97-099-01p
CBI deleted

Monsanto

Monsanto Company
700 Chesterfield Parkway North
St. Louis. Missouri 63198
Phone: (314) 694-1000

July 30th, 1997

Dr. Ray Dobert
BBEP, APHIS, U.S. Department of Agriculture
4700 River Road, Unit 147
Riverdale, MD 20737-1237

Subject: Revised version of Petition 97-099-01p for Determination of Nonregulated Status of Roundup Ready Corn Line GA21.

Dear Dr. Dobert,

Please find attached two copies of a revised version of petition 97-099-01p for Determination of Nonregulated Status of Roundup Ready Corn Line GA21. The present revised version proposed by Monsanto Company and DEKALB Genetics Corporation has been prepared to address the questions and comments raised during the review for completeness. This revised version replaces the Volume I of the petition submitted initially on April 9th, 1997 and all the documents exchanged with you since the beginning of the review. The Volume II of petition 97-099-01p (AnnexIII: USDA Field Trial Termination Reports) remains unchanged. Two revised non-confidential copies are also enclosed.

Should you have any questions, please feel free to contact either Dr. Russ Schneider at 202-383-2866 or myself (314-737-7054).

Sincerely,

J-N Mutz,
Regulatory Affairs Manager, Roundup Ready Corn

CC: Dr R. P. Schneider - Monsanto

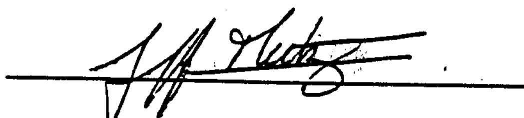
C 3 I DELETED

Petition for Determination of Nonregulated Status:

Roundup Ready Corn Line GA21

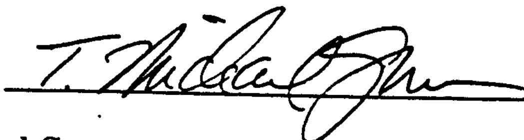
The undersigned submits this petition of 7 CFR 340.6 to request that the Director, BBEP, make a determination that the article should not be regulated under 7 CFR part 340.

Submitted by:



Jean-Noël Mutz, Regulatory Affairs Manager
Monsanto Company
700 Chesterfield Parkway North, BB1K
Chesterfield, MO 63198
Tel: 314-737-7054 / Fax: 314-737-7085

and



T. Michael Spencer, Manager, Regulatory Affairs
DEKALB Genetics Corporation

submitted April 9, 1997
revised July 28, 1997

#97-138-U

Prepared by:

J-N Mutz, T.M. Spencer, D.A. Dixon, R.S. Sidhu, D. Miller.

Contributors:

F. Behr, K.A. Croon, R.L. Fuchs, C. George, J. Gwyn, C.M. Hironaka, B.E. Ledesma, T.C. Lee, K.M. Magin, G.J. Rogan, P.R. Sanders, L. Turner.

Petition For Determination of Nonregulated Status for Roundup Ready™ Corn Line GA21

Summary

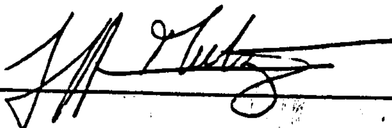
Monsanto Company and DEKALB Genetics Corporation are submitting this Petition for Determination of Nonregulated Status to the Animal Plant Health Inspection Service (APHIS) for corn which is tolerant to the herbicide Roundup®. This petition requests a determination from APHIS that the Roundup Ready corn line GA21, any progenies derived from crosses between this line and other corn varieties, and any progeny derived from crosses of this line with transgenic corn varieties that have also received a determination of nonregulated status, no longer be considered regulated articles under regulations in 7 CFR part 340.

Corn is the largest crop in the United States in terms of planted acreage, total production and crop value. United States production in 1996 was estimated at 236 million metric tons produced on over 73 million acres with the majority of national production concentrated across what is known as the "Corn Belt" in the upper Midwest. Weed management is a critical component to maximize corn yields and retain a high-quality harvest, free of weed seeds. The use of corn plants containing the Roundup Ready gene for corn production would enable the farmer to utilize Roundup herbicide for effective control of weeds during the growing season and to take advantage of this herbicide's environmental and safety characteristics. Glyphosate is highly effective against the majority of annual and perennial grasses and broad-leaved weeds. Glyphosate has excellent environmental features, such as rapid soil binding (resistance to leaching) and biodegradation (which decreases persistence), as well as extremely low toxicity to mammals, birds and fish. The use of Roundup Ready corn can positively impact current agronomic practices in corn by 1) offering the farmer a new, wide-spectrum weed control option, 2) allowing the use of an environmentally acceptable herbicide, 3) increasing flexibility to treat weeds on an "as needed" basis, 4) providing an excellent fit with reduced-tillage systems, which results in increased soil moisture, while reducing soil erosion and fuel use and 5) providing cost-effective weed control.

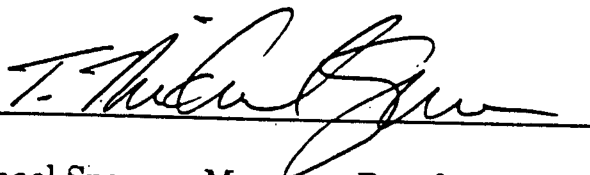
Roundup Ready corn line GA21 has been transformed with a restriction fragment of plasmid pDPG434 that contains only the modified corn EPSPS gene cassette. Roundup Ready corn line GA21 is tolerant to Roundup through the expression of a modified corn EPSPS protein whose sequence is more than 99.3 % identical to the endogeneous corn EPSPS protein. Data and information included in this petition demonstrate that GA21 does not represent a unique plant pest risk. Therefore, Monsanto Company and DEKALB Genetics Corporation request a determination from APHIS that Roundup Ready corn line GA21, any progenies derived from crosses between this line and other corn varieties, and any progeny derived from crosses of this line with transgenic corn varieties that have also received a determination of nonregulated status, no longer be considered regulated articles under regulations in 7 CFR part 340.

Certification

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



Jean-Noël Mutz, Regulatory Affairs Manager
Monsanto Company
700 Chesterfield Parkway North, BB1K
Chesterfield, MO 63198
Tel: 314-737-7054
Fax: 314-737-7085



T. Michael Spencer, Manager, Regulatory Affairs
DEKALB Genetics Corporation

Abbreviations Used in this Petition for the Determination of NonRegulated Status of Roundup Ready Corn Line GA21

APHIS	Animal Plant Health Inspection Service
bp	base pairs
CFR	Code of federal regulations
CTP	Chloroplast transit peptide
df	Degrees of freedom
DNA	Deoxyribonucleic Acid
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPA	Environmental Protection Agency
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
FDA	Food and Drug Administration
FIFRA	Federal Insecticide Fungicide and Rodenticide Act
FR	Federal Register
Ga, ga	Gametophyte
GDD	Growing degree days
Kb	Kilobases
kD	Kilodaltons
mEPSPS	Modified corn EPSPS
M	Million
ml, l	Milliliter, liter
mm, cm, m	millimeter, centimeter, meter
mM, M	Millimolar, Molar
MW	Molecular weight
mssu	Maize small subunit of the RuBisCo gene
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NOS 3'	3' transcriptional termination sequence from nopaline synthase
OTP	Optimized transit peptide
PCR	Polymerase chain reaction
pg, ng, µg, mg, g, kg	Picogram, nanogram, microgram, milligram, gram, kilogram
ppm	Part per million
r-act	5' region of the rice actin 1 gene
RuBisCo	ribulose 1,5-bisphosphate carboxylase oxygenase
SCP	Sodium chloride-phosphate buffer
SDS	Sodium dodecyl sulfate
sp	Species
SSC	Saline-sodium citrate buffer
sssu	Sunflower small subunit of the RuBisCo gene
subsp.	Subspecies
TE	Tris-EDTA buffer (10 mM Tris, pH 8.0, 1 mM EDTA)
Tris	Tris (Hydroxymethyl) Aminomethane
tRNA	Transfer ribonucleic acid
USDA	United States Department of Agriculture

CONFIDENTIAL BUSINESS INFORMATION JUSTIFICATION

The information claimed as confidential within this petition concerns the description of the gene introduced into corn and the sequence of the expressed protein. The gene description category includes information about the modified corn EPSPS sequence.

Legal Background

The Freedom of Information Act ("FOIA"), 5 U.S.C. § 552, specifically exempts from release "trade secrets and commercial or financial information obtained from a person and privileged or confidential" ("Exemption 4"). 5 U.S.C. § 552(b)(4). Exemption 4 applies where the disclosure of information would be likely to cause substantial harm to the competitive position of the owner, or where, in the case of voluntarily submitted information, the submitter would be less likely in the future to share data with the agency voluntarily. National Parks & Conservation Association v. Morton, 498 F.2d 765, 770 (D.C.Cir. 1974); Gulf & Western Industries, Inc. v. U.S., 615 F.2d 527, 530 (D.C.Cir. 1979).

A party seeking to demonstrate "substantial competitive harm" need not show actual competitive harm, but must only demonstrate the presence of competition and the likelihood of substantial competitive injury. Id. at 530; National Parks & Conservation Association v. Kleppe, 547 F.2d 673, 679 (D.C.Cir. 1976); Miami Herald Pub. Co. v. U.S. Small Business Administration, 670 F.2d 610, 614 (5th Cir. Unit B 1982).

For the purposes of FOIA, courts have defined the term "trade secret" to mean a "secret, commercially valuable plan, formula, process, or device that is used for the making, preparing, compounding, or processing of trade commodities and that can be said to be the end product of either innovation or substantial effort. Public Citizen Health Research Group v. FDA, 704 F.2d 1280, 1288 (D.C.Cir. 1983); Anderson v. Dept. of Health & Human Services, 907 F.2d 936, 943-44 (10th Cir. 1990).

Information on gene description falls squarely within this definition, and is the type of information accorded trade secret protection by the courts under Exemption 4 of the Freedom of Information Act. It is well established that information on the formulation and chemistry of a product should be treated as confidential for FOIA purposes. See, e.g., Anderson v. Dept. of Health & Human Services, 907 F.2d 936 (10th Cir. 1990). This is exactly the type of information provided by the gene description category and the sequence of the expressed protein. Where, as in the case of the Monsanto and DEKALB Genetics products subject to this FOIA request, the development time and costs of the product have been substantial and the information can only be obtained by competitors at considerable cost, disclosure is prohibited. Greenberg v. Food and Drug Administration, 803 F.2d at 1213, 1216-1218 (D.C. Cir. 1986); Worthington Compressors, Inc. v. Costie, 622 F.2d 45, 51-52 (D.C.Cir. 1981). The existence of confidentiality agreements binding employees not to reveal the information is another factor considered by the courts. Greenberg v. FDA, 803 F.2d at 1216-1218.

The courts have also been very clear in finding commercial development information covered by Exemption 4 where the release of such information could allow competitors to procure a clear understanding of a company's business practices and allow a

competitor to cause harm to a company's competitive standing. See, e.g., Braintree Electric Light Dept. v. Dept. of Energy, 494 F.Supp. 287, 289-291 (D.D.C. 1980). Information on distribution channels, market strategies, pricing structures, and patterns of competition fall squarely within the Exemption because such information enables a competitor to gain an accurate picture of a company's marketing activities and the competitive structure of the market. Timken v. U.S. Customs Service, 531 F.Supp. 194, 200 (D.D.C. 1981). Typically, information concerning marketing strategies, and the names of independent contractors participating in a company's studies have been accorded confidential treatment. See, e.g., Teich v. Food & Drug Administration, 751 F.Supp. 243, 253 (D.D.C. 1990). Specific justifications for treating information in these two categories as CBI are provided below.

Gene Description

The essence of the commercial value of the Monsanto and DEKALB Genetics biotechnology products is the particular genetic information that confers the desired properties on the plant product, as well as the technical know-how inherent in this information. Monsanto and DEKALB Genetics are at the leading edge in the development of biotechnology products in a rapidly growing and highly competitive industry. This expertise has been gained through many person years of effort, and the expenditure of tens of millions of dollars on biotechnology research.

Monsanto and DEKALB Genetics have been working on the development of glyphosate tolerant corn for more than 6 years, and have expended several million dollars in research and testing costs. Monsanto and DEKALB Genetics can document the development and testing costs by means of monthly summaries of the person hours devoted to these projects, budgetary documents, field test agreements, and project documents for the Chesterfield, MO and the Mystic, CN facilities.

The uniqueness of this product lies in the particular combination of genetic components in the DNA sequence transferred to these plants. The genetic entity has three pieces of information: a promotor region, the gene for the expression of the trait, and a stop signal. Although the information on each of these vector components may be in the public domain, the particular combination of the components put together by Monsanto and DEKALB Genetics are unique and represents years of effort and millions of dollars of expense.

In a case decided by the U.S. Court of Appeals for the District of Columbia Circuit, Critical Mass Energy Project v. NRC, No. 90-5120, August 21, 1992, the court determined that information given to the government voluntarily will be treated as confidential under Exemption 4 if such information is of the kind that the provider would not customarily make available to the public. To the extent any references and other information in the Monsanto applications were submitted voluntarily, such information is accorded protection from disclosure.

To achieve the products which are the subject of this FOIA request, Monsanto and DEKALB Genetics have developed and tested many different plants using different combinations of genetic components. The plant products developed by Monsanto and DEKALB Genetics represent the best combination of the components, and the best mode of gene expression of the desired trait. The specific combination of genetic information on the DNA sequence transferred to the Monsanto and DEKALB Genetics

products has been kept strictly confidential. Monsanto and DEKALB Genetics employees and contractors under contract to Monsanto and DEKALB Genetics are contractually obligated to keep this information confidential.

There are many competitors of Monsanto and DEKALB Genetics, both national and international, who have the expertise not only to replicate Monsanto and DEKALB Genetics's products, but also to use Monsanto and DEKALB Genetics's technology to develop other products which would be competitive with Monsanto and DEKALB Genetics, thereby saving millions of dollars and years of development effort.

Monsanto and DEKALB Genetics's competitors cannot presently duplicate Monsanto and DEKALB Genetics's commercially valuable products from information in the public domain without going through the same painstaking trial and error development and testing of many different combinations of genetic information. It is important to emphasize that although there may be information about Monsanto and DEKALB Genetics products available in patent applications, this information is voluminous and general in nature, and does not identify the specific combinations of genetic information which Monsanto and DEKALB Genetics have found to be most effective. A competitor cannot determine from the patent applications which particular combination of genes and transgenic products will prove to be commercially valuable.

Access to gene description information for Monsanto and DEKALB Genetics's products would allow competitors to create essentially "copy" products (avoiding any technical patent infringement) that would result in a market share loss for Monsanto and DEKALB Genetics of millions of dollars. By performing simple copy work, these competitors would avoid the millions of dollars and many years of research and development effort expended by Monsanto and DEKALB Genetics to develop its commercial products.

The release of gene description information would also provide competitors with commercially valuable knowledge about the particular products that Monsanto and DEKALB Genetics are planning to commercialize and the likely time frame for commercialization. This information would be extremely helpful to these companies in developing their own marketing strategies and development plans in a highly competitive market.

Names And Information About Genes, Promoters And Sequence of the Expressed Protein

The release of information about the genes and promoters in the vectors will directly provide competitors with the knowledge of the precise genetic sequence that Monsanto and DEKALB Genetics have found to be most desirable. If this information is disclosed, the competitors will have access to the structure of the Monsanto and DEKALB Genetics product, with the consequences outlined above. Patents for the products at issue in this matter are pending, but have not been issued.

Information on the sequence of the expressed protein is tantamount to providing the sequence of the modified corn EPSPS gene, and will allow Monsanto and DEKALB Genetics's competitors to readily duplicate the Monsanto and DEKALB Genetics

product. The release of any information relating to changes made to an original gene would explicitly reveal Monsanto and DEKALB Genetics' trade secret technology.

Identification of Items claimed as Confidential Business Information

Items claimed as CBI are enclosed in brackets, "[ ]", in the text and labeled as **CBI**. A non-confidential copy of the application is also included with the confidential item indicated in the text as follows: [CBI - Deleted] and labeled as **CBI**.

Page	Category of information	Justification
24	gene description	See discussion on gene description, names and information about the gene, promoter and expressed protein
26	gene description	See discussion on gene description, names and information about the gene, promoter and expressed protein
27	sequence of the expressed protein	See discussion on gene description, names and information about the gene, promoter and expressed protein

Petition for Determination of Nonregulated Status of Roundup Ready Corn Line GA21

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I. Rationale for Development of Roundup Ready Corn

A. Benefits of Roundup Ready Corn

Corn is the largest crop in the United States in terms of planted acreage, total production, and crop value (National Corn Growers Association, 1997). United States production in 1996 was estimated at 236 million metric tons produced on over 73 million acres with the majority of national production concentrated across what is known as the "Corn Belt" in the upper Midwest.

Glyphosate (N-phosphonomethyl-glycine) (CAS Registry #'s 1071-83-6, 38641-94-0), the active ingredient in the non-selective, foliar-applied, broad-spectrum, post-emergent herbicide Roundup (Baird, 1971; Malik *et al.*, 1989), is the world's most popular herbicide. This is primarily due to its excellent weed control capabilities and its well-known, favorable environmental and safety characteristics. However, the sensitivity of crop plants to glyphosate has prevented the in-season use of this herbicide over-the-top on crops. The extension of the use of Roundup herbicide to allow in-season application in major crops such as corn will provide new weed control options for farmers. Recent advances in plant biotechnology have made it possible to insert genes to provide crop tolerance specifically to the non-selective herbicide glyphosate, and bring the benefits of its use to weed management in corn (Padgette *et al.*, 1996).

Weed management is a critical step to maximize corn yields and retain a high-quality harvest, free of weed seeds. For effective weed control, the farmer typically selects a herbicide based on several factors: weed spectrum, lack of crop injury, cost and environmental characteristics. Few herbicides available today deliver optimal performance in all of these areas. Several classes of herbicides are effective for broad-spectrum weed control, but many are either non-selective and kill crop plants or they significantly injure some crops at the application rates required for effective weed control.

The use of Roundup Ready corn will provide farmers new options for effective weed control. Glyphosate is highly effective against the majority of annual and perennial grasses and broad-leaved weeds. Glyphosate has excellent environmental features, such as rapid soil binding (resistance to leaching) and biodegradation (which decreases persistence), as well as extremely low toxicity to mammals, birds and fish (Malik *et al.*, 1989). Glyphosate is classified by the EPA as Category E (evidence of non-carcinogenicity for humans) (57 FR 8739). Studies separate from those summarized herein have been provided to the EPA in a request to amend the Roundup herbicide label to include in-season application on Roundup Ready corn. This label was approved on March 28th, 1997.

The use of corn plants containing the Roundup Ready genes for corn production would enable the farmer to utilize Roundup herbicide for effective

control of weeds during the growing season and to take advantage of this herbicide's environmental and safety characteristics. No increase of the proportion of corn acreage treated with herbicide is expected since herbicides are currently used on the vast majority of corn acreage in the U.S. Roundup Ready corn can positively impact current agronomic practices in corn by:

- Offering the farmer a new, wide-spectrum weed control option.
- Allowing the use of an environmentally acceptable herbicide.
- Increasing flexibility to treat weeds on an "as needed" basis.
- Providing an excellent fit with reduced-tillage systems, which results in increased soil moisture, while reducing soil erosion and fuel use.
- Providing cost-effective weed control.

B. Regulatory Approvals

Before commercializing Roundup Ready corn line GA21, Monsanto and DEKALB Genetics Corporation will seek the following regulatory approvals in the United States:

1. This determination from USDA/APHIS that Roundup Ready corn line GA21, and all progenies from crosses between this line and other corn varieties, are no longer regulated articles according to 7CFR §340.6.
2. Registration of Roundup herbicide (EPA Reg. No. 524-445) for use over-the-top of Roundup Ready corn. This application has been previously submitted to the EPA and the label was approved on March 28th, 1997.

In addition, Monsanto and DEKALB Genetics Corporation will complete a consultation process on Roundup Ready corn line GA21 with the FDA under their May 29, 1992 policy statement concerning foods derived from new plant varieties. Monsanto and DEKALB Genetics Corporation will consult with the pesticide and, if applicable, biotechnology regulatory officials of the states in which the commercial product will be sold and obtain a state license if such is required.

II. The Corn Family

Prepared by:

Dr. Arnel R. Hallauer, Department of Agronomy, Iowa State University, Ames, IA 50011

Introduction

Corn (*Zea mays* L.), or maize, is one of the few major crop species indigenous to the Western Hemisphere. Corn is grown in nearly all areas of the world and ranks third behind rice (*Oryza sativa* L.) and wheat (*Triticum* sp.) in total production. Corn has been studied extensively, and it seems the probable domestication of corn was in southern Mexico more than 7,000 - 10,000 years ago. The putative parents of corn have not been recovered, but it seems teosinte probably played an important role in the genetic background of corn. 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. Corn, 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, corn is not considered a persistent weed nor one difficult to control. A summary of the history, taxonomy, genetics, and life cycle of corn is presented, followed by a discussion of how the characteristics of cultivated corn affect gene flow between cultivated corn and its wild relatives.

A. History of Corn

Corn originated in the highlands of Mexico 7,000 to 10,000 years ago. By the time Columbus discovered the Western Hemisphere, corn was being grown by the indigenous civilizations from Chile to southern Canada. Columbus noted the presence of corn on the north coast of Cuba November 5, 1492 and introduced corn to Europe upon his return to Spain (Goodman, 1988). After the introduction of corn to Europe, corn became distributed within two generations throughout the world where it could be cultivated. Today, corn ranks third after wheat and rice as one of the world's three leading food crops. Unlike wheat and rice, more corn is consumed by livestock rather than directly by humans. Corn, however, is consumed directly by humans in the tropics and in the Southern Hemisphere.

The original corn 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 corns were 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 corns evolved from the gradual mingling of those settlements that spread north and west from the southeastern North America and those settlements that spread south and west from the northeastern North America.

The corns 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, are early maturing, and are short statured plants with tillers. The southern dent corns grown in the southeast United States seemed to have originated from the southeast coast of Mexico. Southern dent corns are characterized as having tall, late maturing, non-tillered, poorly rooted plants with soft-textured white kernels on many rowed, tapering ears. It seems the Tuxpeno race contributed to the development of southern dents. 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 corns that are used extensively throughout the world today.

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

It has been suggested that modern corn originated from corn grass by a single-gene mutation causing ear development. Other suggestions have included *Coix* and species of the genus *Manisuris* in the tribe *Andropogoneae* for contributing to the genome of corn. 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 the preponderance of evidence supports the hypothesis that corn descended from teosinte (Galinat, 1988). The teosinte genome is similar to corn, teosinte easily crosses with corn, and teosinte has several plant morphological traits similar to corn. Teosinte has a more weedy appearance and more tillers than modern corn varieties. The one major distinguishing difference between corn and teosinte is the female inflorescence, or ear. Modern corn varieties have 1 to 3 lateral branches that terminate in an ear with 8 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*

Corn is a member of the tribe Maydae, which is included in the subfamily Panicoideae of the grass family Gramineae (Table II.1). 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 corn, 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*. Corn (*Zea mays* L.) is a separate species within the subgenus *Zea* along with three subspecies. All of the species within the genus *Zea*, except corn, 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 Corn

Corn 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 ($n = 10$), corn has been accessible for study at all levels of genetics. Corn was one of the first crop species included 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 corn in the U.S. and world economies, and the genetic information obtained since 1900, corn has continued to receive extensive study in modern genetic laboratories.

Molecular geneticists have developed extensive genetic maps of corn to complement those developed by the early corn geneticists. Corn 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, in gene transformation, etc.

Extensive compilations on corn genetics, corn cytogenetics, cell tissue culture, and on molecular genetics were provided (Coe *et al.*, 1988; Carlson, 1988; Phillips *et al.*, 1988; Walbot and Messing, 1988). Rapid advances are being made daily in corn genetics, but these are useful references.

D. Life Cycle of Corn

Corn is an annual and the duration of the life cycle depends on the cultivars and on the environments in which the cultivars are grown (Hanway, 1966). Corn cannot survive temperatures below 0 °C (32 °F) 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, length 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 corn crop will have a ragged appearance because the leaf areas damaged by frost persist until maturity. The completion of the life cycle of corn, therefore, is dictated by the duration of the average number of frost-free days.

The number of frost-free days dictates that corns with differences in length of their life cycles be grown in north-to-south directions of temperate areas. In the United States, corns with relative maturities of 80 days or less are grown in the extreme northern areas, and corns with relative maturities of more than 125 days are grown in the southern areas. Corns having 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 corns is the number of growing degree days (GDD) required from emergence to maturity. Based on GDD required to mature, corns are assigned to areas that have, on the average, less than 1850 GDD in the extreme northern areas of the United States to corns that require more than 2750 GDD in more southern areas. Assume a 115-day maturity hybrid is grown in central Iowa. Average last frost date is May 1 and average first frost date is October 5, resulting in an expected 158 frost-free days. If average emergence is May 15 and average flowering is July 15, 60 days are required from emergence to flowering. Corn 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 time-frame for each stage of corn development could be as follows:

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

These suggested time frames can 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 corn in the United States. Corn evolved as an open-pollinated (cross-fertilizing) crop species and until the 20th century the corn cultivars were what we designate today as open-pollinated corn varieties. Because corn is essentially 100% cross pollinated, the corn 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 races, 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 corn were defined by 1920 (Sprague, 1946). Basic studies on the genetic composition of a corn variety were conducted to determine the effects of selfing (or inbreeding which is the opposite of outcrossing) within a corn variety (Shull, 1908). Because corn is naturally cross fertilizing, the genetic composition of each plant is not known. Continuous selfing of individuals for 7 to 10 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 corn was determined (Shull, 1909): 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.

Hybridization is used in many phases of corn breeding because of the expression of heterosis. Hybridization is used to produce breeding populations (e.g., F2) to develop inbred lines for use in hybrids, and hybridization is used to produce the crosses of superior lines for distribution to growers. Hybridization is easily accomplished either by hand pollinations 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 (2n = 20) and corn (2n = 20) are wind pollinated, tend to outcross, and are highly variable, interfertile species (Wilkes, 1972; 1989). Corn and teosinte are genetically compatible, and in areas of Mexico and Guatemala they freely hybridize when they are in proximity to each other. Teosinte exists primarily as a weed around the margins of the corn fields, and the frequency of hybrids between teosinte and corn has been studied. A frequency of one F_1 hybrid (corn x teosinte) for every 500 corn plants or 2 to 5% of the teosinte population for the Chalco region of the Valley of Mexico was reported (Wilkes, 1972). As stated, this frequency of hybrids represents a significant gene exchange between a wild weedy plant (*i.e.*, teosinte) and a cultivated relative (*i.e.*, corn) (Wilkes, 1972). The F_1 hybrid of teosinte by corn is robust and fertile and is capable of backcrossing to corn. Intercrossing and gene exchange between teosinte and corn occurs freely, and, accompanied by selection, teosinte had a significant role in the evolution of corn.

Corn easily crosses with teosinte, but 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). Except for special plantings, teosinte is not found in the United States, and there have been no instances reported that teosinte occurs as a weed along the margins of corn plantings in the U.S. Corn Belt.

Tripsacum-corn hybrids have not been observed in the field and *Tripsacum*-teosinte hybrids have not been produced (Wilkes, 1972). *Tripsacum* evolved by polyploidy, whereas corn 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 corn or teosinte (Goodman, 1976). *Tripsacum* received greater interest in the evolution of corn after Mangelsdorf and Reeves (1931) successfully crossed corn and *Tripsacum dactyloides* (2n = 36). The cross by Mangelsdorf and Reeves (1931) was made with the diploid *Tripsacum dactyloides* (2n = 36) as the male parent. Silks of the female corn parent were cut to permit successful pollination. The cross had 28 chromosomes and was male sterile. Five other *Tripsacum* species have been crossed with corn, and Galinat (1988) has mapped more than 50 homologous loci on the chromosomes of corn and *Tripsacum*. In contrast with corn and teosinte which can be easily hybridized, both in the wild and by controlled pollinations, it requires special techniques to hybridize corn and *Tripsacum*. Except for *Tripsacum floridanum*, it is difficult to cross *Tripsacum* with corn, 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 (Table II.1). *Tripsacum floridanum* is native to southern tip of Florida. 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

Corn 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 corn pollen can travel up to 3.2 km (2 miles) by wind under favorable conditions. All corns 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 corn hybrids, sweet corn, and popcorn if the popcorn does not carry the dent-sterile gametophyte factor. Corn 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, corn pollen is very promiscuous. It is estimated each corn plant can shed more than 10 million pollen grains.

Certification standards for distances between different corn genotypes have been established to assist in the production of hybrid corn 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 corn of a similar type; i.e., if seed parent is a yellow, dent corn it should be isolated at least 200 m from other yellow, dent corns. 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 from the contaminating field. If the hybrid seed being produced is of a different color or texture from neighboring contaminating fields, the distances and the number of border rows should be increased.

G. Weediness of Corn

Modern-day corn cannot survive as a weed. One does not find volunteer corn growing in fence rows, ditches, and road sides as a weed. Although corn from the previous crop year can overwinter and germinate the following year, they cannot persist as a weed. The appearance of corn in soybean fields following the corn crop from the previous year is a common occurrence. Measures are often taken to either eliminate the plants with the hoe or use of herbicides to kill the plants in soybean fields, but the plants that remain and produce seed usually do not persist in the following years.

It is difficult for the corn to survive as a weed because of past selection in the evolution of corn. In contrast with weedy plants, corn 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 corn. Individual kernels of corn, 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 corn become a troublesome weed. Corn cannot survive without human assistance and is not capable of surviving as a weed.

Table II.1. Taxonomic Classification of Corn and its Closely Related Relatives.

Family - Gramineae

Subfamily - Panicoideae

Tribe - Maydae

Western Hemisphere:

A. Genus - Zea
- I. Subgenus - Luxuriantes
  1. Zea luxurians (2n = 20)
  2. Zea perennis (2n = 40)
  3. Zea diploperennis (2n = 20)
- II. Subgenus - Zea
  1. Zea mays (2n = 20)

  Subspecies
  1. Zea parviglumis (2n = 20)
  2. Zea huehuetenangensis (2n = 20)
  3. Zea mexicana (Schrad.) (2n = 20)

B. Genus - Tripsacum

Species --

andersomii (2n = 64)
australe (2n = 36)
bravum (2n = 36, 72)
cundinamarce (2n = 36)
dactyloides (2n = 72)
floridanum (2n = 36)
intermedium (2n = 72)
manisuroides (2n = 72)
latifolium (2n = 36)
percuvianum (2n = 72, 90, 108)
zopilotense (2n - 36, 72)
jalapense (2n = 72)
lanceolatum (2n = 72)
laxum (2n = 36?)
maizar (2n = 36, 72)
pilosum (2n = 72)

Asia:

Genera --

Chionachne (2n = 20)
Coix (2n = 10, 20)
Polytoca (2n = 20)
Schlerachne (2n = 20)
Trilobachne (2n = 20)

Tribe -- Andropogoneae

A. Genus - Manisuris

H. Characteristics of the Recipient Corn Material

The corn plant tissue that was the recipient of the introduced DNA was a cell culture designated AT824 initiated from immature embryos of an inbred corn line (AT). Transformants were selected by their ability to survive and grow in the presence of glyphosate, the active ingredient in the Roundup herbicide. Transgenic callus line GA21 was selected on medium containing glyphosate, and R0 plants were regenerated from the embryogenic callus by placing the callus on media which stimulate the production of shoots and roots.

III. Description of the Transformation Method Utilized in the Development of Roundup Ready Corn Line GA21

A. Particle Acceleration Transformation System

An agarose gel-isolated restriction fragment of plasmid DNA was introduced into embryogenic corn cells using the particle acceleration method (Klein *et al.*, 1987; Gordon-Kamm *et al.*, 1990). DNA is precipitated onto microscopic gold particles using calcium chloride and spermidine. A drop of the coated particles is then placed onto a plastic macrocarrier, which is accelerated at a high velocity through a barrel by the discharge of compressed helium gas. The macrocarrier hits a metal screen which stops the flight of the macrocarrier but allows continued flight of the DNA-coated particles. The particles penetrate the target plant cells, where the DNA is deposited and incorporated into the cell chromosome. The introduced DNA contains a gene encoding for herbicide tolerance (e.g. the modified corn EPSPS gene conferring tolerance to glyphosate). The plant cells are grown in the presence of glyphosate and only the transformed cells continue to grow.

B. Plasmid Sequence Utilized for Transformation

Roundup Ready corn line GA21 was produced using a 3.4 Kb agarose gel-isolated NotI restriction fragment of the plasmid vector pDPG434 (Figure III.1) and the particle acceleration method identified above. The plasmid vector pDPG434 contains the modified corn EPSPS gene cassette, the *bla* selectable marker gene encoding ampicillin resistance in the bacteria allowing selection of bacteria containing the plasmid, an origin of replication necessary for replicating the plasmid in *E. coli*, and a partial *lacZ* sequence (Table III.1). The agarose gel-isolated NotI restriction fragment of plasmid vector pDPG434 utilized for transformation of Roundup Ready corn line GA21 contains only the modified corn EPSPS gene expression cassette and does not contain the *bla* selectable marker gene, the origin of replication and the partial *lacZ* sequence (Figure III.1).

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Table III.1 Summary of DNA Components of the Plasmid

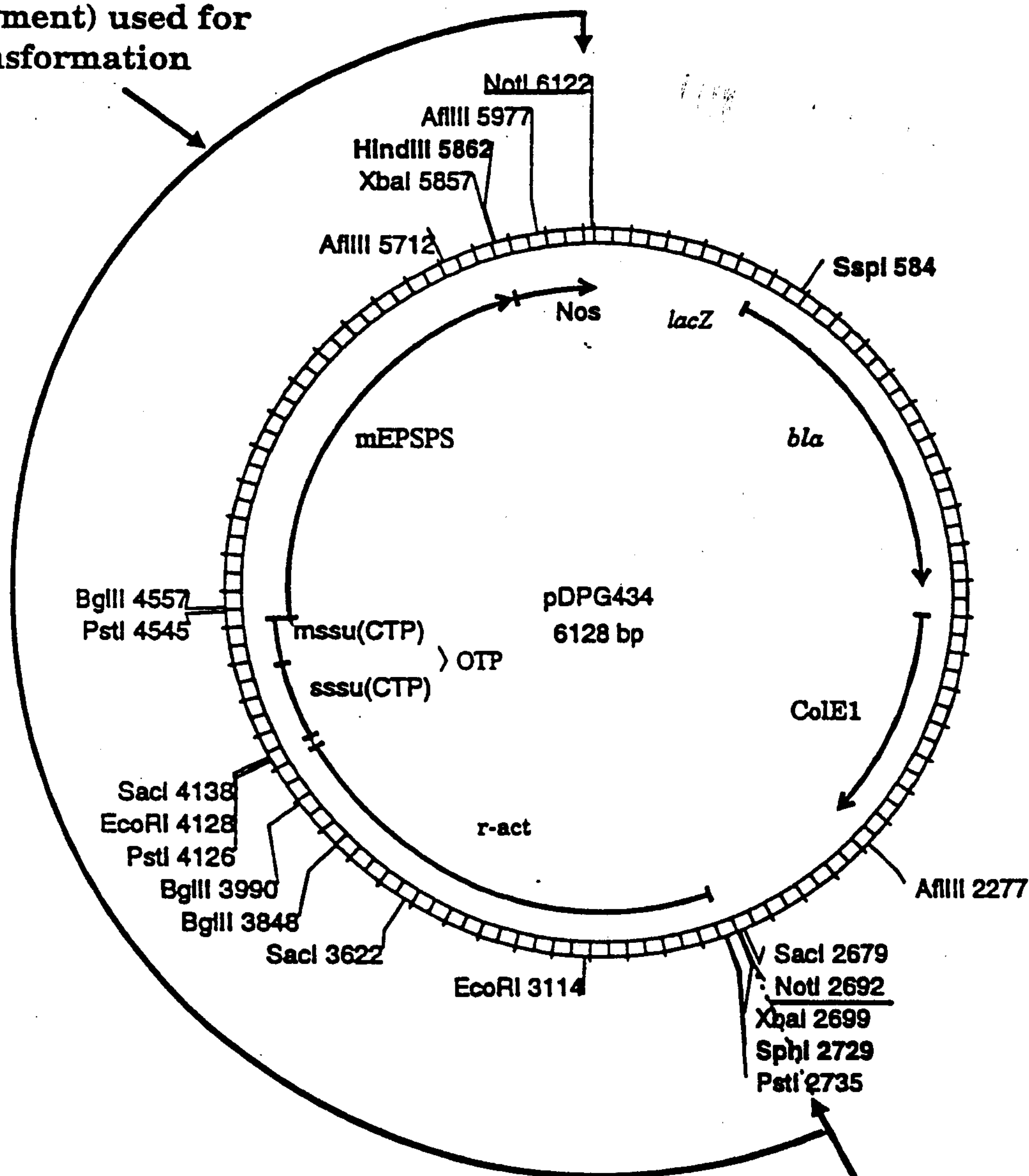
Genetic Element	Size Kb	Function
Genes present in the NotI restriction fragment of pDPG434, used for transformation:		
r-act	1.37	5' region of the rice actin 1 gene containing the promoter and first intron (McElroy *et al.*, 1990).
OTP	0.37	N-terminal chloroplast transit peptide (CTP) sequences based on the CTP sequences from the *Helianthus annuus* and *Zea mays* ribulose 1,5-bisphosphate carboxylase oxygenase (RuBisCo) genes (Lebrun *et al.*, 1996) present to direct the mEPSPS protein to the chloroplast, the site of aromatic amino acid synthesis.
mEPSPS	1.34	The wild type 5-enolpyruvyl-3-phosphoshikimate synthase gene (EPSPS) from *Zea mays* (Lebrun *et al.*, 1991) with [**CBI-Deleted**] to provide tolerance to glyphosate (Roundup) at the whole plant level.
NOS 3'	0.24	A 3' nontranslated region of the nopaline synthase gene which terminates transcription and directs polyadenylation (Fraley, *et al.*, 1983). This sequence was derived from the Ti plasmid of *Agrobacterium*.
Genes present in the pDPG434 plasmid backbone, but not present in the NotI restriction fragment used for transformation:		
lacZ	0.24	A partial *E. coli lacI* coding sequence, the promoter *Plac*, and a partial coding sequence for beta-D-galactosidase protein from pBluescript, a derivative of pUC19 (Yanich-Perron *et al.*, 1985).
ColE1 ori	0.65	The origin of replication from the *E. coli* high copy plasmid pUC19 (Yanich-Perron *et al.*, 1985).
bla	0.86	The β-lactamase gene from *E. coli* plasmid pBR322. This gene is present in the pBR322 derivative, pUC19 (Yanich-Perron *et al.*, 1985), and in the pUC19 derivative, pBluescript SK(+/-) (Short *et al.*, 1988). This enzyme confers resistance to the ampicillin and other penicillin antibiotics (Sutcliffe, 1978) and thereby allows for selection of bacteria containing the plasmid. The coding sequence for the *bla* gene is present under its own bacterial promoter.

CI

Note:
The sequences between positions 584 and 2692 in plasmid pDPG434 (Figure III.1) including the *bla* gene, the ColE1 origin and intervening non-coding sequences, were derived from pUC19. The *lacZ* region and non-coding DNA between the NOS 3' end and the *bla* gene were derived from pBluescript® SK(+/-)(Stratagene), a 2958bp phagemid derived from pUC19. Construction of pBluescript and its relationship to pUC19 is presented in detail in Short *et al.*

pBluescript and its relationship to pUC19 is presented in detail in Short *et al.* (1988). None of these sequences were contained in the agarose gel-isolated NotI restriction fragment used for transformation of GA21.

Figure III.1 Plasmid Map of pDPG434



C B I DELETED

IV. Description of the DNA Sequences Utilized in the Development of Roundup Ready Corn Line GA21

Introduction

Roundup Ready corn line GA21 was generated using a particle acceleration transformation system with an agarose gel-isolated NotI restriction fragment of plasmid vector pDPG434 containing the modified corn EPSPS gene. This gene encodes an EPSPS protein which confers glyphosate (Roundup) tolerance at the whole plant level.

A. The Donor Genes in the Gel-Isolated NotI Restriction Fragment

1. Introduction

A description of the DNA elements present in the NotI restriction fragment used to produce the Roundup ready corn line GA21 is given in Table III.1. The NotI restiction fragment contains the modified corn EPSPS gene under regulation of the rice actin promoter and rice actin intron, and contains the NOS 3' termination sequence (Figure III.1).

2. The modified corn EPSPS gene

The modified corn EPSPS (mEPSPS) gene was used to provide tolerance to glyphosate. The mode of action of glyphosate is the inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme involved in the shikimic acid pathway for aromatic amino acid biosynthesis in plants and microorganisms (Steinruken and Amrhein, 1980). The mEPSPS has been shown to be tolerant to glyphosate (Rodriguez and Padgette, 1997). Thus the I_{50} values for glyphosate (i.e., the concentration of glyphosate required to attain 50% inhibition of EPSPS activity) were determined to be 5 µM and 300 mM for the wild-type maize EPSPS and the mEPSPS, respectively. This indicates that the mEPSPS enzyme has significantly reduced affinity for glyphosate versus the wild-type enzyme.

When corn plants expressing the modified corn EPSPS protein are treated with glyphosate, the plants are unaffected since the continued action of the tolerant EPSPS enzyme meets the plant's need for aromatic amino acids.

The modified corn EPSPS was produced by cloning the wild-type EPSPS gene from corn (Lebrun *et al.*, 1991) and introducing [**CBI-Deleted** CI

]. The mEPSPS gene has been completely sequenced, and encodes a 47.4 kD protein consisting of a single polypeptide of 445 amino acids. The amino acid homology between the mEPSPS protein and the wild-type EPSPS from corn is greater than 99.3 %. Therefore these proteins are expected to be immunologically and functionally equivalent, except for their affinity to glyphosate.

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The modified corn EPSPS gene is fused to chloroplast transit peptide (CTP) sequences based on sequences isolated from corn and sunflower ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCo) genes to produce an optimized transit peptide (OTP) (Lebrun *et al.*, 1996). The CTP directs the EPSPS protein to the chloroplast, the location of EPSPS in plants and the site of aromatic amino acid biosynthesis (Kishore and Shah, 1988). CTPs are typically cleaved from the "mature" protein following delivery to the plastid (della-Cioppa *et al.,* 1986). The modified corn EPSPS gene with its OTP sequence is approximately 1.7 Kb in size. The deduced amino acid sequence of the modified corn EPSPS with the OTP is shown in Figure IV.1. The fusion of the mEPSPS with the OTP results in an additional methionine at the N-terminal end of the mEPSPS protein.

The mEPSPS enzyme has been expressed in *E. coli* and purified. The mEPSPS interacts with the EPSPS substrates, shikimate-3-phosphate and phosphoenolpyruvate, similarly to the plant endogenous EPSPS enzyme, based on kinetic analyses (Rodriguez and Padgette, 1997).

Figure IV.1 **Deduced Amino Acid Sequence of the Modified Corn EPSPS Protein.** Sequence includes the OTP transit peptide (amino acids 1-125 are the transit peptide).

1 [**CBI-Deleted**

51

101

151

201

251

301

351

401

451

501

551]

B. Genes Present in Plasmid pDPG434, Outside of the NotI Fragment

1. *bla* bacterial gene

The β-lactamase gene from *E. coli* plasmid pBR322 is in the plasmid pDPG434. This enzyme confers resistance to the ampicillin and other penicillins antibiotics (Sutcliffe, 1978) and thereby allows for selection of bacteria containing the plasmid during its preparation. The coding sequence for the *bla* gene is present under its own bacterial promoter. The promoter for this gene is only active in bacterial cells. The *bla* sequence is not present in the agarose gel-isolated NotI restriction fragment of pDPG434 that was used for GA21 transformation (see Figure III.1). Southern blot analysis (Figure V.4) confirmed that the *bla* sequence is not present in GA21.

2. Plasmid backbone sequences

A partial sequence of the *lacZ* gene coding for the beta-D-galactosidase, is present under a bacterial promoter (Yanich-Perron *et al.*, 1985). This sequence is followed by the *bla* gene and the 0.65 Kb origin of replication for the pUC19 plasmid (ColE1) which allows for the replication of the plasmid in *E. coli*. The backbone sequences are not present in the agarose gel-isolated NotI restriction fragment of pDPG434 that was used for GA21 transformation (see Figure III.1). Southern blot analysis (Figure V.4) confirmed that the backbone sequences containing the ColE1 origin and the *bla* gene are not present in GA21.

V. Genetic Analysis and Agronomic Performance of Roundup Ready Corn Line GA21

A. Southern Gel Analysis

1. Summary

Roundup Ready corn line GA21 was produced by particle acceleration technology with a 3.4 Kb agarose gel-isolated NotI restriction fragment of the plasmid pDPG434. This fragment contains only the modified corn EPSPS gene fused to an optimized chloroplast transit peptide sequence, under control of the rice actin promoter and intron (McElroy *et al.*, 1990). The map of the plasmid vector is presented in Figure III.1 along with the restriction sites of the enzymes utilized for Southern analyses.

Molecular analysis was performed to characterize the integrated DNA present in the corn line. Using Southern blot analyses (Southern, 1975), the genomic DNA was evaluated for the number of sites into which the plasmid DNA integrated into the corn genome and the integrity of the genes contained within the insert. The results are summarized in Table V.1.

Table V.1 Summary of the Genetic Analysis of Roundup Ready Corn Line GA21

Genetic Element	GA21	Probe	Figure
	≈18.5 Kb insert	3.4 Kb NotI fragment pDPG434	Figure V.2
		0.34 Kb PCR pDPG434 (5089-5432)	Figure V.3
ColE1 origin of replication	absent	SspI/AflIII fragment pBluescript SK(-)	Figure V.4
bla gene	absent	SspI/AflIII fragment pBluescript SK(-)	Figure V.4
rice actin promoter	present	1.4 Kb PstI fragment pDPG434	Figure V.5
modified EPSPS	present	1.3 Kb PstI/XbaI fragment pDPG434	Figure V.6
NOS 3' end	present	0.21 Kb PCR pDPG434 (5902-6114)	Figure V.7

2. Materials and methods

Reference substances.

The reference substances for this investigation included the plasmid DNA used to produce corn line GA21, pDPG434, and a similar plasmid, pDPG427 (this plasmid is identical to pDPG434 except that it contains a corn histone promoter instead of the rice actin promoter). These plasmids and DNA from the nontransgenic control were mixed together then digested with the enzymes. The nontransgenic genomic DNA is used in these samples as a carrier for the plasmid DNA. This technique ensures the equivalent migration of both the plasmid and genomic DNA through the gel, thus establishing accurate size markers when comparing fragments of genomic DNA with plasmid DNA. The plasmid DNAs also served as positive hybridization controls. Additionally, molecular weight (MW) markers from Boehringer Mannheim (molecular weight markers II and IX, catalog #236 250 and catalog #1449 460, respectively) were also used for size estimations. The two MW

markers were mixed together and run on the gel. After gel electrophoresis, all gels were stained with ethidium bromide and photographed in the presence of a fluorescent ruler. Molecular weight size markers were then superimposed upon the scan of the autoradiogram. In some cases holes were poked into the bands of the markers. After transfer, the holes were marked on the nylon membrane with dots of water-proof ink, effectively transferring the correct size standards. These MW markers appear as black dots on the Southern blots. Molecular weights for the genomic fragments for both transgenic and nontransgenic control DNA were then estimated based on the location of the bands relative to the indicated size markers on the autoradiogram. When appropriate, specific restriction fragments from plasmid DNA of known size (based on plasmid maps) were used as reference markers.

DNA isolation.

DNA was isolated from a population of R1 plants that were segregating for the GA21 insert (heterozygous population). Positive and negative segregants were identified by PCR using oligonucleotide primers specific to the pDPG434 fragment used for transformation. Positive segregants were identified using PCR primers specific for the modified EPSPS gene used for transformation. The PCR primers spanned the EPSPS - NOS 3' end junction (see Figure III.1). The sequence of the sense primer is located within the coding sequence of the EPSPS gene. The sequence of the anti-sense primer is located within the NOS 3' end. These primers amplify a fragment of 930 bp. Negative segregants served as negative (nontransgenic) control plants. Negative segregants were chosen as negative controls for Southern blot analysis due to the fact that preliminary Southern blot analysis showed significant polymorphism associated with the endogenous copies of the corn EPSPS genes. Therefore, it was essential to have isogenic material as a control when performing Southern blot analyses using an EPSPS gene probe. The negative segregants in the breeding population were the only option for isogenic negative control material. Young leaf tissue was removed from the plants, placed on dry ice and stored at approximately -80°C prior to use. Genomic DNA was isolated as described in Dellaporta *et al.* (1983). Approximately 1 g of leaf tissue was quick frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. The powder was transferred to a 30 ml Oak Ridge tube containing 15 ml of extraction buffer (100 mM Tris pH 8.0, 50 mM EDTA pH 8.0, 500 mM NaCl, 10 mM mercaptoethanol), 1.0 ml of 20% SDS added and the tubes vigorously shaken and incubated at 65°C for 10 minutes. 5.0 ml of 5 M potassium acetate was added and the tubes were again vigorously shaken and incubated at 0°C for 20 minutes. The samples were centrifuged, the supernatant removed and precipitated with 10 ml of isopropanol for 30 minutes at -20°C. The DNA was then pelleted by centrifugation and the supernatant gently poured off and the pellets allowed to dry. The DNA was redissolved in 50 mM Tris (pH 8.0) and 10 mM EDTA (pH 8.0) and microfuged to remove insoluble debris. The supernatant was transferred to a new tube and the DNA precipitated with 3 M sodium acetate and isopropanol. After centrifugation, the resulting DNA pellet was washed in 80% ethanol, dried, and redissolved in TE buffer, pH 8.0.

DNA quantitation and restriction enzyme digestion.
The DNA samples were quantitated using a fluorimeter. Approximately 10 μg of the isolated genomic DNA from the test and control plants were used for the restriction enzyme digests. DNA was digested in a 200 μl volume for 4 hours at the appropriate temperature. The digests were then precipitated with sodium acetate in ethanol and resuspended in a 30 μl volume. This entire sample was loaded for each analysis, therefore each lane of digested DNA represents 10 μg of sample. All restriction enzymes were purchased from Boehringer Mannheim (Indianapolis, IN).

DNA probe preparation.
Plasmid DNAs [pDPG434 and pBluescript SK(-)] were isolated from an *E. coli* culture. Probes homologous to the NotI transformation fragment, modified EPSPS, rice actin promoter, NOS 3' end, *bla*, and ColEI genetic regions were prepared either by polymerase chain reaction (PCR) or isolated from plasmid DNA using the appropriate restriction enzyme digestion, agarose gel separation and purification. All probes were radioactively labelled with ^{32}P using the random priming method (RadPrime DNA Labelling System, Gibco BRL, Gaithersburg, MD or High Prime DNA Labelling System, Boehringer Mannheim, Indianapolis, IN).

Southern blot analysis.
The analytical procedure used for this investigation was Southern blot analysis, a common tool used for molecular characterizations (Southern, 1975). The samples of DNA treated with restriction enzymes were separated, based on size, using 0.8% or 1.0% agarose gel electrophoresis. The gels were usually electrophoresed for 13 hours at 35 volts and then for 2 hours at approximately 60 volts. The DNA from the agarose gels was transferred to nylon membranes using typical blotting procedures. The DNA was allowed to transfer for approximately 16 hours (using either 20X SSC or 20X SCP as the transfer buffer) and covalently cross-linked to the membrane with ultraviolet light. The blots were prehybridized in either an aqueous solution of 0.5 M $Na_2HPO_4 \cdot 7H_2O$, 7% SDS, 0.1 mg/ml tRNA or a solution consisting of 5X SCP, 2X Denhardt's Solution, 0.05 M Tris, pH 8.0, 0.2% SDS, 10 mM EDTA, 100 mg/L dextran sulfate, and 125 μg/ml denatured salmon sperm DNA. Hybridization with the radiolabeled probe was performed in fresh prehybridization solution typically for 14 to 16 hours at approximately 65°C. Membranes used in Southern blots for Figures V. 2, 3, and 4 were washed four times for 10 minutes in 0.5 X SCP and 0.2% SDS. Membranes in Figures V.5, 6 and 7 were washed in an aqueous solution of 40 mM $Na_2HPO_4 \cdot 7H_2O$, 5% SDS for two 30 minute periods at approximately 65°C. To reprobe blots, probes were removed by treating blots in 0.05 M NaOH, 0.2% SDS for 10 minutes followed by neutralization in 0.2 M Tris, pH 7.5, 0.2% SDS, 0.1 X SCP for 20 minutes at approximately 25°C.

3. Insert number

The genome of the Roundup Ready corn line GA21 was analyzed to determine the number of insertions of the pDPG434 NotI fragment used for transformation. GA21 genomic DNA was digested with EcoRV, a restriction enzyme that does not cut within the NotI EPSPS fragment used for transformation, and probed with the entire NotI EPSPS fragment (Figure V.2). GA21 (lane 1) produced one fragment, approximately 18.5 Kb in size. The nontransgenic control DNA did not produce any fragments which hybridized to the pDPG434 NotI probe (lane 2). Lane 3 shows NotI digested pDPG434 plasmid DNA which was included as a positive hybridization control at the level of approximately one copy per genome. Some background hybridization was seen with the pDPG434 NotI probe in both the transformed line, GA21 (lane 1) and the nontransgenic control DNA (lane 2). This was not unexpected as both the modified EPSPS gene and the chloroplast transit peptide sequences in the probe contain sequences homologous to the endogenous corn sequences.

To further clarify the presence of a single insertion of the pDPG434 NotI fragment in GA21 and to reduce the amount of background hybridization seen with the full-length pDPG434 NotI fragment probe, a 0.34 Kb DNA fragment internal to the EPSPS gene was used as a probe (Figure V.3). GA21 DNA (lane 1), nontransgenic DNA (lane 2) and plasmid pDPG434 mixed with nontransgenic DNA (lane 3) were all digested with EcoRV, which does not cut within the NotI pDPG434 fragment used for transformation. The 0.34 Kb EPSPS probe hybridized strongly to the 18.5 Kb band in GA21 (lane 1), confirming the presence of a single insertion of the NotI EPSPS fragment used for transformation. This small, internal EPSPS probe also hybridized to two smaller molecular weight bands in both the transformed line, GA21 (lane 1) and the nontransgenic control DNA (lane 2). These bands are the endogenous EPSPS sequences.

4. Genetic elements present in the insert

Absence of the ColEI origin of replication and the *bla* gene. DNA from the genetically modified corn line FI35 containing a single copy of the *bla* gene (Figure V.4 lane 1), GA21 DNA (lane 2) and plasmid DNA (lane 3) were digested with BglII and probed with an 1.7 Kb SspI/AflIII fragment from pBluescript SK(-) plasmid (Stratagene, La Jolla, CA) containing the ColE1 origin of replication and the *bla* gene, as described in Table III.1. Hybridization occurred with the SspI/AflIII probe to the *bla*-positive plant (lane 1), demonstrating that a single copy of the *bla* gene could be detected by Southern blot analysis with this probe. As expected from the plasmid map (Annex I), a 4.8 Kb hybridization band was observed with plasmid pDPG427 (lane 3). This plasmid is identical to pDPG434 except that it contains a corn histone promoter instead of the rice actin promoter (see plasmid map in Annex I). No evidence of the plasmid backbone sequence containing either the ColE1 origin of replication or the *bla* gene was detected in GA21 DNA (lane 2). FI35, the transgenic line containing the *bla* gene (lane 1) was transformed with

intact pDPG434 containing the *bla* gene. Southern blot analysis of several transgenic lines derived from transformations with EPSPS genes, with and without the plasmid backbone, has shown that using intact plasmid in most cases leads to integration of at least one copy of *bla*, while using gel-isolated DNA fragments containing only the EPSPS cassette consistently leads to plants that lack *bla*.

Rice actin promoter and intron. GA21 DNA and nontransgenic control DNA were digested with HindIII and probed with a 1.4 Kb PstI fragment from pDPG434 that contained the rice actin promoter sequence (Figure V.5). GA21 DNA (lane 3) produced three specific bands, estimated at 3.4 Kb, 4.5 Kb and 6.5 Kb in size, that were not detected in the nontransgenic control DNA (lane 2). This result demonstrates that GA21 contains at least three copies of the rice actin promoter and intron. The hybridization of the 4.5 Kb band with the rice actin probe appears of lower intensity than the hybridization with the 3.4 and 6.5 Kb bands. Two possible explanations for this difference are either that some secondary structure contained in the flanking plant DNA within this 4.5 Kb fragment (see Figure V.1 for clarification) prevented the rice actin probe from hybridizing efficiently or, the rice actin promoter within the 4.5 Kb DNA fragment may have been rearranged during the transformation process producing either a scrambled or a truncated promoter sequence. Nonetheless, other restriction analysis using SacI, SphI and EcoRI digests to confirm the composition of the insert (data not shown), indicated that these sites are functional and are situated in their predicted location within the rice actin promoter as determined by the plasmid map (Figure III.1).

Modified EPSPS gene. GA21 DNA and nontransgenic control DNA were digested with HindIII. 15 pg of plasmid pDPG434 (in order to approximate the level of one copy per genome) plus nontransgenic control DNA were mixed together and digested with NotI and EcoRV. NotI was used to obtain the size of the NotI fragment used during transformation. In order to facilitate the migration of the genomic DNA through the gel, the plasmid pDPG434 and nontransgenic control DNA mix was also digested with EcoRV, which only digests the plant DNA (there is no EcoRV site in pDPG434). The blot was probed with a 1.3 Kb PstI/XbaI fragment of pDPG434 that contained the full-length modified EPSPS gene (Figure V.6). Plasmid pDPG434 mixed with nontransgenic control DNA as a carrier (lane 1), produced the expected 3.4 Kb band which represents the entire NotI fragment used for transformation of GA21. This was used as a specific size reference when analyzing GA21 digested with HindIII (Figure V.6, lane 3). The highest molecular weight band in the nontransgenic control DNA (lane 2) is the endogenous corn EPSPS. This band is identical to the high molecular weight band seen in the transformed line, GA21 (lane 3) since both samples were digested with HindIII. An endogenous corn EPSPS band of high molecular weight was also seen in the plasmid control (lane 1), but it is not identical in size since EcoRV and NotI were used to cut the plasmid mixed with the nontransgenic control DNA. GA21 DNA (lane 3) produced three fragments unique from the nontransgenic

control (lane 2) when probed with the full-length mEPSPS gene. The 3.4 Kb, 4.5 Kb and 6.5 Kb bands appear to be identical in size to the three bands produced when GA21 was digested with HindIII and probed with the rice actin promoter (Figure V.5, lane 3). The 3.4 Kb band (Figure V.6, lane 3) resulting from the HindIII digestion, represents one copy of the modified EPSPS gene which would be generated if the insert contained two full-length NotI fragments of pDPG434 in tandem (See Figure V.1). The 4.5 Kb and 6.5 Kb bands represent two other fragments containing mEPSPS genes. See Figure V.1 for a schematic representation of the GA21 insert based on the conclusions of these Southern blot analyses.

NOS 3' end. GA21 DNA and the nontransgenic control DNA were digested with HindIII. The nontransgenic DNA mixed with 15 pg of plasmid pDPG434 was digested with NotI in order to obtain the size of the NotI fragment used during transformation, and digested with EcoRV (which does not cut plasmid pDPG434) in order to further digest the plant genomic DNA and facilitate its migration through the gel. The blot was probed with a 0.21 Kb PCR-generated fragment from the NOS 3' end (Figure V.7). The NotI digest of pDPG434 plasmid (lane 1) gave the expected 3.4 Kb band. The GA21 DNA (lane 3) gave two bands estimated at 3.4 Kb and 6.5 Kb in size. These data support the conclusion that GA21 contains two full-length copies of the NotI fragment from pDPG434 in tandem. The 3.4 Kb band (Figure V.7, lane 3) represents the termination site of the first copy of the NotI fragment from pDPG434. The 6.5 Kb band represents the termination site located on the second full-length NotI fragment from pDPG434 (See Figure V.1 for clarification). These data, in conjunction with the blots probed with the rice actin promoter (Figure V.5) and the EPSPS gene (Figure V.6), show that GA21 contains two complete copies of the NotI fragment of pDPG434 including the NOS 3' ends plus an additional tandem copy of the fragment that contains the rice actin promoter and mEPSPS sequence without the NOS 3' end.

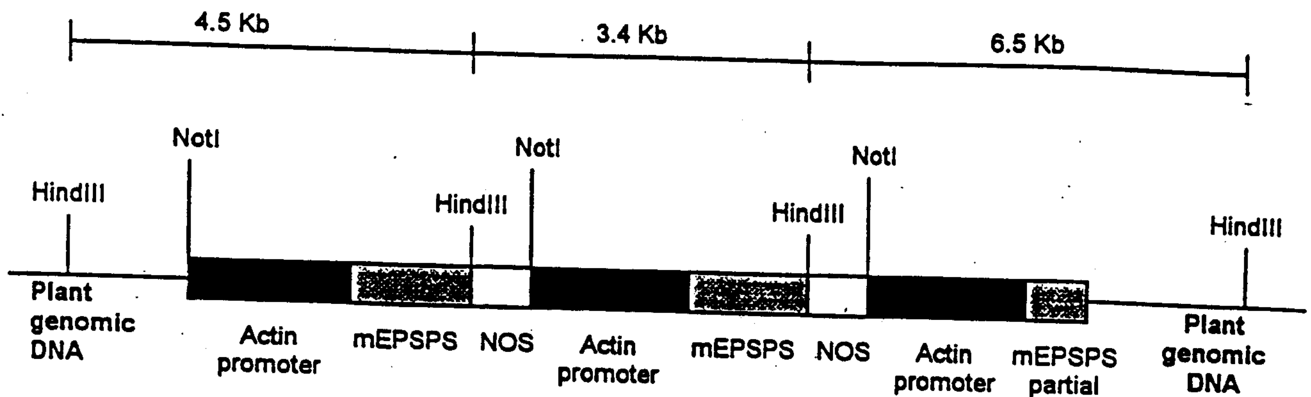
5. Conclusion

Roundup Ready corn line GA21 was produced by particle acceleration technology with a NotI restriction fragment of plasmid pDPG434 containing the modified corn EPSPS gene cassette. Corn line GA21 contains one DNA insert on a EcoRV fragment estimated at 18.5 Kb. This insert contains two copies of the modified corn EPSPS gene cassette, and a third copy which has been demonstrated to contain the rice actin promoter and mEPSPS sequence without the NOS 3' end. As expected from transformation with a gel-isolated NotI restriction fragment containing only the mEPSPS gene cassette, the Roundup Ready corn line GA21 does not contain the *bla* gene nor the sequence containing the ColE1 origin of replication.

Additionally, a western blot analysis (Annex II) conducted in order to assess the equivalence of the modified EPSPS protein (mEPSPS) produced by Roundup Ready corn line GA21 and *E. coli*, supports the molecular analysis by showing that only one immuno-reactive protein of the expected apparent

molecular weight (≈47 kD) is found in crude extracts of Roundup Ready corn line GA21 tissue.

Figure V.1 Schematic Representation of the GA21 Insert



This figure depicts the predicted insert for GA21 based on Southern blot analysis. Actin promoter denotes the 1.74 Kb rice actin promoter and the OTP sequence. mEPSPS denotes the 1.34 Kb modified EPSPS gene. NOS indicates the 0.24 Kb NOS 3' end. There are two copies of the mEPSPS gene cassette in tandem adjacent to a third copy that contains the rice actin promoter and mEPSPS sequence without the NOS 3' end. Directly above the map is a representation of the different size fragments that are produced when GA21 DNA is digested with the HindIII restriction enzyme. When GA21 is digested with EcoRV, a restriction enzyme which does not cut within the inserted DNA (its restriction sites would be situated within the plant genomic DNA in the above schematic), a single approximately 18.5 Kb fragment containing the insert is identified.

Figure V. 2: Insert Analysis Using a Full-length NotI Probe.

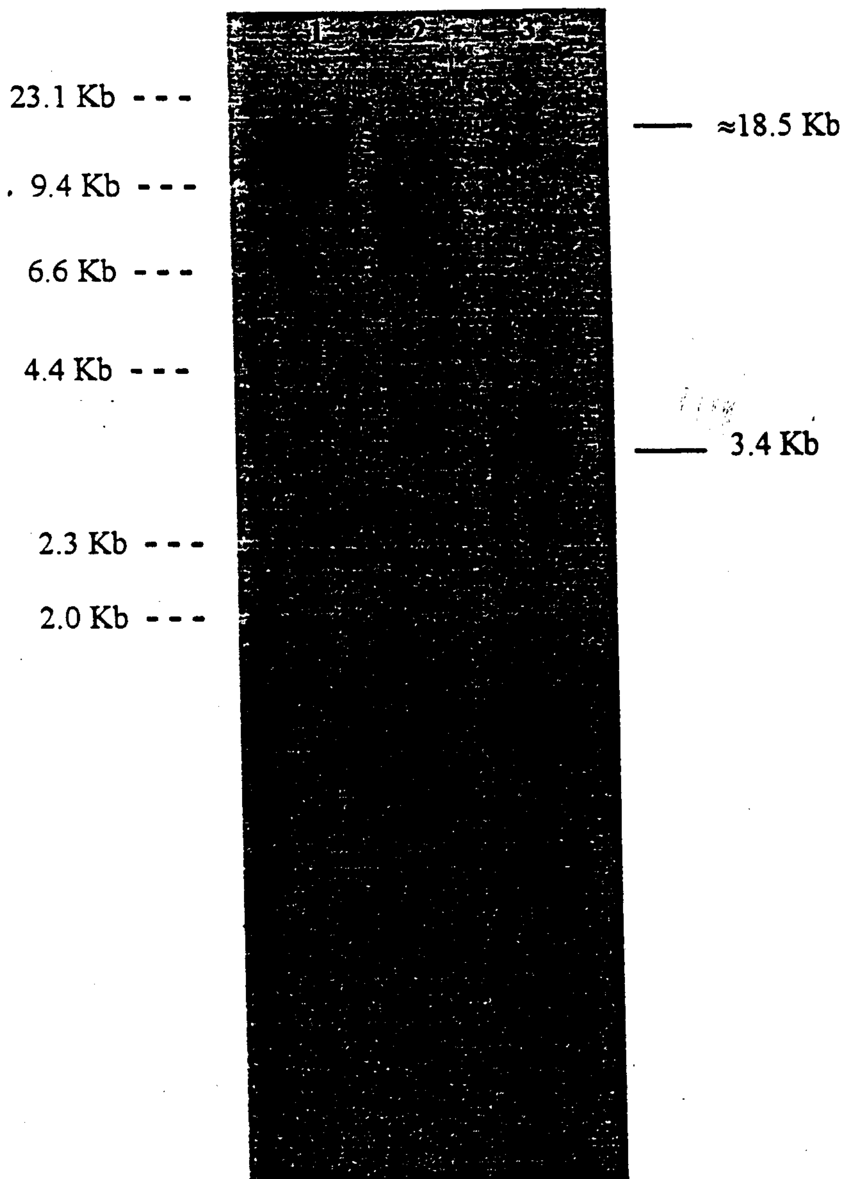


Figure V.2:
Lane 1 contains GA21 DNA digested with EcoRV.
Lane 2 contains nontransgenic control DNA digested with EcoRV.
Lane 3 contains pDPG434 digested with NotI.
The blot was probed with the 3.4 Kb NotI fragment from pDPG434.

- - - Symbol denotes sizes obtained from MW markers on ethidium stained gel.
—— Symbol denotes sizes obtained from plasmid digests.
≈ Symbol denotes a band size estimated from MW markers and plasmid digests.

Figure V.3: Insert Analysis Using an Internal EPSPS Probe.

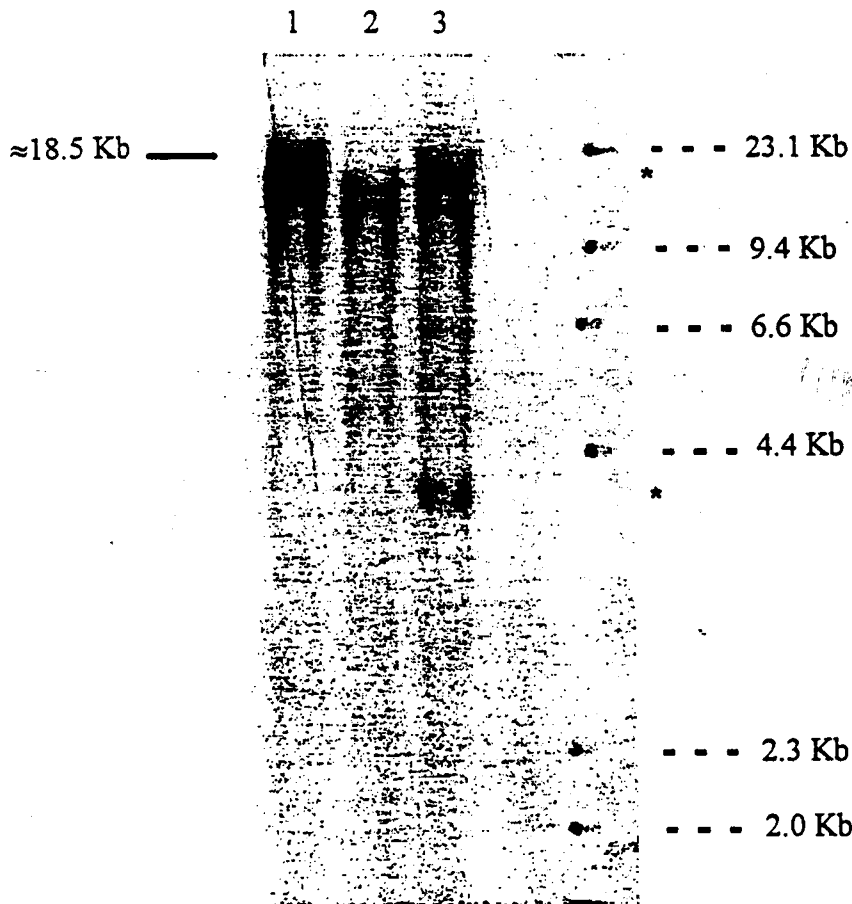


Figure V.3:
Lane 1 contains GA21 DNA digested with EcoRV.
Lane 2 contains nontransgenic control DNA digested with EcoRV.
Lane 3 contains nontransgenic control DNA mixed with 15 pg of pDPG434, both digested with EcoRV. The blot was probed with the 343 bp PCR-generated internal EPSPS fragment (see Table V.1).

- - - Symbol denotes sizes obtained from MW markers on ethidium stained gel.
—— Symbol denotes sizes obtained from plasmid digests.
≈ Symbol denotes a band size estimated from MW markers and plasmid digests.
* Symbol denotes bands (in lane 3) resulting from superhelical and nicked circular forms of the uncut plasmid.

Figure V.4: ColE1 and *bla* Gene Analysis.

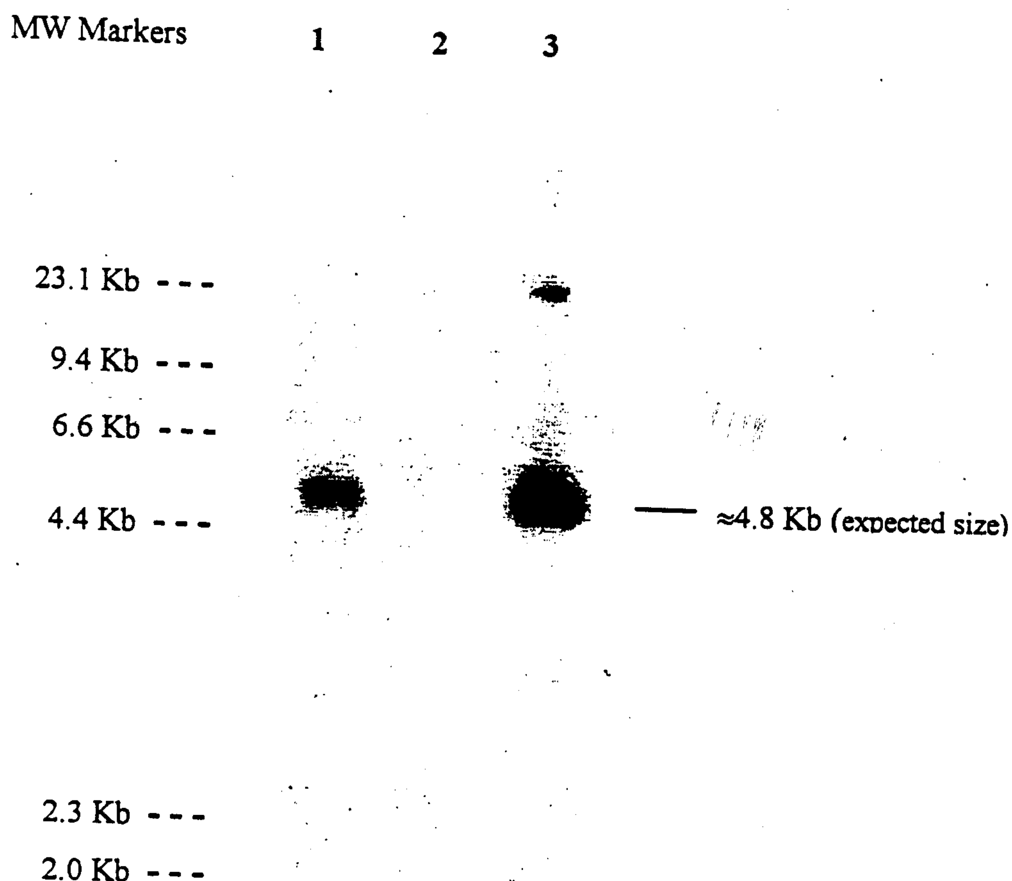


Figure V.4:
Lane 1 contains DNA from FI35, a genetically modified corn line containing a single copy of the *bla* gene, lane 2 contains GA21 DNA and lane 3 is loaded with 6.25 pg of plasmid pDPG427 DNA in order to approximate one copy of the *bla* gene per maize genome (in this particular gel the plasmid DNA has not been spiked into nontransgenic corn DNA). All three lanes contain DNA that was digested with BglII and probed with a 1.7 Kb SspI/AflIII fragment from pBluescript SK(-) that contains the ColEI origin of replication and the *bla* gene.

- - - Symbol denotes sizes obtained from MW markers on ethidium stained gel.
—— Symbol denotes sizes obtained from plasmid digests.
≈ Symbol denotes a band size estimated from MW markers and plasmid digests.

Figure V.5: Rice Actin Promoter and Intron Analysis.

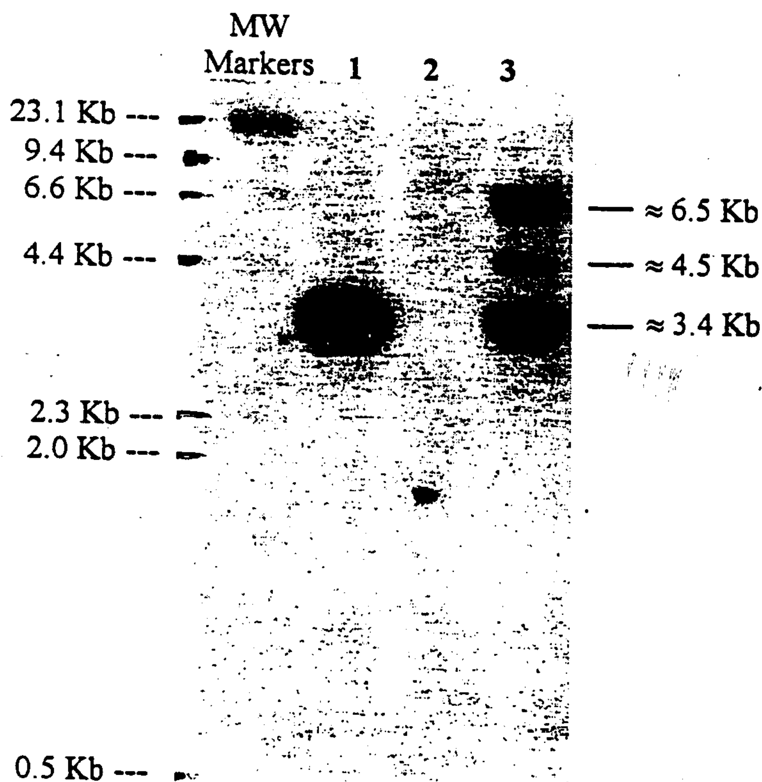


Figure V.5:
Lanes 1, 2 and 3 contain the following DNAs digested with HindIII and probed with the 1.4 Kb PstI fragment of pDPG434 containing the rice actin promoter and intron sequences: lane 1, nontransgenic control DNA mixed with 15 pg of pDPG434; lane 2, nontransgenic control DNA; lane 3, GA21 DNA.

- - - Symbol denotes sizes obtained from MW markers on ethidium stained gel.
—— Symbol denotes sizes obtained from plasmid digests.
≈ Symbol denotes a band size estimated from MW markers and plasmid digests.

Figure V.6: Modified EPSPS Gene Analysis.

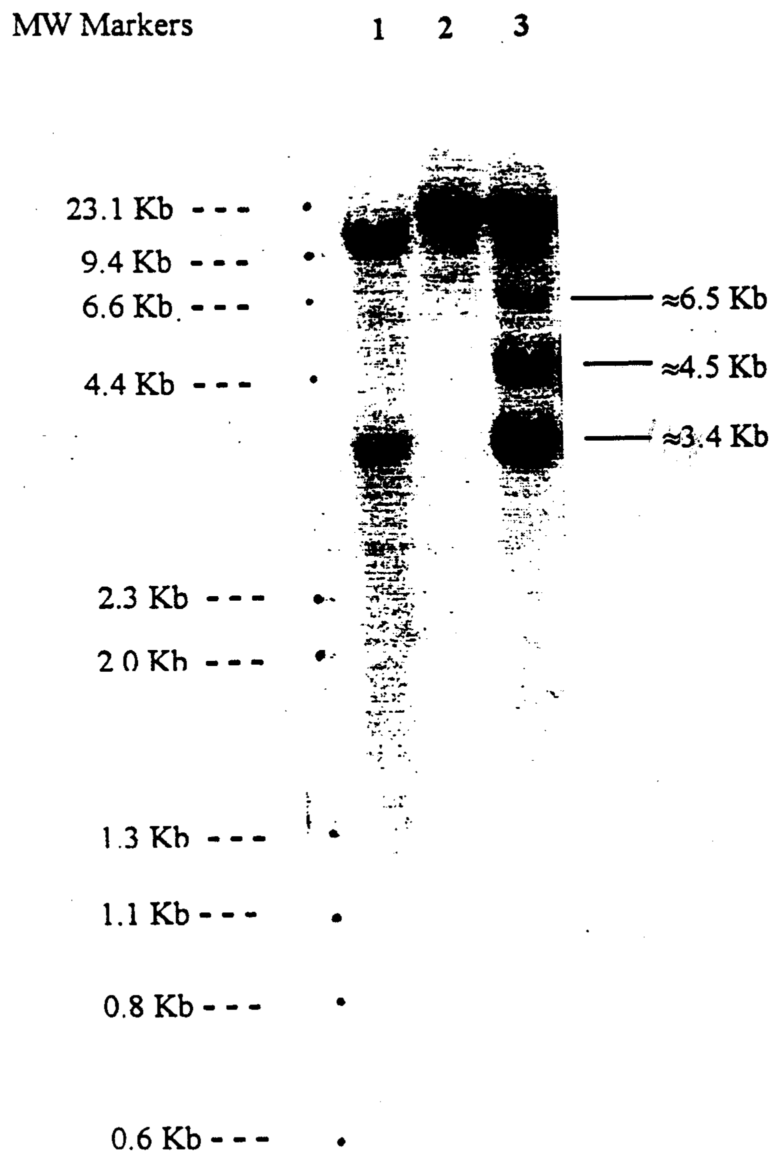


Figure V.6:
Lane 1 contains nontransgenic control DNA mixed with 15 pg of pDPG434 both digested together with NotI and EcoRV.
Lanes 2 and 3 contain nontransgenic control DNA and GA21 DNA respectively, both digested with HindIII only.
This blot was probed with a 1.3 Kb PstI/XbaI fragment of pDPG434 that contains the full-length modified EPSPS gene (see Table V.1).

- - - Symbol denotes sizes obtained from MW markers on ethidium stained gel.
—— Symbol denotes sizes obtained from plasmid digests.
≈ Symbol denotes a band size estimated from MW markers and plasmid digests.

Figure V.7: NOS 3' End Analysis.

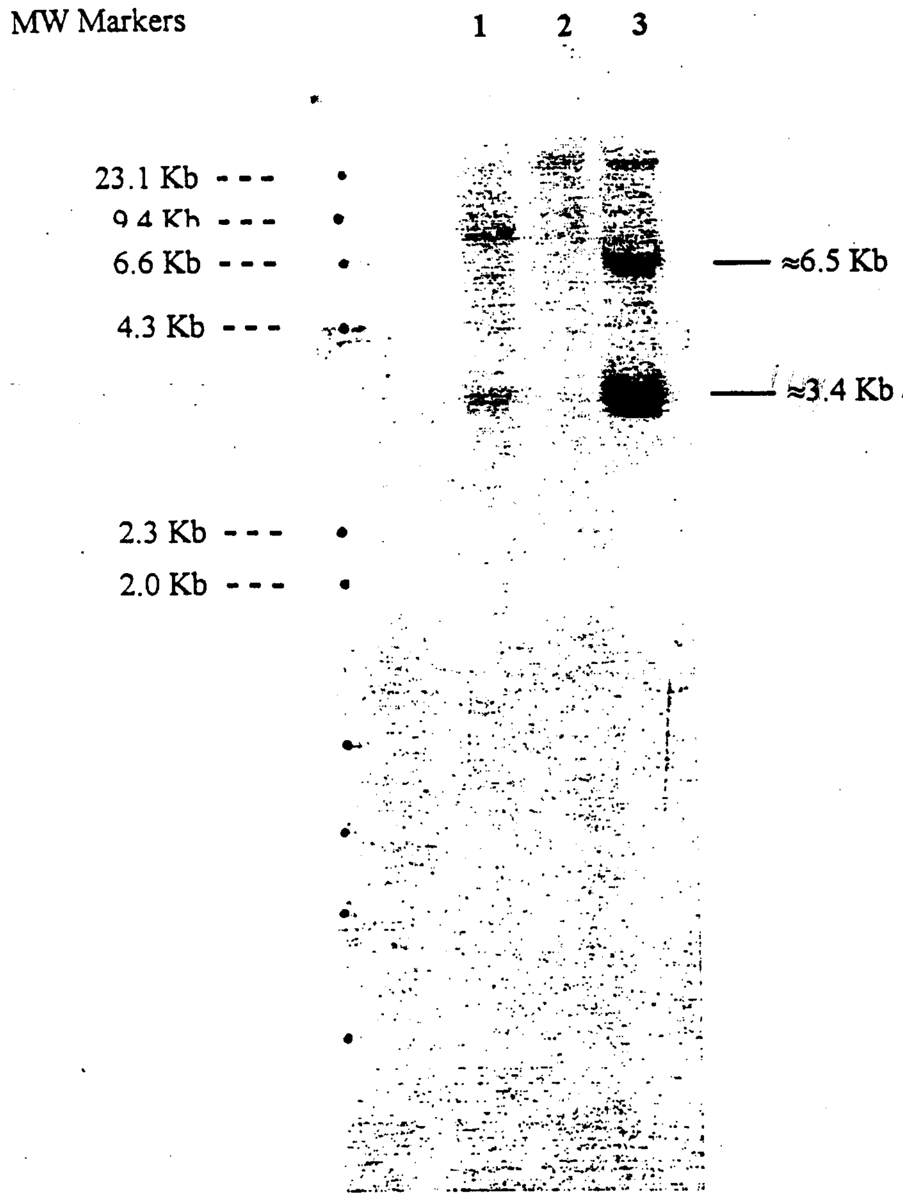


Figure V.7:
Lane 1 contains nontransgenic control DNA mixed with 15 pg of pDPG434 digested with NotI and EcoRV.
Lanes 2 and 3 contain nontransgenic control DNA and GA21 DNA, respectively, both digested with HindIII.
The blot was probed with a 212 bp PCR-generated, internal, partial NOS fragment (see Table V.1).

- - - Symbol denotes sizes obtained from MW markers on ethidium stained gel.
—— Symbol denotes sizes obtained from plasmid digests.
≈ Symbol denotes a band size estimated from MW markers and plasmid digests.

B. Segregation Data and Stability of Gene Transfer of Roundup Ready Corn Line GA21

Segregation data for six generations of line GA21 progeny are presented in Table V.2. Data are presented for the BC0F1 plants (derived from crossing the R0 with an inbred line), BC1F1 plants (derived from crossing the BC0 F1 plants to the same inbred used to cross with the R0 plant), BC2 F1, BC3F1, BC4F1, BC5F1 and BC5F2 progeny (derived from selfing individual BC5F1 plants).

Statistical significance for the segregation data was determined using Chi Square analyses. For these analyses, a Chi Square value (X^2) was determined as follows: $X^2 = \sum [(|o-c|-0.5)^2/c]$ where o = observed frequencies, c = calculated frequencies for the various classes, and 0.5 the Yates correction factor for Chi square analysis with 1 df (Little and Jackson Hills, 1978). Calculated and observed frequencies were determined as described in footnotes of the table. The calculated value was compared to a Table of Chi Square to determine whether the observed frequencies fit the expectation for single insert at p = 0.05 and/or p = 0.01.

Table V.2 Segregation Data and Analysis of Progeny of Roundup Ready Corn Line GA21

Generation	Actual	Expected	ChiSq
BC0F1[1]	64:52	58:58	1.04#
BC1F1[1]	180:165	172.5:172.5	0.57#
BC2F1[1]	55:62	58.5:58.5	0.31#
BC3F1[1]	108:77	92.5:92.5	4.86+
BC4F1[1]	77:76	76.5:76.5	0.00#
BC5F1[1]	60:40	50:50	3.61#
BC5F2[1]	731:262	744.75:248.25	0.94#

[1] Data expressed as number of tolerant plants: number of susceptible plants based on glyphosate sprays.

\# not significant at p = 0.05 (chi square = 3.84, 1 df).

\+ significant at p = 0.05 (chi square = 3.84, 1 df), not significant at p = 0.01 (chi square = 6.63).

The chi square analyses of the segregation results are consistent with a single active site of insertion of the modified corn EPSPS genes into the genomic DNA of Roundup Ready corn line GA21, segregating according to Mendelian genetics. These results are consistent with the molecular analysis described in section V.A. The stability of the insert has been demonstrated through five generations of crossing and one generation of self pollination.

C. Expression of the Modified Corn EPSPS

1. Introduction

The glyphosate tolerant phenotype conferred by the expression of the modified corn EPSPS in GA21 plants has been observed in field trials conducted in the

U.S. in 1994, 1995 and 1996 and has lead to the selection of GA21 as a commercial product. A western blot analysis (Annex II) was conducted to assess the equivalence of the modified EPSPS protein (mEPSPS) produced by Roundup Ready corn line GA21 and *E. coli*. This western blot showed that the mEPSPS protein produced in corn line GA21 and *E. coli* are equivalent and that only one immuno-reactive protein of the expected apparent molecular weight is found in crude extracts of Roundup Ready corn line GA21 tissue. This equivalence justifies the use of the *E. coli*-produced protein as a reference standard in the ELISA (Enzyme Linked Immuno-Sorbent Assay) assay used to estimate the expression levels of the modified EPSPS in corn line GA21, as described in the following section.

2. Expression levels of the modified corn EPSPS protein in Roundup Ready corn line GA21

The expression of the modified corn EPSPS is expected to occur throughout the whole plant since the rice actin promoter has been shown to drive constitutive expression in genetically modified corn (Zhong *et al.*, 1996). Levels of the expressed protein were evaluated in forage and grain samples collected from five field locations during the 1996 growing season using an ELISA assay (Harlow and Lane, 1988). The five field sites established were as follows: Jerseyville, IL; Monmouth, IL; Dekalb, IL, Mystic, CT and Thomasboro, IL. The corn seed planted in the trial was BC_4F_1 progeny (derived from crossing individual BC_4F_1 plants by a non-genetically modified tester); the grain from self-pollinated plants was used for the analysis.

Expression levels of the mEPSPS protein in forage and grain were determined using an antibody sandwich ELISA consisting of anti-petunia polyclonal EPSPS antibody + mEPSPS protein + anti-petunia EPSPS IgG conjugated to horseradish peroxidase (HRP). The levels of the mEPSPS protein in plant extracts were quantitated by comparison to a range of concentrations of the *E. coli*-produced mEPSPS reference standard, which was the positive control in this assay. Plants from plots not treated with glyphosate and identified by PCR as negative segregants were used as negative controls.

The *E. coli*-produced mEPSPS protein was shown to be immunologically equivalent to the mEPSPS expressed in corn leaf tissue by a dilution equivalence (parallelism) ELISA experiment. Thus the responses of the mEPSPS protein derived from both sources were found to be linear and parallel to each other in the concentration range 0.012-0.8 µg/mL. The polyclonal antibody used for the ELISA was produced in goats using petunia EPSPS as the immunogen. Petunia EPSPS was produced in *E. coli* and purified to near homogeneity prior to use for antibody production. Petunia EPSPS shares approximately 84% amino acid sequence identity with both the wild-type maize EPSPS and mEPSPS proteins. Therefore, the goat anti-petunia EPSPS antibody is an appropriate reagent for the detection of these proteins.

Forage samples: Forage was defined as the entire corn plant minus the roots collected at the soft dough stage. At each site, 2 to 4 plants were collected and composited to provide a pooled forage sample. Each pooled forage sample was processed to a fine powder and an aliquot removed for analysis. The estimated expression levels across sites are shown in Table V.3.

Grain samples: There was one pooled grain sample from each site, consisting of grain from 9 to 16 ears, depending on the site. A sample of the pooled grain collected at each site was processed to a fine powder and an aliquot removed for analysis. The estimated expression levels across sites are shown in Table V.3.

Table V.3 Summary of mEPSPS[1] Protein Levels Measured by the ELISA in Tissues of GA21 Corn Plants (µg /g fresh weight)

	mean[2]	range[3]
Forage[4]	118.7	46.6-210.4
Grain[4]	3.2	1.4-4.9

[1]: Values represent the sum of the endogenous and modified corn EPSPS protein expression levels.

[2]: The mean was calculated from the analyses of plant samples, one from each of five sites.

[3]: Minimum and maximum values from the analyses of samples across sites.

[4]: The quantitation limit of the EPSPS ELISA assay was ca. 0.003 µg/mL.

In *grain*, expression of the endogenous EPSPS in nontransgenic negative segregant control samples was nondetectable at all sites. In *forage*, expression of the endogenous EPSPS in nontransgenic negative segregant control samples was nondetectable at one site and detectable but not quantifiable at four sites because the values were below the reference standard calibration curve. These results are consistent with those obtained by the Western blot technique (Annex II) which showed that the expression of mEPSPS protein in GA21 was at least one order of magnitude greater than that of the endogenous EPSPS expressed in the nontransgenic negative segregant control.

D. Disease and Pest Susceptibilities

Roundup Ready corn line GA21 was field tested in the United States in 1994, 1995 and 1996, at the locations listed in Table V.4. Monitoring for the disease and insect susceptibilities of the Roundup Ready corn line GA21 when compared to nontransgenic plants was performed one or more times per season at each of these locations (Annex III).

Table V.4 **Roundup Ready Corn Line GA21 Field Trial Locations**

year	location	USDA Notification
1994	Maui, HI	94-182-03N
1995	Maui, HI	94-283-02N
1995	Champaign, IL	95-074-01N
1996	New London, CT	96-137-02N
1996	Maui, HI	95-158-01N, 96-241-02N
1996	Champaign, IL	96-071-07N
1996	DeKalb, IL	96-071-07N
1996	Jersey, IL	96-071-07N
1996	Warren, IL	96-071-07N
1996	Yauco, PR	96-278-02N

Diseases and insects observed at each locations are summarized in Table V.5 and Table V.6 respectively. No differences in disease severity or insect infestations were detected between Roundup Ready corn line GA21 and non-genetically modified control plants.

E. Agronomic Characteristics of Roundup Ready Corn Line GA21

Based on the observations in field trials conducted in the United States in 1994, 1995 and 1996, Roundup Ready corn line GA 21 has been selected as a commercial product because of its Roundup tolerance performance and the absence of differences between this line and nonmodified plants for the following criteria: seedling emergence, phenotypic characteristics (ear height, plant height, % of root lodged, % of stalks lodged, % of dropped ears, stay green), growth (seedling vigor, plant height), tasseling, pollen and silk GDU (growing degree units), and yield.

F. Conclusion

Roundup Ready corn line GA21 is tolerant to Roundup through the expression of a modified corn EPSPS protein whose sequence is more than 99.3 % identical to the endogeneous corn EPSPS protein. Information included in this section demonstrates that, other than the expression of Roundup tolerance, there are no phenotypic or agronomic differences between Roundup Ready corn line GA21 and nonmodified control corn.

Table V.5 Diseases Observed at Trial Locations with Roundup Ready Corn Line GA21

	Foliar Disease								Ear Rot					Stalk Rot				
	Helminthosporium Leaf Spot	Common Corn Rust	Stewart's Disease	Common Corn Smut	Anthracnose	Southern Corn Leaf Blight	Northern Corn Leaf Blight	Gray Leaf Spot	Fusarium Ear Rot	Gibberella Ear Rot	Penicillium Ear Rot	Trichoderma Ear Rot	Diplodia Ear Rot	Gibberella Stalk Rot	Fusarium Stalk Rot	Charcoal Stalk Rot	Anthracnose Stalk Rot	Red Root Rot
1994 Maui County, HI		X				X	X		X	X	X	X		X	X			
1995 Maui County, HI		X				X	X		X	X	X	X		X	X			
1995 Champaign County, IL	X	X	X	X	X			X	X				X	X		X		X
1996 New London County, CT		X																
1996 Maui County, HI		X				X	X		X	X	X	X		X	X			
1996 Champaign County, IL	X	X	X		X		X	X	X				X	X			X	X
1996 De Kalb County, IL	X	X		X	X	X	X	X	X		X			X	X		X	X
1996 Jersey County, IL				X				X										
1996 Warren County, IL		X		X				X										
1996 Yauco Municipio, PR						X												

X denotes that a particular disease was observed at this location

No differences in disease severity were detected between Roundup Ready corn line GA21 and non-genetically modified control plants

Table V.6 Insects Observed at Trial Locations with Roundup Ready Corn Line GA21

Location	Sugarcane Borer	Corn Rootworm	European Corn Borer	Southwestern Corn Borer	Corn Earworm	Fall Armyworm	Black Cutworm	Aphid	Flea Beetle	Japanese Beetle	Spidermite	Stinkbug	Leafhopper
1994 Maui County, HI	X				X			X		X	X	X	
1995 Maui County, HI	X		X	X	X		X	X		X	X	X	
1995 Champaign County, IL		X							X				
1996 New London County, CT								X					
1996 Maui County, HI	X				X			X		X	X	X	
1996 Champaign County, IL		X	X					X					
1996 DeKalb County, IL			X										
1996 Jersey County, IL		X	X					X					
1996 Warren County, IL			X										
1996 Yauco Municipio, PR					X								X

X denotes that a particular insect was observed at this location

No differences in insect infestation were detected between Roundup Ready corn line GA21 and non-genetically modified control plants

VI. Environmental Consequences of Introduction of Roundup Ready Corn Line GA21

A. The Herbicide Glyphosate

Glyphosate (N-phosphonomethyl-glycine) (CAS Registry #'s 1071-83-6, 38641-94-0), the active ingredient in the non-selective, foliar-applied, broad-spectrum, post-emergent herbicide Roundup (Baird, 1971; Malik *et al.*, 1989), is the world's most popular herbicide. Glyphosate is highly effective against the majority of annual and perennial grasses and broad-leaved weeds. The herbicide glyphosate kills plants cells by inhibition of the enzyme EPSPS, an enzyme involved in the shikimic acid pathway for aromatic amino acid biosynthesis in plants and microorganisms (Steinrücken and Amrhein, 1980). The aromatic amino acid pathway is not present in mammalian metabolic pathways (Cole, 1985). This contributes to the selective action of glyphosate toward plants but not mammals. Glyphosate has favorable environmental and safety characteristics, such as rapid soil binding (resistance to leaching) and biodegradation (which decreases persistence), as well as extremely low toxicity to mammals, birds and fish (Malik *et al.*, 1989). Glyphosate is classified by the EPA as Category E (evidence of non-carcinogenicity for humans) (57 FR 8739). Studies separate from those summarized herein have been provided to the EPA in a request to amend the Roundup herbicide label to include in-season application on Roundup Ready corn. This label was approved on March 28th, 1997.

B. Current Uses of Herbicides on Corn and Impact of the Introduction of Roundup Ready Corn

Corn is very sensitive to weed competition at the early stages of growth. Weed control in corn is predominantly achieved by chemical methods. The choice of the product depends on many factors, but the considerations include the weed species present, herbicide efficacy, crop safety (selectivity), products and application costs, human health and environmental safety considerations and simplicity and flexibility of use. Herbicides are either active by foliar uptake or by root uptake, the latter usually requiring soil persistence in order to sustain weed control over a longer period. Herbicides are applied to corn before planting (preplant applications), at planting (preemergence applications) or after seedling emergence (postemergence applications) at timings usually related to the growth stage of the targeted weeds. Herbicides are applied on 94.3% of the U.S. corn acreage (more than 73 million acres in 1996), with an average of 1.4 treatments per treated acre.

Several postemergence herbicides are registered for use on corn. These herbicides include atrazine, dicamba, imazethapyr, nicosulfuron, rimsulfuron and sethoxydim, used alone or in mixtures. The timing of application is related to the growth stage of the targeted weeds and usually takes place when corn plants are less than 12 inches in height.

The use of corn plants containing the Roundup Ready gene for corn production would enable the farmer to utilize postemergence Roundup application for effective control of weeds during the growing season and to take advantage of this herbicide's environmental and safety characteristics. No increase of the proportion of corn acreage treated with herbicide is expected since herbicides are currently used on the vast majority of corn acreage in the U.S. Roundup Ready corn can positively impact current agronomic practices in corn by:

- Offering the farmer a new, wide-spectrum weed control option.
- Allowing the use of an environmentally acceptable herbicide.
- Increasing flexibility to treat weeds on an "as needed" basis.
- Providing an excellent fit with reduced-tillage systems, which results in increased soil moisture, while reducing soil erosion and fuel use.
- Providing cost-effective weed control, simplicity and flexibility of use.

C. Appearance of Glyphosate-Resistant Weeds

Today there exist some 109 herbicide-resistant weed biotypes, with over half of them resistant to the triazine family of herbicides (Holt and Le Baron, 1990; Le Baron, 1991; Shaner, 1995). Resistance to herbicides has usually developed because of the selection pressure exerted by the repeated use of herbicides with a single target site and a specific mode of action, long residual activity with the capacity to control weeds year-long, and frequent applications without rotation to other herbicides or cultural control practices. Using these criteria and based on current use data, glyphosate is considered to be a herbicide with a low risk for weed resistance (Benbrook, 1991). Nonetheless, it has been questioned whether the introduction of crops tolerant to a specific herbicide, such as glyphosate, may lead to the occurrence of weeds resistant to that particular herbicide. This concern is based on the assumptions that the use of the herbicide will be increased significantly, and possibly that it will be used repeatedly in the same location. However, other increases in glyphosate use over the previous years, have been more significant than the projected increase associated with the introduction of Roundup Ready crops in the U.S. Although it cannot be stated that evolution of resistance to glyphosate will not occur, the development of weed resistance to glyphosate is considered unlikely because:

1. Weeds and crops are inherently not tolerant to glyphosate, and the long history of extensive use of glyphosate has not resulted in resistant weeds. Glyphosate has been used for over 20 years in various preplant, directed, spot and postharvest weed management systems with no verified cases of weed resistance (Holt *et al.*, 1993; Dyer, 1994). A preliminary report was recently presented that discussed annual ryegrass (*Lolium* sp.) seeds collected from a field that, upon germination, demonstrated a rate-related tolerance to glyphosate (Pratley *et al.* 1996). This observation merits further investigation. Insufficient data were reported to define the factors contributing to the observed phenomenon and Monsanto has entered into a collaborative research agreement with Charles Sturt,University of Wagga Wagga in Australia to further investigate these results. Since the source of the 'sensitive' biotype used by Pratley (Pratley *et al.*, 1996) was from a different location than the

'resistant' biotype their genetic relatedness is unclear and additional research to address this question is being initiated.

2. Glyphosate has many unique properties, such as its mode of action (glyphosate is unrelated to triazines and has a differing mode of action from any other herbicide on the market today), chemical structure, limited metabolism in plants, lack of residual activity in the soil and its relatively quick break down by microorganisms in the soil (Malik *et al.*, 1989).

3. Selection for glyphosate resistance using whole plant and cell/tissue culture techniques, including mutagenesis, was largely unsuccessful, and unlikely to be duplicated under normal field conditions. Similarly, the complex genetic transformations required for the development of glyphosate tolerant crops (e.g. modified gene, unique promoters, transit peptide, etc...) would be unlikely to be duplicated in nature to yield glyphosate resistant weeds (Bradshaw *et al.*, 1997).

D. Weediness Potential of Roundup Ready Corn

Modern corn cannot survive as a weed because of past selection in the evolution of corn. In contrast with weedy plants, the corn ear is enclosed with husks. Consequently, seed dispersal of individual kernels does not occur naturally because of the structure of ears of corn. However, even if individual kernels of corn were distributed in the fields and main avenues of travel from the fields to storage facilities, volunteer corn is not found growing in fence rows, ditches, and road sides as a weed (Hallauer, Section II. The Corn Family). Corn cannot survive without human assistance and is not capable of surviving as a weed (Galinat, 1988). Further, although corn seed can overwinter into a crop rotation with soybeans, mechanical and chemical measures can be utilized to control volunteers. Even in the case of a rotation involving Roundup Ready soybeans, it should be considered that a) no-till cultivation which is used on 35% of the soybean acreage, reduces dramatically the occurrence of volunteers and b) control of Roundup Ready corn volunteers will remain possible with an application of grass-killer herbicides such as fluazifop-p, clethodim, quizalofop-ethyl, sethoxydim, currently used in soybean crops for volunteer corn control.

E. Cross Pollination to Wild and Cultivated Related Species

The potential for pollen transfer from corn to other species is addressed in Part II of this document, "The Corn Family", by Arnel R. Hallauer, Ph.D., Department of Agronomy, Iowa State University.

Based upon the report cited above, an extensive review of literature, and a history of nonregulated status for genetically modified corn phenotypes (96-017-01p, 95-195-01p, 95-145-01p, 95-093-01p, 94-357-01p and 93-319-01p), outcrossing from corn to other species and for the Roundup Ready corn to become a weed or pest is considered of very low probability in the United States.

1. Outcrossing to wild *Zea* species

For gene flow to occur via normal sexual transmission, certain conditions must exist: (1) the two parents must be sexually compatible, (2) their fecundity must coincide, (3) a suitable pollen vector must be present and capable of transferring pollen between the two parents, and (4) resulting progeny must be fertile and ecologically fit for the environment in which they are situated.

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

The habitat preferences of *Tripsacum*, another closely related genus, are similar to those of teosinte with twelve of the sixteen species native to Mexico and Guatemala. *Tripsacum floridanum* (Florida Gamagrass) is native to the southern tip of Florida. Outcrossing with *Tripsacum* species is not known to occur in the wild and only with extreme difficulty can corn be crossed with *Tripsacum*. Further, the offspring of this cross show varying levels of sterility (Galinat, 1988; Manglesdorf, 1974; Russell and Hallauer, 1980). No cases of gene flow between corn and its wild relatives are known in the United States.

2. Outcrossing to cultivated *Zea* varieties

Gene exchange between cultivated corn and genetically modified corn would be similar to what naturally occurs at the present time. Wind-blown pollen would move about among plants within the same field and among plants in nearby fields. Free flow of genes would occur similar to what occurs in cultivated corn. The production of the modified corn EPSPS protein in resulting seed would not be of concern since the protein is closely related (more than 99.3 % identical) to the endogeneous corn EPSPS. EPSPS is an enzyme of the shikimate pathway for aromatic amino acid biosynthesis in plants (including corn) and microorganisms (Levin and Sprinson, 1964; Steinrüken and Amrhein, 1980), and is thus ordinarily present in plants and in food derived from plant sources.

F. Transfer of Genetic Information to Species to Which Corn Cannot Interbreed

As stated in the USDA's Interpretative Ruling on Calgene, Inc., Petition for Determination of Regulatory Status (FR 57, No.202, pp 47608-47616, October 19, 1992) "There is no published evidence for the existence of any mechanism, other than sexual crossing" by which genes can be transferred from a plant to other organisms. Evidence presented in the Calgene petition and supplementary information and summarized in the FR Notice suggests that, based on limited DNA homologies, transfer from plants to microorganisms may

have occurred in evolutionary time over many millennia. Even if such transfer were to take place, transfer of an EPSPS gene to a microbe would not pose any plant pest risk. Based on these considerations, transfer to microbes or other living species in nature is extremely unlikely and of no significant consequence from a plant pest point of view.

G. Conclusion

A variety of studies were conducted to characterize the unique trait of the modified corn line and to establish that Roundup Ready corn line GA21 is substantially equivalent to nonmodified corn, except for the inserted genetic sequences, the expressed protein (the modified corn EPSPS protein) and the tolerance of the plant to the herbicide Roundup. These include:

- expression of the modified corn EPSPS protein (section V.C)
- disease and pest susceptibility (section V. D)
- agronomic characteristics (section V. E)
- the potential for outcrossing and weediness (sections VI. D, E and F)

Data and information supplied in these sections demonstrate that Roundup Ready corn line GA21 is not likely to pose a greater plant pest risk than nonmodified corn. This conclusion is based on evaluation of phenotypic characteristics, the similarity of the expressed protein with the endogeneous corn EPSPS (sequences more than 99.3 % identical), and the lack of any deleterious environmental fate/effects.

VII. Statement of Grounds Unfavorable

Monsanto Company and DEKALB Genetics Corporation know of no unfavorable grounds associated with Roundup Ready corn line GA21. Therefore, on the basis of the substantial potential benefits to the grower, the environment, and the consumer, Monsanto and DEKALB request that this corn line no longer be regulated under 7 CFR part 340.6.

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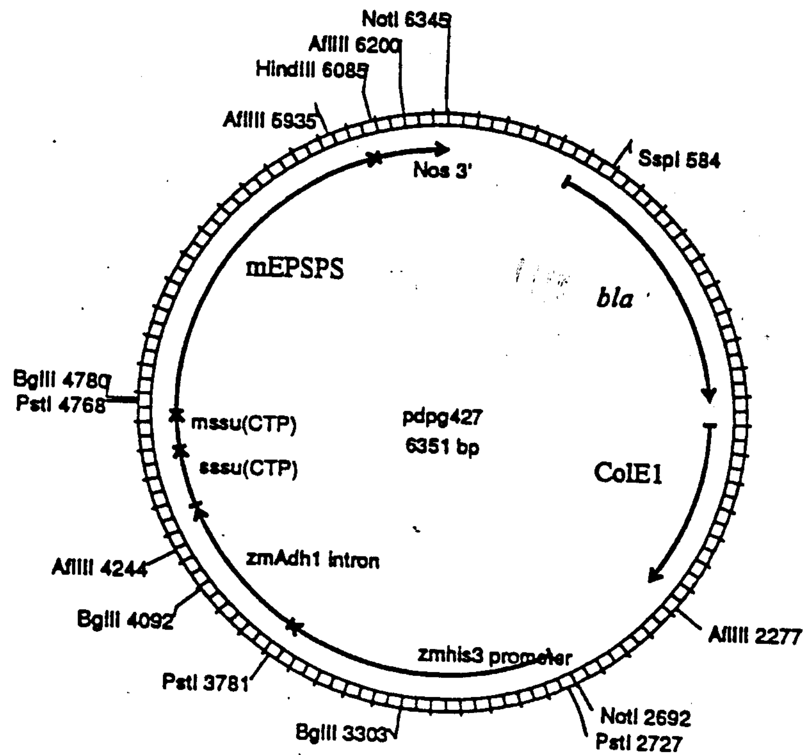
Annex I: Plasmid Map of pDPG427



Annex II. Expression of the Modified Corn EPSPS

1. Introduction

Western blot analysis was conducted to assess the equivalence of the modified EPSPS protein (mEPSPS) produced by Roundup Ready corn line GA21 and *E. coli*. The western blot analytical procedure was chosen for this assessment because it is a highly specific immunological technique which allows for comparison of the apparent molecular weights of proteins possessing immunological cross-reactivity in complex mixtures such as crude protein extracts from corn. The *E. coli*-produced protein was used as a reference standard in the ELISA (Enzyme Linked Immuno-Sorbent Assay) assay to estimate the expression levels of the modified EPSPS as described in section V.C. Additionally, the western blot analysis supports the molecular analysis of Roundup Ready corn line GA21 by showing that only one immuno-reactive protein of the expected apparent molecular weight is found in crude extracts of Roundup Ready corn line GA21 tissue.

2. Material and method

Samples for analysis. There were two test materials in this investigation: (a) mEPSPS protein endogenously expressed by Roundup Ready corn line GA21 and (b) mEPSPS protein produced in and partially purified from *E. coli* (lot # 5981991-A). The *E. coli*-produced protein was produced in strain W3110 containing the plasmid pMON32961, which contained the gene encoding mEPSPS protein. A control sample (negative segregant of GA21) which does not express the mEPSPS protein was also included. Young leaf tissue of Roundup Ready line GA21 and the control sample were obtained as frozen leaves from material harvested from 1996 US field trials. The leaf tissue samples had been continuously stored at -80 °C before use.

3. Preparation of protein extracts for western blot analysis. Leaf samples of line GA21 and the nontransgenic negative segregant were ground to a fine powder in liquid nitrogen and stored at approximately - 80 °C until extracted. Approximately 0.5 gram of frozen powder of each sample was homogenized independently in a 13 ml plastic centrifuge tube with approximately 3.75 ml of an extraction buffer solution containing 50 mM HEPES-NaOH, pH 7.0, 1 mM EDTA, 1 mM DTT, 20% (v/v) glycerol, 1 mM PMSF, 1 mM benzamidine-HCl, 1 mg/ml pepstatin A, 1 mg/ml leupeptin, and 1% (v/v) PVPP. Homogenization was with an Omni 2000 hand held homogenizer at medium speed for 30 seconds. Samples were clarified by centrifugation, and the supernatants decanted to a new plastic tube. Nucleic acids and phospholipids were precipitated by the addition of polyethylenimine to a 0.1% (v/v) final concentration. The extracts were then concentrated approximately 3 to 4 fold using Millipore Ultrafree-15 centrifuge filter units (10 kD MW cut-off) according to the manufacturer's instructions. A 100 µL aliquot of each plant extract was added to 20 µL of 5X Laemmli buffer and heated for 5 min at 100 °C to produce approximately 1X Laemmli samples. The

GA21 1X Laemmli sample was further diluted 7.4 fold with 1X Laemmli buffer and all samples analyzed on the same day.

Two 1X Laemmli samples of 2.0 ng/μL and 0.5 ng/μL of the *E. coli* produced mEPSPS protein standard (lot # 5981991-A, 27.54 ng/μL) were prepared by first diluting the 27.54 ng/μL stock solution with milli Q water and subsequently mixing with an equal amount of 2X Laemmli buffer. These samples were then heated for 5 min at 100 °C and analyzed the same day together with the leaf tissue extracts.

Electrophoresis. Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) was performed on 4 to 20% gradient gels according to SOP PB-EQP-005-01 using the mini gel system of Novex (San Diego, CA). Electrophoresis was conducted at constant voltage (approximately 200 V) for approximately one hour (until the dye front reached the bottom of the gel).

Western blot. Proteins separated by SDS-PAGE were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon P, Millipore Corp., Bedford, MA) at 300 mA constant current for approximately one hour. After blocking non-specific sites, the protein blot was probed with an approximately 1:1000 dilution of goat anti-petunia EPSPS (purified IgGs) antibody.[1] Goat antibody bound to the blot was detected using an approximately 1:7500 dilution of an anti-goat IgG antibody conjugated to horse radish peroxidase (Sigma, P/N A-5420) and the enhanced chemiluminescence (ECL) detection system of Amersham (P/N=RPN 2106, Buckinghamshire, England).

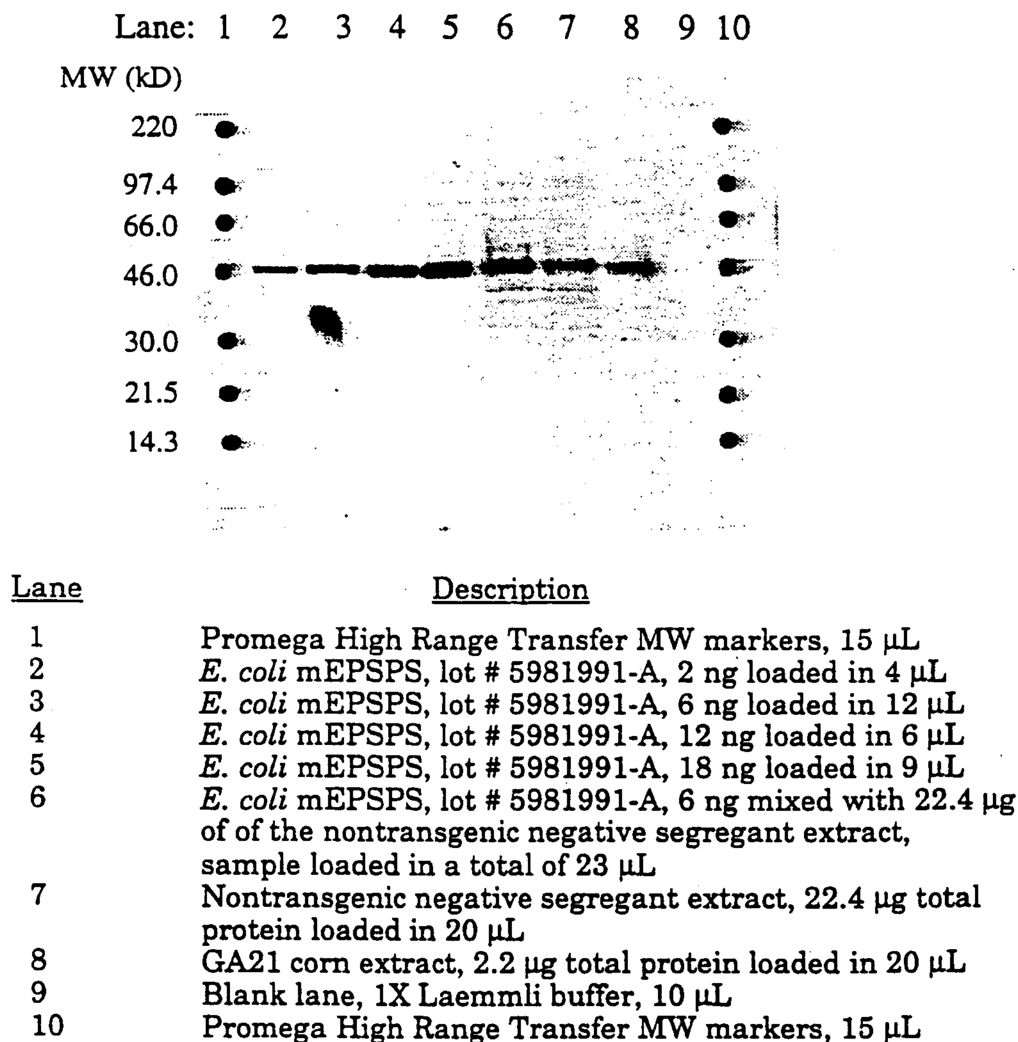
3. Results and conclusions. A western blot comparing the apparent molecular weight (MW) of the mEPSPS protein produced by Roundup Ready corn line GA21 and *E. coli* (lot #5981991-A) is shown in Figure 1 below. Four levels of the *E. coli* sample (2 ng, 6 ng, 12 ng and 18 ng) were loaded in lanes 2, 3, 4 and 5, respectively. Additionally, the *E. coli* protein was spiked into the nontransgenic negative segregant matrix (lane 6, 6 ng *E. coli* protein mixed with 22.4 μg corn protein extract) to assess any bias associated with spiking the standard into a high protein loading of the corn matrix. The nontransgenic negative segregant extract was loaded in lane 7, and the mEPSPS positive GA21 sample was loaded in lane 8. Lane 9 was loaded with 1X Laemmli buffer and lanes 1 and 10 were loaded with high range MW markers (colored). The nontransgenic segregant was used as the negative control because it is isogenic to the transformed line GA21. The expression of endogenous EPSPS in plant tissues is very low relative to microorganisms (Mousdale and Coggins, 1987). Therefore, the nontransgenic negative segregant extract was loaded such that

[1] **The petunia and mEPSPS proteins are 84% identical based on the primary amino acid sequence. Due to this high degree of sequence identity, there would be many epitopes in common between these two proteins that would be recognized by the anti-petunia polyclonal antibody. Therefore, the goat anti-petunia EPSPS is an appropriate antibody to detect the mEPSPS protein.**

its protein level was ten times that of the GA21 extract (22.4 μg of total protein vs. 2.2 μg of total protein).

Immuno-reactive bands at the expected apparent molecular weight (≈47 kD) were observed for lanes loaded with either the *E. coli*-produced protein (#5981991-A, lanes 2 to 5), the nontransgenic negative segregant (lane 7), or extracts prepared from Roundup Ready line GA21 (lane 8). The finding of immuno-reactive bands in lanes 6 and 7 at approximately the apparent molecular weight (≈ 47 kD) demonstrates that the electrophoretic mobility of mEPSPS was not significantly impacted when the amount of total protein loaded/lane was very high (22.4 μg). No other significant immuno-reactive bands were detected in any of the samples loaded.

Figure 1: Equivalence of the mEPSPS Protein in Roundup Ready Corn Line GA21 and the mEPSPS Produced in *E. coli*.



Lane	Description
1	Promega High Range Transfer MW markers, 15 μL
2	*E. coli* mEPSPS, lot # 5981991-A, 2 ng loaded in 4 μL
3	*E. coli* mEPSPS, lot # 5981991-A, 6 ng loaded in 12 μL
4	*E. coli* mEPSPS, lot # 5981991-A, 12 ng loaded in 6 μL
5	*E. coli* mEPSPS, lot # 5981991-A, 18 ng loaded in 9 μL
6	*E. coli* mEPSPS, lot # 5981991-A, 6 ng mixed with 22.4 μg of of the nontransgenic negative segregant extract, sample loaded in a total of 23 μL
7	Nontransgenic negative segregant extract, 22.4 μg total protein loaded in 20 μL
8	GA21 corn extract, 2.2 μg total protein loaded in 20 μL
9	Blank lane, 1X Laemmli buffer, 10 μL
10	Promega High Range Transfer MW markers, 15 μL

● The position of the MW markers was determined after films were developed by overlaying the original blot and marking the position with a pen. The large dark spot located in lane 3 is an artifact of the development process.

In conclusion, based on western blot analysis, the mEPSPS protein produced by *E. coli* and used as an ELISA standard, and the mEPSPS expressed by Roundup Ready line GA21 are equivalent. Additionally, this western blot shows that immuno-reactive bands of similar intensity are observed in both the nontransgenic negative segregant (lane 7) and GA21 extracts (lane 8) when their respective loadings were in the ratio 10:1. This indicates that the expression of mEPSPS in GA21 is at least one order of magnitude greater than that of the endogenous mEPSPS found in the nontransgenic negative segregant. Finally, the absence of any additional immuno-reactive bands in the GA21 extracts suggests that only the protein of the expected size is expressed.

Annex III: USDA Field Trial Termination Reports

see Volume 2

97-099-01p

Petition for Determination of
Nonregulated Status
Roundup Ready Corn LIne

GA21

97-138U

Volume 2: Annex III
USDA Field Trial Termination Reports
97-099-01p

97-099-01p

Annex III:

USDA Field Trial Termination Report

ENVIRONMENTAL RELEASE REPORT

Permit Number: 94-182-03N

Effective Date: July 31, 1994

Permittee: DEKALB Genetics Corporation
3100 Sycamore Road
DeKalb, IL 60115
(815) 758-7333

Transgenic Lines: Corn, *Zea mays*, cultivars B73 derived AT (S70FI, S70FJ, S71FI, S71FK, S74GA, S76GD, S76GE, S71FJ, S78GG, S78GJ, S79IX) and A188 x B73 (HI10)

Sites (with maximum acreage) and periods of release:

Kihei, Maui County, HI (0.01 acres) 8/94 - 11/94

Purpose of Release:

1. Incorporate the transgenic locus into DEKALB germplasm
2. Determine segregation ratios of the transgenic locus

Results:

1. Incorporated the transgenic locus into DEKALB germplasm
2. Determined segregation ratios of the transgenic locus

Monitoring of Field Containment:

Wildlife or livestock invading field:	none
Insect and pest control:	pesticides and herbicides
Adequate isolation:	was maintained
Water management:	irrigation

Plant Characteristics Monitored for Changes:

Reproductive traits:	no change
Disease status	no change
Morphological traits:	no change
Herbicide resistance:	now resistant to glyphosate
Weediness:	no change
Ability to outcross:	no change
Survival of progeny in the field:	no change
Insect resistance:	no change

Any indication that inserted DNA has been transferred to other species, microbial or plant? No

Methods Used to Isolate Transgenic Lines from Other Corn Lines

One (or more) of the following methods were employed:

a) Spatial: Transgenic corn was open-pollinated and spatially isolated by at least 660 feet from other corn
b) Physical: In situations where transgenic plants were shedding pollen, ear shoots of all corn plants within 660 feet were ear-bagged; and/or transgenic plants were either detasselled or tassels were bagged.
c) Temporal: Transgenic plants were planted at a significantly different time from adjacent nontransgenic plants to prevent cross-pollination.

Means of Plant Disposition:

Plant material was either:

a) stored for future use (e.g. seed for breeding purposes), or
b) destroyed by incorporation into the soil (e.g. repeated disking)

Monitoring for Volunteers:

All volunteers were destroyed prior to anthesis.

Additional Comments:

No other wild plant species that can cross-pollinate with corn are present in North America or Hawaii; therefore transfer to wild weedy relatives is not possible.

ENVIRONMENTAL RELEASE REPORT

Permit Number: 94-283-02N

Effective Date: November 9, 1994

Permittee: DEKALB Genetics Corporation
3100 Sycamore Road
DeKalb, IL 60115
(815) 758-7333

Transgenic Lines: Corn, *Zea mays*, lines FI117, FI35, GA21, GA29, FJ15, GG23, GG25, GJ11, GP12, GP15

Sites (with maximum acreage) and periods of release:

Kihei, Maui County, HI (0.6 acres) 11/94 - 11/95

Purpose of Release:

1. Incorporate the transgenic locus into DEKALB germplasm
2. Determine segregation ratios of the transgenic locus

Results:

1. Incorporated the transgenic locus into DEKALB germplasm
2. Determined segregation ratios of the transgenic locus

Monitoring of Field Containment:

Wildlife or livestock invading field:	none
Insect and pest control:	pesticides and herbicides
Adequate isolation:	was maintained
Water management:	irrigation

Plant Characteristics Monitored for Changes:

Reproductive traits:	no change
Disease status	no change
Morphological traits:	no change
Herbicide resistance:	now resistant to glyphosate
Weediness:	no change
Ability to outcross:	no change
Survival of progeny in the field:	no change
Insect resistance:	no change

Any indication that inserted DNA has been transferred to other species, microbial or plant? No

Methods Used to Isolate Transgenic Lines from Other Corn Lines

One (or more) of the following methods were employed:

a) Spatial: Transgenic corn was open-pollinated and spatially isolated by at least 660 feet from other corn
b) Physical: In situations where transgenic plants were shedding pollen, ear shoots of all corn plants within 660 feet were ear-bagged; and/or transgenic plants were either detasselled or tassels were bagged.
c) Temporal: Transgenic plants were planted at a significantly different time from adjacent nontransgenic plants to prevent cross-pollination.

Means of Plant Disposition:

Plant material was either:

a) stored for future use (e.g. seed for breeding purposes), or
b) destroyed by incorporation into the soil (e.g. repeated disking)

Monitoring for Volunteers:

All volunteers were destroyed prior to anthesis.

Additional Comments:

No other wild plant species that can cross-pollinate with corn are present in North America or Hawaii; therefore transfer to wild weedy relatives is not possible.

ENVIRONMENTAL RELEASE REPORT

Permit Number: 95-074-01N

Effective Date: April 17, 1995

Permittee: DEKALB Genetics Corporation
3100 Sycamore Road
DeKalb, IL 60115
(815) 758-7333

Transgenic Lines: FI35, FI177, FJ15, GA01, GA21, GA26, GA29, GG04, GG23, GP15, GJ11, GG22, GG25

Sites (with maximum acreage) and periods of release:

Thomasboro, IL (0.6 acres)
5/95 - 7/95

Purpose of Release:

Herbicide resistance evaluation

Results:

Herbicide resistance was evaluated.

Monitoring of Field Containment:

Wildlife or livestock invading field:	none
Insect and pest control:	pesticides and herbicides
Adequate isolation:	was maintained
Water management:	irrigation

Plant Characteristics Monitored for Changes:

Reproductive traits:	terminated before flowering
Disease status	no change
Morphological traits:	no change
Herbicide resistance:	now resistant to glyphosate
Weediness:	no change
Ability to outcross:	terminated before flowering
Survival of progeny in the field:	terminated before flowering
Insect resistance:	no change

Any indication that inserted DNA has been transferred to other species, microbial or plant? No

Methods Used to Isolate Transgenic Lines from Other Corn Lines

Plants were destroyed (hoed out) before flowering.

Means of Plant Disposition:

Plant material was destroyed by incorporation into the soil (e.g. repeated discing).

Monitoring for Volunteers:

Not applicable, as test plants were destroyed before flowering.

Additional Comments:

No other wild plant species that can cross-pollinate with corn are present in Illinois, and plants were destroyed prior to flowering, therefore transfer to wild weedy relatives is not possible.

ENVIRONMENTAL RELEASE REPORT

Permit Number: 95-158-01N

Effective Date: July 7, 1995

Permittee: DEKALB Genetics Corporation
3100 Sycamore Road
DeKalb, IL 60115
(815) 758-7333

Transgenic Lines: Corn, *Zea mays*, lines S71FI02, S71FI177, S71FI01, S70FI35, S76GC03, S74GA01, S74GA26, AV10EG38, S74GA21, S74GA36, S74GA27, S74GA29, S78GG04, S78GG22, S78GG06, S78GG25, S78GG23, S79IX02, S71FJ15, S71FJ48, S76GE04, S78GJ11, HI10GP12, HI10GP15

Sites (with maximum acreage) and periods of release:

Kihei, Maui County, HI (0.61 acres) 11/95 - 11/96

Purpose of Release:

1. Incorporate the transgenic locus into DEKALB germplasm
2. Determine segregation ratios of the transgenic locus

Results:

1. Incorporated the transgenic locus into DEKALB germplasm
2. Determined segregation ratios of the transgenic locus

Monitoring of Field Containment:

Wildlife or livestock invading field:	none
Insect and pest control:	pesticides and herbicides
Adequate isolation:	was maintained
Water management:	irrigation

Plant Characteristics Monitored for Changes:

Reproductive traits:	no change
Disease status	no change
Morphological traits:	no change
Herbicide resistance:	now resistant to glyphosate
Weediness:	no change

Ability to outcross:	no change
Survival of progeny in the field:	no change
Insect resistance:	no change

Any indication that inserted DNA has been transferred to other species, microbial or plant? No

Methods Used to Isolate Transgenic Lines from Other Corn Lines

One (or more) of the following methods were employed:

a) Spatial: Transgenic corn was open-pollinated and spatially isolated by at least 660 feet from other corn
b) Physical: In situations where transgenic plants were shedding pollen, ear shoots of all corn plants within 660 feet were ear-bagged; and/or transgenic plants were either detasselled or tassels were bagged.
c) Temporal: Transgenic plants were planted at a significantly different time from adjacent nontransgenic plants to prevent cross-pollination.

Means of Plant Disposition:

Plant material was either:

a) stored for future use (e.g. seed for breeding purposes), or
b) destroyed by incorporation into the soil (e.g. repeated disking)

Monitoring for Volunteers:

All volunteers were destroyed prior to anthesis.

Additional Comments:

No other wild plant species that can cross-pollinate with corn are present in North America or Hawaii; therefore transfer to wild weedy relatives is not possible.

ENVIRONMENTAL RELEASE REPORT

Permit Number: 96-071-07N

Effective Date: April 10, 1996

Permittee: DEKALB Genetics Corporation
3100 Sycamore Road
DeKalb, IL 60115
(815) 758-7333

Transgenic Lines: Corn, *Zea mays*, lines FI117, GA21, GG25, and GJ11

Sites (with maximum acreage) and periods of release:

Site	Period
Champaign County, IL (1.5 acres)	5/96 - 11/96
DeKalb County, IL (1.2 acres)	5/96 - 11/96
Warren County, IL (0.4 acres)	5/96 - 11/96
Jersey County, IL (0.04 acres)	5/96 - 11/96

Purpose of Release:

1. Evaluate herbicide resistance of the transgenic line
2. Incorporate the transgenic locus into DEKALB germplasm
3. Determine segregation ratios of the transgenic locus
4. Set up demonstration plots

Results:

1. Evaluated herbicide resistance of the transgenic line
2. Incorporated the transgenic locus into DEKALB germplasm
3. Determined segregation ratios of the transgenic locus
4. Showed demonstration plots to the public

Monitoring of Field Containment:

Wildlife or livestock invading field:	none
Insect and pest control:	pesticides and herbicides
Adequate isolation:	was maintained
Water management:	irrigation

Plant Characteristics Monitored for Changes:

Reproductive traits:	no change
Disease status	no change
Morphological traits:	no change

Herbicide resistance:	now resistant to glyphosate
Weediness:	no change
Ability to outcross:	no change
Survival of progeny in the field:	no change
Insect resistance:	no change

Any indication that inserted DNA has been transferred to other species, microbial or plant? No

Methods Used to Isolate Transgenic Lines from Other Corn Lines

One (or more) of the following methods were employed:

a) Spatial: Transgenic corn was open-pollinated and spatially isolated by at least 660 feet from other corn
b) Physical: In situations where transgenic plants were shedding pollen, ear shoots of all corn plants within 660 feet were ear-bagged; and/or transgenic plants were either detasselled or tassels were bagged.
c) Temporal: Transgenic plants were planted at a significantly different time from adjacent nontransgenic plants to prevent cross-pollination.
d) Early termination: Transgenic corn was destroyed before pollen shed.

Means of Plant Disposition:

Plant material was either:

a) stored for future use (e.g. seed for breeding purposes), or
b) destroyed by incorporation into the soil (e.g. repeated disking)

Monitoring for Volunteers:

This report was prepared before volunteers from the 1996 test season had come up. Volunteers will be monitored during 1997 and destroyed prior to anthesis.

Additional Comments:

No other wild plant species that can cross-pollinate with corn are present in North America or Hawaii; therefore transfer to wild weedy relatives is not possible.

ENVIRONMENTAL RELEASE REPORT

Permit Number: 96-137-02N

Effective Date: June 15, 1996

Permittee: DEKALB Genetics Corporation
3100 Sycamore Road
DeKalb, IL 60115
(815) 758-7333

Transgenic Lines: Corn, *Zea mays*, lines FI117, -GA21, GG25, GJ11

Sites (with maximum acreage) and periods of release:

New London County, CT (1.5 acres) 6/96 - 10/96

Purpose of Release:

1. Evaluate herbicide resistance of the transgenic line
2. Determine segregation ratios of the transgenic locus

Results:

1. Evaluated herbicide resistance of the transgenic line
2. Determined segregation ratios of the transgenic locus

Monitoring of Field Containment:

Wildlife or livestock invading field:	none
Insect and pest control:	pesticides and herbicides
Adequate isolation:	was maintained
Water management:	natural rainfall only

Plant Characteristics Monitored for Changes:

Reproductive traits:	no change
Disease status	not monitored
Morphological traits:	no change
Herbicide resistance:	now resistant to glyphosate
Weediness:	no change
Ability to outcross:	no change
Survival of progeny in the field:	no change
Insect resistance:	no change

Any indication that inserted DNA has been transferred to other species, microbial or plant? no

Methods Used to Isolate Transgenic Lines from Other Corn Lines

Physical: In situations where transgenic plants were shedding pollen, ear shoots of all corn plants within 660 feet were ear-bagged; and/or transgenic plants were either detasselled or tassels were bagged.

Means of Plant Disposition:

Plant material was either:

a) stored for future use (e.g. seed for breeding purposes), or
b) destroyed by incorporation into the soil (e.g. repeated disking)

Monitoring for Volunteers:

This report was prepared before volunteers from the 1996 test season had come up. Volunteers will be monitored during 1997 and destroyed prior to anthesis.

Additional Comments:

No other wild plant species that can cross-pollinate with corn are present in North America; therefore transfer to wild weedy relatives is not possible.

INTERIM
ENVIRONMENTAL RELEASE REPORT

Report Date March 27, 1997

Permit Number: 96-241-02N

Effective Date: September 27, 1996

Permittee: DEKALB Genetics Corporation
3100 Sycamore Road
DeKalb, IL 60115
(815) 758-7333

Transgenic Lines: Corn, *Zea mays*, lines GA21, GG25, GJ11

Sites (with maximum acreage) and periods of release:

Maui County, HI (0.3 acres to date) 11/96 - current

Purpose of Release:

1. Incorporate the transgenic locus into DEKALB germplasm
2. Determine segregation ratios of the transgenic locus

Results:

1. Incorporated the transgenic locus into DEKALB germplasm
2. Determined segregation ratios of the transgenic locus

Monitoring of Field Containment:

Wildlife or livestock invading field:	none
Insect and pest control:	pesticides and herbicides
Adequate isolation:	was maintained
Water management:	irrigation

Plant Characteristics Monitored for Changes:

Reproductive traits:	no change
Disease status	no change
Morphological traits:	no change
Herbicide resistance:	now resistant to glyphosate
Weediness:	no change
Ability to outcross:	no change

Survival of progeny in the field: no change
Insect resistance: no change

Any indication that inserted DNA has been transferred to other species, microbial or plant? No

Methods Used to Isolate Transgenic Lines from Other Corn Lines

One (or more) of the following methods were employed:

a) Spatial: Transgenic corn was open-pollinated and spatially isolated by at least 660 feet from other corn
b) Physical: In situations where transgenic plants were shedding pollen, ear shoots of all corn plants within 660 feet were ear-bagged; and/or transgenic plants were either detasselled or tassels were bagged.
c) Temporal: Transgenic plants were planted at a significantly different time from adjacent nontransgenic plants to prevent cross-pollination.

Means of Plant Disposition:

Plant material was either:

a) stored for future use (e.g. seed for breeding purposes), or
b) destroyed by incorporation into the soil (e.g. repeated disking)

Monitoring for Volunteers:

This report was prepared before volunteers from the winter 1996 test season had come up. Volunteers will be monitored during 1997 and destroyed prior to anthesis.

Additional Comments:

No other wild plant species that can cross-pollinate with corn are present in North America or Hawaii; therefore transfer to wild weedy relatives is not possible.

INTERIM ENVIRONMENTAL RELEASE REPORT
J-N Mutz

Monsanto Company, 4-7-97

Site Location Monsanto Research Farm
Yauco, Puerto Rico 00698

Permit Numbers 96-278-02N

Experiment Description Corn Breeding nursery

Results Good seed yields, and good kernel quality.

General Field Observations
Little insect infestation (Corn Earworm, Leafhopper) and disease severity (Southern Corn Leafblight). Transgenic similar to nontransgenic corn for all comparisons: there was no evidence to suggest that these transgenic plants would be anymore or less weedy than traditional corn. No disease or insect damage was observed to be more or less severe in transgenic plants than in typical non-transgenic corn.

Planting Date 12/5/96 **Harvest/Destroy Date** 3/25/97 and 4/2/97

Trial Size 1 acre in total

Corn Line Numbers GA21 (0.1 acre), GG25 (0.15 acre), 574-04-2 (0.046 acre), 1112-02-1 (0.008 acre), 1163-07-2 (0.008 acre), 599-04-3 (0.0034 acre), 575-07-2 (0.007 acre), 540-04-1 (0.008 acre), 591-03-2 (0.13 acre).

Field Trial Disposal Method Stalks chopped, followed by disking

Isolation Method Used 660 feet isolation

Dates of Monitoring for Volunteer Corn Plants and Number Found

This report was prepared before volunteers had come up (trial destruction: 4/2/97)

Method Used to Destroy Volunteers

Volunteers will be manually or mechanically destroyed before anthesis