximate molecular weights.

Insecticidal Activity

Test solutions for the bioassay were prepared in 50 mM Tris-HCl (pH 9.5) containing 2 mM EDTA from test substances LPMIR162-0106, LPMIR162-0105C and MIR162VIP3A-0106 and tested concurrently. Also a comparison of Vip3Aa20 bioactivity (LPMIR162-0105) to Vip3Aa19 bioactivity (VIP3A-0204) was conducted in separate, but similar insect bioassays. Bioactivity of the proteins was assessed in insect feeding assays using freshly hatched first-instar *S. frugiperda.* The bioassays were conducted in Costar 24-well plates. Each well contained 800 µl insect diet overlayed with 50 µl test solution, containing concentrations ranging from 7.5 to 825 ng Vip3Aa/cm^2 diet surface. Each treatment consisted of 24 individual wells containing one larva/well. Insect diet treated with MilliQ purified (deionized) water alone, insect diet treated with buffer alone, and insect diet treated with control substance LPMIR162-0105C at approximately 4910 µg total protein (an equivalent amount of total protein as applied in the highest test concentration of LPMIR162-0106), were used as negative controls. The wells were covered with silicone stoppers and maintained at room temperature under ambient laboratory conditions. Mortality was assessed after 168 hours and is reported for each treatment as the percent mortality.

Peptide Mass Mapping Analysis

Aliquots, containing 1 µg Vip3Aa20 from either test substance IAPMIR162-0106 or test substance MIR162VIP3A-0106, were subjected to SDS-PAGE using a 4 - 12% Bis-Tris polyacrylamide gradient gel and MES running buffer. The gel was stained with Coomassie and then prepared for peptide mass mapping analysis. Vip3Aa20 bands were excised from the SDS-gel, the protein was reduced, alkylated with iodoacetamide, and digested with trypsin. The mass analysis of the Vip3Aa20-derived peptides was performed on a quadrupole time-of-flight mass spectrometer, fitted with an EPCAS upgrade and connected to a capillary HPLC instrument. Peptide masses were identified by matching the detected peptide masses to a protein database using Mascot software.

Statistical Analysis

The LC_{50} and 95% confidence intervals determined in the *S. frugiperda* larval bioassays were computed by means of a probit analysis.

Results for Vip3Aa20 Characterization and Equivalence

Vip3Aa20 Quantitation and Purity Determination

MIR162VIP3A-0106 was determined to contain 93.0% protein as measured by absorption at 280 nm. Densitometric analysis indicated that Vip3Aa20 represented approximately 90.3% of the total protein in MIR162VIP3A-0106 (Figure C-1). The overall purity of the test substance was therefore, determined to be 84.0% Vip3Aa20 w/w (Table C-2).

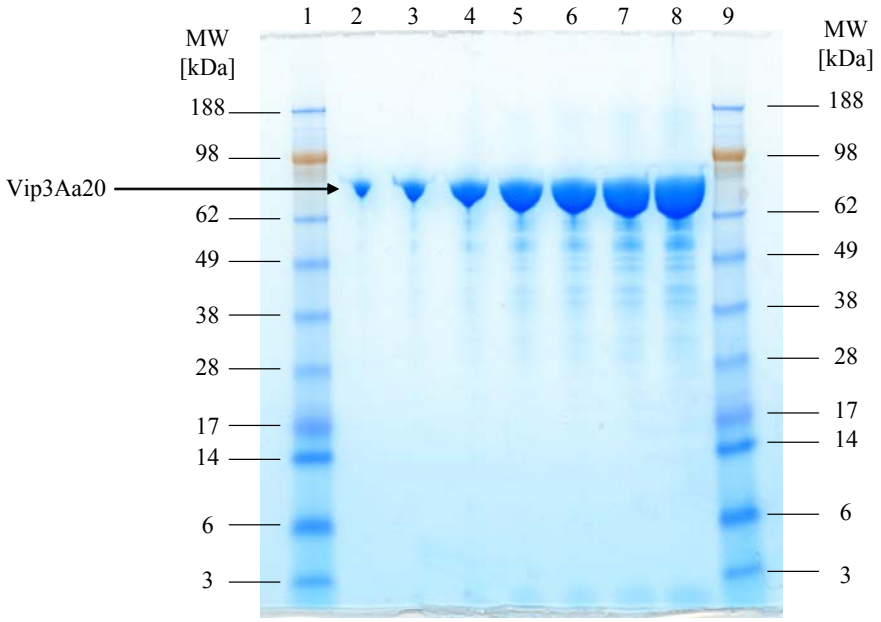


Figure C-1. Purity determination of Vip3Aa20 from *E. coli* (MIR162VIP3A-0106) by SDS-polyacrylamide gel electrophoresis. The molecular weight of Vip3Aa20 corresponds to approximately 89 kDa.

Lanes 1 and 9: Molecular weight standard SeeBlue Plus2
Lanes 2 to 8: 1, 2, 4, 8, 10, 15, 20 µg Vip3Aa20 (MIR162VIP3A-0106)

Table C-2. Characterization of Vip3Aa20 extracted from *E. coli* (MIR162VIP3A-0106).

Total protein [g protein/g extract]	**Densitometric Analysis** [% Vip3Aa20/total protein]	**Purity** [% Vip3Aa20/extract]
0.93 (93.0%)	90.3	84.0

Glycosylation Analysis

No bands representing glycosylated Vip3Aa20 were visible upon DIG Glycan analysis (Figure C-2), providing evidence that, as expected, the *E. coli*-produced Vip3Aa20 is not glycosylated. By comparison, 25 ng of the positive control protein, transferrin, generated a clearly visible band (Figure C-2, lane 3). Transferrin has a molecular weight of approximately 80,000 and contains approximately 5% glycan moieties by weight. This corresponds to approximately 25 glucose equivalents/molecule (the molecular weight of the bound glucose moiety was calculated to be 162). Out of the 25 ng of transferrin loaded on the gel lane, 1.25 ng could be attributed to glycan moieties. Therefore, 1.25 ng represents the approximate limit of detection for this system. Vip3Aa20 from microbial test substance

MIR162VIP3A-0106 was loaded in concentrations of approximately 1000 and 2000 ng/lane. Thus, the 1.25 ng glycan detection limit corresponds to approximately 0.0625% by weight (1.25 ng/2000 ng), or approximately 0.34 glucose equivalents/Vip3Aa20 molecule. Therefore, the results of the DIG Glycan analysis indicate that either Vip3Aa20 is not glycosylated, or that glycan moieties occur at a frequency of less than one glucose equivalent per molecule of Vip3Aa20. Vip3Aa20 extracted from MIR162 maize (IAPMIR162-0105) has also been found not to be glycosylated (data not shown).

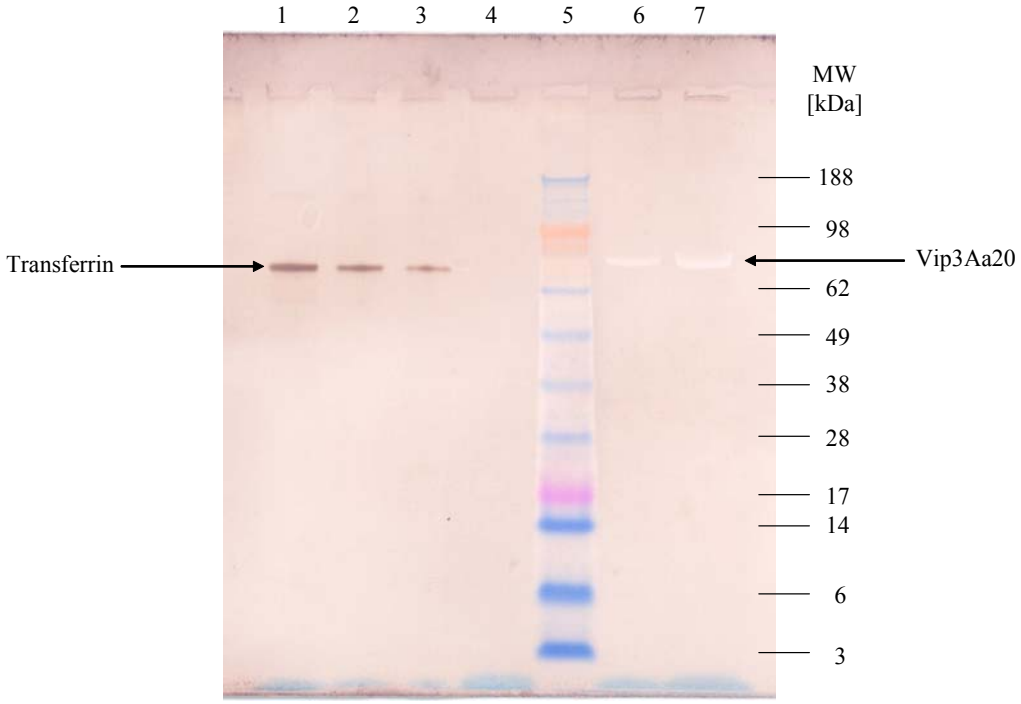


Figure C-2. Glycosylation analysis of Vip3Aa20 produced in recombinant *E. coli* (MIR162VIP3A-0106). Vip3Aa20 from *E. coli* was analyzed for the presence of glycosyl residues using the DIG Glycan Detection Kit. The molecular weight of Vip3Aa20 corresponds to approximately 89 kDa.

Lanes 1, 2 and 3: 100, 50, 25 ng transferrin (positive control)
Lane 4: 2 µg creatinase (negative control)
Lane 5: molecular weight standard SeeBlue Plus2
Lanes 6 and 7: 1 and 2 µg Vip3Aa20 (MIR162VIP3A-0106)

N-Terminal Amino Acid Sequence Analysis

The N-terminal amino acid sequences for the Vip3Aa20 in *E. coli* produced test substance MIR162VIP3A-0106 confirmed the predicted amino acid sequence for the protein, as shown below in Table C-3.

Table C-3. N-terminal sequence of Vip3Aa20 produced in recombinant *E. coli*.

Predicted Amino Acid Sequence	MNKNNTKLSTRA
Vip3Aa20 from MIR162VIP3A-0106	MNKNNTKLSTRA

The N-terminal sequence of the plant derived protein could not be determined due to technical difficulties (most likely N-terminal blockage of the protein). Instead, the sequence of plant-derived Vip3Aa20 protein was determined by peptide mass mapping (described below).

Peptide Mass Mapping Analysis

The analyses of the plant- and microbially-derived Vip3Aa20 by peptide mass mapping covered approximately 40% and 45%, respectively, of the predicted Vip3Aa20 amino acid sequence. Furthermore, the identified peptides were representative of regions throughout the sequence of the Vip3Aa20 protein (including peptides close to the N- and C-termini) and therefore, strongly support the identity of the Vip3Aa20 proteins produced in MIR162 maize and in *E. coli*.

Molecular Weight Determination and Immunoreactivity Analysis

Western blot analysis of the microbial test substances VIP3A-0204 and MIR162VIP3A-0106 as well as the plant-derived test substances (LPMIR162-0105 and IAPMIR162-0105) revealed dominant immunoreactive bands corresponding to the predicted molecular weight of approximately 89 kDa (see Figure C-3). Control substance LPMIR162-0105C showed, as expected, no positive response with the anti-Vip3Aa antibody in the Western blot analysis (Figure C-3, lane 6). Additional, immunoreactive protein bands of higher mobility (lower molecular weight) than intact Vip3Aa20 were also visible in the Western blot analysis (Figure C-3, Lanes 3 to 5); these additional bands most likely represents degradation products of the Vip3Aa20 protein in samples MIR162VIP3A-0106, LPMIR162-0105, and IAPMIR162-0105.

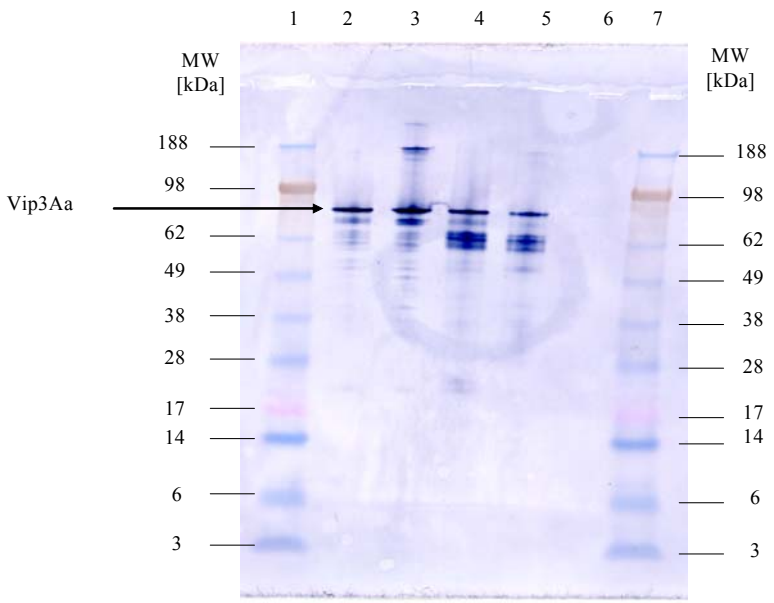


Figure C-3. Western blot of plant and microbially-produced Vip3Aa proteins.
This Western blot shows the immunoreactivity of Vip3Aa19 from *E. coli*, Vip3Aa20 from *E. coli*, and Vip3Aa20 from MIR162 maize. The molecular weights of Vip3Aa19 and Vip3Aa20 correspond to approximately 89 kDa.
Lanes 1 and 7: molecular weight standard
Lane 2: 10 ng Vip3Aa19 from *E. coli*
Lane 3: 10 ng Vip3Aa20 from *E. coli*
Lane 4: 10 ng Vip3Aa20 from MIR162 maize leaf tissue extract
Lane 5: 10 ng immunoaffinity-purified Vip3Aa20 from MIR162 maize leaf tissue
Lane 6: protein extract from control maize leaf tissue.

Insecticidal Activity

The results of the insect bioassays with microbial- and plant-derived Vip3Aa20 are shown in Table C-4; computed LC_{50} estimates are shown in Table C-5. Test substances MIR162VIP3A-0106 and LPMIR162-0106 showed comparable responses in the *S. frugiperda* bioassays, with estimated LC_{50}s of 225 ng Vip3Aa20/cm^2 (95% confidence interval = 155 - 289 ng/cm^2 diet surface) and 318 ng Vip3Aa20/cm2 (95% confidence interval = 232 - 451) after 168 hours, respectively. By comparison, no mortality was observed in the negative control using insect diet treated with water (0%) or insect diet treated with buffer (0%). Furthermore, control substance LPMIR162-0105C showed low mortality (4%). The t-test comparing the two LC_{50} values resulted in a p-value of 0.112. Given that the p-value is greater than 0.05 it can be concluded that there is no significant difference between the LC_{50}s for the Vip3Aa20 proteins from both sources as shown in Table C-6.

Table C-4. Insect mortality results for bioassays with Vip3Aa20 from recombinant *E. coli* (MIR162VIP3A-0106) and Vip3Aa20 from MIR162 maize (LPMIR162-0106) in diet surface bioassays with first-instar *S. frugiperda* (N=24).

Concentration [ng Vip3Aa20/cm^2][a]	Mortality at 168 hours [%]	
	MIR162VIP3A-0106	LPMIR162-0106
7.5	8	0
15	0	0
30	8	4
60	13	4
120	17	42
275	71	33
550	83	58
825	100	88
Water control [b]	0	
Buffer control [c]	0	
LPMIR162-0105C [d]	4	

[a] - Insect diet treated with Vip3Aa20 from *E. coli* or Vip3Aa20 from MIR162 leaf tissue dissolved in 50 mM Tris-HCl (pH 9.5), 2 mM EDTA (50 µl/dish).
[b] - Water control diet: Insect diet treated with water (50 µl/dish).
[c] - Buffer control diet: Insect diet treated with 50 mM Tris-HCl (pH 9.5), 2 mM EDTA (50 µl/dish).
[d] - Insect diet treated with control maize leaf extract in 50 mM Tris-HCl (pH 9.5), 2 mM EDTA.

The computed LC50 estimates for the insect bioassays with plant-derived Vip3Aa20 and *E. coli*-derived Vip3Aa19 are presented in Table C-5. Test substances LPMIR162-0105 and VIP3A-0204 showed comparable responses in the *S. frugiperda* bioassays, with estimated LC_{50} values of 154 ng Vip3Aa20/cm^2 (95% confidence interval = 94 - 222 ng/cm^2 diet surface) and 137 ng Vip3Aa19/cm^2 (95% confidence interval = 82 - 199) after 120 hours, respectively. Table C-7 displays the mortality results by individual test concentration and treatment. By comparison, no mortality was observed in the negative control using insect diet treated with water (0%) and 12% mortality was observed in the group exposed to diet treated with buffer. Furthermore, control substance LPMIR162-0105C showed, as expected, low mortality (4%).

Table C-5. Comparative bioactivity of Vip3Aa proteins from different sources.
Results of mortality bioassays with first instar *S. frugiperda* larvae are presented as LC_{50} values with their 95% confidence intervals. In the first set of bioassays, Vip3Aa20 from MIR162 maize leaf tissue was compared to Vip3Aa20 from *E. coli*. In the second set of bioassays, Vip3Aa20 from maize leaf tissue was compared to Vip3Aa19 from *E. coli*.

Test Substance	LC_{50} (ng protein/cm^2)	95% Confidence Intervals
Vip3Aa20 – MIR162 leaf extract	318	232 – 451
Vip3Aa20 – *E. coli*	225	155 – 289
Vip3Aa20 – MIR162 leaf extract	154	94 – 222
Vip3Aa19 – *E. coli*	137	82 – 199

Table C-6. Statistical comparison of the estimated LC50 values for Vip3Aa20 from recombinant E. coli (MIR162VIP3A-0106) and Vip3Aa20 from MIR162 maize leaf (LPMIR162-0106). LC50 estimates statistically compared by t-test.

Sample	Log(LC_{50}) [a]		Variance of log(LC_{50})	
MIR162VIP3A-0106	2.3530		0.00385	
LPMIR162-0106	2.5019		0.00492	
Comparison				
Difference in log(LC_{50}) [b]	**Variance of Difference**	**Diff /SED**	**P-value**	**Significance**
0.1489	0.00877	1.58981	0.112	nonsignficant

[a] - LC_{50} values are presented in Table B-5.
[b] - Log(LC_{50}) values and their respective variances were taken from SAS output.

Table C-7. Insect mortality results for bioassays with Vip3Aa19 from recombinant *E. coli* (VIP3A-0204) and Vip3Aa20 extracted from MIR162 leaf tissue in diet surface bioassays with first-instar *S. frugiperda* (N=24).

Concentration [ng Vip3Aa/cm^2][a]	Mortality at 120 hours [%]	
	VIP3A-0204	LPMIR162-0105
7.5	8	8
15	4	0
30	17	21
60	38	38
120	54	46
275	75	58
550	83	92
825	92	92
Water control [b]	0	
Buffer control [c]	12	
LPMIR162-0105C [d]	4	

[a] - Insect diet treated with Vip3Aa19 from *E. coli* or Vip3Aa20 from MIR162 leaf tissue extract was dissolved in 50 mM Tris-HCl (pH 9.5), 2 mM EDTA (50 µl/dish).
[b] - Water control diet: Insect diet treated with water (50 µl/dish).
[c] - Buffer control diet: Insect diet treated with 50 mM Tris-HCl (pH 9.5), 2 mM EDTA (50 µl/dish).
[d] - Insect diet treated with protein extract from control leaf tissue (LPMIR162-0105C) in 50 mM Tris-HCl (pH 9.5), 2 mM EDTA.

PMI Test and Control Substances

Leaf Tissue Extractions from MIR162 and Control Maize

Leaf tissue samples used in this study came from greenhouse-grown MIR162 and near-isogenic, nontransgenic maize hybrids. Leaves were collected four to six weeks after the plants emerged, frozen at -80°C ± 10°C and subsequently ground into fine powder and lyophilized. Lyophilized leaf powder was re-suspended in 3 ml PMI extraction buffer containing 50 mM Tris-HCl, pH 9.5, 2 mM EDTA, 100 mM NaCl, 2 mM DTT and 1 µM leupeptin. The mixture was homogenized using a Tomtec Autogizer and placed on ice for 30 min with an additional 3 ml PMI extraction buffer. The supernatant was collected after centrifugation for 15 min at 12,000 x g in a Sorvall RC-5B Refrigerated Superspeed

Centrifuge at 2-8ºC. The supernatant was then concentrated using Centricon 20-Plus centrifugal filters by centrifugation for 25 minutes at 2,600 x g using a Sorvall RC-3B Refrigerated Centrifuge at 2-8ºC. The concentrated supernatant was stored on ice before further use. The test substance derived from MIR162 leaf tissue was designated MIR162(+). The PMI protein in test substance MIR162(+) is 391 amino acids in length and has an expected molecular weight of approximately 42.8 kDa. The nontransgenic control leaf extract, designated MIR162(-), was produced in the same manner as test substance MIR162(+).

PMI from *E. coli*

The *manA* gene from *E. coli* was cloned into an inducible pET-3a over-expression vector in *E. coli* strain BLRDE3. The PMI protein as encoded by this vector is identical in amino acid sequence to that encoded by the vector used to produce MIR162 maize, with the exception of an additional, nonfunctional 16 amino acids at the N-terminus (13 amino acid T7-Tag and three additional amino acids arising from the polylinker), which was added for purification purposes. PMI was purified from the *E. coli* expression system by extraction and differential ammonium sulfate concentration, followed by hydrophobic interaction chromatography on either Phenyl Sepharose or Toyopearl Ether 650M and finally subjected to ion exchange chromatography on Q Sepharose. Pooling of the appropriate fractions was performed prior to dialysis into 50 mM NH_4HCO_3, pH 8.0, followed by lyophilization. The lyophilized products from two preparations were pooled and designated test substance PMI-0198. PMI has been determined to represent approximately 61% of the test substance by weight. The PMI protein in test substance PMI-0198 is 407 amino acids in length and has an expected molecular weight of *approximately* 44.4 kDa. This material has been stored as a lyophilized powder, desiccated at -20 ± 8°C.

Methods for PMI Characterization and Equivalence

Total Protein Determination

Total protein in each of the leaf extracts was quantified with a Coomassie protein assay using BSA as the protein standard (Bradford, 1976).

PMI Quantification

The leaf extracts, test substance MIR162(+) and control substance MIR162(-), were quantitatively analyzed for PMI using sandwich ELISA (Tijssen, 1985). Nunc MaxiSorp plates were coated overnight at 2-8°C with polyclonal goat antibody generated against PMI (diluted to 3 µg per ml in borate buffered saline, 100 mM boric acid, 25 mM sodium borate, 75 mM sodium chloride). Plates were washed five times with ELISA wash solution (100 mM Tris, 0.5% Tween 20, 0.2% sodium azide) using an automatic plate washer. The plates were then blocked with ELISA diluent (phosphate buffered saline + 0.05% Tween 20, 1% BSA, 0.02% sodium azide, pH 7.4) for at least 45 minutes at room temperature. Plates were washed five times with wash solution and samples of each leaf extract preparation and PMI

standard with appropriate dilutions prepared in ELISA diluent were applied (100 µl per well).

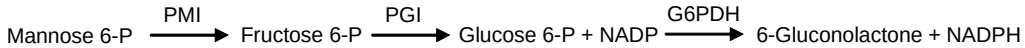
Following incubation for approximately 1.5 hours at ambient temperature, the plates were washed five times and 100 µl of polyclonal rabbit antibody generated against PMI (diluted to 3 µg per ml in ELISA diluent) was added to each well. The plates were incubated for approximately 1 hour at 37± 2ºC and then washed five times prior to the addition of 100 µl of donkey anti-rabbit alkaline phosphatase antibody, diluted to 1 µg per ml in ELISA diluent. After incubation for approximately 1 hour at 37± 2ºC, the plates were washed five times and 100 µl per well of phosphatase substrate solution (two tablets per 20 ml H_2O) was added. Color was allowed to develop for approximately 30 minutes at room temperature and the reaction stopped by the addition of 3N NaOH (100 µl per well). Absorbance at 405 nm was measured using a Tecan Sunrise multi-well plate reader. The results were analyzed using the DeltaSoft Curve Fitting software program. The four parameters algorithm was used to generate a curve.

Molecular Weight Determination and Immunoreactivity Analysis

An aliquot of MIR162(+) and PMI-0198, containing 10 ng PMI, respectively, was subjected to SDS-PAGE using a NuPAGE 4-12% Bis-Tris polyacrylamide gradient gel. For the negative control leaf extract, approximately 58 µg of total soluble protein was loaded per lane on the gel. After electroblotting, the PVDF membrane was probed with polyclonal goat antibody generated against PMI purified from *E. coli*. Donkey anti-goat IgG linked to alkaline phosphatase was used to bind to the primary antibody and visualized by development with an alkaline phosphatase substrate solution. The Western blot was examined for the presence of intact immunoreactive PMI (approximately 42.8 kDa for the plant expressed PMI and approximately 44.4 kDa for the microbially expressed PMI) and other immunoreactive PMI polypeptides.

Enzymatic Activity Determination

The enzymatic activity of PMI was measured in triplicate in a coupled enzyme activity assay (as shown in the diagram below) effectively described in Gill *et al.* (1986). Briefly, the reaction assay was started by adding 4 or 10 µl of MIR162(+), MIR162(-), or PMI-0198 to an assay mixture consisting of 50 mM Tris-HCl (pH 7.0), 0.8 mM NADP, 1.6 mM mannose-6-phosphate, 2 U phosphoglucose isomerase, and 2 U glucose 6-phosphate dehydrogenase in a total volume of 1 ml. PMI activity was measured by monitoring the formation of NADPH at 340 nm on a Thermo Spectronic Genesys6 spectrophotometer at room temperature.



PMI = Phosphomannose isomerase, PGI = Phosphoglucose isomerase, G6PDH = Glucose 6-phosphate dehydrogenase

The specific activity of PMI in test substances MIR162(+) and PMI-0198 was determined using the molar extinction coefficient of NADPH (6.2 x 10^6 cm^2/Mol). One unit of PMI

activity is defined as 1 µmol of NADP reduced to NADPH per min. The concentration of PMI added to the assay was also determined.

Statistical Analysis

ELISA values were analyzed using the DeltaSoft Curve Fitting software program. Mean values and standard deviations for the PMI enzyme assay were calculated using Microsoft Excel.

Results for PMI Characterization and Equivalence

Molecular Weight Determination and Immunoreactivity

A single immunoreactive PMI band corresponding to the anticipated molecular weight of approximately 42.8 kDa was found in the MIR162-derived plant extract, MIR162(+) (Figure C-4; lane 3). The microbially-produced PMI protein in PMI-0198 (Figure C-4; lane 4) showed one major band, with a slightly lower mobility than the plant expressed PMI protein, as expected from its slightly higher predicted molecular weight of approximately 44.4 kDa (resulting from the additional 16 amino acids at the N-terminus). The Western blot analysis of test substance PMI-0198 also revealed another immunoreactive protein band with a molecular weight of approximately 90 kDa (Figure C-4, lane 4). Since this protein also cross reacted with the anti-PMI antibody and the fact that this band showed mobility of a protein corresponding to that of a molecular weight twice that of PMI (*i.e.*, 2 x 44.4 kDA = 89 kDA), this protein band most likely represents a dimer of the PMI protein. No immunoreactive band was found in the extract of control leaf tissue, MIR162(-) (Figure C-4; lane 2).

PMI Enzymatic Activity

The mean PMI specific activity of the MIR162 maize leaf extract was 55.5 ± 4.18 U/mg PMI (Table C-8). PMI extracted from *E. coli* had a mean specific activity of 33.2 ± 2.37 U/mg PMI. Historical activity measurements for maize PMI proteins and test substance PMI-0198, as determined at the Syngenta Biotechnology laboratory are in the ranges of 44 – 113 U/mg maize PMI and 31 – 53 U/mg microbial PMI (see Table C-9). PMI activity measurements in this study are not materially different from activity levels measured in previous Syngenta studies. Considering the expected levels of variability in the measurements it is concluded the PMI extracted from MIR162 maize has similar activity to PMI in *E. coli* test substance PMI-0198. In addition, the control maize leaf extract showed no PMI enzymatic activity.

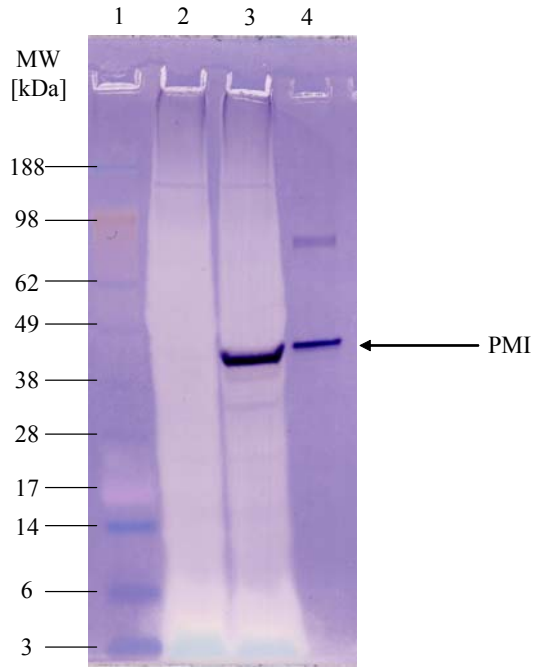


Figure C-4. Western blot of PMI Protein from MIR162 maize leaf tissue and from *E. coli* (PMI-0198). The molecular weights of the plant-produced PMI and the *E. coli*-produced PMI correspond to approximately 42.8 kDa and 44.4 kDa, respectively.

Lane 1: Molecular weight standard SeeBlue Plus2, 8 µl
Lane 2: 58 µg total protein from MIR162(-) maize leaf extract
Lane 3: 10 ng PMI from MIR162(+) maize leaf extract
Lane 4: 10 ng PMI from *E. coli (*PMI-0198)

Table C-8. Enzymatic activity comparison of PMI from MIR162 maize leaf tissue, PMI from *E. coli* (PMI-0198), and from an extract of control maize leaf tissue.

Sample	PMI Conc. [ng PMI/ml]	Specific Activity [U/mg PMI] [a]	Mean [U/mg PMI]	SD [U/mg PMI] [c]
MIR162(+)	557	50.7	55.5	4.18
	557	57.9		
	557	57.9		
PMI-0198	4500	35.8	33.2	2.37
	4500	31.4		
	4500	32.3		
MIR162(-)	ND[b]	ND	ND	ND
		ND		
		ND		

[a] - one unit (U) of PMI activity is defined as 1 µmol of NADP reduced to NADPH per min.
[b] - ND = not detectable
[c] - SD = standard deviation

Table C-9. Enzymatic activity determinations for PMI from maize and *E. coli* as determined in previously conducted Syngenta studies.

Date of Analysis	Maize-derived Range [U/mg PMI]	*E.coli*-derived Range [U/mg PMI]
March 2004	43.45-66.27[a]	35.85-49.27
April 2005	81.6-112.6[a]	52.9
September 2006	50.7-57.9[b]	31.4-35.8

[a] - Extracted from other transgenic maize events containing PMI
[b] - Extracted from MIR162 maize

Appendix D. Quantification of Vip3Aa20 and PMI in MIR162 Maize

Materials and Methods

Source of Plants for Evaluation of Vip3Aa20 and PMI Concentrations

Two MIR162 maize hybrids, designated Hybrid 1 and Hybrid 2, and their corresponding near-isogenic, nontransgenic control hybrid equivalents (see Table 2 and Figure 5) were planted at two locations in 2005 under USDA notification 05-062-02n. The trial with Hybrid 1 was planted in Bloomington, Illinois, and the trial with Hybrid 2 was planted in York, Nebraska. The trials were managed following to local agronomic practices for the 2005 growing season.

Ten plants of each MIR162 hybrid, plus two plants from each of the corresponding control hybrids, were harvested at each of four developmental stages (see Table D-1). Five each of the MIR162 hybrids, plus one plant from each of the corresponding control hybrids, were kept as whole-plant samples for analysis. The remaining plants from each sampling interval were separated into distinct plant parts for analysis (see Table D-1).

Table D-1. Growth stages selected for collection of whole plants and specific plant tissues.

Sampling Interval (Growth Stage)	Tissues Sampled
V9 – V12 (~ 8 weeks after planting)	leaf, root, whole plant
Anthesis	leaf, root, silk, pith, pollen, whole plant
Seed maturity (black layer)	leaf, root, kernels, pith, whole plant
Senescence (~ 23 weeks after planting)	leaf, root, kernels, whole plant

At each sampling interval, whole plants (including roots) were harvested and shipped overnight on ice packs to Syngenta Biotechnology, Inc. Upon receipt, five plants were separated into parts consisting of all the leaves, roots, pith, silk, or kernels. After weighing, all samples were stored at –80 ± 10ºC until the tissue was processed further.

Pollen Collection

At anthesis, pollen was collected and pooled from at least 10 plants of the same genotype, sieved at the collection facility to remove nonpollen debris (*e.g.,* anthers and aphids), air-dried overnight, and stored frozen at –80 ± 10ºC. Prior to analysis, the pollen was sieved again through a 250 µm mesh screen.

Plant Tissue Processing

Whole plants and individual parts (except pollen) were ground to a fine powder in the presence of dry ice. Each ground sample was thoroughly mixed to ensure homogeneity. The powdered samples were weighed, lypophilized, and weighed again. The percent dry weight for each sample was computed based on the difference in pre- and post-lyophilization weights. Equal amounts of lyophilized transgenic samples were pooled to create one composite sample for each tissue type from each hybrid at each sampling stage. Lyophilized samples were stored at –80 ± 10ºC.

Tissue Extraction

Extracts of the lyophilized tissue samples (except pollen) were prepared for Vip3Aa20 and PMI analysis. At least three replicate subsamples of each composite transgenic tissue sample were analyzed to determine the variability of extraction. For each subsample analyzed, an aliquot of approximately 0.1 g of the powdered lyophilized material was transferred into a 15-ml polypropylene tube, suspended in 3 ml extraction buffer (500 mM Tris, 20 mM EDTA, 1 M NaCl, 1 mM AEBSF, 2 mM DTT, 1 µM leupeptin, pH 9.5), and homogenized using an Autogizer homogenizer (6 cycles, setting 4). An additional 3 ml of extraction buffer was added, and samples were vortexed briefly and incubated on wet ice for 30 minutes. After centrifugation for 15 minutes at 10,000 x *g* at 4ºC, the supernatants were used for Vip3Aa20 and PMI quantification by ELISA.

Pollen extracts were prepared by suspending air-dried pollen 1:30 (w/v) in extraction buffer and incubating the samples on ice for 30 minutes. The pollen suspensions were disrupted by three passages through a French pressure cell at 15,000 psi. An additional 3 ml of buffer was added to each sample and the samples were vortexed briefly. After centrifugation for 15 minutes at 10,000 x *g* at 4ºC, the supernatants were used for Vip3Aa20 and PMI quantification by ELISA.

Vip3Aa20 Quantification

Tissue sample extracts were quantitatively analyzed for Vip3Aa20 by ELISA using immunoaffinity-purified goat anti-Vip3Aa and Protein A-purified rabbit anti-Vip3Aa polyclonal antibodies. Microbially produced Vip3Aa20 was used to produce the standard curves for all measurements except extraction efficiency where microbially produced Vip3Aa19 was used.

PMI Quantification

Tissue sample extracts were quantitatively analyzed for PMI by ELISA using immunoaffinity-purified goat anti-PMI and Protein A-purified rabbit anti-PMI polyclonal antibodies. Microbially produced PMI was used to produce the standard curves for all measurements.

Limit of Quantification and Limit of Detection Determination

The limit of quantification (LOQ) of the ELISAs was estimated based on the lowest concentration of pure protein of interest (POI) lying on the linear portion of the standard curve and the dilution factor (DF; based on optical density values from dilutions of negative control tissue extracts). LOQ values are shown in Table D-2 and were calculated as follows:

LOQ (µg POI/g dry-weight) = lowest concentration on the linear portion of standard curve (ng POI/ml) × DF × volume of buffer used in extraction (ml) ÷ g of tissue extracted ÷ 1000

Table D-2. LOQs for Vip3Aa20 and PMI in MIR162 tissues. Values have not been corrected for extraction efficiency.

Limits of Quantification (µg/g dw)				
Tissue	**V9 - V12**	**Anthesis**	**Seed Maturity**	**Senescence**
Vip3Aa20				
Leaf	0.22	0.27	0.33	0.24
Root	0.22	0.27	0.23	0.24
Pith	N/A[a]	0.24	0.21	N/A
Silk	N/A	0.24	N/A	N/A
Kernels	N/A	N/A	0.24	0.30
Pollen	N/A	0.35	N/A	N/A
Whole Plants	0.22	0.24	0.24	0.21
PMI				
Leaves	0.22	0.29	0.22	0.26
Roots	0.21	0.21	0.21	0.23
Pith	N/A[a]	0.24	0.21	N/A
Silk	N/A	0.31	N/A	N/A
Kernels	N/A	N/A	0.21	0.26
Pollen	N/A	0.35	N/A	N/A
Whole Plants	0.41	0.40	0.26	0.27

[a] - N/A = not analyzed at this stage

The limit of detection (LOD) of the ELISAs was estimated based on the mean optical density (OD) + 2 standard deviations of the lowest concentration of pure POI used in the standard curve and the dilution factor (DF; based on OD values from dilutions of negative control tissue extracts). LOD values are shown in Table D-3 and were calculated as follows:

LOD (µg POI/g dw) = mean OD + 2 S.D. of lowest concentration used in the standard curve (expressed as ng POI/ml) × DF × volume of buffer used in extraction (ml) ÷ g of tissue extracted ÷ 1000

Table D-3. LODs for Vip3Aa20 and PMI in MIR162 tissues. Values have not been corrected for extraction efficiency.

Limits of Detection (µg/g dw)				
Tissue	**V9 - V12**	**Anthesis**	**Seed Maturity**	**Senescence**
Vip3Aa20				
Leaves	0.030	0.030	0.030	0.039
Roots	0.030	0.030	0.029	0.045
Pith	N/A[a]	0.030	0.030	N/A
Silk	N/A	0.030	N/A	N/A
Kernels	N/A	N/A	0.030	0.030
Pollen	N/A	0.035	N/A	N/A
Whole Plants	0.030	0.030	0.030	0.030
PMI				
Leaves	0.066	0.049	0.064	0.046
Roots	0.041	0.041	0.041	0.032
Pith	N/A	0.042	0.048	N/A
Silk	N/A	0.057	N/A	N/A
Kernels	N/A	N/A	0.063	0.056
Pollen	N/A	0.112	N/A	N/A
Whole Plants	0.070	0.069	0.090	0.109

[a] - N/A = not analyzed at this stage

Extraction Efficiency for Vip3Aa20 and PMI

Extraction efficiency measurements were performed to estimate the relative amount of Vip3Aa20 and PMI extracted during routine procedures, compared to that which remained associated with the post-extraction solids. Tissues were extracted in triplicate as described above. The insoluble material was then collected and re-extracted twice more, while retaining the supernatant for analysis each time. If an extract was either <LOQ or <LOD, the LOQ or LOD value for the sample, expressed as ng POI/ml, was used to estimate the extraction efficiency for the sample. Percent extraction efficiency was calculated as follows:

[ng POI/ml 1st Extraction ÷ (ng POI/ml 1st extraction + 2nd extraction + 3rd extraction)] x 100 = % extraction efficiency

ELISA values were corrected for extraction efficiency as follows:

µg POI/gdw (uncorrected) ÷ % extraction efficiency = µg POI/gdw (corrected)

µg POI/gdw (corrected) × % dry weight = µg POI/gfw (corrected)

Statistical Methods

The mean and standard deviation of replicate samples were calculated using Microsoft Excel.

Appendix E. Compositional Analysis of MIR162 Forage and Grain

Study Design

Forage and grain for compositional analysis were harvested from multiple locations planted in U.S. in 2005. The locations chosen were representative of major maize producing regions in the country. Trials were planted with a MIR162 hybrid and genetically matched nontransgenic hybrid in a randomized complete block design, at multiple locations, with three replicated plots per location, and were managed following local agronomic practices. See Table 2 and Figure 5 for a description of the hybrids used in this study. The plants were self-pollinated by hand and the developing ears were bagged to avoid cross-pollination. Eight locations were planted for this study to insure that grain and forage from six locations could be harvested in the event of loss due to adverse environmental conditions (early freeze, drought, *etc.*). Six locations that produced sufficient grain and forage were selected for this study (see Table E-1).

Table E-1. Locations selected for planting and harvest of forage and grain.

ID	Location
Location 1	Stanton, Minnesota
Location 2	Janesville, Wisconsin
Location 3	Alleman, Iowa
Location 5	Seward, Nebraska
Location 6	Bloomington, Illinois
Location 8	Bondville, Illinois

Forage Sampling and Processing

The entire above-ground portion from five plants of each hybrid was harvested at dough stage (R4), the stage at which silage would typically be prepared, from each of the three replicated plots at each location. For each hybrid, plants were pooled to create a composite sample for each plot, then ground with a chipper-shredder. A subsample from each composite sample was shipped overnight on ice packs to Syngenta Crop Protection, Inc. (Greensboro, NC USA). Samples were stored frozen at -20±10˚C, then finely ground and shipped on dry ice to Covance Laboratories, Inc. (Madison, WI USA) where they were stored frozen at -20±10˚C until they were analyzed.

Grain Sampling and Processing

Ears were harvested after physiological maturity (R6) at 18-24% moisture content, and then mechanically dried to approximately 10-13% moisture content. Each grain sample represented grain shelled from ears collected from 15 plants of each hybrid grown in each of

the three replicated plots, at each location. A subsample of approximately 500 g of grain from each replicate plot was shipped at ambient temperature to Syngenta Crop Protection, Inc. where it was stored frozen at -20±10˚C, then finely ground and shipped on dry ice to Covance Laboratories, Inc. Samples were stored frozen at -20±10˚C until they were analyzed.

Compositional Analysis

Selection of analytes for measurement in forage and grain was based on recommendations of the OECD for comparative assessment of composition of new varieties of maize. Forage was analyzed for proximates and the minerals calcium and phosphorus (Table 25). Grain was analyzed for major constituents (proximates, including starch, ADF, NDF and TDF), minerals, amino acids, fatty acids, vitamins, selected anti-nutrients, and secondary metabolites (Table 25).

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

Statistical Analysis

An F-test was also used to assess the significance of the location by genotype interaction. An F-test probability of <0.05 suggests that the effect of the genotype was not consistent across locations and that the comparison of genotypes averaged across locations may not be valid.

The analyte composition tables for forage and grain (Tables E-3 through E-11) include the overall averages of each analyte across locations in both the MIR162 and the nontransgenic hybrids. Also included are the F-test probabilities for both the genotype comparisons and the location by genotype interactions. F-test probabilities that were statistically significant ($p<0.05$) are indicated in bold type. For analytes showing statistically significant location by genotype interactions, the means for both the MIR162 and the nontransgenic hybrid at each location are provided in a separate table (Table E-8).

Moisture levels in grain were not subjected to analysis of variance since the moisture analysis was performed on grain that had been mechanically dried, thus altering the original moisture content of the harvested grain. Mechanical drying after harvest is a standard agronomic practice for improving storage characteristics of maize grain.

Analytical Methods and Reference Standards for Compositional Analysis

2-Furaldehyde (Furfural)

Grain samples were extracted with 4% trichloroacetic acid in MilliQ water, centrifuged and filtered. The level of furfural in the extract was determined by reverse phase high performance liquid chromatography with ultra-violet quantitation. The quantitation limit for

this study was calculated to be 0.500 ppm.

Reference Standard:
ACROS 2-Furaldehyde, 99%, Lot Number A018806701

Reference:
Albala-Hurtado S., M. T. Veciana-Nogues, M. Izquierdo-Pulido, and M. C. Vidal-Carou (1997). Determination of free and total furfural compounds in infant milk formulas by high-performance liquid chromatography. *Journal of Agricultural and Food Chemistry* **45**:2128-2133.

Acid Detergent Fiber

The sample was placed in a fritted vessel and washed with an acidic boiling detergent solution that dissolved the protein, carbohydrate, and ash. An acetone wash removed the fats and pigments. Lignocellulose fraction was collected on the frit and determined gravimetrically. The limit of quantitation for this study was 0.1%.

Reference:
USDA (1970). Forage fiber analyses. *In* **Agriculture Handbook No. 379**, United States Department of Agriculture, Washington, DC.

Amino Acids

Total aspartic acid (including asparagine)	Total threonine
Total serine	Total glutamic acid (including glutamine)
Total praline	Total glycine
Total alanine	Total valine
Total isoleucine	Total leucine
Total tyrosine	Total phenylalanine
Total histidine	Total lysine
Total arginine	Total tryptophan

Sulfur-containing amino acids: Total methionine, Total cystine (including cysteine)

The sample was assayed by three methods to obtain the full profile. Tryptophan required a base hydrolysis with sodium hydroxide. The sulfur containing amino acids required an oxidation with performic acid prior to hydrolysis with hydrochloric acid. Analysis of the samples for the remaining amino acids was accomplished through direct acid hydrolysis with hydrochloric acid. Once hydrolyzed, the individual amino acids were then quantitated using an automated amino acid analyzer. The limit of quantitation for each amino acid was 0.100 mg/g.

Reference Standards:
Beckman K18, 2.5 µmol/mL per constituent (except cystine 1.25 µmol/mL),
Lot Number S504255
Sigma L-Tryptophan, >99% (used as 100%), Lot Number 063K0382

Fluka L-Cysteic Acid Monohydrate, 99.9% (used as 100%), Lot Number 1157629
Sigma L-Methionine Sulfone, >99% (used as 100%), Lot Number 012H3349

Reference:
AOAC International (2005). Method 982.30. *In* ***Official Methods of Analysis of AOAC International,* 18th Edition.** AOAC International, Gaithersburg, Maryland.

Ash

The sample was placed in an electric furnace at 550°C and ignited to drive off all volatile organic matter. The nonvolatile matter remaining was quantitated gravimetrically and calculated to determine percent ash. The limit of quantitation for all samples in this study was 0.1%.

Reference:
AOAC International (2005). Method 923.03. *In* ***Official Methods of Analysis of AOAC International,* 18th Edition.** AOAC International, Gaithersburg, Maryland.

Beta Carotene (Vitamin A)

The sample was extracted using alcohol and hexane. The extracts were injected on a reverse phase HPLC system and compared to a standard curve. The limit of quantitation for Beta Carotene in this study was approximately 0.0333 RE/g.

Reference Standard:
Sigma, Beta Carotene, 102%, Lot Number 104K2514

References:
AOAC International (2005). Method 941.15. *In* **Official Methods of Analysis of AOAC International, 18th Edition.** AOAC International, Gaithersburg, Maryland.

Carbohydrates

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

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

The limit of quantitation for this study was 0.1%.

Reference:
USDA (1973). Energy value of foods. Pp 2-11. *In* **Agriculture Handbook No. 74**. United States Department of Agriculture, Washington, DC.

Total Fat by Acid Hydrolysis

The sample was hydrolyzed with hydrochloric acid at an elevated temperature. The fat was extracted using ether and hexane. The extract was washed with a dilute alkali solution and filtered through a sodium sulfate column. The extract was then evaporated, dried, and weighed. The limit of quantitation for all samples in this study was 0.1%.

Reference:
AOAC International (2005). Methods 922.06 and 954.02. *In* **Official Methods of Analysis of AOAC International, 18th Edition.** AOAC International, Gaithersburg, Maryland.

Fatty Acids

The lipid was extracted and saponified with 0.5 N sodium hydroxide in methanol. The saponification mixture was methylated with 14% boron trifluoride:methanol. The resulting methyl esters were extracted with heptane containing an internal standard. The methyl esters of the fatty acids were analyzed by gas chromatography using external standards for quantitation. The limit of quantitation was for each individual fatty acid was 0.004%.

Reference Standards:
Nu Chek Prep GLC Reference Standard Hazleton No. 1, used as 100%,
Lot Number AU22-P
Nu Chek Prep GLC Reference Standard Hazleton No. 2, used as 100%,
Lot Number M13-0
Nu Chek Prep GLC Reference Standard Hazleton No. 3, used as 100%,
Lot Number MA13-0
Nu Chek Prep GLC Reference Standard Hazleton No. 4, used as 100%,
Lot Number JA13-P
Nu Chek Prep Methyl Gamma Linolenate, used as 100%,
Lot Number U-63M-J1-P
Sigma Methyl Tridecanoate, used as 100%, Lot Number 035K1392

Reference:
AOCS (1997). Method Ce 1-62. *In* **Official Methods and Recommended Practices of the AOCS, 5th Edition.** American Oil Chemists' Society, Champaign, Illinois.

Folic acid

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

Reference Standard:
USP Folic acid, 98%, Lot Number P

References:
AOAC International (2005). Methods 960.46 and 992.05. *In* **Official Methods of Analysis of AOAC International, 18th Edition.** AOAC International, Gaithersburg, Maryland.

Infant Formula Council (1973). Section C-2. *In* **Methods of Analysis for Infant Formulas.** Infant Formula Council, Atlanta, Georgia.

Minerals by ICP Emission Spectrometry

Calcium	Copper	Iron
Magnesium	Manganese	Phosphorus
Potassium	Sodium	Zinc

The sample was dried, precharred, and ashed overnight in a muffle set to maintain 500°C. The ashed sample was treated with hydrochloric acid, taken to dryness, and put into a solution of 5% hydrochloric acid. The amount of each element was determined at appropriate wavelengths by comparing the emission of the unknown sample, measured by the inductively coupled plasma, with the emission of the standard solutions. The reference standards and limits of quantification for each mineral are listed in Table E-2.

References:
Dahlquist, R. L. and J. W. Knoll (1978). Inductively Coupled Plasma-Atomic Emission spectrometry: Analysis of biological materials and soils for major, trace, and ultra trace elements. *Applied Spectroscopy* **32**:1-29.

AOAC International (2005). Methods 984.27 and 985.01. *In* **Official Methods of Analysis of AOAC International, 18th Edition.** AOAC International, Gaithersburg, Maryland.

Table E-2. Inorganic Ventures/IV Labs reference standards and limits of quantification.

Mineral	Lot Numbers	Concentration (ppm)	Limit of Quantification (ppm)*
Calcium	Y-MEB198136, Y-MEB198138	200, 1000	20.0
Copper	Y-MEB198136, Y-MEB198137	2, 10	0.50
Iron	Y-MEB198136, Y-MEB198139	10, 50	2.00
Magnesium	Y-MEB198136, Y-MEB198137	50, 250	20.0
Manganese	Y-MEB198136, Y-MEB198137	2, 10	0.30
Phosphorus	Y-MEB198136, Y-MEB198138	200, 1000	20.0
Potassium	Y-MEB198136, Y-MEB198138	200, 1000	100
Sodium	Y-MEB198136, Y-MEB198138	200, 1000	100
Zinc	Y-MEB198136, Y-MEB198137	10, 50	0.40

* Calculated on a fresh weight basis.

Moisture

The sample was dried in a vacuum oven at approximately 100°C to a constant weight. The moisture weight loss was determined and converted to percent moisture. The limit of quantitation for this study was 0.1%.

Reference:
AOAC International (2005). Methods 926.08 and 925.09. *In* **Official Methods of Analysis of AOAC International, 18th Edition.** AOAC International, Gaithersburg, Maryland.

Neutral Detergent Fiber, Enzyme Method

The sample was placed in a fritted vessel and washed with a neutral boiling detergent solution that dissolved the protein, carbohydrate, enzyme, and ash. An acetone wash removed the fats and pigments. Hemicellulose, cellulose, and lignin fractions were collected on the frit and determined gravimetrically. The limit of quantitation for this study was 0.1%.

References:
AACC (1998). Method 32.20. *In* **Approved Methods of the American Association of Cereal Chemists, 9th Edition**.

USDA (1970). Forage fiber analyses. *In* **Agriculture Handbook No.379.** United States Department of Agriculture, Washington, DC.

p-Coumaric Acid and Ferulic Acid

The sample was extracted with methanol using ultrasonication, hydrolyzed using 4 N sodium hydroxide, buffered using acetic acid/sodium hydroxide, acidified with 3 N hydrochloric acid, and filtered. The levels of p-coumaric and ferulic acids in the extract were determined by reverse phase high performance liquid chromatography with ultra-violet quantitation. The limit of quantitation for the p-coumaric acid and ferulic acid assays were approximately 50.0 ppm.

Reference Standards:
Acros, 4-Hydroxy-3-methoxycinnamic Acid (Ferulic Acid), 99.9%, Lot Number A014010401
Acros, p-Hydroxycinnamic Acid (p-Coumaric Acid), 97.9%, Lot Number A018661301

Reference:
Hagerman, A. E. and R. L. Nicholson (1982). High-performance liquid chromatographic determination of hydroxycinnamic acids in maize mesocotyl. *Journal of Agricultural and Food Chemistry* **30** (6):1098-1102.

Phytic Acid

The sample was extracted using 0.5M HCl with ultrasonication. Purification and concentration was done on a silica based anion exchange (SAX) column. Sample analysis was done on a macroporous polymer HPLC column PRP-1, 5µm (150 x 4.1mm) and a refractive index detector. The limit of quantitation for this study was approximately 0.100%.

Reference Standard:
Aldrich, Phytic Acid, Dodecasodium Salt Hydrate, 95%, Lot Number 1913EC

References:
Lehrfeld, J. (1989). High-performance liquid chromatography analysis of phytic acid on a ph-stable, macroporous polymer column. *Cereal Chemistry* **66**(6):510-515.

Lehrfeld, J. (1994). HPLC separation and quantitation of phytic acid and some inositol phosphates in foods: problem and solutions. *Journal of Agricultural Food Chemistry* **42**:2726-2731.

Protein

Nitrogenous compounds in the sample were reduced in the presence of boiling sulfuric acid and a mercury catalyst mixture to form ammonia. The acid digest was made alkaline. The ammonia was distilled and then titrated with a standard acid. The percent nitrogen was

calculated and converted to protein using the factor 6.25. The limit of quantitation for this study was 0.1%.

References:
AOAC International (2005). Methods 955.04 and 979.09. *In* **Official Methods of Analysis of AOAC International, 18th Edition.** AOAC International, Gaithersburg, Maryland.

Bradstreet, R. B. (1965). **The Kjeldahl Method for Organic Nitrogen.** Academic Press, New York, New York.

Kalthoff, I. M., and E. B. Sandell (1948). **Quantitative Inorganic Analysis.** MacMillan, New York, New York.

Raffinose

The sample was extracted with deionized water and the extract treated with a hydroxylamine hydrochloride solution in pyridine, containing phenyl-β-D-glucoside as an internal standard. The resulting oximes were converted to silyl derivatives by treatment with hexamethyldisilazane and trifluoracetic acid and analyzed by gas chromatography using a flame ionization detector. The limit of quantitation (LOQ): The acceptable range for an 8/5 dilution was 0.100-1.800%.

Reference Standard:
Sigma, D(+)-Raffinose Pentahyrate, 99%, Lot Number 073K0938

References:
Mason, B. S., and H. T. Slover (1971). A gas chromatographic method for the determination of sugars in foods. *Journal of Agricultural and Food Chemistry* **19**(3):551-554.

Brobst, K. M. (1972). Gas-liquid chromatography of trimethylsilyl derivatives. *In* **Methods in Carbohydrate Chemistry, Volume 6.** Academic Press, New York, New York.

Selenium by ICP-Mass Spectrometry

An appropriately sized sample was wet-ashed with nitric acid (HNO^3) using open or closed vessel microwave digestion. Samples that were readily soluble in water were diluted with HNO_3 and analyzed straight. The amount of each element was determined using mass spectrometry by comparing the counts generated by the unknowns to those generated by standard solutions of known concentration. The limit of quantitation for this study was 50 ppb.

Reference Standard:
SPEX, Selenium, 100 mg/L, Lot Number 8-175VY

References:
EPA Method 200.8, *Determination of Trace Elements in Waters and Wastes by Inductively*

Coupled Plasma-Mass Spectrometry

Cabrera et al. (1994). Determination of levels of lead contamination in food and feed crops. *Journal of AOAC International* **77** (5):1249-1252.

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Starch

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

Calculations:

% starch = glucose concentration from curve (µg/mL) x dilution factor x initial volume (mL) x 0.9 sample weight (g) x 10,000

Reference Standard:
Sigma, D(+)-Glucose, 99.9% Lot Number 123K0095

Reference:
AOAC International (2005). Method 996.11. *In* **Official Methods of Analysis of AOAC International, 18th Edition.** AOAC International, Gaithersburg, Maryland.

Tocopherols, Total

The product was saponified to break down any fat and release any vitamin E. The saponified mixture was dissolved directly in hexane and then quantified directly by high-performance liquid chromatography on a silica column. The limit of quantitation for this study was approximately 0.500 mg/100g.

Reference Standards:
USP, Alpha Tocopherol, 100%, Lot Number M
Matreya, Beta Tocopherol, 50 mg/mL, Lot Number 22077
Sigma, Delta Tocopherol, 98%, Lot Number 072K1326
Sigma, Gamma Tocopherol, 99%, Lot Number 095K1062

Reference:
Cort, W. M., T. S. Vincente, E. H. Waysek, and B. D. Williams (1983). Vitamin E content of feedstuffs determined by high-performance liquid chromatographic fluorescence. *Journal of Agricultural Food Chemistry* **31**:1330-1333.

Speek, A. J., J. Schijver, and W. H. P. Schreurs (1985). Vitamin E composition of some seed oils as determined by high-performance liquid chromatography with fluorometric quantitation. *Journal of Food Science* **50**(1):121-124.

McMurray, C. H., W. J. Blanchflower, and D. A. Rice (1980). Influence of extraction techniques on determination of alpha-tocopherol in animal feedstuffs. *Journal of the Association of Official Analytical Chemists* **63**(6):1258-1261.

Total Dietary Fiber

Duplicate samples were gelatinized with alpha-amylase and digested with enzymes to break down starch and protein. Ethanol was added to each sample to precipitate the soluble fiber. The samples were filtered, and the residue was rinsed with ethanol and acetone to remove starch and protein degradation products and moisture. Protein content was determined for one of the duplicates; ash content was determined for the other. The total dietary fiber in the sample was calculated using the protein and ash values. The limit of quantitation for this study was 1.0%.

Reference:
AOAC International (2005). Method 985.29. *In* **Official Methods of Analysis of AOAC International, 18th Edition.** AOAC International, Gaithersburg, Maryland.

Trypsin Inhibitor

The sample was ground and/or defatted with petroleum ether, if necessary. A sample of matrix was extracted for 3 hours with 0.01 N sodium hydroxide. Varying aliquots of the sample suspension were exposed to a known amount of trypsin and benzoy1-DL-arginine-p-nitroanalide hydrochloride. The sample was allowed to react for 10 minutes at 37°C. After 10 minutes, the reaction was halted by the addition of acetic acid. The solution was filtered or centrifuged and the absorbance was determined at 410 nm. Trypsin inhibitor activity (TIU) was determined by photometrically measuring the inhibition of trypsin's reaction with benzoyl-DL-arginine-*p*-nitroanalide hydrochloride. The limit of quantification for this study was 1.00 Trypsin Inhibitor Unit/mg.

Reference:
AOCS (1997). Method Ba 12-75. *In* **Official Methods and Recommended Practices of the American Oil Chemists' Society, 5th Edition.** American Oil Chemists' Society, Champaign, Illinois.

Vitamin B_1 (Thiamine)

The sample was autoclaved under weak acid conditions to extract the thiamine. The resulting solution was incubated with a buffered enzyme solution to release any bound thiamine. The solution was purified on an ion-exchange column. An aliquot was taken and reacted with potassium ferricyanide to convert thiamin to thiochrome. The thiochrome was

extracted into isobutyl alcohol and read on a fluorometer against a known standard. The limit of quantification for this study was 0.01 mg/100g.

Reference Standard:
USP, Thiamine, 97.22% after correction for degree of hydration, Lot Number O

Reference:
AOAC International (2005). Methods 942.23, 953.17, and 957.17. *In* **Official Methods of Analysis of AOAC International, 18th Edition.** AOAC International, Gaithersburg, Maryland.

Vitamin B_2 (Riboflavin)

The sample was hydrolyzed with dilute hydrochloric acid (HCl) and the pH was adjusted to remove interferences. The amount of riboflavin was determined by comparing the growth response of the sample, using the bacteria *Lactobacillus casei*, with the growth response of multi-point riboflavin standard curve. This growth response was measured turbidimetrically. The limit of quantification for this study was 0.0200 mg/100g.

Reference Standard:
USP Riboflavin, 100%, Lot Number N0C021

Reference:
AOAC International (2005). Method 940.33. *In* **Official Methods of Analysis of AOAC International, 18th Edition.** AOAC International, Gaithersburg, Maryland.

Vitamin B_3 (Niacin)

The sample was hydrolyzed with sulfuric acid and the pH was adjusted to remove interferences. The amount of niacin was determined by comparing the growth response of the sample, using the bacteria *Lactobacillus plantarum,* with the growth response of a niacin standard. This response was measured turbidimetrically. The limit of quantification for this study was 0.03 mg/100g.

Reference Standard:
USP, Niacin, 100%, Lot Number H2C121

Reference:
AOAC International (2005). Method 944.13. *In* **Official Methods of Analysis of AOAC International, 18th Edition**. AOAC International, Gaithersburg, Maryland.

Vitamin B_6 (Pyridoxine)

The sample was hydrolyzed with dilute sulfuric acid in the autoclave and the pH was adjusted to remove interferences. The amount of B_6 was determined by comparing the growth response of the sample, using the yeast *Saccharromyces carlsbergenesis,* with the

growth response of a B_6 standard. This response was measured turbidimetrically. The limit of quantification for this study was 0.00700 mg/100g.

Reference Standard:
USP Pyridoxine, 100%, Lot Number P

Reference:
AOAC International (2005). Method 961.15. *In* **Official Methods of Analysis of AOAC International, 18th Edition.** AOAC International, Gaithersburg, Maryland.

Results

Results of the forage analyses are presented in Tables E-3 and E-4. Results of grain analyses are presented in Tables E-5 through E-11. Results are presented as the mean values of analyte results across the six locations. The range of individual plot values for both the MIR162 and control genotypes are shown, along with the computed difference between the mean values (expressed as a percentage of the control). The results of the across location statistical comparison and the test for potential genotype by location interaction are presented as their respective *p*-values. Significance was assigned at the standard level of 5%.

The results of the statistical analysis for genotype by location interaction revealed two analytes for which there was a statistically significant interaction ($p<0.05$), vitamin A (β-carotene) and vitamin B_9 (folic acid). For these two analytes, individual location mean values are presented in Table E-8.

Table E-3. Proximate composition of maize forage from a MIR162 hybrid and a control hybrid. For comparison, ILSI Crop Composition database and OECD literature values are also shown. All units as described in column headings except F-test values.

Treatment		Moisture (% fw)	Protein (% dw)	Total Fat (% dw)	Ash (% dw)	Carbohydrates (% dw)	ADF (% dw)	NDF (% dw)
MIR162	$\bar{X}$	71.2	7.2	1.5	4.1	87.3	28.2	43.2
	range	66.2-77.2	3.1-10.1	0.8-1.9	3.1-5.8	82.9-90.7	23.6-34.2	35.1-56.1
Control	$\bar{X}$	70.5	7.3	1.6	4.0	87.1	28.8	38.8
	range	64.1-75.8	3.4-8.9	0.4-2.3	2.9-5.7	83.6-91.7	23.3-34.8	32.1-46.9
% difference[a]		0.99	-1.37	-6.25	2.50	0.23	-2.10	11.34
p-value for genotype[b]		0.184	0.711	0.421	0.796	0.694	0.635	**0.010**
p-value for location x genotype interaction		0.730	0.645	0.100	0.948	0.973	0.687	0.286
ILSI (2006)	$\bar{X}$	70.2	7.78	2.039	4.628	85.6	27.00	41.51
	range	49.1 - 81.3	3.14 - 11.57	0.296 - 4.570	1.527 - 9.638	76.4 - 92.1	16.13 - 47.39	20.29 - 63.71
	N	945	945	921	945	945	945	945
OECD (2002)	range	62 - 78	4.7 - 9.2	1.5 - 3.2	2.9 - 5.7		25.6 - 34	40 - 48.2

[a] – [(MIR162 - control)/control] x 100

[b] - statistically significant F-test ($p<0.05$) indicated in bold type.

Table E-4. Calcium and phosphorus composition of maize forage from a MIR162 hybrid and a control hybrid. For comparison, ILSI Crop Composition database and OECD values are also shown. All units as described in column headings, except F-test values and where noted.

Treatment		Calcium (mg/kg dw)	Phosphorus (mg/kg dw)
MIR162	$\bar{X}$	2106	1997
	range	1720-2930	1270-2240
Control	$\bar{X}$	2039	2079
	range	1440-2620	1760-2560
% difference [a]		3.29	-3.94
p-value for genotype		0.484	0.153
p-value for location by genotype interaction		0.944	0.066
ILSI (2006)	$\bar{X}$	2028.6	2066.1
	range	713.9 - 5767.9	936.2 - 3704.1
	N	481	481
OECD (2002) [b]	range	0.15 - 0.31% dw	0.20 - 0.27% dw

[a] – [(MIR162 - control)/control] x 100
[b] - for conversion, 1% = 10,000 mg/kg.

Table E-5. Proximate composition of maize grain from a MIR162 hybrid and a control hybrid. For comparison, ILSI Crop Composition database and OECD literature values are also shown. All units as described in column headings except F-test values.

Treatment		Moisture[a] (% fw)	Protein (% dw)	Fat (% dw)	Ash (% dw)	Carbohydrates (% dw)	ADF (% dw)	NDF (% dw)	TDF (% dw)	Starch (% dw)
MIR162	$\bar{X}$	10.3	9.8	3.79	1.4	85.0	5.0	11.7	16.8	63.1
	range	9.5-11.5	7.5-11.2	3.3-4.6	1.1-1.6	83.2-87.1	3.3-7.0	10.1-13.0	14.1-19.4	54.8-68.1
Control	$\bar{X}$	10.5	9.6	3.78	1.3	85.3	4.6	11.1	16.3	64.9
	range	9.4-12.0	7.1-11.0	3.0-4.4	1.1-1.5	83.3-88.1	3.3-6.2	9.5-12.8	14.3-17.8	60.6-69.2
% difference [b]		-1.91	2.08	0.26	7.69	-0.36	8.70	5.41	3.18	-2.77
p-value for genotype[c]			0.103	0.904	**0.012**	0.062	0.154	**0.016**	0.092	**0.015**
p-value for location x genotype interaction			0.832	0.538	0.381	0.696	0.309	0.703	0.653	0.455
ILSI (2006)	$\bar{X}$	11.3	10.30	3.555	1.439	84.6	4.05	11.23	16.43	57.7
	range	6.1 - 40.5	6.15 - 17.26	1.742 - 5.823	0.616 - 6.282	77.4 - 89.5	1.82 - 11.34	5.59 - 22.64	8.85 - 35.31	26.5 - 73.8
	N	1434	1434	1174	1410	1410	1350	1349	397	168
OECD (2002)	range	7.0 - 23	6 - 12.7	3.1 - 5.8	1.1 - 3.9	82.2 - 82.9	3.0 - 4.3	8.3 - 11.9	11.1	

[a] - moisture levels in grain not subject to analysis of variance as grain was mechanically dried after harvest
[b] – [(MIR162 - control)/control) x 100
[c] - statistically significant F-test ($p<0.05$) indicated in bold type

Table E-6. Mineral composition of maize grain from a MIR162 hybrid and a control hybrid. For comparison, ILSI Crop Composition database and OECD values are also shown. All units as described in column headings, except F-test values and where noted.

Treatment		Calcium (mg/kg dw)	Copper (mg/kg dw)	Iron (mg/kg dw)	Magnesium (mg/kg dw)	Manganese (mg/kg dw)	Phosphorus (mg/kg dw)	Potassium (mg/kg dw)	Selenium [a,b] (mg/kg dw)	Sodium [a,b] (mg/kg dw)	Zinc (mg/kg dw)
MIR162	$\bar{X}$	38.1	1.3	20.2	1252	6.3	3173	3352	N/A	N/A	21.7
	range	29.4-47.2	0.96-1.95	17.3-22.9	1090-1480	4.14-7.97	2810-3550	3160-3710	<LOQ - 0.414	<LOQ - <LOQ	18.8-24.3
Control	$\bar{X}$	35.3	1.2	19.2	1218	6.1	3073	3357	N/A	N/A	21.5
	range	25.7-44.0	1.00-1.58	15.7-22.5	960-1470	4.59-8.01	2710-3400	2950-3660	<LOQ - 0.531	<LOQ - <LOQ	19.2-23.8
% difference [c]		7.93	8.33	5.21	2.70	3.28	3.25	-0.15	N/A	N/A	0.93
p-value for genotype [d]		**0.002**	0.073	**0.011**	0.102	0.078	**0.030**	0.916			0.606
p-value for location x genotype interaction		0.148	0.360	0.685	0.288	0.697	0.136	0.291			0.326
ILSI (2006)	$\bar{X}$	46.4	1.75	21.81	1193.8	6.18	3273.5	3842	0.2	31.75	21.6
	range	12.7 - 208.4	0.73 - 18.5	10.42 - 49.07	594.0 - 1940.0	1.69 - 14.30	1470.0 - 5330.0	1810.0 - 6030.0	0.05 - 0.75	0.17 - 731.54	6.5 - 37.2
	N	1344	1249	1255	1257	1256	1349	1257	89	223	1257
OECD (2002) [e]	range	3 -100	0.09 - 1.0	0.1 - 10	82 - 1000		234 - 750	320 - 720	0.001 - 0.1	0 - 150	1.2 - 3.0

[a] - where some values were <LOQ, statistical comparison was not possible and only the range is shown
[b] - LOQ for selenium 0.056-0.057 mg/kg dw, LOQ for sodium 110-114 mg/kg dw
[c] – [(MIR162 - control)/control] x 100
[d] - statistically significant F-test ($p<0.05$) indicated in bold type
[e] – units of measure for OECD values: mg/100 g dw; for conversion, 1 mg/100g = 10 mg/kg

Table E-7. Vitamin analysis of maize grain from a MIR162 hybrid and a control hybrid. For comparison, ILSI Crop Composition database and OECD values are also shown. All units as described in column headings, except F-test values and where noted.

Treatment		Vitamin A [a] ß-Carotene (mg/100g dw)	Vitamin B_1 Thiamine (mg/100g dw)	Vitamin B_2 Riboflavin (mg/100g dw)	Vitamin B_3 Niacin (mg/100g dw)	Vitamin B_6 Pyridoxine (mg/100g dw)	Vitamin B_9 Folic Acid (mg/100g dw)	Vitamin E α-Tocopherol (mg/g dw)
MIR162	$\bar{X}$	0.277	0.393	0.190	2.37	0.565	0.028	0.0125
	range	0.241-0.316	0.358-0.433	0.112-0.238	2.11-2.83	0.434-0.694	0.021-0.034	0.0097-0.0154
Control	$\bar{X}$	0.294	0.392	0.180	2.47	0.605	0.028	0.0132
	range	0.244-0.358	0.339-0.443	0.144-0.226	2.03-3.15	0.486-0.738	0.024-0.033	1.10-1.54
% difference [b]		-5.78	0.26	5.56	-4.04	-6.61	0	-5.30
p-value for genotype [c]		**0.001**	0.983	0.314	0.093	**0.002**	0.471	**0.016**
p-value for location by genotype Interaction [c]		**0.048**	0.952	0.278	0.639	0.765	**0.036**	0.917
	$\bar{X}$	0.684	0.530	0.125	2.376	0.644	0.0651	0.0103
ILSI (2006)	Range	0.019 - 4.681	0.126 - 4.000	0.050 - 0.236	1.037 - 4.694	0.368 - 1.132	0.0147 - 0.1464	0.0015 - 0.0687
	N	276	894	704	415	415	895	863
OECD (2002) [d]	Range		2.3 - 8.6	0.25 - 5.6	9.3 – 70	4.6 - 9.6		

[a] - for direct comparison to literature values, original units of RE/g dw were converted to units of mg/100 g dw based on 1 RE = 6 µg beta-carotene. Institute for Laboratory Animal Research (1995).
[b] – [(MIR162 - control)/control] x 100
[c] - statistically significant F-test ($p<0.05$) indicated in bold type
[d] – units of measure for OECD values: mg/kg dw; for conversion, 1 mg/100g = 10 mg/kg

Table E-8. Location means for ß-carotene and folic acid in maize grain from a MIR162 hybrid and a control hybrid.

Treatment		Vitamin A [a] ß-Carotene (mg/100g dw)	Vitamin B_9 Folic Acid (mg/100g dw)	Treatment		Vitamin A [a] ß-Carotene (mg/100g dw)	Vitamin B_9 Folic Acid (mg/100g dw)
Location 1	MIR162	0.283	0.027	Location 5	MIR162	0.266	0.029
	Control	0.296	0.028		Control	0.286	0.032
Location 2	MIR162	0.304	0.032	Location 6	MIR162	0.292	0.024
	Control	0.352	0.029		Control	0.291	0.025
Location 3	MIR162	0.272	0.027	Location 8	MIR162	0.243	0.025
	Control	0.280	0.024		Control	0.260	0.029
ILSI (2006)	$\bar{X}$	0.684	0.0651				
	range	0.019 - 4.681	0.0147 - 0.1464				
	N	276	895				

[a] - for direct comparison to literature values, original units of RE/g dw were converted to units of mg/100 g dw based on 1 RE = 6 µg beta-carotene. Institute for Laboratory Animal Research (1995).

Table E-9. Amino acid composition of maize grain from a MIR162 hybrid and a control hybrid. For comparison, ILSI Crop Composition database and OECD values are also shown. All units as described in column headings, except F-test values and where noted.

Treatment		Aspartic Acid (mg/g dw)	Threonine (mg/g dw)	Serine (mg/g dw)	Glutamic Acid (mg/g dw)	Proline (mg/g dw)	Glycine (mg/g dw)	Alanine (mg/g dw)	Cysteine (mg/g dw)	Valine (mg/g dw)
MIR162	$\bar{X}$	6.66	3.55	5.21	19.54	9.12	3.84	7.70	2.31	4.81
	range	5.29-7.72	2.83-4.06	3.95-6.06	14.0-23.3	6.79-10.8	3.26-4.27	5.59-9.17	1.96-2.62	3.78-5.61
Control	$\bar{X}$	6.54	3.47	5.11	19.16	8.96	3.79	7.55	2.29	4.74
	range	4.85-7.45	2.64-3.96	3.68-5.84	13.2-22.5	6.51-10.3	3.13-4.10	5.24-8.89	1.96-2.65	3.52-5.37
% difference [a]		1.83	2.31	1.96	1.98	1.79	1.32	1.99	0.87	1.48
p-value for genotype		0.171	0.059	0.149	0.129	0.119	0.269	0.135	0.545	0.247
p-value for location x genotype interaction		0.500	0.699	0.307	0.443	0.233	0.265	0.557	0.301	0.329
ILSI (2006)	$\bar{X}$	6.88	3.75	5.12	20.09	9.51	3.85	7.90	2.21	4.90
	range	3.35 - 12.08	2.24 - 6.66	2.35 - 7.69	9.65 - 35.36	4.62 - 16.32	1.84 - 5.39	4.39 - 13.93	1.25 - 5.14	2.66 - 8.55
	N	1350	1350	1350	1350	1350	1350	1350	1350	1350
OECD (2002) [b]	range	0.48 - 0.85	0.27 - 0.58	0.35 - 0.91	1.25 - 2.58	0.63 - 1.36	0.26 - 0.49	0.56 - 1.04	0.08 - 0.32	0.21 - 0.85

[a] – [(MIR162 - control)/control] x 100 (continued)

[b] - unit of measure for OECD values: % dw; for conversion, 1% = 10 mg/g

Table E-9 (cont). Amino acid composition of maize grain from a MIR162 hybrid and a control hybrid. For comparison, ILSI Crop Composition database and OECD values are also shown. All units as described in column headings, except F-test values and where noted.

Treatment		Methionine (mg/g dw)	Isoleucine (mg/g dw)	Leucine (mg/g dw)	Tyrosine [a] (mg/g dw)	Phenyl-alanine (mg/g dw)	Lysine (mg/g dw)	Histidine (mg/g dw)	Arginine (mg/g dw)	Tryptophan (mg/g dw)
MIR162	$\bar{X}$	2.15	3.38	12.85	3.42	5.09	3.05	2.87	4.77	0.570
	range	1.76-2.54	2.55-4.00	8.86-15.6	2.58-4.09	3.70-6.04	2.52-3.44	2.28-3.26	3.89-5.30	0.453-0.645
Control	$\bar{X}$	2.10	3.31	12.57	3.35	4.99	2.96	2.85	4.68	0.562
	range	1.71-2.42	2.35-3.85	8.28-15.1	2.35-3.86	3.43-5.84	2.47-3.29	2.20-3.14	3.64-5.27	0.479-0.636
% difference [b]		2.38	2.11	2.23	2.09	2.00	3.04	0.70	1.92	1.42
p-value for genotype		0.177	0.113	0.115	0.201	0.128	0.095	0.438	0.276	0.349
p-value for location by genotype interaction		0.459	0.372	0.561	0.694	0.483	0.256	0.195	0.157	0.090
ILSI (2006)	$\bar{X}$	2.09	3.68	13.41	3.36	5.25	3.15	2.96	4.33	0.627
	range	1.24 - 4.68	1.79 - 6.92	6.42 - 24.92	1.03 - 6.42	2.44 - 9.30	1.72 - 6.68	1.37 - 4.34	1.19 - 6.39	0.271 – 2.150
	N	1350	1350	1350	1350	1350	1350	1350	1350	1350
OECD (2002) [c]	range	0.1 - 0.46	0.22 - 0.71	0.79 - 2.41	0.12 - 0.79	0.29 - 0.64	0.05 - 0.55	0.15 - 0.38	0.22 - 0.64	0.04 – 0.13

[a] – one transgenic value was an outlier and was excluded from analysis
[b] – [(MIR162 - control)/control] x 100
[c] – units of measure for OECD values: % dw; 1% = 10 mg/g

Table E-10. Fatty acid (FA) composition of maize grain from a MIR162 hybrid and a control hybrid.[q] For comparison, ILSI Crop Composition database and OECD values are also shown. All units as described in column headings, except F-test values and where noted.

Treatment		16:0 Palmitic (% of total FA)	18:0 Stearic (% of total FA)	18:1 Oleic (% of total FA)	18:2 Linoleic (% of total FA)	18:3 Linolenic (% of total FA)
MIR162	$\bar{X}$	12.78	1.84	25.49	56.99	1.81
	range	12.25-13.09	1.56-1.99	22.67-26.57	55.86-59.74	1.72-1.89
Control	$\bar{X}$	12.69	1.88	25.22	57.36	1.75
	range	12.29-13.12	1.625-2.07	23.38-26.77	56.26-59.47	1.64-1.86
% difference [b]		0.71	-2.13	1.07	-0.65	3.43
p-value for genotype [c]		0.275	0.051	0.057	**0.026**	**<0.001**
p-value for location By genotype interaction		0.554	0.934	0.154	0.444	0.163
ILSI (2006)	$\bar{X}$	11.50	1.82	25.8	57.60	1.20
	range	7.94 - 20.71	1.02 - 3.40	17.4 - 40.2	36.2 - 66.5	0.57 - 2.25
	N	1344	1344	1344	1344	1344
OECD (2002) [d]	range	0.29 - 0.79	0.04 - 0.17	0.70 - 1.39	0.67 - 2.81	0.03 - 0.10

[a] - five most abundant fatty acids (FA) in maize grain.
[b] – [(MIR162 - control)/control] x 100
[c] - statistically significant F-test ($p<0.05$) indicated in bold type
[d] – units of measure for OECD values: % dw

Table E-11. Secondary metabolite and anti-nutrient analysis of maize grain from a MIR162 hybrid and a control hybrid. For comparison, ILSI Crop Composition database and OECD values are also shown. All units as described in column headings, except F-test values and where noted.

Treatment		Ferulic Acid (mg/kg dw)	*p*-Coumaric Acid (mg/kg dw)	Inositol (ppm)	Phytic Acid (% dw)	Trypsin Inhibitor (TIU/mg dw)	Furfural [a,b] (mg/kg dw)	Raffinose [a,b] (% dw)
MIR162	$\bar{X}$	2682	179	2957	0.745	2.82	N/A	N/A
	range	2490-2980	148-202	2410-3530	0.621-0.871	2.27-3.72	<LOQ - <LOQ	<LOQ - 0.116
Control	$\bar{X}$	2453	157	2792	0.727	2.92	N/A	N/A
	range	2010-2760	137-179	2180-3610	0.593-0.919	2.38-3.48	<LOQ - <LOQ	<LOQ - 0.148
% difference [c]		9.34	14.01	5.91	2.48	-3.42	N/A	N/A
p-value for genotype [d]		**<0.001**	**<0.001**	0.196	0.334	0.416	N/A	N/A
p-value for location x genotype interaction		0.436	0.073	0.459	0.589	0.671	N/A	N/A
ILSI (2006) [e]	$\bar{X}$	2201.1	218.4	1331.5	0.745	2.73	3.697	0.132
	range	291.9 - 3885.8	53.4 - 576.2	89.0 - 3765.4	0.111 - 1.570	1.09 - 7.18	3.000 - 6.340	0.020 - 0.320
	N	817	817	504	1196	696	14	701
OECD (2002) [f]	range	0.02 - 0.3% dw	0.003 - 0.03% dw		0.45 - 1.0		<0.01 ppm	0.21 - 0.31

[a] - where some values were <LOQ, statistical comparison was not possible so only the range is shown.
[b] - LOQ for furfural 0.55-0.57 mg/kg dw; LOQ for raffinose 0.11-0.15% dw
[c] – [(MIR162 - control)/control) x 100
[d] - statistically significant F-test probability (<0.05) indicated in bold type
[e] - below LOQ values are not included.
[f] – For conversion of ferulic acid, 0.02% dw = 200 mg/kg; of *p*-coumeric acid, 0.003% dw = 30 mg/kg; of furfural, 1ppm = 1 mg/kg

Appendix F. Benefit Analysis for MIR162 Maize

The document contained within this Appendix is entitled "Public Interest Assessment Supporting Registration of MIR162, Bt11xMIR162, and Bt11xMIR162xMIR604 Maize". This document was written for the U.S. EPA application for registration of MIR162 maize and it describes in some detail the agronomic, environmental, and economic benefits of the MIR162 maize technology. Such a benefit assessment addresses the need of the EPA Administrator to make a finding that registration of a new pesticide (plant-incorporated protectant) is in the public's interest. The benefits characterized herein are equally applicable to addressing USDA's obligations under NEPA, 42 U.S.C. §§ 4321 *et seq*.

The attached report is unaltered from the version submitted to EPA. As such, it has its own pagination, table of contents, and references. It comprises pages 182-268 of this petition.

Title

Public Interest Assessment Supporting Registrations of
MIR162, Bt11xMIR162, and Bt11xMIR162xMIR604 Maize

Data Requirement

Federal Register **51**:7626 (1986)

Authors

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Date

May 17, 2007

Submitter

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Report ID

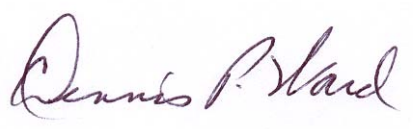
SSB-518-07

Statement of No Data Confidentiality Claim

No claim of confidentiality is being made for information in this application on the basis of its falling within the scope of FIFRA §10(d)(1)(A), (B), or (C).

Syngenta submits this material to the United States Environmental Protection Agency specifically under provisions contained in FIFRA, as amended, and consents to use and disclosure of this material by EPA according to FIFRA. In submitting this material to EPA according to method and format requirements contained in PR Notice 86-5 and 40 CFR §158.33, Syngenta does not waive any protection of rights involving this material that would have been claimed by the company if this material had not been submitted to the EPA.

Company: Syngenta Seeds, Inc.

Agent: 

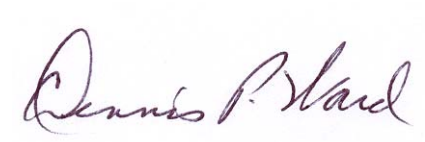
Dennis P. Ward, Ph.D.
Regulatory Affairs Manager

Date: May 17, 2007

Statement Concerning Good Laboratory Practices

This assessment was not conducted in compliance with the Good Laboratory Practice Standards as set forth in 40 CFR §160.

Study Director: There was no GLP Study Director for this investigation

Submitter: 

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Summary

Syngenta is seeking registration for a new plant-incorporated protectant, the Vip3Aa20 derived from *Bacillus thuringiensis*, as produced in maize transformation event MIR162. Syngenta is also seeking registration for two combined trait maize cultivars containing MIR162 and two other registered plant-incorporated protectants, Cry1Ab in Bt11 maize and mCry3A in MIR604 maize. The first combined trait product will be a breeding cross of MIR162 and Bt11, designated Bt11xMIR162, and the second will be a breeding cross of MIR162, Bt11, and MIR604, designated Bt11xMIR162xMIR604. Data has been developed by Syngenta demonstrating that issuance of each of these registrations will be in the public interest.

Field efficacy trials demonstrate that MIR162 maize and Bt11xMIR162 maize hybrids provide improved protection against lepidopteran insect feeding damage when compared to the protection provided by conventional insecticides or Bt11 maize alone. This improved product efficacy is expected to translate into increased maize grain yield and quality. In a time of rising demand for maize grain, the MIR162 trait has the potential to provide U.S. agriculture with an economic benefit exceeding $371 million annually at product maturity. The introduction of the MIR162 trait in combination with Bt11 also has the potential to replace many conventional insecticide applications, reduce greenhouse gas emissions, and reduce mycotoxin contamination of livestock feed. There will also be IRM benefits stemming from the introduction of these combined trait hybrids. The Vip3Aa20 protein contained in MIR162 maize brings a second mode of action against *Helicoverpa zea* and *Spodoptera frugiperda*, two pests that are only suppressed by Cry1Ab. Data has been developed showing that Bt11xMIR162 is high dose against these two pests; accordingly a reduction from the 50% structured refuge requirement in the South is warranted. This will greatly benefit maize growers in the affected counties of the South as it will allow them to protect more of their maize acres against feeding damage from lepidopteran pests.

Adoption of Bt11xMIR162xMIR604 hybrids by growers is predicted to offer crop yield advantages and important new options for control of virtually all the major insect pests of maize, all built into a single seed product. The availability of a new product for lepidopteran and rootworm control will provide choices for growers in the marketplace, and lead to increased price competition for traits, which will benefit growers and others in the maize value chain. Bt11xMIR162xMIR604 maize also offers health and environmental safety advantages over the use of conventional insecticides, as well as insect resistance management benefits that will preserve the durability of this and other *Bt*-based products.

Collectively, the information presented in this document convincingly supports a public interest finding for registration of the plant-incorporated protectants in MIR162, Bt11xMIR162, and Bt11xMIR162xMIR604 maize.

I. Introduction

At the time of application for a §3 pesticide registration under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), it is often not possible to determine if the registration will be granted under special circumstances. Since their introduction, plant-incorporated protectant registrations have only been granted under special circumstances. FIFRA authorizes the Administrator of the Environmental Protection Agency (EPA) to conditionally register a pesticide containing a new active ingredient for a period sufficient to generate and submit additional data. Conditional registrations are granted if the Administrator determines that use of the pesticide during such period will not cause any unreasonable adverse effect on human health or the environment and that use of the pesticide is in the public interest.

The introduction of crops improved through modern biotechnology has been the single most important technological innovation brought to United States (U.S.) agriculture in the past 25 years. Adoption of genetically engineered crops with insect and herbicide tolerance traits has increased dramatically since their commercial introduction in 1995. Net economic benefits at the farm level have been substantial, exceeding a cumulative $27 billion (Brookes and Barfoot, 2005). Improved insect and weed control have led to increased crop yields and reductions in conventional pesticide applications (Marra *et al.*, 2002). The continued development and introduction of these traits is expected to benefit both growers and consumers.

Maize transformation event MIR162 has been developed by Syngenta Biotechnology, Inc. to provide U.S. growers with maize hybrids that are resistant to feeding damage caused by a number of significant lepidopteran insect pests. This assessment will characterize the benefits of MIR162 maize (field corn) hybrids and combined trait Bt11xMIR162 and Bt11xMIR162xMIR604 hybrids, and will show how these benefits support a determination that their registration their insecticidal active ingredients is in the public interest.

II. Overview of Maize

Zea mays Linnaeus, known as maize throughout the world, and as corn in the U.S., is one of the few major crop species indigenous to the Western Hemisphere. It has been cultivated in the Americas since early historic times. Field maize is the leading production crop globally, with the 2005/2006 growing season yielding 695 million metric tons of grain (USDA, 2006a).

A. Importance of Maize to the U.S. Economy

The U.S. accounts for nearly 41% of global maize production. Maize is the largest crop grown in the U.S. in terms of both volume and value. Approximately 78.3 million acres were planted in 2006. In the same year, 70.6 million acres were harvested for grain, yielding 10.5 billion bushels (267 million metric tons) with a gross crop value of $33.71 billion (USDA, 2007a).

Maize is grown for animal feed, human food, vegetable oil, high fructose corn syrups (HFCS), starch, fermentation into ethanol, and a multitude of industrial uses. U.S. maize usage by market segment is shown in Figure 1. Maize as a source of fuel ethanol has increased dramatically over the past two years and is expected to continue doing so as the U.S. continues to focus on finding renewable sources of energy. By 2010, U.S. ethanol production could displace the equivalent of 311,000 barrels of imported crude oil per day. The U.S. is by far the world's largest exporter of maize, accounting for 68% of global exports. Domestic maize production falls on the positive side of the U.S. trade balance sheet.

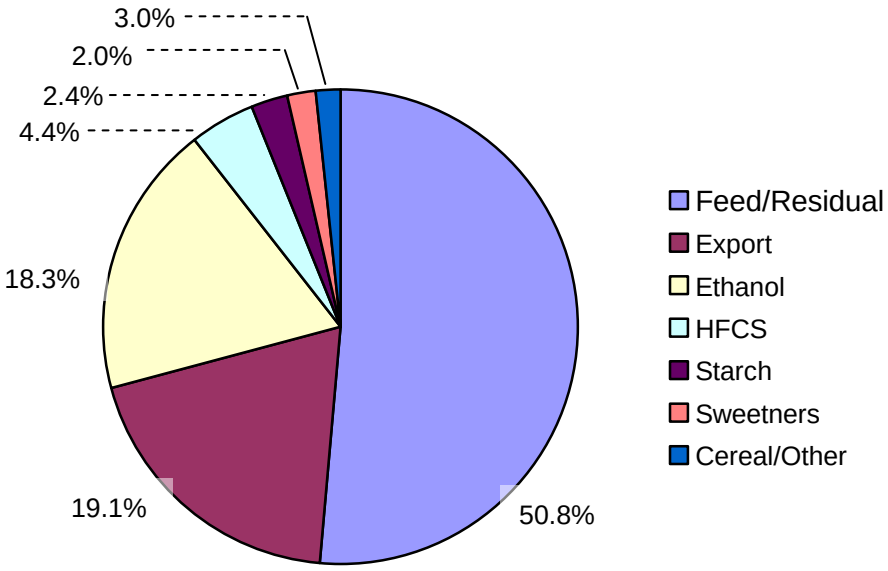


Figure 1. U.S. maize usage by segment, 2006 (Baker and Allen, 2007).

Total U.S. agricultural exports in 2006 were valued at $71 billion, 10% of which was attributable to maize (Brooks, 2007). Agricultural exports generate employment, income, and purchasing power in both farm and nonfarm sectors of the economy. Production from almost one-third of U.S. cropland moved into export channels in 2005 and generated $166.1 billion in business activity. Technology advances increase agricultural productivity and keep domestic growers competitive in the global market.

B. Maize Agronomics

Z. mays is a large, annual monoecious grass; the duration of its life cycle depends on the cultivar and the environment in which the cultivar is grown. The bulk of maize is produced between latitudes 30˚ and 47˚. Practically no maize is grown where the mean midsummer temperature is < 19˚C or where the average nighttime temperature during the summer months falls much below 13˚C. The greatest production occurs where the warmest month isotherms range between 21˚ and 27˚C and the freeze-free season lasts 120 to 180 days. Maize is grown in areas where annual precipitation ranges from 25 to > 500 cm. Summer rainfall of 15 cm is approximately the lower limit for maize production without irrigation.

The upper Midwest region of the U.S. provides an ideal combination of temperature, rainfall, and soil type for the cultivation of maize. Iowa, Illinois, Nebraska, Minnesota, Indiana, Ohio, Wisconsin, Missouri, Kansas, and South Dakota are major maize growing states. Production in these ten states accounts for 84% of total annual production (USDA, 2007a). Figure 2 displays the geographic distribution of acres planted in 2005.

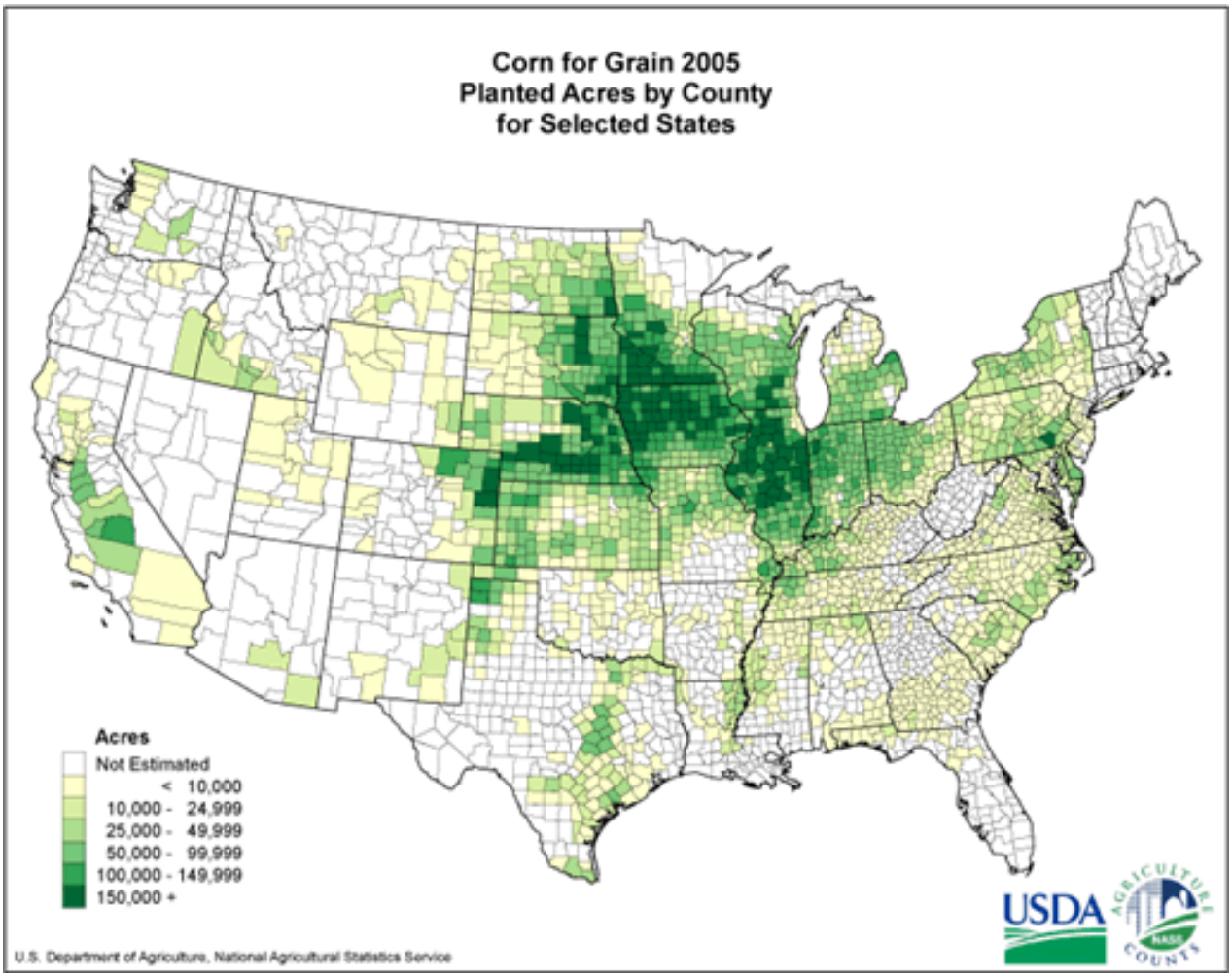


Figure 2. Geographic distribution by county of acres planted with maize in 2005 (source USDA/NASS Charts & Maps).

Farmers have hundreds of maize hybrids from which to choose. Available hybrids differ widely in agronomic characteristics, including length of growing period. Technology providers continue to develop varieties with desirable traits and increasing yield. Maize yields have increased an average of 3.5 bushels per year over the past decade. The average yield reported for the 2006 growing season was 149.1 bushels/acre (NCGA, 2007).

The adoption of new varieties improved through biotechnology has added greatly to farm productivity and profits since their introduction in 1996 (Fernandez-Cornejo and Caswell, 2006). Varieties containing herbicide-tolerance and insect-protection traits have been widely adopted by maize, soybean, and cotton growers because they protect the inherent yield

potential of these crops or typically reduce grower input costs. In 2006, herbicide tolerant varieties accounted for 89% of soybeans planted, 65% of cotton planted, and 36% of maize planted (USDA, 2006b). Insect-protected varieties accounted for 52% of cotton and 40% of maize acres planted. Planting of these varieties has also benefited the environment by displacing conventional pesticide applications and reducing the production of greenhouse gases. The adoption of biotech maize varieties has been estimated to reduce the application of conventional pesticides by more than 20 million pounds annually (NCGA, 2007).

C. Pests of Maize

Yield losses due to weeds, diseases, and insects were huge until the introduction of crop protection chemicals in the 1960s. Weeds compete with crops for light, nutrients, water, and other growth factors. If weeds are left uncontrolled, maize simply cannot be grown successfully. Estimates of maize yield loss caused by pathogens have ranged from 2 to 17% (Smith and White, 1988). In addition, a maize crop is susceptible to attack by a variety of insects from the time it is planted until it is consumed as food or feed.

Insect pests can be categorized as major and consistent pests, major and sporadic, and moderate to minor based on annual destructiveness and their geographic distribution. Table 1 categorizes most of the insect pests of maize found in the U.S. The most economically significant insect pests of maize are: *Ostrinia nubilalis* (European corn borer), *Diatraea saccharalis* (sugarcane borer), *Diatraea grandiosella* (southwestern corn borer), *Elasmopalpus lignosellus* (lesser cornstalk borer), *Helicoverpa zea* (corn earworm/cotton bollworm), *Agrotis ipsilon* (black cutworm), *Spodoptera frugiperda* (fall armyworm), *Rhopalosiphum maidis* (corn leaf aphids), and *Diabrotica spp.* (corn rootworm complex). Pests of secondary economic importance in maize include both soil-dwelling insects that feed on roots or other subterranean tissue (*e.g.,* wireworms, billbugs, webworms, white grubs, corn root aphids, the seed corn maggot, grape colaspis and seedcorn beetles) and aboveground insects that attack the stalk, leaf, and ear (*e.g.,* cutworms, corn leaf aphids, chinch bugs, grasshoppers, corn flea beetles and Japanese beetles). *Striacosta albicosta* (western bean cutworm) is a pest of increasing economic importance because of its movement into Iowa, Illinois, and Indiana.

In addition to direct damage caused by feeding on plant tissue, insects play an important role in the transmission and dissemination of pathogenic organisms during maize development. Soil abounds in microorganisms, particularly fungi, which may infect plant parts injured by soil-dwelling insects. In much of the Corn Belt, pathogenic fungi probably pose more problems in corn production than any other group of organisms. Primary roots of the seedling and the radical and seminal roots are commonly infected with *Fusarium* spp. after they have served their function and become senescent. Feeding by *Diabrotica* rootworms has been associated with increased frequencies of *Fusarium* infection (Dicke and Guthrie, 1988); rootworm feeding may also lead to increased incidences of stalk rots. These pathogenic infections can lead to reduced crop quality, harvestability, and yield.

Ear, kernel, and cob rots occur wherever maize is grown and result in reduced test weight, poor grain quality, and mycotoxin contamination of food and feed. *Fusarium* kernel or ear

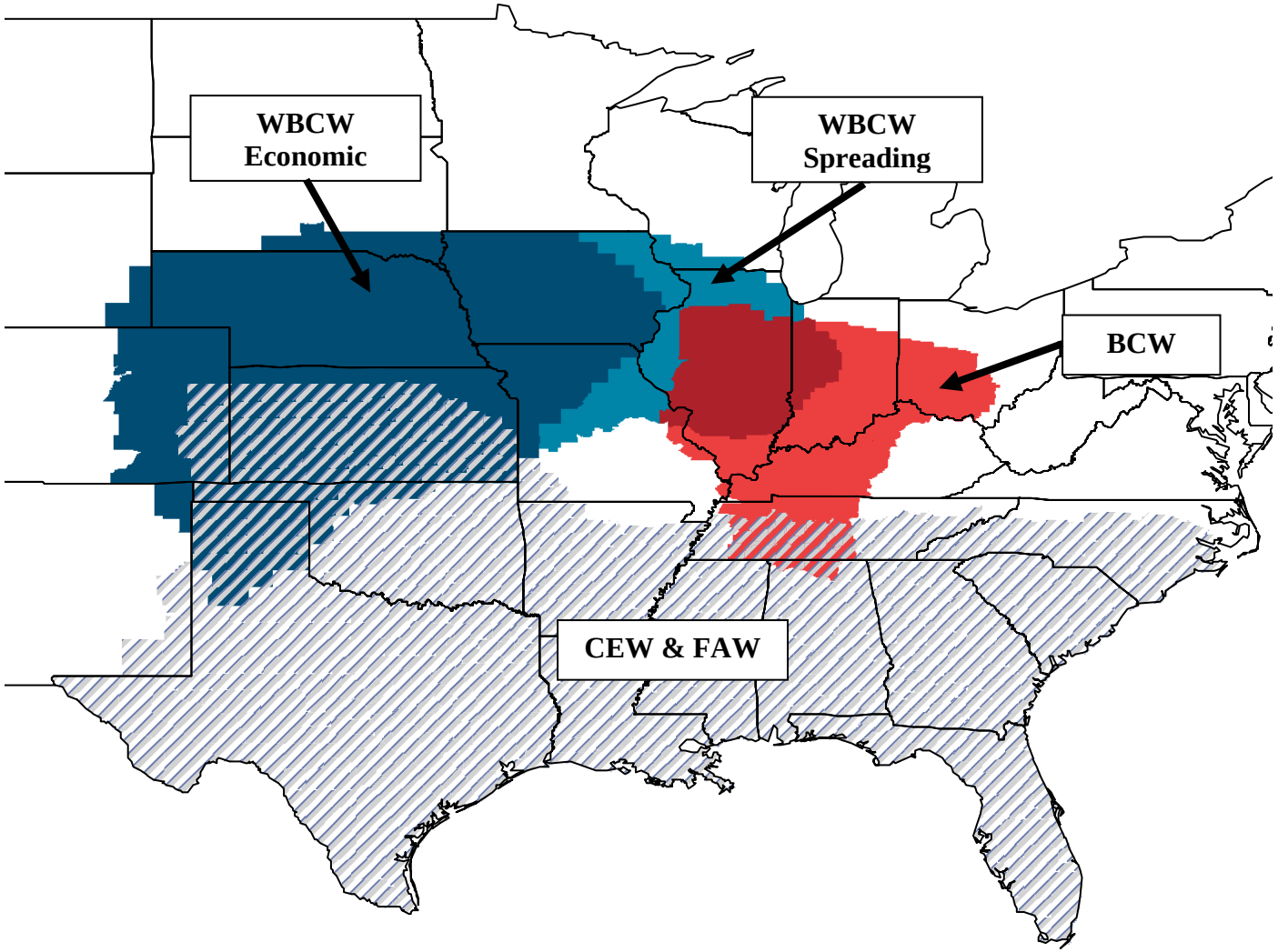
rot is the most widespread disease of maize ears and is frequently associated with insect feeding damage.

Table 1. Categorization of maize insect pests based on their potential for causing economic losses (modified from Gray and Luckmann, 1994).

Major & Consistent Insect Pests	Moderate to Minor Insect Pests (cont.)
Northern corn rootworm (*Diabrotica barberi*)	Cinch bug (*Blissus leucopterus*)
Western corn rootworm (*Diabrotica virgifera virgifera*)	Southern corn rootworm (*Diabrotica undecimpunctata*)
European corn borer (*Ostrinia nubilalis*)	Other cutworms, many species
Corn earworm (*Helicoverpa zea*)	Seedcorn beetle (*Stenolophus lecontei*)
Fall armyworm (*Spodoptera frugiperda*)	Seedcorn maggot (*Delia platura*)
	Banks grass mite (*Oligonychus pratensis*)
Major - Sporadic Insect Pests	Two-spotted spider mite (*Tetranychus urticae*)
Black cutworm (*Agrotis ipsilon*)	Billbugs, many species
Corn leaf aphid (*Rhopalosiphum maidis*)	White grubs, many species
Southwest corn borer (*Diatraea grandiosella*)	Stalk borer (*Papaipema nebris*)
Sugarcane borer (*Diatraea saccharalis*)	Garden symphylan (*Scutigerella immaculata*)
Lesser cornstalk borer (*Elasmopalpus lignosellus*)	Japanese beetle (*Popillia japonica*)
Western bean cutworm (*Srtiacosta albicosta*)	Sod webworms, several species
	Grape colaspis (*Colaspis brunnea*)
Moderate to Minor Insect Pests	Thrips, several species
Wireworms, many species	Dusky sap beetle (*Carpophilus lugubris*)
Armyworm (*Pseudaletia unipunctata*)	Stink bugs, several species
White-fringed beetles (*Graphognathus spp.*)	Southern cornstalk borer (*Diatraea crambidoides*)
Grasshoppers, many species	Corn root aphid (*Anuraphis maidiradicis*)
Corn flea beetle (*Chaetocnema pulicaria*)	

Crop losses attributable to *O. nubilalis* and *Diabrotica* infestations have been well characterized. However, there is surprisingly little quantitative information available on the economic impacts of the other major insect pests of maize, *H. zea*, *S. frugiperda*, *A. ipsilon*, and *S. albicosta*. These pests are not as widely spread as corn borers and rootworms; however, they can be very costly to growers as they have the potential to significantly lower grain yield and quality. Figure 3 shows the approximate geographic distribution of these four insect pests in the continental U.S. As described in Appendix 1, there is compelling evidence

Figure 3. U.S. geographic distribution of the maize insect pests *H. zea* (CEW), *S. frugiperda* (FAW), *A. ipsilon* (BCW), and *S. albicosta* (WBCW). Two regions are shown for WBCW occurrence, one where its presence presents an economic problem for growers and the other where it is spreading into.



that one of these pests, *S. albicosta*, is spreading eastward at an alarming rate. Note the overlap of *S. albicosta* occurrence in Figure 3 with the regions of highest maize production shown in Figure 2.

1. *Helicoverpa zea* (Corn Earworm)

Helicoverpa zea, formerly *Heliothis zea*, is native to the Americas and occurs wherever maize is grown. In its larval stage it is a major economic pest of maize. Larvae feed on leaves, tassels, silk, and ears; refer to Figure 4. Whorl feeding appears as ragged holes in the leaves. Earworm larvae prefer to feed on developing ears during the silking period. Silks are often clipped as larvae tunnel into the ear. The ear will have an incomplete kernel set if the silks are destroyed before pollination is complete. Larval feeding on kernels causes the most significant damage. Larvae feed on the silks and ears, leaving their waste behind. This behavior creates a highly favorable microenvironment for the invasion of secondary pests and for the production of mycotoxins. Ear molds developing in the damaged kernels may cause toxicity problems in livestock.

Figure 4. *H. zea* larva feeding on maize silks (top) and ear (bottom).

Although *H. zea* can be very damaging to maize, injury is so evenly distributed and control is so difficult that farmers generally accept the loss. Chemical control of earworms is prohibitively expensive except to protect sweet maize. Control is difficult because eggs are laid on emerging silks; a protective insecticide coating of the silks cannot be maintained unless sprays are applied every two to three days.

In a study conducted by Hudson and All (1995), 35,000 acres of field maize in Georgia were identified as having *H. zea* infestations at levels warranting control. Yield loss was 1.5 bu/ac on chemically treated acres and 5.6 bu/ac on untreated acres. Losses to Georgia maize growers in 1995 attributable to *H. zea* infestations totaled $510,000 in combined chemical costs and yield loss. Losses in 1996 attributable to *H. zea* were even higher, totaling $872,000.

2. *Agrotis ipsilon* (Black Cutworm)

A. ipsilon (formerly *Agrotis ypsilon*) is by far the most destructive species of the cutworm complex in maize (see Figure 5). *A. ipsilon* occurs throughout North and South America. It feeds on many hosts, including several weed species. Damage caused by *A. ipsilon* larvae is particularly severe in rotated maize that is planted late and in maize planted in flood plains. *A. ipsilon* larvae also cause economic damage to vegetables, cotton, tobacco, and turf grasses.

A. ipsilon does not overwinter in the Midwest; moths migrate from the coastal areas of the Gulf of Mexico in the early spring. Females prefer to deposit eggs on densely growing weeds and debris in fields that have not been tilled. When weed hosts are killed with cultivation or herbicides, the larvae begin feeding on maize. They are nocturnal feeders, cutting seedlings at or just below the soil surface. Older instars can then tunnel into the growing stalk.

Figure 5. *A. ipsilon* larva (top) and cut maize stalk (bottom).

Plants in the one- to four-leaf development stage are particularly susceptible to cutting and the extent of yield reduction is dependent on where the plant is severed. Maize seedlings cut below the soil surface or larger plants that have been tunneled usually do not recover. Seedlings cut above the soil surface often recover if moisture is adequate, but they will not yield as well. While some plants cut at the soil surface produce ears, there is a significant yield loss. Plants cut below the soil surface do not produce ears.

Tillage, removal of winter and early spring vegetation, and insecticide applications at planting can be used to prevent cutworm damage. However, when outbreaks occur, they are difficult to control even with insecticides. Scouting for cutworm feeding injury should be conducted as soon as maize plants emerge from the soil. A rescue post-emergent insecticide application when ≥ 3% cut plants are found is necessary to prevent devastating crop losses.

3. *Striacosta albicosta* (Western Bean Cutworm)

S. albicosta (formerly *Loxagrotis albicosta* and *Richia albicosta*), has been categorized as a pest of moderate significance in the western Corn Belt. Over the past several years it has become established in Iowa and continues to move east. There are numerous university

extension reports documenting its spread into Illinois and Indiana (see Figure 3).

One generation of *S. albicosta* occurs each year. Moth emergence begins in July and after mating, females lay eggs on available host plants such as field maize, sweet maize, or dry beans. In maize, females lay eggs primarily on the upper surface of leaves. After hatching, larvae move to more protected sites on the host plant and progress through five instars. Feeding on maize occurs in the whorls where the larvae feed on the flag leaf, tassel, and other yellow tissue. Larvae then move to feeding on emerging silks. As both larvae and maize develop, they begin feeding on ear tips. *Fusarium* and other secondary pests typically follow kernel injury. Figure 6 shows the nature and extent of damage that can be caused by larvae feeding on ears.

Figure 6. *S. albicosta* larval feeding damage caused in maize ears.

An infestation of more than one larva per ear can occur because, unlike *H. zea*, *S. albicosta* larvae are not cannibalistic. One larva per ear rarely causes economic injury, but studies have shown that even one larva per ear on a field-wide basis can reduce yield by 3.7 bu/ac (Cook, 2004). In years of severe infestation with multiple larvae per ear, 50 to 60% of an ear's kernels can be damaged. This contributes directly to significant reductions in yield and grain quality.

Scouting is essential for effectively reducing the risk of major infestations. Insecticide applications can be effective but timing of application is critical. Once the larvae have entered the silks and are inside the husk, insecticide applications are ineffective and larvae will feed until mature. Few cultural controls are effective against this pest.

4. *Spodoptera frugiperda* (Fall Armyworm)

S. frugiperda is native to tropical regions of North and South America. In the U.S. it overwinters in Florida and Texas and the moths can fly great distances. The insect has been found in virtually all states east of the Rocky Mountains. As a pest of maize, it is typically only a problem in the southeastern states.

Maize, sorghum, and other grassy plants are preferred hosts for *S. frugiperda*. The number of generations per year varies with latitude; up to four generations are common in southern states. The larvae progress through six instars and feed on foliage. Young larvae initially consume leaf tissue from one side, leaving the opposite epidermal layer intact. Feeding by second and third-generation larvae results in holes in leaves. Feeding in the whorl of maize produces a characteristic row of perforations in the leaves (see Figure 7). Older larvae cause extensive defoliation, often leaving only the ribs and stalks of a plant or a very ragged, torn appearance.

Yield loss is proportional to loss of leaf mass but varies with plant development stage. Early whorl-stage feeding produces the least impact on yield; feeding at late whorl stage is very damaging to crop yield. Marenco *et al.* (1992) observed that mean densities of larvae per plant during late whorl stage could reduce yield by 5 to 20%. Larvae can also burrow into ears causing kernel loss and diminished grain quality.

Early planting is an effective cultural practice in some regions for limiting *S. frugiperda* damage. An early harvest allows many maize ears to escape the higher armyworm densities that develop later in the season. Control of armyworms with conventional insecticides is typically not economical, unless infestation is severe and the larvae are feeding on parts of the plant that would bring them into contact with the insecticide. The economic feasibility of conventional insecticide applications will likely change as the price of maize grain increases. Insecticides are most commonly applied in sweet corn production as *S. frugiperda* is the economically most damaging pest to crop yield and quality.

Figure 7. *S. frugiperda* larva feeding on maize leaf (top) and whorl feeding damage (bottom).

D. Current Insect Control Practices

The most widespread and damaging insects of maize in the U.S. Corn Belt have been *O. nubilalis* and *Diabrotica* species. Before the introduction of *Bt* maize hybrids ten years ago growers had few practical options for controlling stalk boring insects; only about 10% of growers applied insecticides for control of corn borers. As a result, they incurred significant

annual yield losses because most fields were not treated and chemical applications were not always effective. Timing of insecticide applications had to be nearly perfect because there was only a very short period of time (two to six days) that these insects would be physically positioned on the plant where they could be exposed to an insecticide application. The introduction of the first *Bt* maize hybrids in 1996 provided growers with an effective means of limiting damage caused by *O. nubilalis*. By plant-incorporating the insecticide, exposure of the insect to the toxin was guaranteed. In 2006, 42% of maize acres were planted with *Bt* corn borer-protected hybrids (Doane, 2006a). These hybrids express either a *cry1Ab* or *cry1F* gene from *B. thuringiensis*, both of which are highly toxic to *O. nubilalis* and *D. grandiosella*.

More options have been available to growers for mitigating damage caused by corn rootworms. In many maize growing regions crop rotation has been effective in limiting *Diabrotica* populations because it breaks the life cycle of the insect. There have also been many effective conventional insecticide products available to growers for control of these pests. Prior to the introduction of rootworm-protected *Bt* varieties in 2003 an estimated 14 million acres were treated annually with conventional insecticides to control corn rootworms. This equated to more than 7.7 million pounds of insecticide active ingredient being applied annually in maize fields for the control of *Diabrotica* species (Ward *et al.*, 2005). Control of *Diabrotica* rootworms accounted for the largest single use of insecticides in the U.S.

Controlling aboveground insects presents a challenge for maize growers. The majority of maize fields are not treated for control of leaf, stalk, and ear feeding insects. A grower decision not to treat is generally not reversible because of the feeding location of the pests (in the soil, under the leaf, inside the stalk, or in the ear); the pests are shielded from aerial chemical applications. Timing of applications is critical for them to be effective.

Data obtained from the Doane 2005 and 2006 AgroTrak studies indicate that growers are currently treating approximately three million acres a year with conventional insecticides for control of *H. zea*, *A. ipsilon*, *S. albicosta*, and *S. frugiperda* (see Table 2). Compared to the total number of maize acres planted annually this represents a relatively small use of conventional pesticides; however, three million acres treated represents a significant use compared to chemical usage in other crops.

III. Characteristics of MIR162 Maize

MIR162 maize was produced by *Agrobacterium*-mediated transformation using elements of a vector (pNOV1300) containing a variant of the *vip3Aa1* gene from *Bacillus thuringiensis.* The *vip3Aa1* gene was isolated from *B. thuringiensis* strain AB88 (Estruch *et al.*, 1996). This gene encodes a vegetative insecticidal protein (Vip) that is highly toxic to numerous lepidopteran pests of maize, including: *A. ipsilon*, *H. zea*, *S. frugiperda*, and *S. albicosta*. A maize-optimized variant of the gene has been incorporated into the genome of MIR162 maize and encodes a protein assigned the following toxin designation: Vip3Aa20. MIR162 maize also contains the *manA* gene from *Escherichia coli* which encodes the selectable marker enzyme, phosphomannose isomerase.

Table 2. Conventional insecticide usage in maize production during 2005 and 2006 for the control of *S. frugiperda*, *A. ipsilon*, *S. albicosta*, and *H. zea* (Doane, 2005 and 2006b).

Pest	2005		2006	
	Acres Treated	Grower Cost ($)	Acres Treated	Grower Cost ($)
S. frugiperda	0	0	20,441	86,550
A. ipsilon	2,721,543	19,457,090	3,064,137	22,328,818
S. albicosta	44,410	363,801	72,373	343,552
H. zea	99,620	825,654	161,002	848,502
Totals	2,865,573	$20,646,545	3,317,953	$23,607,422

Syngenta conducted field trials at multiple locations in 2005 and 2006 to assess the trait efficacy of MIR162 maize hybrids (Huber *et al.*, 2007; White *et al.*, 2007a; White *et al.*, 2007b). Trials were placed in locations to take advantage of natural pest infestations. In some locations trials were artificially infested to test trait performance at high levels of insect pressure. MIR162, Bt11, Bt11xMIR162, conventional maize, and conventional maize with an insecticide application were the treatments employed in most trials. Figure 8 presents a graphic representation of the comparative feeding damage for each of the treatments, expressed as a percentage of damage measured in the control plants, for five of the insects evaluated.

MIR162 alone has no activity against *O. nubilalis* but is very efficacious in limiting feeding damage caused by the other four insect pests. Whereas Bt11 is highly efficacious against *O. nubilalis*, it has limited or no activity against the other four insects. The combined-trait hybrid, Bt11xMIR162, is very efficacious against all five insects. MIR162 addresses the lepidopteran pest control limitations of Syngenta's Bt11 product. In combination with Bt11, it will provide growers with excellent control of the following significant lepidopteran insect pests of maize: *A. ipsilon* (black cutworm), *D. crambidoides* (southern cornstalk borer), *D. grandiosella* (southwestern corn borer), *D. saccharalis* (sugarcane borer), *H. zea* (corn earworm), *O. nubilalis* (European corn borer), *P. nebris* (common stalk borer), *S. exigua* (beet armyworm), *S. frugiperda* (fall armyworm), and *S. albicosta* (western bean cutworm).

Comparative efficacy trials of Bt11xMIR162 hybrids with commercially available lepidopteran-protected *Bt* varieties, specifically *YieldGard CB* (MON 810) and *Herculex I* (TC1507), were not possible due to intellectual property constraints. However, it is reasonable to assume that MON 810 hybrids have the same spectrum of activity limitations that Bt11 hybrids do and, therefore, the Bt11xMIR162 product will provide superior protection against *A. ipsilon*, *D. saccharalis*, *H. zea*, *P. nebris*, *S. exigua*, *S. frugiperda*, and *S. albicosta*. Publicly available information on the performance of *Herculex I* hybrids suggests that Bt11xMIR162 hybrids may outperform them in controlling *H. zea* and *S. albicosta* feeding damage.

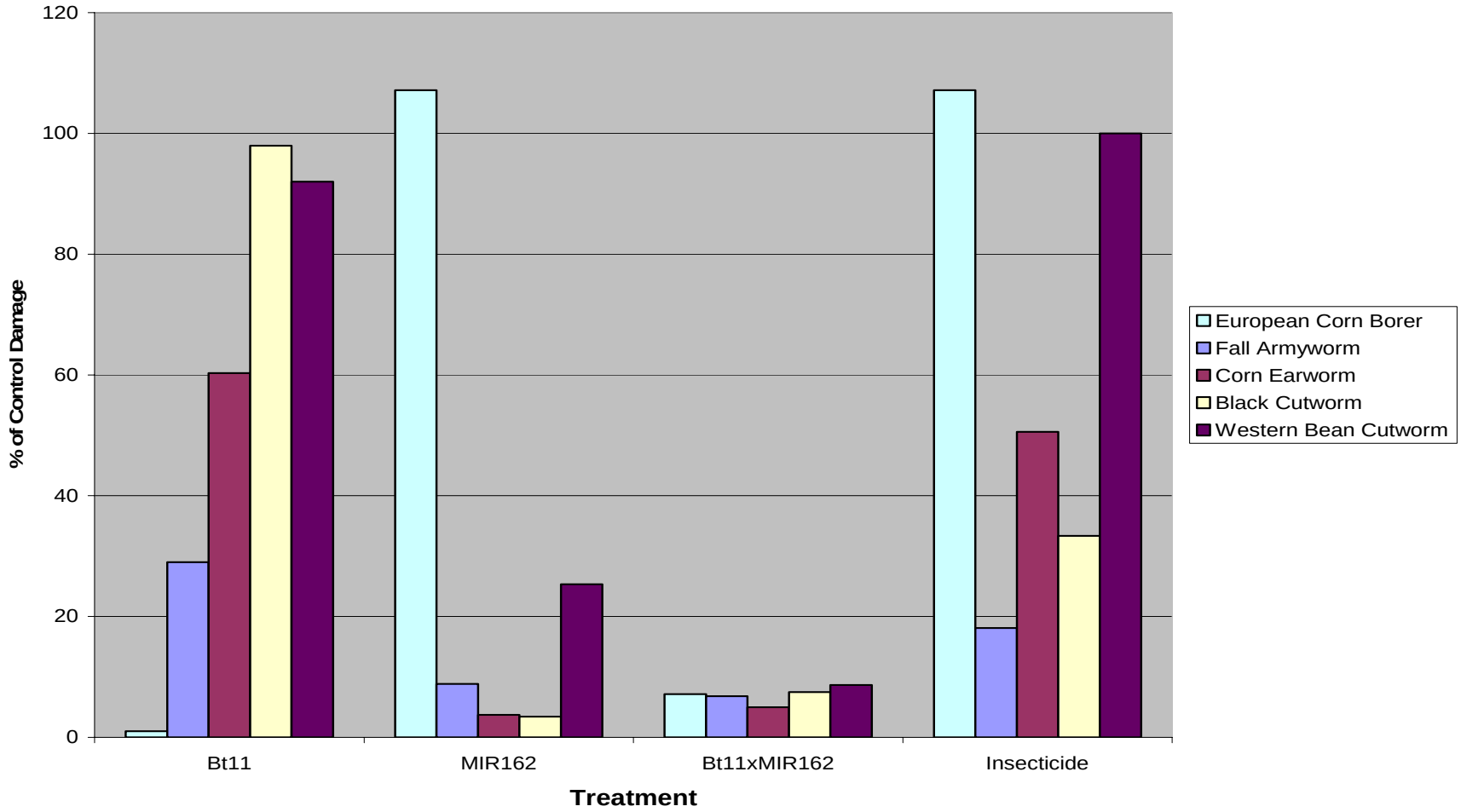


Figure 8. Feeding damage ratings from field efficacy trials conducted in 2005 and 2006 with Bt11, MIR162, Bt11xMIR162, and a conventional insecticide treatment (*Warrior® Insecticide*). Ratings are expressed as a percentage of the damage observed in the control plots.

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Other than its resistance to lepidopteran insect feeding, MIR162 maize hybrids are phenotypically equivalent to conventional maize hybrids. The presence of the MIR162 transgenes will protect the inherent yield potential of varieties that contain them. In 2005 and 2006, Syngenta conducted phenotypic equivalency studies at 16 locations to compare the agronomic performance of two MIR162 hybrids to the performance of two near-isogenic conventional hybrids. These trials were planted in a randomized complete block design with either four or five replications per location. The trial results are presented in Table 3 and demonstrate no difference in yield between MIR162 and conventional hybrids.

Table 3. Yield results from agronomic equivalency studies conducted with two MIR162 hybrids in 2005 and 2006. Average yield (bu/ac) at each location and across locations was subjected to an analysis of variance and least significant difference test. Statistical significance was assigned at $p<0.05$.

Location	MIR162 Yield	Control Yield	Difference	Significance [a]
2005 Trials				
Allen, IA	166.3	145.1	21.2	NS
Seward, NE	152.4	158.4	-6.0	NS
Hudson, IL	196.6	185.9	10.7	NS
Bloomington, IL	202.1	200.3	1.8	NS
Wapella, IL	204.7	187.4	17.3	NS
Mackinaw, IL	187.0	175.8	11.2	NS
Average	**184.9**	**175.5**	**9.4**	**NS**
2006 Trials				
Brookings, SD	152.2	155.4	-3.2	NS
Gaylord, MN	204.6	206.7	-2.1	NS
Janesville, WI	155.4	153.6	1.8	NS
Maxwell, IA	153.6	159.7	-6.1	NS
Monroeville, IN	156.3	164.6	-8.3	NS
Seward, NE	194.1	178.2	15.9	NS
El Paso, IL	200.3	193.4	6.9	NS
Bloomington, IL	201.7	187.8	13.9	*
Sadorus, IL	174.8	183.1	-8.3	NS
Mackinaw, IL	190.6	181.7	8.9	NS
Average	**178.4**	**176.4**	**2.0**	**NS**

a - NS = means not statistically significant; * = means significantly different ($p<0.05$)

In addition to evaluating the performance characteristics of MIR162 maize, Syngenta has examined its potential human and environmental impacts. The standard battery of studies to identify hazards for mammalian species potentially exposed to plant-incorporated Vip3Aa20 has been conducted. These Vip3Aa20 studies include an acute oral toxicity study with mice (Draper, 2007), *in vitro* digestive fate assays (Stacy, 2007a; Stacy, 2007b), a comparison of amino acid sequence to that of known toxins and allergens (Harper, 2006a; Harper, 2006b), and a characterization of the biochemical properties of the Vip3Aa20 protein (Graser and Stacy, 2006). The results of these studies demonstrate that no adverse effects were observed in mice exposed to a maximum attainable oral dose, that the protein is rapidly degraded in a gastric matrix, that the protein does not share sequence homology with known mammalian toxins and allergens, and it does not possess properties suggestive of food allergen potential. A temporary exemption from the requirement of a tolerance has been established for Vip3Aa20 when used as a plant-incorporated protectant in the food and feed commodities of corn (40 CFR §174.458).

A comprehensive set of data has been developed to support an environmental safety assessment for Vip3Aa proteins and MIR162 maize. No harmful effects have been observed in studies with a range of nontarget organisms, which in most cases were exposed to levels of Vip3Aa protein above expected environmental concentrations. A comprehensive environmental assessment describing the results of these studies has been prepared by Raybould (2007a).

This failure to elicit adverse effects in nontarget species is not surprising given the known mode of insecticidal action for Vip proteins and the narrow spectrum of activity for Vip3Aa proteins. Vip3Aa20 is a member of a class of insecticidal proteins that are naturally produced by *B. thuringiensis*, a gram positive bacterium commonly found in soil. Unlike the crystal proteins (Cry) of *B. thuringiensis*, Vip proteins are produced during vegetative bacterial growth and are secreted as soluble proteins into the extracellular environment. *B. thuringiensis* cultures continue to produce Vip protein during the stationary phase of development and sporulation. Unlike the thermostabile nonproteinaceous β-exotoxin secreted by some *B. thuringiensis* strains, Vip proteins are thermolabile.

The mechanism by which Vip proteins exert their insecticidal activity has been studied and been found to be similar to that which has been previously described for the *B. thuringiensis* Cry proteins. Following ingestion, full-length Vip proteins are proteolytically processed to active fragments of approximately 65 kDa which bind to receptors in the midgut epithelium of susceptible insects. Competitive binding assays have shown that Vip proteins and Cry proteins bind to different receptors (Lee *et al.*, 2003). Receptor binding is followed by the formation of selective ion channels (pores) in epithelial membranes. Each of these steps plays a role in establishing the insecticidal specificity of a given protein for different insect species.

Syngenta has conducted mortality bioassays with Vip3Aa protein variants in a range of insect species to identify those that are susceptible. The results of these bioassays and the results of assays reported in the scientific literature clearly demonstrate that activity of Vip3Aa proteins is limited to species within order Lepidoptera, yet all lepidopteran species are not sensitive to

the protein. Table 4 provides a listing of insect species that have been found by Syngenta or reported in the literature as being sensitive to one or more Vip3Aa protein variants.

Table 4. Insect species that have been screened for sensitivity to Vip3Aa protein variants in diet-surface or diet-incorporation bioassays. Activity was determined as evidence of treatment-related mortality.

Order: Family	Genus: Species	Activity
Lepidoptera: Noctuidae	*Agrotis ipsilon*	Active
	Helicoverpa zea	Active
	Helicoverpa armigera	Active
	Helicoverpa punctifera	Active
	Heliothis virescens	Active
	Spodotera exigua	Active
	Spodoptera frugiperda	Active
	Spodoptera litura	Active
	Trichoplusia ni	Active
Lepidoptera: Gelechidae	*Phthorimea operculella*	Active
Lepidoptera: Sphingidae	*Manduca sexta*	Active
Lepidoptera: Pyralidae	*Chilo partellus*	Active
	Ostrinia nubilalis	Not Active
Lepidoptera: Plutellidae	*Plutella xylostella*	Active
Lepidoptera: Danaidae	*Danaus plexippus*	Not Active
Lepidoptera: Pieridae	*Pieris brassicae*	Not Active
Other Lepidoptera	*Bombyx mori*	Not Active
	Earias vittella	Active
	Ephestia kuehniella	Active
Diptera	*Culex pipiens*	Not Active
	Drosophila melanogaster	Not Active
	Culex quinquefasciatus	Not Active
Coleoptera	*Coleomegilla maculata*	Not Active
	Diabrotica virgifera virgifera	Not Active
	Leptinotarsa decemlineata	Not Active
	Tenebrio molitor	Not Active
	Anthonomus grandis grandis	Not Active
	Aleochara bilineata	Not Active

Table 4 (cont.). Insect species that have been screened for sensitivity to Vip3Aa protein variants in diet-surface or diet-incorporation bioassays. Activity was determined as evidence of treatment related mortality.

Order: Family	Genus: Species	Activity
Hymenoptera	*Apis mellifera*	Not Active
Hemiptera: Anthocoridae	*Orius insidious*	Not Active
Thysanoptera	*Frankliniella occidentalis*	Not Active
Isotomidae	*Folsomia candida*	Not Active
Neuroptera	*Chrysoperla carnea*	Not Active

IV. Public Interest Factors Applicable to MIR162 Maize

Registration of the *B. thuringiensis* Vip3Aa20 protein encoded by elements of the pNOV1300 vector in maize transformation event MIR162 can be presumed to be in the public interest because it meets criteria for a conditional registration as delineated in the EPA policy notice regarding approval or denial of applications for conditional registration of pesticide products (EPA, 1986). This criterion states that use of a new pesticide during the period of its conditional registration must be in the public interest. Many factors can be considered in determining whether this public interest criterion has been satisfied. Registration of a new pesticide is presumed to be in the public interest if it is a replacement for another pesticide that is of continuing concern to EPA. Maize varieties containing the MIR162 trait have the potential to replace the use of the numerous conventional insecticides that are of concern to EPA, farmers, and the public due to human and environmental risk factors. Additionally, the safety, convenience, and simplicity of planting MIR162 hybrids compared to the application of conventional insecticides, along with the opportunity to extract an economic benefit through increased crop yield, are expected to make this product attractive to growers.

A. Presumption of Public Interest

In certain circumstances, EPA is empowered to make a presumption that use of a new pesticide is in the public interest. In these instances, the applicant need not substantiate a public interest finding. EPA has defined three uses of high priority and if a new pesticide active ingredient addresses one or more of these uses, it qualifies for a presumption that its registration will be in the public interest. One of the uses that qualifies for a presumption of public interest is "…replacement for another pesticide that is of continuing concern to the Agency".

The most commonly used conventional insecticides registered for control of earworms, cutworms, and armyworms in maize are listed in Table 5. The majority of these insecticides are classified as Restricted Use Pesticides due to human and environmental risk concerns.

Table 5. Most commonly used conventional insecticide products (excluding seed treatments) used in maize for control of *A. ipsilon*, *H. zea*, *S. albicosta*, or *S. frugiperda*. Active ingredients contained in each product, EPA use classification, Signal word, and key precautionary text from each product label are shown.

Product Name	Active Ingredient	Signal Word / Use Classification
Asana XL Insecticide	8.4% esfenvalerate	**WARNING. Restricted Use:** Extremely toxic to fish and aquatic invertebrates. Highly toxic to bees.
Aztec 2.1% Granular Insectide	2.0% tebupirimfos 0.1% cyfluthrin	**WARNING. Restricted Use:** Toxic to fish and wildlife.
Baythroid 2 Emulsifiable Pyrethroid Insecticide	25% cyfluthirn	**DANGER. Restricted Use:** Extremely toxic to fish and aquatic invertebrates. Highly toxic to bees.
Baythroid XL Insecticide	12.7% cyfluthrin	**WARNING. Restricted Use:** Extremely toxic to fish and aquatic invertebrates. Highly toxic to bees.
Brigade 2EC Insecticide/Miticide *&* *Capture 2EC Insecticide/Miticide*	25.1% bifenthrin	**WARNING. Restricted Use:** Extremely toxic to fish and aquatic invertebrates. Highly toxic to bees.
Chlorpyrifos 4E AG Insecticide	40.7% chlorpyrifos	**WARNING. Restricted Use:** Toxic to fish, aquatic invertebrates, small mammals, and birds. Highly toxic to bees.
Decis 1.5EC Insecticide	16.6% deltamethrin	**DANGER. Restricted Use:** Extremely toxic to fish and aquatic invertebrates. Highly toxic to bees.
Empower2 Granular Insecticide	1.15% bifenthrin	**CAUTION. Restricted Use:** Extremely toxic to fish and aquatic invertebrates. Highly toxic to bees.
Entrust Naturalyte Insect Control	80% spinosad	**CAUTION. General Use:** Toxic to aquatic invertebrates. Toxic to bees.
Fanfare 2EC Insecticide/Nematicide	25.1% binfenthrin	**WARNING. Restricted Use:** Extremely toxic to fish and aquatic invertebrates. Highly toxic to bees.
Furadan 4F Insecticide	44% carbofuan	**DANGER. Restricted Use:** Poisonous if swallowed or inhaled. Toxic to fish, birds and other wildlife. Highly toxic to bees.
Hero Insecticide	3.75 zeta-cypermethrin 11.25% bifenthrin	**CAUTION. Restricted Use:** Extremely toxic to fish and aquatic invertebrates. Highly toxic to bees.
Intrepid 2F Insecticide	22.6% methoxyfenozide	**CAUTION. General Use:** Hazardous to sensitive aquatic invertebrates.

Table 5 (cont.). Most commonly used conventional insecticide products (excluding seed treatments) used in maize for control of *A. ipsilon*, *H. zea*, *S. albicosta*, or *S. frugiperda*. Active ingredients contained in each product, EPA use classification, Signal word, and key precautionary text from each product label are shown.

Product Name	Active Ingredient	Signal Word / Use Classification
Lambda-T	11.4% λ-cyhalothrin	**WARNING. Restricted Use:** Extremely toxic to fish and aquatic invertebrates. Highly toxic to bees.
Lannate SP Insecticide	90% methomyl	**DANGER. Restricted Use:** Fatal if swallowed. Toxic to fish, aquatic invertebrates, and mammals. Highly toxic to bees.
Lorsban 4E Insecticide	44.9% chlorpyrifos	**WARNING. General Use:** Toxic to fish, aquatic invertebrates, small mammals, and birds. Highly toxic to bees.
Lorsban 75WG Insecticide	75% chlorpyrifos	**WARNING. General Use:** Toxic to fish, aquatic invertebrates, small mammals, and birds. Highly toxic to bees.
Mocap 15% Granular Nematicide-Insecticide	15% ethoprop	**DANGER. Restricted Use:** Poisonous if swallowed. Toxic to aquatic organisms and wildlife.
Mustang Insecticide	17.1% zeta-cypermethrin	**WARNING. Restricted Use:** Extremely toxic to fish and aquatic invertebrates. Highly toxic to bees.
Pounce 25 WP Insecticide	25% permethrin	**WARNING. Restricted Use:** Extremely toxic to fish and aquatic invertebrates. Highly toxic to bees.
Regent 4 SC Insecticide	39.4% fipronil	**WARNING. Restricted Use:** Toxic to birds, fish, and aquatic invertebrates.
Sevin brand 4F Carbaryl Insecticide	43% carbaryl	**CAUTION. General Use:** Extremely toxic to aquatic and estuarine invertebrates. Highly toxic to bees.
Silencer Insecticide	11.4% λ-cyhalothrin	**WARNING. Restricted Use:** Extremely toxic to fish and aquatic invertebrates. Highly toxic to bees.
Success Naturalyte Insect Control	22.8% spinosad	**CAUTION. General Use:** Toxic to aquatic invertebrates. Toxic to bees.
Warrior with Zeon Technology Insecticide	11.4% λ-cyhalothrin	**WARNING. Restricted Use:** Extremely toxic to fish and aquatic invertebrates. Highly toxic to bees.

The Restricted Use classification, imposed due to adverse environmental effects under normal use practices (40 CFR 152.171(a)), limits the use of these chemicals to certified pesticide applicators who have received special training needed for safe handling and application of these products. Personal protective equipment to reduce occupational exposure, special labeling, and special record keeping are required for the sale and use of Restricted Use Pesticides. Without these restrictions EPA has determined that use of the products may cause unreasonable adverse effects on the environment. This classification is an indication of EPA concern about the safety of these products.

MIR162 has the potential to displace the use of many of the Restricted Use Pesticides that are currently being used for control of lepidopteran pests of maize. Based on this consideration alone, the plant-incorporated Vip3Aa20 pesticidal protein encoded in MIR162 maize is entitled to a presumption of public interest.

B. Factors Affecting a Public Interest Finding

The 1986 policy notice regarding conditional registrations indicates that EPA will consider a variety of factors pertaining to the need for a new pesticide active ingredient, specifically its comparative benefits, risks, and costs. EPA policy (EPA, 1986) states: "The Agency must determine that (1) there is a need for the new chemical that is not being met with other currently registered pesticides or none-pesticide alternatives; (2) the new pesticide is comparatively less risky to health or the environment than currently registered pesticides; or (3) the benefits (including economic benefits) from the use of the new chemical exceed those of alternative registered pesticides and other available non-chemical techniques." A consideration of these factors, as they relate to Vip3Aa20 maize, clearly demonstrates that its registration will be in the public interest.

1. Need Factors

While economic losses attributable to the insects that are effectively controlled by MIR162 maize are not as large as those attributable to *O. nubilalis* and *Diabrotica* rootworms, they are significant when severe infestations occur. As the price of maize grain continues to rise, the economic threshold for growers to respond to infestations of *A. ipsilon*, *H. zea*, *S. albicosta*, or *S. frugiperda* will fall. Even relatively small reductions in crop yield (< 10%) will result in a significant economic loss for growers. Additionally, there is evidence that populations of *S. albicosta* are spreading eastward and will have the potential to cause greater harm in critical maize producing states.

As described in Section II.D, control of aboveground maize insect pests is challenging for growers. Conventional insecticide applications are costly and intensive scouting of fields is required to identify the appropriate timing for applications. Growers only have a very narrow time window during which insecticides can be applied because many of the above-ground feeding insects are shielded from contact with the insecticides by virtue of their feeding location on the plant. Planting of combined-trait hybrids containing MIR162 will provide growers with a more effective means of controlling these economically significant insect pests of maize.

2. Composition Factors

The composition of MIR162 maize is fundamentally different from the composition of conventional insecticide end-use products. The active ingredient, Vip3Aa20, is plant-incorporated. It is safer than all currently registered conventional maize insecticide products. This characteristic of the product virtually eliminates the occupational and environmental risks currently associated with the application of chemical controls for maize insect pests. Registration of this product also provides EPA with an opportunity to reduce the manufacture, transportation, storage, and disposal of millions of pounds of hazardous chemicals annually and to eliminate the greenhouse gas emissions associated with these activities. These product characteristics support a conclusion that registration of MIR162 is in the public interest.

3. Usage Factors

The safety, convenience, and simplicity of planting MIR162 hybrids compared to the application of conventional insecticides, along with the opportunity to extract an economic benefit through increased crop yield, are expected to make this product attractive to growers.

4. Performance Factors

Two years of extensive efficacy field trials, conducted at multiple locations under varying levels of insect pressure, have demonstrated the superior leaf, stalk, and ear protection provided by MIR162 maize compared to hybrids treated with a conventional insecticide product. Vip3Aa20 possesses a number of unique properties that conventional insecticides do not. The protein is efficacious *via* a mode of action that is selective to lepidopteran insects. The protein is expressed throughout all tissues of the maize plant. This ensures protection where it is needed and eliminates the risk of insecticide failures associated with timing of applications or unfavorable environmental conditions. Furthermore, the delivery of Vip3Aa20 in the maize seed and its production in plants eliminates many risks associated with conventional insecticide usage, some of which include improper calibration and maintenance of application equipment, handling of hazardous chemical insecticides, container disposal, chemical misplacement, runoff, and spray drift.

Integrated pest management (IPM) in agriculture includes insect scouting or monitoring to determine pest populations, consideration and application of compatible alternative biological, cultural, mechanical and chemical controls, and the establishment of action thresholds for agricultural inputs. The delivery of pest management interventions on target and on time is a key to successful IPM. Planting of MIR162 hybrids provides much greater accuracy of application compared to chemical treatments due to the localization of Vip3Aa20 within the plant tissues. Timing of application is not a factor with MIR162 hybrids since Vip3Aa20 is present in the plant throughout the growing season. Planting of MIR162 hybrids is compatible with current insect scouting and monitoring programs that provide data upon which to base crop management decisions. The product is also fully compatible with cultural control measures such as crop rotation. MIR162 fits seamlessly into the concept of

integrated pest management for maize. Superior protection of crop yield and a seamless fit with IPM programs indicate that registration of MIR162 maize is in the public interest.

5. Risk Factors

A standard battery of mammalian toxicity studies failed to provide any evidence of Vip3Aa20-induced adverse effects. The protein is rapidly degraded in mammalian digestive systems and it bears no amino acid sequence similarities to known toxins and allergens. Since the insecticidal protein is plant-incorporated, the opportunity for exposure when handling and planting seed is minimal. Planting of MIR162 hybrids will essentially eliminate the occupational health risks currently associated with chemical controls for leaf and ear feeding insect pests.

The selectivity of Vip3Aa20 for lepidopteran pests minimizes risk for nontarget organisms. A series of hazard identification studies has been conducted with nontarget indicator species, including many species that are part of the maize ecosystem. No adverse effects attributable to Vip3Aa proteins were observed in these studies, even at exposure levels exceeding expected environmental concentrations (Raybould, 2007a).

Most currently registered maize insecticides exhibit a broad spectrum of activity and present discrete risks to a multitude of nontarget terrestrial and aquatic species when they are applied. The conventional insecticide products currently used for control of aboveground insects are of concern to growers, consumers, and the EPA for environmental reasons.

Fusarium ear rot is the most common ear rot disease in the Midwest and is closely associated with insect feeding damage to maize ears. Although the disease does not cause significant yield loss, it reduces grain quality, and increases the fungi that can produce mycotoxins, such as fumonisins. Mycotoxin contamination of maize grain presents a potential threat to livestock health and it is occasionally necessary to reject or reformulate field lots because of contamination. Due to the superior protection from insect ear feeding damage that will be afforded by planting MIR162 hybrids there is a potential health benefit for the livestock industry resulting from reduced mycotoxin levels in livestock feed.

Thus, the introduction of MIR162 technology has the potential to reduce applications of conventional insecticides and improve grain quality by reducing mycotoxin levels. These facts indicate that registration of MIR162 is in the public interest.

6. Economic Factors

At the request of Syngenta a study was undertaken by agricultural economists at North Carolina State University to develop an estimate of the value to U.S. farmers of the MIR162 maize trait technology. First they considered the potential economic effects of MIR162 introduction on the market for existing insect-protection trait technologies. Second, they commissioned a grower survey to assess willingness to adopt the new technology. Lastly, they estimated the spatial distribution of the costs of control for *H. zea* and *S. albicosta* and how these costs might change in future years. A full report for this economic study is

contained in Addendix 1. This analysis only examined the potential benefits associated with improved control of *H. zea* and *S. albicosta*.

Following general economic principles, the introduction of a new technology will have an effect on the market for existing technologies that is beneficial to users of either technology. This will come in the form of downward pressure on prices of the competing technologies. This is beneficial to growers because prices of maize traits will tend to remain lower and more stable in the future than would otherwise be the case.

From data collected in a telephone survey of 150 maize growers in 12 states, average yield losses in 2006 attributable to *H. zea* were estimated to be 4.9 bu/ac and losses attributable to *S. albicosta* were estimated to be 4.8 bu/ac. Examination of data provided by these growers for the past five seasons suggests that yield losses attributable to the two pests are increasing. This conclusion is supported by analysis of insecticide use data for 2005 and 2006 which indicate that economically significant infestations of *H. zea* and *S. albicosta* are on the rise in the Corn Belt and Great Plains. As the price of maize grain increases, the amount of feeding damage needed to exceed an economic threshold for applying corrective measures decreases. The grower survey results indicate that 70% of respondents would purchase MIR162 hybrids if they were available and would plant them on an average of 500 acres per farm four years after introduction.

A potential economic benefit for maize growers from the commercial introduction of MIR162 hybrids has been computed in the form of an upper-bound estimate for the three largest maize producing states (IA, IL, and NE). An upper-bound estimate is all that was able to be computed because county-specific information is not available on infestation levels of the insects being investigated. Providing growers with a means to effectively control *H. zea* and *S. albicosta* in these three states alone provides an economic benefit of up to $371 million annually. This is an upper-bound estimate on value available to growers; it assumes a 100% market share for MIR162 hybrids and does not take into account potential price responses for competitive products. Ultimately, some portion of the economic gain derived by growers using this new technology will be passed along to consumers in the form of lower commodity prices. These substantial economic benefits indicate that registration of MIR162 is in the public interest.

V. Public Interest Factors Applicable to Bt11xMIR162 Maize

Concurrent with the application for a FIFRA §3 registration of the Vip3Aa20 plant-incorporated protectant in MIR162 maize, Syngenta is also submitting an application for registration of a new product that contains the combined plant-incorporated protectant active ingredients in Bt11 maize and MIR162 maize. The transgenic traits in Bt11 and MIR162 maize have been combined by traditional breeding methods to create maize hybrids, designated herein as 'Bt11xMIR162 maize', that possess the combined insect-control characteristics of Bt11 and MIR162. Hybrids that contain multiple transgenic traits are often referred to as 'stacked' hybrids or hybrids containing 'pyramided' traits and genes.

The Cry1Ab active ingredient in Bt11 maize was first registered by the EPA in 1996 (EPA

Reg. No. 67979-1; OECD ID SYN-BTØ11-1). At that time, the Agency found that its registration was in the public interest. Upon extension of the Bt11 registration in 2001, EPA reiterated its public interest finding, which is summarized in the Biopesticide Registration Action Document for *Bt* crops (EPA, 2001) and reflects the first few years of grower experience and continued research on the benefits of Bt11 maize.[1] Therefore, the various environmental, health, agricultural, and economic benefits of Bt11 maize will not be restated here.

The benefits of both Bt11 and MIR162 will be reflected in Bt11xMIR162 combined-trait maize hybrids. While it is not necessary to restate the benefits of the traits conferred by Bt11 and MIR162 maize individually, some specific and unique benefits that result from combining the traits in a single product are described below.

A. Product Description and Intended Use

Bt11 maize plants produce a Cry1Ab protein from *B. thuringiensis* for control of certain lepidopteran maize pests. The plants also produce phosphinothricin acetyltransferase, an enzyme that was used as a selectable marker in transformation and which confers tolerance of the plants to glufosinate herbicides. MIR162 maize plants produce a variant of the Vip3Aa protein from *B. thuringienses*.

Bt11xMIR162 hybrids will provide growers with protection and convenience for the control of a wide spectrum of lepidopteran pests. The broad efficacy of the product will allow maize growers across wide geographies to benefit from routinely planting Bt11xMIR162 hybrids to control the majority of lepidopteran maize pests that could be prevalent in any given year. In addition to the insect-control advantages of the product, the herbicide tolerance conferred by the phosphinothricin acetyltransferase protein in Bt11xMIR162 maize will provide growers with the option of using glufosinate products for weed control.

B. Factors Affecting a Public Interest Finding

1. Need Factors

Although conventional chemical insecticides are available and sometimes applied to minimize the yield losses associated with lepidopteran pest damage (see Section II.D and EPA, 2001), the efficacy of such products typically depends on careful scouting of fields for the presence of larvae and on timing of insecticide applications to achieve economically beneficial levels of control. Many maize growers typically do not treat their fields to control lepidopteran infestations for a number of reasons, including inconvenience, lack of awareness of the yield losses being incurred, and the costs and hazards associated with insecticide applications. After initially feeding on external plant tissues, several lepidopteran maize pests bore into the stalk or ear, where they are inaccessible to foliar insecticide applications. Even with carefully timed applications, under typical use conditions the

[1] The registration for the plant-incorporated pesticide active ingredient in Syngenta's Bt11 maize is scheduled to expire on October 15, 2008. Prior to this expiration date, Syngenta will request an amendment to extend the registration of Bt11 maize.

efficacy of foliar or soil-applied insecticide products cannot achieve the consistent level of season-long control that is possible using the technology represented by Bt11xMIR162 maize.

Maize hybrids expressing Cry1F are commercially available and offer a measure of broad-spectrum lepidopteran control. In 2006, Cry1F hybrids accounted for 7.1% of maize acres planted in the U.S. (Doane, 2006a). In contrast, 34.9% of acres in 2006 were planted to Cry1Ab-containing hybrids (event Bt11 or MON 810), which control a narrower spectrum of lepidopteran pests. It is reasonable to predict that, as the benefits of broad-lepidopteran control are more widely recognized and available to growers, the relative proportion of acres planted to such hybrids will change. While it has not been possible to conduct direct side-by-side efficacy comparisons of Cry1F and Bt11xMIR162 hybrids, Bt11xMIR162 hybrids are expected to provide a level of broad lepidopteran control that is unsurpassed by currently available *Bt* hybrids or conventional insecticide products. For *H. zea*, in particular, Bt11xMIR162 hybrids have been shown to provide excellent control that meets EPA insect resistance management criteria for 'high dose' (see V.B.5 below and Kurtz *et al.*, 2007a), whereas Cry1F hybrids provide only 'suppression' of this pest (EPA, 2005).

2. Performance Factors

Bt11 maize provides control of *O. nubilalis* and *D. grandiosella*, two key pests of U.S. maize (see Table 1). Additionally, Bt11 maize provides some control or suppression of *H. zea*, *S. frugiperda*, *D. crambidoides*, *P. nebris*, and *D. saccharalis*. The activity spectrum and efficacy of MIR162 maize for control of several lepidopteran maize pests are described in Section III.

Bt11xMIR162 maize will combine the efficacy of Bt11 maize and MIR162 maize to provide broad-spectrum control of major U.S. lepidopteran maize pests at a level that will outperform current technologies. Figure 8 illustrates the performance benefits of stacking the Cry1Ab and Vip3Aa20 proteins in the same hybrid. Collectively, the results of field efficacy trials demonstrate that Bt11xMIR162 maize will be protected from feeding damage caused by the following insect pests: *O. nubilalis*, *D. grandiosella*, *D. crambidoides*, *H. zea*, *S. frugiperda, P. nebris*, *D. saccharalis*, *A. ipsilon*, *S. albicosta*, and *S. exigua* (Huber *et al.*, 2007; White *et al.*, 2007a, 2007b, 2007c, 2007d, 2007e). As MIR162 maize does not control *O. nubilalis*, a significant maize pest in many areas of the U.S., it will not likely be marketed as a stand-alone product.

Studies to quantify the yield advantage of Bt11xMIR162 maize across multiple geographies, environmental conditions, cropping practices and pest pressure are ongoing. The data available from a limited set of trials indicate that, in the absence of pest pressure, Bt11xMIR162 hybrids will provide the same yield as their nontransgenic counterparts. It is expected that, as a result of superior and season-long control of significant lepidopteran pests, Bt11xMIR162 hybrids will demonstrate significant yield advantages under both low and high pest pressure.

Some lepidopteran pests, such as *A. ipsilon*, can cause major yield losses due to cutting of an

entire stand of maize in a field. If the crop is left untreated, the grower is sometimes forced to replant an entire field. Such catastrophic losses will be prevented by planting Bt11xMIR162 maize. Other, stalk-boring lepidopteran larvae cause physiologic yield loss because the stalk damage interferes with nutrient uptake and increases susceptibility to plant diseases. They can also cause severe stalk lodging and dropped ears, which results in a physical yield loss because the ears cannot be mechanically harvested by the combine. Ear-feeding pests reduce grain yield and quality, and occasionally result in elevated grain fumonisin levels that render the grain unsafe and unusable as food or feed (see Sections IV.B.5 and V.B.3 and EPA, 2001). In the presence of pest pressure, Bt11xMIR162 maize hybrids can be expected to preserve yield potential, grain quality, and silage quality by minimizing the damage that could otherwise be caused by lepidopteran insects.

3. Risk Factors

Syngenta has provided data to the Agency supporting the mammalian safety of the transgenic proteins produced in Bt11xMIR162 maize. Permanent exemptions from the requirement of a tolerance in all crops exist for both Cry1Ab and the marker protein produced in Bt11 maize (40 CFR §174.511 and 40 CFR §174.522, respectively). The selectable marker in MIR162 maize is permanently exempt from tolerances in all crops (40 CFR §174.527) and a temporary tolerance exemption for Vip3Aa20 in MIR162 maize is currently in place (40 CFR §174.528).

The available data further support a conclusion that there will be no harmful interactions between the transgenic proteins in Bt11xMIR162 maize that might trigger a concern for mammalian safety. The proteins are individually not toxic to mammals and the lepidopteran-specific toxins in Bt11xMIR162 maize do not interact in a manner that results in toxicity to nontarget insects (Raybould, 2007b). A FIFRA Scientific Advisory Panel concluded that *Bt* insect toxins that are individually nontoxic to mammals do not pose a mammalian safety concern when present in combination (FIFRA SAP, 2004).

Despite the broad-spectrum lepidopteran activity of Bt11xMIR162 maize, the environmental safety assessment supporting this product (Raybould, 2007b) indicates that it will pose no significant risk to nontarget organisms. The environmental safety assessment also considered the potential for effects on endangered species and for synergistic effects of the Cry1Ab and Vip3Aa20 proteins on nontarget Lepidoptera and other nontarget species.

The combined mammalian and environmental safety profile of Bt11xMIR162 indicates that the product will pose no significant risks. Accordingly, it offers health and environmental advantages over current chemical alternatives for control of lepidopteran pests.

EPA estimated that, prior to the adoption of *Bt* maize hybrids, an annual average of 6.3 million acres were treated with conventional insecticides for control of maize-boring lepidopteran pests (primarily *O. nubilalis*) in the U.S. (EPA, 2001). The Agency further estimated that between the introduction of the first (Cry1Ab) *Bt* maize hybrids in 1996 and 2000, 3.9 million fewer acres of insecticide applications were made to maize for control of the same pests. As detailed in Section III, the additional control spectrum provided by the

Vip3Aa20 protein in MIR162 maize is expected to allow maize growers to further reduce their use of insecticides for lepidopteran control. Significantly, many of the conventional insecticides currently registered for lepidopteran control in maize are Restricted Use Pesticides (see Table 5).

For maize growers who currently rely upon conventional insecticide applications for lepidopteran control, Bt11xMIR162 maize will allow them to significantly reduce, if not eliminate, the need to apply chemical controls for these pests. This will represent both a reduced health and safety risk for agricultural workers, and will reduce the impact of insecticide use on wildlife and the environment.

An additional food and feed safety benefit of Bt11xMIR162 is its potential to reduce the level of fumonisin, a harmful fungal toxin, in maize grain. As summarized by EPA (2001), grain from *Bt* maize hybrids (including Bt11 maize) is associated with significantly reduced levels of fumonisin. This is an indirect benefit of protecting maize ears from feeding damage by lepidopteran pests. The additional control of ear-feeding pests, particularly *H. zea* and *S. albicosta*, that will be provided by Bt11xMIR162 maize will likely further reduce mycotoxin contamination in grain.

4. Economic Factors

Although some maize-growing areas experience significant lepidopteran infestations in most years, it is not possible for individual growers to accurately predict whether lepidopteran pest pressure will be economically significant in any particular growing season. Growers must make seed purchase decisions prior to knowing whether the seed price premium for lepidopteran control in a given year will actually be recouped as higher crop yields when compared to the seed cost for unprotected hybrids or the costs of other control measures that might be applied. Nevertheless, the acres planted to *Bt* maize have continued to increase steadily since the first *Bt* hybrids were introduced in the U.S. in 1996. This is a testament to the actual yield-preserving benefits that *Bt* maize growers have experienced over the long term, and to the value of the built-in 'insurance' against the occasional severe pest outbreak that could otherwise result in high economic losses. For many growers, the broad lepidopteran control offered by Bt11xMIR162 hybrids will represent a higher insurance value than currently available *Bt* products. Additionally, Bt11xMIR162 hybrids will offer unsurpassed convenience to growers by reducing the need to scout fields for pest pressure or to apply other control measures for lepidopteran larvae.

The continued success of *Bt* maize hybrids in the marketplace since their initial introduction attests to their economic benefits to growers. It is difficult at this time to accurately predict the magnitude of economic benefits that growers of Bt11xMIR162 maize will realize beyond the previously described benefits expected from growing either Bt11 (EPA, 2001; Van Duyn, 2005) or MIR162 maize (see Appendix 1). Nevertheless, the improved pest protection profile of Bt11xMIR162 maize can be expected to translate into correspondingly higher overall economic benefits to growers, consumers, and other downstream users of maize products. The magnitude of these economic benefits will necessarily depend upon the seed price premium paid for the pest-control traits, the level of local pest pressure, and the value

of the crop. As discussed in Section II.A, commodity prices for maize grain have dramatically increased recently due to high demand for fuel ethanol production, and sustained demand is predicted for the coming years. Such demand will function to increase the value of a grower's investment in any agricultural practice, technology, or product, including the Bt11xMIR162 traits, that increases or preserves yield.

Another predicted economic benefit for growers and downstream consumers is increased competition in the marketplace for pest-control products, including hybrid seed from multiple providers of lepidopteran-tolerant *Bt* varieties. The commercial availability of Bt11xMIR162 hybrid maize seed will represent a significant new pest control option and tool for growers. Increased grower choice can be expected to exert downward pressure on the cost of all products that offer control of lepidopteran pests.

5. Other Factors

A unique benefit offered by Bt11xMIR162 hybrids relates to their distinct advantages in the area of insect resistance management (IRM). As fully detailed in the report by Kurtz *et al.* (2007a), the Cry1Ab and Vip3Aa20 proteins are present in these hybrids at levels that have been demonstrated to provide a high-dose for control of *O. nubilalis, H. zea* and *S. frugiperda*, thus minimizing the risk of resistance developing in these species. Bt11xMIR162 hybrids offer IRM advantages in comparison to other control options that do not demonstrably provide a 'high dose' against the target pests. Moreover, Vip3Aa20 operates by a mode of action different from that of Cry1Ab or Cry1F and targets a unique binding site(s) in susceptible larvae. The available data support a conclusion that Vip3Aa20 shows no potential for cross-resistance with Cry proteins (Lee *et al.*, 2003). Thus, for *H. zea* and *S. frugiperda*, which are sensitive to both Cry1Ab and Vip3Aa20, Bt11xMIR162 maize is predicted to significantly extend the durability of both traits for control of these pests because local populations are very unlikely to evolve resistance to two proteins that act on independent target sites.

The possibility of resistance development in *H. zea* has been of particular concern to the EPA, as it is also a pest of cotton and has the potential to undergo selection pressure from both *Bt* maize and *Bt* cotton varieties that express similar Cry proteins, where the two crops are grown in the same geographies. The principal reason that the EPA requires growers in cotton-growing areas to plant 50% of their maize acres to non-*Bt* maize hybrids concerns the potential for resistance evolution in *H. zea* populations. In the report by Kurtz *et al.* (2007a), Syngenta provides data and rationale to justify reduction of the maize refuge in cotton-growing areas from 50% to 20% of maize acres for growers of Bt11xMIR162 maize. No other *Bt* product offers comparable IRM advantages in maize.[2]

For growers of Bt11xMIR162 maize hybrids, the reduced refuge requirement in cotton-growing regions will translate into a higher proportion of insect-protected maize acres, with a proportional increase in all the attendant benefits of the product in these areas. As an added advantage, compliance with the refuge requirement for IRM can be predicted to increase

[2] Syngenta's Cry1Ab+Vip3Aa19 stacked cotton product, VipCot™ cotton, also has similar IRM advantages for the same reasons. An application for FIFRA §3 registration of VipCot cotton is currently under review.

because *Bt* maize growers in cotton-growing regions have heretofore not been able to fully experience the benefits enjoyed by *Bt* maize growers in other regions of the U.S. The potential for increased maize acres in cotton-growing regions can also help meet the current high demand for maize grain.

6. Public Interest Finding for Bt11xMIR162

The information presented herein supports a conclusion that registration of Bt11xMIR162 maize is in the public interest. Use of Bt11xMIR162 hybrids by U.S. maize growers is predicted to offer crop yield advantages both for growers who do not currently use insecticides to control lepidopteran pests as well as for growers who use other options for lepidopteran control. Another economic benefit for growers and downstream consumers is anticipated in the form of increased price competition in the marketplace for pest-control traits. Use of Bt11xMIR162 maize offers health and environmental safety advantages over conventional insecticides, as well as insect resistance management benefits that will help to preserve the durability of this and other *Bt*-based products for lepidopteran control.

VI. Public Interest Factors Applicable to Bt11xMIR162xMIR604 Maize

Concurrent with the application for a FIFRA §3 registration of MIR162 maize and Bt11xMIR162 maize, Syngenta is submitting an application for registration of a new product that contains the combined active ingredients in Bt11 maize, MIR162 maize, and MIR604 maize (EPA Reg. No. 67979-5; OECD ID SYN-IR6Ø4-5). The transgenes in Bt11, MIR162, and MIR604 maize have been combined by traditional breeding methods to create maize hybrids, designated herein as 'Bt11xMIR162xMIR604 maize', that express the combined insect-control traits of Bt11, MIR162, and MIR604 maize.

The characteristics and benefits of Bt11xMIR162 maize, and the component traits from Bt11 and MIR162 maize, are referenced or described in previous sections of this document. The significant benefits of MIR604 maize as a stand-alone product for rootworm control have been summarized by EPA (2007) and described in detail by Syngenta in a previous regulatory submission (Steiner *et al.*, 2004).

Additionally, the plant-incorporated protectants in Bt11 maize and MIR604 maize have been combined by traditional breeding in Bt11xMIR604 maize, for combined control of certain lepidopteran and rootworm (coleopteran) pests. The plant-incorporated protectants in Bt11xMIR604 maize were approved by the EPA in 2007 (EPA Reg. No. 67979-8) and the benefits of Bt11xMIR604 maize have been described by Steffens and Tinsworth (2006).

The benefits offered by the component traits in Bt11xMIR162xMIR604 maize will not be restated here. This section will focus on the specific characteristics of Bt11xMIR162xMIR604 maize and the benefits it affords by combining multiple insecticidal traits in a single product.

A. Product Description and Intended Use

Bt11xMIR162xMIR604 maize plants combine the transgenic traits of Bt11, MIR162, and MIR604 maize and thereby produce a total of six transgenic proteins: the *B. thuringiensis*-derived insecticidal proteins Cry1Ab, Vip3Aa20 and modified Cry3A (mCry3A). Bt11xMIR162xMIR604 hybrids will provide growers with performance and convenience in the control of a wide spectrum of lepidopteran pests and important *Diabrotica* rootworm pests of maize. The broad efficacy of the product will allow growers across wide geographies to benefit from routinely planting Bt11xMIR162xMIR604 maize hybrids to control the majority of lepidopteran and rootworm pests that might be prevalent in any given year. In addition to the insect control advantages of the product, the herbicide tolerance conferred by the PAT protein in Bt11xMIR162xMIR604 maize will provide growers with the option of using labeled glufosinate products for weed control.

B. Factors Affecting a Public Interest Finding

1. Need Factors

The available alternative methods of lepidopteran control have been summarized in Sections II.D and IV.A. The alternative methods of maize rootworm control have been summarized separately (EPA, 2007; Steiner *et al.*, 2004), and include soil-applied insecticides, seed treatments, and transgenic maize hybrids expressing coleopteran-specific *cry* genes. Bt11xMIR162xMIR604 hybrids are expected to deliver superior control of lepidopteran pests in comparison to alternative control methods, and rootworm control that is equal to or superior to that of alternative products.

Although other stacked transgenic maize hybrids offering combined lepidopteran and coleopteran control are currently available in the U.S., direct efficacy comparisons with Bt11xMIR162xMIR604 hybrids have not been possible. It is expected that Bt11xMIR162xMIR604 hybrids will provide unsurpassed control of target pests. Their excellent broad-lepidopteran control, particularly for *H. zea* and *S. albicosta*, can potentially result in better performance than competitor offerings.

2. Performance Factors

In addition to the multiple lepidopteran pests controlled by Bt11xMIR162 maize (see Section V.B), the mCry3A protein in Bt11xMIR162xMIR604 maize will provide protection against feeding damage caused by larvae of *Diabrotica virgifera virgifera* (western corn rootworm), *Diabrotica longicornis barberi* (northern corn rootworm), and *Diabrotica virgifera zeae* (Mexican corn rootworm), all of which are major coleopteran pests of maize in the U.S. The mCry3A protein has a relatively narrow spectrum of activity among coleopteran species, and has been demonstrated to have no adverse affects on nontarget beetles or other nontarget arthropods (Raybould, 2007c).

Combining Cry1Ab, Vip3Aa20, and mCry3A traits in a single maize hybrid retains the insect control efficacy of the individual proteins. Accompanying the present submission are

reports of efficacy studies in *O. nubilalis* (White *et al.*, 2006f), *H. zea* (White *et al.*, 2006g), *S. frugiperda* (White *et al.*, 2006h) and *D. virgifera virgifera* (White *et al.*, 2006i) that substantiate the predicted efficacy of combining multiple insecticidal traits in Bt11xMIR162xMIR604 maize hybrids. Therefore, it is reasonable to assume that growers will realize the cumulative benefits of all three insecticidal traits in this product.

Studies to quantify the yield advantage of Bt11xMIR162xMIR604 maize across multiple geographies, environmental conditions, cropping practices and pest pressure are ongoing. Data available from a limited set of trials indicate that, in the absence of pest pressure, Bt11xMIR162xMIR604 hybrids will provide the same yield as their nontransgenic counterparts. It is expected that, as a result of superior and season-long control of multiple significant lepidopteran and coleopteran pests, Bt11xMIR162xMIR604 hybrids will demonstrate significant yield advantages under pest pressure.

3. Risk Factors

The health and environmental benefits previously described for Bt11 maize (EPA, 2001), MIR162 maize (see Section IV.B.5), MIR604 maize (EPA, 2007; Steiner *et al.*, 2004), and Bt11xMIR162 maize (see Section V.B.3) will also apply, in combination, to Bt11xMIR162xMIR604 maize. Therefore, this product offers significant advantages over other pest control methods, as it will allow U.S. farmers to reduce their use of soil-applied insecticides for rootworm control. The EPA has been concerned for several years about the hazard and risk profiles of many of soil-applied rootworm insecticides (EPA, 2007). The availability of Bt11xMIR162xMIR604 maize hybrids will further help the EPA achieve its risk-reduction objectives, and will help growers better protect the safety of their workers and the environment.

Syngenta has provided data to the Agency supporting the mammalian safety of the transgenic proteins produced in Bt11xMIR162xMIR604 maize. The mCry3A protein in Bt11xMIR162xMIR604 maize is permanently exempt from food and feed tolerances in all corn (40 CFR §174.505). The tolerance exemptions supporting the other transgenic proteins in Bt11xMIR162xMIR604 maize are described in Section V.B.

The available data further support a conclusion that there will be no harmful interactions between the transgenic proteins in Bt11xMIR162xMIR604 maize that might trigger a concern for mammalian safety. The proteins are individually not toxic to mammals and the insect-specific toxins in Bt11xMIR162xMIR604 maize do not interact in a manner that results in toxicity to nontarget insects (Raybould, 2007c). A FIFRA Scientific Advisory Panel also concluded that *Bt* insect toxins that are individually nontoxic to mammals do not pose a mammalian safety concern when present in combination (FIFRA SAP, 2004).

Despite the broad-spectrum lepidopteran activity and coleopteran activity of Bt11xMIR162xMIR604 maize, the environmental safety assessment supporting this product (Raybould, 2007b) indicates that it will pose no significant risk to nontarget organisms. This assessment also considered the potential for effects on endangered species and for synergistic effects of the Cry1Ab, Vip3Aa20 and mCry3A proteins on nontarget insects.

The combined mammalian and environmental safety profile of Bt11xMIR162xMIR604 maize indicates that the product will pose no significant safety risks. Accordingly, it offers significant health and environmental advantages over current chemical alternatives for control of lepidopteran and rootworm pests. For maize growers who currently rely upon conventional insecticide applications for lepidopteran and rootworm control, Bt11xMIR162xMIR604 maize will allow them to significantly reduce, if not eliminate, the need to apply chemical controls for these pests. This will represent both a reduced health and safety risk for agricultural workers, and will reduce the impact of insecticide use on wildlife and the environment.

4. Economic Factors

The broad efficacy of Bt11xMIR162xMIR604 hybrids will provide 'insurance' for growers against damage by multiple pests that might otherwise cause significant economic loss in any given year. The same broad efficacy will provide convenience for growers, as they will be able to eliminate the need to apply both a soil insecticide for control of *Diabrotica* rootworms and *A. ipsilon*, and a foliar insecticide later in the season for foliar insects. It will also reduce or eliminate their need to scout fields for pest pressure.

The increasing acreage planted with *Bt* maize hybrids for lepidopteran and rootworm control, attests to their economic benefits to growers. It is difficult at this time to accurately predict the magnitude of economic benefits that growers of Bt11xMIR162xMIR604 maize will realize beyond those previously described benefits expected from growing either Bt11 (EPA, 2001; Van Duyn, 2005), MIR162 maize (see Section IV.B.6), or MIR604 maize (EPA, 2007; Steiner *et al.*, 2004). However, the improved pest-control profile of Bt11xMIR162xMIR604 maize can be expected to translate into correspondingly higher overall economic benefits to growers, consumers, and other downstream users of maize products. The magnitude of these economic benefits will necessarily depend upon the seed price premium paid for the pest-control traits, the level of local pest pressure, and the value of the crop. Commodity prices for maize grain have dramatically increased recently due to high demand for fuel ethanol, and sustained demand is predicted for the coming years. Such demand will operate to increase the value of a grower's investment in any agricultural practice, technology, or maize traits that increase or preserve yield.

Another predicted economic benefit for growers and downstream consumers is increased competition in the marketplace for pest-control products, including hybrid seed from multiple providers of lepidopteran-active and or rootworm-active transgenic varieties. The commercial availability of Bt11xMIR162xMIR604 hybrid maize seed will represent a significant new pest control option and tool available to growers. Increased grower choice can be expected to exert downward pressure on the cost of products that offer control of lepidopteran and rootworm pests.

5. Other Factors

A detailed insect resistance management plan and discussion for Bt11xMIR162xMIR604

maize is provided in an accompanying data volume (Kurtz *et al.*, 2007b). The same insect resistance management benefits described above (Section V.B.) for Bt11xMIR162 maize will also apply to Bt11xMIR162xMIR604 maize. Accordingly, a 20% non-*Bt* maize refuge in cotton-growing regions will be justified. The stacking of three insecticidal proteins in this product is not expected to increase selection pressure for cross-resistance among local pest populations, owing to the different modes-of-action and target sites for the Cry1Ab, Vip3Aa20, and mCry3A proteins. Because the mCry3A trait in Bt11xMIR162xMIR604 maize has good efficacy against its target rootworm pests, introduction of this product is expected to help extend the durability of other commercially available rootworm-protected *Bt* maize products.

6. Public Interest Finding for Bt11xMIR162xMIR604 Maize

The information summarized herein supports a conclusion that registration of Bt11xMIR162xMIR604 maize is in the public interest. Use of Bt11xMIR162xMIR604 hybrids by U.S. maize growers is predicted to offer crop yield advantages and important new options for control of major pests, all built into a single seed product. The availability of a new product for lepidopteran and rootworm control will provide choices for growers in the marketplace, and lead to increased price competition for traits, which will benefit growers and others in the maize value chain. Bt11xMIR162xMIR604 maize also offers health and environmental safety advantages over the use of conventional insecticides, as well as insect resistance management benefits that will preserve the durability of this and other *Bt*-based products.

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Appendix 1

Final Report for a Study Entitled:

The Economic Implications of the Introduction of Syngenta's MIR162 Corn Trait for U.S. Farmers

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The Economic Implications of the Introduction of Syngenta's MIR162 Corn Trait for U.S. Farmers

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EXECUTIVE SUMMARY

This purpose of this study was to examine the available data to attempt to estimate the potential gain at the farm and state levels of the commercial introduction of Syngenta Corporation's MIR162 corn trait. We use several sources of information. First, we examine the introduction of a new technology that is a substitute for an existing technology using the economic theory of supply and demand. The results of this analysis show an unambiguously positive gain to technology buyers of the introduction of a new substitute into the market. The results show that introduction of a new substitute not only puts downward pressure on all substitute technology prices in the market, but also dampens technology supplies' ability to raise prices in the future.

The second part of the study consists of an examination of the spatial distribution of the two insects, the western bean cutworm and the corn earworm, for which the MIR162 trait will provide superior control relative to current Bt traits on the market. Using Doane's Agro-Trak data on acres treated for them in 2005 and 2006, we show that pressure from these insects is likely to spread geographically in the near future. This will increase the value of the MIR162 trait in future years.

Third, we present the results of a grower survey that we designed and commissioned expressly for this study in which we asked corn growers in a 12-state area of the High Plains and the Corn Belt about their experience with the western bean cutworm and the corn earworm over the 2002-2006 time-period. The respondents were asked about the insect densities, treatments, treatment costs, and yield losses on their farms from the two insects of interest. Growers also were read a description of the MIR162 trait and then asked several questions regarding their adoption intentions, once the trait is commercialized. The responses were quite positive, with 70% of the respondents indicating that they would try hybrids containing the MIR162 trait and, after three years, would probably plant those hybrids on a significant portion of their corn acreage.

Lastly, we calculated the total potential gain from the commercial introduction of MIR162 in three states, Nebraska, Iowa, and Illinois, using information generated by the analyses above. We find that an upper bound estimate of value of the introduction of MIR162 per year in the three states is more than $300 million. Given that this estimate is an upper bound estimate as of today because we had no information with which to exclude corn acreage not affected by the insects. At the same time, because of the lack of sufficient data to estimate the value in other states, the estimate may be representative of the total regional value of the introduction of MIR162 today.

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Introduction

A new genetically-engineered corn trait (MIR162) has been developed by Syngenta Corp. that is expected to provide excellent control of several insect species that are not controlled well with existing technology. Chief among these insects are the corn earworm (*Helicoverpa zea*) (CEW) and the western bean cutworm (*Striacosta albicosta*) (WBC). Farmers should benefit anywhere MIR162 is introduced (along with traits for corn borer and corn rootworm, when appropriate), given that it is a viable substitute for the existing technologies on the market today. The two major existing technologies are YieldGard® corn traits from Monsanto Company and Herculex® corn traits from Dow AgroSciences.

The purpose of this study is to develop an estimate of the value to farmers of the introduction of the MIR162 corn trait technology. We approach this task in several ways. First we present a theoretical discussion of the economic effects of the introduction of any new technology on the market for existing technologies. We then discuss the results of a grower survey that we developed for this study that is designed to ascertain the insect pressure, current control measures, and willingness to adopt the new technology. Finally, using Doane's Agro-Trak data, we estimate the spatial distribution of the costs of control for the CEW and WBC and predict changes in that spatial distribution in the future, so that we can estimate the potential total value to farmers of MIR162 for a few years after its introduction.

The Economics of Substitutes and Competition

In economic terms, goods are deemed to be related in consumption if a change in the price of one good affects demand for the other good. One type of related good is a substitute. Two goods are substitutes if a change in the demand for one good changes the price of the other good in the opposite direction. That is, if the price of one of a pair of substitute goods increases due to an increase in demand, then the demand for and, thus, the price of the other good will fall (Nicholson).[3] The related goods in question here are corn trait technologies grown by farmers in the U.S.

The existence of viable substitutes creates competition in the marketplace. First, the mere existence of a new substitute technology will have an effect on the market for the existing technologies that is beneficial to the technology users. Second, as soon as some adoption of the new technology begins to take place, downward pressure on the prices of the competing technologies will be created. Each of these phenomena will be discussed in detail below.

[3] The other types of related goods are called complements, where a change in the price of one good changes the price of the other in the same direction.

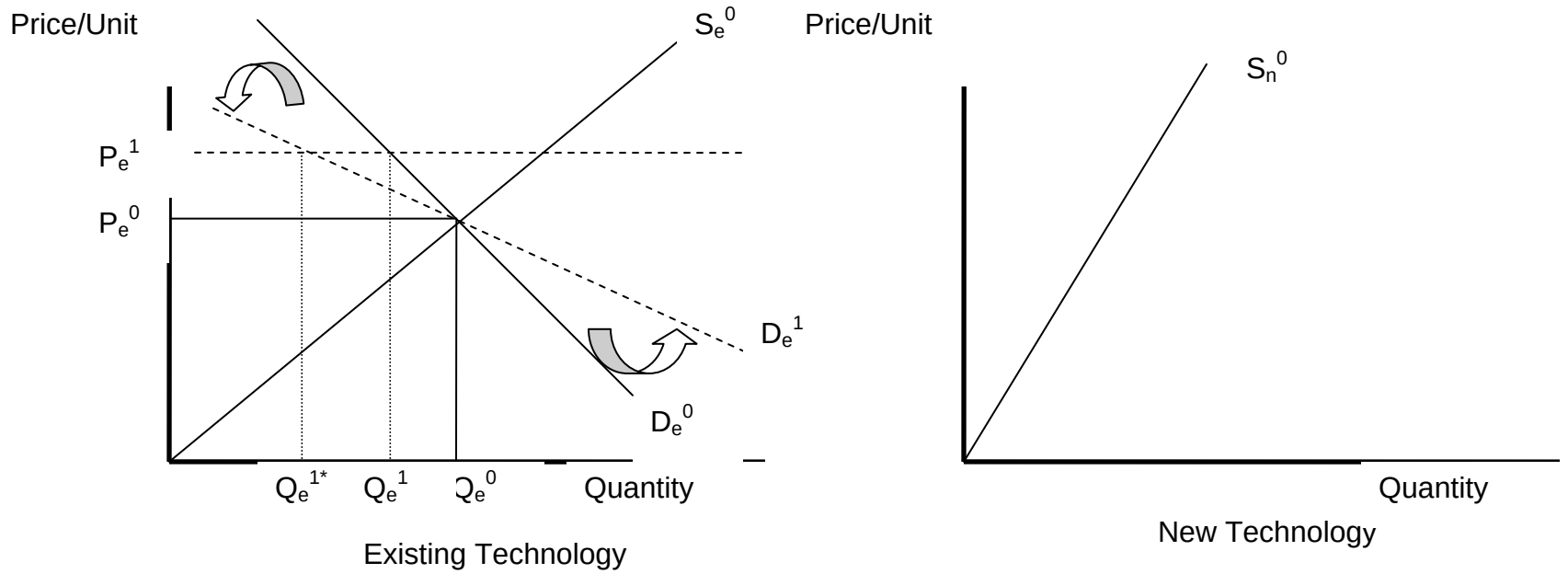
Existence of a New Technology

Consider Figure 1, which illustrates one effect of the introduction of a new, substitute technology into the market for an existing technology. The graph on the left hand side depicts the supply and demand conditions for the existing technology. Originally, before the introduction of the new technology, the supply schedule of the existing technology is shown by $\mathbf{S_e^0}$ and the demand schedule is shown by $\mathbf{D_e^0}$**.** The subscript **e** represents the existing technology and the superscript **0** represents time, with 0 denoting time before the introduction of the substitute. The supply schedule shows the quantity that would be produced at each price per unit. The higher (lower) the price, the more (less) the producers of the technology are willing to produce and sell. The demand schedule shows the quantity demanded by consumers of the existing technology (corn growers) at each price per unit. The higher (lower) the price per unit, the smaller (larger) is the quantity demanded. The intersection of the supply and demand schedules gives the market price, $\mathbf{P_e^0}$ and quantity sold, $\mathbf{Q_e^0}$ of the existing technology before the new technology is introduced.

Now, suppose a new, substitute technology is introduced into the market. The supply schedule for this new technology, $\mathbf{S_n^0}$, is shown in the right hand side graph in Figure 1. Notice that we assume in Figure 1 that there is, as yet, no demand for the new technology; it has just been introduced. Even so, the new technology begins to affect the market for the existing technology because now a new choice exists in the market place. In other words, corn growers' technology choice has become less *constrained*. This flexibility (relaxing of constraints), in and of itself, has value to the corn growers. The fewer the constraints on choice, the better off is the consumer. Although the proof of this assertion is beyond the scope of this report, the economic principle involved is called "Le Chatelier's Principle", and its intuitive appeal is easily understood (See, for example, Silberberg, 1990, for a mathematical proof and theoretical explanation).

The effect of the new choice in the technology market is illustrated by a rotation of the demand schedule of the existing technology on the left hand side graph in Figure 1 so that it is "flatter" or "more elastic" than before. Now, suppose the producers of the existing technology want to raise its price from $\mathbf{P_e^0}$ to $\mathbf{P_e^1}$. The decrease in the quantity demanded will be larger, given the more elastic demand schedule, $\mathbf{D_e^1}$, caused by the existence of the substitute. The decrease in the quantity demanded for the given price change would be from $\mathbf{Q_e^0}$ to $\mathbf{Q_e^1}$ before the introduction of the new technology (based on the original demand schedule). However, the decrease in the quantity demanded after the introduction of the new technology would be greater, from $\mathbf{Q_e^0}$ to $\mathbf{Q_e^{1*}}$. This means that, if the producers of the existing technology decide to raise its price, they would lose more market share than if

Figure 1: The Effect of a New Technology on the Elasticity of Demand for an Existing Technology



the new technology did not exist and, thus they would be more reluctant to do so. This is beneficial to corn growers in that prices of corn traited technologies will tend to remain lower and more stable in the future than otherwise would be the case.

Adoption of a New Technology (A Change in Demand)

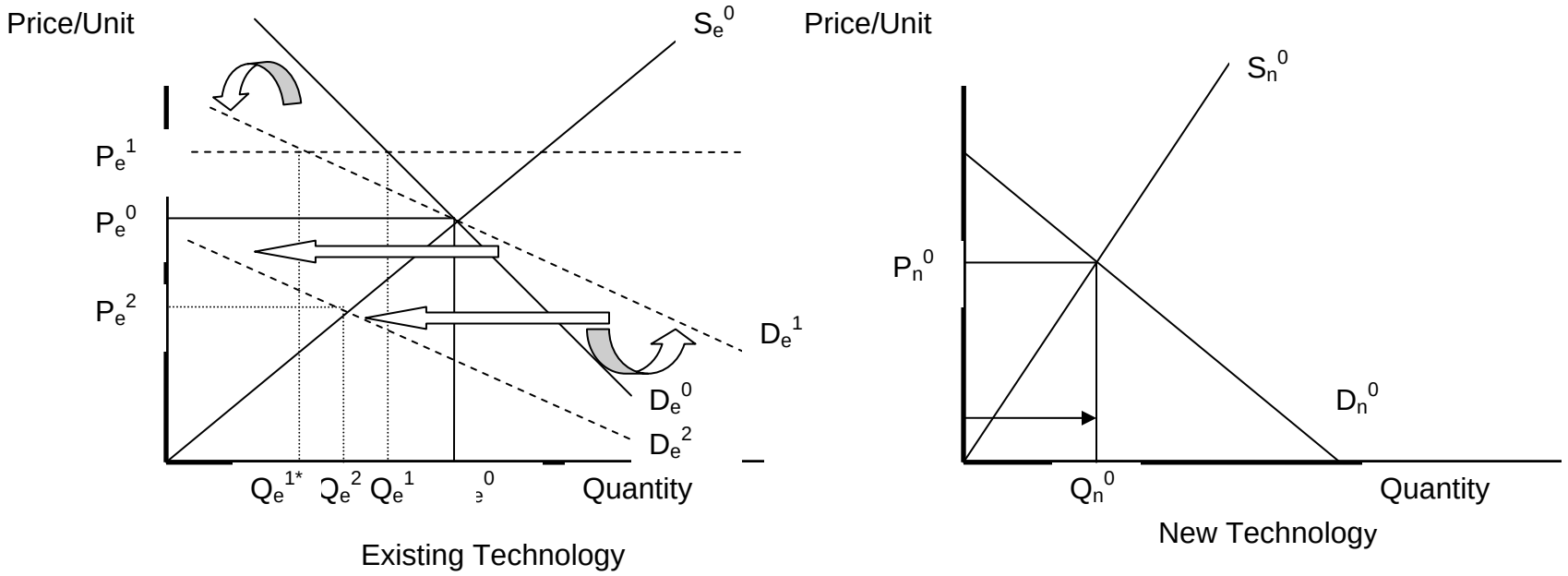
Consider now what happens to market prices and quantities sold when some growers begin to adopt the new technology (a positive level of demand develops). This is shown in Figure 2. Notice that we now have introduced a demand schedule for the new technology, $\mathbf{D_n}$. The initial market price of the new technology is $\mathbf{P_n}$ and the initial quantity sold is $\mathbf{Q_n}$. The initial adoption process of the new technology, because it is a viable substitute for the existing technology, results in a lower quantity demanded for the existing technology at every price and shifts the (already more elastic) demand schedule for the existing technology from $\mathbf{D_e^1}$ to $\mathbf{D_e^2}$**.** The new equilibrium price for the existing technology has fallen to $\mathbf{P_e^2}$, and the existing technology loses market share to the new technology ($\mathbf{Q_n^0}$=$\mathbf{Q_e^2}$**-** $\mathbf{Q_e^0}$). This benefits corn growers because the price of the existing technology is now lower *on every unit sold*.

Note that we have shown the initial market price for the new technology to be lower than that of the existing technology. The effect on the existing technology would be qualitatively the same if the new equilibrium price for the new technology were higher than that of the existing technology as long as some adoption of it takes place. A higher market price for the new technology would simply mean that many growers judge the new technology to be somewhat superior to the existing technology and so are willing to pay a higher price for it. In other words, the two technologies depicted in Figures 1 and 2 need not be perfect substitutes for each other; just substitutes. The more closely related the existing and new technologies are, the stronger will be the effect, however.

The above discussion is necessarily stylized so that the two different effects—the effect of the introduction of an additional choice into the market and the effect of adoption of the new technology—could be explained. In reality, these two effects would be expected to take place at about the same time. That is, it would not be necessary to distinguish the separate changes in the demand curve $\mathbf{D_e^0}$ to $\mathbf{D_e^1}$ to $\mathbf{D_e^2}$ instead it would be $\mathbf{D_e^0}$ to $\mathbf{D_e^2}$.

The conclusion that can be drawn from this section is that farmers are unambiguously better off when a substitute technology is introduced into the market, whether the number of original technologies in the market is one or ten or whether the initial market price for the new technology is the same or different than the price of the existing technology.

Figure 2: The Effect of a New Technology on the Elasticity of Demand for an Existing Technology



Benefits to Farmers from Superior Control of Corn Earworm and Western Bean Cutworm: Information from Secondary Sources

Both the CEW and the WBC feed on the reproductive parts of the corn plant, especially on developing corn kernels. Recommended management of CEW in field corn is limited to planting resistant hybrids (hybrids with very tight husks around the ear) and altering planting dates to avoid peak infestations. Chemical control of CEW in field corn is typically not profitable (Cook and Weinzierl), and losses are typically about 2.5% annually, with bigger losses usually expected in the South due to multi-generational effects. These losses amount to about 3.75 bushels per acre at an average corn yield of 150 bushels per acre and, at corn prices around $3.50 is equal to a loss of about $13.13 per acre.

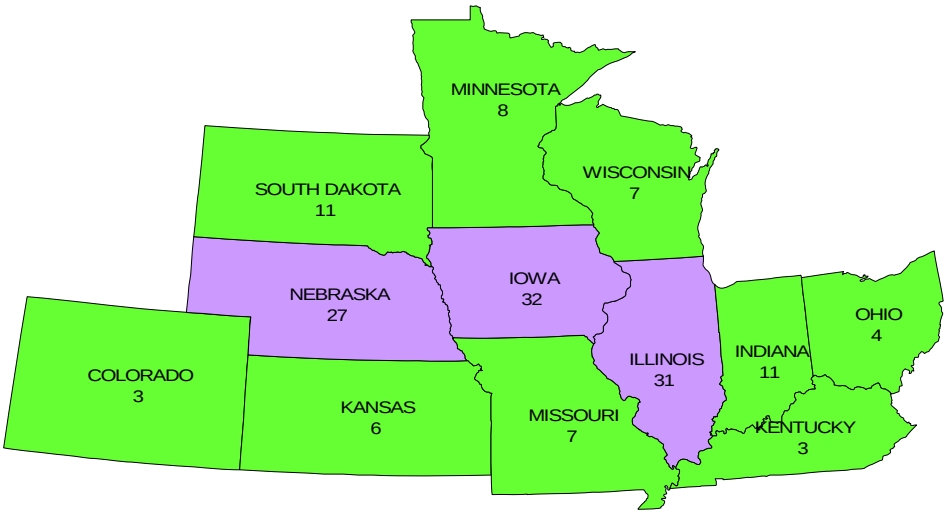
On the other hand, conventional management of the WBC is restricted to chemical pest control and scouting, along with fairly precise timing of insecticide applications, to achieve acceptable control. Estimates suggest that an average of one WBC larvae per ear can result in losses of four bushels per acre (Peairs). This would amount to a loss of about $14 per acre with $3.50 corn.

Benefits to Farmers from Superior Control of Corn Earworm and Western Bean Cutworm: Information from a Grower Survey

A survey of 150 corn growers was conducted in early 2007 to collect information about the incidence of the CEW and WBC in the Midwest and High Plains over the past five years. Growers were randomly selected across the area of the High Plains and the Corn Belt. The 12 states involved in the survey were Colorado, South Dakota, Nebraska, Kansas, Minnesota, Iowa, Missouri, Wisconsin, Illinois, Indiana, Ohio, and Kentucky. Questions were asked about pest infestation, yield losses, treatment for each pest and treatment costs. Then a description of MIR162 was given to each respondent, and questions were asked about growers' interest in the new technology. This section contains the survey results. The survey questionnaire can be found in Appendix A.

Figure 3 shows the number of respondents in the survey from each state. Given that the number of respondents in many of the states is too low to be useful from a statistical standpoint, we decided to concentrate on the states of Nebraska, Iowa, and Illinois for the economic analysis of the survey data. These states each have a larger number of respondents with 27 in Nebraska, 32 in Iowa, and 31 in Illinois, respectively.

Figure 3: Number of respondents by state in survey sample



Descriptive Statistics

In this section, we report the results for all the states in the survey for completeness, even though some of the numbers of respondents at the state level are too small for any type of statistical inference. Table 1 describes the farm and farmer characteristics of the survey respondents. Their total crop acres range from 914 in Wisconsin to over 3,000 in Kentucky, with an average over all respondents of about 1,400 acres. Of those, the acres planted to corn tended to be about one-third to one-half of their total acres, with an average of 740 corn acres planted in 2007. Of the corn acres planted, the percent of those planted to any type of Bt corn ranges from less than 20% to almost 80%, with an average of about 70%. About 70% of those who planted Bt corn hybrids did so, in part, to control the CEW or the WBC, with a range from 25% in Ohio to 100% in Missouri.

Survey respondents' average years of experience operating a farm is about 30 years, with a range of just over 20 years in Colorado to just over 33 years in South Dakota. Almost all of the respondents had at least a high school education (years' schooling = 12) and most of them had some post-secondary education, ranging from an average of 12.5 years of schooling in Ohio to an average of 18 years of schooling in Colorado.

Table 1. Descriptive statistics of survey respondents

State	N	07 Crop Acres	07 Corn Acres	07 Bt Corn Acres	07 Pct. Bt Corn Acres	Bt Planted to Control CEW or WBC	Operator Years' Exp.	Operator Years' School
COLORADO	3	1,065.00	803.33	225.00	32.62	0.67	20.67	18.00
ILLINOIS	31	1,193.39	674.74	445.65	66.37	0.71	30.90	13.87
INDIANA	11	1,483.64	786.36	302.73	45.14	0.64	28.36	12.91
IOWA	32	1,379.41	819.66	632.66	78.12	0.72	31.84	13.56
KANSAS	6	1,616.67	561.67	355.00	61.00	0.83	27.50	14.17
KENTUCKY	3	3,033.33	1423.33	366.67	19.44	0.67	24.00	13.33
MINNESOTA	8	1,368.75	712.50	555.00	79.19	0.88	31.63	13.38
MISSOURI	7	1,260.43	565.71	416.29	73.27	1.00	30.29	13.71
NEBRASKA	27	1,441.11	726.67	480.56	64.88	0.67	30.63	14.26
OHIO	4	950.00	440.00	275.00	64.35	0.25	29.75	12.50
SOUTH DAKOTA	11	2,057.27	894.55	324.55	48.62	0.64	33.18	14.00
WISCONSIN	7	914.00	628.57	527.86	75.13	0.57	32.14	14.29
All States	**150**	**1,406.41**	**740.84**	**508.61**	**69.49**	**0.70**	**30.59**	**13.85**

Pest Pressure and Mean Yield Losses Over Time

All of the survey respondents were asked to estimate the intensity of the pest pressure for each insect by the measure of number of larvae per corn plant. Table 2 shows their responses by state, year, and insect. Overall in the region, the number of CEW larvae per plant was relatively constant over the period 2002-2006 at about 0.25 larvae per plant. On the other hand, the overall WBC pressure as reported by these farmers increased over time from about 0.19 larvae per plant in 2002 to about 0.26 larvae per plant later in the time period. These results are consistent with expectations.

Perhaps a better way to measure the pest pressure is by looking at the respondents' observed yield losses from the two insects in corn. Respondents were asked to give the best estimate of their per acre yield losses from the CEW and from the WBC over the 2002-2006 time period. Table 3 shows average reported yield losses for all the states in the sample. The overall averages for the sample indicate that yield losses from CEW may have been increasing slightly in the region over the past five years, but a significant amount of variability exists across states. There seems to be an upward trend in the overall average yield losses from WBC in the sample region over the past five years. To explore these trends further, we examine more closely three states, the westernmost being Nebraska, then Iowa and the easternmost is Illinois.

Figures 4, 5 and 6 show these results for Nebraska, Iowa, and Illinois based on statistical analysis of the responses. As mentioned above, these are the only states for which there are enough observations for reasonable statistical reliability. Linear regressions were fitted to each series of yield loss to determine if a trend exists. The full regression analyses for each of the three states can be seen in Appendix B. Notice that, when a linear coefficient was not statistically significantly different from zero in the first regression for a state, we used the average yield loss over time as representative of the expected loss in any year.

In Nebraska the CEW seems to be causing increasing yield loss over time. The estimated increase is about 1.04 bu./acre/year, and the predicted yield loss for 2007 from CEW in Nebraska is 4.82 bu./acre. The yield losses caused by the WBC are highly variable over the period and no trend could be detected. This is probably because the WBC moved into Nebraska some time ago, and no upward trend in infestations or yield loss would be expected there. The mean yield loss over the period for the WBC in Nebraska is about 4.89 bu./acre/year.

The increasing trend for yield losses from the CEW is also evident in Iowa. The estimated increase in yield losses from the CEW is about 0.85 bu./acre/year, with the prediction for 2007 of about 3.97 bu./acre. A significant upward trend in yield losses from the WBC is observed in Iowa, as well, at about 0.68 bu./acre/year, with an expected loss in 2007 of 5.51 bu./acre. This is indicative of the fact that the WBC is spreading into Iowa and so the yield losses could be expected to become larger over time.

The results for Illinois also show an increase in yield losses due to the corn earworm. The upward trend is estimated to be about 1.33 bu./acre/year, with a predicted loss of about 6.12 bu./acre in 2007. The results for the WBC for Illinois show significant yield losses, as well. No significant trend was discerned, but the average loss per acre per year is estimated to be 4.54, about one-third bushel lower than the average yield loss due to WBC in Nebraska over the period. Although this finding is somewhat surprising, since the WBC is thought to be moving west to east, it is supported by the reported acres treated in Doane's Agro-Trak data for Illinois and by Pope (2007).

Table 2. Pest pressure as reported by survey respondents by insect, state and year

State		06 CEW larvae/corn plant	05 CEW larvae/corn plant	04 CEW larvae/corn plant	03 CEW larvae/corn plant	02 CEW larvae/corn plant	06 WBC larvae/corn plant	05 WBC larvae/corn plant	04 WBC larvae/corn plant	03 WBC larvae/corn plant	02 WBC larvae/corn plant
Number of Obs.											
COLORADO	3	0.03	0.26	0.03	0.03	0.03	0.16	0.17	0.16	0.18	0.18
ILLINOIS	31	0.35	0.26	0.35	0.33	0.52	0.25	0.46	0.23	0.09	0.09
INDIANA	11	0.51	0.35	0.48	0.40	0.55	0.11	0.08	0.06	0.10	0.10
IOWA	32	0.25	0.25	0.15	0.09	0.35	0.31	0.38	0.35	0.29	0.40
KANSAS	6	0.30	0.07	0.06	0.38	0.29	0.10	0.10	0.07	0.10	0.10
KENTUCKY	3	0.50	0.10	0.10	0.10	0.05	.	.	.	.	.
MINNESOTA	8	0.23	0.03	0.03	0.19	0.03	.	0.05	.	0.05	.
MISSOURI	7	0.12	0.12	0.20	0.25	0.00	.	.	.	0.00	.
NEBRASKA	27	0.18	0.22	0.29	0.29	0.33	0.28	0.18	0.28	0.25	0.10
OHIO	4	0.53	0.50	.	.	.	0.10	.	.	.	.
SOUTH DAKOTA	11	0.11	0.31	0.07	0.11	0.09	.	0.05	0.25	0.15	.
WISCONSIN	7	0.10	0.29	0.08	0.07	0.06	0.33	.	.	.	.
All States	**150**	**0.28**	**0.24**	**0.22**	**0.25**	**0.26**	**0.26**	**0.26**	**0.24**	**0.17**	**0.19**

Table 3. Reported yield losses by insect, state and year

State	N	06 CEW yield loss	05 CEW yield loss	04 CEW yield loss	03 CEW yield loss	02 CEW yield loss	06 WBC yield loss	05 WBC yield loss	04 WBC yield loss	03 WBC yield loss	02 WBC yield loss
COLORADO	3	1.00	1.50	1.00	35.00	1.00	1.00	2.33	2.00	2.00	2.00
ILLINOIS	31	5.13	4.38	4.92	6.18	2.75	2.88	3.86	6.50	5.80	3.67
INDIANA	11	5.61	5.17	6.33	6.00	3.00	6.67	5.17	7.00	7.00	7.00
IOWA	32	2.79	3.71	4.00	3.50	4.00	4.46	4.11	4.56	2.25	2.00
KANSAS	6	3.33	3.00	1.50	2.76	3.20	3.00	3.00	1.50	3.00	3.00
KENTUCKY	3	5.00	2.50	6.00	2.00	2.50	.	.	.	.	.
MINNESOTA	8	2.33	1.50	2.67	1.60	1.50	.	2.00	.	2.00	.
MISSOURI	7	1.00	1.00	5.25	7.50	0.00	.	.	.	0.00	.
NEBRASKA	27	3.73	3.89	3.50	3.25	2.38	4.75	7.36	3.50	6.83	2.00
OHIO	4	11.00	3.00	.	.	.	20.00	.	.	.	.
SOUTH DAKOTA	11	1.33	2.83	1.75	2.50	1.70	.	10.00	2.50	1.50	.
WISCONSIN	7	25.13	4.30	5.08	1.08	0.00	11.50	.	.	.	.
All States	**150**	**4.90**	**3.72**	**3.98**	**4.50**	**2.30**	**4.82**	**5.07**	**4.63**	**3.88**	**3.00**

Figure 4: Nebraska Average Reported Yield Losses by Insect and Year

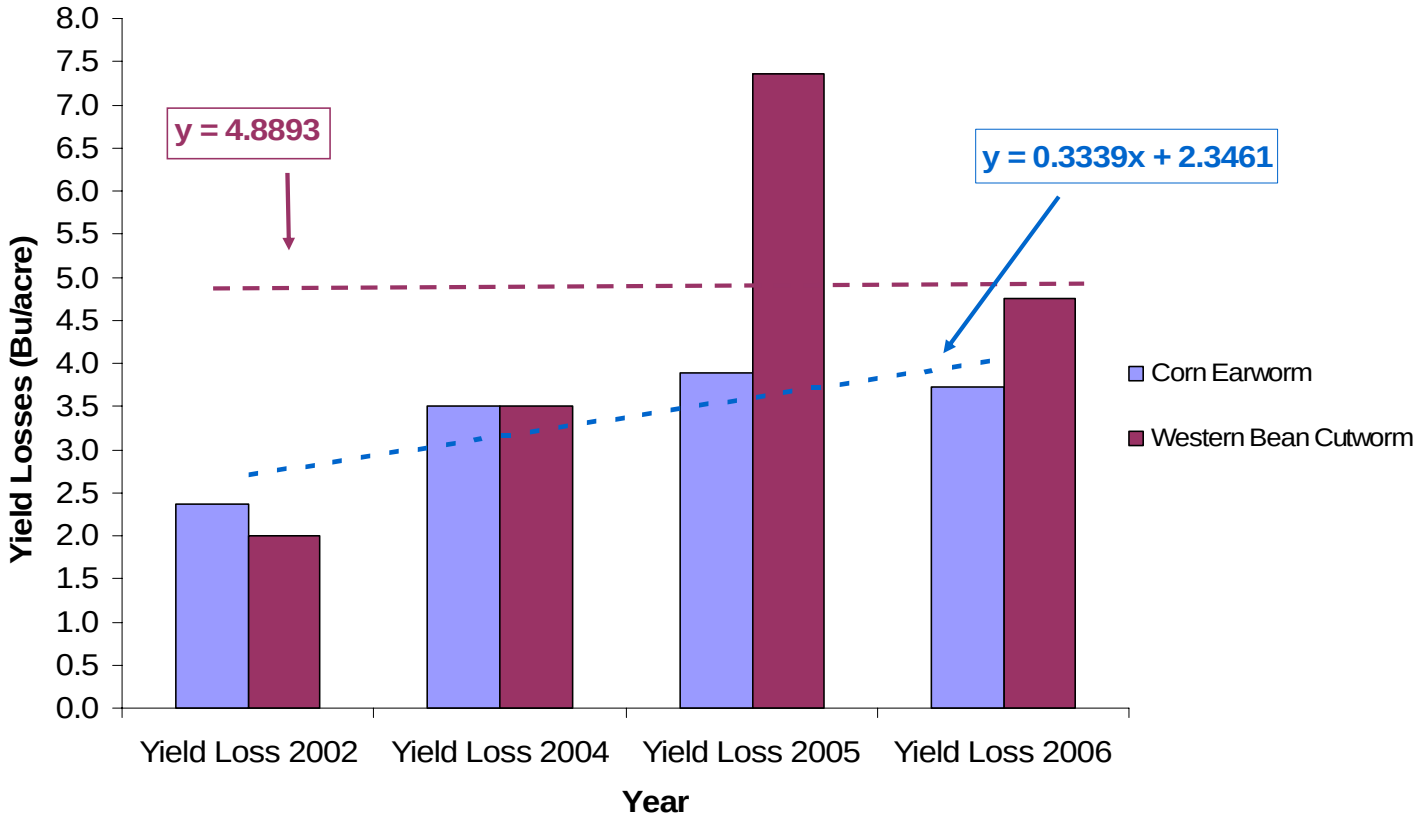


Figure 5: Iowa Average Reported Yield Losses by Insect and Year

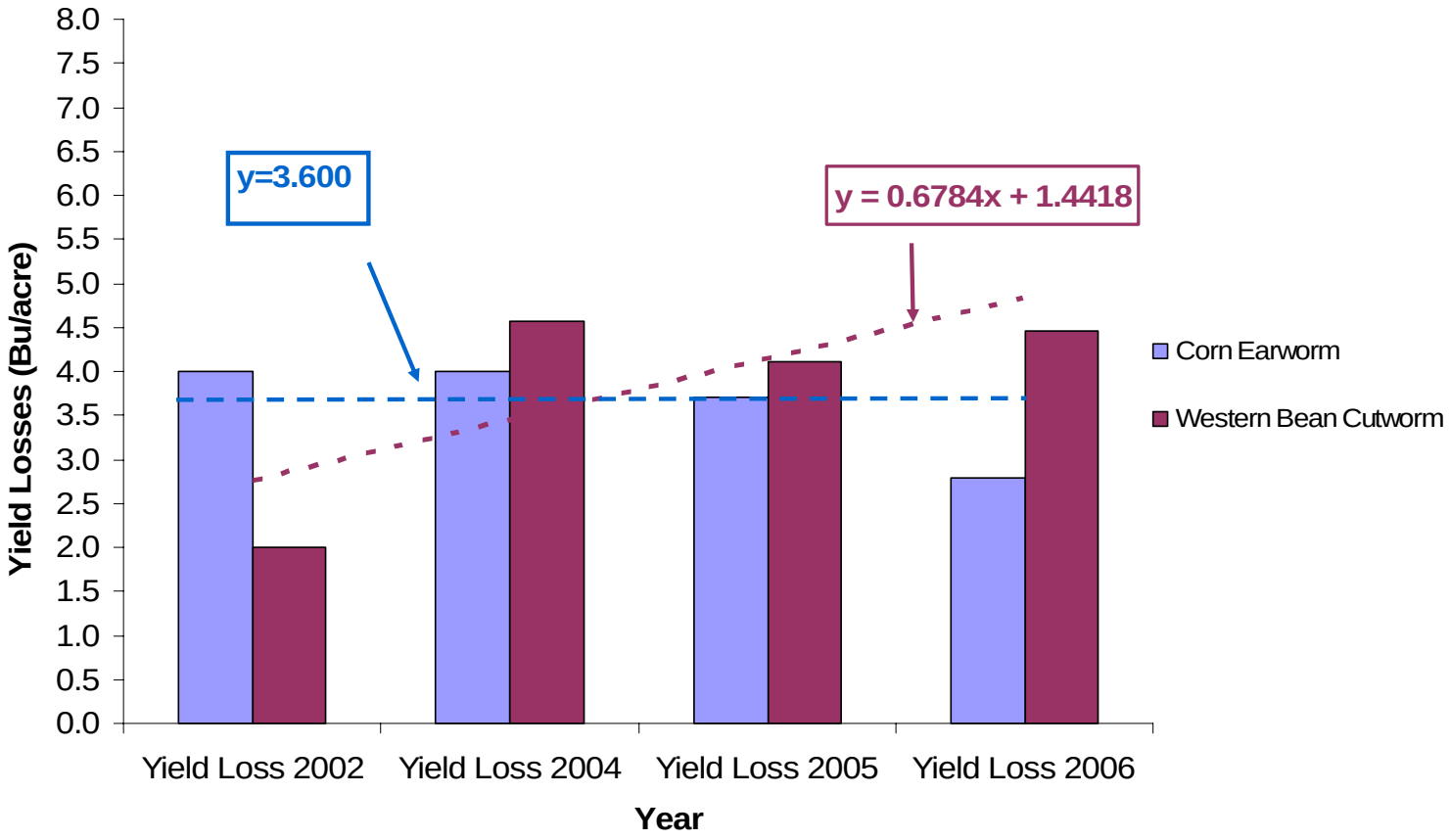
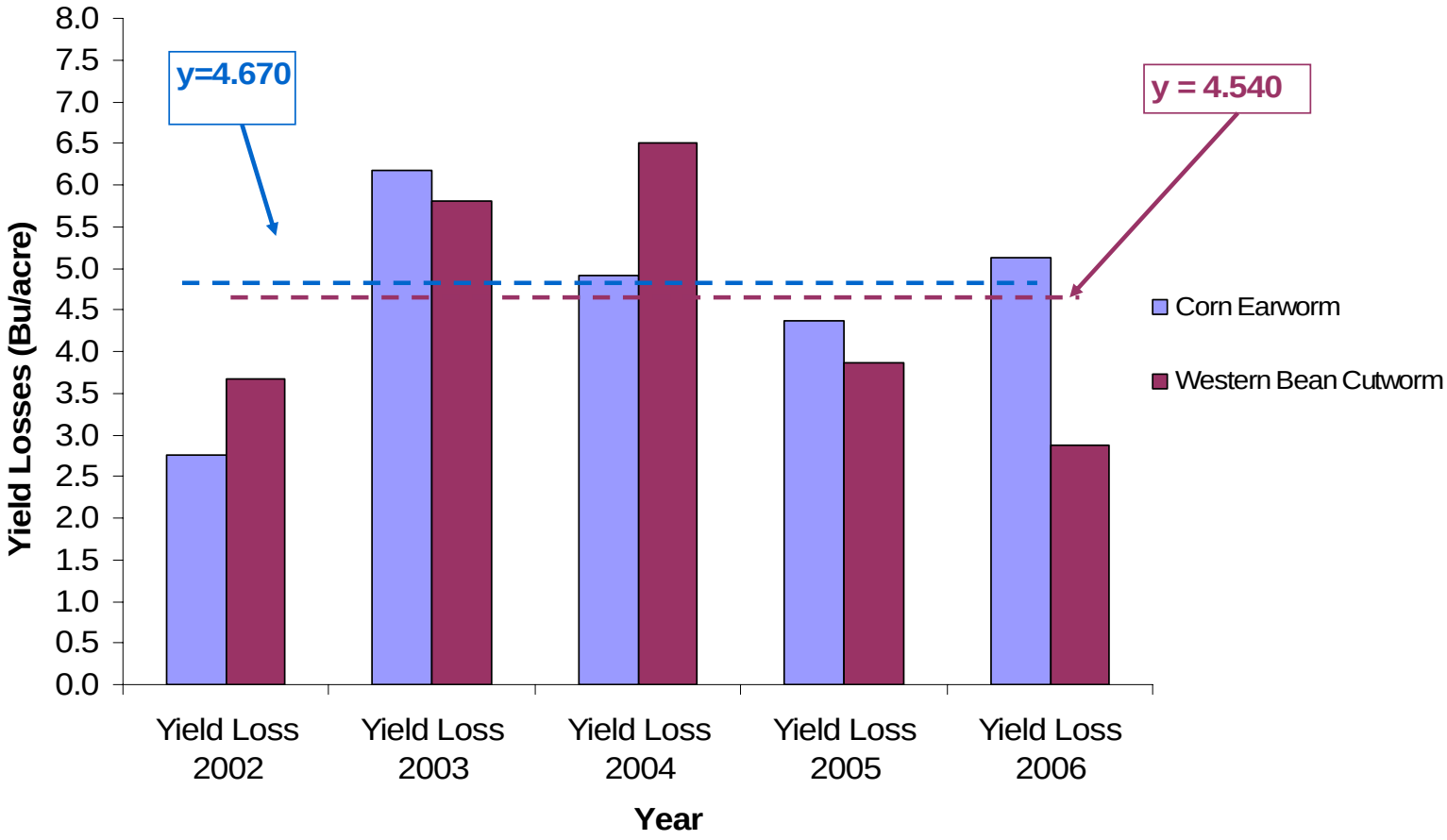


Figure 6: Illinois Average Reported Yield Losses by Insect and Year



Measures and Predictions of Pest Pressure from Doane's Agro-Trak Survey Data

An important component of our analysis involved the development of models that could be used to predict the one-year-ahead levels of insect pressures at various locations in the region of interest. To do so, we used a simple model of expenditures on treatment options for insects in the relevant states included in the survey. These states included Colorado, Illinois, Indiana, Iowa, Kansas, Kentucky, Minnesota, Missouri, Nebraska, Ohio, South Dakota, and Wisconsin. The source for pesticide expenditures data was Doane's Agro-Trak "Treatment for Selected Corn Insect-Pests", which was supplied to us by Syngenta Corp. The Doane's data report expenditures by pesticide brand and the total expenditures for all brands as well as the total number of acres treated. They also report treatment expenditures and number of acres treated by insect (fall armyworm, black cutworm, western bean cutworm, and corn earworm). We can, therefore, isolate expenditures for the insects of interest in this study and calculate the average per-acre expenditures for each insect. These data are reported at the Crop Reporting District (CRD) level. Data were available for the 2005 and 2006 crop years. We also collected total (all practices) corn for grain acreage at the CRD level from the National Agricultural Statistics Service (NASS) website for the 2005 and 2006 crop years. Using the total expenditures on treatment (Grower $) and the total level of harvested acreage in each CRD, we calculated a measure of expenditures per harvested corn acre.[4] We inflated the 2005 totals using the Consumer Price Index to place all expenditures on a 2006 equivalent basis. Our analysis is conducted at the county level, but assumes that the CRD-level average expenditures per acre apply to all counties within each CRD.

Our modeling goal was to use 2005 and 2006 data to predict spatial dispersion in the future—in the current 2007 year. To do so, we adopted a simple set of steps involving extrapolation of each county's expenditures to 2007 and then spatially smoothing the resulting predictions across space. Specifically, expenditures per acre for 2007 were predicted by adding the difference between the 2006 and 2005 levels to the 2006 level. In cases where expenditures declined from 2005 to 2006, 2007 expenditures were constrained to be non-negative.[5]

We then undertook spatial smoothing techniques to generate a smooth response

[4] We use harvested rather than planted acreage for two reasons. First, harvest acreage statistics from NASS tend to be more accurate than planted acreage. Second, we are assuming that abandoned acreage (which is defined by the difference between planted and harvested acreage) is not likely to have received treatment.

[5] This extrapolation process could have resulted in negative estimates of expenditures in cases where the drop in expenditures from 2005 to 2006 was greater than the 2006 level. Thus, we constrained the 2007 estimates to be non-negative.

surface that could be used to predict treatment costs over space and thus to simulate the spatial spread of the pest threat. We considered a number of different spatial smoothing techniques, including inverse distance weighting, global polynomial smoothing, local polynomial smoothing, and radial basis function smoothing.[6] On the basis of superior fit (in terms of minimal root mean squared error) and the reasonableness of the fit, we chose local polynomial interpolation methods to fit the response surface. In particular, we used a first-order (linear) local smoothing function.[7] For any CRDs (counties) that did not have reported treatment costs for a particular pest but did have treatment costs reported for some other pest, the treatment costs were assumed to be zero for the specific pest of interest.[8]

The intuition of our smoothing methods merits additional discussion. The basic approach involves taking weighted averages of values across space to generate predictions. This has two implications. First, high values that neighbor low values tend to be smoothed downward. Likewise, low values that neighbor high values will tend to have a higher prediction than the actual value. An example can be used to illustrate the intuition. Consider a county with costs of $0.50/acre that neighbors a county with zero expenditure. The smoothing methods could result in predicted expenditures of $0.40 in the county with positive costs and $0.10 in the neighboring county that actually had no costs. The idea is to use information from nearby counties to predict the values. The area considered for smoothing (which involves both interpolation and extrapolation in the x- and y-dimensions) is defined by a box constructed by the most extreme latitude and longitude values of the county centroids for districts surveyed by Doane. This implied a rectangular area that roughly encompassed the north central part of the U.S. (see Figure 7 below).

The distribution of actual treatment costs for all insect pests tracked by Doane is illustrated below in Figure 7. High levels of expenditures, indicating significant pest pressures, are apparent in several regions—especially in the lower Corn Belt, Upper Ohio, and South Dakota areas. It is important to again point out that our analysis is confined only to those states targeted by the survey—the absence of treatment expenditures in other states does not necessarily imply that they were zero. Figure 8 presents the spatially smoothed predictions of treatment costs that were derived from the local polynomial interpolation techniques. Note that the patterns are very similar but reflect a wider potential for pest pressures—the effect of having infestations in neighboring areas.

We considered spatial prediction for two specific pests of interest—the Western Bean Cutworm and the Corn Earworm. Figure 9 presents actual treatment costs

[6] Spatial smoothing was accomplished using the ArcMap package.
[7] Higher ordered polynomials sometimes achieved lower RMSE values, but often resulted in negative predicted values.
[8] For example, if treatment costs were reported for black cutworm in a county but nothing was reported for western bean cutworm, we assumed that treatment costs for western bean cutworm were zero.

and Figure 10 presents the spatially smoothed values for the Western Bean Cutworm. Localized infestations are apparent in the Corn Belt and Great Plains. Figures 11 and 12 present actual and spatially-smoothed values for treatment expenditures on the Corn Earworm. Again, the infestations are largely localized but appear to be much more widespread than was the case for the cutworm. The smoothed values encompass a wider area of infestation, again reflecting the spatial smoothing used to predict treatment costs.

Based on these modeling efforts, it can be seen from Figures 10 and 12 that the Western Bean Cutworm and the Corn Earworm are becoming more widespread in the Corn Belt and the Great Plains. Because the existing insect resistant corn traits have no or only moderate activity against these pests and that the cost of scouting and treating them in a timely manner with chemical pesticides is significant or even impractical, it follows that the MIR162 corn trait will provide additional yield protection for an increasing number of corn growers. The additional value to growers of hybrids with the MIR162 trait, assuming those hybrids are priced competitively, likely will be substantial.

Figure 7. Actual Treatment Costs ($/harvested acre) for All Four Pests

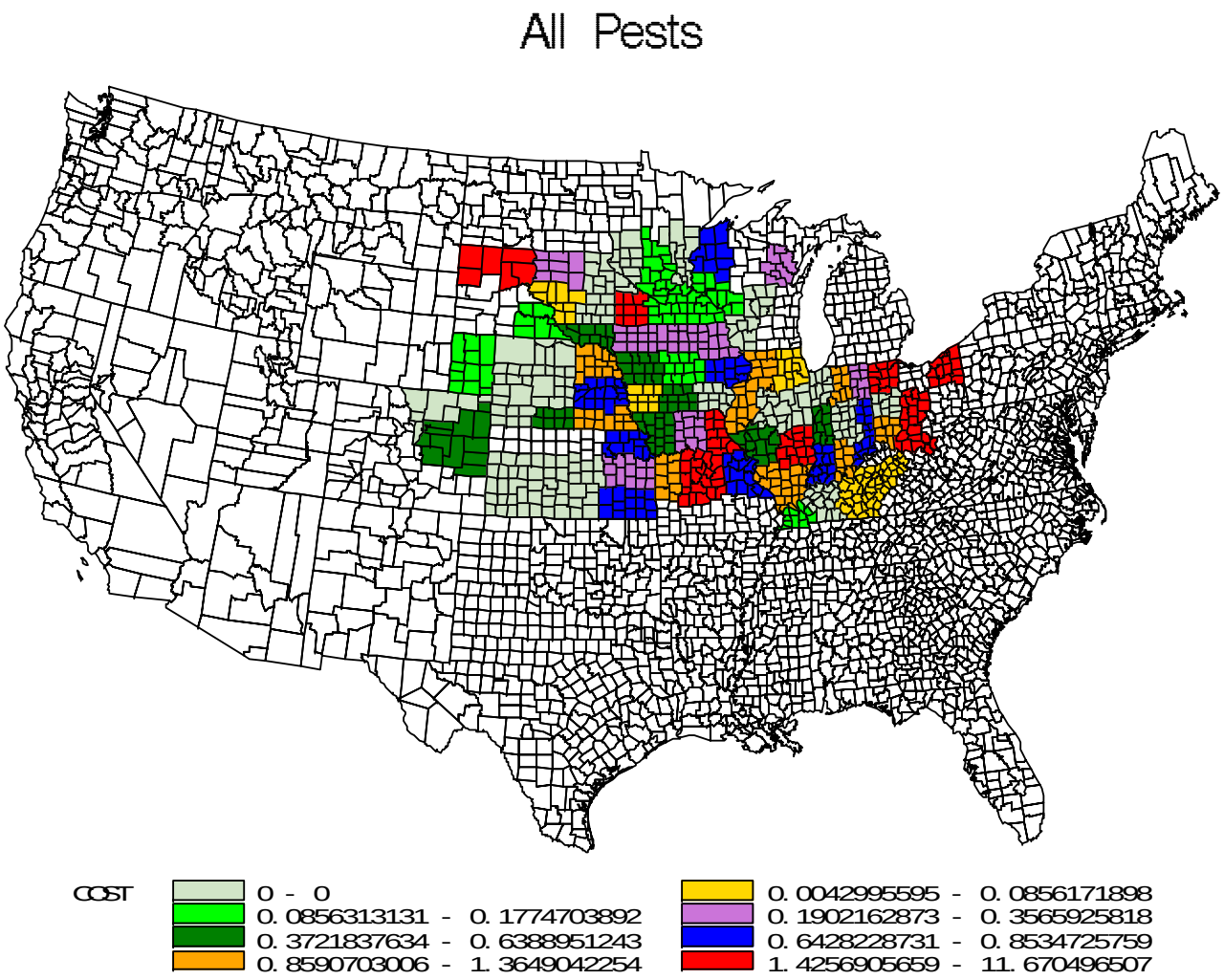


Figure 8. Spatially Smoothed Treatment Costs ($/harvested acre) for All Four Pests

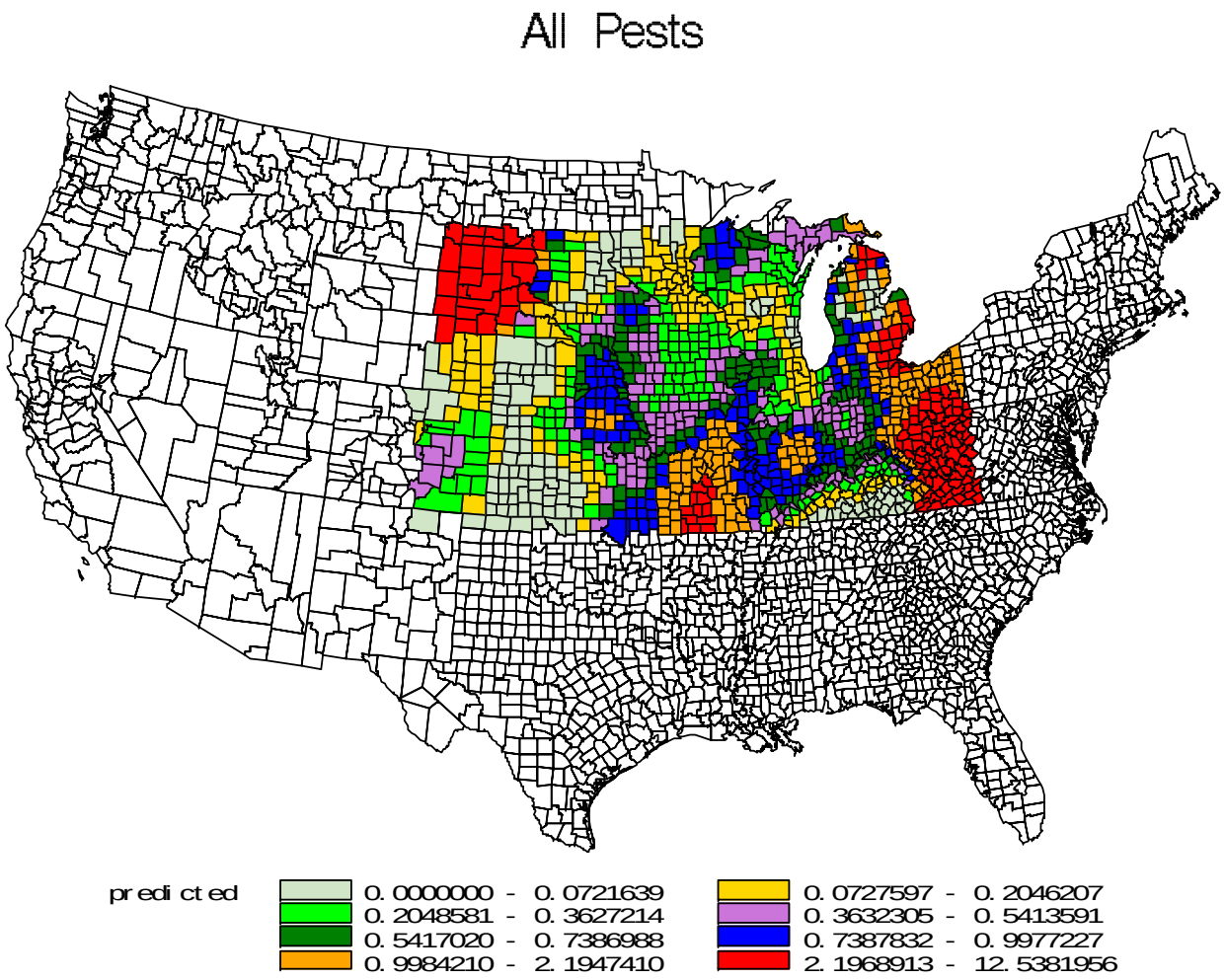


Figure 9. Actual Treatment Costs ($/harvested acre) for Western Bean Cutworm

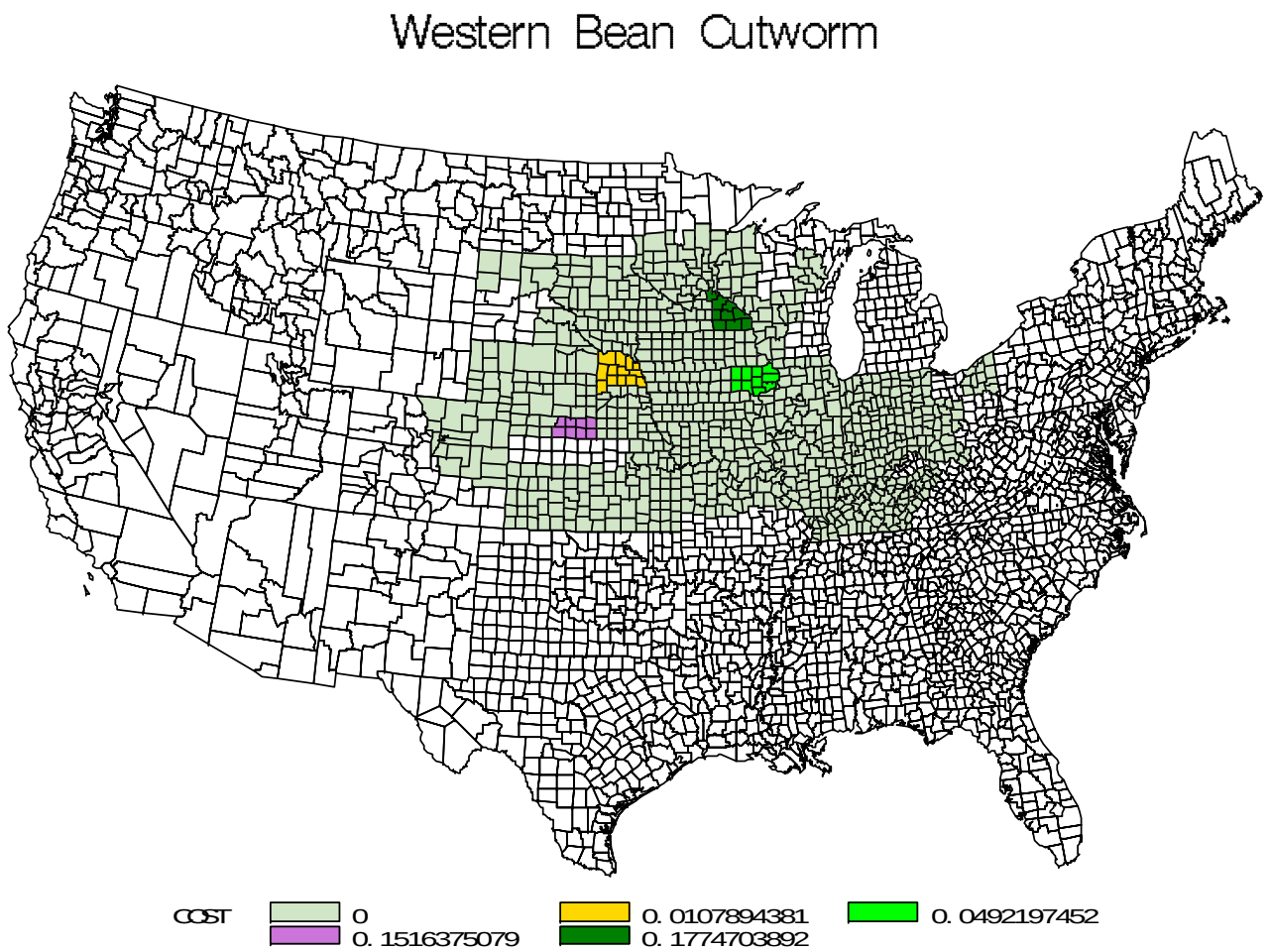


Figure 10. Spatially Smoothed Treatment Costs ($/harvested acre) for Western Bean Cutworm

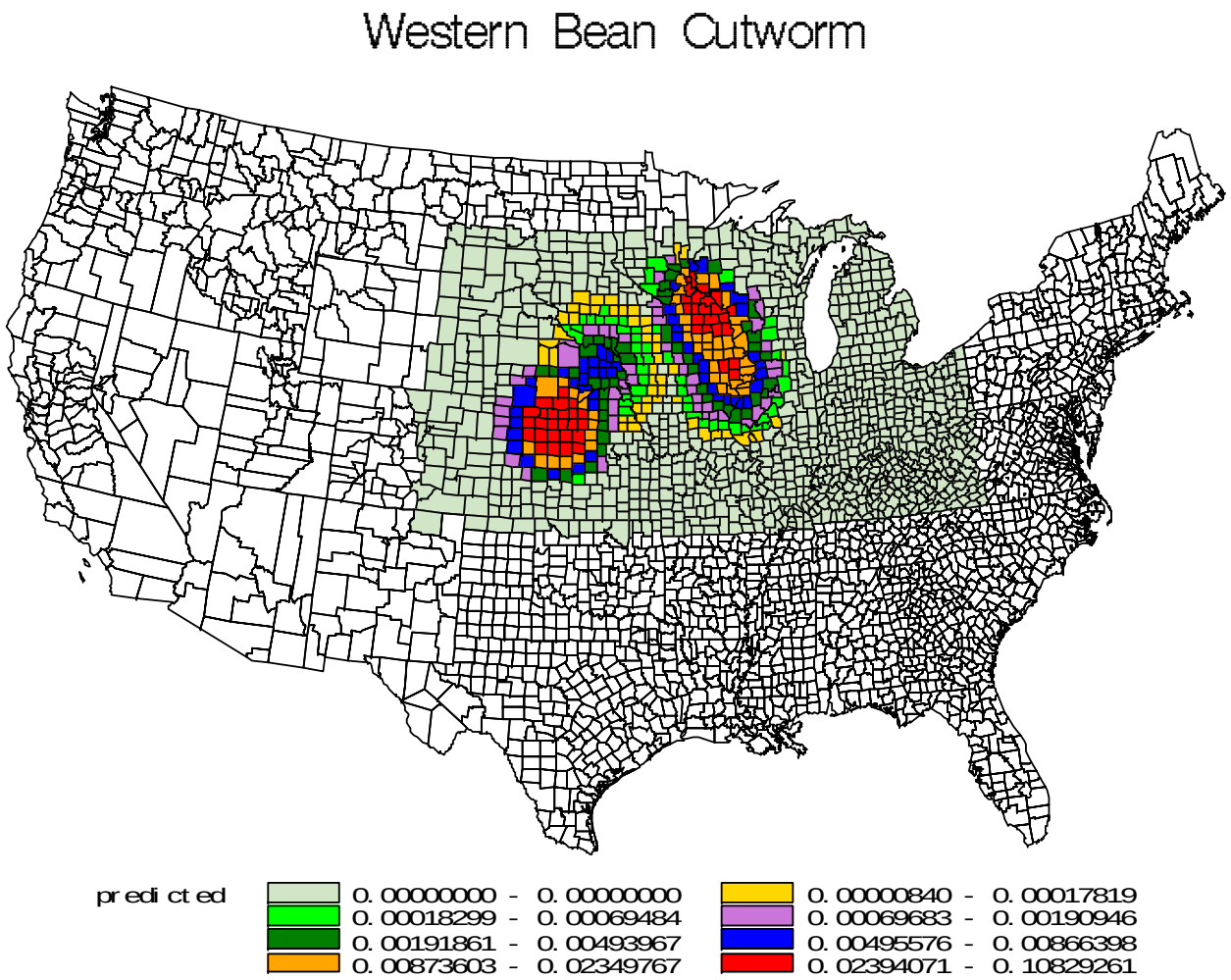


Figure 11. Actual Treatment Costs ($/harvested acre) for Corn Earworm

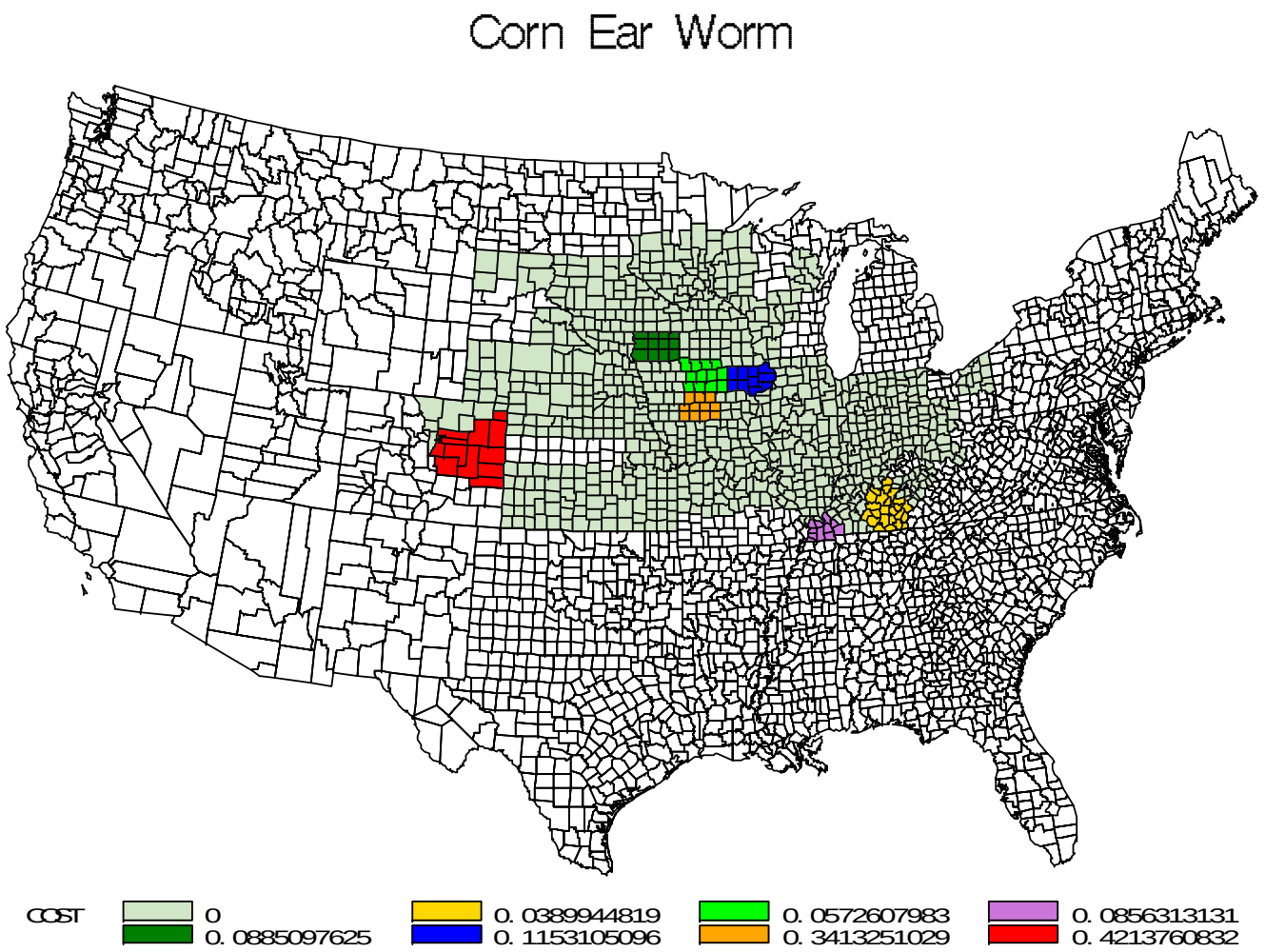
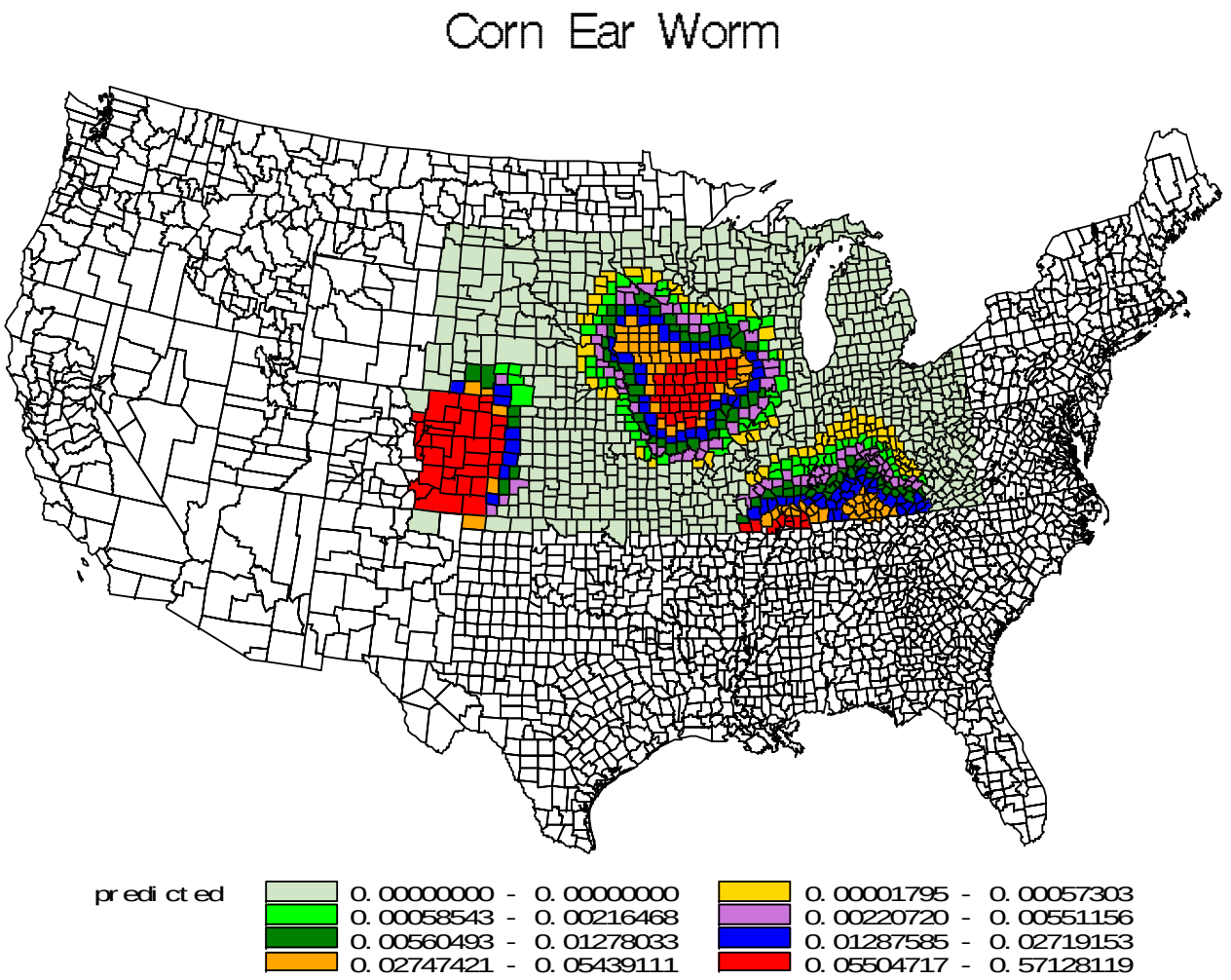


Figure 12. Spatially Smoothed Treatment Costs ($/harvested acre) for Corn Earworm



Respondents' MIR 162 Adoption Intentions

Survey respondents were also given a brief description of the new MIR 162 trait (see Appendix B). After they were read the description twice, they were asked a set of questions about their attitudes and intentions about adopting hybrids with the MIR162 trait. An average of 70 percent of respondents said they would try the MIR162-traited hybrids after listening to its description. The next two questions asked were about the acres of MIR162-traited hybrids they intended to plant in 2007, if it were available and, if it performed as described, how many acres they intended to plant in 2010. Table 4 reports the state-level average answers to those questions.

Table 4: Reported percent willing to adopt and average increase in adoption acreage between 2007 and 2010

State	*N*	Average MIR162 Acres in 2010 [A]	Average MIR162 Acres 2007 [B]	Average Increased Adoption [A]-[B]
KENTUCKY	2	510.00	25.50	484.50
WISCONSIN	6	628.33	153.33	475.00
ILLINOIS	27	474.52	117.96	356.56
IOWA	27	469.70	115.30	354.41
INDIANA	11	431.82	83.64	348.18
COLORADO	2	425.00	105.00	320.00
SOUTH DAKOTA	9	511.11	219.44	291.67
NEBRASKA	20	528.50	243.75	284.75
MINNESOTA	8	461.88	180.63	281.25
MISSOURI	6	441.67	206.67	235.00
KANSAS	5	403.00	170.00	233.00
OHIO	3	305.00	140.00	165.00
ALL STATES	126	478.80	152.41	326.39

Notes:
N denotes number of individuals sampled in each state which comprise this average. The entire sample included 150 individuals but 24 of these oberservations were discarded due to nonsensical responses.

Potential Farm-Level Gain from the Introduction of MIR162-traited Hybrids

The potential gain at the state level is calculated as:

$$\text{Total Corn Acres in State}_i \times \text{Percent Acres Infested with Insect}_j \left[(\text{Percent Acres Treated for Insect}_j \times \text{Treatment Cost per Acre}) + (\text{Percent Acres Not Treated} \times \text{Value of Yield Loss per acre from Insect}_j)\right]$$

We do not have information that would shed light on the percent of acres in a state that has been infested with the insects. Therefore, our calculations here are to be considered to be an upper bound to the potential state-level gain. As the WBC, in particular, infiltrates the Corn Belt, our estimates would become more reflective of the state-level gain in those states. We have made the calculation for only the three states that had a sufficient number of responses for statistical reliability. Table 5 shows the values of the components used in the calculation of the potential state level gains. The potential gain in Nebraska is calculated to be about $84 million per year, or about 2.7% of the total value of corn production, for the western bean cutworm and about $646,000 per year, or 0.03% of the total value of the corn crop, for the corn earworm if the MIR162 trait is adopted by all farmers in the state. The gain in Iowa is estimated to be about $1.7 million for the WBC, or 0.03% of the value of the corn crop and $23 million, or 0.55% of the crop value. In Illinois the total potential gain is about $137 million per year, which is 3.2% of the value of the corn crop, for the WBC and about $124 million per year from the corn earworm, which is about 2.9% of the value of the corn crop.

The upper bound total potential gain in Nebraska, Iowa, and Illinois is estimated to be $222 million per year from the adoption of MIR162 on all corn acres in those states from control of western bean cutworm. This gain is estimated to be about $149 million for the control of corn earworm. The total potential gain in the three states is estimated to be $371 million per year from control of both pests. As the western bean cutworm moves east and populations of the corn earworm rise, the potential gain from adoption of MIR162-traited hybrids will increase in the Corn Belt.

All three of our measures of the value of the introduction of MIR162-traited corn hybrids were positive and, where measured empirically, substantial. These results lend strong support for the trait's success and the potential value to farmers.

Table 5: Components needed to calculate gain from MIR162 for selected states for 2005 and 2006

	% Acres Treated			Total Corn Acres			Treament Cost			Yield Loss			Corn Price			Gain from MIR162		
Year	NE	IL	IA	NE	IL	IA	NE	IL	IA	NE	IL	IA	NE	IL	IA	NEBRASKA	ILLINOIS	IOWA
	[A]			[B]			[C]			[D]			[E]			[A*C+(1-A)*D*E]*B		
	%			Acres			*$/acre*			*Bu/ac*			*$/bu*			*$*		
	Western Bean Cutworm															*Western Bean Cutworm*		
2005	23.24	84.42	3.17	8,500,000	12,100,000	12,800,000	$5.47	$14.80	$0.00	4.38	3.89	0.00	$1.92	$2.08	$1.94	$65,608,452	$166,429,830	$0
2006	37.22	23.66	5.06	8,100,000	11,300,000	12,600,000	$6.69	$0.00	$5.63	5.13	3.73	0.00	$3.15	$3.35	$3.15	$102,275,886	$107,644,628	$3,584,437
Aver.	30.23	54.04	4.12	8,300,000	11,700,000	12,700,000	$6.08	$7.40	$2.81	4.75	3.81	0.00	$2.54	$2.72	$2.55	$83,942,169	$137,037,229	$1,792,218
	Corn Earworm															*Corn Earworm*		
2005	2.16	25.65	22.33	8,500,000	12,100,000	12,800,000	$7.03	$0.00	$15.45	0.00	7.36	0.00	$1.92	$2.08	$1.94	$1,293,017	$137,793,937	$44,150,733
2006	24.50	37.78	5.00	8,100,000	11,300,000	12,600,000	$0.00	$0.00	$4.04	0.00	4.75	0.00	$3.15	$3.35	$3.15	$0	$111,883,529	$2,548,872
Aver.	13.33	31.71	13.67	8,300,000	11,700,000	12,700,000	$3.52	$0.00	$9.74	0.00	6.06	0.00	$2.54	$2.72	$2.55	$646,509	$124,838,733	$23,349,802

Table 6: Gain from MIR162 for selected states, value of production, and percentage gain for 2005 and 2006

	Gain from MIR162			Value of Production			% of Value		
Year	NE	IL	IA	NE	IL	IA	NE	IL	IA
	$			$ millions			%		
	Western Bean Cutworm								
2005	$65,608,452	$166,429,830	$0	$2,439	$3,554	$4,195	2.7%	4.7%	0.0%
2006	$102,275,886	$107,644,628	$3,584,437	$3,711	$6,088	$6,458	2.8%	1.8%	0.1%
Aver.	$83,942,169	$137,037,229	$1,792,218	$3,075	$4,821	$5,327	2.7%	3.2%	0.0%
	Corn Earworm								
2005	$1,293,017	$137,793,937	$44,150,733	$2,439	$3,554	$4,195	0.1%	3.9%	1.1%
2006	$0	$111,883,529	$2,548,872	$3,711	$6,088	$6,458	0.0%	1.8%	0.0%
Aver.	$646,509	$124,838,733	$23,349,802	$3,075	$4,821	$5,327	0.0%	2.9%	0.5%

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APPENDIX A

THE SURVEY QUESTIONNAIRE

MIR162 Value Questionnaire

Hello, my name is _____ with Doane Marketing Research, Inc. We are conducting a survey for researchers at a major land grant university. *Insert usual introduction and offer.*

A.1 Are you the primary decision maker for corn seed and insecticides in your farming operation?

Yes _____ No _____
If Yes, go to A.2.
If No, ask to speak to the principal decision maker.

A.2 Approximately how many acres of corn do you intend to plant this year in 2007?

_____Acres (if "0") thank and terminate

A.3 Have you or your scouts observed any Corn Earworm or Western Bean Cutworm larvae in your corn in the past five years? These pests may also be called earworms, head worms, or tomato fruit worms..

Yes _____ No _____
If Yes, continue with Q1.
If No, thank you and terminate.

I would like to ask you a few questions about your experience with Corn Earworms and Western Bean Cutworms in corn and about a new technology geared toward controlling these pests effectively.

Q.1. How many years out of the last five years, 2006 back to 2002 have you found Corn Earworm larvae on your corn?

_____years
If greater than Zero, answer Q.2 through Q.6
If Zero, skip to Q.7

Note to enumerator: Please get the respondent to answer Q.2. through Q.9. based on the number of years reported in Q.1.

Q1A. In which of the following years did you find Corn Earworm larvae in your corn?

2006
2005
2004
2003
2002

Q.2. In (year), please estimate your Corn Earworm infestation based on 1 larvae for every how many corn plants?

2006,1 larvae per every _____ corn plants

Interviewer note: If respondent can not tell you the infestation this way ask: In (year), please estimate your Corn Earworm infestation based on how many larvae per corn plant you experienced?

2006, _____larvae per corn plant

Q.3. Please give me your <u>best estimate</u> of about how much yield loss in bushels per acre you experienced, from Corn Earworm in (year) *whether or not* you applied an insecticide for control?.

2006, _____ bushels per acre

Q.4. Did you apply an insecticide to control Corn Earworm in (year)?

Yes _____ No _____
If Yes, go to Q.5.
If No, skip to Q.10.

Q.5.What is the product name of the insecticide you applied to control Corn Earworm, in (year)?

<u>2006</u> Insecticide _____

Q.6..What rate in ounces, pints quarts, or pounds per acre did you use for this application of (product)?

<u>2006</u>, ounces per acre_____ or
Pints per acre_____ or
Quarts per acre _____ or
Pounds per acre _____

Q.7. Excluding application costs, how much did you pay per acre, per application for (product) to control Corn Earworm?

<u>2006</u> Insecticide Cost _____ $ per acre

Q.8. How many total applications of (product) did apply to control Corn Earworm in (year)?

2006, number of applications of (product)_____

Q.9. What other insects, if any were you also trying to control with this application of (product) in (year)?

2006, other insects
_____
_____
_____

Repeat questions 2 through 9 for each year the respondent had a Corn Earworm infestation in question 1A.

Now I'd like to ask you a few questions about your experience with Western Bean Cutworm infestations of your corn fields.

Q.1. How many years out of the last five years, 2006 back to 2002 have you found Western bean cutworm larvae on your corn?

_____years

If greater than Zero, answer Q.2 through Q.9

If Zero, skip to Q.10

Note to enumerator: Please get the respondent to answer Q.2. through Q.9. based on the number of years reported in Q.1.

Q1A. In which of the following years did you find Western Bean Cutworm in your corn?

2006
2005
2004
2003
2002

Q.2. In (year), please estimate your Western Bean Cutworm infestation based on 1 larvae for every how many corn plants?

2006,1 larvae per every _____ corn plants

Interviewer note: If respondent can not tell you the infestation this way ask: In (year), please estimate your Western Bean Cutworm infestation based on how many larvae per corn plant you experienced?

2006, _____larvae per corn plant

Q.3. Please give me your best estimate of about how much yield loss in bushels per acre you experienced, from Western Bean Cutworm in (year) *whether or not* you applied an insecticide for control?.

2006, _____ bushels per acre

Q.4. Did you apply an insecticide to control Western Bean Cutworm in (year)?

Yes _____ No _____
If Yes, go to Q.5.
If No, skip to Q.10.

Q.5.What is the product name of the insecticide you applied to control Western Bean Cutworm, in (year)?

2006 Insecticide _____

Q.6..What rate in ounces, pints quarts, or pounds per acre did you use for this application of (product)?
2006, ounces per acre_____ or
Pints per acre_____ or
Quarts per acre _____ or
Pounds per acre _____

Q.7. Excluding application costs, how much did you pay per acre, per application for (product) to control Western Bean Cutworm?
2006 Insecticide Cost _____ $ per acre

Q.8. How many total applications of (product) did apply to control Western Bean Cutworm in (year)?
2006, number of applications of (product)_____

Q.9. What other insects, if any were you also trying to control with this application of (product) in (year)?

2006, other insects
_____
_____
_____

Repeat questions 2 through 9 for each year the respondent had a Western Bean Cutworm infestation in question 1A.

Q.15. Did you plant any Bt varieties to control Corn Earworm or Western Bean Cutworm in 2006?

Yes _____ No _____
If Yes, Q.16
If No, skip to Q.17

Q.16. What Bt brand did you plan tin 2006 and which of the pests we've discussed were you trying to control?

16.a.1. Bt brand _____ 16.a.2. Insects _____

Now I want to describe to you a new corn technology and then ask you a few questions about it.

A new Bt corn variety is being developed that provides superior control of Corn Earworm and Western Bean Cutworm compared with Bt varieties that are currently available. This new variety will be offered in combination with European corn borer resistance, corn rootworm resistance, and herbicide tolerance traits.

Q.17. If this new corn technology were available to you today at a competitive price compared to other technologies that are similar, but do not control effectively for Corn Earworm and Western Bean Cutworm, would you try it on your farm?

Yes _____ No _____ If No, skip to 19.

Q.18. This year, in 2007, on how many of your corn acres would you be likely to try this new technology?

_____ acres

Q.19. If the technology works as advertised, on how many of your corn acres do you think would be planted to the technology three years from now?

_____ acres

Q.20. How many total crop acres will you farm in 2007?

_____ acres

Earlier, you told us you intend to plant (acres) of corn in 2007?

Q.22. On how many of these ___ 2007 corn acres do you intend to plant with seed that has been engineered to be resistant to insects?

_____ acres

Q.23. How many years have you operated a farm?

_____ years

Q.24. How many years of formal schooling have you had?

_____ years

APPENDIX B

REGRESSION RESULTS

Table B.1 Yield loss data by state and year

	All States		ILLINOIS		IOWA		NEBRASKA	
Insect	N	Mean	N	Mean	N	Mean	N	Mean
Year	bushels/acre/year							
	Corn Earworm							
2002	35	2.30	4	2.75	2	4.00	8	2.38
2003	50	4.50	11	6.18	2	3.50	12	3.25
2004	65	3.98	12	4.92	11	4.00	12	3.50
2005	77	3.72	16	4.38	14	3.71	18	3.89
2006	95	4.90	20	5.13	21	2.79	20	3.73
	Western Bean Cutworm							
2002	11	3.00	3	3.67	3	2.00	1	2.00
2003	20	3.88	5	5.80	4	2.25	3	6.83
2004	31	4.63	10	6.50	8	4.56	4	3.50
2005	36	5.07	7	3.86	9	4.11	11	7.36
2006	41	4.82	8	2.88	13	4.46	10	4.75

Source: Grower survey.

Table B.2 Regression results for Illinois

Corn Earworm - Regression 1	
Regression Statistics	
Multiple R	0.3700
R Square	0.1369
Adjusted R Square	-0.1508
Standard Error	1.3492
Observations	5

	Coefficients	*Standard Error*	*t Stat*
Intercept	3.7867	1.4150	2.6761
t	0.2943	0.4266	0.6899

Corn Earworm - Regression 2	
Regression Statistics	
Multiple R	0.0000
R Square	0.0000
Adjusted R Square	-0.2500
Standard Error	1.2577
Observations	5.0000

	Coefficients	*Standard Error*	*t Stat*
Intercept	4.6697	0.5624	8.3025

Western Bean Cutworm - Regression 1	
Regression Statistics	
Multiple R	0.3631
R Square	0.1318
Adjusted R Square	-0.1575
Standard Error	1.6520
Observations	5

	Coefficients	*Standard Error*	*t Stat*
Intercept	5.5976	1.7326	3.2308
t	-0.3526	0.5224	-0.6750

Western Bean Cutworm - Regression 2	
Regression Statistics	
Multiple R	0.0000
R Square	0.0000
Adjusted R Square	-0.2500
Standard Error	1.5355
Observations	5.0000

	Coefficients	*Standard Error*	*t Stat*
Intercept	4.5398	0.6867	6.6112

Source: Grower survey.

Table B.3 Regression results for Iowa

Corn Earworm - Regression 1	
Regression Statistics	
Multiple R	0.6981
R Square	0.4873
Adjusted R Square	0.3164
Standard Error	0.4147
Observations	5

	Coefficients	*Standard Error*	*t Stat*
Intercept	4.2643	0.4349	9.8053
t	-0.2214	0.1311	-1.6887

Corn Earworm - Regression 2	
Regression Statistics	
Multiple R	0.0000
R Square	0.0000
Adjusted R Square	-0.2500
Standard Error	0.5015
Observations	5

	Coefficients	*Standard Error*	*t Stat*
Intercept	3.6000	0.2243	16.0506

Western Bean Cutworm - Regression 1	
Regression Statistics	
Multiple R	0.8590
R Square	0.7380
Adjusted R Square	0.6506
Standard Error	0.7381

	Coefficients	*Standard Error*	*t Stat*
Intercept	1.4418	0.7741	1.8625
t	0.6784	0.2334	2.9067

Source: Grower survey

Table B.4 Regression results for Nebraska

Corn Earworm - Regression 1	
Regression Statistics	
Multiple R	0.8879
R Square	0.7883
Adjusted R Square	0.7178
Standard Error	0.3159
Observations	5

	Coefficients	*Standard Error*	*t Stat*
Intercept	2.3461	0.3313	7.0820
t	0.3339	0.0999	3.3427

Western Bean Cutworm - Regression 1	
Regression Statistics	
Multiple R	0.4243
R Square	0.1800
Adjusted R Square	-0.0933
Standard Error	2.3497
Observations	5

	Coefficients	*Standard Error*	*t Stat*
Intercept	3.0803	2.4643	1.2499
t	0.6030	0.7430	0.8116

Western Bean Cutworm - Regression 2	
Regression Statistics	
Multiple R	0.0000
R Square	0.0000
Adjusted R Square	-0.2500
Standard Error	2.2472

	Coefficients	*Standard Error*	*t Stat*
Intercept	4.8894	1.0050	4.8652

Source: Grower Survey

Courtesy Copy:

Supplemental Information for the Petition for Determination of Nonregulated Status for Insect-Resistant MIR162 Maize

[OECD Unique Identifier: SYN-IR162-4]

USDA Petition 07-253-01p

CBI DELETED

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Submission Date:

November 12, 2007

Syngenta Document ID:

MIR162-USDA-2

Syngenta Biotechnology is submitting this Courtesy Copy of MIR162 maize and Vip3Aa protein technical reports to aid USDA scientists in their review of our petition for a determination of nonregulated status for insect-resistant MIR162 maize:
USDA petition #07-253-01p.

Claim of Data Confidentiality

Syngenta is claiming these technical reports as Confidential Business Information. These reports contain technical information that could be of value to our competitors. These same technical reports have been submitted to the U.S. Environmental Protection Agency as part of the MIR162 application for registration and are afforded confidential business information status under FIFRA.

Report: Author & Title	**Section Number**
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