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DuPont Agriculture & Nutrition
Pioneer Emerson Building
7250 N.W. 62nd Ave.
P.O. Box 552
Johnston, IA 50131-0552
(515) 270-3200 Tel

March 12, 2008

Dr. Natalia Weinsetel
Biotechnology Regulatory Services
USDA-APHIS
4700 River Road
Riverdale, MD 20737

Subject: Addendum to Petition for the Determination of Nonregulated Status for Herbicide Tolerant 98140 Corn (07-152-01p)

Dear Dr. Weinsetel,

This letter is in reference to Pioneer's petition for determination of non-regulated status for herbicide tolerant 98140 corn, submitted to the USDA, Animal and Plant Health Inspection Service/Biotechnology Regulatory Services (APHIS/BRS) on June 5, 2007. APHIS/BRS has assigned this petition the number 07-152-01p.

This addendum clarifies language contained in Appendix 7, page 170 of our petition dated 12/12/07, in which Pioneer predicted that acetylated serine and threonine would not accumulate to detectable levels in 98140 corn. Because of the level of interest that acetylated amino acids received during the public comment period for 356043 soybean (06-271-01p), Pioneer recently analyzed the concentrations of N-acetylthreonine, N-acetylserine and N-acetylglycine in 98140 and control corn grain as part of our ongoing stewardship activities.

As shown in the attached addendum, these acetylated amino acids have been detected at very low levels in both control and 98140 corn grain (each less than 0.0003% on a mean dry weight basis). There are no known safety issues related to the low-level presence of these additional acetylated amino acids in 98140 corn. Humans and other mammals have aminoacylase enzymes in various tissues that deacetylate N-acetylated amino acids. Numerous classes of aminoacylase enzymes have been identified in brain, liver, kidney, intestine, and other mammalian tissues. The finding of additional acetylated amino acids does not change our overall conclusions of the safety of 98140 corn.

This letter and attachment do not contain any confidential business information. If you have questions about the statements and information in this letter, please contact me at 515-270-4036 or tracy.rood@pioneer.com.

Sincerely,

Tracy A. Rood
Registration Manager

cc: hard copy original mailed to Cindy Eck, USDA

3/12/08 Addendum to Pioneer Petition 07-152-01p

Additional N-Acetylated Amino Acids in 98140 Corn

As shown in Appendix 7 of the 07-152-01p petition, Pioneer examined the ability of the GAT4621 enzyme to acetylate free amino acids *in vitro*. The GAT4621 enzyme had low but measurable activity on aspartate, glutamate, serine, threonine and glycine in a substrate specificity end-point assay. A continuous spectrophotometric assay demonstrated that a k_{cat}/K_M could be calculated for aspartate, glutamate, serine and threonine. Based on the very low catalytic efficiency of the GAT4621 protein on serine and threonine, Pioneer predicted that acetylated serine and threonine would not accumulate to detectable levels in 98140 corn.

Because of the level of interest that acetylated amino acids received during the public comment period for 356043 soybean (06-271-01p), Pioneer recently analyzed the concentrations of N-acetylthreonine (NAThr), N-acetylserine (NASer) and N-acetylglycine (NAGly) in 98140 and control corn grain as part of our ongoing stewardship activities.

Results of the acetylated amino acid analyses are shown in Table A. Mean concentrations of N-acetylglutamate (NAGlu) and N-acetylaspartate (NAAsp) in corn grain (previously reported on page 93 of the petition) are included for reference.

Mean values for NAThr, NASer and NAGly were statistically higher than those of control corn grain, although overall concentrations were very low (each less than 0.0003% on a mean dry weight basis). The concentration range for NASer was within the statistical tolerance interval for non-transgenic corn. Historical literature values for acetylated amino acids are not available.

In conclusion, analysis of acetylated amino acids in corn grain demonstrates that concentrations of five acetylated amino acids are higher in 98140 corn grain than in near-isoline control corn grain. This observation is not unexpected, given the ability of the GAT4621 enzyme to acetylate certain amino acids with low catalytic efficiency. Overall levels of NAAsp, NAGlu, NAThr, NASer and NAGly in 98140 corn grain are very low (together less than 0.05% on a dry weight basis).

The finding of additional acetylated amino acids does not change our overall conclusions of safety of 98140 corn for the following reasons:

1. NAThr, NASer and NAGly are present in non-transgenic control corn (Table A of this addendum), demonstrating that these acetylated amino acids are not novel substances;
2. A literature search revealed no toxicological or safety issues associated with the additional acetylated amino acids NAThr, NASer or NAGly;
3. The concentrations of NAThr, NASer and NAGly detected in 98140 corn grain are >100X lower than the previously quantified NAGlu and NAAsp, which were determined to be safe for consumption and the environment;
4. Acetylated amino acids (namely, NAAsp and NAGlu) are components of a wide variety of commonly consumed foods (Appendix 8, Table 1 of the petition);
5. Humans and other mammals have aminoacylase enzymes in various tissues that deacetylate N-acetylated amino acids (Gade and Brown, 1981; Endo, 1980). Numerous classes of aminoacylase enzymes have been identified in brain (Kaul *et*

al., 1991), liver (Kobayashi *et al.*, 1989), kidney (Perrier et *al.*, 2004), intestine (Giardina *et al.*, 1999), and other mammalian tissues (Surendran *et al.*, 2003);

6. It has been reported that acetylated amino acids such as glutamine (Magnusson *et al.*, 1989; Neuhauser and Bassler, 1986; Arnaud *et al.*, 2004), cystine (Du Vigneaud *et al.*, 1934) and threonine and methionine (Boggs, 1978) substitute nutritionally for their constituent amino acids in animal feeding studies; and
7. A poultry study confirmed the nutritional wholesomeness and comparability of feed made from 98140 corn as compared to conventional diets made with non-GM corn (Section VIII-E, page 109 of the petition).

Therefore, Pioneer concludes that 98140 corn is safe for consumption and the environment.

Table A. N-Acetylated Amino Acids in Corn Grain

Analyte (% Dry Weight)		Control	98140 Corn	Tolerance Interval [g, h]
NAAsp	Mean[a]	0.00009	0.0403	0 – 0.000023
	Range[b]	0.0000098 – 0.000732	0.0103 – 0.0926	
	Adjusted P-value[c]		0.0144[f]	
	P-value[d]		0.0072	
NAGlu	Mean	0.00005	0.0079	0.000005 – 0.000083
	Range	<0.0000075[e] – 0.0004	0.0000622 – 0.0195	
	Adjusted P-value		0.033[f]	
	P-value		0.033	
NAThr	Mean	0.000018	0.000299	0.000019 – 0.000152
	Range	0.000013 – 0.000030	0.000177 – 0.000442	
	Adjusted P-value		<0.0001[f]	
	P-value		<0.0001	
NASer	Mean	0.000090	0.000197	0.000030 – 0.000888
	Range	0.000030 – 0.000188	0.000073 – 0.000287	
	Adjusted P-value		<0.0001[f]	
	P-value		<0.0001	
NAGly	Mean	0.000007	0.000023	0.000001 - 0.000020
	Range	0.000003 - 0.000013	0.000011 - 0.000037	
	Adjusted P-value		<0.0001[f]	
	P-value		<0.0001	

[a] Least squares mean
[b] Range denotes the lowest and highest individual value across sites.
[c] False Discovery Rate (FDR) adjusted P-value
[d] Non-adjusted P-value
[e] <Lower Limit of Quantitation (LLOQ); indicates that the values of the sample or samples were detected below the assay's LLOQ. Sample results which were below the LLOQ were assigned a value equal to the LLOQ for statistical analysis.
[f] Statistically significant difference, adjusted P-value < 0.05
[g] Using the data obtained from four reference hybrids, a statistical tolerance interval was calculated to contain, with 95% confidence, 99% of the values contained in the population of commercial corn.
[h] Negative tolerance intervals are set to zero.

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DuPont Agriculture & Nutrition
Pioneer Emerson Building
7250 N.W. 62nd Ave.
P.O. Box 552
Johnston, IA 50131-0552
(515) 270 3202 Tel

October 31, 2007

Dr. Natalia Weinsetel
Biotechnology Regulatory Services
USDA-APHIS
4700 River Road
Riverdale, MD 20737

Subject: Addendum to Pioneer's Response to USDA's Review for Technical Completeness on Pioneer's Petition for the Determination of Nonregulated Status for Herbicide Tolerant 98140 Corn (07-152-01p)

Dear Dr. Weinsetel,

This letter is in reference to Pioneer's letter, dated 10/3/07, containing our responses to USDA's review of the technical completeness of Pioneer's petition for determination of non-regulated status for herbicide tolerant 98140 corn, submitted to the USDA, Animal and Plant Health Inspection Service/Biotechnology Regulatory Services (APHIS/BRS) on June 5, 2007. APHIS/BRS has assigned this petition the number 07-152-01p.

As you requested, we are now submitting an addendum to our responses regarding the deficiencies outlined in USDA's September 13, 2007 letter. This addendum clarifies responses contained in our 10/3/07 letter.

This letter and attachment do not contain any confidential business information.

If you have questions but the statements and information in this letter, please contact me at 515-270-4036 or tracy.rood@pioneer.com.

Sincerely,

Tracy A. Rood
Registration Manager

cc: hard copy original mailed to Cindy Eck, USDA

10/31/07 Addendum to Pioneer Responses of 10/3/07 Regarding USDA's Review of Technical Completeness (9/13/07) 07-152-01p

USDA Question 1.

Where will Table Y of the 10/3/07 response be located in the revised petition?

Pioneer Response 1.

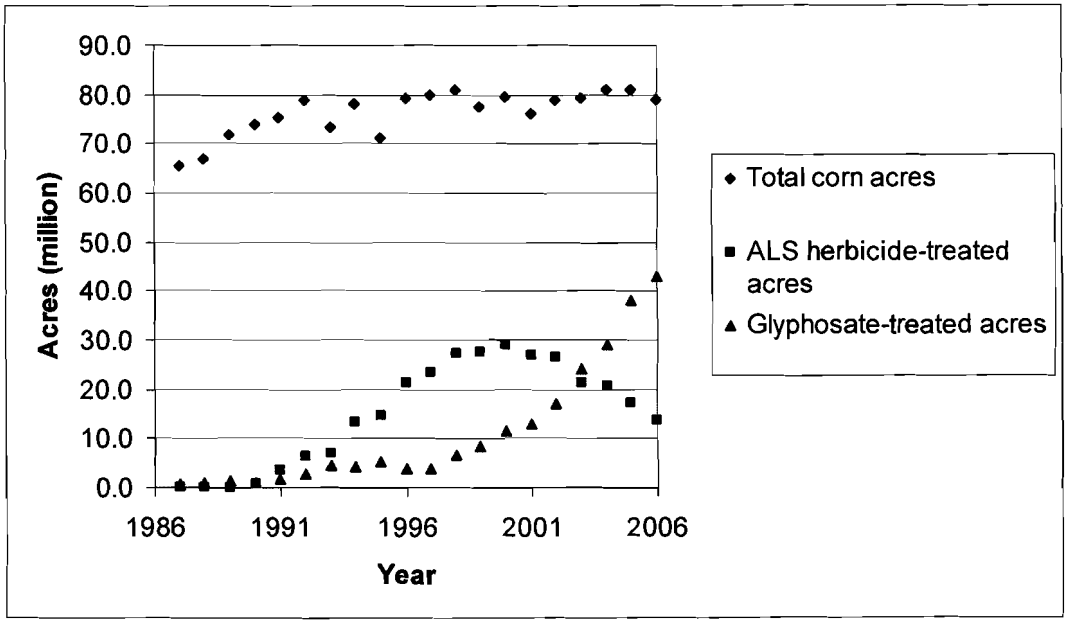
Table Y will be added to Section IX-E2 (Current Agronomic Practices for U.S. Corn—Agricultural Chemical Use in Corn).

USDA Question 2.

Please add glyphosate-treated acres to Figure X.

Pioneer Response 2.

Below is the updated Figure that will be included in the revised petition.



Source: Doane market research study (www.doane.com)

USDA Question 3.

Explain the definition of "biotype" the first time this term is used.

Pioneer Response 3.

The discussion on p. 188 of the original petition will be modified as stated in the 10/3/07 response letter, with the addition of the information below in italics.

Weed biotypes with resistance to ALS inhibitors or glyphosate and ALS inhibitors have been reported. *Weeds biotypes are sub-types of a weed species that have developed resistance to one more herbicides.* However, just because a biotype has been reported *for a given species* does not mean that weed resistance is common, widespread, or persistent. If a grower finds herbicide resistant weeds in his field of 98140 corn, or finds that herbicide resistant weeds have been reported in his area, there are many options for control of herbicide resistant weed biotypes.

USDA Question 4.

Table Z lists only two classes of herbicide-resistant weed biotypes—those with resistance to members of the ALS inhibitor family, and those with resistance to both glyphosate and members of the ALS inhibitor family. Please explain why there are no weeds listed with resistance to glyphosate only.

Pioneer Response 4.

The weeds listed in Table Z are from www.weedscience.org. This website resource lists 12 weeds with biotypes reported to be resistant to glyphosate. Four of these (junglerice, goosegrass, buckhorn plantain and johnsongrass) have occurred outside the U.S. Three others (hairy fleabane, italian ryegrass and rigid ryegrass) have been reported in the U.S., but these weeds do not occur in row crops. For the other five weed species with reported glyphosate resistance, there have also been reports of biotypes with resistance to members of the ALS inhibitor family. In a very few cases, there are biotypes of these five species that have been reported that are resistant to both glyphosate and members of the ALS inhibitor family.

The discussion on "Potential Impact of Stacking Herbicide Tolerance Traits" (p. 118) will be further expanded as follows (new text in italics). In addition, the text in Table Z will be changed (see text in italics in the first column).

Table Z lists various weed species of importance *to row crops in the U.S.* The table lists weed species that have been reported to have biotypes with resistance to ALS inhibitors, *as well as weeds that have been reported to have biotypes with resistance to either glyphosate, members of the ALS inhibitor family, or both. For example, giant ragweed biotypes have been found in Indiana and Ohio that are resistant to glyphosate but not ALS herbicides. On the other hand, there are other biotypes of ragweed in the same states that have been found to be resistant to just ALS herbicides. There are also a very few biotypes that are resistant to both glyphosate and members of the ALS inhibitor family.* Table Z also lists other currently registered herbicides that can be used on corn to control these resistant biotypes, if needed. Although cross resistance to both glyphosate and ALS inhibitor herbicides has been reported in the species noted, it is rare.

Table Z. Most Problematic Herbicide-Resistant Weed Biotypes for Corn in the U.S. and Herbicide Options to Control

Biotypes Identified with Resistance To:	Common Name	Scientific Name	Herbicide Options to Control Resistant Biotype
Members of the ALS inhibitor family	Smooth Pigweed	*Amaranthus hybridus*	glyphosate Dicamba Mesotrione glufosinate
	Redroot Pigweed	*Amaranthus retroflexus*	glyphosate Dicamba Mesotrione glufosinate
	Lambsquarters	*Chenopodium album*	glyphosate bromoxynil Dicamba Mesotrione atrazine glufosinate
	Common Sunflower	*Helianthus annuus*	glyphosate Dicamba Mesotrione glufosinate
	Kochia	*Kochia scoparia*	glyphosate Dicamba glufosinate
	Foxtails	*Setaria* species	glyphosate Metolachlor Acetochlor glyphosate
	Johnsongrass	*Sorghum halepense*	glyphosate
	Common cocklebur	*Xanthium strumarium*	glyphosate dicamba atrazine glufosinate
	Shattercane	*Sorghum bicolor*	glyphosate glufosinate
Glyphosate, members of the ALS inhibitor family, and both glyphosate and members of the ALS inhibitor family	Common Waterhemp	*Amaranthus rudis*	Dicamba Mesotrione Glufosinate
	Horseweed	*Conyza canadensis*	2,4-D dicamba atrizine paraquat glufosinate
	Common Ragweed	*Ambrosia artemisiifolia*	Dicamba Mesotrione atrazine glufosinate
	Giant Ragweed	*Ambrosia trifida*	Dicamba Mesotrione atrazine glufosinate
	Palmer Amaranth	*Amaranthus palmeri*	Dicamba Mesotrione glufosinate

Source: www.weedscience.org

USDA Question 5.

In Table 2 of Appendix 5 (Disease Stressor Comparison between 98140 and Control Corn), counties Freeborn, Stearns and Steele are in Minnesota, not Missouri (p. 160 of the original petition). Please correct.

Pioneer Response 5.

Table 2 of Appendix 5 will be corrected in the revised petition.

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DuPont Agriculture & Nutrition
Pioneer Emerson Building
7250 N.W. 62nd Ave.
P.O. Box 552
Johnston, IA 50131-0552
(515) 270 3202 Tel

September 13, 2007

Cynthia Eck
Document Control Officer
Biotechnology Regulatory Services
USDA-APHIS
4700 River Road, Unit 147 6B72
Riverdale, MD 20737

Subject: Addendum to "Petition for the Determination of Nonregulated Status for Herbicide Tolerant 98140 Corn" (07-152-01p)

Dear Ms. Eck,

At the request of USDA-APHIS, Pioneer Hi-Bred International, Inc. (Pioneer) is submitting an addendum to our petition for determination of nonregulated status for herbicide tolerant corn event DP-Ø9814Ø-6.

USDA-APHIS has requested additional information on the potential exposure levels of acetylated amino acids due to the commercialization of 98140 corn, and additional information on the impact of potential increased dietary exposure of N-acetylaspartate (NAA) to individuals with Canavan Disease (CD). This addendum is provided to supplement the information on safety of acetylated amino acids that is already included in the 98140 corn petition.

Levels of two acetylated amino acids, NAA and N-acetylglutamate (NAG), are slightly elevated in 98140 corn, although overall, levels of NAA and NAG remain low (together less than 0.05% in grain on a dry weight basis and less than 0.5% of the total amino acids in grain). Commercialization of 98140 corn may increase dietary exposure to NAA and NAG. NAA and NAG are common constituents of the human diet present in eggs, chicken, turkey and beef. Acetylated amino acids are readily metabolized and have a history of safe consumption by humans and animals; therefore, no safety issues are expected to result from commercialization of 98140 corn.

The information included in Appendix 1 of this addendum shows that the modest potential increase in dietary exposure to NAA resulting from commercialization of 98140 corn is negligible compared to the amount of NAA being produced in the brain, as evidenced by the large amount of NAA being excreted in the urine of individuals with CD. Thus, we conclude that there will be no adverse impact to persons with CD from the potential increase in dietary NAA as a result of commercializing 98140 corn.

Enclosed please find a paper copy of the addendum as well as a CD containing an electronic copy in Microsoft Word. The addendum contains no confidential business information.

Please feel free to contact me at 515-270-4036 if you have any questions.

Sincerely,

Tracy A. Rood

Tracy A. Rood
Registration Manager

electronic cc: Natalia Weinsetel, USDA

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Addendum to
Petition for the Determination of Nonregulated Status for Herbicide Tolerant 98140 Corn
07-152-01p

Quantitative Dietary Exposure Assessment for NAA and NAG

Submitting Company:

Pioneer Hi-Bred International, Inc.
7100 NW 62nd Avenue
PO Box 1014
Johnston, IA 50131-1014

Submitted by:

Tracy A. Rood, Registration Manager
Pioneer Hi-Bred International, Inc.
7250 NW 62nd Ave.
PO Box 552
Johnston, IA 50131
Telephone: 515-270-4036
Fax: 515-334-4478

Prepared by:

Bryan Delaney, Ray Layton and Tracy Rood

OECD Unique Identifier: DP-Ø9814Ø-6

September 13, 2007

NO CBI

Quantitative Dietary Exposure Assessment for NAA and NAG

Because the concentrations of N-acetylglutamate (NAG) and N-acetylaspartate (NAA) in 98140 corn are higher than the concentrations in control or reference corn lines, an exposure assessment for humans was conducted to compare the dietary intake of NAA and NAG with and without exposure from 98140 corn. An exposure assessment for livestock was also conducted. The expected dietary exposures were then compared with levels of exposure in the broiler study where no adverse effects were seen.

1. Human Exposure Assessment for NAA and NAG

Both conventional and 98140 corn (as well as other foods) contain NAA and NAG. The dietary exposure of NAA and NAG were estimated using DEEM/FCID (Dietary Exposure Evaluation Model – Food Commodity Intake Database, Version 2.14, Exponent Inc., Washington, DC). This model is commonly used by the EPA Office of Prevention, Pesticides and Toxic Substances to estimate human dietary exposure. Annual mean and 90th percentile exposures were calculated for the U.S. population and several subpopulations.

DEEM/FCID categorizes maize (field corn) consumption into nine categories: flour, flour-babyfood, meal, meal-babyfood, bran, starch, starch-babyfood, syrup, syrup-babyfood. In order to conduct the dietary exposure assessment, the concentrations of NAA and NAG in various corn fractions were determined. 98140 corn grain was processed using wet and dry milling methods typical and representative of commercial grain processing facilities. Reductions in NAA and NAG concentrations in various fractions ("processing factors") were calculated.

NAA and NAG concentrations in unprocessed grain were taken from Table 19 on page 93 of the original petition (0.9 mg/kg for non-98140 corn and 403 mg/kg for 98140 corn for NAA, and 0.5 mg/kg for non-98140 corn and 79 mg/kg for 98140 corn for NAG) and adjusted using processing factors (Table 1). For example, NAA levels in flour were 62% of those in unprocessed grain (processing factor = 0.62). NAA and NAG were not found in corn oil. Analyses were not conducted to determine NAA and NAG levels in high fructose corn syrup (HFCS) so starch processing factors were used as a worst-case surrogate since corn starch is the feedstock for producing HFCS.

Two dietary exposure assessments were conducted. The first used NAA or NAG concentrations in control corn ("Baseline" columns in Tables 2 and 3), and the second assessment used NAA or NAG concentrations in 98140 corn combined with control corn ("98140 corn" columns in Tables 2 and 3). In the second assessment, 98140 corn made up 40% of the consumed corn, and non-98140 corn made up the remaining 60%. The results of the assessments for the U.S. population and selected population subgroups are summarized in Table 2 for NAA and Table 3 for NAG.

Table 1. Processing Factors for NAA and NAG in Corn Grain

Processed Fraction	DEEM Category	NAA Processing Factor	NAG Processing Factor
Flaking (large) grits	Corn, field, meal	0.54	0.45
Flour (fines)	Corn, field, flour	0.62	0.81
Bran	Corn, field, bran	0.88	1.09
Starch (dried)	Corn, field, starch	0.02	0.01
Starch (dried)	Corn, field, syrup	0.02	0.01

Table 2. DEEM Dietary Exposure Analysis for NAA

Population Subgroup	NAA Exposure mg/kg body weight/day			
	Baseline[a] Mean	Baseline 90th Percentile	98140 Corn[b] Mean	98140 Corn 90th Percentile
U.S. Population	0.0028	0.0060	0.0246	0.0661
Hispanics	0.0034	0.0073	0.0452	0.1266
Non-hispanic whites	0.0026	0.0055	0.0211	0.0568
Non-hispanic blacks	0.0033	0.0075	0.0290	0.0778
Non-hispanic/non-white/non-black	0.0031	0.0068	0.0218	0.0611
All infants	0.0022	0.0079	0.0169	0.0440
Children 1-6 yrs	0.0057	0.0124	0.0639	0.1700
Children 7-12 yrs	0.0037	0.0080	0.0497	0.1294
Youth 13-19 yrs	0.0027	0.0058	0.0302	0.0811
Adults 20-49 yrs	0.0024	0.0051	0.0180	0.0485
Adults 50+ yrs	0.0020	0.0042	0.0116	0.0351

[a] Baseline exposure calculations used NAA or NAG concentrations in control corn.
[b] 98140 corn exposure calculations used NAA and NAG concentrations as would be measured in a mixture of 60% control corn and 40% 98140 corn.

The estimated annual mean baseline exposure to NAA for the U.S. population was 0.0028 mg/kg body weight per day. With the addition of 98140 corn, the estimated exposure increased to 0.0246 mg/kg body weight per day. The estimated 90th percentile baseline exposure was 0.0060 mg/kg body weight per day, which increased to 0.0661 mg/kg body weight per day with the addition of 98140 corn.

The population subgroup with the highest predicted exposure is the group associated with children ages 1-6 (mean annual exposure 0.0639 mg/kg body weight per day, 90th percentile 0.1700 mg/kg body weight per day). The primary sources of exposure are corn meal and flour.

These estimated exposures can be compared to the poultry study where broiler chickens were exposed to an average NAA level of 21.7 mg/kg body weight per day throughout a 42-day test (see Table 4 of this addendum). The exposures to NAA in the poultry study (where no adverse effects were observed) were over one hundred times greater than the exposures predicted for various human population subgroups.

These results indicate that there is a high margin (>100X) of exposure between expected human dietary exposures to NAA and levels of exposure in the poultry trial where no adverse effects were observed.

Table 3. DEEM Dietary Exposure Analysis for NAG

Population Subgroup	NAG Exposure mg/kg body weight/day			
	Baseline[a] Mean	Baseline 90th Percentile	98140 Corn[b] Mean	98140 Corn 90th Percentile
U.S. Population	0.0020	0.0044	0.0067	0.0169
Hispanics	0.0022	0.0048	0.0124	0.0330
Non-hispanic whites	0.0019	0.0041	0.0057	0.0143
Non-hispanic blacks	0.0022	0.0052	0.0076	0.0194
Non-hispanic/non-white/non-black	0.0025	0.0058	0.0066	0.0173
All infants	0.0021	0.0051	0.0049	0.0109
Children 1-6 yrs	0.0038	0.0080	0.0166	0.0421
Children 7-12 yrs	0.0028	0.0059	0.0128	0.0325
Youth 13-19 yrs	0.0020	0.0042	0.0077	0.0201
Adults 20-49 yrs	0.0018	0.0039	0.0051	0.0126
Adults 50+ yrs	0.0013	0.0029	0.0034	0.0088

[a] Baseline exposure calculations used NAA or NAG concentrations in control corn.
[b] 98140 corn exposure calculations used NAA and NAG concentrations as would be measured in a mixture of 60% control corn and 40% 98140 corn.

The estimated annual mean baseline exposure to NAG for the U.S. population was 0.0020 mg/kg body weight per day. With the addition of 98140 corn, the estimated exposure increased to 0.0067 mg/kg body weight per day. The estimated 90th percentile baseline exposure was 0.0044 mg/kg body weight per day, which increased to 0.0169 mg/kg body weight per day with the addition of 98140 corn.

The population subgroup with the highest predicted exposure is the group associated with children ages 1-6 (mean annual exposure 0.0166 mg/kg body weight per day, 90th percentile 0.0421 mg/kg body weight per day). The primary sources of exposure are corn meal and flour.

These estimated exposures can be compared to the poultry study where broiler chickens were exposed to an average of 8.0 mg/kg body weight/day throughout a 42-day test (see Table 4 of this addendum). The exposures to NAG in the poultry study (where no adverse effects were observed) were over one hundred times greater than the exposures predicted for various human population subgroups.

These results indicate that there is a high margin (>100X) of exposure between expected human dietary exposures to NAA and level of exposure in the poultry study where no adverse effects were observed.

2. Animal Exposure Assessment for NAA and NAG

Because corn is typically fed to livestock as grain (whole or ground) or silage, NAA and NAG are expected to be present in 98140 corn fed to animals. Low levels of NAA and NAG are found in non-transgenic corn grain and forage (Tables 19 and 28 of 07-152-01p), so livestock animals are already being exposed to these compounds. Animal exposure to NAA and NAG would likely increase with commercialization of 98140 corn.

Poultry consume a significant fraction of corn grown in the U.S. The safety of 98140 corn for poultry is supported by the results of the 42-day broiler study where no adverse effects were found. NAA and NAG levels were measured in the diets, and dietary consumption and body weight data were used to calculate exposure. The average exposure to NAA during the 42-day broiler study was 21.7 mg/kg body weight/day, and the average exposure to NAG during the 42-day broiler study was 8.0 mg/kg body weight/day (Table 4). No adverse effects were seen during the study at these levels. In general practice, exposure levels would be expected to be lower due to mixing of 98140 corn in general commerce with non-98140 corn.

For cattle and swine, estimated exposures to NAA and NAG were calculated by assuming that 100% of the corn grain or silage fed was 98140 corn. Concentrations of NAA and NAG in silage are not available, but NAA and NAG concentrations in forage are appropriate to use in estimating exposure since the protein content of forage is generally maintained during the ensiling process.

The greatest percentage of corn harvested for feed use is fed to cattle. Potential exposures were estimated for dairy cattle and beef cattle eating 98140 corn grain and silage.

Assuming a dairy cow weighs 1600 lbs (725.7 kg) and consumes 20 lbs (9.1 kg) of corn grain each day with an NAA and NAG content of 403 mg/kg and 79 mg/kg respectively, the predicted exposures would be approximately 5.0 and 1.0 mg/kg body weight/day for NAA and NAG (Table 4). Dairy cattle eating silage would have similar levels of exposure (1600 lb animal consuming 50 lbs (22.7 kg) of silage containing 20 lbs (9.1 kg) dry matter from silage would have 8.1 and 1.2 mg/kg body weight/day exposure for NAA and NAG, respectively).

Similarly, predicted exposures can also be calculated for feeder steers (~6-7 months of age) and finisher steers (~11-12 months of age). A feeder steer weighing 500 lbs (226.8 kg) eating 35 lbs (15.9 kg) fresh silage (14 lbs or 6.4 kg of silage dry matter based on 40% dry matter) each day containing 650 mg/kg NAA and 93 mg/kg NAG would be exposed to 18.2 mg/kg body weight/day NAA and 2.6 mg/kg body weight/day NAG. Alternatively, feeder steers may also be fed rations containing corn grain. A feeder steer consuming 2.3 kg of corn grain would be exposed to 4.0 mg/kg body weight/day of NAA and 0.8 mg/kg body weight/day of NAG. Predicted exposures are for an 1100 lb (499 kg) steer fed finishing rations containing 20 lb (9.1 kg) corn grain each day are 7.3 mg/kg body weight/day of NAA and 1.4 mg/kg body weight/day of NAG (Table 4).

These predicted exposure levels for dairy and beef cattle are all lower than those used in the 42-day poultry study where no adverse effects were seen. In general practice, exposure levels may be lower due to mixing of 98140 corn grain and silage with non-98140 corn grain and silage. Microbes in the rumen are expected to metabolize NAA and NAG.

Another major use of corn is for swine feed. Corn grain makes up approximately 44% of starter diets and 77% of grow-finish diets (ISU, 2002). Consumption of diet for young pigs (4 to 15 kg) is approximately 583 g/day and for larger pigs (35 to 100 kg) is approximately 2500 g/day (National Research Council, 1998). Assuming an NAA concentration of 403 mg/kg in corn grain (Table 19), this would result in an estimated exposure of 11.5 mg/kg body weight/day in young pigs and 12.8 mg/kg body weight/day in older pigs (Table 4). Assuming an NAG concentration of 79 mg/kg in corn grain, this would result in an estimated exposure of 2.3 mg/kg body weight/day in young pigs and 2.5 mg/kg body weight/day in older pigs. These estimated exposures are much lower than the exposure levels seen in the poultry study where no adverse effects were seen.

Table 4. Livestock Estimated Exposure for NAA and NAG

Animal (Weight)	Form of Corn Consumed	Typical % Corn in Diet	Estimated Amount of 98140 Corn Consumed	Predicted exposure mg/kg body weight/day	
				NAA	NAG
Poultry[a] (0.7 kg)	Grain[b]	59%-72%	55 g/day	21.7	8.0
Dairy cow (726 kg)	Grain	~30-50%	9.1 kg/day	5.0	1.0
	Silage[c]	~16-45%	9.1 kg dry matter/day	8.1	1.2
Feeder steer (227 kg)	Grain	~30-40%	2.3 kg dry matter/day	4.0	0.8
	Silage	~80-95%	6.4 kg dry matter/day	18.2	2.6
Finisher steer (499 kg)	Grain	~66-87%	9.1 kg /day	7.3	1.4
Younger pig (4-15 kg)	Grain	~44%	257 g/day	11.5	2.3
Older pig (35-100kg)	Grain	~77%	1.93 kg/day	12.8	2.5

[a] For the 42-day poultry study conducted with 98140 corn grain (see Section VIII-E of 07-152-01p), NAA and NAG levels were measured in the diets, and dietary consumption and body weight data were used to calculate exposure (42-day average weight was 0.7 kg; 42-day average consumption was 0.055 kg/day).
[b] NAA and NAG content of corn grain on a dry weight basis is 403 mg/kg and 79 mg/kg, respectively (Table 19).
[c] NAA and NAG content of corn forage on a dry weight basis is 650 mg/kg and 93 mg/kg, respectively (Table 28).

3. Conclusions

The estimated daily intake of N-acetylaspartate (NAA) and N-acetylglutamate (NAG) from 98140 corn (assuming 40% of the commodity corn grown in the US was 98140 corn) was calculated for humans. On a body weight basis, the mean and 90th percentile intakes of NAA for the general US population are determined to be 0.0246 and 0.0661 mg/kg body weight/day, respectively. The mean and 90th percentile intakes of NAG are determined to be 0.0067 and 0.0169 mg/kg body weight/day, respectively.

NAA and NAG are normal constituents of the human diet present in soybean, eggs and meat. The current mean and 90th percentile estimated daily intakes of naturally occurring NAA in the general US population are 0.0028 and 0.0060 mg/kg body weight/day, respectively. For NAG, the mean and 90th percentile estimated daily intakes of naturally occurring NAG are 0.0020 and 0.0044 mg/kg body weight/day.

Commercialization of 98140 corn may increase dietary exposure to NAA and NAG above current levels. Because acetylated amino acids are metabolizable and have a history of safe use by humans and animals, there are no safety issues that would be expected to result from this potential increase in exposure.

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Appendix 1

Analysis of Impact of Potential Dietary Increase in NAA from 98140 Corn on Individuals with Canavan Disease

N-acetyl-L-aspartate (NAA) is an essential substance of the human brain. NAA is continuously produced and metabolized within the brain. It is present in relatively high concentrations and is the second most abundant free amino acid in the brain (Tallan *et al.*, 1956; Miyake *et al.*, 1981). NAA is normally converted to acetate and the free amino acid L-aspartate by the enzyme aspartoacylase. The free acetate resulting from the hydrolysis of NAA is required for proper myelination of the neurons of the brain, which is critical for development of a functional central nervous system (Birken and Oldendorf, 1989).

Canavan Disease (CD) is caused by heritable mutations in the aspartoacylase gene (Zeng *et al.,* 2002; Hershfield *et al.,* 2007). These mutations result either in the absence of, or expression of a non-functional, aspartoacylase enzyme (Matalon and Michals-Matalon, 1999). The lack of aspartoacylase activity causes an inability to hydrolyze NAA, resulting in a deficiency of acetate leading to hypomyelination in the brain of infants and children (Madhavarao *et al.,* 2005; Kirmani *et al.,* 2002; and Mehta and Namboodiri, 1995) and an accumulation of excess NAA. Concentrations of NAA in the brain increase approximately two-fold relative to healthy persons (Blüml, 1999; Leone *et al.,* 2000; Janson *et al.,* 2006), and persons with CD excrete large amounts of excess NAA (10-100-fold higher than normal) in the urine (Kvittingen *et al.*, 1986).

NAA is also a natural constituent of commonly consumed foodstuffs (*e.g.,* meat and eggs). Raw, unprocessed 98140 corn grain contains an increased concentration of NAA relative to control corn grain because of the aspartate acetylation activity of the GAT4621 enzyme. This elevated concentration of NAA translates to modest increases in estimated dietary exposure when factors such as processing factors and corn consumption patterns are taken into consideration. The mean baseline dietary exposure of NAA to children ages 1-6 from existing foodstuffs is 0.0057 mg NAA/kg body weight/day, and the predicted mean exposure after commercialization of 98140 corn to children ages 1-6 is 0.0639 mg NAA/kg body weight/day (Table 2 of this addendum).

Since the brain is the principal source of NAA, the levels of NAA being excreted in the urine of persons with CD provides us with a minimum estimate for the body's endogenously produced levels of NAA against which we can evaluate any contribution from dietary NAA. Since CD is primarily a disease of children, and because children ages 1-6 are the population subgroup with the highest predicted exposure to NAA from 98140 corn, we focused our comparison on children ages 1-6. Estimates of total urinary NAA elimination in healthy children and children with CD can be calculated based on reported urinary concentrations of NAA and estimated daily urine output (Table 5). The concentration of NAA excreted by children with CD (31-126 mg/kg body weight/day) relative to healthy children (maximum of 2 mg/kg body weight/day) is indicative of the large amount of NAA naturally synthesized in the body.

To understand the potential impact of NAA dietary exposure on persons with CD, daily dietary NAA exposure estimates were compared to the amount of NAA excreted in the urine. Even using conservative assumptions, both the baseline and predicted daily dietary exposure to NAA are approximately 500-fold lower than quantities excreted in the urine on a daily basis by children with CD. Therefore, the overwhelming majority of NAA in the urine of persons with CD is being produced endogenously, and the background dietary exposure to NAA is insignificant relative to the whole body pool of NAA in persons with CD. Further, the modest potential increase in dietary exposure to NAA from the commercialization of 98140 corn is negligible when compared to the significant concentrations of NAA being synthesized in the body and to the whole body pool of NAA in persons with CD.

In conclusion, NAA synthesized by the brain is an essential substance that provides acetate for proper brain development in healthy individuals. NAA is present at relatively high levels in the brain. Although there could be modest increases in dietary exposure to NAA resulting from commercialization of 98140 corn, this increase is negligible compared to the amount of NAA being produced in the brain, as evidenced by the large amount of NAA being excreted in the urine of individuals with CD. Thus, we conclude that there will be no adverse impact to persons with CD from the potential increase in dietary NAA as a result of commercializing 98140 corn.

Table 5. Comparison of NAA Excretion in Urine

	Amount of NAA in urine (mg NAA/1000 mg of creatinine)[1]	**Typical urine output in children (ml/day)[2]**	**Calculated amount of NAA excreted in urine (mg/kg body weight/day)[3]**
Healthy children	0 – 47	250-500	0 – 2
Children with Canavan Disease	1,239 – 2,529	250-500	31 – 126

[1] From Janson *et al.*, 2006.

[2] Typical urine output in infants and children is in the range of 1-2 ml/kg/hour (WHO). Using a default body weight of 10 kg for infants and small children, they produce approximately 250-500 ml of urine over a 24 hour period.

[3] The mass of NAA excreted in urine was calculated using an assumption of 100 mg/dL of creatinine in the urine (Barr *et al.*, 2004) and the following sample equation:

(1,239 mg NAA/1000 mg creatinine) * (100 mg/dL) * (250 ml/day) * (dL/100 ml) = 310 mg NAA/day per 10 kg body weight of a child or 31 mg NAA/kg body weight/day

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Petition for the Determination of Nonregulated Status for Herbicide Tolerant 98140 Corn

We submit 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 no longer be regulated under 7 CFR §340.

Submitting Company:

Pioneer Hi-Bred International, Inc.
7100 NW 62nd Avenue
PO Box 1014
Johnston, IA 50131-1014

Submitted by:

Tracy A. Rood, Registration Manager
Pioneer Hi-Bred International, Inc.
7250 NW 62nd Ave.
PO Box 552
Johnston, IA 50131
Telephone: 515-270-4036
Fax: 515-334-4478

Prepared by:

Tracy Rood, Kent Brink, Natalie Weber, Annie Tang Gutsche,
Meredith Evans and Maria Fedorova

OECD Unique Identifier: DP-Ø9814Ø-6

May 30, 2007

NO CBI

Release of Information

Pioneer is submitting the information in this assessment for review by the USDA as part of the regulatory process. By submitting this information, Pioneer does not authorize its release to any third party except to the extent it is requested under the Freedom of Information Act (FOIA), 5 U.S.C., Section 552; USDA complies with the provisions of FOIA and USDA's implementation regulations (7 CFR Part 1.4); and this information is responsive to the specific request. Except in accordance with the FOIA, Pioneer does not authorize the release, publication or other distribution of this information (including website posting) without Pioneer's prior notice and consent.

Certification

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

_____

Date

Tracy A. Rood
Registration Manager
Pioneer Hi-Bred International, Inc.
7250 NW 62nd Ave.
PO Box 552
Johnston, IA 50131
Telephone: 515-270-4036
Fax: 515-334-4478

Summary

Pioneer Hi-Bred International, Inc. (Pioneer) is submitting a Petition for Determination of Nonregulated Status for herbicide tolerant corn event DP-Ø9814Ø-6. Corn lines containing the DP-Ø9814Ø-6 event are referred to as 98140 corn. Event DP-Ø9814Ø-6 was developed by Pioneer, a DuPont Company. Pioneer requests a determination from USDA - Animal and Plant Health Inspection Service (APHIS) that 98140 corn and any crosses of this line with nonregulated corn lines no longer be considered regulated articles under 7 CFR §340.

98140 corn a transgenic product that provides tolerance to two different classes of herbicides: glyphosate and acetolactate synthase (ALS)-inhibiting herbicides. The dual herbicide tolerance of 98140 corn will enable growers to choose an optimal combination of these herbicides to best manage their individual weed populations. The availability of 98140 corn will enable growers to proactively manage weed populations while delaying adverse population shifts of troublesome weeds or the development of resistance. Herbicide tolerant 98140 corn will be marketed in the U.S. under the brand name Optimum™ GAT™.[1]

The 98140 corn plants have been genetically modified to express the GAT4621 (glyphosate acetyltransferase) and ZM-HRA (modified version of a maize acetolactate synthase) proteins. The GAT4621 protein, encoded by the *gat4621* gene, confers tolerance to glyphosate-containing herbicides by acetylating glyphosate and thereby rendering it non-phytotoxic. The ZM-HRA protein, encoded by the *zm-hra* gene, confers tolerance to the ALS-inhibiting class of herbicides.

The *gat4621* gene is based on the sequences of three *gat* genes from the common soil bacterium *Bacillus licheniformis*. *B. licheniformis* is widespread in the environment; therefore, animals and humans are regularly exposed without adverse consequences to this organism and its components, such as the glyphosate acetyltransferase (GAT) protein. GAT proteins are members of the GCN 5-related family of N-acetyltransferases (also known as the GNAT family). The GNAT superfamily is one of the largest enzyme superfamilies recognized to date with over 10,000 representatives from plants, animals and microbes. The GAT4621 protein is 75-78% identical and 90-91% similar at the amino acid level to each of the three native GAT enzymes from which it was derived. In 98140 corn, the expression of the *gat4621* gene is driven by the corn ubiquitin promoter.

The ZM-HRA protein is a modified version of the maize ALS protein. ALS is involved in branched chain amino acid (leucine, isoleucine and valine) biosynthesis in the plastid. The herbicide tolerant *zm-hra* gene was made by isolating the herbicide sensitive maize *als* gene and introducing two specific amino acid changes known to confer herbicide tolerance to tobacco ALS. In 98140 corn, the expression of the *zm-hra* gene is driven by the maize ALS promoter.

The allergenic potential of the GAT4621 and ZM-HRA proteins was assessed using a step-wise, weight of evidence approach using guidance from the Codex Alimentarius Commission. Bioinformatic analyses revealed no biologically significant identities to known or putative protein allergens or toxins for either the GAT4621 or ZM-HRA protein sequences. Both the GAT4621 and ZM-HRA proteins were non-glycosylated. Both proteins hydrolyzed rapidly (within 30 seconds) in simulated gastric fluid. In simulated intestinal fluid, the GAT4621 protein hydrolyzed within 5 minutes and the ZM-HRA protein hydrolyzed within 30 seconds. There was no evidence of acute toxicity in mice for either GAT4621 or ZM-HRA at a target dose of 2000 mg protein per

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[1] Optimum™ and GAT™ are trademarks of Pioneer Hi-Bred International, Inc.

kg of body weight (actual dose of 1640 and 1236 mg/kg of body weight, respectively, for GAT4621 and ZM-HRA). Based on the GAT4621 and ZM-HRA protein levels in 98140 corn, exposure levels would be exponentially lower than the tested doses. These data support the food and feed safety of the GAT4621 and ZM-HRA proteins. New Protein Consultation comprehensive safety data packages for the GAT4621 and ZM-HRA proteins were submitted to FDA on January 31, 2007 and February 13, 2007, respectively.

Transformation, via *Agrobacterium tumefaciens*, of immature embryos with a 7440 bp T-DNA region containing the *gat4621* and *zm-hra* expression cassettes resulted in the generation of 98140 corn. Molecular characterization of 98140 corn by Southern blot analysis confirmed that a single, intact fragment was inserted into the corn genome. A single copy of each of the genetic elements of the *gat4621* and *zm-hra* expression cassettes was present, and the integrity of the inserted fragment was demonstrated in four different generations, confirming stability during traditional breeding procedures. Southern blot analysis also verified the absence of plasmid backbone DNA from outside the T-DNA region. Segregation analysis of four generations of 98140 corn confirmed Mendelian inheritance of the *gat4621* and *zm-hra* genes.

98140 corn has been field tested since 2005 in the major corn-growing regions of the continental United States as well as Hawaii and Puerto Rico. All field tests have occurred under field permits and notifications granted by USDA - APHIS. Comprehensive agronomic performance and ecological observation assessments for 98140 corn were conducted in replicated field studies at a total of 15 North American locations over the 2006 growing season. Characteristics such as emergence, seedling vigor, plant height, ear height, stalk lodging, root lodging, stay green, time to silking and pollen shed, yield, disease incidence and insect damage were measured. Seed germination, dormancy, and pollen viability data were also collected in laboratory experiments. All field trials of 98140 corn were observed for opportunistic disease or insect biotic stressors. Analysis of agronomic and ecological data showed no biologically meaningful differences between 98140 corn and control corn lines, indicating no plant pest characteristics for 98140 corn. Likewise, assessment of the ecological data detected no biologically meaningful differences between 98140 corn and control corn lines indicative of a selective advantage that would result 98140 corn having an increased invasiveness potential for natural habitats . These data support a conclusion of agronomic comparability of 98140 corn to conventional corn with respect to the lack of increased weediness and plant pest potential.

Because of the agronomic similarity of 98140 corn to conventional corn and the demonstrated safety of the GAT4621 and ZM-HRA proteins, there is no significant impact expected on raw or processed agricultural commodities, on non-target, beneficial organisms (including threatened and endangered species), or on biodiversity. Impacts on organic or conventional farming are also expected be minimal, as growers' decisions to plant biotechnology-derived, organic or conventional corn are driven largely by market dynamics. Market dynamics, grower choice, and existing corn production practices will not change due to the availability of 98140 corn.

With the introduction of herbicide tolerant 98140 corn, we do not expect a significant change in agronomic practices, with the exception of current weed control practices. We anticipate no increase in the usage of glyphosate, but we do expect an increase in the use of ALS-inhibiting herbicides, as this family of herbicides currently is not as widely used for control of weeds in corn. With the introduction of 98140 corn, new weed control options will be available to growers that are compatible with integrated weed management practices. This technology advances agricultural sustainability by helping growers achieve higher yields per acre while providing improved environmentally sound options for integrated weed management.

Extensive nutrient composition analysis of grain and forage was conducted to compare the composition of 98140 corn to that of a null segregant near isoline and four conventional corn hybrids. In total, data from 83 different analytical components (83 in grain and 53 of those in forage) are presented herein. Compositional analysis of 98140 corn was used to evaluate any changes in the levels of key nutrients, anti-nutrients and secondary metabolites. Along with the

agronomic data included in this petition, compositional comparability is a general indicator that 98140 corn will not exhibit unexpected effects with respect to plant pest risk.

Based on the compositional evaluation, the grain and forage of 98140 corn were considered to be comparable to conventional corn. Levels of two acetylated amino acids, N-acetylaspartate and N-acetylglutamate, were elevated in 98140 corn grain and forage. GAT proteins are known to acetylate certain amino acids under specific *in vitro* conditions. Levels of N-acetylaspartate and N-acetylglutamate were very low in 98140 corn grain and forage (together less than 0.05% in grain and 0.08% in forage, on a dry weight basis; together less than 0.5% of the total amino acids in grain and less than 1.2% of the total amino acids in forage). In addition to being found in conventional corn grain and forage, these two analytes are components of commonly consumed food such as eggs, chicken, turkey, and beef.

A 42-day broiler study confirmed the nutritional wholesomeness and comparability of poultry diets made from 98140 corn and control corn. Based on the food and feed safety assessment of 98140 corn, we conclude there will be no adverse effects to animal or human health. A detailed assessment of the food and feed safety and nutritional value of 98140 corn will be submitted to FDA.

In conclusion, based on the data contained herein, Pioneer requests that APHIS grant the request for a determination of nonregulated status for 98140 corn and any crosses of this line with nonregulated corn lines.

Abbreviations, Acronyms and Definitions

~	approximately
98140 corn	corn lines containing the DP-Ø9814Ø-6 event
ADF	acid detergent fiber
als	native acetolactate synthase gene from maize
ALS	native acetolactate synthase protein from maize
APHIS	Animal and Plant Health Inspection Service
ATCC	American Type Culture Collection
B. licheniformis	*Bacillus licheniformis*
B. subtilis	*Bacillus subtilis*
BAR	phosphinothricin acetyltransferase from *Streptomyces hygroscopicus*
bp	base pair
CaMV	Cauliflower Mosaic Virus
CFIA	Canadian Food Inspection Agency
Da	Dalton
DIG	digoxygenin
DP-Ø9814Ø-6	event DP-Ø9814Ø-6 that confers glyphosate and ALS inhibitor herbicide tolerance to 98140 corn lines
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
E score	expectation score
ELISA	enzyme linked immunosorbent assay
EPA	Environmental Protection Agency
EPSPS	enolpyruvylshikimate-3-phosphate synthase
ERS	Economic Research Service, the primary source of economic and research information in the U.S. Department of Agriculture
ESMS	electrospray ionization mass spectroscopy
FDA	Food and Drug Administration
FDR	false discovery rate
GAT	glyphosate N-acetyltransferase
GAT4621	specific GAT protein
gat4621	specific *gat* gene
GDU	Growing Degree Units
Gly	glyphosate
HRP	horseradish peroxidase

Abbreviations, Acronyms and Definitions, continued

IgG	immunoglobulin G
ISAAA	International Service for the Acquisition of Agri-biotech Applications
ILSI	International Life Sciences Institute
kb	kilobase pair
kDa	kilodalton
LLOQ	lower limit of quantitation
LOD	limit of detection
MALDI-MS	matrix assisted laser desorption ionization mass spectrometry
NAA	N-acetylaspartate
NAG	N-acetylglutamate
NCBI	National Center for Biotechnology Information
NDF	neutral detergent fiber
OD	optical density
OECD	Organisation for Economic Co-operation and Development
PAT	phosphinothricin acetyltransferase from *Streptomyces viridochomogenes*
PCR	polymerase chain reaction
pinII	proteinase inhibitor II
ppm	parts per million
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
USDA	United States Department of Agriculture
UTR	untranslated region
zm-hra	modified version of maize acetolactate synthase gene
ZM-HRA	modified version of maize acetolactate synthase protein

*Abbreviations of units of measurement and of physical and chemical quantities are done according to the standard format described in Instructions to Authors in the Journal of Biological Chemistry (http://www.jbc.org/).

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I. Rationale for the Development of 98140 Corn

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

The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) has responsibility, under the Plant Protection Act (7 U.S.C. 7701-7772) and the Plant Quarantine Act (7 U.S.C. 151-167), to prevent the introduction or dissemination of plant pests into or within the United States. Part 340 regulates introduction of organisms altered or produced through genetic engineering which are plant pests or for which there is a reason to believe are plant pests. The APHIS regulations at 7 CFR §430.6 provide that an applicant may petition APHIS to evaluate submitted data on the genetically engineered crop to determine that a regulated article does not present a plant pest risk and therefore should no longer be regulated.

Pioneer Hi-Bred International, Inc. is submitting data for genetically engineered herbicide tolerant 98140 corn and requests a determination from APHIS that event DP-Ø9814Ø-6 and crosses of corn lines containing this event with nonregulated corn lines no longer be considered regulated articles under 7 CFR 340.

I-B. Benefits of 98140 Corn

The commercialization of herbicide tolerant 98140 corn will have a beneficial impact on weed control practices, providing growers with another tool to address their weed control needs. The availability of 98140 corn will enable growers to choose an optimal combination of glyphosate, ALS-inhibiting herbicides, and other herbicides to best manage their individual weed populations, as well as enabling growers to proactively manage weed populations while delaying development of resistance.

Glyphosate has become a popular herbicide for corn, and combinations of glyphosate with ALS-inhibiting herbicides or other herbicides with different modes of action and inherent crop tolerance will enable more effective management of weed populations. Glyphosate is a post-emergence herbicide used to control weeds after emergence, whereas ALS inhibitors can effectively control weeds when applied post-emergence as well as pre-emergence (prior to weed emergence). Both chemical families are broad-spectrum herbicides and effective in controlling grasses and annual broadleaf weeds. Both herbicides have excellent environmental profiles and low mammalian toxicity. Glyphosate has no residual soil activity, thus it does not control weeds prior to their emergence, whereas the ALS inhibitors can be applied to the soil for pre-emergence activity as well as to emerged weeds in a post-emergence application. Finally, the ALS-inhibiting herbicides are typically applied at very low usage rates.

Alternating herbicides with different modes of actions to control weeds generally is recommended to help avoid the development of herbicide-resistant weeds. Incorporating tolerance to two or more herbicides in the same crop plant may be useful in delaying the development of herbicide-resistant weeds. Pioneer and DuPont Crop Protection businesses actively promote Integrated Weed Management, and the availability of 98140 corn will be a beneficial component of an integrated plan.

Therefore, the availability of 98140 corn with tolerance to both glyphosate and ALS-inhibiting herbicides will be beneficial for growers and the environment.

I-C. Submissions to Other Regulatory Agencies

New Protein Consultations for the GAT4621 and ZM-HRA proteins were submitted to FDA on January 31, 2007 and February 13, 2007, respectively. A safety and nutritional assessment for feed and food derived from 98140 corn will be submitted to FDA in 2nd quarter, 2007. Submissions will also be made in Canada, Japan, Mexico, Taiwan and Korea. Submission of a tolerance petition and supporting residue data to the Environmental Protection Agency (EPA) to amend the corn tolerance expression for glyphosate to include N-acetylglyphosate is targeted for 3rd quarter, 2007.

Pioneer is committed to robust product stewardship prior to and continuing after obtaining a determination of non-regulated status from USDA-APHIS for 98140 corn. Further, a full commercial launch of the Optimum™ GAT™ corn will not occur until import regulatory approvals have been obtained in key corn import markets with functioning regulatory systems.

II. The Biology of Corn

Refer to the OECD Consensus Document on the Biology of *Zea mays* subsp. *mays* (Maize), 2003, for information pertaining to the following aspects of corn biology:

- general description, including information on use of corn as a crop plant;
- taxonomic status of *Zea;*
- identification methods;
- center of origin / diversity and maize diversity;
- reproductive biology, including sexual and asexual reproduction;
- crosses, including intra- and inter-specific crosses and gene flow; and
- agro-ecology, including information about cultivation, volunteers and weediness, soil ecology, and maize-insect interactions.

Characterization of the Recipient Corn Line

A Pioneer proprietary inbred, PHWVZ, was used as the recipient line for the generation of 98140 corn. This inbred was derived from two inbreds: PH09B, which is a Pioneer elite inbred (Williams, 1999), and public line Hi-II (Lowe and Chomet, 2004). Hi-II is a derivative of the A188 and B73 inbred lines of corn that are publicly available from the University of Minnesota and Iowa State University, respectively.

The PHWVZ inbred was chosen because of its receptiveness to transformation. Because PHWVZ is a pure inbred (homozygous at all loci), Pioneer breeders are able to quickly breed and test events in hybrid combinations.

The PHWVZ inbred has yellow dent kernels and a purple cob. It has a stiff stalk background and is characterized as a mid-maturity line.

III. Method of Development of 98140 Corn

III-A. Description of the Transformation System

The T-DNA of plasmid PHP24279 (containing the *gat4621* and *zm-hra* gene cassettes) was used to generate 98140 corn. Refer to section IV for the detailed description of the plasmid.

Immature embryos of corn were aseptically removed from the developing caryopsis 9 - 10 days after pollination and infected with *Agrobacterium tumefaciens* strain LBA4404 containing plasmid PHP24279, essentially as described in Zhao *et al*., 2001. After 6 days of embryo and *Agrobacterium* co-cultivation on solid culture medium with no selection, the embryos were transferred to selective medium that was stimulatory to corn somatic embryogenesis and contained glyphosate for selection of cells expressing the *gat4621* transgene. The medium also contained carbenicillin to kill any remaining *Agrobacterium*.

After two weeks on the selective medium, healthy, growing calli that demonstrated resistance to glyphosate were identified. The putative transgenic calli were continually transferred to fresh selection medium for further growth until the start of the regeneration process.

Callus samples were taken for PCR analysis to verify the presence of both transgenes. The embryonic calli were regenerated into whole transgenic plants and transferred to the greenhouse. The regenerated plants (designated T0 plants) were evaluated for glyphosate and ALS inhibitor herbicide tolerance. Positive plants were sampled for molecular analysis for transgene copy numbers and crossed with inbred lines to obtain seeds for further evaluation. Refer to Figure 1 for a schematic diagram of the development process for 98140 corn and Figure 2 for a breeding diagram.

III-B. Selection of Comparators for 98140 Corn

To ensure the accurate assessment of the impact of transgene insertion on various characteristics of 98140 corn, a proper selection of comparator plants is important. Two types of corn lines—control corn and reference corn, were used as comparators for 98140 corn (Figure 2).

The control plants should have a genetic background similar to that of 98140 corn but lack the transgenic insert. For most analyses, a null segregant near-isoline was used as the control. The null segregants were essentially genetically identical to 98140 corn but did not contain the transgenic insert DNA.

For the molecular analysis, two Pioneer inbreds (PH09B and PHWVZ) were employed as non-transgenic controls. PH09B was one of the elite inbreds used in early breeding, and PHWVZ was the recipient line for 98140 corn.

For the nutrient compositional assessment and poultry study, non-transgenic commercial corn reference hybrids were used to help determine the normal variation seen in corn and to develop the statistical tolerance intervals.

Figure 1. Schematic of the Development of 98140 Corn

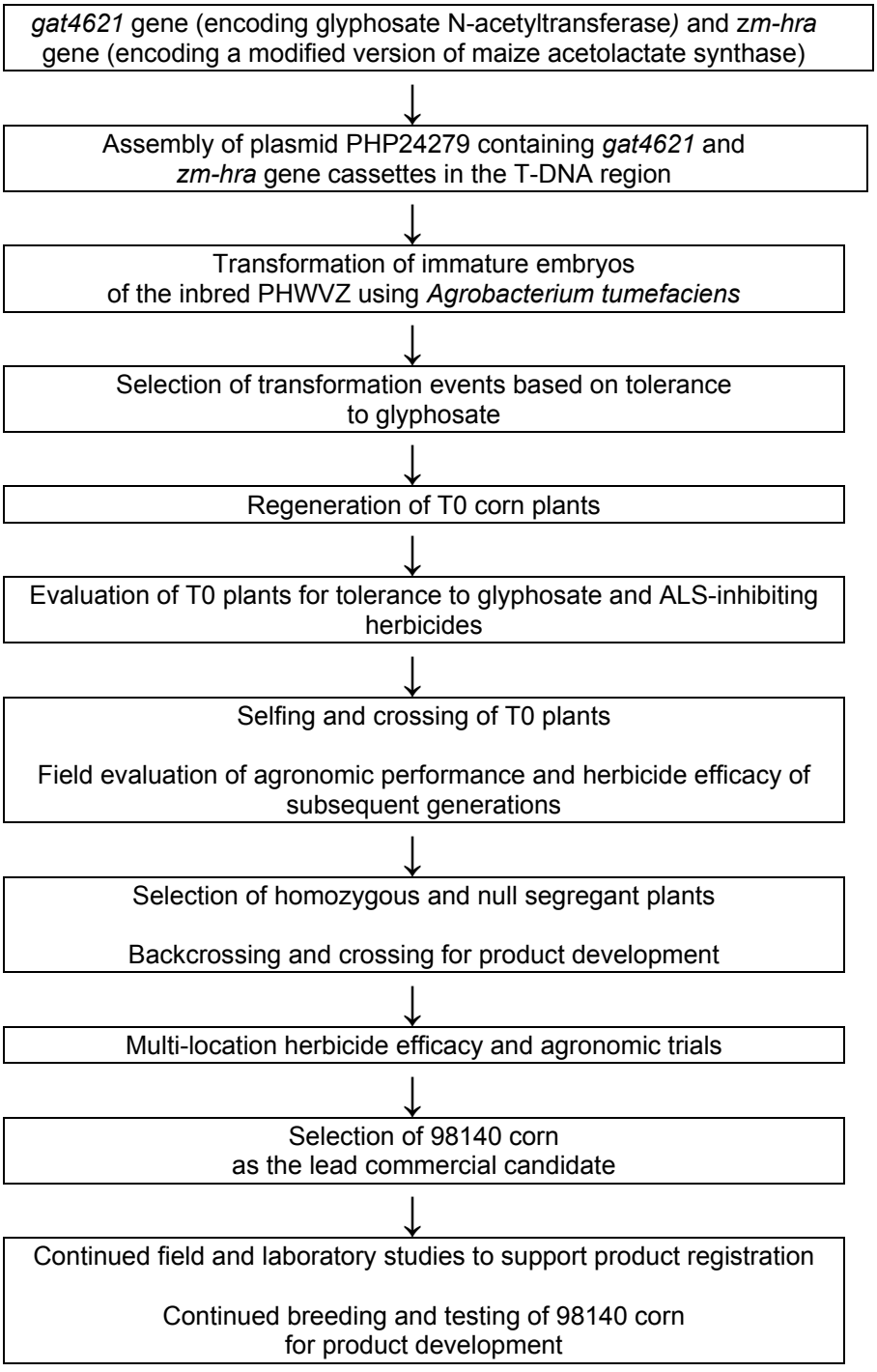
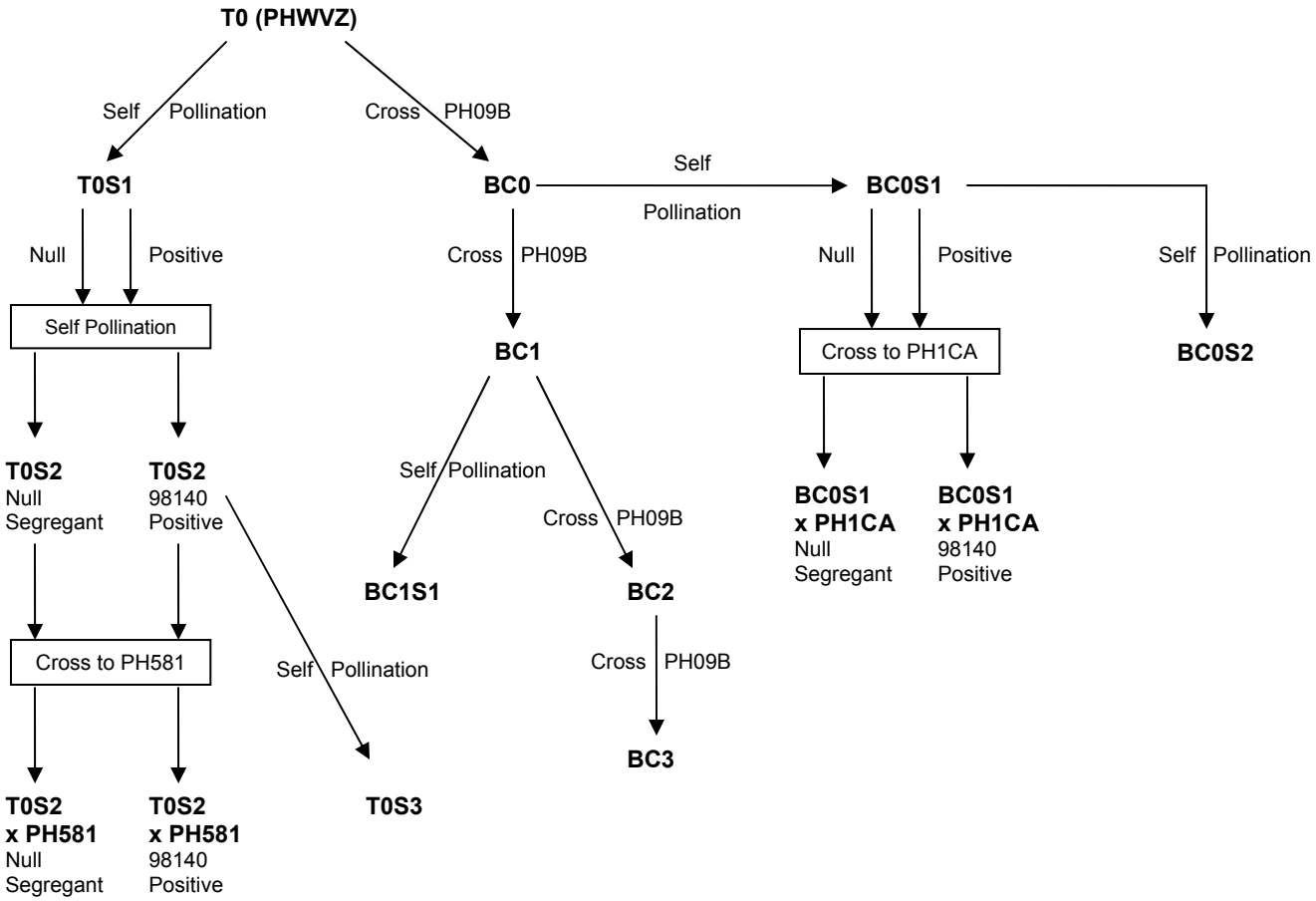


Figure 2. Breeding Diagram for 98140 Corn and Generations Used for Analyses



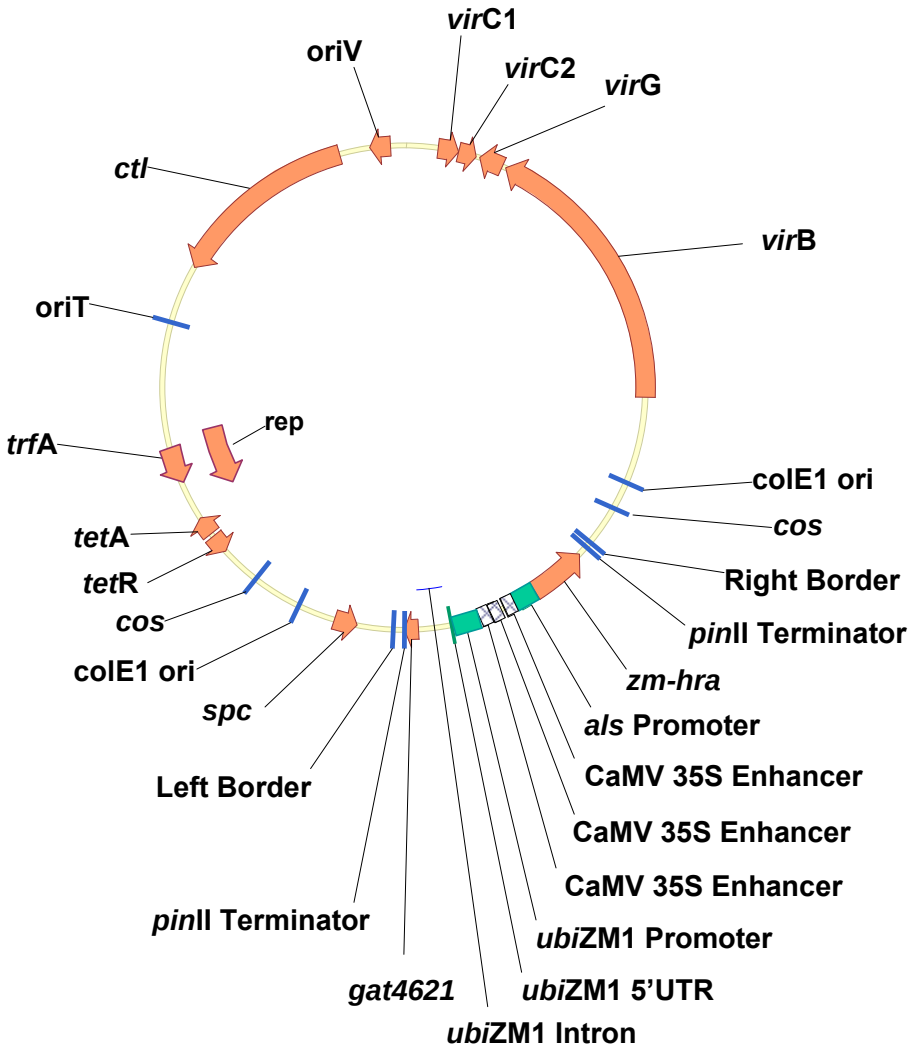
Analysis	Relevant Section of Petition	98140 Corn Generation	Control Corn Generation
Molecular	V-B, V-C and V-D	T0S3, BC1, BC0S2, BC1S1	PH09B, PHWVZ & null segregants of BC1 and BC1S1
Inheritance	V-E	BC0S1, BC1S1, BC2, BC3	Not applicable
Concentrations of GAT4621 and ZM-HRA	VI-C	BC0S1xPH1CA	Null segregant of BC0S1xPH1CA
Germination/ dormancy	VII-A	BC0S1xPH1CA	Null segregant of BC0S1xPH1CA
Field agronomics	VII-B	T0S2xPH581 (Experiment A)	Null segregant of T0S2xPH581
		BC0S1xPH1CA (Experiments B and C)	Null segregant of BC0S1xPH1CA
Compositional assessment	VIII-A, VIII-B, VIII-C and VIII-D	Forage and grain from BC0S1xPH1CA	Forage and grain from null segregant of BC0S1xPH1CA and four commercial reference hybrids
Poultry study	VIII-E	Grain from BC0S1xPH1CA	Grain from null segregant of BC0S1xPH1CA and three commercial reference hybrids

IV. Donor Genes and Regulatory Sequences

IV-A. Plasmid PHP24279 Used in Transformation

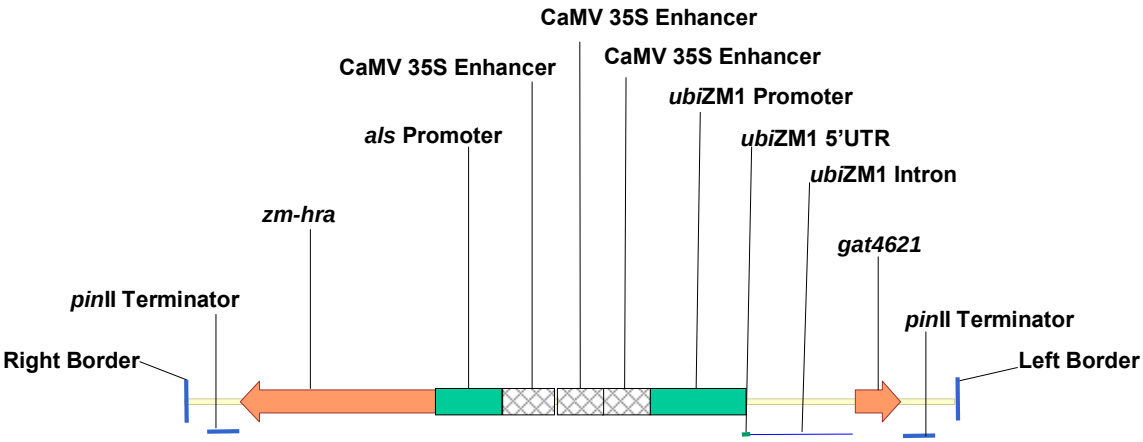
98140 corn was produced by *Agrobacterium tumefaciens*-mediated transformation with PHP24279, which contains the T-DNA region with the *gat4621* and *zm-hra* expression cassettes (Figure 3). A summary of the genetic elements and their position on the T-DNA (Figure 4) is given in Table 1.

Figure 3. Plasmid Map of PHP24279



Schematic diagram of plasmid PHP24279 with the location of genes and regulatory elements indicated. Plasmid size is 50371 base pairs.

Figure 4. Schematic Diagram of the T-DNA Region of PHP24279



Schematic diagram of the T-DNA insert of PHP24279 indicating the *gat4621* gene and the *zm-hra* gene, along with their respective regulatory elements. The size of the T-DNA is 7440 base pairs.

Table 1. Description of Genetic Elements in the T-DNA of PHP24279

Location on T-DNA (base pair position)	Genetic Element	Size (base pairs)	Description
1 to 25	Right Border	25	T-DNA Right Border region, from Ti plasmid of *Agrobacterium tumefaciens*
26 to 177	Ti Plasmid Region	152	Non-functional sequence from Ti plasmid of *A. tumefaciens*
178 to 210	Polylinker Region	33	Region required for cloning genetic elements
211 to 521	*pin*II Terminator	311	Terminator region from *Solanum tuberosum* proteinase inhibitor II gene (Keil *et al.,* 1986; An *et al.*, 1989). *(reverse orientation)*
522 to 537	Polylinker Region	33	Region required for cloning genetic elements
538 to 2454	*zm-hra* Gene	1917	Modified endogenous *Zea mays* acetolactate synthase gene (Fang *et al.*, 1992). *(reverse orientation)*
2455 to 3115	*zm-als* Promoter	661	Promoter region from *Zea mays* acetolactate synthase gene (Fang *et al.*, 1992). *(reverse orientation)*
3116 to 3189	Polylinker Region	74	Region required for cloning genetic elements
3190 to 3625	CaMV 35S Enhancer	436	Enhancer region from the Cauliflower Mosaic Virus genome (Franck *et al.*, 1980; Odell *et al.*, 1985).
3626 to 3648	Polylinker Region	23	Region required for cloning genetic elements
3649 to 4086	CaMV 35S Enhancer	438	Enhancer region from the Cauliflower Mosaic Virus genome (Franck *et al.*, 1980; Odell *et al.*, 1985).
4087 to 4093	Polylinker Region	7	Region required for cloning genetic elements
4094 to 4531	CaMV 35S Enhancer	438	Enhancer region from the Cauliflower Mosaic Virus genome (Franck *et al.*, 1980; Odell *et al.*, 1985).
4532 to 4566	Polylinker Region	35	Region required for cloning genetic elements
4567 to 5466	*ubi*ZM1 Promoter	900	Promoter region from *Zea mays* ubiquitin gene (Christensen *et al.*, 1992).
5467 to 5549	*ubi*ZM1 5' UTR	83	5' untranslated region from *Zea mays* ubiquitin gene (Christensen *et al.*, 1992).
5550 to 6558	*ubi*ZM1 Intron	1009	Intron region from *Zea mays* ubiquitin gene (Christensen *et al.*, 1992).
6559 to 6586	Polylinker Region	28	Region required for cloning genetic elements
6587 to 7030	*gat4621* Gene	444	Synthetic glyphosate N-acetyltransferase gene (Castle *et al.*, 2004. Siehl *et al.,* 2007).
7031 to 7046	Polylinker Region	16	Region required for cloning genetic elements
7047 to 7362	*pin*II Terminator	316	Terminator region from *Solanum tuberosum* proteinase inhibitor II gene (Keil *et al.,* 1986; An *et al.*, 1989).
7363 to 7415	Ti Plasmid Region	53	Non-functional sequence from Ti plasmid of *A. tumefaciens*
7416 to 7440	Left Border	25	T-DNA Left Border region, from Ti plasmid of *Agrobacterium tumefaciens*

IV-B. Identity and Source of Genetic Material in the T-DNA of PHP24279

B1. The *gat4621* Gene Cassette

The T-DNA region of PHP24279 contains two gene cassettes. The first cassette contains the synthetic glyphosate N-acetyltransferase (*gat4621*) gene that encodes the GAT4621 protein. The *gat* genes were isolated from three strains of *Bacillus licheniformis*, and the *gat4621* sequence was generated by functional optimization of these genes using the gene shuffling process to enhance the acetylation activity of the GAT enzyme (see Section VI-A for additional details). The GAT4621 protein acetylates glyphosate, rendering it non-phytotoxic to plants. The insertion of the *gat4621* gene in corn plants confers tolerance to glyphosate-containing herbicides. The *gat4621* gene encodes a protein of 147 amino acids that has a molecular weight of approximately 17 kDa (Castle *et al.*, 2004; Siehl *et al*., 2007).

B. licheniformis, the source organism for the *gat4621* gene, is used for the production of a number of enzymes such as proteases and amylases that have wide application in the detergent industry. *B. licheniformis* has been used in the United States, Canada, and Europe in the fermentation industry for production of food enzymes (*e.g.,* alpha-amylase, cyclodextrin glycosyltransferase, hemicellulase, proteases, and pullulanase; Rey *et al.,* 2004). All *B. licheniformis* cultures available from the American Type Culture Collection (ATCC) are classified as Biosafety Level 1. Items that the ATCC classifies at Biosafety Level 1 have no known history of causing disease in humans or animals based on their assessment of potential risk using U.S. Public Health Service guidelines, with assistance provided by ATCC scientific advisory committees.

The promoter for the *gat4621* coding region is the promoter from the maize ubiquitin gene, including a 5' untranslated region and an intron (Christensen *et al.,* 1992). The terminator for the *gat4621* gene is the 3' terminator sequence from the proteinase inhibitor II gene of *Solanum tuberosum* (Keil *et al*., 1986; An *et al.*, 1989).

B2. The *zm-hra* Gene Cassette

The second cassette of the T-DNA region of PHP24279 contains *zm-hra*, a modified version of the endogenous maize acetolactate synthase gene (*als*). The *zm-hra* gene encodes the ZM-HRA protein, which has two amino acid residues modified in comparison to the wild-type ALS protein. This gene encodes a form of acetolactate synthase that is tolerant to the ALS-inhibiting class of herbicides. The ZM-HRA protein is comprised of 638 amino acids and has a molecular weight of approximately 69 kDa.

The expression of the *zm-hra* gene is controlled by the maize *als* promoter (Fang *et al*., 1992). The terminator for the *zm-hra* gene is the 3' terminator sequence from the proteinase inhibitor II gene of *Solanum tuberosum* (*pin*II terminator) (Keil *et al*., 1986; An *et al.*, 1989).

In addition to the regulatory elements mentioned above for the *gat4621* and *zm-hra* cassettes, three copies of the CaMV 35S enhancer region from Cauliflower Mosaic Virus (Franck *et al*., 1980; Odell *et al*., 1985) serve to enhance expression of both the *gat4621* and *zm-hra* gene cassettes.

V. Genetic Characterization of 98140 Corn

V-A. Molecular Analysis Overview

To characterize the DNA insertion in 98140 corn, Southern blot analysis was conducted. 98140 corn was generated by *Agrobacterium*-mediated transformation with plasmid PHP24279 (Figure 5). The T-DNA from plasmid PHP24279 (Figure 6) contains two expression cassettes: the *gat4621* cassette comprised of the *ubi*ZM1 promoter, *ubi*ZM1 intron, the *gat4621* gene, and the *pin*II terminator, and the *zm-hra* cassette comprised of the *als* promoter, *zm-hra* gene, and *pin*II terminator. Located between the two cassettes are three copies of the CaMV 35S enhancer element, providing transcription enhancement to both cassettes. Individual plants of the T0S3 generation (refer to Figure 2 for the breeding diagram) were analyzed to determine the copy number of each of the genetic elements inserted into 98140 corn and to verify that the integrity of the PHP24279 T-DNA was maintained upon integration. The analysis confirmed a single, intact PHP24279 T-DNA has been inserted into the corn genome to produce 98140 corn (Section V-B).

Southern blot analysis was conducted on three additional generations, BC0S2, BC1, and BC1S1 (refer to Figure 2 for the breeding diagram), to verify that the 98140 corn insertion remained intact and stably integrated as demonstrated by identical hybridization patterns in all generations. Plants from these three generations exhibited the same event-specific hybridization pattern as exhibited by the T0S3 generation. These results confirmed the stability of the insertion in 98140 corn across these four breeding generations (Section V-C).

Both the BC0S2 and BC1 generations were analyzed to confirm the absence of plasmid sequence from PHP24279 outside of the T-DNA region, *i.e.* the plasmid backbone sequence not intended for transformation into corn. The results verified the absence of backbone sequences in 98140 corn (Section V-D).

Genomic DNA samples from leaf material of the PH09B and PHWVZ corn varieties were used as negative controls for all Southern blot analyses (refer to Figure 2 for the breeding diagram). The PH09B and PHWVZ control corn lines were selected due to their presence in the backgrounds of the 98140 corn generations analyzed. The T0S3 generation has a background containing only PHWVZ, while the background of the BC0S2, BC1, and BC1S1 generations consists of PHWVZ and PH09B. Certain Southern probes for the PHP24279 T-DNA (*als* promoter, *zm-hra*, *ubi*ZM1 promoter, and *ubi*ZM1 intron) are highly homologous to corresponding elements in the control corn genome and thus these probes hybridize to sequences in the control corn DNA as well as the 98140 corn insertion. These endogenous bands are identified by their presence in one or both of the control corn samples, and may vary between the control lines and different 98140 corn generations due to allelic differences in the backgrounds of the different generations.

Plasmid PHP24279 was used as a positive control for probe hybridization and to verify fragment sizes internal to the PHP24279 T-DNA. All probes used for the analysis are indicated on the schematic maps of PHP24279 and the PHP24279 T-DNA (Figures 5 and 6, respectively) and outlined in Table 2.

Based on these analyses, a schematic map of the insertion region in 98140 corn was determined and is presented in Figure 7.

Materials and methods used for Southern blot analysis are described in detail in Appendix 1.

V-B. Transgene Copy Number and Insertion Integrity

The integration pattern of the insertion in 98140 corn was investigated with *Eco*R V digestion to determine copy number and with *Spe* I digestion to determine insertion integrity. Southern blots were hybridized with several probes to confirm copy number and integrity of each genetic element. The *ubi*ZM1 promoter, *ubi*ZM1 intron, and *gat4621* probes were used to characterize the *gat4621* cassette (Table 2, Figure 6). The *als* promoter and z*m-hra* probes were used to characterize the *zm-hra* cassette (Table 2, Figure 6). The 35S enhancer and *pin*II terminator probes were used to characterize genetic elements that are associated with both cassettes (Table 2, Figure 6).

Predicted and observed hybridization bands are described in Tables 3, 4, and 5 for probes unique to the *gat4621* cassette, unique to the *zm-hra* cassette, and for probes associated with both cassettes, respectively. Some of the probes used to characterize the 98140 corn insertion are highly homologous to sequences found in the corn genome. The *ubi*ZM1 promoter, *ubi*ZM1 intron, *als* promoter, and z*m-hra* probes all hybridize to sequences in both control and 98140 corn genomic DNA in addition to the PHP24279 T-DNA. Corresponding hybridization bands are identified by their presence in control corn DNA and are thus not part of the T-DNA insertion. They are indicated in Tables 3 and 4 by asterisks (*) and gray shading. Some variation in sample loading, gel electrophoresis and transfer, and hybridization intensity affected the visibility of faint endogenous bands between different generations and samples within generations.

Based on the Southern blot analyses as discussed below, it was determined that a single, intact PHP24279 T-DNA has been inserted into the genome of 98140 corn as diagramed in the insertion map (Figure 7).

Copy Number

The *Eco*R V digest provides information about the number of copies of the genetic elements integrated into the genome of 98140 corn as there is a single restriction enzyme site in the PHP24279 T-DNA at base pair (bp) position 3651 (Figure 6) and any additional sites would fall outside the T-DNA sequence in the corn genome. Hybridization with the probes from each cassette, except for the 35S enhancer probe, would indicate the number of copies of each element found in 98140 corn based on the number of hybridizing bands (*e.g.* one hybridizing band indicates one copy of the element). There are two copies of the *pin*II terminator; one located in each gene cassette on either side of the *Eco*R V site, so two hybridizing bands would be expected with this probe for a single T-DNA insertion. There are three copies of the 35S enhancer element in the T-DNA; however, since the *Eco*R V site is located between two of the copies, only two hybridizing bands would be expected. Predicted and observed fragment sizes for 98140 corn with *Eco*R V are given in Table 3 for the *gat4621* cassette, in Table 4 for the *zm-hra* cassette, and in Table 5 for elements associated with both cassettes.

gat4621 Cassette:

A single copy of the unique elements of the *gat4621* cassette was inserted into 98140 corn. The *ubi*ZM1 promoter, *ubi*ZM1 intron, and *gat4621* probes were hybridized to *Eco*R V-digested genomic DNA from individual 98140 corn plants of the T0S3 generation (Table 3, Figure 8). Each of the probes hybridized to the same single fragment of approximately 6100 bp (Table 3, Figure 8), indicating a single copy insertion with the expected arrangement of genetic elements on the inserted fragment in 98140 corn. The *ubi*ZM1 promoter and *ubi*ZM1 intron probes are homologous to elements endogenous to the corn genome and therefore each probe also hybridized to bands in control corn samples (Table 3, Figure 8).

zm-hra Cassette:

Likewise, a single copy of each element exclusive to the *zm-hra* cassette was inserted into 98140 corn. The two unique elements comprising this cassette - the *als* promoter and *zm-hra* gene - were used as probes to determine number of copies inserted. Each of the two probes hybridized to the same single fragment of greater than 8600 base pairs (bp) (Table 4, Figure 9), indicating a single copy insertion with the expected arrangement of genetic elements on the inserted fragment in 98140 corn. The probes of this cassette are also homologous to elements endogenous to the corn genome and therefore each probe also hybridized to bands in control corn samples.

Elements Associated with Both Cassettes:

The *pin*II terminator is present in both the *gat4621* and *zm-hra* cassettes. The three copies of the 35S enhancer element are located between the two expression cassettes. Due to the location of the *Eco*R V restriction enzyme site between two of the three copies of the 35S enhancer at bp position 3651 (Figure 6), it would be expected that the *pin*II terminator and 35S enhancer probes would hybridize to the same fragments that contain the *gat4621* or *zm-hra* gene cassettes. In both cases, hybridization of the *Eco*R V Southern blots with these probes resulted in the detection of both the 6100 bp band associated with the *gat4621* cassette and the greater than 8600 bp band associated with the *zm-hra* cassette (Table 5, Figure 10). In the case of the 35S enhancer probe, the band of greater than 8600 bp is substantially fainter than the approximately 6100 bp band as it is the band containing a single copy of the enhancer element, compared to the 6100 bp band which contains two copies of the 35S enhancer (Figure 10). The presence of only two hybridizing bands for the *pin*II terminator and 35S enhancer probes, corresponding to the hybridizing bands noted above for the other components of the two gene cassettes, is further indication that there is a single copy of the PHP24279 T-DNA, in its expected arrangement, inserted in the 98140 corn genome.

Insertion Integrity

Spe I digestion was used to verify that the inserted T-DNA containing both of the *gat4621* and *zm-hra* cassettes was complete and intact in 98140 corn. There are two *Spe* I sites in the PHP24279 T-DNA (base pair positions 396 and 7169) which are located within the *pin*II terminator elements found on the ends of each gene expression cassette (Figure 6). Hybridization with the probes of the *gat4621* and *zm-hra* cassettes confirmed that all the elements were found on the predicted internal 6773 bp fragment. In addition, the *pin*II terminator probe hybridized to two other expected border fragments, due to the locations of the *Spe* I sites within the terminator. Predicted and observed fragment sizes with *Spe* I are given in Table 3 for the *gat4621* cassette, Table 4 for the *zm-hra* cassette, and in Table 5 for elements associated with both cassettes.

The *ubi*ZM1 promoter, *ubi*ZM1 intron, and *gat4621* probes for the *gat4621* cassette hybridized to a single insertion-derived band of 6773 bp that matched the plasmid control band (Table 3, Figure 11). Similarly, the probes for the *zm-hra* cassette (*als* promoter and *zm-hra*) hybridized to the same internal 6773 bp band (Table 4, Figure 12). The 35S enhancer and *pin*II terminator probes also hybridized to the predicted internal band of 6773 bp (Table 5, Figure 13). The size of the band for each probe was confirmed by hybridization to the PHP24279 plasmid fragment corresponding to the T-DNA (Figure 11, Lanes 2, 9, and 16; Figure 12, Lanes 2 and 9; Figure 13, Lanes 2 and 9). Because these probes hybridized to the same internal fragment of the predicted size, the PHP24279 T-DNA in 98140 corn was determined to be intact and all elements of the cassette were confirmed on this fragment.

In addition to the internal 6773 bp band, the *pin*II terminator probe hybridized to two additional bands, one of about 4900 bp and one of about 450 bp (Table 5, Figure 13). These additional bands are due to the location of the *Spe* I restriction site within the *pin*II terminator probe region, leading to hybridization of the probe to two border fragments for an intact insertion of the

PHP24279 T-DNA. The presence of the two additional hybridizing bands indicates that the *pin*II terminators in the T-DNA are intact, and serve as additional confirmation that the complete PHP24279 T-DNA was inserted into 98140 corn.

As stated previously, the *ubi*ZM1 promoter, *ubi*ZM1 intron, *als* promoter, and *zm-hra* probes are homologous to elements endogenous to the corn genome and therefore each probe hybridized to bands in control corn samples (Tables 3 and 4, Figures 11 and 12).

V-C. Stability of the Insertion Across Generations

Southern blot analysis was conducted using *Eco*R V on three generations of 98140 corn; BC0S2, BC1, and BC1S1, to verify the stability of the insertion in 98140 corn as demonstrated by identical hybridization patterns in all generations. As discussed earlier, the *Eco*R V restriction enzyme has a single site (bp position 3651) located within the PHP24279 T-DNA (Figure 6) and will generate a unique event-specific hybridization pattern for 98140 corn when hybridized to the *gat4621* and *zm-hra* probes. This analysis would confirm event stability across generations as changes to the insertion structure in 98140 corn would be detected. As discussed in Section V-B, a band of approximately 6100 bp would be predicted with the *gat4621* probe to confirm stability across generations (Table 3). Likewise for the *zm-hra* probe, a band of greater than 8600 bp would be predicted to confirm stability across generations (Table 4). As described in detail below, all three generations analyzed, BC0S2, BC1, and BC1S1, showed identical hybridization patterns consistent with the T0S3 analysis (Section V-B) confirming the stability of inheritance of the insertion in 98140 corn.

Genomic DNA from the BC0S2 and BC1 generations of 98140 corn was digested with *Eco*R V and hybridized to the *gat4621* and *zm-hra* probes to confirm stability across generations (Figures 14 and 15, respectively). A band of approximately 6100 bp specific to 98140 corn hybridized to the *gat4621* probe in both generations (Table 3, Figure 14). With the *zm-hra* probe, a single band of greater than 8600 bp specific to 98140 corn was present in both generations (Table 4, Figure 15). In addition to the greater than 8600 bp band, the *zm-hra* probe also hybridized to additional bands that were determined to be endogenous to the corn genome since these bands were present in both 98140 corn and control corn plants (Figure 15). The consistency of hybridization results from both the *gat4621* and *zm-hra* probes confirmed that the insertion of PHP24279 T-DNA in 98140 corn remained stable across the BC0S2 and BC1 generations. In addition, the bands observed resulting from the 98140 insertion in these two generations were the same size as the bands seen with the same probes on the *Eco*R V Southern blots of the T0S3 generation described above (Figures 8 and 9), indicating the 98140 insertion is stable across all three generations. There is expected variation in the endogenous bands seen with the *zm-hra* probe between the 98140 corn plants from the differing generations, due to allelic differences between the original transformed corn line and the back-cross parent lines that are not associated with the 98140 insertion.

Plants from a segregating BC1S1 generation of 98140 corn were also analyzed by Southern blot. Genomic DNA of the BC1S1 generation was digested with *Eco*R V and hybridized to the *gat4621* and *zm-hra* probes. In plants containing the 98140 corn insertion, a band of approximately 6100 bp was observed with the *gat4621* probe (Table 3, Figure 16, Lanes 6 through 20) and a band of greater than 8600 bp specific to 98140 corn was observed with the *zm-hra* probe (Table 4, Figure 17, Lanes 6 through 20). As in previous analyses, the *zm-hra* probe hybridized to additional bands in 98140 corn and control samples which were due to endogenous sequences within the corn genome (Figure 17). Variations in the endogenous bands in the BC1S1 generation are due to segregation of alleles from the parent lines, and are not due to a change in the 98140 insertion. Null segregant plants did not hybridize to the *gat4621* probe and showed only the endogenous hybridization observed in control plants with the *zm-hra* probe (Figures 16 and 17, Lanes 21 through 25). Hybridization results from both the *gat4621* and *zm-hra* probes

were consistent with the results from the T0S3, BC0S2, and BC1 generations described above and confirmed the stability of inheritance of the insertion during traditional corn breeding.

Thus, Southern blot analysis of the T0S3, BC0S2, BC1, and BC1S1 generations of 98140 corn using the *gat4621* and *zm-hra* probes resulted in identical hybridization patterns on *Eco*R V digests of all four generations. The consistent hybridization patterns indicate that the T-DNA insertion is stably inherited across generations.

V-D. Absence of Plasmid Backbone DNA from Outside the T-DNA Region

Both the BC0S2 and BC1 generations were analyzed to confirm the absence of plasmid sequence from PHP24279 outside of the T-DNA region, *i.e.* the plasmid backbone sequence not intended for transformation into corn. As described below, the analysis completed confirmed the absence of backbone sequences in 98140 corn.

The *vir*G*, tet, spc*, LB, and RB probes were designed to detect key areas of plasmid PHP24279 outside of the T-DNA that may have been inserted during *Agrobacterium*-mediated transformation (Figure 5, probes A through E). Because of the ordered nature of T-DNA integration during transformation, these five probes confirm the lack of any inserted plasmid backbone sequence in 98140 corn. The backbone probes were hybridized to *Eco*R V blots to confirm absence of the plasmid backbone in 98140 corn. Figures 18 through 22 show the results of hybridization with these probes. None of the backbone probes hybridized to 98140 corn samples (Figures 18 through 22, Lanes 5 through 12), confirming the absence of these sequences and hence, the absence of any inserted plasmid backbone. Hybridization of the probes to the plasmid control samples (Figures 18 through 22, Lanes 1 and 17) indicate the probes were able to detect the target sequences and would have identified backbone sequences if they had been present in 98140 corn.

To confirm absence of plasmid backbone sequences, Southern blot analysis was carried out on the BC0S2 and BC1 generations of 98140 corn using probes for regions in the PHP24279 plasmid backbone. There was no hybridization of the probes to genomic DNA from 98140 corn, indicating that plasmid regions outside of the PHP24279 T-DNA were not inserted in 98140 corn.

Table 2. Description of DNA Probes Used for Southern Blot Hybridization

Probe Name	Genetic Element	Figure *Probe*	Position on PHP24279 T-DNA (bp to bp)	Position on PHP24279 Plasmid (bp to bp)	Probe Length (bp)
*ubi*ZM1 promoter	*ubi*ZM1 promoter	Figure 6 *probe 5*	4602 to 5460	22689 to 23457	859
*ubi*ZM1 intron	*ubi*ZM1 5' UTR and intron	Figure 6 *probe 6*	5472 to 6551	23559 to 24638	1080
gat4621	*gat4621* gene	Figure 6 *probe 7*	6587 to 7021	24674 to 25108	435
als promoter	*als* promoter	Figure 6 *probe 3*	2503 to 3101	20590 to 21188	599
zm-hra[1]	*zm-hra* gene	Figure 6 *probe 2*	538 to 1468 1490 to 2259	18625 to 19555 19577 to 20346	931 770
*pin*II terminator	*pin*II terminator	Figure 6 *probe 1*	235 to 468[2] 7100 to 7333[2]	18322 to 18555[2] 25187 to 25420[2]	234
35S enhancer	CaMV 35S enhancer	Figure 6 *probe 4*	3192 to 3611[3] 3653 to 4072[3] 4097 to 4513[3]	21279 to 21698[3] 21740 to 22159[3] 22184 to 22603[3]	420
*vir*G	*vir*G gene	Figure 5 *probe A*	N/A	2512 to 3255	744
tet[1]	Tetracycline resistance gene	Figure 5 *probe B*	N/A	32556 to 33094 33200 to 33657	539 458
spc	Spectinomycin resistance gene	Figure 5 *probe C*	N/A	26707 to 27481	775
LB	Region on the plasmid backbone adjacent to the left T-DNA border	Figure 5 *probe D*	N/A	25552 to 25897	346
RB	Region on the plasmid backbone adjacent to the right T-DNA border	Figure 5 *probe E*	N/A	17654 to 18043	390

Abbreviations

N/A-Not Applicable, these are not present on the PHP24279 T-DNA.

Footnotes

1. Two non-overlapping segments were generated for this probe and were combined for hybridization. The bp positions provided are the positions of each different segment.
2. There are two copies of the *pin*II terminator on PHP24279 and the PHP24279 T-DNA. The bp positions provided are the positions of each separate copy.
3. There are three copies of the 35S enhancer on PHP24279 and the PHP24279 T-DNA. The bp positions provided are the positions of each separate copy.

Table 3. Predicted and Observed Hybridizing Bands on Southern Blots with Probes Unique to the *gat4621* Cassette

Probe	Restriction Enzyme	Figure	Predicted Fragment Size from PHP24279[1] (bp)	Predicted Fragment Size from PHP24279 T-DNA[2] (bp)	Observed Fragment Size in 98140 Corn[3] (bp)
*ubi*ZM1 promoter	*EcoR* V	8	11178	>3800[4]	~6100
					>8600*
*ubi*ZM1 intron	*EcoR* V	8	11178	>3800[4]	~6100
					>8600* >8600* ~7000*
gat4621	*EcoR* V	8, 14, 16	11178	>3800[4]	~6100
*ubi*ZM1 promoter	*Spe* I	11	6773	6773	6773[5]
					>8600*
*ubi*ZM1 intron	*Spe* I	11	6773	6773	6773[5]
					>8600* ~8600* ~7400* ~4000* ~2700*
gat4621	*Spe* I	11	6773	6773	6773[5]

Note: An asterisk (*) and gray shading indicates the designated band is due to probe hybridization to endogenous corn genome sequences, as determined by the presence of the same band in all lanes, both 98140 corn and control. Certain endogenous bands may be difficult to discern on a printed copy but are visible on the original film.

Footnotes:

1. Predicted fragment sizes for hybridization to samples containing the plasmid positive control are based on the PHP24279 plasmid map as shown in Figure 5.
2. Predicted fragment sizes for 98140 corn are based on the map of the PHP24279 T-DNA as shown in Figure 6.
3. Observed fragment sizes are considered approximate from these analyses and are based on the indicated sizes of the DIG-labeled DNA Molecular Weight Marker VII fragments on the Southern blots. Due to incorporation of DIG molecules for visualization, the marker fragments typically run approximately 5-10% larger than their actual indicated molecular weight. The sizes of fragments not corresponding directly to plasmid fragments are rounded to the nearest 100 bp.
4. Minimum fragment size predicted based on an intact insertion of the T-DNA from PHP24279 (Figure 6). Fragment size is rounded to the nearest 100 bp.
5. Observed fragment size is the same as the predicted fragment size based on equivalent migration on the Southern blots.

Table 4. Predicted and Observed Hybridizing Bands on Southern Blots with Probes Unique to the *zm-hra* Cassette

Probe	Restriction Enzyme	Figure	Predicted Fragment Size from PHP24279[1] (bp)	Predicted Fragment Size from PHP24279 T-DNA[2] (bp)	Observed Fragment Size in 98140 Corn[3] (bp)
als promoter	*EcoR* V	9	9691	>3700[4]	>8600
					~7400*
zm-hra	*EcoR* V	9, 15, 17	9691	>3700[4]	>8600
					>8600*[5] >8600*[5] ~7400*[5] ~4700*[5]
als promoter	*Spe* I	12	6773	6773	6773[6]
					~5500*
zm-hra	*Spe* I	12	6773	6773	6773[6]
					~5300* ~5200* ~4700*

Note: An asterisk (*) and gray shading indicates the designated band is due to probe hybridization to endogenous corn genome sequences, as determined by the presence of the same band in all lanes, both 98140 corn and control. Certain endogenous bands may be difficult to discern on a printed copy but are visible on the original film. Not all endogenous bands are the same in all samples due to genomic differences in varieties used in the breeding process to produce the different generations analyzed.

Footnotes:

1. Predicted fragment sizes for hybridization to samples containing the plasmid positive control are based on the PHP24279 plasmid map as shown in Figure 5.
2. Predicted fragment sizes for 98140 corn are based on the map of the PHP24279 T-DNA as shown in Figure 6.
3. Observed fragment sizes are considered approximate from these analyses and are based on the indicated sizes of the DIG-labeled DNA Molecular Weight Marker VII fragments on the Southern blots. Due to incorporation of DIG molecules for visualization, the marker fragments typically run approximately 5-10% larger than their actual indicated molecular weight. The sizes of fragments not corresponding directly to plasmid fragments are rounded to the nearest 100 bp.
4. Minimum fragment size predicted based on an intact insertion of the T-DNA from PHP24279 (Figure 6). Fragment size is rounded to the nearest 100 bp.
5. Not all endogenous bands are observed in all generations due to allelic differences in backgrounds. Also, variations in sample loading, gel electrophoresis and transfer, and hybridization intensity affect the visibility of faint endogenous bands between different generations and samples within generations.
6. Observed fragment size is the same as the predicted fragment size based on equivalent migration on the Southern blots.

Table 5. Predicted and Observed Hybridizing Bands on Southern Blots with Probes Common to *gat4621* and *zm-hra* Cassettes

Probe	Restriction Enzyme	Figure	Predicted Fragment Size from PHP24279[1] (bp)	Predicted Fragment Size from PHP24279 T-DNA[2] (bp)	Observed Fragment Size in 98140 Corn[3] (bp)
*pin*II terminator	*EcoR* V	10	11178 9691	>3800[4] >3700[4]	>8600 ~6100
35S enhancer	*EcoR* V	10	11178 9691	>3800[4] >3700[4]	>8600 ~6100
*pin*II terminator	*Spe* I	13	42785 6773 813	6773 >400[4] >300[4]	6773[5] ~4900 ~450
35S enhancer	*Spe* I	13	6773	6773	6773[5]

Footnotes:

1. Predicted fragment sizes for hybridization to samples containing the plasmid positive control are based on the PHP24279 plasmid map as shown in Figure 5.
2. Predicted fragment sizes for 98140 corn are based on the map of the PHP24279 T-DNA as shown in Figure 6.
3. Observed fragment sizes are considered approximate from these analyses and are based on the indicated sizes of the DIG-labeled DNA Molecular Weight Marker VII fragments on the Southern blots. Due to incorporation of DIG molecules for visualization, the marker fragments typically run approximately 5-10% larger than their actual indicated molecular weight. The sizes of fragments not corresponding directly to plasmid fragments are rounded to the nearest 100 bp if >500 bp, or to the nearest 50 bp if <500 bp.
4. Minimum fragment size predicted based on an intact insertion of the T-DNA from PHP24279 (Figure 6). Fragment size is rounded to the nearest 100 bp.
5. Observed fragment size is the same as the predicted fragment size based on equivalent migration on the Southern blots.

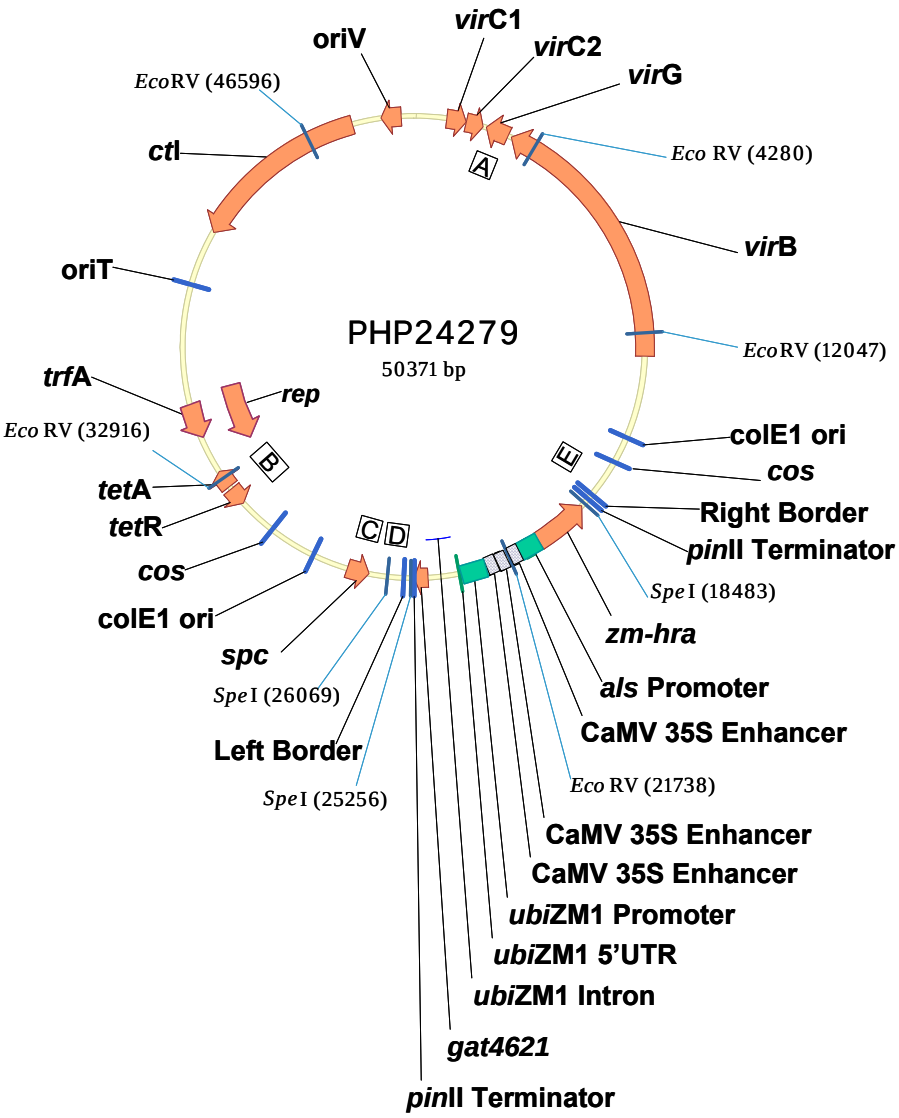


Figure 5. Map of Plasmid PHP24279 with Backbone Probes Indicated

Schematic map of plasmid PHP24279 indicating location of the genetic elements and base pair positions for *Eco*R V and *Spe* I restriction enzyme sites. Plasmid PHP24279 was used for *Agrobacterium*-mediated transformation to produce 98140 corn. The total plasmid size is 50371 base pairs. Approximate locations of the backbone probes used are shown as boxes within the plasmid and are identified below. Additional details on these probes are provided in Table 2.

Letter	Backbone Probe Identification
A	*vir*G probe
B	*tet* probe
C	*spc* probe
D	LB probe
E	RB probe

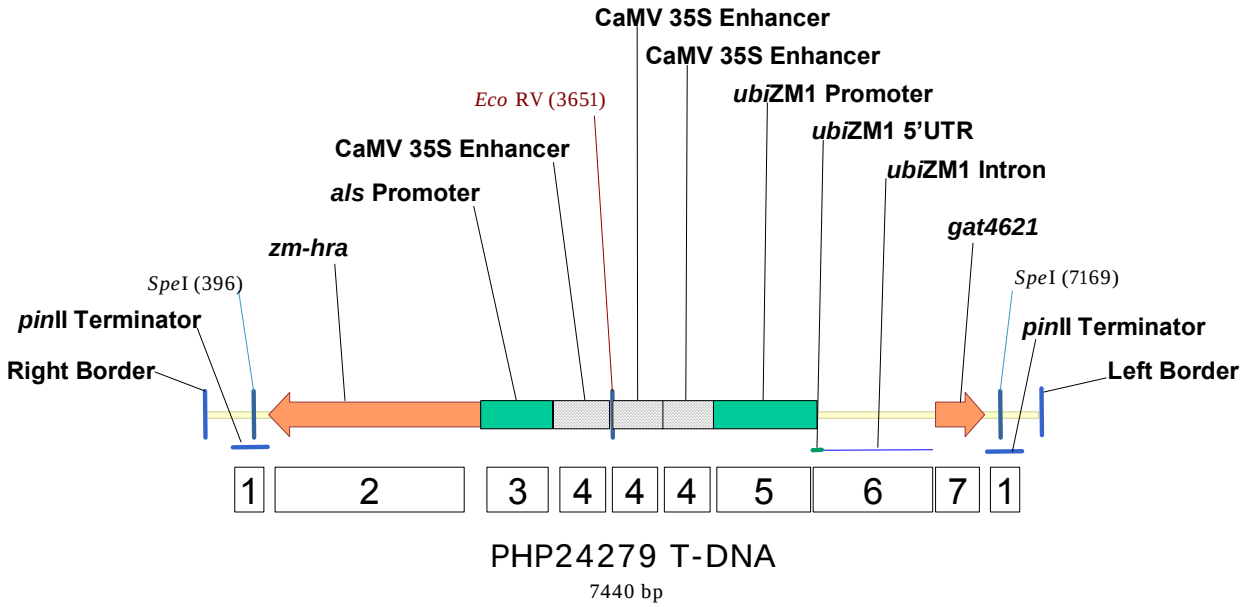


Figure 6. Map of PHP24279 T-DNA with Genetic Element Probes Indicated

Schematic map of the T-DNA from plasmid PHP24279 indicating the location of genetic elements within the *gat4621* and *zm-hra* expression cassettes and base pair positions for restriction enzyme sites for *Eco*R V and *Spe* I. The T-DNA is expected to be transferred from plasmid PHP24279 during *Agrobacterium*-mediated transformation to produce 98140 corn. The total T-DNA size is 7440 base pairs. Probes are indicated schematically as numbered boxes below the map and are identified below. Additional details on these probes are provided in Table 2.

Number	Probe Identification
1	*pin*II terminator probe
2	*zm-hra* probe
3	*als* promoter probe
4	35S enhancer probe
5	*ubi*ZM1 promoter probe
6	*ubi*ZM1 intron probe
7	*gat4621* probe

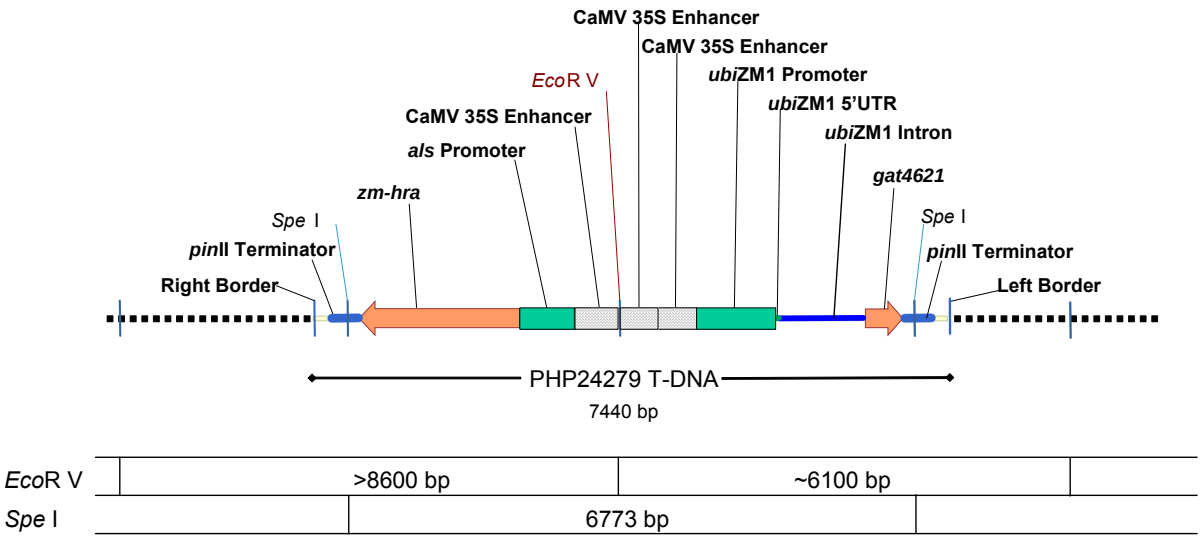


Figure 7. Map of the T-DNA Insertion in 98140 Corn

Schematic map of the insertion in 98140 corn based on Southern blot analysis. The flanking corn genome is represented by the horizontal dotted line. A single, intact copy of the PHP24279 T-DNA integrated into the corn genome. *Eco*R V and *Spe* I restriction enzyme sites are indicated with the sizes of observed fragments on Southern blots shown below the map in base pairs (bp).

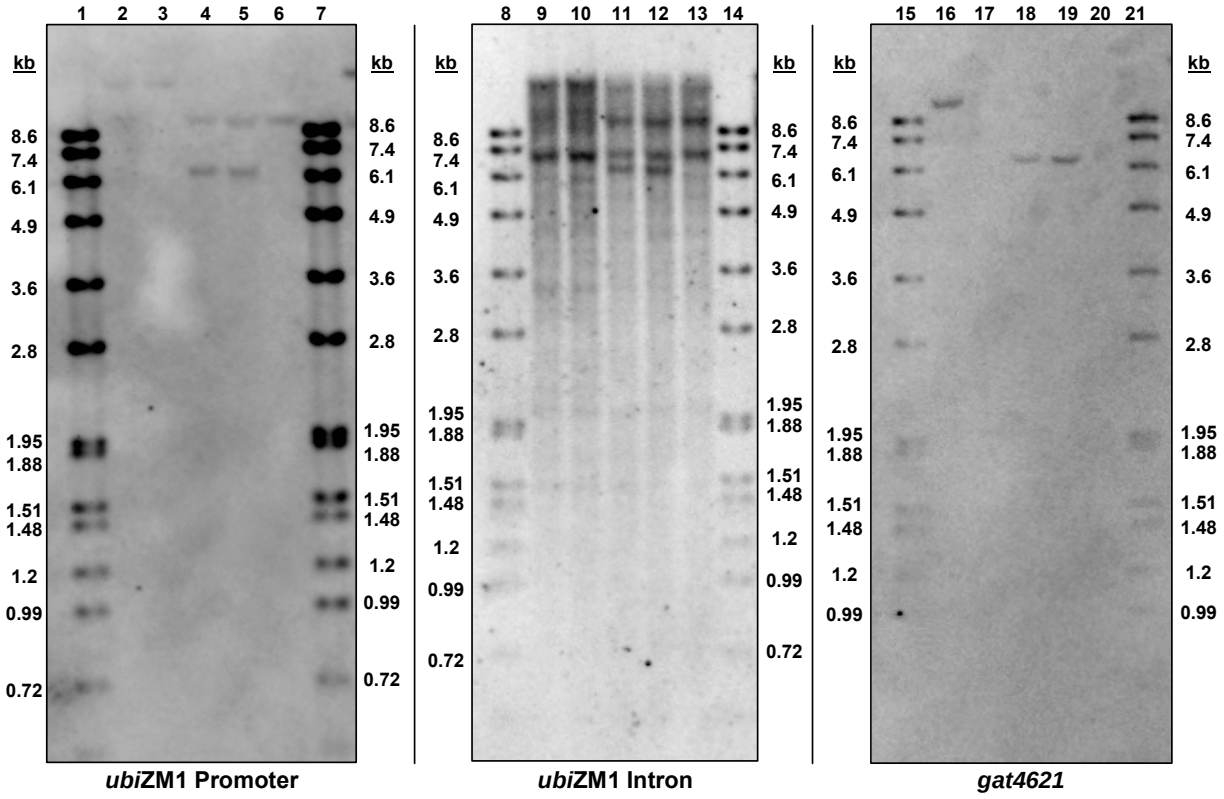


Figure 8. *gat4621* Cassette Probes and *EcoR V* Digest

Genomic DNA isolated from leaf tissue from individual plants of 98140 corn (T0S3 generation) and of control corn (PH09B and PHWVZ) was digested with *Eco*R V and hybridized to the probes unique to the *gat4621* cassette. Probes used are indicated below each panel. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes included plasmid PHP24279 at approximately one gene copy and 4 µg of control corn (PH09B) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	*Sample*
1	Molecular weight markers (DIG VII)
2	control (PH09B) + 1 copy of PHP24279
3	control (PH09B)
4	98140 corn/ plant 9
5	98140 corn/ plant 11
6	control (PHWVZ)
7	Molecular weight markers (DIG VII)
8	Molecular weight markers (DIG VII)
9	control (PH09B) + 1 copy of PHP24279
10	control (PH09B)

Lane	*Sample*
11	98140 corn/ plant 9
12	98140 corn/ plant 11
13	control (PHWVZ)
14	Molecular weight markers (DIG VII)
15	Molecular weight markers (DIG VII)
16	control (PH09B) + 1 copy of PHP24279
17	control (PH09B)
18	98140 corn/ plant 9
19	98140 corn/ plant 11
20	control (PHWVZ)
21	Molecular weight markers (DIG VII)

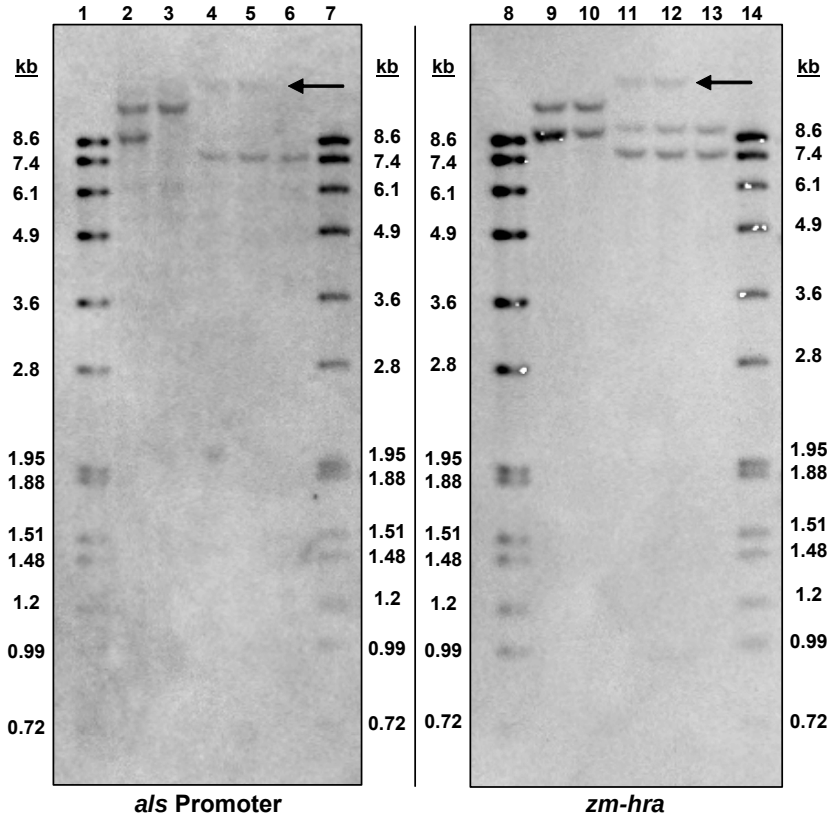


Figure 9. *zm-hra* Cassette Probes and *EcoR* V Digest

Genomic DNA isolated from leaf tissue from individual plants of 98140 corn (T0S3 generation) and of control corn (PH09B and PHWVZ) was digested with *Eco*R V and hybridized to the probes unique to the *zm-hra* cassette. Probes used are indicated below each panel. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes included plasmid PHP24279 at approximately one gene copy and 4 µg of control corn (PH09B) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb). Note: The band at greater than 8600 bp for both *als* promoter and *zm-hra* probes is difficult to discern on a printed copy, but is visible on the X-ray film (arrows). Reduced efficiency of Southern transfer of large fragments caused fainter hybridization to the larger fragments.

Lane	*Sample*
1	Molecular weight markers (DIG VII)
2	control (PH09B) + 1 copy of PHP24279
3	control (PH09B)
4	98140 corn/ plant 9
5	98140 corn/ plant 11
6	control (PHWVZ)
7	Molecular weight markers (DIG VII)

Lane	*Sample*
8	Molecular weight markers (DIG VII)
9	control (PH09B) + 1 copy of PHP24279
10	control (PH09B)
11	98140 corn/ plant 9
12	98140 corn/ plant 11
13	control (PHWVZ)
14	Molecular weight markers (DIG VII)

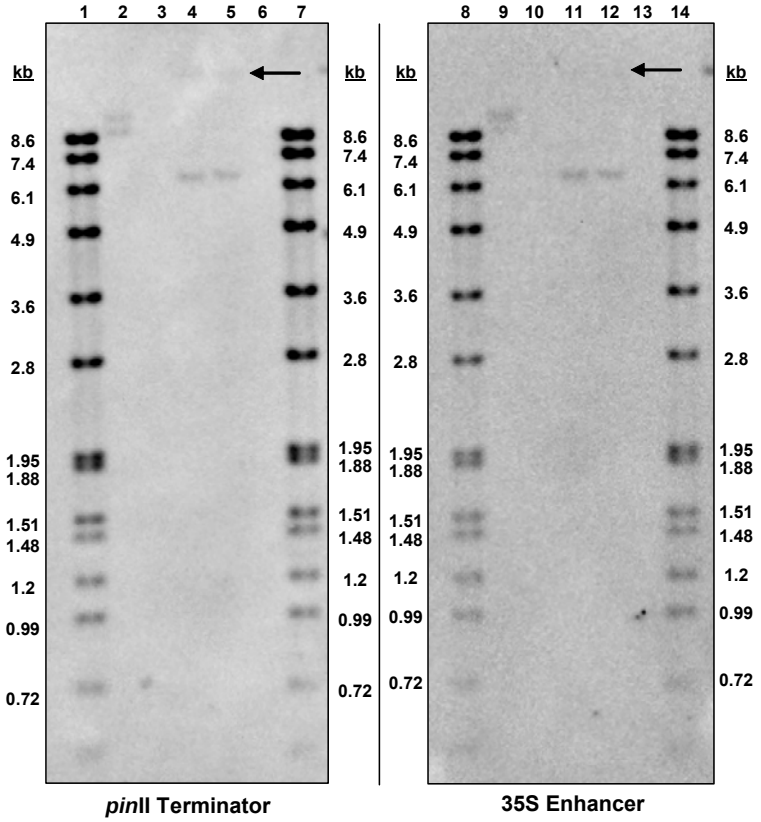


Figure 10. Common Element Probes and *EcoR* V Digest

Genomic DNA isolated from leaf tissue from individual plants of 98140 corn (T0S3 generation) and of control corn (PH09B and PHWVZ) was digested with *Eco*R V and hybridized to the probes common to both cassettes. Probes used are indicated below each panel. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes included plasmid PHP24279 at approximately one gene copy and 4 µg of control corn (PH09B) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb). Note: The band at greater than 8600 bp for both *pin*II Terminator and 35S Enhancer probes is difficult to discern on a printed copy, but is visible on the X-ray films (arrows). Reduced efficiency of Southern transfer of large fragments, as well as the difference in element copy number for the 35S enhancer, caused fainter hybridization to the larger fragments.

Lane	*Sample*
1	Molecular weight markers (DIG VII)
2	control (PH09B) + 1 copy of PHP24279
3	control (PH09B)
4	98140 corn/ plant 5
5	98140 corn/ plant 14
6	control (PHWVZ)
7	Molecular weight markers (DIG VII)

Lane	*Sample*
8	Molecular weight markers (DIG VII)
9	control (PH09B) + 1 copy of PHP24279
10	control (PH09B)
11	98140 corn/ plant 5
12	98140 corn/ plant 14
13	control (PHWVZ)
14	Molecular weight markers (DIG VII)

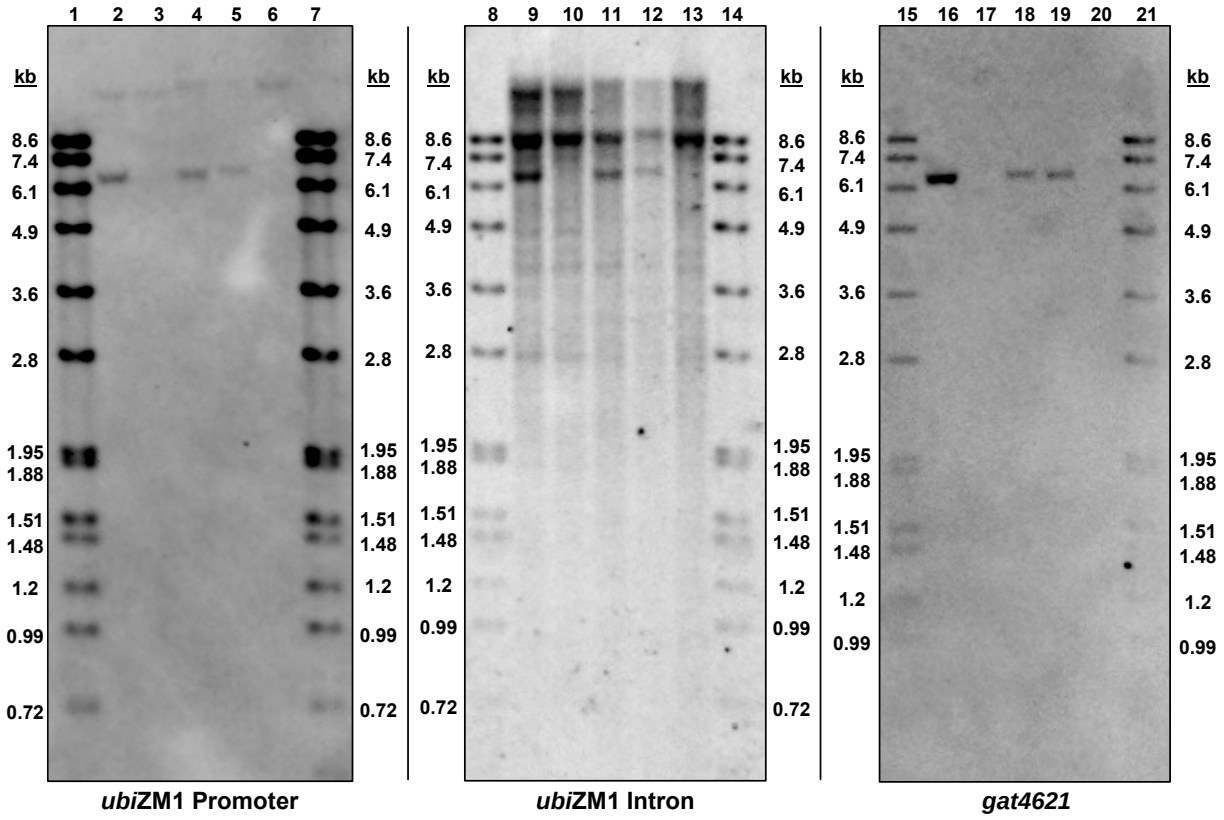


Figure 11. *gat4621* Cassette Probes and *Spe* I Digest

Genomic DNA isolated from leaf tissue from individual plants of 98140 corn (T0S3 generation) and of control corn (PH09B and PHWVZ) was digested with *Spe* I and hybridized to the probes unique to the *gat4621* cassette. Probes used are indicated below each panel. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes included plasmid PHP24279 at approximately one gene copy and 4 µg of control corn (PH09B) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	*Sample*
1	Molecular weight markers (DIG VII)
2	control (PH09B) + 1 copy of PHP24279
3	control (PH09B)
4	98140 corn/ plant 9
5	98140 corn/ plant 11
6	control (PHWVZ)
7	Molecular weight markers (DIG VII)
8	Molecular weight markers (DIG VII)
9	control (PH09B) + 1 copy of PHP24279
10	control (PH09B)

Lane	*Sample*
11	98140 corn/ plant 9
12	98140 corn/ plant 11
13	control (PHWVZ)
14	Molecular weight markers (DIG VII)
15	Molecular weight markers (DIG VII)
16	control (PH09B) + 1 copy of PHP24279
17	control (PH09B)
18	98140 corn/ plant 9
19	98140 corn/ plant 11
20	control (PHWVZ)
21	Molecular weight markers (DIG VII)

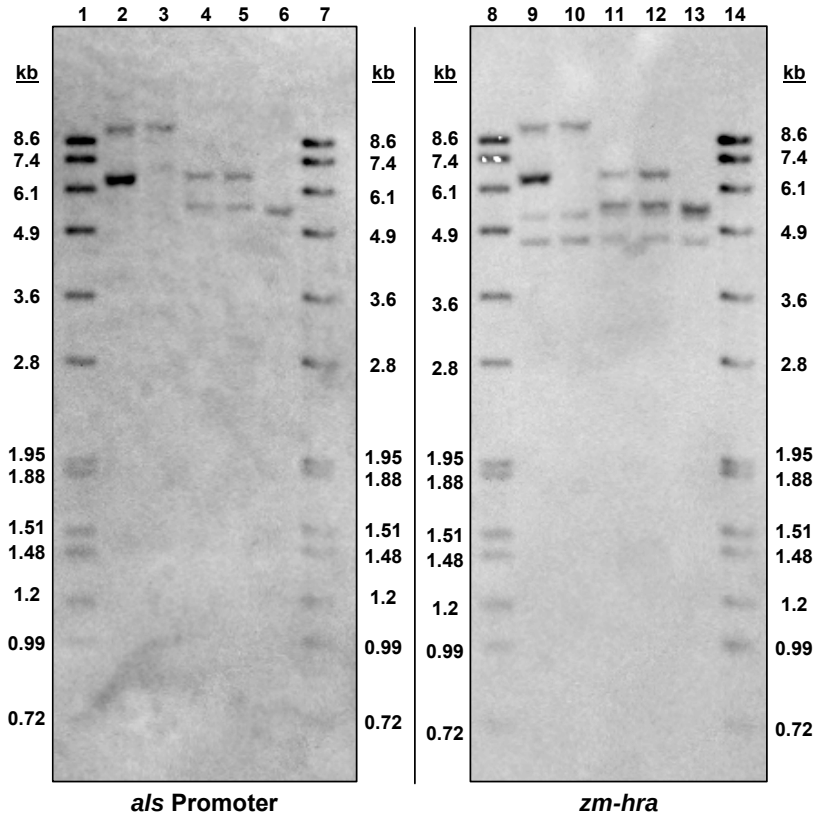


Figure 12. *zm-hra* Cassette Probes and *Spe* I Digest

Genomic DNA isolated from leaf tissue from individual plants of 98140 corn (T0S3 generation) and of control corn (PH09B and PHWVZ) was digested with *Spe* I and hybridized to the probes unique to the *zm-hra* cassette. Probes used are indicated below each panel. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes included plasmid PHP24279 at approximately one gene copy and 4 µg of control corn (PH09B) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	*Sample*
1	Molecular weight markers (DIG VII)
2	control (PH09B) + 1 copy of PHP24279
3	control (PH09B)
4	98140 corn/ plant 9
5	98140 corn/ plant 11
6	control (PHWVZ)
7	Molecular weight markers (DIG VII)

Lane	*Sample*
8	Molecular weight markers (DIG VII)
9	control (PH09B) + 1 copy of PHP24279
10	control (PH09B)
11	98140 corn/ plant 9
12	98140 corn/ plant 11
13	control (PHWVZ)
14	Molecular weight markers (DIG VII)

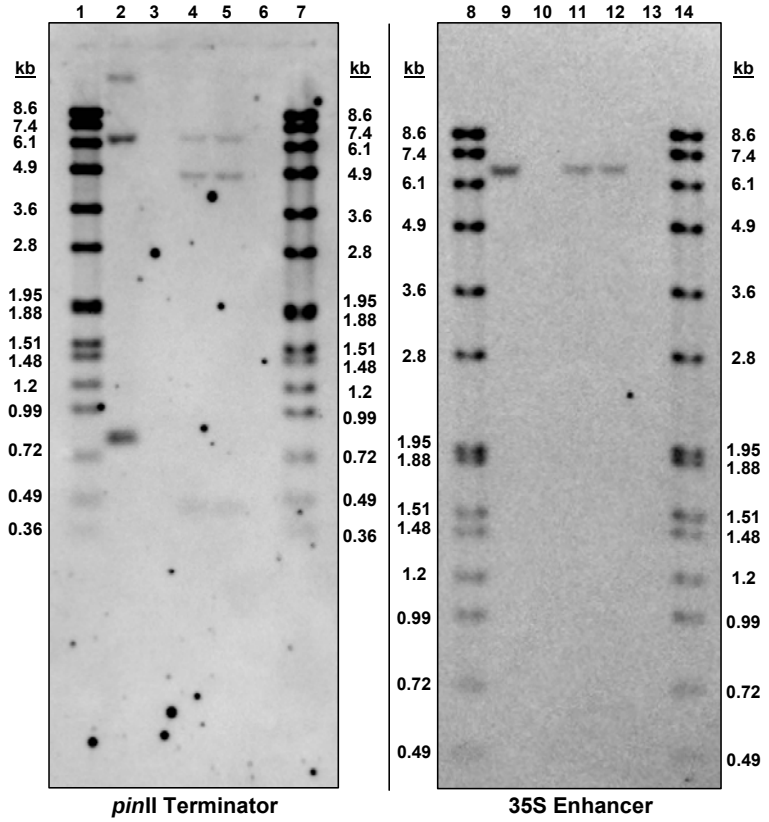


Figure 13. Common Element Probes and *Spe* I Digest

Genomic DNA isolated from leaf tissue from individual plants of 98140 corn (T0S3 generation) and of control corn (PH09B and PHWVZ) was digested with *Spe* I and hybridized to the probes common to both cassettes. Probes used are indicated below each panel. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes included plasmid PHP24279 at approximately one gene copy and 4 µg of control corn (PH09B) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	*Sample*
1	Molecular weight markers (DIG VII)
2	control (PH09B) + 1 copy of PHP24279
3	control (PH09B)
4	98140 corn/ plant 5
5	98140 corn/ plant 14
6	control (PHWVZ)
7	Molecular weight markers (DIG VII)

Lane	*Sample*
8	Molecular weight markers (DIG VII)
9	control (PH09B) + 1 copy of PHP24279
10	control (PH09B)
11	98140 corn/ plant 5
12	98140 corn/ plant 14
13	control (PHWVZ)
14	Molecular weight markers (DIG VII)

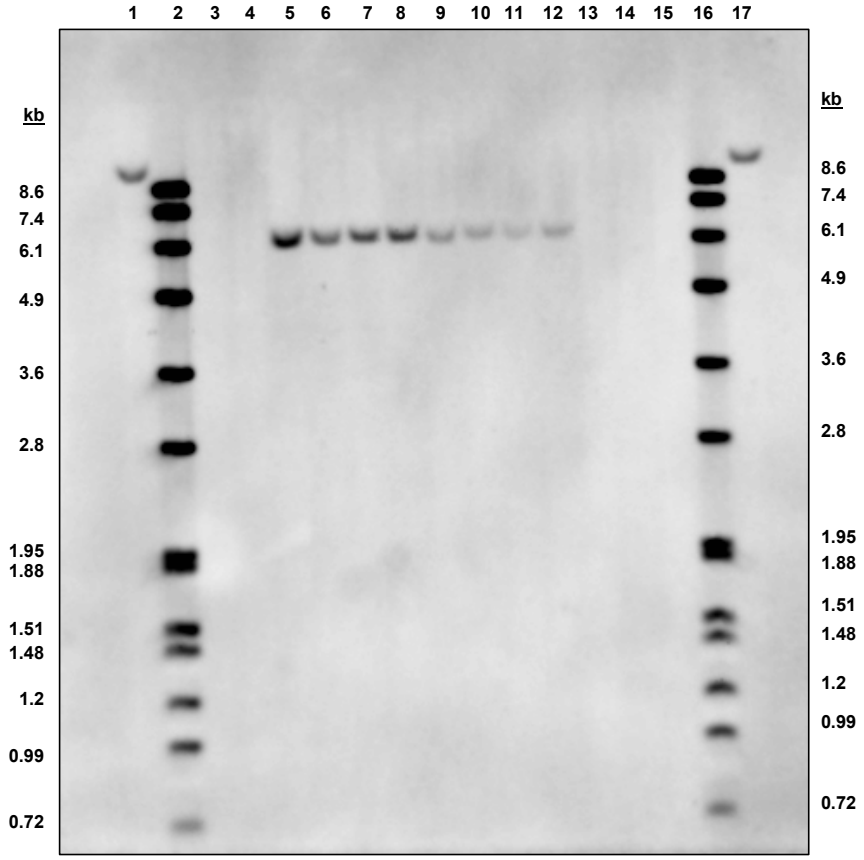


Figure 14. BC0S2 and BC1 Generations: *gat4621* Probe and *EcoR* V Digest

Genomic DNA isolated from leaf tissue from individual plants of 98140 corn (BC0S2 and BC1 generations) and of control corn (PH09B and PHWVZ) was digested with *Eco*R V and hybridized to the *gat4621* probe. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes included plasmid PHP24279 at approximately one gene copy and 4 µg of control corn (PH09B) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	control (PH09B) + 1 copy of PHP24279
2	Molecular weight markers (DIG VII)
3	control (PH09B)
4	control (PHWVZ)
5	98140 corn/ plant 1 (BC0S2 generation)
6	98140 corn/ plant 4 (BC0S2 generation)
7	98140 corn/ plant 7 (BC0S2 generation)
8	98140 corn/ plant 9 (BC0S2 generation)
9	98140 corn/ plant 11 (BC1 generation)
10	98140 corn/ plant 12 (BC1 generation)

Lane	Sample
11	98140 corn/ plant 13 (BC1 generation)
12	98140 corn/ plant 15 (BC1 generation)
13	98140 corn/ plant 20 (BC1 generation; null)
14	control (PHWVZ)
15	control (PH09B)
16	Molecular weight markers (DIG VII)
17	control (PH09B) + 1 copy of PHP24279

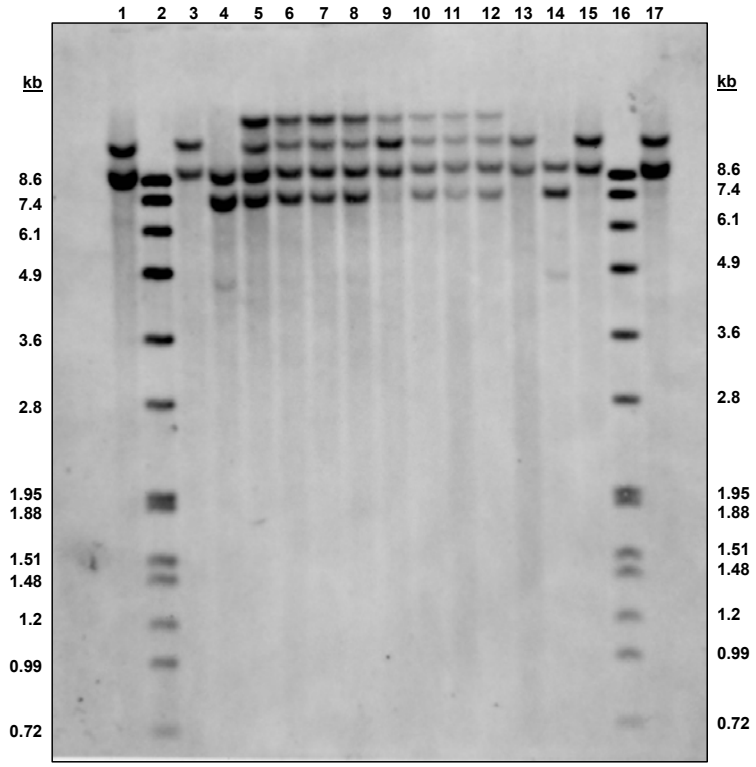


Figure 15. BC0S2 and BC1 Generations: *zm-hra* Probe and *EcoR* V Digest

Genomic DNA isolated from leaf tissue from individual plants of 98140 corn (BC0S2 and BC1 generations) and of control corn (PH09B and PHWVZ) was digested with *Eco*R V and hybridized to the *zm-hra* probe. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes included plasmid PHP24279 at approximately one gene copy and 4 µg of control corn (PH09B) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb). Note: A faint band at about 4700 bp is visible on the X-ray film in Lanes 4-8 and 14. Variations in sample loading and hybridization intensity affected the visibility of faint endogenous bands between different generations and samples within generations.

Lane	Sample
1	control (PH09B) + 1 copy of PHP24279
2	Molecular weight markers (DIG VII)
3	control (PH09B)
4	control (PHWVZ)
5	98140 corn/ plant 1 (BC0S2 generation)
6	98140 corn/ plant 4 (BC0S2 generation)
7	98140 corn/ plant 7 (BC0S2 generation)
8	98140 corn/ plant 9 (BC0S2 generation)
9	98140 corn/ plant 11 (BC1 generation)
10	98140 corn/ plant 12 (BC1 generation)

Lane	Sample
11	98140 corn/ plant 13 (BC1 generation)
12	98140 corn/ plant 15 (BC1 generation)
13	98140 corn/ plant 20 (BC1 generation; null)
14	control (PHWVZ)
15	control (PH09B)
16	Molecular weight markers (DIG VII)
17	control (PH09B) + 1 copy of PHP24279

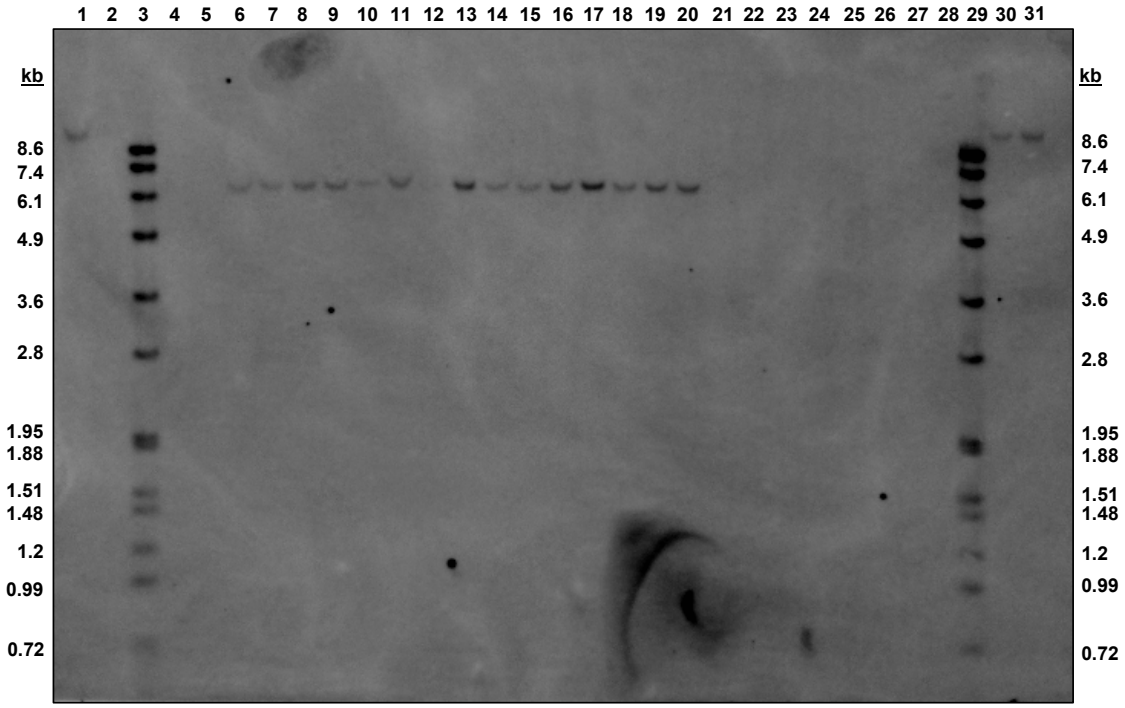


Figure 16. BC1S1 Generation: *gat4621* Probe and *EcoR* V Digest

Genomic DNA isolated from leaf tissue from individual plants of 98140 corn (BC1S1 generation) and of control corn (PH09B and PHWVZ) was digested with *Eco*R V and hybridized to the *gat4621* probe. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes included plasmid PHP24279 at the indicated approximate gene copy numbers and 4 µg of control corn (PH09B) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb). Note: The DNA from plant 14 (Lane 12) was degraded and therefore no hybridizing band was detected.

Lane	Sample
1	control (PH09B) + 3 copies of PHP24279
2	control (PH09B) + 1 copy of PHP24279
3	Molecular weight markers (DIG VII)
4	control (PH09B)
5	control (PHWVZ)
6	98140 corn/ plant 1 (BC1S1 generation)
7	98140 corn/ plant 5 (BC1S1 generation)
8	98140 corn/ plant 6 (BC1S1 generation)
9	98140 corn/ plant 8 (BC1S1 generation)
10	98140 corn/ plant 10 (BC1S1 generation)
11	98140 corn/ plant 11 (BC1S1 generation)
12	98140 corn/ plant 14 (BC1S1 generation)
13	98140 corn/ plant 17 (BC1S1 generation)
14	98140 corn/ plant 20 (BC1S1 generation)
15	98140 corn/ plant 21 (BC1S1 generation)

Lane	Sample
16	98140 corn/ plant 24 (BC1S1 generation)
17	98140 corn/ plant 25 (BC1S1 generation)
18	98140 corn/ plant 26 (BC1S1 generation)
19	98140 corn/ plant 28 (BC1S1 generation)
20	98140 corn/ plant 32 (BC1S1 generation)
21	98140 corn/ plant 4 (BC1S1 generation; null)
22	98140 corn/ plant 9 (BC1S1 generation; null)
23	98140 corn/ plant 12 (BC1S1 generation; null)
24	98140 corn/ plant 13 (BC1S1 generation; null)
25	98140 corn/ plant 15 (BC1S1 generation; null)
26	control (PH09B)
27	control (PHWVZ)
28	blank
29	Molecular weight markers (DIG VII)
30	control (PHWVZ) + 1 copy of PHP24279
31	control (PHWVZ) + 3 copies of PHP24279

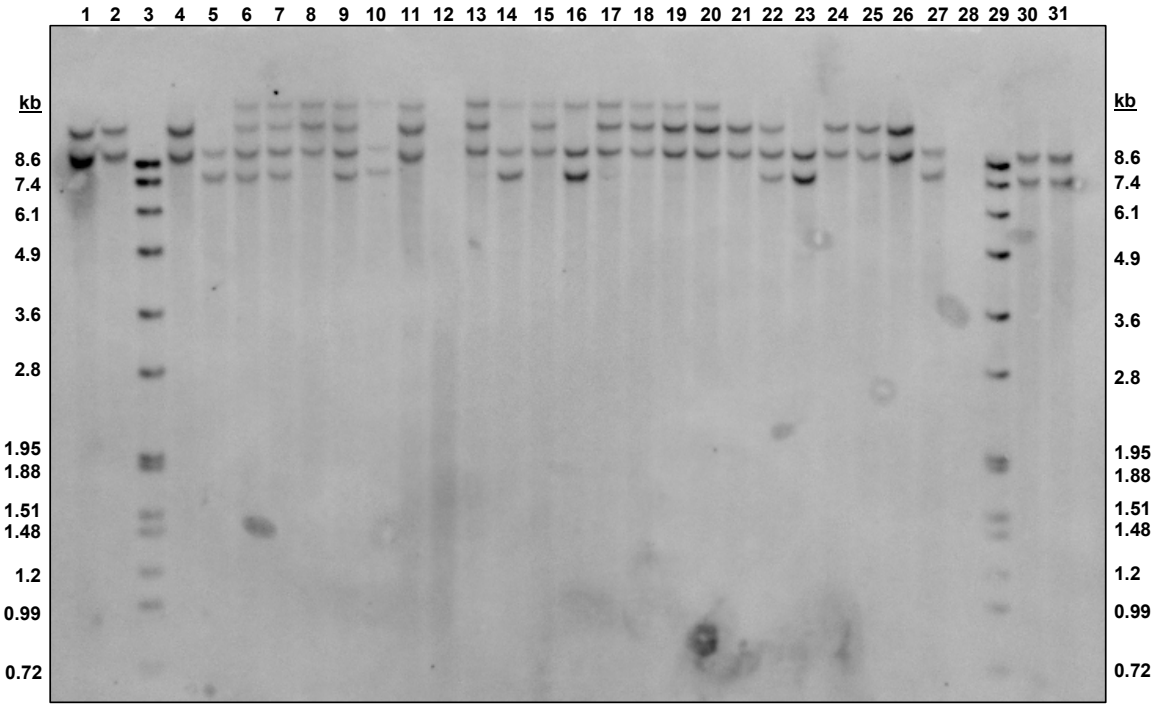


Figure 17. BC1S1 Generation: *zm-hra* Probe and *EcoR* V Digest

Genomic DNA isolated from leaf tissue from individual plants of 98140 corn (BC1S1 generation) and of control corn (PH09B and PHWVZ) was digested with *Eco*R V and hybridized to the *gat4621* probe. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes included plasmid PHP24279 at the indicated approximate gene copy numbers and 4 µg of control corn (PH09B) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb). Note: The DNA from plant 14 (Lane 12) was degraded and therefore no hybridizing band was detected.

Lane	Sample
1	control (PH09B) + 3 copies of PHP24279
2	control (PH09B) + 1 copy of PHP24279
3	Molecular weight markers (DIG VII)
4	control (PH09B)
5	control (PHWVZ)
6	98140 corn/ plant 1 (BC1S1 generation)
7	98140 corn/ plant 5 (BC1S1 generation)
8	98140 corn/ plant 6 (BC1S1 generation)
9	98140 corn/ plant 8 (BC1S1 generation)
10	98140 corn/ plant 10 (BC1S1 generation)
11	98140 corn/ plant 11 (BC1S1 generation)
12	98140 corn/ plant 14 (BC1S1 generation)
13	98140 corn/ plant 17 (BC1S1 generation)
14	98140 corn/ plant 20 (BC1S1 generation)
15	98140 corn/ plant 21 (BC1S1 generation)

Lane	Sample
16	98140 corn/ plant 24 (BC1S1 generation)
17	98140 corn/ plant 25 (BC1S1 generation)
18	98140 corn/ plant 26 (BC1S1 generation)
19	98140 corn/ plant 28 (BC1S1 generation)
20	98140 corn/ plant 32 (BC1S1 generation)
21	98140 corn/ plant 4 (BC1S1 generation; null)
22	98140 corn/ plant 9 (BC1S1 generation; null)
23	98140 corn/ plant 12 (BC1S1 generation; null)
24	98140 corn/ plant 13 (BC1S1 generation; null)
25	98140 corn/ plant 15 (BC1S1 generation; null)
26	control (PH09B)
27	control (PHWVZ)
28	blank
29	Molecular weight markers (DIG VII)
30	control (PHWVZ) + 1 copy of PHP24279
31	control (PHWVZ) + 3 copies of PHP24279

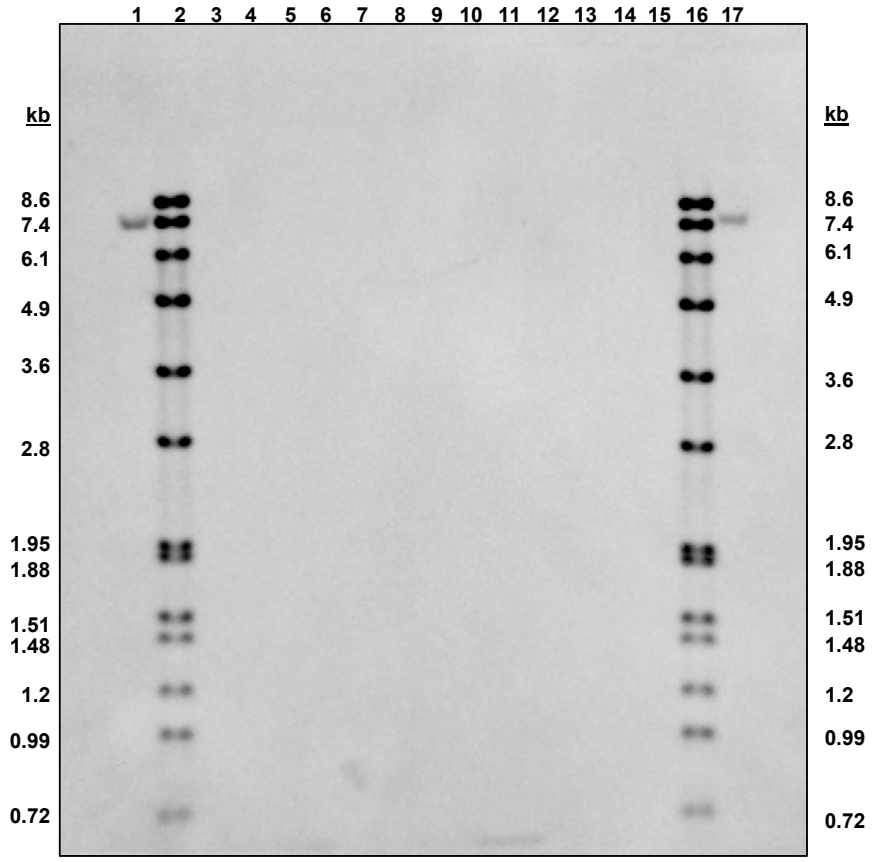


Figure 18. BC0S2 and BC1 Generations: *virG* Probe and *EcoR* V Digest

Genomic DNA isolated from leaf tissue from individual plants of 98140 corn (BC0S2 and BC1 generations) and of control corn (PH09B and PHWVZ) was digested with *Eco*R V and hybridized to the *vir*G probe. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes included plasmid PHP24279 at approximately one gene copy and 4 µg of control corn (PH09B) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	control (PH09B) + 1 copy of PHP24279
2	Molecular weight markers (DIG VII)
3	control (PH09B)
4	control (PHWVZ)
5	98140 corn/ plant 1 (BC0S2 generation)
6	98140 corn/ plant 4 (BC0S2 generation)
7	98140 corn/ plant 7 (BC0S2 generation)
8	98140 corn/ plant 9 (BC0S2 generation)
9	98140 corn/ plant 11 (BC1 generation)
10	98140 corn/ plant 12 (BC1 generation)

Lane	Sample
11	98140 corn/ plant 13 (BC1 generation)
12	98140 corn/ plant 15 (BC1 generation)
13	98140 corn/ plant 20 (BC1 generation; null)
14	control (PHWVZ)
15	control (PH09B)
16	Molecular weight markers (DIG VII)
17	control (PH09B) + 1 copy of PHP24279

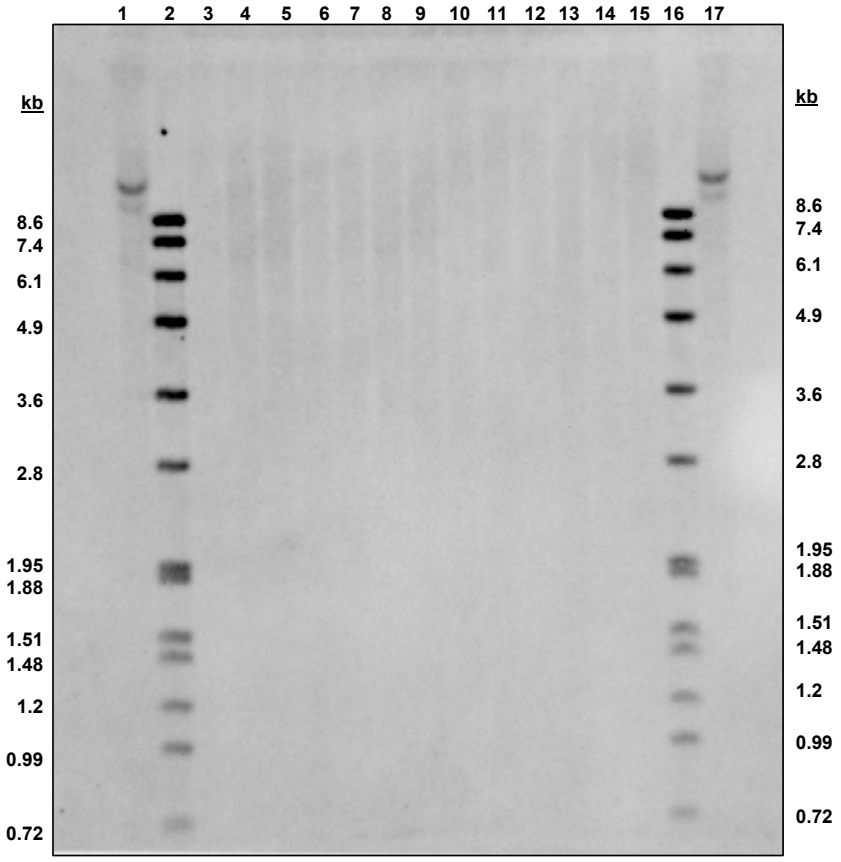


Figure 19. BC0S2 and BC1 Generations: *tet* Probe and *EcoR* V Digest

Genomic DNA isolated from leaf tissue from individual plants of 98140 corn (BC0S2 and BC1 generations) and of control corn (PH09B and PHWVZ) was digested with *Eco*R V and hybridized to the *tet* probe. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes included plasmid PHP24279 at approximately one gene copy and 4 µg of control corn (PH09B) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb). Note: Faint hybridization within the lanes along their entire length is due to non-specific binding of the probe to genomic DNA, as it is observed in all control and 98140 lanes.

Lane	Sample
1	control (PH09B) + 1 copy of PHP24279
2	Molecular weight markers (DIG VII)
3	control (PH09B)
4	control (PHWVZ)
5	98140 corn/ plant 1 (BC0S2 generation)
6	98140 corn/ plant 4 (BC0S2 generation)
7	98140 corn/ plant 7 (BC0S2 generation)
8	98140 corn/ plant 9 (BC0S2 generation)
9	98140 corn/ plant 11 (BC1 generation)
10	98140 corn/ plant 12 (BC1 generation)

Lane	Sample
11	98140 corn/ plant 13 (BC1 generation)
12	98140 corn/ plant 15 (BC1 generation)
13	98140 corn/ plant 20 (BC1 generation; null)
14	control (PHWVZ)
15	control (PH09B)
16	Molecular weight markers (DIG VII)
17	control (PH09B) + 1 copy of PHP24279

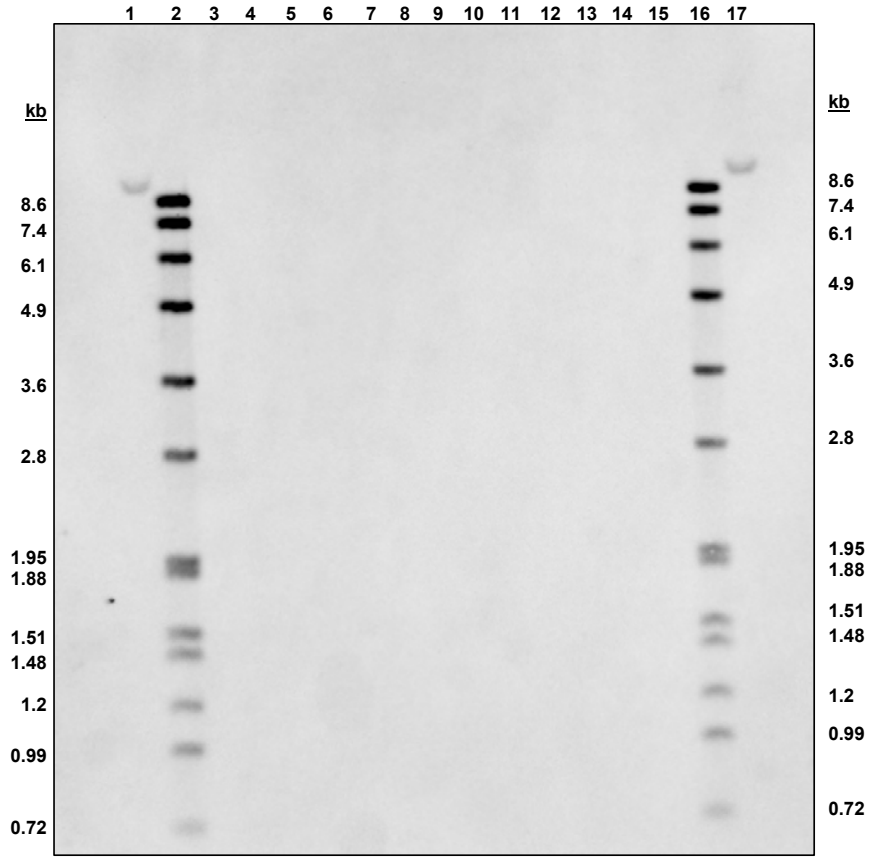


Figure 20. BC0S2 and BC1 Generations: *spc* Probe and *EcoR* V Digest

Genomic DNA isolated from leaf tissue from individual plants of 98140 corn (BC0S2 and BC1 generations) and of control corn (PH09B and PHWVZ) was digested with *Eco*R V and hybridized to the *spc* probe. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes included plasmid PHP24279 at approximately one gene copy and 4 µg of control corn (PH09B) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	control (PH09B) + 1 copy of PHP24279
2	Molecular weight markers (DIG VII)
3	control (PH09B)
4	control (PHWVZ)
5	98140 corn/ plant 1 (BC0S2 generation)
6	98140 corn/ plant 4 (BC0S2 generation)
7	98140 corn/ plant 7 (BC0S2 generation)
8	98140 corn/ plant 9 (BC0S2 generation)
9	98140 corn/ plant 11 (BC1 generation)
10	98140 corn/ plant 12 (BC1 generation)

Lane	Sample
11	98140 corn/ plant 13 (BC1 generation)
12	98140 corn/ plant 15 (BC1 generation)
13	98140 corn/ plant 20 (BC1 generation; null)
14	control (PHWVZ)
15	control (PH09B)
16	Molecular weight markers (DIG VII)
17	control (PH09B) + 1 copy of PHP24279

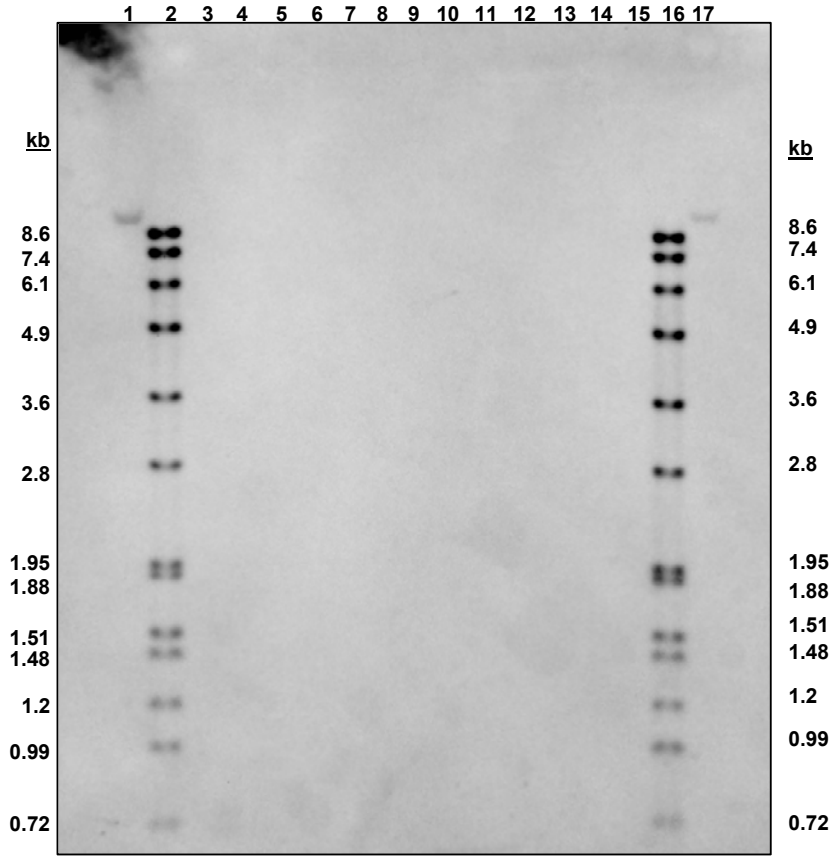


Figure 21. BC0S2 and BC1 Generations: LB Probe and *EcoR* V Digest

Genomic DNA isolated from leaf tissue from individual plants of 98140 corn (BC0S2 and BC1 generations) and of control corn (PH09B and PHWVZ) was digested with *Eco*R V and hybridized to the LB probe. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes included plasmid PHP24279 at approximately one gene copy and 4 µg of control corn (PH09B) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	control (PH09B) + 1 copy of PHP24279
2	Molecular weight markers (DIG VII)
3	control (PH09B)
4	control (PHWVZ)
5	98140 corn/ plant 1 (BC0S2 generation)
6	98140 corn/ plant 4 (BC0S2 generation)
7	98140 corn/ plant 7 (BC0S2 generation)
8	98140 corn/ plant 9 (BC0S2 generation)
9	98140 corn/ plant 11 (BC1 generation)
10	98140 corn/ plant 12 (BC1 generation)

Lane	Sample
11	98140 corn/ plant 13 (BC1 generation)
12	98140 corn/ plant 15 (BC1 generation)
13	98140 corn/ plant 20 (BC1 generation; null)
14	control (PHWVZ)
15	control (PH09B)
16	Molecular weight markers (DIG VII)
17	control (PH09B) + 1 copy of PHP24279

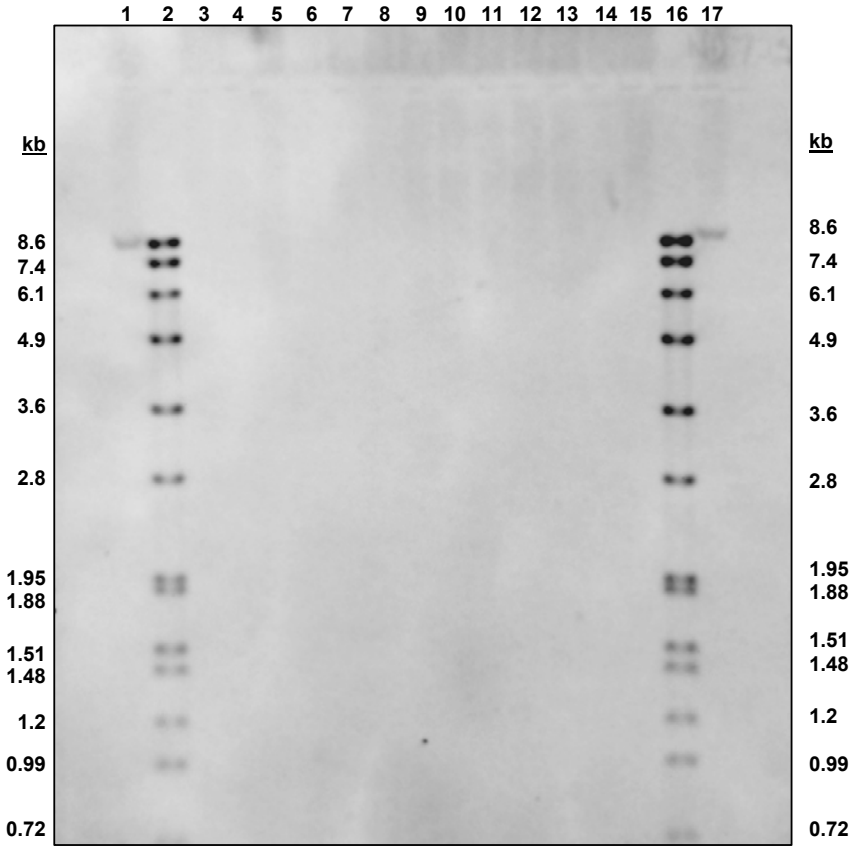


Figure 22. BC0S2 and BC1 Generations: RB Probe and *EcoR* V Digest

Genomic DNA isolated from leaf tissue from individual plants of 98140 corn (BC0S2 and BC1 generations) and of control corn (PH09B and PHWVZ) was digested with *Eco*R V and hybridized to the RB probe. Approximately 4 µg of genomic DNA was digested and loaded per lane. Positive control lanes included plasmid PHP24279 at approximately one gene copy and 4 µg of control corn (PH09B) DNA. Sizes of the DIG VII molecular weight markers are indicated adjacent to the blot image in kilobase pairs (kb).

Lane	Sample
1	control (PH09B) + 1 copy of PHP24279
2	Molecular weight markers (DIG VII)
3	control (PH09B)
4	control (PHWVZ)
5	98140 corn/ plant 1 (BC0S2 generation)
6	98140 corn/ plant 4 (BC0S2 generation)
7	98140 corn/ plant 7 (BC0S2 generation)
8	98140 corn/ plant 9 (BC0S2 generation)
9	98140 corn/ plant 11 (BC1 generation)
10	98140 corn/ plant 12 (BC1 generation)

Lane	Sample
11	98140 corn/ plant 13 (BC1 generation)
12	98140 corn/ plant 15 (BC1 generation)
13	98140 corn/ plant 20 (BC1 generation; null)
14	control (PHWVZ)
15	control (PH09B)
16	Molecular weight markers (DIG VII)
17	control (PH09B) + 1 copy of PHP24279

V-E. Inheritance of the Traits in 98140 Corn

Chi-square analysis of trait inheritance data from four different generations (BC0S1, BC1S1, BC2 and BC3) was performed to determine the heritability and stability of the *gat4621* and *zm-hra* genes in 98140 corn. The breeding history of the four generations evaluated for Mendelian inheritance is described in Figure 2. The plants from the BC0S1 and BC1S1 generations were expected to segregate 3:1, and the plants from the BC2 and BC3 generations were expected to segregate 1:1 for the presence of the *gat4621* and *zm-hra* genes.

In order to confirm the expected segregation ratios, polymerase chain reaction (PCR) analysis was performed on leaf punches from seedlings. Qualitative PCR analysis for the *gat4621* and *zm-hra* genes was conducted on all plants.

Results from the segregation analysis are summarized in Table 6. In every case, plants that were positive for the *gat4621* gene were also positive for the *zm-hra* gene and vice versa, confirming co-segregation of the two genes as expected. To confirm that *gat4621* and *zm-hra* genes segregate according to Mendel's laws of genetics, chi-square analysis was performed. Details of the statistical methodology can be found in Appendix 2. All P-values were greater than 0.05, indicating no statistically significant differences between the observed and expected frequencies of the *gat4621* and/or *zm-hra* genes in four generations of 98140 corn.

The results of this analysis are consistent with the finding of a single locus of insertion of the *gat4621* and *zm-hra* genes that segregates in 98140 corn progeny according to Mendel's laws of genetics. The stability of the insert has been demonstrated in four generations of self- and cross-pollinations.

Table 6. Comparison of Observed and Expected Segregation Ratios for 98140 Corn

Generation	Observed		Expected			Chi-Square Test
	Positive for *gat4621* and *zm-hra* genes	Negative for *gat4621* and *zm-hra* genes	Expected Ratio	Positive for *gat4621* and *zm-hra* genes	Negative for *gat4621* and *zm-hra* genes	P-value
BC0S1	55	22	3:1	57.75	19.25	0.5537
BC1S1	45	20	3:1	48.75	16.25	0.3519
BC2	51	48	1:1	49.5	49.5	0.8407
BC3	52	45	1:1	48.5	48.5	0.5424

V-F. Summary and Conclusions

Southern blot analysis was conducted to characterize the DNA insertion in 98140 corn. The analysis confirmed that a single, intact PHP24279 T-DNA has been inserted into the corn genome to produce 98140 corn. A single copy of each of the elements of the *gat4621* and *zm-hra* expression cassettes was present, along with the three 35S enhancer elements between the two cassettes, and the integrity of the PHP24279 T-DNA was maintained. The analysis confirmed the stability of the insertion in 98140 corn across the T0S3, BC1S1, BC0S2, and BC1 generations, thus confirming stability of inheritance during traditional breeding procedures. In addition, Southern blot analysis verified the absence of plasmid backbone sequences in 98140 corn.

Inheritance studies confirmed that the insert segregated in normal Mendelian fashion. None of the P-values obtained in the studies indicated a statistically significant difference between observed and expected segregation ratios for the *gat4621* and *zm-hra* genes over four different plant generations. The results are consistent with the molecular characterization data, which indicates stable integration of the *gat4621* and *zm-hra* transgenes at a single site in the corn genome.

VI. Characterization of the Introduced GAT4621 and ZM-HRA Proteins

VI-A. The GAT4621 Protein

A1. Identity of the GAT4621 Protein

GAT4621 is a protein based on N-acetyltransferase protein sequences from *Bacillus licheniformis*, a gram positive saprophytic bacterium that is widespread in nature and thought to contribute substantially to nutrient cycling due to the diversity of enzymes produced by members of its species. GAT4621 is 147 amino acids in length and has an approximate molecular weight of 17 kDa (Figure 23).

Figure 23. Deduced Amino Acid Sequence of the GAT4621 Protein

```
  1    MAIEVKPINA EDTYDLRHRV LRPNQPIEAC MFESDLTRSA FHLGGFYGGK
 51    LISVASFHQA EHSELQGKKQ YQLRGVATLE GYREQKAGSS LVKHAEEILR
101    KRGADMIWCN ARTSASGYYR KLGFSEQGEV FDTPPVGPHI LMYKRIT
```

The GAT4621 protein is very similar to the GAT4601 protein. The GAT4601 protein is present in Pioneer's 356043 soybean, which was the subject of a petition submitted to USDA on September 27, 2006 (06-271-01p).

GAT4621 and GAT4601 are 91% identical and 96% similar (Figure 24). The GAT4621 protein has higher catalytic efficiency for glyphosate than GAT4601, as discussed in Section VI, A3.

Figure 24. Sequence Comparison of GAT4621 and GAT4601

```
                1                                                  50
GAT4621    (1)  MAIEVKPINAEDTYDLRHRVLRPNQPIEACMFESDLTRSAFHLGGFYGGK
GAT4601    (1)  -MIEVKPINAEDTYELRHRILRPNQPIEACMFESDLLRGAFHLGGFYRGK

               51                                                 100
GAT4621   (51)  LISVASFHQAEHSELQGKKQYQLRGVATLEGYREQKAGSSLVKHAEEILR
GAT4601   (50)  LISIASFHQAEHSELQGQKQYQLRGMATLEGYREQKAGSTLVKHAEEILR

               101                                            147
GAT4621  (101)  KRGADMIWCNARTSASGYYRKLGFSEQGEVFDTPPVGPHILMYKRIT
GAT4601  (100)  KRGADMLWCNARTSASGYYKKLGFSEQGEIFDTPPVGPHILMYKRIT
```

Note: Shaded amino acids represent conservative amino acid differences between GAT4621 and GAT4601, and boxed amino acids represent non-conservative differences.

A2. Characteristics of GAT Proteins

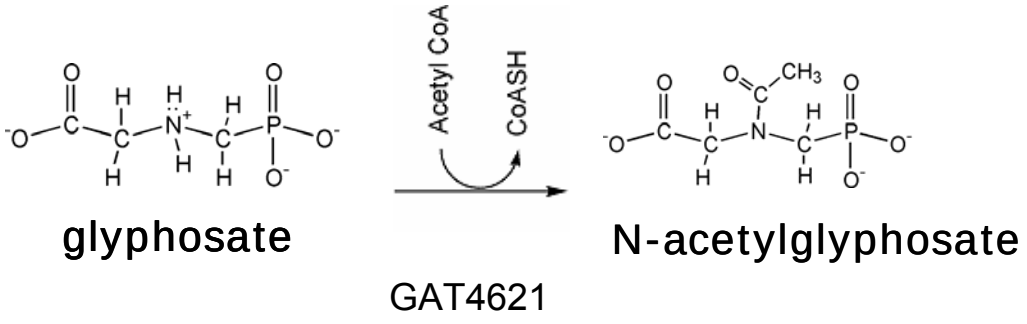
Glyphosate acetyltransferase (GAT) proteins are members of the GCN 5-related family of N-acetyltransferases (also known as the GNAT superfamily). The GNAT superfamily is one of the largest enzyme superfamilies recognized to date with over 10,000 representatives from plants, animals and microbes. Members of the GNAT superfamily all contain highly conserved GNAT motifs but have high sequence diversity (Vetting *et al.,* 2005). GNAT proteins are known to have a number of metabolic functions including detoxification (Dyda *et al*., 2000). In particular, and as

described below, GAT proteins can confer tolerance by detoxification to the broad spectrum herbicide glyphosate.

Glyphosate has proven to be a very popular herbicide because of its effectiveness and safety. Post emergence application of glyphosate effectively controls grasses and annual broadleaf weeds. Glyphosate inhibits the enzyme enolpyruvylshikimate-3-phosphate synthase (EPSPS) in the plant chloroplast-localized pathway that leads to the biosynthesis of aromatic amino acids. Some microbial EPSPS enzyme variants are insensitive to glyphosate inhibition. When expressed in chloroplasts of transgenic plants, the insensitive enzymes confer tolerance to glyphosate. This is the basis of current commercial glyphosate tolerant corn.

GAT proteins provide an alternative mechanism of resistance to glyphosate (Figure 25). They detoxify glyphosate to the non-phytotoxic form N-acetylglyphosate. This detoxification mechanism is similar to that of the phosphinothricin acetyltransferase (PAT or BAR) enzymes from *Streptomyces*, which detoxify glufosinate ammonium herbicides by adding an acetyl group (De Block *et al.,* 1987) and have been deregulated by USDA (Van Wert, 1994). As shown in Figure 25, GAT proteins acetylate the secondary amine of glyphosate using acetyl coenzyme A as an acetyl donor (Castle *et al.,* 2004). Although GAT4621, PAT and BAR enzymes are N-acetyltransferase proteins, expression of the *gat4621* gene in 98140 corn does not confer tolerance to glufosinate ammonium herbicides (Pioneer internal data). Transgenic expression of GAT proteins was shown to confer glyphosate tolerance in several plant species (Castle *et al.,* 2004). This strategy was used to develop glyphosate tolerant 98140 corn.

Figure 25. Enzymatic Activity of GAT4621



A3. Generation of GAT4621

In order to develop a GAT protein that would confer commercial levels of tolerance to the herbicide glyphosate when expressed in plants, an in-house collection of several hundred *Bacillus* isolates was screened. A mass spectrometry method was developed to detect low levels of N-acetylglyphosate, the non-phytotoxic end product of glyphosate acetylation. The *Bacillus* isolates were grown to stationary phase, permeabilized, and incubated with glyphosate and acetyl coenzyme A, and the supernatants were screened for the presence of N-acetylglyphosate by mass spectrometry. Several strains of *Bacillus licheniformis* exhibited GAT activity and had the greatest reproducible accumulation of N-acetylglyphosate (Castle and Lassner, 2004).

To isolate the gene encoding GAT, recombinant *E. coli* expressing genomic DNA fragments from *B. licheniformis* were assayed by the mass spectrometry method. DNA sequences of multiple

genomic fragments specifying GAT activity from *B. licheniformis* strain B6 and *B. licheniformis* strain 401 (purchased from ATCC—catalog number 14580) were determined, and the corresponding genes were cloned. A polymerase chain reaction (PCR) survey of *B. licheniformis* strains revealed a third gene variant in isolate DS3. The B6, 401 and DS3 *gat* genes were used as parents for fragmentation-based multigene shuffling to create enzymes with improved activity on the substrate glyphosate.

DNA shuffling is a process that recombines genetic diversity from parental genes to create libraries of gene variants that are then screened to identify those progeny with improved properties (Stemmer, 1994; Crameri *et al*., 1998). This process of fragmentation and recombination followed by selection can be repeated using those progeny with improved properties as parents for the next round of shuffling. In the case of the *gat4621* gene, this process was repeated eleven times using a combination of multi-gene shuffling and the introduction of genetic diversity via PCR.

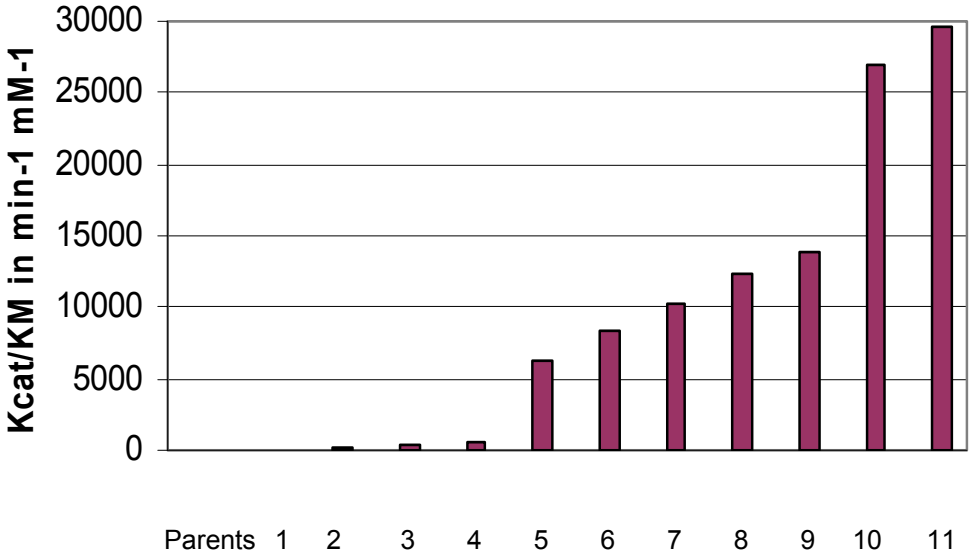
To initiate the first round of gene shuffling, the three native *B. licheniformis gat* genes were used as parental templates. The initial diversity represented among the three native GAT protein sequences occurred at 12 of the 146 total amino acid positions. Libraries of shuffled gene variants were created, expressed in *E. coli*, and screened. Shuffled variants that resulted in the accumulation of more N-acetylglyphosate than the parental controls were selected for further rounds of shuffling. In each round of DNA shuffling, approximately 5,000 gene variants were screened and 24-48 purified enzymes were analyzed to determine their kinetic properties. Typically, three to twelve improved variants exhibiting a high k_{cat}, a low K_M, or a high k_{cat}/K_M ratio were chosen to be the parents for the next round.

In enzyme kinetics, k_{cat} is a measure of the turnover rate or speed of the reaction. The higher the k_{cat}, the faster the enzyme reaction. K_M is the affinity of the enzyme for a substrate or tightness of binding of the substrate to the enzyme. The lower the K_M, the greater the affinity of the enzyme for the substrate. A k_{cat}/K_M ratio is the common way to express the catalytic efficiency of the enzyme.

Several GAT enzymes from the third round of gene shuffling had rate constants (k_{cat}/K_M ratios) about 100-fold improved over the original enzymes, but the gene variants were unable to confer glyphosate tolerance to *E. coli*. At the fifth round of shuffling, two advances were made: 1) the additional introduction of diversity by PCR incorporation of oligonucleotides based on related DNA sequences from *Bacillus cereus* and *Bacillus subtilis* during the fragment reassembly step that allowed for substitutions at 27 amino acid residues; and 2) a functional pre-screen based on resistance to glyphosate of *E. coli* strains expressing GAT (Castle *et al*., 2004). At the sixth round of shuffling, a spectrophotometric assay was used that allowed the variants to be screened for relative k_{cat} and K_M values without protein purification. An additional oligonucleotide incorporation of sequence diversity based on acetyltransferase proteins with 30-60% identity to GAT was introduced in the eighth round of shuffling.

At the end of the eleventh round of gene shuffling, the rate constant for glyphosate of the best round 11 variant analyzed was approximately 7000-fold improved over the native enzymes (Castle *et al*., 2004; Siehl *et al*., 2005). The average k_{cat}/K_M of the parental enzymes was 4.2 min^{-1} mM^{-1}, and the activity of the best round 11 variant was approximately 29,600 min^{-1} mM^{-1} (Figure 26).

Figure 26. Kinetic Improvement of GAT Enzymes Through Shuffling



Taken from Siehl *et al*., 2005.

In order to optimize the gene from the best round 11 variant for plant expression, codon changes in the gene sequence were made to eliminate rare plant codons. These changes did not alter the encoded protein sequence. Additionally, a GCT codon for alanine was inserted at amino acid position 2. This, along with a plant promoter, resulted in a consensus translation initiation site thought to be best for protein production in plants (Joshi *et al.,* 1997). The plant-optimized gene was given the designation *gat4621*.

The GAT4621 protein, encoded by the *gat4621* gene, is 75-78% identical and 90-91% similar at the amino acid level to each of the three native GAT enzymes from which it was derived, compared to 94% identity of each of the native enzymes to each other (Table 7). There are 32-36 amino acid changes (22-23 of which are conservative) between the shuffled GAT4621 protein and any one of the original three native GAT proteins.

Table 7. Comparison of Homology Between Parental GAT Proteins and GAT4621

	GAT from strain 401	**GAT from strain B6**	**GAT from strain DS3**	**GAT4621**
GAT from strain 401	100%	94% identical	94% identical	78% identical 91% similar
GAT from strain B6		100%	94% identical	76% identical 91% similar
GAT from strain DS3			100%	75% identical 90% similar
GAT4621				100%

VI-B. The ZM-HRA Protein

B1. Identity of the ZM-HRA Protein

The ZM-HRA protein is a modified version of the native acetolactate synthase (ALS) protein from maize. Endogenous ALS proteins are the target sites of ALS inhibitor herbicides. Expression of the herbicide tolerant ZM-HRA protein in transgenic crops confers tolerance to the ALS-inhibiting class of herbicides. In addition to sulfonylureas (Levitt, 1978) and imidazolinones, there are three other chemical classes of herbicides identified to date that inhibit the activity of ALS: triazolopyrimidines, pyrimidinylthio (or oxy)-benzoates, and sulfonylamino-carbonyltriazolinones (Tan *et al.,* 2006). The ZM-HRA protein is tolerant to the ALS-inhibiting class of herbicides. Therefore, expression of the herbicide-tolerant ZM-HRA protein in 98140 corn confers plant tolerance to the ALS-inhibiting class of herbicides.

The herbicide tolerant *zm-hra* gene encoding the ZM-HRA protein was made by isolating an endogenous maize *als* gene, which codes for a herbicide-sensitive protein, and introducing two specific amino acid changes in the mature protein. The full-length ZM-HRA protein (including the chloroplast transit peptide sequence) is 638 amino acids in length and has an approximate molecular weight of 69 kDa (Figure 27).

ALS proteins contain N-terminal transit peptides, and the mature protein is formed following transport into the chloroplast and subsequent cleavage of the transit peptide. The mature protein starts at residue S41, resulting in a mature protein of 598 amino acids with a predicted molecular weight of 65 kDa.

Figure 27. Deduced Amino Acid Sequence of the Full-Length ZM-HRA Protein

```
  1    MATAAAASTA LTGATTAAPK ARRRAHLLAT RRALAAPIRC SAASPAMPMA
 51    PPATPLRPWG PTEPRKGADI LVESLERCGV RDVFAYPGGA SMEIHQALTR
101    SPVIANHLFR HEQGEAFAAS GYARSSGRVG VCIATSGPGA TNLVSALADA
151    LLDSVPMVAI TGQVARRMIG TDAFQETPIV EVTRSITKHN YLVLDVDDIP
201    RVVQEAFFLA SSGRPGPVLV DIPKDIQQQM AVPVWDKPMS LPGYIARLPK
251    PPATELLEQV LRLVGESRRP VLYVGGGCAA SGEELRRFVE LTGIPVTTTL
301    MGLGNFPSDD PLSLRMLGMH GTVYANYAVD KADLLLALGV RFDDRVTGKI
351    EAFASRAKIV HVDIDPAEIG KNKQPHVSIC ADVKLALQGM NALLEGSTSK
401    KSFDFGSWND ELDQQKREFP LGYKTSNEEI QPQYAIQVLD ELTKGEAIIG
451    TGVGQHQMWA AQYYTYKRPR QWLSSAGLGA MGFGLPAAAG ASVANPGVTV
501    VDIDGDGSFL MNVQELAMIR IENLPVKVFV LNNQHLGMVV QLEDRFYKAN
551    RAHTYLGNPE NESEIYPDFV TIAKGFNIPA VRVTKKNEVR AAIKKMLETP
601    GPYLLDIIVP HQEHVLPMIP SGGAFKDMIL DGDGRTVY
```

Notes: Amino acid residues boxed in grey are those modified from the endogenous maize acetolactate synthase protein (P165A and W542L).
The underlined sequence indicates the chloroplast transit peptide sequence of the ZM-HRA protein.

The ZM-HRA protein from maize is similar to the GM-HRA protein from soybean. The GM-HRA protein is present in Pioneer's 356043 soybean, which was the subject of a petition submitted to USDA on September 27, 2006 (06-271-01p). The ZM-HRA and GM-HRA mature proteins (after cleavage of transit peptide) are 74% identical and 83% similar at the amino acid level. Both proteins confer ALS inhibitor tolerance in transgenic plants (GM-HRA in dicotyledonous species and ZM-HRA in monocotyledonous species).

B2. Characteristics of ALS Proteins

The native ALS, also known as acetohydroxyacid synthase (AHAS)[2], is a key enzyme that catalyzes the first common step in the biosynthesis of the essential branched-chain amino acids isoleucine, leucine, and valine (LaRossa and Schloss, 1984; LaRossa and Falco, 1984; Duggleby and Pang, 2000; Coruzzi and Last, 2000). Two reactions are catalyzed by ALS enzymes: the conversion of two molecules of pyruvate to form acetolactate leading to the synthesis of leucine and valine, and the condensation of pyruvate with 2-ketobutyrate to form 2-acetohydroxybutyrate in the pathway to isoleucine (Figure 28).

In plants, ALS genes are encoded in the nucleus, and the enzymes contain a chloroplast directed N-terminal transit peptide. The transit peptide serves to import the protein into the chloroplast, where the majority of branched chain amino acid biosynthesis occurs. Natural herbicide tolerance mutations are most commonly found at four amino acid locations in ALS: A122, P197, W574 and S653 (Duggleby and Pang, 2000). Several commercialized crops (soybean, corn, wheat, rice, canola and sunflower) that are tolerant to ALS inhibitors contain mutations derived through mutagenesis or selection (Tan *et al*., 2006).

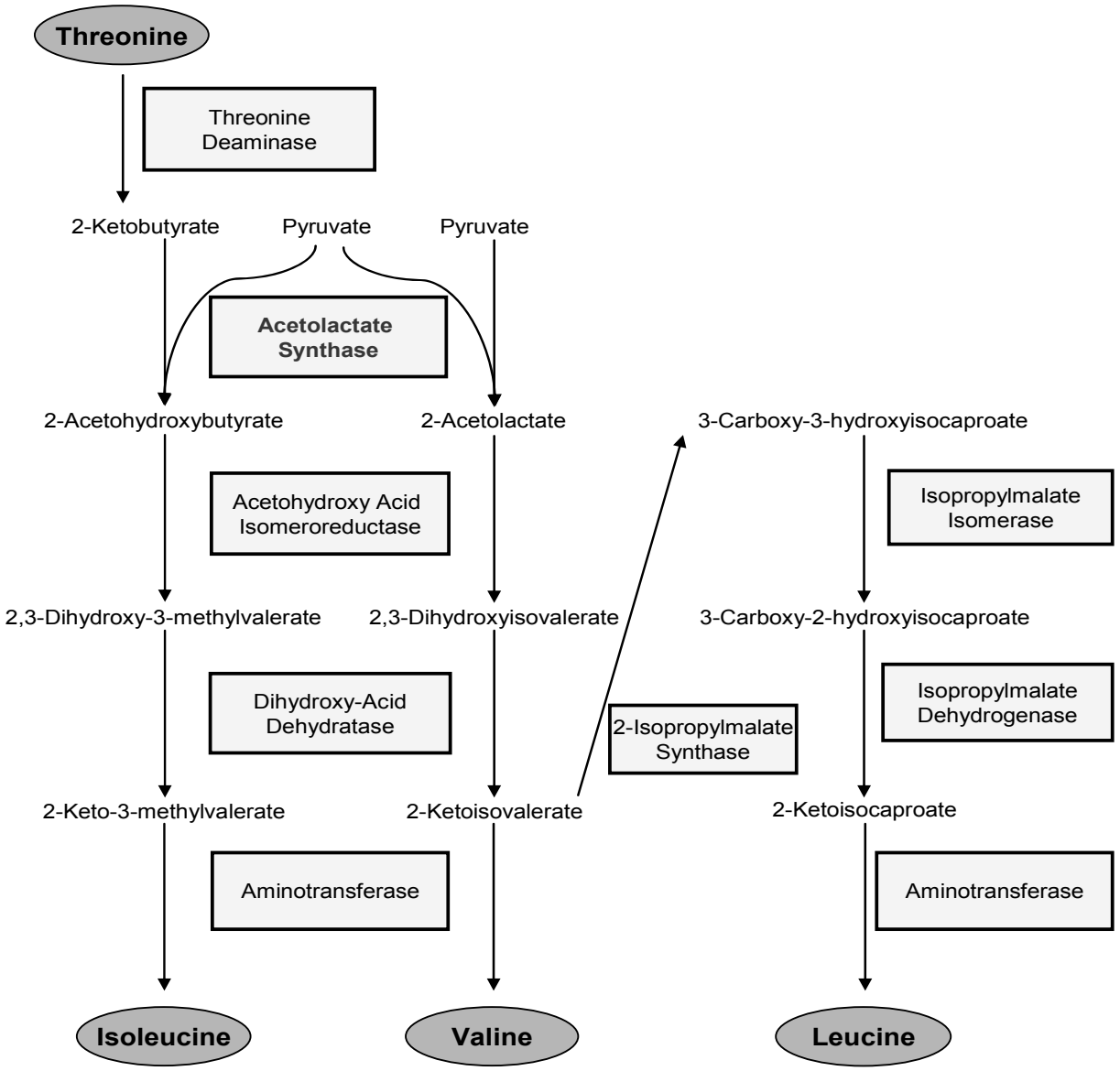
The ALS enzyme was first identified as the target site for sulfonylurea herbicides in the late 1970's (Levitt, 1978), and the first enzymes with herbicide tolerant activity were identified in bacteria (LaRossa and Schloss, 1984), yeast (*Saccharomyces cerevisiae*) (Falco and Dumas, 1985), and plants (Chaleff and Mauvais, 1984). The respective genes were then isolated from various species and amino acid sequence changes accountable for the tolerant phenotype were identified. Several reviews are available on amino acid substitutions that result in tolerance to ALS inhibitors (Hartnett *et al*., 1990, 1991; Falco *et al*., 1989; Duggleby and Pang, 2000). For example, a resistant tobacco (*Nicotiana tabacum*) line was isolated through two successive rounds of tissue culture selection in the presence of sulfonylurea herbicides (Creason and Chaleff, 1988). The gene responsible for the altered ALS was identified, and the sequence of the gene identified two amino acid substitutions that contributed to the herbicide tolerant activity: mutations P196A and W573L (Lee *et al*., 1988a). The locations of these two mutations are equivalent to the locations of the commonly found natural tolerance mutations, P197 and W574 (Duggleby and Pang, 2000). Both individual mutations conferred tolerance to herbicides, but the two mutations combined together typically resulted in a higher level of tolerance (Mazur and Falco, 1989; Hartnett *et al*., 1990; Creason and Chaleff, 1988).

B3. Generation of ZM-HRA

In the case of ZM-HRA, mutations analogous to those described above in the double mutant tobacco enzyme were introduced into the sensitive version of the maize *als* gene (P165A and W542L) by site-directed mutagenesis in order to produce the *zm-hra* gene encoding the ZM-HRA herbicide tolerant enzyme.

[2] Although both 'ALS' and 'AHAS' are acceptable nomenclature for acetohydroxyacid synthase, recent literature more commonly refers to the enzyme in plants as 'AHAS' (EC 2.2.1.6, formerly EC 4.1.3.18). However, 'ALS' will be used throughout this submission for consistency with the early literature.

Figure 28. Branched Chain Amino Acid Biosynthesis in Plants



Adapted from Coruzzi and Last, 2000.

VI-C. Concentrations of GAT4621 and ZM-HRA Proteins in 98140 Corn

98140 corn contains the *gat4621* and *zm-hra* genes encoding the GAT4621 and ZM-HRA proteins, respectively. The transcription of the *gat4621* gene is under the control of the maize ubiquitin promoter, which directs GAT4621 protein expression constitutively. The transcription of the *zm-hra* gene is under the control of the maize acetolactate synthase promoter, which directs ZM-HRA protein expression constitutively.

Concentrations of the GAT4621 and ZM-HRA proteins in 98140 corn were measured in tissue samples collected from a replicated field study grown at six field locations in North America in 2006. The BC0S1xPH1CA generation of 98140 was planted (Figure 2).

Tissue samples were collected at various developmental growth stages (Ritchie *et al*., 1993). Timepoints were chosen for their relevance to commercial corn production practices. The V9 developmental stage is the stage just after glyphosate application. The R1 stage is the normal developmental stage of pollen shed, and the R4 stage is the normal developmental harvest stage of forage for ensiling. Grain is normally harvested at the R6 stage of development. Samples of leaf and root tissues were collected over the growing season to provide data on the concentrations of GAT4621 and ZM-HRA proteins over time.

Leaf tissue was collected at the V9, R1, R4 and R6 developmental growth stages. Root tissue was collected at the R1, R4 and R6 developmental growth stages. Pollen was collected at the R1 stage of development, and whole plant tissue was collected at the R1 and R6 stages of development. Forage was collected at the R4 stage of development, and grain was collected at the R6 stage of development.

Three replicated samples per tissue per location were collected for 98140 corn, and one sample per tissue per location was collected for control corn. Tissue samples were processed and analyzed using enzyme linked immunosorbent assay (ELISA) methods described in Appendix 3.

Results of the expression analyses are presented in Table 8 for GAT4621 and Table 9 for ZM-HRA. Mean concentrations of GAT4621 protein in various tissues of 98140 corn throughout the growing season ranged from 2.6 ng/mg tissue on a dry weight basis (root at the R6 stage) to 51 ng/mg (leaf at the R1 stage). Mean concentrations of ZM-HRA protein in various tissues of 98140 corn throughout the growing season ranged from below the limit of quantitation (pollen at the R1 stage) to 6.7 ng/mg on a dry weight basis (leaf at the V9 stage). As expected, all GAT4621 and ZM-HRA protein concentrations were below the limit of quantification in control corn tissues.

These results demonstrate that protein concentrations of GAT4621 can be measured at various developmental growth stages in all six tissues analyzed. ZM-HRA protein concentrations can also be measured at various developmental growth stages in all tissues analyzed with the exception of pollen, where the concentration of ZM-HRA protein was below the lower limit of quantitation.

Table 8. Concentration of GAT4621 Protein Measured in 98140 Corn

Tissue	Growth Stage[b]	ng/mg Tissue Dry Weight[a]			
		LLOQ[c]	98140 Corn Mean	98140 Corn Range[d]	Number of samples <LLOQ/Number of Samples Analyzed
Leaf	V9	0.22	44	18 - 67	0/17
	R1		51	38 - 59	0/18
	R4		34	3.3 - 51	0/18
	R6		4.3	0 - 41	9/18
Root	R1	0.11	11	0.63 - 22	0/18
	R4		6.9	0.28 - 14	0/18
	R6		2.6	0 - 11	3/18
Pollen	R1	0.43	13	11 - 18	0/18
Whole Plant	R1	0.072	28	19 - 36	0/18
	R6		3.5	0 - 8.0	2/18
Forage	R4	0.072	16	5.6 - 28	0/18
Grain	R6	0.11	7.9	3.6 - 20	0/18

[a] For results below the sample Lower Limit of Quantitation, a value of zero was assigned for calculation purposes.
[b] Ritchie *et al*., 1993
[c] Lower Limit of Quantitation for the assay and tissue type
[d] Range denotes the lowest and highest individual value across sites.

Table 9. Concentration of ZM-HRA Protein Measured in 98140 Corn

Tissue	Growth Stage[b]	ng/mg Tissue Dry Weight[a]			
		LLOQ[c]	98140 Corn Mean	98140 Corn Range[d]	Number of samples <LLOQ/Number of Samples Analyzed
Leaf	V9	0.54	6.7	0.66 - 11	0/18
	R1		5.9	2.2 - 13	0/18
	R4		5.5	0 - 13	3/18
	R6		0.048	0 - 0.87	17/18
Root	R1	0.27	0.36	0 - 1.3	7/18
	R4		0.17	0 - 0.67	11/18
	R6		0.026	0 - 0.46	17/18
Pollen	R1	0.54	0	0	18/18
Whole Plant	R1	0.36	4.0	2.4 - 5.2	0/18
	R6	0.18	0.23	0 - 0.90	10/18
Forage	R4	0.36	2.7	0.73 - 4.8	0/18
Grain	R6	0.14	0.34	0 - 0.92	1/18

[a] For results below the sample Lower Limit of Quantitation, a value of zero was assigned for calculation purposes.
[b] Ritchie *et al*., 1993
[c] Lower Limit of Quantitation for the assay and tissue type
[d] Range denotes the lowest and highest individual value across sites.

VI-D. Characterization of the GAT4621 and ZM-HRA Proteins

In order to obtain sufficient protein to conduct safety assessment studies, GAT4621 and ZM-HRA were produced in and purified from an *E. coli* protein expression system. For ZM-HRA, the mature form of the protein (excluding the chloroplast transit peptide sequence) was produced.

Prior to the use of the purified microbially produced GAT4621 and ZM-HRA proteins in toxicological and biochemical studies, equivalency of the microbially produced proteins to the 98140 corn-derived proteins was demonstrated. Small amounts of GAT4621 and ZM-HRA proteins were purified from 98140 corn leaf tissue by immunoaffinity chromatography.

Characterization of the microbially produced GAT4621 and ZM-HRA proteins was achieved through the determination of purity (utilizing a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method), concentration (utilizing an amino acid composition analysis method), and identity (utilizing Western blot analysis, electrospray mass spectroscopy, mass determination of tryptic peptides by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS), N-terminal amino acid sequencing, and enzyme activity assay methods).

Equivalence of the microbially expressed and plant-derived GAT4621 and ZM-HRA proteins was achieved using the following analyses:

1) SDS-PAGE to confirm equivalent molecular weight;
2) Western blot analysis to confirm equivalent molecular weight and immunoreactivity;
3) N-terminal amino acid sequence analysis to determine the identity of the proteins;
4) Mass determination of tryptic peptides by MALDI-MS to confirm the identity of the proteins;
5) Glycoprotein staining to determine potential post-translational modification (glycosylation).

A detailed description of the methods used in the equivalency studies and the resulting data are included in Appendix 4.

Utilizing the above analyses, the equivalency of GAT4621 and ZM-HRA proteins expressed in *E. coli* to the GAT4621 and ZM-HRA proteins expressed *in planta* in 98140 corn was demonstrated. Therefore, the GAT4621 and ZM-HRA proteins derived from the microbial expression system were appropriate for utilization in safety assessment studies as a proxy for the GAT4621 and ZM-HRA proteins expressed in 98140 corn plants. Microbial GAT4621 and ZM-HRA were used subsequently for *in vitro* and *in vivo* safety assessment studies summarized in Sections VI-E and VI-F.

VI-E. Summary of the Food and Feed Safety Assessment for the GAT4621 Protein

A food and feed safety assessment was conducted to assess the allergenicity and toxicity potential of the GAT4621 protein. The data and assessment form the basis of the conclusion that the GAT4621 protein is unlikely to cause an allergic reaction in humans or be a toxin in humans or animals, and therefore is safe for human and animal consumption. A detailed assessment of animal and human safety of the GAT4621 protein was submitted to FDA on January 31, 2007 as part of New Protein Consultation 005. The conclusions of the safety assessment are summarized below. Further information on 98140 corn will be submitted to FDA as part of the consultation process for bioengineered foods.

1) The donor organism, *B. licheniformis*, is a common soil bacterium widely distributed in the environment. Due to its ubiquitous presence as spores in soil and dust, *B. licheniformis* is widely known as a contaminant of food but is not associated with any adverse effects.
2) *B. licheniformis* has a history of safe use for the production of food enzymes in the United States, Canada, and Europe (*e.g.*, alpha-amylase, cyclodextrin glycosyltransferase, hemicellulase, proteases, pullulanase), biocontrol agents (FDA, 2001; EU Commission, 2000) and as a probiotic (Kritas *et al*., 2006; Alexopoulos *et al.,* 2004a and b). *B. licheniformis* was determined by EPA to present low risk of adverse effects to human health and the environment and was subsequently granted an exemption for use in certain industrial fermentation processes (Federal Register, 1997).
3) The amino acid sequence of the GAT4621 protein was compared to a database of allergens from the Food Allergy Research and Resource Program (FARRP), University of Nebraska, Allergen Database (Version 7.0, January 2007), which contains the amino acid sequences of known and putative allergenic proteins. Potential identities between the GAT4621 amino acid sequence and those of proteins in the allergen database were evaluated using the FASTA34 sequence alignment program (Pearson and Lipman, 1988) set to the default parameters (word size = 2, scoring matrix = BLOSUM50, gap creation penalty = -10, gap extension penalty = -2, *E* score cutoff = 10). The resulting alignments were returned and reviewed for identities of eight or more contiguous identical amino acid matches as well as identities greater than or equal to 35% over 80 or greater residues. None of the alignments met or exceeded the thresholds. These data indicate the lack of both amino acid identity and immunologically relevant similarities between the GAT4621 protein and known protein allergens.
4) The GAT4621 protein is rapidly hydrolyzed in both simulated gastric and intestinal fluids (less than 30 seconds in simulated gastric fluid containing pepsin at pH 1.2 as demonstrated by SDS-PAGE analysis, and less than 5 minutes in simulated intestinal fluid containing pancreatin at pH 7.5 as demonstrated by SDS-PAGE analysis).
5) Bioinformatic analyses demonstrated that the GAT4621 protein retains the characteristics found in other N-acetyltransferases that are ubiquitous in plants and microorganisms (Neuwald and Landsman, 1997). GAT4621 contains the definitive motif for the GNAT family of N-acetyltransferases (Marchler-Bauer *et al.,* 2005). This superfamily of enzymes is present in all organisms, including plants, mammals, fungi, algae, and bacteria (Dyda *et al*., 2000).
6) No biologically relevant amino acid sequence identities were observed between known protein toxins and the GAT4621 protein sequence.
7) There was no evidence of acute toxicity in mice at a target dose of 2000 mg purified protein preparation per kg of body weight (equivalent to approximately 1640 mg of the full-length GAT4621 protein per kg of body weight). Based on expression levels of GAT4621 protein in 98140 corn grain, a child weighing 10 kg would have to consume 2,076 kg of 98140 corn grain to match the dose used in the mouse acute toxicity test. An adult weighing 60 kg would have to consume 12,456 kg of 98140 corn grain. Based on these simplistic worse-case calculations, it is clear there is a wide margin of safety for the GAT4621 protein, especially if other factors such as market share are taken into account.

In addition, the GAT4621 protein is not glycosylated (see Appendix 4). Results of the safety assessment indicate that the ZM-HRA protein is unlikely to cause an allergic reaction in humans or be a toxin in humans or animals, and therefore is safe for animal and human consumption.

VI-F. Summary of the Food and Feed Safety Assessment for the ZM-HRA protein

A food and feed safety assessment was conducted to assess the allergenicity and toxicity potential of the GAT4621 protein. The data and assessment form the basis of the conclusion that the GAT4621 protein is unlikely to cause an allergic reaction in humans or be a toxin in humans or animals, and therefore is safe for human and animal consumption. A detailed assessment of animal and human safety of the ZM-HRA protein was submitted to FDA on February 13, 2007 as part of New Protein Consultation 006. The conclusions of the safety assessment are summarized below. Further information on 98140 corn will be submitted to FDA as part of the consultation process for bioengineered foods.

1) The donor organism is *Zea mays*. The ZM-HRA protein contains only two amino acids differences from the endogenous maize ALS protein from which it was derived.
2) Corn has a long history of safe food and feed use for humans and animals. Corn is not considered a common allergenic food (Hefle *et al*., 1996; Moneret-Vautrin *et al.,* 1998), although in a few case studies allergenic reactions were reported and corn allergens identified (Pasterello *et al.,* 2000, 2003; Pasini *et al.,* 2002). The maize ALS protein from which the ZM-HRA protein is derived, however, has not been characterized as an allergen (Weichel *et al*., 2006). Herbicide-insensitive ALS proteins are components of certain varieties of several crops (soybean, corn, wheat, rice, canola and sunflower) for which no instances of food allergies to such ALS proteins have been reported.
3) Bioinformatic analyses revealed ZM-HRA to be similar to other acetolactate synthase proteins found in bacteria, fungi, algae and plants (Friden *et al.,* 1985; Falco *et al.,* 1985; Reith and Munholland, 1995; Mazur *et al.,* 1987).
4) The amino acid sequence of the ZM-HRA protein was compared to a database of allergens from the Food Allergy Research and Resource Program (FARRP), University of Nebraska, Allergen Database (Version 7.0, January 2007), which contains the amino acid sequences of known and putative allergenic proteins. Potential identities between the ZM-HRA amino acid sequence and those of proteins in the allergen database were evaluated using the FASTA34 sequence alignment program (Pearson and Lipman, 1988) set to the default parameters (word size = 2, scoring matrix = BLOSUM50, gap creation penalty = -10, gap extension penalty = -2, *E* score cutoff = 10). The resulting alignments were returned and reviewed for identities of eight or more contiguous identical amino acid matches as well as identities greater than or equal to 35% over 80 or greater residues. None of the alignments met or exceeded the thresholds. These data indicate the lack of both amino acid identity and immunologically relevant similarities between the ZM-HRA protein and known protein allergens.
5) The ZM-HRA protein is rapidly hydrolyzed in both simulated gastric and intestinal fluids (less than 30 seconds in simulated gastric fluid containing pepsin at pH 1.2 as demonstrated by SDS-PAGE analysis, and less than 30 seconds in simulated intestinal fluid containing pancreatin at pH 7.5 as demonstrated by SDS-PAGE analysis).
6) No biologically relevant amino acid sequence identities were observed between known protein toxins and the ZM-HRA protein sequence.
7) There was no evidence of acute toxicity in mice at a target dose of 2000 mg purified protein preparation per kg of body weight (equivalent to approximately 1236 mg of full-length ZM-HRA protein per kg of body weight). Based on expression levels of the ZM-HRA protein in 98140 corn grain, a child weighing 10 kg would have to consume 36,353 kg of 98140 corn grain to match the dose used in the mouse acute toxicity test. An adult weighing 60 kg would have to consume 218,118 kg of 98140 corn grain. Based on these simplistic worse-case calculations, it is clear there is a wide margin of safety for the ZM-HRA protein, especially if other factors such as market share are taken into account.

In addition, the ZM-HRA protein is not glycosylated (see Appendix 4). Results of the safety assessment indicate that the ZM-HRA protein is unlikely to cause an allergic reaction in humans or be a toxin in humans or animals, and therefore is safe for animal and human consumption.

VII. Agronomic Performance and Ecological Observations

Agronomic evaluations were conducted to assess the agronomic comparability of 98140 corn to conventional corn. This evaluation also included observed responses to insect and disease stressors. These evaluations form the basis for the conclusion that 98140 corn is comparable to conventional corn and is therefore no more likely to pose a plant pest risk than conventional corn.

The agronomic evaluations were based on both laboratory experiments and replicated, multi-site field trials conducted by agronomists and scientists who are considered experts in the production and evaluation of corn. In each of these assessments, 98140 corn was compared to null segregant near-isoline that did not carry any recombinant DNA.

To evaluate the agronomic characteristics of 98140 corn, data were collected on representative characteristics that influence reproduction and survival of the crop.

VII-A. Germination and Dormancy Evaluations

In order to test germination and potential dormancy, seeds from the BC0S1xPH1CA generation (see Figure 2) of 98140 corn were tested for germination under both cold and warm growing conditions using standard laboratory tests. The control was near-isoline seed.

The cold germination test consisted of four replications with 50 seeds from each of the 98140 and isoline corn. The seeds were placed into a saturated soil/sand mixture and placed in an approximately 10°C chamber for four days. After four days, the seed was moved to an approximately 25°C chamber for a three-day grow-out. After three days, the average percentage of germination was calculated.

The warm germination test consisted of four replications with 50 seeds from each of the 98140 and isoline corn. The seeds were placed between sheets of germination toweling and placed in an approximately 25°C chamber at approximately 90% relative humidity for five days. After five days, the average percentage of germination was calculated.

Germination rates were reported as a percentage of germinating seed as follows: (number of germinated seeds/total seeds)*100.

Seeds that did not germinate during the cold germination test were tested for viability using a Tetrazolium Chloride (TZ) test. The TZ test was developed as a color test for seed viability; viable but non-germinated seed would be an indicator of potential dormancy. The color phase of the test occurs when the colorless testing solution consisting of water or buffer and 2, 3, 5-triphenyltetrazolium chloride is added to the seed. Once this solution penetrates into living cells, the TZ solution is reduced to a reddish (pink), water insoluble compound. The absence or presence of and variation of color characteristics within the tissues permit recognition and location of functional or non-functional tissues within embryo structures. Seed viability was reported as a percentage of non-viable seed as follows: (number of non-viable seeds/total seeds)*100.

Means were calculated for all germination data (Table 10). The warm test germination results between the 98140 and isoline corn lines were similar and were ≥ 95% (met the United States standard for warm test germination results). The cold test germination results between the 98140 and isoline corn lines were similar and were ≥ 80% (met the Pioneer Hi-Bred International, Inc. standard for cold test germination results).

These results indicate that the germination and dormancy characteristics of 98140 corn were not altered when compared to non-transgenic corn. 98140 corn exhibits no dormancy tendencies.

Based on this information, 98140 corn is unlikely to present an increased potential for weediness as compared to conventional corn.

Table 10. Summary of Germination Results for 98140 Corn

Analysis	**98140 Corn**	**Control Corn**
Average Germination Rate[1] (% germination)		
Warm Test	100	100
Cold Test	100	99
Seed Viability (% non-viable seed)		
Warm Test	0	0
Cold Test	0	1

[1] Seed is considered germinated with the uptake of water (*i.e.,* when there is visible elongation of embryonic axis and visible penetration of the structures surrounding the embryo by the radicle).

VII-B. Field Trial Evaluations

98140 corn has been field tested in the United States since 2005 as authorized by USDA permits listed in Appendix 5. The list compiles a number of test sites in diverse regions of the U.S. including the major corn-growing areas and off-season nurseries in Hawaii and Puerto Rico. Agronomic data were collected to assess agronomic comparability as it relates to plant pest potential.

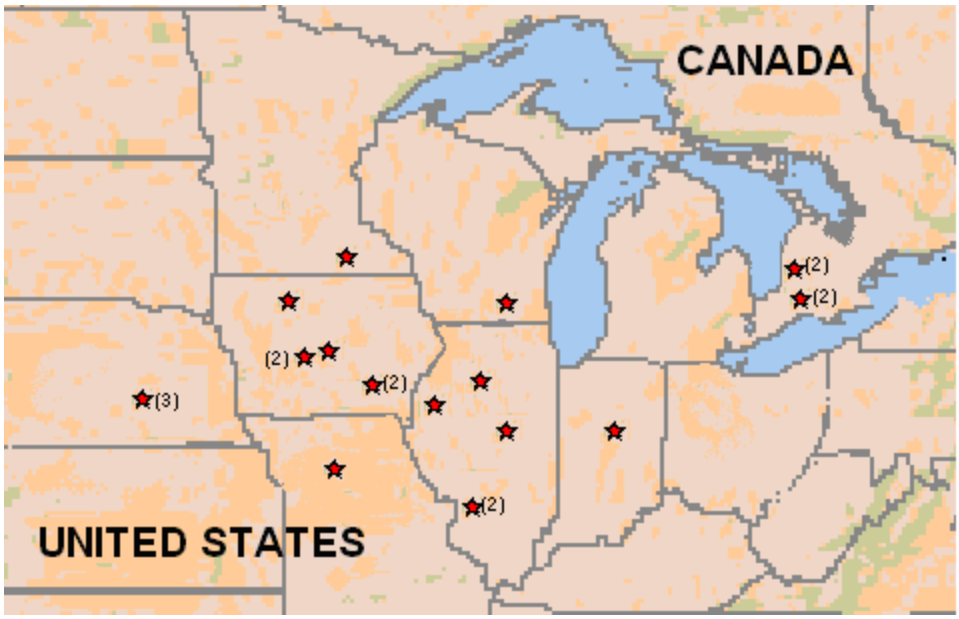
Also throughout the development process, 98140 corn was observed for unexpected differences in responses to abiotic stress (*e.g*, drought, excess moisture, temperature extremes, *etc*.). These monthly observations were qualitative and opportunistic, and indicated that 98140 corn and near-isoline controls were similar with respect to their response to abiotic stress.

Agronomic data were collected from 98140 and control corn within three experiments (denoted A, B, and C) that were conducted at 15 total field locations in 2006 (Figure 29). The trial locations provided a range of environmental and agronomic conditions representative of the major corn growing regions in the U.S. and Canada, where commercial production of 98140 corn is expected. Agronomic practices used to prepare and maintain each field site were characteristic of each respective region.

A statistical analysis of agronomic data was conducted to test for differences in the mean values between the 98140 corn and the near-isoline control (see Appendix 2 for statistical model). When numerous comparisons are being made, it is important to control the rate of false positive results. Since the introduction of the false discovery rate (FDR) approach in the mid-1990's, it has been widely employed across a number of scientific disciplines, including genomics, ecology, medicine, plant breeding, epidemiology, dairy science and signal/image processing (*e.g*., Pawitan *et al*., 2005; Spelman and Bovenhuis, 1998). A false positive result occurs when two means are deemed significantly different when, in fact, they are not. If one uses a 5% type I error rate for each agronomic characteristic measured, then the number of false positives increases as the number of characteristics increase. In order to help manage the false positive rate, the FDR method of Benjamini and Hochberg was applied to account for making multiple comparisons (Benjamini and Hochberg, 1995; Westfall *et al*., 1999). P-values were adjusted accordingly. This resulted in the false positive rate being held to 5%. Both adjusted and unadjusted P-values are provided for the agronomic data.

Table 11 describes the quantitative field agronomic characteristics that were collected during the 2006 growing season.

Figure 29. Map of Locations for Agronomic Data Collection for 98140 Corn



Numbers in parentheses on the map indicate the number of experiments (listed below) that were conducted at a given location.

Experiment A, 2006
Purpose: Agronomic and yield evaluation

1. Algona, IA
2. Johnston, IA
3. York, NE
4. Miami, MO
5. Princeton, IL
6. Champaign, IL
7. Windfall, IN

Experiment B, 2006
Purpose: Agronomic and yield evaluation

1. Richland, IA
2. Bagley, IA
3. Carlyle, IL
4. Wyoming, IL
5. Geneva, MN
6. York, NE
7. Delavan, WI
8. Thorndale, ON, Canada
9. Branchton, ON, Canada

Experiment C, 2006
Purpose: Nutrient composition, protein expression and agronomic evaluation

1. Bagley, IA
2. Richland, IA
3. Carlyle, IL
4. York, NE
5. Thorndale, ON, Canada
6. Branchton, ON, Canada

Table 11. Field Agronomic Characteristics Measured

General Characteristic	Characteristic Measured	Evaluation timing*	Data Description	Scale
Germination / Emergence	Early Population	V2-V4	Number of plants emerged per plot	Actual count per plot
	Seedling Vigor	V2-V4	Visual estimate of average vigor of emerged plants per plot	From 1 to 9, where 1=short plants with small leaves, and 9=tall plants with large leaves
Vegetative Parameters	Plant Height	Approximately R6	Height from the soil surface to the tip of the tassel	Height in cm
	Ear Height	Approximately R6	Height from the soil surface to the base of the primary ear	Height in cm
	Stalk Lodging	Approximately R6	Visual estimate of percent of plants in the plot with stalks broken below the primary ear.	0 to 100%
	Root Lodging	Approximately R6	Visual estimate of percent of plants in the plot leaning approximately 30˚ or more in the first 2 feet (0.6 m) above the soil surface.	0 to 100%
	Final Population	Approximately R6	The number of plants remaining per plot	Actual count per plot
	Stay Green	Approximately R6	Overall plant health	Ranging from 1-9 where 1 = No visible green tissue; 5 = approximately 50% green tissue remaining; 9 = very green, approximately 90% or greater green tissue remaining.
	Percent Moisture	Harvest Maturity	Percent moisture in grain at maturity	Percent moisture
	Test Weight	Harvest Maturity	Weight of a bushel of corn	Weight in pounds

Table 11, continued. Field Agronomic Characteristics Measured

General Characteristic	Characteristic Measured	Evaluation timing*	Data Description	Scale
Reproductive Parameters	Time to Silking	Approximately 50% silking	From the time of planting until approximately 50% of the plants have emerged silks.	Number of accumulated Growing Degree Units (GDU)
	Time to Pollen Shed	Approximately 50% pollen shed	From the time of planting until approximately 50% of the plants have tassels shedding pollen.	Number of accumulated Growing Degree Units (GDU)
	Yield	Approximately R6	Harvest weight per area adjusted to 13% moisture content	Bushels per acre
	Pollen Viability	Approximately 50% pollen shed	Pollen Shape	Percentage of pollen grains with collapsed walls at 0, 30, 60 & 120 minutes
			Pollen Color	Percentage of pollen grains with intense yellow color at 0, 30, 60 & 120 minutes
Ecological Interactions	Disease Incidence	Approximately R6	Visual estimate of foliar disease incidence.	Ranging from 1-9 where 1 = poor disease resistance or high infection; 9 = best disease resistance or low infection.
	Insect Damage	Approximately R6	Visual estimate of insect damage.	Ranging from 1-9 where 1 = poor insect resistance or high damage; 9 = best insect resistance or low damage.

*Refer to Ritchie *et al*., 1993 for a description of corn growth stages.

Experiment A was planted at seven locations in the major corn growing regions of the Midwest during the 2006 growing season. The purpose of Experiment A was to evaluate the agronomic characteristics and yield of 98140 corn (Figure 29). Seed of the T0S2xPH581 generation of 98140 corn was used (Figure 2). The control plants were near isolines of the T0S2xPH581 generation.

The following characteristics were measured: time to silking, time to pollen shed, plant height, ear height, final population, percent moisture, test weight and yield. Descriptions of the characteristics and their measurement are found in Table 11.

Seed was planted in rows 17.5 feet long and 30 inches apart, with 35 seeds per row. The two-row plots were thinned at each location to reach a target final stand. Depending upon the location, final stand counts targets were 27 to 30 plants per row or 54 to 60 plants per two row plot. Normal agronomic practices were employed throughout the growing season. There were three replicates of 98140 corn and three replicates of near-isoline control corn at each location.

Results of Experiment A are summarized in Table 12. For all characteristics measured (time to silking, time to pollen shed, plant height, ear height, final population, percent moisture, test weight and yield), no statistical differences in mean values were seen between 98140 and control corn across locations (adjusted P-value > 0.05). The results from Experiment A indicate that 98140 corn is agronomically comparable to the near-isoline control.

Table 12. Summary of Experiment A: Agronomic Performance of 98140 Corn across Seven Locations

Agronomic Characteristic[a] (Unit)		Control	98140 Corn
Time to Silking (growing degree units)	Mean[b]	1400	1400
	Range[c]	1300-1520	1300-1520
	Adjusted P-value[d]		0.6860
	P-value[e]		0.3293
Time to Pollen Shed (growing degree units)	Mean	1360	1360
	Range	1250-1520	1250-1490
	Adjusted P-value		0.9395
	P-value		0.8889
Plant Height (cm)	Mean	307	307
	Range	277-343	279-335
	Adjusted P-value		0.9395
	P-value		0.9395
Ear Height (cm)	Mean	116	111
	Range	107-130	104-121
	Adjusted P-value		0.6356
	P-value		0.1589
Final Population (number of plants)	Mean	54.6	54.9
	Range	49-60	52-60
	Adjusted P-value		0.686
	P-value		0.343
Moisture (% in grain)	Mean	24.4	25.2
	Range	18.5-33.8	19.2-34.3
	Adjusted P-value		0.1752
	P-value		0.0219
Test Weight (weight in pounds of a bushel of corn)	Mean	54.0	53.8
	Range	50.6-58.7	51.7-58
	Adjusted P-value		0.9395
	P-value		0.8028
Yield (bushels/acre)	Mean	127	129
	Range	96.3-152	79.9-156
	Adjusted P-value		0.9395
	P-value		0.6662

[a] Refer to Table 11 for descriptions of each agronomic characteristic measured.
[b] Least squares mean
[c] Range denotes the lowest and highest individual value across sites.
[d] False Discovery Rate (FDR) adjusted P-value
[e] Non-adjusted P-value

Experiment B was planted at nine locations in the major corn growing region of North America during the 2006 growing season (Figure 29). The purpose of Experiment B was to evaluate the agronomic characteristics and yield of 98140 corn. Seed from the BC0S1xPH1CA generation was used (Figure 2). The control plants were near isolines of the BC0S1xPH1CA generation.

The following characteristics were measured: early population, final population, seedling vigor, time to silking, time to pollen shed, stalk lodging, root lodging, stay green, disease incidence, insect damage, yield, plant height and ear height. Descriptions of the characteristics and their measurement are found in Table 11.

Seed was planted in rows 25 feet long and spacing between rows was approximately 30 inches, with 30 seeds per row. Plants were not thinned. Normal agronomic practices were employed throughout the growing season. Each site employed a randomized complete block design containing four blocks. Each block contained two-row plots of 98140 and control corn. Each two-row plot was bordered on each side by one row of non-transgenic, commercial corn of a similar relative maturity.

Results of Experiment B are summarized in Table 13. For all characteristics measured (early population, final population, seedling vigor, time to silking, time to pollen shed, stalk lodging, root lodging, stay green, disease incidence, insect damage, yield, plant height and ear height), no statistical differences in mean values were seen between 98140 and control corn across locations (adjusted P-value > 0.05). The results from Experiment B indicate that 98140 corn is agronomically comparable to the near-isoline control.

Table 13. Summary of Experiment B: Agronomic Performance of 98140 Corn across Nine Locations

Agronomic Characteristic[a] (Unit)		Control	98140 Corn
Early Population (number of plants)	Mean[b]	54	55
	Range[c]	32 - 61	18 - 60
	Adjusted P-value[d]		0.7017
	P-value[e]		0.4034
Final Population (number of plants)	Mean	53	53
	Range[f]	33 - 64	29 - 66
	Adjusted P-value		0.9459
	P-value		0.8688
Seedling Vigor (1-9 scale)	Mean	8	8
	Range	7 - 9	6 - 9
	Adjusted P-value		0.7017
	P-value		0.3122
Time to Silking (growing degree units)	Mean	1350	1370
	Range	1220 - 1460	1240 - 1490
	Adjusted P-value		0.7017
	P-value		0.105
Time to Pollen Shed (growing degree units)	Mean	1370	1380
	Range	1240 - 1470	1270 - 1470
	Adjusted P-value		0.7017
	P-value		0.4318
Stalk Lodging (% plants broken below primary ear)	Mean	2.1	1.7
	Range	0 - 15	0 - 25
	Adjusted P-value		0.8707
	P-value		0.6028
Root Lodging (% plants leaning ~30˚ or more)	Mean	3.7	3.8
	Range	0 - 30	0 - 30
	Adjusted P-value		0.9575
	P-value		0.9575

Table 13, continued. Summary of Experiment B: Agronomic Performance of 98140 Corn across Nine Locations

Agronomic Characteristic (Unit)		Control	98140 Corn
Stay Green (1-9 scale)	Mean	4	4
	Range	1 - 9	2 - 8
	Adjusted P-value		0.7017
	P-value		0.3972
Disease Incidence (1-9 scale)	Mean	7	7
	Range	3 - 9	4 - 9
	Adjusted P-value		0.7017
	P-value		0.195
Insect Damage (1-9 scale)	Mean	7	7
	Range	5 - 9	5 - 9
	Adjusted P-value		0.7017
	P-value		0.1602
Yield (bushels/acre)	Mean	114	117
	Range	21.4 - 156	15.7 - 175
	Adjusted P-value		0.7017
	P-value		0.3560
Plant Height (cm)	Mean	285	285
	Range	220 - 330	200 - 330
	Adjusted P-value		0.9459
	P-value		0.8731
Ear Height (cm)	Mean	110	110
	Range	68.0 - 155	55.0 - 150
	Adjusted P-value		0.9459
	P-value		0.7515

[a] Refer to Table 11 for descriptions of each agronomic characteristic measured.
[b] Least squares mean
[c] Range denotes the lowest and highest individual value across sites.
[d] False Discovery Rate (FDR) adjusted P-value
[e] Non-adjusted P-value
[f] Final population range exceeded early population range in some instances due to seed that germinated following the early population reading.

Experiment C was planted at six locations in the major corn growing regions of North America during the 2006 growing season. The purpose of Experiment C was to evaluate the agronomic characteristics of 98140 corn and collect tissue samples for transgenic protein levels and nutrient composition analyses (Figure 29). Seed from the BC0S1xPH1CA generation was used (Figure 2). The control plants were near isolines of the BC0S1xPH1CA generation.

The following characteristics were measured: early population, final population, seedling vigor, time to silking, time to pollen shed, stalk lodging, root lodging, stay green, disease incidence, insect damage, plant height, ear height and pollen viability (shape & color) over time. Descriptions of the characteristics and their measurement are found in Table 11.

Pollen collected from Experiment C was evaluated using a magnifying lens for shape and color at various timepoints in order to evaluate pollen viability of 98140 corn and control corn over time. The correlation between visual appearance and viability of pollen is well documented (Luna *et al*., 2001).

Seed was planted in rows 25 feet long and spacing between rows was approximately 30 inches, with 30 seeds per row. Plants were not thinned. Normal agronomic practices were employed throughout the growing season. Each site employed a randomized complete block design containing four blocks, of which 3 blocks were used for analysis of agronomic characteristics. Each block contained two-row plots of 98140 and control corn. Each two-row plot was bordered on each side by one row of non-transgenic, commercial corn of a similar relative maturity.

Results of Experiment C are summarized in Table 14. For all characteristics measured (early population, final population, seedling vigor, time to silking, time to pollen shed, stalk lodging, root lodging, stay green, disease incidence, insect damage, plant height, ear height and pollen viability (shape and color) over time), no statistical differences in mean values were seen between 98140 and control corn across locations (adjusted P-value > 0.05). The results from Experiment C indicate that 98140 corn is agronomically comparable to near-isoline control corn.

Table 14. Summary of Experiment C: Agronomic Performance of 98140 Corn across Six Locations

Agronomic Characteristic[a] (Unit)		Control	98140 Corn
Early Population (number of plants)	Mean[b]	55	55
	Range[c]	36 - 60	41 - 60
	Adjusted P-value[d]		0.9851
	P-value[e]		0.6201
Final Population (number of plants)	Mean	54	54
	Range[f]	37 - 67	39 - 65
	Adjusted P-value		0.9851
	P-value		0.7382
Seedling Vigor (1-9 scale)	Mean	8	8
	Range	7 - 9	7 - 9
	Adjusted P-value		0.586
	P-value		0.0293
Time to Silking (growing degree units)	Mean	1380	1380
	Range	1290 - 1500	1290 - 1520
	Adjusted P-value		0.9851
	P-value		0.9851
Time to Pollen Shed (growing degree units)	Mean	1400	1400
	Range	1310 - 1480	1310 - 1510
	Adjusted P-value		0.9851
	P-value		0.8054
Stalk Lodging (% plants broken below primary ear)	Mean	1.8	1.8
	Range	0 - 5.0	0 - 5.0
	Adjusted P-value		0.9851
	P-value		0.9632
Root Lodging (% plants leaning ~30˚ or more)	Mean	1.2	2.8
	Range	0 - 5.0	0 - 25
	Adjusted P-value		0.9851
	P-value		0.2717
Stay Green (1-9 scale)	Mean	4	4
	Range	1 - 6	1 - 6
	Adjusted P-value		0.9851
	P-value		0.929
Disease Incidence (1-9 scale)	Mean	7	7
	Range	3 - 9	4 - 9
	Adjusted P-value		0.9851
	P-value		0.4618
Insect Damage (1-9 scale)	Mean	7	7
	Range	4 - 9	5 - 9
	Adjusted P-value		0.9851
	P-value		0.9049
Plant Height (cm)	Mean	278	281
	Range	231 - 315	240 - 316
	Adjusted P-value		0.9851
	P-value		0.2368

Table 14, continued. Summary of Experiment C: Agronomic Performance of 98140 Corn across Six Locations

Agronomic Characteristic (Unit)		Control	98140 Corn
Ear Height (cm)	Mean	116	119
	Range	75.0 - 150	85.0 - 155
	Adjusted P-value		0.9851
	P-value		0.2626
Pollen Viability (Shape) - %(0 to 100) pollen grains with collapsed walls			
0 minutes (%)	Mean	6.4	5.3
	Range	0 - 25	0 - 20
	Adjusted P-value		0.9851
	P-value		0.4461
30 minutes (%)	Mean	54	55
	Range	10 - 95	10 - 100
	Adjusted P-value		0.9851
	P-value		0.9578
60 minutes (%)	Mean	80	82
	Range	40 - 100	40 - 100
	Adjusted P-value		0.9851
	P-value		0.2941
120 minutes (%)	Mean	96	95
	Range	80 - 100	80 - 100
	Adjusted P-value		0.9851
	P-value		0.6652
Pollen Viability (Color) - %(0 to 100) pollen grains with intense yellow color			
0 minutes (%)	Mean	10	9.0
	Range	1.0 - 30	1.0 - 30
	Adjusted P-value		0.9851
	P-value		0.3369
30 minutes (%)	Mean	51	52
	Range	25 - 95	20 - 95
	Adjusted P-value		0.9851
	P-value		0.8256
60 minutes (%)	Mean	81	80
	Range	60 - 100	60 - 100
	Adjusted P-value		0.9851
	P-value		0.8733
120 minutes (%)	Mean	96	96
	Range	85 - 100	80 - 100
	Adjusted P-value		0.9851
	P-value		0.6383

[a] Refer to Table 11 for descriptions of each agronomic characteristic measured.
[b] Least squares mean
[c] Range denotes the lowest and highest individual value across sites.
[d] False Discovery Rate (FDR) adjusted P-value
[e] Non-adjusted P-value
[f] Final population range exceeded early population range in some instances due to seed that germinated following the early population reading.

VII-C. Ecological Observations

Ecological observations (plant interactions with insects and diseases) were recorded for all USDA-APHIS permitted field trials of 98140 corn during the 2005 and 2006 growing seasons. Plant breeders and field staff familiar with plant pathology and entomology observed 98140 corn and control lines at least every four weeks for insect and disease pressure and recorded the severity of any stressor seen. Any unexpected differences in response between 98140 corn and various control lines (null segregants near isolines and/or conventional corn lines) were recorded.

The following scale was used when recording observations:

- mild – very little disease or insect injury (<10%) visible;
- moderate – noticeable plant tissue damage (10% to 30%);
- severe – significant plant tissue damage (>30%).

A summary of the insect and disease ecological observations is presented in Appendix 5. In every case, the severity of insect or disease stress on 98140 corn was not qualitatively different from various control lines growing at the same location. These results support the conclusion that the ecological interactions for 98140 corn were comparable to control corn lines with similar genetics or to conventional corn lines.

VII-D. Conclusions on Agronomic Performance and Ecological Observations

98140 corn was observed in laboratory experiments and at 15 field locations to measure agronomic data. Data generated from these studies represent observations that are typically recorded by plant breeders and agronomists to evaluate the characteristics of corn over a broad range of environmental conditions that 98140 corn would encounter. The measured characteristics provide crop biology data useful in establishing a basis to assess agronomic comparability and familiarity of 98140 corn compared to conventional corn in the context of ecological risk assessment.

The agronomic data showed no biologically meaningful differences between 98140 corn and control corn (near isolines of 98140 corn and/or conventional corn lines) with respect to germination/emergence, vegetative growth, reproductive parameters, yield and ecological interactions. These data support the conclusion that 98140 corn is comparable in agronomic characteristics to conventional corn.

Opportunistic monthly observations of all USDA-permitted field trials for responses of 98140 corn to insect and disease stressors showed no unexpected differences from control corn. In addition, assessment of the ecological data detected no biologically significant differences between 98140 corn and control corn lines indicative of a selective advantage that would result in increased weed potential or plant pest risk for 98140 corn.

VIII. Compositional Assessment

Compositional analysis of 98140 corn grain and forage was used to evaluate any changes in the levels of key nutrients, anti-nutrients, and secondary metabolites compared to the null segregant near-isoline control. Along with agronomic data, compositional similarity is a general indicator that 98140 corn will not exhibit unexpected effects with respect to plant pest risk. The U.S. FDA will review the details of the compositional analyses as a component of the safety assessment of 98140 corn.

Comprehensive compositional analyses were performed on forage and grain from 98140 corn and a non-transgenic near-isoline control grown in 2006 at six field locations in corn-growing areas of North America (Experiment C, Figure 29).

In a separate experiment, forage and grain were also collected from four conventional (*i.e.*, non-modified) commercial corn hybrids ("reference hybrids") grown in 2003 at six field locations in corn-growing areas of North America (Bagley, IA; York, NE; Chula, GA; New Holland, OH; Larned, KS and Hereford, PA). The reference hybrids were planted, harvested, processed, and analyzed using similar methods to those employed for the near-isoline control and 98140 corn. Composition analysis of the reference varieties was used to help determine the normal variation for the measured analytes.

In both experiments, seed was planted in rows 25 feet long; spacing between rows was approximately 30 inches, with 30 seeds per row. There were three and four replicates at each location of the 2006 and 2003 studies, respectively. Each experimental block was planted in a randomized complete block design of two-row plots. Each two-row plot was bordered on each side by one row of non-transgenic, commercial corn of a similar relative maturity.

The compositional assessment was conducted in accordance with the OECD consensus document on compositional considerations for new varieties of corn (OECD, 2002). Compositional analyses of grain samples included protein, fat, acid detergent fiber (ADF), neutral detergent fiber (NDF), ash, carbohydrates, fatty acids, amino acids, vitamins and minerals, key anti-nutrients (raffinose, phytic acid and trypsin inhibitor) and key secondary metabolites (furfural, ferulic acid and *p*-coumaric acid). Compositional analyses of forage samples included protein, fat, ADF, NDF, ash, carbohydrates, calcium, phosphorus and amino acids.

Statistical analysis of nutrient composition data was conducted to test for differences in the analyte mean values between 98140 corn and the near-isoline control. (For details of the statistical methodology, refer to Appendix 2.) When numerous analytes are being evaluated on the same samples, controlling false positive outcomes is important. A false positive outcome occurs when an analyte mean of the transgenic line is deemed significantly different from the analyte mean of the control line, when in fact the two means are not different. If one uses a 5% type I error rate for each analyte, then the number of false positives increases as the number of analytes increase. In order to help manage the false positive rate, the false discovery rate (FDR) method of Benjamini and Hochberg was applied to account for making multiple comparisons (Benjamini and Hochberg, 1995; Westfall *et al*., 1999). P-values were adjusted accordingly, resulting in the false positive rate being held to 5%. Both adjusted and non-adjusted P-values are provided in this petition. A significant difference between the mean of 98140 corn and that of the near isoline was established with an FDR-adjusted P-value <0.05.

Using the data obtained from the reference hybrids, a statistical tolerance interval was calculated to contain, with 95% confidence, 99% of the values contained in the population of commercial corn. This statistical tolerance interval and the combined range of values for each analyte from the published literature, where available, provided further context for interpretation of the composition results for 98140 corn. 98140 corn analyte ranges that fell within the tolerance

interval and/or combined literature range for that analyte were considered to be within the range of normal variability of commercial corn hybrids.

VIII-A. Key Nutrients in Corn Grain

In the U.S., corn grain is used for food, feed, and fuel. About half of the corn grown in the U.S. is used domestically for animal feed (primarily beef, poultry, pork and dairy) because of its high nutrient value and relative low cost. Approximately 10% of corn grain is used for food products, including high fructose corn syrup, cereal and starch (NCGA, 2007). The remainder is used for ethanol (18%) or exported (19%).

A1. Proximates in Corn Grain

Proximates were analyzed in 98140 and near-isoline control corn grain. Results are shown in Table 15. No statistically significant differences were observed between the 98140 and control corn mean values for protein, fat, acid detergent fiber (ADF), neutral detergent fiber (NDF), ash and carbohydrates (adjusted P-values were > 0.05).

In conclusion, analysis of proximates in corn grain demonstrates that 98140 corn is comparable to near-isoline and reference corn hybrids.

Table 15. Proximates in Corn Grain

Analyte (% Dry Weight)		Control	98140 Corn	Tolerance Interval	Literature Range[a]
Protein	Mean[b]	10.8	10.6	4.11 - 15.6	6 - 17.3
	Range[c]	8.95 - 14.0	9.14 - 11.9		
	Adjusted P-value[d]		0.8513		
	P-value[e]		0.6939		
Fat	Mean	4.16	4.13	1.04 - 6.48	2.47 - 5.90
	Range	3.18 - 4.89	3.02 - 4.91		
	Adjusted P-value		0.8854		
	P-value		0.7555		
ADF	Mean	3.12	3.08	0.958 - 6.49	1.82 - 11.3
	Range	1.81 - 4.23	1.82 - 4.02		
	Adjusted P-value		0.7978		
	P-value		0.6276		
NDF	Mean	9.94	9.29	2.34 - 20.6	5.59 - 22.6
	Range	6.53 - 13.6	6.24 - 11.6		
	Adjusted P-value		0.7324		
	P-value		0.166		
Ash	Mean	1.54	1.47	0.338 - 2.54	0.616 - 6.28
	Range	1.14 - 2.07	1.05 - 1.76		
	Adjusted P-value		0.7434		
	P-value		0.2226		
Carbohydrates (calculated)	Mean	83.5	83.8	78.2 - 91.6	77.4 - 89.5
	Range	79.3 - 85.8	81.5 - 86.7		
	FDR		0.7681		
	P-value		0.5467		

[a] Literature ranges are taken from published literature for corn (Watson, 1982, 1987; Codex, 1996; Codex, 2001; OECD, 2002 and ILSI, 2006).
[b] Least squares mean
[c] Range denotes the lowest and highest individual value across locations.
[d] False Discovery Rate (FDR) adjusted P-value
[e] Non-adjusted P-value

A2. Fatty Acids in Corn Grain

Levels of 28 fatty acids were measured in 98140 and near-isoline control corn grain. Levels of 17 fatty acids were below the limit of quantitation for the assay: caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), myristoleic acid (C14:1), pentadecanoic acid (C15:0), pentadecenoic acid (C15:1), heptadecenoic acid (C17:1), heptadecadienoic acid (C17:2), γ-linolenic acid (C18:3), nonadecanoic acid (C19:0), eicosadienoic acid (C20:2), eicosatrienoic acid (C20:3), arachidonic acid (C20:4), heneicosanoic acid (C21:0), erucic acid (C22:1) and tricosanoic acid (C23:0). Therefore, no statistical analyses were conducted on these fatty acids and data are not shown.

Results of the fatty acid analysis are shown in Table 16. No statistically significant differences were observed between the 98140 and near-isoline control corn for any of the fatty acid mean values (adjusted P-values were > 0.05).

In conclusion, fatty acid analysis of corn grain demonstrates that 98140 corn is comparable to near-isoline and reference corn hybrids.

Table 16. Major Fatty Acids in Corn Grain

Analyte (% Total Fatty Acids)		Control	98140 Corn	Tolerance Interval[a]	Literature Range[b]
Palmitic acid (C16:0)	Mean[c]	14.1	14.2	4.85 - 19.3	7 - 20.7
	Range[d]	13.2 - 15.6	13.4 - 15.4		
	Adjusted P-value[e]		0.5365		
	P-value[f]		0.076		
Palmitoleic acid (C16:1)	Mean	0.0989	0.0974	NA[g]	0 - 1
	Range	0 - 0.187	0 - 0.164		
	Adjusted P-value		0.9134		
	P-value		0.7916		
Heptadecanoic acid (C17:0)	Mean	0.0645	0.0846	NA	0 - 0.111
	Range	0 - 0.125	0 - 0.150		
	Adjusted P-value		0.4575		
	P-value		0.0367		
Stearic acid (C18:0)	Mean	1.39	1.36	0.635 - 2.04	0 - 4.0
	Range	1.12 - 1.75	1.06 - 1.68		
	Adjusted P-value		0.4575		
	P-value		0.0549		
Oleic acid (C18:1)	Mean	20.8	21.2	0 - 73.4	17.4 - 50
	Range	19.5 - 21.4	20.0 - 22.9		
	Adjusted P-value		0.7434		
	P-value		0.2897		
Linoleic acid (C18:2)	Mean	61.4	61.0	21.4 - 97.3	34.0 - 70
	Range	60.7 - 63.4	59.6 - 62.0		
	Adjusted P-value		0.7434		
	P-value		0.307		
Linolenic acid (C18:3)	Mean	1.21	1.16	0 - 2.91	0 - 2.25
	Range	1.03 - 1.44	1.02 - 1.39		
	Adjusted P-value		0.4575		
	P-value		0.0323		
Arachidic acid (C20:0)	Mean	0.380	0.360	NA	0 - 2
	Range	0.339 - 0.477	0.292 - 0.444		
	Adjusted P-value		0.3725		
	P-value		0.0149		
Eicosenoic acid (C20:1)	Mean	0.328	0.317	NA	0 - 1.92
	Range	0.241 - 0.456	0.240 - 0.501		
	Adjusted P-value		0.7681		
	P-value		0.5218		
Behenic acid (C22:0)	Mean	0.0421	0.0307	NA	0 - 0.5
	Range	0 - 0.196	0 - 0.189		
	Adjusted P-value		0.7434		
	P-value		0.2878		
Lignoceric acid (C24:0)	Mean	0.203	0.151	NA	0 - 0.5
	Range	0 - 0.302	0 - 0.268		
	Adjusted P-value		0.5365		
	P-value		0.0884		

[a] Negative tolerance limits have been set to zero.
[b] Literature ranges are taken from published literature for corn (Watson, 1982, 1987; Codex, 1996; Codex, 2001; OECD, 2002 and ILSI, 2006).
[c] Least squares mean
[d] Range denotes the lowest and highest individual value across locations.
[e] False Discovery Rate (FDR) adjusted P-value
[f] Non-adjusted P-value
[g] Tolerance interval not available (NA).

A3. Total Amino Acids in Corn Grain

Corn grain is generally a good source of essential and non-essential amino acids for most domestic animal species. Total levels of 18 amino acids were measured in 98140 and near-isoline control corn grain.

Results are shown in Table 17. No statistically significant differences were observed between the 98140 corn and near-isoline control corn for any of the amino acid mean values (adjusted P-values were > 0.05).

In conclusion, total amino acid analysis of corn grain demonstrates that 98140 corn is comparable to near-isoline and reference corn hybrids.

Table 17. Total Amino Acids in Corn Grain

Analyte (% Dry Weight)		Control	98140 Corn	Tolerance Interval[a]	Literature Range[b]
Methionine	Mean[c]	0.255	0.254	0.0923 - 0.535	0.10 - 0.468
	Range[d]	0.153 - 0.369	0.142 - 0.345		
	Adjusted P-value[e]		0.9272		
	P-value[f]		0.8655		
Cystine	Mean	0.245	0.249	0.0831 - 0.360	0.08 - 0.514
	Range	0.166 - 0.346	0.186 - 0.344		
	Adjusted P-value		0.8421		
	P-value		0.6737		
Lysine	Mean	0.348	0.352	0.214 - 0.537	0.05 - 0.668
	Range	0.248 - 0.462	0.265 - 0.478		
	Adjusted P-value		0.867		
	P-value		0.7283		
Tryptophan	Mean	0.0653	0.0681	0 - 0.134	0.0271 - 0.215
	Range	0.0566 - 0.0803	0.0592 - 0.0908		
	Adjusted P-value		0.4575		
	P-value		0.0391		
Threonine	Mean	0.455	0.455	0.158 - 0.660	0.224 - 0.666
	Range	0.353 - 0.540	0.350 - 0.556		
	Adjusted P-value		0.9967		
	P-value		0.9834		
Isoleucine	Mean	0.359	0.351	0.121 - 0.532	0.179 - 0.71
	Range	0.305 - 0.485	0.313 - 0.392		
	Adjusted P-value		0.7978		
	P-value		0.625		
Histidine	Mean	0.292	0.286	0.142 - 0.389	0.137 - 0.434
	Range	0.217 - 0.343	0.220 - 0.358		
	Adjusted P-value		0.7593		
	P-value		0.4309		
Valine	Mean	0.483	0.472	0.179 - 0.616	0.21 - 0.855
	Range	0.424 - 0.620	0.437 - 0.549		
	Adjusted P-value		0.7681		
	P-value		0.553		
Leucine	Mean	1.36	1.30	0.333 - 2.10	0.642 - 2.49
	Range	1.11 - 1.96	1.15 - 1.52		
	Adjusted P-value		0.7681		
	P-value		0.4896		
Arginine	Mean	0.496	0.509	0.162 - 0.620	0.119 - 0.64
	Range	0.418 - 0.560	0.426 - 0.572		
	Adjusted P-value		0.7434		
	P-value		0.3093		

Table 17, continued. Total Amino Acids in Corn Grain

Analyte (% Dry Weight)		Control	98140	Tolerance Interval	Literature Range
Phenylalanine	Mean	0.556	0.548	0.180 - 0.774	0.244 - 0.930
	Range	0.461 - 0.804	0.493 - 0.635		
	Adjusted P-value		0.8513		
	P-value		0.7037		
Glycine	Mean	0.381	0.371	0.205 - 0.528	0.184 - 0.539
	Range	0.323 - 0.445	0.333 - 0.438		
	Adjusted P-value		0.7373		
	P-value		0.1966		
Alanine	Mean	0.852	0.816	0.298 - 1.27	0.439 - 1.39
	Range	0.636 - 1.16	0.716 - 0.918		
	Adjusted P-value		0.7681		
	P-value		0.4978		
Aspartic Acid	Mean	0.819	0.797	0.332 - 1.02	0.335 - 1.21
	Range	0.689 - 1.08	0.661 - 0.921		
	Adjusted P-value		0.7593		
	P-value		0.4480		
Glutamic Acid	Mean	2.19	2.10	0.742 - 3.26	0.965 - 3.54
	Range	1.64 - 2.89	1.66 - 2.52		
	Adjusted P-value		0.7533		
	P-value		0.3917		
Proline	Mean	1.02	0.992	0.501 - 1.84	0.462 - 1.63
	Range	0.873 - 1.26	0.854 - 1.19		
	Adjusted P-value		0.7978		
	P-value		0.6071		
Serine	Mean	0.543	0.522	0.209 - 0.780	0.235 - 0.91
	Range	0.464 - 0.734	0.434 - 0.599		
	Adjusted P-value		0.7533		
	P-value		0.3613		
Tyrosine	Mean	0.296	0.284	0.138 - 0.435	0.103 - 0.79
	Range	0.214 - 0.359	0.225 - 0.340		
	Adjusted P-value		0.7434		
	P-value		0.3278		

[a] Negative tolerance limits have been set to zero.
[b] Literature ranges are taken from published literature for corn (Watson, 1982, 1987; Codex, 1996; Codex, 2001; OECD, 2002 and ILSI, 2006).
[c] Least squares mean
[d] Range denotes the lowest and highest individual value across locations.
[e] False Discovery Rate (FDR) adjusted P-value
[f] Non-adjusted P-value

A4. Free Amino Acids in Corn Grain

Amino acids in corn seed are distributed between those that are incorporated into proteins (typically >99%) and those in the free amino acid pool (typically <1%) (Takahashi *et al*., 2003). Free amino acids are found in cells in amounts that vary according to the tissue and to the amino acid. The amino acids that play key roles in the incorporation and transfer of ammonia, such as glutamic acid, aspartic acid, and their amides, are often present in relatively high amounts, but the concentrations of the other free amino acids are very low.

Because a GAT enzyme similar to GAT4621 has been shown to acetylate some amino acids with low efficiency under certain *in vitro* conditions (Siehl *et al.,* 2005), the levels of the free amino acids were measured in 98140 and control corn grain. This analysis was done to confirm that the individual free amino acids amounts and the composition of the free amino acid pool in 98140 corn grain were unchanged from near-isoline control corn grain.

Results are presented in Table 18. No statistically significant differences were observed between the 98140 and near-isoline control corn grain (adjusted P-values were > 0.05) for any of the 23 individual free amino acids or the two related compounds (ethanolamine and ammonia) measured. Literature values and statistical tolerance intervals for free amino acids in corn grain are not available.

In conclusion, free amino acid analysis of corn grain demonstrates that 98140 corn is comparable to near-isoline control corn.

Table 18. Free Amino Acids in Corn Grain

Analyte (mg/g Dry Weight)		Control	98140 Corn
L-Aspartic Acid	Mean[a]	0.235	0.238
	Range[b]	0.00832 - 0.453	0.0117 - 0.524
	Adjusted P-value[c]		0.9513
	P-value[d]		0.933
L-Threonine	Mean	0.035	0.0386
	Range	0.00129 - 0.124	0.00238 - 0.0996
	Adjusted P-value		0.7125
	P-value		0.5083
L-Serine	Mean	0.0509	0.0603
	Range	0.00434 - 0.163	0.00691 - 0.146
	Adjusted P-value		0.4647
	P-value		0.0537
L-Asparagine	Mean	0.333	0.312
	Range	0.000246 - 0.600	0 - 0.555
	Adjusted P-value		0.6672
	P-value		0.227

Table 18, continued. Free Amino Acids in Corn Grain

Analyte (mg/g Dry Weight)		Control	98140 Corn
L-Glutamic Acid	Mean	0.117	0.127
	Range	0.0118 - 0.253	0.00853 - 0.279
	Adjusted P-value		0.7125
	P-value		0.5344
L-Glutamine	Mean	0.0342	0.0381
	Range	0.00188 - 0.117	0 - 0.101
	Adjusted P-value		0.7125
	P-value		0.4146
Cysteine	Mean	0.00750	0.0186
	Range	0 - 0.0278	0 - 0.0359
	Adjusted P-value		0.4647
	P-value		0.0877
L-Proline	Mean	0.488	0.469
	Range	0.00309 - 1.13	0.00336 - 0.907
	Adjusted P-value		0.8422
	P-value		0.7841
L-Glycine	Mean	0.0432	0.0409
	Range	0.00194 - 0.101	0.00150 - 0.0883
	Adjusted P-value		0.7125
	P-value		0.4955
L-Alanine	Mean	0.105	0.109
	Range	0.00199 - 0.300	0.00205 - 0.243
	Adjusted P-value		0.8422
	P-value		0.7035
L-Valine	Mean	0.0387	0.0519
	Range	0 - 0.125	0.00272 - 0.106
	Adjusted P-value		0.4647
	P-value		0.0656
L-Cystine	Mean	0.000225	0.00214
	Range	0 - 0.00406	0 - 0.0386
	Adjusted P-value		0.7125
	P-value		0.3865
L-Methionine	Mean	0.0122	0.0155
	Range	0 - 0.0368	0 - 0.0642
	Adjusted P-value		0.7125
	P-value		0.3856
L-Isoleucine	Mean	0.0218	0.0289
	Range	0.00125 - 0.0701	0.00291 - 0.0634
	Adjusted P-value		0.4923
	P-value		0.1136
Leucine	Mean	0.0378	0.0472
	Range	0.00236 - 0.132	0.00707 - 0.200
	Adjusted P-value		0.7125
	P-value		0.4351

Table 18, continued. Free Amino Acids in Corn Grain

Analyte (mg/g Dry Weight)		Control	98140 Corn
L-Tyrosine	Mean	0.0490	0.0599
	Range	0.000725 - 0.113	0.00547 - 0.0910
	Adjusted P-value		0.4647
	P-value		0.0966
L-Phenylalanine	Mean	0.0312	0.0372
	Range	0.00349 - 0.0803	0.00135 - 0.120
	Adjusted P-value		0.7125
	P-value		0.434
γ-Amino-n-Butyric Acid	Mean	0.192	0.124
	Range	0.00128 - 0.497	0 - 0.286
	Adjusted P-value		0.6772
	P-value		0.3172
Ethanolamine	Mean	0.0198	0.0218
	Range	0 - 0.0420	0 - 0.0363
	Adjusted P-value		0.7125
	P-value		0.4529
Ammonia	Mean	0.0342	0.0480
	Range	0.00964 - 0.0855	0.0209 - 0.165
	Adjusted P-value		0.6068
	P-value		0.1867
L-Ornithine	Mean	0.00788	0.00891
	Range	0 - 0.0201	0 - 0.0661
	Adjusted P-value		0.8422
	P-value		0.7872
L-Tryptophan	Mean	0.00615	0.00570
	Range	0 - 0.0225	0 - 0.0225
	Adjusted P-value		0.8994
	P-value		0.8648
L-Lysine	Mean	0.104	0.155
	Range	0.00505 - 0.381	0.00783 - 0.512
	Adjusted P-value		0.4647
	P-value		0.0983
L-Histidine	Mean	0.0379	0.0468
	Range	0 - 0.0951	0 - 0.0795
	Adjusted P-value		0.3757
	P-value		0.0289[e]
L-Arginine	Mean	0.116	0.247
	Range	0 - 0.326	0 - 0.469
	Adjusted P-value		0.3137
	P-value		0.0122[e]

[a] Least squares mean
[b] Range denotes the lowest and highest individual values across sites
[c] False Discovery Rate (FDR) adjusted P-value
[d] Non-adjusted P-value
[e] Statistically significant difference, non-adjusted P-value <0.05

A5. Acetylated Amino Acids in Corn Grain

Because a similar GAT enzyme has been shown to acetylate some amino acids with low efficiency under certain *in vitro* conditions (Siehl *et al.,* 2005), the levels of the acetylated amino acids N-acetylglutamate (NAG) and N-acetylaspartate (NAA) were measured in 98140 and control corn grain. Refer to Appendix 7 for more information on the potential for certain amino acids to act as substrates for GAT4621.

Results of the acetylated amino acid analyses are shown in Table 19. Mean values for NAA and NAG were statistically higher than those of control corn grain. Literature values and statistical tolerance intervals for NAA and NAG are not available.

NAA and NAG are normal components of human diets. NAA and NAG are present in a variety of foods including eggs, chicken, turkey, beef, bouillon, mushrooms, hydrolyzed yeast, and soy sauce (Pioneer analysis of foods purchased in local grocery stores or through mail order suppliers; Appendix 8). There is no evidence to indicate that exposure to either NAA or NAG from these sources is associated with adverse effects in humans. A poultry study confirmed the nutritional wholesomeness and comparability of feed made from 98140 corn to conventional diets (see Section VIII-E). For additional information about the safety of acetylated amino acids and their presence in commonly consumed foods, see Appendix 8.

In conclusion, analysis of NAA and NAG in corn grain demonstrates that these two acetylated amino acids are elevated in 98140 corn grain when compared to near isoline control corn grain. This observation is not unexpected, given the ability of GAT enzymes to acetylate glutamate and aspartate with low catalytic efficiency. Overall levels of NAA and NAG in 98140 corn grain are still very low (together less than 0.05% on a dry weight basis), and these two acetylated amino acids are components of commonly consumed foods.

Table 19. N-Acetylaspartate and N-Acetylglutamate in Corn Grain

Analyte (% Dry Weight)		Control	98140 Corn
NAA	Mean[a]	0.00009	0.0403
	Range[b]	0.0000098 – 0.000732	0.0103 – 0.0926
	Adjusted P-value[c]		0.0144[f]
	P-value[d]		0.0072
NAG	Mean	0.00005	0.0079
	Range	<0.0000075[e] – 0.0004	0.0000622 – 0.0195
	Adjusted P-value		0.033[f]
	P-value		0.033

[a] Least squares mean
[b] Range denotes the lowest and highest individual value across sites.
[c] False Discovery Rate (FDR) adjusted P-value
[d] Non-adjusted P-value
[e] <Lower Limit of Quantitation (LLOQ); indicates that the values of the sample or samples were detected below the assay's LLOQ. Sample results which were below the LLOQ were assigned a value equal to the LLOQ for statistical analysis.
[f] Statistically significant difference, adjusted P-value < 0.05

Conclusions on Amino Acid Composition of 98140 Corn Grain

The distribution of total amino acids into three categories (amino acids that are incorporated into proteins, free amino acids, and acetylated amino acids) was calculated for 98140 and near-isoline control corn grain (Table 20). As expected, the majority of amino acids are incorporated into proteins, and a small proportion of amino acids are in the free amino acid pool for both 98140 and control corn grain. Together, N-acetylaspartate and N-acetylglutamate make up less than 0.5% of the total amino acids in 98140 corn grain. In addition, the levels of protein in grain for 98140 corn are comparable to near-isoline control corn (Table 15). Therefore, the low levels of acetylation of aspartate and glutamate in 98140 corn (Table 19) are not affecting amino acid incorporation into proteins or the level or composition of the free amino acid pool (Table 18), and 98140 corn is comparable to near-isoline control corn with respect to amino acids.

Table 20. Distribution of Amino Acids in 98140 and Control Corn Grain

Amino Acid Category (mg/g Dry Weight)	Control		98140 Corn	
	Mean (Range)	% Total Amino Acids	Mean (Range)	% Total Amino Acids
Total amino acids[a]	110.15 (87.51 – 463.52)	100%	107.26 (89.24 – 127.66)	100%
Free amino acids	2.158 (0.0587 – 5.299)	1.96%	2.351 (0.0847 – 5.298)	2.19%
Acetylated amino acids NAA + NAG[b]	0.00139 (<0.00017 - 0.0114)	0.0013%	0.482 (0.103 - 1.121)	0.449%
Incorporated amino acids (by subtraction[c])	107.99	98.04%	104.43	97.36%

[a] Concentrations of individual amino acids from Table 17 (% dry weight of tissue) were totaled and converted to mg/g dry weight.
[b] Concentrations of NAA and NAG from Table 19 (% dry weight of tissue) were totaled and converted to mg/g dry weight.
[c] The amount of incorporated amino acids was calculated by subtracting total free amino acids and acetylated amino acids from the total amino acid amount.

A6. Vitamins and Minerals in Corn Grain

Corn is a nutritional source of vitamins and minerals for both humans and animals; therefore vitamins and minerals were measured in 98140 and near-isoline control corn grain. Based on OECD guidance, the following vitamins were analyzed: beta-carotene, vitamin B1 (thiamin), vitamin B2 (riboflavin), vitamin B6 (pyridoxine), vitamin B3 (niacin), folic acid and α-tocopherol (OECD, 2002). The following minerals were also analyzed, based on OECD guidance: calcium, copper, iron, magnesium, phosphorus, potassium, sodium and zinc (OECD, 2002).

Vitamin results are shown in Table 21 and mineral results are shown in Table 22. No statistically significant differences were observed between the 98140 and control corn mean values for any of the vitamins or minerals analyzed (adjusted P-values were > 0.05).

In conclusion, vitamin and mineral analyses of corn grain demonstrate that 98140 corn is comparable to near isoline and reference corn hybrids.

Table 21. Vitamins in Corn Grain

Analyte(% Dry Weight)		Control	98140 Corn	Tolerance Interval[a]	Literature Range[b]
Beta-carotene	Mean[c]	14.9	13.9	0 - 30.9	0.19 - 46.8
	Range[d]	3.86 - 28.8	4.17 - 26.6		
	Adjusted P-value[e]		0.7434		
	P-value[f]		0.2744		
Vitamin B1 (Thiamin)	Mean	3.85	3.28	0 - 33.4	1.26 - 40.0
	Range	2.18 - 4.93	1.85 - 4.58		
	Adjusted P-value		0.075		
	P-value		0.002		
Vitamin B2 (Riboflavin)	Mean	1.05	<1.00[g]	NA[h]	0.25 - 5.6
	Range	<1.00[g] - 1.85	<1.00[g]		
	Adjusted P-value		0.7434		
	P-value		0.337		
Vitamin B6 (Pyridoxine)	Mean	3.80	3.80	NA	3.68 - 11.3
	Range	2.23 - 5.88	2.22 - 5.85		
	Adjusted P-value		1		
	P-value		1		
Vitamin B3 (Niacin)	Mean	18.2	19.8	NA	9.3 - 70
	Range	6.90 - 22.9	14.8 - 25.7		
	Adjusted P-value		0.7434		
	P-value		0.2421		
Folic acid	Mean	1.15	0.800	0.114 - 1.49	0.147 - 683
	Range	0.484 - 4.23	0.470 - 1.24		
	Adjusted P-value		0.7366		
	P-value		0.1866		
α-tocopherol	Mean	4.17	4.42	0 - 53.6	1.5 - 68.7
	Range	<0.500[g] - 9.52	<0.500[g] - 13.6		
	Adjusted P-value		0.7681		
	P-value		0.5389		

[a] Negative tolerance limits have been set to zero.
[b] Literature ranges are taken from published literature for corn (Watson, 1982, 1987; Codex, 1996; Codex, 2001; OECD, 2002 and ILSI, 2006).
[c] Least squares mean
[d] Range denotes the lowest and highest individual value across locations.
[e] False Discovery Rate (FDR) adjusted P-value
[f] Non-adjusted P-value
[g] <Lower Limit of Quantitation (LLOQ); Indicates that the values of the sample or samples were detected below the assay's LLOQ. Sample results which were below the LLOQ were assigned a value equal to the LLOQ for statistical analysis.
[h] Tolerance interval not available (NA).

Table 22. Minerals in Corn Grain

Analyte (% Dry Weight)		Control	98140 Corn	Tolerance Interval[a]	Literature Range[b]
Calcium	Mean[c]	0.00470	0.00475	0 - 0.00961	0.00127 - 0.100
	Range[d]	0.00117 - 0.0103	0.00257 - 0.0105		
	Adjusted P-value[e]		0.9272		
	P-value[f]		0.8952		
Copper	Mean	0.000218	0.000190	0 - 0.00114	0.000073 - 0.00185
	Range	0.000145 - 0.000490	0.000138 - 0.000249		
	Adjusted P-value		0.7533		
	P-value		0.3802		
Iron	Mean	0.00215	0.00222	0.000898 - 0.00274	0.0001 - 0.010
	Range	0.00142 - 0.00362	0.00138 - 0.00352		
	Adjusted P-value		0.7681		
	P-value		0.5442		
Magnesium	Mean	0.149	0.140	0.0613 - 0.193	0.0594 - 1.00
	Range	0.103 - 0.195	0.0607 - 0.185		
	Adjusted P-value		0.7533		
	P-value		0.3854		
Phosphorus	Mean	0.379	0.363	0.103 - 0.533	0.147 - 0.750
	Range	0.261 - 0.463	0.181 - 0.501		
	Adjusted P-value		0.7593		
	P-value		0.4391		
Potassium	Mean	0.459	0.467	0 - 0.835	0.181 - 0.720
	Range	0.270 - 0.699	0.251 - 0.766		
	Adjusted P-value		0.9272		
	P-value		0.8535		
Sodium	Mean	0.000949	0.00140	0 - 0.000999	0.0 - 0.150
	Range	0.0000417 - 0.00334	0.0000417 - 0.00509		
	Adjusted P-value		0.7324		
	P-value		0.1489		
Zinc	Mean	0.00189	0.00181	0.00113 - 0.00254	0.00065 - 0.00372
	Range	0.00133 - 0.00293	0.00101 - 0.00291		
	Adjusted P-value		0.7593		
	P-value		0.4155		

[a] Negative tolerance limits have been set to zero.
[b] Literature ranges are taken from published literature for corn (Watson, 1982, 1987; Codex, 1996; Codex, 2001; OECD, 2002 and ILSI, 2006).
[c] Least squares mean
[d] Range denotes the lowest and highest individual value across locations.
[e] False Discovery Rate (FDR) adjusted P-value
[f] Non-adjusted P-value

VIII-B. Key Anti-nutrients in Corn Grain

Corn grain contains several key anti-nutrients: raffinose, phytic acid and trypsin inhibitor (OECD, 2002). Raffinose is a low molecular weight carbohydrate that is non-digestible. Phytic acid binds most of the phosphorus in corn, which results in reduced bioavailability of phosphorus for non-ruminant animals. In addition, phytic acid chelates mineral nutrients including calcium, magnesium, potassium, iron and zinc, rendering them unavailable to monogastric animals. Trypsin inhibitor can interfere with the digestion of proteins, resulting in decreased animal growth.

Levels of key anti-nutrients were measured in 98140 corn and near-isoline control grain. Results are shown in Table 23. No statistically significant differences were observed between the mean values for 98140 and near-isoline control corn for any of the anti-nutrient measured (adjusted P-values were > 0.05).

In conclusion, anti-nutrient analysis of corn grain demonstrates that 98140 corn is comparable to near isoline control corn and reference corn hybrids.

Table 23. Key Anti-nutrients in Corn Grain

Analyte (% Dry Weight or as Indicated)		Control	98140 Corn	Tolerance Interval[a]	Literature Range[b]
Raffinose	Mean[c]	0.168	0.163	0 - 0.495	0.020 - 0.320
	Range[d]	<0.160[g] - 0.226	<0.160[g] - 0.205		
	Adjusted P-value[e]		0.7533		
	P-value[f]		0.3629		
Phytic acid	Mean	0.821	0.825	0.188 - 1.29	0.111 - 1.57
	Range	0.651 - 1.22	0.629 - 1.09		
	Adjusted P-value		0.9272		
	P-value		0.9025		
Trypsin Inhibitor (TIU[h]/mg)	Mean	3.69	3.61	1.26 - 5.05	1.09 - 7.18
	Range	2.47 - 5.80	2.61 - 5.41		
	Adjusted P-value		0.7593		
	P-value		0.4655		

[a] Negative tolerance limits have been set to zero.
[b] Literature ranges are taken from published literature for corn (Watson, 1982, 1987; Codex, 1996; Codex, 2001; OECD, 2002 and ILSI, 2006).
[c] Least squares mean
[d] Range denotes the lowest and highest individual value across locations.
[e] False Discovery Rate (FDR) adjusted P-value
[f] Non-adjusted P-value
[g] <Lower Limit of Quantitation (LLOQ); Indicates that the values of the sample or samples were detected below the assay's LLOQ. Sample results which were below the LLOQ were assigned a value equal to the LLOQ for statistical analysis.
[h] Abbreviation: TIU = trypsin inhibitor units

VIII-C. Key Secondary Plant Metabolites in Corn Grain

Secondary plant metabolites are neither nutrients nor anti-nutrients, but can be analyzed as indicators of the absence of unintended effects of the genetic modification on metabolism (OECD, 2002). Characteristic plant metabolites in corn are furfural and phenolic acids (ferulic acid and *p*-coumaric acid).

Furfural, *p*-coumaric acid and ferulic acid were measured in 98140 and near-isoline control corn grain. Results are shown in Table 24. No statistically significant differences were observed between the mean values for 98140 and near-isoline control corn for any of the secondary plant metabolites measured (adjusted P-values were > 0.05).

In conclusion, metabolite analysis of corn grain demonstrates that 98140 corn is comparable to near-isoline control corn and reference corn hybrids.

Table 24. Key Secondary Plant Metabolites in Corn Grain

Analyte (% Dry Weight)		Control	98140 Corn	Tolerance Interval[a]	Literature Range[b]
Furfural	Mean[c]	0.000119	0.000127	NA[g]	0 - 0.000634
	Range[d]	<0.000100[h] - 0.000367	<0.000100[h] - 0.000412		
	Adjusted P-value[e]		0.7978		
	P-value[f]		0.609		
p-Coumaric acid	Mean	0.0109	0.00977	0 - 0.0415	0.003 - 0.0576
	Range	0.00450 - 0.0182	0.00547 - 0.0137		
	Adjusted P-value		0.7593		
	P-value		0.4657		
Ferulic acid	Mean	0.126	0.117	0.0585 - 0.300	0.02 - 0.389
	Range	<0.0400[h] - 0.239	<0.0400[h] - 0.193		
	Adjusted P-value		0.7978		
	P-value		0.5942		

[a] Negative tolerance limits have been set to zero
[b] Literature ranges are taken from published literature for corn (Watson, 1982, 1987; Codex, 1996; Codex, 2001; OECD, 2002 and ILSI, 2006).
[c] Least squares mean
[d] Range denotes the lowest and highest individual value across sites.
[e] False Discovery Rate (FDR) adjusted P-value
[f] Non-adjusted P-value
[g] Tolerance interval not available (NA).
[h] <Lower Limit of Quantitation (LLOQ); Indicates that the values of the sample or samples were detected below the assay's LLOQ. Sample results which were below the LLOQ were assigned a value equal to the LLOQ for statistical analysis.

VIII-D. Key Nutrients in Corn Forage

Corn silage is an important feed ingredient for feedlot cattle and dairy cattle. In the U.S., approximately 10% of the corn crop is harvested as forage. The whole corn plant contains about one and one-half times the nutrients of the grain, and the ensiling process preserves more than 90% of the nutrients (OECD, 2002).

D1. Proximates and Minerals in Corn Forage

Protein, fat, ADF, NDF, ash, carbohydrates and the minerals calcium and phosphorus were measured in 98140 and near-isoline control corn forage.

Results are shown in Table 25. For each analyte measured, there was no statistical difference between 98140 corn and the near-isoline control corn (adjusted P-values were > 0.05).

In conclusion, proximate and mineral analysis of corn forage demonstrated that 98140 corn forage is comparable to near-isoline and reference corn forage.

Table 25. Proximates and Minerals in Corn Forage

Analyte (% Dry Weight)		Control	98140 Corn	Tolerance Interval[a]	Literature Range[b]
Protein	Mean[c]	7.41	7.70	1.60 - 14.8	3.14 - 15.9
	Range[d]	4.41 - 10.1	4.18 - 9.77		
	Adjusted P-value[e]		0.7434		
	P-value[f]		0.2959		
Fat	Mean	3.03	2.83	0.998 - 3.92	0.296 - 6.7
	Range	1.99 - 4.02	1.78 - 3.61		
	Adjusted P-value		0.5365		
	P-value		0.093		
ADF	Mean	30.8	31.0	12.9 - 48.7	16.1 - 47.4
	Range	26.1 - 35.9	27.1 - 34.7		
	Adjusted P-value		0.9272		
	P-value		0.8728		
NDF	Mean	50.4	51.7	21.3 - 81.9	20.3 - 63.7
	Range	44.9 - 57.4	45.9 - 55.9		
	Adjusted P-value		0.4575		
	P-value		0.0471		
Ash	Mean	4.42	4.25	0 - 9.97	1.3 - 10.5
	Range	3.12 - 5.78	2.99 - 5.57		
	Adjusted P-value		0.7434		
	P-value		0.2253		
Carbohydrates (calculated)	Mean	85.1	85.2	74.5 - 95.0	76.4 - 92.1
	Range	80.6 - 89.3	81.9 - 90.1		
	FDR		0.9272		
	P-value		0.8657		
Calcium	Mean	0.250	0.226	0 - 0.529	0.0714 - 0.6
	Range	0.137 - 0.336	0.143 - 0.388		
	Adjusted P-value		0.5365		
	P-value		0.0846		
Phosphorus	Mean	0.207	0.205	0 - 0.542	0.0936 - 0.55
	Range	0.152 - 0.267	0.159 - 0.303		
	Adjusted P-value		0.9272		
	P-value		0.8616		

[a] Negative tolerance limits have been set to zero.
[b] Literature ranges are taken from published literature for corn (Watson, 1982 and ILSI, 2006).
[c] Least squares mean
[d] Range denotes the lowest and highest individual value across locations.
[e] False Discovery Rate (FDR) adjusted P-value
[f] Non-adjusted P-value

D2. Total Amino Acids in Corn Forage

Total amino acids were measured in 98140 and near-isoline control corn forage. The results are shown in Table 26. There were no statistical differences between the mean values of 98140 corn and the near-isoline control corn (adjusted P-values were > 0.05) for all 18 of the amino acids measured. Literature values and statistical tolerance intervals for amino acids in corn forage were not available.

In conclusion, amino acid analysis of corn forage demonstrated that 98140 corn forage is comparable to near-isoline and reference corn forage.

Table 26. Total Amino Acids in Corn Forage

Analyte (% Dry Weight)		Control	98140 Corn
Methionine	Mean[a]	0.151	0.152
	Range[b]	0.0871 - 0.215	0.0873 - 0.188
	Adjusted P-value[c]		0.9078
	P-value[d]		0.8574
Cystine	Mean	0.108	0.0989
	Range	0.0654 - 0.168	0.0535 - 0.161
	Adjusted P-value		0.5062
	P-value		0.242
Lysine	Mean	0.217	0.235
	Range	0.134 - 0.300	0.146 - 0.296
	Adjusted P-value		0.5062
	P-value		0.2531
Tryptophan	Mean	0.0679	0.0637
	Range	0.0318 - 0.105	0.0270 - 0.0901
	Adjusted P-value		0.5062
	P-value		0.185
Threonine	Mean	0.290	0.313
	Range	0.185 - 0.380	0.210 - 0.364
	Adjusted P-value		0.5126
	P-value		0.3066
Isoleucine	Mean	0.243	0.273
	Range	0.165 - 0.318	0.166 - 0.312
	Adjusted P-value		0.229
	P-value		0.0539
Histidine	Mean	0.135	0.152
	Range	0.0962 - 0.175	0.112 - 0.184
	Adjusted P-value		0.229
	P-value		0.0508
Valine	Mean	0.320	0.366
	Range	0.219 - 0.420	0.227 - 0.431
	Adjusted P-value		0.229
	P-value		0.0602

Table 26, continued. Total Amino Acids in Corn Forage

Analyte (% Dry Weight)		Control	98140 Corn
Leucine	Mean	0.608	0.642
	Range	0.445 - 0.812	0.442 - 0.806
	Adjusted P-value		0.5926
	P-value		0.4609
Arginine	Mean	0.268	0.289
	Range	0.189 - 0.356	0.225 - 0.353
	Adjusted P-value		0.5126
	P-value		0.3702
Phenylalanine	Mean	0.321	0.346
	Range	0.194 - 0.418	0.218 - 0.404
	Adjusted P-value		0.5062
	P-value		0.2452
Glycine	Mean	0.293	0.331
	Range	0.198 - 0.369	0.217 - 0.410
	Adjusted P-value		0.229
	P-value		0.0636
Alanine	Mean	0.527	0.561
	Range	0.317 - 0.711	0.342 - 0.727
	Adjusted P-value		0.618
	P-value		0.515
AsparticAcid	Mean	0.619	0.745
	Range	0.352 - 0.926	0.467 - 0.966
	Adjusted P-value		0.229
	P-value		0.0583
Glutamic Acid	Mean	1.02	1.04
	Range	0.664 - 1.48	0.800 - 1.28
	Adjusted P-value		0.9154
	P-value		0.9154
Proline	Mean	0.486	0.499
	Range	0.334 - 0.653	0.390 - 0.619
	Adjusted P-value		0.7551
	P-value		0.6712
Serine	Mean	0.316	0.343
	Range	0.160 - 0.439	0.225 - 0.422
	Adjusted P-value		0.5126
	P-value		0.3489
Tyrosine	Mean	0.141	0.151
	Range	0.0820 - 0.197	0.127 - 0.200
	Adjusted P-value		0.5126
	P-value		0.3144

[a] Least squares mean
[b] Range denotes the lowest and highest individual value across locations.
[c] False Discovery Rate (FDR) adjusted P-value
[d] Non-adjusted P-value

D3. Free Amino Acids in Corn Forage

Amino acids in corn seed are distributed between those which are incorporated into proteins and those in the free amino acid pool (Takahashi *et al*., 2003). Free amino acids are found in cells in amounts that vary according to the tissue and to the amino acid.

In order to assess whether acetylation of aspartate and glutamate affected the overall amount or composition of the free amino acid pool, individual free amino acid levels were measured in 98140 and near-isoline control corn forage.

Results of the analysis are presented in Table 27. No statistically significant differences were observed between the 98140 and near-isoline control corn forage (adjusted P-values were > 0.05) for any of the 23 individual free amino acids or two related compounds (ethanolamine and ammonia) measured. Literature values and statistical tolerance intervals for free amino acids in corn forage are not available.

In conclusion, free amino acid analysis of corn forage demonstrates that 98140 corn forage is comparable to near-isoline corn forage.

Table 27. Free Amino Acids in Corn Forage

Analyte (mg/g Dry Weight)		Control	98140 Corn
L-Aspartic Acid	Mean[a]	0.819	0.989
	Range[b]	0.467 - 1.29	0.386 - 1.80
	Adjusted P-value [c]		0.4647
	P-value[d]		0.0874
L-Threonine	Mean	0.212	0.232
	Range	0.115 - 0.358	0.107 - 0.352
	Adjusted P-value		0.6772
	P-value		0.3157
L-Serine	Mean	0.264	0.284
	Range	0.175 - 0.474	0.142 - 0.500
	Adjusted P-value		0.7125
	P-value		0.4769
L-Asparagine	Mean	0.375	0.442
	Range	0 - 0.903	0 - 0.991
	Adjusted P-value		0.6772
	P-value		0.3241
L-Glutamic Acid	Mean	0.399	0.381
	Range	0.186 - 0.711	0.249 - 0.551
	Adjusted P-value		0.8297
	P-value		0.6542

Table 27, continued. Free Amino Acids in Corn Forage

Analyte (mg/g Dry Weight)		Control	98140 Corn
L-Glutamine	Mean	0.154	0.182
	Range	0.0477 - 0.397	0.0376 - 0.403
	Adjusted P-value		0.6672
	P-value		0.2566
Cysteine	Mean	0.0174	0.0208
	Range	0 - 0.0524	0 - 0.0535
	Adjusted P-value		0.7125
	P-value		0.4617
L-Proline	Mean	0.309	0.320
	Range	0.236 - 0.399	0.219 - 0.523
	Adjusted P-value		0.7125
	P-value		0.5290
L-Glycine	Mean	0.0705	0.0689
	Range	0.0578 - 0.0888	0.0442 - 0.117
	Adjusted P-value		0.8422
	P-value		0.7862
L-Alanine	Mean	0.664	0.647
	Range	0.308 - 1.22	0.324 - 0.954
	Adjusted P-value		0.8422
	P-value		0.7936
L-Valine	Mean	0.205	0.287
	Range	0.00274 - 0.331	0.150 - 0.541
	Adjusted P-value		0.4647
	P-value		0.0911
L-Cystine	Mean	0.0340	0.00943
	Range	0 - 0.312	0 - 0.0649
	Adjusted P-value		0.7125
	P-value		0.5020
L-Methionine	Mean	0.0193	0.0159
	Range	0.00956 - 0.0290	0.00613 - 0.0284
	Adjusted P-value		0.6672
	P-value		0.2532
L-Isoleucine	Mean	0.115	0.116
	Range	0.0762 - 0.167	0.0704 - 0.154
	Adjusted P-value		0.8422
	P-value		0.7499
L-Leucine	Mean	0.186	0.203
	Range	0.130 - 0.256	0.136 - 0.321
	Adjusted P-value		0.6672
	P-value		0.2386
L-Tyrosine	Mean	0.145	0.144
	Range	0.0688 - 0.348	0.0691 - 0.317
	Adjusted P-value		0.9561
	P-value		0.9561

Table 27, continued. Free Amino Acids in Corn Forage

Analyte (mg/g Dry Weight)		Control	98140 Corn
L-Phenylalanine	Mean	0.102	0.0992
	Range	0.0616 - 0.156	0.0588 - 0.162
	Adjusted P-value		0.8389
	P-value		0.6776
γ-Amino-n-Butyric Acid	Mean	0.0629	0.0575
	Range	0.0381 - 0.0962	0.0337 - 0.0893
	Adjusted P-value		0.6068
	P-value		0.1763
Ethanolamine[e]	Mean	0.126	0.191
	Range	0.0737 - 0.238	0.0947 - 0.346
	Adjusted P-value		0.3137
	P-value		0.0181
Ammonia[e]	Mean	0.0779	0.0727
	Range	0.0395 - 0.227	0.0537 - 0.113
	Adjusted P-value		0.8297
	P-value		0.6454
L-Ornithine	Mean	0.00513	0.00294
	Range	0 - 0.0298	0 - 0.00821
	Adjusted P-value		0.6010
	P-value		0.1618
L-Tryptophan	Mean	0.0243	0.0203
	Range	0.0112 - 0.0678	0.00781 - 0.0332
	Adjusted P-value		0.6772
	P-value		0.3229
L-Lysine	Mean	0.111	0.0953
	Range	0.0635 - 0.207	0.0552 - 0.165
	Adjusted P-value		0.601
	P-value		0.1577
L-Histidine	Mean	0.0282	0.0248
	Range	0.0173 - 0.0513	0.0145 - 0.0544
	Adjusted P-value		0.6772
	P-value		0.3256
L-Arginine	Mean	0.108	0.115
	Range	0.0566 - 0.168	0.0593 - 0.183
	Adjusted P-value		0.7125
	P-value		0.4820

[a] Least squares mean
[b] Range denotes the lowest and highest individual values across sites
[c] False Discovery Rate (FDR) adjusted P-value
[d] Non-adjusted P-value
[e] Ammonia and ethanolamine are not amino acids but are typically measured as part of a free amino acid analysis. γ-amino-n-butyric acid and ornithine are amino acids but are not incorporated into proteins. All four compounds are included in the total free amino acid calculation.

D4. Acetylated Amino Acids in Corn Forage

Because a similar GAT enzyme has been shown to acetylate some amino acids with low efficiency under certain *in vitro* conditions (Siehl *et al.,* 2005), the levels of the acetylated amino acids N-acetylglutamate (NAG) and N-acetylaspartate (NAA) were measured in 98140 and near-isoline control corn forage. Refer to Appendix 7 for more information on the potential for certain amino acids to act as substrates for GAT4621.

Results of the acetylated amino acid analyses are shown in Table 28. The mean values for NAA and NAG in 98140 corn forage were statistically higher than those of near-isoline control corn forage (adjusted P-value was <0.05). Literature values and statistical tolerance intervals for NAA and NAG are not available.

Beef and dairy cattle are the primary consumers of corn forage and silage. Because microorganisms in the rumen make microbial protein using nitrogen from both protein and non-protein sources, NAA and NAG are expected to be readily metabolized in the rumen. A poultry study confirmed the nutritional wholesomeness and comparability of feed made from 98140 corn to conventional diets (see Section VIII-E). For additional information about the safety of acetylated amino acids and their presence in commonly consumed foods, see Appendix 8.

In conclusion, analysis of N-acetylaspartate and N-acetylglutamate in corn forage demonstrates that these two acetylated amino acids are elevated in 98140 corn forage when compared to near-isoline control corn forage. This observation is not unexpected, given the ability of GAT enzymes to acetylate glutamate and aspartate with low catalytic efficiency. Overall levels of NAA and NAG in 98140 corn forage are still very low (less than 0.08% on a dry weight basis).

Table 28. N-Acetylaspartate and N-Acetylglutamate in Corn Forage

Analyte (% Dry Weight)		Control	98140 Corn
NAA	Mean[a]	0.0005	0.0650
	Range[b]	0.00006 – 0.00447	0.0387 – 0.1620
	Adjusted P-value[c]		0.0002[e]
	P-value[d]		0.0001
NAG	Mean	0.0006	0.0093
	Range	0.00005 – 0.0022	0.0040 – 0.0147
	Adjusted P-value		0.0002[e]
	P-value		0.0001

[a] Least squares mean
[b] Range denotes the lowest and highest individual value across sites.
[c] False Discovery Rate (FDR) adjusted P-value
[d] Non-adjusted P-value
[e] Statistically significant difference, adjusted P-value < 0.05

Conclusions on Amino Acid Composition of 98140 Corn Forage

The distribution of total amino acids into three categories (amino acids that are incorporated into proteins, free amino acids, and acetylated amino acids) was calculated for 98140 and near-isoline control corn forage (Table 29). As expected, the majority of amino acids are incorporated into proteins, and a small proportion of amino acids are in the free amino acid pool for both 98140 and control corn forage. Together, N-acetylaspartate and N-acetylglutamate make up less than 1.2% of the total amino acids in 98140 corn forage. In addition, the levels of protein in grain for 98140 corn are comparable to near-isoline control corn (Table 25). Therefore, the low levels of acetylation of aspartate and glutamate in 98140 corn (Table 28) are not affecting amino acid incorporation into proteins or the level or composition of the free amino acid pool (Table 27), and 98140 corn is comparable to near-isoline control corn with respect to amino acids.

Table 29. Distribution of Amino Acids in 98140 and Control Corn Forage

Amino Acid Category (mg/g Dry Weight)	Control		98140 Corn	
	Mean (Range)	% Total Amino Acids	Mean (Range)	% Total Amino Acids
Total amino acids[a]	61.31 (39.19 – 84.42)	100%	66.01 (44.82 – 82.13)	100%
Free amino acids	4.634 (2.185 – 8.409)	7.56%	5.021 (2.259 – 8.642	7.61%
Acetylated amino acids NAA + NAG[b]	0.0110 0.0011 - 0.0667	0.018%	0.7430 0.4270 - 1.767	1.13%
Incorporated amino acids (by subtraction[c])	56.67	92.4%	60.25	91.3%

[a] Concentrations of individual amino acids from Table 26 (% dry weight of tissue) were totaled and converted to mg/g dry weight.
[b] Concentrations of NAA and NAG from Table 28 (% dry weight of tissue) were totaled and converted to mg/g dry weight.
[c] The amount of incorporated amino acids was calculated by subtracting total free amino acids and acetylated amino acids from the total amino acid amount.

VIII-E. Poultry Feeding Study

To assess the wholesomeness of 98140 corn grain when used as animal feed, a 42-day study was conducted in rapidly growing broiler chickens. The broiler chicken is a recognized model for assessing the wholesomeness of feedstuffs (ILSI, 2003).

The nutritional comparability of 98140 corn was evaluated by comparing growth performance and carcass yield variables of broiler chickens fed diets containing grain from the BC0S1xPH1CA generation of 98140 corn (see Figure 2) with those fed diets produced with grain from near-isoline control corn.

Diets produced from control corn, 98140 corn, and three non-transgenic commercial corn hybrids were fed to Ross x Cobb broilers (n = 120/group, 50% male and 50% female) for a period of 42 days. Diets were fed in three phases in accordance with standard commercial poultry farming practice: starter (days 0-21), grower (days 22-35), and finisher (days 36-42). Starter diets contained 58.5% corn grain, grower diets contained 64% corn grain, and finisher diets contained 71.5% corn grain. Body weights were collected and feed intakes calculated every seven days during the growing period; weight gain, feed intake, and corrected feed:gain ratio (feed efficiency) were calculated for days 0 to 42. Standard carcass and organ yield data were determined at the end of the feeding trial. Growth performance and carcass trait tolerance intervals were constructed using data from reference (non-transgenic commercial corn) groups. Statistical differences between broilers fed control corn and 98140 corn were evaluated at $P < 0.05$. Data from control and 98140 corn treatment groups were also evaluated to determine if observed values were within the statistical tolerance intervals from this study.

No statistically significant differences were observed in mortality, weight gain, feed efficiency (corrected for mortalities), and carcass yield variables between broilers consuming diets produced with 98140 corn and those consuming diets produced with near isoline control corn. Additionally, all response variables evaluated in control and 98140 corn groups fell within the statistical tolerance intervals of the values observed in broilers fed diets produced with the reference corn hybrids. Based on the results from this study, 98140 corn was nutritionally comparable to control corn and commercial corn hybrids.

VIII-F. Conclusions on Compositional Assessment of 98140 Corn

Extensive nutritional compositional analyses of grain and forage were conducted to evaluate the composition of 98140 corn as compared to a non-transgenic near isoline and four conventional corn hybrids. In total, data from 83 different analytical components (83 in grain, 53 of these in forage) were presented. Compositional analysis of 98140 corn was used to evaluate any changes in the levels of key nutrients, anti-nutrients or secondary metabolites.

Compositional analyses of grain included protein, fat, acid detergent fiber (ADF), neutral detergent fiber (NDF), ash, carbohydrates, fatty acids, total amino acids, free amino acids, two acetylated amino acids, key anti-nutrients (raffinose, phytic acid and trypsin inhibitor) and key secondary metabolites (furfural, ferulic acid and *p*-coumaric acid). Compositional analyses of forage included protein, fat, ADF, NDF, carbohydrates, calcium, phosphorus, total amino acids, free amino acids and two acetylated amino acids. Based on the compositional evaluation, the grain and forage of 98140 corn are considered to be comparable to conventional corn with respect to nutrient composition.

Levels of two acetylated amino acids, aspartate and glutamate, were elevated in 98140 corn grain and forage. This was not unexpected because GAT proteins are known to acetylate certain amino acids under specific conditions *in vitro*. Levels of N-acetylaspartate and N-acetylglutamate were very low in 98140 corn grain and forage (together less than 0.05% in grain and 0.08% in forage, on a dry weight basis; together less than 0.5% of the total amino acids in grain and less than 1.2% of the total amino acids in forage). These two analytes are components of commonly consumed foods including eggs, chicken, turkey, and beef.

In conclusion, 98140 corn is comparable to near-isoline control corn and commercial corn hybrids with respect to nutrient composition. A 42-day poultry broiler study confirmed the wholesomeness and nutritional comparability of 98140 corn to control and conventional corn.

IX. Environmental Assessment and Impact on Agronomic Practices

IX-A. Environmental Assessment of the GAT4621 and ZM-HRA Proteins

The GAT4621 protein sequence is derived from N-acetyltransferase protein sequences from *Bacillus licheniformis*, a gram positive saprophytic bacterium that is ubiquitous in soil and has a history of safe use for the production of food enzymes in the United States, Canada, and Europe (*e.g.,* alpha-amylase, cyclodextrin glycosyltransferase, hemicellulase, protease, pullulanase), biocontrol agents (FDA, 2001; EU Commission, 2000) and as a probiotic (Kritas *et al*., 2006; Alexopoulos *et al.,* 2004a and b). This indicates a previous history of exposure to and safe use of the source organism for GAT4621.

The GAT4621 protein in 98140 corn is a member of the GNAT acetyltransferase superfamily that contains more than 10,000 representatives from plants, animals, bacteria, and fungi. Members of the GNAT superfamily all contain a highly conserved GNAT motif and high sequence diversity (Vetting *et al.,* 2005). GAT4621 is 75-78% identical and 90-91% similar at the amino acid level to the translated protein sequences of each of the three original *gat* genes from *Bacillus licheniformis* from which the *gat4621* gene was derived. GAT4621 retains the acetyltransferase enzyme function of the native proteins. The GAT4621 protein sequence does not have any homology to proteins that are toxic to humans or animals. Therefore, the GAT4621 protein is highly unlikely to pose a safety risk to beneficial organisms or the environment. (Refer to Section VI-E for a summary of the safety assessment for the GAT4621 protein.)

The ZM-HRA protein in 98140 corn encodes a modified version of the maize acetolactate synthase (ALS) enzyme. ALS proteins are ubiquitous in bacteria, fungi, algae and plants (Friden *et al.,* 1985; Falco *et al*., 1985; Reith and Munholland, 1995; Mazur *et al*., 1987). Moreover, naturally occurring mutations in plant ALS proteins that confer herbicide tolerance have also been identified (for a review, see Duggleby and Pang, 2000). Several crops with herbicide tolerant ALS genes have been commercialized without any unexpected environmental consequences (for example, Clearfield®[3] wheat, Clearfield® sunflower, Clearfield® corn, Clearfield® lentils, and STS®[4] soybean). None of these ALS enzymes or herbicide tolerant crops is known to pose an environmental safety concern. This indicates a previous history of exposure to and safe use of proteins similar to ZM-HRA.

The ZM-HRA protein introduced into 98140 corn has minimal modifications (two amino acid differences) compared to the endogenous maize ALS enzyme from which it was derived. In addition to being derived from a corn protein and having a high degree of similarity to other ALS plant enzymes, the ZM-HRA protein sequence does not have any homology to proteins that are toxic to humans or animals. Therefore, the ZM-HRA protein is highly unlikely to pose a safety risk to beneficial organisms or the environment. (Refer to Section VI-F for a summary of the safety assessment for the ZM-HRA protein.)

In conclusion, there is not likely to be any environmental effects due to the presence of the GAT4621 and ZM-HRA proteins introduced in 98140 corn.

IX-B. Fate of Transgenic DNA in Humans and Animals

Transgenic DNA is no different from other DNA consumed as part of the normal diet. Genetically engineered organisms have been used in drug production (insulin, http://pubs.acs.org/cen/coverstory/83/8325/8325insulin.html) and microbial fermentation (cheese) since the late 1970's (http://www.ncbe.reading.ac.uk/NCBE/GMFOOD/chymosin.html, and http://vm.cfsan.fda.gov/~lrd/biopolcy.html#summary). More than 1.4 billion cumulative acres of

[3] Clearfield® is a registered trademark of BASF.
[4] STS® is a registered trademark of DuPont or its affiliates.

engineered food and feed crops have been grown and consumed worldwide over the past seven years (ISAAA, 2006). The FDA has not reported any significant concerns with bioengineered food and feed currently on the market. The EPA has exempted from a tolerance DNA that encodes currently registered plant incorporated protectants because of a lack of toxicity (Federal Register, 2001).

Studies in humans and animals following the fate of DNA once consumed have shown that the majority of DNA is degraded in the gastrointestinal tract. There is evidence that DNA can move from the gastrointestinal tract lumen to other areas of the body, but this is considered to be a normal occurrence and no risks have been identified as a result of absorption (Einspanier *et al*., 2001; Duggan *et al*., 2003).

IX-C. Weediness Potential of 98140 Corn

Commercial corn varieties in the United States are not effective in invading established ecosystems (CFIA, 1994). Corn hybrids have been domesticated for such a long period of time that the seeds cannot be disseminated without human intervention, nor can corn seed readily survive in the U.S. from one growing season to the next because of the poor dormancy. Any volunteer corn plants are easily identified and controlled through manual or chemical means.

There is little probability that 98140 corn could become a problem weed. Various characteristics that might impart weediness potential were evaluated for 98140 and control corn in comparative studies (Section VII). No differences were seen in characteristics such as seed germination, emergence, seedling vigor, yield, and disease/insect susceptibility. Assessment of these data detected no biologically significant differences between 98140 and control corn indicative of a selective advantage that would result in increased weediness potential. Furthermore, post-harvest monitoring of field trial plots containing 98140 corn have shown no differences in survivability or persistence of 98140 corn as compared to conventional corn.

IX-D. Gene Flow Assessment

D1. Vertical Transfer of the Introduced Genetic Material

Corn is an open pollinated monoecious plant that produces abundant pollen. Corn pollen is among the largest and heaviest of the wind-dispersed pollen grains, thus limiting the distance it can travel. The potential transfer of traits in pollen to other corn plants is sometimes called pollen-mediated gene flow. There are many factors that affect pollen-mediated gene flow in corn including isolation distance between pollen source and recipient field, size of pollen source and recipient field, shape and orientation of pollen source and recipient field, wind direction and velocity, rain, temperature and humidity, pollen viability, silk receptivity, synchrony of flowering between source and recipient fields, pollen competition and physical barriers (Devos *et al*., 2005). The level of gene flow decreases with distance, with a rapid decrease within the first 20 meters of the source and thereafter at a decreased rate (Henry *et al*., 2003). 98140 corn is unchanged with respect to pollen characteristics (see Table 14).

An intra-specific cross of cultivated corn with non-cultivated Zea mays such as annual teosinte is possible; however, non-cultivated *Z. mays* species are not found in the United States. The genus most closely related to *Zea* is *Tripsicum*, a genus of eleven species. Three *Tripsicum* species occur in the U.S. Inter-specific crosses can be made between *Z. mays* and *T. dactyloides*, but these require human intervention and progeny are frequently sterile or genetically unstable. Therefore, cross-pollination between *Z. mays* and *T. dactyloides* in the natural environment is not expected to occur (CFIA, 1994).

D2. Horizontal Transfer of the Introduced Genetic Material

There is no known mechanism for, or definitive demonstration of, DNA transfer from plants to microbes (Nap *et al.*, 1992; Redenbaugh *et al.*, 1994). Many genomes have been sequenced from bacteria that are closely associated with plants (*e.g.* *Agrobacterium* and *Rhizobium*), and there is no evidence that these organisms contain genes derived from plants (Kaneko *et al.*, 2002, Wood *et al.*, 2001). Where sequence data indicate that horizontal gene transfer may have occurred, these events are estimated to occur on an evolutionary time scale on the order of millions of years (Koonin, 2001; Brown, 2003). In addition, transgene DNA promoters and coding sequences are optimized for plant expression and not bacterial expression, and it is therefore very unlikely that a protein corresponding to the transgene would be produced. Even if such a transfer were to take place and protein produced, the DNA and protein would not present a human health or plant pest risk. Genes encoding acetyl transferases are already found in nature, and the *zm-hra* gene is endogenous to corn.

IX-E. Current Agronomic Practices for U.S. Corn

E1. Corn Production

The United States is the world's leading corn producer and exporter. Farm value of U.S. corn production in 2006 was $33.8 billion, the highest value among U.S.-produced crops (http://www.nass.usda.gov/QuickStats/). The total expected corn planting in 2007 is 90.5 million acres, up from 78.3 million acres over 2006, and the highest acreage since 1944. This increase is driven by the demand for fuel ethanol produced from corn (USDA/NASS, 2007). Field corn is planted in almost every state in the U.S., but the majority is grown in the North Central region. About 80% of the total area planted to corn is concentrated in 10 states – Iowa, Illinois, Nebraska, Minnesota, Indiana, South Dakota, Wisconsin, Kansas, Ohio, and Missouri. The average annual yield in 2006 was 149.1 bushels/acre; this number can vary considerably, from 72 bu/acre in Alabama to a high of 210 bu/acre in Washington, due to differences in rainfall, climate and soil productivity (USDA/NASS, 2006).

Corn is typically grown in the United States as a row crop, with an average row width of about 30 inches. Planting usually begins in April or May, and harvesting generally occurs from mid-to-late September through November. In 2001, 43% of corn acres were grown under some form of conservation tillage (no-till, ridge-till or mulch-till) (USDA/NASS, 2006). Conservation tillage practices provide the advantages of decreased soil compaction and fuel costs through reduction in use of heavy machinery, reduced soil erosion and better soil moisture conservation. Corn is not generally irrigated; in 2005 about 11% of planted corn acres were irrigated, with the Northern Plains states, particularly Nebraska, irrigating the most (25% of planted acres) (USDA-ERS ARMS, 2005).

E2. Agricultural Chemical Use in Corn

Corn is intensively managed, as evidenced by the chemical usage data from the 2005 USDA-NASS Agricultural Chemical Usage Report (http://www.nass.usda.gov/). Nineteen states (93% of the total US corn acreage) were included in this report: Colorado, Georgia, Illinois, Indiana, Iowa, Kansas, Kentucky, Michigan, Minnesota, Missouri, Nebraska, New York, North Carolina, North Dakota, Ohio, Pennsylvania, South Dakota, Texas, and Wisconsin.

Fertilizers were extensively applied to corn acres in 2005. Corn growers used nitrogen on 96% of all corn acres, applying an average of 138 pounds per acre per crop year. Phosphate was used on 81% of total acres, at an application rate of 58 pounds per acre per crop year. Potash was applied at 84 pounds per acre per crop year on 65% of acres. Sulfur was also applied, but on only 13% of acres, at 12 pounds per acre per crop year.

Insecticide treatments were used on 23% of the corn planted acreage in the Program States. Tefluthrin, Cyfluthrin, and Tebupirimphos were the most widely applied insecticides, at 7, 7, and 6% of planted acres, respectively.

Herbicides were applied to 97% of the corn planted acreage in 2005 in the Program States surveyed. Atrazine continues to be the most widely applied herbicide, with 66% of the planted acreage being treated and applied at an average rate of 1.133 pounds per acre per crop year. Glyphosate was applied to 31% of planted acres, at an average rate of 0.963 pounds per acre per crop year. It was followed closely by *S*-metolachlor and acetochlor, at 23% of the planted corn acreage treated in the Program States.

E3. Weeds in Corn

Growers must control weeds because they compete with their crops for water, nutrients and sunlight and reduce yield. Control of weeds in corn is especially critical during the first 3 to 5 weeks following crop emergence, before weeds reach 6-8 inches in height. Weed species such as giant foxtail and barnyardgrass can reduce corn yields by up to 13 and 35%, respectively (Bosnic and Swanton, 1997; Fausay *et al*., 1997). Late-season weed infestations do not have as much negative impact on yields, but they can be a vector for diseases and other pests such as thrips and armyworm. They can also reduce silage feed quality, slow mechanical harvest, raise grain moisture content, and be a seed source that will infect subsequent crops. More than 200 weed species, including 32 confirmed herbicide resistant species, threaten corn yields in the United States each year (Weed Science Society of America, www.weedscience.org).

Common weed problems in corn fields include annuals (velvetleaf, common cocklebur, common lambsquarters) and perennials such as quackgrass and Johnsongrass. The perennials are particularly competitive and difficult to control, as these weeds re-grow every year from rhizomes or root systems. Table 30 lists some of the most problematic weeds for corn growers in 2004.

Table 30. Troublesome Weeds in Corn (2004)

Weed Species	Total Area Treated[1] (Acres)
Annuals – Broadleaves	
Velvetleaf	26,336,966
Cocklebur, Common	22,001,594
Lambsquarters, Common	20,708,011
Pigweed, Redroot	19,631,482
Waterhemp, Common	16,455,018
Ragweed, Common	10,458,268
Ragweed, Giant	8,431,913
Sunflower	7,765,456
Kochia	5,583,059
Morningglory species	4,982,289
Smartweed, Pennsylvania	3,060,008
Grasses	
Foxtail species	17,573,674
Crabgrass, Large	4,155,522
Panicum, Fall	2,767,510
Barnyardgrass	2,507,698
Shattercane	2,453,032
Perennials	
Johnsongrass	5,598,643
Quackgrass	4,368,668
Thistle, Canada	2,927,943

[1] The total corn acreage in 2004 was 72 million acres. However, the total corn herbicide-treated acreage is much more than 72 million, due to multiple sprayings on each acre. Data from Doane Market Research, 2004.

E4. Weed Management in Corn

A total of 76 million acres of corn were planted in 2005 in the United States, and nearly all of these acres are treated with herbicide. Data from the 2005 USDA-ERS Agricultural Resource Management Survey survey indicate that there were almost 214 million herbicide treated acres, indicating that most acres were treated multiple times with herbicides (USDA-ERS ARMS, 2005). Products can be applied pre-plant, pre-emergence and post-emergence. In general, corn receives a soil applied herbicide application followed by a post-emergence application. The survey indicated that in 2005, 18.6%of planted acres used a burn down herbicide, 61.3% of planted acres used pre-emergence weed control and 66.5% of planted acres used post-emergence weed control. Growers often use mixtures of herbicide active ingredients (*e.g*. tank mixes) to control all the weeds in their fields.

The concerns associated with the use of herbicides and herbicide resistant crops include the evolution of resistant weeds, misapplication, herbicide drift, crop injury, carryover between growing seasons, cost, and the need for timely application. Decisions about weed management may be the most complex ones that growers make, because each weed control option has trade-offs and affects the feasibility of using other options. Generally, growers must manage a wide array of broadleaf and grass weeds simultaneously. In selecting a weed management strategy, growers choose the most economical means to control weeds that does not decrease the quality or quantity of the crop.

Growers will often use a combination of weed management techniques, including application of different herbicides, to effectively control weeds in their fields. After herbicide usage, scouting was the most prevalent form of weed management practice for corn, with 88.9% of those surveyed scouting through general observation or deliberate scouting activities (USDA-ERS ARMS, 2005). 15.3% of planted acres were cultivated for weed control. The combination that a grower chooses to use depends upon factors such as: weed spectrum, level of infestation, soil type, cropping system, weather, and time and labor available for the treatment option.

E5. Crop Rotation Practices

Approximately 60% of corn is grown in rotation following soybean, with another 13% in rotation with other row crops and small grains (USDA-ERS ARMS, 2005). Crop rotation aids in the management of diseases, insects and weeds and increases organic matter and soil fertility. In addition, crop rotation allows growers to diversify farm production to minimize market risks. Even with the advantages of crop rotation, about 26% of corn was grown continuously in 2005. This number will increase if the high demand for corn for fuel ethanol continues.

IX-F. Potential Impact of the Introduction of 98140 Corn on Agronomic Practices

F1. Potential Impact on Cultivation and Management Practices

No negative impact is expected from the introduction of 98140 corn on current cultivation and management practices for corn. 98140 corn has been shown to be comparable to conventional corn in phenotypic, ecological and compositional characteristics (Sections VII and VIII). 98140 corn is expected to be similar in its agronomic characteristics and have the same levels of resistance to insects and diseases as other commercially available corn.

F2. Potential Impact on Weed Control Practices

The commercialization of herbicide tolerant 98140 corn is expected to have a beneficial impact on weed control practices, as growers will have more herbicide options available to address their regional weed problems. 98140 corn will enable growers to choose an optimal combination of glyphosate, ALS-inhibiting herbicides, and other complementary herbicides to best manage their individual weed populations. Growers value the glyphosate-resistant crop trait and the utility of

glyphosate. The availability of 98140 corn will enable growers to proactively manage weed populations while delaying population shifts to troublesome weeds or the evolution of resistant weeds.

Glyphosate has proven to be a very popular herbicide, and combinations of glyphosate with ALS-inhibiting herbicides or other herbicides with different modes of action will enable even more effective weed management options. Glyphosate is used to control weeds after emergence, whereas ALS inhibitors can be applied pre- or post-emergence. Both glyphosate and the ALS-inhibiting herbicide family have broad spectrum activity. Both have excellent environmental profiles and low mammalian toxicity. Glyphosate has no soil residual, whereas the ALS inhibitors have a wide range of residual soil activity, which can be useful in controlling late-emerging weeds. Finally, ALS inhibitor herbicides are typically applied at very low use rates, in the ounces/acre or less range.

Approximately 40% of the U.S. corn crop is already planted with herbicide tolerant varieties developed through biotechnology (http://usda.mannlib.cornell.edu/usda/nass/Acre/2000s/2006/Acre-06-30-2006.pdf). No increase in the use of glyphosate over corn due to the commercialization of 98140 corn is expected, although an increase in corn planting in general is forecast because of the high demand for biofuels. The total per-season labeled use rate for 98140 corn will not be higher than those used on other glyphosate tolerant corn.

Several ALS-inhibiting herbicides are currently registered for use on corn and are used to control troublesome weeds. However, corn is not highly tolerant to all ALS inhibitors. With 98140 corn, crop tolerance will no longer be a problem. Because current combined use of ALS inhibitors on corn is relatively low (5.3% of all acres, USDA-ERS ARMS, 2005), we would expect the number of acres treated with ALS-inhibiting herbicides to increase with the introduction of 98140 corn. Use rates of ALS inhibitors with 98140 corn will be comparable to those currently labeled for use on corn and other crops.

Growers will still select the best weed management for their specific situation, but will have more options available with 98140 corn.

F3. Potential Impact on the Development of Herbicide Resistant Weeds

Growers have been dealing with the issue of herbicide-resistant weeds for decades. The commercialization of herbicide tolerant 98140 corn is expected to help growers address their regional weed problems and delay adverse population shifts of troublesome weeds or the development of herbicide resistant weeds. Alternating herbicides with different modes of actions to control weeds generally is recommended to help delay the evolution of herbicide-resistant weeds. Therefore, incorporating tolerance to two herbicides with different modes of action in corn will be useful. 98140 corn will enable growers to choose an optimal combination of glyphosate, ALS herbicides, and herbicides with other modes of action to manage their individual weed populations.

F4. Potential Impact of Stacking Herbicide Tolerance Traits

The availability of 98140 corn and the potential for stacking with other herbicide tolerance traits will not significantly change current agronomic practices. The factors that need to be considered in evaluating the potential impact of stacking herbicide tolerance traits (USDA/APHIS, draft Environmental Assessment for MON 89788 soybean, 06-178-01p) include:

(1) *The availability of deregulated herbicide resistance events and their level of commercial production:*
Approximately 40% of corn acres were planted with herbicide tolerant corn in 2006. This number includes glufosinate- and glyphosate-tolerant corn developed through recombinant

technology. Imidazolinone-tolerant corn was also commercially available, and was planted on an additional 3.5% of acres in 2004, although it was developed through selected mutagenesis and traditional plant breeding.

(2) *The effect of stacked traits on the plant and on herbicide use:*
Other previously deregulated herbicide tolerance traits have not had an impact on any other plant characteristics, so the stacking of two or more herbicide tolerance traits into one plant should not make the plant more weedy or change the level of natural herbicide tolerance in the plant. Corn varieties with two herbicide tolerance traits stacked together are already commercially available, and there are no known negative environmental consequences of such stacked traits.

(3) *The number of effective alternative herbicides for corn production:*
There are other effective alternative herbicides available for use in corn for controlling a wide array of weeds, as described in Section IX-E2. Pioneer and DuPont's Crop Protection business advocate Integrated Weed Management (Section G2), utilizing a range of weed control methods, to maintain effective weed control and to properly preserve the effectiveness of herbicides for growers.

(4) *The probability of developing weeds with multiple resistance to various herbicide modes of action:*
The development of herbicide resistant weeds is addressed in several of the previous sections as well as in Appendix 9. The combination of 'frequent use' and 'similar mode of action' is the single most important factor in the development of herbicide resistance. One of the strategies to delay weed resistance is therefore to alternate herbicides with different modes of action. 98140 corn could be helpful in delaying the evolution of weeds with multiple resistance, as growers can safely alternate several modes of action without crop injury. In addition, corn varieties with two herbicide resistant traits stacked together are already commercially available, and their use has not been noted to increase the evolution of weeds with herbicide resistance to multiple modes of action.

(5) *The probability of cross pollination in the field:*
Corn can cross pollinate in the field. If 98140 corn should cross with corn lines expressing resistance to herbicides with different modes of action, corn volunteers with multiple herbicide resistance may emerge. However, various agronomic practices including appropriate variety selections, crop rotation, cultural practices, and rotation of herbicides with different modes of action can be used to manage volunteer corn resistant to one or a few herbicides.

(6) *The probability of a stacked corn becoming a weed:*
A stacked corn is unlikely to become a problem weed as corn is non-invasive in natural habitats, and corn hybrids have been domesticated for such a long period of time that the seeds cannot be disseminated without human intervention. As addressed in section IX-C, 98140 corn does not display weediness potential greater than control corn. Post-harvest monitoring of field trial plots containing 98140 have shown no differences in survivability or persistence of 98140 corn as compared to conventional corn

F5. Potential Impact on Volunteer Management

We do not expect changes in volunteer management practices. Data from a warm and cold germination study (Section VII-A) demonstrated that germination of 98140 corn has not changed and that 98140 corn does not display dormancy characteristics. Corn in general grows as a volunteer in the year following cultivation only under certain environmental conditions. When this occurs, volunteers do not compete well with the succeeding crop and can easily be controlled mechanically or chemically with herbicides other than glyphosate or ALS inhibitors. 98140 corn remains susceptible to other herbicides normally used to control corn, should it appear as a

volunteer weed in other crops. For example, in soybean, the crop most commonly rotated with corn, herbicides based on lipid biosynthesis inhibitors could be used to control corn volunteers.

F6. Potential Impact on Insect Control Practices

98140 corn has been shown to be no different than conventional corn in agronomic characteristics, and ecological observations have shown no changes in susceptibility to insect damage (Section VII). Therefore, there are no expected impacts on insect control practices for 98140 corn.

F7. Potential Impact on Crop Rotation Practices

We do not expect changes in crop rotation practices because of the commercialization of 98140 corn. There would be no expected increase in corn-after-corn planting due to the availability of 98140 corn, and no projected increase in overall demand for glyphosate tolerant seed as the result of the introduction of an alternate tolerance gene. ALS-inhibiting herbicides can have recrop restrictions. However, as the ALS-inhibiting herbicides for 98140 corn will be the same as the ones currently labeled for corn, we would not expect any changes in crop rotation practices.

IX-G. Weed Resistance Management

G1. Evolution of resistant weeds

Crop pests respond to the repeated use of any mechanism that attempts to control them by evolving biological tactics to escape control. The widespread use of herbicides can lead to weed populations that are no longer susceptible. The first documented case of a weed evolving resistance in response to repeated use of a herbicide occurred in the mid 1960's (Ryan, 1970). During the 1970's, growers in the U.S. and Europe began to realize that one class of herbicides (triazines) that had successfully controlled many different weeds was no longer effective against certain populations of as many as 30 different weed species (LeBaron and McFarland, 1990; Bandeen *et al*., 1982). By 1990, weed scientists had evidence that at least 81 weed species contained individuals (biotypes) that had evolved resistance to one or more herbicides; 15 different classes of herbicides were no longer effective against at least one weed species (Holt and LeBaron, 1990). Currently more than 314 biotypes of herbicide resistant weeds occur around the world. Data from the international survey of herbicide resistant weeds can be found at http://www.weedscience.org/in.asp. Additional information about the evolution of herbicide resistant weeds and stewardship of herbicide tolerant corn can be found in Appendix 9.

The key factors influencing a plants potential to develop resistance have been outlined by the Herbicide Resistance Action Committee (HRAC), an industry initiative that fosters cooperation between plant protection manufacturers, government, researchers, advisors and farmers. These factors given below (from http://www.plantprotection.org/hrac/Guideline.html):

Biology and Genetic Makeup of the Weed Species in Question

Number or density of weeds: As resistant plants are assumed to be present in all natural weed populations, the higher the density of weeds, the higher the chance that some resistant individuals will be present.

Natural frequency of resistant plants in the population: Some weed species have a higher propensity toward resistance development; this relates to genetic diversity within the species and, in practical terms, refers to the frequency of resistant individuals within the natural population.

Seed soil dormancy potential: Plant species with a longer soil dormancy will tend to exhibit a slower resistance development under a selection pressure as the germination of new, susceptible, plants will tend to dilute the resistant population.

Crop Management Practices That May Enhance Resistance Development

Frequent use of herbicides with a similar mode of action: The combination of 'frequent use' and 'similar mode of action' is the single most important factor in the development of herbicide resistance.

Cropping rotations with reliance primarily on herbicides for weed control: The crop rotation is important in that it will determine the frequency and type of herbicide able to be applied. It is also the major factor in the selection of non-chemical weed control options. Additionally, the cropping period for the various crops will have a strong impact on the weed flora present.

Lack of non-chemical weed control practices: Cultural or non-chemical weed control techniques, incorporated into an integrated approach are essential to the development of a sustainable crop management system.

G2. Herbicide Resistance and Integrated Weed Management

Herbicides are important tools for growers, and they should be used properly to preserve their effectiveness. Preventing resistance from occurring is an easier and cheaper option than managing a confirmed resistance situation. Simply changing herbicides is not enough to overcome resistance in the mid to long term, and a sustainable, integrated system needs to be developed that is appropriate for the individual farm or region.

Any weed management option that reduces herbicide-imposed selection pressure will reduce the rate of resistance development to the herbicide. By adopting practices such as mixing herbicides with different modes of action and crop rotation, selection pressure for resistant weeds can be reduced and the usefulness of herbicides preserved. These approaches are often part of Integrated Weed Management (IWM) programs. IWM is modeled after the more familiar Integrated Pest Management (IPM) used to control insects and plant pathogens. Both IWM and IPM are based on ecological and evolutionary principles.

IWM utilizes a range of weed control methods, including the following:

a. Avoid using the same herbicide or herbicides with the same mode of action multiple times per year or year after year.
b. Use tank-mixtures consisting of different herbicide types with overlapping weed spectra.
c. Use crop rotations because different crops allow different cultural and tillage options that compete much differently with weeds.
d. When using herbicides, use full label rates and tank mix partners.
e. Use clean seed and clean equipment to minimize spread of weed seed.
f. Monitor fields after herbicide applications for appearance of resistant weeds.
g. Control weeds before they form seed.
h. Where practical, use cover crops, set-aside programs, and other methods to reduce weed seed in soil.

IWM relies on using a variety of control measures to slow the evolution of resistance to a single control measure; therefore IWM is maximized when growers have access to the widest possible array of weed control tools. Pioneer and DuPont through its Crop Protection Chemicals business actively promote IWM techniques through communication, research, education and participation in industry coalitions such as the Herbicide Resistance Action Committee (HRAC, see Appendix 9, Section 9.4 on stewardship of 98140 corn). With the introduction of 98140 corn, new weed

control options will be available to growers that are compatible with integrated weed management practices. This technology advances agricultural sustainability by helping growers achieve higher profits per acre while providing improved environmentally sound options for integrated weed management.

IX-H. Potential Impact on Organic or Conventional Farming

Herbicide tolerant corn developed through biotechnology currently comprises ~40% of corn acres, so organic and conventional corn production remain viable options for growers. Growers choose to grow organic, conventional or biotechnology-derived corn primarily based on economic and market factors. Growers of organic corn and conventional corn for non-biotechnology markets are generally paid a premium for their products, making the additional production costs worthwhile. Conventional and organic corn seed is readily available to growers who decide to plant them. The introduction of 98140 corn will not change growers' choice of production, as they are based on market factors.

IX-I. Potential Impacts on Raw or Processed Agricultural Commodities

Data submitted on agronomic performance, disease and insect susceptibility, and compositional analyses of 98140 corn show no significant differences between 98140 corn and non-transgenic control corn that would be expected to cause either a direct or indirect plant pest effect on any raw or processed plant commodity. 98140 corn will also be reviewed by the FDA for use in food and feed. Based on the analyses above, we expect no significant impact on raw or processed agricultural commodities based on the introduction of 98140 corn.

IX-J. Potential Impact on Non-target Organisms, Including Beneficial Organisms and Threatened or Endangered Species

Based on the safety of the proteins expressed in 98140 corn described in Section IX-A and the compositional analysis described in Section VIII, we would expect no effect on non-target organisms, including beneficial organisms and threatened or endangered species. Both the native GAT protein produced by *Bacillus licheniformis* and a wide variety of ALS proteins are already present in the environment. The two expressed proteins, GAT4621 and ZM-HRA, are not potential food allergens. Based on tests conducted in rodents (mouse acute study) and poultry (42-day broiler study) these proteins are not toxic to either mammals or birds. Observations made during field testing have revealed no effects on invertebrate populations. The compositional analysis of 98140 corn grain and forage showed that 98140 corn is nutritionally comparable to conventional corn. Levels of two acetylated amino acids, aspartate and glutamate, were slightly elevated. These two analytes are natural compounds commonly found in meats, eggs and seeds such as corn and soybeans, and would not be expected to impact an organism that might feed on 98140 corn. Acetylases, including the native GAT protein produced by *Bacillus licheniformis,* are commonly present in the environment.

We would expect that 98140 corn would replace some to many of the corn acres currently planted, but do not expect that 98140 corn will cause new corn acres to be planted in areas that are not already in agricultural use. Threatened or endangered species are generally found outside of agricultural fields. Any habitat disruption within fields will be comparable to other glyphosate tolerant (or other no-till) cropping systems and will be less than conventional tillage systems. Based on this information, we would not expect cultivation of 98140 corn to have an effect on threatened or endangered species, or expect it to adversely change designated critical habitats compared to current agricultural practices.

IX-K. Potential Impact on Biodiversity

98140 corn does not have an increased weediness potential, and unconfined cultivation of 98140 corn should not lead to increased weediness of other sexually compatible relatives, as non-

cultivated *Zea mays* species are not found in the United States (Section IX-C). Therefore, it is unlikely to have effects on non-target organisims common to the agricultural ecosystem or threatened or endangered species, and there is no apparent potential for significant impact to biodiversity.

IX-L. Overall Environmental and Agronomic Practices Conclusions

A thorough characterization of 98140 corn was performed, including molecular analysis, GAT4621 and ZM-HRA protein concentration analysis, phenotypic and ecological evaluation, and nutrient composition evaluation. Assessment of the data generated supports the conclusion of no increased plant pest potential, phenotypic comparability, and familiarity as they relate to ecological risk assessment.

Due to the previous history of exposure to and safe use of organisms containing proteins similar to GAT4621 and ZM-HRA, as well as the safety assessments on the GAT4621 and ZM-HRA proteins, no environmental effects due to the presence of the GAT4621 and ZM-HRA proteins introduced in 98140 corn are expected. Likewise, there is no impact on public health and safety expected due to the DNA introduced in 98140 corn.

98140 corn has been shown to be agronomically and ecologically similar to conventional corn, which has no weedy tendencies and are non-invasive in natural habitats. No differences were seen in characteristics such as seed germination and dormancy, emergence, seedling vigor, plant height, lodging, stay green, time to flowering, pollen viability, yield, disease incidence and insect damage. Assessment of these data detected no biologically significant differences between 98140 and control corn indicative of a selective advantage that would result in increased weediness or outcrossing potential. On the basis of these data, it is concluded that there is no increased plant pest potential of 98140 corn.

Because of the agronomic similarity of 98140 corn to conventional corn, there is no significant impact expected on raw or processed agricultural commodities, on non-target, beneficial organisms (including threatened and endangered species), or on biodiversity. Impacts on organic or conventional farming are also expected be minimal, as growers' decisions to plant biotechnology-derived, organic or conventional corn are driven largely by market dynamics. Market dynamics, grower choice, and existing corn production practices will not change due to the availability of 98140 corn.

With the introduction of herbicide tolerant 98140 corn, we do not expect a significant change in agronomic practices, with the exception of current weed control practices. We anticipate no increase in the usage of glyphosate, but we do expect an increase in the use of ALS-inhibiting herbicides, as this family of herbicides currently is not as widely used for control of weeds in corn. With the introduction of 98140 corn, new weed control options will be available to growers that are compatible with integrated weed management practices. This technology advances agricultural sustainability by helping growers achieve higher yields per acre while providing improved environmentally sound options for integrated weed management.

X. Adverse Consequences of Introduction

Pioneer Hi-Bred International, Inc. is unaware of any information indicating that 98140 corn may pose a greater plant pest risk than conventional corn. There are no adverse environmental consequences anticipated with its introduction. Thus we make the statement “unfavorable information: NONE,” and on the basis of the substantial benefits that this product offers for weed control options, Pioneer requests that 98140 corn be granted nonregulated status under 7 CFR Part 340.

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XII. Appendices 1-7

Appendix 1. Materials and Methods for Molecular Characterization of 98140 Corn

To characterize the DNA insertion in 98140 corn, Southern blot analysis was conducted. Individual plants of the T0S3 generation were analyzed by Southern blot to determine the number of each of the genetic elements of the expression cassettes inserted and to verify the integrity of the PHP24279 T-DNA was maintained upon integration. The integration pattern of the insertion in 98140 corn was investigated with *Eco*R V and *Spe* I restriction enzymes. Southern blot analysis was conducted on individual plants of two generations, BC0S2 and BC1 to confirm insert stability across generations and to verify the absence of backbone sequences from plasmid PHP24279. The BC1S1 generation was also analyzed by Southern blot to confirm insert stability within a fourth generation.

1.1. 98140 Corn Material

Seeds from the T0S3, BC0S2, BC1, and BC1S1 generations of 98140 corn were planted and leaf tissue harvested from individual plants was used for genomic DNA extraction.

1.2. Control Corn Material

Seeds from the unmodified corn varieties PHWVZ and PH09B were planted and leaf tissue harvested from individual plants was used for genomic DNA extraction. PHWVZ and PH09B control DNA was used as a negative control to help interpret hybridization results since several probes (*zm-hra, als* promoter, *ubi*ZM1 promoter, and *ubi*ZM1 intron) cross-hybridize with endogenous corn sequences.

1.3. Reference Material

Plasmid DNA from PHP24279 was prepared from *E. coli* (Invitrogen, Carlsbad, CA) and was used as a positive control for Southern analysis to verify probe hybridization and to verify sizes of internal fragments. The plasmid stock was a copy of the plasmid used for *Agrobacterium*-mediated transformation experiments to produce 98140 corn and was digested with restriction enzymes to confirm the plasmid map. The probes used in this study were derived from plasmid PHP24279 or from a plasmid containing equivalent genetic elements.

DNA molecular weight markers for gel electrophoresis and Southern blot analysis were used to determine approximate molecular weights. For Southern analysis, DNA Molecular Weight Marker VII, digoxigenin (DIG) labeled (Roche, Indianapolis, IN), was used as a size standard for hybridizing fragments. ΦX174 RF DNA/Hae III Fragments (Invitrogen, Carlsbad, CA) was used as a molecular weight standard to determine sufficient migration and separation of the fragments on the gel.

1.4. Genomic DNA Extraction

Genomic DNA was extracted from leaf tissue harvested from individual plants as described above. The tissue was pulverized in tubes containing grinding beads using a Geno/Grinder™ (SPEX CertiPrep, Inc., Metuchen, NJ) instrument and the genomic DNA isolated using a urea-based procedure (modification from Chen and Dellaporta, 1994). Approximately 1 gram of ground tissue was extracted with 5 ml Urea Extraction Buffer (7 M Urea, 0.34 M NaCl, 0.05 M Tris-HCl, pH 8.0, 0.02 M EDTA, 1% N-Lauroylsarcosine) for 12-30 minutes at 37°C, followed by two extractions with phenol/chloroform/isoamyl alcohol (25:24:1) and one extraction with water saturated chloroform. The DNA was precipitated from the aqueous phase by the addition of 1/10 volume of 3 M NaOAc (pH 5.2) and 1 volume of isopropyl alcohol, followed by centrifugation to pellet the DNA. After washing the pellet twice with 70% ethanol, the DNA was dissolved in 0.5

ml TE buffer (10mM Tris, 1 mM EDTA, pH 7.5) and treated with 10 μg Ribonuclease A for 15 minutes at 37°C. The sample was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) and once with water saturated chloroform, followed by precipitation with isopropyl alcohol and washing with 70% ethanol. After drying, the DNA was re-dissolved with 0.5 ml TE buffer and stored at 4°C.

1.5. Quantitation of Genomic DNA

Following extraction, the DNA was quantified on a spectrofluorometer using PicoGreen® reagent (Molecular Probes, Inc., Eugene, OR) following a standard procedure. The DNA was also visualized on an agarose gel to confirm quantitation values from the PicoGreen® analysis and to determine DNA quality.

1.6. Phenotypic Identification of 98140 Corn

Phenotypic analysis of 98140 corn plants and control plants was carried out by the use of lateral flow devices able to detect the GAT4621 protein to confirm the absence or presence of the GAT4621 protein in material used for Southern blot analysis.

Leaf extract was prepared by grinding leaf punches to homogeneity in 500 μl of extraction buffer (20mM Tris (pH 7.5), 67mM NaCl, 0.5% Tween 20). Lateral flow devices (EnviroLogix, Inc., Portland, ME) were placed in the homogenate and allowed to develop. After incubation, the results were read from the lateral flow devices. A single stripe indicated a negative result and a double stripe indicated the sample was positive for the GAT4621 protein.

A preliminary Southern blot analysis of DNA isolated from all 98140 corn plants from generations T0S3, BC0S2, and BC1 was used to verify the presence of both the *gat4621* and *zm-hra* genes. Methods for this preliminary characterization are described below. Final Southern blot analysis was carried out on a subset of 98140 corn plants from these three generations and the BC1S1 generation.

1.7. Digestion of DNA for Southern Blot Analyses

Genomic DNA samples extracted from selected 98140 corn and control corn plants were digested with restriction enzymes following a standard procedure. Approximately 4 μg of genomic DNA was digested in a volume of 100 μl using 50 units of enzyme according to manufacturer's recommendations. The digestions were carried out at 37°C for three hours, followed by ethanol precipitation with 1/10 volume of 3 M NaOAc (pH 5.2) and 2 volumes of 100% ethanol. After incubation at 4°C and centrifugation, the DNA was allowed to dry and re-dissolved in TE buffer. The reference plasmid, PHP24279, was spiked into a control plant DNA sample in an amount equivalent to approximately one or three gene copies per corn genome and digested with the same enzyme to serve as a positive control for probe hybridization and to verify sizes of internal fragments on the Southern blot.

1.8. Electrophoretic Separation and Southern Transfer

Following restriction enzyme digestion, the DNA fragments produced were electrophoretically separated by size through an agarose gel and a molecular weight standard [ΦX174 RF DNA/Hae III Fragments (Invitrogen)] was used to determine sufficient migration and separation of the fragments on the gel. DIG labeled DNA Molecular Weight Marker VII (Roche), visible after DIG detection as described below, was used to determine hybridizing fragment size on the Southern blots.

Agarose gels containing the separated DNA fragments were depurinated, denatured, and neutralized in situ, and transferred to a nylon membrane in 20x SSC buffer (3M NaCl, 0.3 M

Sodium Citrate) using the method as described for the TURBOBLOTTER™ Rapid Downward Transfer System (Schleicher & Schuell, Keene, NH). Following transfer to the membrane, the DNA was bound to the membrane by UV crosslinking (Stratalinker, Stratagene, La Jolla, CA).

1.9. DNA Probe Labeling for Southern Blot Hybridization

Probes for the *ubi*ZM1 promoter, *ubi*ZM1 intron, *gat4621*, *pin*II terminator, *als* promoter, *zm-hra*, and 35S enhancer were used to detect genes and elements within the insertion. Backbone regions (*vir*G*, tet, spc,* LB, and RB probes) of the PHP24279 plasmid were used to verify absence of plasmid backbone DNA in 98140 corn. DNA fragments of the probe elements were generated by PCR from plasmid PHP24279 or a plasmid with equivalent elements using specific primers. PCR fragments were electrophoretically separated on an agarose gel, excised and purified using a gel purification kit (Qiagen, Valencia, CA). DNA probes were generated from these fragments by PCR that incorporated a DIG labeled nucleotide, [DIG-11]-dUTP, into the fragment. PCR labeling of isolated fragments was carried out according to the procedures supplied in the PCR DIG Probe Synthesis Kit (Roche).

1.10. Probe Hybridization and Visualization

The DNA fragments bound to the nylon membrane were detected as discrete bands when hybridized to a labeled probe. Labeled probes were hybridized to the target DNA on the nylon membranes for detection of the specific fragments using the procedures essentially as described for DIG Easy Hyb solution (Roche). After stringent washes, the hybridized DIG-labeled probes and DIG-labeled DNA standards were visualized using CDP-Star Chemiluminescent Nucleic Acid Detection System with DIG Wash and Block Buffer Set (Roche). Blots were exposed to X-ray film for one or more time points to detect hybridizing fragments and to visualize molecular weight standards. Images were digitally captured by detection with the Luminescent Image Analyzer LAS-3000 (Fujifilm Medical Systems, Stamford, CT). Digital images were compared to original X-ray film exposures as verification for use in this report. The sizes of detected bands were documented for each digest and each probe.

1.11. Stripping of Probes and Subsequent Hybridizations

Following hybridization and detection, membranes were stripped of DIG-labeled probe to prepare the blot for subsequent re-hybridization to additional probes. Membranes were rinsed briefly in distilled, de-ionized water and then stripped in a solution of 0.2 M NaOH and 0.1% SDS at 37-40°C with constant shaking. The membranes were then rinsed in 2x SSC and either used directly for subsequent hybridizations or stored at 4°C or -20°C for later use. The alkali-based stripping procedure effectively removes probes labeled with the alkali-labile DIG.

Appendix 2. Description of Statistical Analyses

2.1. Trait Inheritance Data (Section V-E, Table 6)

Based on Mendel's segregation law, the expected segregation ratios in Table 6 were tested by the statistic:

$$\chi^2 = [\Sigma(|o - e| - 0.5)^2 / e]$$

where o = observed frequency of the genotype,
e = expected frequency of the genotype, and
0.5 = Yates correction for continuity (Yates, 1934).

For the binomial case, chi-squared values are calculated by

$$\chi^2 = \Sigma [(|o - e| - 0.5)^2 / e]$$
$$= (| n_{obs(pos)} - n_{exp(pos)} | - 0.5)^2 / n_{exp(pos)} + (| n_{obs(neg)} - n_{exp(neg)} | - 0.5)^2 / n_{exp(neg)}$$

where n is the total of the positive ($n_{obs(pos)}$) and negative ($n_{obs(neg)}$) frequencies.

χ^2 follows a chi-squared distribution with one degree of freedom (df) for binomial cases (Agresti, 2002).

χ^2 values >= 3.84 (χ^2(df=1)) have probability p<= 0.05, indicate a significant difference between observed and expected frequencies.

χ^2 values <3.84 (χ^2(df=1)) have probability p> 0.05, indicate no evidence of a significant difference between observed and expected frequencies.

2.2. Agronomic Data (Section VII-B)

Agronomic data (Tables 12, 13 and 14) were analyzed using a linear mixed model designed to account for the design effects of location and blocks within location. The linear mixed model assumed the entries were a fixed effect while the locations, blocks within locations and the entry by location interaction were random effects. A significant difference between the mean of 98140 corn and the means of near-isoline control was established with an FDR-adjusted P-value <0.05.

Early population, seedling vigor, time to silking, time to pollen shed, pollen viability, stalk lodging, root lodging, final population, stay green, yield, disease incidence and insect damage data were analyzed using the following linear mixed model:

$$y_{ijk} = \mu_i + \ell_j + r_{k(j)} + (\mu\ell)_{ij} + \varepsilon_{ijk}$$

$\ell_j \sim iid\ N(0, \sigma^2_{Loc})$, $r_{k(j)} \sim iid\ N(0,\sigma^2_{Rep})$, $(\mu\ell)_{ij} \sim iid\ N(0,\sigma^2_{Loc\times Ent})$, and $\varepsilon_{ijk} \sim iid\ N(0,\sigma^2_{plot})$

Where μ_i denotes the mean of the i^{th} entry (fixed effect), ℓj denotes the effect of the j^{th} site (random effect), $r_{k(j)}$ denotes the effect of the k^{th} block within the j^{th} site (random effect), $(\mu\ell)_{ij}$ denotes the interaction between the entries and sites (random effect) and ε_{ijk} denotes the effect of the plot assigned the i^{th} entry in the k^{th} block of the j^{th} site (random

effect or residual). Notation ~iid $N(0,\sigma^2_a)$ indicates random variables that are identically independently distributed (iid) as normal with zero mean and variance σ^2_a.

Plant height and ear height data were analyzed using the following linear mixed model:

$$y_{ijkl} = \mu_i + \ell_j + r_{k(j)} + (\mu\ell)_{ij} + \varepsilon_{ijk} + \delta_{ijkl}$$

$\ddot{y}_j \sim iid\ N(0,\sigma^2_{Loc})$, $r_{k(j)} \sim iid\ N(0,\sigma^2_{Rep})$, $(\mu\ell)_{ij} \sim iid\ N(0,\sigma^2_{Loc\times Ent})$, $\varepsilon_{ijk} \sim iid\ N(0,\sigma^2_{plot})$, and $\delta_{ijkl} \sim iid\ N(0,\sigma^2_{observations})$

Where μ_i denotes the mean of the ith entry (fixed effect), ℓj denotes the effect of the jth site (random effect), $r_{k(j)}$ denotes the effect of the kth block within the jth site (random effect), $(\mu\ell)_{ij}$ denotes the interaction between the ith entries and jth sites (random effect), ε_{ijk} denotes the effect of the plot assigned the ith entry in the kth block of the jth site (random plot), and δ_{ijkl} denotes the effect of the plant assigned the lth plant in the ith entry in the kth block of the jth site (observational error).

2.3. Nutrient Composition Data (Section VIII-A through VIII-D)

Data were analyzed using a linear mixed model designed to account for the design effects of location and blocks within location. The linear mixed model assumes the entries are a fixed effect while the locations, blocks within locations and the entry by location interaction are random effects. A significant difference between the mean of 98140 corn and the means of the near-isoline control was established with an FDR-adjusted P-value <0.05 for each analyte.

Composition data presented in Tables 15-19 and Tables 21-28 were analyzed using the following linear mixed model:

$$y_{ijk} = \mu_i + \ell_j + r_{k(j)} + (\mu\ell)_{ij} + \varepsilon_{ijk}$$

$\ell_j \sim iid\ N(0,\sigma^2_{Loc})$, $r_{k(j)} \sim iid\ N(0,\sigma^2_{Rep})$, $(\mu\ell)_{ij} \sim iid\ N(0,\sigma^2_{Loc\times Ent})$, and $\varepsilon_{ijk} \sim iid\ N(0,\sigma^2_{plot})$

Where μ_i denotes the mean of the ith entry (fixed effect), ℓj denotes the effect of the jth site (random effect), $r_{k(j)}$ denotes the effect of the kth block within the jth site (random effect), $(\mu\ell)_{ij}$ denotes the interaction between the entries and sites (random effect) and ε_{ijk} denotes the effect of the plot assigned the ith entry in the kth block of the jth site (random effect or residual). Notation ~iid $N(0,\sigma^2_a)$ indicates random variables that are identically independently distributed (iid) as normal with zero mean and variance σ^2_a.

Appendix 3. Materials and Methods for Determination of the GAT4621 and ZM-HRA Protein Concentrations

Corn plant tissue concentrations of the GAT4621 and ZM-HRA proteins were determined using enzyme linked immunosorbent assays (ELISA) developed at Pioneer Hi-Bred International, Inc., Johnston, IA, USA.

3.1 Storage and Processing of ELISA samples

Upon receipt, all plant tissue samples were stored in temperature-monitored freezers at <-10°C.

Forage samples were coarsely homogenized on dry ice using a Stephan VCM 12 (Stephan Machinery Singapore Pte Ltd, Singapore) blender for approximately 2 minutes and sub-sampled.

All samples were lyophilized at <-12°C under vacuum until dry. The lyophilization time varied between 18 to 72 hours depending on the sample size and tissue type.

Forage and grain tissues were finely ground for approximately 60 seconds using a GenoGrinder (BT&C/OPS Diagnostics, Metuchen, NJ, USA). Root samples were finely ground for approximately two minutes using a Harbil 5G High-Speed Mixer (IDEX Corporation, Northbrook, IL).

Between lyophilization and grinding, samples were stored frozen in temperature-monitored freezers at <-10°C.

3.2. Protein Extraction from Processed Corn Tissues

Processed corn tissues were weighed into 1.2 ml tubes at the following target weights: 5 milligrams (mg) for pollen, 10 mg for leaf, 20 mg for grain and root, and 30 mg for stalk, whole plant, and forage tissues. Each sample was extracted with 600 microliters (µl) of chilled (2-8°C) H5 buffer solution which is comprised of 90 millimolar (mM) HEPES, 140 mM NaCl, 1% PVP-40, 0.3% Tween-20, 1% Bovine Serum Albumin, 1% polyethylene glycol, and 0.007% Thimerosal. Two 5/32" steel balls were added to each tube, and the samples were homogenized with a single 30 second cycle at a setting of 1500 strokes per minute using a SPEX Certiprep GenoGrinder (BT&C/OPS Diagnostics). Following centrifugation, supernatants were removed, diluted and analyzed for extractable GAT4621 and ZM-HRA concentrations using specific enzyme linked immunosorbent assay (ELISA) for each protein.

3.3. Determination of GAT4621 Protein Concentrations

The GAT4621 ELISA utilizes a sequential "sandwich" format for the quantitation of GAT4621 in corn plant tissue extracts. Standards (analyzed in triplicate wells) and diluted tissue extracts (analyzed in duplicate wells) were incubated for one hour in a 96-well stabilized ELISA plate that was pre-coated with a GAT-specific antibody. Unbound substances were washed from the plate and a different GAT-specific antibody that was conjugated to the enzyme horseradish peroxidase (HRP) was added to each well. Bound GAT4621 protein was sandwiched between the antibody coated on the plate and the antibody-HRP conjugate. At the end of a one hour incubation, unbound substances were washed from the plate. Detection of the bound GAT4621-antibody complex was accomplished by the addition of a substrate that generated a colored product in the presence of HRP. The reaction was stopped after 30 minutes with stop solution (hydrochloric acid) and the optical density of each well was determined using a Molecular Devices plate reader (Molecular Devices Corporation, Sunnyvale, CA) with a wavelength setting of 450 nm minus 650

nm. SoftMax Pro software (Molecular Devices Corporation) was used to perform the calculations that generated the quadratic fit of the standard curve and converted the sample optical density (OD) values to GAT4621 protein concentration values. The mean duplicate well values in ng/ml were used in the calculation of the reported GAT4621 concentration of each sample (ng/mg dry weight). A characterized sample extract (QCE) was included on each plate as a control for data acceptance.

The quantitative range for the assay was 0.36 to 8.8 ng/ml. The lower limit of quantitation (LLOQ) in ng/mg dry weight for each tissue was based on extraction volume (µl) to weight ratios, the limit of quantitation for the ELISA in ng/ml, and the dilutions used for analysis. The sample LLOQs on a ng/mg dry weight basis for GAT4621 were 0.072 ng/mg dry weight for stalk, forage and whole plant; 0.11 ng/mg dry weight for root and grain; 0.22 ng/mg dry weight for leaf; and 0.43 ng/mg dry weight for pollen.

3.4. Determination of ZM-HRA Protein Concentrations

The ZM-HRA ELISA method utilized a sequential “sandwich” format for the determination of the presence of ZM-HRA protein in corn plant tissue extracts. In this assay, sample extracts were incubated for one hour in stabilized 96-well plates that were precoated with a ZM-HRA-specific antibody. Unbound substances were washed from the plate, and a different ZM-HRA- antibody that had been conjugated to the enzyme horseradish peroxidase (HRP) was added to the wells. Bound ZM-HRA protein was sandwiched between the antibody coated on the plate and the antibody-HRP conjugate. At the end of the one hour incubation, unbound substances were washed from the plate. Detection of the bound ZM-HRA protein-antibody complex was accomplished by the addition of a substrate solution, which generated a colored product in the presence of HRP. The reaction was stopped after 30 minutes with stop solution (hydrochloric acid) and the optical density of each well was determined using a Molecular Devices (Molecular Devices Corporation) plate reader with a wavelength setting of 450 nm minus 650 nm. SoftMax Pro software (Molecular Devices Corporation) was used to perform the calculations that generated the quadratic fit for the standard curve and converted the sample OD values to ZM-HRA protein concentration values. The mean concentration from the duplicate wells in ng/ml was used in the calculation of the reported ZM-HRA concentration of each sample (ng/mg dry weight).

The quantitative range for the assay was 0.90 to 22 ng/ml. The lower limit of quantitation (LLOQ) in ng/mg dry weight for each tissue was based on extraction volume (µl) to weight ratios, the limit of quantitation for the ELISA in ng/ml, and the dilutions used for analysis. The sample LLOQs on a ng/mg dry weight basis for ZM-HRA were 0.14 ng/mg dry weight for grain; 0.18 ng/mg dry weight for whole plant at R6, 0.36 ng/mg dry weight for forage and whole plant at R1; 0.27 ng/mg dry weight for root; and 0.54 ng/mg dry weight for leaf and pollen.

Appendix 4. Equivalency of Plant- and Microbial-Expressed GAT4621 and ZM-HRA Proteins

4.1. Methods for Purification of GAT4621 and ZM-HRA Proteins from 98140 Corn Tissue

The GAT4621 protein was extracted from 80 grams of 98140 corn leaf tissue in 250 ml of extraction buffer consisting of 50 mM HEPES, pH 7.8, 150 mM NaCl, 1 mM EDTA, 5% glycerol, and Complete Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN). Particulate leaf material in the resulting slurry was removed by filtering through four layers of cheese cloth. The extract was further clarified by centrifugation for 30 minutes at 30,500 *g*.

Proteins in the extract were fractionated with ammonium sulfate precipitation (40-85% saturation). The precipitated protein with 85% saturated ammonium sulfate was resuspended in extraction buffer and then desalted with 10DG columns (Bio-Rad Laboratories, Inc., Hercules, CA). The GAT4621 protein was further purified using immunoaffinity chromatography. The column was prepared by coupling GAT-specific mouse monoclonal antibody to AminoLink Plus Coupling Gel (Pierce Biotechnology, Inc., Rockford, IL) according to the manufacturer's instructions. The desalted sample was loaded onto three affinity columns (2 ml each) which were pre-equilibrated with the extraction buffer. Unbound material was removed by washing with the same buffer. Bound GAT4621 protein was then eluted using ImmunoPure IgG Elution Buffer (Pierce Biotechnology, Inc.). Fractions (2 ml each) were collected and the protein concentration was estimated by Bradford assay with BSA as the standard. Fractions containing the GAT4621 protein were pooled and concentrated using iCon 9K concentrators (Pierce Biotechnology, Inc.).

The ZM-HRA protein was extracted from 100 grams of 98140 corn leaf tissue in 300 ml of homogenization buffer consisting of 50 mM Tris-HCl, pH 7.5, 5 mM sodium pyruvate, 10 µM flavin adenine dinucleotide, 1 mM EDTA, 5% glycerol, 5 mM magnesium chloride, 50 mM sodium chloride, 5% (w/w) polyvinylpolypyrrolidone, and EDTA-free Complete Protease Inhibitor Cocktail (Roche Applied Science). Particulate leaf material in the resulting slurry was removed by filtering through four layers of cheese cloth. The extract was further clarified by centrifugation for 30 minutes at 30,500 *g*.

The ZM-HRA protein was partially purified from the extract using immunoaffinity columns that were prepared by coupling HRA-specific mouse monoclonal antibodies to AminoLink Plus Coupling Gel (Pierce Biotechnology, Inc.) according to the manufacturer's instructions. The entire clarified leaf extract was loaded onto two affinity columns (3 ml each), which were pre-equilibrated with the homogenization buffer. Unbound material was removed by washing with the homogenization buffer. Bound ZM-HRA protein was eluted using ImmunoPure IgG Elution Buffer (Pierce Biotechnology, Inc.). The total protein concentration in the eluted fractions was estimated by Bradford assay with BSA as the standard. The fraction containing ZM-HRA protein was concentrated using iCon 20K concentrators (Pierce Biotechnology, Inc.).

4.2. Methods for Purification of GAT4621 and ZM-HRA Proteins Expressed in *E. coli*

The GAT4621 protein was expressed in *E. coli* strain BL21(DE3) as a soluble protein and was purified at Aldveron, LLC (Fargo, ND) using cation exchange chromatography, anion exchange chromatography and hydrophobic interaction chromatography, followed by diafiltration into 0.1 M ammonium bicarbonate, pH 7.8. The purified protein was then lyophilized. For characterization, the GAT4621 protein was used in the following form: 1 mg of the lyophilized powder was dissolved in 1 ml of 100 mM KCl, 10% methanol, and 25 mM HEPES, pH 7.2.

The ZM-HRA protein was expressed in *E. coli* strain BL21(DE3) RIPL as a soluble protein with an N-terminal His-T7 fusion tag and was purified at Aldveron (Fargo, ND) using an immobilized metal affinity column (Ni-NTA His•Bind resin, EMD Biosciences, Inc., San Diego, CA). The His-T7 tag was cleaved from the affinity purified protein with thrombin (EMD Biosciences, Inc.) and diafiltration was used to remove the cleaved tag and thrombin. The purified microbially expressed ZM-HRA protein was dialyzed into 50 mM ammonium bicarbonate pH 7.5 and then lyophilized.

4.3. Method for SDS-PAGE Analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by first mixing protein samples with Laemmli sample buffer (Gradipore Limited, NSW 2086, Australia) containing 100 mM dithiothreitol and heating the solution at 100°C for approximately five minutes. The prepared protein samples were loaded into a 10-20% gradient Ready Gel Tris-HCl gel (Bio-Rad Laboratories, Inc., Hercules, CA). Molecular weight markers were loaded into the gel to provide a visual estimate of molecular weight. Electrophoresis was conducted using the Ready Gel Cell system (Bio-Rad Laboratories, Inc) with Tris-glycine running buffer (Gradipore Limited) at a constant 150 volts for 60 minutes or until the dye front neared the bottom of the gel.

Upon completion of electrophoresis, the gels were removed from the gel cassette. Then the gels were either prepared for Western blot analysis or washed three times with deionized water for five minutes each. The washed gels were stained for 60 minutes with GelCode Coomassie Blue stain reagent (Pierce Biotechnology, Inc., Rockford, IL), and rinsed with deionized water two to three times for ten minutes each.

4.4. Methods for Western Blot Analysis

Following SDS-PAGE, the resulting gel was soaked in Transfer buffer (48 mM Tris-HCl, pH 8.6, 39 mM glycine, 0.0375% sodium dodecyl sulfate, and 20% methanol) for 20 minutes. A polyvinylidene difluoride (PVDF, Bio-Rad Laboratories, Inc.) membrane was briefly placed in 100% methanol, followed by immersion in Transfer buffer for 10-15 minutes. A Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell system (Bio-Rad Laboratories, Inc.) was used to transfer proteins from the gel to the membrane at 10 volts for 45 minutes.

For GAT4621, membranes were washed four times for five minutes each in Classic buffer (50 mM Tris-HCl, pH 7.0, 500 mM NaCl, 0.5% Tween-20), and blocked by incubating in phosphate-buffered saline solution with Tween-20 (PBST: 8.1 mM phosphate buffer, pH 7.4, 137 mM NaCl, 2.7 mM KCl, and 0.05% Tween-20) containing 5% (w/v) non-fat dry milk for approximately 60 minutes. The blocked membrane was washed three times for five minutes each in Classic buffer, and then incubated for 60 minutes with a mouse monoclonal primary antibody diluted 1:5,000 in PBST containing 5% non-fat dry milk. The unbound antibody was rinsed from the membrane with three washes of Classic buffer for five minutes each. The membrane was then incubated for 60 minutes with a secondary antibody (anti-mouse IgG horseradish peroxidase conjugate) diluted 1:10,000 in Classic buffer. The membrane was then washed three times with Classic buffer for five minutes each and developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc.) according to the manufacturer's instructions.

For ZM-HRA, membranes were washed four times for five minutes each in Classic buffer), and then blocked by incubating in phosphate-buffered saline solution with Tween®-20 (PBST: 8.1 mM phosphate buffer, pH 7.4, 137 mM NaCl, 2.7 mM KCl, and 0.05% Tween-20) containing 5% non-fat dry milk for approximately 60 minutes. The blocked membranes were washed four times for approximately five minutes each in Classic buffer. The membranes were then incubated for 90 minutes with primary antibodies diluted in PBST containing 5% non-fat dry milk. One membrane was incubated with a rabbit polyclonal antibody R144 (diluted 1:3,000) which is able to recognize both ZM-ALS and ZM-HRA proteins. Another membrane was incubated with a rabbit polyclonal antibody R6961 (diluted 1:2,000) which only recognizes the ZM-HRA protein. This antibody was used to detect and confirm the ZM-HRA protein in the partially purified maize ZM-HRA sample. The unbound antibodies were rinsed from the membranes with four washes of Classic buffer for approximately five minutes each. The membranes were then incubated for 60 minutes with a secondary antibody (anti-rabbit IgG horseradish peroxidase conjugate) diluted 1:10,000 in Classic buffer. The membranes were then washed four times with Classic buffer for approximately five minutes each. The blots were soaked in phosphate buffered saline solution (PBS: 8.1 mM phosphate buffer, pH 7.4, 137 mM NaCl, and 2.7 mM KCl) for at least five minutes and developed with SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc.) according to the manufacturer's instructions.

4.5. Methods for N-Terminal Amino Acid Sequence Analysis

The microbially expressed GAT4621 protein sample was sent to Crop Genetics (E. I. du Pont de Nemours and Company, DuPont Experimental Station, Wilmington, DE) in 25mM HEPES, pH 7.2, 100mM KCl, 10% methanol. The protein was transferred to PVDF membrane using ProSorb Inserts (Applied Biosystems, Foster City, CA) and washed with 200 µl 0.1% trifluoroacetic acid in water. The membrane was removed and used for Edman N-terminal amino acid sequencing with the Procise 494 LC analyzer (Applied Biosystems) equipped with an online high performance liquid chromatography system.

The microbially expressed and 98140 corn-derived ZM-HRA protein samples and the 98140 corn-derived GAT4621 sample were separated by SDS-PAGE and electrophoretically transferred to a PVDF membrane as described in Sections 4.3 and 4.4. After transfer, the PVDF membrane was stained with Ponceau S solution (Sigma-Aldrich, St. Louis, MO) or GelCode Coomassie Blue stain reagent (Pierce Biotechnology, Inc.) to visualize the protein band. The resulting ZM-HRA protein band was excised and shipped to the Keck Biotechnology Resource Laboratory (Yale University, New Haven, CT) for Edman N-terminal amino acid sequencing using the Procise 494 cLC analyzer (Applied Biosystems, Inc.) equipped with an online high performance liquid chromatography (HPLC) system.

4.6. Method for MALDI-MS Identification of Tryptic Peptides

Gel slices containing either the GAT4621 or ZM-HRA protein bands were placed in labeled tubes and shipped overnight on dry ice to the Keck Biotechnology Resource Laboratory (Yale University). The protein in the gel slice was digested with trypsin for 18 hours at 37°C. An aliquot of the digest was analyzed by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) on an Applied Biosystems/MDS SCIEX 4700 MALDI-Tof-Tof instrument (Applied Biosystems) in the reflectron mode of operation. Detected peptide peaks were considered a match if they were within 100 parts per million (ppm) of the theoretical mass of peptides determined via *in silico* trypsin cleavage of the protein sequence.

Allowances were made for the following potential modifications to the peptides: oxidation of methionine or tryptophan residues (observed value is 15.995 Da greater than the theoretical value) and modification of cysteine residues by acrylamide free radicals during SDS-PAGE (observed value is 71.037 Da greater than the theoretical value).

4.7 Methods for Protein Glycosylation Analysis

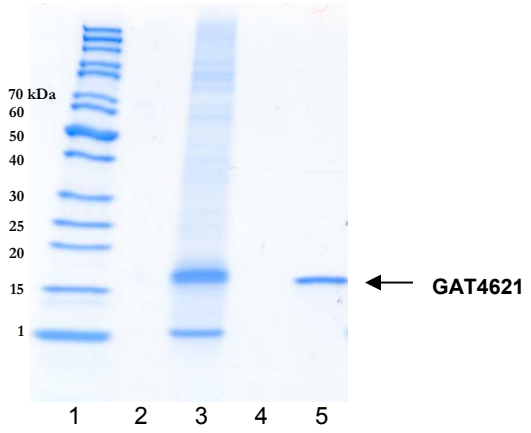
A GelCode glycoprotein staining kit (Pierce Biotechnology, Inc.) was used according to the manufacturer's instructions to determine whether the GAT4621 and ZM-HRA proteins were glycosylated. The microbially expressed and 98140 corn-derived GAT4621 or ZM-HRA proteins, a positive control protein (horseradish peroxidase), and a negative control protein (soybean trypsin inhibitor) were run on SDS-PAGE as described in Section 4.3. Following electrophoresis, the gel was fixed with 50% methanol for 30 minutes and washed with 3% acetic acid. The gel was then incubated with oxidizing solution for 15 minutes and washed three times with 3% acetic acid. The gel was incubated with GelCode glycoprotein staining reagent (Pierce Biotechnology, Inc.) for 15 minutes and then treated with reducing reagent. The gel was then extensively washed with 3% acetic acid and deionized water. Glycoproteins were detected as magenta colored bands on the gel. Following glycoprotein detection, the gel was scanned and the image was captured electronically. The same gel was then stained with Coomassie Blue to visualize the total protein content of all protein bands.

4.8. Results of SDS-PAGE and Western Analyses of GAT4621

SDS-PAGE analysis indicated that the partially purified 98140 corn GAT4621 protein and the microbially expressed GAT4621 protein sample had predominant bands migrating as expected at approximately 16 kDa (Figure 1). The 98140 corn GAT4621 protein sample contained an additional unidentified band that migrated at approximately 10 kDa. Western blot analysis of the 98140 corn-derived and microbially expressed GAT4621 proteins resulted in detection of a single immunoreactive band migrating at approximately 16 kDa (Figure 2).

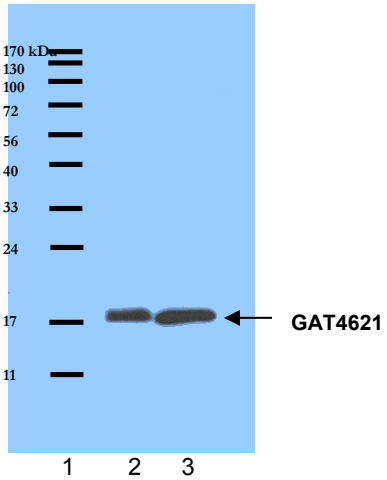
Therefore, SDS-PAGE and Western analyses confirmed that both microbially expressed and 98140 corn plant-derived GAT4621 proteins had the expected molecular weight and immunoreactivity and were equivalent as demonstrated by these analyses.

Figure 1. SDS-PAGE Gel of the Microbially Expressed and 98140 Corn-Derived GAT4621 Proteins



Lane	Sample ID
1	Fermentas PageRuler Unstained Protein Ladder (#SM0661)
2	Blank
3	GAT4621 Derived from 98140 Corn (~1.7 µg)
4	Blank
5	GAT4621 Derived from Microbial Expression System (~1µg)

Figure 2. Western Blot of the Microbially Expressed and 98140 Corn-Derived GAT4621 Proteins



Lane	Sample ID
1	Fermentas PageRuler Prestained Protein Ladder (#SM0671)
2	GAT4621 Derived from 98140 Corn (~7 ng)
3	GAT4621 Derived from Microbial Expression System (~15 ng)

4.9 Results of N-Terminal Sequencing of GAT4621

N-terminal sequence analysis of the 98140 corn-derived GAT4621 protein yielded thirteen amino acid residues that matched residues 2 through 14 of the expected GAT4621 sequence (Table 1). The N-terminal sequence analysis of the microbially expressed GAT4621 protein yielded ten amino acid residues that matched residues 2 through 11 of the expected GAT4621 sequence. The data indicated that the N-terminal methionine residue was not present for both the plant-derived and the microbially expressed GAT4621 proteins.

N-terminal sequence analysis confirmed that both microbially expressed and 98140 corn-derived GAT4621 proteins had identical N-terminal amino acid sequences.

Table 1. N-terminal Amino Acid Sequence of the Microbially Expressed and 98140 Corn-Derived GAT4621 Proteins

Theoretical GAT4621 Sequence	M - A - I - E - V - K - P - I - N - A - E - D - T - Y
Microbial GAT4621 Sequence	A - I - E - V - K - P - I - N - A - E
98140 Corn-Derived GAT4621 Sequence	A - I - E - V - K - P - I - N - A - E - D - T - Y

4.10 Results of the MALDI-MS Identification of Tryptic Peptides of GAT4621

A tryptic digest of the microbially expressed and 98140 corn GAT4621 proteins each produced eight peptides that matched theoretical GAT4621 peptide masses, resulting in coverage of 76% and 83%, respectively, of the GAT4621 amino acid sequence (Table 2).

The high percentage coverage of both the microbially expressed and 98140 corn-derived GAT4621 protein sequences demonstrates that both proteins were expressed as intended.

Table 2. Matching Peptides from the Microbially Expressed and 98140 Corn-Derived GAT4621 Protein Identified using MALDI-MS

Amino Acid Residue #	Theoretical Mass [M+H]	Observed Microbially Expressed GAT4621 Mass [M+H]	Observed 98140 Corn-Derived GAT4621 Mass [M+H]	Identified Sequence
2-17	1846.95	1846.97[a]	1846.96[a]	AIEVKPINAEDTYDLR
39-50	1240.60	1240.61	1240.61	SAFHLGGFYGGK
51-68	1981.01	1981.02	1981.01	LISVASFHQAEHSELQGK
69-74	835.47	835.48	835.48	KQYQLR
75-83	965.50	965.51	965.51	GVATLEGYR
84-102	2179.22	ND[b]	2179.13	EQKAGSSLVKHAEEILRKR
94-100	867.46	867.47	ND	HAEEILR
101-121	2434.18	2434.21	ND	KRGADMIWCNARTSASGYYRK
102-121	2306.08	ND	2306.10	RGADMIWCNARTSASGYYRK
122-144	2561.27	2577.27[c]	2577.29[c]	LGFSEQGEVFDTPPVGPHILMYK

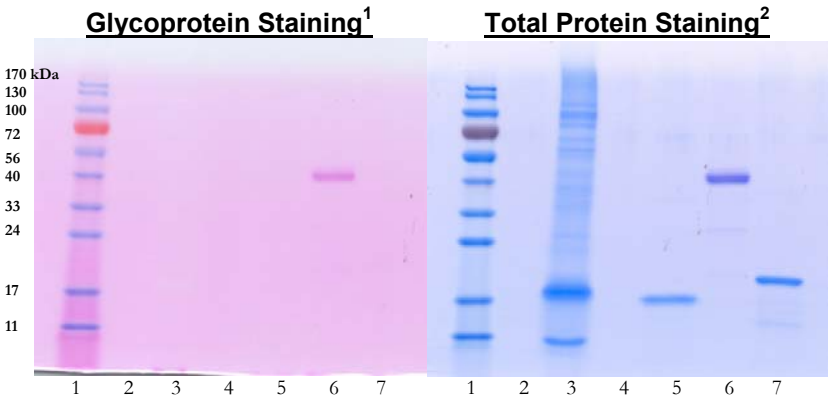
[a] This peptide match (amino acids 2-17) was made by allowing for the absence of the N-terminal methionine.
[b] ND is an abbreviation for not detected
[c] Peptide identified following correction of observed mass for methionine oxidation during SDS-PAGE (15.995 Da was added to the theoretical mass).

4.11. Results of Protein Glycosylation Analysis of GAT4621

Glycosylation was not detected for either the 98140 corn-derived or microbially expressed GAT4621 proteins (Figure 3). The glycoprotein positive control (horseradish peroxidase) was stained and clearly visible as a magenta colored band and the negative control (soybean trypsin inhibitor) was not stained.

The results show that the microbially expressed and 98140 corn-derived GAT4621 proteins do not posess detectable carbohydrates and are equivalent with respect to protein glycosylation.

Figure 3. Glycosylation Analysis of the Microbially Expressed and 98140 Corn-Derived GAT4621 Proteins



Lane	Sample ID
1	Fermentas PageRuler Prestained Protein Ladder (#SM0671)
2	Blank
3	GAT4621 from 98140 Corn (~4 µg)
4	Blank
5	Microbially Expressed GAT4621 (~1µg)
6	Horseradish Peroxidase (~1µg)
7	Soybean Trypsin Inhibitor (~1µg)

[1] This gel was stained with the glycoprotein staining kit
[2] This gel was stained with the glycoprotein staining kit followed by staining with Coomassie Blue

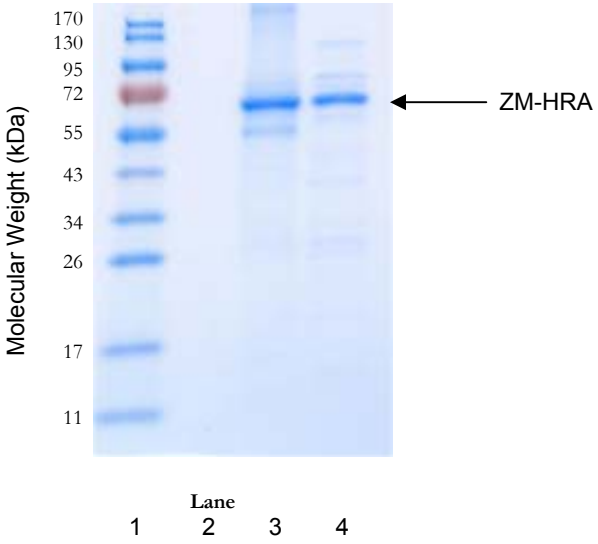
4.12 Results of SDS-PAGE and Western Analyses of ZM-HRA

SDS-PAGE analysis indicated that the microbially expressed and 98140 corn plant-derived ZM-HRA proteins had predominant bands migrating as expected at approximately 65 kDa (Figure 4).

A predominant immunoreactive band migrating at approximately 65 kDa was detected using maize ALS/ZM-HRA antibody (Figure 5) and ZM-HRA-specific antibody (Figure 6) for both the microbially expressed and 98140 corn plant derived ZM-HRA proteins. As expected, the ZM-HRA-specific antibody did not recognize the maize ALS protein.

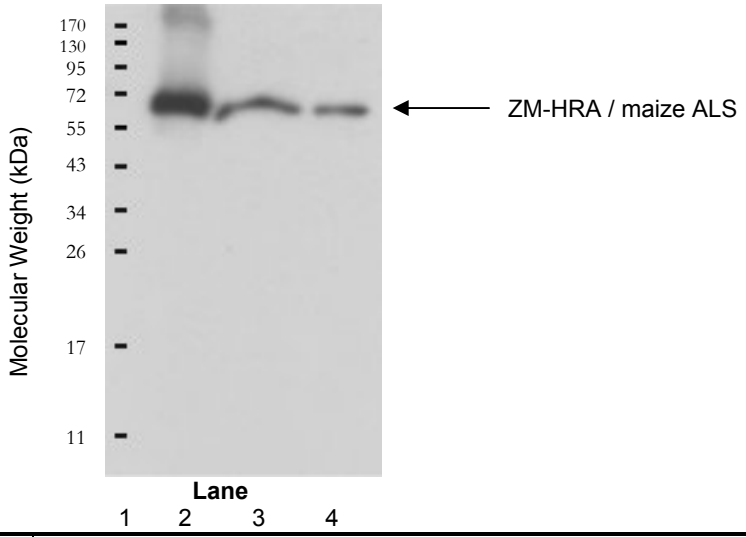
Therefore, SDS-PAGE and Western analyses confirmed that both microbially expressed and 98140 corn plant-derived ZM-HRA proteins had the expected molecular weight and immunoreactivity and were equivalent in these analyses.

Figure 4. SDS-PAGE Gel of the Microbially Expressed and 98140 Corn-Derived ZM-HRA Proteins



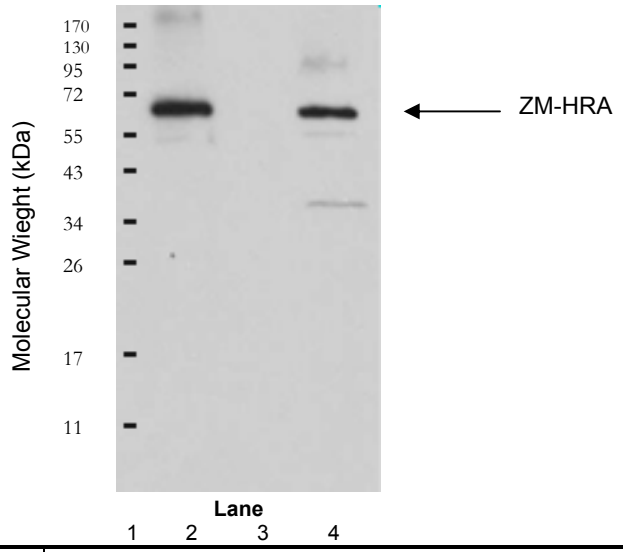
Lane	Sample ID
1	Fermentas PageRuler Prestained Protein Ladder (#SM0671)
2	Blank
3	ZM-HRA Protein Purified from the 98140 Corn Tissues (~1.5 µg)
4	ZM-HRA Protein Derived from a Microbial Expression System (~1 µg)

Figure 5. Western Blot of the Microbially Expressed and 98140 Corn-Derived ZM-HRA Proteins Using Antibody R144 that Recognizes both Maize ALS and ZM-HRA



Lane	Sample ID
1	Fermentas PageRuler Prestained Protein Ladder (#SM0671)
2	ZM-HRA Protein Purified from 98140 Corn Tissues (~25 ng)
3	Maize ALS Protein Derived from a Microbial Expression System (~20 ng)
4	ZM-HRA Protein Derived from a Microbial Expression System (~20 ng)

Figure 6. Western Blot of the Microbially Expressed and 98140 Corn-Derived ZM-HRA Proteins Using Antibody R6961 that Recognizes ZM-HRA but Not Maize ALS



Lane	Sample ID
1	Fermentas PageRuler Prestained Protein Ladder (#SM0671)
2	ZM-HRA Protein Purified from 98140 Maize Tissues (~25 ng)
3	Maize ALS Protein Derived from a Microbial Expression System (~20 ng)
4	ZM-HRA Protein Derived from a Microbial Expression System (~20 ng)

4.13 Results of N-Terminal Sequencing of ZM-HRA

N-terminal sequencing demonstrated that the N-terminal sequences of the microbially expressed and 98140 corn-derived ZM-HRA proteins were identical to the N-terminal sequence expected for each (Table 3). As expected, the primary sequence of the microbially expressed ZM-HRA protein possessed N-terminal glycine, serine and cysteine residues resulting from thrombin cleavage of the His-T7 fusion tag used for protein purification. Therefore, after accounting for the three N-terminal amino acid residues of the microbially expressed ZM-HRA, the primary N-terminal sequences of the microbially expressed and 98140 corn-derived ZM-HRA proteins matched the expected sequence of the mature ZM-HRA protein.

Table 3. N-terminal Amino Acid Sequence of the Microbially Expressed and 98140 Maize Plant Derived ZM-HRA Proteins

Expected Maize ZM-HRA Sequence:	S - A - A - S - P - A - M - P - M - A - P - P - A
98140 Maize Plant-Derived ZM-HRA Primary Sequence:	S - A - A - S - P - A - M - P - M - A - P - P - A
Microbially Expressed ZM-HRA Primary Sequence:	(G - S - C)[b] - S - A - A - S - P - A - M - P - M - A

Note: The N-terminal glycine, serine, and cysteine residues are not part of the mature form of the maize plant derived ZM-HRA protein sequence but remain following thrombin cleavage of the His-T7 fusion tag used for protein purification of the microbially expressed ZM-HRA protein.

4.14 Results of the MALDI-MS Identification of Tryptic Peptides of ZM-HRA

MALDI-MS analysis of the trypsin digest of ZM-HRA protein purified from 98140 corn tissue identified 21 unique peptides that were within 100 ppm of the theoretical peptide masses predicted from the *in silico* trypsin digestion of the ZM-HRA protein (Table 4). Seven additional peptide matches could be made by allowing for modification of cysteine residues by acrylamide (observed mass increases by 71.037 Da compared to the theoretical) and for oxidation of methionine residues (observed mass increases by 15.995 Da compared to the theoretical). The 28 identified peptides account for 460 of the 598 amino acids, or 77% of the mature ZM-HRA amino acid sequence. A peptide containing the expected tryptophan to leucine amino acid change specific for ZM-HRA was among the identified peptides.

MALDI-MS analysis of the trypsin digest of microbially expressed ZM-HRA protein identified 23 unique peptides that were within 100 ppm of theoretical peptide masses predicted from the *in silico* trypsin digestion of the microbially expressed ZM-HRA protein (Table 4). Three additional peptides matches could be made by allowing for modification of cysteine residues by acrylamide free radicals during SDS-PAGE (observed mass increases by 71.037 Da). The 26 identified peptides account for 439 of the 601 amino acids, or 73% of the microbially expressed ZM-HRA amino acid sequence. A peptide containing the expected tryptophan to leucine amino acid change specific for ZM-HRA was among the identified peptides.

The high percentage coverage of both the microbially expressed and 98140 corn-derived ZM-HRA protein sequences demonstrates that both proteins were expressed as intended.

Table 4. Matching Peptides from the Microbially Expressed and 98140 Corn-Derived ZM-HRA Proteins Identified Using MALDI-MS

ZM-HRA Amino Acid Residue #	Theoretical Mass [M+H]	Observed Microbially Expressed ZM-HRA Mass [M+H]	Observed 98140 Corn-Derived ZM-HRA Mass [M+H]	Identified Sequence
(GSC)[a]+ 1-25	2833.35	2904.38[c]	ND	GSCSAASPAMPMAPPATPLRPWGPTEPR
1-25	2586.29	ND	2618.29[b]	SAASPAMPMAPPATPLRPWGPTEPR
26-37	1329.73	1329.73	1329.74	KGADILVESLER
42-60	2062.99	2063.00	2063.05	DVFAYPGGASMEIHQALTR
61-70	1153.64	1153.64	1153.65	SPVIANHLFR
71-84	1493.67	1493.67	1493.68	HEQGEAFAASGYAR
127-144	2063.05	2063.00	2063.05	RMIGTDAFQETPIVEVTR
149-161	1568.80	1568.79	1568.81	HNYLVLDVDDIPR
162-174	1410.73	1410.73	1410.74	VVQEAFFLASSGR
175-184	1034.62	1034.61	1034.62	PGPVLVDIPK
185-207	2643.33	2643.32	2675.34[b]	DIQQQMAVPVWDKPMSLPGYIAR
208-222	1704.00	1704.00	1704.01	LPKPPATELLEQVLR
229-247	1990.02	2061.05[d]	2061.06[c]	RPVLYVGGGCAASGEELRR
248-275	2990.55	2990.53	2990.58	FVELTGIPVTTTLMGLGNFPSDDPLSLR
276-291	1769.83	1769.83	1801.83[b]	MLGMHGTVYANYAVDK
292-301	1040.64	1040.64	1040.65	ADLLLALGVR
306-333	3006.67	3006.56	3006.56[d]	VTGKIEAFASRAKIVHVDIDPAEIGKNK
332-344	1438.74	ND	1509.68[c, d]	NKQPHVSICADVK
334-344	1196.60	1267.63[c]	ND	QPHVSICADVK
345-360	1632.86	1632.84	1632.88	LALQGMNALLEGSTSK
361-376	1943.87	ND	1943.89[d]	KSFDFGSWNDELDQQK
361-377	2099.97	2100.02	ND	KSFDFGSWNDELDQQKR
362-377	1971.88	ND	1971.89[d]	SFDFGSWNDELDQQKR
378-384	853.44	853.44	853.44	EFPLGYK
385-404	2319.16	2319.15	2319.18	TSNEEIQPQYAIQVLDELTK
405-427	2572.22	2572.21	2572.24	GEAIIGTGVGQHQMWAAQYYTYK
481-505	2904.57	2904.38	ND	IENLPVKVFVLNNQHLGMVVQLEDR[e]
488-505	2111.10	ND	2111.11	VFVLNNQHLGMVVQLEDR[e]
512-534	2608.25	2608.24	2608.27	AHTYLGNPENESEIYPDFVTIAK
535-546	1329.79	1329.73	1329.74	GFNIPAVRVTKK
556-586	3399.78	ND	3431.78[b]	MLETPGPYLLDIIVPHQEHVLPMIPSGGAFK
587-595	991.44	991.46	1007.44[b]	DMILDGDGR

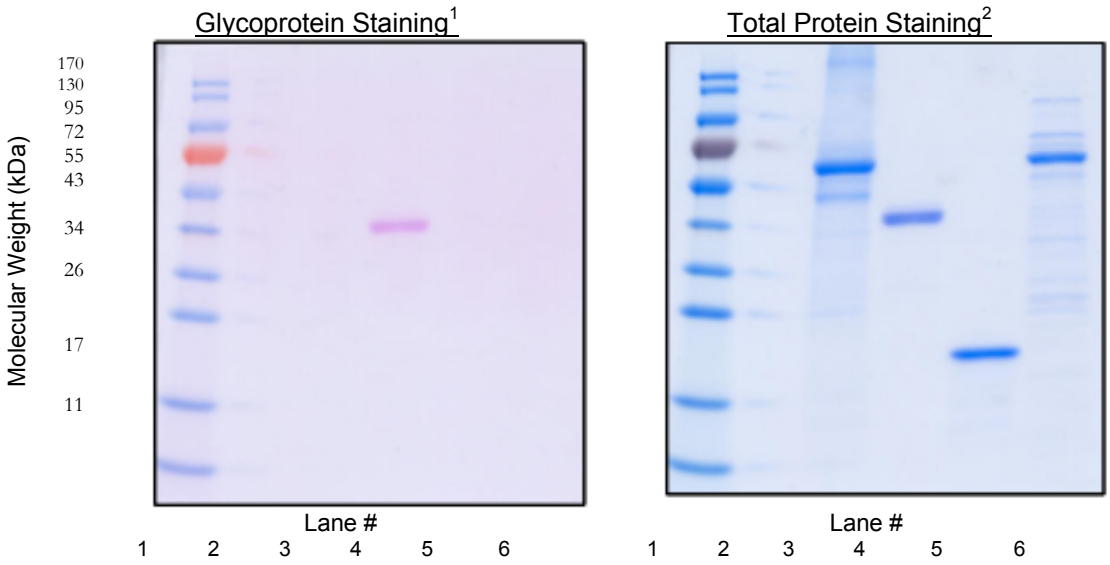
[a] The N-terminal glycine, serine, and cysteine residues are not part of the mature form of the 98140 corn-derived ZM-HRA sequence but remain following thrombin cleavage of the His-T7 fusion tag used for protein purification of the microbially expressed ZM-HRA protein.
[b] Peptide identified following correction of observed mass for methionine oxidation during SDS-PAGE (subtraction of 15.995 Da from observed mass)
[c] Peptide identified following correction of observed mass for modification of a cysteine by acrylamide during SDS-PAGE (subtraction of 71.037 Da from observed mass)
[d] This peptide has overlapping sequence with another tryptic peptide.
[e] This peptide contains the expected tryptophan to leucine (highlighted) amino acid change specific for ZM-HRA

4.15 Results of Protein Glycosylation Analysis of ZM-HRA

Glycosylation was not detected for the microbially expressed and 98140 corn-derived ZM-HRA proteins using a glycoprotein staining kit (Figure 7). The glycoprotein positive control (horseradish peroxidase) was stained and clearly visible as a magenta colored band and the negative control (soybean trypsin inhibitor) was not stained.

These results show that the microbially expressed and 98140 corn-derived ZM-HRA proteins did not posess detectable carbohydrates and are equivalent with respect to protein glycosylation.

Figure 7. Glycosylation Analysis of Microbially Expressed and 98140 Corn-Derived ZM-HRA Proteins



Lane	Sample ID
1	Fermentas PageRuler Prestained Protein Ladder (#SM0671)
2	Blank
3	ZM-HRA derived from the 98140 corn tissue (~1.5 µg)
4	Horseradish Peroxidase (~1 µg)
5	Soybean Trypsin Inhibitor (~1 µg)
6	Microbially Expressed ZM-HRA (~1 µg)

[1] This gel was stained with the glycoprotein staining kit
[2] This gel was stained with the glycoprotein staining kit followed by staining with Coomassie Blue

Appendix 5. Ecological Observations of 98140 Corn

Table 1. Insect Stressor Comparison between 98140 and Control Corn

Year of planting	Permit number	State	County	Stressor	Range of severity in 98140 corn	Difference with control?
2005	05-024-04r	HI	Kauai	Ants (Formicidae)	mild	no
				Beet armyworm (*Spodoptera exigua*)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
				Leafhopper (Cicadellidae)	mild	no
				Rose beetle (*Adoretus sinicus*)	mild	no
2005	05-024-03r	PR	Salinas	Corn earworm (*Helicoverpa zea*)	mild	no
				Fall armyworm (*Spodoptera frugiperda*)	mild	no
			Santa Isabel	Corn earworm (*Helicoverpa zea*)	mild	no
				Fall armyworm (*Spodoptera frugiperda*)	severe	no
2006	05-024-03r	PR	Juana Diaz	Corn earworm (*Helicoverpa zea*)	mild	no
				Fall armyworm (*Spodoptera frugiperda*)	mild	no
2006	05-024-04r	HI	Kauai	Ants (Formicidae)	mild	no
				Common thrips (Thripidae)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild to moderate	no
				Leafhopper (Cicadellidae)	mild	no
				Rose beetle (*Adoretus sinicus*)	mild	no
2006	06-019-03r	CA	Yolo	Painted lady (*Vanessa cardui*)	mild	no

Table 1, continued. Insect Stressor Comparison between 98140 and Control Corn

Year of planting	Permit number	State	County	Stressor	Range of severity in 98140 corn	Difference with control?
2006 (cont.)	06-019-03r (cont.)	CO	Weld	Corn rootworm (*Diabrotica* spp.)	mild to moderate	no
				Twospotted spider mite (*Tetranychus urticae*)	moderate	no
		GA	Brooks	Corn earworm (*Helicoverpa zea*)	mild to moderate	no
				Fall armyworm (*Spodoptera frugiperda*)	mild	no
			Turner	Corn earworm (*Helicoverpa zea*)	mild to moderate	no
		IL	Bureau	Corn earworm (*Helicoverpa zea*)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild to moderate	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
				Grasshoppers (Orthoptera)	mild	no
				Japanese beetle (*Popillia japonica*)	mild	no
				Spiders (Arachnida)	mild	no
			Champaign	Corn earworm (*Helicoverpa zea*)	moderate	no
				Corn rootworm (*Diabrotica* ssp.)	mild to moderate	no
				European corn borer *(Ostrinia nubilalis)*	mild to moderate	no
				Japanese beetle (*Popillia japonica*)	mild	no
			Clinton	Grasshoppers (Orthoptera)	mild	no
				Japanese beetle *Popillia japonica*)	mild	no
			Effingham	Black cutworm (*Agrotis ipsilon)*	moderate	no
				Corn flea beetle (*Chaetocnema pulicaria*)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
				Japanese beetle (*Popillia japonica*)	mild to moderate	no
			Ogle	Corn rootworm (*Diabrotica* ssp.)	mild to severe	no
				Grasshoppers (Orthoptera)	mild	no
				Japanese beetle (*Popillia japonica*)	mild	no
			Stark	Aphids (Aphididae)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
				Grasshoppers (Orthoptera)	mild	no
		IN	Tipton	Corn rootworm (*Diabrotica* spp.)	mild	no

Table 1, continued. Insect Stressor Comparison between 98140 and Control Corn

Year of planting	Permit number	State	County	Stressor	Range of severity in 98140 corn	Difference with control?
2006 (cont.)	06-019-03r (cont.)	IN	Tipton	Japanese beetle (*Popillia japonica*)	mild	no
		IA	Guthrie	Aphids (Aphididae)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
				Grasshoppers (Orthoptera)	mild	no
				Western bean cutworm (*Richia albicosta*)	mild	no
			Jefferson	Aphids (Aphididae)	mild	no
				Common thrips (Thripidae)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
				Leafhopper (Cicadellidae)	mild	no
			Keokuk	Aphids (Aphididae)	mild	no
				Common thrips (Thripidae)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
				Leafhopper (Cicadellidae)	mild	no
			Kossuth	Beetles (Coleoptera)	mild	no
				Corn rootworm (*Diabrotica* ssp.)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
				Grasshoppers (Orthoptera)	mild	no
			Linn	Corn rootworm (*Diabrotica* spp.)	mild to moderate	no
				European corn borer *(Ostrinia nubilalis)*	mild to moderate	no
				Japanese beetle (*Popillia japonica*)	mild	no
				Western bean cutworm (*Richia albicosta*)	mild	no
			Polk	Corn flea beetle (*Chaetocnema pulicaria*)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
				Western bean cutworm (*Richia albicosta*)	mild	no
		KS	Kingman	Aphids (Aphididae)	mild	no
				Corn earworm (*Helicoverpa zea*)	moderate	no
				Lady beetles (Coccinellidae)	mild	no
				Southwestern corn borer (*Diatraea grandiosella)*	moderate	no

Table 1, continued. Insect Stressor Comparison between 98140 and Control Corn

Year of planting	Permit number	State	County	Stressor	Range of severity in 98140 corn	Difference with control?
2006 (cont.)	06-019-03r (cont.)	MN	Blue Earth	Aphids (Aphididae)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
			Freeborn	Aphids (Aphididae)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
			Stearns	Aphids (Aphididae)	mild	no
				Grasshoppers (Orthoptera)	mild	no
			Steele	European corn borer *(Ostrinia nubilalis)*	mild	no
		MO	Adair	Aphids (Aphididae)	mild	no
				Common thrips (Thripidae)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
				Leafhopper (Cicadellidae)	mild	no
			Saline	Corn rootworm (*Diabrotica* spp.)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
		NE	Antelope	Corn earworm (*Helicoverpa zea*)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
				Fall armyworm (*Spodoptera frugiperda*)	mild	no
			Polk	Corn earworm (*Helicoverpa zea*)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
				Fall armyworm (*Spodoptera frugiperda*)	mild	no
				Twospotted spider mite (*Tetranychus urticae*)	mild	no
				Western bean cutworm (*Richia albicosta*)	mild	no
			York	Aphids (Aphididae)	mild	no
				Beetles (Coleoptera)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild to moderate	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
				Fall armyworm (*Spodoptera frugiperda*)	mild	no
				Green lacewing *(Chrysoperla carnea)*	mild	no
				Lady beetles (Coccinellidae)	mild	no
				Twospotted spider mite (*Tetranychus urticae*)	mild to severe	no

Table 1, continued. Insect Stressor Comparison between 98140 and Control Corn

Year of planting	Permit number	State	County	Stressor	Range of severity in 98140 corn	Difference with control?
2006 (cont.)	06-019-03r (cont.)	NE	York (cont.)	Western bean cutworm (*Richia albicosta*)	mild	no
		OK	Caddo	Corn earworm (*Helicoverpa zea*)	moderate to severe	no
				Fall armyworm (*Spodoptera frugiperda*)	moderate to severe	no
				Wasps (Hymenoptera)	mild	no
			Custer	Corn earworm (*Helicoverpa zea*)	moderate	no
				Fall armyworm (*Spodoptera frugiperda*)	moderate to severe	no
				Southwestern corn borer (*Diatraea grandiosella)*	moderate	no
				Wasps (Hymenoptera)	mild	no
		PA	Lancaster	Beetles (Coleoptera)	mild	no
				Corn earworm (*Helicoverpa zea*)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild to moderate	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
				Japanese beetle (*Popillia japonica*)	mild to moderate	no
				Spiders (Arachnida)	mild	no
			Lehigh	European corn borer *(Ostrinia nubilalis)*	moderate to severe	no
		PR	Guayama	Fall armyworm (*Spodoptera frugiperda*)	mild to moderate	no
			Salinas	Corn earworm (*Helicoverpa zea*)	mild	no
				Fall armyworm (*Spodoptera frugiperda*)	moderate	no
		VA	Louisa	Corn earworm (*Helicoverpa zea*)	mild	no
		WI	Rock	Corn rootworm (*Diabrotica* ssp.)	mild	no
				European corn borer *(Ostrinia nubilalis)*	mild	no
			Walworth	Twospotted spider mite (*Tetranychus urticae*)	mild	no
				Corn rootworm (*Diabrotica* spp.)	mild	no
				Lady beetles (Coccinellidae)	mild	no
				Picnic beetle *(Glischrochilus quadrisignatus)*	mild	no
2006	06-019-04r	HI	Kauai	Corn earworm (*Helicoverpa zea*)	mild to severe	no
				Leafhopper (Cicadellidae)	mild	no
				Rose beetle (*Adoretus sinicus*)	mild	no
				Seedcorn beetle *(Stenolophus lecontei)*	moderate	no
2006	06-097-101n	MS	Washington	Chinch bug *(Blissus leucopterus)*	moderate to severe	no
				Corn earworm (*Helicoverpa zea*)	moderate	no
				Fall armyworm (*Spodoptera frugiperda*)	moderate	no

Table 2. Disease Stressor Comparison between 98140 and Control Corn

Year of planting	Permit number	State	County	Stressor	Range of severity in 98140 corn	Difference with control?
2005	05-024-04r	HI	Kauai	Aspergillus ear and kernel rot (*Aspergillus* sp.)	mild	no
				Fusarium (*Fusarium* sp.)	mild	no
				Gibberella ear rot (*Gibberella zeae*)	mild	no
2006	05-024-04r	HI	Kauai	Common corn rust (*Puccinia sorghi*)	moderate	no
				Maize mosaic virus	mild	no
2006	06-019-03r	CA	Yolo	Common smut (*Ustilago zeae*)	mild	no
		CO	Weld	Common smut (*Ustilago zeae*)	mild	no
		GA	Brooks	Common corn rust (*Puccinia sorghi*)	mild	no
			Turner	Common corn rust (*Puccinia sorghi*)	moderate	no
				Common smut (*Ustilago zeae*)	mild	no
		IL	Bureau	Anthracnose stalk rot (*Colletotrichum graminicola*)	moderate	no
				Common corn rust (*Puccinia sorghi*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	moderate	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild to moderate	no
				Southern corn rust (*Puccinia polysora*)	mild	no
			Champaign	Anthracnose stalk rot (*Colletotrichum graminicola*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild to moderate	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild to moderate	no
			Effingham	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Southern corn leaf blight (*Bipolaris maydis*)	mild	no
				Stewart's seedling wilt (*Pantoea stewartii*)	mild	no
			Stark	Anthracnose (*Colletotrichum graminicola*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
		IN	Tipton	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
		IA	Guthrie	Common smut (*Ustilago zeae*)	mild	no
				Eyespot (*Aureobasidium zeae*)	mild	no

Table 2, continued. Disease Stressor Comparison between 98140 and Control Corn

Year of planting	Permit number	State	County	Stressor	Range of severity in 98140 corn	Difference with control?
2006 (cont.)	06-019-03r (cont.)	IA	Guthrie	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
			Jefferson	Common corn rust (*Puccinia sorghi*)	mild	no
				Common smut (*Ustilago zeae*)	mild	no
			Keokuk	Common corn rust (*Puccinia sorghi*)	mild	no
				Common smut (*Ustilago zeae*)	mild	no
				Northern corn leaf blight (*Exserohilum turcicum*)	moderate	no
			Kossuth	Common smut (*Ustilago zeae*)	mild	no
			Linn	Anthracnose (*Colletotrichum graminicola*)	mild	no
				Gibberella ear rot (*Gibberella zeae*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
			Polk	Common corn rust (*Puccinia sorghi*)	mild	no
				Common smut (*Ustilago zeae*)	mild to severe	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild to moderate	no
				Southern corn rust (*Puccinia polysora*)	mild	no
				Stewart's seedling wilt (*Pantoea stewartii*)	mild to severe	no
		MO	Freeborn	Common corn rust (*Puccinia sorghi*)	mild	no
				Common smut (*Ustilago zeae*)	mild	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
			Stearns	Common corn rust (*Puccinia sorghi*)	mild	no
				Common smut (*Ustilago zeae*)	mild	no
				Northen corn leaf spot (*Bipolaris zeicola*)	mild	no
			Steele	Common corn rust (*Puccinia sorghi*)	mild	no

Table 2, continued. Disease Stressor Comparison between 98140 and Control Corn

Year of planting	Permit number	State	County	Stressor	Range of severity in 98140 corn	Difference with control?
2006 (cont.)	06-019-03r (cont.)	MO	Adair	Common smut (*Ustilago zeae*)	mild	no
				Southern corn leaf blight (*Bipolaris maydis*)	mild	no
			Saline	Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
		NE	Antelope	Common corn rust (*Puccinia sorghi*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
			Polk	Common corn rust (*Puccinia sorghi*)	mild	no
			York	Common corn rust (*Puccinia sorghi*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Southern corn rust (*Puccinia polysora*)	moderate	no
		PA	Lancaster	Anthracnose (*Colletotrichum graminicola*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no
				Northern corn leaf blight (*Exserohilum turcicum*)	mild	no
				Northen corn leaf spot (*Bipolaris zeicola*)	mild	no
			Lehigh	Common smut (*Ustilago zeae*)	mild	no
		WI	Rock	Common corn rust (*Puccinia sorghi*)	mild	no
				Gray leaf spot (*Cercospora zeae-maydis*)	mild	no

Appendix 6. USDA Field Trials of 98140 Corn[1,2]

Year of Planting	Permit Name	Permit Valid Date	State	Number of Counties Where 98140 Corn Was Planted	Acreage[4]
2005	05-024-04r	5/25/2005	HI	1	1.60
2005	05-024-03r	5/25/2005	PR	2	9.55
2006[3]	05-024-03r	5/25/2005	PR	1	1.23
2006[3]	05-024-04r	5/25/2005	HI	1	15.47
2006[3]	06-019-03r	5/2/2006	CA	1	0.18
			CO	1	0.37
			GA	2	0.35
			IL	6	5.14
			IN	1	0.57
			IA	6	3.61
			KS	1	0.05
			MN	4	2.25
			MO	2	0.75
			NE	3	3.59
			ND	1	0.13
			OK	2	0.39
			PA	2	1.21
			PR	2	3.39
			VA	1	0.13
			WI	2	0.61
2006[3]	06-019-04r	5/3/2006	HI	1	5.54
2006[3]	06-097-101n	5/9/2006	MS	1	0.24
2007[3]	06-019-04r	5/3/2006	HI	1	2.17
2007[3]	06-019-03r	5/2/2006	PR	1	4.72
2007[3]	07-040-101rm	5/3/2007	multiple	NA[5]	NA

[1] Plantings through March 22, 2007 are listed.
[2] In USDA final reports, 98140 corn (event DP-Ø9814Ø-6) is called DP-098140-6.
[3] Final field test report not yet due to USDA.
[4] Total acreage for all experiments that contain 98140 corn (acreage includes controls and other non-98140 corn plants)
[5] Data not yet available (NA)

Appendix 7. Survey of Potential Substrates for GAT4621

In enzyme kinetics, k_{cat} is a measure of the turnover rate or speed of the reaction. The higher the k_{cat}, the faster the enzyme reaction. K_M is the affinity of the enzyme for a substrate or tightness of binding of the substrate to the enzyme. The lower the K_M, the greater the affinity of the enzyme for the substrate. A k_{cat}/K_M ratio is the common way to express the catalytic efficiency of the enzyme. The greater the k_{cat}/K_M ratio, the greater the catalytic efficiency of the enzyme for a given substrate. Specificity of an enzyme can be judged by comparing the values of k_{cat}/K_M for various enzyme substrates.

A survey of enzyme substrates was undertaken to determine the kinetic properties and specificity of GAT enzymes, including a Round 11 GAT protein that only differed from GAT4621 in 98140 corn by the absence of an alanine residue in the second position of the protein sequence (Siehl *et al*., 2005). The survey was done, in part, to determine the physiological substrate of the native enzymes from *Bacillus licheniformis*. A broad sampling of amino acids, antibiotics and nucleotides was surveyed with the native enzyme. Among antibiotics, no detectable activity was seen with D,L-phosphinothricin or kanamycin, erythromycin, carbenicillin, spectinomycin, streptomycin, chloramphenicol or ampicillin. Other biological amines that supported no activity were D-glucosamine, serotonin, anthranilate, ornithine, purine and pyrimidine bases, nucleosides, nucleotides, histone and tRNA. None of the amino acids, antibiotics or nucleotides surveyed exhibited high catalytic efficiency with the native enzyme, from which it was concluded that the physiological role of GAT in *B. licheniformis* is still unknown.

Native GAT enzymes from *Bacillus licheniformis* strains did exhibit weak activity with seven of the common amino acids: L-aspartate, L-serine, phospho-L-serine, L-threonine, L-glutamate, L-asparagine and L-cysteine (Siehl *et al.,* 2005). Activity with the other protein amino acids was either nil or less than 3% of that of glyphosate. Shuffling of the enzyme towards an improved k_{cat}/K_M for glyphosate resulted in increased activity toward aspartate, although overall catalytic efficiency was still very low (~0.5% of glyphosate for a Round 11 GAT protein). Activity with serine and phosphoserine almost completely vanished.

In order to examine the specific catalytic efficiency of the GAT4621 enzyme expressed in 98140 corn, a substrate specificity study was done with the microbial GAT4621 protein that had previously been determined to be equivalent to plant-derived GAT4621 protein (see Section VI-D and Appendix 4). A dithiobis-2-nitrobenzoate enzymatic end-point assay was used to detect any enzyme activity in a survey of various substrates (Siehl *et al*., 2005) using assay buffer containing 100 mM KCl to represent physiological conditions in the plant cytosol and chloroplast (Coruzzi and Last, 2000; Cuin *et al*., 2003). Twenty agrochemicals, 21 amino acids and 11 antibiotics were tested as potential substrates for the GAT4621 protein. As expected from the previous survey, no significant activity was seen with the majority of the substrates.

Of the substrates surveyed for activity with GAT4621, only five amino acid substrates indicated low but measurable enzyme activity (sufficiently above the limit of quantitation of the assay): L-aspartate, L-glutamate, L-serine, L-glycine and L-threonine. The remaining amino acids and substrates surveyed (other than glyphosate) produced levels of end product near or below the limit of quantitation of the end-point assay.

GAT4621 activity on the five amino acids was further characterized using a continuous spectrophotometric assay (Siehl *et al*., 2005) for characterization of kinetic properties (Table 1). The affinity of the GAT4621 enzyme for L-glycine was so low that K_M values could not be accurately estimated. Therefore, a k_{cat}/K_M ratio could not be calculated.

Table 1. k_{cat}/K_M for the GAT4621 Enzyme on Glyphosate and Selected Amino Acids

Substrate	k_{cat}/K_M (+/- standard deviation) min^{-1} mM^{-1}	% k_{cat}/K_M of glyphosate
Glyphosate	1063 (+/- 31.8)	100
Aspartate	12.1 (+/- 1.57)	1.14
Glutamate	8.32 (+/- 0.46)	0.78
Serine	0.57 (+/- 0.05)	0.05
Threonine	0.60 (+/- 0.01)	0.06
Glycine	ND[1]	ND[1]

[1] ND – Not able to determine because the K_M was too high to estimate.

The results of the GAT4621 substrate survey confirmed the results of the earlier published substrate survey for the GAT enzyme. The GAT4621 enzyme was able to use five amino acids as substrates (aspartate, glutamate, serine, threonine and glycine), although inefficiently (~0.05 - 1% of that of GAT4621 on glyphosate). Further kinetic characterization indicated that the K_M of GAT4621 for glycine was too high to estimate (low affinity of the GAT4621 enzyme for glycine as substrate). The level of catalytic efficiency of GAT4621 on aspartate, glutamate, serine, and threonine was about 1%, 0.8%, 0.05 and 0.06%, respectively, of the activity on glyphosate.

Due to the low levels of GAT4621 enzyme activity on aspartate and glutamate as substrates, the presence of N-acetylaspartate and N-acetylglutamate in 98140 and control corn grain was examined in the nutrient composition studies. From the very low catalytic efficiency of GAT4621 on serine and threonine (0.05-0.06% of that of GAT4621 on glyphosate) in comparison with that of aspartate and glutamate, we predicted that acetylated serine and threonine would not accumulate to detectable levels.

Appendix 8. Safety and History of Consumption of Acetylated Amino Acids

8.1 Safety of Acetylated Amino Acids

Acetylated amino acids are naturally occurring substances that have been identified in many biological systems including plants and animals. N-acetylglutamate is the first intermediate in the biosynthesis of arginine in prokaryotes, lower eukaryotes and plants (Caldovic and Tuckman, 2003).

Acetylation of N-terminal amino acids is the most commonly observed posttranslational modification of cytosolic proteins (Persson *et al.,* 1985; Polevoda and Sherman, 2002). It has been estimated that up to 80% of all cytosolic proteins in mammalian systems are α-N-acetylated (Brown and Roberts, 1976; Driessen *et al*., 1985). Enzymatic acetylation of amino acids using acetyl-CoA as the acetyl donor group can occur either cotranslationally or posttranslationally depending on the biological system (Polevoda and Sherman, 2000). Enzymes responsible for intracellular acetylation of amino acids (N^{α}-acetyltransferases) have been identified in rat, yeast, and other eukaryotic organisms (Lee *et al*., 1988a and 1989; Mullen *et al*., 1989; Yamada and Bradshaw, 1991a and 1991b). The biological role of acetylation of N-terminal amino acids of cytosolic proteins has been investigated and evidence indicates that this modification protects proteins from proteolysis by intracellular aminopeptidases (Jörnvall, 1975; Brown, 1979; Berger *et al*., 1981). A variety of additional roles for N-acetylation of amino acids in biological systems have been established (Polevoda and Sherman, 2002).

In light of the wide distribution and biological roles of acetylation, it is not surprising that a number of enzymes responsible for deacetylation of N-acetylated amino acids (*i.e*., acylases) have also been described. It has long been speculated that enzymatic deacetylation of amino acids is a "general" phenomenon in mammals because this reaction has been observed in numerous organs (Neuberger and Sanger, 1943). It was later hypothesized that this enzymatic reaction plays a role in the salvage of N-acetylated amino acids formed during the metabolic degradation of N-terminal acetylated proteins (Endo, 1980; Gade and Brown, 1981). To date, four classes of acylases (Types I – IV) that mediate deacetylation of N-acetylated amino acids have been described in mammalian systems that differ with regard to distribution and specificity.

N-acetylaspartate (NAA) is commonly found in mammals and is utilized as an acetyl group donor during development of nerve tissue. An elevated level of NAA in human urine or serum is used as a biochemical marker for a rare disease known as Canavan Disease (http://www.ninds.nih.gov/disorders/canavan/canavan.htm). In individuals with Canavan Disease, NAA levels increase because the enzyme needed to utilize NAA is inactive due to a genetic defect. Consumption of acetylated amino acids is not the cause of Canavan Disease, nor is the avoidance of acetylated amino acids in foodstuffs a remediation for the disease.

Acetylation of proteins is commonly employed in the food industry to alter the solubility, water absorption capacity and emulsifying properties of protein concentrates (*e.g*., El-Adawy, 2000; Ramos and Bora, 2004). Another well-characterized use of acetylated amino acids is in the livestock industry in cases when it is unsuitable to use free amino acids in feed. For example, the quality of soy protein fractions can be limited by the concentrations of the essential amino acid L-methionine. This deficiency can be overcome by supplementation of diets with free L-methionine; however this can lead to development of objectionable odors and flavors from conversion of L-methionine to methional by Strecker degradation (Ballance, 1961). Therefore, feed may be supplemented with N-acetyl-L-methionine. In rats fed diets produced with soy protein isolates, growth and weight gains were similar regardless of whether they were supplemented with L-methionine or N-acetyl-L-methionine (Boggs *et al*., 1975; Amos *et al*., 1975).

In the case of α-N-acetyl-L-methionine, the basis of substitution for L-methionine is metabolic deacetylation. Metabolism studies have demonstrated that N-acetyl-^{14}C-L-methionine is readily metabolized to L-methionine in rats and in human infants (Boggs, 1978; Stegink *et al.,* 1980 and 1982). Similarly, metabolic deacetylation of α-N-acetyl-L-methionine has been reported in *in vitro* studies using rabbit intestinal epithelial cells (Brachet *et al.*, 1991). While these reports demonstrate that this enzymatic deacetylation occurs within the digestive system, there is also evidence that N-acetyl amino acids are deacetylated in other tissues (Yoshida and Lin, 1972).

Nutritional and metabolic studies with the α-N-acetyl forms of some other amino acids have been conducted in humans, rats, and pigs. In most cases, these studies have reported that the α-N-acetyl form of amino acids substitute for the constituent amino acid via metabolic deacetylation. Such results have been reported for glutamate (Magnusson *et al*., 1989; Neuhauser and Bassler, 1986; Arnaud *et al*., 2004), phenylalanine and tryptophan (Du Vigneaud *et al.,* 1934) and threonine (Boggs, 1978). Although specific information is not available for aspartate and glutamate, there is no reason to believe these amino acids would not also be biologically available when acetylated.

8.2 History of Consumption of Acetylated Amino Acids

Several different foods were analyzed for NAA and NAG content. These foods were selected because they had high concentrations of aspartic acid and glutamic acid (USDA, 2006) and it was assumed that this might be correlated with high levels of NAA and NAG, respectively. Foods were purchased either at local grocery stores or from on-line retailers. NAA was found to be present in a variety of foods including autolyzed yeast, chicken bouillon, eggs, ground turkey, ground chicken and ground beef (Table 1). NAG was found to be present in a variety of foods including autolyzed yeast, ground beef, ground turkey, and dried egg powder (Table 2). Since only a small number of selected foods were tested, it is likely NAA and NAG can be found in many other commonly consumed foods. In addition, non-transgenic corn and soybean grain has been found to contain measurable levels of NAA and NAG.

In conclusion, NAA and NAG are normal components of human diets, based on their presence in common foods. There is no evidence to indicate that exposure to either NAA or NAG from these sources is associated with adverse effects in humans.

Table 1. Levels of NAA in Selected Foods

Description	NAA (mg/kg fresh weight)	NAA (% dry weight)
Autolyzed Yeast	12.57	0.0013
Chicken Bouillon (vegan)	12.11	0.0012
Dried Egg Powder	6.94	0.0007
Ground Turkey	3.98	0.0014
Ground Chicken	1.53	0.0006
Whole Egg	1.38	0.0006
Ground Beef	1.07	0.0003
Egg White	0.55	0.0005
98140 Corn Grain	NA[1]	0.0403

Table 2. Levels of NAG in Selected Foods

Description	NAG (mg/kg fresh weight)	NAG (% dry weight)
Autolyzed Yeast	159.75	0.0160
Ground Beef	1.53	0.0004
Ground Turkey	0.79	0.0003
Dried Egg Powder	0.70	0.00007
Chicken Bouillon (vegan)	0.36	0.00004
98140 Corn Grain	NA[1]	0.0079

[1] Data are not available

Appendix 9. Herbicide Resistant Weeds

9.1. Evolution of Herbicide Resistant Weeds

Weeds will eventually adapt and circumvent any single control mechanism. Herbicide resistant weeds are a well-established aspect of weed control for many herbicide classes including acetyl-CoA carboxylase and ALS inhibitors, glyphosate, hormone, triazines and other photosystem II inhibitors.

Herbicide resistance usually evolves in only one or two weed species in an area, even though a much larger number of weeds are exposed to the same herbicide selection intensity. Nonetheless, weed resistance to herbicides currently affects hundreds of thousands of fields and the most widely used herbicides (Heap, 2007). According to a recent survey, more than 314 herbicide resistant weed biotypes are present in agricultural fields around the world (www.weedscience.org). Resistant weeds often increase the cost of crop production and limit the effectiveness of herbicides that can be used and the crops that can be grown. Growers use a variety of approaches to limit the impact of resistant weeds on crop productivity.

When growers say that their "weeds have become resistant," they really mean that the population of resistant weed biotypes has increased to an unacceptable level. The spread of a resistance phenotype depends primarily upon the exposure or selection applied by the herbicide. When a herbicide is applied, most of the susceptible weeds die while the resistant weeds survive, mature, and produce seed. Even though the resistant population may be small, repeated application of the same herbicide continues to increase the proportion of resistant weeds in the population.

Not all weed shifts are driven by a genetically based biochemical capacity to survive exposure. For example, weeds with delayed emergence and slower development are also able to avoid exposure to the herbicide (Hilgenfield *et al*., 2004).

Herbicide resistance can become an ecological problem if the resistant weed biotype replaces the non-resistant biotype in the weed population. Even then, the shift to a herbicide resistant population of weeds has ecological consequence only if the resistant population cannot be controlled with other herbicides or other control practices. This is generally not the case. Many hundreds of cases of resistant weeds have been documented worldwide, but resistance is usually not a limiting factor for crop production. There are examples, such as biotypes of rigid ryegrass (*Lolium rigidum*) in Australia are resistant to many herbicides in several different classes (Heap, 2007).

In spite of the evolution of herbicide resistance in weed populations, US corn growers continue to have many herbicides and management options for weed control. Even so, growers must always be concerned about herbicide sustainability and the economic consequences of losing any herbicide technology due to the evolution of resistant weeds.

9.2. Characteristics of Glyphosate and ALS-Inhibiting Herbicides

9.2a. Glyphosate

Glyphosate is a broad spectrum herbicide that was introduced in the 1970s for management of annual, perennial and biennial herbaceous grasses, sedges, and broadleaves, as well as woody brush and trees (Franz *et al*., 1996).

Glyphosate controls plants by inhibiting the enzyme EPSPS (5-enolpyruvylshikimate-3-phosphate synthase). EPSPS is an essential enzyme in the shikimate pathway that ultimately leads to the

production of aromatic amino acids (tryptophan, tyrosine, and phenylalanine). The shikimate pathway for synthesizing aromatic amino acids, and therefore the enzyme EPSPS, is found in plants, bacteria and fungi, but not animals.

The structure of glyphosate resembles the structure of the substrate for EPSPS, which is phosoenolpyruvate (PEP). Therefore, glyphosate competes with PEP for the enzyme's active site and prevents conversion of PEP to the precursor that is required in the synthesis of aromatic amino acids. Aromatic amino acids are essential for many plant processes such as protein synthesis, cell wall formation, pathogen defense and hormone production. At high rates, glyphosate is toxic to virtually all plants.

9.2b. ALS-Inhibiting Herbicides

ALS-inhibiting herbicides were discovered in 1975 (Stetter, 1994; Shaner and O'Connor, 2000; Tan *et al*., 2005). They inhibit a plant enzyme called acetolactate synthase (ALS) that is required for the production of essential branched-chain amino acids such as valine, leucine, and isoleucine. There are five different chemical classes of ALS inhibitor herbicides that have been commercialized: sulfonylureas (SU), imidazolinones (IMI), triazolopyrimidines (TP), pyrimidinylthiobenzoates (PTB), and sulfonylamino-carbonyl-triazolinones (SCT).

ALS inhibitors control a wide spectrum of grass and broadleaf weeds at very low application rates (typically, fractions of ounces per acre). In addition, they generally have very low mammalian toxicity and possess a favorable environmental profile. Today, about 56 different ALS inhibitor active ingredients are marketed with registrations in all major crops. Significant changes in herbicide potency, crop selectivity, and weed control can be made with small chemical alterations within the ALS-inhibiting herbicide class.

9.3. Evolution of Resistance to Glyphosate and ALS-Inhibiting Herbicides

9.3a. Glyphosate

For more than two decades, the evolution of glyphosate resistance was not perceived as a problem (Bradshaw *et al*., 1997). This opinion was based on the difficulty of discovering a fully functional 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) that was insensitive to glyphosate, the inability of plant species to enzymatically deactivate glyphosate, the lack of soil activity of glyphosate (thus reducing the selection pressure due to exposure), and the empirical observation that no resistant weeds had appeared after at least 20 years of use.

The most likely theoretical ways for weeds to develop resistance did not seem likely for glyphosate (Jasieniuk, 1995). Over-production of the EPSPS target site did not increase resistance enough for plants to survive glyphosate amounts used in agricultural settings (Kishore and Shah, 1988). The EPSPS modifications that conferred glyphosate resistance in bacteria were inside the enzyme's active site, which reduced its catalytic efficiency and thus would probably reduce plant fitness (Padgette *et al*., 1995). No higher plants could be found metabolically inactivate glyphosate (Dyer, 1994).

However, the views about the ability of weeds to evolve glyphosate resistance changed in 1996 when glyphosate-resistant *Lolium rigidum*, was discovered in Australia (Powles *et al*., 1998; Pratley *et al.*, 1999). Since then, glyphosate-resistant biotypes in at least ten other weed species have been confirmed (Green, 2007; Heap, 2007), and more than a million hectares are now affected by resistant weeds. Some of these biotypes exhibit resistance at application rates four to 13 times higher than susceptible populations.

The most widespread glyphosate-resistant weed is marestail (*Conyza canadensis*). Glyphosate-resistant marestail was confirmed in 2000 (VanGessel, 2001). Marestail produces very large numbers of light, wind dispersed seed and can cross-pollinate, leading to rapid spreading of

resistant weeds to no-tillage crop and non-crop land. As a result, five years after its first occurrence, it was found on a half-million hectares across the U.S. Midwest, South and Atlantic states (VanGessel, 2001; Heap, 2007). Dose response analysis showed these populations were eight to 13-fold more resistant than susceptible marestail populations.

The molecular basis for weed resistance to glyphosate is not understood in most cases. Initial studies of various weed species revealed EPSPS target site insensitivity (Braerson *et al*., 2002). Differences in translocation and transport to the chloroplast are also important in some weeds (Lorraine-Colwill *et al*., 2003; Feng *et al*., 2004). The mechanisms of glyphosate resistance in other weeds appear to be complex and polygenic.

The effectiveness, economic benefits, and ease of using glyphosate have led to repeated applications, year after year, in areas where glyphosate tolerant biotech crops are grown. This intensive use has resulted in a high selection pressure for weeds that inherently are difficult to control with glyphosate (Culpepper, 2004). Eventually, this selection pressure can lead the spectrum of weeds in the fields to a shift to those weeds that inherently can tolerate glyphosate.

As predicted, spectrum shifts to weed populations with endogenous glyphosate resistance have occurred more rapidly than evolved resistance in response to glyphosate exposure (Shaner, 2000). For example, in Iowa, common waterhemp (*Amaranthus rudis*) and velvetleaf (*Abutilon theophrasti*) became a concern in glyphosate-resistant soybean soon after crop commercialization (Owen, 1997).

9.3b. ALS-Inhibiting Herbicides

The five classes of ALS-inhibiting herbicides differ chemically, but all bind to the same target site on the ALS enzyme. The primary molecular basis of weeds resistant to ALS inhibitors is reduction of target site sensitivity, although there are biotypes that are resistant through more rapid detoxification of the herbicide to inactive metabolites.

To date, weed scientists have identified 95 weed species containing biotypes that are resistant to ALS-inhibiting herbicides (Heap, 2007). The large number of resistant biotypes is due, in part, to the relatively large number of amino acid substitutions that can change the ALS enzyme from a sensitive to a resistant form. Five different mutations sites have been identified in naturally occurring resistant weed populations (Bernasconi *et al.,* 1995; Tranel and Wright, 2002). However, not all resistant weeds are resistant to all classes of ALS-inhibiting herbicides (Tranel and Wright, 2002). ALS inhibitor resistance generally falls into three categories:

1. Broad resistance to sulfonylureas (SU), imidazolinones (IMI), triazolopyrimidines (TP), and pyrimidinylthiobenzoates (PTB);
2. Resistance to IMI and PTB only; and
3. Resistance to SU and TP only.

The evolution of ALS inhibitor-resistant weeds has typically been after five to eight years of repeated, if not continuous, use of herbicides with that mode of action. Resistance has generally not been selected where ALS-inhibiting herbicides have been used as part of an integrated program (Shaner *et al*., 1997). Fortunately, most ALS inhibitor-resistant weed populations are localized. Exceptions include ALS-resistant kochia (*Kochia scoparia*) and Russian thistle (*Salsola iberica*) that are now present in over 60% of the wheat fields in the northern United States (Heap and LeBaron, 2001), wild sunflowers (*Helianthus annuus*) in the upper Midwest, and waterhemp species in the Midwest. To control these weeds, growers mix herbicides with different modes of action. Fortunately, most resistant weed populations can still be controlled with commonly used, low cost mixtures and the value of this mode of action has largely been retained. Therefore, despite the evolution of resistance, ALS-inhibiting herbicides are still among the most efficacious and widely used weed control agents in the world (Shaner and Heap, 2002). These products account for about 17.5% of the total worldwide herbicide market with new products still being

commercialized. Within the past five years, eight new active ingredients from the ALS-inhibiting herbicide family have been introduced.

9.4. Stewardship of Herbicide Tolerant 98140 Corn

DuPont's Pioneer Hi-Bred International, Inc. and Crop Protection Chemicals businesses both have long histories of product stewardship. Because of the unique nature of the dual herbicide tolerance in 98140 corn, stewardship efforts for this product will be a joint collaboration between the two businesses. Stewardship principles will be incorporated into marketing, positioning, promotional and communications strategies for herbicide tolerant 98140 corn. Examples of these efforts are detailed below.

9.4a. Local Weed Management

In collaboration with university investigators, private consultants, other manufacturers, and growers, DuPont Crop Protection field development personnel conduct hundreds of field trials annually to refine existing recommendations and to investigate potential new active ingredients and herbicide combinations deployed in an Integrated Weed Management context to improve solutions for existing and emerging weed problems. Within Pioneer, field agronomists give presentations to local audiences about resistant weeds and best management practices. In addition, they assist customers in making crop management decisions, including options for managing weeds.

9.4b. Product Labeling

For at least 20 years, all DuPont herbicide labels have carried voluntary statements regarding resistant weed management. An example of the language used on the labels follows. These labels actively promote an integrated weed management philosophy as seen in the second paragraph:

RESISTANCE

When herbicides that affect the same biological site of action are used repeatedly over several years to control the same weed species in the same field, naturally-occurring resistant biotypes may survive a correctly applied herbicide treatment, propagate, and become dominant in that field. Adequate control of these resistant weed biotypes cannot be expected. If weed control is unsatisfactory, it may be necessary to retreat the problem area using a product affecting a different site of action.

To better manage herbicide resistance through delaying the proliferation and possible dominance of herbicide resistant weed biotypes, it may be necessary to change cultural practices within and between crop seasons such as using a combination of tillage, retreatment, tank-mix partners and/or sequential herbicide applications that have a different site of action. Weed escapes that are allowed to go to seed will promote the spread of resistant biotypes.

It is advisable to keep accurate records of pesticides applied to individual fields to help obtain information on the spread and dispersal of resistant biotypes. Consult your agricultural dealer, consultant, applicator, and/or appropriate state agricultural extension service representative for specific alternative cultural practices or herbicide recommendations available in your area.

9.4c. Training and Education of Sales Representatives and Agronomists

DuPont Crop Protection: In addition to the formal academic training of technical, marketing, and sales professionals, DuPont Crop Protection has a mandatory on-line interactive training course with intensive and comprehensive coverage of product stewardship generally and herbicide resistance specifically. This training is administered globally to all employees involved in weed management recommendations and is also provided to other interested parties as a community education contribution. The technical, marketing, and sales professionals are required to be certified in this training by receiving a passing grade of 90% or higher in each of the eight modules. A person unfamiliar with this topic is expected to take about twelve hours to complete the internal version of the training. This is the first training of its kind to be awarded Certified Crop Adviser (CCA) credits by the American Society of Agronomy. The CCA standard allows growers, employers, and other organizations to help manage risk by enabling them to have assurance that a person has the appropriate professional qualifications.

Pioneer: The Pioneer sales force, made up of agronomists, account managers and sales representatives, and customers receive ongoing mandatory and voluntary stewardship training throughout the sales season through a variety of tactics. These include stewardship training sessions administered in person or communicated electronically by Pioneer Stewardship personnel. The Pioneer sales force also utilizes a proprietary computer software system to access information about products, traits, crop management practices, sales transactions and required stewardship documentation. This formal training is in addition to the practical experience of the Pioneer sales force working with growers on the positioning and use of products containing biotech traits, including herbicide resistant traits.

9.4d. Technical Bulletins Provided to Seed Customers and the Public

Direct Mail

Pioneer's Growing Point magazine is mailed to all Pioneer customers, prospects and employees. This magazine contains information about products, marketing programs, production and management practices and advice on agronomic application of traits and technologies. Information on 98140 corn and weed resistance management practices will be included in future issues of Growing Point.

Information on Websites

- Pioneer's "Growing Point" site (https://www.pioneer.com/growingpoint/login/login.jsp) has an extensive agronomy section, with access to information about many different crops and crop management practices. Technical bulletins such as "Crop Insights" have been published for the past 15 years, and are a good example of how Pioneer makes the latest information available to growers.

- The DuPont Biotechnology website (http://www2.dupont.com/Biotechnology/en_US/) contains an in-depth "Science Knowledge" section addressing "Herbicide Resistant Crops and Weed Management: Scientific Summary and the DuPont Perspective", with a section devoted to Integrated Weed Management, as well as an FAQ section that addresses weed control and Integrated Weed Management. There is also a Scientific Summary on Integrated Weed Management and Herbicide Resistance: (http://www2.dupont.com/Biotechnology/en_US/science_knowledge/herbicide_resistance/moreinfo8.html).

9.4e. Involvement in Industry Groups

DuPont Crop Protection is a long-standing participant in the Herbicide Resistance Action Committee (HRAC), an industry-based group supported by Croplife International. Their stated

mission is to “Facilitate the effective management of herbicide resistance by fostering communication and co-operation between industry, government and farmers.” They work towards fostering responsible attitudes towards herbicide use, communicating herbicide resistance management strategies and support their implementation through practical guidelines, and they seek active collaboration with public and private researchers, especially in the areas of problem identification and devising and implementing management strategies.

9.4f. Involvement with Academic Groups

Pioneer and DuPont personnel interact with academic weed scientists in conducting trials at university sites as well as seeking input from them regarding weed management strategies.

9.4g. Customer Satisfaction and Weed Resistance Management Plan

Pioneer and DuPont Crop Protection are committed to active market presence wherever we sell our products. Consistent with our stewardship principles, all of our business teams are required to maintain an active contact with customers and awareness of end-user practices and a capability to respond rapidly to issues arising. DuPont Crop Protection maintains a customer satisfaction database to track and address any complaints, including for resistant weeds. Field employees are trained and provided tools and processes for responding to inquiries regarding product performance (or any potential impacts on human health or environment).