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Ann Tuttle
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January 10, 2007

Neil E. Hoffman, Ph.D.
Director, Regulatory Programs
Biotechnology Regulatory Services
USDA, Animal and Plant Health Inspection Service
4700 River Road
Unit 147
Riverdale, MD 20737

Subject: Response to APHIS/BRS Review for Technical Completeness of Syngenta's petition for a Determination of Non-regulated Status for Corn Event 3272, assigned APHIS number 05-280-01p.

Dear Dr. Hoffman:

This letter is in response to the APHIS/BRS review of the Petition for Determination of Non-Regulated Status for Corn Event 3272, received in a letter dated November 29, 2006. Syngenta has provided the additional information and clarifications in the attached responses.

Please do not hesitate to contact me should you have any questions regarding these responses.

Sincerely,

Ann Tuttle

Ann Tuttle
Syngenta Seeds, Inc.
Regulatory Affairs Manager

Attachment

Chapter 1

Question: Page 17 – Are the data in Table 1-1 correct? Presumably the 2005 trials have been planted but field data reports are still in progress. Please clarify.

Response: The data in Table 1-1 have been updated. Most of the 2005 field trial reports are still in progress and will be submitted to USDA-APHIS. The individual trial sites that were not planted in 2005 will be noted in the field trial reports.

Table 1-1. Summary of USDA APHIS Notifications/Permits and Field Trial Reports for Event 3272

Year	USDA Notification or Permit No.	Trial Sites by State	Status of USDA field trial reports
2002	02-022-02 r/m	HI	Submitted
2003	03-021-01 r/m	FL,IA,IL,MN,PR,SD,WI	Submitted
	03-021-02 r/m	HI	Submitted
2004	04-051-08n	IA	Submitted
	04-064-04n	FL,HI,IA,ID,IL,IN,KY,MN,NE,PA,PR,SD,WI	Submitted
	04-082-03n	IA	Submitted
	04-126-03n	NE	Submitted
	04-203-03n	PR	Submitted
	04-216-02n	HI	Submitted
2005	05-042-09n	HI, NE	Submitted
	05-049-10n	CO,FL,HI,IA,ID,IL,IN,KY,MN,MO,NE,PR,SD,WI	In Progress
	05-102-02n	IL	In Progress
	05-104-08n	HI	In Progress
	05-255-01n	HI	In Progress

Chapter 3

Question: Page 24 – Under the description of the transformation system and method you do not indicate that the *Agrobacterium tumefaciens* used for transformation was disarmed. Was it disarmed?

Response: Yes, the *Agrobacterium tumefaciens* strain used for transformation was disarmed.

Question: Page 42 – The figure on this page was truncated on the right-hand side. Please provide the information that is missing.

Response: On the right-hand side of the figure on page 42, the information that has been truncated is provided as follows:

NOS terminator (253 bp)
gDNA (335 bp)

Question: Page 43 – Third paragraph. Do you have an explanation as to why the AMY797E protein was detected in one leaf sample during the senescence stage?

Response: A low level of AMY797E protein was detected in one of the ten senescence stage leaf samples and is likely due to contamination in sample handling. It is unlikely that this represents expression in the leaves as AMY797E protein was not detectable in the other nine senescence stage leaf samples.

Question: Page 43 and Page 46 – Fourth paragraph and Table 3-5. The levels of PMI reported in pollen are the highest level for all tissues tested. In addition wildlife could be exposed to PMI through pollen so pollen data should also be included in Table 3-5 on page 46.

***Response*:** The first sentence on page 46 should read, "Wildlife will be exposed to AMY797E through consumption of kernels and PMI through consumption of leaves, kernels or pollen."

Table 3-5 on page 46 has been revised to include pollen and is provided below.

Table 3-5. AMY797E and PMI levels for wildlife exposure assessment

Protein	Tissue	Development Stage	Highest Level (µg/g)	Extraction Efficiency (%)	Risk Assessment Exposure Level (µg/g)
AMY797E	kernels	kernel dough	1951	91.0	2144
PMI	leaves	kernel dough	5.7	78.8	7.2
PMI	kernels	kernel dough	0.9	100	0.9
PMI	pollen	anthesis	8.5	92.6	9.2

Question:
Page 50 - Under Alpha-amylases in other organisms:
There are no levels given in this summary that indicate what concentration of alpha-amylase one expects to find in nature. And these levels are not compared to those found in event 3272. Please expand this section and give examples of levels found in the references cited and compare those to that found in the event (see comment on page 93).

Page 93 – Section C3. Under section C3i for alpha-amylase in plants and in soil, it is indicated that "no harmful effects of such exposure to naturally occurring concentrations are known." However, there is no comparison made between the levels in event 3272 and the levels that occur in nature. Please clarify this statement and provide levels that occur in nature. See comment on page 50 above.

***Response*:**

AMY797E alpha-amylase as expressed in Event 3272 maize is not expected to have any impact on human and animal health or have any harmful environmental effects based upon characterization of the AMY797E amylase, a detailed assessment of human and animal safety (also being reviewed by FDA) and an environmental assessment (see Chapter 4, Section A6 and Chapter 8, respectively). The safety and environmental assessments included conclusions of no treatment-related effects in an acute oral mouse toxicity study, no adverse nutritional or toxic effects in a 49-day feeding trial in broiler chickens, no significant sequence identity to any proteins identified as, or known to be, toxins, rapid degradation in simulated gastric fluid, and that Event 3272 maize is highly unlikely to be more harmful to wildlife than is conventional maize based on consideration of the safety of the AMY797E amylase, composition of Event 3272 maize, the environmental fate of AMY797E amylase, the routes of exposure to wildlife, and data in the published literature indicating that amylases are not toxic to wildlife.

In addition, consideration of the history of safe consumption and/or exposure to alpha-amylases found naturally and added to a variety of human food and animal feed sources and functional homology of AMY797E alpha-amylase to other alpha-amylases further supports the conclusion that AMY797E alpha-amylase as expressed in Event 3272 maize is not expected to have any impact on human and animal health or have any harmful environmental effects.

In general, proteins that function as enzymes are not known to be toxic (Pariza and Foster, 1983; FDA, 1992; Pariza and Johnson, 2001). More specifically, many enzyme proteins have a safe history of being consumed and used in food and food processing (Pariza and Foster, 1983; Pariza and Johnson, 2001). Alpha-amylases, including thermostable and recombinant alpha-amylases, are one of several specific classes of enzymes produced by many different fungal and bacterial sources that have a long history of safe use in food and feed and are found in a variety of human food and animal feed sources (see Chapter 4, Section A5; Pariza and Foster, 1983; Pariza and Johnson, 2001; FDA GRAS Notices; AAFCO, 2007). Within a known safe family of enzymes having the same fundamental catalytic activity, such as alpha-amylases, the natural variation of enzymes from different source organisms or changes to the enzyme introduced by molecular techniques do not pose specific safety concerns (Kessler *et al.*, 1992; Pariza and Johnson, 2001). The AMY797E alpha-amylase is functionally homologous to other alpha-amylases including characteristic catalytic activity, shared conserved sequences and the preservation of

protein folding domains for this class of alpha-amylases. (Pujadas and Palau, 2001; Janeček *et al.*, 1999; Lévêque *et al.*, 2000; Richardson *et al.*, 2002).

Alpha-amylases are ubiquitous in the environment, being naturally present in microorganisms, plants, and animals as well as being commercially produced and used for a number of food processing, feed ingredient and industrial applications, including starch hydrolysis for the production of fuel and potable alcohol (brewing, distillation processes) and corn syrups (high fructose, maltose, dextrose, and glucose corn syrups) (see Chapter 4, Section A5; Pariza and Foster, 1983; Pariza and Johnson, 2001; FDA GRAS Notices; AAFCO, 2007).

Alpha-amylases play a key role in many cellular metabolic processes by catalyzing the breakdown of starch, including the hydrolysis of starch reserves to metabolizable sugars in germinating seed of cereal plants and the microbial decomposition of organic matter containing starch to provide carbon and energy to support microbial growth. In these two examples, one of plant alpha-amylase activity and the other of widespread microbial hydrolytic enzyme activity, the enzyme is synthesized in response to substrate availability, specific inducers, availability of other nutrients, physical and chemical parameters such as moisture, temperature, pH, and seasonal or diurnal variation. These factors vary in time and space and from one microenvironment to another. Naturally occurring concentrations of alpha-amylase protein from all of these organisms and in the general environment have not been determined. Therefore, it is not possible to quantitate these and compare individual or aggregate levels of naturally occurring alpha-amylases to the AMY797E alpha-amylase levels contained in kernels of Event 3272 maize.

However, as summarized above, the AMY797E alpha-amylase, as expressed in Event 3272 maize, is not expected to have any impact on human and animal health or have any harmful environmental effects. This conclusion is based on the assessment of human and animal safety, an environmental assessment, and characterization of the AMY797E amylase and its functional homology to other alpha-amylases that are ubiquitous in nature and alpha-amylases found naturally and added to a variety of human food and animal feed sources that have a history of safe consumption and/or exposure.

References:

AAFCO (2007). Official Publication Association of American Feed Control Officials Incorporated. Feed ingredient definitions, 30.1 Enzymes/source organisms acceptable for use in animal feeds. pp. 275-278. Association of American Feed Control Officials.

FDA (1992). Statement of policy: foods derived from new plant varieties. *Fed. Reg*. 57(104), 22984-23005, May 29, 1992.

FDA GRAS notices. (http://www.cfsan.fda.gov/~rdb/opa-gras.html).

Janeček, S., Lévêque, E., Belarbi, A., Haye, B. (1999). Close evolutionary relatedness of α-amylases from archaea and plants. *J. Mol. Evol.* 48, 421-426.

Kessler, D.A., Taylor, M.R., Maryanski, J.H., Flamm, E.L., Kahl, L.S. (1992). The safety of foods developed by biotechnology. *Science* 256:1747-1749.

Lévêque, E., Janeček, S., Haye, B., Belarbi, A. (2000). Thermophilic archael amylolytic enzymes. *Enzyme and Microbial Technology* 26, 3-14.

Pariza, M.W. and Foster, E.M. (1983). Determining the safety of enzymes used in food processing. *J. Food Prot.* 46, 453-468.

Pariza, M.W. and Johnson, E.A. (2001). Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. *Regul. Toxicol. Pharmacol.* 33(2), 173-186.

Pujadas, G. and Palau, J. (2001). Evolution of α-amylases: architectural features and key residues in the stabilization of the (β/α)8 scaffold. *Mol. Biol. Evol.* 18, 38-54.

Richardson, T.H., Tan, X., Frey, G., Callen, W., Cabell, M., Lam, D., *et al*. (2002). A novel, high performance enzyme for starch liquefaction. *J. Biol. Chem.* 277(29), 26501-26507.

Question: Page 50 and Page 51 – Under the summary data in section A6:
Item 2: This section needs to be expanded to include information on the how the studies were conducted. Short summary data could be given in this section or in the Appendix.

Item 3: As above please provide a short summary outlining how the studies were conducted.

Item 4: Please provide a short summary outlining how the studies were conducted.

Item 7: Please provide a short summary outlining how this study was conducted.

Item 9: Please provide a short summary outlining how this study was conducted.

***Response*:**

Item 2 - A short summary outlining how the studies in Item 2 were conducted is provided below.

Acute Oral Mouse Toxicity Study of the AMY797E Alpha-Amylase Protein

The potential toxicity of the AMY797E alpha-amylase protein was evaluated in a single dose oral toxicity study in the mouse using AMY797E alpha-amylase protein prepared from Event 3272 maize grain. Groups of five male and five female mice were dosed orally by gavage with 0 (control) or 1511 mg AMY797E protein/kg body weight as a single dose using 0.5% w/v aqueous carboxymethylcellulose as the control substance and vehicle. Clinical observations, body weight and food consumption were measured throughout the study. Fourteen days after dosing, the animals were sacrificed and subjected to an examination *post mortem*. Blood samples were taken for clinical pathology, selected organs were weighed and specified tissues were processed and examined for histopathological changes. There were no treatment-related effects of AMY797E. The conclusion of the study was that AMY797E alpha-amylase protein was nontoxic.

Item 3 - A short summary outlining how the study in Item 3 was conducted is provided below.

Nutritional Quality of Event 3272 Maize Grain in a Poultry Feeding Trial

The nutritional quality of Event 3272 maize grain was assessed in a 49-day feeding trial in broiler chickens. Broiler chickens are highly sensitive to small nutrient changes within their diets because of their extremely rapid growth. Male and female broiler chickens were fed diets containing Event 3272 grain (52% to 65%), near-isogenic control grain (53% to 67%) or commercially available grain (51% to 64%). Each treatment group was assigned 150 male and 150 female chickens. The diets were formulated based on the individual nutrient analyses for each of the grain sources to meet standard nutritional recommendations for poultry. All diets supported rapid broiler chicken growth at low mortality rates and excellent feed conversion ratios without significant impact on overall carcass yield or quality. The absence of any adverse nutritional or toxic effects confirms the nutritional equivalence of Event 3272 maize to maize from near-isogenic and commercial controls and supports the conclusion that consumption of Event 3272 maize in animal feed does not pose a safety concern.

Item 4 - A short summary outlining how the study in Item 4 was conducted is provided below.

Evaluation of AMY797E Alpha-Amylase Amino Acid Sequence Identity with Known Toxins

To determine whether the AMY797E alpha-amylase protein had any significant amino acid sequence identity with protein sequences identified as toxins, the amino acid sequence was systematically compared to the National Center for Biotechnology Information (NCBI, 2006) Entrez Protein Database containing all publicly available protein sequences. No significant sequence identity to any proteins identified as, or known to be, toxins was identified.

Reference:

NCBI (2006) National Center for Biotechnology Information GenBank Database (http://www.ncbi.nlm.nih.gov/BLAST/); non-redundant GenBank coding sequence translations (PDB+SwissProt+PIR+PRF) containing 3,284,262 sequences. Accessed on March 11, 2006.

Item 7 - A short summary outlining how the study in Item 7 was conducted is provided below.

In vitro Digestibility Study of AMY797E Alpha-Amylase Protein

The susceptibility of an alpha-amylase protein, AMY797E, to proteolytic degradation in simulated mammalian gastric fluid (SGF) containing pepsin was evaluated. AMY797E protein purified from Event 3272 maize grain was incubated in SGF at 37 ºC for 0, 1, 5, 10, 20 and 30 minutes. The intact AMY797E protein (*ca.* 50.2 kDa) was not detected after 1 minute incubation in SGF and no immunoreactive fragments were detected following digestion in SGF for 5 minutes, as assessed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses. These data support the conclusion that AMY797E expressed in transgenic plants will be readily digested under typical mammalian gastric conditions.

Item 9 - A short summary outlining how the study in Item 9 is provided below.

Glycosylation Determination of AMY797E Alpha-Amylase Protein

To determine whether the AMY797E alpha-amylase protein is glycosylated, AMY797E protein purified from Event 3272 maize grain was analyzed using the DIG Glycan Detection Kit (Roche Molecular Biochemicals, Indianapolis, IN, USA). After subjecting an AMY797E sample and the control proteins supplied in the kit (creatinase, a non-glycosylated protein and transferrin, a glycosylated protein) to SDS-polyacrylamide gel electrophoresis and blotting, glycan moieties associated with glycosylated proteins were oxidized using periodate, labeled with

digoxigenin, and detected with an anti-digoxigenin antibody coupled to alkaline phosphatase. The results of the DIG Glycan analysis indicate that the AMY797E alpha-amylase protein is not glycosylated.

Question:

Page 53-54 - Under PMI Enzymes in Other Organisms: There are no levels given in this summary that indicate what concentration of PMI one expects to find in nature. And these levels are not compared to those found in event 3272. Please expand this section and give examples of levels found in the references cited and compare those to that found in the event (see comment on page 93).

Page 93 – Section C3.
Under section C3i for phosphomannose isomerase it is indicated that "no harmful effects of such exposure to naturally occurring concentrations are known." However, there is no comparison made between the levels in event 3272 and the levels that occur in nature. Please clarify this statement and provide levels that occur in nature. See comment on page 53-54 above.

Response:

Phosphomannose isomerase (PMI) protein as expressed in Event 3272 maize is not expected to have any impact on human and animal health or have any harmful environmental effects based upon the extensive characterization of the PMI protein, including a detailed assessment of human and animal safety (also being reviewed by the FDA, see Chapter 4, section B6 for a summary) and an environmental assessment (see Chapter 8). In addition, the EPA has granted an "exemption from the requirement of a tolerance" for PMI in all crops (EPA, 2004). The EPA was required to consider a requirement for tolerance because PMI is expressed in some transgenic plants that produce pesticidal proteins, and therefore in these circumstances PMI is regarded as an 'inert' component of a pesticide and regulated under the Federal Food, Drug and Cosmetic Act (FFDCA) (among other legislation). To grant an exemption from tolerances, the EPA must be satisfied that there is a "reasonable certainty that no harm will result from aggregate exposure to the [substance], including all anticipated dietary exposures and all other exposures for which there is reliable information".

Reference:

EPA (2004). Phosphomannose isomerase and the genetic material necessary for its production in all plants; Exemption from the requirement of a tolerance. 40 CFR Part 180. *Fed. Reg*. 69(94), 26770-26775, May 14, 2004.

Question: Page 57 – Table 5-1. It is not clear to what Trial Series # refers, presumably these are those shown in the breeding chart on page 111.

Response: Yes, on page 57, Table 5-1, Trial Series # refers to the AgTrial # shown on the breeding chart on page 111.

Question: Page 66 – Second paragraph under section B7. How many replications were conducted in the seed viability and germination study?

Response: All experiments in the seed viability and germination study on page 66 utilized four replications.

Question: Page 88 – Second paragraph – the Connor et al., 2003 reference is missing in the list of references. Please provide the reference.

Response: The reference on page 88, second paragraph, should read as "Conner *et al.*, 2003" and is as follows:

Conner, A.J., Glare, T.R., Nap, J.P. (2003). The release of genetically modified crops into the environment. Part II. Overview of ecological risk assessment. *Plant J.* 33, 19-46.

Question:

Page 93 – Section C3.

Under section C3ii it is indicated that "AMY797E and PMI do not have significant amino acid sequence identity to any proteins known to be toxins" but there are no references or data provided. Please provide a reference and/or data.

Response: The summary data for the toxin identity search for the AMY797E protein is provided under the response to the question for page 50 and page 51, item 4. The summary data for the toxin identity search for the PMI protein is provided below.

Evaluation of PMI Protein Amino Acid Sequence Identity with Known Toxins

To determine whether the PMI protein had any significant amino acid sequence identity with protein sequences identified as toxins, the amino acid sequence was systematically compared to the National Center for Biotechnology Information (NCBI, 2006) Entrez Protein Database containing all publicly available protein sequences. No significant sequence identity to any proteins identified as, or known to be, toxins was identified.

Reference:

NCBI (2006) National Center for Biotechnology Information GenBank Database (http://www.ncbi.nlm.nih.gov/BLAST/); non-redundant GenBank coding sequence translations (PDB+SwissProt+PIR+PRF) containing 3,284,262. Accessed on February 10, 2006.

Question: Page 101 - first Hallauer reference. There appears to be missing information in this reference.

Response: The first Hallauer reference on page 101 is complete. However, the second Hallauer reference on page 101 should be deleted along with the Hallauer reference on page 22.

Question: Page 130 - 131 – Appendix 5-Table 2. In the row for ILSI (2006) the numbers provided are not in the form of a range.

Response: On pages 130 and 131, Appendix 5-Table 2 should read as follows for the ILSI (2006) numbers:

range for calcium:	713.9 – 5767.9
range for phosphorus:	936.2 – 3704.1

Question: Page 132 - 134 – Appendix 5-Table 3. In the cell for OECD level for TDF the number provided is not in the form of a range.

Response: The OECD (2002) reference only provided one number without a range.

Petition for the Determination of Nonregulated Status

Maize Event 3272

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

Submitted by:

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Contributors:

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OECD Unique Identifier: SYN-E3272-5

Date:

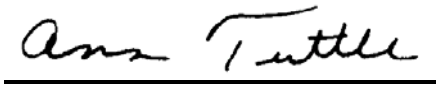
September 10, 2006

Release of Information

Syngenta is submitting the information in this petition for purposes of review by USDA pursuant to the regulations at 7 CFR Part 340. By submitting this information, Syngenta does not authorize its release to any third party except to the extent that the following three conditions are satisfied: (i) it is requested under the Freedom of Information Act (FOIA), 5 U.S.C., §§ 551 *et seq*.; (ii) USDA complies with the provisions of FOIA and USDA's implementation regulations (7 CFR Part 1.4); and (iii) this information is responsive to the specific request. Except in accordance with the Freedom of Information Act, Syngenta does not authorize the release, publication or other distribution of this information (including website posting) without Syngenta's prior notice and consent. The information contained in this document may not be used or cited by any third party, including but not limited to any regulatory authority other than USDA, to support the regulatory approval of this product or any other product, or for any other purpose, without Syngenta's prior written consent.

Statement of Grounds Unfavorable

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



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ABBREVIATIONS USED IN THIS PETITION

aadA	adenylyltransferase gene
ADF	acid detergent fiber
amy797E	gene in Event 3272 encoding the AMY797E alpha-amylase protein
AMY797E	AMY797E alpha-amylase protein expressed in Event 3272
AOSA	Association of Official Seed Analysts
APHIS	Animal and Plant Health Inspection Service
ATP	adenosine 5'-triphosphate
A. tumefaciens	*Agrobacterium tumefaciens*
bp	base pair
bw	body weight
BC	backcross
CAS	Chemical Abstracts Service
CFIA	Canadian Food Inspection Agency
CFR	Code of Federal Regulations
ColE1ori	*E. coli* origin of replication
DDD	daily dietary dose
DNA	deoxyribonucleic acid
dw	dry weight
EC	Enzyme Commission
ECB	European corn borer
E. coli	*Escherichia coli*
EINECS	European Inventory of Existing Chemical Substances
ELISA	enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FFDCA	Federal Food Drug and Cosmetic Act
FIR	food intake rate
fw	fresh weight
GC	guanosine, cytosine
GDP	guanosine diphosphate
gdw	gram dry weight
GRAS	generally recognized as safe
GTP	guanosine triphosphate
GZein	promoter from the maize gamma-zein gene
Gzein ss	signal sequence from the maize gamma-zein gene
ILSI	International Life Sciences Institute
IUBMB	International Union of Biochemistry and Molecular Biology
kb	kilobase
kDa	kilodalton

kg	kilogram
LB	left border
LOD	limit of detection
LOQ	limit of quantitation
manA	phosphomannose isomerase gene from *Escherichia coli*
MIR604	Syngenta corn rootworm protected event
µg	microgram
mg	milligram
NASS	National Agricultural Statistics Service
ND	not detectable
NDF	neutral detergent fiber
NOED	no observed effect dose
NOS	nopaline synthase terminator
OECD	Organization for Economic Cooperation and Development
PBN	FDA Pre-market Biotechnology Notification
PCR	polymerase chain reaction
PEPC	maize phosphoenolpyruvate carboxylase gene
pg	picogram
pmi	gene in Event 3272 encoding the selectable marker protein
PMI	selectable marker protein phosphomannose isomerase
pNOV7013	plasmid employed to create Event 3272
PPA	Plant Protection Act
RB	right border
RepA	bacterial replication protein
RNA	ribonucleic acid
SBI	Syngenta Biotechnology, Inc.
SD	standard deviation
Spec	spectinomycin resistance gene
TDF	total dietary fiber
T-DNA	transfer DNA
Ti plasmid	tumor-inducing plasmid
TIU	trypsin inhibitor unit
USDA	United States Department of Agriculture
VS1ori	*Agrobacterium* origin of replication
WHO	World Health Organization
ZmUbiInt	promoter from maize polyubiquitin gene

Chapter 1. SYNGENTA SEEDS PETITION FOR THE DETERMINATION OF NONREGULATED STATUS OF MAIZE EVENT 3272

A. Summary

This petition is submitted to USDA APHIS to determine that Syngenta Seeds' Event 3272 maize (corn), genetically engineered to produce the AMY797E alpha-amylase protein, as well as an associated plant selectable marker protein, phosphomannose isomerase (PMI), neither poses a plant pest risk nor has an adverse effect in the environment and should therefore no longer be considered a regulated article under 7 CFR 340.

Syngenta's maize transformation Event 3272 contains two transgenes: 1) the *amy797E* gene encoding the thermostable AMY797E alpha-amylase protein and 2) the *pmi* (*manA*) gene from *Escherichia coli*, which encodes the enzyme phosphomannose isomerase, used as a plant selectable marker. The AMY797E alpha-amylase enzyme is a chimeric enzyme derived from three wild-type alpha-amylases from the archael order *Thermococcales*. This enzyme was selected for development due to its increased thermostability and activity during the high temperatures required for starch hydrolysis in dry-grind ethanol production from corn. Expression of the *amy797E* gene in Event 3272 is driven by the promoter from a maize seed storage (gamma-zein) gene and the *pmi* gene is driven by the maize polyubiquitin promoter. Data from Southern analyses demonstrate that Event 3272 plants 1) contain a single copy of both the *amy797E* and *pmi* genes, 2) contain a single copy of both the gamma-zein and ubiquitin promoters and 3) do not contain any of the backbone sequences from the transformation plasmid pNOV7013. DNA sequencing of Event 3272 confirmed that the overall integrity of the intended insert and the contiguousness of the functional elements had been maintained. Statistical analyses over multiple generations confirmed that the *amy797E* gene is stably inherited in the expected Mendelian ratio.

Event 3272-derived (hereafter referred to as "Event 3272") maize hybrids have been field tested for two years (2003 and 2004) at more than 25 locations in several states across the U.S. Corn Belt. Agronomic traits that encompass the entire life cycle of the plant were assessed in Event 3272 hybrids and near-isogenic, non-transgenic control hybrids. The agronomic performance and phenotypic data generated demonstrate that the genetic modification resulting in Event 3272 did not have any unintended effects on seed germination, dormancy, plant growth habit and general morphology, life-span, vegetative vigor, flowering and pollination, grain yield, stress adaptations or disease susceptibility. These data support the conclusion that Event 3272 hybrids are unlikely to form feral persistent populations, or to be more invasive or weedy than conventional maize hybrids, and would not display higher rates of outcrossing than unmodified maize.

Compositional analysis of forage and grain from Event 3272 hybrids were compared over two years (2003 and 2004) to near-isogenic, non-transgenic control hybrids. Compositional analysis of the forage samples included proximates (protein, fat, ash,

carbohydrates and moisture), acid detergent fiber (ADF), neutral detergent fiber (NDF), and minerals (calcium, phosphorus). Compositional analysis of the grain samples included proximates (protein, fat, ash, carbohydrates), acid detergent fiber (ADF), neutral detergent fiber (NDF), total dietary fiber (TDF), starch, minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, zinc and selenium), vitamins (beta-carotene (provitamin A), folic acid, B1 (thiamine), B2 (riboflavin), B3 (niacin), B6, and E), fatty acids (16:0 palmitic, 18:0 stearic, 18:1 oleic, 18:2 linoleic and 18:3 linolenic), amino acids, antinutrients (phytic acid, raffinose and trypsin inhibitor) and secondary maize metabolites (furfural, ferulic acid and *p*-coumaric acid). The conclusion based on the compositional analysis data is that Event 3272 hybrids are nutritionally equivalent in composition to other commercial corn hybrids and will not pose an increased plant pest risk or have increased weediness potential over conventional maize hybrids.

A thorough mammalian safety assessment was conducted for both the AMY797E and PMI proteins as expressed in Event 3272 maize. No adverse effects were observed for either protein. The large body of data and information described herein support the conclusion that the AMY797E alpha-amylase and PMI proteins as expressed in Event 3272 maize will pose no hazard to humans or animals. Additionally, the U.S. Environmental Protection Agency (EPA) has granted a permanent exemption for the requirement of a tolerance for the PMI protein in all crops when used as a plant-incorporated protectant inert ingredient. A detailed assessment of human and animal safety of the AMY797E alpha-amylase and PMI proteins has been provided to the Food and Drug Administration (FDA) as part of a food and feed safety and nutritional assessment for maize Event 3272.

An environmental safety assessment was conducted for the AMY797E and PMI proteins expressed in maize Event 3272. Two objectives were set to demonstrate minimal environmental risk of cultivating maize Event 3272: to show that Event 3272 is highly unlikely to be more harmful to wildlife than is conventional maize; and to show that Event 3272 is highly unlikely to be a worse weed of agriculture, or to be more invasive of non-agricultural habitats, than is conventional maize. The assessment concluded that no significant risks to the diversity and abundance of wildlife within and outside maize fields, or to crop yield, have been identified from the proposed cultivation of maize Event 3272.

Based on the information and data contained in this petition, Syngenta requests that USDA APHIS make a determination of nonregulated status for Event 3272, any progeny derived from crosses between Event 3272 and other maize varieties, and progeny derived from crosses of Event 3272 with other transgenic maize varieties that have received nonregulated status under 7 CFR Part 340.

B. Rationale for the Development of Maize Event 3272

Corn or maize (*Zea mays* L.) is the most widely distributed cereal grain grown worldwide. It is the most important crop in the United States, occupying double the area of any other crop. Corn is used for three main purposes: (1) as a staple human food, particularly in the tropics; (2) as feed for livestock, particularly in temperate and advanced countries, providing over two-thirds of the total world wide trade in feed grains; (3) as a raw material for many industrial products which include corn starch, corn syrup and sugars, corn oil and numerous alcohol products.

The fuel ethanol industry has grown tremendously in the United States over the last 25 years for many reasons, including the increased interest in the environment. Ethanol used as either a fuel additive or gasoline replacement offers numerous environmental benefits and is considered a "renewable" fuel. Ethanol used as a fuel additive produces a cleaner-burning fuel and improves air quality by reducing carbon monoxide and volatile organic compound emissions. In addition to the environmental benefits, increased use of renewable fuels lessens the U.S. dependence on foreign oil sources. The Energy Policy Act of 2005, signed on August 8, 2005, includes a Renewable Fuels Standard that directs the doubling of the use of ethanol and biodiesel in the U.S. fuel supply over the next seven years to 7.5 billion gallons.

Syngenta has developed a thermostable alpha-amylase enzyme (AMY797E) expressed in Event 3272 maize grain for use in the dry-grind fuel ethanol process in the United States. Microbially produced alpha-amylases are commonly used commercially in the starch-processing step during corn dry-grind and wet milling processing. Syngenta has expressed a thermostable alpha-amylase enzyme in the endosperm of maize grain with enzyme characteristics suitable for the starch processing step of dry-grind ethanol production. Ideally amylases for this industry should work at high temperature and have low calcium requirements. Syngenta's amylase enzyme expressed in Event 3272 matches these criteria. The product concept is that the Event 3272 grain will serve as the source of amylase enzyme in the dry-grind ethanol process, replacing the addition of microbially produced enzyme. The Event 3272 grain expressing the AMY797E alpha-amylase enzyme will be mixed with conventional corn at the processing plant.

Starch is a major component of corn grain. It consists of a mixture of amylose (linear glucose chains) and amylopectin (branched glucose chains) and is processed using enzymes such as alpha-amylases, glucoamylases, pullulanases, alpha-glucosidases and glucose isomerases, depending on the intended use. Alpha-amylase enzymes catalyze the hydrolysis of starch by cleaving the internal α–1,4-glucosidic bonds of amylose and amylopectin into dextrins (starch fragments which range in size from 5-50 glucose units), maltose and glucose (van der Maarel *et al.*, 2002). In the dry-grind ethanol process, glucoamylase is added after the alpha-amylase step to completely hydrolyze dextrins to glucose. Glucose, a fermentable sugar, is then used as the substrate for yeast fermentation in the production of ethanol. Dry-grind ethanol production involves these starch enzymatic hydrolysis steps, a fermentation step and a distillation step and produces three final coproducts: fuel ethanol, CO_2 and distillers grains/solubles. The coproducts obtained after the removal of ethanol by distillation

from the yeast fermentation are distillers grains and solubles, which can be used either separately or combined. Distillers grains and solubles are commonly used in animal feed, with the distillers grains either in a wet or dry form. One of these products, dried distillers grains with solubles (DDGS), is high in protein, fiber and fat content and is sold as an animal feed ingredient, primarily in ruminant diets.

A thorough mammalian safety assessment was conducted for both the AMY797E and PMI proteins as expressed in maize Event 3272. The large body of data and information support the conclusion that the AMY797E alpha-amylase and PMI proteins as expressed in Event 3272 maize will pose no hazard to humans or animals.

C. Status with Federal Agencies

Event 3272 maize falls within the scope of the U.S. Food and Drug Administration's 1992 Statement of Policy: Foods Derived from New Plant Varieties, including genetically engineered varieties pursuant to 21 CFR Section 192.25 of the Federal Food, Drug, and Cosmetic Act (FFDCA). Before offering Event 3272 maize hybrids for commercial sale in the United States, Syngenta will complete the voluntary consultation process with the FDA. Syngenta has initiated a consultation with the FDA and has filed an Early Food Safety Evaluation of New Non-Pesticidal Proteins Produced by New Plant Varieties Intended for Food Use: AMY797E Alpha-Amylase and Phosphomannose Isomerase Proteins Produced by Corn Transformation Event 3272 (NPC 001 and NPC 002 respectively) on April 19, 2005. A Pre-market Biotechnology Notification (PBN) consultation for Event 3272 was submitted to the FDA on August 31, 2005.

D. Regulatory Permit Status with USDA APHIS

Syngenta is pursuing field trial applications in the Unites States (see Table 1-1) to facilitate commercial development and regulatory approvals for Event 3272 hybrids in the United States. Event 3272 hybrids have been planted in several states under USDA APHIS comprehensive permits and notifications since 2002.

Table 1-1. Summary of USDA APHIS Notifications/Permits and Field Trial Reports for Event 3272

Year	USDA Notification or Permit No.	Trial Sites by State	Status of USDA field trial reports
2002	02-022-02 r/m	HI	Submitted
2003	03-021-01 r/m	FL,IA,IL,MN,PR,SD,WI	Submitted
	03-021-02 r/m	HI	Submitted
2004	04-051-08n	IA	Submitted
	04-064-04n	FL,HI,IA,ID,IL,IN,KY,MN,NE,PA,PR,SD,WI	Submitted
	04-082-03n	IA	Submitted
	04-126-03n	NE	Submitted
	04-203-03n	PR	Submitted
	04-216-02n	HI	Submitted
2005[1]	05-042-09n	HI, NE	In Progress
	05-049-10n	CO,FL,HI,IA,ID,IL,IN,KY,MN,MO,NE,PR,SD,WI	In Progress
	05-102-02n	IL	In Progress
	05-104-08n	HI	In Progress

[1] Trial sites may be approved, but not all trial sites planted.

Chapter 2. THE MAIZE FAMILY

The following was excerpted, with minor edits, from USDA APHIS Environmental Assessment 92-042-01 (authored by Dr. James Lackey), the Canadian Food and Inspection Agency (CFIA) Regulatory Directive Dir94-11 CFIA, 1994) and the Organization for Economic Cooperation and Development (OECD) Consensus Document on the Biology of *Zea mays* subsp. mays (Maize) (OECD, 2003). Full descriptions and complete references can be obtained from these documents.

A. General Description of *Zea mays* L. (Maize)

Zea is a genus of the family Graminae (Poaceae), commonly known as the grass family. Maize (*Zea mays* L.) is a tall, monecious annual grass with overlapping sheaths and broad conspicuously distichous blades. Plants have staminate spikelets in long spike-like racemes that form large spreading terminal panicles (tassels) and pistillate inflorescences in the leaf axils, in which the spikelets occur in 8 to 16 rows, approximately 30 cm long, on a thickened, almost woody axis (cob). The whole structure (ear) is enclosed in numerous large foliaceous bracts and long styles (silks) protrude from the tip of the ear as a mass of silky threads (Hitchcock and Chase, 1971). Pollen is produced entirely in the staminate inflorescence and eggs, entirely in the pistillate inflorescence. Maize is wind-pollinated and both self and cross-pollination are usually possible. Shed pollen usually remains viable for 10 to 30 minutes, but can remain viable for longer durations under favorable conditions (Coe *et al*., 1988). Cultivated maize is presumed to have been derived from teosinte (*Z. mexicana*) and is thought to have been introduced into the old world in the sixteenth century. Maize is cultivated worldwide and represents a staple food for a significant proportion of the world's population. No significant native toxins are reported to be associated with the genus *Zea* (International Food Biotechnology Council, 1990).

B. Origin of the Species *Zea mays* L.

It is generally agreed that teosinte (*Z. mexicana*) is an ancestor of maize, although opinions vary as to whether maize is a domesticated version of teosinte (Galinat, 1988; for reviews see OECD, 2003; CFIA, 1994). Teosinte is an ancient wild grass found in Mexico and Guatemala. Because it has differentiated into various races, species and plant habits, taxonomic classification is still a matter of controversy. Doebley and Iltis (1980) and Iltis and Doebley (1980) classified the annual teosintes into two subspecies of *Z. mays*: ssp. *mexicana* (including races Chalco, Central Plateau and Nobogame) and ssp. *parviglumis*-var. *parviglumis* (race Balsas) and var. *huehuetenangensis* (race Huehuetenango) and the species *Z. luxurians* (race Guatemala). The perennial teosintes from Jalisco, Mexico are separated into two more species according to ploidy, *Z. perennis* and *Z. diploperennis*. The Meso-American region located within middle South Mexico and Central America is recognized as one of the main centers of origin and development of agriculture as well as center of origin and diversification of more than one hundred crops (Vavilov, 1951; Smith, 1995; Harlan, 1992). At the present time, there is no agreement about where exactly maize was domesticated and there are

several proposals in this regard. Based on the findings of archaeological materials from the maize plant (pollen, cobs, husks, and other remnants) in the United States and Mexico, which are older than those found in South America, Randolph (1959) proposed that maize was domesticated, independently, in the southwestern United States, Mexico, and Central America. Mangelsdorf (1974) proposed that "corn had not one origin but several in both Mexico and South America", because the archaeological evidences are found in Mexico and several morphological characteristics of extant populations are found in the maize races of South America (Andes region) in comparison to those races of Meso-America.

C. Cultivation and Use of Maize

As discussed above, maize has been cultivated by the indigenous peoples of North America for thousands of years. The modern era of maize hybrid production began in the United States where research conducted in the early part of this century proved that hybrid maize could produce a yield superior to open-pollinated varieties (Sprague and Eberhart, 1977). Gradually, hybrid-derived varieties replaced the open-pollinated types in the 1930's and 1940's. Almost all maize grown in the United States now comes from hybrid seed that is obtained every planting season from private enterprises; the older open-pollinated varieties are virtually unknown in commerce (Hallauer *et al*., 1988).

The production of hybrid seed requires the development and maintenance of inbred lines and subsequent controlled crosses to produce commercial seed. Self-pollination is essential for inbred development while controlled cross-pollination is mandatory for hybrid seed production. Mechanisms have been developed to ensure the correct form of pollination for each process and to prevent genetic contamination of seed stocks (Wych, 1988). Breeder or foundation seed is produced from self-pollinated seed after the eighth or ninth generation of inbreeding. A high degree of self-pollination is assured by planting in blocks that are isolated by a distance of at least 200 meters (~660 ft.) from any other contaminating source of pollen. Hybrid seed production is accomplished by inter-planting rows of the male and female inbred parents (*e.g.*, one row of male to four female rows). Hybrid seed production requires isolation similar to that for foundation seed. Self-pollination of the female parent is prevented through detasseling prior to pollen shed or by the use of male sterile females. Genetic conformity of inbreds and hybrids is monitored and assured through grow-outs of representative seed lots and laboratory screening using such criteria as isozyme profiles.

Maize is planted when soil temperatures are warm (greater than or equal to 10 ^{o}C) usually mid to late April through to mid-May in the U.S. Corn Belt. Optimum yields occur when the appropriate hybrid maturity and population density are chosen. In addition, exogenous sources of nitrogen fertilizer are generally applied and weed and insect control measures are generally recommended. Choice of the appropriate hybrid for the intended growing area helps to ensure that the crop will mature before frost halts the growth of the plant at the end of the season; hybrids are categorized according to the amount of "heat units" that will be required for maturity. Therefore, a

hybrid developed for a specific heat unit zone, will not mature in (cooler) areas that receive fewer "heat units". Traditional cultivation practices in maize often result in bare soil that is susceptible to erosion by wind or water; increasingly, "no till" maize is being grown in an effort to reduce this soil loss.

In 2003, there were more than 78 million acres planted to maize in the United States producing over 10 billion bushels of grain (USDA, 2004b). Maize grown in the United States is predominantly of the yellow dent type, a commodity crop largely used to feed domestic animals, either as grain or silage. The remainder of the crop is exported or processed by wet or dry milling to yield products such as high fructose corn syrup and starch or oil, grits and flour. These processed products are used extensively in the food industry. For example, maize starch serves as a raw material for an array of processed foods, and in industrial manufacturing processes. Since the early 1980's, a significant amount of grain has also been used for fuel ethanol production. The by-products from these processes are often used in animal feeds. For a full discussion of the uses of maize see Watson (1988).

D. Pollination of Maize

Pollination, fertilization, and caryopsis development of maize follows the same pattern for chasmogamous wind-pollinated grasses, with the following exceptions:

1. Pollen is produced entirely in the staminate inflorescences. Eggs are produced entirely in the pistillate inflorescences.
2. Self-pollination and fertilization and cross-pollination and fertilization are usually possible, and frequencies of each are usually determined by physical proximity and other environmental influences on pollen transfer. A number of complicating factors, such as genetic sterility factors and differential growth rates of pollen tubes may also influence the frequencies of self-fertilization versus cross-fertilization.
3. Maize styles and maize pollen tubes are the longest known in the plant kingdom.
4. Shed pollen typically remains viable for 10 to 30 minutes, but may remain viable for much longer under refrigerated conditions (Coe *et al*., 1988).
5. Pollen dispersal is limited due to its large size (0.1 mm diameter) and spherical nature. Numerous studies have shown that greater than 98% of pollen settles to the ground within 60 meters of its source (Raynor *et al*., 1972; Luna *et al*., 2001; Burris, 2002).
6. The staminate and pistallate inflorescences do not develop at the same time. The pistillate inflorescence is precocious. However, there is the appearance of slight protandry because the elongating styles (silks) are delayed for about seven days in emergence from the bracts of the pistillate inflorescence, while the later-developing staminate inflorescence is fully visible. The silks are receptive to pollen up to 10 days after emergence, but receptivity decreases rapidly thereafter (Walden and Everett, 1961).

Maize is primarily wind-pollinated; insects are responsible for insignificant amounts of pollen dispersal (Russell and Hallauer, 1980).

E. Inter-Species/Genus Hybridization

Maize and other species and subspecies of teosinte are sexually compatible and can produce fertile hybrids (Wilkes, 1977). Related *Zea* species are geographically restricted and occur only in Mexico and Guatemala. The closest known relative of *Zea* is *Tripsacum*, a genus of eleven species, widely distributed between 42oN and 24oS latitude (de Wet *et al*., 1981). Three species occur in the United States, two of which, *Tripsacum floridanum* (Florida Gamagrass) and *Tripsacum lanceolatum* (Mexican Gamagrass), are confined to the southernmost states of the United States. Only one, *Tripsacum dactyloides* (Eastern Gamagrass), has a distribution that includes the northern (U.S.) maize belt (Gould, 1968).

F. Potential for Introgression from *Z. mays* into Relatives

An examination of the literature prior to 1980 would lead to the conclusion that there is constant gene flow between maize and teosinte, and that the weedy teosinte (*Z. mays* ssp. *mexicana*) is a hybrid of the two sub-species, and functions as a genetic bridge between the two (de Wet and Harlan, 1972; de Wet, 1975; Galinat, 1973). However, this premise has been re-evaluated using techniques of gene mapping, which failed to show any evidence of recent introgression between maize and teosinte (Smith *et al*., 1985). Moreover, *Z. mays* ssp. *mexicana* seems not to be a hybrid of the wild and cultivated forms of *Zea* and therefore probably does not serve as a genetic bridge as the physical similarities appear to be due to parallel adaptation to the same habitat (Doebley, 1984). There is evidence of highly restricted gene flow between *Zea* spp. that apparently occurs predominantly from teosinte into maize (Doebley *et al.*, 1987). *Tripsacum* and *Zea* have different chromosome numbers (n = 9 versus n = 10). Crosses between *Z. mays* and *T. dactyloides* can be made, but only through human intervention and, even then, only with extreme difficulty. Moreover, the progeny are frequently sterile or genetically unstable (Mangelsdorf, 1974). The process of transferring *Tripsacum* germplasm into maize is technically difficult. The transmission rate of the single extra *Tripsacum* chromosome added to the genome is so low and the rate of maize *Tripsacum* crossing over so reduced, as to practically exclude the general use of experimentally introduced *Tripsacum* germplasm in maize improvement (Galinat, 1988).

G. Volunteers and Weediness in Maize

Maize has lost the ability to survive in the wild due to its long process of domestication, and needs human intervention to disseminate its seed. Although maize from the previous crop year can overwinter and germinate the following year, it cannot persist as a weed. The presence of maize in soybean fields following the maize crop from the previous year is a common occurrence. Measures are often taken to either eliminate the plants with the hoe or use of herbicides to kill the plants in soybean fields, but the plants that remain and produce seed usually do not persist

during the following years. Volunteers are common in many agronomic systems, but they are easily controlled; however, maize is incapable of sustained reproduction outside of domestic cultivation. Maize plants are non-invasive in natural habitats (Gould, 1968). Some *Zea* species are successful wild plants in Central America, but they have no pronounced weedy tendencies (Galinat, 1988). In contrast to weedy plants, maize has a pistillate inflorescence (ear) with a cob enclosed with husks. Consequently seed dispersal of individual kernels does not occur naturally. Individual kernels of maize, however, are distributed in fields and main avenues of travel from the field operations of harvesting the crop and transporting the grain from the harvested fields to storage facilities (Hallauer, 2000).

Chapter 3. IDENTIFICATION AND CHARACTERIZATION OF THE INTRODUCED GENETIC MATERIAL IN EVENT 3272

A. Summary

Data from Southern analyses and DNA sequencing demonstrated that single copies of the alpha-amylase (*amy797E*) gene, phosphomannose isomerase (*pmi*) gene, maize gamma-zein (GZein) promoter and maize ubiquitin (ZmUbiInt) promoter derived from the transformation plasmid pNOV7013 (Figure 3-1) are present in maize derived from Syngenta's Event 3272. Event 3272 does not contain any of the backbone sequences from the transformation plasmid pNOV7013. Additionally, Southern analysis demonstrated that the T-DNA insert is stable over several generations in Event 3272. Sequence analysis of the entire T-DNA insert present in Event 3272 confirms the overall integrity of the insert and that contiguousness of the functional elements has been maintained. Statistical analyses confirmed the expected Mendelian inheritance ratio for the *amy797E* gene.

B. Introduction

This chapter is being submitted to the USDA in support of a petition for nonregulated status for Syngenta Seeds' maize transformation Event 3272. These plants express a synthetic thermostable alpha-amylase protein, AMY797E. Alpha-amylases are enzymes that catalyze the hydrolysis of starch into soluble sugars. Additionally, the plants express the selectable marker phosphomannose isomerase (PMI). The present chapter represents a summary of data and information relevant to the molecular characterization of the T-DNA insert and the genetic material required for its production (*via* transformation plasmid pNOV7013) in transgenic maize plants derived from Syngenta Seeds' transformation Event 3272. Included herein are data and information describing the genetic elements that have been introduced into Event 3272, the process used for transformation, and a molecular and genetic characterization of Event 3272 maize plants.

C. Event 3272 Maize

Syngenta's Event 3272 maize has been transformed with a synthetic *amy797E* gene whose expression produces the thermostable AMY797E alpha-amylase protein. The *amy797E* gene is a chimeric gene derived from sequences of three alpha-amylase genes originating from three hyperthermophilic microorganisms of the archael order *Thermococcales.* This chimeric alpha-amylase gene was assembled from the parental sequences using GeneReassembly™ technology (Diversa Corporation, San Diego, CA) and was selected for the encoded amylase protein's thermostability properties needed during the starch liquefaction step of corn processing. The alpha-amylase sequence was codon-optimized for expression in maize. The *amy797E* gene additionally codes for the fusion of this chimeric alpha-amylase with an N-terminal maize gamma-zein signal sequence and a C-terminal SEKDEL sequence for targeting to and retention of the protein in the endoplasmic reticulum. The *amy797E* gene is expressed in Event 3272 using the maize gamma-zein promoter, which confers expression of the AMY797E

protein only in the endosperm tissue of the corn kernel. Kernels produced from maize plants transformed with the *amy797E* gene from pNOV7013 contain the thermostable AMY797E alpha-amylase protein and are efficient at starch hydrolysis under the high temperatures used during corn processing. A complete description of the *amy797E* gene and AMY797E protein is found in Chapter 4.

Event 3272 maize also contains the *pmi* gene, which was introduced *via* the same pNOV7013 transformation vector. This gene represents the *manA* gene from *Escherichia coli* and encodes the enzyme phosphomannose isomerase, which was employed as a selectable marker during the process of regenerating plant material following transformation (Negrotto *et al*., 2000). Maize cells expressing *pmi* can utilize mannose as a primary carbon source and therefore survive on media in which mannose is the sole source of carbon, whereas cells lacking *pmi* expression will fail to proliferate in a mannose-based culture medium. A complete description of the *pmi* gene and PMI protein is found in Chapter 4.

D. Description of the Transformation System and Method

Transformation of Syngenta's AMY797E-expressing maize Event 3272 was conducted using immature maize embryos derived from a proprietary *Zea mays* line (Negrotto *et al*., 2000), via *Agrobacterium tumefaciens*-mediated transformation. By this method, genetic elements within the left and right border regions of the transformation vector are efficiently transferred and integrated into the genome of the plant cell, while genetic elements outside these border regions are generally not. Immature embryos were excised from 8 - 12 day old ears and rinsed with fresh medium in preparation for transformation. Embryos were mixed with the suspension of *A. tumefaciens* cells harboring the transformation vector pNOV7013, vortexed for 30 seconds, and allowed to incubate for an additional five minutes. Excess *A. tumefaciens* solution was aspirated and embryos were then moved to plates containing a non-selective culture medium. Embryos were co-cultured with the remaining *A. tumefaciens* at 22 ^{o}C for 2-3 days in the dark. Embryos were transferred to culture medium supplemented with ticarcillin (100 mg/ml) and silver nitrate (1.6 mg/l) and incubated in the dark for ten days. Embryos producing embryogenic callus were transferred to cell culture medium containing mannose. The phosphomannose isomerase gene, *pmi*, was employed as a selectable marker during the transformation process (Negrotto *et al*., 2000). After initial incubation with *A. tumefaciens*, transformed tissue was transferred to and grown for four months on selective media containing 500 mg/l of the broad-spectrum antibiotic cefotaxime, insuring that the *A. tumefaciens* was cleared from the transformed tissue. Regenerated plantlets were tested for the presence of both the *pmi* and *amy797E* genes, as well as for the absence of the spectinomycin antibiotic resistance gene (*aadA*), by TaqMan® PCR analyses (Ingham *et al.*, 2001). The TaqMan® PCR method is described in Appendix 1. Plants positive for both the *pmi* and *amy797E* genes, and negative for *aadA*, were transferred to the greenhouse for further propagation.

E. The Donor Genes and Regulatory Sequences

E1. Active ingredient cassette:

GZein promoter (677 bp): Promoter region from the *Zea mays* 27-kDa storage protein (*zein*) gene (GenBank® Accession Number X56117, NCBI, 2005; Das *et al.*, 1991). Provides endosperm-specific expression in *Zea mays* (Russell and Fromm, 1997).

***amy797E* (1383 bp):** Chimeric, thermostable 797GL3 alpha-amylase gene, derived from alpha-amylase genes from three hyperthermophilic microorganisms of the archael order *Thermococcales.* The *amy797E* gene includes the fusion of the 797GL3 amylase with a 19 amino acid N-terminal maize gamma-zein signal sequence (GZein ss) and a C-terminal SEKDEL endoplasmic reticulum retention signal (Lanahan *et al*., 2003). The maize gamma-zein signal sequence and the endoplasmic reticulum retention signal provide for protein targeting to and retention in the endoplasmic reticulum of the cell, respectively. The N-terminal maize gamma-zein signal sequence is cleaved from the precursor protein to yield the mature alpha-amylase protein. The alpha-amylase coding region of the *amy797E* gene was synthesized to accommodate the preferred codon usage for corn (Murray *et al.*, 1989). The alpha-amylase enzyme catalyzes the hydrolysis of starch by cleaving the internal α-1,4-glucosidic bonds into dextrins, maltose and glucose.

PEPC9 (108 bp): Intron #9 from the phosphoenolpyruvate carboxylase gene (GenBank Accession Number X15239) from *Zea mays* (Matsuoka and Minami, 1989).

35S terminator (70 bp): Terminator sequence from the cauliflower mosaic virus genome (Similar to GenBank Accession Number AF140604). Its function is to provide a polyadenylation sequence (Franck *et al*., 1980).

E2. Selectable marker cassette:

ZmUbiInt (1993 bp): Promoter region from *Zea mays* polyubiquitin gene, contains the first intron (GenBank Accession Number S94464). Provides constitutive expression in monocots (Christensen *et al*., 1992).

***pmi* (1176 bp):** *E. coli manA* gene encoding phosphomannose isomerase (GenBank Accession Number M15380). Catalyzes the isomerization of mannose-6-phosphate to fructose-6-phosphate (Negrotto *et al*., 2000).

NOS (253 bp): Terminator sequence from the nopaline synthase gene of *Agrobacterium tumefaciens* (GenBank Accession Number V00087). Its function is to provide a polyadenylation site (Depicker *et al*., 1982).

E3. Vector backbone components:

Spec (789 bp): Streptomycin adenylyltransferase, *aadA* gene from *E. coli* Tn7 (GenBank Accession Number X03043). Confers resistance to erythromycin, streptomycin, and spectinomycin; used as a bacterial selectable marker (Fling *et al*., 1985).

VS1ori (405 bp): Consensus sequence for the origin of replication and partitioning region from plasmid pVS1 of *Pseudomonas aeruginosa* (similar to GenBank Accession Number U10487). Serves as origin of replication in *Agrobacterium tumefaciens* host (Itoh *et al*., 1984).

ColE1ori (807 bp): Origin of replication that permits replication of plasmid in *E. coli*. (similar to GenBank Accession Number V00268) (Itoh and Tomizawa, 1978).

LB (25 bp): Left border region of T-DNA from *Agrobacterium tumefaciens* nopaline Ti plasmid (GenBank Accession Number J01825). 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).

RB (25 bp): Right border region of T-DNA from *Agrobacterium tumefaciens* nopaline Ti plasmid (GenBank Accession Number J01826). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell (Wang *et al*., 1984).

***virG* (726 bp):** VirGN54D from pAD1289 (similar to GenBank Accession Number AF242881). The N54D substitution results in a constitutive *virG* phenotype. VirG is part of the two-component regulatory system for the vir regulon in *Agrobacterium tumefaciens* (Hansen *et al*., 1994).

***repA* (1074 bp):** pVS1 replication protein from *Pseudomonas aeruginosa*, which is a part of the minimal pVS1 replicon that is functional in gram-negative plant-associated bacteria (GenBank Accession Number AF133831) (Heeb *et al*., 2000).

F. Genetic Analysis of Event 3272

Genetic analysis of Event 3272 included Southern analyses for both functional element copy number and generational stability, Mendelian inheritance studies and sequencing of the T-DNA insert. Backcross generations of Event 3272 were used for these studies (see Figure 3-2 and Appendix 1-Figure 1). In backcross generations, the linked transgenes segregate 1:1, with 50% of the plants containing the linked transgenes. TaqMan® PCR analyses for the *amy797E* and *pmi* transgenes were used to identify the positive and negative plant segregants in each segregating backcross population. All plants were positive for the assay's internal control, the endogenous maize *adhl* gene.

F1. Functional element copy number Southern analyses

Materials and Methods

All plants used for molecular characterization studies were individually analyzed using TaqMan® PCR. Genomic DNA was isolated from pooled leaf tissue of ten plants representing positive segregants of the backcross four (BC4) generation (see Figure 3-2 for pedigree) of Event 3272 using the method of Thomas *et al*. (1993). For the negative controls, genomic DNA was isolated from pooled leaf tissue of ten plants representing negative segregants of the BC4 generation of Event 3272.

Southern analysis was performed using standard molecular biology techniques (Chomczynski, 1992). Genomic DNA (7.5 µg) was digested with a restriction enzyme that cuts within the Event 3272 T-DNA insert from plasmid pNOV7013 (Figure 3-1), but not within the functional element that corresponds to the specific probe used in the experiment. This approach allows for determination of the number of copies of the element, corresponding to the specific probe used for each Southern analysis, which have been incorporated into Event 3272. This results in one hybridization band per copy of the element present in Event 3272. A second series of digests was performed in which the T-DNA insert was digested with a restriction enzyme(s) that would release a known size fragment for the entire T-DNA insert. This approach provides additional evidence for the presence of a single copy of each functional element present in Event 3272. Following agarose gel electrophoresis and alkaline transfer to a Nytran® membrane, hybridizations were carried out using element specific full-length PCR-generated probes (see Figures 3-3, 3-5, 3-7, 3-9 and 3-11). The probes were labeled with ^{32}P *via* random priming using the Rediprime II™ system (Amersham Biosciences, Cat. No. RPN1633).

Included in each Southern analysis were three control samples:

1. DNA from a negative (non-transformed) segregant used to identify any endogenous *Zea mays* sequences that may cross-hybridize with the element-specific probe.
2. DNA from a negative segregant into which is introduced an amount of digested pNOV7013 plasmid that is equal to varying copy equivalents based on probe length (Table 3-1), to demonstrate the sensitivity of the experiment

in detecting the indicated copy equivalent within the *Zea mays* genome. An example calculation for the *amy797E* probe is included.[1]

3. Digested pNOV7013 plasmid that is equal to the indicated copy equivalent based on probe length, to demonstrate a positive control for hybridization as well as the sensitivity of the experiment.

Table 3-1. Copy number equivalents for the probes employed in Southern analysis of Event 3272.

Probe	Size in base pairs	pg loaded on the Southern blot	Copy number equivalent
amy797E	1383	1.94	0.12
pmi	1176	1.65	0.10
GZein	677	0.95	0.06
ZmUbiInt	1993	2.80	0.17
Backbone	5309	7.46	0.46

Results

These hybridization data provide evidence that Event 3272 contains a single copy of the *amy797E* gene and the *pmi* gene. Additionally, the data demonstrate that Event 3272 contains a single copy of the GZein promoter and ZmUbiInt promoter. As expected for the *amy797E* (Figure 3-4), *pmi* (Figure 3-6), GZein (Figure 3-8) and ZmUbiInt (Figure 3-10) probes, the restriction enzyme digests resulted in a single hybridization band specific to the Event 3272 insert, demonstrating that a single copy of each element is present in Event 3272. The second hybridization bands seen in Event 3272 with the Gzein and ZmUbiInt probes represent hybridization to the endogenous maize sequences. Additionally for the backbone probe (Figure 3-12), lack of hybridization demonstrates the absence of any pNOV7013 vector backbone sequences being incorporated into Event 3272 during the transformation process.

[1] Formula to determine copy number equivalent based on probe length

((Probe size/(Genome size*Ploidy))*µg loaded)*1.00E+06) =	pg for copy equivalent
Example	
Zea mays genome size in bp:	2.67E+09
Ploidy:	2
Amy797E probe size in bp:	1383
µg *KpnI* digested DNA loaded on Southern:	7.5
Calculation for *amy797E*: ((1383/(2.67E+09*2))*7.5)*1.00E+06)=	1.94 pg

F2. Generational stability Southern analysis

Materials and Methods

All plants used for generational stability Southern analysis were individually analyzed using TaqMan® PCR. Genomic DNAs were isolated from pooled leaf tissue of ten positive segregant plants each from the backcross one (BC1), backcross two (BC2) and backcross three (BC3) generations of Event 3272 (see Figure 3-2 for pedigrees) using the method of Thomas *et al*. (1993). For the negative controls, genomic DNA was isolated from pooled leaf tissue of ten plants representing negative segregants of the BC3 generation of Event 3272.

Results

The hybridization pattern over three generations of Event 3272 was identical using an *amy797E* probe (Figure 3-14). The hybridization data demonstrated that the T-DNA insert from pNOV7013 incorporated into Event 3272 is stable over several generations.

F3. T-DNA insert sequencing

Materials and Methods

The nucleotide sequence of the entire T-DNA insert present in Event 3272 was determined to demonstrate overall integrity of the insert, contiguousness of the functional elements and to detect any individual base pair changes. The Event 3272 insert was amplified from DNA derived from the BC4 generation as two individual overlapping fragments (Figure 3-15). Each fragment was amplified using one oligonucleotide complementary to plant genomic sequences flanking the Event 3272 insert and one oligonucleotide complementary to the T-DNA insert. PCR amplification was carried out using the Expand High Fidelity PCR system (Roche, Cat. No. 1732650). Each sequencing fragment was individually cloned into the pCR®-XL-TOPO vector (Invitrogen, Cat. No. K4700-20) and three separate clones for each fragment were identified and sequenced. Sequencing was carried out using the ABI3730XL analyzer using ABI BigDye® 1.1 or Big Dye 3.1 dGTP (for GC rich templates) chemistry. The sequence analysis was done using the Phred, Phrap, and Consed package from the University of Washington and was carried out to an error rate of less than 1 in 10,000 bases (Ewing and Green, 1998, Gordon *et al.* 1998). The final consensus sequence was determined by combining the sequence data from the six individual clones (three for each sequencing fragment) to generate one consensus sequence of the Event 3272 insert. 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).

Results

The consensus sequence data for the Event 3272 T-DNA insert demonstrated the overall integrity of the insert and that contiguousness of the functional elements within the insert as intended in pNOV7013 has been maintained. Sequence analysis revealed that some truncation occurred at the RB and LB ends of the T-DNA insert most likely during the transformation process that resulted in Event 3272. The RB portion of the T-DNA insert was truncated by 23 bp and the LB end of the T-DNA insert was truncated by 7 bp. These deletions have no effect on the efficacy of the T-DNA insert, and this phenomenon has also been previously observed in *A. tumefaciens* transformation (Tinland and Hohn, 1995).

F4. Mendelian inheritance of transgene insert

Materials and Methods

The inheritance pattern of the T-DNA insert derived from pNOV7013 in Event 3272 was investigated. An Event 3272 plant (T1 generation) was crossed with maize inbred line NP2222, creating the F1 generation (Figure 3-2). A single Event 3272 plant from this F1 generation was backcrossed to the recurrent inbred parent NP2222 to yield the BC1 generation. A single Event 3272 plant from this BC1 generation was backcrossed to the recurrent inbred parent NP2222 to yield the BC2 generation. A single Event 3272 plant from this BC2 generation was backcrossed to the recurrent inbred parent NP2222 to yield the BC3 generation. Finally, a single Event 3272 plant from this BC3 generation was backcrossed to the recurrent inbred parent NP2222 to yield the BC4 generation.

Individual plants from these BC1, BC2, BC3 and BC4 generations were assayed for the presence of the *amy797E* gene by TaqMan® PCR analysis (Table 3-2). The expected Mendelian inheritance ratio of positive and negative plants for a hemizygous trait in these populations is 1:1.

Results

Genotypic data (Table 3-2) were used to assess the goodness-of-fit of the observed genotypic ratio to the expected genotypic ratio using Chi Square (X^2) analysis with Yates correction factor (Strickberger, 1976).

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

This analysis tested the hypothesis that the genetic trait is segregating in a Mendelian fashion. The critical value to reject the hypothesis at the 5% level is 3.84 (Strickberger, 1976). Since the Chi squared value is less than 3.84 for all generations tested the hypothesis that the genetic trait is behaving in a Mendelian fashion is accepted for all generations.

Table 3-2. Observed vs. expected* genotype for multiple 3272 generations as determined by Taqman® PCR analysis

	BC1		BC2		BC3		BC4	
	O*	**E***	**O***	**E***	**O***	**E***	**O***	**E***
Trait Positive	95	90.5	15	17.5	122	119	105	102.5
Trait Negative	86	90.5	20	17.5	116	119	100	102.5
Total	181	181	35	35	238	238	205	205
X^2 value	0.354		0.457		0.105		0.078	

* O = Observed values and E = Expected values

F5. Conclusions for the genetic analysis of Event 3272

Data from Southern analyses and DNA sequencing demonstrated that single copies of the *amy797E* gene, phosphomannose isomerase (*pmi*) gene, GZein promoter and ZmUbiInt promoter are present in Syngenta's maize Event 3272. Event 3272 does not apparently contain any of the backbone sequences from the transformation plasmid pNOV7013. Additionally, the T-DNA insert is stable over several generations of Event 3272. Sequence analysis of the entire T-DNA insert present in Event 3272 confirms that the overall integrity of the insert and contiguousness of the functional elements has been maintained. A 23 bp truncation at the right border (RB) junction of the T-DNA insert and 7 bp truncation at the left border (LB) junction of the T-DNA insert were identified. Statistical analyses confirmed the expected Mendelian inheritance ratio of the *amy797E* gene.

Figure 3-1. Plasmid map of pNOV7013 indicating the restriction sites used for Southern analysis

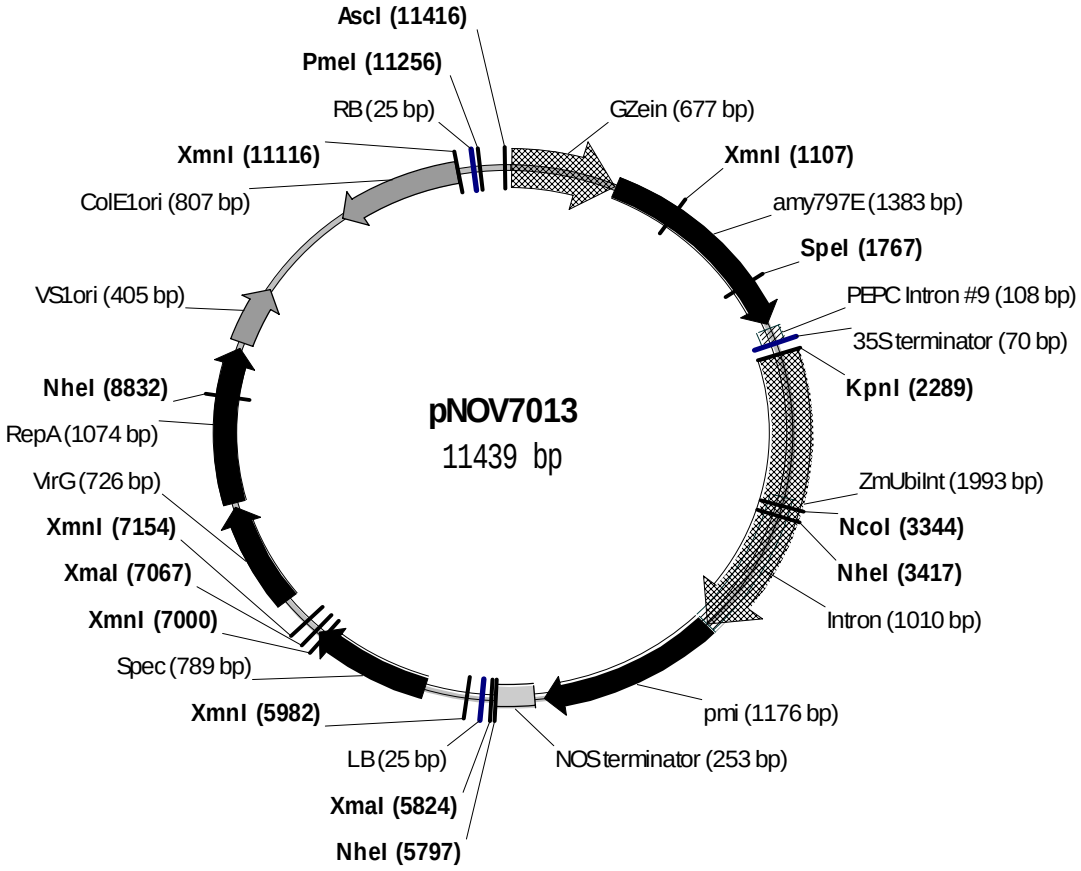
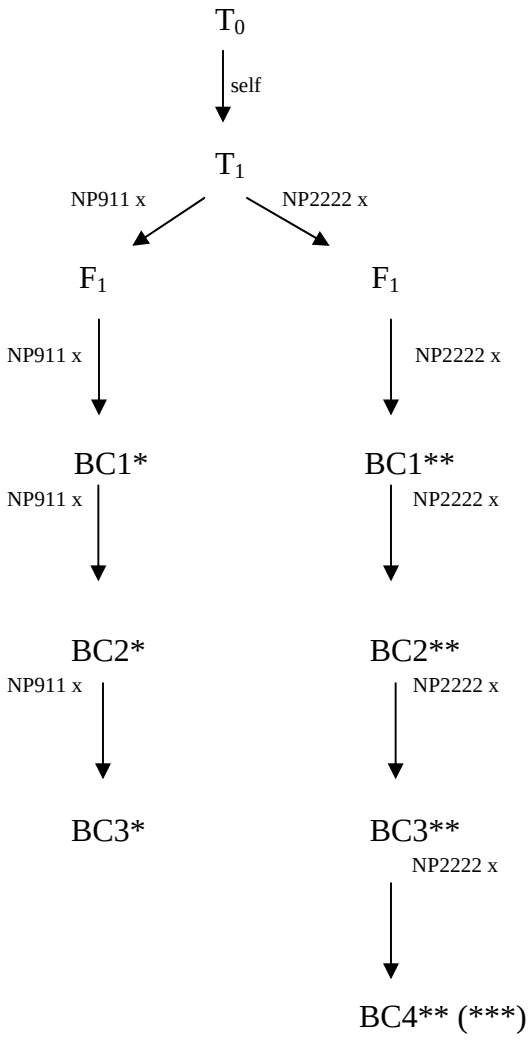


Figure 3-2. Breeding pedigree indicating the generations tested in the molecular analysis of Event 3272



*Materials used for generational stability Southern analysis

**Materials used for Mendelian inheritance studies

***Materials used for all functional element Southern analyses, T-DNA insert sequencing

T_0 = initial Event 3272 transformed plant
BC = backcross generation
NP911 and NP2222 are maize inbreds

Figure 3-3. Location of *Asc*I, *Kpn*I and *Xma*I restriction sites and position of *amy797E* probe in the T-DNA region of transformation vector pNOV7013 introduced into Event 3272

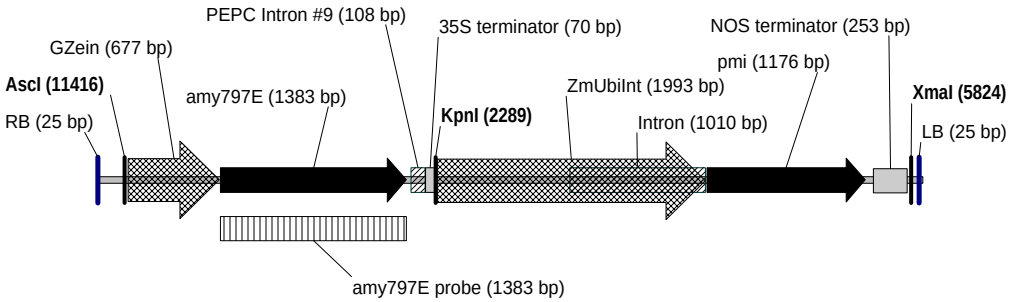


Figure 3-4. Southern analysis of Event 3272 with *amy797E*-specific probe

Maize genomic DNA (7.5 µg) was digested with *Asc*I, *Kpn*I and *Xma*I restriction enzymes and, following electrophoresis and transfer to a Nytran membrane, hybridized to an *amy797E*-specific probe (1383 bp). Lane 1: Molecular weight markers (Kb DNA ladder, Stratagene Cat. No. 201115-81; The molecular weight markers were measured from the center of each marker to determine bp size.); Lane 2: Blank; Lane 3: BC4 generation of Event 3272 digested with *Kpn*I; Lane 4: Negative segregants from BC4 generation of Event 3272 digested with *Kpn*I; Lane 5: BC4 generation of Event 3272 digested with *Asc*I/*Xma*I; Lane 6: Negative segregants from BC4 generation of Event 3272 digested with *Asc*I/*Xma*I; Lane 7: Negative segregants from BC4 generation of Event 3272 digested with *Asc*I/*Xma*I and spiked with 1.94 pg of *Asc*I/*Xma*I digested pNOV7013 DNA; Lane 8: Blank; Lane 9: 1.94 pg *Asc*I/*Xma*I digested pNOV7013 DNA (equivalent to 0.12 copies based on probe length).

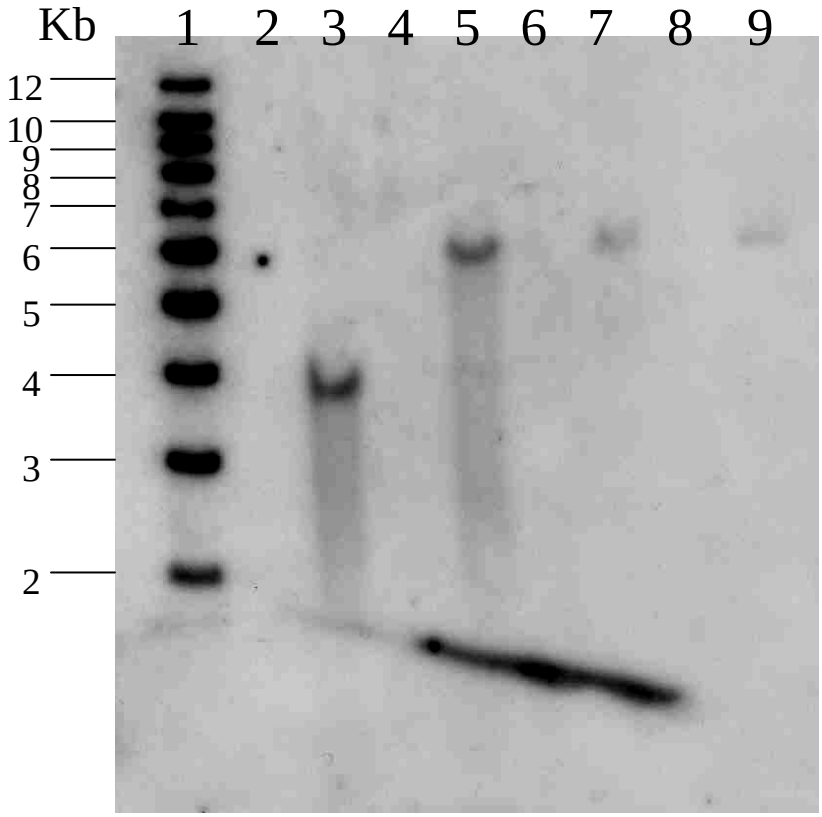


Figure 3-5. Location of *Asc*I, *Xma*I and *Xmn*I restriction sites and position of *pmi* probe in the T-DNA region of transformation vector pNOV7013 introduced into Event 3272

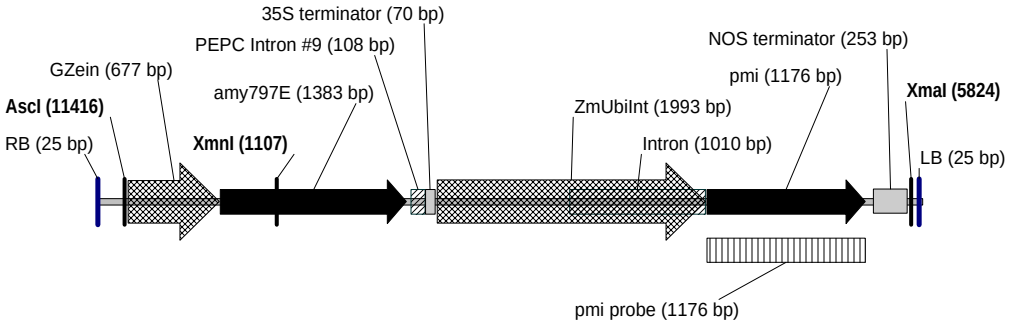


Figure 3-6. Southern analysis of Event 3272 with *pmi*-specific probe

Maize genomic DNA (7.5 µg) was digested with *Asc*I, *Xma*I and *Xmn*I restriction enzymes and, following electrophoresis and transfer to a Nytran membrane, hybridized to a *pmi*-specific probe (1176 bp). Lane 1: Molecular weight markers (Kb DNA ladder, Stratagene Cat. No. 201115-81; The molecular weight markers were measured from the center of each marker to determine bp size.); Lane 2: Blank; Lane 3: BC4 generation Event 3272 digested with *Xmn*I; Lane 4: Negative segregants from BC4 generation of Event 3272 digested with *Xmn*I; Lane 5: BC4 generation of Event 3272 digested with *Asc*I/*Xma*I; Lane 6: Negative segregants from BC4 generation of Event 3272 digested with *Asc*I/*Xma*I; Lane 7: Negative segregants from BC4 generation of Event 3272 digested with *Asc*I/*Xma*I and spiked with 1.65 pg of *Asc*I/*Xma*I digested pNOV7013 DNA; Lane 8: Blank; Lane 9: 1.65 pg of *Asc*I/*Xma*I digested pNOV7013 DNA (equivalent to 0.10 copies based on probe length).

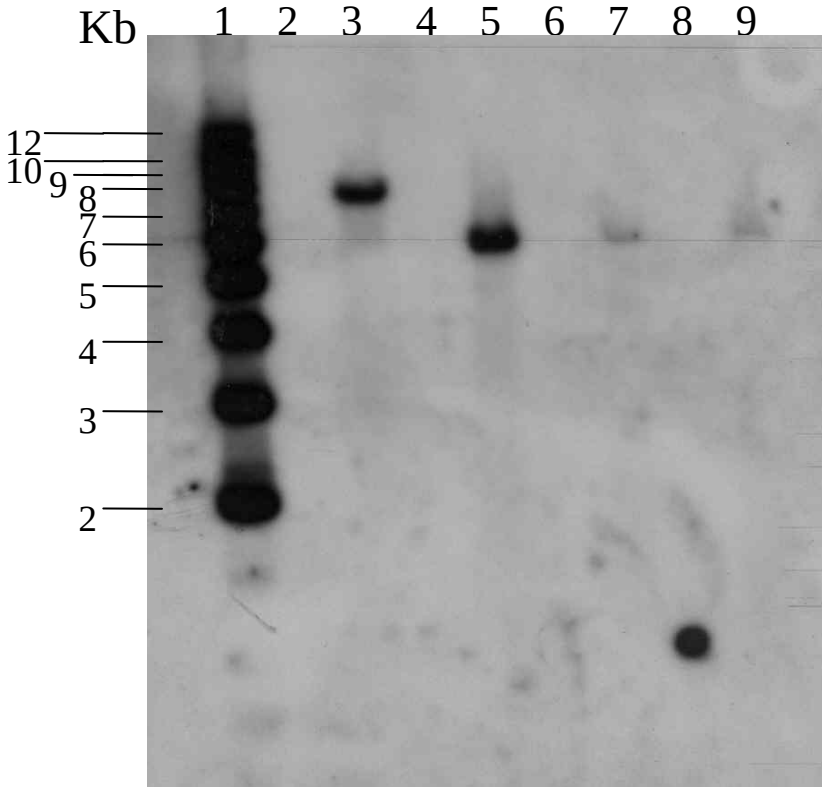


Figure 3-7. Location of *Asc*I, *Nco*I and *Xma*I restriction sites and position of GZein probe in the T-DNA region of transformation vector pNOV7013 introduced into Event 3272

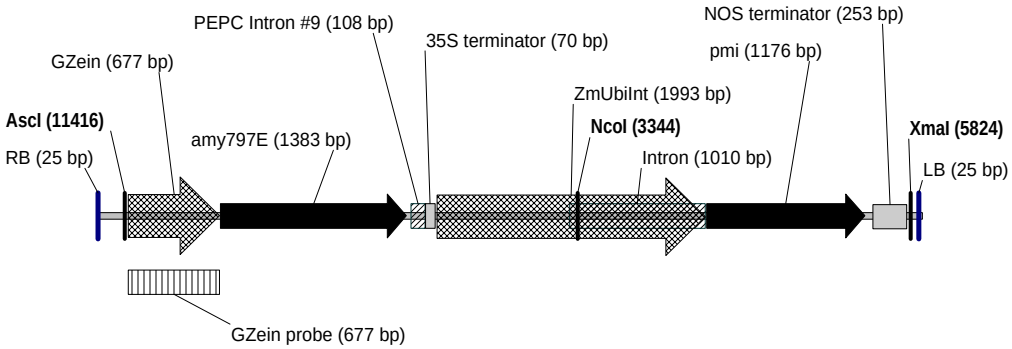


Figure 3-8. Southern analysis of Event 3272 with Gzein-specific probe

Maize genomic DNA (7.5 µg) was digested with *Asc*I, *Nco*I and *Xma*I restriction enzymes and, following electrophoresis and transfer to a Nytran membrane, hybridized to a Gzein-specific probe (677 bp). Lane 1: Molecular weight markers (Kb DNA ladder, Stratagene Cat. No. 201115-81; The molecular weight markers were measured from the center of each marker to determine bp size.); Lane 2: Blank; Lane 3: BC4 generation of Event 3272 digested with *Nco*I; Lane 4: Negative segregants from BC4 generation of Event 3272 digested with *Nco*I; Lane 5: BC4 generation of Event 3272 digested with *Asc*I/*Xma*I; Lane 6: Negative segregants from BC4 generation of Event 3272 digested with *Asc*I/*Xma*I; Lane 7: Negative segregants from BC4 generation of Event 3272 digested with *Asc*I/*Xma*I and spiked with 0.9 pg of *Asc*I/*Xma*I digested pNOV7013 DNA; Lane 8: Blank; Lane 9: 0.9 pg of *Asc*I/*Xma*I digested pNOV7013 DNA (equivalent to 0.06 copies based on probe length).

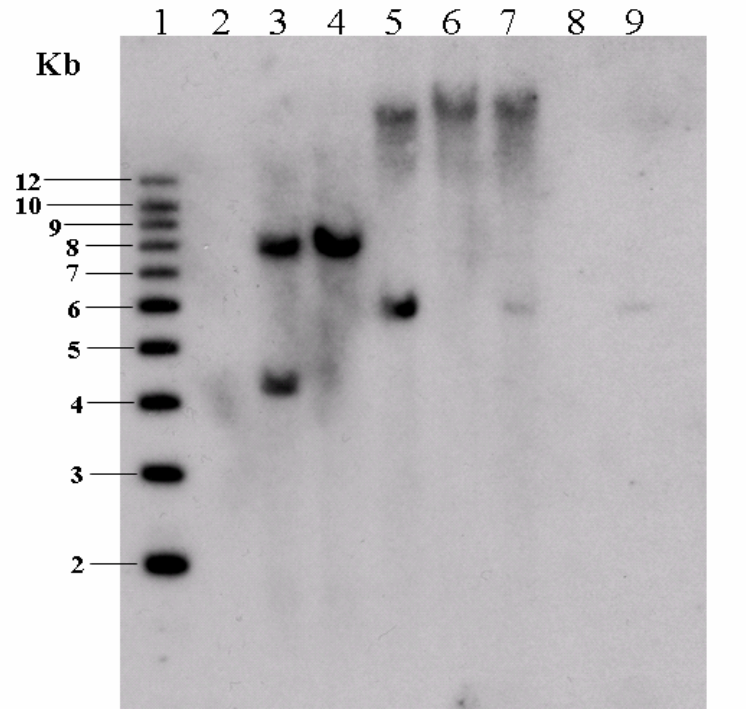


Figure 3-9. Location of *Asc*I, *Spe*I and *Xma*I restriction sites and position of ZmUbiInt probe in the T-DNA region of transformation vector pNOV7013 introduced into Event 3272

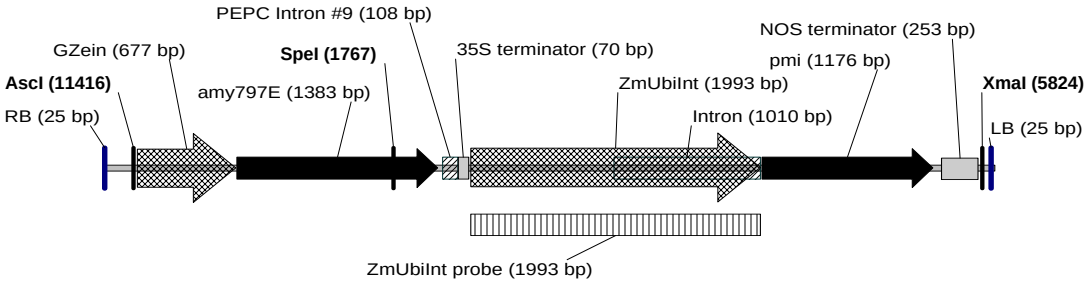


Figure 3-10. Southern analysis of Event 3272 with ZmUbiInt-specific probe

Maize genomic DNA (7.5 µg) was digested with *Asc*I, *Spe*I and *Xma*I restriction enzymes and, following electrophoresis and transfer to a Nytran membrane, hybridized to a ZmUbiInt-specific probe (1993 bp). Lane 1: Molecular weight markers (Kb DNA ladder, Stratagene Cat. No. 201115-81; The molecular weight markers were measured from the center of each marker to determine bp size.); Lane 2: Blank; Lane 3: BC4 generation Event 3272 digested with *Spe*I; Lane 4: Negative segregants from BC4 generation of Event 3272 digested with *Spe*I; Lane 5: BC4 generation of Event 3272 digested with *Asc*I/*Xma*I; Lane 6: Negative segregants from BC4 generation of Event 3272 digested with *Asc*I/*Xma*I; Lane 7: Negative segregants from BC4 generation of Event 3272 digested with *Asc*I/*Xma*I and spiked with 2.80 pg of *Asc*I/*Xma*I digested pNOV7013 DNA; Lane 8: Blank; Lane 9: 2.80 pg of *Asc*I/*Xma*I digested pNOV7013 DNA (equivalent to 0.17 copies based on probe length).

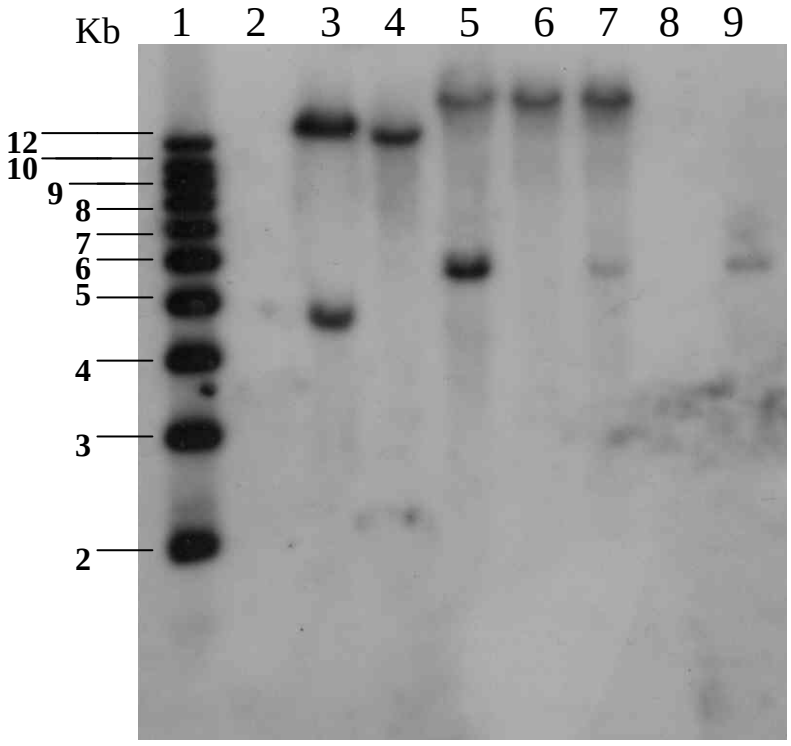


Figure 3-11. Location of *Kpn*I, *Nhe*I and *Pme*I restriction sites and position of backbone probe in the transformation vector pNOV7013

Encompasses all base pairs outside of the LB and RB regions.

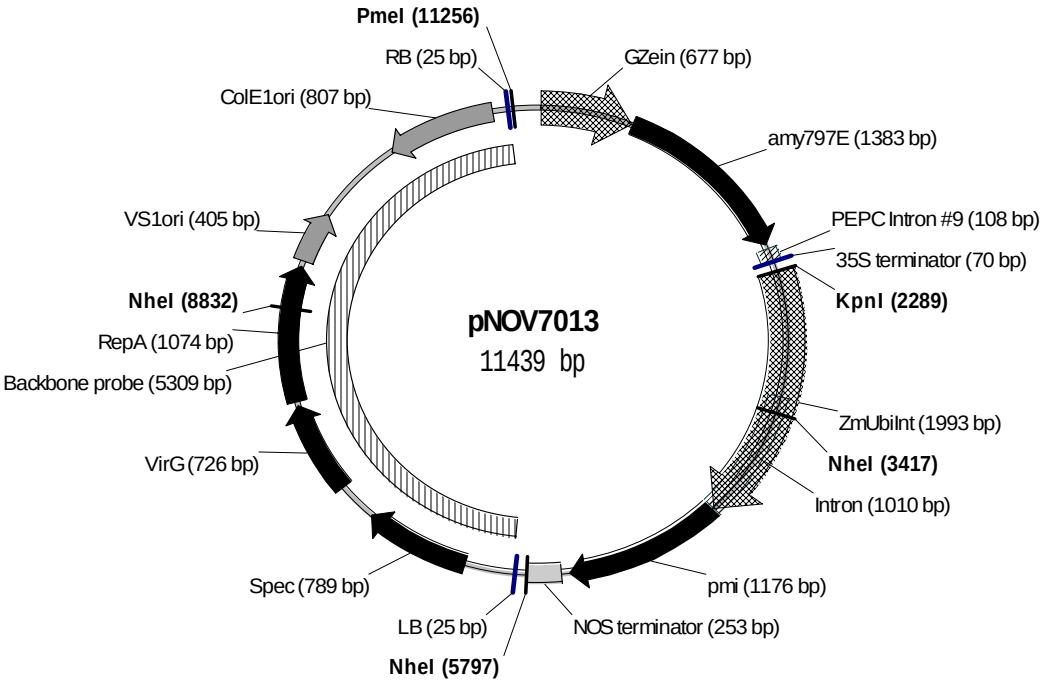


Figure 3-12. Southern analysis of Event 3272 with backbone-specific probe

Maize genomic DNA (7.5 µg) was digested with *Kpn*I restriction enzyme and, following electrophoresis and transfer to a Nytran membrane, hybridized to a backbone-specific probe (5309 bp). Lane 1: Molecular weight markers (Kb DNA ladder, Stratagene Cat. No. 201115-81; The molecular weight markers were measured from the center of each marker to determine bp size.); Lane 2: Blank; Lane 3: BC4 generation Event 3272; Lane 4: Negative segregants from BC4 generation of Event 3272; Lane 5: Negative segregants from BC4 generation of Event 3272 spiked with 7.46 pg of *Nhe*I/*Pme*I digested pNOV7013 DNA; Lane 6: Blank; Lane 7: 7.46 pg *Nhe*I/*Pme*I digested pNOV7013 DNA (equivalent to 0.46 copies based on probe length).

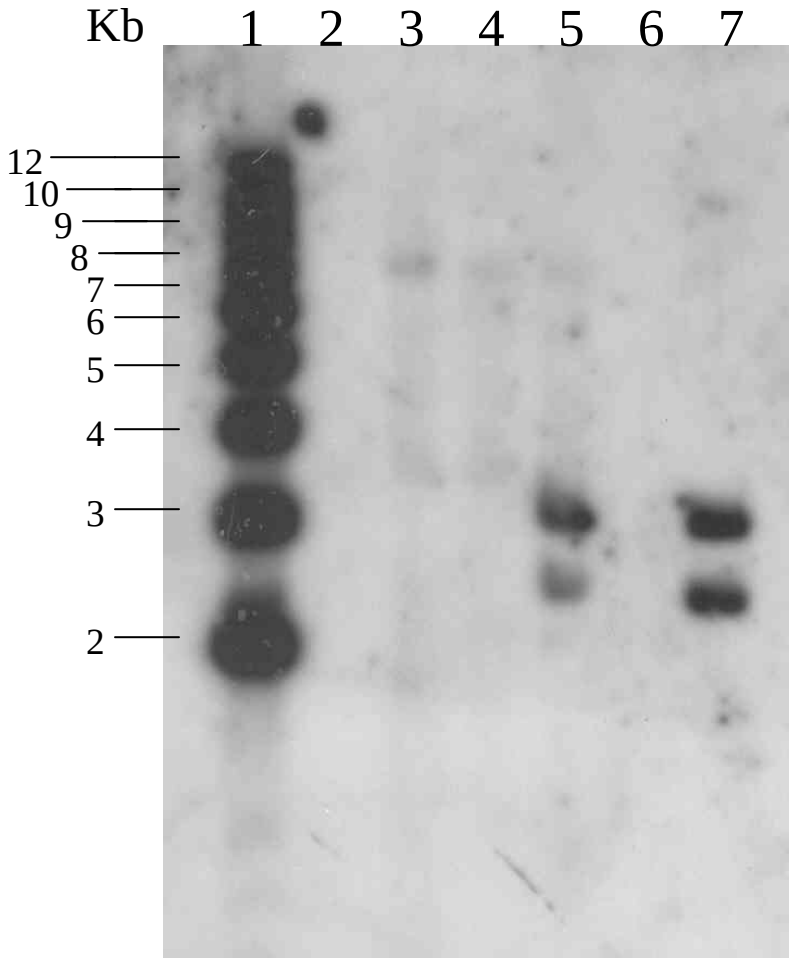


Figure 3-13. Location of *Kpn*I restriction site and position of *amy797E* probe in the T-DNA region of transformation vector pNOV7013 introduced into Event 3272.

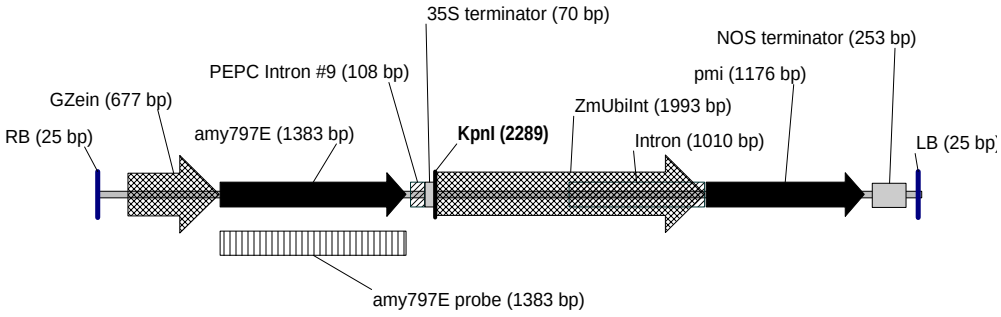


Figure 3-14. Generational stability Southern analysis of backcrosses one (BC1), two (BC2) and three (BC3) of Event 3272 with *amy797E*-specific probe

Maize genomic DNA (7.5 µg) were digested with *Kpn*I restriction enzyme and, following electrophoresis and transfer to a Nytran membrane, hybridized to an *amy797E*-specific probe (1383 bp). Lane 1: Molecular weight markers (Kb DNA ladder, Stratagene Cat. No. 201115-81; The molecular weight markers were measured from the center of each marker to determine bp size.); Lane 2: Blank; Lane 3: BC1 generation Event 3272; Lane 4: BC2 generation Event 3272; Lane 5: BC3 generation Event 3272; Lane 6: Negative segregants from BC3 generation of Event 3272; Lane 7: Negative segregants from BC3 generation of Event 3272 spiked with 1.94 pg of *Kpn*I digested pNOV7013 DNA; Lane 8: Blank; Lane 9: 1.94 pg of *Kpn*I digested pNOV7013 DNA (equivalent to 0.12 copies based on probe length).

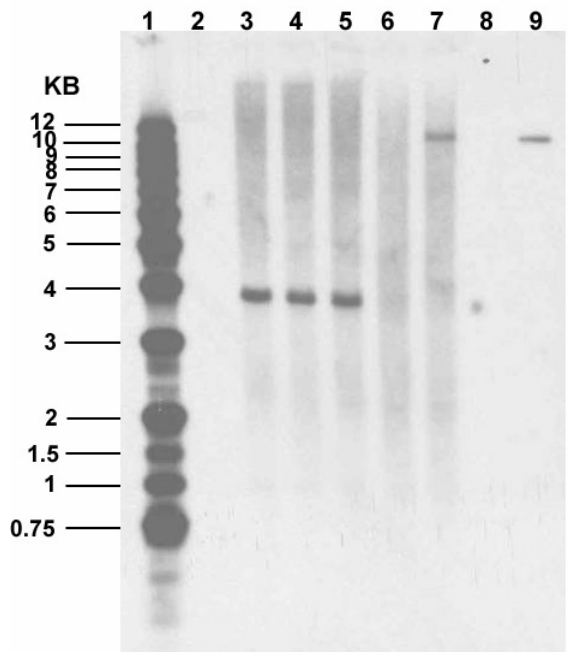
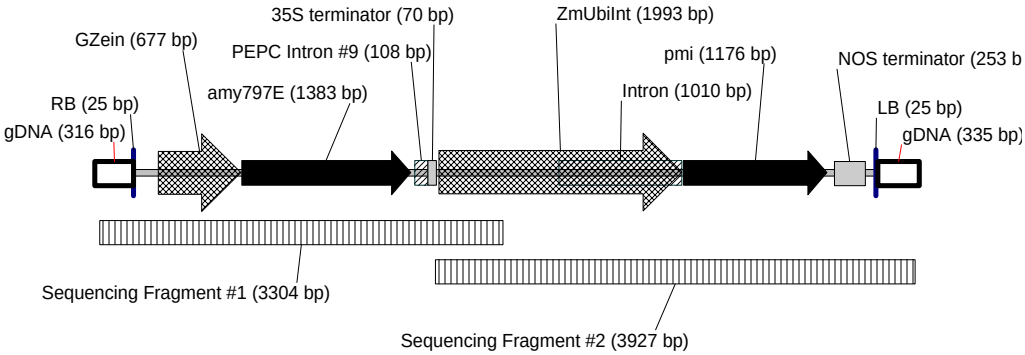


Figure 3-15. Location of fragments amplified from Event 3272 to determine insert sequence



G. Expression of AMY797E and PMI Proteins in Event 3272

Expression of the AMY797E alpha-amylase protein in Event 3272 plants is driven by the maize gamma-zein promoter, which directs expression to the maize kernel, and PMI expression is driven by the constitutive maize polyubiquitin promoter. Confirmation of the expression patterns and levels of the AMY797E and PMI proteins in Event 3272 was conducted for the purpose of determining potential exposure of wildlife to AMY797E and PMI for environmental risk assessment (see Chapter 8).

AMY797E and PMI protein expression in Event 3272 and near-isogenic, non-transgenic control plants was evaluated using ELISA methods as described in Appendix 2. Two Event 3272 maize hybrids (hybrids A1 and B1) and corresponding control maize hybrids were grown in Bloomington, IL in 2003 (see Appendix 1-Figure 1). Several plant tissues (leaves, roots, kernels, and pollen) at five developmental stages (whorl, anthesis, kernel dough, kernel maturity and senescence) were evaluated. AMY797E and PMI protein levels were determined on a microgram (µg) per gram (g) fresh weight (fw) basis and are presented as the means of five replicate tissue samples for each developmental stage.

As expected, the highest levels of AMY797E protein in Event 3272 were detected in maize kernels at the dough stage, maturity stage, and at senescence (Table 3-3). Mean AMY797E levels in kernels of both Event 3272 hybrids at all three stages ranged from 838 to 1627 µg/g fw. Quantifiable levels of AMY797E protein below 0.4 µg/g fw were detected in four of the ten root samples of whorl-stage plants (0.01, 0.06, 0.09, and 0.36 µg/g fw) but was either not detectable (ND) or below the limit of quantitation (<LOQ) for all other root samples at whorl stage and other stages of development. AMY797E protein was not detectable in pollen and detected in only one leaf sample at 1 µg/g fw from senescence stage plants. Control sample levels from non-transgenic, near-isogenic plants were either ND or <LOQ for all stages and tissues. These results confirm kernel-specific expression of AMY797E in Event 3272 plants. The low levels of AMY797E detected in some root samples of whorl-stage plants were more than two thousand fold less than levels detected in kernels. This low level detected in some root samples at the whorl stage is likely due to AMY797E protein in remnants of the germinated seed attached to the root ball.

PMI was detected in all of the plant tissues analyzed, except for some senescence-stage leaf samples (Table 3-4). Control sample levels from non-transgenic, near-isogenic plants were either ND or <LOQ for all stages and tissues. Mean PMI levels measured in leaves at whorl, anthesis and kernel dough stages ranged from 1.3 to 5.0 µg/g fw. PMI levels in leaves at kernel maturity stage ranged from <LOQ - 1.9 µg/g fw. PMI protein levels in leaves from the senescence stage were either not detected ND or <LOQ. PMI levels measured at all development stages in kernels ranged from <LOQ to 0.9 µg/g fw and in root tissue ranged from <LOQ to 1.0 µg/g fw. PMI levels detected in pollen were 8.0 µg/g fw (hybrid A1) and 8.5 µg/g fw (hybrid B1).

These results confirm expression of the AMY797E and PMI proteins in Event 3272 and that AMY797E is predominantly expressed in grain, while PMI is expressed throughout most maize tissues.

Table 3-3. AMY797E levels (µg/g fresh weight) in Event 3272 plant tissue[1]

Tissue	Hybrids	Developmental Stage									
		Whorl		Anthesis		Kernel Dough		Kernel Maturity		Senescence	
		mean ± SD[2] (range)	LOQ/ LOD[3] (µg/g)	mean ± SD (range)	LOQ/ LOD (µg/g)	mean ± SD (range)	LOQ/ LOD (µg/g)	mean ± SD (range)	LOQ/ LOD (µg/g)	mean ± SD (range)	LOQ/ LOD (µg/g)
Leaves	A1	ND[4] (ND)	0.007/ 0.003	ND (ND)	0.01/ 0.004	ND (ND)	0.01/ 0.006	ND (ND)	0.02/ 0.01	ND (ND)	0.02/ 0.01
	B1	ND (ND)		ND (ND)		ND (ND)		(ND - <LOQ)		<1[5] (ND - 1)	
Roots	A1	<0.1[5] (ND - 0.1)	0.004/ 0.002	ND (ND)	0.007/ 0.004	ND (ND)	0.01/ 0.004	ND (ND)	0.01/ 0.004	ND (ND)	0.01/ 0.004
	B1	<0.1[5] (ND - 0.4)		(ND - <LOQ)		(ND - <LOQ)		(ND - <LOQ)		not analyzed	
Kernels	A1					1627 ± 338 (1177 - 1951)	0.02/ 0.007	905 ± 208 (659 - 1147)	0.04/ 0.01	955 ± 225 (679 - 1294)	0.04/ 0.01
	B1					874 ± 160 (638 - 1057)		924 ± 201 (686 - 1130)		838 ± 268 (512 - 1168)	
Pollen	A1			ND							
	B1			ND							

[1] Number of samples analyzed (N), N = 5 except pollen, which was a single pooled sample.

[2] SD = standard deviation [3] LOQ = limit of quantitation, LOD = limit of detection [4] ND = not detectable, below the LOD

[5] Some of the values were <LOQ or ND. The mean shown is expressed as < (average of the quantifiable values)

Table 3-4. PMI levels (µg/g fresh weight) in Event 3272 plant tissue[1]

Tissue	Hybrids	Developmental Stage									
		Whorl		Anthesis		Kernel Dough		Kernel Maturity		Senescence	
		mean ± SD[2] (range)	LOQ/ LOD[3] (µg/g)	mean ± SD (range)	LOQ/ LOD (µg/g)	mean ± SD (range)	LOQ/ LOD (µg/g)	mean ± SD (range)	LOQ/ LOD (µg/g)	mean ± SD (range)	LOQ/ LOD (µg/g)
Leaves	A1	2.3 ± 0.3 (1.9 - 2.7)	0.04/ 0.001	2.8 ± 0.5 (2.2 - 3.4)	0.06/ 0.002	5.0 ± 0.7 (4.0 - 5.7)	0.09/ 0.003	<1.9[5] (<LOQ - 1.9)	0.15/ 0.005	(ND[4] - <LOQ)	0.16/ 0.006
	B1	1.3 ± 0.5 (0.6 - 1.8)		2.3 ± 0.5 (1.9 - 3.0)		3.5 ± 1.1 (2.3 - 5.1)		1.1 ± 0.7 (0.2 - 1.9)		(ND - <LOQ)	
Roots	A1	0.3 ± 0.2 (0.2 - 0.5)	0.03/ 0.001	0.2 ± 0.1 (0.1 - 0.3)	0.06/ 0.002	0.3 ± 0.1 (0.2 - 0.4)	0.07/ 0.002	<0.2[5] (<LOQ - 0.3)	0.06/ 0.002	<0.1[5] (<LOQ - 0.1)	0.07/ 0.001
	B1	0.8 ± 0.1 (0.6 - 1.0)		0.3 ± 0.1 (0.2 - 0.5)		0.2 ± 0.1 (0.1 - 0.3)		0.3 ± 0.2 (0.2 - 0.5)		not analyzed	
Kernels	A1					0.4 ± 0.02 (0.3 - 0.4)	0.15/ 0.005	<0.4[5] (<LOQ - 0.5)	0.22/ 0.008	<0.4[5] (<LOQ - 0.4)	0.26/ 0.01
	B1					0.8 ± 0.1 (0.6 - 0.9)		0.5 ± 0.2 (0.4 - 0.7)		0.4 ± 0.1 (0.3 - 0.6)	
Pollen	A1			8.0	0.14/ 0.01						
	B1			8.5							

[1] Number of samples analyzed (N), N = 5 except pollen, which was a single pooled sample

[2] SD = standard deviation [3] LOQ = limit of quantitation, LOD = limit of detection [4] ND = not detectable, below the LOD

[5] Some of the values were <LOQ or ND. The mean shown is expressed as < (average of the quantifiable values)

G1. Estimates of AMY797E and PMI Levels for Wildlife Exposure Assessment

Wildlife will be exposed to AMY797E through consumption of kernels and PMI through consumption of leaves or kernels. A highly conservative estimate of potential wildlife exposure to these proteins was calculated based on the highest level detected in the tissues that would be consumed. In addition, those highest levels in Table 3-3 and 3-4 were adjusted for extraction efficiency (see Appendix 2). The resulting values presented in Table 3-5 are used in the environmental risk assessment described in Chapter 8.

Table 3-5. AMY797E and PMI levels for wildlife exposure assessment

Protein	**Tissue**	**Development Stage**	**Highest Level (µg/g)**	**Extraction Efficiency (%)**	**Risk Assessment Exposure Level (µg/g)**
AMY797E	kernels	kernel dough	1951	91.0	2144
PMI	leaves	kernel dough	5.7	78.8	7.2
PMI	kernels	kernel dough	0.9	100	0.9

Chapter 4. AMY797E ALPHA-AMYLASE AND PHOSPHOMANNOSE ISOMERASE PROTEINS

A. AMY797E Alpha-Amylase Protein

A1. Enzyme name

The alpha-amylase protein in Event 3272 maize is designated AMY797E.

A2. Enzyme identity

The enzyme properties of the AMY797E alpha-amylase protein are described below:

Classification:	alpha-amylase
Systematic name:	1,4-α-D-glucan glucanohydrolase
Other name(s):	alpha-amylase; endoamylase; glycogenase
IUBMB classification:	EC 3.2.1.1
CAS No.:	9000-90-2
EINECS No.:	232-565-6
Specificity:	1,4-α-glucosidic linkages in amylose and amylopectin
Molecular weight:	50.2 kDa

A3. Source and amino acid sequence

The alpha-amylase enzyme, AMY797E, is a chimeric enzyme derived from three wild-type alpha-amylases from the archael order *Thermococcales*. This enzyme was selected for development due to its increased thermostability and activity during the high temperatures required for starch hydrolysis in corn processing.

The three parental alpha-amylases used to develop AMY797E came from three naturally-occurring different sources. The source of the parental alpha-amylases and the Gene Reassembly™ (Diversa Corporation, San Diego, CA) technique used to create AMY797E are described in detail elsewhere (Richardson *et al.*, 2002). Two of the parental alpha-amylase genes, BD5031 and BD5064, were isolated from microbial DNA libraries constructed from pure cultures of *Thermococcus* organisms isolated from samples taken from shallow marine hydrothermal systems at 95 ^{o}C, pH 7.0 and 85 ^{o}C, pH 6.0, respectively. The third parental alpha-amylase gene, BD5063, originated from a microbial DNA library constructed from a primary enrichment culture containing an undetermined number of high temperature organisms isolated from the Deep Sea Pacific Ocean at 90 ^{o}C, pH 6.5. Based on sequence comparisons, the most likely source of BD5063 is either a *Pyrococcus* or *Thermococcu*s species within the order *Thermococcales*. These three alpha-amylase enzymes were selected for their superior activity under either high temperature, low calcium or low pH conditions, all relevant to the starch liquefaction step of corn processing. In order to combine the best features of all three enzymes, a "gene reassembly" recombinant technique was performed in which fragments from the three parental genes were

combined (in the same relative position) to create a library of recombinant alpha-amylase enzymes. The chimeric AMY797E alpha-amylase enzyme (alternatively known as "797GL3") was identified by screening these recombined enzymes and is composed of four fragments from BD5031, two fragments from BD5064 and three fragments from BD5063. Targeting signals were added at the N and C-terminus of the alpha-amylase sequence (maize gamma-zein signal sequence and SEKDEL sequence, respectively, for targeting to and retention in the endoplasmic reticulum of the cell) to optimize protein expression. Cleavage of the N-terminal signal sequence from the preprotein (i.e. precursor protein) yields the mature AMY797E protein (*ca*. 50.2 kDa). The amino acid sequence of the AMY797E precursor protein is shown in Figure 4-1.

A4. Sequence comparison to other alpha-amylases

The AMY797E alpha-amylase enzyme is most similar to the BD5088 alpha-amylase developed by Innovase LLC (Innovase) for use as an enzyme in the hydrolysis of edible starch to produce various food products and for ethanol production for use in alcoholic beverages. BD5088 was produced using the same gene reassembly technique from the same three parental alpha-amylase genes as AMY797E (Richardson *et al.*, 2002). The AMY797E alpha-amylase protein has 93% amino acid identity to BD5088 (Figure 4-2). BD5088 was the subject of GRAS Notice No. GRN 000126 and a peer-reviewed publication on its safety (Landry *et al*., 2003). The FDA has reviewed the notice and in its response letter did not question the use of the substance as GRAS (FDA, 2003).

Figure 4-1. Amino acid sequence of AMY797E alpha-amylase precursor protein including the maize gamma-zein signal sequence and endoplasmic reticulum retention signal as expressed in transgenic maize Event 3272

The location of the maize gamma-zein signal sequence (amino acids 1 – 19) and endoplasmic reticulum retention signal sequences (amino acids 455 – 460) are indicated by bold text.

```
  1 MRVLLVALAL LALAASATSA KYLELEEGGV IMQAFYWDVP SGGIWWDTIR
 51 QKIPEWYDAG ISAIWIPPAS KGMSGGYSMG YDPYDYFDLG EYYQKGTVET
101 RFGSKQELIN MINTAHAYGI KVIADIVINH RAGGDLEWNP FVGDYTWTDF
151 SKVASGKYTA NYLDFHPNEL HAGDSGTFGG YPDICHDKSW DQYWLWASQE
201 SYAAYLRSIG IDAWRFDYVK GYGAWVVKDW LNWWGGWAVG EYWDTNVDAL
251 LNWAYSSGAK VFDFPLYYKM DAAFDNKNIP ALVEALKNGG TVVSRDPFKA
301 VTFVANHDTD IIWNKYPAYA FILTYEGQPT IFYRDYEEWL NKDKLKNLIW
351 IHDNLAGGST SIVYYDSDEM IFVRNGYGSK PGLITYINLG SSKVGRWVYV
401 PKFAGACIHE YTGNLGGWVD KYVYSSGWVY LEAPAYDPAN GQYGYSVWSY
451 CGVGSEKDEL
```

Figure 4-2 Amino acid alignment of AMY797E and BD5088 alpha-amylases

Positives 95.0%, Identity 93.2%. Identical residues are indicated with the following text format: MAT. Similar residues are indicated by the following text format: MAT. Alignment was performed using the FASTA algorithm employing a pairwise alignment with the following parameters: gap opening penalty 10, gap extension penalty 0.1.

```
                1                                                  60
AMY797E     (1) -AKYLELEEGGVIMQAFYWDVPSGGIWWDTIRQKIPEWYDAGISAIWIPPASKGMSGGYS
 BD5088     (1) MAKYSELEKGGVIMQAFYWDVPSGGIWWDTIRQKIPEWYDAGISAIWIPPASKGMGGAYS
               61                                                 120
AMY797E    (60) MGYDPYDYFDLGEYYQKGTVETRFGSKQELINMINTAHAYGIKVIADIVINHRAGGDLEW
 BD5088    (61) MGYDPYDFFDLGEYDQKGTVETRFGSKQELVNMINTAHAYGMKVIADIVINHRAGGDLEW
              121                                                 180
AMY797E   (120) NPFVGDYTWTDFSKVASGKYTANYLDFHPNELHAGDSGTFGGYPDICHDKSWDQYWLWAS
 BD5088   (121) NPFVNDYTWTDFSKVASGKYTANYLDFHPNELHAGDSGTFGGYPDICHDKSWDQYWLWAS
              181                                                 240
AMY797E   (180) QESYAAYLRSIGIDAWRFDYVKGYGAWVVKDWLNWWGGWAVGEYWDTNVDALLNWAYSSG
 BD5088   (181) QESYAAYLRSIGIDAWRFDYVKGYAPWVVKDWLNWWGGWAVGEYWDTNVDAVLNWAYSSG
              241                                                 300
AMY797E   (240) AKVFDFPLYYKMDAAFDNKNIPALVEALKNGGTVVSRDPFKAVTFVANHDTDIIWNKYPA
 BD5088   (241) AKVFDFALYYKMDEAFDNKNIPALVSALQNGQTVVSRDPFKAVTFVANHDTDIIWNKYPA
              301                                                 360
AMY797E   (300) YAFILTYEGQPTIFYRDYEEWLNKDKLKNLIWIHDNLAGGSTSIVYYDSDEMIFVRNGYG
 BD5088   (301) YAFILTYEGQPTIFYRDYEEWLNKDKLKNLIWIHENLAGGSTDIVYYDNDELIFVRNGYG
              361                                                 420
AMY797E   (360) SKPGLITYINLGSSKVGRWVYVPKFAGACIHEYTGNLGGWVDKYVYSSGWVYLEAPAYDP
 BD5088   (361) DKPGLITYINLGSSKAGRWVYVPKFAGACIHEYTGNLGGWVDKYVYSSGWVYLEAPAYDP
              421          442
AMY797E   (420) ANGQYGYSVWSYCGVGSEKDEL
 BD5088   (421) ANGQYGYSVWSYCGVG------
```

A5. Alpha-amylases in other organisms and prior exposure to AMY797E alpha-amylase protein

Alpha-amylases (EC 3.2.1.1) are ubiquitous, occurring in all three Domains of life (Bacteria, Archaea and Eukaryota) (Pujadas and Palau, 2001), including humans (Horii *et al*., 1987) and plants such as barley, rice and maize (Huang *et al*., 1992; Kramhoft *et al*., 2005; O'Neill *et al*., 1990; Young *et al*., 1994). Syngenta is unaware of any previous human or animal dietary exposure to the AMY797E alpha-amylase protein. However, alpha-amylases from fungal and bacterial sources (*Aspergillus niger*, *Aspergillus oryzae*, *Rhizopus oryzae*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus stearothermophilus*) have a long history of safe use for starch processing in the food processing industry (Pariza and Foster, 1983; Pariza and Johnson, 2001) and alpha-amylases are present in a variety of human food and animal feed sources.

AMY797E alpha-amylase as expressed in Event 3272 maize is not expected to have any impact on human and animal health based upon the extensive characterization of the AMY797E protein (summarized below) and its functional homology to other alpha-amylases found in a variety of human food and animal feed sources which have a history of safe consumption and/or exposure.

A6. Summary of AMY797E alpha-amylase protein food and feed safety assessment

A detailed assessment of human and animal safety of the AMY797E alpha-amylase protein has been provided to the FDA as part of a food and feed safety and nutritional assessment for Event 3272 maize. A summary of the data and information are presented below:

1. A history of safe consumption and/or exposure to alpha-amylases found naturally and added to a variety of human food and animal feed sources and functional homology of AMY797E alpha-amylase to other alpha-amylases.
2. Results of an acute oral mouse toxicity study concluded that the AMY797E alpha-amylase protein prepared from Event 3272 maize is non-toxic to mice and there were no treatment-related effects at a single dose of 1511 mg AMY797E protein/kg body weight.
3. Results from a 49-day feeding trial in broiler chickens fed diets containing 52% to 65% Event 3272 maize grain demonstrated that there were not any adverse nutritional or toxic effects associated with consumption of poultry diets containing Event 3272 maize with AMY797E compared to broiler chickens consuming diets made with commercially available grain containing no AMY797E.
4. No significant sequence identity to any proteins identified as, or known to be, toxins was identified.

5. The donor organisms (*Thermococcus*/*Pyrococcus*) used to develop the AMY797E alpha-amylase protein are not known to be sources of allergenic proteins.
6. No significant amino acid sequence identity to known or putative allergenic protein sequences that are biologically relevant or have implications for allergenic potential was identified (see Appendix 3).
7. The AMY797E alpha-amylase protein is rapidly degraded (within 5 minutes) in simulated gastric fluid containing pepsin and can be expected to be digested under typical mammalian gastric conditions as conventional dietary protein, therefore posing no safety concerns.
8. The AMY797E alpha-amylase protein is a thermostable protein. This enzyme was selected for development due to its increased thermostability and activity during the high temperatures required for starch hydrolysis in dry-grind corn processing for fuel ethanol production. The stability of AMY797E alpha-amylase to heat has no implications, in and of itself, for human safety.
9. Analysis of the AMY797E alpha-amylase protein as expressed in Event 3272 corn revealed no evidence of post-translational glycosylation.

B. Phosphomannose Isomerase Protein

B1. Source and function

The PMI protein produced in Event 3272 plants is encoded by the native *pmi* gene (also referred to elsewhere as the "*manA*" gene) from *E. coli* (strain K-12; Miles and Guest, 1984). The *pmi* gene (GenBank® Accession No. M15380; NCBI, 2003) encodes a protein (GenBank Accession No. AAA24109.1) of 391 amino acids and *ca*. 45,000 molecular weight. PMI [1, 2] catalyzes the reversible inter-conversion of mannose-6-phospate and fructose-6-phosphate (Figure 4-3), and requires zinc for activity. The PMI reaction is specific for mannose-6-phospate and fructose-6-phosphate with a K_{eq} near 1.0. No other natural substrates for PMI are known (Freeze, 2002). Plant cells expressing the *pmi* gene are capable of survival and growth in the presence of mannose as the only or primary carbon source. Under the same conditions, plant cells lacking PMI accumulate mannose-6-phosphate and fail to grow.

B2. Selection of PMI-expressing plant cells by mannose

Mannose and mannose derivatives (*e.g.*, carbohydrate polymers, glycoproteins and glycolipids) are common constituents of living cells and are key components or products of intermediary metabolism (Figure 4-4; Reed *et al.*, 2001). Mannose is phosphorylated by hexokinase to mannose-6-phosphate and in the presence of PMI

[1] The enzyme is also referred to in the literature by the alternate name "mannose-6-phosphate isomerase" (MPI).

[2] IUBMB enzyme classification: EC 5.3.1.8.

enters the glycolytic pathway after isomerization to fructose-6-phosphate. Mannose has also been identified as a precursor for ascorbate synthesis (Wheeler *et al.*, 1998).

Figure 4-3. Reaction catalyzed by phosphomannose isomerase

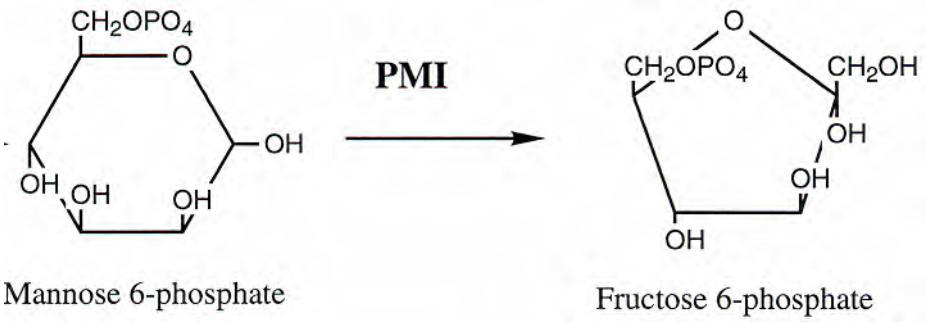
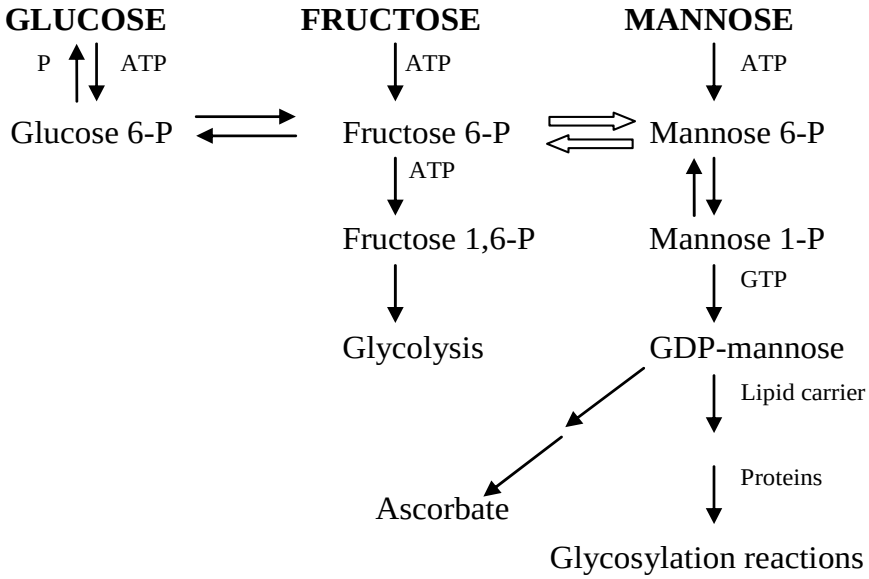


Figure 4-4. The basic intermediary metabolism involving mannose in plant cells
The open arrows indicate the reaction catalyzed by PMI



The effect of mannose on plants was first described over 50 years ago as inhibiting respiration in wheat and tomato (Stenlid, 1954; Morgan and Street, 1959; reviewed by Reed et al., 2001). This appears to result from the accumulation of mannose-6-phosphate, a consequence of which is the inhibition of phosphoglucose isomerase, thereby blocking glycolysis (Goldsworthy and Street, 1965). Other impacts include (1) depletion of the pyrophosphate required for ATP production (Goldsworthy and Street, 1965; Herold and Lewis, 1977); (2) transcriptional repression of genes associated with photosynthesis and the glyoxylate cycle (Jang and Sheen, 1994, 1997); and (3) apoptosis in maize cells (Stein and Hansen, 1999).

Plant cells that produce PMI are able to convert mannose to a readily metabolized compound, fructose-6-phosphate, thus improving the energy status of the cells and avoiding the accumulation of derivatized mannose (Joersbo *et al.,* 1999). PMI has been shown to be an effective selectable marker *via* multiple transformation methods in several plants, including maize, wheat, barley, sugar beet, tomato, rice, cassava and *Arabidopsis thaliana* (Reed *et al.,* 2001; Negrotto *et al.*, 2000; Wright *et al.,* 2001; Joersbo *et al*., 1999; Lucca *et al.,* 2001; Zhang *et al.,* 2000; Todd and Tague, 2001). The use of PMI as a selectable marker and mannose as the selective agent offers an efficient alternative selection system to the more traditional markers that confer resistance to antibiotics or herbicides. In the PMI selection system, mannose is employed only during selection of transformed cells in culture; it is not applied to mature plants as a selective agent.

B3. Equivalency of the PMI protein in test substance PMI-0198 and Event 3272 maize

The PMI protein produced in Event 3272 maize was compared by analysis of several biochemical and functional parameters to the PMI protein expressed in recombinant *E. coli* (test substance PMI-0198) used for the acute oral toxicity mouse study summarized in section B6. The PMI protein was extracted from Event 3272 maize leaf tissue, and its size, immunoreactivity and specific enzymatic activity were compared to the PMI protein in test substance PMI-0198. The PMI proteins from recombinant *E. coli* and Event 3272-derived corn were determined to be substantially equivalent and the microbial test substance PMI-0198 was considered a suitable surrogate for PMI protein produced in Event 3272 maize.

B4. Lack of unintended effects of PMI expression in plants

Expression of the *pmi* gene in transformed plants does not appear to adversely affect plant morphology, growth or agronomic characteristics. For example, Reed *et al.* (2001) reported that grain nutrient composition, grain yield and agronomic characteristics were not demonstrably different between transgenic PMI-producing hybrid maize plants derived from seven different transformation events as compared to PMI-negative segregants from the same lines. They also observed no changes in glycoprotein profiles in PMI-transformed maize or sugar beet varieties as compared to non-transgenic controls. In the absence of exogenous mannose, Joersbo (2001) found that the levels of mannose and mannose-6-phosphate were below the detection limit in the roots of both PMI-transformed sugar beets and their non-transgenic controls, and no differences were observed in the levels of sucrose, glucose or fructose. This suggests that the presence of PMI activity *per se* in transformed plants does not affect the concentrations of simple carbohydrates.

B5. PMI proteins in other organisms and prior exposure to PMI proteins

PMI proteins are ubiquitous in nature, occurring widely among prokaryotes and eukaryotes. Although Syngenta is unaware of documented dietary exposure to PMI, it is conceivable, and indeed likely, that small amounts of PMI proteins from various

sources have always been present in the food and feed supply due to the occurrence of PMI proteins in food plants and animals. PMI proteins have been found in such diverse plant species as tobacco (Barb *et al.,* 2002), pine (Kara *et al*., 1997), walnut (Malvoti *et al*., 1993), lily bulbs (Miller, 1989), *Brassica* species (Chen *et al*., 1989), *A. thaliana* (thale cress; Fujiki *et al*., 2001), as well as in seeds of soybeans and other legumes (Lee and Matheson, 1984). Genes encoding putative PMI proteins have also been identified in rice (Sasaki *et al*., 2002) and guar (Joersbo *et al*., 1997). PMI proteins have been purified and characterized from many other organisms, including bacteria, yeast, rats, pigs and humans (Proudfoot *et al*., 1994a, b; Davis *et al*., 2002) and have been demonstrated to be essential for many organisms, including humans (Freeze, 2002; de Lonlay *et al*., 1998; Keir *et al*., 1999; Hendriksz *et al*., 2001). Proudfoot *et al.* (1994b) proposed three classes of PMI enzymes (Types I, II and III) on the basis of their amino acid identity. The *E. coli* PMI enzyme was classified as Type I, in the same category as PMI enzymes from *Salmonella typhimurium, Saccharomyces cerevisiae, Aspergillus nidulans, Candida albicans* and humans.

PMI as expressed in Event 3272 maize is not expected to have any impact on human and animal health based upon the extensive characterization of the PMI protein (summarized below) and its functional homology to other PMI proteins found in a variety of human food and animal feed sources which have a history of safe consumption and/or exposure.

B6. Summary of PMI protein food and feed safety assessment

A detailed assessment of human and animal safety of the PMI protein has been provided to the FDA as part of a food and feed safety and nutritional assessment for Event 3272 maize. A summary of the data and information are presented below:

1. A history of safe consumption and/or exposure to PMI proteins found naturally in a variety of human food and animal feed sources and functional homology to other PMI proteins.
2. Results of an acute oral mouse toxicity study concluded that the PMI protein prepared from Event 3272 maize is non-toxic to mice and there were no treatment-related effects at a dose of 3080 mg PMI protein/kg body weight.
3. Results from a 49-day feeding trial in broiler chickens fed diets containing 52% to 65% Event 3272 maize grain demonstrated that there were not any adverse nutritional or toxic effects associated with consumption of poultry diets containing Event 3272 maize with PMI compared to broiler chickens consuming diets made with commercially available grain containing no PMI.
4. No significant sequence identity to any proteins identified as, or known to be, toxins was identified.
5. The donor organism (*E. coli*) used to develop the PMI protein is not known to be a source of allergenic proteins.

6. No significant amino acid sequence identity to known or putative allergenic protein sequences that are biologically relevant or have implications for allergenic potential was identified (see Appendix 3).
7. The PMI protein is rapidly degraded (not detected at immediate sampling, time 0) in simulated gastric fluid containing pepsin and can be expected to be digested under typical mammalian gastric conditions as conventional dietary protein, therefore posing no safety concerns.
8. The PMI protein is not highly stable at high temperatures. PMI was inactivated after incubation at 65 ^{o}C for 30 minutes.
9. It is unlikely that PMI as expressed in Event 3272 plants is post-translationally glycosylated. The PMI protein contains no consensus amino acid sequences for *N*-glycosylation[3], although *O*-glycosylation could theoretically occur at the serine or threonine residues present in the protein (Privalle, 2002). However, PMI is not expected to be post-translationally glycosylated in Event 3272 maize, because its expression is not targeted to a cellular glycosylation pathway. PMI as expressed in *E. coli* is not glycosylated; bacteria are not capable of eukaryotic post-translational protein modifications.

[3] *N*-glycosylation sites have the amino acid sequence –NX(S or T)X–, where X can be any amino acid except proline, N = asparagines, S = serine and T = threonine. *N*-glycosylation is the most common form of protein glycosylation.

Chapter 5. PHENOTYPIC EVALUATION

This chapter provides a phenotypic assessment of Event 3272 for the purpose of contributing to the determination that Event 3272 does not have any unintended or unanticipated traits and will not present a plant pest risk when released into the environment.

The genetic modification resulting in transgenic maize Event 3272 was not intended to affect a specific agronomic characteristic or to result in any change in cultivation practices for maize. To confirm that Event 3272 hybrids do not differ in agronomic characteristics, apart from the introduced traits, to the corresponding near-isogenic, non-transgenic hybrids, grain yield and other agronomic (phenotypic) measurements were compared in multi-year trials at multiple locations representing the major growing regions of maize (corn) in the United States.

A. Agronomic Trials and Assessment Methods

Agronomic and phenotypic characterization of Event 3272 maize hybrids was carried out during 2003 and 2004 in a series of agronomic field trials (USDA Comprehensive Permit number 03-021-01 r/m and Notification number 04-064-04n). Agronomic studies were conducted in four trial series, with each trial series composed of Event 3272 hybrids and corresponding near-isogenic, non-transgenic control hybrids (Appendix 1-Figure 1). Phenotypic data were collected from replicated field trials at 8 locations in 2003 and 17 locations in 2004. These 25 locations covered seven states in the U.S. Corn Belt (Table 5-1) and were selected to represent a range of diverse growing environments where Event 3272 maize hybrids are expected to be commercially grown. Field husbandry at all of the trial sites, including irrigation use, fertilization rate, and pest control methods, were consistent with best agronomic practices in the area.

Table 5-1. Field trial locations for agronomic evaluations of Event 3272 hybrids

USDA APHIS #	City	State	Location Code	Trial Series #
2003 Agronomic Trials				
03-021-01 r/m	Brookings	South Dakota	4343	1
03-021-01 r/m	Mankato	Minnesota	4441	1
03-021-01 r/m	Le Roy	Minnesota	4527	1
03-021-01 r/m	Owatonna	Minnesota	4528[a]	1
03-021-01 r/m	Stanton	Minnesota	4538	1
03-021-01 r/m	Hampton	Iowa	5504	1
03-021-01 r/m	Rochelle	Illinois	5625[a]	1
03-021-01 r/m	Janesville	Wisconsin	5628	1
2004 Agronomic Trials				
04-064-04n	Brookings	South Dakota	4343	2, 3, 4
04-064-04n	Nicollet	Minnesota	4441	2, 3, 4
04-064-04n	Faribault	Minnesota	4526	2, 4
04-064-04n	Le Roy	Minnesota	4527	2, 4
04-064-04n	Meriden	Minnesota	4528[a]	2, 3, 4
04-064-04n	Randolph	Minnesota	4538	2
04-064-04n	Rochelle	Illinois	5625[a]	2, 3, 4
04-064-04n	Janesville	Wisconsin	5629	2, 3, 4
04-064-04n	Glidden	Iowa	6427[a]	2, 3, 4
04-064-04n	Leesburg	Illinois	6725	2
04-064-04n	Seward	Nebraska	7334	2
04-064-04n	Washington	Iowa	7532	2
04-064-04n	Hudson	Illinois	7618	2
04-064-04n	Bloomington	Illinois	7627	2, 4
04-064-04n	St Joseph	Illinois	7633	2
04-064-04n	Shirley	Illinois	7638	2, 4
04-064-04n	Bondville	Illinois	7647	2

a. Trial not harvested due to poor late-season growing conditions.

In addition to inspections for disease and insect damage, qualitative and quantitative comparisons for a number of morphological and agronomic traits were made between Event 3272 and control hybrids. The traits chosen for agronomic comparison were those typically observed by professional maize breeders and agronomists, covering a broad range of characteristics that encompass the entire life cycle of the maize plant. These agronomic traits included data on germination and seedling emergence, growth habit, vegetative vigor, reproduction, insect and disease susceptibility, biotic and abiotic stress factors, and yield (Table 5-2). Up to 26 different agronomic characteristics were assessed at each location, but not all traits were assessed at all locations. Dormancy and overwintering ability were also assessed with laboratory germination assays.

Table 5-2. List and definitions of characteristics assessed in Event 3272 agronomic field trials

Brief Description	Trait Code	Description
Percent Barren Plants	BRRNP	Percent of plants per plot that do not develop an ear.
Percent Dropped Ears	DROPP	Percent of plants per plot that have dropped a developed ear prior to harvest. Does not include STKLP.
Harvest Loss from ECB	ECBBN	Number of plants out of 10 plants per plot broken at harvest due to European corn borer infestation.
Early ECB Damage	ECBLR	Rating of first-generation European corn borer damage per plot. Taken between V6 and V8 stage of corn development. 1=no infestation. 9=all plants infested.
Days to 50% Emergence	EMERN	Number of days from planting to 50% of plants per plot emerged.
Percent Emerged Plants	EMRGP	Percent of sowed kernels that resulted in emerged plants within 14 days after planting.
Early Vigor	EMRGR	Early emergence vigor rating. Data collected prior to V3 stage of corn development. 5=same as commercial check. 1=more vigorous. 9=less vigorous
Early Growth	ERGRR	Early growth rating recorded at V6. 1=more vigorous. 9=less vigorous. 4=the commercial checks.
Ear Height	ERHTN	Ear height from base of plant to node where ear connects to plant (cm). Taken at R2-R6 stage of corn development.
Early Root Lodging	ERTLP	Percent of plants per plot leaning greater than 30 degrees from vertical at the root prior to anthesis.
Grain Moisture	GMSTP	Grain moisture % measured at harvest.
Gray Leaf Spot	GRLSR	Rating of Gray Leaf Spot disease progression. Taken between R2 and R6 stage of corn development. 1=no infestation. 9=all leaves fully infested.
Percent Snapped Plants	GRSNP	Percent of plants per plot broken prior to anthesis due to adverse environmental conditions, such as high wind speeds.
Plant Population at Harvest	HAVPN	Harvest population (plants per acre).
Heat Units to 50% Silking	HUS5N	Heat units to 50% of plants extruding silks.
Heat Units to 50% Pollen Shed	HUPSN	Heat units to 50% of plants shedding pollen.
Late Season Intactness	INTLR	Rating of late-season integrity of the plant above the ear. 1=all plants parts intact at harvest. 9=100% of plants in the plot are broken at the ear node prior to harvest.
Leaf Color Rating	LFCLR	Leaf color rating taken between R4 and R6 stage of corn development. 5=same as commercial check. 1=darker. 9=severely chlorotic.
Late Root Lodging	LRTLP	Percent of plants per plot leaning greater than 30 degrees from vertical at the root after anthesis.
Northern Corn leaf Blight	NCLMR	Rating of Northern Corn Leaf Blight disease progression. Taken between R2 and R6 stage of corn development. 1=no infestation. 9=all leaves fully infested.
Plant Height	PLHTN	Plant height from base of plant to collar of flag leaf (cm). Taken between R2 and R6 stage of corn development.
Push Test	PUSXN	Number of plants out of 10 plants tested that break at the stalk or have root failure after pushing to 45 degrees from vertical.
Southern Corn leaf Blight	SCLBR	Rating of Southern Corn leaf Blight disease progression. Taken between R2 and R6 stage of corn development. 1=no infestation. 9=all leaves fully infested.
Percent Broken Stalks	STKLP	Percent of plants per plot with broken stalks below the ear at harvest.
Test Weight	TWSMN	Grain test weight (pounds/bushel) converted to standard 15.5% moisture.
Grain Yield	YGSMN	Grain yield (bushels/acre) converted to standard 15.5% grain moisture.

At all 2003 agronomic field trial locations, four blocks, each block containing one plot for each comparator, were planted in a randomized complete block design (all 2004 trials used three blocks). Each plot consisted of two rows of maize, approximately 17.5 feet in length, spaced approximately 30 inches apart.

Phenotypic data were analyzed across locations for each year using analysis of variance methods to test for differences between Event 3272 and control hybrids (Appendix 4). A genotype pair is defined as consisting of an Event 3272 hybrid and the corresponding near-isogenic, non-transgenic control hybrid. Data not suitable for formal statistical analysis are presented as combined location means in Tables 5-3 to 5-7.

B. Phenotypic trait assessment of event 3272

B1. Growth habit

The following agronomic traits were assessed in the 2003 and 2004 field trials as indicators of basic morphology and growth habit: ERTLP (early root lodging); GRSNP (percent snapped plants); INTLR (late season intactness); LFCLR (leaf color rating); LRTLP (late root lodging); PUSXN (push test); and STKLP (percent broken stalks). These agronomic traits are presented as combined location means for each genotype (Table 5-3). In trial series #3, genotype 1 (3272) had less favorable late season intactness and push test scores than genotype 2 (control). However, percent broken stalks, another characteristic related to late season plant integrity, was favorable to the Event 3272 genotype 1. Overall, characteristics evaluating growth habit did not differ in comparisons of Event 3272 and control hybrids in any of the trial series. In addition, there were no remarkable morphological differences between Event 3272 and control hybrids recorded in field trial reports submitted to USDA APHIS.

B2. Vegetative vigor

Comparisons of vegetative vigor between Event 3272 and control hybrids were based on assessments of: EMRGR (early emergence vigor); ERGRR (early growth rating); ERHTN (ear height); and PLHTN (plant height). There were small but statistically significant differences in plant height between Event 3272 and control hybrids, with Event 3272 plants being shorter (hybrid pairs 3,4 and 5,6 from trial series #2, hybrid pair 1,2 from trial series #4) (Table 5-4). However, there was no trend in the trials to suggest a consistent difference in height. Furthermore, reduction in height is unlikely to be associated with increased weediness. There were not corresponding significant differences in ear height measurements, and ratings of early emergence vigor and early growth were comparable. Overall, the vegetative vigor data show no indication of increased weediness potential of Event 3272 maize.

Table 5-3. Genotype means of Event 3272 and control hybrids for morphological traits

	ERTLP[a] (%)	GRSNP (%)	LRTLP (%)	STKLP (%)	INTLR (1–9)	LFCLR (1–9)	PUSXN (per 10 plants)
Trial series #1 – 2003 hybrid pairs							
Genotype 1 (3272)		1.1		6			
Genotype 2 (control)		2.2		5			
N[b]		2		5			
Genotype 3 (3272)		2.9		8			
Genotype 4 (control)		0.8		10			
N		2		4			
Genotype 5 (3272)		1.3		6			
Genotype 6 (control)		1.4		9			
N		2		5			
Genotype 7 (3272)		1.0		4			
Genotype 8 (control)		1.8		6			
N		1		2			
Trial series #2 – 2004 hybrid pairs							
Genotype 1 (3272)	0.7	0.0	1.4	2.8	3.3	4.9	2.6
Genotype 2 (control)	0.8	0.2	1.1	3.8	3.3	4.8	2.4
N	14	12	13	16	15	17	14
Genotype 3 (3272)	0.9	0.2	1.3	3.4	3.2	5.0	2.1
Genotype 4 (control)	0.8	0.1	1.2	3.6	3.4	4.8	2.3
N	14	12	13	16	15	17	14
Genotype 5 (3272)	0.8	0.0	1.4	2.6	3.0	4.9	1.9
Genotype 6 (control)	0.7	0.4	1.1	2.2	3.4	4.9	2.1
N	13	12	12	15	14	15	13
Genotype 7 (3272)	0.6	0.1	1.0	3.1	3.4	4.8	2.6
Genotype 8 (control)	0.5	0.0	1.1	2.1	3.4	4.8	2.6
N	14	12	13	16	15	17	14
Trial series #3 – 2004 hybrid pairs							
Genotype 1 (3272)	0	0.2	2.2	3.6	7.1	5.3	6.2
Genotype 2 (control)	0	2.1	0	4.9	6.4	5.3	5.4
N	3	3	2	4	4	6	4
Trial series #4 – 2004 hybrid pairs							
Genotype 1 (3272)	0.4	0.14	0.8	3.6	3.7	5	2.1
Genotype 2 (control)	0.6	0.16	1.3	3.5	3.2	5	2.4
N	6	6	5	7	8	10	7

a. ERTLP = early root lodging; GRSNP = percent snapped plants; LRTLP = late root lodging; STKLP = percent broken stalks; INTLR = late season intactness; LFCLR = leaf color rating; PUSXN = push test. These agronomic traits were not amenable to formal statistical analysis.

b. N = number of locations with data.

Table 5-4. Assessment of vegetative vigor of Event 3272 and control hybrids

	ERHTN[a] (cm)	PLHTN (cm)	EMRGR (1–9)	ERGRR (1–9)
Trial series #1 – 2003 hybrid pairs				
Genotype 1 (3272)	120	275	3.6	
Genotype 2 (control)	119	278	4.1	
N[b]	2	2	2	
Genotype 3 (3272)	119	273	4.1	
Genotype 4 (control)	119	269	4.1	
N	2	2	2	
Genotype 5 (3272)	106	273	4.9	
Genotype 6 (control)	113	276	4.3	
N	2	2	2	
Genotype 7 (3272)	117	274	3.9	
Genotype 8 (control)	113	275	5.0	
N	2	2	2	
Trial series #2 – 2004 hybrid pairs				
Genotype 1 (3272)	107.8	280.4	4.3	4.1
Genotype 2 (control)	109.4	278.9	4.0	3.9
N	17	17	16	17
Genotype 3 (3272)	113.1*	273.6†	4.1	4.2
Genotype 4 (control)	111.9	279.6	4.0	4.0
N	17	17	16	17
Genotype 5 (3272)	108.5	272.7†	4.3	4.1
Genotype 6 (control)	110.2	279.3	3.9	3.9
N	15	15	15	17
Genotype 7 (3272)	106.4	273.1	4.0	4.0
Genotype 8 (control)	108.4	275.0	4.0	4.1
N	17	17	16	17
Trial series #3 – 2004 hybrid pairs				
Genotype 1 (3272)	99.5	246.4	4.4	3.9
Genotype 2 (control)	101.4	249.7	4.3	3.6
N	6	6	6	6
Trial series #4 – 2004 hybrid pairs				
Genotype 1 (3272)	107.3	263.8†	4.2	4.2
Genotype 2 (control)	106.2	268.9	4.1	3.8
N	10	10	9	10

† Indicates that the difference between the genotypes was statistically significant at the 95 percent confidence level ($p < 0.05$).

* Indicates that a statistically significant genotype x location interaction undermines the validity of the comparison of genotypes across locations. Individual location means are presented in Appendix 4.

a. ERHTN = ear height; PLHTN = plant height; EMRGR = early emergence vigor; ERGRR = early growth rating. The agronomic traits of EMRGR and ERGRR were not amenable to formal statistical analysis. Statistical analysis was performed on ERHTN and PLHTN values measured in trial series #2–#4, but not on values from trial series #1.

b. N = number of locations with data.

B3. Reproductive characteristics

The relevant indicators of potential changes to seed dormancy, pollination or fertility were: EMERN (days to 50 percent emergence); EMRGP (percent emerged plants); HUS5N (heat units to 50 percent silking); HUPSN (heat units to 50 percent pollen shed); and BRRNP (percent barren plants). The interval between planting and

flowering is reported as heat units or growing degree days. Since maize development is strongly dependent on temperature, the use of heat units allows for a comparison across sites. With respect to EMERN, EMRGP, HUS5N or HUPSN data, there was no indication of a difference between Event 3272 and control hybrids (Table 5-5). In trial series #2, it appeared that percent barren plants could be greater in Event 3272 hybrids. However, this was observed at only a few locations out of the 13–14 locations assessed. This same parameter was either less than or the same as control values in trial series #3 and #4. This observation of potentially higher barrenness under some environmental conditions would not increase the plant pest risk or weediness potential of Event 3272.

Table 5-5. Assessment of reproductive characteristics of Event 3272 and control hybrids

	EMERN[a] (days)	EMRGP (%)	HUS5N (heat units)	HUPSN (heat units)	BRRNP (%)
Trial series #1 – 2003 hybrid pairs					
Genotype 1 (3272)		93	1226	1214	
Genotype 2 (control)		84	1218	1209	
N[b]		8	5	5	
Genotype 3 (3272)		93	1241	1221	
Genotype 4 (control)		89	1247	1217	
N		7	4	4	
Genotype 5 (3272)		97	1268	1247	
Genotype 6 (control)		98	1261	1237	
N		8	5	5	
Genotype 7 (3272)		95	1250	1224	
Genotype 8 (control)		87	1264	1243	
N		7	4	4	
Trial series #2 – 2004 hybrid pairs					
Genotype 1 (3272)	8.6	88.0	1261	1267	0.2
Genotype 2 (control)	8.4	88.4	1258	1271	0.4
N	13	17	17	17	14
Genotype 3 (3272)	8.4	87.7	1262	1266	0.5
Genotype 4 (control)	8.2	89.5	1264	1271	0.2
N	13	17	17	17	14
Genotype 5 (3272)	8.2	83.9	1256	1262	0.4
Genotype 6 (control)	8.2	86.7	1247	1252	0.1
N	12	15	17	17	13
Genotype 7 (3272)	8.3	89.3	1260	1261	0.7
Genotype 8 (control)	8.2	89.1	1262	1269	0.4
N	13	17	17	17	14
Trial series #3 – 2004 hybrid pairs					
Genotype 1 (3272)	9.7	90.4	1253	1233	0
Genotype 2 (control)	9.8	93.6	1250	1241	0.3
N	4	2	6	6	2
Trial series #4 – 2004 hybrid pairs					
Genotype 1 (3272)	9.5	87.4	1265	1270	0.3
Genotype 2 (control)	9.2	85.6	1269	1278	0.3
N	7	10	10	10	4

a. EMERN = days to 50 percent emergence; EMRGP = percent emerged plants; HUS5N = heat units to 50 percent silking; HUPSN = heat units to 50 percent pollen shed; BRRNP = percent barren plants. These agronomic traits were not amenable to formal statistical analysis and are presented as means.

b. N = number of locations with data.

No trials were conducted to specifically study pollen production or pollen dispersal. However, based on the fact that pollen production and pollen viability [as measured by yield (see section B4) and germination (viability of the embryo) of progeny] were unchanged by the genetic modification, the outcrossing frequency is unlikely to be different for Event 3272 hybrids when compared to other commercial maize hybrids.

Overall, there were no indications that the reproductive characteristics or fertility of Event 3272 hybrids had been changed as a result of the genetic modification.

B4. Yield and grain characteristics

Parameters used to evaluate yield and grain characteristics included: YGSMN (grain yield); HAVPN (plant population at harvest); DROPP (percent dropped ears); TWSMN (grain test weight); and GMSTP (grain moisture percent). Grain moisture at harvest also provides an indication of maturity. Although there were some statistically significant differences in grain yield and grain moisture between Event 3272 and control hybrids, the direction of the differences varied and no trends were noted that would contribute to an increased plant pest risk or weediness potential (Table 5-6).

There were no remarkable differences in plants per acre or percent dropped ears across Event 3272 and control hybrids indicating that the proportion of plants surviving from seedling to reproduction and maturity had not been affected by the genetic modification.

Table 5-6. Assessment of yield and grain characteristics of Event 3272 and control hybrids

	YGSMN[a] (bu/acre)	HAVPN (plants/acre)	DROPP (%)	TWSMN (lb/bu)	GMSTP (%)
Trial series #1 – 2003 hybrid pairs					
Genotype 1 (3272)	122.8	28273		47.6	22.1*
Genotype 2 (control)	123.1	25607		48.6	20.2
N[b]	5	8		5	5
Genotype 3 (3272)	125.5†	28210		51.8†	18.3†
Genotype 4 (control)	107.5	27741		53.2	18.8
N	4	7		4	4
Genotype 5 (3272)	119.4	29122		50.6	21.6
Genotype 6 (control)	124.9	29252		50.2	21.3
N	5	8		5	5
Genotype 7 (3272)	100.8	28618		56.0	20.2
Genotype 8 (control)	105.9	27038		55.8	20.8
N	2	7		2	2
Trial series #2 – 2004 hybrid pairs					
Genotype 1 (3272)	155.4	29521	0.00	56.9	27.7†
Genotype 2 (control)	159.4	29465	0.04	56.5	26.9
N	16	15	13	15	16
Genotype 3 (3272)	151.3†	29709	0.00	56.3	27.7
Genotype 4 (control)	160.9	29703	0.12	56.0	27.3
N	16	15	13	15	16
Genotype 5 (3272)	148.8*	28280	0.00	55.9	28.2†
Genotype 6 (control)	159.7	29033	0.00	56.2	27.2
N	14	14	13	13	14
Genotype 7 (3272)	165.0†	29902	0.00	56.7*	26.8
Genotype 8 (control)	154.9	29969	0.04	56.3	27.0
N	16	15	13	15	16
Trial series #3 – 2004 hybrid pairs					
Genotype 1 (3272)	120.8	27464	0	54.6	25.0
Genotype 2 (control)	120.2	28044	0	54.0	24.6
N	5	4	3	5	5
Trial series #4 – 2004 hybrid pairs					
Genotype 1 (3272)	135.5*	29123	0.0	54.1	30.1†
Genotype 2 (control)	139.5	28812	0.1	54.4	31.0
N	9	8	5	9	9

† Indicates that the difference between the genotypes was statistically significant at the 95 percent confidence level ($p < 0.05$).

* Indicates that a statistically significant genotype x location interaction undermines the validity of the comparison of genotypes across locations. Individual location means are presented in Appendix 4.

a. YGSMN = grain yield; HAVPN = plant population at harvest; DROPP = percent dropped ears; TWSMN = test weight; GMSTP = grain moisture. Parameters HAVPN and DROPP were not amenable to formal statistical analysis.

b. N = number of locations with data.

B5. Disease and abiotic stress factor observations

Disease observations of northern corn leaf blight (NCLMR) and southern corn leaf blight (SCLBR) were conducted at all trial sites in 2004. However, diagnostic symptoms characteristic of these diseases were not expressed at any of the trial locations. This could have been due to either a lack of disease inoculum, unfavorable environmental conditions for disease development, or that the plants used in the trial were not susceptible to the causal organisms. Similarly, there was no early infestation damage by European corn borer (ECB) at either of the two locations evaluated in trial series #2 (Table 5-7). No differences in harvest losses from ECB between Event 3272 and the control hybrids were noted (only two of the nine sites had losses). Gray leaf spot (GRLSR) disease presence in 2004 trials was recorded and no remarkable differences were observed between Event 3272 and control hybrids.

Agronomic parameters used to evaluate tolerance to abiotic stress factors, such as high wind, at different growth stages included early and late root lodging (ERTLP and LRTLP) and green snap percentages (GRSNP). No trends were observed in comparisons of Event 3272 and control plants (Table 5-3) across sites that would indicate an enhanced plant pest risk or weediness potential.

Table 5-7. Disease observations of Event 3272 and control hybrids

	GRLSR[a] (1–9)	ECBBN (number/10)	ECBLR (1–9)
Trial series #2 – 2004 hybrid pairs			
Genotype 1 (3272)	1.8	0.3	1.0
Genotype 2 (control)	1.8	0.4	1.0
N[b]	8	9	2
Genotype 3 (3272)	1.8	0.3	1.0
Genotype 4 (control)	2.0	0.3	1.0
N	8	9	2
Genotype 5 (3272)	1.9	0.3	1.0
Genotype 6 (control)	1.8	0.3	1.0
N	8	9	2
Genotype 7 (3272)	1.9	0.4	1.0
Genotype 8 (control)	1.9	0.3	1.0
N	8	9	2
Trial series #3 – 2004 hybrid pairs			
Genotype 1 (3272)	2.7		
Genotype 2 (control)	2.7		
N	2		
Trial series #4 – 2004 hybrid pairs			
Genotype 1 (3272)	1.6		
Genotype 2 (control)	1.7		
N	4		

a. GRLSR = gray leaf spot rating; ECBBN = harvest loss from European corn borer (ECB); ECBLR = early ECB damage. None of these agronomic traits were amenable to formal statistical analysis and are presented as means.

b. N = number of locations with data.

B6. Life-span

Maize is an annual crop. In addition, maize lacks the winter hardiness generally characteristic of biennial or perennial species. The expression of AMY797E alpha-amylase and PMI proteins in Event 3272 hybrids would not be anticipated to alter their life-span. In addition, there were no remarkable differences in flowering time, seed production capacity, and general morphology between Event 3272 and control hybrids that would be indicative of a shift towards greater perenniality.

B7. Seed dormancy and germination assessment

Weedy plants use many complex mechanisms to disperse seeds and propagate (Anderson, 1996). Dormancy mechanisms[1], such as hard seeds, are used to spread seed germination across many growing seasons. Some weed species can germinate seeds at low temperature in order to produce seeds when competition from other species is low, giving their offspring a competitive advantage. Primary dormancy is extremely rare or nonexistent in most field crops, including maize (Gould, 1968; Galinat, 1988).

Standardized germination assays are routinely conducted to measure the seed viability and germination potential of maize seeds (AOSA, 1998). A laboratory experiment was conducted to determine if the germination and overwintering ability of Event 3272 differed from that of the non-transgenic near-isogenic control line. Three temperature regimes were used: an AOSA-recommended standard warm germination assay to simulate optimum germination conditions (25 ^{o}C for 7 days), an AOSA-recommended saturated cold germination assay to simulate normal spring germination conditions in the field (10 ^{o}C for 4 days, 25 ^{o}C for 3 days) and a non-AOSA germination assay (5 ^{o}C for 7 days) to simulate germination conditions during a temperate-zone winter. The first two treatments test for differences in primary dormancy (as the conditions are suitable for germination); the 5 ^{o}C treatment does not test for primary dormancy as maize seeds cannot germinate at this temperature, but it serves as a test for changes in cold-induced secondary dormancy. Seeds were rolled in towels and arranged in a completely random design in temperature-controlled incubators. Seed germination and dormancy differences between Event 3272 and the control were assessed by evaluating the percent germinated (normal and abnormal), percent viable hard (dormant), percent dead and percent viable firm swollen seed.

Germination and dormancy testing results are shown in Table 5-8. No differences at $p \leq 0.05$ were detected for germination characteristics between Event 3272 and the non-transgenic near-isogenic control at either of the biologically active temperatures. In the 5 ^{o}C assay, a significantly greater number of dead seeds were observed in Event 3272 as compared to the control, but this would not increase the secondary dormancy

[1] Primary dormancy is conferred by physical or physiological traits of the seed that prevent immediate germination; secondary dormancy occurs when the seed is capable of germination, but environmental conditions are unsuitable to induce germination. Overwintering of maize seed occurs *via* secondary dormancy.

ability of Event 3272. No viable hard seed were detected in any seed sample. These results demonstrate that Event 3272 seed does not have altered germination, dormancy or overwintering characteristics as compared to the non-transgenic near-isogenic control that would result in an increased potential for weediness when released into the environment.

Table 5-8. Germination and dormancy testing results for Event 3272

Temperature (°C)	Seed Material	Normal Germinated (%)	Abnormal Germinated (%)	Viable Hard (%)	Dead (%)	Viable Firm Swollen (%)
25	3272[1]	97.5	1.75	0	0.75	0
	control	98.8	0.50	0	0.75	0
10 / 25	3272[1]	87.8	11.25	0	1.00	0
	control	88.0	10.75	0	1.25	0
5	3272[2]	0	0	0	8.75	91.25
	control	0	0	0	3.50	96.50

[1] No significant differences between 3272 and the control at $p \leq 0.05$.

[2] Significant differences at $p \leq 0.05$.

C. Conclusions of phenotypic assessment

The agronomic performance and phenotypic data generated suggest that the genetic modification resulting in Event 3272 did not have any unintended effects on seed germination, dormancy, plant growth habit and general morphology, life-span, vegetative vigor, flowering and pollination, grain yield, stress adaptations or disease susceptibility. These data support the conclusion that Event 3272 hybrids are unlikely to form feral persistent populations, or to be more invasive or weedy than conventional maize hybrids, and would not display higher rates of outcrossing than unmodified maize.

Chapter 6. COMPOSITIONAL ASSESSMENT

Compositional analysis of Event 3272 was performed in order to investigate whether the levels of various key nutrients or antinutrients were comparable with conventional counterparts, and within the normal range of variation reported for maize. For transgenic plants without purposefully altered nutritional properties, the nutritional evaluation is part of the weight-of-evidence approach for evaluating whether there were any unanticipated consequences of the genetic modification and is used as part of the assessment of plant pest risk and weediness potential. The detailed description of compositional analysis has been submitted to the FDA as part of the food and feed safety evaluation of Event 3272.

Key nutritional components in maize forage and grain derived from Event 3272 and near-isogenic, non-transgenic control plants were compared. Replicate trials of transgenic and control plants were planted in multiple locations in the U.S. Corn Belt during 2003 (six locations – Bloomington, IL; Bondville, IL; Fairbault, MN; Glidden, IA; Shirley, IL; and Stanton, MN) and 2004 (seven locations – Bloomington, IL; Bondville, IL; Brookings, SD; Glidden, IA; Janesville, WI; Stanton, MN; and Washington, IA). The locations of the trials were selected to be representative of the range of environmental conditions under which the hybrid varieties would be grown. Two hybrid pairs (a hybrid pair consisting of hybrid Event 3272 maize plants and hybrid near-isogenic, non-transgenic maize plants) were grown at each of the six locations during the 2003 growing season and one hybrid pair was grown at each of the seven locations in 2004 (Appendix 1-Figure 1). At each field site location, Event 3272 and near-isogenic, non-transgenic control plants were planted in a randomized complete block design with three blocks, each block containing one plot for each comparator. All plants were grown using local agronomic practices for the respective regions. Forage was harvested at the dough stage and grain was harvested at physiological maturity. The compositional evaluation was guided by the OECD consensus document on compositional considerations for new varieties of maize (OECD, 2002).

Compositional analysis of the forage samples included proximates (protein, fat, ash, carbohydrates and moisture), acid detergent fiber (ADF), neutral detergent fiber (NDF), and minerals (calcium, phosphorus). Compositional analysis of the grain samples included proximates (protein, fat, ash, carbohydrates), acid detergent fiber (ADF), neutral detergent fiber (NDF), total dietary fiber (TDF), starch, minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, zinc and selenium), vitamins (beta-carotene (provitamin A), folic acid, B1 (thiamine), B2 (riboflavin), B3 (niacin), B6, and E), fatty acids (16:0 palmitic, 18:0 stearic, 18:1 oleic, 18:2 linoleic and 18:3 linolenic), amino acids, antinutrients (phytic acid, raffinose and trypsin inhibitor) and secondary maize metabolites as recommended by OECD (furfural, ferulic acid and *p*-coumaric acid).

Statistical analyses of the compositional data were conducted using analysis of variance across locations (see Appendix 5). Three sets of analyses were made based

on data from each of the three hybrid pairs, with data for each analyte in forage and grain of an Event 3272 hybrid compared to that of the corresponding near-isogenic, non-transgenic control hybrid. Statistically significant differences were determined at the 5% level of significance. For those comparisons in which Event 3272 was statistically different from the control, the Event 3272 mean was compared to the ranges in the ILSI crop composition database (ILSI, 2006) and to the ranges reported in the OECD consensus document on compositional considerations for new varieties of maize (OECD, 2002). The ILSI crop composition database provides information on the natural variability in composition of conventional crops (Ridley *et al.*, 2004). This database is an up-to-date compilation of data on the nutrients, antinutrients and secondary metabolites for maize and is currently the most comprehensive database for maize composition. Summaries of the statistically significant differences in forage and grain composition are found in Tables 6-1 to 6-6. Tables 6-7 to 6-13 present summaries of the individual location means in cases where the validity of the comparison across locations was undermined by a statistically significant genotype x location interaction. Appendix 5 - Tables 1 to 8 provide a summary of all forage and grain compositional values.

Results of the forage analyses showed that there were very few statistically significant differences between Event 3272 hybrids and the corresponding near-isogenic, non-transgenic control hybrids and all values for these Event 3272 forage components fell within the reported ranges in the ILSI composition database (Tables 6-1 and 6-7). Grain proximate and fiber analyses results showed statistically significant differences in some components in some of the hybrid pairs. However, all Event 3272 values fell within the ranges reported in the ILSI and OECD databases (Tables 6-2 and 6-8). Results of grain analyses for mineral and vitamin composition showed very few statistically significant differences; all Event 3272 mineral and vitamin values fell within the ILSI and OECD database ranges (Tables 6-3, 6-4, 6-9 and 6-10). All Event 3272 location mean values for fatty acid composition were contained within the range of the OECD database (Table 6-11). Grain amino acid analyses showed statistically significant differences in one of the three Event 3272 hybrids, with higher amino acid levels than the non-transgenic control. However, all Event 3272 amino acid levels still fell within the ranges of the ILSI and OECD databases (Tables 6-5 and 6-12).

There are generally no recognized toxicants or antinutrients in maize at levels considered to be harmful. For the purpose of safety assessment, OECD recommends testing for antinutrients and secondary metabolites (OECD, 2002). Results of the grain analysis for antinutrients and secondary metabolites showed very few statistically significant differences between Event 3272 and the non-transgenic control. All Event 3272 values fell within the ranges in the ILSI and OECD databases (Tables 6-6 and 6-13).

These compositional data support the conclusion that forage and grain produced from Event 3272 maize hybrids do not have any biologically meaningful differences in terms of plant pest risk from non-transgenic maize hybrids and will not pose an increased plant pest risk or have increased weediness potential over conventional maize hybrids.

Table 6-1. Summary of statistically significant differences in proximate composition of Event 3272 and non-transgenic maize forage

(All values % dry weight)

Data from ILSI crop composition database and OECD consensus document included for comparison (no OECD data for carbohydrates).

	Protein	Carbohydrates	ADF
A1 (3272)	8.18	85.8	27.6
A2 (Control)	8.11	86.1	28.8
F-test Probability for Genotype	*	*	48.0%
B1 (3272)	8.77	84.6	27.1
B2 (Control)	8.02	86.0	30.4
F-test Probability for Genotype	***3.6%***	6.1%	***3.9%***
B3 (3272)	7.08	87.1	26.4
B4 (Control)	6.96	87.6	27.0
F-test Probability for Genotype	56.6%	***4.9%***	59.5%

ILSI (2006)	**average**	7.78	85.6	27.00
	range	3.14 - 11.57	76.4 - 92.1	16.13 - 47.39
	N	945	945	945
OECD (2002)	**range**	4.7 - 9.2		25.6 - 34

* Statistically significant genotype x location interaction undermines the validity of the comparison of genotypes across locations. Individual location means are presented in Table 6-7.

Table 6-2. Summary of statistically significant differences in proximate composition of Event 3272 and non-transgenic maize grain

(All values % dry weight)

Data from ILSI crop composition database and OECD consensus document included for comparison (no OECD data for starch).

	Protein	Ash	Carbohydrates	ADF	NDF	TDF	Starch
A1 (3272)	10.88	1.46	84.52	4.20	10.96	11.83	63.28
A2 (Control)	10.54	1.37	84.86	4.18	11.22	12.44	60.84
F-test Probability for Genotype	6.0%	***4.9%***	11.6%	95.0%	57.4%	*	*
B1 (3272)	10.74	1.35	84.22	4.73	11.24	12.29	63.29
B2 (Control)	10.05	1.29	85.02	4.79	12.00	13.94	60.28
F-test Probability for Genotype	***0.2%***	6.0%	***0.6%***	*	8.0%	***0.4%***	***0.2%***
B3 (3272)	9.57	1.54	85.06	4.69	11.40	13.85	50.50
B4 (Control)	9.43	1.54	85.20	5.32	12.61	16.24	47.20
F-test Probability for Genotype	*	100.0%	45.3%	***4.3%***	***2.3%***	*	47.9%

	average	10.30	1.439	84.6	4.05	11.23	16.43	57.7
ILSI (2006)	**range**	6.15 - 17.26	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	1410	1410	1350	1349	397	168
OECD (2002)	**range**	6 - 12.7	1.1 - 3.9	82.2 - 82.9	3.0 - 4.3	8.3 - 11.9	11.1	

* Statistically significant genotype x location interaction undermines the validity of the comparison of genotypes across locations. Individual location means are presented in Table 6-8.

Table 6-3. Summary of statistically significant differences in mineral composition of Event 3272 and non-transgenic maize grain

(All values mg/kg dry weight)

Data from ILSI crop composition database included for comparison.

	Manganese
A1 (3272)	6.50
A2 (Control)	6.19
F-test Probability for Genotype	14.6%
B1 (3272)	5.68
B2 (Control)	5.15
F-test Probability for Genotype	***0.2%***
B3 (3272)	5.66
B4 (Control)	5.42
F-test Probability for Genotype	11.4%

	average	6.18
ILSI (2006)	**range**	1.69 - 14.3
	N	1256

Table 6-4. Summary of statistically significant differences in vitamin composition of Event 3272 and non-transgenic maize grain

(All values mg/100g dry weight unless otherwise indicated)

Data from ILSI crop composition database and OECD consensus document included for comparison.

	Vitamin B1 Thiamine	Vitamin B6
A1 (3272)	0.40	0.61
A2 (Control)	0.41	0.64
F-test Probability for Genotype	31.0%	***4.4%***
B1 (3272)	0.44	0.61
B2 (Control)	0.41	0.62
F-test Probability for Genotype	***1.0%***	72.7%
B3 (3272)	0.45	0.50
B4 (Control)	0.47	0.53
F-test Probability for Genotype	*	***4.2%***

ILSI (2006)	**average**	0.530	0.644
	range	0.126 - 4.000	0.368 - 1.132
	N	894	415
OECD (2002) (mg/kg = (mg/100g) x 10)	**range**	2.3 - 8.6 mg/kg	4.6 - 9.6 mg/kg

* Statistically significant genotype x location interaction undermines the validity of the comparison of genotypes across locations. Individual location means are presented in Table 6-10.

Table 6-5. Summary of statistically significant differences in amino acid composition of Event 3272 and non-transgenic maize grain

(All values % dry weight unless otherwise indicated)

Data from ILSI crop composition database and OECD consensus document included for comparison.

	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Cys	Val
A1 (3272)	0.717	0.343	0.546	2.058	0.908	0.394	0.809	0.201	0.488
A2 (Control)	0.711	0.331	0.554	2.047	0.896	0.395	0.807	0.205	0.487
F-test Probability for Genotype	60.7%	12.5%	35.9%	77.1%	39.5%	86.8%	84.5%	13.0%	83.1%
B1 (3272)	0.729	0.334	0.559	2.094	0.893	0.387	0.827	0.204	0.489
B2 (Control)	0.663	0.311	0.520	1.927	0.840	0.367	0.762	0.196	0.453
F-test Probability for Genotype	***< 0.1%***	***< 0.1%***	***< 0.1%***	***< 0.1%***	***< 0.1%***	***< 0.1%***	***< 0.1%***	***1.9%***	***< 0.1%***
B3 (3272)	0.666	0.323	0.496	1.851	0.808	0.365	0.734	0.184	0.449
B4 (Control)	0.651	0.322	0.488	1.826	0.813	0.361	0.720	0.186	0.437
F-test Probability for Genotype	18.2%	81.9%	39.4%	53.0%	70.1%	53.7%	35.5%	51.6%	21.3%

ILSI (2006) (mg/g = % dw x 10)	**average**	6.88 mg/g	3.75 mg/g	5.12 mg/g	20.09 mg/g	9.51 mg/g	3.85 mg/g	7.90 mg/g	2.21 mg/g	4.90 mg/g
	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)	**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

Table 6-5. Summary of statistically significant differences in amino acid composition of Event 3272 and non-transgenic maize grain

(All values % dry weight unless otherwise indicated)

Data from ILSI crop composition database and OECD consensus document included for comparison.

	Ile	Leu	Phe	His	Lys	Arg	Trp
A1 (3272)	0.368	1.365	0.546	0.294	0.319	0.488	0.0683
A2 (Control)	0.365	1.355	0.544	0.296	0.328	0.491	0.0637
F-test Probability for Genotype	60.4%	68.6%	84.2%	58.9%	11.3%	78.6%	*
B1 (3272)	0.371	1.404	0.554	0.293	0.318	0.471	0.0673
B2 (Control)	0.339	1.284	0.511	0.277	0.301	0.448	0.0604
F-test Probability for Genotype	***< 0.1%***	***< 0.1%***	***< 0.1%***	***< 0.1%***	***< 0.1%***	***0.7%***	***< 0.1%***
B3 (3272)	0.335	1.227	0.480	0.275	0.315	0.424	0.0643
B4 (Control)	0.326	1.203	0.469	0.273	0.310	0.423	0.0586
F-test Probability for Genotype	18.2%	36.8%	22.0%	76.7%	45.4%	87.9%	***3.4%***

ILSI (2006) (mg/g = % dw x 10)	**average**	3.68 mg/g	13.41 mg/g	5.25 mg/g	2.96 mg/g	3.15 mg/g	4.33 mg/g	0.627 mg/g
	range	1.79 - 6.92	6.42 - 24.92	2.44 - 9.30	1.37 - 4.34	1.72 - 6.68	1.19 - 6.39	0.271 - 2.150
	N	1350	1350	1350	1350	1350	1350	1350
OECD (2002)	**range**	0.22 - 0.71	0.79 - 2.41	0.29 - 0.64	0.15 - 0.38	0.05 - 0.55	0.22 - 0.64	0.04 - 0.13

* Statistically significant genotype x location interaction undermines the validity of the comparison of genotypes across locations.

Individual location means are presented in Table 6-12.

Table 6-6. Summary of statistically significant differences in secondary metabolites of Event 3272 and non-transgenic maize grain

(All values mg/kg dry weight unless otherwise indicated)

Data from ILSI crop composition database and OECD consensus document included for comparison.

		Ferulic Acid
A1 (3272)		1939
A2 (Control)		1999
F-test Probability for Genotype		32.6%
B1 (3272)		2277
B2 (Control)		2404
F-test Probability for Genotype		11.9%
B3 (3272)		3034
B4 (Control)		3153
F-test Probability for Genotype		***3.0%***
ILSI (2006)	**average**	2201.1
	range	291.9 - 3885.8
	N	817
OECD (2002) (% dw x 10^4 = mg/kg)	**range**	0.02 - 0.3 % dw

Table 6-7. Location means for proximate composition of Event 3272 and non-transgenic maize forage

(All values % dry weight)

Data from ILSI crop composition database and OECD consensus document included for comparison (no OECD data for carbohydrates).

Location	Genotype	Protein	Carbohydrates
Bloomington, IL	**A1 (3272)**	9.20	84.4
Bloomington, IL	**A2 (Control)**	10.17	83.5
Bondville, IL	**A1 (3272)**	8.77	85.1
Bondville, IL	**A2 (Control)**	8.67	84.9
Fairbault, MN	**A1 (3272)**	7.83	87.3
Fairbault, MN	**A2 (Control)**	7.40	87.4
Glidden, IA	**A1 (3272)**	8.40	84.7
Glidden, IA	**A2 (Control)**	6.63	87.4
Shirley, IL	**A1 (3272)**	7.73	85.9
Shirley, IL	**A2 (Control)**	8.60	84.6
Stanton, MN	**A1 (3272)**	7.17	87.4
Stanton, MN	**A2 (Control)**	7.20	88.5
A1 (3272)		8.18	85.8
A2 (Control)		8.11	86.1
F-test Probability for Genotype		*	*
F-test Probability for Location x Genotype Interaction		***2.2%***	***1.6%***
	average	7.78	85.6
ILSI (2006)	**range**	3.14 - 11.57	76.4 - 92.1
	N	945	945
OECD (2002)	**range**	4.7 - 9.2	

* Statistically significant genotype x location interaction undermines the validity of the comparison across locations.

Table 6-8. Location means for proximate composition of Event 3272 and non-transgenic maize grain

(All values % dry weight)

Data from ILSI crop composition database and OECD consensus document included for comparison (no OECD data for starch).

Location	Genotype	TDF	Starch
Bloomington, IL	**A1 (3272)**	13.40	62.9
Bloomington, IL	**A2 (Control)**	15.97	52.1
Bondville, IL	**A1 (3272)**	11.70	63.8
Bondville, IL	**A2 (Control)**	14.60	54.9
Fairbault, MN	**A1 (3272)**	12.03	61.6
Fairbault, MN	**A2 (Control)**	10.33	67.3
Glidden, IA	**A1 (3272)**	10.47	68.1
Glidden, IA	**A2 (Control)**	11.70	67.0
Shirley, IL	**A1 (3272)**	10.87	65.0
Shirley, IL	**A2 (Control)**	11.43	62.8
Stanton, MN	**A1 (3272)**	12.53	58.3
Stanton, MN	**A2 (Control)**	10.63	61.0
A1 (3272)		11.83	63.3
A2 (Control)		12.44	60.8
F-test Probability for Genotype		*	*
F-test Probability for Location x Genotype Interaction		***0.1%***	***< 0.1%***
	average	16.43	57.7
ILSI (2006)	**range**	8.85 - 35.31	26.5 - 73.8
	N	397	168
OECD (2002)	**range**	11.1	

Location	Genotype	ADF
Bloomington, IL	**B1 (3272)**	5.97
Bloomington, IL	**B2 (Control)**	4.20
Bondville, IL	**B1 (3272)**	6.93
Bondville, IL	**B2 (Control)**	6.53
Fairbault, MN	**B1 (3272)**	3.63
Fairbault, MN	**B2 (Control)**	4.83
Glidden, IA	**B1 (3272)**	4.27
Glidden, IA	**B2 (Control)**	3.73
Shirley, IL	**B1 (3272)**	4.27
Shirley, IL	**B2 (Control)**	3.70
Stanton, MN	**B1 (3272)**	3.30
Stanton, MN	**B2 (Control)**	5.77
B1 (3272)		4.73
B2 (Control)		4.79
F-test Probability for Genotype		*
F-test Probability for Location x Genotype Interaction		***3.0%***
	average	4.05
ILSI (2006)	**range**	1.82 - 11.34
	N	1350
OECD (2002)	**range**	3.0 - 4.3

Location	Genotype	Protein
Bondville, IL	**B3 (3272)**	8.43
Bondville, IL	**B4 (Control)**	8.50
Brookings, SD	**B3 (3272)**	8.80
Brookings, SD	**B4 (Control)**	9.03
Glidden, IA	**B3 (3272)**	10.00
Glidden, IA	**B4 (Control)**	9.23
Janesville, WI	**B3 (3272)**	11.33
Janesville, WI	**B4 (Control)**	10.17
Stanton, MN	**B3 (3272)**	10.33
Stanton, MN	**B4 (Control)**	10.87
Washington, IA	**B3 (3272)**	8.53
Washington, IA	**B4 (Control)**	8.80
B3 (3272)		9.57
B4 (Control)		9.43
F-test Probability for Genotype		*
F-test Probability for Location x Genotype Interaction		***2.7%***
	average	10.30
ILSI (2006)	**range**	6.15 - 17.26
	N	1434
OECD (2002)	**range**	6 - 12.7

* Statistically significant genotype x location interaction undermines the validity of the comparison across locations.

Table 6-9. Location means for mineral composition of Event 3272 and non-transgenic maize grain

(All values mg/kg dry weight unless otherwise indicated)

Data from ILSI crop composition database and OECD consensus document included for comparison.

Location	Genotype	Calcium	Copper	Magnesium	Phosphorus	Zinc
Bondville, IL	**B3 (3272)**	31.5	1.93	1040	2730	18.5
Bondville, IL	**B4 (Control)**	32.2	1.92	1040	2650	18.5
Brookings, SD	**B3 (3272)**	53.4	2.20	1260	3110	18.1
Brookings, SD	**B4 (Control)**	54.1	1.99	1350	3280	18.8
Glidden, IA	**B3 (3272)**	39.7	2.39	1230	3040	22.8
Glidden, IA	**B4 (Control)**	41.7	2.03	1090	2660	20.0
Janesville, WI	**B3 (3272)**	43.8	1.91	1470	3400	22.7
Janesville, WI	**B4 (Control)**	51.0	1.64	1370	3160	21.6
Stanton, MN	**B3 (3272)**	52.9	1.52	1250	3470	20.5
Stanton, MN	**B4 (Control)**	53.4	1.58	1390	3800	21.7
Washington, IA	**B3 (3272)**	49.1	1.59	1270	3390	22.9
Washington, IA	**B4 (Control)**	41.1	2.04	1270	3370	22.0
B3 (3272)		45.1	1.92	1250	3190	20.9
B4 (Control)		45.6	1.87	1250	3150	20.4
F-test Probability for Genotype		*	*	*	*	*
F-test Probability for Location x Genotype Interaction		***0.3%***	***0.5%***	***3.1%***	***2.6%***	***1.4%***

ILSI (2006)	**average**	46.4	1.75	1193.8	3273.5	21.6
	range	12.7 - 208.4	0.73 - 18.5	594.0 - 1940	1470 - 5330	6.5 - 37.2
	N	1344	1249	1257	1349	1257
OECD (2002) ((mg/100g) x 10 = mg/kg)	**range**	3 - 100 mg/100 g	0.09 - 1.0 mg/100 g	82 - 1000 mg/100g	234 - 750 mg/100g	1.2 - 3.0 mg/100g

* Statistically significant genotype x location interaction undermines the validity of the comparison across locations.

Table 6-10. Location means for vitamin composition of Event 3272 and non-transgenic maize grain

(All values mg/100g dry weight unless otherwise indicated)

Data from ILSI crop composition database and OECD consensus document included for comparison (no OECD data for beta-carotene).

Location	Genotype	Beta-carotene	Vitamin B1 Thiamine
Bondville, IL	**B3 (3272)**	0.111	0.44
Bondville, IL	**B4 (Control)**	0.119	0.42
Brookings, SD	**B3 (3272)**	0.099	0.46
Brookings, SD	**B4 (Control)**	0.122	0.52
Glidden, IA	**B3 (3272)**	0.068	0.45
Glidden, IA	**B4 (Control)**	0.098	0.47[1]
Janesville, WI	**B3 (3272)**	0.074	0.50
Janesville, WI	**B4 (Control)**	0.127	0.47
Stanton, MN	**B3 (3272)**	0.097	0.42
Stanton, MN	**B4 (Control)**	0.093	0.46
Washington, IA	**B3 (3272)**	0.122	0.44
Washington, IA	**B4 (Control)**	0.082	0.46
B3 (3272)		0.095	0.45
B4 (Control)		0.107	0.47
F-test Probability for Genotype		*	*
F-test Probability for Location x Genotype Interaction		***<0.1%***	***0.2%***
ILSI (2006)	**average**	0.684	0.530
	range	0.019 - 4.681	0.126 - 4.000
	N	276	894
OECD (2002) (mg/kg = (mg/100g) x 10)	**range**		2.3 - 8.6 mg/kg

[1] One replication data point not included because determined to be an outlier by scatter plot.

* Statistically significant genotype x location interaction undermines the validity of the comparison across locations.

Table 6-11. Location means for fatty acid composition of Event 3272 and non-transgenic maize grain

(All values % dry weight)

Data from OECD consensus document included for comparison.

Location	Genotype	18:0 Stearic
Bloomington, IL	**A1 (3272)**	0.054
Bloomington, IL	**A2 (Control)**	0.058
Bondville, IL	**A1 (3272)**	0.055
Bondville, IL	**A2 (Control)**	0.073
Fairbault, MN	**A1 (3272)**	0.061
Fairbault, MN	**A2 (Control)**	0.057
Glidden, IA	**A1 (3272)**	0.053
Glidden, IA	**A2 (Control)**	0.059
Shirley, IL	**A1 (3272)**	0.061
Shirley, IL	**A2 (Control)**	0.067
Stanton, MN	**A1 (3272)**	0.058
Stanton, MN	**A2 (Control)**	0.052
A1 (3272)		0.057
A2 (Control)		0.061
F-test Probability for Genotype		*
F-test Probability for Location x Genotype Interaction		***4.0%***
OECD (2002)	**range**	0.04 - 0.17

* Statistically significant genotype x location interaction undermines the validity of the comparison across locations.

Table 6-12. Location means for amino acid composition of Event 3272 and non-transgenic maize grain

(All values % dry weight unless otherwise indicated)

Data from ILSI crop composition database and OECD consensus document included for comparison.

Location	Genotype	Tryptophan
Bloomington, IL	**A1 (3272)**	0.0755
Bloomington, IL	**A2 (Control)**	0.0660
Bondville, IL	**A1 (3272)**	0.0653
Bondville, IL	**A2 (Control)**	0.0563
Fairbault, MN	**A1 (3272)**	0.0680
Fairbault, MN	**A2 (Control)**	0.0723
Glidden, IA	**A1 (3272)**	0.0640
Glidden, IA	**A2 (Control)**	0.0530
Shirley, IL	**A1 (3272)**	0.0708
Shirley, IL	**A2 (Control)**	0.0633
Stanton, MN	**A1 (3272)**	0.0664
Stanton, MN	**A2 (Control)**	0.0709
A1 (3272)		0.0683
A2 (Control)		0.0637
F-test Probability for Genotype		*
F-test Probability for Location x Genotype Interaction		***1.0%***
	average	0.627 mg/g
ILSI (2006)	**range**	0.271 - 2.150
(mg/g = % dw x 10)	**N**	1350
OECD (2002)	**range**	0.04 - 0.13

Location	Genotype	Methionine
Bloomington, IL	**B1 (3272)**	0.239
Bloomington, IL	**B2 (Control)**	0.207
Bondville, IL	**B1 (3272)**	0.200
Bondville, IL	**B2 (Control)**	0.193
Fairbault, MN	**B1 (3272)**	0.254
Fairbault, MN	**B2 (Control)**	0.228
Glidden, IA	**B1 (3272)**	0.164
Glidden, IA	**B2 (Control)**	0.170
Shirley, IL	**B1 (3272)**	0.213
Shirley, IL	**B2 (Control)**	0.207
Stanton, MN	**B1 (3272)**	0.216
Stanton, MN	**B2 (Control)**	0.209
B1 (3272)		0.214
B2 (Control)		0.202
F-test Probability for Genotype		*
F-test Probability for Location x Genotype Interaction		***1.4%***
	average	2.09 mg/g
ILSI (2006)	**range**	1.24 - 4.68
(mg/g = % dw x 10)	**N**	1350
OECD (2002)	**range**	0.10 - 0.46

Location	Genotype	Tyrosine
Bondville, IL	**B3 (3272)**	0.316
Bondville, IL	**B4 (Control)**	0.275
Brookings, SD	**B3 (3272)**	0.348
Brookings, SD	**B4 (Control)**	0.353
Glidden, IA	**B3 (3272)**	0.373
Glidden, IA	**B4 (Control)**	0.359
Janesville, WI	**B3 (3272)**	0.436
Janesville, WI	**B4 (Control)**	0.365
Stanton, MN	**B3 (3272)**	0.370
Stanton, MN	**B4 (Control)**	0.428
Washington, IA	**B3 (3272)**	0.313
Washington, IA	**B4 (Control)**	0.291
B3 (3272)		0.359
B4 (Control)		0.345
F-test Probability for Genotype		*
F-test Probability for Location x Genotype Interaction		***4.1%***
	average	3.36 mg/g
ILSI (2006)	**range**	1.03 - 6.42
(mg/g = % dw x 10)	**N**	1350
OECD (2002)	**range**	0.12 - 0.79

* Statistically significant genotype x location interaction undermines the validity of the comparison across locations.

Table 6-13. Location means for antinutrients and secondary metabolites of Event 3272 and non-transgenic maize grain

(All values mg/kg dry weight unless otherwise indicated)

Data from ILSI crop composition database version and OECD consensus document included for comparison (no OECD data for trypsin inhibitor).

Location	Genotype	*p*-Coumaric Acid
Bloomington, IL	**A1 (3272)**	158
Bloomington, IL	**A2 (Control)**	175
Bondville, IL	**A1 (3272)**	152
Bondville, IL	**A2 (Control)**	199
Fairbault, MN	**A1 (3272)**	195
Fairbault, MN	**A2 (Control)**	144
Glidden, IA	**A1 (3272)**	90
Glidden, IA	**A2 (Control)**	123
Shirley, IL	**A1 (3272)**	73
Shirley, IL	**A2 (Control)**	91
Stanton, MN	**A1 (3272)**	197
Stanton, MN	**A2 (Control)**	169
A1 (3272)		144
A2 (Control)		150
F-test Probability for Genotype		*
F-test Probability for Location x Genotype Interaction		***4.0%***
ILSI (2006)	**average**	218.4
	range	53.4 - 576.2
	N	817
OECD (2002) (% dw x 10^4 = mg/kg)	**range**	0.003 - 0.03 % dw

Location	Genotype	Phytic Acid (% dw)	Trypsin Inhibitor (TIU[1]/mg)
Bondville, IL	**B3 (3272)**	0.706	2.69
Bondville, IL	**B4 (Control)**	0.638	3.07
Brookings, SD	**B3 (3272)**	0.811	2.69
Brookings, SD	**B4 (Control)**	0.822	2.53
Glidden, IA	**B3 (3272)**	0.717	2.71
Glidden, IA	**B4 (Control)**	0.600	2.53
Janesville, WI	**B3 (3272)**	0.866	2.42
Janesville, WI	**B4 (Control)**	0.664	2.82
Stanton, MN	**B3 (3272)**	0.984	3.10
Stanton, MN	**B4 (Control)**	1.069	3.14
Washington, IA	**B3 (3272)**	0.740	3.10
Washington, IA	**B4 (Control)**	0.822	2.83
B3 (3272)		0.804	2.79
B4 (Control)		0.769	2.82
F-test Probability for Genotype		*	*
F-test Probability for Location x Genotype Interaction		***1.6%***	***0.3%***
ILSI (2006)	**average**	0.745 % dw	2.73 TIU/mg
	range	0.111 - 1.570	1.09 - 7.18
	N	1196	696
OECD (2002)	**range**	0.45 - 1.0 % dw	

* Statistically significant genotype x location interaction undermines the validity of the comparison across locations.

[1] TIU= Trypsin Inhibitor Unit

Chapter 7. CULTIVATION PRACTICES

A. Intended Cultivation Area

A1. Regions where the plant will be grown

Event 3272 will be grown in the United States for domestic use by the dry-grind fuel ethanol production market. Field maize is planted in almost every state in the United States; however, the majority of maize (88 percent in 2003) is grown in the region which extends from western New York to western Nebraska and from the Canadian border to the panhandle of Texas. It is anticipated that Event 3272 hybrids will be grown in the same areas as current commercial maize hybrids.

A2. New ecosystems where the plant will be grown

The introduced traits in Event 3272 were not intended to confer any competitive advantage or extend the range of maize cultivation outside of existing cultivation areas.

B. Cultivation Practices

B1. Standard cultivation practices for maize

Maize is intensively managed, as evidenced by the chemical usage data from the USDA-NASS Agricultural Chemical Usage Report that examined chemical usage in 18 states in 2003 (USDA, 2004a). Nitrogen was applied to 96 percent of the 2003 maize-planted acreage in the states of: Colorado, Illinois, Indiana, Iowa, Kansas, Kentucky, Michigan, Minnesota, Missouri, Nebraska, New York, North Carolina, North Dakota, Ohio, Pennsylvania, South Dakota, Texas, and Wisconsin. Corn growers used an average of 1.7 applications per acre while applying 78 pounds of nitrogen per treatment. This computes to a crop year rate per acre of 136 pounds per acre. In the surveyed states, 79 percent of the maize-planted acreage received a phosphate application, while potash was applied to 64 percent of the planted acreage.

Herbicides were applied to 95 percent of the maize-planted acreage in 2003 in the USDA-NASS Program States. Atrazine continued to be the most widely applied herbicide with 68 percent of the planted acreage being treated. It was applied at a rate of 1.04 pounds per acre. Acetochlor, at 26 percent of the planted acres treated, was the second most widely applied herbicide, followed by glyphosate and S-metolachlor, both applied to 19 percent of the planted maize acreage treated in the Program States.

In 2003, 29 percent of the maize-planted acreage was treated with insecticides. Cyfluthrin and tebupirimphos were the most widely applied insecticides, both applied to 7 percent of the acres planted to maize in the states surveyed.

During the domestication process, maize lost its ability to disperse seed and to compete in unmanaged environments, and now relies entirely on human intervention for its survival (Gould 1968). Plants occasionally grow in uncultivated fields and by

roadsides or occur as volunteers in cultivated crops in the year following cultivation of a maize crop (CFIA, 1994), but these plants are generally not competitive and rarely produce viable seed for the next growing season. Control of volunteer maize is commonly achieved by mechanical means and herbicides.

B2. Cultivation practices for Event 3272 hybrids

No changes to agronomic practices typically applied in management of conventional maize are required for Event 3272 hybrids. Specifically, no increases in pesticides and fertilizers are required as well as no changes in cultivation, planting, harvesting, or volunteer control.

B3. Specific deployment strategies

There are no specific deployment strategies (*e.g.*, insect resistance management or herbicide tolerance management) required for Event 3272 hybrids as they do not display the traits of insect resistance or herbicide tolerance. However, the Event 3272 harvested grain will need to be directed to dry-grind ethanol producers to allow capture of the value resulting from the introduced alpha-amylase enzyme.

Chapter 8. ENVIRONMENTAL CONSEQUENCES OF INTRODUCTION

A. Introduction

The purpose of this chapter is to summarize data on the environmental safety of field maize derived from Event 3272 and to draw conclusions about the likely environmental impact of its cultivation. The environmental safety of commercial cultivation of Event 3272 is considered in two parts: the likelihood that Event 3272 maize will harm wildlife, including species beneficial to agriculture and endangered and threatened species; and the likelihood that Event 3272 maize will become a serious weed of agriculture or non-agricultural habitats.

B. Assumptions and objectives

A fundamental assumption of this chapter is that the cultivation of non-transgenic maize poses no currently unacceptable environmental risks, and hence if it can be shown that Event 3272 maize does not increase those risks significantly, Event 3272 maize can be regarded as environmentally safe. Throughout the chapter, therefore, the risks of Event 3272 maize relative to conventional maize are assessed, rather than the absolute risks posed by Event 3272 maize.

The objectives of the environmental assessment are twofold: to show that Event 3272 maize is highly unlikely to be more harmful to wildlife than is conventional maize; and to demonstrate that Event 3272 maize is highly unlikely to display weediness characteristics in agricultural habitats, or to be more invasive of non-agricultural habitats than is conventional maize.

If these objectives are met, the protection of two assessment endpoints are demonstrated. The first is the diversity and abundance of wildlife within and outside maize fields. This endpoint includes, but is not limited to, animals that consume maize or pests of maize; insects that benefit agriculture; animals that ingest or are otherwise exposed to maize or its derivatives; and plants that grow in habitats that could be invaded by maize. The second endpoint is the yield of crops in which maize is a potential weed. Protection of these endpoints ensures that we meet the objectives of several environmental protection statutes, including the Federal Plant Protection Act, the Endangered Species Act and the National Environmental Policy Act.

C. The safety of Event 3272 maize to wildlife

The likelihood that wildlife will be exposed to harmful amounts of toxic substances present in Event 3272 maize is evaluated in this section.

The safety assessment for wildlife first compares the composition of Event 3272 maize with that of near-isogenic non-transgenic maize, and with maize in general. The aim is to identify substances in Event 3272 maize that show changed concentration relative to non-transgenic maize. The routes of exposure of wildlife to these substances are then identified, followed by an assessment of their hazard (toxicity). The hazard and exposure data are then combined to assess the risk, defined as the likelihood that Event 3272 maize will be associated with reduced abundance or diversity of wildlife.

C1. The composition of Event 3272 maize

Studies of composition and nutritional quality (Chapter 6) support the conclusion that Event 3272 maize does not differ from conventional maize, apart from the presence of AMY797E and PMI, and the DNA insert required for their production. The results of the compositional analysis demonstrate that any risk from toxicity of Event 3272 maize to wildlife will arise from exposure to AMY797E and PMI (DNA and RNA pose no safety concerns; FDA, 1992).

C2. The environmental fate of AMY797E and PMI

The environmental fate of AMY797E and PMI, including presence of these proteins resulting from gene flow of *amy797E* and *pmi* from Event 3272 maize, will be considered in order to identify routes of exposure to wildlife.

C2i. AMY797E and PMI in cultivated plants

The pattern of expression of AMY797E in Event 3272 maize is described in Chapter 3. During cultivation of Event 3272 maize, AMY797E will be present in kernels at all stages of kernel development. PMI will be present in all tissues during cultivation of Event 3272 maize, although the concentration of PMI will decline significantly throughout the plant at kernel maturity. PMI is expressed in pollen, and therefore exposure to PMI off-crop may occur *via* dispersed pollen.

C2ii. AMY797E and PMI in soil

Plants exude proteins from their roots (*e.g.*, Rengel, 2002) and it is possible, therefore, that Event 3272 maize will exude AMY797E or PMI, or both, into the soil during cultivation. AMY797E and PMI may also enter soil from plant debris during and immediately after harvest of Event 3272 maize, and if Event 3272 maize is not harvested for any reason. Most proteins do not persist or accumulate in the soil because they are inherently degradable by proteases in soils that have healthy microbial activity (Burns, 1982; Marx *et al*., 2005, and references therein). Alpha-amylases and phosphomannose isomerases are common in soil organisms (see section

C3i below) and these proteins are not known to persist or accumulate in soil. No studies have addressed specifically the stability of AMY797E or PMI in soil. However, both proteins show susceptibility to protease degradation in simulated gastric fluid containing a single protease, pepsin; therefore there is no evidence that AMY797E and PMI will be less inherently degradable than other alpha-amylases or phosphomannose isomerases present in soil.

Another theoretical route of entry to the soil is horizontal gene flow of *amy797E* and *pmi* leading to expression of these proteins in soil micro-organisms. Recent reviews (EPA, 2001; Connor *et al*., 2003) conclude that there is minimal likelihood of horizontal gene transfer between transgenic plants and soil micro-organisms. Should *amy797E* or *pmi* from Event 3272 maize be integrated into a plasmid or chromosome of a bacterium, AMY797E and PMI are extremely unlikely to be produced because their plant-derived promoters are unlikely to function in bacteria. In addition, codon use in *amy797E* is optimized for expression in maize, not bacteria. Therefore AMY797E and PMI are extremely unlikely to be produced in soil *via* transformation of bacteria with genes from Event 3272 maize.

C2iii. Volunteers of Event 3272 maize

Maize seed spilled during harvest can overwinter and germinate in a subsequent crop as a volunteer weed; for example, maize is a common volunteer in soybeans. However, several features of maize make it unlikely to form self-sustaining weedy populations in agriculture: it is easily controlled in subsequent crops with selective herbicides; seed dispersal is limited because seeds are held inside the husks of the cob; and the seeds lack primary dormancy[1] so that young plants are exposed to harsh winter conditions. Volunteer maize can be controlled by good agronomic practice and it not usually a problem to farmers.

Event 3272 maize hybrids did not differ from near-isogenic, non-transgenic hybrids in agronomic characteristics typically used by breeders and agronomists to evaluate maize (Chapter 5), and therefore Event 3272 maize is highly unlikely to be associated with an increase in the abundance of maize volunteers or be more difficult to control than conventional maize volunteers.

It is possible that some exposure to PMI will occur *via* volunteers of Event 3272 maize. However, exposure will be negligible compared with that during cultivation of Event 3272 maize. Exposure to AMY797E is much less likely than exposure to PMI because volunteer maize is likely to be destroyed before kernels are formed: AMY797E expression in Event 3272 maize is almost completely restricted to kernels.

[1] Primary dormancy is conferred by physical or physiological traits of the seed that prevent immediate germination; secondary dormancy occurs when the seed is capable of germination, but environmental conditions are unsuitable to induce germination. Overwintering of maize seed occurs *via* secondary dormancy.

C2iv. Feral populations of Event 3272 maize

During selection for desirable agronomic traits, maize lost the ability to survive outside cultivation (OECD, 2003). In addition to a lack of dormancy and limited dispersal, maize requires disturbed ground to germinate and is uncompetitive with perennial plants and hence self-sustaining feral populations of maize do not form. Agronomic data (Chapter 5) provide no evidence that Event 3272 maize will form persistent feral populations. The occasional feral plant of Event 3272 maize on roadsides as a result of spillage of seed is possible. However, compared with cultivation of Event 3272 maize, exposure to AMY797E and PMI *via* feral plants is likely to be minimal. In addition, because expression of AMY797E is targeted to the seeds, any feral maize plants must flower and set seed for significant exposure to AMY797E to occur.

C2v. Gene flow from Event 3272 maize to wild relatives of maize

Maize (*Zea mays* ssp. *mays*) will hybridize with a group of wild 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 (EPA, 2001). Teosinte species are not natives of the United States, 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 (EPA, 2001). Teosinte species are grown in botanical gardens, but fertilization of these plants with pollen from Event 3272 maize is extremely unlikely. There are no records of teosinte from the border of the United States and Mexico (OECD, 2003); therefore any cultivation of Event 3272 maize near the Mexican border is unlikely to result in fertilization of teosinte.

Species of the genus *Tripsacum* are considered close relatives of maize. There are sixteen species of *Tripsacum* worldwide, of which three occur in the United States: *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) and therefore substantial effort has been made to obtain and characterize maize X *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 United States would interbreed with commercial maize.

The data indicate a very low probability of transfer of the *amy797E* and *pmi* genes from Event 3272 maize to wild relatives of maize: species of *Zea* other than maize are not recorded outside botanical gardens in the United States; *Tripsacum dactyloides* is widespread, but does not hybridize readily with maize; and the probability of backcross or F2 progeny of *Tripsacum* X *Zea* hybrids being produced in the field is negligible. Therefore, exposure to AMY797E and PMI *via* expression in wild relatives of maize in the United States is extremely unlikely.

C2vi. Outcrossing between Event 3272 maize and other cultivated maize

Maize is an outcrossing species and almost all varieties are interfertile (OECD, 2003). Therefore Event 3272 maize is likely to cross-fertilize other maize varieties. However, the extent of cross fertilization will be limited. Maize pollen is heavy and tends to be deposited close to the source plant (*e.g.*, Pleasants *et al*., 2001); for instance, work in Canada by Sears and Stanley-Horn (2000) estimated that 100% of pollen from 7 maize fields in Ontario, was deposited within 100 meters of the field margin, and 95% was deposited within 10 meters. Also, pollen dispersal is not the only determinant of fertilization; receptive silks of the potential female parent need to be present during anthesis of the potential male parent and therefore different planting times or developmental rates may prevent cross-fertilization between varieties that are grown adjacently.

The rate of outcrossing between maize varieties varies according to the size of the source of pollen, wind direction and speed, other weather conditions such as rainfall, and the presence of barriers. Viable maize pollen can travel at least 800 meters, and probably much farther (Eastham and Sweet, 2002), but on average cross-fertilization is low beyond a few hundred meters: in the EU, 200 meters is deemed sufficient to maintain inbred lines at 99.9% purity (Eastham and Sweet, 2002); in the United States, inbred maize lines that produce foundation seed must be 660 feet (*ca*. 200 meters) from sources of contaminating pollen (7 CFR 201.76). Therefore, due to fertilization by pollen from Event 3272 maize, expression of AMY797E and PMI may occur in kernels of maize other than hybrids specifically intended to express these proteins. However, expression will be predominantly within 200 meters of fields where hybrids derived from Event 3272 maize are grown, and wildlife will be exposed to much lower concentrations of AMY797E and PMI than by exposure through intentional cultivation of Event 3272 maize.

The introduced traits in Event 3272 were not intended to have any effect on the range or frequency of maize outcrossing. In addition, there were no phenotypic data indicating that the reproductive characteristics or fertility of Event 3272 hybrids had been changed as a result of the genetic modification.

C2vii. Potential consequences of gene flow

The traits introduced into Event 3272 were not intended to alter reproductive fitness nor confer any selective advantage over conventional maize. Additionally, there was no evidence from phenotypic and agronomic data that these characteristics had been

unintentionally altered as a result of the genetic modification. Therefore, there are no anticipated environmental or agronomic consequences arising from the potential for limited gene flow from Event 3272 to other cultivated maize varieties.

C2viii. Summary – identification of wildlife exposed to AMY797E and PMI

Wildlife will be exposed to AMY797E and PMI mainly during cultivation of Event 3272 maize. Exposure to the highest concentrations of AMY797E will occur *via* consumption of kernels. Another route of exposure to AMY797E is contact with or ingestion of soil in fields of Event 3272 maize during and immediately after harvest. Concentrations of AMY797E are expected to be low and transient as the protein is likely to be degraded by soil proteases during decomposition of plant residues, and the probability of expression of AMY797E in soil bacteria following horizontal transfer of *amy797E* is minimal. The only other realistic route of exposure to AMY797E is consumption of kernels of maize in fields within 200 meters of fields of Event 3272 maize. Organisms exposed *via* this route will be the same as in fields of Event 3272 maize, and doses of AMY797E will be much lower; only a small proportion of kernels will express the protein, and compositional analysis indicates that Event 3272 maize should be as palatable as any other maize. Exposure *via* volunteers or feral plants will be insignificant compared with the crop, as these plants are unlikely to flower and AMY797E is expressed principally in kernels. AMY797E is not detectable in pollen of Event 3272 maize and therefore negligible exposure will occur *via* contact with or consumption of pollen. Negligible exposure will also occur through hybridization of Event 3272 maize with wild relatives of maize.

The patterns of exposure of wildlife to PMI will be similar to AMY797E, with slightly higher probabilities of exposure through certain routes. During cultivation of Event 3272 maize, exposure to PMI can occur through consumption of any tissue, not merely kernels. The probability of exposure *via* volunteers and feral plants is higher than for AMY797E, as exposure to PMI can occur *via* vegetative tissue, but is still low. Finally, off crop exposure through pollen is possible for PMI.

Many insects will be exposed to AMY797E by eating kernels. Common insects feeding on maize kernels include Angoumois grain moths (*Sitotroga cerealella*)[2], corn earworms (*Helicoverpa zea*), European corn borers (*Ostrinia nubilalis*), pink scavenger caterpillars (*Pyroderces rileyi*) and wheat curl mites (*Aceria tosichella*) (Steffey *et al*., 1999). Insects may also be exposed by eating planted seeds; potentially exposed species include fire ants (*Solenopsis invicata*), thief ants (*Solenopsis molesta*), seedcorn beetles (*Stenolophus lecontei* and *Clivina impressifrons*), wireworms (*Aeolus* ssp., *Conoderus* species, *Limonius* species, *Hemicrepidius* species, *Agriotes* species and *Melanotus* species), seedcorn maggots (*Delia platura* and *Anthomyia zeae*) and slugs (in particular *Derocera reticulatum*) (Steffey *et al*., 1999). However, these species are regarded as pests. It is possible that beneficial insects that control pests of maize kernels will be exposed to AMY797E in the bodies of their prey: *Orius insidiosus* and braconid parasitic wasps are important natural enemies of corn

[2] Angoumois grain moths infest corn kernels in the field and are the most serious pest of stored corn.

earworms. Natural enemies are not regarded as important controls of other pests of maize kernels (Steffey et al., 1999).

Mammals and birds also consume maize kernels in the field. Mammals can be serious pests of maize. Rodents such as thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*), deer mice (*Peromysus maniculatus*), house mice (*Mus domesticus*), and prairie and meadow voles (*Microtus* species) will feed on germinating maize seeds. Frequently these species remove so many seeds that the field needs to be replanted. Woodchucks (*Marmota monax*) also feed on sprouting maize seed, but because they feed along the edges of fields, they usually cause less serious damage than other rodents. Larger mammals such as white-tailed deer (*Odocoilus virginianus*) and raccoons (*Procyon lotor*) cause injury to ripening ears. Deer typically nip off ear tips, whereas raccoons chew through husks. In some areas these species are hunted specifically to reduce damage to maize fields (Steffey *et al*., 1999).

Birds such as crows (*Corvus brachyrhynchos*), grackles (*Quiscalus quiscula*) and sandhill cranes (*Grus canadensis*) uproot sprouting maize to feed on the germinating kernels (*e.g*., Steffey *et al*., 1999; Blackwell *et al*., 2001; Sterner *et al*., 2003). Red-winged blackbirds (*Agelaius phoeniceus*) and grackles destroy over 360,000 metric tons per annum of ripening field maize in the United States 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 maize stubble where they forage for spilled maize kernels and weed seeds (Linz *et al*., 2003).

Soil in maize fields contains an enormous number of invertebrate and microbial species. Among the more important for agriculture are earthworms for their role in improving soil structure (*e.g.*, Six *et al*., 2004), organisms that decompose organic matter (*e.g.*, Knacker *et al*., 2003), organisms involved in nutrient cycling (*e.g.*, Schloter *et al*., 2003) and predators of pests of maize, notably ground and rove beetles (Carabidae and Staphylinidae respectively) (Steffey *et al*., 1999).

Organisms exposed to AMY797E will also be exposed to PMI. Additional species and groups will be exposed to PMI because of its expression in vegetative tissue and pollen. In maize there are many pests that feed on vegetative tissue. Among the more important economically are corn rootworms (*Diabrotica* species), armyworms (*Pseudaletia unipunctata* and *Spodoptera* species), black cutworms (*Agrotis ipsilon*), European corn borers (*Ostrinia nubilalis*) , grasshoppers (*e.g.*, *Melanoplus* species), southwestern corn borer (*Diatraea grandiosella*) and aphids (*e.g.*, *Aphis* species and *Rhopalosiphum* species). Predators and parasitoids of these pests that might be exposed to PMI through the body of their prey or host include tachinid flies, braconid, ichneumonid wasps, ground and rove beetles, ladybird beetles, bug (including *Orius insidiosus*) and green and brown lacewings (Neuroptera: Chrysopidae and Neuroptera: Hemerobiidae respectively). Ladybird beetles, *Orius* and lacewings may also be exposed to PMI in pollen.

Species not exposed to AMY797E, but potentially exposed to PMI because of expression in pollen, include honeybees and monarch butterflies. Although maize does not produce nectar, honeybees forage in maize for pollen (*e.g.*, Bonmatin *et al*., 2005). Monarch butterflies are potentially exposed to PMI when they feed on milkweed leaves dusted with maize pollen in, or adjacent to, maize fields (*e.g.*, Pleasants *et al*., 2001).

C3. Hazards of AMY797E and PMI to wildlife

C3i. Prior exposure to alpha-amylases and phosphomannose isomerases

Alpha-amylases are ubiquitous, occurring widely in plants, and are common in crops including maize (see Chapter 4A5.) Exposure to AMY797E will occur mainly through consumption or contact with Event 3272 maize or soil in which Event 3272 maize is cultivated (section C2). It is likely, therefore, that species exposed to AMY797E *via* plant tissue have prior exposure to proteins with similar function. No harmful effects of such exposure to naturally-occurring concentrations of alpha-amylases are known.

Alpha-amylases are common in soils (Ross, 1983; Kumari and Singaracharya, 1998; Fioretto *et al*., 2000). There is an enormous diversity of alpha-amylases in soil micro-organisms (*e.g.*, Rondon *et al*., 2000), and many heat-stable alpha-amylases have been isolated from soil micro-organisms (*e.g.*, Medda and Chandra, 1980; Mellouli *et al*., 2005). Therefore, species exposed to AMY797E *via* soil are likely to have been exposed previously to enzymes with similar function. No harmful effects of such exposure are known.

Phosphomannose isomerase proteins are ubiquitous, occurring widely among prokaryotes and eukaryotes (see Chapter 4B5). Data on the distribution of phosphomannose isomerases indicate that species that will be exposed to PMI from Event 3272 maize are highly likely to have prior exposure to proteins with similar function. No harmful effects of such exposure to naturally-occurring concentrations of PMI are known.

C3ii. Assessment of similarity of AMY797E and PMI to known toxins

AMY797E and PMI do not have significant amino acid sequence identity to any proteins known to be toxins.

C3iii. Potential toxicity of AMY797E and PMI proteins

Rodents are the mammals most likely to be exposed to AMY797E (section C2). The daily dietary dose of AMY797E for rodents can be calculated using the methods of Crocker *et al*. (2002). The formula for daily dietary dose (DDD) is

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

where FIR = food intake rate, bw = body weight and C = concentration of AMY797E in food.

Crocker *et al*. (2002) estimated the ratio of food intake rate and body weight (*FIR/bw*) for several rodent species. The values for the harvest mouse (*Micromys minutus*) and

the wood mouse (*Apodemus sylvaticus*) consuming cereal seeds are 0.33 and 0.28, respectively. The highest concentration of AMY797E recorded in kernels of Event 3272 maize is 2144 µg AMY797E/g fresh weight (Table 3-5); therefore a worst-case DDD for rodents eating a diet comprising 100% kernels of Event 3272 maize is approximately 708 mg AMY797E/kg body weight. An acute oral mouse toxicity study concluded that AMY797E protein is non-toxic to mice (see Chapter 4A6). The no observed effect dose (NOED) in the mouse study (1511 mg AMY797E protein/kg body weight) therefore represents about 2.1X the worst-case DDD. Other mammals, such as deer, will be exposed to lower amounts of AMY797E than rodents because they nibble the tips of maize ears rather than kernels (Steffey *et al*., 1999).

The effects of AMY797E (and PMI) on birds was investigated in a long-term (49 days) feeding study, in which broiler chickens were fed diets containing 52% to 65% grain of Event 3272 maize (see Chapter 4A6). No harmful effects of exposure to grain of Event 3272 maize were observed. Wild birds are unlikely to be exposed to a diet of 65% grain of Event 3272 maize for 49 consecutive days: exposure to planted seed will be limited because of seed germination and exposure to kernels in ears will be limited by harvest.

The highest recorded concentration of PMI in kernels of Event 3272 maize is 0.9 µg/g fresh weight (Table 3-5). Using the calculation for rodents described above, this represents a DDD of about 0.3 mg PMI/kg body weight. An acute oral mouse toxicity study concluded that AMY797E protein is non-toxic to mice (see Chapter 4B6). The NOED in the mouse study (3080 mg PMI protein/kg body weight) is therefore over 10,000X the worst-case DDD for rodents.

The highest recorded concentration of PMI in leaves of Event 3272 maize is 7.2 µg/g fresh weight (Table 3-5). Crocker *et al*. (2002) estimated the *FIR*/*bw* for fallow deer eating grass to be 0.09. Therefore the worst-case DDD for deer browsing Event 3272 maize ears or leaves is 0.65 mg PMI/kg body weight; the NOED in the mouse study is therefore over 4,700X the worst case DDD for deer.

Other data add to the weight of evidence that AMY797E will not harm birds or mammals, and will not harm invertebrates or soil micro-organisms that might be exposed *via* routes discussed in section C2. The protein has no significant sequence identity to known toxins; and agronomic data do not suggest that Event 3272 maize has altered sensitivity (resistance) to pests and diseases. In addition, alpha-amylases are ubiquitous and diverse, and have a history of safe use for starch processing in the food industry (*e.g.*, Pariza and Foster, 1983; Pariza and Johnson, 2001). The diversity of alpha-amylases is particularly high in soil (Rondon *et al*., 2000). Invertebrate wildlife and soil micro-organisms are constantly exposed to a variety of alpha-amylases without apparent ill effects.

The effects on soil microbial diversity of cultivating transgenic alfalfa (*Medicago sativa*) with high expression of an alpha-amylase from *Bacillus licheniformis* in its roots (Austin *et al*., 1994) were examined by Di Giovanni *et al*. (1999) and Donegan *et al*. (1999). Alfalfa plants expressing the transgenic amylase were grown for two

years in a field plot at Oregon State University; transgenic alfalfa expressing lignin peroxidase and non-transgenic alfalfa (the parent line of the two transgenic lines) were also grown. The three alfalfa genotypes were inoculated with a non-transgenic strain of the nitrogen fixing bacterium *Sinorhizobium meliloti*, or one of two transgenic strains of *S. meliloti*, or were not inoculated; this gave 12 treatments, three genotypes and four types of inoculation. The treatments were compared for a variety of parameters: metabolic fingerprints and DNA fingerprints of soil bacterial communities; soil microbial respiration; population counts of indigenous soil bacteria, fungi, nematodes, protozoa and micro-arthropods; plant shoot weight and composition; and soil chemistry and enzyme activities. In general, there were no statistically significant differences among the treatments. Although there were statistically significant differences between principal component analysis scores of the transgenic lignin peroxidase-producing alfalfa and the parental line, the amylase line and parental line scores were not significantly different. This suggests that expression of amylases in transgenic plants may have little effect on microbial populations because of the existing high diversity of these enzymes in soil. No differences in soil function were detected. This is particularly interesting because it supports the theory that species diversity in soil is a poor predictor of function because of high functional redundancy in soil (Ritz, 2005).

The lack of detectable toxicity of AMY797E in birds and mammals, and the weight of evidence that AMY797E is not toxic to other wildlife, indicates minimal risk of significant reduction in the abundance or diversity of wildlife resulting from exposure to this protein in Event 3272 maize.

The EPA has granted an "exemption from the requirement of a tolerance" for PMI in all crops (EPA, 2004). The EPA was required to consider a requirement for tolerance because PMI is expressed in some transgenic plants that produce pesticidal proteins, and therefore in these circumstances PMI is regarded as an 'inert' component of a pesticide and regulated under the Federal Food, Drug and Cosmetic Act (FFDCA) (among other legislation). To grant an exemption from tolerances, the EPA must be satisfied that there is a "reasonable certainty that no harm will result from aggregate exposure to the [substance], including all anticipated dietary exposures and all other exposures for which there is reliable information".

The conclusion that PMI is regarded as safe under FFDCA has bearing on the risk of PMI to wildlife because mammals are likely to be exposed to PMI *via* Event 3272 maize; PMI is highly unlikely to be toxic to these organisms. Birds may also be exposed to low amounts of PMI *via* leaves and kernels of Event 3272 maize; the lack of detectable harm to broilers fed grain from Event 3272 maize for 49-days indicates that PMI is unlikely to be toxic birds. Low probability of harm to other wildlife is indicated by weight of evidence: PMI has no significant sequence identity to known toxins; organisms potentially exposed to PMI in Event 3272 maize, including those such as honeybees that may be exposed *via* pollen, have prior exposure to diverse phosphomannose isomerases without apparent ill effect; and agronomic data do not suggest that Event 3272 maize has altered sensitivity (resistance) to pests and diseases.

In summary, Event 3272 maize has similar composition and nutritional quality to conventional maize, apart from the presence of AMY797E and PMI. As a consequence, reduction in the abundance or diversity of wildlife through cultivation of Event 3272 maize will occur only if AMY797E or PMI, or both, are toxic. Laboratory studies carried out by Syngenta, and the weight of evidence from published studies, indicate that PMI and AMY797E are highly unlikely to be toxic to wildlife exposed to these proteins *via* Event 3272 maize. Therefore, cultivation of Event 3272 maize poses minimal risk to wildlife.

D. Risk of increased weediness of Event 3272 maize

This risk assessment seeks to demonstrate the protection of two assessment endpoints: the diversity and abundance of wildlife within and outside maize fields, and crop yield.

Wild plants can also be regarded as wildlife, and they could be harmed if Event 3272 maize is weedier than conventional maize. The abundance and diversity of wild plants could be reduced if feral populations of Event 3272 maize, or hybrids of Event 3272 maize with wild species, establish and spread into semi-natural or natural habitats; organisms that rely on these wild plants for food or shelter could also be harmed (*e.g.*, Raybould and Wilkinson, 2005). If Event 3272 maize is more likely to be a volunteer weed than conventional maize, the yield of other crops may be affected. Volunteers reduce crop yield directly through competition, and indirectly by acting as "green bridges" for pests and pathogens, and are therefore regarded as plant pests (Froud-Williams *et al*., 1993; Raybould, 2005).

A simple way to test whether a transgenic variety is likely to be weedier than its corresponding non-transgenic variety is to compare their performance in agronomic trials (White, 2002; Raybould, 2005). If agronomic characteristics are similar, then it is likely that the potential to form weedy populations is unchanged. If the risks to endpoints potentially affected by weediness are acceptable for the non-transgenic crop, it follows that the risks should be acceptable for the transgenic crop (Raybould, 2005).

The risks to wildlife and agricultural productivity from weedy maize populations are low, provided that the maize does not contain substances not intended for general human or animal consumption (Miller, 2003). Maize is a highly domesticated crop that has lost the ability to persist in the wild: it has no primary dormancy, requires disturbed ground to germinate and has extremely limited seed dispersal because kernels are retained on the cob. Maize can volunteer in subsequent crops, particularly soybeans, but it is easily controlled with selective herbicides or tillage; any plants that escape control usually do not produce viable progeny (OECD, 2003). Maize plants are not invasive of natural habitats (Gould, 1968), and although plants can establish outside cultivation from seed spilled during transport, feral maize is unable to form self-sustaining populations (OECD, 2003). If there are no changes in Event 3272 maize in characters associated with weediness, it is highly unlikely that it will pose significantly greater risks to agricultural productivity or to semi-natural and natural habitats than non-transgenic maize. These weediness characteristics were evaluated in extensive agronomic trials comparing Event 3272 to near-isogenic, non-transgenic

plants and no substantial differences were observed in seed germination, dormancy, plant growth habit, life-span, vegetative vigor, flowering characteristics, grain yield, stress adaptations and disease susceptibility (Chapter 5).

These data support the conclusion that Event 3272 will not be weedier than conventional maize and that there is minimal risk to the abundance or diversity of populations of wild plants and to crop yield from the cultivation of Event 3272.

E. Summary environmental risk assessment

Two objectives to demonstrate minimal environmental risk of cultivating Event 3272 maize were listed: to show that Event 3272 maize is highly unlikely to be more harmful to wildlife than is conventional maize; and to show that Event 3272 maize is highly unlikely to display more weediness characteristics, or be more invasive of non-agricultural habitats, than is conventional maize.

Wildlife is highly unlikely to suffer toxicity from exposure to Event 3272 maize. The composition of Event 3272 maize is no different from non-transgenic maize, apart from the presence of AMY797E and PMI. Studies performed by Syngenta, and extensive data in the published literature indicate that these proteins are not toxic to wildlife at concentrations found in Event 3272 maize. Risks to the abundance and diversity of wildlife from exposure to toxic substances in Event 3272 maize therefore are minimal.

Conventional maize is incapable of forming self-sustaining populations without cultivation, and field trial data support that Event 3272 maize is highly unlikely to increase the potential of maize to become weedy. The risks to wild plants from invasions of feral or wild populations derived from Event 3272 maize, and the risks to yields of crops potentially affected by volunteer maize, are minimal.

In summary, no significant risks to the diversity and abundance of wildlife within and outside maize fields, or to crop yield, have been identified from the proposed cultivation of Event 3272 maize.

Chapter 9. REFERENCES

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Appendix 1. Event 3272 Pedigree

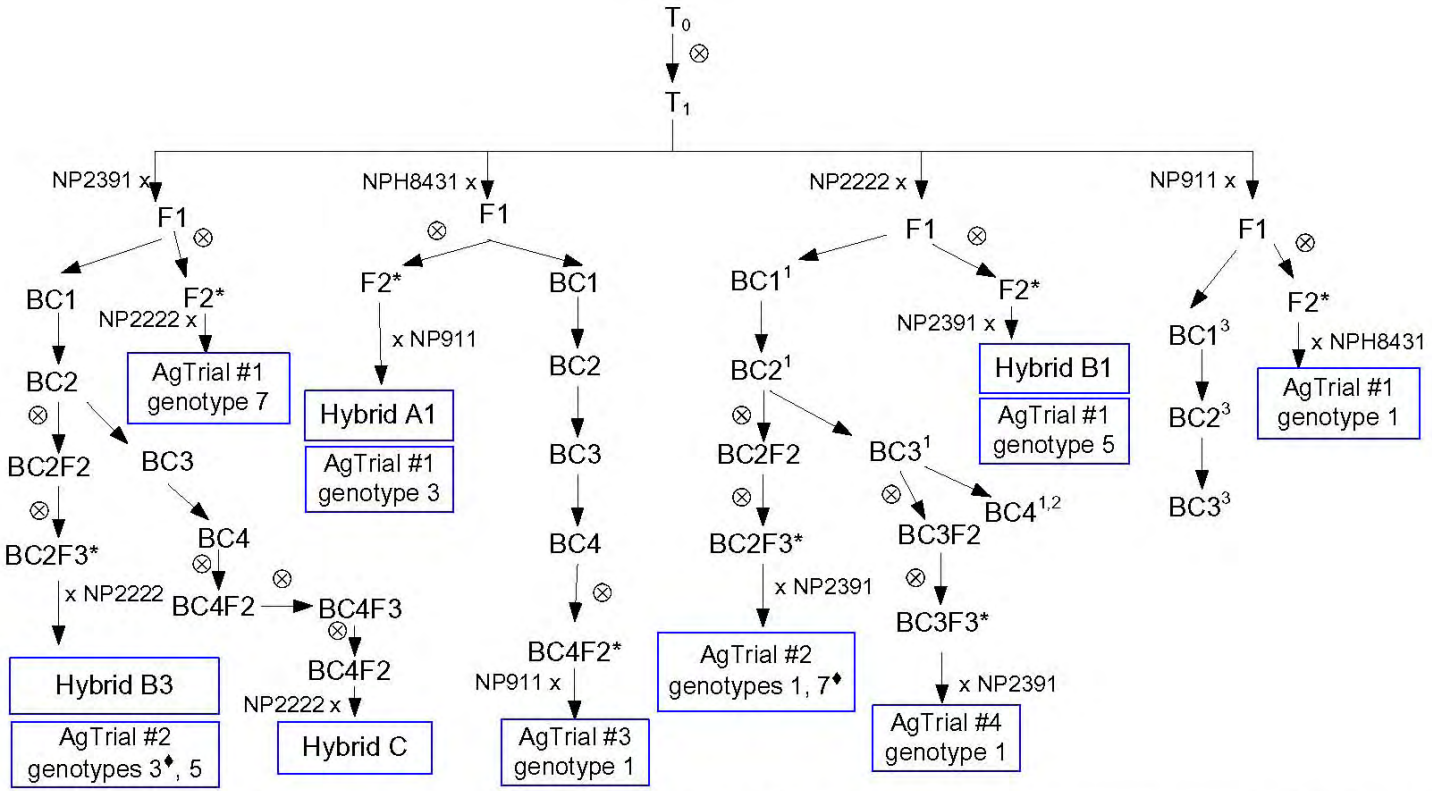
A. Breeding History of Event 3272

A single maize transformation event, designated Event 3272, was utilized for the introgression of the linked *amy797E* and *pmi* transgenes into several elite maize inbreds. Event 3272 was generated from *Agrobacterium*-mediated transformation of immature maize embryos (see Chapter 3 for details) from a heterogenous genetic background (NP2499 x NP2500) derived from a cross between two dent corn inbred lines. This background was selected for its suitability to transformation by *Agrobacterium*. One transformed maize plant, designated Event 3272, was selfed and then crossed to several elite maize inbreds (NP2391, NPH8431, NP2222 and NP911) (see Appendix 1-Figure 1 for schematic diagram). Several backcrosses were performed in order to introgress the transgenes into the respective inbred germplasms. Maize hybrids are typically made by crossing two different inbreds, and the hybrids utilized in the studies in this petition involved hybrids made by crossing inbred NPH8431 to inbred NP911 and by crossing inbred NP2391 to inbred NP2222. Event 3272 maize hybrids were made by crossing an Event 3272-containing inbred to a conventional second inbred. In trial series #2 of the agronomic studies, some of the hybrids were made by reciprocal crosses, with the Event 3272-containing inbred used as either the pollen donor or pollen recipient in the cross to the conventional second inbred. Control hybrids (near-isogenic, non-transgenic) were made by crossing the negative segregants identified in the inbred introgressions to elite maize inbred lines. A hybrid pair is defined as consisting of a hybrid Event 3272-derived maize line and the corresponding hybrid near-isogenic, non-transgenic maize line. Taqman PCR assays were used to confirm the Event 3272 and control hybrid lines during the breeding process.

B. Description of Taqman® PCR Method

TaqMan® is a PCR-based reaction that allows quantification of amplified products in real-time (Ingham *et al*., 2001). Target-specific oligonucleotides are designed to amplify a short product (~50-200 bp) for which the concentration is determined by incorporating a third fluorescently-labeled, target-specific oligonucleotide referred to as the probe. The amount of fluorescence incorporated into the PCR product is measured. Comparing these results with copy control standards allows for the determination of copy number for a gene.

Appendix 1-Figure 1. Diagram of Event 3272 breeding pedigree



[1] Used for Mendelian Inheritance studies.
[2] Used for all functional element Southern analyses, T-DNA insert sequencing.
[3] Used for generational stability Southern analysis.

♦Signifies that this is a reciprocal cross of the corresponding genotype, with the Event 3272 inbred used as the pollen donor.
* Near-isogenic negative segregants were selected and used to make control hybrid lines.
NP2391, NPH8431, NP2222 and NP911 are elite maize inbreds.

Compositional analysis and protein expression were performed on Hybrids A1 and B1 grown in 2003. Compositional analysis was also performed on Hybrid B3 grown in 2004. Hybrid genotypes in Agronomic trial (AgTrial) series #1 were grown in 2003; hybrid genotypes in trial series #2 - #4 were grown in 2004. Germination laboratory tests performed on Hybrid C. T_0 = initial Event 3272 transformed plant; ⊗ = self-pollination; BCn = backcross generation; Fn = filial generation

Appendix 2. Materials and Methods for Quantification[1] of AMY797E And PMI Proteins in Transgenic Tissues from Event 3272 Maize

The levels of alpha-amylase (AMY797E) protein and phosphomannose isomerase (PMI) protein expressed in Event 3272 maize plants were determined in several plant tissues by ELISA at five growth stages (whorl, anthesis, kernel dough, kernel maturity and senescence).

Plant material. Plants representing two Event 3272 maize hybrids (A1 and B1, see Appendix 1), plus their respective near-isogenic, non-transgenic controls, were field-grown concurrently in Bloomington, Illinois, using standard local agronomic practices. Prior to anthesis, silks were bagged to ensure self-pollination. Plants were harvested at each of five developmental stages: whorl, anthesis, kernel dough, kernel maturity, and senescence.

For each stage, the harvested whole plants were shipped on ice overnight to Syngenta Biotechnology Regulatory Science & Product Support, Research Triangle Park NC. Upon receipt, five transgenic plants per genotype were separated by tissue type into individual samples of leaves, roots, and kernels (when present). One control plant per genotype was separated into tissue types.

At anthesis, pollen was collected and pooled from multiple plants of the same genotype, sieved at the collection facility to remove non-pollen debris (*e.g.*, anthers and aphids), air-dried overnight and stored frozen until shipped overnight on dry ice to Regulatory Science & Product Support. All plant tissue samples were stored frozen at -80 ^{o}C until processed.

Plant tissue processing. Individual tissue samples (except pollen) were reduced to a fine powder by processing in the presence of dry ice. Samples were mixed well after grinding and again prior to sampling. Processed samples were stored at -80 ^{o}C until lyophilization.

AMY797E extraction. Lyophilized tissue and whole plant samples were extracted in accordance with a validated standardized procedure. For each sample analyzed, a 0.1 g aliquot of the powdered lyophilized material was suspended in 3 ml extraction solution [20% v/v glycerol/water purified by a MilliQ® system], and extracted using an Autogizer® homogenizer (Tomtek; Hamden, CT). Then each sample was incubated at 90 ^{o}C for 45 min. After centrifugation for 15 min at 10,000-14,000 x *g* at room temperature, the supernatants were retained at 2-8 ^{o}C for AMY797E quantitation by ELISA.

Pollen extracts were prepared by suspending pollen 1:30 (w/v) in extraction solution, disrupting the pollen grains by three passages through a French pressure cell at *ca*. 15,000 psi. and placing on ice for 30 min. The pollen extracts were then incubated at 90 ^{o}C for 45 min, centrifuged at 10,000 x *g* for 5 min at room temperature and the supernatants retained at 2-8 ^{o}C for ELISA quantitation of AMY797E.

[1] Measurement and quantity abbreviations are described in the instructions to authors of the Journal of Biological Chemistry.

PMI extraction. For each sample analyzed, a 0.1 g aliquot of the powdered lyophilized material was suspended in 3 ml extraction buffer [50 mM Tris, 0.1 M NaCl, 2 mM EDTA, 0.5 mM dithiothreitol, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride HCl, 1 mM leupeptin, pH 9.5], and extracted using an Autogizer® homogenizer. An additional 3 ml extraction buffer was added to each sample and placed on ice for 30 min. Extracts were vortexed and then centrifuged at 14,000 x *g* for 15 min at room temperature. The supernatants were retained at 2-8 ^{o}C for PMI quantitation by ELISA.

Pollen extracts were prepared by suspending pollen 1:60 (w/v) in extraction buffer. The pollen grains in the suspensions were disrupted by three passages through a French pressure cell at ca. 15,000 psi, and then placed on ice for 30 min. The pollen extracts were centrifuged at 10,000 x g for 15 min at room temperature and the supernatants retained at 2-8 ^{o}C for ELISA quantitation of PMI.

ELISA Reagents and Methods

Buffers

Borate Buffered Saline (BBS)
100 mM Boric acid
25 mM Sodium borate
75 mM Sodium chloride
pH 8.4 – 8.5

Diluent
10 mM Sodium phosphate
140 mM Sodium chloride
0.05% Tween-20
1% Bovine serum albumin (BSA)
0.02% Sodium azide
pH 7.4

Wash Buffer
10 mM Tris-HCl
0.05% Tween-20
0.02% Sodium azide
pH 8.0

Phosphatase Substrate Buffer
Prepared as described in
manufacturer's manual
Sigma catalog #N-2770

AMY797E ELISA method. The AMY797E extracts were quantitatively analyzed for AMY797E by ELISA (Tijssen, 1985) as follows: Nunc-Immuno™ Maxisorp™ 96-well plates (Fisher Scientific; Agawam, MA) were coated with immuno-affinity purified goat anti-AMY797E polyclonal antibodies (1 μg/ml BBS). After incubation overnight at 4 ^{o}C, the plates were washed five times with wash buffer and blocked for at least 45 min at room temperature with diluent. Plates were washed five times and triplicate aliquots of each tissue extract (appropriate dilutions prepared in diluent) were applied (total volume 100 μl per well). Following incubation at room temperature for 1.5 h, the plates were washed five times and coated with immuno-affinity purified rabbit anti-AMY797E polyclonal antibodies (2 μg/ml diluent). The plates were incubated for 1 h at 37 ^{o}C and then washed prior to coating with donkey anti-rabbit alkaline phosphatase conjugated antibody (1 μg/ml; Jackson ImmunoResearch Laboratories, Inc.; West Grove, PA). Plates were incubated for 1 h at 37 ºC then washed five times and phosphatase substrate (1.0 mg p-nitrophenyl phosphate/ml phosphatase substrate buffer) was added. Color was allowed to develop for 30 min at room temperature and the reaction stopped by the addition of 3N NaOH. Absorbance at 405 nm was measured using a Sunrise™ multi-well plate reader (Tecan; Research Triangle Park, NC).

The results were analyzed using the DeltaSoft® KC3 Curve fitting software program, version 1.71.2 (BioMetallics, Inc.; Princeton, NJ). The four parameters algorithm was used to generate a curve. Standard curves, prepared from purified AMY797E, were run on each plate. Data points were only considered acceptable if the mean "Delta" OD value obtained lay within the linear range of the standards (approximately 1.8 to 10 ng/ml) and the coefficient of variance was less than 10%.

PMI ELISA method. The extracts were quantitatively analyzed for PMI by ELISA (Tijssen, 1985) as follows: Nunc-Immuno™ Maxisorp™ 96-well plates (Fisher Scientific; Agawam, MA) were coated with immuno-affinity purified goat anti-PMI polyclonal antibodies (3 µg/ml BBS). After incubation overnight at 4 °C, the plates were washed five times with wash buffer and blocked for at least 45 min at room temperature with diluent. Plates were washed and triplicate aliquots of each tissue extract (appropriate dilutions prepared in diluent) were applied (total volume 100 µl per well). Following incubation at room temperature for 1.5 h, the plates were washed and coated with Protein A-purified rabbit anti-PMI polyclonal antibodies (5 µg/ml diluent). The plates were incubated for 1 h at 37 °C and then washed prior to coating with donkey anti-rabbit alkaline phosphatase conjugated antibody (1 µg/ml, Jackson ImmunoResearch Laboratories, Inc.; West Grove, PA). The plates were incubated for 1 h at 37 °C then washed five times and phosphatase substrate (1.0 mg p-nitrophenyl phosphate/ml phosphatase substrate buffer) was added. Color was allowed to develop for 30 min at room temperature and the reaction stopped by the addition of 3N NaOH. Absorbance at 405 nm was measured using a Sunrise™ multi-well plate reader (Tecan; Research Triangle Park, NC).

The results were analyzed using the DeltaSoft Curve fitting software program, version 1.71.2 (BioMetallics, Inc.; Princeton, NJ). The four parameters algorithm was used to generate a curve. Standard curves prepared from purified PMI were run on each plate. Data points were only considered acceptable if the mean "Delta" OD value obtained lay within the linear range of the standards (approximately 5.0 to 27 ng/ml) and the coefficient of variance was less than 10%.

AMY797E extraction efficiency. Extraction efficiency measurements were performed to estimate the relative amount of AMY797E extracted during routine procedures, compared to that which remained associated with the post extraction solids. Kernels from hybrid A1 at kernel dough stage were extracted as described above in AMY797E extraction and the insoluble material was collected and re-extracted multiple times. The quantity of AMY797E recovered in each extraction was measured by ELISA. Extraction efficiency of the first extraction was calculated as follows:

$$\% \text{ efficiency} = (((\mu g \text{ AMY797E/ml}) \text{ in } 1^{st} \text{ extract}) / (\text{total } (\mu g \text{ AMY797E/ml}) \text{ extracted})) \times 100$$

PMI extraction efficiency. Extraction efficiency measurements were performed to estimate the relative amount of PMI extracted during routine procedures, compared to that which remained associated with the post extraction solids. Leaves and kernels from hybrid A1 at kernel dough stage were extracted as described above in PMI tissue extraction and the insoluble material was collected and re-extracted multiple times. The quantity of PMI recovered in each extraction was measured by ELISA. Extraction efficiency of the first extraction was calculated as follows:

$$\% \text{ efficiency} = (((\mu g \text{ PMI/ml}) \text{ in } 1^{st} \text{ extract})/ (\text{total } \mu g \text{ PMI/ml}) \text{ extracted})) \times 100$$

Appendix 3. Evaluation of AMY797E Alpha-Amylase and PMI Amino Acid Sequence Identity with Known or Putative Allergens

A. Syngenta Biotechnology, Inc. Allergen Database

The Syngenta Biotechnology, Inc. (SBI) Allergen Database contains the amino acid sequences of known and putative protein allergens, including gliadins, and was initially compiled from entries in the database sources listed below. The SBI Allergen Database is updated on an annual basis.

1. All entries identified as allergens or putative allergens in the publicly available GenPept, PIR or SWISS-PROT protein databases. Allergen sequence entries from these public databases were identified using the program Lookup from the GCG Wisconsin Package version 10.1 as part of the SeqWeb Bioinformatics package (Accerlys, Inc., 2001);
2. Entries in the SWISS-PROT Allergen database (SWISS-PROT, 2001);
3. Entries in the List of Allergens database (International Union of Immunological Societies, 2001);
4. Entries in the FARRP Protein Allergen database (Food Allergy Research and Resource Program, 2001); and
5. Additional entries identified in the scientific literature as putative allergens, but which are not found in the public databases.

B. Evaluation of AMY797E Alpha-Amylase Amino Acid Sequence Identity with Known or Putative Allergens

Two different sequence identity searches were performed for the AMY797E alpha-amylase protein. First, sequence identity was examined by comparing sequential 80-amino acid peptides of the AMY797E alpha-amylase protein sequence to the allergen sequences. Any 80-amino acid peptide of the query sequence having greater than 35% amino acid identity to an allergen sequence was defined as having significant identity to the allergen sequence. Second, the AMY797E alpha-amylase protein sequence was screened for matches of eight or more contiguous amino acids using a program that compared every possible peptide of eight contiguous amino acids between the AMY797E alpha-amylase sequence and the allergen sequences. The purpose of this analysis was to screen for short, local regions of amino acid identity that might indicate the presence of common IgE-binding epitopes.

The results of these analyses revealed that there was no significant sequence identity between any of the sequential AMY797E alpha-amylase 80-amino acid peptides and any entries in the SBI Allergen Database. There was one region of eight contiguous identical amino acids between AMY797E alpha-amylase and a known species-specific allergen, Per a 3, from the American cockroach. The IgE-binding epitopes of Per a 3 have been identified (Wu *et al*., 2003) and there is no overlap between these binding epitopes and the region of sequence identity with AMY79E alpha-amylase. Therefore, the observed sequence identity between AMY797E alpha-amylase and Per a 3 is not biologically

relevant and has no implication for the allergenic potential of the AMY797E alpha-amylase**.**

There is evidence that *Aspergillus oryzae* alpha amylases used in the baking industry are, in some subjects, associated with IgE-mediated occupational rhinitis, asthma and dermatitis. Although fungal amylases used in the baking industry have been implicated as respiratory sensitizers, there is no reason to suppose that proteins with this enzyme specificity, *per se*, are necessarily allergenic. In fact, AMY797E alpha-amylase shows only limited (less than 17%) identity with a range of amylases derived from *Aspergillus oryzae*. Despite functional similarity, there is little amino acid identity between AMY797E alpha-amylase and known amylase respiratory allergens. Moreover, it has been established that the AMY797E alpha-amylase protein has no identity with *Aspergillus oryzae* allergens at the level of eight or more contiguous amino acids, indicating an absence of shared epitopes.

C. Evaluation of PMI Amino Acid Sequence Identity with Known or Putative Allergens

Two different sequence identity searches were performed for the PMI protein. First, sequence identity was examined by comparing sequential 80-amino acid peptides of the PMI protein sequence to the allergen sequences. Any 80-amino acid peptide of the query sequence having greater than 35% amino acid identity to an allergen sequence was defined as having significant identity to the allergen sequence. Second, the PMI protein sequence was screened for matches of eight or more contiguous amino acids using a program that compared every possible peptide of eight contiguous amino acids between the PMI sequence and the allergen sequences. The purpose of this analysis was to screen for short, local regions of amino acid identity that might indicate the presence of common IgE-binding epitopes.

There was no significant identity between any of the sequential 80-amino acid peptides and any entries in the SBI Allergen Database. There was one region of eight contiguous identical amino acids between PMI and a known allergen, α-parvalbumin from *Rana* species CH2001 (unidentified edible frog species from Indonesia) (Hilger *et al*., 2002). Further investigation using sensitive serum screening methodology (Codex Alimentarius Commission, 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 the PMI protein and α-parvalbumin from *Rana* species CH2001 is not biologically relevant and has no implications for the potential allergenicity of the PMI protein.

Appendix 4. Agronomic Field Trials for Phenotypic Evaluation

A. Statistical Analyses Methods

At all 2003 agronomic field trial locations, four blocks, each block containing one plot for each comparator, were planted in a randomized complete block design (all 2004 trials used three blocks).

For each variable, data for each genotype pair were considered separately, and were subjected to analysis of variance across locations 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 location, LT_{ij} is the location x genotype interaction effect and e_{ijk} is the residual error.

In all cases, the structure of the analysis of variance table and the corresponding degrees of freedom (assuming there are no missing values) are as follows:

Source of Variation	Degrees of Freedom
Genotype	t-1
Location	l-1
Block within location	l(b-1)
Location x genotype	(l-1)(t-1)
Residual error	l(t-1)(b-1)

t = number of genotypes, l = the number of locations and b = the number of blocks per location.

For each variable, the statistical significance of the genotype effect was determined using a standard F-test. An F-test probability of <5% indicates that the difference between the genotypes was statistically significant at the customary 5% level. An F-test was also used to assess the significance of the location x genotype interaction. A significant outcome (F-test probability <5%) indicates that the effect of genotype was not consistent across all locations, and undermines (to a greater or lesser extent depending on the nature of the interaction) the validity of the comparison of genotypes across locations. No overall genotype comparison is provided in cases where the location x genotype interaction is significant.

All means reported following statistical analysis are least square means.

Certain variables were not suitable for formal statistical analysis because the data did not conform to the assumptions upon which the validity of the analysis depends. The most common concern was that, within a given variable, the data were not sufficiently continuous but instead tended to take one of a very limited range of values. For example, data for EMERN (number of days to 50% emergence) from Trial Series #2, location 4343 are all recorded as either 11 or 12. Summary tables of the genotype means for these variables are presented in Chapter 5, Tables 5-3 to 5-7.

B. Statistical Analyses of Field Trial Phenotypic Data Tables

Appendix 4-Table 1. Detailed statistical data for 2003 Agronomic Trial Series #1

Means of Event 3272 and control hybrids.

	YGSMN	GMSTP	TWSMN
Location 4343 Genotype 1	104.8	30.6	40.4
Location 4343 Genotype 2	118.2	25.3	39.8
Location 4441 Genotype 1	166.7	17.7	47.7
Location 4441 Genotype 2	156.7	17.1	53.0
Location 4527 Genotype 1	107.6	23.6	44.2
Location 4527 Genotype 2	106.8	21.7	43.4
Location 5504 Genotype 1	143.2	18.4	54.8
Location 5504 Genotype 2	141.8	17.8	55.3
Location 5628 Genotype 1	91.6	20.2	51.1
Location 5628 Genotype 2	91.8	19.4	51.5
Genotype 1 (3272)	122.8	22.1	47.6
Genotype 2 (control)	123.1	20.2	48.6
F-test Probability for Genotype	94.6%	*	25.4%
F-test Probability for Location x Genotype Interaction	56.7%	***1.5%***	20.2%

* Statistically significant genotype x location interaction undermines the validity of the comparison across locations.

	YGSMN	GMSTP	TWSMN
Location 4343 Genotype 3	122.0	24.7	39.5
Location 4343 Genotype 4	N/P	N/P	N/P
Location 4441 Genotype 3	166.4	16.7	52.5
Location 4441 Genotype 4	137.0	17.7	51.9
Location 4527 Genotype 3	113.5	20.3	45.0
Location 4527 Genotype 4	115.2	21.1	49.3
Location 5504 Genotype 3	140.0	17.3	57.4
Location 5504 Genotype 4	106.4	17.7	58.2
Location 5628 Genotype 3	82.1	19.1	52.4
Location 5628 Genotype 4	72.6	19.1	53.2
Genotype 3[1] (3272)	125.5	18.3	51.8
Genotype 4 (control)	107.5	18.8	53.2
F-test Probability for Genotype	***0.4%***	***2.6%***	***4.4%***
F-test Probability for Location x Genotype Interaction	7.5%	17.7%	8.1%

[1] Genotype mean does not include data from location 4343.

N/P = Not planted due to seed shortage.

YGSMN = grain yield; GMSTP = grain moisture; TWSMN = test weight

Appendix 4-Table 1. Detailed statistical data for 2003 Agronomic Trial Series #1

Means of Event 3272 and control hybrids.

	YGSMN	GMSTP	TWSMN
Location 4343 Genotype 5	118.9	24.8	39.4
Location 4343 Genotype 6	120.8	24.7	38.9
Location 4441 Genotype 5	172.0	19.2	54.5
Location 4441 Genotype 6	168.0	19.2	53.5
Location 4527 Genotype 5	110.4	22.9	47.2
Location 4527 Genotype 6	115.9	22.1	48.7
Location 5504 Genotype 5	115.5	19.7	57.9
Location 5504 Genotype 6	129.3	19.6	56.8
Location 5628 Genotype 5	80.3	21.3	53.9
Location 5628 Genotype 6	90.4	20.8	53.2
Genotype 5 (3272)	119.4	21.6	50.6
Genotype 6 (control)	124.9	21.3	50.2
F-test Probability for Genotype	20.6%	32.5%	60.5%
F-test Probability for Location x Genotype Interaction	69.6%	87.9%	69.5%

	YGSMN	GMSTP	TWSMN
Location 4343 Genotype 7	N/P	N/P	N/P
Location 4343 Genotype 8	103.4	28.3	43.5
Location 4441 Genotype 7	N/P	N/P	N/P
Location 4441 Genotype 8	158.4	20.1	53.8
Location 4527 Genotype 7	N/P	N/P	N/P
Location 4527 Genotype 8	114.9	23.5	47.2
Location 5504 Genotype 7	114.7	18.6	58.1
Location 5504 Genotype 8	129.6	18.9	57.6
Location 5628 Genotype 7	86.9	21.7	53.9
Location 5628 Genotype 8	82.3	22.8	53.9
Genotype 7 (3272)	100.8	20.2	56.0
Genotype 8[1] (control)	105.9	20.8	55.8
F-test Probability for Genotype	29.9%	6.7%	66.0%
F-test Probability for Location x Genotype Interaction	7.5%	28.4%	61.0%

[1] Genotype mean does not include data from locations 4343, 4441 and 4527.

N/P = Not planted due to seed shortage.

YGSMN = grain yield; GMSTP = grain moisture; TWSMN = test weight

Appendix 4-Table 2. Detailed statistical data for 2004 Agronomic Trial Series #2
Means of Event 3272 and control hybrids.

	ERHTN	PLHTN	YGSMN	GMSTP	TWSMN
Location 4343 Genotype 1	119.3	294.3	118.5	27.4	54.9
Location 4343 Genotype 2	129.3	295.0	123.4	27.9	55.4
Location 4441 Genotype 1	87.5	262.5	84.2	38.5	59.6
Location 4441 Genotype 2	87.5	247.5	83.7	35.9	58.5
Location 4526 Genotype 1	113.3	310.0	103.1	38.4	46.7
Location 4526 Genotype 2	113.3	310.0	129.6	37.5	48.4
Location 4527 Genotype 1	121.7	305.0	134.1	45.5	53.5
Location 4527 Genotype 2	106.7	292.5	150.3	45.2	50.1
Location 4528 Genotype 1	118.3	295.0			
Location 4528 Genotype 2	111.7	295.0			
Location 4538 Genotype 1	106.7	291.7	126.6	36.2	
Location 4538 Genotype 2	109.2	289.2	126.1	35.9	
Location 5625 Genotype 1	103.3	306.7	136.3	27.5	55.1
Location 5625 Genotype 2	85.0	290.0	153.7	25.4	53.7
Location 5629 Genotype 1	111.7	241.7	126.4	24.3	55.1
Location 5629 Genotype 2	115.0	233.3	128.7	24.4	55.4
Location 6427 Genotype 1	112.7	244.7	130.6	21.5	57.3
Location 6427 Genotype 2	126.0	260.7	128.8	20.7	55.3
Location 6725 Genotype 1	103.3	261.7	149.4	29.4	62.0
Location 6725 Genotype 2	113.3	261.7	166.4	28.5	62.4
Location 7334 Genotype 1	115.0	298.7	197.5	20.8	56.5
Location 7334 Genotype 2	104.3	308.0	192.0	19.4	56.7
Location 7532 Genotype 1	111.7	315.0	162.0	22.4	61.2
Location 7532 Genotype 2	113.3	313.3	162.7	21.2	61.6
Location 7618 Genotype 1	98.3	265.0	200.3	24.8	58.5
Location 7618 Genotype 2	108.3	276.7	192.1	24.2	58.0
Location 7627 Genotype 1	106.7	245.0	218.7	24.3	59.1
Location 7627 Genotype 2	115.0	239.0	214.8	23.6	58.1
Location 7633 Genotype 1	81.7	306.7	178.3	19.1	57.6
Location 7633 Genotype 2	101.7	306.7	179.1	18.5	58.9
Location 7638 Genotype 1	108.3	258.3	222.8	23.7	59.4
Location 7638 Genotype 2	106.7	258.3	224.0	23.0	59.3
Location 7647 Genotype 1	113.3	265.0	198.0	19.0	57.5
Location 7647 Genotype 2	113.3	265.0	195.3	18.5	56.0
Genotype 1 (3272)	107.8	280.4	155.4	27.7	56.9
Genotype 2 (control)	109.4	278.9	159.4	26.9	56.5
F-test Probability for Genotype	59.1%	46.7%	26.0%	***<0.1%***	29.4%
F-test Probability for Location x Genotype Interaction	73.7%	38.3%	90.6%	63.3%	74.3%

For all genotypes, location 4528 was not harvested and equipment malfunctioned for 4538 TWSMN.
ERHTN = ear height; PLHTN = plant height; YGSMN = grain yield; GMSTP = grain moisture; TWSMN = test weight

Appendix 4-Table 2. Detailed statistical data for 2004 Agronomic Trial Series #2
Means of Event 3272 and control hybrids.

	ERHTN	PLHTN	YGSMN	GMSTP	TWSMN
Location 4343 Genotype 3	112.3	279.0	106.7	26.6	57.0
Location 4343 Genotype 4	124.3	296.0	127.9	27.2	56.1
Location 4441 Genotype 3	90.0	256.7	83.3	38.8	57.2
Location 4441 Genotype 4	93.3	260.0	69.9	38.8	53.8
Location 4526 Genotype 3	123.3	286.7	104.3	40.2	42.7
Location 4526 Genotype 4	113.3	291.7	119.3	38.9	48.5
Location 4527 Genotype 3	147.5	305.8	145.9	44.6	51.5
Location 4527 Genotype 4	115.0	303.3	147.9	44.9	54.0
Location 4528 Genotype 3	108.3	283.3			
Location 4528 Genotype 4	120.0	290.0			
Location 4538 Genotype 3	96.7	280.0	116.2	37.2	
Location 4538 Genotype 4	111.7	290.0	122.6	35.6	
Location 5625 Genotype 3	103.3	295.0	141.1	27.1	55.9
Location 5625 Genotype 4	100.0	306.7	154.5	25.8	54.2
Location 5629 Genotype 3	113.3	230.0	107.3	24.8	57.1
Location 5629 Genotype 4	120.0	236.7	129.9	24.8	54.7
Location 6427 Genotype 3	122.0	248.7	120.3	20.8	51.5
Location 6427 Genotype 4	120.7	251.3	125.2	20.4	51.8
Location 6725 Genotype 3	110.0	250.0	147.7	29.1	62.7
Location 6725 Genotype 4	100.0	248.3	167.2	29.7	61.1
Location 7334 Genotype 3	100.0	298.0	196.3	19.3	56.0
Location 7334 Genotype 4	128.7	308.3	209.2	19.1	54.6
Location 7532 Genotype 3	115.0	313.3	135.1	21.7	60.8
Location 7532 Genotype 4	116.7	321.7	161.5	21.5	60.0
Location 7618 Genotype 3	118.3	251.7	202.0	25.9	59.2
Location 7618 Genotype 4	115.0	261.7	206.5	25.4	58.1
Location 7627 Genotype 3	123.3	243.3	210.3	24.3	59.2
Location 7627 Genotype 4	113.3	245.0	224.1	24.0	59.8
Location 7633 Genotype 3	95.0	306.7	187.0	18.7	56.9
Location 7633 Genotype 4	88.3	305.0	174.4	18.4	57.4
Location 7638 Genotype 3	120.0	253.3	217.9	24.0	60.9
Location 7638 Genotype 4	113.3	266.7	226.1	24.2	58.2
Location 7647 Genotype 3	125.0	270.0	200.0	18.8	56.5
Location 7647 Genotype 4	108.3	271.7	207.8	18.8	57.1
Genotype 3 (3272)	113.1	273.6	151.3	27.7	56.3
Genotype 4 (control)	111.9	279.6	160.9	27.3	56.0
F-test Probability for Genotype	*	***1.5%***	***0.2%***	27.4%	37.3%
F-test Probability for Location x Genotype Interaction	***1.0%***	98.7%	43.7%	98.9%	15.4%

* Statistically significant genotype x location interaction undermines the validity of the comparison across locations.
ERHTN = ear height; PLHTN = plant height; YGSMN = grain yield; GMSTP = grain moisture; TWSMN = test weight

Appendix 4-Table 2. Detailed statistical data for 2004 Agronomic Trial Series #2

Means of Event 3272 and control hybrids.

	ERHTN	PLHTN	YGSMN	GMSTP	TWSMN
Location 4343 Genotype 5	116.7	288.7	111.7	28.4	56.2
Location 4343 Genotype 6	118.7	295.7	127.3	26.1	55.8
Location 4441 Genotype 5	85.0	241.7	73.4	39.6	55.7
Location 4441 Genotype 6	96.7	263.3	94.1	37.3	60.5
Location 4526 Genotype 5	115.0	306.7	132.6	36.9	48.4
Location 4526 Genotype 6	111.7	313.3	140.2	35.7	52.3
Location 4527 Genotype 5	133.3	295.0	143.2	43.4	56.1
Location 4527 Genotype 6	130.0	306.7	150.3	43.2	55.4
Location 4528 Genotype 5	111.7	285.0			
Location 4528 Genotype 6	105.0	286.7			
Location 4538 Genotype 5	100.0	277.5	107.6	37.9	
Location 4538 Genotype 6	105.0	287.5	137.8	36.2	
Location 5625 Genotype 5	95.0	288.3	151.1	26.2	52.3
Location 5625 Genotype 6	91.7	305.0	149.1	25.5	52.2
Location 5629 Genotype 5	116.7	243.3	129.3	24.8	56.2
Location 5629 Genotype 6	121.7	245.0	143.2	23.7	55.6
Location 6427 Genotype 5	123.3	242.7	133.3	21.2	53.4
Location 6427 Genotype 6	120.0	239.3	124.8	19.9	51.0
Location 6725 Genotype 5	105.0	263.3	149.1	29.6	62.2
Location 6725 Genotype 6	110.0	256.7	155.5	28.3	62.0
Location 7334 Genotype 5	107.7	283.3	173.8	20.2	54.7
Location 7334 Genotype 6	102.0	292.0	154.0	20.0	54.1
Location 7618 Genotype 5	106.7	268.3	187.6	25.7	59.8
Location 7618 Genotype 6	110.0	268.3	208.5	25.3	59.2
Location 7627 Genotype 5	110.0	246.0	220.0	24.2	57.8
Location 7627 Genotype 6	106.7	245.0	225.6	23.5	57.3
Location 7633 Genotype 5	91.7	300.0	168.6	18.5	57.2
Location 7633 Genotype 6	93.3	305.0	197.7	18.2	57.6
Location 7647 Genotype 5	110.0	260.0	202.1	18.6	57.3
Location 7647 Genotype 6	130.0	280.0	227.7	18.0	58.1
Genotype 5 (3272)	108.5	272.7	148.8	28.2	55.9
Genotype 6 (control)	110.2	279.3	159.7	27.2	56.2
F-test Probability for Genotype	43.3%	***0.2%***	*	***<0.1%***	66.7%
F-test Probability for Location x Genotype Interaction	63.8%	33.1%	***2.5%***	62.4%	72.2%

* Statistically significant genotype x location interaction undermines the validity of the comparison across locations.
Genotypes 5 and 6 not planted at locations 7532 and 7638 due to seed shortages.
ERHTN = ear height; PLHTN = plant height; YGSMN = grain yield; GMSTP = grain moisture; TWSMN = test weight

Appendix 4-Table 2. Detailed statistical data for 2004 Agronomic Trial Series #2
Means of Event 3272 and control hybrids.

	ERHTN	PLHTN	YGSMN	GMSTP	TWSMN
Location 4343 Genotype 7	115.3	285.3	117.5	26.7	55.6
Location 4343 Genotype 8	113.7	286.3	110.5	28.3	56.1
Location 4441 Genotype 7	87.5	252.5	95.0	33.8	55.7
Location 4441 Genotype 8	95.0	247.5	75.1	37.1	52.8
Location 4526 Genotype 7	115.0	303.3	150.4	36.5	51.2
Location 4526 Genotype 8	115.0	303.3	124.5	38.6	46.0
Location 4527 Genotype 7	113.3	298.3	140.1	44.7	51.4
Location 4527 Genotype 8	121.7	301.7	133.8	45.4	57.7
Location 4528 Genotype 7	106.7	276.7			
Location 4528 Genotype 8	115.0	285.0			
Location 4538 Genotype 7	98.3	273.3	126.4	36.1	
Location 4538 Genotype 8	105.0	281.7	132.1	35.4	
Location 5625 Genotype 7	100.0	295.0	153.4	26.6	53.0
Location 5625 Genotype 8	108.3	288.3	151.0	25.9	55.3
Location 5629 Genotype 7	103.3	223.3	127.4	24.6	56.4
Location 5629 Genotype 8	106.7	233.3	127.5	25.0	56.3
Location 6427 Genotype 7	109.3	237.3	127.8	20.7	56.5
Location 6427 Genotype 8	116.0	244.7	123.9	19.8	51.5
Location 6725 Genotype 7	113.3	265.0	174.4	26.9	61.6
Location 6725 Genotype 8	108.3	256.7	169.8	27.5	61.5
Location 7334 Genotype 7	104.3	294.7	196.3	19.9	55.7
Location 7334 Genotype 8	94.0	294.7	189.1	19.6	55.2
Location 7532 Genotype 7	110.0	315.0	164.8	22.2	61.5
Location 7532 Genotype 8	118.3	313.3	148.1	21.2	61.2
Location 7618 Genotype 7	115.0	265.0	210.7	24.3	58.5
Location 7618 Genotype 8	110.0	275.0	212.9	24.0	58.7
Location 7627 Genotype 7	106.7	240.0	225.1	24.6	58.1
Location 7627 Genotype 8	115.0	247.7	218.1	24.2	59.7
Location 7633 Genotype 7	91.7	301.7	187.5	18.2	58.4
Location 7633 Genotype 8	85.0	301.7	153.3	18.9	57.8
Location 7638 Genotype 7	105.0	258.3	227.1	24.3	60.8
Location 7638 Genotype 8	105.0	258.3	215.3	23.1	58.0
Location 7647 Genotype 7	113.3	258.3	215.7	18.4	56.3
Location 7647 Genotype 8	110.0	255.0	193.7	18.9	57.2
Genotype 7 (3272)	106.4	273.1	165.0	26.8	56.7
Genotype 8 (control)	108.4	275.0	154.9	27.0	56.3
F-test Probability for Genotype	40.6%	28.0%	***<0.1%***	23.6%	*
F-test Probability for Location x Genotype Interaction	95.5%	70.7%	30.3%	6.9%	***0.5%***

* Statistically significant genotype x location interaction undermines the validity of the comparison across locations.
ERHTN = ear height; PLHTN = plant height; YGSMN = grain yield; GMSTP = grain moisture; TWSMN = test weight

Appendix 4-Table 3. Detailed statistical data for 2004 Agronomic Trial Series #3

Means of Event 3272 and control hybrids.

	ERHTN	PLHTN	YGSMN	GMSTP	TWSMN
Location 4343 Genotype 1	121.3	285.7	124.7	26.2	56.5
Location 4343 Genotype 2	121.3	285.0	127.4	25.9	56.3
Location 4441 Genotype 1	91.7	231.7	87.3	33.4	58.2
Location 4441 Genotype 2	93.3	238.3	94.9	33.0	59.6
Location 4528[1] Genotype 1	88.3	240.0			
Location 4528[1] Genotype 2	93.3	240.0			
Location 5625 Genotype 1	90.0	283.3	157.5	23.2	54.1
Location 5625 Genotype 2	90.0	290.0	151.2	22.8	55.7
Location 5629 Genotype 1	101.7	211.7	115.1	22.7	56.1
Location 5629 Genotype 2	103.3	208.3	116.7	22.6	56.2
Location 6427 Genotype 1	104.0	226.0	119.7	19.7	48.0
Location 6427 Genotype 2	107.3	236.7	110.6	18.5	42.2
Genotype 1 (3272)	99.5	246.4	120.8	25.0	54.6
Genotype 2 (Control)	101.4	249.7	120.2	24.6	54.0
F-test Probability for Genotype	65.1%	37.3%	82.6%	21.0%	58.3%
F-test Probability for Location x Genotype Interaction	99.9%	85.4%	44.8%	90.1%	30.4%

[1] Location not harvested due to poor late-season growing conditions

ERHTN = ear height; PLHTN = plant height; YGSMN = grain yield; GMSTP = grain moisture; TWSMN = test weight

Appendix 4-Table 4. Detailed statistical data for 2004 Agronomic Trial Series #4

Means of Event 3272 and control hybrids.

	ERHTN	PLHTN	YGSMN	GMSTP	TWSMN
Location 4343 Genotype 1	116.0	290.3	110.4	26.9	54.4
Location 4343 Genotype 2	118.3	295.7	109.9	30.2	55.5
Location 4441 Genotype 1	93.3	246.7	89.5	37.6	58.5
Location 4441 Genotype 2	93.3	260.0	77.4	40.3	55.8
Location 4526 Genotype 1	101.7	290.0	111.7	41.1	43.6
Location 4526 Genotype 2	118.3	305.0	121.7	41.4	46.1
Location 4527 Genotype 1	123.3	303.3	159.0	45.0	52.6
Location 4527 Genotype 2	126.7	303.3	127.7	45.3	51.1
Location 4528[1] Genotype 1	91.7	253.3			
Location 4528[1] Genotype 2	81.7	253.3			
Location 5625 Genotype 1	110.0	310.0	132.2	26.2	53.5
Location 5625 Genotype 2	100.0	301.7	158.2	26.3	52.9
Location 5629 Genotype 1	113.3	223.3	100.0	24.2	55.6
Location 5629 Genotype 2	115.0	238.3	107.7	25.2	55.7
Location 6427 Genotype 1	106.7	230.7	118.1	19.9	51.1
Location 6427 Genotype 2	91.3	240.0	121.2	20.0	51.7
Location 7627 Genotype 1	116.7	243.3	209.8	24.7	59.9
Location 7627 Genotype 2	119.0	245.0	217.9	26.1	60.8
Location 7638 Genotype 1	100.0	246.7	188.6	25.6	57.7
Location 7638 Genotype 2	98.3	246.7	213.8	24.5	59.7
Genotype 1 (3272)	107.3	263.8	135.5	30.1	54.1
Genotype 2 (Control)	106.2	268.9	139.5	31.0	54.4
F-test Probability for Genotype	58.6%	1.0%	*	***2.7%***	82.7%
F-test Probability for Location x Genotype Interaction	7.0%	10.8%	***3.6%***	19.1%	98.5%

* Statistically significant genotype x location interaction undermines the validity of the comparison across locations.

[1] Location not harvested due to poor late-season growing conditions

ERHTN = ear height; PLHTN = plant height; YGSMN = grain yield; GMSTP = grain moisture; TWSMN = test weight

Appendix 5. Forage and Grain Composition

A. Statistical Analyses Methods

For each analyte, data for each genotype pair were considered separately, and were subjected to analysis of variance across locations 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 location, LT_{ij} is the location x genotype interaction effect and e_{ijk} is the residual error.

In all cases, the structure of the analysis of variance table and the corresponding degrees of freedom (assuming there are no missing values) are as follows:

Source of Variation	Degrees of Freedom
Genotype	t-1
Location	l-1
Block within location	l(b-1)
Location x genotype	(l-1)(t-1)
Residual error	l(t-1)(b-1)

t = number of genotypes, l = the number of locations and b = the number of blocks per location.

For each analyte, the statistical significance of the genotype effect was determined using a standard F-test. An F-test probability of <5% indicates that the difference between the genotypes was statistically significant at the customary 5% level. An F-test was also used to assess the significance of the location x genotype interaction. A significant outcome (F-test probability <5%) indicates that the effect of genotype was not consistent across all locations, and undermines (to a greater or lesser extent depending on the nature of the interaction) the validity of the comparison of genotypes across locations. No overall genotype comparison is provided in cases where the location x genotype interaction is significant.

Prior to analysis, data for each variable were checked for homogeneity of variance and reasonable Normality, and all were found to conform adequately to these assumptions (apart from those in which one or more data values were less than the limit of quantification – see below).

All means reported following statistical analysis are least square means.

In datasets where some but not all of the values were less than the limit of quantification (<LOQ), the average is represented as less than (<) the mean of the quantifiable values and the known LOQ value for that analyte. Such data were not suitable for further statistical analysis.

B. Statistical Analyses of Forage and Grain Composition

Appendix 5-Table 1. Proximate composition of Event 3272 and non-transgenic maize forage

(All values % dry weight unless otherwise indicated)

Location	Genotype	Protein	Fat	Ash	Carbohydrates[2]	Moisture (%fw)	ADF	NDF
Bloomington, IL	**A1 (3272)**	9.20	1.53	4.87	84.4	75.9	30.2	41.6
Bloomington, IL	**A2 (Control)**	10.17	1.20	5.10	83.5	75.2	26.9	34.3
Bondville, IL	**A1 (3272)**	8.77	1.00	5.20	85.1	73.0	25.7	40.1
Bondville, IL	**A2 (Control)**	8.67	1.47	4.97	84.9	70.4	25.5	40.1
Fairbault, MN	**A1 (3272)**	7.83	0.97	3.93	87.3	65.8	26.6	40.5
Fairbault, MN	**A2 (Control)**	7.40	1.27	3.90	87.4	64.3	26.0	39.6
Glidden, IA	**A1 (3272)**	8.40	2.83	4.13	84.7	65.9	26.1	40.2
Glidden, IA	**A2 (Control)**	6.63	1.90	4.00	87.4	66.6	26.5	39.2
Shirley, IL	**A1 (3272)**	7.73	2.20	4.13	85.9	59.5	27.6	40.7
Shirley, IL	**A2 (Control)**	8.60	2.10	4.70	84.6	67.0	33.5	42.3
Stanton, MN	**A1 (3272)**	7.17	1.70	3.77	87.4	59.5	29.2	43.5
Stanton, MN	**A2 (Control)**	7.20	1.43	2.93	88.5	59.4	34.1	46.7
A1 (3272)		8.18	1.71	4.34	85.8	66.6	27.6	41.1
A2 (Control)		8.11	1.56	4.27	86.1	67.1	28.8	40.4
F-test Probability for Genotype		*	46.9%	75.4%	*	57.9%	48.0%	70.4%
F-test Probability for Location x Genotype Interaction		***2.2%***	40.4%	61.4%	***1.6%***	9.6%	60.3%	67.8%
ILSI[1] (2006)	**average**	7.78	2.039	4.628	85.6	70.2	27.00	41.51
	range	3.14 - 11.57	0.296 - 4.570	1.527 - 9.638	76.4 - 92.1	49.1 -81.3	16.13 - 47.39	20.29 - 63.71
	N	945	921	945	945	945	945	945
OECD (2002)	**range**	4.7 - 9.2	1.5 - 3.2	2.9 - 5.7		62 - 78	25.6 - 34	40 - 48.2

* Statistically significant genotype x location interaction undermines the validity of the comparison across locations.

Appendix 5-Table 1. Proximate composition of Event 3272 and non-transgenic maize forage

(All values % dry weight unless otherwise indicated)

Location	Genotype	Protein	Fat	Ash	Carbohydrates[2]	Moisture (%fw)	ADF	NDF
Bloomington, IL	**B1 (3272)**	9.87	1.07	4.03	85.0	75.5	27.5	35.6
Bloomington, IL	**B2 (Control)**	9.57	1.03	3.87	85.5	74.2	28.5	35.6
Bondville, IL	**B1 (3272)**	8.67	2.23	5.20	83.9	69.8	24.9	38.6
Bondville, IL	**B2 (Control)**	8.63	2.00	4.73	84.7	70.9	30.2	40.7
Fairbault, MN	**B1 (3272)**	8.90	3.53	4.53	83.0	67.7	24.1	41.6
Fairbault, MN	**B2 (Control)**	7.67	2.17	3.70	86.5	66.5	25.7	38.3
Glidden, IA	**B1 (3272)**	8.93	2.53	4.47	84.1	68.5	28.0	38.3
Glidden, IA	**B2 (Control)**	8.23	2.50	4.30	84.9	68.1	26.5	36.4
Shirley, IL	**B1 (3272)**	9.37	1.70	4.93	84.0	66.8	27.3	42.1
Shirley, IL	**B2 (Control)**	8.67	1.40	4.60	85.3	64.6	32.4	42.6
Stanton, MN	**B1 (3272)**	6.90	1.73	3.63	87.7	58.0	30.7	42.6
Stanton, MN	**B2 (Control)**	5.35	1.58	3.93	89.1	57.2	39.0	47.2
B1 (3272)		8.77	2.13	4.47	84.6	67.7	27.1	39.8
B2 (Control)		8.02	1.78	4.19	86.0	66.9	30.4	40.1
F-test Probability for Genotype		***3.6%***	11.6%	34.3%	6.1%	16.4%	***3.9%***	85.1%
F-test Probability for Location x Genotype Interaction		77.3%	42.4%	92.0%	79.4%	58.0%	47.6%	85.4%
	average	7.78	2.039	4.628	85.6	70.2	27.00	41.51
ILSI[1] (2006)	**range**	3.14 - 11.57	0.296 - 4.570	1.527 - 9.638	76.4 - 92.1	49.1 - 81.3	16.13 - 47.39	20.29 - 63.71
	N	945	921	945	945	945	945	945
OECD (2002)	**range**	4.7 - 9.2	1.5 - 3.2	2.9 - 5.7		62 - 78	25.6 - 34	40 - 48.2

Appendix 5-Table 1. Proximate composition of Event 3272 and non-transgenic maize forage

(All values % dry weight unless otherwise indicated)

Location	Genotype	Protein	Fat[†]	Ash	Carbohydrates[2]	Moisture (%fw)	ADF	NDF
Bloomington, IL	**B3 (3272)**	5.70	0.83	3.90	89.60	68.23	27.23	41.57
Bloomington, IL	**B4 (Control)**	5.60	1.10	3.87	89.43	69.67	28.20	43.33
Bondville, IL	**B3 (3272)**	7.03	1.80	3.93	87.23	70.17	24.93	41.87
Bondville, IL	**B4 (Control)**	7.67	1.50	3.40	87.40	66.63	22.97	36.70
Brookings, SD	**B3 (3272)**	7.77	3.23	4.40	84.63	70.33	26.73	45.00
Brookings, SD	**B4 (Control)**	7.47	2.33	3.93	86.30	71.10	26.73	44.07
Glidden, IA	**B3 (3272)**	7.03	2.30	4.00	86.67	63.97	23.10	41.27
Glidden, IA	**B4 (Control)**	6.97	1.53	4.70	86.80	66.57	27.17	44.00
Janesville, WI	**B3 (3272)**	7.17	2.27	3.53	87.00	63.87	24.37	40.87
Janesville, WI	**B4 (Control)**	6.83	2.33	3.87	87.00	64.07	25.53	41.83
Stanton, MN	**B3 (3272)**	7.73	< 0.50[3]	3.50	88.30	69.50	29.07	42.27
Stanton, MN	**B4 (Control)**	6.93	< LOQ	3.63	89.43	70.57	28.90	41.77
Washington, IA	**B3 (3272)**	7.13	2.07	4.83	85.97	62.73	29.43	45.17
Washington, IA	**B4 (Control)**	7.27	1.33	4.43	86.97	64.73	29.17	44.17
B3 (3272)		7.08	2.08	4.01	87.06	66.97	26.41	42.57
B4 (Control)		6.96	1.69	3.98	87.62	67.62	26.95	42.27
F-test Probability for Genotype		56.6%	8.6%	81.3%	***4.9%***	51.0%	59.5%	76.2%
F-test Probability for Location x Genotype Interaction		66.7%	52.5%	35.6%	44.9%	70.7%	80.1%	48.0%
ILSI[1] (2006)	**average**	7.78	2.039	4.628	85.6	70.2	27.00	41.51
	range	3.14 - 11.57	0.296 - 4.570	1.527 - 9.638	76.4 - 92.1	49.1 - 81.3	16.13 - 47.39	20.29 - 63.71
	N	945	921	945	945	945	945	945
OECD (2002)	**range**	4.7 - 9.2	1.5 - 3.2	2.9 - 5.7		62 - 78	25.6 - 34	40 - 48.2

[†] Data from Stanton location excluded from statistical analysis because some values were < LOQ. LOQ for fat = 0.1% fw.

Appendix 5-Table 2. Mineral composition of Event 3272 and non-transgenic maize forage

(All values mg/kg dry weight unless otherwise indicated)

Location	Genotype	Calcium	Phosphorus
Bloomington, IL	**A1 (3272)**	2440	2800
Bloomington, IL	**A2 (Control)**	2540	2700
Bondville, IL	**A1 (3272)**	2150	2230
Bondville, IL	**A2 (Control)**	2300	2000
Fairbault, MN	**A1 (3272)**	2380	1120
Fairbault, MN	**A2 (Control)**	2270	1140
Glidden, IA	**A1 (3272)**	2030	2150
Glidden, IA	**A2 (Control)**	2350	1270
Shirley, IL	**A1 (3272)**	2150	2070
Shirley, IL	**A2 (Control)**	2730	1740
Stanton, MN	**A1 (3272)**	2020	1440
Stanton, MN	**A2 (Control)**	2050	1880
A1 (3272)		2200	1970
A2 (Control)		2370	1790
F-test Probability for Genotype		22.1%	24.2%
F-test Probability for Location x Genotype Interaction		75.7%	26.5%
ILSI[1] (2006)	**average**	2028.6	2066.1
	range	5767.9	3704.1
	N	481	481
OECD (2002) (% dw x 10^4 = mg/kg)	**range**	0.15 - 0.31 % dw	0.20 - 0.27 % dw

Location	Genotype	Calcium	Phosphorus
Bloomington, IL	**B1 (3272)**	2170	2220
Bloomington, IL	**B2 (Control)**	2190	2260
Bondville, IL	**B1 (3272)**	1980	1970
Bondville, IL	**B2 (Control)**	2020	1960
Fairbault, MN	**B1 (3272)**	2770	1320
Fairbault, MN	**B2 (Control)**	2170	1120
Glidden, IA	**B1 (3272)**	2390	1600
Glidden, IA	**B2 (Control)**	2410	1680
Shirley, IL	**B1 (3272)**	2180	1750
Shirley, IL	**B2 (Control)**	2560	2010
Stanton, MN	**B1 (3272)**	2010	1490
Stanton, MN	**B2 (Control)**	2370	1460
B1 (3272)		2250	1730
B2 (Control)		2290	1750
F-test Probability for Genotype		82.3%	85.6%
F-test Probability for Location x Genotype Interaction		57.8%	86.1%
ILSI[1] (2006)	**average**	2028.6	2066.1
	range	5767.9	3704.1
	N	481	481
OECD (2002) (% dw x 10^4 = mg/kg)	**range**	0.15 - 0.31 % dw	0.20 - 0.27 % dw

Appendix 5-Table 2. Mineral composition of Event 3272 and non-transgenic maize forage

(All values mg/kg dry weight unless otherwise indicated)

Location	Genotype	Calcium	Phosphorus
Bloomington, IL	**B3 (3272)**	1430	2000
Bloomington, IL	**B4 (Control)**	1230	2070
Bondville, IL	**B3 (3272)**	1680	2050
Bondville, IL	**B4 (Control)**	1270	2170
Brookings, SD	**B3 (3272)**	2730	1820
Brookings, SD	**B4 (Control)**	2580	1990
Glidden, IA	**B3 (3272)**	1860	1790
Glidden, IA	**B4 (Control)**	1880	1490
Janesville, WI	**B3 (3272)**	1850	1750
Janesville, WI	**B4 (Control)**	1770	1690
Stanton, MN	**B3 (3272)**	1850	2140
Stanton, MN	**B4 (Control)**	1680	1820
Washington, IA	**B3 (3272)**	2250	2500
Washington, IA	**B4 (Control)**	2190	2290
B3 (3272)		1950	2010
B4 (Control)		1800	1930
F-test Probability for Genotype		6.7%	21.3%
F-test Probability for Location x Genotype Interaction		81.4%	19.1%
ILSI[1] (2006)	**average**	2028.6	2066.1
	range	5767.9	3704.1
	N	481	481
OECD (2002) (% dw x 10^4 = mg/kg)	**range**	0.15 - 0.31 % dw	0.20 - 0.27 %dw

Appendix 5-Table 3. Proximate composition of Event 3272 and non-transgenic maize grain

(All values % dry weight)

Location	Genotype	Protein	Fat	Ash	Carbohydrates	ADF	NDF	TDF	Starch[2]
Bloomington, IL	**A1 (3272)**	12.17	3.07	1.83	82.90	4.37	11.67	13.40	62.9
Bloomington, IL	**A2 (Control)**	11.77	3.00	1.67	83.57	5.60	12.53	15.97	52.1
Bondville, IL	**A1 (3272)**	10.67	2.97	1.50	84.93	5.90	11.77	11.70	63.8
Bondville, IL	**A2 (Control)**	10.00	3.73	1.60	84.63	4.20	12.40	14.60	54.9
Fairbault, MN	**A1 (3272)**	11.07	3.27	1.20	84.43	4.30	10.33	12.03	61.6
Fairbault, MN	**A2 (Control)**	10.63	3.27	1.10	85.07	3.27	10.07	10.33	67.3
Glidden, IA	**A1 (3272)**	10.33	2.97	1.30	85.40	2.87	8.80	10.47	68.1
Glidden, IA	**A2 (Control)**	10.00	3.10	1.13	85.73	3.33	9.90	11.70	67.0
Shirley, IL	**A1 (3272)**	10.77	3.57	1.43	84.23	3.20	9.30	10.87	65.0
Shirley, IL	**A2 (Control)**	10.30	3.57	1.30	84.80	4.30	9.00	11.43	62.8
Stanton, MN	**A1 (3272)**	10.30	3.00	1.50	85.23	4.57	13.87	12.53	58.3
Stanton, MN	**A2 (Control)**	10.53	2.70	1.40	85.37	4.37	13.43	10.63	61.0
A1 (3272)		10.88	3.14	1.46	84.52	4.20	10.96	11.83	63.3
A2 (Control)		10.54	3.23	1.37	84.86	4.18	11.22	12.44	60.8
F-test Probability for Genotype		6.0%	41.9%	***4.9%***	11.6%	95.0%	57.4%	*	*
F-test Probability for Location x Genotype Interaction		72.7%	16.4%	51.4%	71.5%	15.9%	86.8%	***0.1%***	***< 0.1%***
ILSI[1] (2006)	**average**	10.30	3.555	1.439	84.6	4.05	11.23	16.43	57.7
	range	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	1174	1410	1410	1350	1349	397	168
OECD (2002)	**range**	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	

* Statistically significant genotype x location interaction undermines the validity of the comparison across locations.

Appendix 5-Table 3. Proximate composition of Event 3272 and non-transgenic maize grain

(All values % dry weight)

Location	Genotype	Protein	Fat	Ash	Carbohydrates	ADF	NDF	TDF	Starch[2]
Bloomington, IL	**B1 (3272)**	11.83	4.03	1.70	82.50	5.97	13.93	15.17	58.7
Bloomington, IL	**B2 (Control)**	10.60	4.07	1.57	83.80	4.20	14.53	15.77	53.5
Bondville, IL	**B1 (3272)**	9.47	3.80	1.53	85.20	6.93	13.33	13.07	63.6
Bondville, IL	**B2 (Control)**	9.40	3.83	1.53	85.27	6.53	12.60	16.93	57.4
Fairbault, MN	**B1 (3272)**	11.70	3.73	1.10	83.50	3.63	9.97	11.17	67.3
Fairbault, MN	**B2 (Control)**	10.93	3.53	1.07	84.50	4.83	10.83	12.93	63.5
Glidden, IA	**B1 (3272)**	10.40	3.53	1.10	84.90	4.27	9.57	11.63	67.8
Glidden, IA	**B2 (Control)**	9.77	3.47	1.10	85.67	3.73	10.87	13.97	64.1
Shirley, IL	**B1 (3272)**	10.83	3.73	1.27	84.10	4.27	8.07	10.17	62.9
Shirley, IL	**B2 (Control)**	10.00	3.67	1.20	85.13	3.70	9.47	11.70	62.6
Stanton, MN	**B1 (3272)**	10.20	3.27	1.40	85.13	3.30	12.60	12.57	59.4
Stanton, MN	**B2 (Control)**	9.60	3.37	1.27	85.73	5.77	13.70	12.37	60.5
B1 (3272)		10.74	3.68	1.35	84.22	4.73	11.24	12.29	63.3
B2 (Control)		10.05	3.66	1.29	85.02	4.79	12.00	13.94	60.3
F-test Probability for Genotype		***0.2%***	82.7%	6.0%	***0.6%***	*	8.0%	***0.4%***	***0.2%***
F-test Probability for Location x Genotype Interaction		59.1%	98.5%	62.0%	74.0%	***3.0%***	66.0%	23.9%	10.4%

	average	10.3	3.555	1.439	84.6	4.05	11.23	16.43	57.7
ILSI[1] (2006)	**range**	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	1174	1410	1410	1350	1349	397	168
OECD (2002)	**range**	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	

* Statistically significant genotype x location interaction undermines the validity of the comparison across locations.

Appendix 5-Table 3. Proximate composition of Event 3272 and non-transgenic maize grain

(All values % dry weight)

Location	Genotype	Protein	Fat	Ash	Carbohydrates	ADF	NDF	TDF	Starch[2]
Bondville, IL	**B3 (3272)**	8.43	4.17	1.73	85.67	4.07	10.17	12.67	56.9
Bondville, IL	**B4 (Control)**	8.50	3.97	1.60	85.93	4.40	11.93	17.43	43.8
Brookings, SD	**B3 (3272)**	8.80	4.30	1.73	85.20	4.73	11.40[4]	14.93	49.6
Brookings, SD	**B4 (Control)**	9.03	4.53	1.57	84.83	5.40	13.60	17.93	52.8
Glidden, IA	**B3 (3272)**	10.00	4.03	1.33	84.53	4.73	12.93	14.37	56.7
Glidden, IA	**B4 (Control)**	9.23	3.87	1.30	85.60	5.00	12.33	16.07	62.4
Janesville, WI	**B3 (3272)**	11.33	3.47	1.47	83.70	4.73	12.37	12.97	39.6
Janesville, WI	**B4 (Control)**	10.17	3.77	1.47	84.67	6.77	13.43	16.90	40.8
Stanton, MN	**B3 (3272)**	10.33	2.53	1.60	85.43	4.33	11.13	12.97	47.9
Stanton, MN	**B4 (Control)**	10.87	2.53	1.90	84.70	5.47	12.90	15.93	40.0
Washington, IA	**B3 (3272)**	8.53	4.20	1.40	85.80	5.57	11.27	15.20	52.5
Washington, IA	**B4 (Control)**	8.80	4.33	1.43	85.47	4.87	11.47	13.20	43.5
B3 (3272)		9.57	3.78	1.54	85.06	4.69	11.40	13.85	50.5
B4 (Control)		9.43	3.83	1.54	85.20	5.32	12.61	16.24	47.2
F-test Probability for Genotype		*	73.7%	100.0%	45.3%	***4.3%***	***2.3%***	*	47.9%
F-test Probability for Location x Genotype Interaction		***2.7%***	87.9%	30.4%	7.4%	17.4%	37.8%	***< 0.1%***	78.8%
ILSI[1] (2006)	**average**	10.3	3.555	1.439	84.6	4.05	11.23	16.43	57.7
	range	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	1174	1410	1410	1350	1349	397	168
OECD (2002)	**range**	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	

* Statistically significant genotype x location interaction undermines the validity of the comparison across locations.

Appendix 5-Table 4. Mineral composition[#] of Event 3272 and non-transgenic maize grain

(All values mg/kg dry weight unless otherwise indicated)

Location	Genotype	Calcium	Copper	Iron	Magnesium	Manganese[2]	Phosphorus	Potassium	Zinc	Selenium[§]
Bloomington, IL	**A1 (3272)**	35.2	1.39	25.1	1170	7.11	3330	3840	24.0	< LOQ
Bloomington, IL	**A2 (Control)**	37.9	1.43	25.6	1190	6.84	3450	4050	23.4	< LOQ
Bondville, IL	**A1 (3272)**	34.2	1.48	23.6	1090	6.43	3120	3700	22.4	< LOQ
Bondville, IL	**A2 (Control)**	40.1	1.66	25.8	1130	6.04	3190	3900	23.6	< LOQ
Fairbault, MN	**A1 (3272)**	55.2	1.90	21.0	1030	4.68	2380	3480	22.6	0.23
Fairbault, MN	**A2 (Control)**	52.1	2.06	21.8	1060	4.70	2410	3510	22.7	0.21
Glidden, IA	**A1 (3272)**	41.4	1.79	22.5	1190	6.67	3060	3780	22.6	0.10
Glidden, IA	**A2 (Control)**	44.3	1.71	20.0	1010	6.14	2590	3340	20.4	0.09
Shirley, IL	**A1 (3272)**	39.8	1.47	25.5	1110	6.50	3080	3560	22.4	0.16
Shirley, IL	**A2 (Control)**	44.1	1.63	26.1	1190	6.41	3200	3750	23.7	0.12
Stanton, MN	**A1 (3272)**	50.0	2.05	27.5	1140	7.57	2790	3440	23.5	< 0.08[3]
Stanton, MN	**A2 (Control)**	46.9	2.04	25.8	1080	6.99	2720	3350	22.3	< 0.07[3]
A1 (3272)		42.6	1.68	24.2	1120	6.50	2960	3630	22.9	< 0.11[5]
A2 (Control)		44.2	1.76	24.2	1110	6.19	2930	3650	22.7	< 0.10[5]
F-test Probability for Genotype		30.6%	38.8%	97.0%	79.8%	14.6%	74.2%	87.7%	77.6%	♦
F-test Probability for Location x Genotype Interaction		41.3%	92.0%	47.2%	56.6%	93.4%	54.7%	60.6%	82.4%	♦
ILSI[1] (2006)	**average**	46.4	1.75	21.81	1193.8	6.18	3273.5	3842	21.6	0.2
	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	6.5 - 37.2	0.05 - 0.75
	N	1344	1249	1255	1257	1256	1349	1257	1257	89
OECD (2002) ((mg/100g) x 10 = mg/kg)	**range**	3 - 100 mg/100 g	0.09 - 1.0 mg/100 g	0.1 - 10 mg/100 g	82 - 1000 mg/100 g		234 - 750 mg/100 g	320 - 720 mg/100 g	1.2 - 3.0 mg/100 g	0.001 - 0.1 mg/100g

[#] Sodium levels were < LOQ in all samples; LOQ for sodium = 100 mg/kg.

[§] LOQ for selenium = 0.05 mg/kg

♦ Statistical analysis was not performed on estimated averages.

Appendix 5-Table 4. Mineral composition[#] of Event 3272 and non-transgenic grain

(All values mg/kg dry weight unless otherwise indicated)

Location	Genotype	Calcium	Copper	Iron	Magnesium	Manganese[2]	Phosphorus	Potassium	Zinc	Selenium[§]
Bloomington, IL	**B1 (3272)**	35.9	1.57	23.4	1150	6.40	3220	3800	20.4	< 0.05[3]
Bloomington, IL	**B2 (Control)**	31.7	1.52	21.2	1060	5.27	3060	3840	19.7	< LOQ
Bondville, IL	**B1 (3272)**	36.1	1.67	19.9	1000	5.00	2720	3540	18.8	< LOQ
Bondville, IL	**B2 (Control)**	37.0	1.75	20.1	1060	5.05	2940	3790	20.6	< LOQ
Fairbault, MN	**B1 (3272)**	51.8	1.93	20.9	1160	4.77	2420	3290	20.5	0.18
Fairbault, MN	**B2 (Control)**	42.9	1.84	20.0	1040	4.24	2170	3190	20.8	0.20
Glidden, IA	**B1 (3272)**	37.7	1.77	18.6	970	4.93	2520	3230	17.5	0.13
Glidden, IA	**B2 (Control)**	37.3	1.74	17.9	1020	5.01	2610	3420	18.4	0.13
Shirley, IL	**B1 (3272)**	39.8	1.70	14.6	1160	6.14	3010	3520	20.3	0.18
Shirley, IL	**B2 (Control)**	47.8	1.71	24.8	1120	5.51	3010	3750	20.2	0.15
Stanton, MN	**B1 (3272)**	48.0	2.13	27.3	1150	6.83	2830	3540	23.2	< 0.06[3]
Stanton, MN	**B2 (Control)**	49.9	2.16	25.8	1070	5.86	2610	3370	21.2	< LOQ
B1 (3272)		41.60	1.80	22.5	1100	5.68	2790	3490	20.4	< 0.108[5]
B2 (Control)		41.10	1.79	21.7	1060	5.15	2730	3560	20.2	< 0.105[6]
F-test Probability for Genotype		86.6%	92.8%	9.3%	13.2%	***0.2%***	45.8%	35.3%	58.1%	♦
F-test Probability for Location x Genotype Interaction		53.0%	99.1%	56.5%	26.2%	9.5%	34.6%	48.3%	26.9%	♦
ILSI[1] (2006)	**average**	46.4	1.75	21.81	1193.8	6.18	3273.5	3842	21.6	0.2
	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	6.5 - 37.2	0.05 - 0.75
	N	1344	1249	1255	1257	1256	1349	1257	1257	89
OECD (2002) ((mg/100g) x 10 = mg/kg)	**range**	3 - 100 mg/100 g	0.09 - 1.0 mg/100 g	0.1 - 10 mg/100 g	82 - 1000 mg/100 g		234 - 750 mg/100 g	320 - 720 mg/100 g	1.2 - 3.0 mg/100 g	0.001 - 0.1 mg/100g

[#] Sodium levels were < LOQ in all samples; LOQ for sodium = 100 mg/kg.

[§] LOQ for selenium = 0.05 mg/kg.

♦ Statistical analysis was not performed on estimated averages.

Appendix 5-Table 4. Mineral composition[#] of Event 3272 and non-transgenic grain

(All values mg/kg dry weight unless otherwise indicated)

Location	Genotype	Calcium	Copper	Iron	Magnesium	Manganese[2]	Phosphorus	Potassium	Zinc	Selenium[§]
Bondville, IL	**B3 (3272)**	31.5	1.93	18.20	1040	4.79	2730	3440	18.5	< LOQ
Bondville, IL	**B4 (Control)**	32.2	1.92	12.00	1040	4.53	2650	3380	18.5	< LOQ
Brookings, SD	**B3 (3272)**	53.4	2.20	20.20	1260	5.50	3110	4480	18.1	0.227
Brookings, SD	**B4 (Control)**	54.1	1.99	22.00	1350	5.65	3280	4650	18.8	0.203
Glidden, IA	**B3 (3272)**	39.7	2.39	22.10	1230	5.76	3040	3640	22.8	0.610
Glidden, IA	**B4 (Control)**	41.7	2.03	19.40	1090	5.26	2660	3270	20.0	0.433
Janesville, WI	**B3 (3272)**	43.8	1.91	22.80	1470	7.24	3400	3940	22.7	< LOQ
Janesville, WI	**B4 (Control)**	51.0	1.64	22.20	1370	6.60	3160	3870	21.6	< LOQ
Stanton, MN	**B3 (3272)**	52.9	1.52	25.50	1250	7.04	3470	4260	20.5	< LOQ
Stanton, MN	**B4 (Control)**	53.4	1.58	26.50	1390	7.10	3800	4480	21.7	< LOQ
Washington, IA	**B3 (3272)**	49.1	1.59	23.40	1270	3.63	3390	3880	22.9	0.450
Washington, IA	**B4 (Control)**	41.1	2.04	21.40	1270	3.37	3370	3760	22.0	0.447
B3 (3272)		45.1	1.92	22.00	1250	5.66	3190	3940	20.9	< 0.240[6]
B4 (Control)		45.6	1.87	20.60	1250	5.42	3150	3900	20.4	< 0.206[6]
F-test Probability for Genotype		*	*	24.4%	*	11.4%	*	48.7%	*	♦
F-test Probability for Location x Genotype Interaction		***0.3%***	***0.5%***	45.0%	***3.1%***	58.8%	***2.6%***	6.7%	***1.4%***	♦
ILSI[1] (2006)	**average**	46.4	1.75	21.81	1193.8	6.18	3273.5	3842	21.6	0.2
	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	6.5 - 37.2	0.05 - 0.75
	N	1344	1249	1255	1257	1256	1349	1257	1257	89
OECD (2002) ((mg/100g) x 10 = mg/kg)	**range**	3 - 100 mg/100 g	0.09 - 1.0 mg/100 g	0.1 - 10 mg/100 g	82 - 1000 mg/100 g		234 - 750 mg/100 g	320 - 720 mg/100 g	1.2 - 3.0 mg/100 g	0.001 - 0.1 mg/100g

[#] Sodium levels were < LOQ in all samples; LOQ for sodium = 100 mg/kg.

[§] LOQ for selenium = 0.05 mg/kg.

♦ Statistical analysis was not performed on estimated averages.

* Statistically significant genotype x location interaction undermines the validity of the comparison across locations.

Appendix 5-Table 5. Vitamin composition of Event 3272 and non-transgenic maize grain

(All values mg/100 g dry weight unless otherwise indicated)

Location	Genotype	Beta-carotene[2]	Folic Acid[2]	Vitamin B1 Thiamine	Vitamin B2 Riboflavin	Vitamin B3 Niacin	Vitamin B6	Vitamin E[2,&] Alpha-tocopherol (mg/g)
Bloomington, IL	**A1 (3272)**	0.046	0.078	0.43	0.11	2.07	0.63	0.0082
Bloomington, IL	**A2 (Control)**	0.099	0.074	0.43	0.12	2.03	0.68	0.0087
Bondville, IL	**A1 (3272)**	0.043	0.066	0.42	0.13	2.46	0.60	0.0092
Bondville, IL	**A2 (Control)**	0.077	0.059	0.45	0.13	2.48	0.64	0.0078
Fairbault, MN	**A1 (3272)**	0.127	0.055	0.36	0.13	2.56	0.58	0.0086
Fairbault, MN	**A2 (Control)**	0.087	0.057	0.38	0.13	2.63	0.58	0.0066
Glidden, IA	**A1 (3272)**	0.052	0.034	0.37	0.14	2.03	0.61	0.0069
Glidden, IA	**A2 (Control)**	0.080	0.058	0.37	0.14	2.09	0.66	0.0071
Shirley, IL	**A1 (3272)**	0.065	0.077	0.40	0.12	2.40	0.55	0.0077
Shirley, IL	**A2 (Control)**	0.119	0.072	0.39	0.11	2.34	0.63	0.0083
Stanton, MN	**A1 (3272)**	0.140	0.072	0.44	0.12	2.46	0.66	< 0.0074[3]
Stanton, MN	**A2 (Control)**	0.112	0.057	0.44	0.11	2.31	0.65	0.0073
A1 (3272)		0.079	0.064	0.40	0.13	2.33	0.61	< 0.0080[7]
A2 (Control)		0.095	0.063	0.41	0.12	2.31	0.64	0.0076
F-test Probability for Genotype		13.1%	79.8%	31.0%	12.0%	72.6%	***4.4%***	♦
F-test Probability for Location x Genotype Interaction		8.0%	9.8%	22.7%	63.2%	76.2%	55.0%	♦
ILSI[1] (2006)	**average**	0.684	0.0651	0.530	0.125	2.376	0.644	0.0103
	range	0.019 - 4.681	0.0147 - 0.1464	0.126 - 4.000	0.050 - 0.236	1.037 - 4.694	0.368 - 1.132	0.0015 - 0.0687
	N	276	895	894	704	415	415	863
OECD (2002) (mg/kg = (mg/100g) x 10)	**range**			2.3 - 8.6 mg/kg	0.25 - 5.6 mg/kg	9.3 - 70 mg/kg	4.6 - 9.6 mg/kg	

[&] LOQ for α-Tocopherols = 0.005 mg/g.

♦ Statistical analysis was not performed on estimated averages.

Appendix 5-Table 5. Vitamin composition of Event 3272 and non-transgenic maize grain

(All values mg/100 g dry weight unless otherwise indicated)

Location	Genotype	Beta-carotene[2]	Folic Acid[2]	Vitamin B1 Thiamine	Vitamin B2 Riboflavin	Vitamin B3 Niacin	Vitamin B6	Vitamin E[2,&] Alpha-tocopherol (mg/g)
Bloomington, IL	**B1 (3272)**	0.146	0.079	0.45	0.11	2.04	0.66	0.0093
Bloomington, IL	**B2 (Control)**	0.167	0.077	0.43	0.12	2.14	0.65	0.0118
Bondville, IL	**B1 (3272)**	0.109	0.082	0.43	0.13	2.17	0.59	0.0088
Bondville, IL	**B2 (Control)**	0.115	0.062	0.40	0.13	2.35	0.60	0.0109
Fairbault, MN	**B1 (3272)**	0.191	0.043	0.40	0.12	2.19	0.54	< 0.0060[3]
Fairbault, MN	**B2 (Control)**	0.175	0.043	0.39	0.11	2.06	0.60	0.0074
Glidden, IA	**B1 (3272)**	0.130	0.056	0.41	0.13	1.68	0.61	0.0075
Glidden, IA	**B2 (Control)**	0.170	0.051	0.38	0.13	1.85	0.59	0.0097
Shirley, IL	**B1 (3272)**	0.156	0.085	0.45	0.11	2.04	0.55	0.0078
Shirley, IL	**B2 (Control)**	0.161	0.066	0.37	0.10	2.08	0.63	0.0098
Stanton, MN	**B1 (3272)**	0.152	0.061	0.47	0.10	2.29	0.71	0.0086
Stanton, MN	**B2 (Control)**	0.155	0.056	0.47	0.12	2.61	0.64	< 0.0077[3]
B1 (3272)		0.148	0.068	0.44	0.12	2.07	0.61	< 0.0080[7]
B2 (Control)		0.157	0.059	0.41	0.12	2.18	0.62	< 0.0095[7]
F-test Probability for Genotype		40.0%	5.9%	***1.0%***	84.0%	18.8%	72.7%	♦
F-test Probability for Location x Genotype Interaction		78.7%	61.2%	36.2%	74.3%	71.5%	43.0%	♦
ILSI[1] (2006)	**average**	0.684	0.0651	0.530	0.125	2.376	0.644	0.0103
	range	0.019 - 4.681	0.0147 - 0.1464	0.126 - 4.000	0.050 - 0.236	1.037 - 4.694	0.368 - 1.132	0.0015 - 0.0687
	N	276	895	894	704	415	415	863
OECD (2002) (mg/kg = (mg/100 g) x 10)	**range**			2.3 - 8.6 mg/kg	0.25 - 5.6 mg/kg	9.3 - 70 mg/kg	4.6 - 9.6 mg/kg	

[&] LOQ for α-Tocopherols = 0.005 mg/g.

♦ Statistical analysis was not performed on estimated averages.

Appendix 5-Table 5. Vitamin composition of Event 3272 and non-transgenic maize grain

(All values mg/100 g dry weight unless otherwise indicated)

Location	Genotype	Beta-carotene[2]	Folic Acid[2]	Vitamin B1 Thiamine	Vitamin B2 Riboflavin	Vitamin B3 Niacin	Vitamin B6	Vitamin E[2,&] Alpha-tocopherol (mg/g)
Bondville, IL	**B3 (3272)**	0.111	0.047	0.44	0.15	2.29	0.48	0.0083
Bondville, IL	**B4 (Control)**	0.119	0.054	0.42	0.14	2.35	0.51	0.0079
Brookings, SD	**B3 (3272)**	0.099	0.079	0.46	0.18	2.99	0.49	0.0068
Brookings, SD	**B4 (Control)**	0.122	0.094	0.52	0.20	3.05	0.51	0.0069
Glidden, IA	**B3 (3272)**	0.068	0.090	0.45	0.23	2.28	0.54	0.0086
Glidden, IA	**B4 (Control)**	0.098	0.082	0.47[4]	0.21	2.31	0.61[1]	0.0083
Janesville, WI	**B3 (3272)**	0.074	0.047	0.50	0.18	2.39	0.52	0.0058
Janesville, WI	**B4 (Control)**	0.127	0.067	0.47	0.17	2.44	0.52	< 0.0055[3]
Stanton, MN	**B3 (3272)**	0.097	0.085	0.42	0.18	2.84	0.48	0.0075
Stanton, MN	**B4 (Control)**	0.093	0.068	0.46	0.17	2.68	0.58	0.0084
Washington, IA	**B3 (3272)**	0.122	0.054	0.44	0.14	2.39	0.49	< 0.0060[3]
Washington, IA	**B4 (Control)**	0.082	0.052	0.46	0.17	2.20	0.46	< LOQ
B3 (3272)		0.095	0.067	0.45	0.18	2.53	0.50	< 0.0072[7]
B4 (Control)		0.107	0.069	0.47	0.18	2.50	0.53	< 0.0070[5]
F-test Probability for Genotype		*	68.2%	*	90.1%	54.4%	***4.2%***	♦
F-test Probability for Location x Genotype Interaction		***< 0.1%***	41.2%	***0.2%***	32.8%	29.7%	12.6%	♦
ILSI[1] (2006)	**average**	0.684	0.0651	0.530	0.125	2.376	0.644	0.0103
	range	0.019 - 4.681	0.0147 - 0.1464	0.126 - 4.000	0.050 - 0.236	1.037 - 4.694	0.368 - 1.132	0.0015 - 0.0687
	N	276	895	894	704	415	415	863
OECD (2002) (mg/kg = (mg/100 g) x 10)	**range**			2.3 - 8.6 mg/kg	0.25 - 5.6 mg/kg	9.3 - 70 mg/kg	4.6 - 9.6 mg/kg	

[&] LOQ for α-Tocopherols = 0.005 mg/g.

♦ Statistical analysis was not performed on estimated averages.

* Statistically significant genotype x location interaction undermines the validity of the comparison across locations.

Appendix 5-Table 6. Fatty acid composition of Event 3272 and non-transgenic maize grain

(All values % dry weight)

Location	Genotype	16:0 Palmitic	18:0 Stearic	18:1 Oleic	18:2 Linoleic	18:3 Linolenic
Bloomington, IL	**A1 (3272)**	0.393	0.054	0.639	1.627	0.050
Bloomington, IL	**A2 (Control)**	0.401	0.058	0.649	1.627	0.055
Bondville, IL	**A1 (3272)**	0.369	0.055	0.629	1.543	0.049
Bondville, IL	**A2 (Control)**	0.465	0.073	0.818	1.920	0.063
Fairbault, MN	**A1 (3272)**	0.417	0.061	0.671	1.750	0.061
Fairbault, MN	**A2 (Control)**	0.417	0.057	0.646	1.703	0.060
Glidden, IA	**A1 (3272)**	0.382	0.053	0.645	1.570	0.052
Glidden, IA	**A2 (Control)**	0.399	0.059	0.682	1.627	0.054
Shirley, IL	**A1 (3272)**	0.428	0.061	0.712	1.887	0.056
Shirley, IL	**A2 (Control)**	0.435	0.067	0.728	1.903	0.058
Stanton, MN	**A1 (3272)**	0.375	0.058	0.617	1.547	0.054
Stanton, MN	**A2 (Control)**	0.347	0.052	0.548	1.377	0.047
A1 (3272)		0.394	0.057	0.652	1.654	0.054
A2 (Control)		0.411	0.061	0.679	1.693	0.056
F-test Probability for Genotype		20.6%	*	30.6%	50.4%	12.6%
F-test Probability for Location x Genotype Interaction		17.7%	***4.0%***	13.8%	19.7%	9.7%
OECD (2002)	**range**	0.29 - 0.79	0.04 - 0.17	0.70 - 1.39	0.67 - 2.81	0.03 - 0.10

* Statistically significant genotype x location interaction undermines the validity of the comparison across locations.

Appendix 5-Table 6. Fatty acid composition of Event 3272 and non-transgenic maize grain

(All values % dry weight)

Location	Genotype	16:0 Palmitic	18:0 Stearic	18:1 Oleic	18:2 Linoleic	18:3 Linolenic
Bloomington, IL	**B1 (3272)**	0.480	0.078	0.919	2.073	0.059
Bloomington, IL	**B2 (Control)**	0.488	0.078	0.997	2.023	0.058
Bondville, IL	**B1 (3272)**	0.463	0.076	0.882	1.920	0.057
Bondville, IL	**B2 (Control)**	0.473	0.077	0.932	1.927	0.059
Fairbault, MN	**B1 (3272)**	0.454	0.066	0.798	1.990	0.061
Fairbault, MN	**B2 (Control)**	0.428	0.062	0.778	1.813	0.061
Glidden, IA	**B1 (3272)**	0.440	0.070	0.816	1.877	0.058
Glidden, IA	**B2 (Control)**	0.425	0.067	0.837	1.800	0.055
Shirley, IL	**B1 (3272)**	0.437	0.074	0.854	1.917	0.054
Shirley, IL	**B2 (Control)**	0.441	0.072	0.875	1.860	0.054
Stanton, MN	**B1 (3272)**	0.406	0.065	0.722	1.663	0.054
Stanton, MN	**B2 (Control)**	0.425	0.066	0.744	1.737	0.057
B1 (3272)		0.447	0.071	0.832	1.907	0.057
B2 (Control)		0.447	0.070	0.861	1.860	0.057
F-test Probability for Genotype		100.0%	69.7%	41.2%	48.1%	97.2%
F-test Probability for Location x Genotype Interaction		94.1%	98.7%	97.3%	91.3%	92.3%

OECD (2002)	**range**	0.29 - 0.79	0.04 - 0.17	0.70 - 1.39	0.67 - 2.81	0.03 - 0.10

Appendix 5-Table 6. Fatty acid composition of Event 3272 and non-transgenic maize grain

(All values % dry weight)

Location	Genotype	16:0 Palmitic	18:0 Stearic	18:1 Oleic	18:2 Linoleic	18:3 Linolenic
Bondville, IL	**B3 (3272)**	0.484	0.082	1.023	2.050	0.070
Bondville, IL	**B4 (Control)**	0.454	0.072	0.931	1.943	0.065
Brookings, SD	**B3 (3272)**	0.510	0.068	0.872	2.153	0.072
Brookings, SD	**B4 (Control)**	0.506	0.067	0.915	2.293	0.073
Glidden, IA	**B3 (3272)**	0.488	0.069	0.902	2.033	0.066
Glidden, IA	**B4 (Control)**	0.472	0.062	0.827	1.940	0.063
Janesville, WI	**B3 (3272)**	0.433	0.061	0.775	1.723	0.058
Janesville, WI	**B4 (Control)**	0.456	0.063	0.845	1.837	0.061
Stanton, MN	**B3 (3272)**	0.319	0.038	0.492	1.317	0.046
Stanton, MN	**B4 (Control)**	0.323	0.039	0.485	1.313	0.045
Washington, IA	**B3 (3272)**	0.513	0.076	1.017	2.077	0.068
Washington, IA	**B4 (Control)**	0.515	0.079	1.046	2.127	0.071
B3 (3272)		0.458	0.066	0.847	1.892	0.063
B4 (Control)		0.454	0.064	0.841	1.909	0.063
F-test Probability for Genotype		83.6%	46.2%	84.5%	81.4%	94.4%
F-test Probability for Location x Genotype Interaction		94.7%	49.6%	53.5%	85.9%	92.1%

OECD (2002)	**range**	0.29 - 0.79	0.04 - 0.17	0.70 - 1.39	0.67 - 2.81	0.03 - 0.10

Appendix 5-Table 7. Amino acid composition of Event 3272 and non-transgenic maize grain

(All values % dry weight unless otherwise indicated)

Location	Genotype	Aspartic Acid	Threonine	Serine	Glutamic Acid	Proline	Glycine	Alanine	Cystine	Valine
Bloomington, IL	**A1 (3272)**	0.794	0.393	0.611	2.33	1.024	0.431	0.917	0.212	0.542
Bloomington, IL	**A2 (Control)**	0.780	0.346	0.645	2.31	1.015	0.437	0.908	0.213	0.544
Bondville, IL	**A1 (3272)**	0.699	0.339	0.530	1.96	0.889	0.409	0.776	0.205	0.488
Bondville, IL	**A2 (Control)**	0.662	0.334	0.503	1.87	0.839	0.393	0.739	0.210	0.466
Fairbault, MN	**A1 (3272)**	0.691	0.328	0.550	2.12	0.919	0.389	0.821	0.217	0.487
Fairbault, MN	**A2 (Control)**	0.736	0.358	0.573	2.20	0.949	0.402	0.855	0.221	0.502
Glidden, IA	**A1 (3272)**	0.756	0.310	0.525	1.86	0.808	0.360	0.739	0.163	0.440
Glidden, IA	**A2 (Control)**	0.710	0.276	0.542	1.86	0.817	0.347	0.743	0.163	0.424
Shirley, IL	**A1 (3272)**	0.684	0.354	0.534	2.06	0.932	0.388	0.810	0.205	0.488
Shirley, IL	**A2 (Control)**	0.673	0.336	0.525	2.00	0.875	0.395	0.794	0.205	0.492
Stanton, MN	**A1 (3272)**	0.680	0.331	0.524	2.02	0.876	0.388	0.791	0.203	0.485
Stanton, MN	**A2 (Control)**	0.703	0.336	0.534	2.04	0.879	0.399	0.801	0.218	0.492
A1 (3272)		0.717	0.343	0.546	2.06	0.908	0.394	0.809	0.201	0.488
A2 (Control)		0.711	0.331	0.554	2.05	0.896	0.395	0.807	0.205	0.487
F-test Probability for Genotype		60.7%	12.5%	35.9%	77.1%	39.5%	86.8%	84.5%	13.0%	83.1%
F-test Probability for Location x Genotype Interaction		31.9%	8.6%	37.7%	77.5%	47.6%	61.0%	69.9%	57.6%	69.0%
	average	6.88 mg/g	3.75 mg/g	5.12 mg/g	20.09 mg/g	9.51 mg/g	3.85 mg/g	7.90 mg/g	2.21 mg/g	4.90 mg/g
ILSI[1] (2006)	**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
(mg/g = % dw x 10)	**N**	1350	1350	1350	1350	1350	1350	1350	1350	1350
OECD (2002)	**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

Appendix 5-Table 7. Amino acid composition of Event 3272 and non-transgenic maize grain

(All values % dry weight unless otherwise indicated)

Location	Genotype	Methionine	Isoleucine	Leucine	Tyrosine	Phenylalanine	Histidine	Lysine	Arginine	Tryptophan
Bloomington, IL	**A1 (3272)**	0.208	0.413	1.57	0.452	0.635	0.335	0.349	0.542	0.0755
Bloomington, IL	**A2 (Control)**	0.200	0.412	1.54	0.445	0.627	0.339	0.362	0.553	0.0660
Bondville, IL	**A1 (3272)**	0.201	0.361	1.29	0.396	0.532	0.299	0.343	0.499	0.0653
Bondville, IL	**A2 (Control)**	0.195	0.342	1.22	0.350	0.503	0.292	0.336	0.487	0.0563
Fairbault, MN	**A1 (3272)**	0.212	0.368	1.40	0.416	0.538	0.286	0.302	0.494	0.0680
Fairbault, MN	**A2 (Control)**	0.220	0.389	1.46	0.447	0.570	0.302	0.316	0.482	0.0723
Glidden, IA	**A1 (3272)**	0.155	0.339	1.24	0.385	0.513	0.269	0.318	0.452	0.0640
Glidden, IA	**A2 (Control)**	0.155	0.327	1.26	0.373	0.513	0.259	0.304	0.436	0.0530
Shirley, IL	**A1 (3272)**	0.209	0.369	1.40	0.424	0.553	0.296	0.308	0.475	0.0708
Shirley, IL	**A2 (Control)**	0.200	0.357	1.34	0.324	0.537	0.300	0.335	0.490	0.0633
Stanton, MN	**A1 (3272)**	0.207	0.356	1.30	0.371	0.505	0.276	0.294	0.463	0.0664
Stanton, MN	**A2 (Control)**	0.222	0.364	1.31	0.384	0.517	0.283	0.316	0.496	0.0709
A1 (3272)		0.199	0.368	1.37	0.407	0.546	0.294	0.319	0.488	0.0683
A2 (Control)		0.199	0.365	1.36	0.387	0.544	0.296	0.328	0.491	0.0637
F-test Probability for Genotype		98.6%	60.4%	68.6%	13.0%	84.2%	58.9%	11.3%	78.6%	*
F-test Probability for Location x Genotype Interaction		20.5%	30.6%	63.9%	10.0%	42.6%	46.8%	22.1%	75.3%	***1.0%***
	average	2.09 mg/g	3.68 mg/g	13.41 mg/g	3.36 mg/g	5.25 mg/g	2.96 mg/g	3.15 mg/g	4.33 mg/g	0.627 mg/g
ILSI[1] (2006)	**range**	1.24 - 4.68	1.79 - 6.92	6.42 - 24.92	1.03 - 6.42	2.44 - 9.30	1.37 - 4.34	1.72 - 6.68	1.19 - 6.39	0.271 - 2.150
(mg/g = % dw x 10)	**N**	1350	1350	1350	1350	1350	1350	1350	1350	1350
OECD (2002)	**range**	0.10 - 0.46	0.22 - 0.71	0.79 - 2.41	0.12 - 0.79	0.29 - 0.64	0.15 - 0.38	0.05 - 0.55	0.22 - 0.64	0.04 - 0.13

* Statistically significant genotype x location interaction undermines the validity of the comparison across locations.

Appendix 5-Table 7. Amino acid composition of Event 3272 and non-transgenic maize grain

(All values % dry weight unless otherwise indicated)

Location	Genotype	Aspartic Acid	Threonine	Serine	Glutamic Acid	Proline	Glycine	Alanine	Cystine	Valine
Bloomington, IL	**B1 (3272)**	0.801	0.323	0.661	2.32	0.979	0.422	0.908	0.218	0.532
Bloomington, IL	**B2 (Control)**	0.718	0.301	0.586	2.02	0.888	0.408	0.797	0.204	0.482
Bondville, IL	**B1 (3272)**	0.657	0.323	0.484	1.84	0.811	0.371	0.731	0.198	0.453
Bondville, IL	**B2 (Control)**	0.627	0.322	0.463	1.77	0.796	0.358	0.707	0.189	0.433
Fairbault, MN	**B1 (3272)**	0.772	0.364	0.601	2.29	0.978	0.400	0.894	0.222	0.516
Fairbault, MN	**B2 (Control)**	0.738	0.348	0.589	2.23	0.949	0.388	0.871	0.214	0.502
Glidden, IA	**B1 (3272)**	0.764	0.294	0.554	1.96	0.820	0.352	0.784	0.165	0.438
Glidden, IA	**B2 (Control)**	0.665	0.270	0.522	1.86	0.796	0.331	0.733	0.171	0.408
Shirley, IL	**B1 (3272)**	0.680	0.362	0.526	2.10	0.886	0.381	0.840	0.203	0.500
Shirley, IL	**B2 (Control)**	0.590	0.321	0.479	1.85	0.802	0.349	0.739	0.196	0.444
Stanton, MN	**B1 (3272)**	0.701	0.338	0.529	2.05	0.884	0.395	0.805	0.216	0.495
Stanton, MN	**B2 (Control)**	0.642	0.305	0.481	1.83	0.812	0.369	0.726	0.203	0.450
B1 (3272)		0.729	0.334	0.559	2.09	0.893	0.387	0.827	0.204	0.489
B2 (Control)		0.663	0.311	0.520	1.93	0.840	0.367	0.762	0.196	0.453
F-test Probability for Genotype		***< 0.1%***	***< 0.1%***	***< 0.1%***	***< 0.1%***	***< 0.1%***	***< 0.1%***	***< 0.1%***	***1.9%***	***< 0.1%***
F-test Probability for Location x Genotype Interaction		11.1%	22.8%	14.9%	14.4%	22.7%	32.4%	10.9%	36.4%	8.2%

	average	6.88 mg/g	3.75 mg/g	5.12 mg/g	20.09 mg/g	9.51 mg/g	3.85 mg/g	7.90 mg/g	2.21 mg/g	4.90 mg/g
ILSI[1] (2006)	**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
(mg/g = % dw x 10)	**N**	1350	1350	1350	1350	1350	1350	1350	1350	1350
OECD (2002)	**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

Appendix 5-Table 7. Amino acid composition of Event 3272 and non-transgenic maize grain

(All values % dry weight unless otherwise indicated)

Location	Genotype	Methionine	Isoleucine	Leucine	Tyrosine	Phenylalanine	Histidine	Lysine	Arginine	Tryptophan
Bloomington, IL	**B1 (3272)**	0.239	0.409	1.56	0.459	0.626	0.331	0.345	0.528	0.0746
Bloomington, IL	**B2 (Control)**	0.207	0.358	1.32	0.396	0.541	0.311	0.340	0.519	0.0629
Bondville, IL	**B1 (3272)**	0.200	0.341	1.22	0.324	0.497	0.278	0.310	0.436	0.0639
Bondville, IL	**B2 (Control)**	0.193	0.321	1.18	0.315	0.477	0.266	0.293	0.428	0.0571
Fairbault, MN	**B1 (3272)**	0.254	0.398	1.54	0.398	0.587	0.306	0.326	0.456	0.0678
Fairbault, MN	**B2 (Control)**	0.228	0.386	1.51	0.451	0.580	0.299	0.312	0.451	0.0621
Glidden, IA	**B1 (3272)**	0.164	0.341	1.32	0.398	0.531	0.264	0.301	0.438	0.0595
Glidden, IA	**B2 (Control)**	0.170	0.319	1.25	0.365	0.500	0.251	0.274	0.407	0.0534
Shirley, IL	**B1 (3272)**	0.213	0.365	1.44	0.417	0.563	0.299	0.316	0.483	0.0669
Shirley, IL	**B2 (Control)**	0.207	0.315	1.25	0.363	0.494	0.273	0.290	0.446	0.0591
Stanton, MN	**B1 (3272)**	0.216	0.369	1.34	0.363	0.519	0.283	0.309	0.484	0.0712
Stanton, MN	**B2 (Control)**	0.209	0.336	1.20	0.294	0.471	0.262	0.296	0.437	0.0678
B1 (3272)		0.214	0.371	1.40	0.393	0.554	0.293	0.318	0.471	0.0673
B2 (Control)		0.202	0.339	1.28	0.364	0.511	0.277	0.301	0.448	0.0604
F-test Probability for Genotype		*****	***< 0.1%***	***< 0.1%***	9.1%	***< 0.1%***	***< 0.1%***	***< 0.1%***	***0.7%***	***< 0.1%***
F-test Probability for Location x Genotype Interaction		***1.4%***	11.3%	7.5%	29.9%	5.6%	39.5%	26.3%	43.5%	60.7%

	average	2.09 mg/g	3.68 mg/g	13.41 mg/g	3.36 mg/g	5.25 mg/g	2.96 mg/g	3.15 mg/g	4.33 mg/g	0.627 mg/g
ILSI[1] (2006)	**range**	1.24 - 4.68	1.79 - 6.92	6.42 - 24.92	1.03 - 6.42	2.44 - 9.30	1.37 - 4.34	1.72 - 6.68	1.19 - 6.39	0.271 - 2.150
(mg/g = % dw x 10)	**N**	1350	1350	1350	1350	1350	1350	1350	1350	1350
OECD (2002)	**range**	0.10 - 0.46	0.22 - 0.71	0.79 - 2.41	0.12 - 0.79	0.29 - 0.64	0.15 - 0.38	0.05 - 0.55	0.22 - 0.64	0.04 - 0.13

* Statistically significant genotype x location interaction undermines the validity of the comparison across locations.

Appendix 5-Table 7. Amino acid composition of Event 3272 and non-transgenic maize grain

(All values % dry weight unless otherwise indicated)

Location	Genotype	Aspartic Acid	Threonine	Serine	Glutamic Acid	Proline	Glycine	Alanine	Cystine	Valine
Bondville, IL	**B3 (3272)**	0.579	0.291	0.437	1.600	0.734	0.346	0.632	0.172	0.395
Bondville, IL	**B4 (Control)**	0.581	0.295	0.444	1.623	0.742	0.349	0.639	0.176	0.396
Brookings, SD	**B3 (3272)**	0.616	0.318	0.441	1.680	0.767	0.354	0.687	0.182	0.427
Brookings, SD	**B4 (Control)**	0.627	0.322	0.453	1.700	0.783	0.357	0.688	0.183	0.424
Glidden, IA	**B3 (3272)**	0.731	0.331	0.511	1.883	0.799	0.345	0.752	0.156	0.444
Glidden, IA	**B4 (Control)**	0.696	0.317	0.484	1.797	0.779	0.330	0.721	0.151	0.427
Janesville, WI	**B3 (3272)**	0.803	0.376	0.600	2.277	0.962	0.417	0.899	0.202	0.545
Janesville, WI	**B4 (Control)**	0.703	0.346	0.538	2.040	0.890	0.381	0.801	0.197	0.484
Stanton, MN	**B3 (3272)**	0.708	0.346	0.552	2.067	0.898	0.397	0.809	0.205	0.480
Stanton, MN	**B4 (Control)**	0.718	0.358	0.568	2.130	0.932	0.404	0.832	0.228	0.484
Washington, IA	**B3 (3272)**	0.562	0.276	0.433	1.600	0.686	0.333	0.627	0.185	0.404
Washington, IA	**B4 (Control)**	0.582	0.292	0.441	1.663	0.752	0.345	0.641	0.183	0.407
B3 (3272)		0.666	0.323	0.496	1.851	0.808	0.365	0.734	0.184	0.449
B4 (Control)		0.651	0.322	0.488	1.826	0.813	0.361	0.720	0.186	0.437
F-test Probability for Genotype		18.2%	81.9%	39.4%	53.0%	70.1%	53.7%	35.5%	51.6%	21.3%
F-test Probability for Location x Genotype Interaction		5.9%	18.3%	12.7%	27.3%	12.7%	28.4%	24.6%	35.0%	34.2%

	average	6.88 mg/g	3.75 mg/g	5.12 mg/g	20.09 mg/g	9.51 mg/g	3.85 mg/g	7.90 mg/g	2.21 mg/g	4.90 mg/g
ILSI[1] (2006)	**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
(mg/g = % dw x 10)	**N**	1350	1350	1350	1350	1350	1350	1350	1350	1350
OECD (2002)	**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

Appendix 5-Table 7. Amino acid composition of Event 3272 and non-transgenic maize grain

(All values % dry weight unless otherwise indicated)

Location	Genotype	Methionine	Isoleucine	Leucine	Tyrosine	Phenylalanine	Histidine	Lysine	Arginine	Tryptophan
Bondville, IL	**B3 (3272)**	0.188	0.291	1.043	0.316	0.414	0.247	0.287	0.401	0.0647
Bondville, IL	**B4 (Control)**	0.182	0.290	1.043	0.275	0.409	0.252	0.292	0.401	0.0607
Brookings, SD	**B3 (3272)**	0.232	0.309	1.107	0.348	0.435	0.254	0.301	0.393	0.0603
Brookings, SD	**B4 (Control)**	0.221	0.312	1.117	0.353	0.437	0.257	0.317	0.400	0.0603
Glidden, IA	**B3 (3272)**	0.168	0.346	1.280	0.373	0.504	0.257	0.319	0.420	0.0630
Glidden, IA	**B4 (Control)**	0.153	0.332	1.223	0.359	0.487	0.251	0.305	0.408	0.0513
Janesville, WI	**B3 (3272)**	0.244	0.406	1.520	0.436	0.577	0.321	0.358	0.490	0.0787
Janesville, WI	**B4 (Control)**	0.218	0.357	1.347	0.365	0.512	0.296	0.322	0.448	0.0587
Stanton, MN	**B3 (3272)**	0.205	0.358	1.353	0.370	0.525	0.301	0.333	0.460	0.0670
Stanton, MN	**B4 (Control)**	0.220	0.356	1.390	0.428	0.537	0.308	0.331	0.493	0.0600
Washington, IA	**B3 (3272)**	0.187	0.300	1.060	0.313	0.424	0.269	0.291	0.379	0.0520
Washington, IA	**B4 (Control)**	0.195	0.310	1.100	0.291	0.435	0.277	0.294	0.388	0.0607
B3 (3272)		0.204	0.335	1.227	0.359	0.480	0.275	0.315	0.424	0.0643
B4 (Control)		0.198	0.326	1.203	0.345	0.469	0.273	0.310	0.423	0.0586
F-test Probability for Genotype		18.5%	18.2%	36.8%	*	22.0%	76.7%	45.4%	87.9%	***3.4%***
F-test Probability for Location x Genotype Interaction		11.9%	15.5%	21.4%	***4.1%***	13.8%	33.7%	25.7%	16.8%	6.1%
	average	2.09 mg/g	3.68 mg/g	13.41 mg/g	3.36 mg/g	5.25 mg/g	2.96 mg/g	3.15 mg/g	4.33 mg/g	0.627 mg/g
ILSI[1] (2006)	**range**	1.24 - 4.68	1.79 - 6.92	6.42 - 24.92	1.03 - 6.42	2.44 - 9.30	1.37 - 4.34	1.72 - 6.68	1.19 - 6.39	0.271 - 2.150
(mg/g = % dw x 10)	**N**	1350	1350	1350	1350	1350	1350	1350	1350	1350
OECD (2002)	**range**	0.10 - 0.46	0.22 - 0.71	0.79 - 2.41	0.12 - 0.79	0.29 - 0.64	0.15 - 0.38	0.05 - 0.55	0.22 - 0.64	0.04 - 0.13

* Statistically significant genotype x location interaction undermines the validity of the comparison across locations.

Appendix 5-Table 8. Antinutrients and secondary metabolites[¶] of Event 3272 and non-transgenic maize grain

(All values mg/kg dry weight unless otherwise indicated)

2003		Phytic Acid (% dw)	Raffinose[¥] (% dw)	Trypsin Inhibitor[2] (TIU[8]/mg)	Ferulic Acid	*p* -Coumaric Acid
Bloomington, IL	**A1 (3272)**	0.899	0.151	2.64	2203	158
Bloomington, IL	**A2 (Control)**	0.780	0.138	2.92	2303	175
Bondville, IL	**A1 (3272)**	0.476	0.158	2.78	2140	152
Bondville, IL	**A2 (Control)**	0.400	0.167	2.54	2280	199
Fairbault, MN	**A1 (3272)**	0.343	< 0.115[3]	2.58	1913	195
Fairbault, MN	**A2 (Control)**	0.423	< 0.111[3]	2.50	1813	144
Glidden, IA	**A1 (3272)**	0.584	0.120	2.71	1510	90
Glidden, IA	**A2 (Control)**	0.624	< 0.103[3]	3.12	1830	123
Shirley, IL	**A1 (3272)**	0.666	0.157	2.81	1483	73
Shirley, IL	**A2 (Control)**	0.613	0.154	2.86	1637	91
Stanton, MN	**A1 (3272)**	0.562	< 0.107[3]	3.01	2383	197
Stanton, MN	**A2 (Control)**	0.588	< 0.109[3]	2.83	2133	169
A1 (3272)		0.588	< 0.135[7]	2.76	1939	144
A2 (Control)		0.571	< 0.130[7]	2.79	1999	150
F-test Probability for Genotype		60.6%	♦	68.3%	32.6%	*
F-test Probability for Location x Genotype Interaction		48.4%	♦	33.2%	15.6%	***4.0%***
ILSI[1] (2006)	**average**	0.745 % dw	0.132 % dw	2.73 TIU /mg	2201.1	218.4
	range	0.111 - 1.570	0.020 - 0.320	1.09 - 7.18	291.9 - 3885.8	53.4 - 576.2
	N	1196	701	696	817	817
OECD (2002) (% dw x 10^4 = mg/kg)	**range**	0.45 - 1.0 % dw	0.21 - 0.31 % dw		0.02 - 0.3 % dw	0.003 - 0.03 % dw

[¶] Furfural levels were < LOQ in all samples. LOQ for furfural = 0.500 mg/kg.

[¥] LOQ for raffinose = 0.100 % dw.

♦ Statistical analysis was not performed on estimated averages.

* Statistically significant genotype x location interaction undermines the validity of the comparison across locations.

Appendix 5-Table 8. Antinutrients and secondary metabolites[¶] of Event 3272 and non-transgenic maize grain

(All values mg/kg dry weight unless otherwise indicated)

Location	Genotype	Phytic Acid[£] (% dw)	Raffinose[¥] (% dw)	Trypsin Inhibitor[2] (TIU[8]/mg)	Ferulic Acid	*p* -Coumaric Acid
Bloomington, IL	**B1 (3272)**	0.867	0.138	3.01	2953	220
Bloomington, IL	**B2 (Control)**	0.623	< 0.119[3]	2.72	2983	221
Bondville, IL	**B1 (3272)**	< 0.263[3]	0.137	2.71	2577	222
Bondville, IL	**B2 (Control)**	< 0.368[3]	0.132	2.75	2760	202
Fairbault, MN	**B1 (3272)**	0.416	< 0.121[3]	2.44	2120	129
Fairbault, MN	**B2 (Control)**	< 0.246[3]	< LOQ	2.34	2070	141
Glidden, IA	**B1 (3272)**	0.591	0.120	2.71	1860	150
Glidden, IA	**B2 (Control)**	0.435	0.114	2.67	2137	152
Shirley, IL	**B1 (3272)**	< 0.374[3]	0.145	2.41	1827	128
Shirley, IL	**B2 (Control)**	< 0.450[3]	0.127	2.40	2013	135
Stanton, MN	**B1 (3272)**	0.522	< 0.115[3]	2.71	2327	209
Stanton, MN	**B2 (Control)**	0.455	< 0.127[3]	2.69	2460	194
B1 (3272)		< 0.506[7]	< 0.129[7]	2.66	2277	176
B2 (Control)		< 0.430[7]	< 0.120[5]	2.60	2404	174
F-test Probability for Genotype		♦	♦	31.4%	11.9%	74.9%
F-test Probability for Location x Genotype Interaction		♦	♦	73.8%	83.4%	74.5%
ILSI[1] (2006)	**average**	0.745 % dw	0.132 % dw	2.73 TIU/mg	2201.1	218.4
	range	0.111 - 1.570	0.020 - 0.320	1.09 - 7.18	291.9 - 3885.8	53.4 - 576.2
	N	1196	701	696	817	817
OECD (2002) (% dw x 10^4 = mg/kg)	**range**	0.45 - 1.0 % dw	0.21 - 0.31 % dw		0.02 - 0.3 % dw	0.003 - 0.03 % dw

[¶] Furfural levels were < LOQ in all samples. LOQ for furfural = 0.500 mg/kg.

[£] LOQ for phytic acid = 0.100 % dw.

[¥] LOQ for raffinose = 0.100 % dw.

♦ Statistical analysis was not performed on estimated averages.

Appendix 5-Table 8. Antinutrients and secondary metabolites[¶] of Event 3272 and non-transgenic maize grain

(All values mg/kg dry weight unless otherwise indicated)

Location	Genotype	Phytic Acid (% dw)	Raffinose[¥] (% dw)	Trypsin Inhibitor[2] (TIU[8]/mg)	Ferulic Acid	*p* -Coumaric Acid
Bondville, IL	**B3 (3272)**	0.706	0.157	2.69	2940	345
Bondville, IL	**B4 (Control)**	0.638	0.153	3.07	2950	283
Brookings, SD	**B3 (3272)**	0.811	< LOQ	2.69	3557	399
Brookings, SD	**B4 (Control)**	0.822	< LOQ	2.53	3753	401
Glidden, IA	**B3 (3272)**	0.717	0.163	2.71	3037	313
Glidden, IA	**B4 (Control)**	0.600	0.143	2.53	3240	334
Janesville, WI	**B3 (3272)**	0.866	0.161	2.42	2970	370
Janesville, WI	**B4 (Control)**	0.664	0.149	2.82	3220	352
Stanton, MN	**B3 (3272)**	0.984	< LOQ	3.10	2567	246
Stanton, MN	**B4 (Control)**	1.069	< LOQ	3.14	2793	255
Washington, IA	**B3 (3272)**	0.740	0.119	3.10	3137	275
Washington, IA	**B4 (Control)**	0.822	0.127	2.83	2963	291
B3 (3272)		0.804	< 0.133[6]	2.79	3034	325
B4 (Control)		0.769	< 0.129[6]	2.82	3153	319
F-test Probability for Genotype		*	♦	*	***3.0%***	62.3%
F-test Probability for Location x Genotype Interaction		***1.6%***	♦	***0.3%***	15.5%	29.9%

ILSI[1] (2006)	**average**	0.745 % dw	0.132 % dw	2.73 TIU[4]/mg	2201.1	218.4
	range	0.111 - 1.570	0.020 - 0.320	1.09 - 7.18	291.9 - 3885.8	53.4 - 576.2
	N	1196	701	696	817	817
OECD (2002)	**range**	0.45 - 1.0 % dw	0.21 - 0.31 % dw		0.02 - 0.3 % dw	0.003 - 0.03 % dw
(% dw x 10^4 = mg/kg)						

[¶] Furfural levels were < LOQ in all samples. LOQ for furfural = 0.500 mg/kg.

[¥] LOQ for raffinose = 0.100 % dw.

♦ Statistical analysis was not performed on estimated averages.

* Statistically significant genotype x location interaction undermines the validity of the comparison across locations.

[1] Values < LOQ not included.

[2] No OECD data.

[3] Where some sample values were < LOQ, the average is estimated as < (average of the quantifiable values).

[4] One replication data point not included because determined to be an outlier by scatter plot.

[5] An estimated genotype average is calculated using the known LOQ for locations where all sample values were < LOQ and the estimated < (average of the quantifiable values) for locations where some sample values were <LOQ.

[6] An estimated genotype average is calculated using the known LOQ for locations where all sample values were < LOQ.

[7] An estimated genotype average is calculated using the estimated < (average of the quantifiable values) for locations where some sample values were <LOQ.

[8] TIU = Trypsin Inhibitor Units