Petition for Determination of Nonregulated Status
for Lysine Maize LY038

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

Submitted by

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On behalf of Renessen LLC

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NO CBI

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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.

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Summary

Lysine maize LY038 was developed, through the use of recombinant DNA techniques, to integrate the *cordapA* coding sequence into the maize genome. The *cordapA* sequence is under the control of the maize Glb1 promoter to direct expression of the *Corynebacterium glutamicum*-derived lysine-insensitive dihydrodipicolinate synthase (cDHDPS) enzyme predominantly in the germ, to increase the level of lysine in grain for animal feed applications. Maize-soybean meal based broiler diets formulated to include animal protein products and/or corn gluten meal and typical maize-soy based swine diets are characteristically deficient in lysine and require the addition of supplemental lysine for optimal animal growth and production (NRC, 1994; 1998). The supplemental lysine is most commonly provided from commercially available lysine sources in the form of lysine monohydrochloride or lysine sulphate (Leuchtenberger, 1996) produced via fermentation by *Corynebacterium glutamicum* or *Brevibacterium lactofermentum* (Eggeling, 1994). When added to animal diets at nutritional levels, lysine is Generally Recognized As Safe (GRAS) by the U.S. Food and Drug Administration (21 CFR 582.5411) and may be used safely as a human food additive when provided at nutrient levels (21 CFR 172.320).

Development of LY038 provides an alternative to direct addition of supplemental lysine to poultry and swine diets by increasing the amount of lysine in the maize component of feed. Introduction of the *cordapA* gene into the maize genome produces a maize grain with higher lysine content and higher nutritional value for use as a feed ingredient for animals, primarily poultry (broilers and turkeys) and swine. Total lysine content of conventional maize, most of which is present as protein-incorporated lysine, typically ranges from 2500 to 2800 ppm on a dry weight basis. Levels of free lysine are targeted to be in the range of 1000 to 2500 ppm in LY038 grain, compared to levels of <100 ppm in conventional maize grain. Therefore, in LY038, the expected total lysine would range from 3500 to 5300 ppm.

The 5.9 kb *Xho* I linear fragment of plasmid vector PV-ZMPQ76 used for the initial maize biolistic transformation contained the *cordapA* coding sequence as well as an *nptII* cassette encoding resistance to the antibiotic paromomycin to facilitate selection of plants carrying both the *cordapA* and *nptII* coding sequences. The *nptII* cassette was flanked by *lox*P sites that allowed the *nptII* cassette to be excised by Cre recombinase when plants regenerated from transformation were crossed with maize plants expressing the *cre* gene. The *cre* gene was then segregated out by conventional breeding to produce the LY038 product from which the *nptII* gene was eliminated.

Molecular characterization by Southern blot analysis demonstrated that LY038 contains one intact copy of the *cordapA* gene cassette inserted at a single site in the maize genome. PCR analyses confirmed the organization of the genetic elements in the LY038 insert to be identical to that in plasmid PV-ZMPQ76. No additional elements, including intact or partial DNA fragments of the *nptII* cassette or backbone sequence, from plasmid

vector PV-ZMPQ76, linked or unlinked to the intact gene cassette, were detected in LY038. Also, no intact or partial DNA fragments of the *cre* cassette or backbone sequence from plasmid PV-ZM003 used to generate *cre*-expressing plants used in excision of the *nptII* cassette were detected in LY038. The presence of the *cordapA* gene cassette and absence of both the *cre* and *nptII* gene cassettes in LY038 was further confirmed by Southern blot generational stability analyses over multiple generations representing each branch point of the LY038 breeding tree. Therefore, no gene products from the *cre* and *nptII* coding sequences are expected to be present in LY038. Based on the detailed molecular characterization, LY038 contains only one newly inserted coding sequence, *cordapA*, that encodes the cDHDPS enzyme from *Corynebacterium glutamicum*.

Corynebacterium glutamicum is a common soil bacterium that is widespread in the environment; therefore, animals and humans are regularly exposed without adverse consequences to this organism and its components, such as the cDHDPS protein. In addition, DHDPS proteins functionally related to cDHDPS in LY038 are present in plants and microbes that make lysine, many of which are consumed as feed and/or food, such as maize. Consistent with the fact that all of these DHDPS proteins catalyze the first enzymatic step in lysine biosynthesis in a wide range of organisms, it is to be expected that an algorithm to identify local amino acid homology along the entire length of the protein showed 27 - 37% sequence identity and 36 – 47% similarity between cDHDPS and DHDPS proteins from other species.

Bioinformatic analyses revealed no biologically relevant structural or immunological similarities of the cDHDPS protein sequence to known allergens, toxins, or pharmacologically active proteins. Furthermore, no short (eight amino acid) polypeptide matches were shared between the cDHDPS protein sequence and known protein allergens. These data establish the lack of both structurally and immunologically relevant similarities between allergens and the cDHDPS protein sequence. The cDHDPS protein purified from *E. coli* was shown to be physicochemically and functionally equivalent to cDHDPS produced in LY038. The demonstrated rapid degradation of the *E. coli*-produced cDHDPS protein in simulated gastric fluid (greater than 96% of cDHDPS was degraded within 30 seconds) supports the lack of allergenic potential of the expressed cDHDPS protein. No adverse effects were detected in a mouse gavage acute oral toxicity study at the highest dose tested (NOEL of ≥ 800 mg/kg for *E. coli*-produced cDHDPS protein). These data support the feed and food safety of the cDHDPS protein.

LY038 maize grain will be identity preserved and used as a feed ingredient in poultry and possibly swine diets. Because LY038 is not intended to be used for food, human consumption of the cDHDPS protein from processed grain products is expected to be low. Possible human exposure to cDHDPS from LY038 is further decreased by the fact that expression of the cDHDPS protein in LY038 is primarily in the germ portion of the grain, while the endosperm is the predominant maize fraction consumed by humans from typical grain processing (wet and dry milling).

As expected, the levels of cDHDPS in grain were higher than those in other plant tissues (26, 0.081 and 0.94 μg/g dwt in grain, whole plant at V2-V4 growth stage and forage at the R5 growth stage, respectively) when measured by ELISA. This is consistent with the fact that *cordapA* gene expression is predominantly targeted to the germ by the Glb1 promoter in LY038. Based on cDHDPS levels in grain and the determined NOEL from the mouse acute oral toxicity evaluation, large margins of exposure were calculated for cDHDPS for livestock (>500 for broiler chickens and pigs) and humans (>45,000 for the highest consuming U.S. subpopulation, using conservative assumptions). This assessment leads to the conclusion that there is no meaningful risk to animal or human health from dietary exposure to cDHDPS from LY038.

A comprehensive phenotypic and ecological assessment based on both laboratory experiments and replicated, multisite field trials was conducted for LY038. Field trials were conducted at a total 17 total locations over two years, 2002 and 2003. Characteristics for dormancy and germination, emergence and vegetative growth, reproductive growth, seed retention, and disease, insect, and abiotic stressor-plant interactions were evaluated in each trial and provided a basis for assessment of pest potential of LY038. In each of these assessments, LY038 was compared to its negative segregant control [LY038(-)] and conventional maize to provide benchmark values common to conventional maize for each measured phenotypic and ecological characteristic.

Analysis of seed dormancy and germination data showed no differences between LY038 and LY038(-) for percent germinated, percent normal germinated, percent abnormal germinated, percent viable hard, percent dead, or percent viable firm swollen seed at any of the five temperature regimes evaluated. These results indicate no increased weed potential for LY038 from increased dormancy via hard seed or from changes in germination characteristics.

The phenotypic characterization data showed no biologically meaningful differences between LY038 and LY038(-), or several conventional reference maize hybrids with the exception of an infrequent white leaf phenotype. Differences detected in a characteristic were considered alone and in the context of whether or not trends were observed across locations, differences detected in other measured characteristics, contributions to enhanced pest potential of the crop itself, and potential effects of the transfer of the trait to a sexually compatible species. The white leaf phenotype observed in a small percentage of the plants was determined to be associated with the LY038 trait of increased lysine in the seed. The effects of the white leaf characteristic did not result in significant changes in other growth and development characteristics on a whole-plot basis and it would not contribute to increased pest potential. Therefore, assessment of the phenotypic data detected no biologically meaningful differences between LY038 and LY038(-) indicative of a selective advantage that would result in increased weed potential for LY038 or other plants if the trait were transferred to a sexually compatible species. These data support a conclusion of phenotypic equivalence as it relates to familiarity and a lack of increased weed potential. Furthermore, monitoring of field trial plots containing

LY038 after harvest has not revealed differences in survivability or persistence relative to the control or conventional maize varieties.

Insect-plant interactions were evaluated as part of the plant phenotypic studies conducted under a broad range of environmental conditions. Results from these field studies revealed no biologically meaningful differences in damage caused by insects for LY038, LY038(-), and conventional reference maize.

Evaluation of morphology and viability of pollen from LY038 detected no difference as compared to pollen from LY038(-). The lack of significant difference in evaluated characteristics of pollen from LY038 plants and control LY038(-) plants supports a conclusion of phenotypic equivalence for a component of reproductive development.

Extensive compositional analyses of forage (whole plant at early-dent stage) and grain were conducted to evaluate the composition of LY038 compared to LY038(-) and conventional reference maize. LY038 forage samples were subjected to compositional analysis for proximates (protein, fat, ash, and moisture), acid detergent fiber (ADF), neutral detergent fiber (NDF), lysine, and minerals (calcium, phosphorus), as well as carbohydrates by calculation. Compositional analysis of LY038 grain samples included proximates (protein, fat, ash, moisture), ADF, NDF, total dietary fiber (TDF), amino acids, free lysine, fatty acids (C8-C22), vitamins (B_1, B_2, B_6, E, niacin, and folic acid), antinutrients (phytic acid and raffinose), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc), carbohydrates by calculation, secondary maize metabolites per OECD concensus (furfural, ferulic acid, and p-coumaric acid) and additional lysine-related metabolites (cadaverine, α-aminoadipic acid, saccharopine, homoserine, L-pipecolic acid, and 2,6-diaminopimelic acid). In all, 85 different analytical components (75 in grain, ten in forage) were analyzed.

Based on this extensive compositional evaluation, the grain and forage of LY038 are considered to be compositionally equivalent to those of conventional maize except for the intended increase in grain lysine content and an associated increase in lysine-related catabolites, saccharopine and α-aminoadipic acid in grain. These two metabolites are normal components of the lysine metabolic pathway found in plants and animals (including humans) and have also been found as measurable components of certain common foods. Thus, there is a history of exposure / consumption of these two metabolites. Therefore, there is a reasonable certainty that the levels of α-aminoadipic acid and saccharopine in LY038 are not harmful to animal or human health. A detailed assessment of the feed and food safety and nutritional value of LY038 and these two metabolites has been submitted to the FDA.

The cDHDPS protein expressed in LY038 has the same enzymatic activity as other DHDPSs that are ubiquitous in plants and microorganisms. Therefore, we conclude that the presence of the cDHDPS protein in LY038 does not pose a hazard to organisms in the environment based on the history of safe exposure to the family of DHDPS proteins, including the cDHDPS protein.

A thorough characterization of LY038 was performed including molecular, cDHDPS protein expression, phenotypic, and compositional evaluations. Assessment of the data generated from this extensive characterization supports conclusions of no increased pest potential, phenotypic equivalence, and familiarity compared to conventional maize as they relate to ecological risk assessment. There are no biologically meaningful differences between LY038 and the negative segregant control with the exception of the presence of cDHDPS protein that effects the intended increase in grain lysine content and the related increase in the lysine-related catabolites, saccharopine and α-aminoadipic acid. The phenotypic data supports the conclusion that LY038 is not different from its negative segregant control or conventional reference maize hybrids grown in the same field trials, with the exception of the white leaf phenotype. The white leaf phenotype observed in a small percentage of the plants was determined to be associated with the LY038 trait of increased lysine in the seed. The effects of the white leaf characteristic did not result in significant changes in other growth and development characteristics on a whole-plot basis and it would not contribute to increased pest potential. On the basis of these data and the safety of DHDPS proteins in the environment, it is concluded that there is no increased pest potential of LY038 and that other than the intentional compositional change caused by the introduced trait, the phenotype of LY038 has not been unintentionally changed.

Data and information presented in this request demonstrate that LY038 does not pose a unique plant pest risk. Therefore, Monsanto Company, on behalf of Renessen LLC, requests that APHIS grant the request for a determination of nonregulated status for Lysine maize LY038.

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Key to Abbreviations

AD4	allergen, gliadin, and glutenin protein sequence database
ADF	acid detergent fiber
ALLPEPTIDES	protein sequence database comprised of GenBank and SwissProt
amp	coding sequence for the enzyme □-lactamase
AOSA	Association of Official Seed Analysts
APHIS	Animal and Plant Health Inspection Service (United States)
ble	gene for bleomycin resistance
bp	base pair
BSA	bovine serum albumin
CaMV 35S	cauliflower mosaic virus (CaMV) 35S promoter
CaMV e35S	cauliflower mosaic virus (CaMV) 35S promoter containing a duplication of the [-90 to –300] enhancer region
CBI	confidential business information
CFR	Code of Federal Regulations (United States)
cDHDPS	lysine-insensitive *Corynebacterium glutamicum* dihydrodipicolinate synthase enzyme
cordapA	coding sequence for the enzyme dihydrodipicolinate synthase from *Corynebacterium glutamicum*
cre	coding sequence of the Cre recombinase (rec3) gene from bacteriophage P1
Cre	Cre recombinase enzyme from bacteriophage P1
CTP	chloroplast transit peptide
DapA	another name for dihydrodipicolinate synthase (DHDPS)
DW	dry weight
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
E score	Expectation score
FA	fatty acid
FAO	Food and Agriculture Organization of the United Nations
FASTA	algorithm used to find local high scoring alignments between a pair of protein or nucleotide sequences
FDA	Food and Drug Administration
FW	fresh weight
Glb1 promoter	promoter from the *Globulin 1* gene from *Zea mays*
Glb1 3' UTR	3' nontranslated region of the *Globulin 1* gene from *Zea mays*
HRP	horseradish peroxidase
I-DNA	integrated DNA
IgG	immunoglobulin class G
LOD	limit of detection
LOQ	limit of quantitation
*lox*P	recombination site recognized by Cre recombinase
*lox*P-2/1	recombined *lox*P site

LY038	Lysine maize event name
LY038(-)	negative segregant control of LY038
mDHDPS	native *Zea mays* dihydrodipicolinate synthase enzyme
mRNA	messenger ribonucleic acid
NDF	neutral detergent fiber
NOEL	no observable effect level
NOS 3'	nopaline synthase 3' polyadenylation sequence
nptII	coding sequence for the enzyme neomycin phosphotransferase type II from Tn5
OECD	Organization for Economic Co-operation and Development
PCR	polymerase chain reaction
rAct1 intron	intron from the rice actin gene
rAct1 promoter	promoter from the rice actin gene
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
S.E.	standard error of the mean
SGF	simulated gastric fluid
TDF	total detergent fiber
T-DNA	transfer(ed) DNA
TMB	3,3',5,5'- tetramethyl-benzidine
TOXIN5	toxin protein sequence database
USDA	United States Department of Agriculture
UTR	untranslated region
Zm.hsp70 intron	intron from the *Zea mays* hsp (heat-shock protein) 70 gene

Standard abbreviations (e.g., units of measure) are used according to the format described in 'Instructions to Authors' in the Journal of Biological Chemistry.

I. Rationale for Development of Lysine Maize LY038

A. Basis for Determination of Nonregulated Status Under 7 CFR Part 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 U.S. The APHIS regulations at 7 CFR 430.6 provide that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and should no longer be regulated. If APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article.

B. Lysine Maize LY038

Lysine maize LY038 was generated through application of techniques of modern biotechnology by integrating the *cordapA* coding sequence into the maize genome using a biolistic transformation system. The nucleotide sequence of *cordapA* isolated from *Corynebacterium glutamicum* encodes the lysine-insensitive dihydrodipicolinate synthase (cDHDPS) enzyme. The transcription of *cordapA* is under the control of the maize Glb1 promoter, which directs cDHDPS expression predominantly in the germ, resulting in accumulation of lysine in grain.

C. Benefits of Lysine Maize LY038

Maize-soybean meal based broiler diets formulated to include animal protein products and/or corn gluten meal and typical maize-soy based swine diets are characteristically deficient in lysine and require the addition of supplemental lysine for optimal animal growth and production (NRC, 1994; 1998). The supplemental lysine is most commonly provided from commercially available lysine sources in the form of lysine monohydrochloride or lysine sulphate (Leuchtenberger, 1996). Commercial lysine production is primarily via fermentation by *Corynebacterium glutamicum* or *Brevibacterium lactofermentum* (Eggeling, 1994). When added to animal diets at nutritional levels, lysine is Generally Recognized As Safe (GRAS) by the U.S. Food and Drug Administration (21 CFR 582.5411) and may be used safely as a human food additive when provided at nutrient levels (21 CFR 172.320).

Development of LY038 provides an alternative to direct addition of supplemental lysine to poultry and swine diets by increasing the amount of lysine in the maize component of feed. Introduction of the *cordapA* gene into the maize genome produces a maize grain with higher lysine content and higher nutritional value for use as a feed ingredient for animals, primarily poultry (broilers and turkeys) and swine. Total lysine content of conventional maize, most of which is present as protein-incorporated lysine, typically ranges from 2500 to 2800 ppm on a dry weight basis. Levels of free lysine are targeted to be in the range of 1000 to 2500 ppm in LY038 grain, compared to levels of <100 ppm in conventional maize grain. Therefore, in LY038, the expected total lysine would range from 3500 to 5300 ppm.

D. Submissions to Other Regulatory Agencies

1. Submissions to U.S. Agencies

Lysine maize LY038 falls within the scope of the U.S. Food and Drug Administration's (FDA) policy statement concerning regulation of products derived from new plant varieties, including those produced through genetic engineering (FDA, 1992). Monsanto has voluntarily initiated and will complete, on behalf of Renessen LLC, a consultation process with FDA prior to commercial distribution of this product. A safety and nutritional assessment for feed and food derived from LY038 will be submitted to FDA in 2004.

2. Submissions to Foreign Agencies

An assessment of the environmental safety of LY038 has been submitted in Argentina to the National Advisory Committee on Agricultural Biotechnology (CONABIA) with a request for environmental deregulation (flexibilization) to allow LY038 production in Argentina. A feed and food safety assessment for LY038 will be submitted to Argentina's National Service of Agricultural and Food Health and Quality (SENASA). In Canada, submissions will be made to Health Canada (HC) and the Canadian Food Inspection Agency (CFIA), although no Canadian production of LY038 is intended. Submissions in support of approval to import LY038 will be made to the Japan Ministry of Health, Labor and Welfare (MHLW) and Ministry of Agriculture, Forestry and Fisheries (MAFF), the EU under the Regulation on GM Food and Feed, and to other countries that may be identified as target markets for production or import of LY038 for use as animal feed.

II. The Biology of Maize

The biology of maize has been reviewed in a number of publicly available documents including:

- Consensus Document on the Biology of *Zea mays* subsp. *mays* (Maize) developed as part of the Organization for Economic Co-operation and Development (OECD) Series on Harmonization of Regulatory Oversight in Biotechnology (OECD, 2003) accessible at http://www.olis.oecd.org/olis/2003doc.nsf/LinkTo/env-jm-mono(2003)11; and
- A summary prepared by the USDA-APHIS Biotechnology Regulatory Services group available at http://www.aphis.usda.gov/brs/corn.html.

Maize (*Zea mays* L.) is one of the few major crop species indigenous to the Western Hemisphere. Maize is grown in nearly all areas of the world and ranks third behind rice (*Oryza sativa* L.) and wheat (*Triticum* sp.) in total production. Maize has been studied extensively, and it seems the probable domestication of maize was in southern Mexico more than 7,000 - 10,000 years ago. The putative parents of maize have not been recovered, but it is likely teosinte played an important role in the genetic background of maize. The transformation from a wild, weedy species to one dependent on humans for its survival probably evolved over a long period of time by the indigenous inhabitants of the Western Hemisphere. Maize, as we known it today, cannot survive in the wild, because the female inflorescence (the ear) restricts seed dispersal. Although grown extensively throughout the world, maize is not considered a persistent weed nor one difficult to control. A summary of the history, taxonomy, genetics, and life cycle of maize is presented, followed by a discussion of how the characteristics of cultivated maize affect gene flow between cultivated maize and sexually compatible species.

A. History of Maize

Maize originated in the highlands of Mexico 7,000 to 10,000 years ago. By the time Columbus discovered the Western Hemisphere, maize was being grown by the indigenous civilizations from Chile to southern Canada. Columbus noted the presence of maize on the north coast of Cuba on November 5, 1492 and introduced maize to Europe upon his return to Spain (Goodman, 1988). Within two generations after the introduction of maize to Europe, maize became distributed throughout those regions of the world where it could be cultivated. Today, maize ranks third after wheat and rice as one of the world's three leading food crops. However, unlike wheat and rice, the majority of maize produced in the U.S. is consumed by livestock. In the tropics and in the Southern Hemisphere maize is a significant component of the human diet.

The original maize growing areas did not include the north-central area (U.S. Corn Belt) of the United States. The highly productive U.S. Corn Belt dent maize was derived after the colonization of North America. The European settlers accepted the local native American varieties and incorporated them with other crops to provide food, feed, and fuel for their survival. The current U.S. Corn Belt dent maize evolved from the gradual mingling of those

settlements that spread north and west from southeastern North America and those settlements that spread south and west from northeastern North America.

The maize types grown in the northeast are called northern flints; their origin is not clear, but races from the highlands of Guatemala have similar ear morphology (Goodman and Brown, 1988). Northern flints are largely eight-rowed with cylindrical ears, early maturing, and short statured plants with tillers. The southern dent maize races grown in the southeast United States seemed to have originated from the southeast coast of Mexico. Southern dent maize races are characterized as having tall, late maturing, nontillered, poorly rooted plants with soft-textured white kernels on many-rowed, tapering ears. It seems the Tuxpeno race contributed to the development of southern dent races. The intentional and/or unintentional crossing between the early northern flints and late southern dents led eventually to the highly productive U.S. Corn Belt dent maize races that are used extensively throughout the world today.

The origin of maize has been studied extensively, and hypotheses for the origin and for the parentage of maize have been advanced (Mangelsdorf, 1974). Hypotheses suggested for the origin of maize include the following: 1) cultivated maize is a descendent of pod maize; 2) maize originated by direct selection from teosinte; 3) maize, teosinte, and *Tripsacum* descended independently from a common, unknown ancestor; and 4) the tripartite theory: a) maize originated from pod maize, b) teosinte derived from a cross of maize and *Tripsacum*, and c) modern maize varieties evolved by maize intercrossing with teosinte or *Tripsacum* or both (Mangelsdorf, 1974).

It has been suggested that modern maize originated from maize grass by a single-gene mutation causing ear development. Other suggestions have included *Coix* and species of the genus *Manisuris* in the tribe *Andropogoneae* as contributors to the genome of maize. The hypotheses have been tested by the study of crosses for genome commonality, fertility, variation, and segregation of morphological plant traits, by archeological evidence, and by use of molecular genetic markers.

Evidence has been reported to support the different hypotheses, but it seems that the preponderance of evidence supports the hypothesis that maize descended from teosinte (Galinat, 1988). The teosinte genome is similar to maize, teosinte easily crosses with maize, and teosinte has several plant morphological traits similar to maize. Teosinte has a more weedy appearance and more tillers than modern maize varieties. The one major distinguishing difference between maize and teosinte is the female inflorescence, or ear. Modern maize varieties have one to three lateral branches that terminate in an ear with eight to 24 kernel rows of 50 seeds, and the ear is enclosed in modified leaves or husks. Teosinte also has lateral branches, but they terminate in two-rowed spikes of perhaps 12 fruit cases, with each fruit case having one seed enclosed by an indurated glume (Goodman, 1988).

B. Taxonomy of the Genus *Zea*

Maize is a member of the tribe Maydae, which is included in the subfamily Panicoideae of the grass family Gramineae [see the Consensus Document on the Biology of *Zea mays* subsp. *mays* (Maize) (OECD, 2003) for taxonomic classification of maize and its closely related relatives]. The genera included in the tribe Maydae include *Zea* and *Tripsacum* in the Western Hemisphere and *Coix*, *Polytoca*, *Chionachne*, *Schlerachne*, and *Trilobachne* in Asia. Although the Asian genera have been implicated by some in the origin of maize, the evidence for them is not as extensive and convincing as for the genera located in the Western Hemisphere.

There has been some fluctuation in Latin binomial designations of the species included in *Zea* in recent years and the classification will be used herein (Doebley and Iltis, 1980). The genus *Zea* includes two subgenera: *Luxuriantes* and *Zea*. Maize (*Zea mays* L.) is a separate species within the subgenus *Zea,* along with three subspecies. All of the species within the genus *Zea*, except maize, are different species of teosinte. Until recently, the teosinte species were included in the genus *Euchlaena* rather than the genus *Zea*.

The other genus included in the Maydae tribe is *Tripsacum*. *Tripsacum* includes 16 species with a basic set of 18 chromosomes (n = 18), and the different species of *Tripsacum* include multiples of 18 chromosomes ranging from 2n = 36 to 2n = 108.

Five genera are included in the tribe Maydeae that originated in Asia. Except for *Coix*, the basic chromosome number is n = 10. Within *Coix*, n = 5 and n = 10 have been reported.

C. Genetics of Maize

Maize is genetically one of the best developed and best characterized of the higher plants. Because of the separation of male and female inflorescence, number of seeds produced on female inflorescence, ease in handling (growing and hand pollinating), nature of the chromosomes, and low basic chromosome number (n = 10), maize has been accessible for study at all levels of genetics.

Maize was one of the first crop species studied in genetic laboratories to obtain a basic understanding of mitosis, meiosis, chromosome segregation, linkage and effects of crossing-over, and transposable elements. Because of the importance of maize in the U.S. and world economies, and the genetic information obtained since 1900, maize has continued to receive extensive study in modern genetic laboratories.

Molecular geneticists have developed extensive genetic maps of maize to complement those developed by the early maize geneticists. Maize has been used in tissue culture research, in extensive studies to relate molecular markers to qualitative and quantitative traits, in sequencing of genes, in study of transposable elements for gene tagging and generating genetic variability, and in gene transformation (Coe et al*.,* 1988; Carlson, 1988; Phillips et al*.,* 1988; Walbot and Messing, 1988).

D. Life Cycle of Maize

Maize is an annual plant and the duration of its life cycle depends on the cultivars and on the environments in which the cultivars are grown (Hanway and Ritchie, 1982). Maize cannot survive temperatures below 0°C for more than 6 to 8 hours after the growing point is above ground (5- to 7-leaf stage). Damage from freezing temperatures, however, depends on the extent of temperatures below 0°C, soil condition, residue, duration of freezing temperatures, wind movement, relative humidity, and stage of plant development. Light frosts in the late spring of temperate areas can cause leaf burning, but the extent of the injury usually is not great enough to cause permanent damage, although the maize crop will have a ragged appearance because the leaf areas damaged by frost persist until maturity. The completion of the life cycle of maize, therefore, is dictated by the duration of the average number of frost-free days.

The number of frost-free days dictates that maize that differs in length of life cycles be grown in north-to-south directions of temperate areas. In the United States, maize with relative maturities of 80 days or less are grown in the extreme northern areas, and maize with relative maturities of more than 125 days are grown in the southern areas. Maize with relative maturities of 100 to 115 days are typically grown in the U.S. Corn Belt. Relative maturities, however, are not parallel lines east-to-west because they are dependent on prevailing weather patterns, topography, large bodies of water, and soil types (Troyer, 1994).

Another measure used to judge the relative maturities of maize races is the number of growing degree units (GDU) required from emergence to maturity. Based on GDU required to mature, maize cultivars are assigned to areas that have, on the average, less than 1850 GDU in the extreme northern areas of the United States to cultivars that require more than 2750 GDU in more southern areas. Assume a 115-day maturity hybrid is grown in central Iowa. The average last frost date is May 1 and the average first frost date is October 5, resulting in an expected 158 frost-free days. If average emergence occurs May 15 and average flowering occurs July 15, 60 days are required from emergence to flowering. Maize requires 50 to 60 days to attain physiological maturity. If physiological maturity occurs 55 days after flowering, physiological maturity will occur on or about September 10, or 115 days from emergence to physiological maturity.

If one considers the central U.S. Corn Belt as an example, the following timeframe for each stage of maize development could be as follows:

Planting date: May 1 ± 10 days
Date of emergence: May 10 ± 4 days
Date of flowering: July 20 ± 10 days
Physiological maturity: September 10 ± 5 days
Harvest maturity: October 10 ± 10 days

These estimated timeframes could vary within the same year among locations and among years at the same location, depending on the environmental conditions experienced from planting to harvesting.

E. Hybridization

Hybridization is a fundamental concept used in the breeding, production, and growing of maize in the United States. Maize evolved as an open-pollinated (cross-fertilizing) crop species and until the 20th century the maize cultivars were what we designate today as open-pollinated maize varieties. Because maize is essentially 100% cross pollinated, the maize varieties were a collection of heterozygous and heterogeneous individuals (genotypes). Varieties were developed by simple mass selection by the indigenous natives prior to the arrival of Columbus. Their methods of selection were simple by present-day standards, but they were obviously effective in developing varieties and strains to satisfy their food, fuel, feed, and cultural needs. Hybridization occurred between varieties as cultures moved within the Western Hemisphere, releasing genetic variability to develop other unique varieties.

The fundamental concepts for development of hybrid maize were defined by 1920 (Sprague, 1946). Basic studies on the genetic composition of a maize variety were conducted to determine the effects of self pollinating (or inbreeding, which is the opposite of outcrossing) within a maize variety (Shull, 1908). Because maize naturally cross-fertilizes, the genetic composition of each plant is not known. Continuous selfing of individuals for seven to ten generations resulted in pure lines (or inbred lines) within which every plant had similar traits. The correct interpretation of what occurred during inbreeding was based on Mendelian genetics: the heterozygous loci were eliminated by inbreeding to homozygous loci of either one of the two alleles at each locus. The fixation of alleles in pure lines caused a general reduction in vigor and productivity.

It was found upon crossing two pure lines that vigor was restored. If no selection occurred during inbreeding, the average performance (e.g., grain yield) of all possible crosses was similar to performance of the original variety in which inbreeding was initiated. Some crosses, however, were better than the original open-pollinated variety and could be reproduced from the cross of the pure-line parents of the cross. Hence, the concept of hybrid maize was determined: self to develop pure lines, cross the pure lines to produce hybrids, evaluate hybrids to determine the best hybrid, and use of pure-line parents to reproduce the superior hybrid and distribute it for use by the growers (Shull, 1909).

Hybridization is used in many phases of maize breeding because of the expression of heterosis. Hybridization is used to produce breeding populations (e.g., F_2) to develop inbred lines for use in hybrids and to produce the crosses of superior lines for distribution to growers. Hybridization is easily accomplished either by hand pollination or by wind pollination in large crossing fields (male and female inbred lines) to produce large quantities of high quality hybrid seed.

F. Pollination

1. Outcrossing with Wild *Zea* Species

Annual teosinte (*Zea mays* subsp. *mexicana*) and maize are wind pollinated, self-compatible, and are highly variable, interfertile species (Wilkes, 1972 and 1989). Maize and teosinte are genetically compatible, and in areas of Mexico and Guatemala they freely hybridize when they are in proximity to each other and other conditions are favorable. Teosinte exists primarily as a weed around the margins of maize fields, and the frequency of hybrids between teosinte and maize has been studied. A frequency of one F_1 hybrid (maize × teosinte) for every 500 maize plants or 2 to 5% of the teosinte population for the Chalco region of the Valley of Mexico has been reported (Wilkes, 1972). This frequency of hybrids represents a significant gene exchange between a wild weedy plant (i.e., teosinte) and a cultivated relative (i.e., maize). The F_1 hybrid of teosinte by maize is robust and fertile and is capable of backcrossing to maize. Intercrossing and gene exchange between teosinte and maize occurs freely, and, accompanied by selection, teosinte had a significant role in the evolution of maize.

Although maize easily crosses with teosinte, teosinte is not present in the U.S. Corn Belt. The natural distribution of teosinte is limited to the seasonally dry, subtropical zone with summer rain along the western escarpment of Mexico and Guatemala and the Central Plateau of Mexico (Wilkes, 1972; Gonzalez and Corral, 1997). Dependent upon the human characterization of teosinte with its local environment, it may be considered a weed. However, it has been noted that populations of teosinte have been in decline for several decades due to increased grazing and urbanization in Mexico (Wilkes, 1995). Except for special plantings, there are no reports of teosinte occurring in the United States.

Tripsacum-maize hybrids have not been observed in the field and *Tripsacum*-teosinte hybrids have not been produced (Wilkes, 1972). *Tripsacum* evolved by polyploidy, whereas maize and teosinte have undergone introgressive hybridization at the diploid level (2n = 20). The diploid forms of *Tripsacum* (2n = 36) are morphologically distinct and allopathic in their distribution (Wilkes, 1989). *Tripsacum* species are perennials and seem to be more closely related to the genus *Manisuris* than to either maize or teosinte (Goodman, 1976). *Tripsacum* received greater interest in the evolution of maize after Mangelsdorf and Reeves (1931) successfully crossed maize and *Tripsacum dactyloides* (2n = 36). The cross was made with the diploid *Tripsacum dactyloides* (2n = 36) as the male parent. Silks of the female maize parent were cut to permit successful pollination. The cross had 28 chromosomes and was male sterile. Five other *Tripsacum* species have been crossed with maize, and Galinat (1988) has mapped more than 50 homologous loci on the chromosomes of maize and *Tripsacum*. In contrast with maize and teosinte, which can be easily hybridized, both in the wild and by controlled pollinations, special techniques are required to hybridize maize and *Tripsacum*. Except for *Tripsacum floridanum*, it is difficult to cross *Tripsacum* with maize, and the offspring of the cross show varying levels of sterility. Small portions of *Tripsacum* genome can be incorporated by backcrossing.

Sixteen species of *Tripsacum* have been described, five of which are found in the U.S. (USDA, 2001). *Tripsacum floridanum* is native to the southern tip of Florida. *Tripsacum lanceolatum* is native to Arizona and New Mexico. *Tripsacum dactyloides* is native to the Midwest, Eastern and Southern U.S. *Tripsacum fasciculatum* is native to Puerto Rico. *Tripsacum hermaphrodita* (*Anthephora hermaphrodita*) is native to Florida, Puerto Rico, the Virgin Islands and Hawaii. Twelve of 16 *Tripsacum* species are native to Mexico and Guatemala. *Tripsacum australe* and two other species are native to South America. The center of variation for *Tripsacum* is the western slopes of Mexico, the same area where teosinte is frequently found. The habitat preferences of *Tripsacum* are similar to those for teosinte: seasonally dry, summer rains, elevation of 1500 m, and limestone soils (Wilkes, 1972).

2. Outcrossing with Cultivated *Zea* Varieties

Maize is wind pollinated, and the distances that viable pollen can travel depend on prevailing wind patterns, humidity, and temperature. Occasionally it has been found that maize pollen can travel up to 3.2 km (2 miles) by wind under favorable conditions. All maize will interpollinate, except for certain popcorn varieties and hybrids that have one of the gametophyte factors (Ga^s, Ga, and ga allelic series on chromosome 4). Pollen of a specific hybrid can be carried by wind to pollinate other dent maize hybrids, sweet corn, and popcorn if the popcorn does not carry the dent-sterile gametophyte factor. Maize pollen, therefore, moves freely within an area, lands on silks of the same cultivar or different cultivars, germinates almost immediately after pollination, and within 24 hours completes fertilization. Although there may be some minor differences in rate of pollen germination and pollen tube elongation on some genotypes, maize pollen is very promiscuous. It is estimated each maize plant can shed more than 10 million pollen grains.

Certification standards for distances between different maize genotypes have been established to assist in the production of hybrid maize having desired levels of purity. A specific isolation field to produce commercial hybrid seed shall be located so that the seed parent is no less than 200 m (640 feet or 40 rods) from other maize of a similar type (i.e., if seed parent is a yellow, dent maize it should be isolated at least 200 m from other yellow, dent maize). The distance of 200 m can be modified because of size of field, number of border rows, and different maturity dates of flower, provided no receptive silks are available at the time pollen is being shed. If the hybrid seed being produced is of a different color or texture from neighboring fields, the distances and the number of border rows should be increased.

G. Weediness of Maize

Modern day maize cannot survive outside of cultivation (Gould, 1968). One does not find volunteer maize growing in fencerows, ditches, and roadsides as a weed. Although maize from the previous crop year can overwinter and germinate the following year, it cannot persist as a weed. The appearance of maize in soybean fields following the maize crop from the previous year is a common occurrence. Measures are often taken to either eliminate the

plants with a hoe or use of herbicides to kill the maize plants in soybean fields, but the plants that remain and produce seed usually do not persist in the following years.

It is difficult for maize to survive as a weed because of past selection in the evolution of maize. In contrast with weedy plants, maize has a polystichous female inflorescence (or ear) on a stiff central spike (or cob) enclosed with husks (modified leaves). Consequently, seed dispersal of individual kernels naturally does not occur because of the structure of the ears of maize. Individual kernels of maize, however, are distributed in fields and main avenues of travel from the field operations of harvesting the crop and transporting the grain from the harvested fields to storage facilities. In neither instance (natural or mechanical harvesting) does maize become a troublesome weed. Maize cannot survive without human assistance and is not capable of surviving as a weed.

H. Characteristics of the Recipient Maize Material

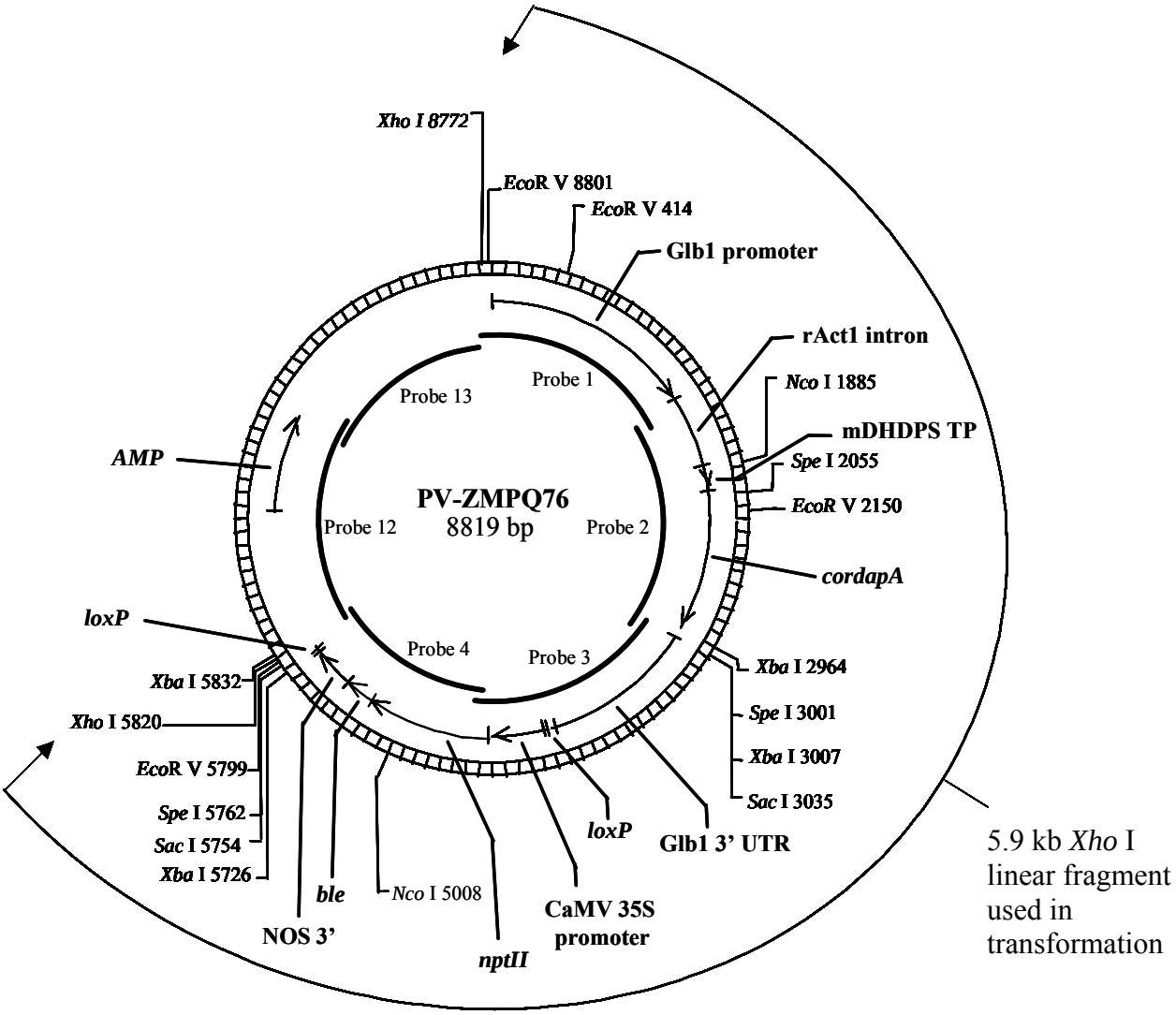
The germplasm that was the recipient of the transgenes in LY038 is a publicly available inbred line of maize, H99. This inbred line was used because it responds well to particle bombardment transformation and tissue culture regeneration.

The inbred line H99 was released in 1974 by the Indiana Agricultural Experiment Station at Purdue University. H99 is a non-stiff-stalk yellow Lancaster maize that was derived from Illinois Synthetic 60C (USDA Blight Resistant Double Double X B8, Ia 55:1473, M14, Oh43, Oh45, Oh51A, R160, and R168).

III. Description of the Transformation System

A 5.9 kb *Xho* I linear fragment of DNA derived from plasmid vector PV-ZMPQ76 was used for transformation of maize to produce LY038. Figures III-1a and III-1b display a plasmid map of PV ZMPQ76. This plasmid was amplified in *E. coli* and purified from bacterial lysate.

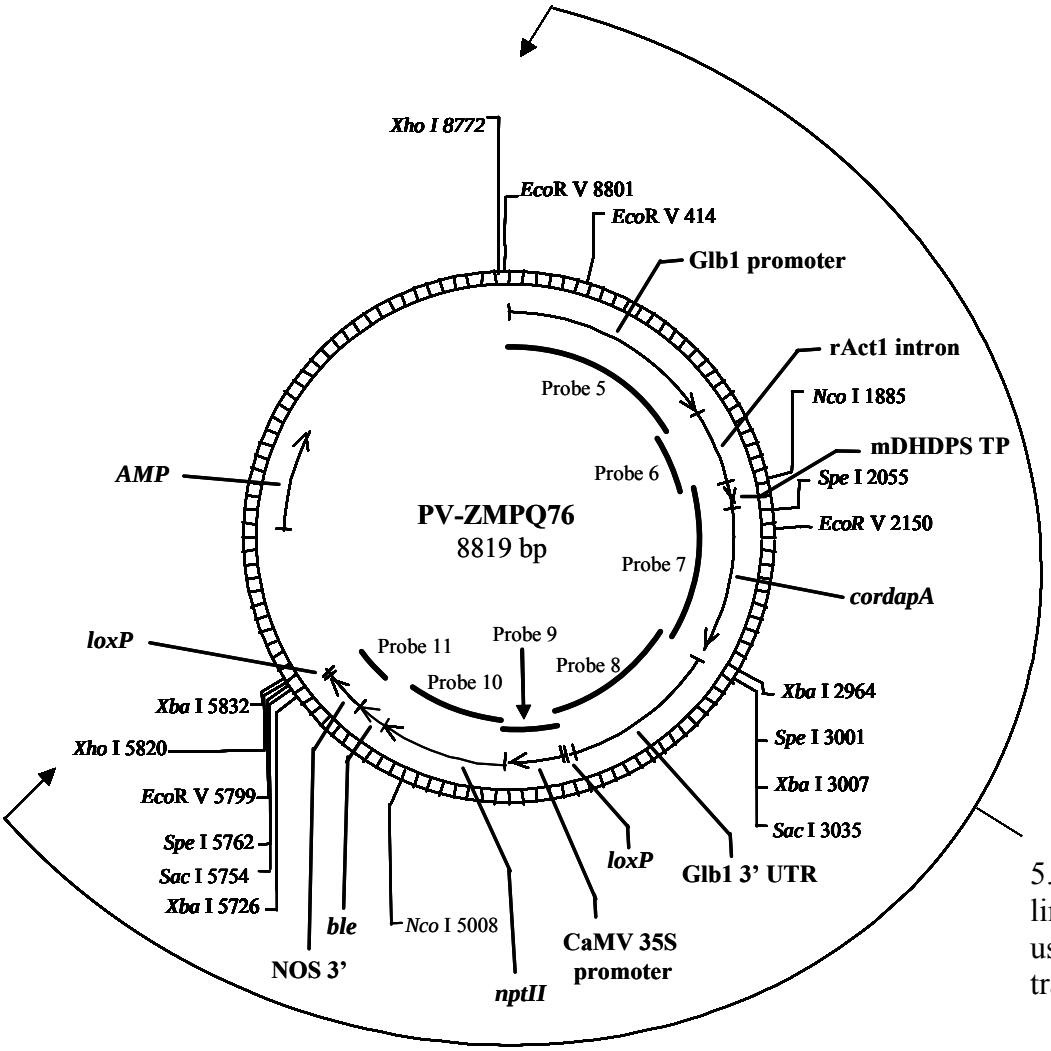
DNA was introduced into callus tissue from maize inbred line H99 by a particle acceleration methodology (Klein et al., 1987; Gordon-Kamm et al., 1990). Briefly, the procedure used DNA that was precipitated onto microscopic gold particles using calcium chloride and spermidine. The particles with precipitated DNA were placed onto a plastic macrocarrier and then accelerated at high velocity such that the macrocarrier was retained and particles with DNA were permitted to continue their flight with eventual penetration into the plant callus tissue cells. The particle-delivered DNA was incorporated into one or more plant chromosomes. The plant cells were incubated on tissue culture medium containing 2,4 dichlorophenoxyacetic acid that supported callus growth. The introduced DNA contained the *nptII* gene, encoding resistance to the antibiotic, paromomycin. When cultured in the presence of paromomycin, only successfully transformed plant callus tissue cells continued to grow. Plants regenerated from the callus tissue cells growing in the presence of paromomycin were assayed for the presence of the *cordapA* gene using standard PCR methodology and only positive plants were continued to be propagated.



Note: *Nde* I is a non-cutting enzyme

Probe	DNA Probe	Start Position	End Position	Total Length (~kb)
1	I-DNA 1	8773	1508	1.6
2	I-DNA 2	1426	3070	1.6
3	I-DNA 3	3039	4559	1.5
4	I-DNA 4	4485	5820	1.3
12	Backbone 1	5821	7411	1.6
13	Backbone 2	7291	8772	1.5

Figure III-1a. Map of plasmid vector PV-ZMPQ76
Circular map of the plasmid vector PV-ZMPQ76 used to generate the 5.9 kb *Xho* I linear fragment (*Xho* I $_{8722}$ to *Xho* I $_{5820}$) for biolistic transformation to produce LY038. Genetic elements are annotated in the interior of the map and restriction sites (with positions relative to the size of the plasmid vector) are shown for enzymes used in the Southern analysis on the exterior. Probes used in Southern analyses are illustrated in the interior of the map and are detailed in the accompanying table.



Note: *Nde* I is a non-cutting enzyme

Probe	DNA Probe	Start Position	End Position	Total Length (~kb)
5	Glb1 promoter	3	1397	1.4
6	rAct1 intron	1405	1885	0.5
7	mDHDPS TP / *cordapA* coding region	1887	2968	1.1
8	Glb1 3' UTR / *loxP*	3037	4081	1.0
9	CaMV e35S promoter*	-	-	0.6
10	*nptII* coding region	4448	5242	0.8
11	NOS 3' polyadenylation sequence*	-	-	0.3

* Probe generated using plasmid PV-ZM003 as a template (see Figures V-11a and V-11b)

Figure III-1b. Map of plasmid vector PV-ZMPQ76
Circular map of the plasmid vector PV-ZMPQ76 used to generate the 5.9 kb *Xho* I linear fragment (*Xho* I $_{8722}$ to *Xho* I $_{5820}$) for biolistic transformation to produce LY038. Genetic elements are annotated in the interior of the map and restriction sites (with positions relative to the size of the plasmid vector) are shown for enzymes used in the Southern analysis on the exterior. Probes used in Southern analyses are illustrated in the interior of the map and are detailed in the accompanying table.

IV. Donor Genes and Regulatory Sequences

A. Vector PV-ZMPQ76 and the 5.9 kb *Xho* I DNA Fragment for Transformation

The plasmid vector PV-ZMPQ76 is an 8.8 kb *E. coli*-expression plasmid. Vector PV-ZMPQ76 includes three expression cassettes, each with a single copy of a gene: *cordapA*, *nptII*, and *amp*. The *cordapA* gene cassette enables the expression of cDHDPS predominantly in grain, and the *nptII* gene cassette confers the paromomycin resistance that permits the selection of cells containing the expression cassette. The third gene cassette contains the *amp* gene under the control of a bacterial promoter that enables the propagation and selection of the transformed *E. coli* harboring the vector. The circular map of the PV-ZMPQ76 vector is presented in Figures III-1a and III-1b and the genetic elements contained are listed in Table IV-1.

Maize callus tissue was transformed with a 5.9 kb *Xho* I linear DNA fragment of the PV-ZMPQ76 plasmid prepared by digestion with the restriction endonuclease *Xho* I. This 5.9 kb *Xho* I fragment contained both the *cordapA* and *nptII* gene cassettes but did not contain any other plasmid backbone DNA including the *amp* gene cassette. The *cordapA* gene cassette consisted of a *Zea mays globulin 1* (Glb1) promoter, a rice actin (rAct1) intron, a *cordapA* coding sequence with maize DHDPS chloroplast transit peptide (CTP), and a *globulin 1* 3′ untranslated region (Glb1 3′ UTR). The second cassette consisted of the *nptII* coding region regulated by the CaMV 35S promoter and the nopaline synthase 3′ (NOS 3′) transcription termination sequence. The *nptII* cassette was cloned between two *lox*P sites to allow for its subsequent removal by Cre recombinase. The genetic elements discussed above are listed in Table IV-1.

B. Marker Removal Through Cre/*lox* Recombination System

The use of the Cre-*lox* recombination system for marker removal has been previously described (Russell et al., 1992; Zhang et al., 2003; Hare and Chua, 2002). The Cre-*lox* recombination system is derived from the bacteriophage P1 and consists of the 38.5 kDa Cre recombinase and a stretch of DNA flanked by two copies of the 34-bp *lox*P sites. The *lox*P site is 34 bp in length and consists of two 13-bp inverted repeats and an asymmetrical 8-bp spacer (Gilbertson, 2003). The 13-bp inverted repeats are the Cre recombinase binding sequence, and the 8-bp spacer is essential for the recombination reaction. Cre recombinase binds to the inverted repeat sequences in the *lox*P sites, catalyzing a crossover in the 8-bp spacer regions of the two *lox*P sites. The results of this crossover are two-fold: one is the excision of the DNA fragment flanked by the two half-*lox*P sites forming a circular extra-genomic DNA fragment; the other is the recombination of linear DNA between the remaining two half-*lox* P sites within the maize genome (Gilbertson, 2003).

Maize plants expressing Cre recombinase were crossed with plants positive for the *cordapA* gene that were regenerated from callus tissue transformed with the 5.9 kb linear DNA fragment described above. The resulting hybrid underwent excision of the DNA

fragment containing the *nptII* cassette flanked by the *lox*P sites (Figure IV-1). The excised *nptII* gene cassette (circular extra-genomic DNA), which did not contain an origin of replication, was subsequently lost, most likely during cell division. Through extensive PCR screening of subsequent maize breeding progeny, the *cre* gene was segregated away from the *cordapA* gene such that F3 progeny containing only the *cordapA* gene cassette in the inserted DNA (I-DNA) and lacking both the *nptII* and the *cre* gene cassettes were identified and designated as LY038. The LY038 development process is illustrated in Figure IV-2.

The absence of the *cre* and *nptII* gene cassettes in LY038 was demonstrated by event-specific PCR analyses conducted during the LY038 development process and by extensive Southern blot analyses (see Section V.A.2.a.iii and V.A.2.b). The absence of *cre* and *nptII* gene cassettes was further confirmed by Southern blot generational stability analyses over multiple generations representing each branch point of the breeding tree as reported in Section V.B.2. Therefore, the *cre* and *nptII* gene cassettes and their respective expression products are not expected to be present in LY038.

Table IV-1. Summary of genetic elements in PV-ZMPQ76

Genetic Element	Position in Plasmid	Function and/or Reference
Intervening sequence	8773-5	Synthetic linker sequence.
Glb1 promoter	6-1397	The promoter from the *Globulin 1* (*Glb1*) gene from *Zea mays* (Belanger and Kriz, 1991).
Intervening sequence	1398-1404	Synthetic linker sequence.
rAct1 intron	1405-1885	Intron from the rice actin gene (McElroy et al., 1990).
Intervening sequence	1886	Synthetic linker sequence.
mDHDPS TP	1887-2057	The chloroplast targeting sequence from dihydrodipicolinate synthase (DHDPS) from *Zea mays* (Frisch et al., 1991).
cordapA	2058-2960	The coding region from dihydrodipicolinate synthase (*dapA*) from *Corynebacterium glutamicum* in the lysine biosynthetic pathway, conferring resistance to lysine feedback inhibition. (Bonnassie et al., 1990).
Intervening sequence	2961-3036	Synthetic linker sequence.
Glb1 3′ UTR	3037-4036	The 3′ nontranslated region from the *Globulin 1* (*Glb1*) gene from *Zea mays* which directs the polyadenylation of the mRNA (Belanger and Kriz, 1991).
Intervening sequence	4037-4047	Synthetic linker sequence.
*lox*P	4048-4081	Recombination site recognized by Cre recombinase (Russell et al., 1992).
Intervening sequence	4082-4090	Synthetic linker sequence.
CaMV 35S promoter	4091-4414	Cauliflower mosaic virus (CaMV) promoter (Odell et al., 1985).
Intervening sequence	4415-4447	Synthetic linker sequence.
nptII	4448-5242	The gene for the enzyme neomycin phosphotransferase type II from Tn5, a transposon isolated from *Escherichia coli* (Beck et al., 1982).
Intervening sequence	5243-5262	Synthetic linker sequence.
ble	5263-5415	A 0.153 kb portion of the 0.378 kb *bleomycin* gene from Tn5 (Mazodier et al., 1985).
Intervening sequence	5416-5426	Synthetic linker sequence.
NOS 3′	5427-5682	3′ nontranslated region of the nopaline synthase (NOS) coding sequence from *Agrobacterium tumefaciens* which directs polyadenylation of the mRNA (Bevan et al., 1983).
Intervening sequence	5683-5691	Synthetic linker sequence.
*lox*P	5692-5725	Recombination site recognized by Cre recombinase (Russell et al., 1992).
Backbone sequence	5726-6670	Derived from *E. coli* with polylinker sequences (Fling et al., 1985; Sutcliffe, 1978).
AMP	6671-7291	Bacterial promoter and coding sequence for the enzyme β-lactamase which confers resistance to ampicillin resistance in *E. coli* (Sutcliffe, 1978).
Backbone sequence	7292-8772	Derived from *E. coli* with polylinker sequences (Fling et al., 1985; Sutcliffe, 1978).

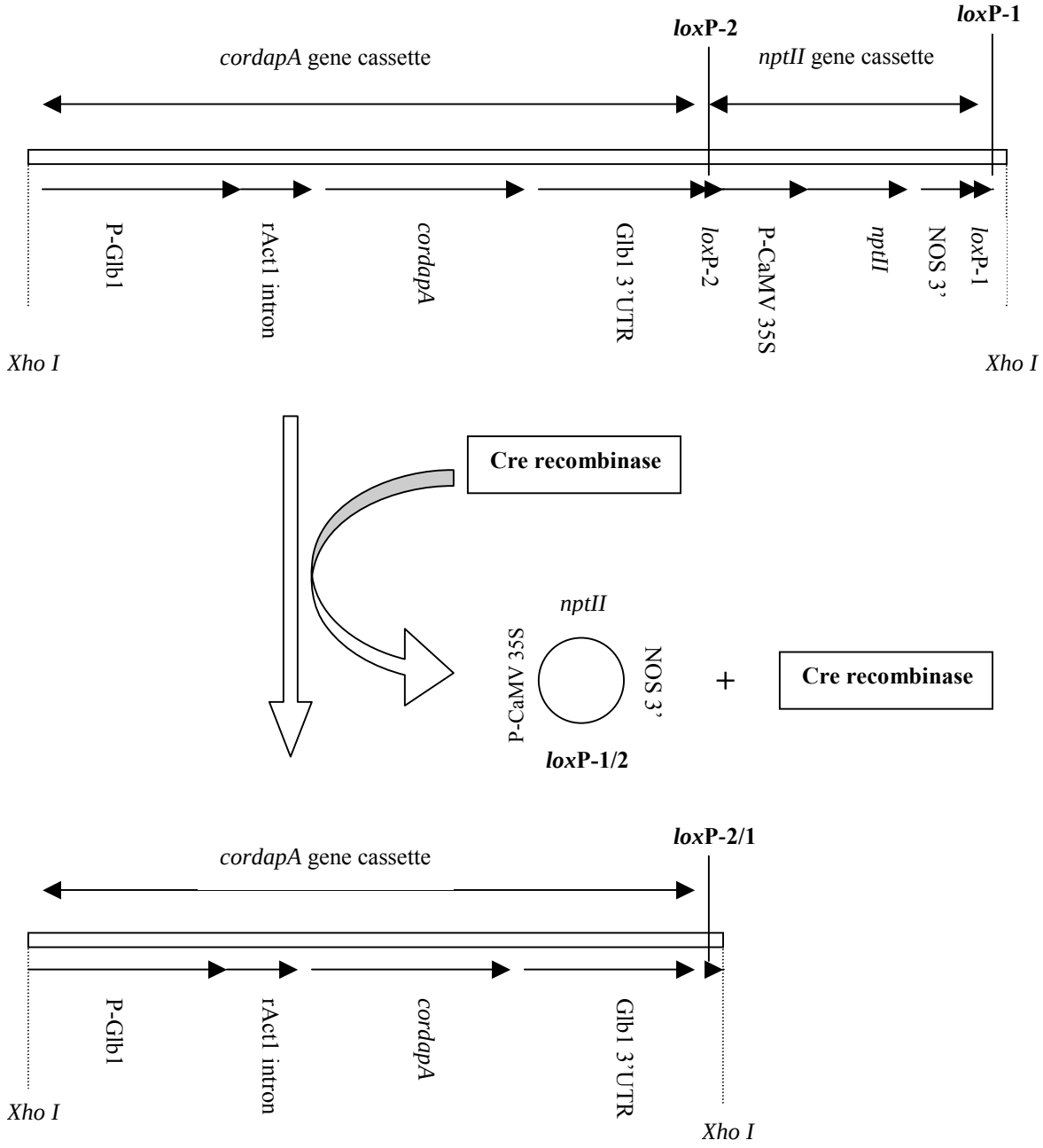


Figure IV-1. Cre-*lox*P recombination system
The 5.9 kb *Xho* I DNA fragment (*Xho* I $_{8722}$ to *Xho* I $_{5820}$) of PV-ZMPQ76 was used in the transformation and was incorporated in the maize genome. Subsequent breeding with a Cre recombinase-containing plant resulted in the excision of the *nptII* gene cassette. The circular *nptII* gene cassette as well as the Cre recombinase was further segregated through breeding, which resulted in LY038 in which the inserted DNA consists only of the *cordapA* gene cassette plus the single recombined *lox*-P 2/1 site.

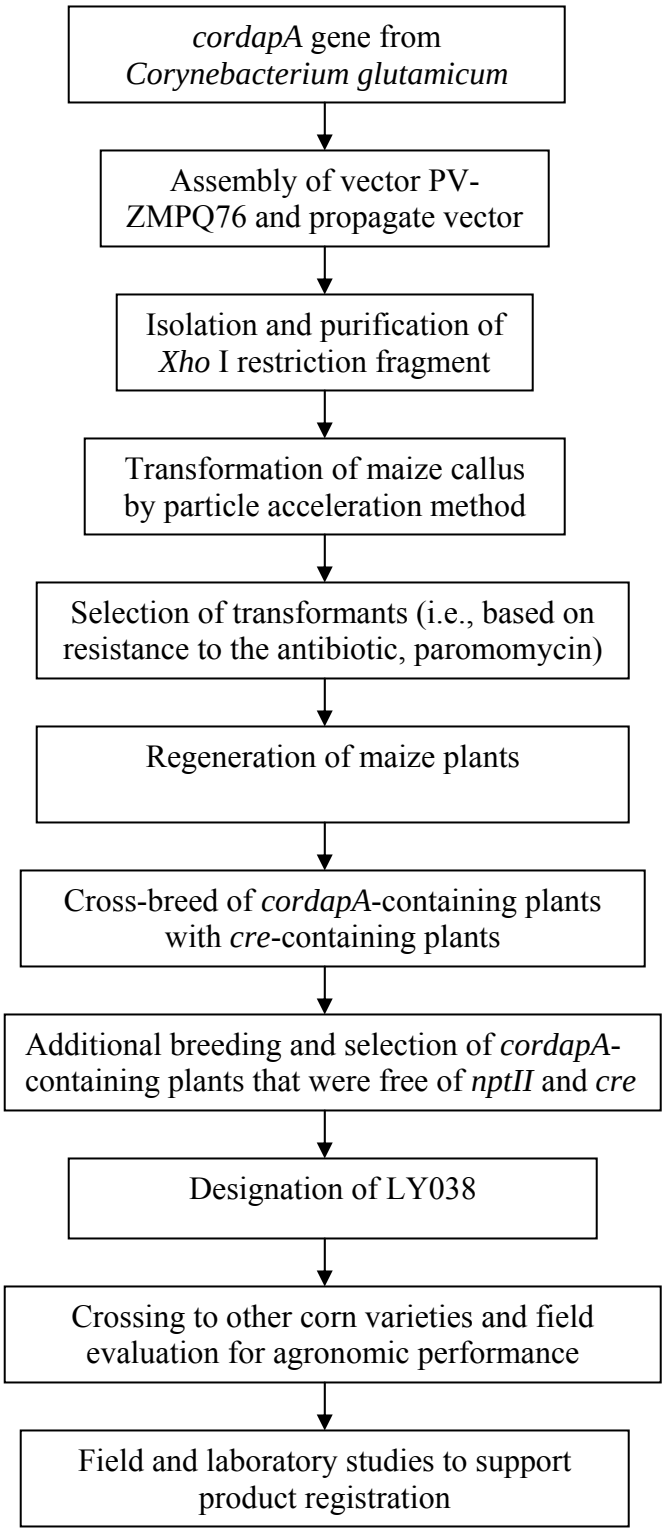


Figure IV-2. LY038 development process

As recently reviewed (Gilbertson, 2003), one of the advantages of the Cre–*lox* system is the specificity of the enzyme for the wild-type *lox*P 34-bp recognition sequence. Although DNA sequences within the maize genome have been detected with limited, although not full, homology to wild-type *lox*P sites, it has been shown that the frequency of Cre recombinase-mediated DNA recombination is significantly reduced when only a few nucleotides are changed in specific regions of the *lox*P sequence (Hoess et al., 1986; Hartung and Kisters-Woike, 1998; Lee and Saito, 1998). Also, the potential for recombination involving a marginally homologous maize sequence and the remaining recombined *lox*P site in the I-DNA is markedly diminished as the physical distance that separates them on a chromosome increases (Golic and Golic, 1996; Stuurman et al., 1996). Additionally, it has been shown that wild-type *lox*P sites recombine much less efficiently when they are located on unlinked chromosomal locations than when they are closely linked (e.g., ≤ 10 kb apart) (Qin et al., 1994; Medberry et al., 1995). Therefore, neither the specific DNA insert nor the usage of the Cre-*lox* system was expected to negatively influence the stability of the I-DNA in LY038 across breeding generations, which has been confirmed, and is described in Section V.B.2 of this petition.

C. Regulatory Sequence

In the *cordapA* cassette, the cDHDPS coding sequence is under the control of *Zea mays* globulin 1 (Glb1) promoter, which in wild-type maize directs expression of the most abundant embryo-specific protein in maize grain (Belanger and Kriz, 1991). The utilization of the Glb1 promoter for *cordapA* transcription results in the expression of cDHDPS and the accumulation of lysine predominantly in the germ portion of the grain. The intron sequence following the Glb1 promoter is derived from the rice *actin-1* gene, and the purpose of this element is to enhance DNA transcription (McElroy et al., 1990). The *cordapA* gene coding sequence was preceded by the *Zea mays* dihydrodipicolinate synthase chloroplast transit peptide (mDHDPS CTP), which resulted in the translation of cDHDPS with the mDHDPS CTP at the N-terminus of the protein. The purpose of the mDHDPS chloroplast transit peptide is to translocate cDHDPS to the plastid where the majority of amino acid biosynthesis occurs (Frisch et al., 1991). The 3′ nontranslated region of the *globulin 1* gene following the *cordapA* gene contains the polyadenylation signal that directs the termination and maturation of the *cordapA* transcript (Belanger and Kriz, 1991).

D. The *cordapA* Coding Sequence

The *cordapA* gene encodes a dihydrodipicolinate synthase (DHDPS, EC4.2.1.52) from *Corynebacterium glutamicum,* which has been utilized for the industrial production of lysine for decades (Eggeling et al., 1998). DHDPS is the first and major rate-limiting enzyme for lysine biosynthesis in plants and bacteria. The enzyme catalyzes the condensation of L-aspartate-4-semialdehyde and pyruvate to form 2,3-dihydrodipicolinate that is converted to lysine through a series of subsequent enzymatic reactions. As the first committed enzyme in lysine biosynthesis, DHDPS is highly susceptible to lysine feedback inhibition, particularly DHDPSs isolated from plants (Ki of ~5-50 μM). DHDPS isolated from bacteria is either less sensitive or insensitive to lysine

inhibition compared to plant DHDPS enzymes (>50-fold), including the DHDPS from *C. glutamicum* (cDHDPS) (Vauterin et al., 2000).

Lysine biosynthesis in plants occurs via the aspartate pathway where another branch of this pathway leads to the synthesis of three additional essential amino acids, threonine, methionine and isoleucine. The entire aspartate pathway, except the last step of methionine synthesis, occurs in the plastid. Because cDHDPS is a bacterial enzyme, a maize chloroplast transit peptide (CTP) was inserted at the N-terminus of the protein to target its localization to the plastid. The deduced amino acid sequence of CTP + cDHDPS is shown in Figure IV-3.

1 **MVSPTNLLPA RKITPVSNGG AATASPSSPS VAARPRRLPS GLQSVTGRGK**

51 **VSLAAIT**STG LTAKTGVEHF GTVGVAMVTP FTESGDIDIA AGREVAAYLV

101 DKGLDSLVLA GTTGESPTTT AAEKLELLKA VREEVGDRAK LIAGVGTNNT

151 RTSVELAEAA ASAGADGLLV VTPYYSKPSQ EGLLAHFGAI AAATEVPICL

201 YDIPGRSGIP IESDTMRRLS ELPTILAVKD AKGDLVAATS LIKETGLAWY

251 SGDDPLNLVW LALGGSGFIS VIGHAAPTAL RELYTSFEEG DLVRAREINA

301 KLSPLVAAQG RLGGVSLAKA ALRLQGINVG DPRLPIMAPN EQELEALRED

351 MKKAGVL

Figure IV-3. Deduced amino acid sequence of the CTP + cDHDPS
The above sequence represents the translated protein sequence for the mDHDPS CTP + *cordapA* coding sequence. This protein sequence was deduced from the DNA sequence of the inserted DNA for LY038. Amino acids 1 – 57 (in bold) represent the mDHDPS CTP sequence, and amino acids 58 – 357 represent the cDHDPS sequence.

V. Genetic Analysis

A. Molecular Characterization of LY038

Molecular analysis was performed to characterize the I-DNA in LY038. Genomic DNA was analyzed using Southern blot analysis (Southern, 1975) to determine the insert number (number of integration sites within the maize genome); the copy number (number of DNA segments used for transformation integrated within one insertion site); the integrity of the inserted promoters, introns, coding regions, and polyadenylation sequences; and the presence or absence of the plasmid backbone sequence. In addition, molecular analyses were conducted to confirm the absence of the *cre* gene cassette that was introduced into another corn transformation event to enable the excision of the *nptII* gene cassette through conventional breeding. Polymerase chain reaction (PCR) (Saiki, 1990) was performed to verify the linkages of the individual elements of the insert. A linear map of the LY038 insert is shown in Figure V-1.

1. Molecular Analysis Methods

Genomic DNA from LY038 was digested with a variety of restriction enzymes and subjected to Southern blot analysis to characterize the I-DNA that was integrated into the maize genome (see Appendix 1 for detailed molecular characterization methods). The PV-ZMPQ76 plasmid map annotated with the probes used in the Southern analyses is presented in Figures III-1a and III-1b. The determination of the insert number, copy number, integrity of the inserted *cordapA* gene cassette, and confirmation of the absence of plasmid backbone sequences, selectable marker sequences, and *cre* cassette sequences are presented in the following sections.

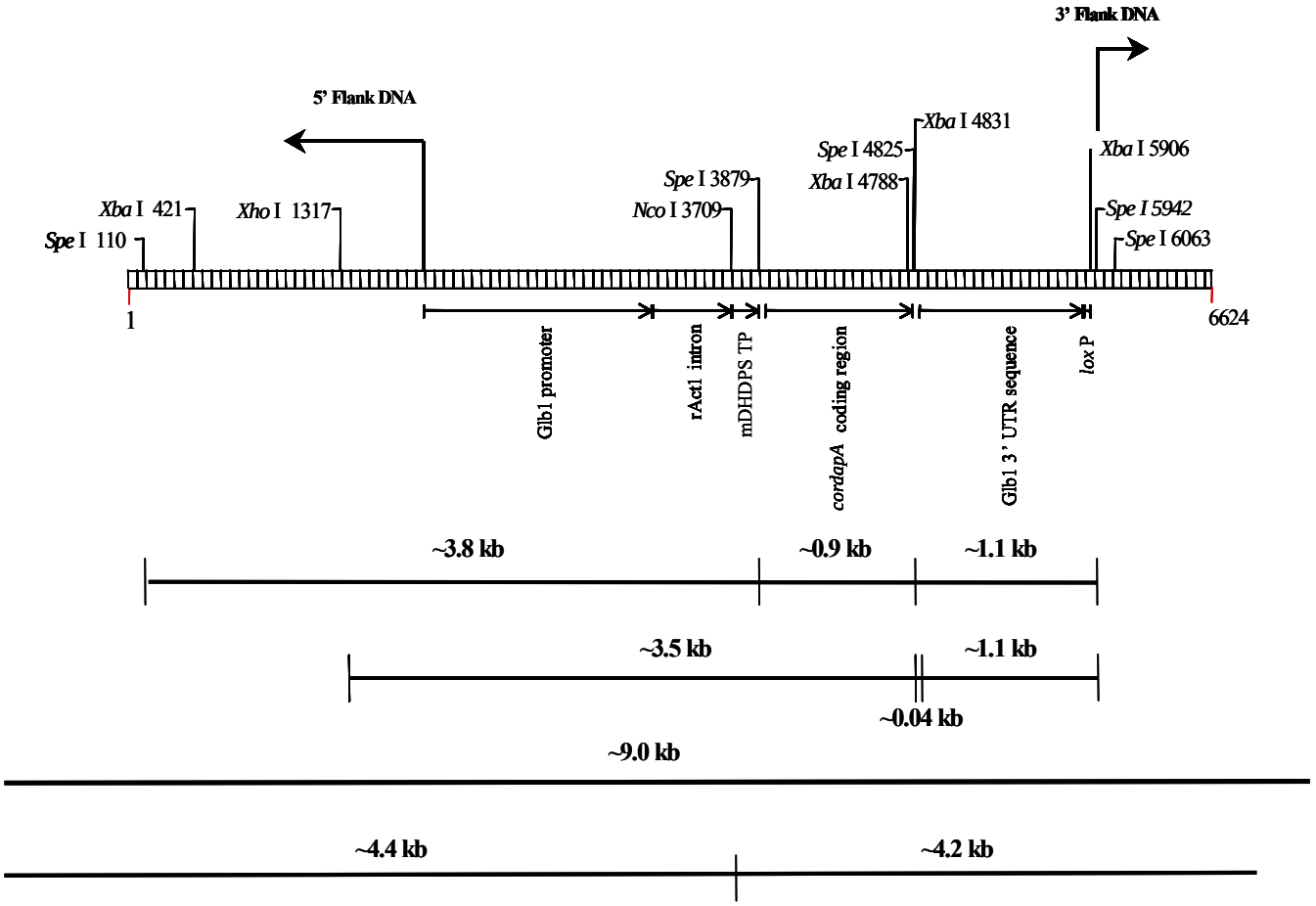


Figure V-1. Linear map of I-DNA
A linear map of the insert and adjacent DNA flanking the insert in LY038 is shown. Arrows indicate the end of the insert and beginning of maize genomic flanking sequence. Identified on the map are genetic elements within the insert, as well as restriction sites with positions relative to the size of the linear map for enzymes used in the Southern analysis.

2. Molecular Analysis Results

2.a. Southern Blot Analyses of Genetic Elements from *cordapA* Plasmid PV-ZMPQ76

2.a.i. Insert and Copy Number

Probes including I-DNA 1 through I-DNA 4 (Figure III-1a) were radiolabeled with ^{32}P and were utilized in the determination of I-DNA insert number. Genomic DNA from LY038 and the negative segregant control [subsequently referred to as LY038(-)] were digested with *NdeI* or the combination of *Nde* I and *Nco* I, and the DNA fragments were separated by gel electrophoresis and transferred to a nylon membrane for Southern analysis. Two Southern blots were generated (Figure V-2). Blot A was probed with I-DNA 1 and I-DNA 2 that span 75% of the I-DNA, and Blot B was probed with I-DNA 3 and I-DNA 4, which span the remainder of the I-DNA. A single unique hybridization band (~9.0 kb) was observed comparing LY038 and LY038(-) samples (Lane 3 versus Lane 1 in both blots). DNA subjected to shorter duration of electrophoresis displayed the same pattern (Short run, Lane 9 versus Lane 5 in both blots). These data support the conclusion that there is only one I-DNA present in the genome at a single locus in LY038.

Digestion of the LY038 genomic DNA with the combination of *Nde I* and *Nco I* gave rise to two unique bands upon hybridization with I-DNA 1 and I-DNA 2 probes because of the presence of an internal *Nco I* site (Blot A, Lane 4 versus Lane 2). Since I-DNA 3 and I-DNA 4 probes only hybridize to the Glb 1 3′ UTR in the I-DNA, the presence of this internal *Nco I* site resulted in the detection of only the *Nco I-Nde I* fragment (Blot B, Lane 4). The hybridization pattern produced by *Nde I* (cleavage site external to I-DNA) and the combination of *Nde I* plus *Nco I* (cleavage sites external and internal to I-DNA) is consistent with the presence of one *cordapA* cassette in LY038.

2.a.ii. *cordapA* Cassette Intactness

The intactness of the inserted *cordapA* gene cassette and its associated genetic elements was assessed by digestion of the test DNA with the restriction enzyme *Spe* I, or the combination of restriction enzymes *Xho I* and *Xba* I. Digestion of LY038 DNA with *Spe* I releases three fragments: 0.9 kb, 3.8 kb, and 1.1 kb (Figure V-1). Digestion of LY038 DNA with the combination of *Xho I* and *Xba* I will generate two border fragments: 3.5 kb and 1.1 kb. Plasmid PV-ZMPQ76 DNA previously digested with *Eco*R V was used as positive control while LY038(-) DNA digested with either *Spe* I or a combination of *Xho* I and *Xba* I were utilized as negative controls. Individual Southern blots probed with the Glb1 promoter, the rAct1 intron, the mDHDPS CTP / *cordapA* coding region, and the Glb1 3′ UTR / *lox*P sequences (Probes 5, 6, 7 and 8, respectively, Figure III-1b) are presented below. Results from the Southern blotting analysis support the conclusion that the genetic elements of the intact *cordapA* cassette are present in LY038 and there are no unexpected genetic elements present in LY038.

2.a.ii(a). Glb1 Promoter Southern Blot Analysis

Figure V-3 depicts the Southern blot analysis of LY038 DNA using the Glb1 promoter probe (probe 5, Figure III-1b). Hybridization of *Spe* I-digested LY038 DNA (Lanes 3 and 9) produced a unique 3.8 kb band compared to that of LY038(-) DNA (Lanes 1 and 5). Similarly, hybridization of LY038 DNA digested with the combination of *Xho* I *and Xba* I (lanes 4 and 10) produced the single predicted band of 3.5 kb in addition to the background observed in LY038(-) (Lanes 2 and 6). No unexpected bands were detected in the LY038 DNA samples probed with Glb1 promoter sequences, supporting the conclusion that LY038 contains no additional Glb1 promoter elements other than those associated with the intact *cordapA* cassette.

2.a.ii(b). rAct1 Intron Southern Blot Analysis

Southern blot analysis with the rAct1 intron probe (Probe 6, Figure III-1b) generated the expected single unique band of 3.8 kb on *Spe* I-digested LY038 DNA (Figure V-4, Lane 3 and 9). In addition, the probe also recognized a unique band of 3.5 kb in LY038 DNA digested with the combination of *Xho* I and *Xba* I (lanes 4 and 10). These data support the conclusion that there is only one copy of the rAct1 intron element present in LY038 that is associated with the *cordapA* cassette.

2.a.ii(c). mDHDPS CTP + *cordapA* Gene Southern Blot Analysis

The Southern blot analysis utilizing the mDHDPS CTP and *cordapA* coding sequence probe (Probe 7, Figure III-1b) is presented in Figure V-5. Hybridization of LY038 DNA digested with *Spe* I (Lanes 3 and 9) generated two expected unique bands of 3.8 kb and 0.9 kb in addition to the background observed in LY038(-) DNA (Lanes 1 and 5). The 0.9 kb band is not observed in the long run (Lane 3) because it was not retained on the gel during electrophoresis. Similarly, hybridization of LY038 DNA digested with the combination of *Xho* I *and Xba* I (Lanes 4 and 10) generated a single unique band of 3.5 kb in addition to the background observed in LY038(-) DNA (Lanes 2 and 6). These results are consistent with the conclusion that LY038 contains no additional mDHDPS CTP or *cordapA* coding elements other than those associated with the intact *cordapA* cassette.

2.a.ii(d). Globulin1 3' UTR / *lox*P Southern Blot Analysis

Southern blot analysis with the Glb1 3′ UTR / *lox*P probe (Probe 8, Figure III-1b) is shown in Figure V-6. Hybridization of the *Spe* I-digested LY038 DNA (Lanes 5 and 11) generated a unique band of 1.1 kb in addition to the background observed in LY038(-) DNA (Lanes 1 and 7). Similarly, hybridization of LY038 DNA digested with *Xho* I and *Xba* I (Lanes 6 and 12) produced a unique 1.1 kb band in addition to the background bands observed in LY038(-) DNA (Lanes 2 and 8). Hybridization of positive control DNA in Lanes 9 and 10 generated the expected size bands at 3.6 kb in addition to bands observed in LY038(-) DNA (Lane 7). These results support the conclusion that LY038 does not contain any additional Glb1 3′ UTR or *lox*P elements other than those associated with the intact *cordapA* cassette.

2.a.iii. Absence of *nptII* Cassette

As previously described (Section IV.B and Figures IV-1 and IV-2), the Cre recombination system was used to remove the *nptII* antibiotic resistance marker present in plants generated from the initial transformation with plasmid vector PV-ZMPQ76. The following Southern blot analyses were performed on LY038 DNA to confirm the absence of the *nptII* cassette. LY038 or LY038(-) DNA was digested with either *Spe* I or a combination of *Xho* I and *Xba* I. Linear plasmid DNA (PV-ZMPQ76 or PV-ZM003) was used as the positive control, and LY038(-) DNA was utilized as the negative control. Individual Southern blots were examined with the CaMV e35S promoter, the *nptII* coding region, and the NOS 3′ polyadenylation sequence (Probes 9, 10, and 11, respectively, Figure III-1b). Results from these Southern blot analyses described below support the conclusion that the *nptII* cassette and associated partial or intact genetic elements are absent in LY038 as no hybridization signals were observed.

2.a.iii(a). CaMV e35S Promoter Probe

Hybridization of the positive control DNA (Lanes 9 and 10, Figure V-7) with the CaMV e35S probe (Probe 9, Figure III-1b) generated the expected size band at 3.0 kb. Hybridization of LY038 DNA digested with *Spe* I or a combination of *Xho* I and *Xba* I (Lanes 3, 7 and 4, 8, respectively) with this same probe showed no detectable hybridization. These results demonstrate that LY038 does not contain detectable CaMV 35S or CaMV e35S promoter elements.

2.a.iii(b). *nptII* Coding Region Probe

Hybridization of the positive control DNA with the *nptII* coding sequence probe (Probe 10, Figure III-1b) produced the expected size band at 3.6 kb (Figure V-8, Lane 7 and 8). Hybridization of LY038 DNA digested with *Spe* I (Figure V-8, Lanes 3 and 9) or the combination of *Xho* I and *Xba* I (Figure V-8, Lanes 4 and 10) with this same probe showed no detectable hybridization, which demonstrates that LY038 does not contain detectable *nptII* coding region.

2.a.iii(c). NOS 3' Polyadenylation Sequence Probe

Hybridization of the positive control DNA (Lanes 7 and 8) with the NOS 3′ probe (Probe 11, Figure III-1b) produced the expected size band at approximately 3.6 kb, while hybridization of LY038 DNA digested with *Spe* I (Figure V-9, Lanes 3 and 9) or the combination of *Xho* I and *Xba* I (Figure V-9, Lanes 4 and 10) with this same probe showed no detectable hybridization. These results demonstrate that LY038 does not contain detectable NOS 3′ polyadenylation sequence.

2.a.iv. Absence of Plasmid PV-ZMPQ76 Backbone

Southern blot analysis was performed with two overlapping probes (Probes 12 and 13, Figure III-1a) that span the backbone present in PV-ZMPQ76. Hybridization of positive

control DNA (Figure V-10, Lanes 7 and 8) produced one expected size band at 3.0 kb. Hybridization of LY038 DNA digested with either *Spe* I (Figure V-10, Lanes 3 and 9), or a combination of *Xho* I and *Xba* I (Figure V-10, lanes 4 and 10) with these same probes showed no detectable hybridization signal, demonstrating that LY038 does not contain detectable backbone sequence from the PV-ZMPQ76 vector.

2.b. Southern Blot Analyses of Genetic Elements from *cre* Plasmid PV-ZM003

As described in Section IV.B and diagramed in Figures IV-1 and IV-2, the Cre recombination system was used for removal of the *nptII* antibiotic resistance marker present in plants generated from the initial transformation with plasmid vector PV-ZMPQ76 by crossing them with Cre recombinase expressing plants produced by transformation with plasmid PV-ZM003. The following Southern blot analyses were conducted to confirm the absence of DNA from *cre* plasmid PV-ZM003 in antibiotic resistance marker free LY038.

2.b.i. Absence of T-DNA

Four T-DNA (transferred DNA) probes spanning the entire *cre* cassette were used in Southern blot analysis (Probes 14 - 17, Figure V-11a) and the linear plasmid PV-ZM003, containing the *cre* cassette, was used as positive control DNA. As shown in Figure V-12, hybridization of the positive control DNA (Lanes 7 and 8) generated the expected size bands at 3.0 kb, 2.0 kb, and 1.0 kb in addition to those produced by the LY038(-) control DNA (Lane 5). Hybridization of LY038 DNA digested with *Spe* I (Lanes 3 and 9) produced an expected unique band of approximately 3.8 kb in addition to those produced by the LY038(-) control DNA (Lanes 1 and 5). This band resulted from the hybridization of the rAct1 intron portion of the probe with the rAct1 intron associated with the *cordapA* cassette and is expected to be present in LY038. Hybridization of *Xho* I and *Xba* I-digested LY038 DNA (Lanes 4 and 10) produced a single unique band of 3.5 kb in addition to the background observed in LY038(-) (Lanes 2 and 6). These results support the conclusion that the *cre* cassette and associated partial or intact genetic elements are absent in LY038.

2.b.ii. Absence of *cre* Cassette

As shown in Figure V-13, hybridization of the positive control DNA (Lanes 7 and 8) with a *cre* coding region probe (Probe 19, Figure V-11b) produced the expected size bands at 1.0 kb and 2.0 kb. LY038 DNA digested with *Spe* I (Lanes 3 and 9) or a combination of *Xho* I and *Xba* I (Lanes 4 and 10) showed no detectable hybridization. These results confirmed the previous conclusion that LY038 does not contain any detectable *cre* coding region sequence.

2.b.iii. Absence of *nptII* Cassette

See prior Section V.A.2.a.iii of this petition for Southern analysis which demonstrates that the *nptII* cassette from either plasmid PV-ZMPQ76 or PV-ZM003, and associated partial or intact genetic elements, are absent in LY038.

2.b.iv. Absence of Plasmid PV-ZM003 Backbone

Southern analysis was performed with three overlapping probes (Probes 21, 22 and 23, Figure V-11a) that span the backbone present in PV-ZM003. Hybridization of the positive control DNA (Figure V-14, Lanes 7 and 8) generated one expected size band at 5.3 kb. Hybridization of LY038 DNA digested with either *Spe* I (Lanes 3 and 9), or the combination of *Xho* I and *Xba* I (Figure V-14, Lanes 4 and 10) showed no detectable band. This result is consistent with the conclusion that PV-ZM003 backbone sequence is absent in LY038.

2.c. Confirmation of the Organization of the Insert in LY038

The organization of the elements within the LY038 insert was confirmed by PCR analysis of four overlapping regions of DNA that span the entire length of the insert and the maize genomic DNA flanking the 5′ and 3′ ends of the insert. The size and position of the PCR products in relation to the insert as well as the results of the PCR analyses are shown in Figure V-15.

Upon PCR amplification, LY038 DNA produced the expected PCR products of 4.1 kb for Product A (Lane 4); 3.3 kb for Product B (Lane 7); 3.6 kb for Product C (Lane 11); and 3.8 kb for Product D (Lane 14). The control reactions with either LY038(-) template (Lanes 3, 6, 10,13) or lacking template DNA (Lanes 2, 5, 9, 12) did not produce PCR products with any of the primer sets except for a nonspecific band in Lane 3. These results support the conclusion that the genetic elements in the insert did not undergo rearrangements or transpositions when compared to their arrangement in plasmid PM-ZMPQ76 as shown in Figures III-1a and III-1b.

3. Conclusions from Molecular Characterization

In summary, the Southern blot analyses support the conclusion that LY038 contains one intact copy of the *cordapA* gene cassette inserted at a single site in the maize genome. No additional elements from vector PV-ZMPQ76, linked or unlinked to the intact gene cassette, were detected in LY038. LY038 does not contain either intact or partial DNA fragments of the *nptII* cassette or the *cre* cassette, and also lacks detectable backbone sequence from plasmids PV-ZMPQ76 and PV-ZM003. The presence of the *cordapA* gene cassette and absence of both *cre* and *nptII* gene cassettes in LY038 was further confirmed by Southern blot generational stability analyses over multiple generations representing each branch point of the LY038 breeding tree. PCR analyses confirmed the organization of the genetic elements of the I-DNA in LY038 to be identical to that in plasmid PM-ZMPQ76.

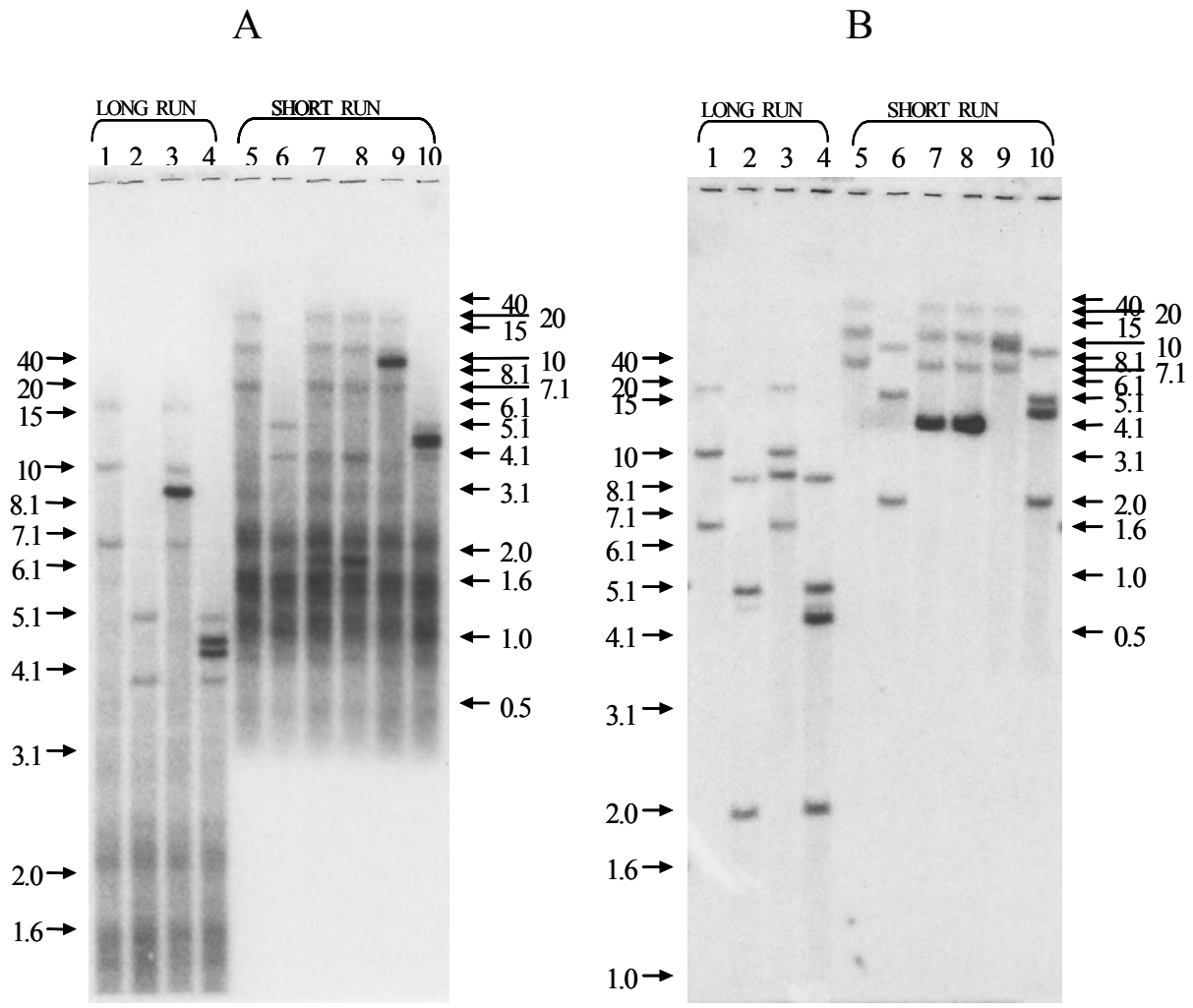


Figure V-2. Southern blot analysis of LY038: Insert and copy number probes
Each blot was examined simultaneously with two ^{32}P-labeled probes that spanned a portion of the I-DNA (Panel A, I-DNA 1 and I-DNA 2, Figure III-1a), (Panel B, I-DNA 3 and I-DNA 4, Figure III-1a). Each lane contains ~10 µg of digested genomic DNA isolated from grain.

Lane 1: LY038(-) (*Nde* I)
2: LY038(-) (*Nde* I and *Nco* I)
3: LY038 (*Nde* I)
4: LY038 (*Nde* I and *Nco* I)
5: LY038(-) (*Nde* I)
6: LY038(-) (*Nde* I and *Nco* I)
7: LY038(-) (*Nde* I) spiked with PV-ZMPQ76 (*EcoR* V) [0.5 copy]
8: LY038(-) (*Nde* I) spiked with PV-ZMPQ76 (*EcoR* V) [1.0 copy]
9: LY038 (*Nde* I)
10: LY038 (*Nde* I and *Nco* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

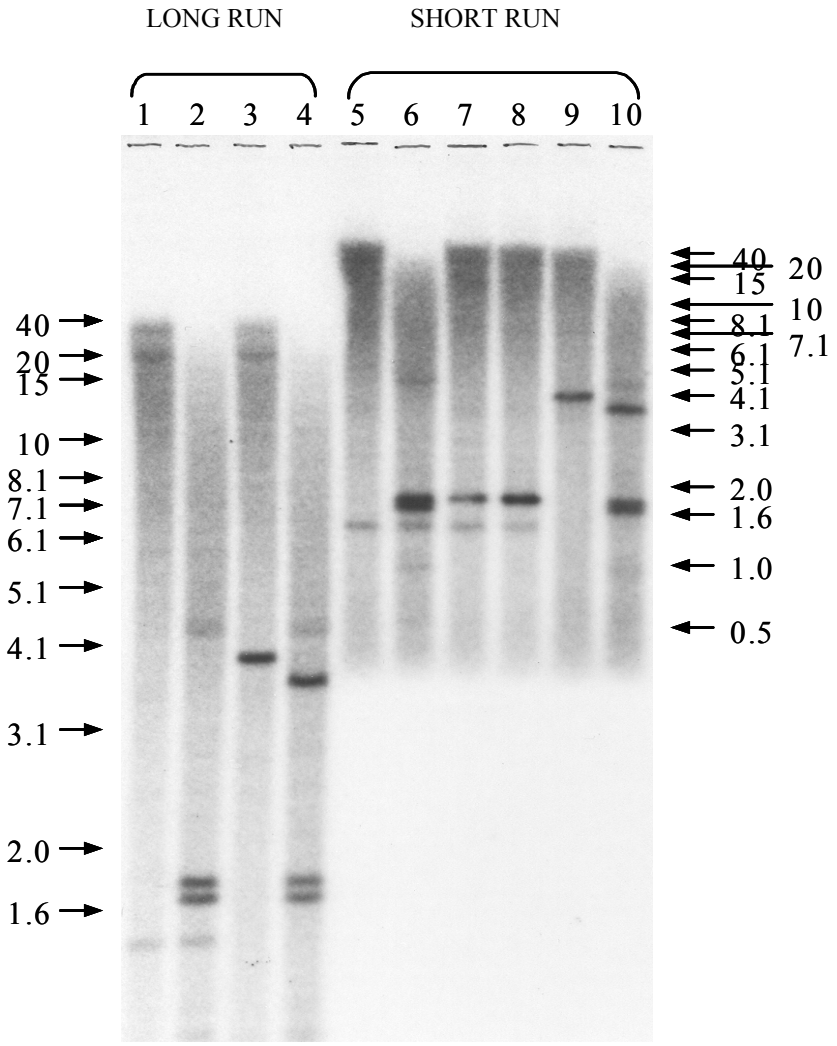


Figure V-3. Southern blot analysis of LY038: Glb 1 promoter probe
The blot was examined with a ^{32}P-labeled probe that spanned the Glb 1 promoter (probe 5, Figure III-1b). Each lane contains ~10 μg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: LY038(-) (*Spe* I)
2: LY038(-) (*Xho* I and *Xba* I)
3: LY038 (*Spe* I)
4: LY038 (*Xho* I and *Xba* I)
5: LY038(-) (*Spe* I)
6: LY038(-) (*Xho* I and *Xba* I)
7: LY038(-) (*Spe* I) spiked with PV-ZMPQ76 (*EcoR* V) [0.5 copy]
8: LY038(-) (*Spe* I) spiked with PV-ZMPQ76 (*EcoR* V) [1.0 copy]
9: LY038 (*Spe* I)
10: LY038 (*Xho* I and *Xba* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

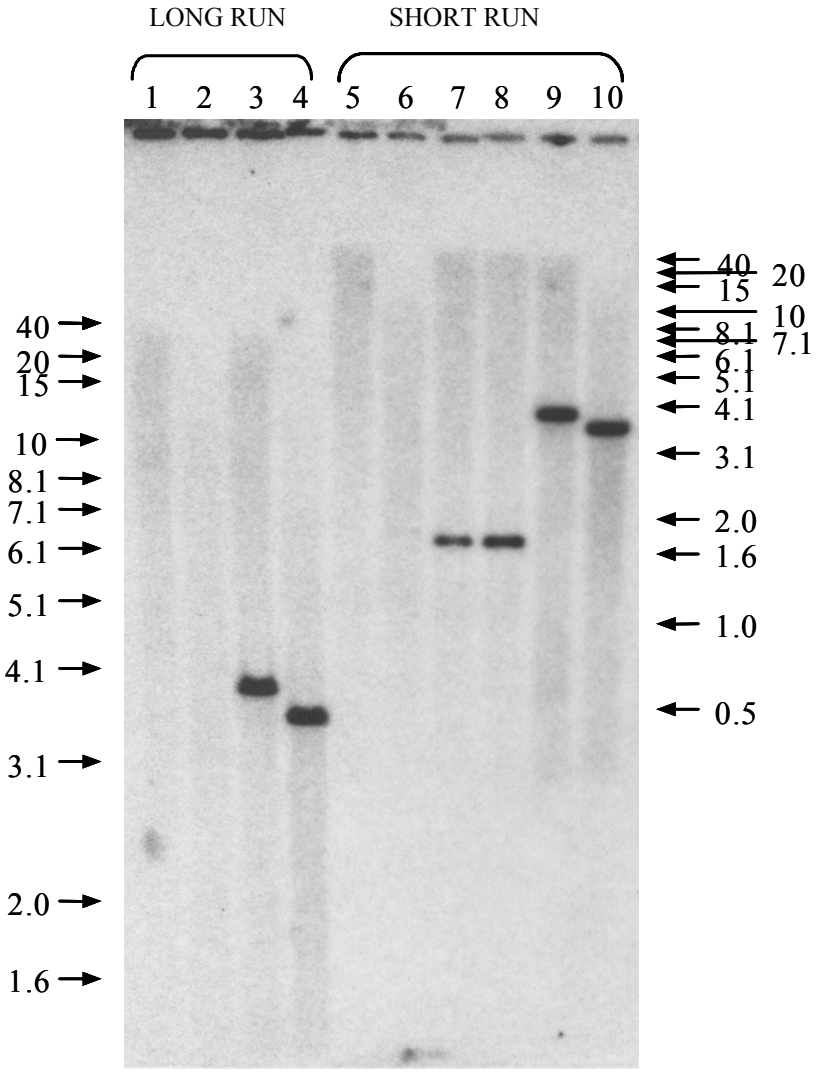


Figure V-4. Southern blot analysis of LY038: rAct1 intron probe
The blot was examined with a ^{32}P-labeled probe that spanned the rAct1 intron (probe 6, Figure III-1b). Each lane contains ~10 μg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: LY038(-) (*Spe* I)
2: LY038(-) (*Xho* I and *Xba* I)
3: LY038 (*Spe* I)
4: LY038 (*Xho* I and *Xba* I)
5: LY038(-) (*Spe* I)
6: LY038(-) (*Xho* I and *Xba* I)
7: LY038(-) (*Spe* I) spiked with PV-ZMPQ76 (*EcoR* V) [0.5 copy]
8: LY038(-) (*Spe* I) spiked with PV-ZMPQ76 (*EcoR* V) [1.0 copy]
9: LY038 (*Spe* I)
10: LY038 (*Xho* I and *Xba* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

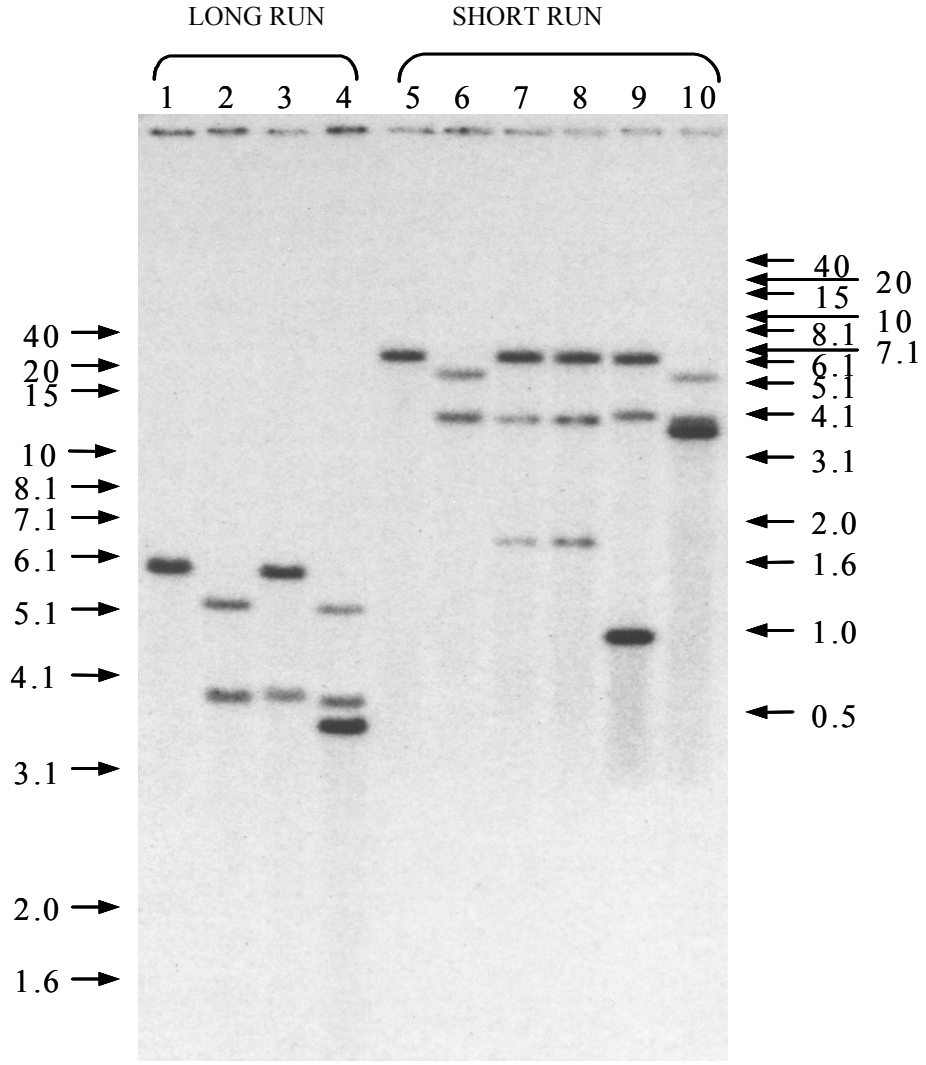


Figure V-5. Southern blot analysis of LY038: mDHDPS CTP / *cordapA* coding region probe

The blot was examined with a ^{32}P-labeled probe that spanned the mDHDPS TP/*cordapA* coding region (probe 7, Figure III-1b). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: LY038(-) (*Spe* I)
2: LY038(-) (*Xho* I and *Xba* I)
3: LY038 (*Spe* I)
4: LY038 (*Xho* I and *Xba* I)
5: LY038(-) (*Spe* I)
6: LY038(-) (*Xho* I and *Xba* I)
7: LY038(-) (*Spe* I) spiked with PV-ZMPQ76 (*EcoR* V) [0.5 copy]
8: LY038(-) (*Spe* I) spiked with PV-ZMPQ76 (*EcoR* V) [1.0 copy]
9: LY038 (*Spe* I)
10: LY038 (*Xho* I and *Xba* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

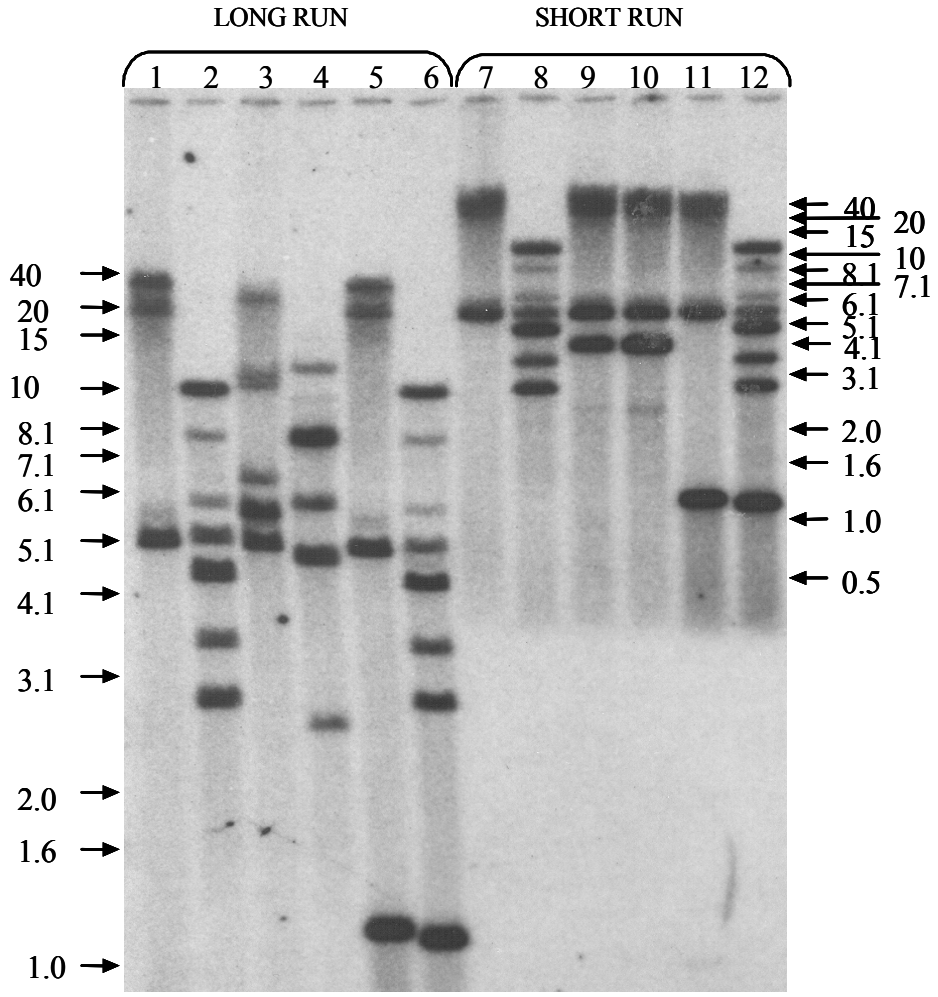


Figure V-6. Southern blot analysis of LY038: Globulin 1 3' UTR / *loxP* probe
The blot was examined with a ^{32}P-labeled probe that spanned the Globulin 1 3' UTR and *loxP* elements (probe 8, Figure III-1b). Each lane contains ~10 μg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: LY038(-) (*Spe* I)
2: LY038(-) (*Xho* I and *Xba* I)
3: 91INH2 (*Spe* I)
4: 91INH2 (*Xho* I and *Xba* I)
5: LY038 (*Spe* I)
6: LY038 (*Xho* I and *Xba* I)
7: LY038(-) (*Spe* I)
8: LY038(-) (*Xho* I and *Xba* I)
9: LY038(-) (*Spe* I) spiked with PV-ZMPQ76 (*EcoR* V) [0.5 copy]
10: LY038(-) (*Spe* I) spiked with PV-ZMPQ76 (*EcoR* V) [1.0 copy]
11: LY038 (*Spe* I)
12: LY038 (*Xho* I and *Xba* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

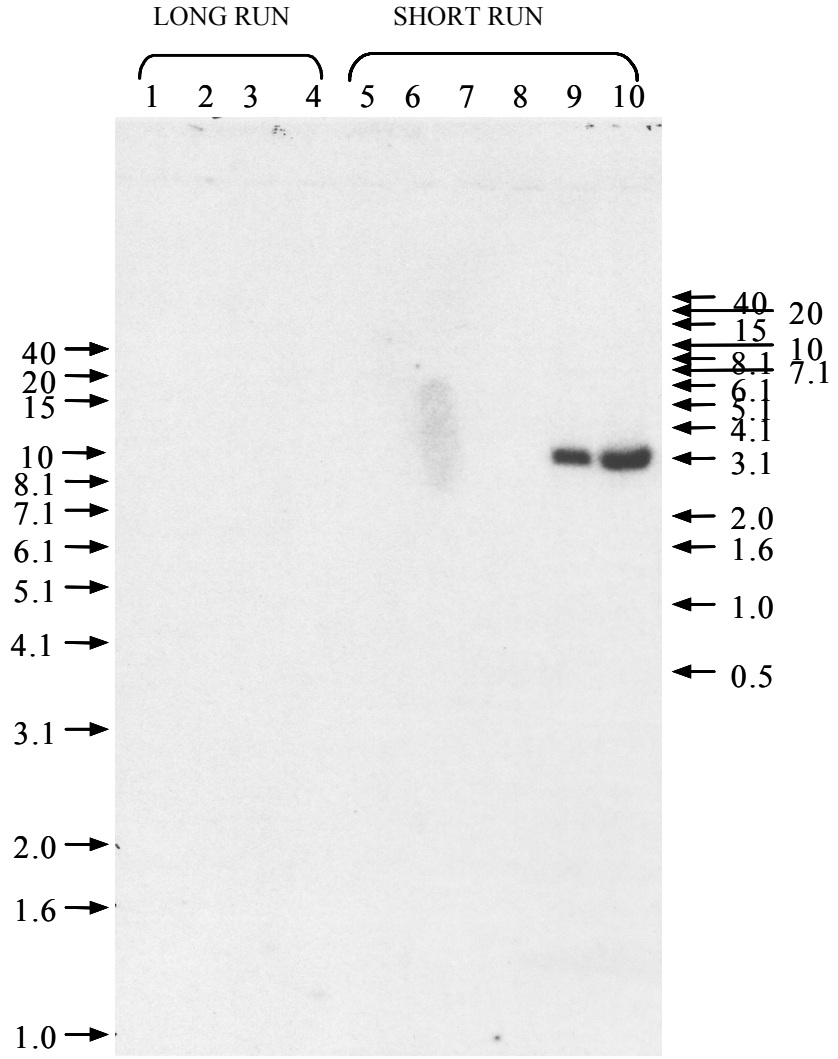


Figure V-7. Southern blot analysis of LY038: CaMV e35S promoter probe
The blot was examined with a ^{32}P-labeled probe that spanned the CaMV e35S promoter (probe 9, Figures III.1 and V-11). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: LY038(-) (*Spe* I)
2: LY038(-) (*Xho* I and *Xba* I)
3: LY038 (*Spe* I)
4: LY038 (*Xho* I and *Xba* I)
5: LY038(-) (*Spe* I)
6: LY038(-) (*Xho* I and *Xba* I)
7: LY038 (*Spe* I)
8: LY038 (*Xho* I and *Xba* I)
9: LY038(-) (*Spe* I) spiked with PV-ZM003 (*Hin*d III and *Mun* I) [0.5 copy]
10: LY038(-) (*Spe* I) spiked with PV-ZM003 (*Hin*d III and *Mun* I) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

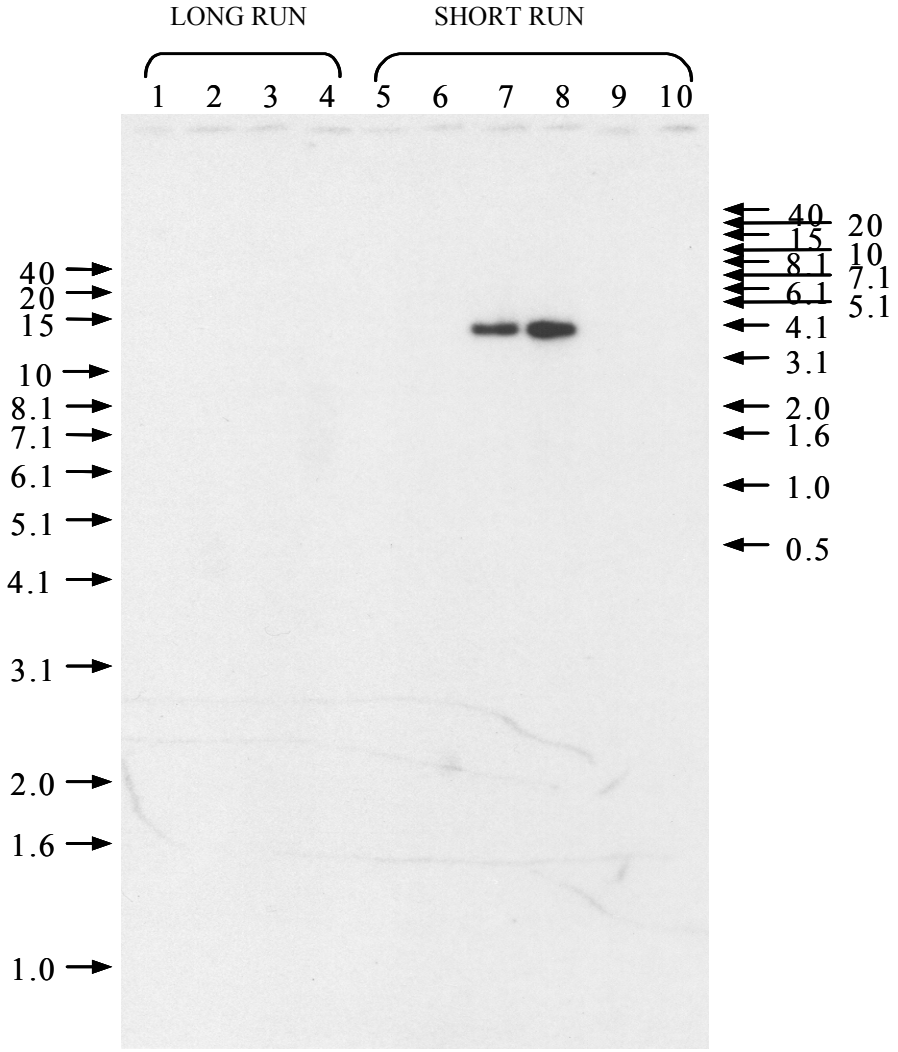


Figure V-8. Southern blot analysis of LY038: *nptII* coding region probe
The blot was examined with a ^{32}P-labeled probe that spanned the entire length of the *nptII* coding region (probe 10, Figure III-1b and V-11b). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: LY038(-) (*Spe* I)
2: LY038(-) (*Xho* I and *Xba* I)
3: LY038 (*Spe* I)
4: LY038 (*Xho* I and *Xba* I)
5: LY038(-) (*Spe* I)
6: LY038(-) (*Xho* I and *Xba* I)
7: LY038(-) (*Spe* I) spiked with PV-ZMPQ76 (*EcoR* V) [0.5 copy]
8: LY038(-) (*Spe* I) spiked with PV-ZMPQ76 (*EcoR* V) [1.0 copy]
9: LY038 (*Spe* I)
10: LY038 (*Xho* I and *Xba* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

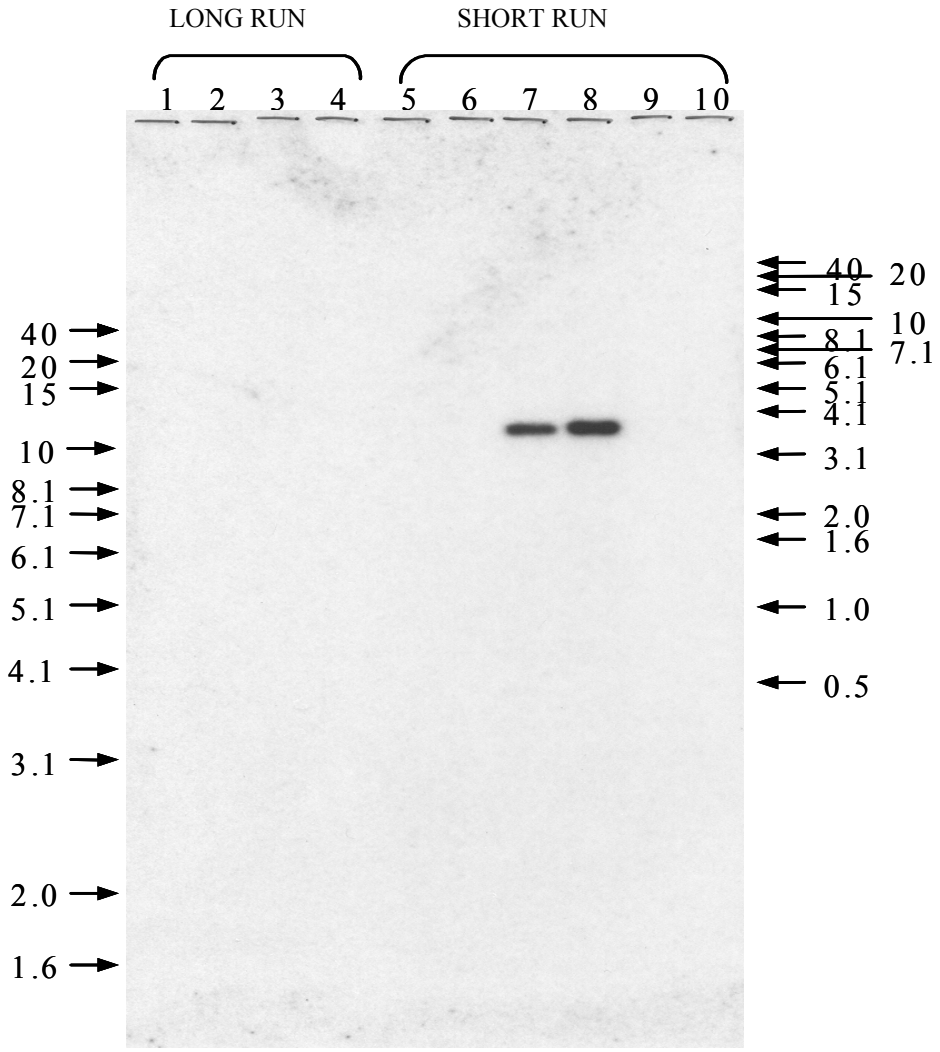


Figure V-9. Southern blot analysis of LY038: NOS 3' polyadenylation sequence probe

The blot was examined with a ^{32}P-labeled probe that spanned the NOS 3' polyadenylation sequence (probe 11, Figures III-1 and V-11). Each lane contains ~10 μg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: LY038(-) (*Spe* I)
2: LY038(-) (*Xho* I and *Xba* I)
3: LY038 (*Spe* I)
4: LY038 (*Xho* I and *Xba* I)
5: LY038(-) (*Spe* I)
6: LY038(-) (*Xho* I and *Xba* I)
7: LY038(-) (*Spe* I) spiked with PV-ZMPQ76 (*EcoR* V) [0.5 copy]
8: LY038(-) (*Spe* I) spiked with PV-ZMPQ76 (*EcoR* V) [1.0 copy]
9: LY038 (*Spe* I)
10: LY038 (*Xho* I and *Xba* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

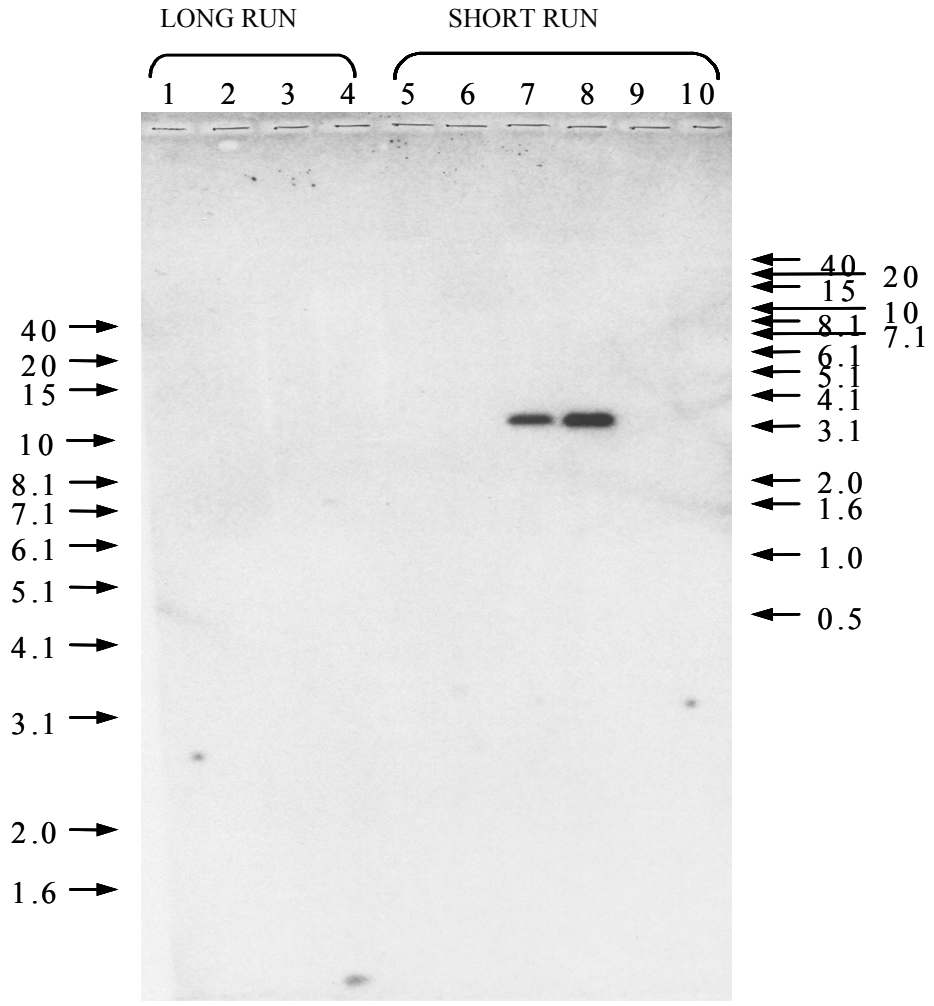
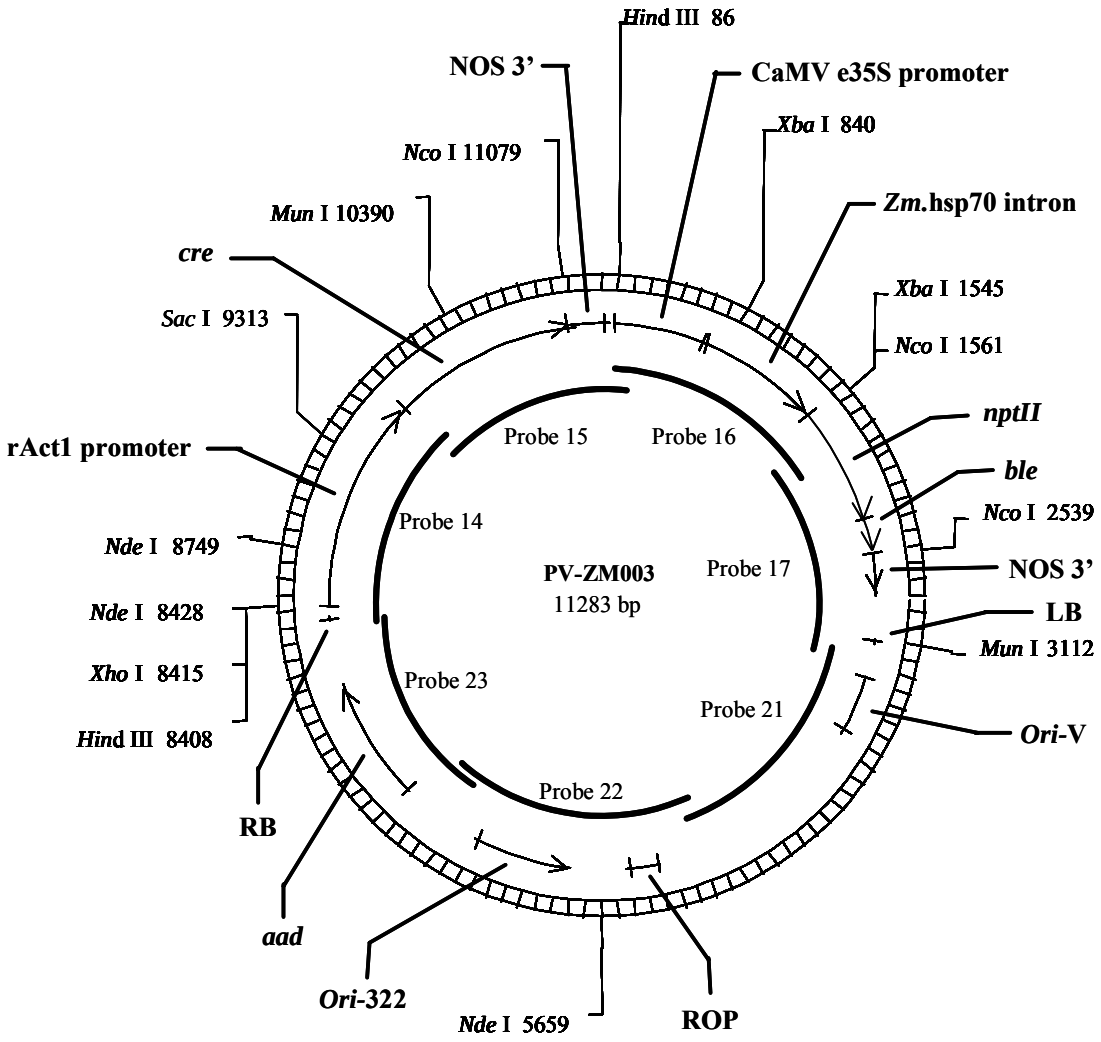


Figure V-10. Southern blot analysis of LY038: PV-ZMPQ76 backbone probes
The blot was examined simultaneously with two ^{32}P-labeled probes that spanned the entire backbone sequence of plasmid PV-ZMPQ76 (probes 12 and 13, Figure III-1a). Each lane contains ~10 μg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: LY038(-) (*Spe* I)
2: LY038(-) (*Xho* I and *Xba* I)
3: LY038 (*Spe* I)
4: LY038 (*Xho* I and *Xba* I)
5: LY038(-) (*Spe* I)
6: LY038(-) (*Xho* I and *Xba* I)
7: LY038(-) (*Spe* I) spiked with PV-ZMPQ76 (*EcoR* V) [0.5 copy]
8: LY038(-) (*Spe* I) spiked with PV-ZMPQ76 (*EcoR* V) [1.0 copy]
9: LY038 (*Spe* I)
10: LY038 (*Xho* I and *Xba* I)

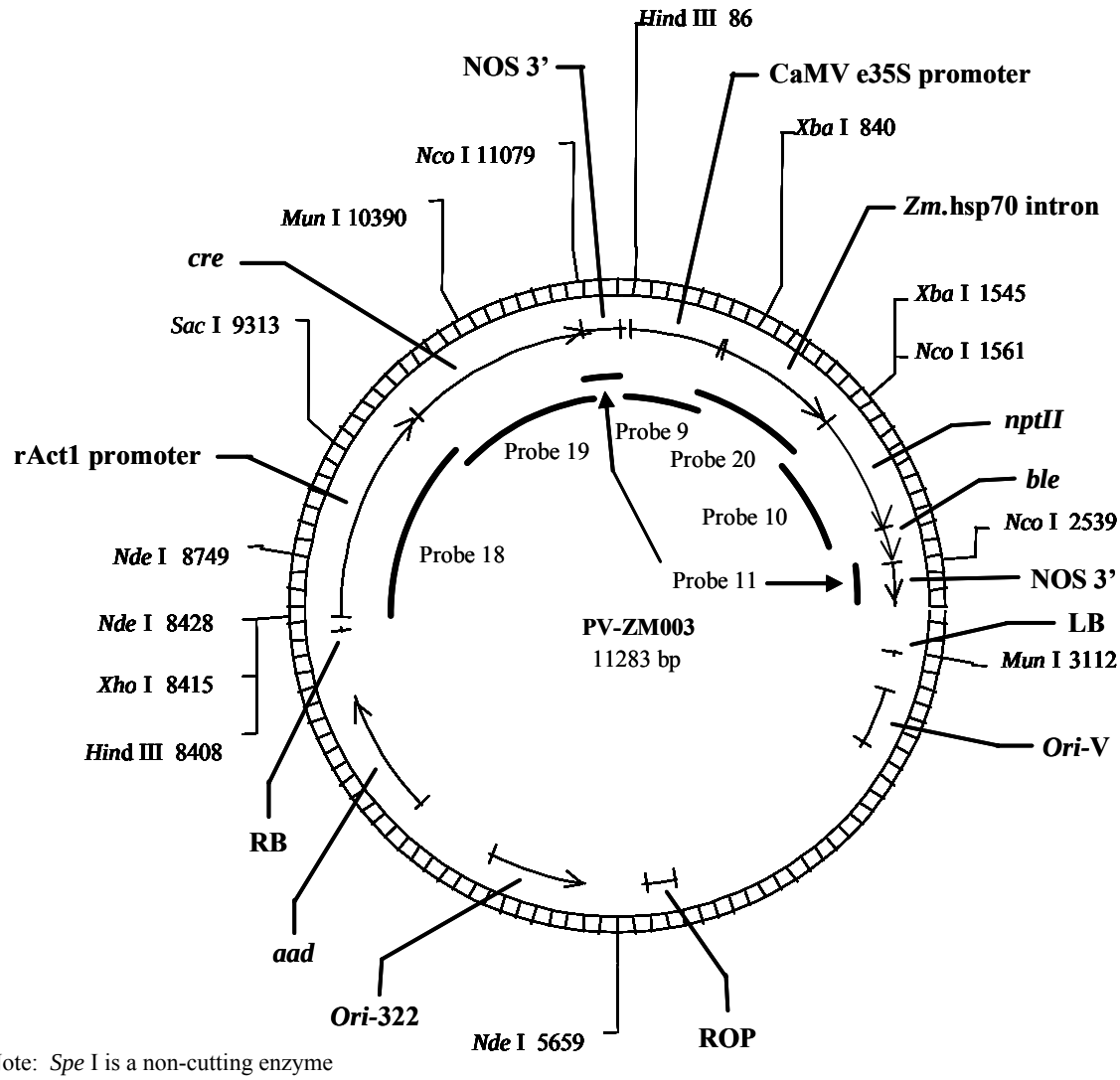
→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.



Note: *Spe* I is a non-cutting enzyme

Probe	DNA Probe	Start Position	End Position	Total Length (~kb)
14	T-DNA 1	8316	9791	1.5
15	T-DNA 2	9729	242	1.8
16	T-DNA 3	87	1971	1.9
17	T-DNA 4	1821	3234	1.4
21	Backbone 3	3231	5135	1.9
22	Backbone 4	5082	6915	1.8
23	Backbone 5	6797	8331	1.5

Figure V-11a. Map of plasmid vector PV-ZM003
Circular map of the plasmid vector PV-ZM003 used in *Agrobacterium sp.*-mediated transformation to produce the *cre*-containing maize event used in the excision of the *nptII* cassette. Genetic elements are annotated in bold on the exterior of the map and restriction sites (with positions relative to the size of the plasmid vector) for enzymes used in the Southern analysis are shown. Probes used in Southern analyses are illustrated in the interior of the map and are detailed in the accompanying table.



Note: *Spe* I is a non-cutting enzyme

Probe	DNA Probe	Start Position	End Position	Total Length (~kb)
18	rAct1 promoter	8412	9807	1.4
19	*cre* coding region	9852	11089	1.2
11	NOS 3' polyadenylation sequence	11051	63	0.3
9	CaMV e35S promoter	87	712	0.6
20	*Zm*.hsp70 intron	687	1555	0.9
10	*nptII* coding region	1563	2357	0.8

Figure V-11b. Map of plasmid vector PV-ZM003
Circular map of the plasmid vector PV-ZM003 used in *Agrobacterium sp.*-mediated transformation to produce the *cre*-containing maize event used in the excision of the *nptII* cassette. Genetic elements are annotated in bold on the exterior of the map and restriction sites (with positions relative to the size of the plasmid vector) for enzymes used in the Southern analysis are shown. Probes used in Southern analyses are illustrated in the interior of the map and are detailed in the accompanying table.

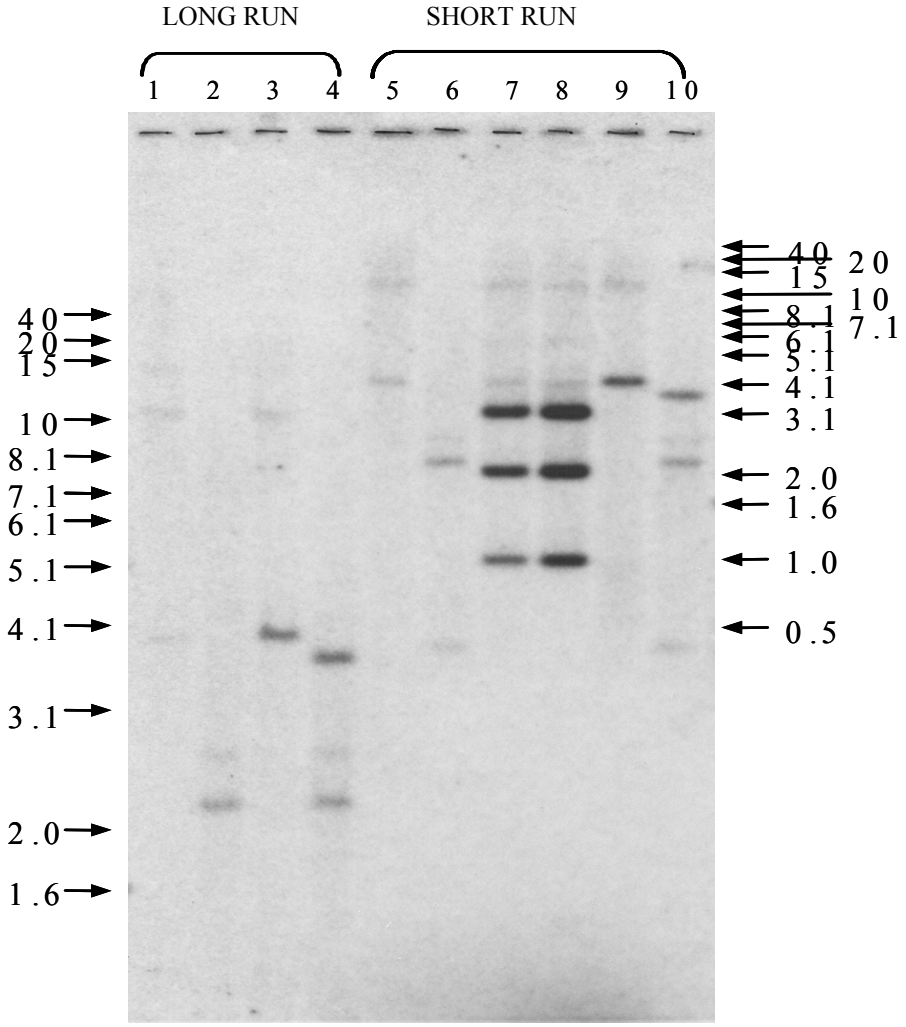


Figure V-12. Southern blot analysis of LY038: PV-ZM003 T-DNA probes
The blot was examined simultaneously with four ^{32}P-labeled probes that spanned the entire length of the T-DNA (probes 14, 15, 16, and 17, Figure V-11a). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: LY038(-) (*Spe* I)
2: LY038(-) (*Xho* I and *Xba* I)
3: LY038 (*Spe* I)
4: LY038 (*Xho* I and *Xba* I)
5: LY038(-) (*Spe* I)
6: LY038(-) (*Xho* I and *Xba* I)
7: LY038(-) (*Spe* I) spiked with PV-ZM003 (*Hin*d III *and Mun* I) [0.5 copy]
8: LY038(-) (*Spe* I) spiked with PV-ZM003 (*Hin*d III *and Mun* I) [1.0 copy]
9: LY038 (*Spe* I)
10. LY038 (*Xho* I and *Xba* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

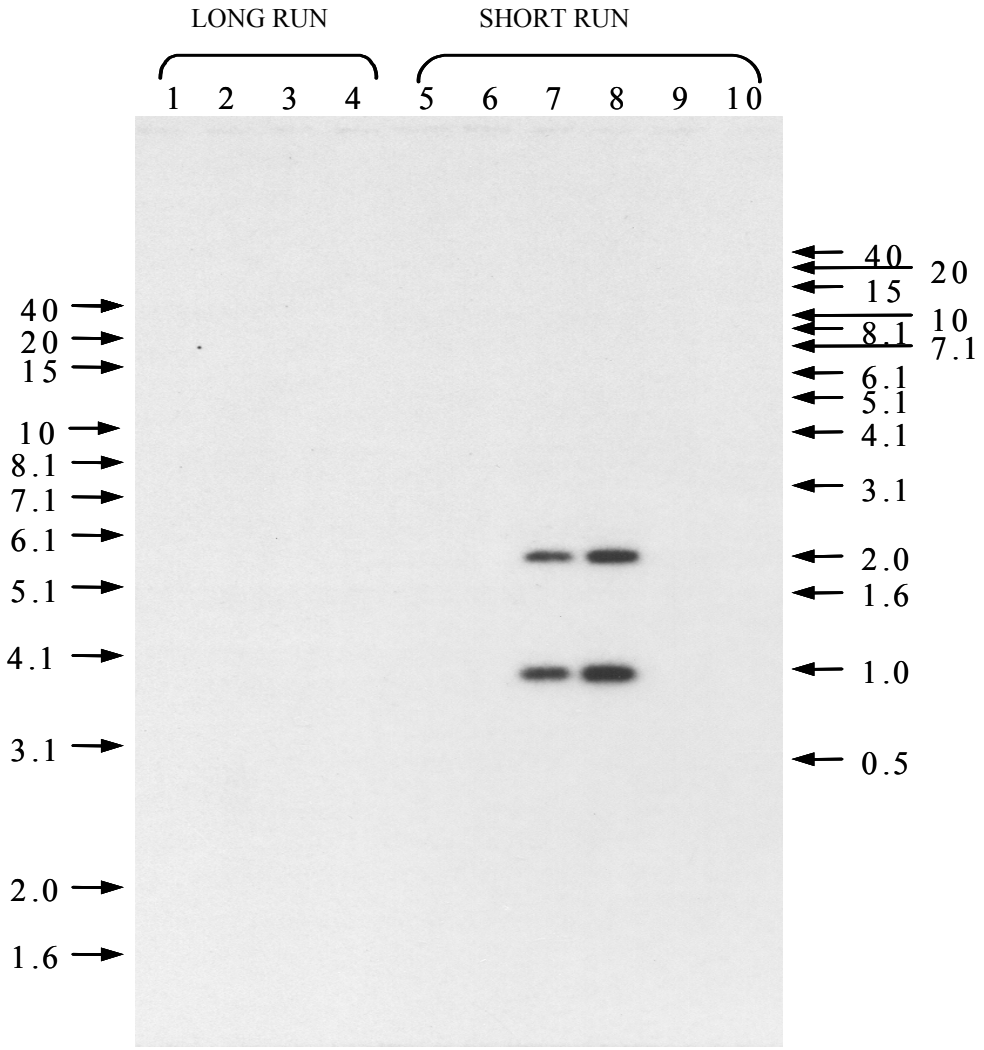


Figure V-13. Southern blot analysis of LY038: *cre* coding region probe
The blot was examined with a ^{32}P-labeled probe that spanned the entire length of the *cre* coding region (probe 19, Figure V-11b). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: LY038(-) (*Spe* I)
2: LY038(-) (*Xho* I and *Xba* I)
3: LY038 (*Spe* I)
4: LY038 (*Xho* I and *Xba* I)
5: LY038(-) (*Spe* I)
6: LY038(-) (*Xho* I and *Xba* I)
7: LY038(-) (*Spe* I) spiked with PV-ZM003 (*Hin*d III *and Mun* I) [0.5 copy]
8: LY038(-) (*Spe* I) spiked with PV-ZM003 (*Hin*d III *and Mun* I) [1.0 copy]
9: LY038 (*Spe* I)
10: LY038 (*Xho* I and *Xba* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

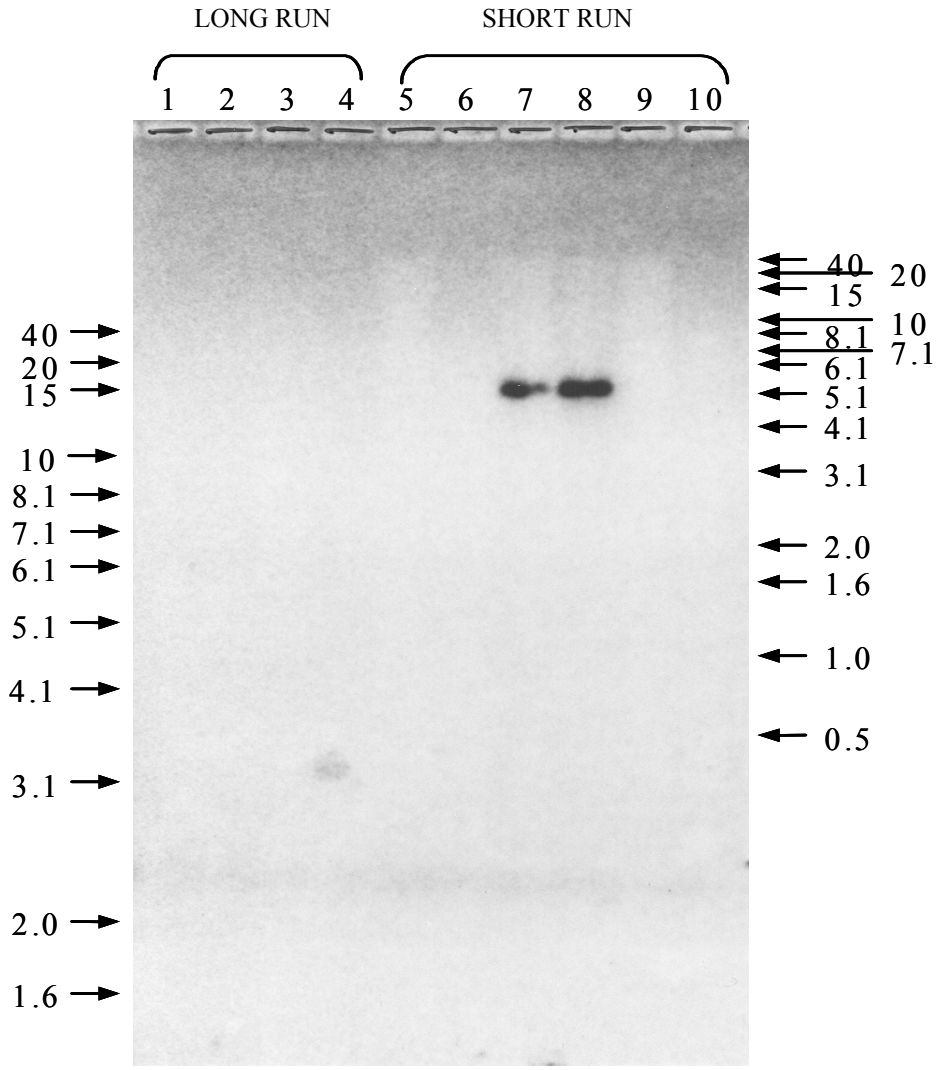


Figure V-14. Southern blot analysis of LY038: PV-ZM003 backbone probes
The blot was examined simultaneously with two ^{32}P-labeled probes that spanned the entire plasmid PV-ZM003 backbone sequence (probes 21, 22, and 23, Figure V-11a). Each lane contains ~10 μg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: LY038(-) (*Spe* I)
2: LY038(-) (*Xho* I and *Xba* I)
3: LY038 (*Spe* I)
4: LY038 (*Xho* I and *Xba* I)
5: LY038(-) (*Spe* I)
6: LY038(-) (*Xho* I and *Xba* I)
7: LY038(-) (*Spe* I) spiked with PV-ZM003 (*Hin*d III *and Mun* I) [0.5 copy]
8: LY038(-) (*Spe* I) spiked with PV-ZM003 (*Hin*d III *and Mun* I) [1.0 copy]
9: LY038 (*Spe* I)
10: LY038 (*Xho* I and *Xba* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

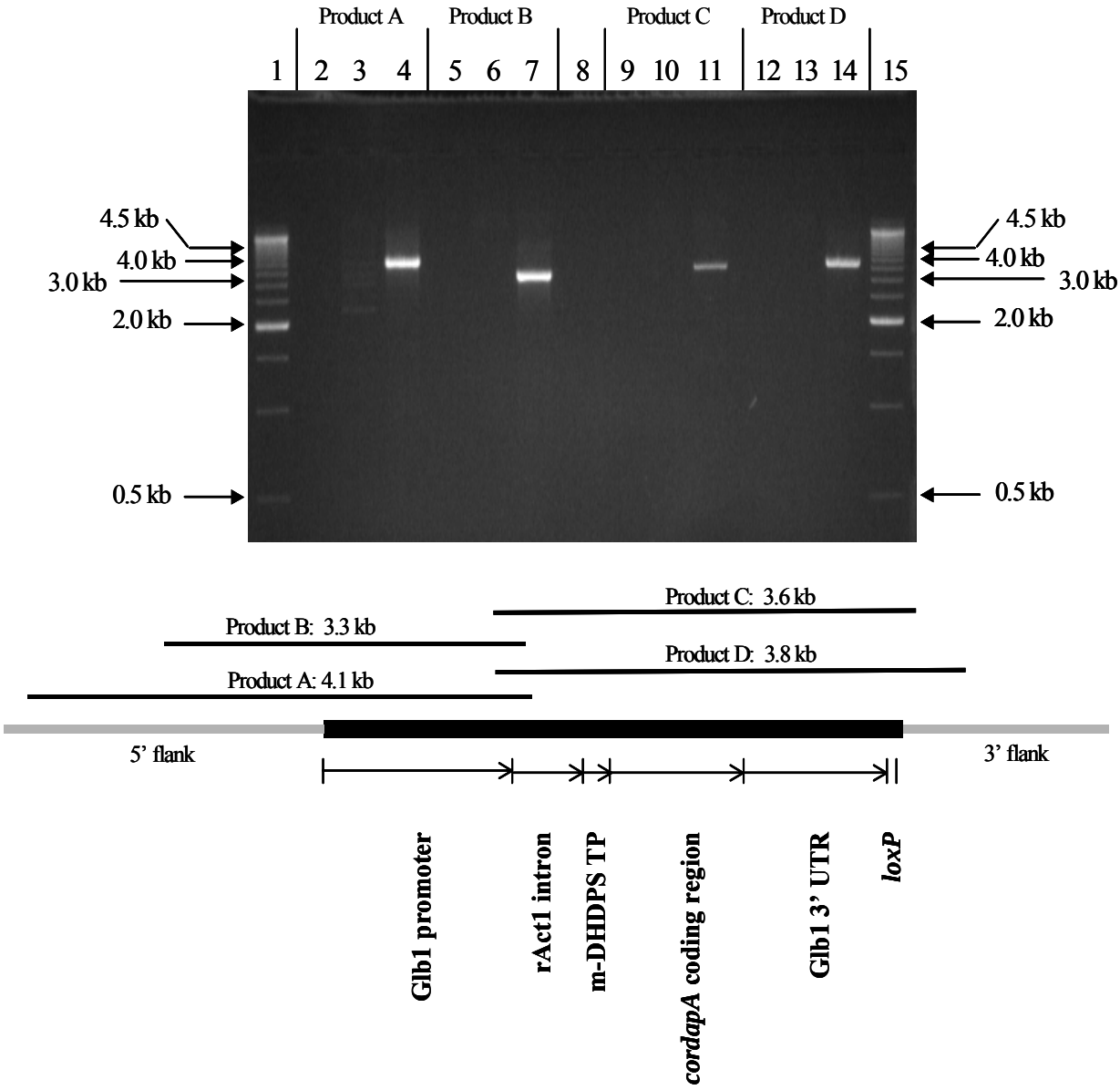


Figure V-15. Overlapping PCR analysis across the insert in LY038
PCR analyses demonstrating the linkages of the individual genetic elements within the insert in LY038 were performed on LY038 genomic DNA extracted from grain (Lanes 4, 7, 11, and 14). Lanes 3, 6, 10, and 13 contain reactions with LY038(-) control DNA while lanes 2, 5, 9, and 12 are reactions containing no template DNA. Lanes 1 and 15 contain Invitrogen 500 bp DNA ladder. Lanes are marked to show which product has been loaded and are visualized on the agarose gel. The expected product size for each amplicon is provided in the illustration of the insert in LY038 that appears at the bottom of the figure. Five microliters of each of the PCR products were loaded on the gel.
⟶ Symbol denotes sizes obtained from MW markers on ethidium bromide stained gel.

B. Heritability and Stability of Gene Transfer for LY038

1. Heritability

Heritability of the *cordapA* gene in LY038 was evaluated by determining segregation ratios at three generational stages: in the F1′ generation, prior to excision of the *nptII* marker gene by Cre-mediated recombination; in the F3 generation which was obtained after *nptII* marker excision; and in the F4 generation which had subsequently undergone two rounds of backcrossing to conventional inbred lines. For analysis of the F3 generation, individual plants homozygous for the *cordapA* gene cassette were crossed to a conventional inbred line (P2), and progeny resulting from this cross were subsequently self-pollinated to yield (P2×F3)F2 progeny. For analysis of the F4 generation, plants homozygous for the *cordapA* gene cassette were crossed to each of three different conventional inbred lines (P3, P4 and P5) and then backcrossed to the corresponding inbred line for two subsequent generations while selecting for individual plants that carried *cordapA*. BC2 plants heterozygous for the *cordapA* gene cassette were subsequently self-pollinated to yield the BC2F2 generation (Table V-1). Figure V-16 illustrates the breeding history of LY038 and indicates the generations used for segregation analysis.

Chi-square (χ^2) analysis was performed to determine the heritability and stability of the *cordapA* gene cassette in LY038 (Table V-1). The Chi-square analysis is based on testing the observed segregation ratio to the expected ratio according to Mendelian principles. The F1′ generation was obtained by crossing the original LY038 transformant, which was hemizygous for the *cordapA* gene cassette, with a conventional inbred line (P1). The expected segregation ratio for the F1′ generation is 1:1 (transgene-positive progeny: transgene-negative progeny), whereas the expected segregation ratio for subsequent generations, which were evaluated by analyzing progeny obtained by self-pollinating heterozygous plants, is 3:1 (transgene-positive progeny : transgene-negative progeny). The Chi-square test was computed as: $\chi^2 = \Sigma [(|o - e| - 0.5)^2 / e]$, where o = observed frequency of the genotype, e = expected frequency of the genotype, and 0.5 is the Yates correction factor for analysis with one degree of freedom (Little and Hills, 1978).

Table V-1. Segregation frequency of positive and negative entries -- Chi-Square test (χ^2)

Generation	Observed Positives	Observed Negatives	Expected Positives	Expected Negatives	χ^2
(R0xP1)=F1′	49	44	46.50	46.50	0.17ns
(P2xF3)F2	145	46	143.25	47.75	0.04ns
(P3xF4)BC2F2	348	110	343.50	114.50	0.19ns
(P4xF4)BC2F2	586	176	571.50	190.50	1.37ns
(P5xF4)BC2F2	460	175	476.25	158.75	2.08ns

ns = nonsignificant

In the F1′ generation, which was analyzed prior to Cre recombinase-mediated excision of the *nptII* gene cassette, it was possible to identify transgene-positive and transgene-negative segregants by screening plants for the presence of the *nptII* gene product. This was possible because the *cordapA* gene cassette and the *nptII* gene cassette were physically linked in the original transformation vector and, therefore, constituted a single Mendelian locus in the F1′ generation. The identification of positive and negative plants in the F1′ generation was performed by using a *nptII* assay on intact plants (Howe and Feng, 2003). For analysis of *cordapA* segregation in generations subsequent to Cre-mediated excision of the *nptII* gene cassette, transgene-positive and transgene-negative segregants were identified by screening for the presence of the *cordapA* gene sequence by using *cordapA*-specific oligonucleotides in standard DNA analytical procedures employing either TaqMan® (Gloffke, 2003) or Invader® (Wilkinson, 1999) technology.

None of the χ^2 values obtained in these studies indicated a significant difference between observed and expected segregation ratios for the LY038 *cordapA* gene cassette over five plant generations. These results are consistent with molecular characterization data, which indicates stable integration of the *cordapA* transgene at a single site in the genome.

® TaqMan is a registered trademark of Applied Biosystems, Inc.
® Invader is a registered trademark of Third Wave Technologies, Inc.

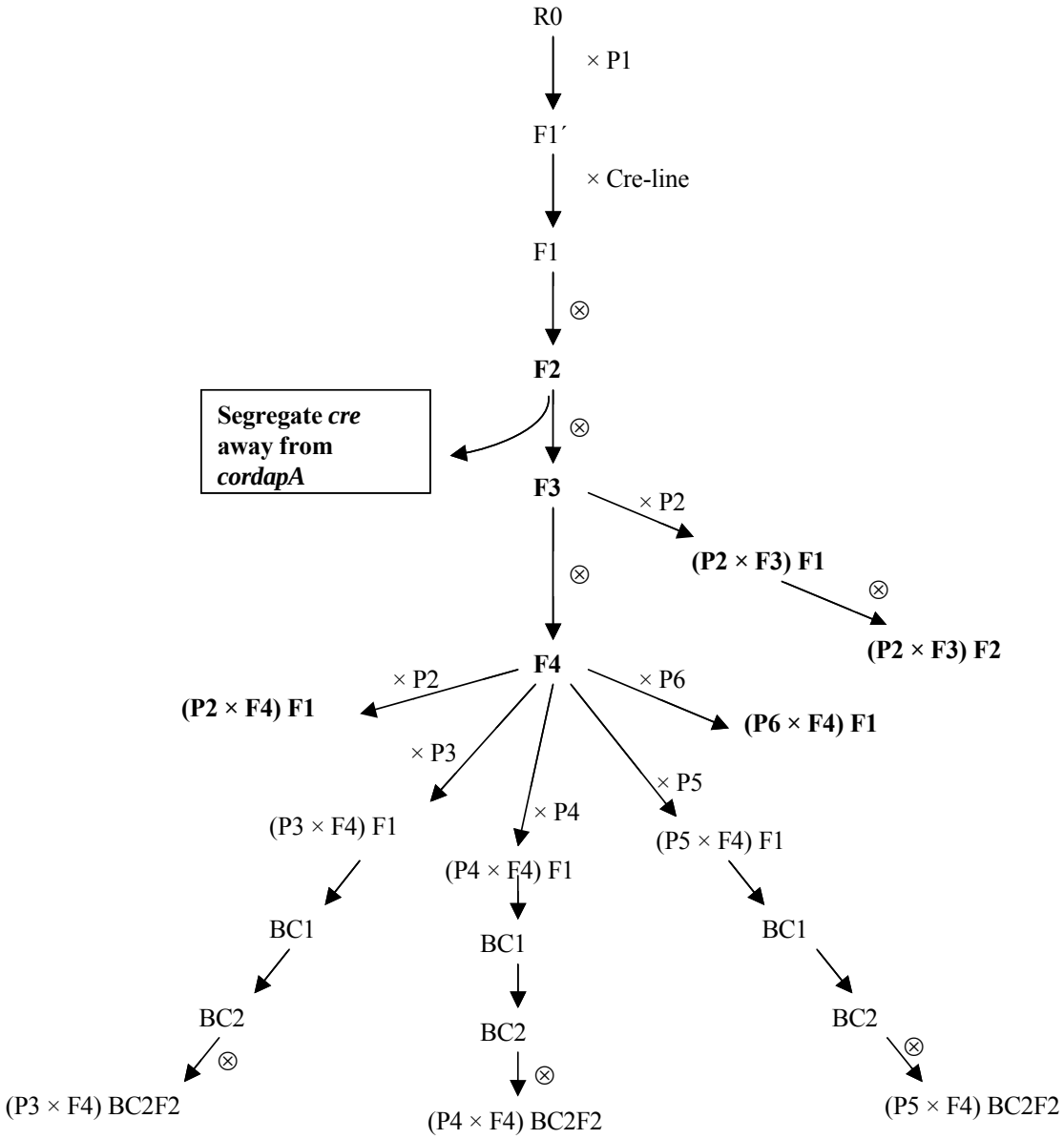


Figure V-16. Diagrammatic representation of LY038 breeding tree
The generation immediately prior to the one in which the *nptII* antibiotic-resistance marker was excised by Cre-mediated recombination is designated F1′. Segregation analysis was performed on F1′, (P2×F3)F2, (P3×F4)BC2F2, (P4×F4)BC2F2, and (P5×F4)BC2F2. Molecular generational stability analysis was performed on F2, F3, (P2×F3)F1, (P2×F3)F2, F4, (P2×F4)F1, and (P6×F4)F1 (all shown in bold font in figure). Molecular characterization was performed on (P2×F3)F2. Gene expression and composition analysis was performed on (P2×F4)F2.
R0, transformed plant; Pn, nontransgenic inbred line; Fn, filial generation;
⊗, self-pollination; BCn, backcross generation.

2. Southern Blot Generational Stability

To demonstrate the stability of the I-DNA in LY038, Southern blot analyses were performed using DNA obtained from multiple generations, including F2, F3, (P2×F3)F1, (P2×F3)F2, F4, (P2×F4)F1, and (P6×F4)F1 (Figure V-16). DNA samples were digested with the combination of *Nde* I and *Nco* I that would give rise to 4.4 kb and 4.2 kb fragments upon hybridization with the I-DNA probes. Two Southern blots were generated where one was probed with I-DNA 1 and I-DNA 2 (Figure III-1a) that span approximately 75% of the I-DNA, and the second blot was probed with I-DNA 3 and I-DNA 4 (Figure III-1a) that span the remaining 25% of the I-DNA. The results of these analyses are presented in Figures V-17 and V-18, respectively.

Hybridization of the positive control DNA with I-DNA 1 and I-DNA 2 generated two unique bands of 3.6 kb and 1.7 kb (Figure V-17, Lane 11). In all generations examined, the expected 4.4 kb and 4.2 kb bands were observed (Lanes 2-6, 8, 10) in addition to background hybridization bands present in the control DNA lanes (Lanes 1, 7, 9).

Hybridization of the positive control DNA with I-DNA 3 and I-DNA 4 generated a 3.6 kb band (Figure V-18, Lane 11) in addition to the background bands in LY038(-) DNA. In all generations examined, the expected 4.2 kb band was observed (Lanes 2-6, 8, 10) in addition to background hybridization bands present in the control DNA lanes (Lanes 1, 7, 9). Two additional hybridization bands of 1 kb and 1.8 kb were observed in the F2 generation (Lane 3). Since F2 represented the generation before *cre* and *nptII* were segregated, the presence of the additional 1.8 kb band (CaMV e35S promoter in PV-ZM003) and the 1.0 kb band (*nptII* coding region in PV-ZMPQ76 and PV-ZM003) were anticipated. These bands were not observed in any of the subsequent generations, confirming that both *cre* and *nptII* cassettes were successfully segregated in the following generations.

In summary, the Southern blot analysis successfully established the stability of the inserted DNA over multiple generations representing each branch point of the breeding history.

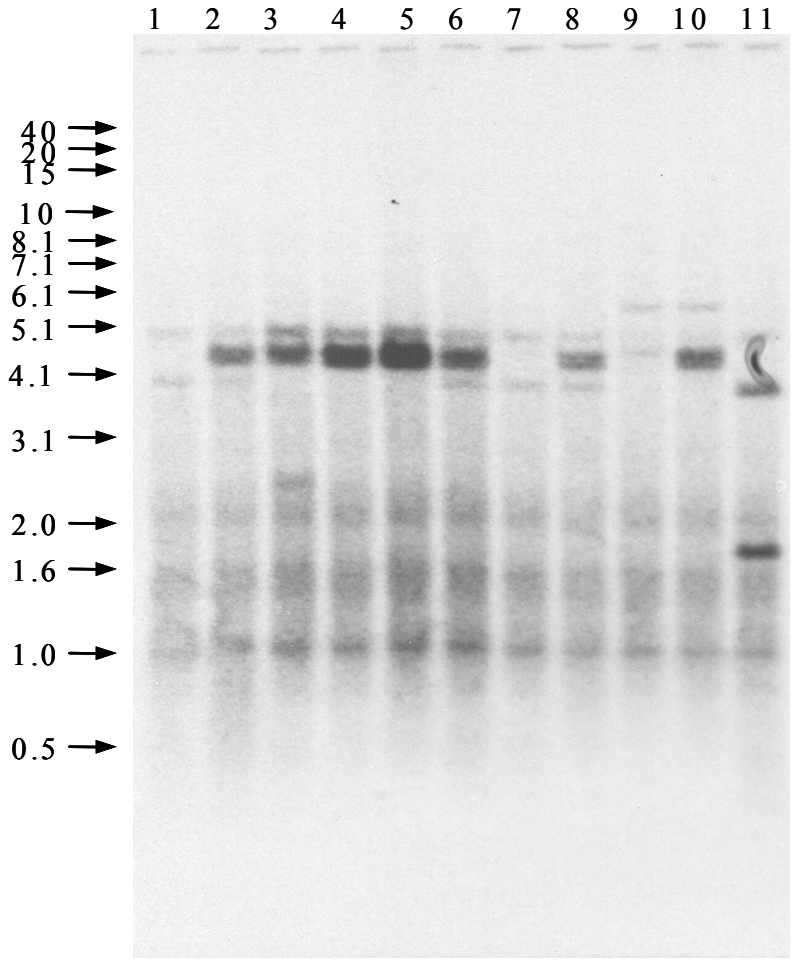


Figure V-17. Generational stability of LY038: I-DNA 1 and I-DNA 2 probes
The blot was examined simultaneously with two ^{32}P-labeled probes that spanned a portion of the I-DNA (probes 1 and 2, Figure III-1a). Each lane contains ~10 μg of digested genomic DNA isolated from seed, grain, or leaf material. Lane designations are as follows:

Lane 1: [LH195 x LY038(-)-91INH2 F_3]F_2 (*Nde* I and *Nco* I)
2: [LH195 x LY038-91INH2 F_3]F_2 (*Nde* I and *Nco* I)
3: LY038-91INH2 F_2 (*Nde* I and *Nco* I)
4: LY038-91INH2 F_3 (*Nde* I and *Nco* I)
5: LY038-91INH2 F_4 (*Nde* I and *Nco* I)
6: [LH195 x LY038-91INH2 F_3] $_{(H)}$ (*Nde* I and *Nco* I)
7: [LH195 x LY038(-)-91INH2 F_4] $_{(H)}$ (*Nde* I and *Nco* I)
8: [LH195 x LY038-91INH2 F_4] $_{(H)}$ (*Nde* I and *Nco* I)
9: [HOI002 x LY038(-)-91INH2 F_4] $_{(H)}$ (*Nde* I and *Nco* I)
10: [HOI002 x LY038-91INH2 F_4] $_{(H)}$ (*Nde* I and *Nco* I)
11: [LH195 x LY038(-)-91INH2 F_3]F_2 (*Nde* I and *Nco* I) spiked with PV-ZMPQ76 (*Eco*R V) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

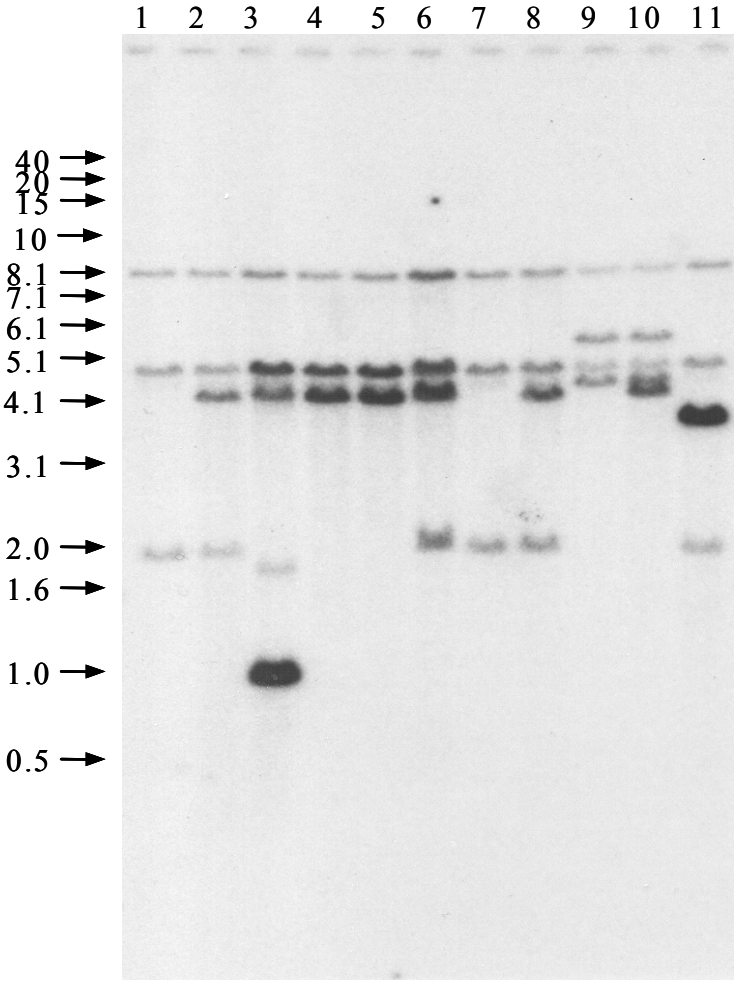


Figure V-18. Generational stability of LY038: I-DNA 3 and I-DNA 4 probes
The blot was examined simultaneously with two ^{32}P-labeled probes that spanned a portion of the I-DNA (probes 3 and 4, Figure III-1a). Each lane contains ~10 μg of digested genomic DNA isolated from seed, grain or leaf material. Lane designations are as follows:

Lane 1: [LH195 x LY038(-)-91INH2 F_3]F_2 (*Nde* I and *Nco* I)
2: [LH195 x LY038-91INH2 F_3]F_2 (*Nde* I and *Nco* I)
3: LY038-91INH2 F_2 (*Nde* I and *Nco* I)
4: LY038-91INH2 F_3 (*Nde* I and *Nco* I)
5: LY038-91INH2 F_4 (*Nde* I and *Nco* I)
6: [LH195 x LY038-91INH2 F_3] $_{(H)}$ (*Nde* I and *Nco* I)
7: [LH195 x LY038(-)-91INH2 F_4] $_{(H)}$ (*Nde* I and *Nco* I)
8: [LH195 x LY038-91INH2 F_4] $_{(H)}$ (*Nde* I and *Nco* I)
9: [HOI002 x LY038(-)-91INH2 F_4] $_{(H)}$ (*Nde* I and *Nco* I)
10. [HOI002 x LY038-91INH2 F_4] $_{(H)}$ (*Nde* I and *Nco* I)
11. [LH195 x LY038(-)-91INH2 F_3]F_2 (*Nde* I and *Nco* I) spiked with PV-ZMPQ76 (*Eco*R V) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

C. Expression of the Inserted *cordapA* Gene

Lysine maize LY038 contains the *cordapA* coding sequence isolated from *Corynebacterium glutamicum* that encodes the lysine-insensitive dihydrodipicolinate synthase (cDHDPS) enzyme. The transcription of *cordapA* is under the control of the maize Glb1 promoter, which directs cDHDPS expression predominantly in the germ, resulting in accumulation of free lysine in grain.

Expression of the cDHDPS protein in LY038 and control LY038(-) tissue samples from plants grown in the U.S. at five field sites, three replicated plots per site, in 2002 (USDA-APHIS notification number 02-052-05n) was evaluated using ELISA methods described in Appendix 2. cDHDPS protein levels for all tissue types were determined on a microgram (μg) per gram (g) fresh weight (fwt) basis. Moisture content was then measured for all tissue types and protein levels were converted to a dry weight (dwt) basis.

The mean cDHDPS protein levels across five sites in LY038 grain, forage, whole plant (V2-V4), forage root, root (V2-V4), and pollen tissues were 26, 0.94, 0.081, 0.069, 1.5, and 0.78 μg/g dwt, respectively (Table V-2). Levels of cDHDPS protein in LY038 leaf tissues harvested at four time points throughout the growing season were less than the assay limit of detection of leaf tissue (LOD, 0.013 μg/g fwt).

These results confirm transcription of the *cordapA* gene expressing the cDHDPS protein and that cDHDPS expression is predominantly in grain tissue.

Table V-2. Summary of cDHDPS protein levels in LY038 tissues collected from multi-site U.S. field trials in 2002

(USDA-APHIS notification number 02-052-05n)

Tissue Type	cDHDPS Mean Protein Level µg/g fwt[1] (SD)	Range[2] (µg/g fwt)	cDHDPS Mean Protein Level µg/g dwt[3] (SD)	Range (µg/g dwt)	LOQ / LOD[4] (µg/g fwt)
Grain[5]	24 (9.1)	13 – 43	26 (10)	14 – 49	0.044 / 0.021
Forage	0.25 (0.21)	0.034 – 0.79	0.94 (0.75)	0.15 – 2.8	0.0025 / 0.00056
Whole Plant V2-V4	0.0093 (0.0083)	0.0026 – 0.026	0.080 (0.068)	0.024 – 0.22	0.0025 / 0.00056
Forage Root	0.010 (0.0043)	0.0052 – 0.019	0.069 (0.031)	0.031 – 0.11	0.0050 / 0.0050
Root V2-V4	0.14 (0.23)	0.011 – 0.62	1.5 (2.2)	0.099 – 6.2	0.0050 / 0.0050[6]
Pollen	0.43 (0.14)	0.27 – 0.67	0.78 (0.24)	0.45 – 1.1	0.025 / 0.0052
OSL 1-4	< LOD	–	n/a[7]	–	0.038 / 0.013

[1] Protein levels are expressed as micrograms (µg) of protein per gram (g) of tissue on a fresh weight (fwt) basis. The mean and standard deviation (SD) were calculated for each tissue type across sites.

[2] Minimum and maximum values were determined for each tissue type across sites.

[3] Protein levels are expressed as µg/g of tissue on a dry weight (dwt) basis. The dwt values were calculated by dividing the fwt values by the dry weight conversion factors obtained from moisture analysis data.

[4] The cDHDPS assay limits of quantitation (LOQ) and detection (LOD) are presented in µg/g fwt.

[5] The number of samples used for data analysis (n) is as follows:

a. Grain, forage, forage root, pollen, and OSL 1-4 (n = 15)
b. Whole plant (n = 16)
c. Root (n = 12)

[6] The LOQ and LOD for the cDHDPS protein in root tissues are identical.

[7] Protein levels that were < LOD on a fwt basis were not converted to dwt values.

VI. Dihydrodipicolinate synthase (DHDPS) Proteins

The *cordapA* gene introduced into the maize genome to produce LY038 encodes expression of the lysine-insensitive dihydrodipicolinate synthase (cDHDPS) enzyme. A detailed discussion of the development of LY038 and the genetic elements involved is included in Section IV and data on levels of expression of the cDHDPS protein in several tissue types from LY038 are included in Section V.C. of this petition. The function of the cDHDPS protein, characterization of cDHDPS protein extracted from LY038, comparision of cDHDPS to other DHDPSs with a history of safe exposure, and a summary of the feed and food safety assessment for cDHDPS provided to FDA are summarized in this section.

A. Specificity of DHDPS Protein

The mechanism of action for dihydrodipicolinate synthase (DHDPS; EC4.2.1.52) has been well characterized (Karsten, 1997). The DHDPS protein is a member of the lyase subfamily of pyruvate-dependent class I aldolases (Lawrence et al., 1997) found in a wide range of organisms including bacteria, rodents, and humans. The DHDPS enzyme mediates a critical rate-limiting step in the lysine biosynthetic pathway that in maize is controlled by lysine feedback inhibition. The enzyme catalyzes the condensation of L-aspartate-4-semialdehyde and pyruvate to form 2,3-dihydrodipicolinate that is converted to lysine through a series of subsequent enzymatic reactions. In contrast to the native maize DHDPS, the variant of this enzyme from *C. glutamicum* (cDHDPS) is less sensitive to lysine feedback inhibition. As expected based on the detailed description of the genetic elements present in LY038 in Section IV and confirmed by cDHDPS expression data presented in Section V.C., cDHDPS expression is predominantly in the grain portion of LY038, producing the desired effect of increased grain lysine levels compared to lysine levels in conventional maize grain.

B. Characterization of the cDHDPS Protein in LY038

The physicochemical and functional characteristics of the cDHDPS protein produced in and purified from LY038 and the equivalence of this plant-produced cDHDPS protein to a previously characterized *E. coli*-produced cDHDPS protein were determined in laboratory studies. Detailed descriptions of materials and methods used in these protein characterization studies are included in Appendix 3.

The identity of the plant-produced cDHDPS protein was confirmed using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry, and N-termninal sequence and immunoblot analyses. Approximately 58% of the expected 303 amino acid sequence comprising the *in planta* cDHDPS protein was identified using MALDI-TOF mass spectrometry. N-terminal sequence analysis (15 cycles) identified two sequences in the plant-produced cDHDPS; both were consistent with the sequence of the cDHDPS protein. The primary identified sequence contained three additional amino acids (residues 1-3) derived from the C-terminus of the maize DHDPS chloroplast transit

peptide (CTP) sequence, as well as the first twelve amino acids (residues 4-15) expected for the N-terminus of the cDHDPS protein. The second minor sequence corresponded to the N-terminal sequence of the cDHDPS protein without the three additional amino acids derived from the CTP. Both MALDI-TOF and N-terminal sequence analysis yielded peptide sequences consistent with the sequence obtained for *E. coli*-produced reference standard cDHDPS protein. On the basis of western blot analysis, electrophoretic mobility and inmmuoreactivity, the plant-produced cDHDPS protein was similar to the *E coli*-produced cDHDPS reference standard.

Purity and apparent molecular weight of the plant-produced cDHDPS protein, estimated using densitometric analysis of Colloidal Brilliant Blue G stained SDS-polyacrylamide gels, were observed to be approximately 98% and 32 kDa, respectively. The mass average (MH+) molecular weight of the intact protein was determined using MALDI-TOF mass spectrometry to be 31,376 Da. Both the SDS-PAGE and MALDI-TOF results are consistent with the molecular weight estimations for *E. coli*-produced cDHDPS reference standard protein.

The functional activity of the plant-produced and *E. coli*-produced cDHDPS proteins was determined using a coupled enzyme assay. The specific activity for the plant-produced and *E. coli*-produced cDHDPS protein was estimated to be 68 ± 3 and 84 ± 5 U/mg total protein, respectively.

Glycosylation analysis demonstrated that there was no detectable glycosylation of the plant-produced cDHDPS protein, confirming equivalence to the *E. coli*-produced cDHDPS reference standard with respect to glycosylation.

These data provide a detailed characterization of the cDHDPS protein isolated from LY038 and establish the equivalence of the cDHDPS protein produced in maize to an *E. coli*-produced cDHDPS reference standard protein, the latter of which was subsequently used for *in vitro* and *in vivo* safety assessment studies summarized in Section VI.E.

C. Similarity of cDHDPS to DHDPSs Derived from Food Sources with a Long History of Safe Exposure

The cDHDPS protein belongs to the family of related DapA (DHDPS) proteins. DHDPS is the first enzyme unique to lysine biosynthesis in bacteria and higher plants (Galili, 1995). DHDPS proteins isolated from a number of species including spinach, pea, maize, *E. coli*, and *Bacillus subtilis* have been extensively characterized (Wallsgrove and Mazelis, 1981; Dereppe et al., 1992; Frisch et al., 1991; Karsten, 1997). Assessment of the potential impact of cDHDPS on animal and human health is based upon extensive characterization of the cDHDPS protein and its functional homology to other DHDPS proteins commonly found in a wide variety of animal feed and human food sources, which have a history of safe consumption / exposure. Since all of these proteins catalyze the first enzymatic step in lysine biosynthesis in all of these organisms, it is predictable that they share reasonable sequence identity and similarity when evaluated using a

"BestFit" program such as that in the Wisconsin Package (Table VI-1). The Best Fit program makes an optimal alignment of two sequences using a local homology algorithm by Smith and Waterman (1981). Consistent with the fact that all of these DHDPS proteins catalyze the first enzymatic step in lysine biosynthesis in a wide range of organisms, it is to be expected that an algorithm to identify local amino acid homology along the entire length of the protein showed 27 - 37% sequence identity and 36 – 47% similarity between cDHDPS and DHDPS proteins from other species.

Table VI-1. Comparison of amino acid sequence of cDHDPS and representative DHDPS proteins

DHDPS sequence (Accession)	% Identity to cDHDPS	% Similarity to cDHDPS
C. glutamicum (BAB99364)	100.0	100.0
E. coli (AAO43656)	36.8	46.9
Maize (1718320A)	29.4	38.5
Soy (AAA73555)	28.0	38.5
Wheat (AAA34264)	28.9	36.4
Rice (AAF44718)	27.0	36.1

D. Current Uses of *Corynebacterium glutamicum* Expressing cDHDPS

Corynebacterium glutamicum, a nonpathogenic species of coryneform bacteria, are rod-shaped, nonsporulating Gram-positive bacteria that are widely distributed in the environment (Abe and Takayama, 1972; Aida et al., 1986; Eggeling, 1994; Hodgson, 1994; Leuchtenberger, 1996; Nakayama, 1972). Commercial lysine production is primarily via fermentation of *Corynebacterium glutamicum* strains expressing dihydrodipicolinate synthase (cDHDPS) that is less sensitive to lysine feedback inhibition than DHDPS enzymes from other species (Eggeling, 1994; Eggeling et al., 1998). The DHDPS enzyme catalyzes the condensation of L-aspartate-4-semialdehyde and pyruvate to 2,3-dihydrodipicolinate (Bryan, 1980; Schrumpf et al., 1991). The decreased sensitivity of the cDHDPS enzyme from *Corynebacterium glutamicum* to lysine feedback inhibition facilitates increased flux through this enzymatic step in the lysine biosynthetic pathway resulting in accumulation of free lysine (Eggeling et al., 1998).

E. Summary of cDHDPS Feed and Food Safety Assessment

A detailed assessment of animal and human safety of the cDHDPS protein has been provided to the FDA as part of a feed and food safety and nutritional assessment for LY038. The conclusions of the safety assessment are summarized below:

a) The donor organism, *Corynebacterium glutamicum*, is a common soil bacterium widely distributed in the environment.
b) The donor organism, *Corynebacterium glutamicum*, is not a human or animal pathogen.

c) A history of the safe exposure for the cDHDPS protein has been demonstrated, based on the similarity of the cDHDPS protein in LY038 to DHDPSs naturally present in feed and food (e.g., maize, rice, soy and wheat).
d) The cDHDPS protein purified from *E. coli* was found to be physicochemically and functionally equivalent to the protein produced in LY038.
e) The cDHDPS protein is rapidly degraded in simulated gastric fluid indicating that it would be unlikely to elicit allergenic or toxic effects.
f) No biologically relevant structural similarities were observed between the cDHDPS protein present in LY038 or any eight amino acid peptide sequences derived from cDHDPS and toxins, allergens or pharmacologically active proteins that are known to cause adverse health effects in humans or animals.
g) Results of a mouse acute oral toxicity study demonstrate that the cDHDPS protein is not acutely toxic at a dose of 800 mg/kg and does not cause any adverse effects.
h) Based on cDHDPS levels in LY038 grain and the determined NOEL from a mouse acute oral toxicity evaluation, large margins of exposure were calculated for cDHDPS protein for livestock species (> 500 for broilers and pigs) and humans (> 100,000 for the overall U.S. population) indicate that there is no meaningful risk to animal or human health from dietary exposure to cDHDPS from LY038.

Results of the safety assessment demonstrate that the cDHDPS protein in LY038 is safe for animal and human consumption.

VII. Phenotypic Evaluation

This section provides a phenotypic, ecological and compositional assessment that provides the basis for the determination that Lysine maize LY038 is no more likely to pose a plant pest risk than conventional maize.

A phenotypic evaluation of LY038 was conducted to assess its phenotypic equivalence and familiarity compared to conventional maize. The phenotypic evaluation was 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 maize. In each of these assessments, LY038 was compared to LY038(-) as well as several conventional reference maize hybrids. To evaluate the phenotypic characteristics of LY038, data were collected that address specific characteristics that are considered by USDA-APHIS. These phenotypic characteristics have been grouped into five general categories: 1) dormancy, germination, and emergence; 2) vegetative growth; 3) reproductive growth; 4) seed retention on plant; and 5) plant interactions with disease, insect, and abiotic stressors. An overview of the phenotypic characteristics assessed is presented in Table VII-1. To evaluate the forage and grain compositional characteristics of LY038, data were collected that address several plant components including natural toxicants and significant nutrients.

Table VII-1. Phenotypic characteristics measured for Lysine maize LY038

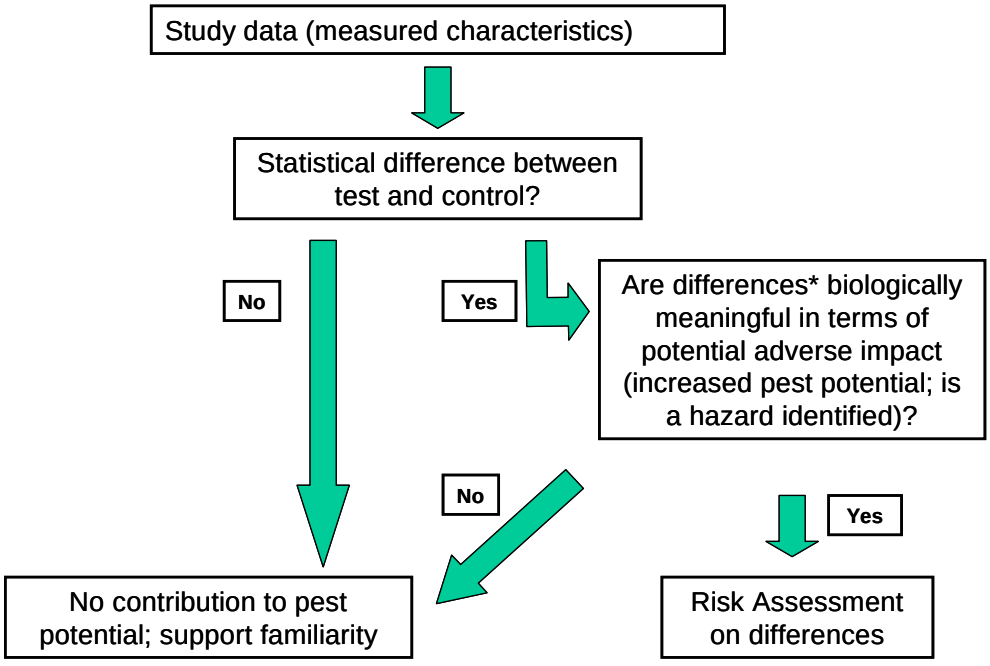
General Assessment Characteristic	Characteristic Measured	Evaluation timing	Evaluation description
Dormancy / Germination / Emergence	Dormancy, Germination	After 4 and 7 days	Percent normal germinated, abnormal germinated, viable hard (dormant), dead, and viable firm swollen seed
	Seedling vigor	Stage V2 – V4	Rated on a 0-9 scale, where 0 = dead and 9 = most vigorous growth
	Early stand count	Stage V2 - V4	Number of emerged plants per plot
Vegetative Growth	Final stand count	Pre-harvest	Number of plants
	Stay green	Maturity	Rated on a 0-9 scale, where 0 = entire plant is dried and 9 = entire plant is green
	Ear height	Maturity	Distance from the soil surface at the base of the plant to the ear attachment node
	Plant height	Maturity	Distance from the soil surface at the base of the plant to the flag leaf collar
	Stalk lodged plants	Pre-harvest	Number of plants broken below the ear
	Root lodged plants	Pre-harvest	Number of plants leaning at the soil surface greater than 30° from vertical
Reproductive Growth	Days to 50% pollen shed	Pollen shed	Days from planting until 50% of the plants have begun to shed pollen
	Days to 50% silking	Silking	Days from planting until 50% of the plants have silks exposed
	Pollen viability	Tasseling	Viable and nonviable pollen based on pollen grain staining characteristics
	Pollen morphology	Tasseling	Diameter of viable pollen grains
	Grain moisture	Harvest	Moisture percentage of harvested shelled grain
	Test weight	Harvest	Test weight of harvested shelled grain
	Yield	Harvest	Harvested shelled grain, adjusted to 15.5% moisture
Seed Retention	Dropped ears	Pre-harvest	Number of mature ears dropped from plants
Plant Interactions with Disease, Insect, and Abiotic Stressors	Differential susceptibility to pests or abiotic stressors	Planting to harvest	Qualitative assessment of each plot, with rating on a 0-9 scale for insect, disease, and abiotic stressors

A. Interpretation of Phenotypic and Ecological Interaction Data

Phenotypic and ecological interaction data are essential to assess phenotypic equivalence and familiarity of LY038 compared to conventional maize as outlined in the USDA-APHIS guidance documents on agronomic performance. On the basis of these data, it can established whether there is an increased pest potential of LY038 and whether the phenotype has been unintentionally changed beyond the introduced traits.

Measurement of phenotypic characteristics and ecological interactions (plant interactions with insect pest, disease, and abiotic stressors) provides data for a comparative assessment of ecological risk (pest potential) between a biotechnology-derived crop and an appropriate control. A tiered approach is used to assess whether a difference is, or is not, biologically meaningful. As such, evaluation of phenotypic characteristics is designed according to the biology of the crop using replicated plots at multiple locations with appropriate controls and commercial crop references. If no statistically significant differences are detected between the biotechnology-derived crop and appropriate controls, a conclusion of "no difference in pest potential" can be made. If a statistically significant and biologically meaningful difference in a characteristic is observed, the magnitude of the difference is considered (relative to the known ranges of values for the crop), and its effect on pest potential is assessed, as shown in the schematic diagram below.

Schematic diagram of data interpretation methods:



*Consider direction and magnitude of change and interaction of differences

A statistically significant difference in one characteristic is considered in terms of the direction of the difference (contributing to or detracting from pest potential), its magnitude (outside the range of the control or reference organisms), and within the context of other observed difference. Interpretation of detected differences in ecological risk assessment data should focus on those differences that are biologically meaningful (i.e., contribute to pest potential). Differences detected in a characteristic are considered alone and in the context of: 1) whether or not trends were observed across locations; 2) differences that are detected in other measured characteristics; 3) contributions to enhanced pest potential of the crop itself; and 4) potential effects of the transfer of the trait to a sexually compatible species.

For example, a significant difference in growth characteristics may not be biologically meaningful in terms of weed potential if it is not outside the range typical for that crop or if a change in another measured characteristic is in the direction toward lower weed potential. A careful assessment must be used to distinguish between meaningful differences toward increased pest potential and differences associated with natural plant variation or random experimental error. A finding of no meaningful difference can be concluded only after an evaluation of all the data collected on the measured characteristics.

B. Assessment of Phenotypic and Ecological Interactions of LY038

The following sections describe the experiments conducted for the phenotypic and ecological evaluation of LY038. The purpose of these experiments was to assess whether the phenotypic and ecological characteristics of LY038 are altered in comparison to the control, LY038(-). Several conventional reference maize hybrids were also evaluated in each experiment to provide a benchmark of values common to conventional maize for each measured phenotypic and ecological characteristic. The sites selected for these evaluations represent a wide range of environments that would be encountered for maize grown in the U.S. The USDA notification number for each trial site is identified in Table VII-2.

Table VII-2. USDA Notifications and Field Trial Locations for Phenotypic and Ecological Assessment of LY038

USDA-APHIS #	Site State	Site County	Location Code
Dormancy/Germination			
01-267-03n[1]	HI	na	na
02-280-06n[1]	WI	na	na
2002 Phenotypic Trials			
02-037-05n	Missouri	Shelby	BE
02-037-05n	Missouri	Shelby	CL
02-046-32n	Illinois	Clinton	CR
02-046-32n	Illinois	Warren	MN
02-037-05n	Indiana	Hamilton	NB
02-037-05n	Iowa	Jefferson	RL
02-037-05n	Iowa	Benton	VH
02-037-05n	Iowa	Hamilton	WC
02-066-15n	Illinois	Stark	WY
02-037-05n	Nebraska	York	YK
2003 Phenotypic Trials			
03-052-17n	Missouri	Shelby	BE
03-052-17n	Illinois	Clinton	CR
03-052-17n	Illinois	Warren	MN
03-052-17n	Indiana	Hamilton	NB
03-052-17n	Iowa	Jefferson	RL
03-052-17n	Iowa	Benton	VH
03-052-17n	Nebraska	York	YK

[1]USDA notification for movement of seed only; na = not applicable.

1. Dormancy and Germination Assessment

Seed dormancy is an important characteristic that is often associated with plants that are weeds (Anderson, 1996). Dormancy mechanisms, including hard seed, vary with species and tend to include complex processes. For most crops, including maize, the number of hard seed is negligible or nonexistent. Standardized germination assays are routinely used to measure the germination potential of maize seed (AOSA, 1998).

A laboratory study was conducted to assess whether the dormancy and germination characteristics of LY038 were altered compared to LY038(-). LY038, LY038(-) and conventional reference seed (three commercial hybrids) used for the evaluation were produced in Maui County, Hawaii (HI) in 2002. The identity of the test and control

substance starting seed was verified by event-specific polymerase chain reaction (PCR) analyses.

The experimental design involved incubating test, control, and reference seeds in rolled towels in temperature-controlled growth chambers at five temperature regimes ranging from 5 to 35^{o} C (Table VII-3). In addition to two AOSA-recommended temperature regimes, three temperature regimes (non-AOSA) were included to extend the range of temperatures under which the seed were evaluated. Seed were arranged in a completely randomized design. LY038 was compared to LY038(-) for the following dormancy and germination characteristics: percent germinated (categorized as percent normal germinated and percent abnormal germinated for AOSA-recommended temperature regimes), percent viable hard (dormant), percent dead, and percent viable firm swollen seed.

Results of dormancy and germination testing are presented in Table VII-3. No differences at $P \leq 0.05$ were detected between LY038 and LY038(-) in any of the germination characteristics evaluated. No viable hard seed were detected for LY038, LY038(-) or conventional reference maize at any temperature regime. These results indicate that the dormancy and germination characteristics of LY038 seeds were not altered when compared to LY038(-) seeds, supporting the conclusion that LY038 does not present an increased weed potential.

Table VII-3. Germination results for LY038, LY038(-) and reference seed

AOSA Temp. Regime (C)	Seed Substance	Normal Germinated (%)	Abnormal Germinated (%)	Viable Hard (%)	Dead (%)	Viable Firm Swollen (%)
25	LY038[1]	98.8	0	0	1.3	0
	LY038(-)	98.5	0.8	0	0.8	0
	Reference Range[2]	91-100	0-2	0-0	0-7	0-0
20/30	LY038[1]	98.5	0	0	1.5	0
	LY038(-)	99.0	0.3	0	0.8	0
	Reference Range	94-100	0-3	0-0	0-4	0-0

Non-AOSA Temp. Regime (C)	Seed Substance	Germinated (%)	Viable Hard (%)	Dead (%)	Viable Firm Swollen (%)
5	LY038[1]	2.0	0	3.5	94.5
	LY038(-)	1.3	0	4.3	94.3
	Reference Range[1]	0-10	0-0	0-11	84-100
15	LY038[1]	98.5	0	1.5	0
	LY038(-)	99.8	0	0.3	0
	Reference Range	96-100	0-0	0-4	0-1
35	LY038[1]	99.5	0	0.5	0
	LY038(-)	98.5	0	1.5	0
	Reference Range	96-100	0-0	0-4	0-0

[1] No significant differences were detected between LY038 and the control at $P \leq 0.05$.
[2] Minimum and maximum values among three reference hybrids. In cases where there were no seed in a particular seed characteristic category for any of the reference substances, the reference range is designated as "0-0".

2. Phenotypic and Ecological Interaction Assessment

Phenotypic and ecological data were collected to assess phenotypic equivalence as it relates to pest potential and familiarity. A subset of the comparative phenotypic data (e.g., certain growth, reproductive, and preharvest seed loss characteristics) can be used for an assessment of enhanced weed potential of the modified crop.

Phenotypic data were collected from 17 field trials conducted in two consecutive years (Table VII-2). Quantitative and qualitative data were collected from LY038, LY038(-) and conventional reference maize hybrids established at ten field locations in 2002 and an additional seven field locations in 2003. The trial locations provided a range of

environmental and agronomic conditions representative of major maize growing regions where commercial production of LY038 is expected. The principal investigator at each site was familiar with the growth, production, and evaluation of the maize characteristics required by this study. Agronomic practices used to prepare and maintain each field site were characteristic of each respective region.

The test and control substances for 2002 and 2003 trials were LY038 and the negative segregant, LY038(-), respectively. Four commercially available conventional maize hybrids (DK537, DKC60-15, RX708 and RX772) were also grown at each test site during both years to provide benchmark values common to maize for each measured phenotypic and ecological characteristic. Identity of LY038 and LY038(-) maize seed planted was verified by trait-specific PCR analyses. Analysis of all seed lots was as expected with exception of the LY038(-) seed lot used in 2002 trials that contained a low level (< 3.05%) of MON 810. The low level of MON 810 was deemed to have no negative effect on the quality of the study or interpretation of the results.

At all 2002 and 2003 trial sites three replicate plots of each test, control and reference substance were established in a randomized complete block design. Each plot consisted of two rows of maize spaced approximately 30 inches apart and approximately 17.5 feet in length. Planting information, soil description and cropping history for 2002 and 2003 trials are presented in Table VII-4. All plots at each site were maintained according to standard maize production practices for the respective region. At each site, soil insecticide was applied at planting to control corn rootworm larvae and an insecticide spray program was used throughout the growing season to control all above ground lepidopteran pests including European corn borer, corn earworm, and fall armyworm.

The following 14 phenotypic characteristics were evaluated during 2002 and 2003: seedling vigor, early stand count, days to 50% pollen shed, days to 50% silking, ear height, plant height, staygreen, final stand count, dropped ears, stalk lodging, root lodging, grain test weight, grain moisture and yield. In 2003 trials, one additional characteristic, white leaf phenotype, was evaluated based on the unanticipated observation of this phenotype at the V1 – V2 growth stage at six of ten trial sites in 2002. The timing of evaluations and a description of the measurements taken are presented in Table VII-1. In addition, each plot was evaluated qualitatively for differential responses of LY038 compared to LY038(-) for observed ecological interactions with insect, disease, and abiotic stressors (e.g., drought) that may have occurred during the growing season. The observed stressors were not induced artificially; therefore, they were not the same at each field site.

Phenotypic data were analyzed using Statistical Analysis Software (SAS). Analysis of variance methods were used to test for differences between LY038 and LY038(-) for each of the characteristics evaluated (Table VII-1). Comparisons were conducted within each test site and across test sites within year. Differences were considered significant at the 5% level ($P \leq 0.05$).

In addition to phenotypic data, each plot was evaluated qualitatively for differential response to observed insect, disease, and abiotic stressors (e.g., drought) that may have occurred during the growing season. The observed stressors were not induced artificially; therefore, they were not the same at each field site. The ecological evaluation was based on qualitative rating of field sites on a standardized 0 – 9 rating scale for insect pests, diseases, and abiotic stressors at specified times during the growing season appropriate for assessment of the respective stressor. A mean was calculated for each pest or stressor rating among the three replications per site and the numerical mean value was converted to a categorical value (i.e., none, slight, moderate, or severe) for reporting. These qualitative data could not be subjected to statistical analyses; therefore, their significance was assessed by the expert opinion of the principle investigator. The incidence range observed among four commercially available reference maize hybrids provides qualitative assessment data common to maize for each characteristic.

Results for the 2002 and 2003 phenotypic and ecological evaluations of LY038 are summarized in the following two sections. Results for individual sites within year are presented in Appendix 4.

Table VII-4. Field site planting information, soil description, and cropping history for 2002 and 2003 field trials

Field site	Planting date	Planting rate (seeds/plot)	Planting Depth (in)	Plot Size (ft)	Reps	Soil series description; organic matter (%); and pH	2 years prior crop	Prior year crop
2002 phenotypic trials								
BE	05/21/02	70	1.0	5 x 17.5	3	Putnam silt loam; 1.8%; 6.9	Soybean	Soybean
CL	06/06/02	70	1.0	5 x 17.5	3	Putnam silt loam; 1.0%; 6.1	Soybean	Maize
CR	05/22/02	70	1.5	5 x 17.5	3	Pike silt loam; 1.8%; 6.5	Alfalfa	Alfalfa
MN	05/20/02	70	2.0	5 x 17.5	3	Sable silty clay loam; 3.8%; 6.5	Maize	Soybean
NB	05/21/02	70	1.5	5 x 17.5	3	Crosby/Brookston loam; 2.6%; 6.5	Wheat	Soybean
RL	05/31/02	70	1.5	5 x 17.5	3	Taintor silty clay loam; 4.0%; 7.0	Soybean	Soybean
VH	05/24/02	70	2.0	5 x 17.5	3	Muscatine silty clay loam; 3.4%; 6.2	Maize	Soybean
WC	05/18/02	70	1.8	5 x 17.5	3	Webster silty clay loam; 4.7%; 7.8	Maize/Soybean	Oats
WY	05/29/02	70	1.5	5 x 17.5	3	Harpster silty clay loam; 5.2%; 6.0	Maize	Maize
YK	05/16/02	70	1.5	5 x 17.5	3	Hastings silt loam; 3.0%; 5.8	Soybean	Maize
2003 phenotypic trials								
BE	05/24/03	70	1.0	5 x 17.5	3	Putnam silt loam; 1.8%; 6.9	Soybean	Maize
CR	05/24/03	70	1.5	5 x 17.5	3	Cisne silt loam; 1.9%; 6.5	Wheat	Soybean
MN	05/14/03	70	2.0	5 x 17.5	3	Sable silty clay loam; 3.8%; 6.8	Maize	Soybean
NB	05/22/03	70	1.5	5 x 17.5	3	Brookston/Crosby loam; 2.6%; 6.5	Maize	Soybean
RL	05/13/03	70	1.5	5 x 17.5	3	Taintor silty clay loam; 4.0 – 4.3%; 6.8	Soybean	Soybean
VH	05/18/03	70	1.5	5 x 17.5	3	Tama silty clay loam; 3.0%; 6.9	Maize	Soybean
YK	05/17/03	70	1.5	5 x 17.5	3	Hastings silt loam; 3.6%; 6.0	Soybean	Maize

2.a. 2002 Field Trial Results

Results for individual phenotypic characteristic comparisons of LY038 to LY038(-) pooled across ten 2002 trial sites are presented in Table VII-5. Minimum and maximum values observed among the four commercially available reference maize hybrids across all ten sites are also included in Table VII-5 to provide benchmark values for maize for each measured phenotypic characteristic.

A total of 140 statistical comparisons were made between LY038 and LY038(-) (14 characteristics at ten field sites). There were no differences detected between LY038 and LY038(-) for any of the measured characteristics at three of the ten sites and only 11 differences ($P \leq 0.05$) in phenotypic characteristics of LY038 and LY038(-) were detected among the ten sites (see Appendix 4, Tables 1 – 10 for individual site data). The frequency of observed differences (11/140 = 7.9%) between LY038 and LY038(-) among the ten field sites was above the rate expected due to random experimental effects (5%). However, the detected differences between LY038 and LY038(-) in the within-site analysis were randomly distributed among the measured characteristics with no trend across sites.

Pooled analysis of phenotypic data across all ten field sites detected a difference ($P \leq 0.05$) for only one of the measured characteristics between LY038 and LY038(-) (Table VII-5). Seedling vigor rating for LY038 was lower compared to LY038(-) (7.1 vs. 7.5). In the within-site analysis, seedling vigor was significantly lower for LY038 compared to LY038(-) at one of the ten sites and was not different at the other nine sites (Appendix 4, Tables 1 through 10). This difference detected at a single site contributed largely to the difference detected across sites and was not indicative of a trend in the data. In addition, the seedling vigor rating values for LY038 and LY038(-) were within the range of values observed for the reference hybrids (4.0 - 9.0) (Table VII-5). Therefore, the small difference detected in seedling vigor across sites is likely not biologically meaningful. Furthermore, decreased seedling vigor would not contribute to increased weed potential.

Results from the within-site and across-site analyses for LY038 support the conclusion that the plant phenotype was not unintentionally altered by genetic modification. Furthermore, no increase in lodging was observed, which supports the conclusion of no increased weed potential. A small increase in the number of dropped ears was detected at the WY and YK sites. A consistent trend toward an increased number of dropped ears may indicate an increase in weed potential; however, no difference in the number of dropped ears was observed at the other eight sites.

Ecological evaluations (plant interactions with insect pest, disease, and abiotic stressors) revealed qualitative differences between LY038 and LY038(-) for anthracnose, leaf spot, seedling blight, and leaf curl incidence (Table VII-6). Although not observed in prior field releases of LY038 and LY038(-), principle investigators at six of the ten sites (BE, MN, NB, VH, WC, and YK) reported a white leaf phenotype in young maize seedlings among the test and control plots. These symptoms did not appear to persist past the V2

growth stage. Two of the 2002 trial sites (NB and VH) rated each plot for white leaf symptoms (Table VII-6). The white leaf symptoms had not been observed for LY038 plants in any previous testing. As a result of these observations, a systematic assessment of the incidence of white leaf symptoms was added to the list of phenotypic observations collected from an additional seven field trials conducted in 2003 and summarized in section VII.2.b.

Qualitative differences between LY038 and LY038(-) were of small magnitude and the incidence of each pest or stressor was within the range of incidence observed for the reference hybrids (Table VII-6). Since there were no trends in susceptibility or tolerance to the observed pests and stressors across sites, a more quantitative assessment was not warranted. Differences noted in the table are likely artifacts of the assessment method (i.e., qualitative assessment of spatially variable pests and stressors among replications) and do not necessarily indicate a biologically meaningful result with respect to its impact on weed potential of the crop. These results support the conclusion that the ecological interactions with insect pests, diseases, and abiotic stressors for LY038 were not unintentionally altered compared to the control.

Table VII-5. Phenotypic comparison of LY038 to control LY038(-) across all sites for 2002 field trials

Phenotypic characteristic	LY038		Ref range[1]	
	Test	Control	Min	Max
Seedling vigor	7.1*	7.5	4.0	9.0
Early stand count (#/plot)	65.2	66.4	53.0	73.0
Days to 50% pollen shed	63.4	63.4	53.0	67.0
Days to 50% silking	63.4	63.0	54.0	68.0
Stay green	4.1	3.9	0	9.0
Ear height (in)	33.6	33.5	22.0	51.8
Plant height (in)	69.8	69.4	37.2	104.2
Dropped ears (#/plot)	0.3	0.2	0	4.0
Stalk lodged plants (#/plot)	1.0	1.5	0	21.0
Root lodged plants (#/plot)	0.7	0.7	0	34.0
Final stand count (#/plot)	53.0	54.2	29.0	65.0
Grain moisture (%)	21.5	20.4	8.2	29.9
Test weight (lbs/bu)	56.2	55.3	46.3	60.6
Yield (bu/a)	104.1	112.9	11.2	266.1

* Indicates a statistically significant difference between a test and control hybrid at $P \leq 0.05$.
[1] Ref range = Minimum and maximum values observed among three replications of four commercially available reference maize hybrids, DK537, DKC60-15, RX708, RX772.

Table VII-6. Insect, disease, and abiotic stressor incidence comparison of LY038 to its control LY038(-) across all sites for 2002 field trials

Insect/disease/abiotic stressor	Sites[1]	LY038		Ref range[2]	
		Test	Control	Min	Max
Insect					
Black cutworm	BE, CR, MN, VH, WY	Slight	Slight	None	Slight
Corn rootworm	MN	None	None	None	None
Flea beetle	CR, CL	None	None	None	None
White grub	MN	None	None	None	None
Wireworm	CR	None	None	None	None
Disease					
Anthracnose	MN	None	Slight	None	Slight
Maize dwarf mosaic virus (MDM)	CR	None	None	None	None
Ear rot	All ten sites	Slight	Slight	None	Moderate
Fusarium	RL	None	None	None	Slight
Leaf blight	RL	None	None	None	None
Leaf rust	CR	None	None	None	None
Leaf spot	BE, CR, NB	Slight	Moderate	None	Moderate
Northern corn leaf blight	CL	Moderate	Moderate	Moderate	Moderate
Penicillium	CR, MN	None	None	None	None
Pythium	MN	None	None	None	None
Rhizoctonia	CR	None	None	None	None
Seedling blight	BE, CR	Slight	None	None	Slight
Southern corn leaf blight	CR	Slight	Slight	None	Slight
Stalk rot	All ten sites	Slight	Slight	None	Moderate
Abiotic stressor					
Chemical	CL	Severe	Severe	Moderate	Severe
Chlorosis	NB, VH	Slight	Slight	None	Severe
Cold	CR	None	None	None	None
Compaction	WY	Moderate	Moderate	Slight	Moderate
Crusting	BE, CL	Slight	Slight	None	Slight
Drought	CR, WY	Severe	Severe	Moderate	Severe
Flood	CR	None	None	None	None
Heat	BE, RL, YK	Severe	Severe	Slight	Severe
Leaf curl	VH	Slight	None	None	Slight
Poor emergence	NB	Slight	Slight	None	Slight
Wind	VH	None	None	None	Slight

[1] Sites that were rated for specific insect, disease, or abiotic stressor. Note that not all sites were rated for each pest or stressor. If more than one site is listed, the incidence corresponds to the mean numerical rating across sites.

[2] Ref range = Minimum and maximum incidence observed among three replications of four commercially available reference maize hybrids, DK537, DKC60-15, RX708, RX772 per site.

2.b. 2003 Field Trial Results

Characteristics evaluated in the 2003 trials were identical to those assessed in the ten trials conducted in 2002 (Table VII-1) with addition of the number of plants per plot exhibiting the white leaf phenotype, recorded at plant growth stage V1 – V2.

Results for individual phenotypic characteristic comparisons of LY038 to LY038(-) pooled across seven 2003 trial sites are presented in Table VII-7. Minimum and maximum values observed among the four reference maize hybrids across all seven sites are also included in Table VII-7 to provide benchmark values for maize for each measured phenotypic characteristic.

A total of 105 within-site comparisons were made between LY038 and LY038(-) (15 characteristics at seven field sites). No differences were detected between LY038 and LY038(-) at any of the sites for early stand count, days to 50% pollen shed, days to 50% silking, ear height, dropped ears, stalk lodged plants, and root lodged plants (see Appendix 4, Tables 11 – 17 for individual site data). Furthermore, no differences were detected between LY038 and LY038(-) for any of the measured characteristics at the CR site. A total of 15 differences ($P \leq 0.05$) in phenotypic characteristics within site were detected between LY038 and LY038(-). The frequency of observed differences (14.3%) was above the rate expected due to random experimental effects (5%). However, no consistent trends were observed among the seven individual sites for the detected differences.

Pooled analysis of phenotypic data across the seven 2003 sites detected differences for three of the measured characteristics between LY038 and LY038(-). Seedlings were less vigorous (7.5 vs. 8.0), plant height was greater (82.3 vs. 79.2 inches), and the number of white leaf plants was greater (2.1 vs. 0 plants/plot), for LY038 compared to LY038(-) ($P \leq 0.05$ (Table VII-7).

A trend toward slightly reduced seedling vigor was observed at several sites; however,the small magnitude of the difference detected in the pooled analysis is likely not biologically meaningful with respect to weed potential. Furthermore, a consistent trend toward reduced seedling vigor would not contribute to increased weed potential.

A consistent trend toward increased plant height may be agronomically desirable within limits but could indicate increased weed potential if the trait were transferred to a sexually compatible species. The observed increase in plant height was not manifested in other characteristics of growth, fitness, and reproduction such as the number of days to 50% pollen shed, the number of days to 50% silking, or yield. The small magnitude of the increase in plant height is likely not biologically meaningful with respect to weed potential as the observed values were all within the range of plant height for reference maize.

Based on data from the 2003 trials, the white leaf phenotype appears to be associated with the LY038 trait as it was only observed in the LY038 plants and not the control LY038(-) or reference maize plants at each of the four sites (NB, RL, VH, YK) where it was observed. The incidence of the white leaf phenotype was significantly higher for LY038 plants than for LY038(-) plants at three sites (NB, VH, YK) and follow-up observations were collected at those sites. Follow-up observations included plant height measurement and number of leaves at V4 -V6 and VT growth stages and grain yield evaluation based on comparison of ten pairs of white leaf and normal LY038 plants at two sites (NB and VH) and comparison of five plant pairs at a third site (YK). The mean percentage of white leaf plants observed per plot across the three sites with higher ($P \leq 0.05$) incidence of white leaf plants in LY038 plots than LY038(-) plots was 7.3%, or approximately five plants per plot (Table VII-8). At each site, this phenotype typically did not persist past the V2 growth stage. Mean plant height was similar for the white leaf plants compared to the paired normal plants at the V4 – V6 growth stage (21 vs. 22 in) but slightly less for the white leaf plants at the VT growth stage (73 vs. 78 in). The difference in plant height at the VT growth stage was most prominent at the YK site. The mean number of leaves for white leaf and normal plants was similar at both growth stages across sites. The mean grain yield was numerically lower for white leaf plants compared to paired normal plants and most prominent at the YK site. However, statistical comparison of whole-plot grain yield for LY038 and LY038(-) across all seven sites did not detect a significant difference (129.5 vs. 129.6 bu/a, respectively,Table VII-7).

A similar white leaf phenotype, including decreased chlorophyll content and altered vegetative development, has been observed in other plant species that accumulate high concentrations of lysine (Coruzzi and Last, 2000). Results of our investigation indicate that the growth and development of LY038 plants expressing the early white leaf phenotype were not severely affected compared to LY038 plants from the same plot that did not exhibit this phenotype. The small percentage of white plants that were observed among three of the seven sites with a significant incidence of white leaf phenotype did not result in significant changes in other growth and development characteristics on a whole-plot basis (e.g., days to 50% pollen shed, days to 50% silking, plant height, and yield) with exception of the YK site, Appendix 4, Tables 11 - 17.

With regard to plant pest potential of LY038 plants, the white leaf phenotype would not contribute to increased weed potential. Therefore, it was concluded that no further ecological risk assessment was required.

Qualitative differences observed in ecological interactions between LY038 and LY038(-) were of small magnitude and the incidence of each pest or stressor was within the range of incidence observed for the reference hybrids (Table VII-9). Since there were no trends in susceptibility or tolerance to the observed pests and stressors across sites, a more quantitative assessment was not warranted. Small qualitative differences indicated in Table VII-9 do not necessarily indicate a biologically meaningful result with respect to its

impact on weed potential of the crop. The results support the conclusion that the ecological interactions for LY038 were not unintentionally altered compared to the control.

Table VII-7. Phenotypic comparison of LY038 to its control across all sites in 2003 field trials

Phenotypic characteristic	LY038		Ref range[1]	
	Test	Control	Min	Max
Seedling vigor	7.5*	8.0	6.0	9.0
Early stand count (#/plot)	68.0	67.3	56.0	76.0
White leaf plants (#/plot)	2.1*	0	0	0
Days to 50% pollen shed	69.3	69.8	60.0	71.0
Days to 50% silking	69.6	69.7	61.0	71.0
Stay green	4.7	4.6	1.0	8.0
Ear height (in)	39.7	39.4	28.6	57.2
Plant height (in)	82.3*	79.2	73.4	103.0
Dropped ears (#/plot)	0.3	0.2	0	15.0
Stalk lodged plants (#/plot)	2.0	3.4	0	25.0
Root lodged plants (#/plot)	0.2	0.2	0	1.0
Final stand count (#/plot)	54.3	54.8	45.0	60.0
Grain moisture (%)	23.3	22.1	13.1	30.5
Test weight (lbs/bu)	55.0	54.5	44.9	61.6
Yield (bu/a)	129.5	129.6	43.9	261.4

* Indicates a statistically significant difference between the test and control at $P \leq 0.05$.

[1] Ref range = Minimum and maximum values observed among three replications of four commercially available reference maize hybrids, DK537, DKC60-15, RX708, RX772.

Table VII-8. Phenotypic data for LY038 plants expressing the white leaf phenotype in 2003 field trials

	Plant number[2]		Plant height[3]				Leaf number[4]				Yield[5]	
			V4 – V6		VT		V4 – V6		VT			
Site[1]	n	n′	White	Normal	White	Normal	White	Normal	White	Normal	White	Normal
		%	in				no				lbs	
NB	10	8.0	13	14	77	79	5	5	15	15	0.19	0.22
VH	10	7.3	29	30	78	81	6	7	13	14	0.21	0.23
YK	5	6.5	20	21	65	75	6	6	16	16	0.12	0.27
Mean		7.3	21	22	73	78	6	6	15	15	0.17	0.24

[1] Sites where LY038 plants expressing the white leaf phenotype were observed.

[2] A maximum of five LY038 (test) plants per plot expressing the white leaf phenotype were selected for evaluation. For each white leaf plant selected, an adjacent normal LY038 plant was also selected for evaluation. n = number of white leaf and normal plant pairs measured per site (maximum of 15). n′ = number of white leaf plants observed per plot as a percentage of the total number of plants per plot.

[3] Plant height for white leaf and normal plants at two different growth stages. V4 – V6 = 4 – 6 leaf growth stage, VT = tasseled growth stage.

[4] Number of leaves for white leaf and normal plants at two different growth stages.

[5] Shelled grain yield of individual ears for white leaf and normal plants at harvest.

Table VII-9. Insect, disease, and abiotic stressor incidence for LY038 compared to its control across all sites in 2003 field trials

Insect/disease/abiotic stressor	Sites[1]	LY038		Ref range[2]	
		Test	Control	Min	Max
Insect					
Armyworm	CR	None	None	None	None
Black cutworm	BE, CR, MN, NB, VH, YK	None	None	None	Slight
Corn rootworm	VH	None	None	None	None
Flea beetle	BE, CR, VH	None	None-Slight	None	Slight
White grub	MN, NB, YK	None	None	None	None
Wireworm	BE, CR, MN, NB, YK	None	None	None	None
Disease					
Brown spot	RL	None	None	None	None
Ear rot	All seven sites	None-Slight	None-Slight	None	Slight
Eye spot	BE, VH	None	None	None	None
Fusarium	BE, MN, RL	None	None	None	None
Leaf rust	MN, VH	None	None	None	None
Leaf spot	BE, CR, MN, NB, RL	None-Slight	None-Slight	None	Slight
Maize dwarf mosaic virus	BE, CR, MN, RL	None	None-Slight	None	None
Necrosis	RL, VH	None	None	None	None
Northern corn leaf blight	NB	None	None	None	None
Penicillium	CR	None	None	None	None
Pythium	MN, RL	None	None	None	None
Seedling blight	BE, CR, NB	None	None	None	Slight
Southern corn leaf blight	CR	None	None	None	None
Stalk rot	All seven sites	None-Slight	None-Moderate	None	Severe
Wheat streak mosaic virus	BE, CR, MN, NB	None	None	None	None

Table VII-9. Insect, disease, and abiotic stressor incidence for LY038 compared to its control across all sites in 2003 field trials

Insect/disease/abiotic stressor	Sites[1]	LY038		Ref range[2]	
		Test	Control	Min	Max
Abiotic stressor					
Chemical	NB, VH	None	None	None	None
Cold	CR, NB	None-Slight	None-Slight	None	Slight
Compaction – early season	BE	None-Slight	None-Slight	None	Slight
Compaction – late season	BE	None	None-Slight	None	None
Drought	CR, MN, RL, VH	None-Slight	None-Slight	None	Slight
Flood	CR, NB	None	None	None	Slight
Hail	VH	None	None	None	None
Nutrient deficiency	VH	None	None	None	None
Stunting	NB, VH	None	None	None	None
Wind – early season	BE, NB, VH	None-Slight	None	None	None
Wind – late season	BE, NB, RL, VH	None	None	None	Slight
Wrapped whorl	MN	None	None	None	Slight

[1] Sites that were rated for specific insect, disease, or abiotic stressor. Note that not all sites were rated for each pest or stressor. If more than one site is listed, the incidence rating corresponds to the minimum and maximum incidence observed across sites.

[2] Ref range = Minimum and maximum incidence observed among three replications of four commercially available reference maize hybrids, DK537, DKC60-15, RX708, RX772 per site and across sites where applicable.

3. Pollen Morphology

Pollen characteristics (morphology and viability) of LY038 were compared to those of LY038(-) to assess phenotypic equivalence for this component of reproductive development as it relates to familiarity. The test substance was maize pollen collected from LY038 plants while the control substance was maize pollen collected from LY038(-) plants. Pollen from four reference hybrids was included to provide benchmark values common to maize for each measured pollen characteristic.

Pollen from LY038, LY038(-), and reference maize plants was collected in 2003 in Jersey County, IL (USDA-APHIS notification number 03-052-17n). The plots were arranged according to a randomized complete block design with three replications, and pollen was collected from five individual maize plants per plot. The samples were stained and viewed microscopically under 100X magnification. Pollen viability was evaluated for each of the 15 pollen samples per test, control and reference substances. When exposed to the staining solution, viable pollen grains stained red (due to the presence of vital cytoplasmic content), while dead pollen grains stained light blue. Pollen morphology was evaluated for three samples per hybrid by measuring the diameter of ten viable pollen grains. Variance analysis was conducted according to a randomized complete block design comparing LY038 to LY038(-) for average pollen grain diameter and percent viable pollen. Differences detected were significant at $P \leq 0.05$.

No differences were detected between the LY038 pollen and LY038(-) pollen for average pollen diameter or percent viable pollen (Table VII-10). Micrographs of LY038 and LY038(-) pollen are presented in Figures VII-1 and VII-2. No differences in the overall morphology of the pollen samples were visually noted between LY038 pollen and LY038(-) pollen.

Table VII-10. Pollen diameter and viability

Hybrid	Average Pollen Diameter[1] (µm)	Viable Pollen[1] (%)
LY038	85.3	97.4
LY038(-)	81.3	96.5
Reference Range[2]	65.0 - 100.0	92.4 - 100.0

[1] No significant differences were detected between LY038 and the corresponding control substance at $P \leq 0.05$.
[2] Range of values observed in the four reference pollen samples. Values represent the average of x-axis and y-axis diameters for a single pollen grain.

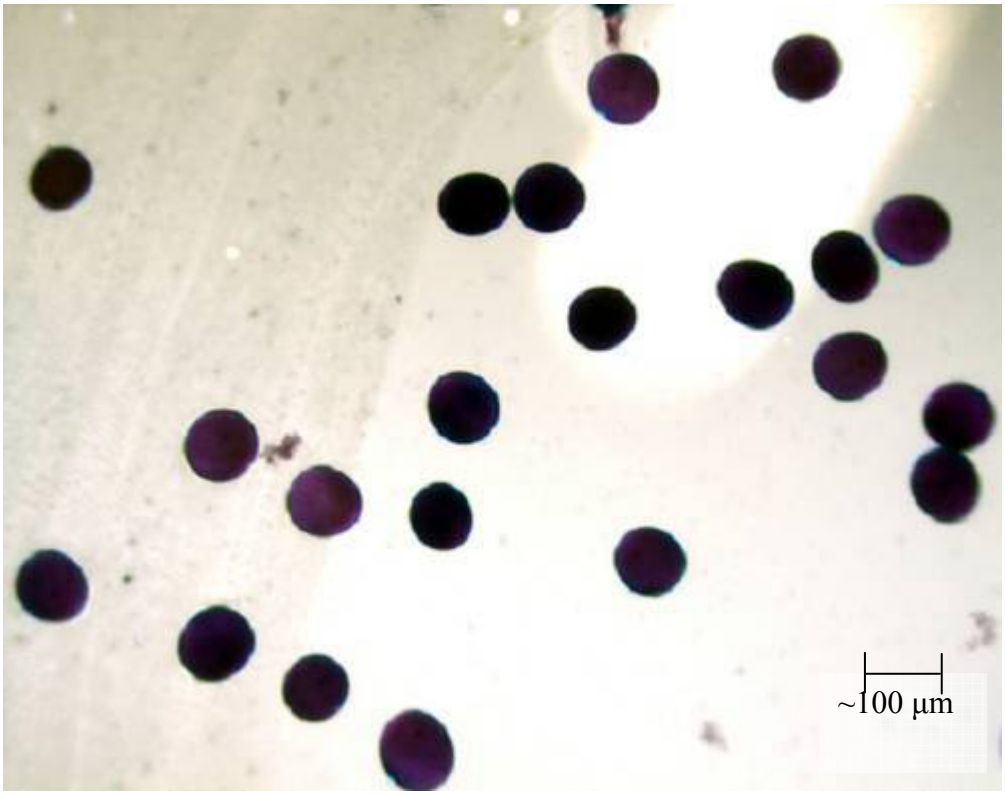


Figure VII-1. Photograph of LY038 pollen under 100X magnification.

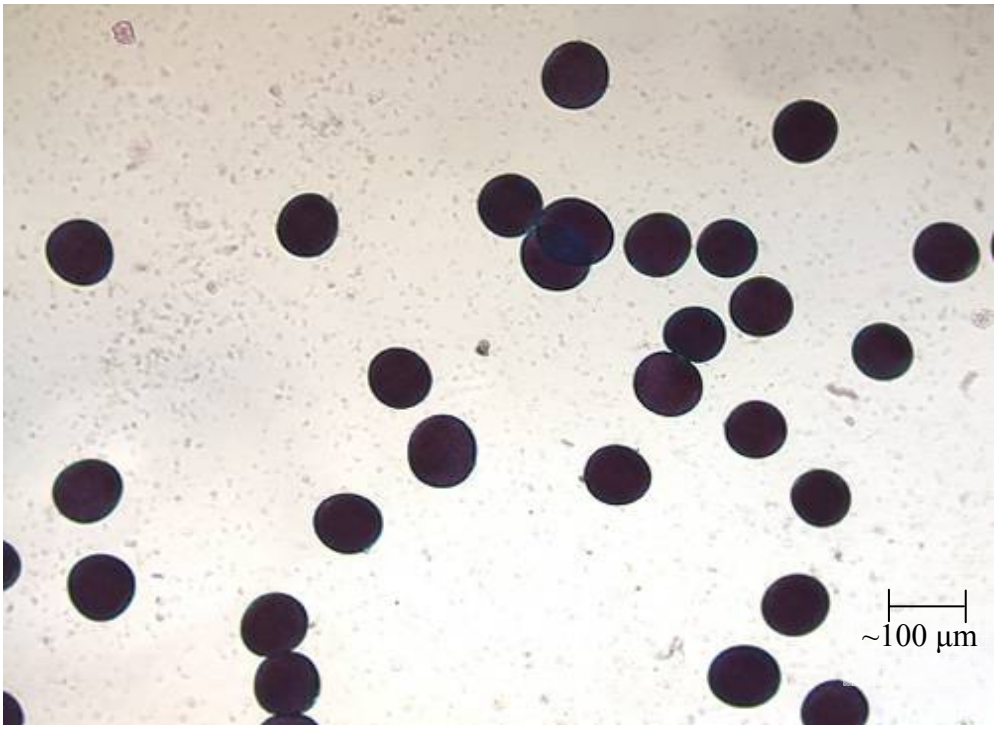


Figure VII-2. Photograph of LY038(-) pollen under 100X magnification.

These results indicate that the overall morphology and viability of pollen from LY038 is not altered when compared to its control. The lack of significant difference in evaluated characteristics of pollen from LY038 plants and LY038(-) plants supports a conclusion of phenotypic equivalence for a component of reproductive development as it relates to familiarity.

4. Conclusions of Dormancy, Phenotypic, and Pollen Morphology Assessments

Data generated from these studies represent observations that are typically recorded by plant breeders and agronomists to evaluate the qualities of maize over a broad range of environmental conditions and agronomic practices that LY038 likely would encounter. The measured characteristics provide crop biology data useful in establishing a basis to assess phenotypic equivalence and familiarity of LY038 compared to conventional maize in the context of ecological risk assessment. Detected differences were considered alone, in consideration of other observed differences, and for trends across locations. The phenotypic characteristic data showed no biologically meaningful differences between LY038 and LY038(-), or a selection of conventional reference maize hybrids, and support a conclusion of phenotypic equivalence as it relates to familiarity and a lack of increased weed potential. Likewise, assessment of the phenotypic data detected no biologically significant differences between LY038 and LY038(-) indicative of a selective advantage that would result in increased weed potential for LY038 or other plants if the trait were transferred to a sexually compatible species.

C. Crop Compositional Assessment

Compositional analysis of the crop is used in assessing plant pest risk of a biotechnology-derived crop by evaluating the significance of any differences in natural toxicants, significant nutrients and other components of the test, control and representative commercial varieties of the conventional crop. In the case of a quality trait crop such as LY038, with exception of the intended increase in grain lysine content and any associated changes in lysine-related metabolites, compositional equivalence further supports a conclusion of lack of altered weediness potential.

1. Lack of Toxicants in Maize

Maize has a long history of safety in terms of production and as a feed and food source. Toxicants are not considered a significant component of healthy maize (White and Pollack, 1995; Watson, 1987). As summarized in the following section (VII.C.2), comprehensive compositional analyses were performed on grain and forage tissues collected from LY038, LY038(-), and 18 unique commercial maize hybrids grown at five replicated field sites in the U.S. Corn Belt in 2002. Details of the compositional assessment have been submitted to the U.S. FDA as a component of the safety assessment of LY038.

2. Intended and Unintended Compositional Changes

Compositional equivalence of LY038, its negative segregant control LY038(-) and 18 unique commercial varieties of conventional maize, grain and forage was assessed using tissues collected from five replicated field sites in the U.S. (Site 1, Jefferson County, Iowa-1; Site 2, Benton County, Iowa-2; Site 3, Clinton County, Illinois-1; Site 4, Warren County, Illinois-2; and Site 5, York County, Nebraska-1) in 2002 (USDA-APHIS notification number 02-052-05n). Three replicate plots of each of the test, control and reference substances were grown at each test site, with either three or four unique reference varieties grown at each of the five test sites to provide samples for a total of 18 unique conventional varieties. The compositional assessment was conducted in accordance with the recent OECD consensus document on compositional considerations for new varieties of maize (OECD, 2002) plus analysis of free lysine and six lysine-related metabolites from the lysine biosynthetic and catabolic pathways in plants (see Appendix 5 for a description of the process and rationale for selecting lysine-related metabolites for analysis).

Compositional analyses of the forage samples included proximates (protein, fat, ash, and moisture), acid detergent fiber (ADF), neutral detergent fiber (NDF), lysine, minerals (calcium, phosphorus), and carbohydrates by calculation. Compositional analysis of the grain samples included proximates (protein, fat, ash, moisture), ADF, NDF, total dietary fiber (TDF), amino acids, free lysine, fatty acids (C8-C22), vitamins (B1, B2, B6, E, niacin, and folic acid), antinutrients (phytic acid and raffinose), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc), carbohydrates by calculation, secondary maize metabolites per OECD concensus (furfural, ferulic acid, and p-coumaric acid) and additional lysine-related metabolites (cadaverine, α-aminoadipic acid, saccharopine, homoserine, L-pipecolic acid, and 2,6-diaminopimelic acid). Lysine refers to the total lysine in the sample including protein-bound and free lysine. In all, 85 different analytical components (75 in grain, ten in forage) were assessed. Of these evaluated components, 18 analytes had more than 50% of the observations below the assay LOQ and, therefore, were excluded from the statistical analysis. In addition, values for the lysine catabolite, α-aminoadipic acid, were summarized separately, as almost all of the control and reference values were below the LOQ, precluding statistical analysis. Data for α-aminoadipic acid were summarized as the mean and range of values for those hybrids having values above the LOQ (5 ppm). Therefore, 66 components were statistically assessed (56 in grain and ten in forage) and the results for one component (α-aminoadipic acid in grain) were summarized.

Statistical analyses of the compositional data were conducted using a mixed model analysis of variance on six sets of comparisons: data were analyzed separately for each of the five trial sites as well as pooled across all five field sites (i.e., combined site). Data for each analyte in LY038 forage and grain were compared to that for the respective sample of control LY038(-). Using the data obtained for each component from the 18 unique conventional maize varieties, a 99% tolerance interval was calculated to contain, with 95% confidence, 99% of the values contained in the population of commercial maize. For any statistically significant difference between the test and comparator, the test range was then compared to the 99% tolerance interval in order to determine if the test range was within the interval and, therefore, considered part of the population of the commercial maize varieties. A summary of compositional components for which

statistically significant differences were detected between LY038 and LY038(-) is presented in Table VII-11 and a summary of grain composition pooled across the five sites (combined site) is summarized in Appendix 6. A summary of α-aminoadipic acid levels in grain is presented in Table VII-12.

Of the 396 comparisons made between LY038 and LY038(-), 94.4% or 374 comparisons, were either not different ($P > 0.05$) or they were within the calculated 99% tolerance interval for the population of conventional reference varieties. For all forage components for which a difference ($P < 0.05$) was detected between LY038 and LY038(-), the range of values determined for LY038 samples was within the calculated 99% tolerance interval for the population of conventional reference varieties. All of the statistically significant differences for forage were within the calculated 99% tolerance interval for the population of conventional reference varieties. Fourteen of the 22 statistically significant differences between LY038 and LY038(-) grain outside of the tolerance interval representing the population of commercial varieties were attributed to differences in grain lysine or free lysine content, as intended, and the associated lysine catabolite, saccharopine. The remaining eight statistically significant differences in grain were only detected at either one or two of the five sites or combined site and the test values fell within ranges reported historically or in the scientific literature with the exception of Total Dietary Fiber (TDF). Statistically significant differences for TDF were detected at only two of the five sites and for the combined site, and the difference in the range of test values from the tolerance interval of commercial reference varieties was very small (0.7% DW), with the TDF value for only one of fifteen LY038 samples falling outside the calculated tolerance interval. Therefore, the eight differences mentioned above were not considered to be biologically relevant. Levels of the lysine catabolite, α-aminoadipic acid, were numerically higher in LY038 compared to LY038(-) grain, which had α-aminoadipic acid values that were below the LOQ (5 ppm) of the assay in all but two of the 15 samples.

The grain and forage of LY038 are considered to be compositionally equivalent to conventional maize except for the intended increase in lysine and free lysine content in grain and the associated increase in lysine-related catabolites, saccharopine and α-aminoadipic acid.

These compositional data support the conclusion that no biologically meaningful phenotypic changes are associated with Lysine maize LY038. Compositional analysis of the crop is used in assessing plant pest risk of a genetically modified crop by evaluating the significance of differences in natural toxicants, significant nutrients and components of the genetically modified crop, its control and representative conventional varieties of the crop. In the case of a quality trait crop such as Lysine maize LY038, with exception of the intended increase in grain lysine content and related increases in grain content of two lysine catabolites, saccharopine and α-aminoadipic acid, compositional equivalence indicates a lack of altered weediness potential for LY038. The impact of the intentionally higher free lysine and the increase in the two lysine catabolites is discussed in Section VIII.B.2.

Table VII-11. Summary of statistically significant compositional differences between LY038 and control LY038(-)

Component	Comparator	Test Mean	Comparator Mean	Mean Diff. (Test minus Comparator) % of Comparator	Mean Diff. (Test minus Comparator) Signif. (p-Value)	Test (Range)	Reference Maize (99% T.I.[1])
Site 1 Grain							
Histidine (% Total AA)	LY038(-)	2.70	2.81	-3.86	0.010	(2.67 - 2.75)	[2.32,3.64]
Lysine (% Total AA)	LY038(-)	3.78	2.55	48.37	<0.001	(3.67 - 3.88)	[1.85,4.29]
Methionine (% Total AA)	LY038(-)	2.07	1.92	7.79	0.012	(2.03 - 2.10)	[1.47,2.46]
Proline (% Total AA)	LY038(-)	9.03	9.38	-3.73	0.024	(8.80 - 9.17)	[7.89,10.23]
16:0 Palmitic (% Total FA)	LY038(-)	10.89	11.14	-2.22	0.002	(10.84 - 10.93)	[7.42,15.14]
18:0 Stearic (% Total FA)	LY038(-)	2.36	2.19	7.94	<0.001	(2.35 - 2.38)	[1.26,2.67]
18:1 Oleic (% Total FA)	LY038(-)	31.51	30.02	4.97	0.005	(31.36 - 31.70)	[9.97,43.10]
18:2 Linoleic (% Total FA)	LY038(-)	53.47	54.91	-2.63	0.016	(53.33 - 53.55)	[42.12,74.18]
20:0 Arachidic (% Total FA)	LY038(-)	0.42	0.40	5.91	0.003	(0.42 - 0.43)	[0.31,0.52]
20:1 Eicosenoic (% Total FA)	LY038(-)	0.26	0.28	-8.55	<0.001	(0.26 - 0.27)	[0.16,0.41]

Table VII-11. Summary of statistically significant compositional differences between LY038 and control LY038(-)

Component	Comparator	Test Mean	Comparator Mean	Mean Diff. (Test minus Comparator) % of Comparator	Signif. (p-Value)	Test (Range)	Reference Maize (99% T.I.[1])
Calcium (% DW)	LY038(-)	0.0039	0.0049	-20.34	<0.001	(0.0039 - 0.0039)	[0.0013,0.0076]
Copper (mg/kg DW)	LY038(-)	1.94	1.61	20.10	0.003	(1.85 - 2.08)	[0.45,2.97]
Manganese (mg/kg DW)	LY038(-)	6.40	7.16	-10.62	0.022	(6.01 - 7.13)	[0.26,12.49]
Potassium (% DW)	LY038(-)	0.36	0.38	-5.87	0.014	(0.35 - 0.37)	[0.28,0.46]
Free Lysine (µg/g DW)[2]	LY038(-)	1514.23	38.54	3828.81	<0.001	(1470.43 - 1584.33)	[0,104.89]
Homo-serine (µg/g DW)	LY038(-)	27.13	35.02	-22.52	0.003	(24.98 - 29.32)	[0,83.82]
L-Pipecolinic Acid (µg/g DW)	LY038(-)	25.59	13.59	88.25	<0.001	(22.37 - 27.76)	[0,45.15]
Saccharopine (µg/g DW)[2]	LY038(-)	590.95	6.91	8452.58	<0.001	(532.35 - 628.77)	[0,23.00]
Folic Acid (mg/kg DW)	LY038(-)	0.57	0.52	9.31	0.047	(0.53 - 0.62)	[0.13,0.59]
Site 2 Grain							
Histidine (% Total AA)	LY038(-)	2.81	2.98	-5.78	0.024	(2.80 - 2.82)	[2.32,3.64]
Isoleucine (% Total AA)	LY038(-)	3.35	3.56	-5.94	0.012	(3.29 - 3.41)	[3.13,3.87]

Table VII-11. Summary of statistically significant compositional differences between LY038 and control LY038(-)

Component	Comparator	Test Mean	Comparator Mean	Mean Diff. (Test minus Comparator) % of Comparator	Mean Diff. (Test minus Comparator) Signif. (p-Value)	Test (Range)	Reference Maize (99% T.I.[1])
Lysine (% Total AA)	LY038(-)	4.34	2.99	45.19	0.003	(4.04 - 4.50)	[1.85,4.29]
Tryptophan (% Total AA)	LY038(-)	0.57	0.65	-13.44	0.042	(0.51 - 0.64)	[0.29,0.89]
Valine (% Total AA)	LY038(-)	4.65	4.82	-3.57	0.040	(4.58 - 4.69)	[4.15,5.51]
Calcium (% DW)	LY038(-)	0.0045	0.0058	-21.79	<0.001	(0.0043 - 0.0047)	[0.0013,0.0076]
Magnesium (% DW)	LY038(-)	0.14	0.12	19.32	0.021	(0.13 - 0.16)	[0.075,0.16]
Zinc (mg/kg DW)	LY038(-)	24.82	20.97	18.34	0.026	(22.01 - 28.53)	[8.94,39.24]
Free Lysine (µg/g DW)[2]	LY038(-)	1317.71	23.76	5446.50	<0.001	(1277.99 - 1364.44)	[0,104.89]
L-Pipecolinic Acid (µg/g DW)	LY038(-)	31.58	20.60	53.27	<0.001	(29.96 - 33.10)	[0,45.15]
Saccharopine (µg/g DW)[2]	LY038(-)	678.53	7.37	9107.07	<0.001	(663.51 - 694.70)	[0,23.00]
Neutral Detergent Fiber (% DW)	LY038(-)	17.27	10.84	59.36	0.010	(15.85 - 18.28)	[5.82,21.51]
Total Dietary Fiber (% DW)	LY038(-)	31.41	18.40	70.71	0.007	(26.08 - 39.65)	[3.77,39.08]

Table VII-11. Summary of statistically significant compositional differences between LY038 and control LY038(-)

Component	Comparator	Test Mean	Comparator Mean	Mean Diff. (Test minus Comparator) % of Comparator	Mean Diff. (Test minus Comparator) Signif. (p-Value)	Test (Range)	Reference Maize (99% T.I.[1])
Protein (% DW)	LY038(-)	11.49	10.23	12.39	0.041	(11.44 - 11.58)	[3.86,17.17]
Site 3 Grain							
Alanine (% Total AA)	LY038(-)	7.90	8.04	-1.77	0.012	(7.77 - 7.96)	[6.90,8.67]
Glutamic Acid (% Total AA)	LY038(-)	20.24	20.90	-3.18	0.002	(19.90 - 20.42)	[16.76,22.36]
Histidine (% Total AA)	LY038(-)	2.73	2.86	-4.60	0.033	(2.68 - 2.76)	[2.32,3.64]
Lysine (% Total AA)	LY038(-)	3.57	2.59	37.90	0.001	(3.08 - 3.84)	[1.85,4.29]
Phenylalanine (% Total AA)	LY038(-)	5.21	5.34	-2.51	0.017	(5.16 - 5.25)	[4.49,5.68]
18:3 Linolenic (% Total FA)	LY038(-)	0.99	0.89	11.14	0.004	(0.97 - 1.02)	[0.61,1.81]
20:1 Eicosenoic (% Total FA)	LY038(-)	0.28	0.30	-7.62	0.001	(0.27 - 0.29)	[0.16,0.41]
22:0 Behenic (% Total FA)	LY038(-)	0.18	0.16	11.68	0.033	(0.17 - 0.19)	[0.030,0.28]
Copper (mg/kg DW)	LY038(-)	2.78	1.74	60.06	0.014	(1.88 - 3.91)	[0.45,2.97]

Table VII-11. Summary of statistically significant compositional differences between LY038 and control LY038(-)

Component	Comparator	Test Mean	Comparator Mean	Mean Diff. (Test minus Comparator) % of Comparator	Signif. (p-Value)	Test (Range)	Reference Maize (99% T.I.[1])
Free Lysine (µg/g DW) [2]	LY038(-)	994.42	19.41	5023.87	<0.001	(921.86 - 1042.10)	[0,104.89]
Homo-serine (µg/g DW)	LY038(-)	7.55	2.76	173.22	<0.001	(7.22 - 8.01)	[0,83.82]
L-Pipecolinic Acid (µg/g DW)	LY038(-)	29.25	11.65	151.19	0.014	(23.13 - 35.35)	[0,45.15]
Saccharopine (µg/g DW) [2]	LY038(-)	583.16	2.76	21014.6	<0.001	(499.30 - 661.37)	[0,23.00]
p-Coumaric Acid (µg/g DW)	LY038(-)	199.84	141.42	41.31	0.015	(181.09 - 222.63)	[17.22,472.67]
Vitamin B2 (mg/kg DW)	LY038(-)	1.58	1.41	11.70	0.032	(1.47 - 1.69)	[0.77,2.16]
Moisture (% FW)	LY038(-)	7.77	9.48	-18.00	<0.001	(7.47 - 8.07)	[6.32,11.00]
Site 4 Forage							
Acid Detergent Fiber (% DW)	LY038(-)	28.12	32.80	-14.27	0.009	(27.63 - 28.63)	[17.65,36.77]
Site 4 Grain							
Lysine (% Total AA)	LY038(-)	3.87	2.88	34.03	0.005	(3.54 - 4.19)	[1.85,4.29]
16:0 Palmitic (% Total FA)	LY038(-)	10.65	10.91	-2.43	0.008	(10.58 - 10.74)	[7.42,15.14]

Table VII-11. Summary of statistically significant compositional differences between LY038 and control LY038(-)

Component	Comparator	Test Mean	Comparator Mean	Mean Diff. (Test minus Comparator) % of Comparator	Signif. (p-Value)	Test (Range)	Reference Maize (99% T.I.[1])
18:1 Oleic (% Total FA)	LY038(-)	32.45	31.05	4.50	0.002	(31.71 - 33.00)	[9.97,43.10]
18:2 Linoleic (% Total FA)	LY038(-)	52.88	54.04	-2.16	0.014	(52.37 - 53.51)	[42.12,74.18]
20:1 Eicosenoic (% Total FA)	LY038(-)	0.27	0.30	-9.25	0.003	(0.27 - 0.27)	[0.16,0.41]
Calcium (% DW)	LY038(-)	0.0047	0.0062	-25.01	<0.001	(0.0043 - 0.0049)	[0.0013,0.0076]
Manganese (mg/kg DW)	LY038(-)	5.43	6.56	-17.22	0.038	(5.16 - 5.78)	[0.26,12.49]
Free Lysine (µg/g DW)[2]	LY038(-)	1349.11	23.50	5639.73	<0.001	(1200.09 - 1496.78)	[0,104.89]
L-Pipecolinic Acid (µg/g DW)	LY038(-)	29.84	16.24	83.68	<0.001	(24.87 - 34.31)	[0,45.15]
Saccharopine (µg/g DW)[2]	LY038(-)	625.55	5.94	10434.4	<0.001	(552.04 - 702.50)	[0,23.00]
Niacin (mg/kg DW)	LY038(-)	19.34	22.92	-15.65	0.036	(18.82 - 19.97)	[5.17,37.49]
Total Fat (% DW)	LY038(-)	3.59	4.47	-19.78	0.008	(3.20 - 4.32)	[1.36,4.67]
Site 5 Grain							
Lysine (% Total AA)	LY038(-)	3.49	2.49	40.21	<0.001	(3.33 - 3.58)	[1.85,4.29]

Table VII-11. Summary of statistically significant compositional differences between LY038 and control LY038(-)

Component	Comparator	Test Mean	Comparator Mean	Mean Diff. (Test minus Comparator) % of Comparator	Mean Diff. (Test minus Comparator) Signif. (p-Value)	Test (Range)	Reference Maize (99% T.I.[1])
18:0 Stearic (% Total FA)	LY038(-)	2.29	2.18	5.23	0.017	(2.23 - 2.37)	[1.26,2.67]
18:1 Oleic (% Total FA)	LY038(-)	32.85	31.07	5.74	<0.001	(31.88 - 33.39)	[9.97,43.10]
18:2 Linoleic (% Total FA)	LY038(-)	52.37	54.17	-3.33	<0.001	(51.77 - 53.33)	[42.12,74.18]
20:0 Arachidic (% Total FA)	LY038(-)	0.44	0.42	4.95	0.013	(0.43 - 0.44)	[0.31,0.52]
20:1 Eicosenoic (% Total FA)	LY038(-)	0.27	0.29	-6.93	<0.001	(0.26 - 0.27)	[0.16,0.41]
Manganese (mg/kg DW)	LY038(-)	7.54	8.85	-14.82	0.002	(7.32 - 7.81)	[0.26,12.49]
Phosphorus (% DW)	LY038(-)	0.34	0.38	-9.52	0.039	(0.31 - 0.36)	[0.21,0.44]
Potassium (% DW)	LY038(-)	0.33	0.37	-12.61	0.012	(0.29 - 0.35)	[0.28,0.46]
Free Lysine (µg/g DW)[2]	LY038(-)	1580.20	24.75	6284.14	<0.001	(1502.10 - 1696.61)	[0,104.89]
L-Pipecolinic Acid (µg/g DW)	LY038(-)	27.34	12.70	115.34	<0.001	(26.39 - 29.05)	[0,45.15]
Saccharopine (µg/g DW)[2]	LY038(-)	773.28	6.40	11979.0	<0.001	(730.73 - 818.42)	[0,23.00]

Table VII-11. Summary of statistically significant compositional differences between LY038 and control LY038(-)

Component	Comparator	Test Mean	Comparator Mean	Mean Diff. (Test minus Comparator) % of Comparator	Mean Diff. (Test minus Comparator) Signif. (p-Value)	Test (Range)	Reference Maize (99% T.I.[1])
p-Coumaric Acid (µg/g DW)	LY038(-)	165.44	137.01	20.75	0.022	(155.25 - 173.18)	[17.22,472.67]
Folic Acid (mg/kg DW)	LY038(-)	0.54	0.39	38.81	0.014	(0.43 - 0.76)	[0.13,0.59]
Acid Detergent Fiber (% DW)	LY038(-)	7.42	5.39	37.52	0.037	(6.85 - 8.44)	[2.64,10.00]
Neutral Detergent Fiber (% DW)	LY038(-)	13.73	10.30	33.25	0.004	(12.18 - 15.86)	[5.82,21.51]
Total Dietary Fiber (% DW)	LY038(-)	23.22	17.78	30.59	0.040	(18.32 - 26.40)	[3.77,39.08]
Combined Sites Forage							
Phosphorus (% DW)	LY038(-)	0.20	0.22	-8.35	0.012	(0.13 - 0.27)	[0.10,0.30]
Combined Sites Grain							
Glutamic Acid (% Total AA)	LY038(-)	19.98	20.35	-1.83	0.002	(19.14 - 20.55)	[16.76,22.36]
Histidine (% Total AA)	LY038(-)	2.76	2.88	-4.12	<0.001	(2.63 - 2.89)	[2.32,3.64]
Isoleucine (% Total AA)	LY038(-)	3.41	3.52	-3.15	0.014	(3.21 - 3.54)	[3.13,3.87]
Lysine (% Total AA)	LY038(-)	3.81	2.70	41.09	<0.001	(3.08 - 4.50)	[1.85,4.29]

Table VII-11. Summary of statistically significant compositional differences between LY038 and control LY038(-)

Component	Comparator	Test Mean	Comparator Mean	Mean Diff. (Test minus Comparator) % of Comparator	Mean Diff. (Test minus Comparator) Signif. (p-Value)	Test (Range)	Reference Maize (99% T.I.[1])
Phenylalanine (% Total AA)	LY038(-)	5.14	5.22	-1.55	0.009	(4.97 - 5.25)	[4.49,5.68]
18:1 Oleic (% Total FA)	LY038(-)	31.81	30.59	4.00	<0.001	(30.62 - 33.39)	[9.97,43.10]
18:2 Linoleic (% Total FA)	LY038(-)	53.24	54.48	-2.27	<0.001	(51.77 - 54.41)	[42.12,74.18]
18:3 Linolenic (% Total FA)	LY038(-)	0.96	0.91	5.21	0.003	(0.89 - 1.02)	[0.61,1.81]
20:0 Arachidic (% Total FA)	LY038(-)	0.44	0.42	3.21	0.005	(0.42 - 0.48)	[0.31,0.52]
20:1 Eicosenoic (% Total FA)	LY038(-)	0.27	0.29	-7.12	<0.001	(0.26 - 0.29)	[0.16,0.41]
Calcium (% DW)	LY038(-)	0.0046	0.0054	-15.15	0.001	(0.0039 - 0.0059)	[0.0013,0.0076]
Copper (mg/kg DW)	LY038(-)	2.20	1.78	23.11	0.018	(1.85 - 3.91)	[0.45,2.97]
Manganese (mg/kg DW)	LY038(-)	6.98	7.72	-9.49	0.001	(5.16 - 9.30)	[0.26,12.49]
Zinc (mg/kg DW)	LY038(-)	26.19	24.27	7.92	0.002	(22.01 - 31.22)	[8.94,39.24]
Free Lysine (µg/g DW)[2]	LY038(-)	1351.13	25.99	5098.13	<0.001	(921.86 - 1696.61)	[0,104.89]

Table VII-11. Summary of statistically significant compositional differences between LY038 and control LY038(-)

Component	Comparator	Test Mean	Comparator Mean	Mean Diff. (Test minus Comparator) % of Comparator	Signif. (p-Value)	Test (Range)	Reference Maize (99% T.I.[1])
L-Pipecolinic Acid (µg/g DW)	LY038(-)	28.72	14.96	92.02	<0.001	(22.37 - 35.35)	[0,45.15]
Saccharopine (µg/g DW) [2]	LY038(-)	650.29	5.88	10966.5	<0.001	(499.30 - 818.42)	[0,23.00]
Folic Acid (mg/kg DW)	LY038(-)	0.47	0.40	17.39	0.006	(0.35 - 0.76)	[0.13,0.59]
Vitamin E (mg/kg DW)	LY038(-)	9.04	10.63	-15.01	0.025	(6.35 - 12.25)	[0.26,24.84]
Neutral Detergent Fiber (% DW)	LY038(-)	12.56	10.19	23.21	0.025	(8.01 - 18.28)	[5.82,21.51]
Total Dietary Fiber (% DW)	LY038(-)	20.77	15.99	29.87	0.042	(11.90 - 39.65)	[3.77,39.08]
Protein (% DW)	LY038(-)	12.90	12.12	6.42	0.002	(11.44 - 14.48)	[3.86,17.17]
Total Fat (% DW)	LY038(-)	3.86	4.42	-12.66	<0.001	(3.00 - 4.72)	[1.36,4.67]

[1] T.I. = tolerance interval specified to contain with 95% confidence, 99% of the population of conventional maize, negative limits set to zero
[2] These analytes have large mean differences (as a % of comparator) due to the intended changes in the lysine metabolic pathway.

Table VII-12: Summary of α-aminoadipic acid levels (µg/g DW) in maize grain from LY038, control LY038(-), and reference lines			
Site	**LY038 Mean (µg/g DW) (Range)**	**LY038(-)[1] Mean (µg/g DW) (Range)**	**Reference Lines [1] Mean (µg/g DW) (Range)**
1	82.34	6.33	8.76
	(78.58 - 89.32)	(6.19 - 6.46)	(5.59 - 13.45)
2	39.65	--	--
	(36.59 - 42.41)	(-- - --)	(-- - --)
3	50.66	--	--
	(46.56 - 54.68)	(-- - --)	(-- - --)
4	59.93	--	8.59
	(44.62 - 67.74)	(-- - --)	(7.83 - 9.36)
5	50.36	--	--
	(48.27 - 51.79)	(-- - --)	(-- - --)
Combined Site	56.59	6.33	8.73
	(36.59 - 89.32)	(6.19 - 6.46)	(5.59 - 13.45)
[1] "--" indicates values below the LOQ			

D. USDA-APHIS Compliance Monitoring During Field Testing of LY038

Field trials of LY038 have been conducted in the U.S. since 2000. These field trials were established for a variety of purposes, including yield testing, efficacy evaluation (increased grain lysine content), genotype evaluation, etc. The field designs for these trials varied, with some field trials being replicated at multiple sites, while other trials were nonreplicated single sites. A listing of field trials conducted under USDA-APHIS notifications between 2000 and 2004, including identification of trials for which a field test report has been submitted to APHIS, is presented in Appendix 7. Observations collected from these trials over five growing seasons provide confirmatory information to the quantitative agronomic characterization data provided in Sections VII.B of this petition.

The broad geographic distribution of the LY038 test sites in the U.S. has exposed the test, control and reference materials to a wide range of diseases and insects. The results of the disease and pest susceptibility observations were provided in the final reports submitted to USDA-APHIS at the conclusion of the notification period for each field trial listed in Appendix 7. The results from these observations consistently showed no meaningful differences in the disease and insect susceptibility between LY038, and its control, LY038(-). While occasional differences were noted at some field sites, there were no concurrent trends of differences across field sites or years, which indicates the few observed differences were likely due to random experimental variation.

These observational data corroborate the conclusion of no enhanced pest or weediness potential of LY038 compared to its control, as previously discussed in this section (Section VII).

VIII. Environmental Consequences of Introduction

A. Agronomic Practices

1. Field Maize Production

Field maize (*Zea mays*) is the third most planted field crop globally after wheat and rice. In 2003, maize was planted on more acres in the United States than any other field crop. The value of the maize crop reached $23.3 billion in the United States in 2003 exceeding soybeans and wheat with a value of $17.5 and $7.8 billion, respectively (NCGA, 2004). The United States produced approximately 42% of the world maize production in 2003-2004. China follows with 19% of the maize production. The principal uses of the 2003 U.S. maize crop were feed (57%), export (19%), high-fructose corn syrup (5%) and ethanol (11%) (NCGA, 2004).

Approximately 79 million acres were planted to field maize in 2003 in the United States. The maize acreage was very similar in 2002, but was up 4% from the 75.8 million acres planted in 2001. Seventy one million acres or slightly over 90% of the total planted acres were harvested for grain in 2003. The remaining harvested maize acres are used for silage. In 2003, approximately 6.5 million acres or 9% of the total planted acres were harvested for silage.

Total U.S. maize production was approximately 10.1 billion bushels in 2003, which was a record high. Yield was also at a record high of 142.2 bushels per acre, which was up significantly from the yields recorded in 2001 and 2002 of 138.2 and 130.0 bushels per acre, respectively. The value of maize production in the United States has ranged from $16.03 to $25.15 billion in the past ten years.

Field maize is planted in almost every state in the United States. However, the majority of the maize (88% in 2003) is grown in the Midwestern region (NCGA, 2004). Yields vary considerably from state to state due to rainfall/irrigation, climatic conditions and soil productivity. Yields in the Midwestern states averaged 147 bushels/acre in 2003 compared to the Northeastern states with 119 bushels/acre, the Mid-Atlantic states with 128 bushels/acre and the Southeastern states with 127 bushels/acre. The Northwest and Southwest states grow a high percentage of the maize under irrigation resulting in higher average yields of 195 and 170 bushels/acre, respectively. The Plains states also grow a very significant percentage of maize under irrigation, resulting in an average yield of 124 bushels per acre (USDA-NASS, 2003).

2. Fertilizer and Herbicide Use In Field Maize

Maize is intensively managed, particularly in the Midwest, as evidenced by the chemical usage data from the 2002 USDA-NASS Agricultural Chemical Usage Report that quantified the chemical usage in seven Midwest states (IL, IN, IA, MN, NE, OH and WI). Fertilizers are used extensively to optimize production and profitability. Nitrogen was applied on 96% of the maize acres at an average use rate of 137 lbs/acre per year. Phosphate and potassium fertilizers were applied on 79 and 68% of the maize acres, respectively. Herbicides were applied on 89% of the planted maize acres in 2002. Atrazine was the most widely used herbicide, with applications on 62% of the maize acres. Acetochlor, s-metolachlor and nicosulfuron were the next three most widely used herbicides with applications on 25%, 15% and 13% of the maize acres, respectively. Insecticides were applied on 24% of the maize acres, with tefluthrin being the most widely used insecticide with applications on 6% of the maize acres.

3. Volunteer Maize Management

Volunteer maize can be a weed in fields in which rotational crops follow maize harvested for grain. However, on the maize acreage grown for silage purposes (approximately 9%), volunteer maize is not an issue. In the warmer climates of the Southeast and Southwest, volunteer maize is rarely an issue because the volunteer maize will germinate in the fall and will be controlled by tillage or freezing temperatures prior to planting the following crop. On the maize acreage harvested for grain in the Northern regions, volunteer maize does not always occur as a weed problem in the rotational crop due to seed decomposition over winter, efficient harvest procedures, good standing hybrids and tillage prior to planting rotational crops.

The first step to management of volunteer maize in rotational crops is to minimize or reduce the potential for volunteers. The following practices should be implemented to reduce volunteer maize in rotational crops: 1) adjust harvest equipment to minimize the amount of maize grain loss in the field, 2) plant maize hybrids with reduced ear drop, 3) choose maize hybrids with superior stalk strength and reduced lodging, and 4) practice no-till production to significantly reduce the potential for volunteer growth in the rotational crop through predation, weathering, and reduced germination of seed remaining on the soil surface.

Preplant tillage or in-crop cultivation is very effective in managing volunteer maize in subsequent crops. In addition, a wide range of herbicides is available for use to control volunteers in subsequent crops.

4. Conclusions for Agronomic Practices

No changes to agronomic practices typically applied in management of conventional maize are required for LY038. Specifically no increases in pesticides and fertilizers are required as well as no changes in cultivation, planting, or harvesting. One exception is that the harvested grain will need to be identity preserved to allow capture of the increased animal feed value resulting from the increased lysine content for this grain product as compared to conventional commodity maize grain.

B. Environmental Consequences of the Crop

1. Characteristics of the Trait

Corynebacterium glutamicum is widespread in the environment. The cDHDPS protein expressed in LY038 has the same enzymatic activity as other DHDPSs that are ubiquitous in plants and microorganisms. Since DHDPSs are present in all plants and many microorganisms, they have no toxic mode of action and a history of safety to nontarget organisms and the environment; thus, the anticipated environmental consequences of the introduction of LY038 are negligible.

Maize-soybean meal based broiler diets formulated to include animal protein products and/or corn gluten meal and typical maize-soy based swine diets are characteristically deficient in lysine and require the addition of supplemental lysine for optimal animal growth and production (NRC, 1994; 1998). When added to animal diets at nutritional levels, the essential amino acid, lysine, is Generally Recognized As Safe (GRAS) by the U.S. Food and Drug Administration (21 CFR 582.5411) and may be used safely as a human food additive when provided at nutrient levels (21 CFR 172.320).

Development of LY038 provides an alternative to direct addition of supplemental lysine to poultry and swine diets by increasing the amount of lysine in the maize component of feed. Introduction of the *cordapA* gene into the maize genome produces a maize grain with higher lysine content and higher nutritional value for use as a feed ingredient for animals, primarily poultry (broilers and turkeys) and swine. Total lysine content of conventional maize, most of which is present as protein-incorporated lysine, typically ranges from 2500 to 2800 ppm on a dry weight basis. Levels of free lysine are targeted to be in the range of 1000 to 2500 ppm in LY038 grain, compared to levels of <100 ppm in conventional maize grain. Therefore, in LY038, the expected total lysine would range from 3500 to 5300 ppm.

2. Characterization of the Plant

Comparisons of phenotypic and compositional characterisitics between LY038 and LY038(-) were conducted to evaluate the phenotypic equivalence and familiarity of LY038 compared to conventional maize. Data generated from the phenotypic studies represent observations that are typically recorded by plant breeders and agronomists to evaluate the qualities of maize over a broad range of environmental conditions and agronomic practices that LY038 likely would encounter. Assessments of the phenotypic data detected no biologically meaningful differences between LY038 and LY038(-) and support conclusions of phenotypic equivalence and familiarity of LY038 compared to conventional maize.

Data generated from compositional analysis of LY038 was used in assessing its plant pest risk by comparing levels of natural toxicants, significant nutrients and other components between LY038 and LY038(-). These compositional data also support the conclusion that no biologically meaningful phenotypic changes were associated with LY038. In the case of a quality trait crop like Lysine maize, LY038, with exception of the intended increase in grain lysine content and related increases in grain content of two lysine catabolites,

saccharopine and α-aminoadipic acid, compositional equivalence indicates a lack of altered weediness potential for LY038. Considered in combination with the measured phenotypic characteristics and observational data on plant interactions with insect, disease, and abiotic stressors, the observed changes in levels of lysine and two lysine catabolites has no biologically meaningful effect on the phenotype of LY038.

3. Potential for LY038 to Become a Weed

Commercial maize varieties in the U.S. are not considered weeds and are not effective in invading established ecosystems. Maize does not possess any of the attributes commonly associated with weeds, such as long soil persistence, the ability to invade and become a dominant species in new or diverse landscapes, or the ability to compete well with native vegetation. It is recognized that in some agricultural systems, maize can volunteer in a subsequent rotational crop. However, volunteers are easily controlled through tillage or use of appropriate herbicides.

There is little probability that LY038 or maize plants resulting from the crossing of any maize variety with LY038 could become a problem weed. In the comparative studies between LY038 and LY038(-), dormancy, germination, phenotypic, and pollen morphology and viability characteristics were evaluated for changes that would impact plant pest potential and, in particular, plant weed potential. Assessment of these data detected no biologically significant differences between LY038 and LY038(-) indicative of a selective advantage that would result in increased weed potential for LY038 or other plants if the trait were transferred to a sexually compatible species. Furthermore, monitoring of field trial plots containing LY038 after harvest has not revealed differences in survivability or persistence relative to the control or conventional maize.

4. Impacts on Pest / Nonpest Organisms

Plant interactions with insect pests and diseases were evaluated as part of the plant phenotypic studies conducted under a broad range of environmental conditions. Qualitative differences observed in ecological interactions between LY038 and LY038(-) were of small magnitude, and the incidence of each pest or stressor was within the range of incidence observed for the conventional reference hybrids. These results support the conclusion that the ecological interactions for LY038 were not unintentionally altered compared to the control. The anticipated environmental consequences of the introduction of LY038 would be negligible, and there is no reason to believe that LY038 would have an adverse impact on organisms beneficial to plants or to “nontarget” organisms, including threatened or endangered organisms. In addition, DHDPS proteins are not known to be associated with feeding behavior or preference in their host organisms. A history of the safe exposure for the cDHDPS protein has been demonstrated, based on the similarity of the cDHDPS protein in LY038 to DHDPSs naturally present in feed and food (e.g., maize, rice, soy and wheat) (Section VII).

C. Consequences of Gene Flow

1. Vertical Gene Flow

1.a. Pollen

For gene flow, in the form of successful introgression, to occur via normal sexual transmission, certain conditions must exist: 1) the two parents must be sexually compatible; 2) there must be overlapping phenology; and 3) a suitable pollen vector must be present and capable of transferring pollen between the two parents.

Maize and annual teosinte (*Zea mays* subsp. *mexicana* Schrad.) are genetically compatible, wind-pollinated and, in areas of Mexico and Guatemala, freely hybridize when in close proximity to each other. Maize easily crosses with teosinte; however, teosinte is not present in the U.S. other than as occasional botanical garden specimens. These specimens would only flower at the same time as maize (due to photoperiod reaction) if they were subjected to artificial day length shortening for several weeks at a time (Wilkes, 1967). Differences in factors such as flowering time, geographical separation and development, make natural crosses in the United States speculative at best.

Outcrossing with *Tripsacum* species, another related genus, is not known to occur in the wild. Only with extreme difficulty can maize be crossed with *Tripsacum* species. Offspring created in forced crosses show varying levels of sterility (Galinat, 1988; Mangelsdorf, 1974; Russell and Hallauer, 1980). The habitat preferences of *Tripsacum*, are similar to those of teosinte, with 12 of the 16 species native to Mexico and Guatemala. *T. dactyloides* is widespread in the U.S. but crosses in nature are unknown. *T. floridanum* (Florida Gamagrass) is native to the southern tip of Florida. No cases of gene flow between maize and sexually compatible species are known to occur in the U.S.

Gene exchange between cultivated maize and biotechnology-derived maize would be similar to that which occurs between conventional maize varieties. Wind blown pollen would move among plants within the same field and among plants in nearby fields. Free flow of genes would occur in a manner similar to that which occurs in cultivated maize. The production of the cDHDPS lysine feedback-insensitive enzyme, predominantly in grain, and resultant increased grain lysine content would not be of concern due to the lack of potential to cause harm, given its demonstrated safety to humans and nontarget organisms.

1.b. Seed

The long domestication of maize has resulted in the seed being the only structure capable of perpetuating the species and, as such, requires human assistance to persist or be disseminated (Gould, 1968, Troyer, 2001, OECD, 2002). Maize plants are noninvasive in natural habitats and have lost their ability to survive in the wild (Gould, 1968). In contrast to weedy plants, maize has a pistillate inflorescence (ear) with a cob enclosed with husks. Consequently, seed dispersal of individual kernels does not typically occur. Individual kernels of maize, however, can be distributed in fields and along main avenues of travel

from the field operations during harvesting the crop and transporting the grain from the fields to storage facilities (Hallauer, 2000).

There are no asexual structures in *Zea mays*, and its seed are not considered to have dormancy characteristics. Maize seed survival depends on temperature, seed moisture, genotype, pericarp protection, and degree of seed development. Temperatures under 0°C affect germination, and this condition was identified as the main risk during seed production. Temperatures over 45°C also negatively impact seed viability (Shaw, 1988).

Although maize from the previous crop year can over-winter and germinate the following year under some environmental conditions, it cannot persist as a perennial weed. The presence of volunteer maize plants in soybean fields following a prior year maize crop is a common observation. However, volunteer plants are common in many agronomic systems and they are easily controlled. Measures are often taken to eliminate the volunteer plants with the hoe or use of selective herbicides to kill the plants in the alternate crop field, and any residual volunteer plant that produce seed usually do not persist during the following years.

2. Horizontal Gene Flow

There is no evidence of transfer of genetic material from maize to other organisms through sexual mechanisms. The occurrence of potential horizontal transfer (bacteria, pathogens, etc.) has been studied and is unlikely (Jonas et al., 2001). Those studies include different environments such as soil, water, and mammalian digestive tracts. Conclusions are that the risk of a possible transfer is irrelevant to its contribution to the environmental risk assessment of the release of biotechnology-derived maize plants (Bogosian and Kane, 1991; Prins and Zadoks, 1994; Schluter et al., 1995).

D. Conclusions on Environmental Consequences of the Crop

A thorough characterization of Lysine maize LY038 was performed including molecular, cDHDPS protein expression, phenotypic, and compositional evaluations. Assessment of the data generated from this extensive characterization supports conclusions of no increased pest potential, phenotypic equivalence, and familiarity as they relate to ecological risk assessment. There are no biologically meaningful differences between LY038 and its control with the exception of the intended increase in grain lysine content and the related increase in the lysine-related catabolites, saccharopine and α-aminoadipic acid. The phenotypic data support the conclusion that LY038 is not different from its control or conventional reference maize hybrids grown in the same field trials, with the exception of the white leaf phenotype. The white leaf phenotype observed in a small percentage of the plants was determined to be associated with the LY038 trait of increased lysine in the seed. The effects of the white leaf characteristic did not result in significant changes in other growth and development characteristics on a whole-plot basis and it would not contribute to increased pest potential. On the basis of these data, it is concluded that there is no increased pest potential of LY038 and that, other than the intentional compositional change caused by the introduced trait, the phenotype of LY038 has not been unintentionally changed.

IX. Adverse Consequences of Introduction

Monsanto Company is unaware of any information indicating that LY038 may pose a greater plant pest risk than conventional maize. 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 as an animal feed ingredient, Monsanto requests, on behalf of Renessen LLC, that LY038 be granted nonregulated status under 7 CFR Part 340.

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XI. Appendices

APPENDIX 1. Molecular Characterization Materials and Methods

APPENDIX 2. Methods and Materials of the cDHDPS Protein Expression Analysis in LY038 Tissues

APPENDIX 3. Materials and Methods: cDHDPS Protein Characterization

A. Characterization of the Physicochemical and Functional Properties of E. *coli*-Produced cDHDPS Protein

B. Characterization of the cDHDPS Protein Purified from Grain of Lysine Maize LY038 and Assessment of the Physicochemical and Functional Equivalence of the Plant-Produced cDHDPS Protein and *E. coli*–produced cDHDPS Protein

APPENDIX 4. Field Trial Individual Site Phenotypic Data Tables

A. 2002 Field Trial Individual Site Phenotypic Data Tables

B. 2003 Field Trial Individual Site Phenotypic Data Tables

APPENDIX 5. Lysine-Related Metabolite Selection

APPENDIX 6. Forage and Grain Composition Across All Sites (Combined Site)

APPENDIX 7. USDA Corn Field Trial Notifications for LY038

APPENDIX 1. Molecular Characterization Materials and Methods

The integrated DNA in Lysine maize LY038 was characterized using Southern blot and PCR methods. Genomic DNA was analyzed using Southern blot methods to determine the insert number (number of integration sites within the maize genome), the copy number (the number of copies of the integrated DNA within one locus), the integrity of the inserted *cordapA* gene cassette, and evaluate the presence or absence of plasmid backbone sequences, selectable marker sequences, and *cre* cassette sequences. Generational stability analysis was performed to determine the stability of the transgene insertion across multiple generations of LY038. Additionally, PCR analyses were performed which confirmed the organization of the elements within the insert and determined the 5' and 3' insert-to-plant junctions.

A. Test Substance

The test substance was Lysine maize LY038 grain or leaf tissue.

B. Control Substances

The control substance was negative segregant control maize LY038(-) grain or leaf tissue.

C. Reference Substances

The reference substances included the plasmid PV-ZMPQ76 that was used to produce LY038. For Southern blot analyses of maize genomic DNA, digested DNA of plasmid PV-ZMPQ76 (approximately 0.5 and 1 genome copy equivalents) was mixed with digested DNA from the control substance and separated by electrophoresis on agarose gels. Plasmid PV-ZM003 was included as a reference standard (serving as a positive hybridization control) for Southern blots that were examined with elements derived from that plasmid. As additional reference standards, the 1 kb DNA Extension Ladder from Life Technologies was used for size estimations on Southern blots and the 500 bp DNA Ladder and High DNA Mass Ladder from Invitrogen were used for size estimations for some PCR analyses.

D. Characterization of Test and Control Substances

The identities of the test and control substances were verified by chain-of-custody documentation. Event-specific PCR assays were used to confirm the identity of the test substances and verify that the control substances did not contain unintended transformation events prior to use in the study, except for the 91INH2 control substance which was tested after its first use in the study. The stability of the test and control substances was determined in each Southern analysis by observation of the digested DNA sample on an ethidium bromide stained agarose gel. The identities of test and control substances used in generational stability analyses were confirmed by the molecular fingerprint generated from the Southern blot stability analyses.

E. Genomic DNA Isolation for Southern Blot Analyses

Genomic DNA from the test and control substances was extracted from maize grain by first processing the grain to a fine powder and followed by a standardized procedure based on the CTAB DNA extraction method described by Rogers and Bendich (1985). Some of the DNA samples used to perform the Southern blot analyses to examine generational stability were subjected to an additional ethanol precipitation using approximately 1/10 volume of 3 M NaOAc (pH 5.2) and 2 volumes (relative to the starting volume of DNA solution) of 100% ethanol. The precipitated DNA was again spooled into a microcentrifuge tube containing 70% (v/v) ethanol. The DNA was precipitated in a microcentrifuge for ~7 minutes, vacuum-dried, and redissolved in TE buffer (pH 8.0). Genomic DNA samples were routinely incubated at 50-60°C prior to quantitation (typically for 1 hour). All genomic DNA was stored in a 4°C refrigerator.

F. Quantitation of Genomic DNA

Quantitation of DNA samples was performed using a Hoefer DyNA Quant 200 Fluorometer with Roche molecular size marker IX or Roche pBR322 DNA as a DNA calibration standard per a standardized procedure.

G. Restriction Enzyme Digestion of Genomic DNA

Approximately 20 µg of genomic DNA from the test substance and ~20 µg of genomic DNA from the control substance were used for restriction enzyme digestions. Overnight digests were performed at 37°C according to a standardized procedure in a total volume of 500-510 µl using 100 units of the appropriate restriction enzyme(s). After digestion, the samples were precipitated by adding 1/10 volume (50 µl) of 3 M NaOAc (pH 5.2) and 2 volumes (1 ml relative to the original digest volume) of 100% ethanol, followed by incubation in a -20°C freezer for at least 60 minutes or a -80°C freezer for at least 30 minutes. The digested DNA was precipitated at maximum speed in a microcentrifuge, washed with 70% (v/v) ethanol, vacuum dried, and redissolved in water or TE.

H. DNA Probe Preparation for Southern Blot Analyses

Probe template DNA containing sequences of either plasmid PV-ZMPQ76 or plasmid PV-ZM003 (Figures III-1a and III-1b, and Figures V-11a and V-11b of this petition) were prepared by PCR amplification. Approximately 25-27 ng of each probe template (except the NOS 3' polyadenylation sequence) were labeled with ^{32}P-dCTP (~6000 Ci/mmol) by a random priming method (RadPrime DNA Labeling System, Life Technologies). The NOS 3' polyadenylation sequence was labeled by PCR using 25-27 ng of DNA probe template in a total reaction volume of 20 µl containing the following components and final concentrations: sense and antisense primers specific to the template (0.25 µM each); 2.0 mM $MgCl_2$; 3 µM each of dATP, dGTP, and dTTP; ~100 µCi of ^{32}P-dCTP (6000 Ci/mmol); and 2.5 units of *Taq* DNA polymerase. The cycling conditions were as follows: 1 cycle at 94°C for 3 minutes; 2 or 5 cycles at 94°C for 45 seconds, 52°C for 30 seconds, 72°C for 2 minutes; and 1 cycle at 72°C for 10 minutes. All radiolabeled probes were purified using a Sephadex G-50 column (Roche).

I. Southern Blot Analyses of Genomic DNA

Samples of DNA digested with restriction enzymes were separated, based on size, using 0.8% (w/v) agarose gel electrophoresis according to a standardized procedure. A 'long run' and 'short run' were performed during this gel electrophoresis. The ~20 μg samples of digested test substance DNA were divided in half for loading, ~10 μg on the long run and ~10 μg on the short run. The long run enabled greater separation of higher molecular weight DNAs while the short run allowed smaller molecular weight DNAs to be retained on the gel. The long run samples were loaded onto the gel and typically subjected to electrophoresis for 15-18 hours at 30-35 volts. The short run samples were then loaded in adjacent lanes on the same gel and typically the gel was subjected to electrophoresis for 3-6 additional hours at 75-80 volts. Southern blot analyses (Southern, 1975) were performed according to a standardized procedure with the exception that some of the gels were incubated in depurinating solution for up to 30 minutes instead of the usual 10-15 minutes described in the standard procedure. Multiple exposures of each blot were then generated using Kodak Biomax MS-2 film in conjunction with one Kodak Biomax MS intensifying screen in a -80°C freezer.

J. PCR Analyses

Overlapping PCR products were generated that span the insert in LY038 (Products A-D, Figure V-15 of this petition). The PCR analyses were conducted using 100 ng of genomic DNA template in a 50 μl reaction volume containing a final concentration of 2 mM $MgSO_4$, 0.2 μM of each primer, 0.2 mM each dNTP, and 1 μl of DNA polymerase mix. The specific DNA polymerase mix used to amplify the products was Elongase Enzyme Mix (Invitrogen), a mixture of *Taq* and *Pyrococcus species* GB-D thermostable DNA polymerases. The amplification of Products A, B, C, and D was performed under the following cycling conditions: 94°C for 3 minutes; 38 cycles at 94°C for 30 seconds, either 55°C or 56°C for 30 seconds, 68°C for 4 minutes; 1 cycle at 68°C for 10 minutes. The PCR products from LY038 were purified using ExoSAP-IT (USB). Following the ExoSAP-IT purification procedure, aliquots of each product were separated on 1.0 % (w/v) agarose gels and visualized by ethidium bromide staining to verify the products were of the expected size and to determine approximate concentration.

APPENDIX 2. Methods and Materials of the cDHDPS Protein Expression Analysis in LY038 Tissues

The purpose of this study was to assess by ELISA methods the levels of cDHDPS protein in LY038 tissues. Tissue samples were collected from plants grown in the U.S. at five field sites in 2002.

Materials

Test Substances. The test substance for this study was LY038. Tissue samples collected from test plants were stored in a -80°C freezer throughout the study.

Control Substances. The control substance for this study was the negative segregant LY038(-). Tissue samples collected from the control plants were stored in a -80°C freezer throughout the study.

Characterization of Test and Control Substances. The identities of the test and control substances were confirmed by verifying the chain-of-custody documentation. To further verify the identities of the test and control substances, event-specific polymerase chain reaction (PCR) analyses were conducted on grain samples to determine the presence of LY038 and to confirm the absence of other transformation events that were planted at the field production sites. The identities of starting seed and grain samples harvested from the field were confirmed by PCR analysis.

Reference Substances. An *E. coli*-produced cDHDPS protein standard was used as the reference substance for analysis of cDHDPS protein levels. This standard was a working dilution of a previously characterized parental cDHDPS protein standard. The total protein concentration of the purified standard was 1.22 mg/ml by amino acid analysis. The purity was 94.5% as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Methods

Generation of Plant Samples

Summary of Field Design. Test and control tissue samples were obtained from five 2002 U.S. field production sites [Jefferson County, IA (IA1), Benton County, IA (IA2), Clinton County, IL (IL1), Warren County, IL (IL2), and York County, NE (NE)]. These field sites provided a range of environmental and agronomic conditions representative of locations where LY038 is expected to be produced commercially. At each site, three replicated plots of LY038 and LY038(-) were planted using a randomized complete block field design. Grain, forage, whole plant (V2-V4), forage root, root (V2-V4), pollen, and overseason leaf (OSL) tissues were collected from each replicated plot at all field sites. Throughout the field production, sample identity was maintained by using unique sample identifiers and proper chain-of-custody documentation. Upon collection, all tissue samples were placed in

uniquely labeled bags or containers. All tissue samples, with the exception of grain tissue which was stored and shipped at ambient temperature, were stored on dry ice and shipped frozen on dry ice to Monsanto's processing facility in St. Louis, Missouri.

Overseason Leaf. The youngest immature whorl leaf (2 - 4 inches) samples were collected from 15 plants from each of the test and control plots. The first overseason leaf (OSL 1) samples were collected at the V2 to V4 growth stage; OSL 2 samples were collected at the V6 to V7 stage; OSL 3 samples were collected at the V11 to V12 stage; and OSL 4 samples were collected at the V13 to V18 stage. The leaves corresponding to each growth stage were pooled from each plot during collection.

Whole Plant (V2-V4). Whole plant (whole aerial portion of the plant minus the roots) samples were collected from each test and control plot when the plants were at the V2 - V4 growth stage. The samples were pooled from each plot during collection.

Root (V2-V4). Root (the below-ground root mass that was cut from the plants sampled for OSWP) samples were collected from each test and control plot when the plants were at the V2 to V4 growth stage. The samples were pooled from each plot during collection.

Forage. Forage samples (whole aerial portion of the plant minus the roots) were collected from each test and control plot when the plants were at the R5 growth stage. Two plants from each plot were cut into small segments, combined, and thoroughly mixed.

Forage Root. Forage root samples (below-ground root ball from the plants sampled for forage) were collected from each test and control plot when the plants were at the R5 growth stage. The roots from two plants from each plot were cut at the soil surface, washed to remove the soil, and combined to make one sample.

Grain. Grain samples were collected from all test and control plots. The ears were dried to a moisture content of 10 to 17%. All ears were shelled and grain composited within test plot prior to shipping to the Monsanto processing facility in St. Louis, Missouri.

Pollen. Pollen samples were collected from each test and control plot at all five sites while the plants were pollinating (during the R1 growth stage). A minimum of 10 ml of pollen (approximately 5 g) was collected per plot.

Tissue Processing and Protein Extraction Methods

Processing Method. All tissue samples produced at the field sites were shipped to Monsanto's processing facility. During the processing step, dry ice was combined with the samples (except pollen) and vertical cutters or mixers were used to thoroughly grind and mix the tissues. Processed tissue samples were transferred into 50 ml tubes and stored in a -80°C freezer until shipped on dry ice to Monsanto's analytical facility in Chesterfield, Missouri. All processed tissue samples were stored in a -80°C freezer during the study.

Extraction Methods. cDHDPS protein extraction from each tissue was accomplished according to a validated standardized procedure by adding an appropriate volume of

cDHDPS Extraction Buffer (CEB) and shaking in a Harbil mixer. The CEB buffer consisted of 0.05 M $Na_2B_4O_7 \cdot 10H_2O$, 0.75 M KCl, 0.2 % (v/v) Tween-20, and 0.2 % (w/v) L-ascorbic acid, pH 10.5. Insoluble material was removed from the extracts using a Serum Filter System (Fisher Scientific, Pittsburgh, PA) or by centrifugation. The extracts were aliquoted and stored at -80°C until ELISA analyses.

When several control root tissues gave unexpected positive results, PCR and western blot analyses were conducted. The results of the PCR and western blot analyses confirmed the samples did not contain cDHDPS protein. Based on these results, it was suspected that the root samples contained a level of soil that was interfering with the ELISA. Because of this, all root extracts were centrifuged to pellet any remaining soil that was present and the clarified extract was transferred to a clean tube prior to loading in the cDHDPS ELISA. All cDHDPS ELISA data that were generated before this change were rejected.

cDHDPS ELISA Reagents and Methods

cDHDPS Antibodies. Goat polyclonal antibody specific for the cDHDPS protein was purified using Protein-G Agarose affinity chromatography. The concentration of the purified IgG was determined to be 6.3 mg/ml by spectrophotometric methods. The purified antibody was stored in a buffer containing 1 mM KH_2PO_4, 10 mM $Na_2HPO_4 \cdot 7H_2O$, 137 mM NaCl, and 2.7 mM KCl, pH 7.4 (1X PBS).

The purified antibody was coupled with biotin (Sigma, St. Louis, MO) according to the manufacturer's instructions and assigned a unique lot number. The detection reagent was NeutrAvidin (Pierce, Rockford, IL) conjugated to horseradish peroxidase (HRP).

cDHDPS ELISA Method. The validated cDHDPS ELISA was performed using an automated robotic workstation (Tecan, Research Triangle Park, NC) according to standardized laboratory procedures. Goat anti-cDHDPS antibody was diluted in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and immobilized onto 96-well microtiter plates at 2.0 μg/ml followed by incubation in a 4°C refrigerator for $\geq$ 8 h. Plates were washed in 1X PBS with 0.05% (v/v) Tween-20 (1X PBST) and blocked for 30 min at 37°C with the addition of 0.1% (w/v) non-fat dry milk in Tris-Borate buffer [100 mM Tris, 100 mM $Na_2B_4O_7 \cdot 10\ H_2O$, 10 mM $MgCl_2$, 0.05% (v/v) Tween-20, pH 7.8, with 0.2% (w/v) L-ascorbic acid]. Plates were washed as before, followed by the addition of 100 μl per well of protein standard or sample, and incubated for 1 h at 37°C. The procedure was completed after sequential incubations (1 h at 37°C each) with 100 μl per well of biotinylated goat anti-cDHDPS antibody and NeutrAvidin-HRP. Plates were developed by adding 100 μl per well of HRP substrate, 3,3′,5,5′- tetramethyl-benzidine (TMB; Kirkegaard & Perry, Gaithersburg, MD). The enzymatic reaction was terminated by the addition of 100 μl per well of 6 M H_3PO_4. Quantitation of cDHDPS protein levels was accomplished by interpolation from a cDHDPS protein standard curve that ranged from 0.05 - 1.6 ng/ml.

Moisture Analysis. All tissues were analyzed for moisture content using an IR-200 Moisture Analyzer (Denver Instrument Company, Arvada, CO) according to a standardized procedure. A homogeneous tissue-specific site pool (TSSP) was prepared by mixing approximately equal portions of the respective tissue type from each test and control plot

within each field site. These pools were prepared for all tissues analyzed in this study. Each TSSP was analyzed in triplicate. The mean percent moisture for each TSSP was calculated and used to convert the fresh weight (fwt) protein levels for the test and control substances at each site to dry weight (dwt) protein levels. The mean percent moisture for each TSSP was calculated from three moisture analyses of a given pool. A tissue-specific Dry Weight Conversion Factor (DWCF) was calculated for each site as follows:

$$DWCF = 1 - \left[(Mean \ \% \ TSSP \ \ Moisture \ ) \Big/ 100 \right]$$

The DWCF was only applied to samples with protein levels greater than the assay limit of quantitation (LOQ). All protein levels calculated on a fresh weight basis were converted into protein levels reported on a dry weight basis using the following calculation:

$$Protein\ Level\ in\ Dry\ Weight = \frac{(Protein\ Level\ in\ Fresh\ Weight)}{(DWCF)}$$

Data Reduction and Statistical Analyses. All cDHDPS ELISA plates were analyzed on a SPECTRAFluor Plus (Tecan) microplate reader using dual wavelengths. The cDHDPS protein absorbance readings were determined at a wavelength of 450 nm with a simultaneous reference reading of 620 nm that was subtracted from the 450 nm reading. Data reduction analyses were performed using Molecular Devices SOFTmax PRO version 2.4.1. Absorbance readings and protein standard concentrations were fitted with a four-parameter logistic curve fit. Following the interpolation from the standard curve, the amount of protein (ng/ml) in the tissue was reported on a μg/g fwt basis. For both proteins, this conversion utilized a sample dilution factor and tissue-to-buffer ratio. The protein values in μg/g fwt were also converted to μg/g dwt by applying the DWCF. Microsoft Excel 2000 (Version 9.0.4402 SR-1, Microsoft, Redmond, WA) was used to calculate the cDHDPS protein levels in maize tissues.

APPENDIX 3. Materials and Methods: cDHDPS Protein Characterization

Table of Contents

A. Characterization of the Physicochemical and Functional Properties of *E. coli*-Produced cDHDPS Protein

Materials

Description of the E. coli–produced cDHDPS protein. The cDHDPS protein was isolated from a 230 L fermentation of *E. coli* containing the *pET23b(+)/cordapA* expression plasmid. The protein was stored in a –80 °C freezer in a buffer solution containing 20 mM Tris-HCl, 100 mM KCl, and 10 mM pyruvate, pH 8.0.

Description of assay controls. Protein molecular weight markers (Bio-Rad broad range, Bio-Rad Precision, Hercules, CA) were used to calibrate SDS-PAGE gels and verify protein transfer to PVDF and nitrocellulose membranes. A β-lactoglobulin protein standard and PTH amino acid standards (both from Applied Biosystems, Foster City, CA) were used to verify the performance of the amino acid sequencer. A peptide mixture (Sequazyme Peptide Mass Standards kit, Applied Biosystems) and analytical BSA standard (National Institutes of Standards and Technology - NIST, Gaithersburg, MD) were used to calibrate the MALDI-TOF mass spectrometer.

Methods

Protein purification. The cDHDPS was produced in and purified from *E. coli* cells, BL21 (DE3), using a combination of cell lysis, anion exchange chromatography and hydrophobic interaction chromatography. Two batches of the cDHDPS protein were purified and combined into a final batch.

Molecular weight and purity determination – SDS-PAGE. An aliquot of the *E. coli*-produced cDHDPS protein was diluted with buffer containing 20 mM Tris-HCl, 100 mM KCl, 10 mM pyruvate, pH 8.0 to a concentration of ~0.65 mg/mL. Aliquots of this dilution were combined with 2× Laemmli (Laemmli, 1970) sample buffer [62.5 mM Tris-HCl, pH 6.8, 5% (v/v) β-mercaptoethanol, 2% (w/v) SDS, 25% (v/v) glycerol and 0.01% (w/v) Bromophenol Blue]. Markers (Bio-Rad broad range, Hercules, CA) were used

to estimate molecular weight. All samples were heated at ~87 °C for five min and applied to pre-cast tris-glycine polyacrylamide gradient (4→20%) mini-gels (Invitrogen, Carlsbad, CA). Samples of approximately 1.0, 2.1 and 3.1 μg (total protein) were loaded into three separate lanes. Electrophoresis was performed at constant voltage (150 V for 15 min followed by 200 V for 55 min), until the dye front reached the bottom of the gel. Proteins were fixed in the gels by gentle shaking with 40% (v/v) methanol and 7% (v/v) glacial acetic acid, stained with Colloidal Brilliant Blue G stain (Sigma Chemical Co., St. Louis, MO) for 2 h, briefly destained with a solution containing 10% (v/v) acetic acid and 25% (v/v) methanol and finally destained overnight with 25% (v/v) methanol.

Analysis of the gel was performed using a Bio-Rad Laboratories GS-710 densitometer with the supplied Quantity One software (version 4.3.0, Hercules, CA). Molecular weight values supplied by the manufacturer were used to estimate the molecular weight of each observed band. The optical density of all visible bands within each lane were measured. Purity was estimated as the percent optical density of the ~32 kDa band relative to all bands detected in the lane. Molecular weight and purity were reported as an average of values obtained from all three lanes containing the *E. coli*-produced cDHDPS protein.

Molecular weight determination – MALDI-TOF MS. Prior to analysis, the *E. coli*-produced cDHDPS protein and BSA reference protein (NIST, Gaithersburg, MD) were desalted using drop dialysis (Görisch, 1988). Briefly, a Millipore microdialysis disk (Bedford, MA) was floated on water, spotted with 4 μL of each protein and dialyzed for 60 min. A portion of each sample (0.3-1 μL) was spotted on an analysis plate, mixed with 0.75 μL sinapinic acid (Sigma Chemical Co., St. Louis, MO) and air-dried. Mass spectral analysis of the *E. coli*-produced cDHDPS protein was performed using an Applied Biosystems Voyager DE-Pro Biospectrometry Workstation Matrix Assisted Laser Desorption and Ionization (MALDI) Time of Flight (TOF) instrument with the supplied Data Explorer software (Applied Biosystems, version 4.0.0.0) (Foster City, CA). Samples were analyzed in the 2,000 to 100,000 Da range in linear mode using 200 laser shots per spectrum at a laser intensity setting of 3174 (a unit-less MALDI-TOF instrument specific value). Mass calibration of the instrument was performed using the desalted NIST BSA reference protein. Mass of the *E. coli*-produced cDHDPS protein was reported as an average of three separate mass spectral acquisitions. For comparison, the mass of the cDHDPS protein was calculated from the expected amino acid sequence of the protein using the GPMAW software (Applied Biosystems, version 4.23).

N-terminal sequence analysis. SDS-PAGE was used to separate proteins for N-terminal sequencing. Proteins (~7.7 μg total protein) were loaded into three separate lanes, subjected to electrophoresis and then electrotransferred to a PVDF membrane (Invitrogen) for 60 min at a constant 300 mA in buffer containing 10 mM CAPS, pH 11 and 10% (v/v) methanol. Pre-stained molecular weight markers (Bio-Rad, Hercules CA) were used to verify electrotransfer of proteins to the membrane. Protein bands were stained by briefly soaking the membrane with Coomassie stain (Bio-Rad) and visualized by brief destaining with a solution containing 10% (v/v) acetic acid and 25% (v/v) methanol, and finally destaining with 25% (v/v) methanol overnight.

The major band, with a molecular weight of approximately 31-32 kDa, and the minor band, with a molecular weight of approximately 34 kDa, observed in each sample lane were excised from the membrane. N-terminal sequence analysis was performed for 15 cycles using automated Edman degradation chemistry (Hunkapillar et al., 1983). An Applied Biosystems 494 Procise Sequencing System with 140C Microgradient and 785A Programmable Absorbance Detector and Procise Control Software (version 1.1a) were used. Chromatographic data were collected using Atlas 99 software (version 3.59a, LabSystems, Altrincham, Cheshire, England). A PTH-amino acid standard mixture (Applied Biosystems, Foster City, CA) was used to chromatographically calibrate the instrument for each analysis. This mixture served to verify system suitability criteria such as percent peak resolution and relative amino acid chromatographic retention times. A control protein (10 pmole β-lactoglobulin, Applied Biosystems) was analyzed before and after the test protein to verify that the sequencer met acceptable performance criteria for repetitive yield and sequence identity.

MALDI-TOF analysis. MALDI-TOF mass spectrometry was used to investigate the identity of the *E. coli*-produced cDHDPS protein.

> ***SDS-PAGE separation of proteins.*** An aliquot of the *E. coli*–produced cDHDPS protein was subjected to electrophoresis on an SDS-polyacrylamide gel. Protein markers (Precision broad range, Bio-Rad, Hercules, CA) were used to estimate molecular weight. All samples were heated at approximately 100 °C for 5 min and then applied to a 4→20% pre-cast polyacrylamide gradient mini-gels. Approximately 7.7 μg of protein was loaded into each of three separate lanes. Electrophoresis was performed at constant voltage (150 V for 15 min followed by 200 V for 60 min), until the dye front reached the bottom of the gel. Proteins were stained by gentle shaking with Colloidal Brilliant Blue G stain (Sigma Chemical Co., St. Louis, MO) for 2 h, briefly destained with a solution containing 10% (v/v) acetic acid and 25% (v/v) methanol and finally destained with 25% (v/v) methanol.
>
> ***In-gel protein digestion.*** Protein bands with an apparent molecular mass of ~31-32 kDa were excised, destained, reduced, alkylated and subjected to an in-gel trypsin (Promega, Madison, WI) digest (Williams et al., 1997). Briefly, each gel band was destained by incubation in 200 μL of destaining buffer [40% (v/v) methanol and 10% (v/v) glacial acetic acid] three times, for 30 min each. Following destaining, gel fragments were incubated for 30 min in 100 μL buffer containing 100 mM ammonium bicarbonate. Proteins were reduced in 100 μL solution of 100 mM ammonium bicarbonate containing 10 mM dithiothreitol for 2 h at 37 °C. Proteins were alkylated by the addition of 50 μL of buffer containing 100 mM ammonium bicarbonate and 200 mM iodoacetic acid. The alkylation reaction was allowed to proceed at room temperature for 20 min in the dark. Gel fragments were incubated in 100 μL buffer containing 100 mM ammonium bicarbonate for 30 min and stored overnight at 2 to 8 ºC. The following day, 100 μL acetonitrile was added (50% (v/v) final concentration) and the gel fragments were incubated for 30 min. The ammonium bicarbonate/acetonitrile incubations were repeated two additional times to remove reducing and alkylating reagents from the gel, but the concentration of ammonium bicarbonate was reduced to 25 mM. The gel bands were dried in a SpeedVac concentrator (Savant, Holbrook, NY), rehydrated with 40 μL 25 mM

ammonium bicarbonate solution containing 33 μg/mL trypsin and incubated for 16 h at 37 °C. Digested peptides were extracted three times for one h each at room temperature with 50 μL 70% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA). Extraction supernatants from each sample were then combined in a single tube, dried in a SpeedVac concentrator, stored frozen and reconstituted in a final volume of 8 μL of 0.1% (v/v) TFA.

Sample preparation. A portion of the digested sample was desalted (Bagshaw et al., 2000) using Millipore (Bedford, MA) ZipTip$_{C18}$ pipette tips. Samples (5 μL) were applied to a ZipTip and washed with 5 μL of Wash 1 [0.1% (v/v) TFA], 5 μL of Wash 2 [20% (v/v) acetonitrile containing 0.1% (v/v) TFA], 5 μL of Wash 3 [50% (v/v) acetonitrile containing 0.1% (v/v) TFA] and finally with 5 μL of Wash 4 [90% (v/v) acetonitrile containing 0.1% (v/v) TFA].

MALDI-TOF instrumentation and mass analysis. Mass calibration of the instrument was performed using an external peptide mixture from a Sequazyme Peptide Mass Standards kit (Applied Biosystems). Samples (0.2-0.3 μL) from each desalting step were co-crystallized with 0.75 μL α-cyano-4-hydroxy cinnamic acid (Ciphergen Biosystems, Palo Alto, CA) on the analysis plate. Samples were analyzed in the 500 to 5000 Da range in reflector mode using 100 laser shots per spectrum at a laser intensity setting of 2525 (a unit-less MALDI-TOF instrument specific value). Protonated (MH+) peptide masses were observed monoisotopic in reflector mode (Aebersold, 1993; Billeci and Stults, 1993). GPMAW software (Applied Biosystems, version 4.23) was used to generate a theoretical trypsin digest of the expected protein sequence. Masses were calculated for each theoretical peptide and compared to the raw mass data. Experimental masses (MH+) were assigned to peaks when three (or more) isotopically resolved ion peaks were observed in the raw mass data. Peaks were ignored if there were less than three isotopically resolved peaks in the spectra, when peak heights were less than approximately twice the baseline noise or when a mass could not be assigned because of overlap with a stronger signal ±2 Da from the mass analyzed. Known autocatalytic fragments from trypsin were ignored.

Functional activity assay. Prior to analysis, a sample of the *E. coli*-produced cDHDPS protein was diluted to approximately 0.01 mg/mL in buffer containing 20 mM Tris-HCl, 100 mM KCl, and 10 mM pyruvate, pH 8.0. Addition of glycerol to a final concentration of 25% (v/v) resulted in a final buffer composition of 15.4 mM Tris-HCl, pH 8.0, 76.9 mM KCl, 7.7 mM pyruvate and 25% (v/v) glycerol. Aliquots of diluted protein were stored at -80 ^{o}C. The functional activity assay utilizes a coupled enzyme system and monitors the change in absorbance at 340 nm, which is associated with the oxidation of β-nicotinamide adenine dinucleotide phosphate (NADPH) by dihydrodipicolinate reductase (DapB). The amount of NADPH oxidized during the reaction is quantitated based on comparison with a NADPH standard curve. Briefly, reaction mixtures contained 100 mM Tris-HCl, 5 mM pyruvate, 0.7 mM NADPH, 6.25 μg of DapB protein, 2.8 mM L-ASA and 0.01 to 0.1 μg of cDHDPS. The cDHDPS-dependent oxidation of NADPH was monitored ($A_{340\ nm}$) for 30 min at 20 sec intervals at room temperature using a PowerWave X_i (Bio-Tek) microplate reader. The specific activity of cDHDPS enzyme was calculated relative to a NADPH

standard curve. For cDHDPS, one unit (U) of enzyme activity was defined as the amount of NADPH oxidized per minute. Specific activity of cDHDPS was defined as the amount of NADPH oxidized per minute of reaction time by 1 mg of cDHDPS protein at room temperature.

Immunoblot analysis. An aliquot of the *E. coli*–produced cDHDPS protein was diluted first with Milli Q water to a concentration of ~0.5 mg/mL and then further diluted with 1X sample loading buffer to a concentration of ~0.01 mg/mL. All samples were heated at ~96 °C for five min and applied to pre-cast tris-glycine polyacrylamide gradient (4→20%) mini-gels (Invitrogen, Carlsbad, CA). Samples of approximately 0.5, 1, 2.5 and 5 ng (total protein) were loaded into separate lanes. Electrophoresis was performed at constant voltage (150 V for 15 min followed by 200 V for 50 min), until the dye front reached the bottom of the gel. Proteins separated by the electrophoresis were electrotransferred to the nitrocellulose membrane (Invitrogen) for 60 min at a constant 300 mA. Pre-stained molecular weight markers (Bio-Rad, Hercules CA) were used to verify electrotransfer of proteins to the membrane. The membrane was blocked by incubation in 5% (w/v) non-fat dry milk (NFDM) in PBST for 60 min. Goat anti–cDHDPS serum was used to probe the membrane for 1 h at a dilution of 1:4,000 in 1% (w/v) NFDM in PBST. Excess serum was removed by three 5 min washes with PBST. The membrane was incubated with HRP–conjugated anti-goat IgG (Sigma) at a dilution of 1:10,000 in 1% (w/v) NFDM in PBST for 45 min, and again washed (three 5 min washes). All incubations were performed at room temperature. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) detection system according to the manufacturer's instructions (Amersham Pharmacia) and exposed (5 and 30 sec and one and two min) to Hyperfilm ECL high performance chemiluminescence film (Amersham Pharmacia). Films were developed using a Konica SRX101A automated film processor (Tokyo, Japan).

Protein concentration. Total protein concentration was estimated by amino acid analysis. Aliquots of the *E. coli*–produced cDHDPS protein were analyzed using a Hitachi L-8800 Amino Acid Analyzer with AAA System Manager Software. Test samples, NIST BSA (used as a system suitability standard) and NIST amino acid calibration control standard (Gaithersburg, MD) were spiked with an internal reference (norvaline, Sigma Chemical Co.) and dried in a Savant SpeedVac (Holbrook, NY). Vapor phase acid hydrolysis [6 N HCl containing 1% (v/v) phenol] was performed at ~150 °C for approximately 90 min. Cooled samples were again evaporated, reconstituted in protein hydrolyzate buffer PH-1 (Hitachi Instruments) and loaded onto the instrument. Amino acids were detected using post-column ninhydrin derivatization. Protein sample was analyzed in triplicate. Protein concentration was determined from acceptable runs and averaged.

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B. Characterization of the cDHDPS Protein Purified from Grain of Lysine Maize LY038 and Assessment of the Physicochemical and Functional Equivalence of the Plant-Produced cDHDPS Protein and *E. coli*–Produced cDHDPS Protein

Materials

Description of plant-produced cDHDPS protein. The cDHDPS protein was purified from grain of Lysine maize LY038. The isolated protein was stored in a −80 °C freezer in a buffer containing 20 mM Tris-HCl, 100 mM KCl and 10 mM pyruvate, pH 8.0. The identity of LY038 grain was confirmed using event-specific PCR.

Description of E. coli-produced cDHDPS reference standard protein. Previously characterized *E. coli*-produced cDHDPS protein was used as a reference standard to establish equivalence for the molecular weight and functional activity assay, as a reference and a negative control in glycosylation analysis, and as a reference and a positive control in western blot analysis. This reference standard was stored in a –80°C freezer in a buffer solution containing 20 mM Tris-HCl, 100 mM KCl and 10 mM pyruvate, pH 8.0 at 9.9 mg/ml total protein.

Description of assay controls. Protein molecular weight markers (Bio-Rad broad range, Bio-Rad Precision, Hercules, CA) were used to calibrate SDS-PAGE gels and verify protein transfer to PVDF and nitrocellulose membranes. Transferrin protein was used as a positive control in the glycosylation analysis. A β-lactoglobulin protein standard and PTH amino acid standards (both from Applied Biosystems, Foster City, CA) were used to verify the performance of the amino acid sequencer. A peptide mixture (Sequazyme Peptide Mass Standards kit, Applied Biosystems) and analytical BSA standard (National Institutes of Standards and Technology - NIST, Gaithersburg, MD) were used to calibrate the MALDI-TOF mass spectrometer.

Methods

Protein purification. The plant-produced cDHDPS protein was purified from LY038 using a combination of cell extraction, anion exchange chromatography and hydrophobic interaction chromatography. The purification procedure preceded the characterization of the cDHDPS protein and assessment of the physicochemical and functional equivalence of the plant-produced and *E. coli*–produced cDHDPS proteins. Briefly, the grain powder was defatted and soluble proteins were extracted with a buffer containing 20 mM Tris-HCl, 100 mM KCl and 10 mM pyruvate, pH 8.0. The extracted proteins were concentrated by ammonium sulfate precipitation, desalted and applied to DEAE Sepharose (anion-exchange chromatography). Proteins were eluted with a potassium chloride (KCl) gradient from to 100 to 950 mM. The cDHDPS-containing fractions were pooled, brought to 0.5 M ammonium sulphate and applied to a hydrophobic interaction column of phenyl sepharose. Proteins bound to the column were eluted with a linear gradient from 0.5 M to 0 M ammonium sulfate. Fractions containing the cDHDPS protein were pooled, concentrated and desalted. The cDHDPS protein was further purified on a high-resolution anion

exchange Mono Q column. The purified cDHDPS protein solution was concentrated, aliquoted and assigned a unique lot number.

N-terminal sequence analysis. SDS-PAGE was used to separate proteins for N-terminal sequencing. Proteins (~5.2 μg total protein) were loaded into three separate lanes, subjected to electrophoresis and then electrotransferred to a PVDF membrane (Invitrogen) for 60 min at a constant 300 mA in buffer containing 10 mM CAPS, pH 11 and 10% (v/v) methanol. Pre-stained molecular weight markers (Bio-Rad, Hercules CA) were used to verify electrotransfer of proteins to the membrane. Protein bands were stained by briefly soaking the membrane with Coomassie stain (Bio-Rad) and visualized by destaining with a solution containing 25% (v/v) methanol overnight.

The protein band with a molecular weight of approximately 32 kDa observed in each sample lane was excised from the membrane. N-terminal sequence analysis was performed for 15 cycles using automated Edman degradation chemistry (Hunkapillar et al., 1983). An Applied Biosystems 494 Procise Sequencing System with 140C Microgradient and 785A Programmable Absorbance Detector and Procise Control Software (version 1.1a) were used. Chromatographic data were collected using Atlas 99 software (version 3.59a, LabSystems, Altrincham, Cheshire, England). A PTH-amino acid standard mixture (Applied Biosystems, Foster City, CA) was used to chromatographically calibrate the instrument for each analysis. This mixture served to verify system suitability criteria such as percent peak resolution and relative amino acid chromatographic retention times. A control protein (10 pmole β-lactoglobulin, Applied Biosystems) was analyzed before and after the test protein to verify that the sequencer met acceptable performance criteria for repetitive yield and sequence identity.

MALDI-TOF analysis. MALDI-TOF mass spectrometry was used to investigate the identity of the plant-produced cDHDPS protein.

> ***SDS-PAGE separation of proteins.*** Aliquots of the plant–produced cDHDPS protein were subjected to electrophoresis on an SDS-polyacrylamide gel. Approximately 5.2 μg of total protein was loaded into each of three separate lanes. Protein markers (Precision Plus Protein Standards, Bio-Rad, Hercules, CA) were used to estimate molecular weight. All samples were heated at 90.8 °C for 5 min and then applied to a 4→20% pre-cast polyacrylamide gradient mini-gel. Electrophoresis was performed at constant voltage (150 V for 15 min followed by 200 V for 60 min), until the dye front reached the bottom of the gel. Proteins were stained by gentle shaking with Colloidal Brilliant Blue G stain (Sigma Chemical Co., St. Louis, MO) for 2 h, briefly destained with a solution containing 10% (v/v) acetic acid and 25% (v/v) methanol and finally destained with 25% (v/v) methanol.
>
> ***In-gel protein digestion.*** Protein bands with an apparent molecular mass of ~32 kDa were excised, destained, reduced, alkylated and subjected to an in-gel trypsin digest (Williams et al., 1997). Briefly, each gel band was destained by incubation in 100 μl of destaining buffer [40% (v/v) methanol and 10% (v/v) glacial acetic acid] for 30 min. Following destaining, gel fragments were incubated for 30 min in 100 μl buffer containing 100 mM ammonium bicarbonate. Proteins were reduced in 100 μl

solution of 100 mM ammonium bicarbonate containing 10 mM dithiothreitol for 2 h at 37 °C. Proteins were alkylated by the addition of 100 µl of buffer containing 100 mM ammonium bicarbonate and 100 mM iodoacetic acid. The alkylation reaction was allowed to proceed at room temperature for 20 min in the dark. Gel fragments were incubated in 100 µl buffer containing 100 mM ammonium bicarbonate for 30 min and then 100 µl acetonitrile was added (50% (v/v) final concentration) and the gel fragments were incubated for 30 min. The ammonium bicarbonate/acetonitrile incubations were repeated two additional times to remove reducing and alkylating reagents from the gel. The gel bands were dried in a SpeedVac concentrator (Savant, Holbrook, NY), rehydrated with 50 µl 25 mM ammonium bicarbonate solution containing 33 µg/ml trypsin (Promega, Madison, WI) and incubated for 16 h at 37 °C. Digested peptides were extracted three times for one h each at room temperature with 50 µl 70% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA). Extraction supernatants from each sample were then combined in a single tube, dried in a SpeedVac concentrator, and reconstituted in a final volume of 5 µl of 0.1% (v/v) TFA.

Sample preparation. A portion of the digested sample was desalted using Millipore (Bedford, MA) ZipTip$_{C18}$ pipette tips. Samples (~5 µL) were applied to a ZipTip and washed with 5 µl of Wash 1 [0.1% (v/v) TFA], 5 µl of Wash 2 [20% (v/v) acetonitrile containing 0.1% (v/v) TFA], 5 µl of Wash 3 [50% (v/v) acetonitrile containing 0.1% (v/v) TFA] and finally with 5 µl of Wash 4 [90% (v/v) acetonitrile containing 0.1% (v/v) TFA].

MALDI-TOF instrumentation and mass analysis. Mass spectral analysis of the plant-produced cDHDPS protein was performed using an Applied Biosystems Voyager DE-Pro Biospectrometry Workstation Matrix Assisted Laser Desorption and Ionization (MALDI) Time of Flight (TOF) instrument with the supplied Data Explorer software (Foster City, CA). Mass calibration of the instrument was performed using an external peptide mixture from a Sequazyme Peptide Mass Standards kit (Applied Biosystems). Samples (0.3 µl) from each desalting step and sample (0.3 µl) that was not desalted were co-crystallized with 0.75 µl α-cyano-4-hydroxy cinnamic acid (Ciphergen Biosystems, Palo Alto, CA) on the analysis plate. Samples were analyzed in the 500 to 5000 Da range in reflector mode using 150 laser shots per spectrum at a laser intensity setting of 2781 (a unit-less MALDI-TOF instrument specific value). Protonated (MH+) peptide masses were observed monoisotopically in reflector mode (Aebersold, 1993; Billeci and Stults, 1993). GPMAW software (Applied Biosystems, version 4.23) was used to generate a theoretical trypsin digest of the expected protein sequence. Masses were calculated for each theoretical peptide and compared to the raw mass data. Experimental masses (MH+) were assigned to peaks when three (or more) isotopically resolved ion peaks were observed in the raw mass data. Peaks were ignored if there were less than three isotopically resolved peaks in the spectra, when peak heights were less than approximately twice the baseline noise or when a mass could not be assigned because of overlap with a stronger signal ±2 Da from the mass analyzed. Known autocatalytic fragments from trypsin were ignored.

Immunoblot analysis. Aliquots of the test substance and reference standard proteins were first diluted to a concentration of ~0.01 mg/ml with buffer containing 20 mM Tris-HCl, 100 mM KCl, and 10 mM pyruvate, pH 8.0, and were further sequentially diluted to concentrations of ~5, 2.5, 1 and 0.5 ng/µl with 1× Laemmli buffer (Laemmli, 1970). The test substance and reference standard were loaded at approximately 5, 2.5, 1 and 0.5 ng total protein per lane. All samples were heated at approximately 89 °C for 5 min and applied to a pre-cast tris-glycine polyacrylamide gradient (4→20%) 10-well mini-gel (Invitrogen, Carlsbad, CA). Electrophoresis was performed at constant voltage (150 V for 15 min followed by 200 V for 60 min) until the dye front reached the bottom of the gel. Following electrophoresis, pre-stained molecular weight markers (Bio-Rad, Hercules CA) were used to verify electrotransfer of proteins to the membrane. Electrotransfer to the nitrocellulose membrane (Invitrogen) was performed for 60 min at a constant current of 300 mA.

The membrane was blocked overnight at 4 ºC with 5% (w/v) non-fat dry milk (NFDM) in PBST buffer, but all subsequent incubations (described below) were performed at room temperature. Goat anti–cDHDPS serum was used to probe the membrane for 1 h at a dilution of 1:4,000 in 1% (w/v) NFDM in PBST. Excess serum was removed by three 5 min washes with PBST. The membrane was incubated with HRP–conjugated anti-goat IgG (Sigma) at a dilution of 1:10,000 in 1% (w/v) NFDM in PBST for 1h, and again washed (three 5 min washes) with PBST. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) detection system according to the manufacturer's instructions (Amersham Pharmacia) and exposed (30 sec, two and five min) to Hyperfilm ECL high performance chemiluminescence film (Amersham Pharmacia). Films were developed using a Konica SRX101A automated film processor (Tokyo, Japan).

Protein concentration. Total protein concentration was estimated by amino acid analysis. Aliquots of the plant-produced cDHDPS protein were analyzed using a Hitachi L-8800 Amino Acid Analyzer with AAA System Manager Software. Test samples, NIST BSA (used as a system suitability standard) and NIST amino acid calibration control standard (Gaithersburg, MD), were spiked with an internal reference (norvaline, Sigma Chemical Co.) and dried in a Savant SpeedVac (Holbrook, NY). Vapor phase acid hydrolysis [6 N HCl containing 1% (v/v) phenol] was performed at ~150 °C for approximately 90 min. Cooled samples were again evaporated, reconstituted in protein hydrolyzate buffer PH-1 (Hitachi Instruments) and loaded onto the instrument. Amino acids were detected using ninhydrin derivatization. Each protein sample was analyzed in triplicate. Protein concentration was determined from acceptable runs and averaged.

Molecular weight and purity determination – SDS-PAGE. Aliquots of stock solutions of the purified test and reference standard proteins were diluted with buffer containing 20 mM Tris-HCl, 100 mM KCl, 10 mM pyruvate, pH 8.0, to a concentration of ~0.5 mg/ml. Aliquots of these dilutions were combined with 2× Laemmli (Laemmli, 1970) sample buffer. The plant-produced protein was analyzed at approximately 1, 2 and 3 µg total protein per lane. The *E. coli*-produced cDHDPS reference standard was analyzed concurrently at 1 µg total protein per lane. Molecular weight markers (4.5 µg total protein per lane, Bio-Rad broad-range, Hercules, CA) were used to estimate the molecular weight. All samples were heated at ~85 ºC for 5 min and applied to pre-cast tris-glycine polyacrylamide gradient (4→20%) mini-gel (Invitrogen, Carlsbad, CA). Electrophoresis was performed at constant

voltage (150 V for 15 min followed by 200 V for 65 min), until the dye front reached the bottom of the gel. Proteins were fixed in the gels by gentle shaking with 40% (v/v) methanol and 7% (v/v) glacial acetic acid, stained with Colloidal Brilliant Blue G stain (Sigma Chemical Co., St. Louis, MO) for 2 h, briefly destained with a solution containing 10% (v/v) acetic acid and 25% (v/v) methanol and finally destained overnight with 25% (v/v) methanol.

Analysis of the gel was performed using a Bio-Rad Laboratories GS-800 densitometer with the Quantity One software (version 4.3.0, Hercules, CA). Molecular weight values supplied by the manufacturer were used to estimate the molecular weight of each observed band. The optical density of all visible bands within each lane was measured. Purity was estimated as the percent optical density of the ~32 kDa band relative to all bands detected in the lane. Molecular weight and purity were reported as an average of values obtained from all three lanes containing the plant-produced cDHDPS protein.

Molecular weight determination – MALDI-TOF MS. Prior to analysis, the plant–produced cDHDPS protein and BSA reference protein (NIST, Gaithersburg, MD) were desalted using drop dialysis (Görisch, 1988). Briefly, a Millipore microdialysis disk (Bedford, MA) was floated on water, spotted with 5 µl of each protein and dialyzed for 45 min. Aliquots of each sample, 0.3 and 0.5 µl, were spotted on an analysis plate, mixed with 0.75 µl sinapinic acid (Sigma Chemical Co., St. Louis, MO) and air-dried. Mass spectral analysis was performed using an Applied Biosystems Voyager DE-Pro Biospectrometry Workstation Matrix Assisted Laser Desorption and Ionization (MALDI) Time of Flight (TOF) instrument with the supplied Data Explorer software (Foster City, CA). Samples were analyzed in the 2,000 to 100,000 Da range in linear mode using 200 laser shots per spectrum at a laser intensity setting of 3374 (a unit-less MALDI-TOF instrument specific value). Mass calibration of the instrument was performed using the desalted NIST BSA reference protein. The mass of the plant-produced cDHDPS protein was reported as an average of three separate mass spectral acquisitions. For comparison, the mass of the cDHDPS protein was calculated from the expected amino acid sequence of the protein using the GPMAW software (Applied Biosystems, version 4.23).

Functional activity assay. Prior to analysis, aliquots of the plant-produced cDHDPS test substance and *E.coli*-produced cDHDPS reference standard were diluted to approximately 0.01 mg/ml in buffer containing 15 mM Tris-HCl, 75 mM KCl, 7.5 mM pyruvate, and 25% (v/v) glycerol, pH 8.0. Aliquots of diluted protein were frozen at –80 ^{o}C prior to functional assay. The test substance and reference standard were assayed at 0.02, 0.04, 0.06 and 0.08 µg of total protein per reaction mixture. The functional activity assay utilized a coupled enzyme system and monitors the change in absorbance at 340 nm, which is associated with the oxidation of β-nicotinamide adenine dinucleotide phosphate (NADPH) by dihydrodipicolinate reductase (DapB). The amount of NADPH oxidized during the reaction was quantitated based on comparison with a NADPH standard curve. Briefly, reaction mixtures contained 100 mM Tris-HCl, 5 mM pyruvate, 0.7 mM NADPH, 6.25 µg of DapB protein, 2.8 mM L-ASA and 0.02 – 0.08 µg of cDHDPS. The cDHDPS-dependent oxidation of NADPH was monitored ($A_{340\ nm}$) for 30 min at 20 sec intervals at room temperature using a PowerWave X_i (Bio-Tek) microplate reader. The specific activity of cDHDPS enzyme was calculated relative to a NADPH standard curve. For cDHDPS, one

unit (U) of enzyme activity was defined as the amount of NADPH oxidized per minute. Specific activity of cDHDPS was defined as the amount of NADPH oxidized per minute of reaction time by 1 mg of cDHDPS protein at room temperature.

Glycosylation analysis: This analysis was used to determine if the plant-produced cDHDPS protein had been post-translationally modified with carbohydrates. Proteins bound to a membrane were oxidized with periodate and reacted with biotin-hydrazide. Biotinylated carbohydrates were detected using a streptavidin-HRP conjugate and visualized with the luminol-based ECL reagents. Aliquots of the test and reference standard proteins were diluted with buffer containing 20 mM Tris-HCl, 100 mM KCl, 10 mM pyruvate, pH 8.0, to a concentration of ~0.5 mg/ml. Aliquots of these dilutions were combined with 1× Laemmli (Laemmli, 1970) sample buffer. The plant-produced cDHDPS test substance was analyzed at approximately 0.5 and 1 μg total protein per lane. The *E. coli*-produced cDHDPS reference standard, which was used as a negative control, was analyzed concurrently at 0.5 and 1 μg total protein per lane. The positive control, transferrin, was initially prepared as a 1 mg/ml stock solution in purified water and was analyzed concurrently at 0.5 and 1 μg total protein per lane. Dilutions of the *E. coli*- and plant-produced cDHDPS proteins and the transferrin protein were applied to an SDS-polyacrylamide gel. Electrophoresis was performed at constant voltage (150 V for 15 min followed by 200 V for 60 min), until the dye front reached the bottom of the gel. Following electrophoresis, pre-stained molecular weight markers (Bio-Rad, Hercules CA) were used to verify electrotransfer of proteins to the membrane. Electrotransfer to the PVDF membrane (Invitrogen) was performed for 60 min at a constant current of 300 mA.

Carbohydrate detection was performed directly on the PVDF membrane. The PVDF membrane was gently shaken for approximately 10 min in PBS and transferred to a solution of 100 mM sodium acetate buffer, pH 5.5, containing the oxidation reagent, 10 mM sodium metaperiodate. The membrane was incubated in the dark for 20 min. The oxidation solution was removed from the membrane by two brief rinses in PBS followed by three sequential 10 min washes with PBS. The membrane was transferred to a solution of 100 mM sodium acetate buffer, pH 5.5, containing 0.125 mM biotin hydrazide and incubated for 60 minutes. Biotin hydrazide solution was removed by washing in PBS, as previously described for the removal of the 10 mM sodium metaperiodate solution. Blocking was performed by overnight incubation of the membrane in a 4 °C refrigerator in 5% (w/v) NFDM blocking agent in PBS. The blocking solution was removed by washing in PBS as previously described. The membrane was incubated with streptavidin-HRP conjugate (diluted ~1:6000) in PBS for approximately 30 min to detect carbohydrate moieties bound to biotin. Excess streptavidin-HRP was removed by washing in PBS as previously described. Bands were visualized using the ECL detection system (Amersham Pharmacia) and exposed (0.5, 1, 3 and 5 min) to Hyperfilm ECL high performance chemiluminescence film (Amersham Pharmacia). Films were developed using a Konica SRX-101A automated film processor (Tokyo, Japan).

Control of Bias and Quality Measures

Controls and standards were included with each analysis. A protein standard (β-lactoglobulin) was sequenced before and after N-terminal sequence analysis to assure the

performance of the sequencer. Additionally, instrument repetitive yield (>94% required) data for the analysis of β-lactoglobulin were calculated and found to meet standard procedure specifications. A four-peptide mixture from the Sequazyme Peptide Mass Standards kit (Applied Biosystems) was used to calibrate the MALDI-TOF mass spectrometer for peptide analysis for masses observed between 500-5000 Da. BSA (NIST) was used to calibrate the MALDI-TOF mass spectrometer for native protein molecular weight determinations. BSA (NIST), as a system suitability standard, and an NIST amino acid calibration control standard (Gaithersburg, MD) were used in amino acid analysis. Transferrin was included in the glycoprotein analysis as a positive control.

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APPENDIX 4. Field Trial Individual Site Phenotypic Data Tables

A. 2002 Field Trial Individual Site Phenotypic Data Tables

Appendix 4, Table 1. Phenotypic comparison of LY038 to its control at BE
(USDA-APHIS notification 02-037-05n)

Phenotypic characteristic	LY038		Ref range[1]	
	Test	Control	Min	Max
Seedling vigor	7.3	8.0	7.0	9.0
Early stand count	67.3	67.0	63.0	69.0
Days to 50% pollen shed	64.7	64.7	59.0	63.0
Days to 50% silking	63.7	63.0	57.0	62.0
Stay green	6.0	6.3	3.0	7.0
Ear height (in)	39.7	38.9	36.4	46.2
Plant height (in)	73.0	73.6	80.6	88.2
Dropped ears (#/plot)	0	0.3	0	2.0
Stalk lodged plants (#/plot)	1.0	1.0	0	2.0
Root lodged plants (#/plot)	0	0	0	0
Final stand count	56.3	56.0	56.0	57.0
Grain moisture (%)	25.9	26.3	16.0	24.4
Test weight (lbs/bu)	51.4	50.6	49.3	55.7
Yield (bu/a)	101.7	112.4	106.2	158.0

***** Indicates a statistically significant difference between a test and control hybrid at $P \leq 0.05$.

[1] Ref range = Minimum and maximum values observed among three replications of four commercially available reference maize hybrids, DK537, DKC60-15, RX708, RX772.

Appendix 4, Table 2. Phenotypic comparison of LY038 to its control at CL
(USDA-APHIS notification 02-037-05n)

Phenotypic characteristic	LY038		Ref range[1]	
	Test	Control	Min	Max
Seedling vigor	5.3	5.3	4.0	6.0
Early stand count	63.0	65.3	56.0	67.0
Days to 50% pollen shed	57.3	58.0	53.0	55.0
Days to 50% silking	59.7	59.0	56.0	58.0
Stay green	5.3	5.0	4.0	6.0
Ear height (in)	30.9	32.2	29.4	44.4
Plant height (in)	70.3	70.8	71.0	81.6
Dropped ears (#/plot)	0.3	0.3	0	4.0
Stalk lodged plants (#/plot)	4.3	2.7	1.0	12.0
Root lodged plants (#/plot)	0	0	0	0
Final stand count	48.7	49.3	46.0	55.0
Grain moisture (%)	16.9	16.3	14.9	16.5
Test weight (lbs/bu)	57.0	56.8	52.0	58.0
Yield (bu/a)	78.0	84.3	65.1	112.9

***** Indicates a statistically significant difference between a test and control hybrid at $P \leq 0.05$.

[1] Ref range = Minimum and maximum values observed among three replications of four commercially available reference maize hybrids, DK537, DKC60-15, RX708, RX772.

Appendix 4, Table 3. Phenotypic comparison of LY038 to its control at CR
(USDA-APHIS notification 02-046-32n)

Phenotypic characteristic	LY038		Ref range[1]	
	Test	Control	Min	Max
Seedling vigor	5.3	7.0	5.0	8.0
Early stand count	68.7	71.7	64.0	73.0
Days to 50% pollen shed	63.3	64.7	59.0	67.0
Days to 50% silking	61.3	62.0	59.0	61.0
Stay green	8.0	7.0	5.0	9.0
Ear height (in)	27.0	29.1	26.2	40.4
Plant height (in)	51.7	55.1	63.8	71.8
Dropped ears (#/plot)	0	0	0	1.0
Stalk lodged plants (#/plot)	0	0.3	0	2.0
Root lodged plants (#/plot)	0	0	0	0
Final stand count	49.3	53.3	47.0	57.0
Grain moisture (%)	16.5	15.9	12.6	15.7
Test weight (lbs/bu)	60.6*	53.6	52.2	60.6
Yield (bu/a)	18.5	24.5	11.2	58.0

* Indicates a statistically significant difference between a test and control hybrid at $P \leq 0.05$.

[1] Ref range = Minimum and maximum values observed among three replications of four commercially available reference maize hybrids, DK537, DKC60-15, RX708, RX772.

Appendix 4, Table 4. Phenotypic comparison of LY038 to its control at MN
(USDA-APHIS notification 02-046-32n)

Phenotypic characteristic	LY038		Ref range[1]	
	Test	Control	Min	Max
Seedling vigor	6.7*	7.7	7.0	8.0
Early stand count	65.0	63.3	64.0	68.0
Days to 50% pollen shed	62.3	62.3	58.0	62.0
Days to 50% silking	60.7	60.7	58.0	60.0
Stay green	6.3*	5.3	6.0	7.0
Ear height (in)	35.0	34.0	34.4	47.8
Plant height (in)	78.3	75.2	88.2	93.6
Dropped ears (#/plot)	0.3	0	0	2.0
Stalk lodged plants (#/plot)	0	0.3	0	1.0
Root lodged plants (#/plot)	0	0	0	0
Final stand count	56.3	55.0	53.0	60.0
Grain moisture (%)	22.0*	17.7	15.3	21.3
Test weight (lbs/bu)	57.3	57.7	56.0	58.5
Yield (bu/a)	141.4	139.9	173.9	221.8

* Indicates a statistically significant difference between a test and control hybrid at $P \leq 0.05$.

[1] Ref range = Minimum and maximum values observed among three replications of four commercially available reference maize hybrids, DK537, DKC60-15, RX708, RX772.

Appendix 4, Table 5. Phenotypic comparison of LY038 to its control at NB
(USDA-APHIS notification 02-037-05n)

Phenotypic characteristic	LY038		Ref range[1]	
	Test	Control	Min	Max
Seedling vigor	8.3	8.7	8.0	9.0
Early stand count	57.0	68.0	64.0	70.0
Days to 50% pollen shed	67.3	65.7	62.0	67.0
Days to 50% silking	67.3	66.3	62.0	68.0
Stay green	3.0	3.0	2.0	4.0
Ear height (in)	37.5	38.8	36.6	49.8
Plant height (in)	84.9	88.9	89.3	104.2
Dropped ears (#/plot)	0.7	1.7	0	4.0
Stalk lodged plants (#/plot)	1.3	2.7	0	21.0
Root lodged plants (#/plot)	0	0	0	0
Final stand count	49.3	55.3	48.0	59.0
Grain moisture (%)	18.2	17.5	16.1	17.9
Test weight (lbs/bu)	58.5	58.2	56.0	58.0
Yield (bu/a)	122.4	149.7	117.6	183.9

* Indicates a statistically significant difference between a test and control hybrid at $P \leq 0.05$.

[1] Ref range = Minimum and maximum values observed among three replications of four commercially available reference maize hybrids, DK537, DKC60-15, RX708, RX772.

Appendix 4, Table 6. Phenotypic comparison of LY038 to its control at RL
(USDA-APHIS notification 02-037-05n)

Phenotypic characteristic	LY038		Ref range[1]	
	Test	Control	Min	Max
Seedling vigor	6.7	7.0	6.0	8.0
Early stand count	66.7	66.7	66.0	70.0
Days to 50% pollen shed	58.3	58.3	54.0	56.0
Days to 50% silking	59.0	59.0	54.0	57.0
Stay green	3.3	4.0	3.0	7.0
Ear height (in)	36.1	35.1	39.0	48.0
Plant height (in)	74.5	72.8	81.0	86.2
Dropped ears (#/plot)	0	0	0	0
Stalk lodged plants (#/plot)	0.7	0	0	5.0
Root lodged plants (#/plot)	0	0	0	1.0
Final stand count	57.7	56.7	54.0	65.0
Grain moisture (%)	23.5	22.1	15.5	22.1
Test weight (lbs/bu)	55.0	55.3	55.0	55.0
Yield (bu/a)	139.8*	155.0	162.5	196.6

* Indicates a statistically significant difference between a test and control hybrid at $P \leq 0.05$.

[1] Ref range = Minimum and maximum values observed among three replications of four commercially available reference maize hybrids, DK537, DKC60-15, RX708, RX772.

Appendix 4, Table 7. Phenotypic comparison of LY038 to its control at VH
(USDA-APHIS notification 02-037-05n)

Phenotypic characteristic	LY038		Ref range[1]	
	Test	Control	Min	Max
Seedling vigor	8.0	8.0	7.0	9.0
Early stand count	60.3	61.3	53.0	66.0
Days to 50% pollen shed	61.0	61.0	58.0	59.0
Days to 50% silking	61.7*	60.7	58.0	60.0
Stay green	3.7	3.3	4.0	5.0
Ear height (in)	31.5	33.3	33.2	46.8
Plant height (in)	74.8	73.7	81.2	90.0
Dropped ears (#/plot)	0.3	0	0	1.0
Stalk lodged plants (#/plot)	1.7	5.7	3.0	13.0
Root lodged plants (#/plot)	7.3	6.7	5.0	34.0
Final stand count	56.0	56.0	54.0	56.0
Grain moisture (%)	21.9	20.4	17.7	22.3
Test weight (lbs/bu)	56.0	56.0	53.5	56.0
Yield (bu/a)	170.0	174.6	185.9	266.1

* Indicates a statistically significant difference between a test and control hybrid at $P \leq 0.05$.

[1] Ref range = Minimum and maximum values observed among three replications of four commercially available reference maize hybrids, DK537, DKC60-15, RX708, RX772.

Appendix 4, Table 8. Phenotypic comparison of LY038 to its control at WC
(USDA-APHIS notification 02-037-05n)

Phenotypic characteristic	LY038		Ref range[1]	
	Test	Control	Min	Max
Seedling vigor	8.0	8.0	7.0	9.0
Early stand count	68.7	68.7	64.0	70.0
Days to 50% pollen shed	66.0	66.0	61.0	64.0
Days to 50% silking	66.0	66.0	61.0	64.0
Stay green	0	0	0	3.0
Ear height (in)	43.3*	40.3	34.8	51.8
Plant height (in)	84.8*	80.9	89.8	98.6
Dropped ears (#/plot)	0.3	0	0	2.0
Stalk lodged plants (#/plot)	1.3	2.0	0	5.0
Root lodged plants (#/plot)	0	0	0	0
Final stand count	57.3	56.7	53.0	59.0
Grain moisture (%)	11.7	10.3	8.2	12.9
Test weight (lbs/bu)	62.3*	61.4	58.8	60.4
Yield (bu/a)	167.3	162.0	188.9	246.1

* Indicates a statistically significant difference between a test and control hybrid at $P \leq 0.05$.

[1] Ref range = Minimum and maximum values observed among three replications of four commercially available reference maize hybrids, DK537, DKC60-15, RX708, RX772.

Appendix 4, Table 9. Phenotypic comparison of LY038 to its control at WY
(USDA-APHIS notification 02-066-15n)

Phenotypic characteristic	LY038		Ref range[1]	
	Test	Control	Min	Max
Seedling vigor	8.0	8.0	8.0	8.0
Early stand count	70.0	68.3	63.0	70.0
Days to 50% pollen shed	64.0	63.7	59.0	64.0
Days to 50% silking	63.7	63.0	59.0	64.0
Stay green	1.0	1.0	1.0	3.0
Ear height (in)	23.6	22.2	22.0	36.0
Plant height (in)	42.0	41.3	37.2	58.4
Dropped ears (#/plot)	1.0*	0	0	1.0
Stalk lodged plants (#/plot)	0	0.3	0	1.0
Root lodged plants (#/plot)	0	0	0	0
Final stand count	43.7	48.0	29.0	55.0
Grain moisture (%)	30.9	30.1	20.6	29.9
Test weight (lbs/bu)	50.0	49.1	46.3	51.2
Yield (bu/a)	46.7	58.4	13.8	112.0

* Indicates a statistically significant difference between a test and control hybrid at $P \leq 0.05$.

[1] Ref range = Minimum and maximum values observed among three replications of four commercially available reference maize hybrids, DK537, DKC60-15, RX708, RX772.

Appendix 4, Table 10. Phenotypic comparison of LY038 to its control at YK
(USDA-APHIS notification 02-037-05n)

Phenotypic characteristic	LY038		Ref range[1]	
	Test	Control	Min	Max
Seedling vigor	7.3	7.7	7.0	8.0
Early stand count	65.0	64.0	58.0	68.0
Days to 50% pollen shed	69.7	70.0	63.0	65.0
Days to 50% silking	70.7	70.3	63.0	66.0
Stay green	4.0	4.0	3.0	5.0
Ear height (in)	31.7	30.7	30.0	40.2
Plant height (in)	63.7	61.9	66.6	77.0
Dropped ears (#/plot)	0.3*	0	0	0
Stalk lodged plants (#/plot)	0	0	0	1.0
Root lodged plants (#/plot)	0	0	0	0
Final stand count	55.7	55.3	52.0	56.0
Grain moisture (%)	27.3	27.5	13.5	22.8
Test weight (lbs/bu)	53.7	54.4	53.4	57.1
Yield (bu/a)	55.6	68.1	102.6	160.1

* Indicates a statistically significant difference between a test and control hybrid at $P \leq 0.05$.

[1] Ref range = Minimum and maximum values observed among three replications of four commercially available reference maize hybrids, DK537, DKC60-15, RX708, RX772.

B. 2003 Field Trial Individual Site Phenotypic Data Tables

Appendix 4, Table 11. Phenotypic comparison of LY038 to its control at BE
(USDA-APHIS notification number 03-052-17n)

Phenotypic characteristic	LY038		Ref range[1]	
	Test	Control	Min	Max
Seedling vigor	8.7	9.0	8.0	9.0
Early stand count	69.0	66.3	58.0	70.0
White leaf plants (#/plot)	0	0	0	0
Days to 50% pollen shed	64.7	65.7	60.0	65.0
Days to 50% silking	66.0	67.0	61.0	65.0
Stay green	5.3	5.7	2.0	6.0
Ear height (in)	44.6	43.3	39.8	52.0
Plant height (in)	83.2	79.5	77.8	92.0
Dropped ears (#/plot)	0.3	0.3	0	3.0
Stalk lodged plants (#/plot)	4.7	6.7	1.0	25.0
Root lodged plants (#/plot)	0	0	0	1.0
Final stand count	56.0*	56.7	56.0	56.0
Grain moisture (%)	20.5	21.6	14.6	21.1
Test weight (lbs/bu)	54.4	53.8	52.3	54.8
Yield (bu/a)	128.0	118.6	101.7	154.2

* Indicates a statistically significant difference between the test and control at $P \leq 0.05$.

[1] Ref range = Minimum and maximum values observed among three replications of four commercially available reference maize hybrids, DK537, DKC60-15, RX708, RX772.

Appendix 4, Table 12. Phenotypic comparison of LY038 to its control at CR
(USDA-APHIS notification number 03-052-17n)

Phenotypic characteristic	LY038		Ref range[1]	
	Test	Control	Min	Max
Seedling vigor	7.0	7.7	6.0	8.0
Early stand count	70.7	69.3	56.0	72.0
White leaf plants (#/plot)	0	0	0	0
Days to 50% pollen shed	68.3	68.3	65.0	68.0
Days to 50% silking	67.3	66.3	64.0	66.0
Stay green	6.3	6.7	7.0	8.0
Ear height (in)	37.1	36.4	30.2	46.0
Plant height (in)	72.8	75.0	75.7	85.5
Dropped ears (#/plot)	0	0	0	0
Stalk lodged plants (#/plot)	6.3	14.7	6.0	17.0
Root lodged plants (#/plot)	0.7	1.0	0	1.0
Final stand count	48.7	49.0	45.0	54.0
Grain moisture (%)	31.9	31.4	23.5	30.5
Test weight (lbs/bu)	47.0	44.9	44.9	49.1
Yield (bu/a)	90.1	95.7	101.2	145.9

* Indicates a statistically significant difference between the test and control at $P \leq 0.05$. None were detected for these comparisons.

[1] Ref range = Minimum and maximum values observed among three replications of four commercially available reference maize hybrids, DK537, DKC60-15, RX708, RX772.

Appendix 4, Table 13. Phenotypic comparison of LY038 to its control at MN
(USDA-APHIS notification number 03-052-17n)

Phenotypic characteristic	LY038		Ref range[1]	
	Test	Control	Min	Max
Seedling vigor	8.3*	9.0	7.0	9.0
Early stand count	68.7	67.7	65.0	70.0
White leaf plants (#/plot)	0	0	0	0
Days to 50% pollen shed	69.3	70.0	64.0	68.0
Days to 50% silking	70.0	70.0	64.0	68.0
Stay green	6.0*	4.7	4.0	7.0
Ear height (in)	47.0	45.9	39.2	57.2
Plant height (in)	95.5*	88.3	86.4	103.0
Dropped ears (#/plot)	0	0	0	0
Stalk lodged plants (#/plot)	0.3	0	0	2.0
Root lodged plants (#/plot)	0	0	0	0
Final stand count	55.0	54.3	53.0	58.0
Grain moisture (%)	24.0*	19.2	15.9	20.9
Test weight (lbs/bu)	61.4	60.0	47.3	61.6
Yield (bu/a)	154.9*	127.5	190.5	250.3

* Indicates a statistically significant difference between the test and control at $P \leq 0.05$.

[1] Ref range = Minimum and maximum values observed among three replications of four commercially available reference maize hybrids, DK537, DKC60-15, RX708, RX772.

Appendix 4, Table 14. Phenotypic comparison of LY038 to its control at NB
(USDA-APHIS notification number 03-052-17n)

Phenotypic characteristic	LY038		Ref range[1]	
	Test	Control	Min	Max
Seedling vigor	7.7	8.0	8.0	8.0
Early stand count	70.3	70.7	66.0	74.0
White leaf plants (#/plot)	5.7*	0	0	0
Days to 50% pollen shed	70.3	71.0	67.0	71.0
Days to 50% silking	70.3	70.7	66.0	71.0
Stay green	3.7	4.3	3.0	5.0
Ear height (in)	36.9	37.9	28.6	49.2
Plant height (in)	82.4*	77.0	75.4	85.0
Dropped ears (#/plot)	1.7	1.3	0	15.0
Stalk lodged plants (#/plot)	0.3	0.3	0	23.0
Root lodged plants (#/plot)	0	0	0	0
Final stand count	56.0	55.3	49.0	56.0
Grain moisture (%)	18.1	18.6	16.0	19.8
Test weight (lbs/bu)	54.7*	52.7	51.5	55.0
Yield (bu/a)	102.6	110.6	43.9	122.5

* Indicates a statistically significant difference between the test and control at $P \leq 0.05$.

[1] Ref range = Minimum and maximum values observed among three replications of four commercially available reference maize hybrids, DK537, DKC60-15, RX708, RX772.

Appendix 4, Table 15. Phenotypic comparison of LY038 to its control at RL
(USDA-APHIS notification number 03-052-17n)

Phenotypic characteristic	LY038		Ref range[1]	
	Test	Control	Min	Max
Seedling vigor	6.7	7.0	6.0	7.0
Early stand count	72.0	71.7	68.0	76.0
White leaf plants (#/plot)	0.3	0	0	0
Days to 50% pollen shed	70.0	70.3	65.0	69.0
Days to 50% silking	70.0	70.0	65.0	69.0
Stay green	2.7	2.3	1.0	3.0
Ear height (in)	41.0	40.9	38.8	52.2
Plant height (in)	86.9	84.7	84.2	97.8
Dropped ears (#/plot)	0	0	0	0
Stalk lodged plants (#/plot)	0.3	0	0	0
Root lodged plants (#/plot)	0.3	0.3	0	1.0
Final stand count	54.0*	58.3	51.0	60.0
Grain moisture (%)	26.7	25.7	19.0	27.0
Test weight (lbs/bu)	57.7	57.0	54.0	58.0
Yield (bu/a)	165.8	186.7	188.9	261.4

* Indicates a statistically significant difference between the test and control at $P \leq 0.05$.

[1] Ref range = Minimum and maximum values observed among three replications of four commercially available reference maize hybrids, DK537, DKC60-15, RX708, RX772.

Appendix 4, Table 16. Phenotypic comparison of LY038 to its control at VH
(USDA-APHIS notification number 03-052-17n)

Phenotypic characteristic	LY038		Ref range[1]	
	Test	Control	Min	Max
Seedling vigor	7.3	7.7	7.0	8.0
Early stand count	64.7	65.3	57.0	71.0
White leaf plants (#/plot)	4.7*	0	0	0
Days to 50% pollen shed	71.0	71.0	65.0	71.0
Days to 50% silking	71.0	71.0	64.0	71.0
Stay green	3.0	2.3	3.0	3.0
Ear height (in)	37.5	37.4	34.8	50.2
Plant height (in)	81.8*	77.7	81.6	92.4
Dropped ears (#/plot)	0	0	0	0
Stalk lodged plants (#/plot)	1.7	2.3	0	3.0
Root lodged plants (#/plot)	0.3	0.3	0	1.0
Final stand count	55.0	54.7	53.0	55.0
Grain moisture (%)	16.6	15.5	14.6	16.0
Test weight (lbs/bu)	59.2	58.3	56.5	59.0
Yield (bu/a)	155.8	136.4	167.6	223.0

* Indicates a statistically significant difference between the test and control at $P \leq 0.05$.

[1] Ref range = Minimum and maximum values observed among three replications of four commercially available reference maize hybrids, DK537, DKC60-15, RX708, RX772.

Appendix 4, Table 17. Phenotypic comparison of LY038 to its control at YK
(USDA-APHIS notification number 03-052-17n)

Phenotypic characteristic	LY038		Ref range[1]	
	Test	Control	Min	Max
Seedling vigor	6.7*	8.0	8.0	8.0
Early stand count	61.0	60.0	57.0	66.0
White leaf plants (#/plot)	4.0*	0	0	0
Days to 50% pollen shed	71.3	72.0	65.0	68.0
Days to 50% silking	72.3	72.7	65.0	69.0
Stay green	5.7	6.0	3.0	5.0
Ear height (in)	33.9	34.1	30.6	42.2
Plant height (in)	73.9	72.5	73.4	79.2
Dropped ears (#/plot)	0	0	0	0
Stalk lodged plants (#/plot)	0	0	0	2.0
Root lodged plants (#/plot)	0	0	0	0
Final stand count	55.7	55.3	54.0	57.0
Grain moisture (%)	25.1	22.6	13.1	26.4
Test weight (lbs/bu)	51.0	54.6	47.3	54.7
Yield (bu/a)	109.5*	131.5	143.2	188.2

* Indicates a statistically significant difference between the test and control at $P \leq 0.05$.

[1] Ref range = Minimum and maximum values observed among three replications of four commercially available reference maize hybrids, DK537, DKC60-15, RX708, RX772.

APPENDIX 5. Lysine-Related Metabolite Analysis

APPENDIX 5. Lysine-Related Metabolite Analysis

A. Introduction

Human and monogastric animals can synthesize only ten of the 20 common amino acids found in proteins and, therefore, they need to obtain half of them from their diets. Among the "essential" amino acids, lysine is exceedingly important because it is one of the most limiting essential amino acids in cereal grains, which represent the largest source of food worldwide. Lysine amounts are limited in most major crop plants because of the feedback inhibition, by lysine, of the lysine biosynthesis enzyme, dihydrodipicolinate synthase (DHDPS). The nutritional value of cereal grain protein is a critical constraint to animal and human health and nutrition as proteins present in cereal are deficient in essential amino acids such as lysine, tryptophan and methionine. The requirement for protein supplementation is costly and energy inefficient for animal nutrition. Therefore, increasing lysine levels in grain has been a primary objective of breeding since the 1960s (Mertz et al., 1964). Because of its nutritional importance, the regulation of lysine metabolism has been studied extensively at the biochemical, genetic and molecular level in a wide range of organisms such as bacteria, plants and mammals. In addition, several laboratories have studied lysine-related metabolites in crop plants and tissues in which they over-expressed a form of DHDPS insensitive to lysine feedback inhibition (Mazur et al., 1999).

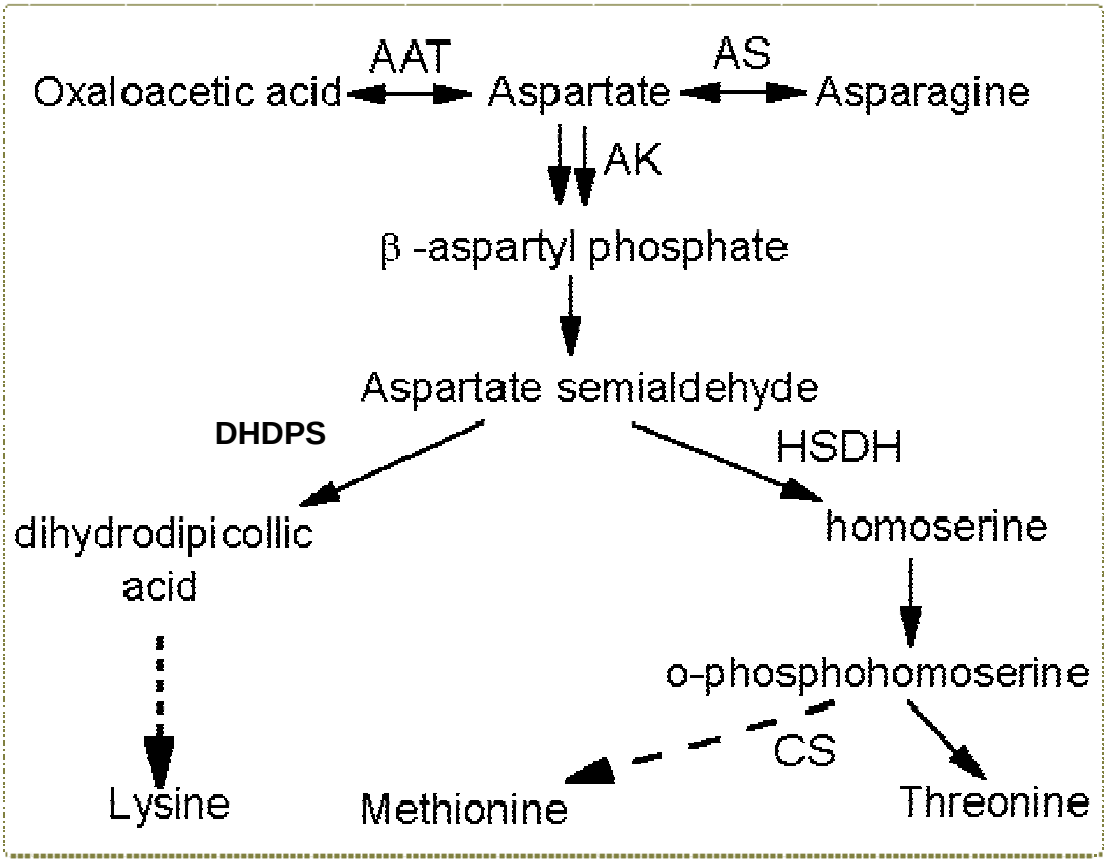
B. Rationale for Analysis of Lysine-related Metabolites

As a key component in the compositional analyses of Lysine maize, the levels of lysine and key lysine-related metabolites were analyzed and compared to the levels of these metabolites in the negative segregant control as well as in several conventional commercial reference maize hybrids. The process for selecting specific lysine metabolites for analysis was based on two factors: 1) a comprehensive understanding of the synthesis and degradation pathway of lysine in plants and 2) selection of stable analytes that are known or expected to change. Lysine biosynthesis is one of the most comprehensively studied amino acid pathways in plant systems. A detailed summary of lysine biosynthesis and catabolism in plants is reviewed below.

1. Lysine Synthesis

Two pathways of lysine synthesis exist in nature: the diaminopimelate (DAP) pathway and the α-aminoadipate (AAA) pathway. For yeast and fungi, lysine is synthesized from 2-oxoglutarate through AAA. In plants and some bacteria, lysine is synthesized via the aspartate pathway that also leads to the synthesis of two additional essential amino acids, methionine and threonine (Figure 1). The enzyme dihydrodipicolinate synthase (DHDPS) catalyzes the first committed step specific to the lysine biosynthesis (Azevedo and Lea, 2001). DHDPS appears to be the key regulatory enzyme that prevents the accumulation of excess free lysine by being sensitive to lysine feedback inhibition (Galili, 1995). Feedback inhibitory regulation of this key step in lysine biosynthesis can be overcome through the insertion of the *cordapA* gene from *Corynebacterium glutamicum* that encodes a variant

form of dihydrodipicolinate synthase (cDHDPS) that is not as sensitive to accumulated free lysine as the maize enzyme (Falco et al., 1995). The net effect of expressing cDHDPS is increased flux through this enzymatic step in the lysine biosynthetic pathway resulting in the accumulation of free lysine. A diagram outlining the complex feedback regulation of lysine biosynthesis is shown in Figure 2. The synthesis of lysine is tightly regulated: DHDPS and aspartate kinase (AK) from plants are sensitive to lysine inhibition (dotted lines, Figure 2).

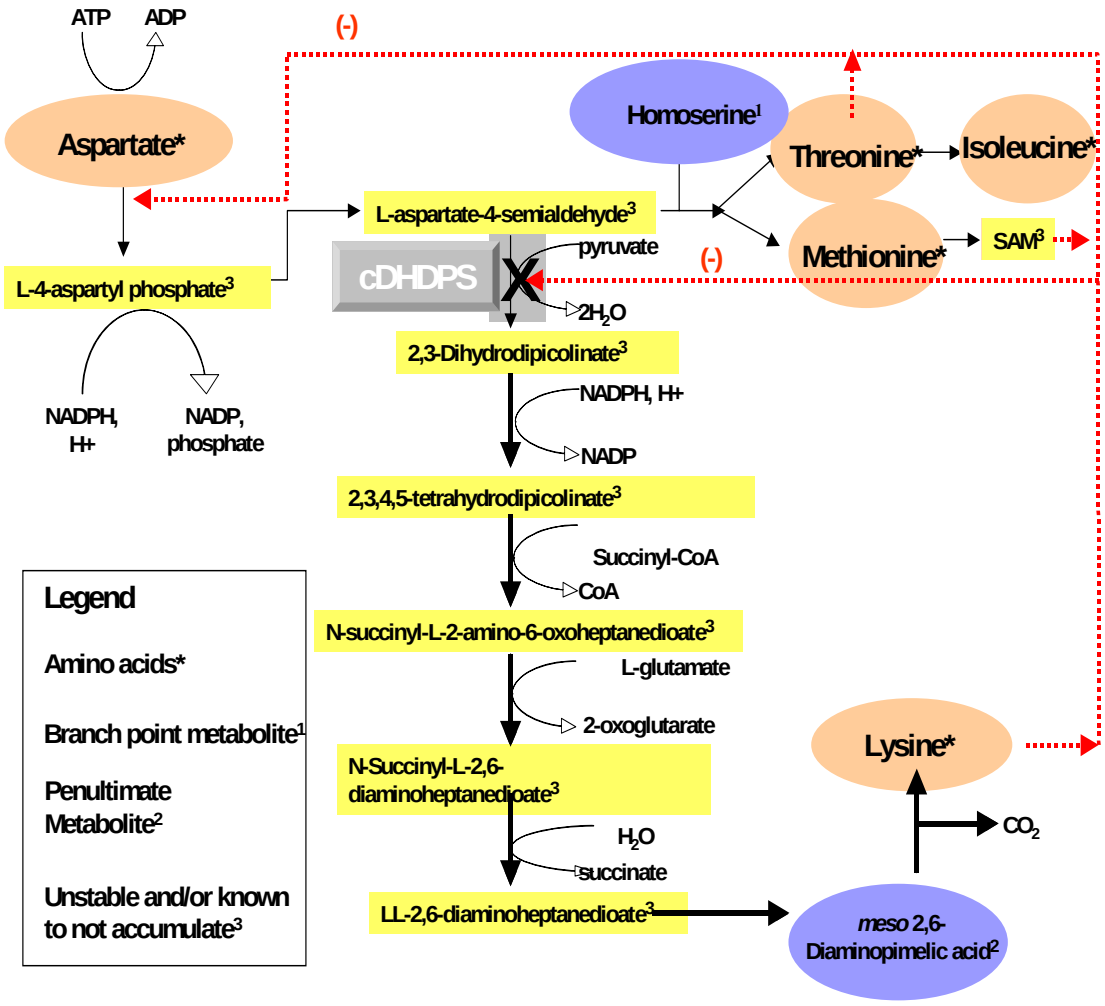


Appendix 5, Figure 1. Aspartate pathway of amino acid biosynthesis

2. Selection of Metabolites in the Lysine Synthesis Pathway

The lysine biosynthetic metabolites identified for analysis in this study were chosen based on whether they were key amino acids, stable branch point metabolites or penultimate metabolites. Many amino acids in the aspartate amino acid biosynthesis pathway related to lysine are currently part of the composition protocol and were analyzed. These include aspartate, threonine, isoleucine, methionine and lysine (indicated with an asterisk, Figure 2). In addition, the branch point analyte homoserine was included because it is the first branch point after aspartate dedicated to the synthesis of methionine, threonine and isoleucine and serves as an indicator of changes in these pathways (superscript[1], Figure 2). The penultimate metabolite in lysine synthesis is 2-6 diaminopimelic acid: penultimate metabolites are often excellent indicators of pathway flux (superscript [2], Figure 2). All of the other intermediary metabolites in the pathway are potentially unstable or known to be present at very low levels

in plant species (superscript[3], Figure 2) and were not analyzed (Glawischnig et al., 2001; Moller, 1974; and Larsen and Norris, 1976). Therefore, key amino acids, branch point, and penultimate metabolites were chosen for analysis.



Appendix 5, Figure 2. Lysine synthesis pathway and metabolites selected for analysis

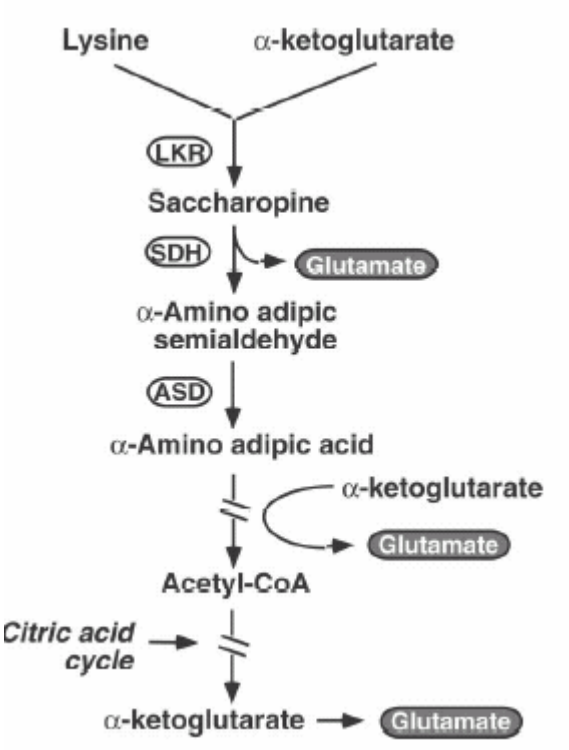
3. Lysine Catabolism

The lysine catabolic pathway has been extensively studied in plant, bacterial and mammalian systems (Galili et al., 2001; Fellows and Lewis, 1973; and Eggling, 1994). In plant cells, excess lysine is catabolized into glutamate and acetyl-CoA via the α-aminoadipic acid pathway (Figure 3). In maize, it has been shown that lysine catabolism is a major mechanism controlling the concentration of free lysine in the endosperm (Arruda et al., 1982). Two- to three-fold higher levels of lysine are transported into developing endosperm (Arruda and Silva, 1983), yet free lysine levels do not increase. Lysine catabolism is important for controlling free lysine levels in plants.

The pathway of lysine catabolism in plants appears to be similar to that operating in human cells (Figure 3). In both organisms, lysine is first converted to saccharopine by lysine-ketoglutarate reductase (LKR) and than saccharopine is converted into α-aminoadipic semialdehyde by saccharopine dehydrogenase (SDH) (Galili, 2002). Several additional enzymatic reactions result in the conversion of α-aminoadipic semialdehyde into acetyl-CoA and glutamate.

The expression of a nonfeedback-inhibited form of DHDPS in tobacco leaf and seed, canola seed, soybean seed, and corn endosperm and embryo resulted in differences in lysine accumulation as well as differences in the presence of the lysine catabolic metabolites saccharopine and α- aminoadipic acid (Mazur et al., 1999) (Table 1). Therefore, it is known that lysine and lysine catabolite levels after expression of a nonfeedback-inhibited form of DHDPS are dependent on the context (plant type and tissue).

In some plant species, lysine can be decarboxylated to the metabolite cadaverine through the action of the enzyme lysine decarboxylase. Lysine decarboxylase activity was detected in 30 *Leguminosae* species, 17 non-*Leguminosae* and seven different plant callus or suspension cultures (Shoofs and Teichmann, 1983). Also, pipecolic acid (PA) can be produced from saccharopine in some plant species. PA was first discovered to be a prominent component of leguminous fruits and seeds in 1952 (Zacharius, 1952) and is abundant in a number of halophytic and sand dune plants subjected to permanent water stress. Therefore, both cadaverine and PA were selected for analysis in this study since they were known to be products of lysine catabolism in many plant species.



Appendix 5, Figure 3. Lysine catabolism via the α-aminoadipic acid pathway (LKR=lysine ketoglutarate reductase, SDH=saccharopine dehydrogenase, ASD= aminoadipic semialdehyde dehydrogenase) (Galili, 2002)

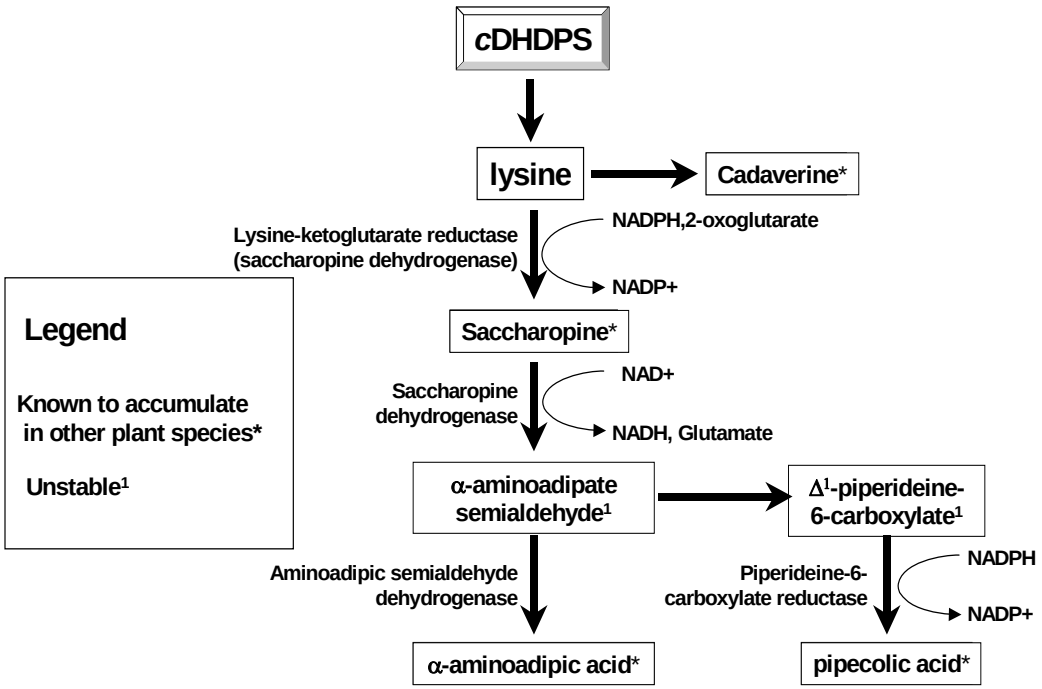
Appendix 5, Table 1. Summary of results from the over-expression of a feedback insensitive DHDPS enzyme in different species and organs.

Tissue	Lysine Increase	Saccharopine	A-Aminoadipic Acid
Tobacco leaf	Yes	No	No
Tobacco seed	No	No	Transient
Canola seed	Yes	No	High
Soybean seed	Yes	High	Low
Corn endosperm	No	No	No
Corn embryo	Yes	Low	Low

Modified from Mazur et al., 1999

4. Selection of Metabolites in the Lysine Catabolic Pathway

To ensure complete coverage of the catabolic products, all the major metabolites that are possible end products of lysine catabolism were analyzed (asterisk, Figure 4). This includes cadaverine, saccharopine, α-aminoadipic acid and pipecolic acid (superscript[1], Figure 4). The additional two intermediates, α-aminoadipate semialdehyde and Δ^1-piperideine-6-carboxylate, spontaneously cyclize, and the direction of the equilibrium under physiological and extraction conditions is unknown; therefore they were not selected for analysis.



From: Galili, G. 1995 Regulation of lysine and threonine synthesis. The Plant Cell 7:899-906

Appendix 5, Figure 4. Lysine catabolism pathway and metabolites selected for analysis

C. Summary

The analytes evaluated in the compositional assessment cover greater than 70 key nutritional and antinutritional components related to feed and food safety. This provides a rational scientific basis for understanding the compositional equivalence of these components in Lysine maize compared to conventional maize. Specific lysine associated metabolites that were analyzed came from the biosynthetic and catabolic pathways. These metabolites include free lysine, cadaverine, α-aminoadipic acid, saccharopine, homo-serine, L-pipecolic acid, and 2,6-diaminopimelic acid. The addition of these metabolites provides a comprehensive assessment of changes associated with presence and production of high lysine levels in maize grain.

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APPENDIX 6. Forage and Grain Composition Across All Sites (Combined Site)

Appendix 6, Table 1. Summary of combined sites maize forage lysine, fiber, calcium, phosphorus, and proximate content for LY038 vs. LY038(-)

			Difference (LY038 minus Comparator)			
Analytical Component	**Hybrid**	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower,Upper)**	**p-Value**	**Reference (Range) [99% T. I.[1]]**
Amino Acid						
Lysine (% Total Prot. DW)	LY038	4.70 ± 0.21 (4.00 - 6.46)				(3.28 - 6.11) [3.17,5.56]
	LY038(-)	4.54 ± 0.21 (3.70 - 5.94)	0.16 ± 0.18 (-0.64 - 1.04)	-0.20,0.51	0.379	
Fiber						
Acid Detergent Fiber (% DW)	LY038	26.59 ± 1.18 (23.51 - 29.20)				(19.73 - 35.22) [17.65,36.77]
	LY038(-)	26.25 ± 1.18 (19.85 - 34.61)	0.34 ± 1.36 (-6.98 - 8.08)	-2.46,3.15	0.803	
Neutral Detergent Fiber (% DW)	LY038	40.62 ± 1.38 (34.00 - 46.89)				(28.00 - 55.71) [27.86,54.72]
	LY038(-)	41.12 ± 1.38 (34.92 - 49.80)	-0.49 ± 1.64 (-10.32 - 8.15)	-3.76,2.77	0.766	

Appendix 6, Table 1. Summary of combined sites maize forage lysine, fiber, calcium, phosphorus, and proximate content for LY038 vs. LY038(-)

			Difference (LY038 minus Comparator)			
Analytical Component	**Hybrid**	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower,Upper)**	**p-Value**	**Reference (Range) [99% T. I.[1]]**
Mineral						
Calcium (% DW)	LY038	0.22 ± 0.014 (0.14 - 0.29)				(0.12 - 0.34) [0.11,0.31]
	LY038(-)	0.21 ± 0.014 (0.15 - 0.25)	0.014 ± 0.011 (-0.037 - 0.076)	-0.0086,0.037	0.218	
Phosphorus (% DW)	LY038	0.20 ± 0.015 (0.13 - 0.27)				(0.14 - 0.29) [0.10,0.30]
	LY038(-)	0.22 ± 0.015 (0.17 - 0.29)	-0.018 ± 0.0071 (-0.059 - 0.021)	-0.032,-0.0039	0.012	
Proximate						
Ash (% DW)	LY038	4.34 ± 0.43 (2.76 - 6.85)				(1.98 - 5.94) [0.63,6.85]
	LY038(-)	4.22 ± 0.43 (2.96 - 6.39)	0.13 ± 0.28 (-1.05 - 1.01)	-0.42,0.67	0.646	
Carbohydrates (% DW)	LY038	87.47 ± 0.95 (83.65 - 92.50)				(83.17 - 92.17) [81.74,94.75]

Appendix 6, Table 1. Summary of combined sites maize forage lysine, fiber, calcium, phosphorus, and proximate content for LY038 vs. LY038(-)

Analytical Component	Hybrid	Mean ± S.E. (Range)	Difference (LY038 minus Comparator) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Reference (Range) [99% T. I.[1]]
	LY038(-)	86.87 ± 0.95 (82.47 - 91.67)	0.60 ± 0.50 (-2.14 - 2.35)	-0.43,1.64	0.238	
Moisture (% FW)	LY038	73.42 ± 1.04 (69.60 - 76.10)				(60.00 - 76.30) [58.09,78.25]
	LY038(-)	72.99 ± 1.04 (67.30 - 76.20)	0.43 ± 0.65 (-2.00 - 5.90)	-0.87,1.72	0.514	
Protein (% DW)	LY038	6.60 ± 0.59 (2.69 - 8.63)				(3.85 - 9.34) [2.42,10.42]
	LY038(-)	6.99 ± 0.59 (3.33 - 8.74)	-0.39 ± 0.33 (-1.48 - 2.01)	-1.06,0.29	0.252	
Total Fat (% DW)	LY038	1.58 ± 0.24 (0.77 - 2.96)				(0.55 - 3.54) [0,3.45]
	LY038(-)	1.93 ± 0.24 (0.73 - 2.83)	-0.35 ± 0.18 (-1.53 - 0.64)	-0.71,0.013	0.058	

[1] T.I. = tolerance interval specified to contain with 95% confidence, 99% of the population of conventional maize, negative limits set to zero.

Appendix 6, Table 2. Summary of combined sites maize grain amino acid, fatty acid, fiber, mineral, proximate, secondary metabolite, vitamin, phytic acid, and raffinose content for LY038 vs. LY038(-)

			Difference (LY038 minus Comparator)			
Analytical Component	**Hybrid**	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower,Upper)**	**p-Value**	**Reference (Range) [99% T. I.[1]]**
Amino Acid						
Alanine (% Total AA)	LY038	7.81 ± 0.065 (7.62 - 8.03)				(7.22 - 8.33) [6.90,8.67]
	LY038(-)	7.88 ± 0.065 (7.49 - 8.05)	-0.069 ± 0.043 (-0.27 - 0.20)	-0.16,0.020	0.122	
Arginine (% Total AA)	LY038	4.26 ± 0.10 (3.79 - 4.73)				(3.88 - 6.00) [3.32,6.04]
	LY038(-)	4.32 ± 0.10 (3.84 - 4.77)	-0.066 ± 0.095 (-0.60 - 0.55)	-0.26,0.12	0.491	
Aspartic Acid (% Total AA)	LY038	6.20 ± 0.048 (5.89 - 6.44)				(5.84 - 7.13) [5.86,7.16]
	LY038(-)	6.24 ± 0.048 (5.87 - 6.53)	-0.033 ± 0.051 (-0.41 - 0.46)	-0.14,0.069	0.523	
Cystine (% Total AA)	LY038	2.03 ± 0.053 (1.90 - 2.13)				(1.76 - 2.55) [1.48,2.80]
	LY038(-)	2.07 ± 0.053 (1.90 - 2.33)	-0.037 ± 0.038 (-0.28 - 0.11)	-0.12,0.042	0.344	

Appendix 6, Table 2. Summary of combined sites maize grain amino acid, fatty acid, fiber, mineral, proximate, secondary metabolite, vitamin, phytic acid, and raffinose content for LY038 vs. LY038(-)

			Difference (LY038 minus Comparator)			
Analytical Component	**Hybrid**	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower,Upper)**	**p-Value**	**Reference (Range) [99% T. I.[1]]**
Glutamic Acid (% Total AA)	LY038	19.98 ± 0.21 (19.14 - 20.55)				(18.02 - 21.86) [16.76,22.36]
	LY038(-)	20.35 ± 0.21 (19.22 - 20.96)	-0.37 ± 0.12 (-1.01 - 0.46)	-0.61,-0.13	0.002	
Glycine (% Total AA)	LY038	3.43 ± 0.081 (3.22 - 3.86)				(3.27 - 4.61) [2.65,4.98]
	LY038(-)	3.51 ± 0.081 (3.16 - 3.99)	-0.076 ± 0.048 (-0.47 - 0.27)	-0.18,0.023	0.125	
Histidine (% Total AA)	LY038	2.76 ± 0.040 (2.63 - 2.89)				(2.63 - 3.39) [2.32,3.64]
	LY038(-)	2.88 ± 0.040 (2.68 - 3.06)	-0.12 ± 0.030 (-0.25 - 0.096)	-0.18,-0.059	<0.001	
Isoleucine (% Total AA)	LY038	3.41 ± 0.043 (3.21 - 3.54)				(3.24 - 3.92) [3.13,3.87]
	LY038(-)	3.52 ± 0.043 (3.32 - 3.76)	-0.11 ± 0.043 (-0.35 - 0.044)	-0.20,-0.023	0.014	

Appendix 6, Table 2. Summary of combined sites maize grain amino acid, fatty acid, fiber, mineral, proximate, secondary metabolite, vitamin, phytic acid, and raffinose content for LY038 vs. LY038(-)

			Difference (LY038 minus Comparator)			
Analytical Component	**Hybrid**	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower,Upper)**	**p-Value**	**Reference (Range) [99% T. I.[1]]**
Leucine (% Total AA)	LY038	13.53 ± 0.19 (12.76 - 14.19)				(11.13 - 14.35) [10.15,15.62]
	LY038(-)	13.64 ± 0.19 (12.63 - 14.48)	-0.11 ± 0.13 (-0.87 - 0.70)	-0.38,0.16	0.420	
Lysine (% Total AA)	LY038	3.81 ± 0.14 (3.08 - 4.50)				(2.38 - 4.07) [1.85,4.29]
	LY038(-)	2.70 ± 0.14 (2.14 - 3.23)	1.11 ± 0.12 (0.49 - 1.98)	0.87,1.34	<0.001	
Methionine (% Total AA)	LY038	2.13 ± 0.046 (1.88 - 2.40)				(1.54 - 2.41) [1.47,2.46]
	LY038(-)	2.05 ± 0.046 (1.85 - 2.37)	0.072 ± 0.054 (-0.35 - 0.41)	-0.039,0.18	0.193	
Phenylalanine (% Total AA)	LY038	5.14 ± 0.048 (4.97 - 5.25)				(4.67 - 5.43) [4.49,5.68]
	LY038(-)	5.22 ± 0.048 (4.86 - 5.41)	-0.081 ± 0.031 (-0.27 - 0.23)	-0.14,-0.020	0.009	

Appendix 6, Table 2. Summary of combined sites maize grain amino acid, fatty acid, fiber, mineral, proximate, secondary metabolite, vitamin, phytic acid, and raffinose content for LY038 vs. LY038(-)

			Difference (LY038 minus Comparator)			
Analytical Component	**Hybrid**	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower,Upper)**	**p-Value**	**Reference (Range) [99% T. I.[1]]**
Proline (% Total AA)	LY038	8.87 ± 0.10 (8.04 - 9.34)				(7.92 - 10.18) [7.89,10.23]
	LY038(-)	9.08 ± 0.10 (8.38 - 9.40)	-0.22 ± 0.11 (-1.23 - 0.51)	-0.44,0.0077	0.058	
Serine (% Total AA)	LY038	5.06 ± 0.054 (4.84 - 5.32)				(4.79 - 5.55) [4.73,5.60]
	LY038(-)	5.11 ± 0.054 (4.90 - 5.37)	-0.056 ± 0.042 (-0.31 - 0.23)	-0.14,0.027	0.186	
Threonine (% Total AA)	LY038	3.11 ± 0.039 (2.91 - 3.26)				(2.84 - 3.62) [2.73,3.82]
	LY038(-)	3.20 ± 0.039 (2.93 - 3.46)	-0.095 ± 0.053 (-0.38 - 0.33)	-0.20,0.013	0.082	
Tryptophan (% Total AA)	LY038	0.52 ± 0.024 (0.40 - 0.64)				(0.45 - 0.90) [0.29,0.89]
	LY038(-)	0.55 ± 0.024 (0.43 - 0.72)	-0.032 ± 0.018 (-0.20 - 0.096)	-0.069,0.0054	0.090	

Appendix 6, Table 2. Summary of combined sites maize grain amino acid, fatty acid, fiber, mineral, proximate, secondary metabolite, vitamin, phytic acid, and raffinose content for LY038 vs. LY038(-)

			Difference (LY038 minus Comparator)			
Analytical Component	**Hybrid**	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower,Upper)**	**p-Value**	**Reference (Range) [99% T. I.[1]]**
Tyrosine (% Total AA)	LY038	3.34 ± 0.18 (2.26 - 3.85)				(1.83 - 3.82) [2.04,4.17]
	LY038(-)	3.02 ± 0.18 (2.17 - 4.68)	0.32 ± 0.26 (-1.29 - 1.38)	-0.22,0.85	0.234	
Valine (% Total AA)	LY038	4.62 ± 0.051 (4.37 - 4.85)				(4.42 - 5.22) [4.15,5.51]
	LY038(-)	4.65 ± 0.051 (4.41 - 4.87)	-0.028 ± 0.041 (-0.29 - 0.17)	-0.11,0.057	0.509	
Fatty Acid						
16:0 Palmitic (% Total FA)	LY038	10.86 ± 0.061 (10.58 - 11.83)				(9.27 - 13.15) [7.42,15.14]
	LY038(-)	10.96 ± 0.061 (10.78 - 11.25)	-0.10 ± 0.075 (-0.41 - 0.96)	-0.26,0.052	0.184	

Appendix 6, Table 2. Summary of combined sites maize grain amino acid, fatty acid, fiber, mineral, proximate, secondary metabolite, vitamin, phytic acid, and raffinose content for LY038 vs. LY038(-)

			Difference (LY038 minus Comparator)			
Analytical Component	**Hybrid**	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower,Upper)**	**p-Value**	**Reference (Range) [99% T. I.[1]]**
18:0 Stearic (% Total FA)	LY038	2.26 ± 0.031 (2.06 - 2.42)				(1.65 - 2.42) [1.26,2.67]
	LY038(-)	2.20 ± 0.031 (2.12 - 2.27)	0.058 ± 0.037 (-0.21 - 0.26)	-0.018,0.13	0.127	
18:1 Oleic (% Total FA)	LY038	31.81 ± 0.41 (30.62 - 33.39)				(21.44 - 35.65) [9.97,43.10]
	LY038(-)	30.59 ± 0.41 (29.08 - 31.49)	1.22 ± 0.31 (-0.30 - 2.66)	0.58,1.87	<0.001	
18:2 Linoleic (% Total FA)	LY038	53.24 ± 0.40 (51.77 - 54.41)				(50.16 - 64.33) [42.12,74.18]
	LY038(-)	54.48 ± 0.40 (53.61 - 55.93)	-1.23 ± 0.30 (-2.86 - 0.44)	-1.85,-0.62	<0.001	
18:3 Linolenic (% Total FA)	LY038	0.96 ± 0.017 (0.89 - 1.02)				(0.83 - 1.53) [0.61,1.81]
	LY038(-)	0.91 ± 0.017 (0.86 - 0.97)	0.048 ± 0.015 (-0.0020 - 0.13)	0.017,0.078	0.003	

Appendix 6, Table 2. Summary of combined sites maize grain amino acid, fatty acid, fiber, mineral, proximate, secondary metabolite, vitamin, phytic acid, and raffinose content for LY038 vs. LY038(-)

			Difference (LY038 minus Comparator)			
Analytical Component	**Hybrid**	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower,Upper)**	**p-Value**	**Reference (Range) [99% T. I.[1]]**
20:0 Arachidic (% Total FA)	LY038	0.44 ± 0.0066 (0.42 - 0.48)				(0.35 - 0.48) [0.31,0.52]
	LY038(-)	0.42 ± 0.0066 (0.39 - 0.45)	0.014 ± 0.0045 (-0.016 - 0.036)	0.0043,0.023	0.005	
20:1 Eicosenoic (% Total FA)	LY038	0.27 ± 0.0034 (0.26 - 0.29)				(0.20 - 0.35) [0.16,0.41]
	LY038(-)	0.29 ± 0.0034 (0.28 - 0.31)	-0.021 ± 0.0027 (-0.033 - 0.012)	-0.026,-0.015	<0.001	
22:0 Behenic (% Total FA)	LY038	0.16 ± 0.010 (0.14 - 0.19)				(0.071 - 0.27) [0.030,0.28]
	LY038(-)	0.14 ± 0.010 (0.13 - 0.17)	0.014 ± 0.0085 (-0.0020 - 0.041)	-0.0033,0.032	0.107	

Appendix 6, Table 2. Summary of combined sites maize grain amino acid, fatty acid, fiber, mineral, proximate, secondary metabolite, vitamin, phytic acid, and raffinose content for LY038 vs. LY038(-)

			Difference (LY038 minus Comparator)			
Analytical Component	**Hybrid**	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower,Upper)**	**p-Value**	**Reference (Range) [99% T. I.[1]]**
Fiber						
Acid Detergent Fiber (% DW)	LY038	6.57 ± 0.42 (4.66 - 11.31)				(4.29 - 9.56) [2.64,10.00]
	LY038(-)	5.80 ± 0.42 (4.20 - 6.82)	0.77 ± 0.43 (-1.56 - 4.49)	-0.11,1.65	0.083	
Neutral Detergent Fiber (% DW)	LY038	12.56 ± 1.08 (8.01 - 18.28)				(9.93 - 20.57) [5.82,21.51]
	LY038(-)	10.19 ± 1.08 (7.89 - 13.03)	2.37 ± 1.00 (-4.36 - 6.75)	0.32,4.42	0.025	
Total Dietary Fiber (% DW)	LY038	20.77 ± 2.48 (11.90 - 39.65)				(12.58 - 35.31) [3.77,39.08]
	LY038(-)	15.99 ± 2.48 (10.96 - 21.30)	4.78 ± 2.25 (-4.26 - 18.35)	0.16,9.39	0.042	

Appendix 6, Table 2. Summary of combined sites maize grain amino acid, fatty acid, fiber, mineral, proximate, secondary metabolite, vitamin, phytic acid, and raffinose content for LY038 vs. LY038(-)

			Difference (LY038 minus Comparator)			
Analytical Component	**Hybrid**	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower,Upper)**	**p-Value**	**Reference (Range) [99% T. I.[1]]**
Mineral						
Calcium (% DW)	LY038	0.0046 ± 0.00022 (0.0039 - 0.0059)				(0.0030 - 0.0075) [0.0013,0.0076]
	LY038(-)	0.0054 ± 0.00022 (0.0043 - 0.0064)	-0.00082 ± 0.00023 (-0.0022 - 0.0016)	-0.0013,-0.00036	0.001	
Copper (mg/kg DW)	LY038	2.20 ± 0.12 (1.85 - 3.91)				(1.12 - 2.58) [0.45,2.97]
	LY038(-)	1.78 ± 0.12 (1.53 - 2.03)	0.41 ± 0.17 (-0.029 - 2.24)	0.073,0.75	0.018	
Iron (mg/kg DW)	LY038	24.15 ± 0.74 (20.29 - 37.09)				(15.39 - 27.88) [11.29,30.67]
	LY038(-)	23.40 ± 0.74 (20.13 - 29.75)	0.75 ± 1.03 (-2.85 - 16.96)	-1.36,2.87	0.471	

Appendix 6, Table 2. Summary of combined sites maize grain amino acid, fatty acid, fiber, mineral, proximate, secondary metabolite, vitamin, phytic acid, and raffinose content for LY038 vs. LY038(-)

			Difference (LY038 minus Comparator)			
Analytical Component	**Hybrid**	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower,Upper)**	**p-Value**	**Reference (Range) [99% T. I.[1]]**
Magnesium (% DW)	LY038	0.14 ± 0.0036 (0.13 - 0.16)				(0.087 - 0.15) [0.075,0.16]
	LY038(-)	0.14 ± 0.0036 (0.12 - 0.15)	0.0045 ± 0.0035 (-0.017 - 0.043)	-0.0028,0.012	0.214	
Manganese (mg/kg DW)	LY038	6.98 ± 0.52 (5.16 - 9.30)				(3.33 - 10.22) [0.26,12.49]
	LY038(-)	7.72 ± 0.52 (5.70 - 9.64)	-0.73 ± 0.22 (-2.42 - 1.27)	-1.17,-0.29	0.001	
Phosphorus (% DW)	LY038	0.37 ± 0.010 (0.31 - 0.44)				(0.25 - 0.41) [0.21,0.44]
	LY038(-)	0.37 ± 0.010 (0.31 - 0.43)	0.00041 ± 0.0095 (-0.087 - 0.099)	-0.020,0.019	0.966	

Appendix 6, Table 2. Summary of combined sites maize grain amino acid, fatty acid, fiber, mineral, proximate, secondary metabolite, vitamin, phytic acid, and raffinose content for LY038 vs. LY038(-)

			Difference (LY038 minus Comparator)			
Analytical Component	**Hybrid**	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower,Upper)**	**p-Value**	**Reference (Range) [99% T. I.[1]]**
Potassium (% DW)	LY038	0.37 ± 0.011 (0.29 - 0.44)				(0.32 - 0.46) [0.28,0.46]
	LY038(-)	0.38 ± 0.011 (0.34 - 0.45)	-0.012 ± 0.0078 (-0.10 - 0.066)	-0.028,0.0041	0.136	
Zinc (mg/kg DW)	LY038	26.19 ± 1.04 (22.01 - 31.22)				(15.94 - 33.80) [8.94,39.24]
	LY038(-)	24.27 ± 1.04 (20.53 - 28.18)	1.92 ± 0.59 (-1.50 - 8.00)	0.72,3.12	0.002	
Proximate						
Ash (% DW)	LY038	1.44 ± 0.033 (1.19 - 1.73)				(1.05 - 1.75) [0.92,1.84]
	LY038(-)	1.44 ± 0.033 (1.29 - 1.73)	0.0014 ± 0.045 (-0.28 - 0.36)	-0.088,0.091	0.974	

Appendix 6, Table 2. Summary of combined sites maize grain amino acid, fatty acid, fiber, mineral, proximate, secondary metabolite, vitamin, phytic acid, and raffinose content for LY038 vs. LY038(-)

			Difference (LY038 minus Comparator)			
Analytical Component	**Hybrid**	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower,Upper)**	**p-Value**	**Reference (Range) [99% T. I.[1]]**
Carbohydrates (% DW)	LY038	81.80 ± 0.62 (80.24 - 84.13)				(80.26 - 87.96) [78.12,92.06]
	LY038(-)	82.02 ± 0.62 (80.34 - 84.75)	-0.22 ± 0.25 (-1.21 - 0.97)	-0.71,0.27	0.375	
Moisture (% FW)	LY038	8.91 ± 0.40 (7.47 - 10.50)				(7.68 - 11.10) [6.32,11.00]
	LY038(-)	9.40 ± 0.40 (8.48 - 11.30)	-0.49 ± 0.26 (-2.03 - 0.99)	-1.03,0.051	0.074	
Protein (% DW)	LY038	12.90 ± 0.56 (11.44 - 14.48)				(7.61 - 14.69) [3.86,17.17]
	LY038(-)	12.12 ± 0.56 (9.59 - 13.79)	0.78 ± 0.23 (-0.24 - 1.86)	0.31,1.24	0.002	
Total Fat (% DW)	LY038	3.86 ± 0.20 (3.00 - 4.72)				(2.03 - 4.53) [1.36,4.67]
	LY038(-)	4.42 ± 0.20 (4.00 - 5.16)	-0.56 ± 0.12 (-1.41 - 0.067)	-0.81,-0.31	<0.001	

Appendix 6, Table 2. Summary of combined sites maize grain amino acid, fatty acid, fiber, mineral, proximate, secondary metabolite, vitamin, phytic acid, and raffinose content for LY038 vs. LY038(-)

			Difference (LY038 minus Comparator)			
Analytical Component	**Hybrid**	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower,Upper)**	**p-Value**	**Reference (Range) [99% T. I.[1]]**
Secondary Metabolite						
Ferulic Acid (µg/g DW)	LY038	2285.73 ± 86.33 (1988.83 - 2701.83)				(1935.84 - 3638.14) [1138.95,3687.86]
	LY038(-)	2257.63 ± 92.44 (1970.88 - 2551.97)	28.09 ± 126.48 (-295.21 - 543.47)	-244.52,300.71	0.827	
Free Lysine (µg/g DW)	LY038	1351.13 ± 109.52 (921.86 - 1696.61)				(14.69 - 108.52) [0,104.89]
	LY038(-)	25.99 ± 3.18 (18.39 - 40.21)	1325.14 ± 109.57 (903.47 - 1671.07)	1043.64,1606.65	<0.001	
Homo-serine (µg/g DW)	LY038	11.18 ± 3.86 (5.48 - 29.32)				(2.72 - 92.67) [0,83.82]
	LY038(-)	12.01 ± 5.55 (2.75 - 37.84)	-0.83 ± 6.76 (-12.86 - 5.26)	-16.67,15.02	0.906	

Appendix 6, Table 2. Summary of combined sites maize grain amino acid, fatty acid, fiber, mineral, proximate, secondary metabolite, vitamin, phytic acid, and raffinose content for LY038 vs. LY038(-)

			Difference (LY038 minus Comparator)			
Analytical Component	**Hybrid**	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower,Upper)**	**p-Value**	**Reference (Range) [99% T. I.[1]]**
L-Pipecolinic Acid (µg/g DW)	LY038	28.72 ± 1.37 (22.37 - 35.35)				(2.71 - 42.14) [0,45.15]
	LY038(-)	14.96 ± 1.58 (10.06 - 21.82)	13.76 ± 2.09 (9.05 - 25.30)	9.28,18.24	<0.001	
Saccharopine (µg/g DW)	LY038	650.29 ± 36.40 (499.30 - 818.42)				(2.71 - 20.85) [0,23.00]
	LY038(-)	5.88 ± 0.90 (2.75 - 8.26)	644.42 ± 36.41 (496.54 - 812.58)	554.29,734.54	<0.001	
p-Coumaric Acid (µg/g DW)	LY038	179.86 ± 22.83 (94.40 - 322.23)				(141.55 - 433.26) [17.22,472.67]
	LY038(-)	150.70 ± 19.38 (76.22 - 217.80)	29.16 ± 29.95 (-22.13 - 107.38)	-37.57,95.89	0.353	
Vitamin						
Folic Acid (mg/kg DW)	LY038	0.47 ± 0.029 (0.35 - 0.76)				(0.24 - 0.60) [0.13,0.59]
	LY038(-)	0.40 ± 0.029 (0.33 - 0.54)	0.069 ± 0.023 (-0.0077 - 0.35)	0.022,0.12	0.006	

Appendix 6, Table 2. Summary of combined sites maize grain amino acid, fatty acid, fiber, mineral, proximate, secondary metabolite, vitamin, phytic acid, and raffinose content for LY038 vs. LY038(-)

			Difference (LY038 minus Comparator)			
Analytical Component	**Hybrid**	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower,Upper)**	**p-Value**	**Reference (Range) [99% T. I.[1]]**
Niacin (mg/kg DW)	LY038	19.49 ± 1.08 (17.40 - 21.81)				(14.81 - 39.93) [5.17,37.49]
	LY038(-)	20.84 ± 1.08 (17.82 - 23.87)	-1.35 ± 1.00 (-5.05 - 0.85)	-3.33,0.62	0.177	
Vitamin B1 (mg/kg DW)	LY038	4.07 ± 0.12 (3.52 - 4.64)				(2.51 - 4.34) [1.80,4.83]
	LY038(-)	4.11 ± 0.12 (3.51 - 4.57)	-0.038 ± 0.091 (-0.38 - 0.30)	-0.22,0.15	0.677	
Vitamin B2 (mg/kg DW)	LY038	1.50 ± 0.068 (1.10 - 1.74)				(0.98 - 1.85) [0.77,2.16]
	LY038(-)	1.42 ± 0.068 (1.12 - 1.74)	0.081 ± 0.050 (-0.22 - 0.41)	-0.021,0.18	0.116	
Vitamin B6 (mg/kg DW)	LY038	5.93 ± 0.27 (4.63 - 6.95)				(3.68 - 8.46) [2.50,9.89]
	LY038(-)	5.63 ± 0.27 (4.85 - 8.00)	0.30 ± 0.24 (-1.35 - 1.42)	-0.18,0.78	0.220	

Appendix 6, Table 2. Summary of combined sites maize grain amino acid, fatty acid, fiber, mineral, proximate, secondary metabolite, vitamin, phytic acid, and raffinose content for LY038 vs. LY038(-)

			Difference (LY038 minus Comparator)			
Analytical Component	**Hybrid**	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**95% CI (Lower,Upper)**	**p-Value**	**Reference (Range) [99% T. I.[1]]**
Vitamin E (mg/kg DW)	LY038	9.04 ± 0.87 (6.35 - 12.25)				(6.94 - 19.26) [0.26,24.84]
	LY038(-)	10.63 ± 0.87 (8.30 - 13.35)	-1.60 ± 0.67 (-4.36 - 1.02)	-2.98,-0.21	0.025	
Miscellaneous						
Phytic Acid (% DW)	LY038	0.68 ± 0.038 (0.36 - 0.90)				(0.11 - 0.83) [0.12,0.98]
	LY038(-)	0.77 ± 0.038 (0.51 - 0.97)	-0.088 ± 0.053 (-0.51 - 0.14)	-0.19,0.017	0.099	
Raffinose (% DW)	LY038	0.13 ± 0.013 (0.078 - 0.18)				(0.053 - 0.18) [0.0094,0.22]
	LY038(-)	0.15 ± 0.013 (0.12 - 0.21)	-0.015 ± 0.0085 (-0.064 - 0.019)	-0.032,0.0025	0.089	

[1] T.I. = tolerance interval specified to contain with 95% confidence, 99% of the population of conventional maize, negative limits set to zero.

APPENDIX 7. USDA Field Trial Notifications for LY038

Appendix 7, Table 1. USDA field trial notifications approved for LY038

USDA Reference Number	Effective Date	Approved Release Sites (by state) Covered by notification # of release sites in a state in parentheses.
2000 Field Trials		
00-098-02n	05/22/2000	CT, HI (3), IA (2), IL (4), PR (5)
00-256-06n	10/19/2000	HI (3), PR (2)
2001 Field Trials		
01-047-10n	03/18/2001	CT, IA (5), IL (6)
01-088-04n	05/17/2001	HI (3), PR (2)
01-267-01n	11/16/2001	PR (2)
01-267-03n	11/19/2001	HI (6)
01-332-02n	01/10/2002	HI (5)
2002 Field Trials		
02-031-01n	03/02/2002	IL (5)
02-037-05n	03/26/2002	IA (3), IN (3), KS, MN (2), MO (2), NE, WI
02-042-12n	03/18/2002	IL (4)
02-046-32n	03/13/2002	IL (5)
02-052-05n	03/23/2002	IA (2), IL (4), NE, OH (2)
02-058-05n	03/29/2002	IL (2)
02-066-15n	04/10/2002	IL
02-087-08n	04/26/2002	IA (5)
02-087-09n	04/27/2002	IL (4)
02-212-07n	09/10/2002	HI (5)
02-212-10n	08/30/2002	PR (2)
02-220-09n	09/13/2002	HI (12)
02-220-11n	09/13/2002	PR (3)
02-220-12n	09/13/2002	CA
02-263-08n	11/19/2002	HI (3)
2003 Field Trials		
03-052-17n	03/23/2003	IA (3), IL (5), IN, KS (2), MO (3), NE
03-052-31n	03/26/2003	IA (9)
03-052-32n	04/15/2003	IL (2)
03-052-33n	03/26/2003	IA (7)

Appendix 7, Table 1. USDA field trial notifications approved for LY038

USDA Reference Number	Effective Date	Approved Release Sites (by state) Covered by notification # of release sites in a state in parentheses.
03-052-34n	03/23/2003	IL (14)
03-052-35n	03/23/2003	IN (5)
03-052-36n	03/26/2003	MO (2)
03-052-37n	03/23/2003	NE (7)
03-052-38n	03/26/2003	TN (3)
03-052-39n	03/23/2003	WI (2)
03-052-40n	03/25/2003	OH (2)
03-052-41n	03/28/2003	HI (6)
03-058-08n	03/29/2003	IL
03-133-06n	07/15/2003	HI (5)
03-133-07n	06/30/2003	PR (2)
03-258-15n*	10/27/2003	HI (6)
03-258-17n*	10/15/2003	PR (3)
03-338-04n*	01/21/2004	CA
2004 Field Trials		
04-006-01n*	02/05/2004	HI (9)
04-006-02n*	02/05/2004	PR (4)
04-014-04n*	02/26/2004	GA
04-014-07n*	02/13/2004	TN (2)
04-022-08n*	02/25/2004	IA (12)
04-022-09n*	02/25/2004	IA (4)
04-022-10n*	02/25/2004	IA (8)
04-022-11n*	02/25/2004	KS (2)
04-022-12n*	02/25/2004	IN (4)
04-022-13n*	02/27/2004	MI (2)
04-023-12n*	02/25/2004	IL (15)
04-023-15n*	02/25/2004	MO (2)
04-023-16n*	02/25/2004	NE (4)
04-023-17n*	02/25/2004	WI (3)
04-028-08n*	03/24/2004	MN (5)
04-028-20n*	02/27/2004	OH (2)
04-030-01n*	03/05/2004	IL (2)
04-030-12n*	03/26/2004	HI (6), PR (2)
04-070-09n*	04/09/2004	CT (2)
04-099-02n*	06/01/2004	MD (2)

* Indicates that field test report has not yet been submitted to APHIS.

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May 5, 2005

Neil Hoffman, Ph.D.
Director, Regulatory Division
Biotechnology Regulatory Services
USDA-APHIS
Regulatory Division Unit 147
4700 River Road
Riverdale, MD 20737

Subject: Response to APHIS/BRS' Letter of Clarification Regarding Lysine Maize LY038, Petition Number 004-229-01p

Dear Dr. Hoffman:

Thank you for your letter of clarification dated March 16, 2005, concerning the Petition for a Determination of Nonregulated Status for Lysine Maize LY038, petition number 004-229-01p. Responses by Monsanto, on behalf of Renessen LLC, a joint venture between Monsanto Company and Cargill Incorporated, are presented in the attached addendum to the petition.

Should you have any questions concerning the attached addendum, please contact either Dr. Russell Schneider, Regulatory Affairs Lead, Washington DC, at 202-383-2866, or me at 314-694-6542.

Sincerely

Donald M. Lucas, Ph.D.
Regulatory Affairs Manager

Attachment

CC: Dr. R.P. Schneider
Dr. S.A. Schuette
Regulatory Files

04-CR-114U

Addendum to Petition number 004-229-01p for a Determination of Nonregulated Status for Lysine Maize LY038

May 6, 2005

This addendum provides responses to APHIS/BRS' letter of clarification dated March 16, 2005, concerning Monsanto's request on behalf of Renessen LLC, a joint venture between Monsanto Company and Cargill Incorporated, for a Determination of Nonregulated Status for Lysine maize LY038.

Summary
QUESTION: Page 6. The first sentence on the page is confusing. It is not clear to what the "26" refers. Please re-write the sentence for clarity.

RESPONSE: "26" is the µg/g dwt of cDHDPS in LY038 grain. cDHDPS protein levels in LY038 tissues are presented in Table V-2 (page 68 of the petition).

Key to Abbreviations
QUESTION: Page 17. There is a typo for *amp*

RESPONSE: The key to the abbreviation for *amp* should read as follows:
"*amp*: coding sequence for the enzyme β-lactamase"

Section II. Biology of Maize
QUESTION: Page 26. Last two sentences. Please provide a reference for the crossing of *Tripsacum floridanum* with maize. This species occurs in southern Florida and can cross with maize. Please provide data to support why this will not be a problem.

RESPONSE: *Tripsacum* spp. (*floridanum*) and others have been tested in experimental crosses with domestic maize as a potential source for genetic improvement to increase resistance to plant diseases and insect pests. Crosses between *Z. mays* and *Tripsacum* have resulted in male sterile progeny with full reproductive fertility only restored after several generations of backcrossing to remove (segregate away) *Tripsacum* chromosomes.

The karyotypes of the maize hybrids possessing the *Tripsacum floridanum* extranuclear genes are summarized in the following table.

Generation	Tripsacum Chromosomes	Maize Chromosomes	% Male Fertility
F_1	18	10	0
BC_1	18	20	0
BC_2	6-18	20	0
BC_3	0-6	20	0
BC_4	0	20 +	100

If LY038 plants were to naturally outcross with *Tripsacum floridanum,* the resulting F_1 progeny are expected to be male sterile based on these data. The male sterile plants would have no obvious advantage in the environment and lack of pollen dispersal would severely limit the ability of the LY038 gene to persist.

Reference: United States Patent. Dewald et al. Patent No.: US 6,657,110 B1 (Dec 2, 2003), Page 7.

Section IV: Donor Genes and Regulatory Sequences
QUESTION: Page 33. For clarity, in the second paragraph, please indicate when these data were collected.

RESPONSE: The absence of the *cre* and *nptII* gene cassettes in LY038 was demonstrated by event-specific PCR analyses conducted during the LY038 development process and by extensive Southern blot analyses conducted during subsequent molecular characterization of LY038 (see Section V.A.2.a.iii and V.A.2.b). The absence of *cre* and *nptII* gene cassettes was further confirmed by Southern blot generational stability analyses over multiple generations representing each branch point of the breeding tree as reported in Section V.B.2. Therefore, the *cre* and *nptII* gene cassettes and their respective expression products are not expected to be present in LY038.

Section V: Genetic Analysis
QUESTION: Page 40, Figure V-I. Please label each fragment with the enzyme combinations used to cut them. It is not clear what restriction enzymes were used to generate the 2nd fragment.

RESPONSE: See following modified Figure V-1 containing the requested restriction enzymes:

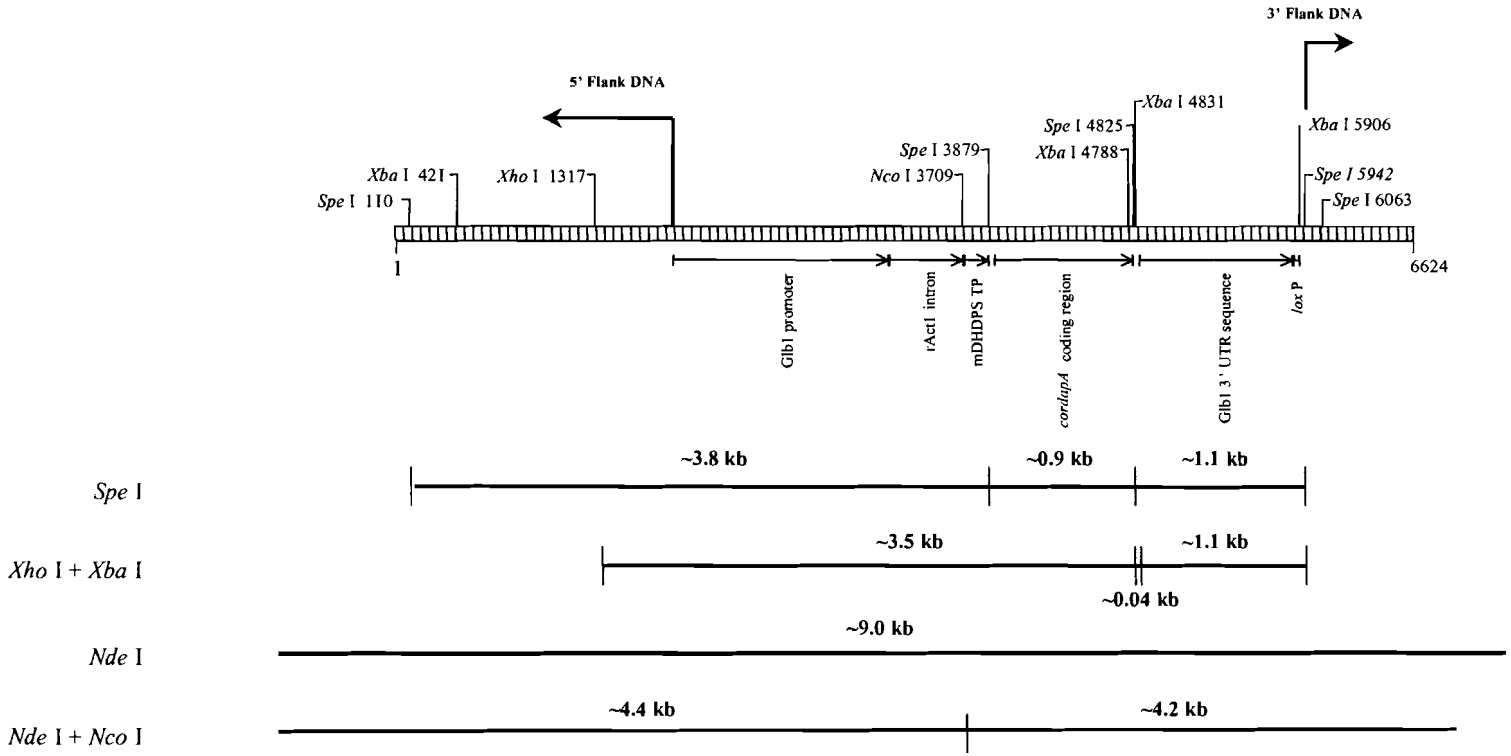

Figure V-1. Map of the insert in LY038
A linear map of the proposed insert and adjacent DNA flanking the insert in LY038 is shown. Arrows indicate the end of the insert and beginning of maize genomic flanking sequence. Identified on the map are genetic elements within the insert, as well as restriction sites with positions relative to the size of the linear map for enzymes used in the Southern analysis.

QUESTION: Page 42 – Section 2.a.ii(b) – The last sentence is not clear. We assume that there is no rice actin intron present and there is only one copy of the intron present in the cassette. It says "that is associated" which implies that there are others not associated with the cassette. Please clarify.

RESPONSE: Because no unexpected bands were detected in Figure V-4 (page 48), the conclusion was that LY038 contains no additional rAct1 intron elements other than those associated with the intact *cordapA* cassette.

QUESTION: Page 43 – Section 2.a.iii(a) – It is not stated in Table IV-1 that an enhanced 35S promoter was used, but from the text one assumes that it was. However, in the last sentence in this section it states that .....LY038 does not contain detectable CaMV 35S or CaMV e35S promoter elements. Were both types of promoters used? Please clarify.

RESPONSE: Yes, both promoters were used. The CaMV 35S promoter was included in the *nptII* cassette of plasmid vector PV-ZMPQ76 (Figures III-1a and 1b, pages 30 and 31 of the petition), and the CaMV e35S promoter was included in the *cre* cassette of plasmid vector PV-ZM003 (Figures V-11a and 11b, pages 55 and 56 of the petition).

QUESTION: Page 46 – Please label Figure V-2 to point out the bands of interest or comparator bands to guide the reader to the relevant bands.

RESPONSE: Symbols (⇦) were added to Figure V-2 to point out the bands of interest (e.g., those bands resulting from hybridization of the probe to the LY038 insert specific DNA fragments).

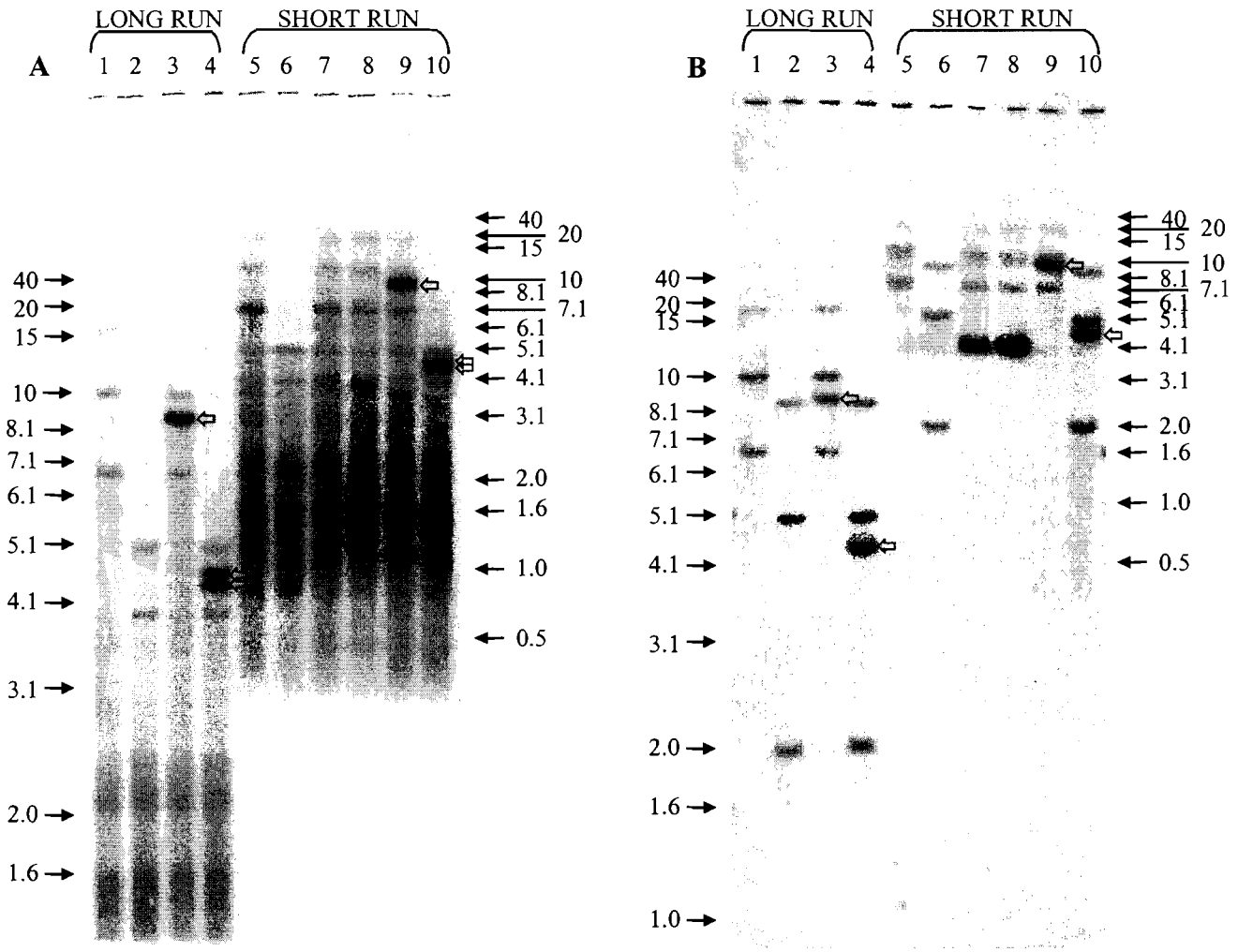

Figure V-2. Southern blot analysis of LY038: Insert and copy number probes
Each blot was examined simultaneously with two ^{32}P-labeled probes that spanned a portion of the I-DNA (Panel A, I-DNA 1 and I-DNA 2, Figure 1), (Panel B, I-DNA 3 and I-DNA 4, Figure 1). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: LY038(-) (*Nde* I)
2: LY038(-) (*Nde* I and *Nco* I)
3: LY038 (*Nde* I)
4: LY038 (*Nde* I and *Nco* I)
5: LY038(-) (*Nde* I)
6: LY038(-) (*Nde* I and *Nco* I)
7: LY038(-) (*Nde* I) spiked with PV-ZMPQ76 (*Eco*R V) [~0.5 copy]
8: LY038(-) (*Nde* I) spiked with PV-ZMPQ76 (*Eco*R V) [~1.0 copy]
9: LY038 (*Nde* I)
10: LY038 (*Nde* I and *Nco* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide-stained gel.

QUESTION: Page 47 – Figure V-3. Below 1.6 in the Long Run there are two bands in lanes 1 and 2 that are not in lanes 3 and 4. Please explain these bands.

RESPONSE: An additional hybridization band of ~1.5 kb was observed in the LY038(-) containing lanes (lanes 1 and 2) that was not observed in the LY038 containing lanes (lanes 3 and 4). The genetic backgrounds associated with LY038 and LY038(-) are complex and the various inbreds that make up these lines are polymorphic. Because this band is not observed in LY038, it is likely not associated with the inserted DNA. Figure V-3 shows that no unexpected bands were detected in the test material.

QUESTION: Page 50 – Figure V-6. Lanes 3 and 4. What is 91INH2? The restriction patterns are different for these. Is this relevant? 91INH2 is discussed in a number of sections but is never defined. Please define and explain its relevance.

RESPONSE: 91INH2 is an elite inbred maize line (indentified as "P1" in Figure V-16, page 63) with which transformants (shown as "R0" at the top of Figure V-16) were crossed to produce the "F1'" generation containing the *nptII* gene cassette that was subsequently excised by Cre-mediated recombination as described on page 62 and in the legend to Figure V-16.

QUESTION: Page 59 – Figure V-14, The legend indicates that two probes were used but three are listed. Were two or three used?

RESPONSE: As reported correctly in Section 2.b.iv. (page 45) "Southern analysis was performed with three overlapping probes (Probes 21, 22 and 23, Figure V-11a) that span the backbone present in PV-ZM003." The title and first sentence of the legend of Figure V-14 (page 59) should read as follows:

Figure V-14. Southern blot analysis of LY038: PV-ZM003 backbone probes The blot was examined simultaneously with three ^{32}P-labeled probes that spanned the entire plasmid PV-ZM003 backbone sequence (probes 21, 22, and 23, Figure V-11a).

A corrected Figure 14 is provide on the following page.

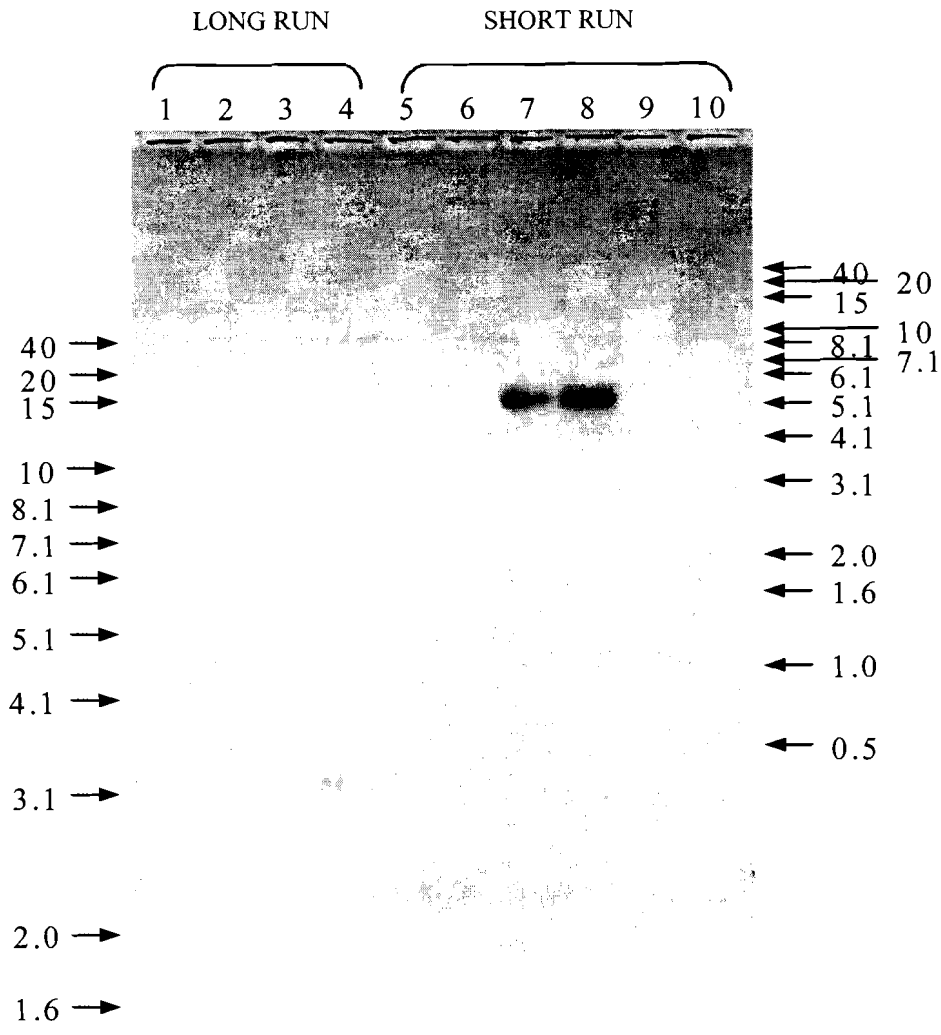

Figure V-14. Southern blot analysis of LY038: PV-ZM003 backbone probes
The blot was examined simultaneously with three ^{32}P-labeled probes that spanned the entire plasmid PV-ZM003 backbone sequence (probes 21, 22, and 23, Figure V-11a). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: LY038(-) (*Spe* I)
2: LY038(-) (*Xho* I and *Xba* I)
3: LY038 (*Spe* I)
4: LY038 (*Xho* I and *Xba* I)
5: LY038(-) (*Spe* I)
6: LY038(-) (*Xho* I and *Xba* I)
7: LY038(-) (*Spe* I) spiked with PV-ZM003 (*Hin*d III *and Mun* I) [0.5 copy]
8: LY038(-) (*Spe* I) spiked with PV-ZM003 (*Hin*d III *and Mun* I) [1.0 copy]
9: LY038 (*Spe* I)
10: LY038 (*Xho* I and *Xba* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

QUESTION: Page 60 – Figure V-15. What are the bands in lane 3?

RESONSE: Polymerase chain reaction with LY038(-) control DNA produced faint products; however, none of these products was the expected 4.1 kb in size. Because one of the primers used to generate Product A was located in the genomic DNA sequence flanking the insert, this PCR primer is most likely amplifying sequences in the maize genome. These products were not observed using the LY038 test material. This is likely because the intended target sequence preferentially hybridizes with the PCR primers compared to the endogenous maize sequence, thereby impairing observation of the maize genomic amplicons.

QUESTION: Page 63 – The nomenclature used on the chart on page 63 does not correspond to that used on Figure V-17, page 65. Please make the two consistent. Also on page 63 (P2xF4)F2 is not on Figure V-16, but it is in the legend.

RESPONSE: Clarification of nomenclature used in Figure V-16 and Figure V-17 is provided in the table below.

Figure V-16 Designation	=	**Figure V-17 and 18 Designation**
F2	=	**LY038-91INH2 F_2**
F3	=	**LY038-91INH2 F_3**
(P2×F3)F1	=	**[LH195 × LY038-91INH2 F_3] $_{(H)}$**
(P2×F3)F2 Control	=	**[LH195 × LY038(-)-91INH2 F_3]F_2**
(P2×F3)F2	=	**[LH195 × LY038-91INH2 F_3]F_2**
F4	=	**LY038-91INH2 F_4**
(P2×F4)F1 Control	=	**[LH195 × LY038(-)-91INH2 F_4] $_{(H)}$**
(P2×F4)F1	=	**[LH195 × LY038-91INH2 F_4] $_{(H)}$**
(P6×F4)F1	=	**[HOI002 × LY038-91INH2 F_4] $_{(H)}$**

RESPONSE (continued): The (P2×F4)F2 generation has been added to Figure V-16 shown on the next page.

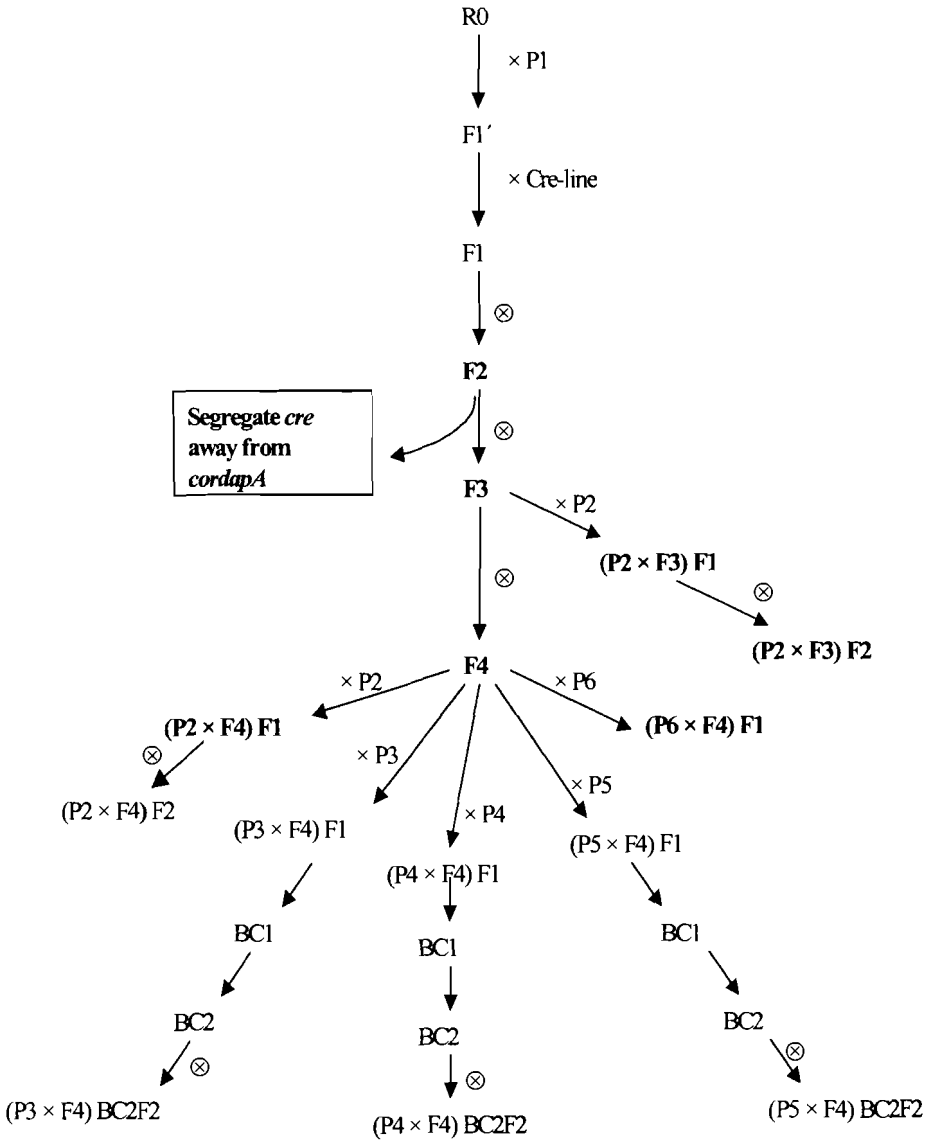

Figure V-16. Diagrammatic representation of LY038 breeding tree
The generation immediately prior to the one in which the *nptII* antibiotic-resistance marker was excised by Cre-mediated recombination is designated F1′. Segregation analysis was performed on F1′, (P2×F3)F2, (P3×F4)BC2F2, (P4×F4)BC2F2, and (P5×F4)BC2F2. Molecular generational stability analysis was performed on F2, F3, (P2×F3)F1, (P2×F3)F2, F4, (P2×F4)F1, and (P6×F4)F1 (all shown in bold font in figure). Molecular characterization was performed on (P2×F3)F2. Gene expression and composition analysis was performed on (P2×F4)F2.
R0, transformed plant; Pn, nontransgenic inbred line; Fn, filial generation;
⊗, self-pollination; BCn, backcross generation.

QUESTION: Page 64 – Second sentence, which lane on Figure V-17 is (P2xF3)F1?

RESPONSE: Lane 6 on Figure V-17 corresponds to (P2×F3)F1.

QUESTION: Page 65 – Figure V-17. What does the "H" mean in lanes 6-10? Also the bands differ in intensity. Please explain.

RESPONSE: Lanes 3, 4, and 5 contain inbred generations, F2, F3 and F4, respectively, while lanes 6, 8 and 10 contain hybrid generations. The "H" designation in lanes 6-10 refers to these lanes containing material from hybrids. Inbreds are expected to contain two copies of the insert while the hybrids should contain only a single copy, which is the most likely explanation for the difference in band intensity between lanes 3-5 and 6-10.

QUESTION: Page 66 – Figure V-18. Same comment as on Figure V-17. Please make the nomenclature used on the chart on page 63 correspond to that used on page 66.

RESPONSE: See the table included in our response to Page 63 question.

QUESTION: Page 68 – Table V-2 shows data for the cDHDPS protein in the plant parts of the transgenic. However there are no data for a nontransgenic comparator. Please provide data for a comparator. Also, lysine levels are not given for plant parts other than the grain and forage. Please provide data showing lysine levels in the plant parts shown in Table V-2 for LY038 and the nontransgenic comparator.

RESPONSE: The control substance was LY038(-), which does not contain the *cordapA* coding sequence and, therefore, does not produce the cDHDPS protein. Overseason leaf (OSL-1-4), whole plant, root, forage, forage root, grain, and pollen tissues collected from LY038(-) control plots were analyzed for the cDHDPS protein and did not contain detectable cDHDPS protein (LOD for respective tissues included in Table V-2), therefore, no data for the control are provided in Table V-2.

Lysine levels in test (LY038) and control [LY038(-)] forage tissue (the entire aerial portion of the plant at approximately the R5 growth stage) collected from five U.S. test sites (three replicate plots per site) in 2002 are presented in the table below. The combined-site lysine level in the forage tissue was not statistically different between LY038 and LY038(-). The cDHDPS levels in the same LY038 grain and forage samples used for lysine analysis averaged 26 and 0.94 µg/g DW, respectively. Although a low concentration of cDHDPS was detected in LY038 forage tissue, there was no change in forage lysine level from that measured in LY038(-) forage tissue, as shown in the table below.

The cDHDPS level in LY038 grain averaged 26 µg/g DW, approximately 28-fold greater than that measured in LY038 forage tissue, and resulted in a significant increase in grain lysine content. This is expected since expression of the *cordapA* gene in LY038 is under the direction of the Glb1 promoter that has been shown to

direct expression predominantly to the germ portion of maize grain (Belanger and Kriz, 1991). cDHDPS concentrations in LY038 young root (V2-V4 growth stage) and pollen , 1.5 and 0.78 μg/g DW, respectively, are in a similar range to those measured in LY038 forage (0.94 μg/g DW) and would also not be expected to differ in lysine concentration from that of control tissues. As discussed in more detail in response to the question identified as Page 92, preliminary analysis showed no differences in lysine between LY038 and LY038(-) pollen, consistent with the prediction that the cDHDPS levels in this tissue are too low to impact total lysine concentation.

Tissue type	**LY038 Mean (Range)**		**LY038(-) Mean (Range)**	
	cDHDPS (μg/g DW)	**Lysine (% DW)**	**cDHDPS (μg/g DW)**	**Lysine (% DW)**
Grain	26 (14 – 49)	0.48 (0.42 – 0.52)	<LOD	0.32 (0.25 – 0.36)
Forage	0.94 (0.15 – 2.8)	0.30 (0.17 – 0.36)	<LOD	0.31 (0.20 – 0.41)

Reference: Belanger, F. C. and A. L. Kriz. 1991. Molecular Basis for Allelic Polymorphism of the Maize Globulin 1 Gene. Genetics 129:863-872.

Section VI: Dihydrodipicolinate synthase (DHDPS) Proteins

QUESTION: Page 70 – In the paragraph on glycosylation the location of these data is not given. Insert a reference indicating that these data are located in Appendix 3, Section 5.

RESPONSE: The materials and methods used for characterization of cDHDPS protein produced in *E. coli* and LY038 maize grain are presented in Sections A and B, respectively, of Appendix 3. Additional detail regarding the cDHDPS protein glycosylation assessment is presented below.

Many eukaryotic proteins are post-translationally modified with carbohydrate moieties (Rademacher et al., 1988). These carbohydrate moieties may be complex branched polysaccharide structures or simple monosaccharides. In contrast, prokaryotic organisms, such as *E. coli* lack the necessary biochemical mechanisms required for protein glycosylation. To test whether potential post-translational glycosylation of the plant-produced cDHDPS protein occurred, the isolated plant-produced cDHDPS protein was analyzed for the presence of covalently-bound carbohydrate. The *E. coli*-produced cDHDPS reference standard, a negative control in this experiment, and transferrin protein, a positive control, were analyzed concurrently. The glycosylation analysis demonstrated that there was no detectable glycosylation of the plant-produced cDHDPS protein and thus the plant-produced

protein is equivalent to the *E. coli*-produced cDHDPS reference standard with respect to glycosylation.

QUESTION: Page 72 – Section e – Please provide data to support the statement: The cDHDPS protein is rapidly degraded in simulated gastric fluid indicating that it would be unlikely to elicit allergenic or toxic effects. What is rapid? Please provide numerical data.

RESPONSE: The demonstrated rapid degradation of the cDHDPS protein in simulated gastric fluid (greater than 96% of the protein was degraded within 30 seconds) provides evidence of the lack of allergenic potential of the expressed cDHDPS protein.

Section VII: Phenotypic Evaluation

QUESTION: Page 80 – Second paragraph – Please define control "substances." Be a little more specific.

RESPONSE: For each of the studies presented in Section VII, the test substance was LY038 and the control substance was LY038(-), a negative-segregant of LY038. For the phenotypic assessment study presented in Section VII.B.2, the growth and development of hybrid plants of LY038 and LY038(-) were evaluated.

QUESTION: Page 87 – Second paragraph – Can you provide more data on the expression of the white leaf phenotype and what is meant by "severely affected?" What is the expression of the phenotype over time and how does it affect plant health/vigor in the field?

RESPONSE: The effect of the white leaf phenotype on LY038 growth and development is discussed in detail in the first and second paragraphs on page 87. The white leaf phenotype is transiently observed upon germination of a minority of LY038 plants and only under certain planting conditions. The statement "not severely affected" within that discussion emphasizes that the subsequent growth and development of LY038 plants that initially expressed the white leaf phenotype was comparable to other LY038 plants that did not exhibit the white leaf phenotype.

QUESTION: Page 92 – Second paragraph – How much lysine is found in corn pollen? Is it the same level in the transgenics? See comment on page 68 above.

RESPONSE: As discussed above for the page 68 question, the level of cDHDPS protein detected in LY038 pollen supports a conclusion that the level of lysine in pollen from LY038 is not different than the level in conventional maize. The Glb1 promoter that is used to drive expression of the *cordapA* gene in LY038 shows a high bias for grain (specifically the germ) over other tissues. Protein expression data are consistent with this expectation, with the concentration of cDHDPS protein in LY038 grain, forage and pollen averaging 26 μg/g DW, 0.94 μg/g DW, and 0.78 μg/g DW, respectively. In grain, with the highest expression level of cDHDPS, it is noteworthy

that the increase in lysine levels is ~ 50% [0.48 ± 0.01 and 0.32 ± 0.01, for LY038 and LY038(-), respectively]. Therefore, it is not surprising that lysine levels were not significantly different for forage when comparing LY038 to LY038(-) (0.30% DW vs. 0.31% DW, respectively) since the expression level of cDHDPS in forage is ~28-fold lower than in LY038 grain. Since the concentration of cDHDPS in LY038 pollen is slightly lower than forage, it would similarly be unexpected to observe a significant difference in lysine content between LY038 and LY038(-) pollen. Indeed, preliminary data show that mean lysine content for LY038 and LY038(-) pollen was 16.54 +/- 0.23 and 16.36 +/- 0.08 mg/g DW, respectively. These values are similar to those reported in the literature for field corn pollen of 18.4 +/- 0.3 mg/g DW (Lundgren and Wiedermann, 2004).

Reference: Lundgren, J.D. and R.N. Wiedenmann. Nutritional suitability of corn pollen for the predator *Coleomegilla maculate* (Coleoptera: Coccinellidae). Journal of Insect Physiology. 50:567-575 (2004).

QUESTION: Page 96 – Last paragraph – It would be more accurate to say that no biologically meaningful phenotypic changes *in terms of a plant pest risk*.... In various parts of the document the term "biologically meaningful" is used but it should be used in the context of a plant pest.

RESPONSE: Section VII discusses several studies that were conducted to evaluate the effects of the lysine trait on the growth, development, and composition of LY038 compared to LY038(-). In each of these studies, statistical comparisons were made between LY038 and LY038(-) for each characteristic measured. Within each study, each significant difference ($p<0.05$) detected was evaluated for its effect on plant pest potential. Each statement that described the "biological meaning" of a detected difference was in the context of its effect on plant pest risk.

Section VIII: Environmental Consequences of Introduction

QUESTION: Page 111 – Last sentence – It is not clear that a "history of the safe exposure for the cDHDPS protein has been demonstrated, based on the similarity of the cDHDPS protein in LY038 to DHDPSs naturally present in feed and food (e.g., maize, rice, soy and wheat)" is from data are presented in Section VII. Can you provide a reference?

RESPONSE: The cDHDPS protein belongs to the family of related DapA (DHDPS) proteins. DHDPS is the first enzyme unique to lysine biosynthesis in bacteria and higher plants (Galili, 1995). DHDPS proteins isolated from a number of species including spinach, pea, maize, *E. coli*, and *Bacillus subtilis* have been extensively characterized (Wallsgrove and Mazelis, 1981; Dereppe et al., 1992; Frisch et al., 1991; Karsten, 1997). Assessment of the potential impact of cDHDPS on animal and human health is based upon extensive characterization of the cDHDPS protein and its functional homology to other DHDPS proteins commonly found in a wide variety of animal feed and human food sources, which have a history of safe consumption / exposure. Since all of these proteins catalyze the first enzymatic step in lysine biosynthesis in all of these organisms, it is predictable that they share reasonable

sequence identity and similarity when evaluated using a "BestFit" program such as that in the Wisconsin Package. The BestFit program makes an optimal alignment of two sequences using a local homology algorithm by Smith and Waterman (1981).

A comparison of amino acid sequence of cDHDPS and representative DHDPS proteins is presented in the following table.

DHDPS sequence (Accession)	% Identity to cDHDPS	% Similarity to cDHDPS
C. glutamicum (BAB99364)	100.0	100.0
E. coli (AAO43656)	36.8	46.9
Maize (1718320A)	29.4	38.5
Soy (AAA73555)	28.0	38.5
Wheat (AAA34264)	28.9	36.4
Rice (AAF44718)	27.0	36.1

References:

Dereppe, C., G. Bold, O. Ghisalba, E. Ebert, and H.P. Schär. 1992. Purification and characterization of dihydrodipicolinate synthase from pea. Plant Physiol. 98:813-821.

Frisch, D.A., A.M. Tommey, G.B. Gengenbach, and D.A. Somers. 1991. Direct genetic selection of a maize cDNA for dihydrodipicolinate synthase in an Escherichia coli dapA⁻auxotroph. Mol. Gen. Genet. 228: 287-293.

Galili, G. 1995. Regulation of lysine and threonine synthesis. Plant Cell 7:899-906.

Karsten, W.E. 1997. Dihydrodipicolinate Synthase from Escherichia coli: pH Dependent Changes in the Kinetic Mechanism and Kinetic Mechanisim of Allosteric Inhibition by L-Lysine. Biochemistry 36:1730-1739.

Smith, T.F. and M.S. Waterman. 1981. Comparison of Biosequences. Advances in Applied Mathematics 2, 482-489.

Wallsgrove, R.M. and M. Mazelis. 1981. Spinach leaf dihydrodipicolinate synthase: partial purification and characterization. Phytochem. 20:2651-2655.

APPENDIX 1. Molecular Characterization Materials and Methods
QUESTION: Page 124 – Section B – Please explain how the negative control was derived and how you determined that it was negative.

RESPONSE: Seed from a single ear on a single F1 plant were used to plant the F2 generation. The seeds on the F3 ears harvested from the F2 generation plants were planted in a separate row for each of the F3 ears. In the resulting F3 generation of plants, LY038 was selected from one of the aforementioned advanced ears, and the near isogenic control, LY038(-), was selected from another of the aforementioned

advanced ears, based on its phenotypic similarity to LY038, and the absence of the LY038 trait based on event-specific PCR analysis.

QUESTION: Page 125 – Line 3 – Rogers and Bendich (1985) reference missing in the reference list.

RESPONSE: The Rogers and Bendich (1985) reference is as follows:

Rogers, S.O. and A.J. Bendich. 1985. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Plant Mol. Biol. 5:69-76.

QUESTION: Page 126 – Line 2 – ...according to a standardized procedure." What procedure? Provide a reference.

RESPONSE: The sentence should read, "Samples of DNA digested with restriction enzymes were separated, based on size, using 0.8% (w/v) agarose gel electrophoresis according to Sambrook and Russell (2001)."

Sambrook, J., and D. Russell. 2001. Molecular Cloning, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. Chapter 5, Protocol 1: Agarose gel electrophoresis.

APPENDIX 3. Materials and Methods: cDHDPS Protein Characterization
QUESTION: Page 132 – Third paragraph, line 3 – Gorisch, 1988 reference is missing in the reference list.

RESPONSE: The Görisch, 1988 reference is as follows:

Görisch, H. 1988. Drop dialysis: time course of salt and protein exchange. Anal Biochem 173:393-398.

APPENDIX 5. Lysine-Related Metabolite Analysis
QUESTION: Page 165 – Line 2 – Eggling 1994 reference is missing in the reference list.

RESPONSE: The Eggling, 1994 reference is as follows:

Eggeling, L. 1994. Biology of L-lysine overproduction by Corynebacterium glutamicum. Amino Acids 6:261-272.

APPENDIX 7. USDA Field Trial Notifications for LY038

QUESTION: Page 190 - Appendix 7: There are a number of lysine level increased corn notifications listed in our database that are not listed here. Were these studies not used to support this petition?

RESPONSE: The field release notifications listed in Appendix 7, Table 1 include all field release notifications for LY038, the subject of this petition.