



A company of Hoechst and NOR-AM

[REDACTED]
Pioneer Hi-Bred International, Inc.
Department of Regulatory Affairs
11252 Aurora Avenue
Des Moines, IA 50322

February 20, 1995

Dear [REDACTED]

Enclosed please find a copy of AgrEvo USA Company's "Petition for Determination of Nonregulated Status: Glufosinate Resistant Corn Transformation Events T14 and T25". USDA/APHIS determined that the petition appears to be complete, and has assigned it the number 94-357-01p. A Federal Register notice announcing receipt of the AgrEvo petition and providing the designated comment period of 60 days has not yet been published.

Please do not distribute the AgrEvo petition outside of Pioneer Hi-Bred. We would prefer that the petition not be distributed outside Pioneer's Regulatory Affairs Department until the Federal Register notice has been published. Feel free to contact me if you have any questions or comments regarding this petition.

Sincerely,

[REDACTED]
[REDACTED]
[REDACTED]
Manager, Regulatory Affairs - Biotechnology

Enclosure

cc: cooperator file

From: GW1::"S001354@swais.access.gpo.gov" 16-MAR-1995 10:08:35.96
To: [REDACTED]
Subject:

Federal Register: February 27, 1995]

DEPARTMENT OF AGRICULTURE
Animal and Plant Health Inspection Service
Docket No. 95-011-1]

Receipt of Petition for Determination of Nonregulated Status for
Genetically Engineered Corn

AGENCY: Animal and Plant Health Inspection Service, USDA.

ACTION: Notice.

*Citation for
Submission*

SUMMARY: We are advising the public that the Animal and Plant Health Inspection Service has received a petition from AgrEvo USA Company seeking a determination of nonregulated status for corn designated as 'Glufosinate Resistant Corn Transformation Events T14 and T25' genetically engineered for tolerance to the herbicide glufosinate. The petition has been submitted in accordance with our regulations concerning the introduction of certain genetically engineered organisms and products. In accordance with those regulations, we are soliciting public comments on whether this corn presents a plant pest risk.

DATES: Written comments must be received on or before April 28, 1995.

ADDRESSES: Please send an original and three copies of your comments to Docket No. 95-011-1, Animal and Plant Health Inspection Service, Policy and Program Development, Regulatory Analysis and Development, 4700 River Road Unit 118, Riverdale, MD 20737-1238. Please state that your comments refer to Docket No. 95-011-01. A copy of the petition and any comments received may be inspected at USDA, room 1141, South Building, 14th Street and Independence Avenue SW., Washington, DC, between 8 a.m. and 4:30 p.m., Monday through Friday, except holidays. Persons wishing access to that room to inspect the petition or comments are asked to call in advance of visiting at (202) 690-2817.

FOR FURTHER INFORMATION CONTACT:

Dr. David Heron, Biotechnologist, Animal and Plant Health Inspection Service, Biotechnology, Biologics, and Environmental Protection, Biotechnology Permits, 4700 River Road Unit 147, Riverdale, MD 20737-1237; (301) 734-7612. To obtain a copy of the petition, contact Ms. Kay Peterson at (301) 734-7601.

SUPPLEMENTARY INFORMATION: The regulations in 7 CFR part 340, 'Introduction of Organisms and Products Altered or Produced Through Genetic Engineering Which Are Plant Pests or Which There Is Reason to Believe Are Plant Pests,' regulate, among other things, the introduction (importation, interstate movement, or release into the environment) of organisms and products altered or produced through

genetic engineering that are plant pests or that there is reason to believe are plant pests. Such genetically engineered organisms and products are considered "regulated articles."

The regulations in Sec. 340.6(a) provide that any person may submit a petition to the Animal and Plant Health Inspection Service (APHIS) seeking a determination that an article should not be regulated under 7 CFR part 340. Paragraphs (b) and (c) of Sec. 340.6 describe the form that a petition for determination of nonregulated status must take and the information that must be included in the petition.

On December 23, 1994, APHIS received a petition (APHIS Petition No. 1-857-01p) from AgrEvo Company USA (AgrEvo) of Wilmington, DE, requesting a determination of nonregulated status under 7 CFR part 340 for herbicide-tolerant corn designed as "Gulfosinate Resistant Corn (GRC) Transformation Events T14 and T25." As described in the petition, GRC Events T14 and T25 are yellow dent corn plants genetically engineered with a stably integrated gene that encodes the enzyme phosphinothricin-N-acyltransferase (PAT). The PAT enzyme catalyzes the conversion of L-phosphinothricin, the active ingredient in glyphosate-ammonium, to an inactive form, thereby conferring resistance to herbicides in the phosphinothricin class. The PAT gene in GRC Events T14 and T25 is a synthetic version of the gene isolated from the bacterium *Streptomyces viridochromogenes*. Expression of the gene is regulated by the 35S promoter and the 35S terminator derived from the plant pathogen cauliflower mosaic virus.

The subject of corn is currently considered a regulated article under the regulations in 7 CFR part 340 because it contains gene sequences (promoters, and terminators) derived from a plant pathogen. GRC Events T14 and T25 [[Page 10538]] were evaluated in field trials conducted under APHIS permits in 1992 and 1993, and under APHIS notifications in 1993 and 1994. In the process of reviewing the applications for those field trials, APHIS determined that these plants could not present a risk of plant pest introduction or dissemination.

In the Federal Plant Pest Act, as amended (7 U.S.C. 150aa et seq.), "plant pest" is defined as "any living stage of: Any insect, mites, nematodes, slugs, snails, protozoa, or other invertebrate animals, bacteria, fungi, other parasitic plants or reproductive parts thereof, viruses, or any organisms similar to or allied with any of the foregoing, or any infectious substances, which can directly or indirectly injure or cause disease or damage in any plants or parts thereof, or any processed, manufactured or other products of plants." APHIS views this definition very broadly. The definition covers direct or indirect injury, disease or damage not just to agricultural crops, but also to plants in general, for example, native species, as well as organisms that may be beneficial to plants, for example, honeybees, mycorrhizobia, etc.

Several issues associated with GRC Events T14 and T25 are also currently subject to regulation by other agencies. The U.S. Environmental Protection Agency (EPA) is responsible for the regulation of pesticides under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), as amended (7 U.S.C. 135 et seq.). FIFRA requires that all pesticides, including herbicides, be registered prior to distribution or sale, unless exempt by regulation. Plants that have been genetically modified for tolerance or resistant to herbicides are not regulated under FIFRA because the plants themselves are not themselves considered pesticides.

In cases in which the genetically modified plants allow for a new use of an herbicide or involve a different use pattern for the

herbicide, EPA must approve the new or different use. In conducting such an approval, EPA considers the possibility of adverse effects to human health and the environment from the use of this herbicide.

When the use of the herbicide on the genetically modified plant could result in an increase in the residues of the herbicide in a food or feed crop for which the herbicide is currently registered, or in new residues in a crop for which the herbicide is not currently registered, establishment of a new tolerance or a revision of the existing tolerance would be required. Residue tolerances for pesticides are established by the EPA under the Federal Food, Drug, and Cosmetic Act (FFDCA) (21 U.S.C. 201 et seq.), and the Food and Drug Administration (FDA) enforces tolerances set by the EPA under the FFDCA.

The FDA publishes a statement of policy on foods derived from new plant varieties in the Federal Register on May 29, 1992 (57 FR 22984-3005). The FDA statement of policy includes a discussion of the FDA's authority for ensuring food safety under the FFDCA, and provides guidance to industry on the scientific considerations associated with the development of foods derived from new plant varieties, including those developed through the techniques of genetic engineering.

In accordance with Sec. 340.6(d) of the regulations, we are publishing this notice to inform the public that APHIS will accept written comments regarding the Petition for Determination of Unregulated Status from any interested person for a period of 60 days from the date of this notice. The petition and any comments received are available for public review, and copies of the petition may be ordered (see the "ADDRESSES" section of this notice).

After the comment period closes, APHIS will review the data submitted by the petitioner, all written comments received during the comment period, and any other relevant information. Based on the available information, APHIS will furnish a response to the petitioner, either approving the petition in whole or in part, or denying the petition. APHIS will then publish a notice in the Federal Register announcing the regulatory status of AgrEvo's GRC Events T14 and T25 and the availability of APHIS' written decision.

Authority: 7 U.S.C. 150aa-150jj, 151-167, and 1622n; 31 U.S.C. 701; 7 CFR 2.17, 2.51, and 371.2(c).

Done in Washington, DC, this 21st day of February 1995.

erry L. Medley,

Acting Administrator, Animal and Plant Health Inspection Service.

FR Doc. 95-4741 Filed 2-24-95; 8:45 am]

ILLING CODE 3410-34-M

**Petition for Determination of
Nonregulated Status:**

Glufosinate Resistant Corn Transformation Events T14 and T25

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

Submitted by:

[REDACTED]

Manager, Regulatory Affairs - Biotechnology

AgrEvo USA Company
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FAX: [REDACTED]

Contributors:

[REDACTED]

December 23, 1994

Contains No Confidential Business Information

Summary

AgrEvo USA Company is submitting a Petition for Determination of Nonregulated Status to the Animal and Plant Health Inspection Service (APHIS) for Glufosinate Resistant Corn (GRC) Transformation Events T14 and T25. AgrEvo requests a determination from APHIS that GRC transformation events T14 and T25, and any progeny derived from crosses of events T14 and T25 with traditional corn varieties, and any progeny derived from crosses of events T14 and T25 with transgenic corn varieties that have also received a determination of nonregulated status, no longer be considered regulated articles under 7 CFR Part 340. Events T14 and T25 are considered regulated articles because they contain sequences from the plant pest, cauliflower mosaic virus (CaMV).

Glufosinate-ammonium (GA) is in the phosphinothricin class of herbicides. It is a non-systemic, non-selective herbicide that provides effective post-emergence control of many broadleaf and grassy weeds. GA controls weeds through the inhibition of glutamine-synthetase (GS), which leads to the accumulation of phytotoxic levels of ammonia in the plant. GS is responsible for the synthesis of the amino acid glutamine from glutamic acid and ammonia. It is the only enzyme in plants that can detoxify ammonia released by photorespiration, nitrate reduction, and amino acid degradation.

Transformation events T14 and T25 are yellow dent corn material that contain a stably integrated gene which encodes phosphinothricin-N-acetyltransferase (PAT). The PAT enzyme catalyzes the conversion of L-phosphinothricin (PPT), the active ingredient in GA, to an inactive form, thereby conferring resistance to the herbicide. The *pat* gene in events T14 and T25 is a synthetic version of the gene isolated from *Streptomyces viridochromogenes*, strain Tü 494. The nucleotide sequence has been modified to provide codons preferred by plants without changing the amino acid sequence of the enzyme. The gene was introduced through direct uptake of plasmid DNA by corn protoplasts. Southern blot and polymerase chain reaction (PCR) analyses show events T14 and T25 contain 3 and 1 copy of the *pat* gene, respectively.

Genetically engineered GRC will provide a new weed management tool to corn growers. GA is currently registered in the United States as a nonselective herbicide for both non-crop and crop uses. It is highly biodegradable, has no residual activity, and has very low toxicity for humans and wild fauna. GRC may positively impact current agronomic practices in corn by 1) offering a broad spectrum, post-emergence weed control system; 2) providing the opportunity to continue to move away from pre-emergent and residually active compounds; 3) providing a new herbicidal mode of action that allows for improved weed resistance management in corn acreage; 4) offering the use of an environmentally sound and naturally occurring herbicide; 5) encouraging herbicide use on an as needed basis; 6) decreasing cultivation needs; and 7)

allowing the application of less total pounds of active ingredient than used presently.

Events T14 and T25 have been field tested by AgrEvo USA Company, formerly Hoechst-Roussel Agri-Vet Company, since 1992 in the primary corn growing regions of the United States. These tests have occurred at approximately 78 sites under field release authorizations granted by APHIS (USDA authorizations: permits 92-017-04, 92-043-01, 93-021-10, 93-021-11; notifications 93-120-17, 93-120-27, 94-074-03). Data collected from these trials, laboratory analyses, an expert letter and reports, and literature references presented herein demonstrate that GRC events T14 and T25: 1) exhibit no plant pathogenic properties; 2) are no more likely to become a weed than non-modified corn; 3) are unlikely to increase the weediness potential of any other cultivated plant or native wild species; 4) do not cause damage to processed agricultural commodities; and 5) are unlikely to harm other organisms that are beneficial to agriculture. Transformation events T14 and/or T25 have also been field tested in Germany, France, Italy, Canada and Chile.

Primary transformation events T14 and T25 were selected for commercial development. They have been crossed with both commercially available public inbred lines and proprietary inbred lines of the yellow dent type. The primary transformation events and their progeny are collectively referred to as GRC transformation events T14 and T25 in this petition.

AgrEvo USA Company requests a determination from APHIS that GRC transformation events T14 and T25, and any progeny derived from crosses of events T14 and T25 with traditional corn varieties, and any progeny derived from crosses of events T14 and T25 with transgenic corn varieties that have also received a determination of nonregulated status, no longer be considered regulated articles under 7 CFR Part 340.

Certification

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

[REDACTED]

Manager, Regulatory Affairs - Biotechnology

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ACRONYMS AND SCIENTIFIC TERMS

ampR - ampicillin resistance gene
CaMV - cauliflower mosaic virus
ELISA - enzyme linked immunosorbent assay
GA - glufosinate-ammonium
GRC - glufosinate resistant corn
GS - glutamine synthetase
HPLC -high pressure liquid chromatography
PAT - phosphinothricin acetyltransferase
pat - phosphinothricin acetyltransferase gene
PPT - phosphinothricin
PCR - polymerase chain reaction
TLC - thin layer chromatography

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Statement of Grounds for Nonregulated Status

I. Rationale for Development of Glufosinate Resistant Corn

Corn, *Zea mays* L., because of its many divergent types, is produced between latitudes 30° and 55°, with relatively little grown at latitudes higher than 47° anywhere in the world (Shaw, 1988). According to the 1993 and 1994 projected production statistics (USDA, 1994), approximately 22% of the total world's corn production is planted in the United States, yielding 45% of the world production. In the United States corn exceeds all other major crops with regard to acres harvested and crop value.

Several herbicides are currently available to the grower for weed management in corn. Weed management is critical to maximum corn yield and is used on most corn acreage grown in the United States. The grower is typically interested in applying a herbicide for weed control that has a broad weed spectrum, does not injure the crop, is cost effective, and has positive environmental attributes. Several classes of herbicides have effective broad spectrum weed control if used either singly or in combination, however, they may injure or kill some crops when used at the application rates suggested for weed control.

Glufosinate-ammonium (GA), the active ingredient in Basta®, Ignite®, Rely® and Finale™, is a broad spectrum, non-systemic, non-selective herbicide. It has very favorable environmental and safety features. Resistance to the herbicide has now been achieved, through the insertion of a resistance gene, in over 20 commercially important plant species including corn. Genetically engineered Glufosinate Resistant Corn (GRC) will provide a selective use for GA and a valuable new weed management tool to corn producers.

For years pre-emergence herbicides have been the major tool used for weed control in conventional production. Entire fields were treated prior to, or at planting, and before the crop and weeds emerged. However, with the increase in no-tillage corn and the advent of excellent post-emergence herbicides, a shift has occurred toward the treatment of weeds when and where they emerge. Applications may be made over the entire field, or as spot spraying, dependent on the weed density. GA, in concert with GRC, can positively impact current agronomic practices by participating in the shift toward the use of post-emergence herbicides. AgrEvo believes that GRC offers the grower the choice and advantages of using a modern herbicide which features broad-spectrum weed control and favorable environmental features, such as low residual activity, low soil leaching, and low toxicity to nontarget organisms, to manage weeds in production fields.

II. The Corn Family

A. History and Uses of Corn

Cultivated corn is a member of the family Gramineae (grass family). The genus *Zea* consists of four species but, only corn, *Zea mays* has been developed so dramatically from the other members of the genus and from its wild ancestors. This is particularly true in regard to the structure, the ear, which bears the female flowers. Natural selection and plant breeding have brought about cultivars in this species which produce many fold more kernels arranged in rows on the corn ear. These kernels remain tightly within the ear which allows for maximum grain harvest. It also prevents any widespread dissemination of the seed. This makes corn unique in its grain producing characteristics (Mangelsdorf, 1986).

The increase in rows (ranks) of kernels on the ear was begun by the ancient Indian tribes of South, Central and North America. This process may have begun as long ago as 8,000 years. Corn is native to the Americas. Many people place its origin in a small valley south of Mexico City (Tehuacan Valley). By the time of Columbus' expedition to the Americas' corn development and production had spread from Chile to Canada. It was Columbus who brought corn to Europe where it spread within two generations to all the world where corn growth was possible.

Some argument still exists regarding the role of teosinte versus *Tripsacum* in the genetic contributions to modern day corn. However, the past 100 years and especially the past 60 years have shown the strong role played by man in the production of the hybrid corn of today (Galinat, 1988; USDA-APHIS, 1992a; Mangelsdorf, 1986).

Of the crops grown in the United States, corn has the highest value of production with an estimated 16.6 billion dollars for 1993. Soybeans rank second at 11.7 billion dollars (National Corn Growers Association, 1994). Maize is now grown in almost every continent of the world. It is used primarily for animal feed, human food, and for the production of materials used in industry.

On a tonnage basis, 33% of the corn grown in the United States in 1992 was used for the production of silage. Only 1% of the corn crop is utilized for forage (Agricultural Statistics, 1993). Much of the corn used as forage is cut during its vegetative period of growth, and fed primarily to dairy cattle.

Silage is corn which is usually harvested at the late milk to early dough stage of growth. It is chopped and blown into upright silos, or placed in plastic covered trenches, or plastic tube silos where anaerobic fermentation occurs, preserving

the corn as silage. Silage is used in the feeding of ruminant cattle, primarily dairy animals, during the winter months.

Field corn is grown for grain on about twelve times more acreage than that used to grow corn for silage (Agricultural Statistics, 1993). Of the total grain produced, between 8-9% is used for seed, human food products and chemicals. Corn exports account for 21% of the corn produced and 69-70% is used for livestock and poultry feed (Considine and Considine, 1982). Much of the feed corn remains on the farm where it was produced.

In the wet milling process, one bushel, or 56 pounds of corn, is converted into 32.0 pounds of starch, 14.5 pounds of feed and feed products, 2.0 pounds of oil and 7.5 pounds of water. Concentrated steep water, coming from water which was used in the original soaking of the corn, contains 45% protein and is used as a feed supplement. Gluten, germ meal and bran are also sources of protein. Other products of the wet milling process include dextrose, lactic acid, sorbitol, mannitol, zein and soapstock. The dry milling of corn only produces grits, meal and flour with a much greater percentage of waste (USDA-APHIS, 1992a; Considine and Considine, 1982).

Two other types of corn familiar to the consumer are used for human food. These include sweet corn and popcorn. The genetics for sweet corn are such that it contains a soft pericarp (hull) and higher sugar content. Plant breeding has also introduced the ability for the slower conversion of sugar to starch. Sweet corn is considered a vegetable. It ranks second in farm value for processing, and fourth in commercial value among all the vegetable crops.

Popcorn is a derivative of the flint race of corn. It has been modified in order to maximize popping expansion. Once popped the kernel becomes a large unit of puffy, soft endosperm. Another key in the characteristic of popcorn is the pericarp which breaks into many small pieces upon popping. Popcorn must be harvested carefully and dried slowly with low heat to prevent breakage of the seed hulls which would reduce the quality for popping. Popcorn is a well known snack food in the United States (Alexander, 1988).

B. Taxonomy of the Genus *Zea*

Corn is a strong growing annual grass with large cylindrical stems (stalks) enclosed with overlapping leaf sheaths. The broad blade-like leaves have prominent midribs and are arranged in an alternate fashion along the stem. The plant terminates with male flowers located in spikelets within branched racemes called the tassel. The female flowers are located along a thickened, almost woody axis called the cob. These pistillate flowers occur in rows along the cob and usually number from 8-16, but can number as many as 30 rows. The whole cob with its female flowers (ear) is enclosed in a series of foliar looking bracts

(spathes). These spathes are commonly referred to as the husk. The female (pistillate) organ has an extremely long style termed the silk. These styles extend from the top of each pistil along the ear and protrude from the spathes at the time of fertilization.

Pollination occurs primarily as the result of wind movements. The fact that corn contains separate male and female flowers and that pollination occurs via wind are important considerations in the development of corn as a major crop. Wind blowing across a field of corn can cause pollen from the tassel to fall on the silk located on the same plant. This self-pollination leads to a concentration of the genetic characteristics within the single plant. But, the wind can cause pollen from plants to fall on the silks of other adjoining plants. This cross-pollination combines the genetic traits of many plants and leads to diversity of the offspring. Plant breeders used both the self-pollination and cross pollination techniques to produce the hybrid corn we know today.

Initially corn was produced under an open pollination system. Seed produced in this manner, when planted, developed into a non-uniform stand of corn with a wide range of genetic variability existing in each plant. In the 1930's plant breeders began to develop inbred lines using self pollination methods. After several generations (usually seven) these lines were more uniform in their characteristics although yield and vigor had been sacrificed. However, by now combining two inbred lines, first generation hybrids were produced, which were extremely uniform and which contained good agronomic traits. Yield losses were more than offset, increases of six fold being the case. Hybrid corn was now much higher yielding and of better quality than the initially developed open pollinated corn. Today, hybrid corn is almost exclusively the type grown for commercial production (USDA-APHIS, 1992a; Considine and Considine, 1982).

C. Genetics of Corn

In the late 19th century corn was classified by the composition of the endosperm. As time went on more research indicated the need for a broader definition of the nature of corn. The genetic variability of corn was likened to that of humankind. Thus, corn is now classified by races with each race being a group of individuals with many similar characteristics.

The races of corn in the United States can be grouped into the following classes: flour, flint, semi-dent, dent. The major race grown in the United States Corn Belt consists of yellow dent cultivars. They contain ears slightly tapered consisting of 14-22 straight rows. Each kernel is distinctly dented at the tip. Cobs are usually red and the kernels contain a yellow endosperm. While the Corn Belt Dents did not exist prior to the 19th century they now comprise most of the germplasm used to produce new cultivars all over the temperate regions of the world (Goodman and Brown, 1988).

American plant breeders, through their innovation and advances in corn production technology have led the way in making corn the key feeding component in the rearing of animals for milk, egg and meat production. It is these technological advances which are allowing other nations of the world to move toward increasing their diet of protein via the consumption of meat. This would not be possible without high levels of crop production such as that exhibited by the culture of maize.

D. Weediness Potential of Corn

Several key agronomic characteristics were genetically introduced into the species *Zea mays*. These included the reactivation of the second female spikelet, development of many ranked central spikes, growth and elevation of each kernel above the chaff and development of a non-shattering rachis (cob) (Galiant, 1988). While these changes from teosinte and wild type maize led to a domestic plant with high yielding capacity, non-shattering of mature seed and ease in harvest they also led to a species unable to exist on its own in the wild. Also lost was a perennial nature and the inability of domestic maize seed to remain viable in the soil for long periods. The many agronomic traits which make maize an outstanding crop species make it completely dependent on man for its survival. In discussing the potential weediness traits of crop plants the Union of Concerned Scientists (Rissler and Mellon, 1993) have stated "Millennia of breeding have transformed corn into a crop that is completely dependent on human intervention for survival and productivity".

In the Corn Belt of the United States corn, grown in rotation with soybeans, may volunteer on occasion. Insect damage or wind damage may cause some of the mature ears to fall to the ground and not be harvested. The grain from these dropped ears will often germinate in the following soybean crop. However, this volunteer corn can be readily controlled with an array of commercial graminicides registered for use in soybeans.

E. Potential for Outcrossing

As late as 1971 Hitchcock and Chase classified the corn-like cousins of cultivated field corn into the genus *Euchlaena*. More recent taxonomic classification place all these relatives into the genus *Zea*. This genus is subdivided into two sections. The first section, *Luxuriantes*, consists of the following three species:

- Zea diploperennis*: a diploid perennial from Jalisco, Mexico
- Zea perennis*: a tetraploid perennial from Jalisco, Mexico
- Zea luxurians*: an annual from southeastern Guatemala and Honduras

The second section, *Zea mays* consists of three subspecies:

Zea mays subsp. *mexicana*: a large spikeleted annual from the high elevations of central-northern Mexico.

Zea mays subsp. *parviglumis*: a small spikeleted annual from the lower elevations of southwestern Mexico.

Zea mays subsp. *mays*: the cultigen (modern cultivars of field corn).

More distant relatives to *Zea mays* belong to the genus *Tripsacum*. The common name for the three species found in the United States is gamagrass. These are rhizomeaceous perennials which produce a great deal of foliage in relation to seed. This growth habit suits their use as forage crops. The three species include *Tripsacum dactyloides* (Eastern gamagrass), *Tripsacum floridanum* (Florida gamagrass) and *Tripsacum lanceolatum* (Mexican gamagrass). These species grow in wasteland areas such as the low, wet pinelands in Florida and the rocky hills and mountains in Arizona (Hitchcock and Chase, 1971). *Tripsacum* species have a chromosome number of ($n=9$) while that of the *Zea* species have a chromosome number of ($n=10$) (Galinat, 1988). Thus, the species in these genera can only be crossed with great difficulty and produce sterile offspring.

There is no unanimity of agreement as to the biological origin of maize. Some scientists believe that maize was developed from a cross between an ancient wild type maize with *Tripsacum*. When a perennial *Zea* was discovered this theory then was modified to state that maize developed from a cross between an ancient wild type maize and *Zea diploperennis*. Others still believe that maize developed directly from annual teosinte, partly by natural selection and partly by man's interaction and selection for yield of grain and ease of harvest (Mangelsdorf, 1986).

Teosinte and maize both have the same chromosome number ($n=10$). Crosses between these species are made readily and produce fertile F_1 offspring. In Mexico, where teosinte grows in fields with maize, limited hybridization occurs (Doebley, 1984). However, in the United States the wild *Zea* species do not occur widely. Differences in such factors as flowering time, geographic separation, block inheritance, development morphology and timing of the reproductive structures make crossing in nature in the United States only speculative (deWet and Harlan, 1972; deWet, 1975; Doebley and Iltis, 1980; USDA-APHIS, 1992a; Doebley, 1984).

Introgression is the incorporation of genes from one population of close genetic relatives into another with a different adaptive norm. This outcrossing between domestic maize and its teosinte relatives has been suggested by some to

proceed bidirectionally (Doebley, 1984). In these discussions it should be remembered that similarities may exist due to evolutionary convergence and not hybridization. Correlations have been made between maize, teosinte and the hybrids of these *Zea* subspecies as regards fruit case shape, sheath color and pilosity, disease resistance and chromosome knobs. When these factors were considered there appeared little or no clear-cut evidence to support the idea that teosinte has been affected by maize introgression. The patterns of variation can best be ascribed to the effects of ecology and phylogeny (Doebley, 1984).

Populations of maize grown in Mexico and the weedy species, teosinte growing near these corn populations were assayed for their various enzyme systems, additional enzyme loci and alleles. There were enough key differences observed to show that teosinte and maize represent different germplasm. The surprising result in these observations was that the race of teosinte which showed the greatest similarity to maize grow in areas of Mexico in which they have the least contact with maize. This Balsa race of teosinte is also morphologically the least like maize (Smith et al., 1985).

An analysis of the pattern of variation for 21 isozyme loci between Mexican maize and Mexican annual teosinte shows that some introgression exists between *Zea mays* subsp. *mexicana* and the cultigen *Zea mays* subsp. *mays* (Doebley et al., 1987). There are so few of the same alleles in teosinte and maize and they occur at low frequency suggesting only low level introgression. The teosinte is always quite distinct genetically from the maize grown in the same region. The data support the view that teosinte is little affected by gene transfer from maize.

III. The Transformation System and Plasmid Used

The GRC transformation events T14 and T25 contain a synthetic version of the *pat* gene derived from *Streptomyces viridochromogenes*, strain Tü 494 (Bayer et al., 1972). The *pat* gene encodes the enzyme phosphinothricin acetyltransferase (PAT), which confers resistance to the herbicide GA. Since the native *pat* gene has a high G:C content, which is atypical for plants, a modified nucleotide sequence was synthesized using codons preferred by plants. The amino acid sequence of the enzyme remains unchanged. The synthetic *pat* gene is fused to a 35S promoter and terminator from CaMV forming a *pat* gene cassette. The plasmid, p35S/AC, used to transform the parental tissue culture line He/89, contains no other plant expressible genes. The plasmid was transferred to the genome of He/89 through direct uptake of plasmid DNA by corn protoplasts. Stable insertion of the *pat* gene cassette into the corn genome results in the expression of the PAT enzyme.

A. Protoplast Transformation System

AgrEvo GmbH, formerly Hoechst Ag, Frankfurt, Germany, introduced the plasmid DNA into corn protoplasts by a direct uptake technique. In this technique protoplasts and DNA are mixed together in a buffered solution and a polyethylene glycol solution is added dropwise. After gentle mixing and incubation at room temperature the protoplasts are gently pelleted, washed and resuspended in a protoplast culture medium. The putatively transformed protoplasts are cultivated in various conditions until microcolonies of more than 20-50 cells are formed. The microcolonies are then transferred to solid medium. For selection of transformants the microcolonies are transferred several weeks later to medium containing L-PPT. Fertile corn plants are regenerated from corn protoplasts as described by Mórocz et al. (1990). The transformed corn was developed at AgrEvo GmbH.

B. Parent Tissue Culture Line He/89

Tissue culture line He/89 was used for transformation. It was developed at AgrEvo GmbH from parents developed at the Cereal Breeding Institute in Szeged, Hungary. Primary transformation events T14 and T25 were selected for commercial development. They have been crossed with both commercially available public inbred lines and proprietary inbred lines of the yellow dent type. The commercialization strategy for GRC is to use traditional backcrossing and breeding to transfer the glufosinate resistance locus from events T14 and T25 to a wide range of varieties with a wide range of maturities.

C. Construction of the Plasmid Used for Transformation

The plasmid, p35S/AC, was used to transform the parental tissue culture line He/89. To construct p35S/AC, the synthetic *pat* gene was cloned into the *Sa*I site, between the CaMV derived 35S gene promoter and terminator sequences, of the pUC derived plasmid pDH51 (Pietrzak et al., 1986). The chimeric *pat* gene cassette (35S promoter::*pat*::35S terminator) can be isolated as a 1.3 kb *Eco*R1 fragment. The construct contains no other plant expressible genes. The pUC sequences include an ampicillin resistance (*ampR*) gene and a bacterial origin of replication. The *ampR* gene has regulatory signals recognized in bacteria but not functional in transgenic corn cells.

The complete sequence of p35S/AC is shown in Appendix 1 and a map of the vector is shown in Figure III.1. A comparison of the native *pat* nucleotide sequence with that of the synthetic sequence is shown in Figure III. 2. The description of the DNA elements in p35S/AC is shown in Table III.1.

D. Open Reading Frames and Associated Regulatory Regions in p35S/AC

Although p35S/AC contains two open reading frames, *ampR* and *pat*, only the *pat* reading frame is intact and functional in transformation events T14 and T25, as will be shown in Section IV. The GRC transformation events T14 and T25 have been considered regulated articles because they contain DNA sequences from CaMV, an organism which is considered to be a plant pest. This section contains a more thorough description of the inserted genetic material responsible for expression of the glufosinate resistance trait. The *ampR* gene is also addressed. Refer to Table III.1. for a description of all other introduced genetic sequences.

1. CaMV 35S promoter and terminator The 35S promoter and terminator sequences are derived from CaMV and control expression of the *pat* gene. CaMV is a doublestranded DNA caulimovirus with a host range restricted primarily to cruciferous plants. The region of the CaMV genome used correspond to nucleotides 6909 to 7437 for the promoter and nucleotides 7439 to 7632 for the terminator (Pietrzak et al., 1986). The 35S promoter directs high level constitutive expression and is widely used as a promoter for high expression of genes (Harpster et al., 1988). The CaMV sequences, as used in the GRC, do not cause the corn to become a plant pest.

2. *pat* The *pat* gene is a synthetic version of the *pat* gene isolated from *Streptomyces viridochromogenes*, strain Tü 494 (Bayer et al., 1972). It encodes the enzyme phosphinothricin acetyltransferase (PAT), which imparts resistance to the phytotoxic activity of GA. Since the native *pat* gene has a high G:C content, which is atypical for plants, a modified nucleotide sequence was

synthesized using codons preferred by plants. The amino acid sequence of the enzyme remains unchanged. The nucleotide sequences of the native and synthetic gene share 70% homology. Genes encoding PAT enzymes have been isolated from *S. viridochromogenes* (Hara et al., 1991) and *S. hygroscopicus* (Thompson et al., 1987).

Table III.1. Genetic Elements of the Vector p35S/Ac

Genetic element	Position in vector	Size (Kb)	Function
pUC18 vector	1747-399	2.63	High copy <i>E.coli</i> plasmid pUC18 used for cloning of DNA sequences. (Yanisch-Perron et al., 1985)
<i>ampR</i>	3783-2923	0.86	Ampicillin resistance gene of pUC18 expresses a β -lactamase only in bacteria. (Yanisch-Perron et al., 1985)
ori-pUC	2164	0.001	Origin of replication of pUC18. (Yanisch-Perron et al., 1985)
P-35S	1746-1217	0.52	The CaMV promoter of the 35S transcript. (Pietrzak et al., 1986)
<i>pat</i>	1188-637	0.53	The synthetic glufosinate resistance gene. (Eckes et al., 1989)
T-35S	618-412	0.20	The CaMV 3'-nontranslated region of the 35S transcript. (Pietrzak et al., 1986)

Members of the genus *Streptomyces* are gram-positive sporulating soil bacteria. These organisms synthesize numerous unique compounds, secondary metabolites, that often possess antibacterial, antitumor, or antiparasitic activity (Demain et al., 1983). One such compound, the antibiotic bialaphos, is produced by both *S. viridochromogenes* and *S. hygroscopicus*. Bialaphos (syn. L-phosphinothricyl-L-alanyl-L-alanine) is an herbicidally active tripeptide consisting of two L-alanine molecules and an analog of L-glutamic acid called phosphinothricin. When it is released by peptidases, the L-PPT moiety, is a potent inhibitor of GS (Bayer et al. 1972). L-PPT is the active component of the commercial herbicides, Herbiace® (Meiji Seika Ltd.) and Basta®, Ignite®, Rely® and Finale™ (AgrEvo GmbH). Herbiace® is bialaphos that is commercially produced using *S. hygroscopicus*. The other herbicides are the ammonium salts of phosphinothricin, common name GA, and are chemically synthesized.

L-PPT is a potent inhibitor of the enzyme GS in both bacteria and plants, where it apparently binds competitively to the enzyme by displacing L-glutamate from the active site. Evidently GS binds L-PPT better than the substrate. GS plays a central role in nitrogen metabolism of higher plants where it is the only enzyme

in plants that can detoxify ammonia released by nitrate reduction, amino acid degradation and photorespiration (Miflin and Lea, 1976). Ammonia, although a plant nutrient and metabolite, is toxic in excess and leads to death of plant cells (Tachibana et al., 1986).

Although the GS from both *S. viridochromogenes* and *S. hygroscopicus* are sensitive to L-PPT, the bacteria produce an inactivating enzyme, PAT. PAT catalyzes the conversion of L-PPT to N-acetyl-L-PPT in the presence of acetyl CoA as a co-substrate. N-acetyl-L-PPT does not inactivate GS, and, thus, has no herbicidal activity. Therefore, plants expressing the PAT enzyme are resistant to the phosphinothricin class of herbicides. The PAT enzyme is encoded by the *bar* (bialaphos-resistance) gene in *S. hygroscopicus*, and by the *pat* gene in *S. viridochromogenes*. These genes function both as an integral part of the biosynthetic pathway of bialaphos and as an enzyme which confers resistance (Kumada, 1986).

3. *ampR* The ampicillin resistance gene was isolated from pBR322, a plasmid of *Escherichia coli* (Yanisch-Perron et al., 1985). It encodes a β -lactamase. β -lactamase genes are found throughout nature (Sykes and Smith, 1979). The *ampR* gene is expressed in bacteria where it is used in the selection of transformed bacteria which are then used to amplify the plasmid vector.

Figure III.1. Vector Map of p35S/AC

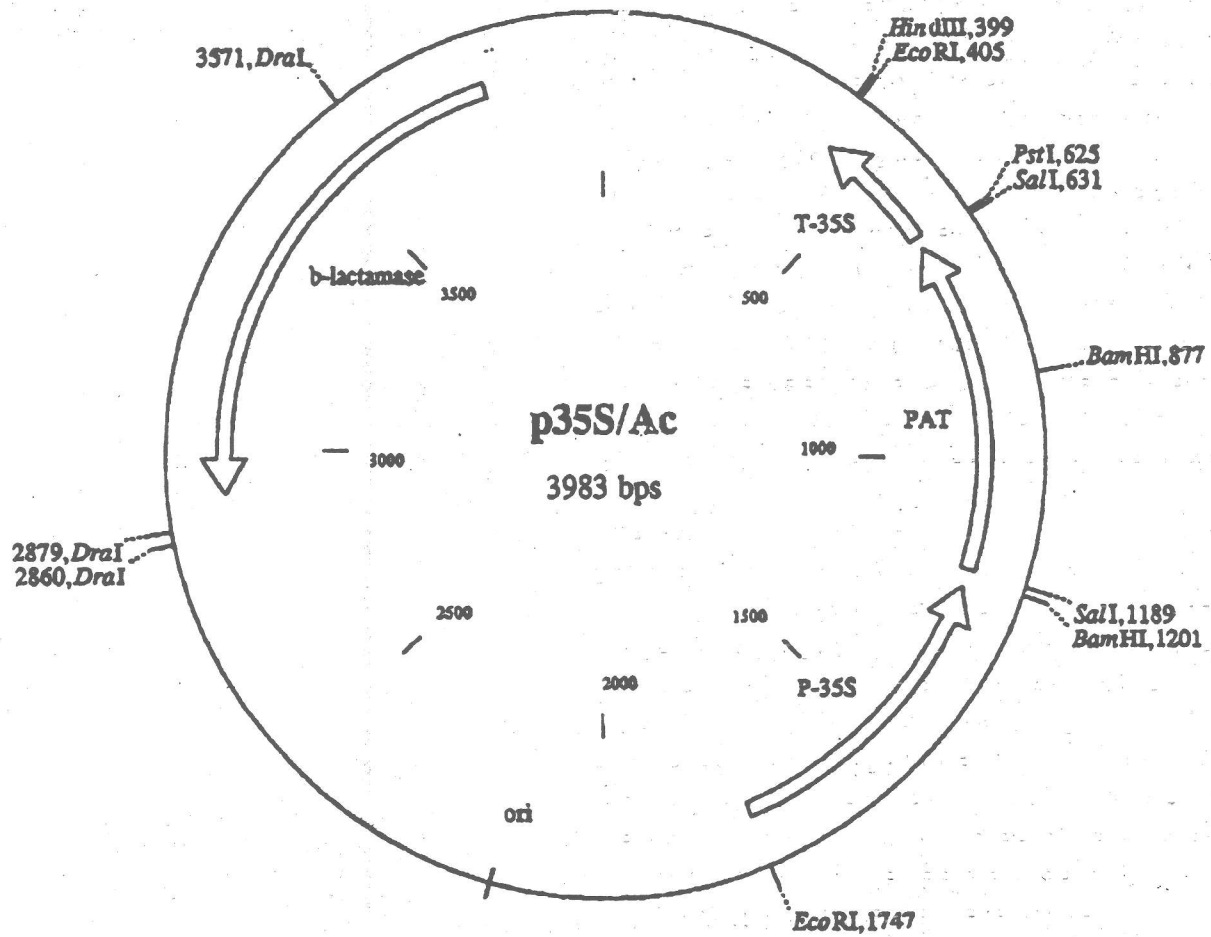


Figure III.2. Comparison of the synthetic *pat* nucleotide sequence (capital letters) with that of the native sequence (small letters)

```

1  ATGTCTCCGGAGAGGAGACCAGTTGAGATTAGGCCAGCTACAGCAGCTGA  50
   ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||
1  gtgagcccagaacgacgcccggtcgagatccgtcccgccaccgcccga  50

51  TATGGCCGCGGTTTGTGATATCGTTAACCATTACATTGAGACGTCTACAG  100
   ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||
51  catggcggcggtctgcgacatcgtcaatcactacatcgagacgagcacgg  100

101  TGAACTTTAGGACAGAGCCACAAACACCACAAGAGTGGATTGATGATCTA  150
   ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||
101  tcaacttccgtacggagccgcagactccgcaggagtggatcgacgacctg  150

151  GAGAGGTTGCAAGATAGATACCCTTGTTGGTTGCTGAGGTTGAGGGTGT  200
   ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||
151  gagcgctccaggaccgctacccctggctcgtcgccgaggtggagggcgt  200

201  TGTGGCTGGTATTGCTTACGCTGGGCCCTGGAAGGCTAGGAACGCTTACG  250
   ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||
201  cgtcgccggcatcgctacgcccggcccctggaaggcccgcaacgcctacg  250

251  ATTGGACAGTTGAGAGTACTGTTTACGTGTACATAGGCATCAAAGGTTG  300
   ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||
251  actggaccgtcgagtcgacggtgtacgtctcccaccggcaccagcggctc  300

301  GGCCTAGGATCCACATTGTACACACATTTGCTTAAGTCTATGGAGGCGCA  350
   ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||
301  ggactgggctccaccctctacacccacctgctgaagtccatggaggccca  350

351  AGGTTTTAAGTCTGTGGTTGCTGTTATAGGCCTTCAAACGATCCATCTG  400
   ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||
351  gggcttcaagagcgtggtcgccgtcatcggactgcccacgacccgagcg  400

401  TTAGGTTGCATGAGGCTTTGGGATACACAGCCCGGGGTACATTGCGCGCA  450
   ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||
401  tgcgcctgcacgaggcgctcggatacaccgcgcgcgggacgctgcgggca  450

451  GCTGGATACAAGCATGGTGGATGGCATGATGTTGGTTTTTGGCAAAGGGA  500
   ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||
451  gccggctacaagcacgggggctggcacgacgtggggttctggcagcgca  500

501  TTTTGAGTTGCCAGCTCCTCCAAGGCCAGTTAGGCCAGTTACCCAGATCT  550
   ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||
501  cttcgagctgccggccccgccccgccccgtccggcccgtcacacagatct  550

551  GA  552
   ||
551  ga  552

```


IV. Molecular Characterization of Transformation Events T14 and T25

A. Description, History and Mendelian Inheritance of Events T14 and T25

Primary transformation events T14 and T25 are derived from the transformation of tissue culture line He/89 as described in Section III. These have been crossed with both commercially available public inbred lines and proprietary inbred lines. Through traditional breeding with these fertile transformation events individuals homozygous at the *pat* locus have been produced. Traditional backcrossing and breeding will be used to continue to transfer the glufosinate resistance locus events in T14 and T25 to a wide range of corn varieties with a wide range of maturities.

Transformation events T14 and T25 have been field tested by AgrEvo USA Company, formerly Hoechst-Roussel Agri-Vet Company, since 1992 in the primary corn growing regions of the United States. These tests have occurred at approximately 78 sites under field release authorizations granted by APHIS (USDA authorizations: permits 92-017-04, 92-043-01, 93-021-10, 93-021-11; notifications 93-120-17, 93-120-27, 94-074-03). Transformation events T14 and/or T25 have also been field tested in Germany, France, Italy, Canada and Chile. The great majority of the trials have been efficacy trials in which the plants have been sprayed with different rates of GA. When sprayed with the herbicide, all plants exhibited a high level of glufosinate resistance, indicating that the gene is stably integrated and expressed.

The *pat* locus has been stabilized in T14 and T25 homozygotes for several generations. To incorporate these transformation events the original hemizygous transformed plants were crossed to inbred lines. This resulted in progeny segregating in a 1:1 fashion with respect to glufosinate resistance. Resistant progeny were selected from a population of young corn plants by spraying with GA. These hemizygous resistant individuals were then self-pollinated producing progeny which segregated 3:1 with respect to glufosinate resistance. The resistant progeny were either homozygous or hemizygous for the *pat* locus. Homozygous ears were selected by self-pollinating a sample of plants grown from the resistant ears, growing up the progeny and treating with GA. Homozygous ears were those from which all progeny from the 2nd self-pollination were unharmed by GA. The seed from the homozygous ears were again self-pollinated and the progeny were sprayed with GA. If the *pat* locus is stable, then all progeny should be resistant to GA, as has been the case with successive self-pollinations for 2 additional generations. Further evidence supporting stable integration is shown by Southern blot analysis of several generations of T14 and T25 (See Section IV.B.3).

Stability has also been confirmed by evaluating the segregation of the glufosinate resistance phenotype in crosses of hemizygous transformation

events T14 and T25 with nontransgenic inbreds. Mendelian inheritance of the *pat* locus in transformation events T14 and T25 has been confirmed in the field (Table IV.1.). All data available indicate that the glufosinate resistance trait is stably inserted and transmitted to progeny as a normal dominant gene.

Table IV.1. Segregation Data for Progeny of Crosses between Hemizygous Events and Nontransgenic Inbreds

Event	Cross ^a	Resistant	Sensitive	χ^2 ^b
T14	1	339	339	0
	2	223	204	0.83
T25	1	305	337	1.60
	2	192	165	2.04

^a 1 = homozygous event crossed to 16 different inbreds; 2 = homozygous event crossed to more than 2 dozen different inbreds.

^b No significant difference ($p=0.05$) for the Chi square goodness-of-fit test for hypothesis of 1:1 segregation. (Significance at $p=0.05$ for $\chi^2 \geq 3.84$, $df = 1$).

B. DNA Analysis of Glufosinate Resistant Corn Events T14 and T25

To determine the nature, number and molecular stability of insertions which occurred in transformation events T14 and T25, Southern hybridization and PCR analysis were used. Southern analysis was used to determine the copy number of the insertions and the stability of these insertions over several generations. Both Southern and PCR analyses were used to map the inserted DNA and lend further confirmation to the copy number.

1. Copy Number

Reconstruction experiments were performed to determine the number of copies of the *pat* and *ampR* genes present in progeny of transformation events T14 and T25. In the reconstruction experiments restriction digested genomic DNA from transgenic plants hemizygous for the integrated DNA were run in parallel with a dilution series of digested p35SVAC vector on an agarose gel. After blotting and hybridization with a *pat*- or an *ampR*- probe the number of copies of the two genes in the corn genome was quantified by comparing the hybridization intensity of the corn DNA with the hybridization intensity of the diluted probe. Such reconstruction experiments can only give a rough estimate of the copy number since the parameters for calculation (mass of maize genome,

spectrophotometric quantification of vector and plant DNA, dilution of DNAs, visual comparison of band intensity) are not absolutely precise.

DNA was digested with Sal1 (for quantification of the *pat* gene) or Dra1 (for quantification of the *ampR* gene). See Figure III.1. to locate restriction sites in p35SVAC. After separation of the DNA by electrophoresis, the DNA was transferred to a nylon membrane and hybridized with a ^{32}P -labeled synthetic *pat* gene (552 bp Sal1 fragment) (Figure IV. 1a) or with a ^{32}P -labeled *ampR* gene fragment (692 bp Dra1 fragment) (Figure IV. 1b). Lanes 1 and 2 contain 5 ug of restricted T14 and T25 DNA, respectively. The amount of restricted p35SVAC in lanes 3 through 8 is equivalent to 50, 10, 5, 1, 0.5, and 0.1 copies, respectively, of the plasmid integrated in 5 ug of maize DNA. In Figure IV. 1a. the intensity of the *pat* band in the T25 lane (lane 2) corresponds with the intensity in lane 6 (1 copy). This means that approximately 1 copy of the *pat* gene is present in the T25 genome. The intensity of the *pat* band in the T14 lane (lane 1) is weaker than that of lane 5 (5 copies) but stronger than that of lane 6 (1 copy). We estimate that approximately 3 copies of the gene are present per genome of T14.

In Figure IV. 1.b. the intensity of the *ampR* band in the T25 lane (lane 2) corresponds with the intensity in lane 6 (1 copy). This indicates that approximately 1 copy of the *ampR* gene is present in the T25 genome. The intensity of the smaller *ampR* band in the T14 lane (lane 1) is much weaker than that of lane 5 (5 copies) but stronger than that of lane 6 (1 copy). We estimate that this represents approximately 2 copies of the gene. There is also a larger hybridizing band in lane 1 of about the same intensity as the lower band. Therefore, there seems to be up to 4 copies of the *ampR* gene per genome of T14. PCR analysis (Section IV. B. 2) show that the *ampR* genes in T14 are either truncated or have a DNA insertion.

2. Verification of Insert Integrity

When transforming a plant with intact, circular vector DNA there is no way to predict at which site or sites on the vector recombination will initiate. We have therefore used a combination of Southern blot and PCR analyses to examine the integrity of the inserted vector in transformation events T14 and T25. These analyses also serve to verify the copy number results obtained in the reconstruction experiments (Section IV.B.1).

a. *Southern blot analysis*

The DNA from hemizygous progeny of transformation events T14 and T25 was isolated and digested with several enzymes. Digested DNA was separated on agarose gels, transferred to nylon filters and hybridized with the ^{32}P -labeled

synthetic *pat* gene (552 bp Sal1 fragment). The Southern blot is shown in Figure IV. 2. The hybridizing fragments expected and observed when using the *pat* gene as probe are listed in Table IV. 2.

Table IV. 2. Hybridizing Fragments in Southern Blots of T14 and T25 DNA Probed with the *pat* Gene

Restriction Enzyme	Expected Fragment (kb) ^a	Observed T14	Fragment (kb) T25
Sal1	0.5	0.5	0.5
EcoR1	1.3	1.3, 2.0, 6.0	1.3
BamH1	0.3, 1 unknown	0.3, 2.6, 4.0, 7.0	0.3, 1.5
Dra1	1 unknown	3.0, 3.5, 5.5	>5
HindIII	1 unknown	2.7, 3.3, 5.0	>10

^a Expected fragment sizes for 1 copy of inserted vector.

The sizes of some hybridizing fragments can be predicted by the location of restriction enzyme cleavage sites internal to the inserted vector. Those hybridizing fragments whose sizes cannot be predicted result from cleavage in the integrated vector and in the adjacent plant DNA.

Transformation event T25. Digestion of T25 DNA with BamH1 (lane 6) gives 2 hybridizing fragments. The 0.3 kb fragment is internal to the vector p35SVAC; the 1.5 kb fragment results from cleavage in the integrated vector and in the adjacent plant DNA. This single additional band is evidence that only one copy of the vector has inserted into the plant genome. The additional band of 2.5 kb is derived from incomplete digestion. Only one hybridizing fragment is detected in EcoR1 (lane 7) digests. This band corresponds to the internal EcoR1 vector fragment. The single hybridizing fragments in the Dra1 (lane 8) and HindIII (lane 9) digests result from cleavage of the respective site in the vector and in the adjacent plant DNA. When the DNA was digested with Sal1 (lane 10) only the expected 0.5 kb fragment internal to the vector was detected. These data provide good evidence that only one copy of vector p35SVAC integrated into the plant genome in transformation event T25. The point of recombination on the vector is somewhere in the pUC18 sequences, as the *pat* cassette is intact (see EcoR1 digest). The results are summarized in Figure IV. 3.

Transformation event T14. The hybridization pattern is more complex for transformation event T14 (Figure IV. 2). The pattern indicates that more than one copy of the vector, probably 3, has integrated into the corn genome. Digestion of T14 DNA with BamH1 (lane 1) gives 4 hybridizing fragments. The

internal fragment of 0.3 kb can again be detected. However, three additional fragments are visible indicating integration of 3 copies of the vector plasmid. These fragments result from cleavage of the BamH1 site in the *pat* gene and another cleavage in the adjacent plant genome at each integration site. The 3 fragments detected in the Dra1 (lane 2) digest result from the cleavage at site internal to the vector and in the adjacent plant DNA, again providing evidence for the integration of 3 copies of the vector. The EcoR1 (lane 3) digest reveals 3 different fragments, again indicating 3 integration sites. The additional 2.6 kb fragment may be the result of incomplete digestion. The 1.3 kb band corresponds to the internal EcoR1 vector fragment. The other fragments must result from integration events where the EcoR1 site at the 35S promoter or terminator has been destroyed during integration. A deletion at the EcoR1 site near the 35S terminator is supported by PCR data (see Section IV.B.2.b). The 3 fragments detected in the HindIII (lane 4) digests result from cleavage at the HindIII site in the vector and in the adjacent plant DNA, again providing evidence for 3 integrated copies of the vector. When the DNA was digested with Sal1 (lane 5) the expected 0.5 kb fragment internal to the vector was detected. The weakly hybridizing fragment of 2.5 kb is the result of incomplete digestion. These data provide strong evidence that three copies of vector p35S/AC integrated into the plant genome in transformation event T14. In at least one of the copies the point of recombination on the vectors is somewhere in the pUC18 sequences, as the *pat* cassette is intact (see EcoR1 digest). However, parts of these integrated vectors appear to have been deleted.

b. PCR analysis

The DNA from hemizygous progeny of transformation events T14 and T25 and a nontransformed parent were isolated and subjected to PCR analysis along with p35S/AC vector DNA. For these experiments up to eleven different primer pairs were used. PCR products were separated on agarose gels and stained with ethidium bromide. The location of primers on the vector is shown in Figures IV. 4 and 6. The gels of PCR products when corn DNA was the template are shown in Figures IV. 5 and 7. Table IV. 3. gives the PCR products obtained with vector DNA as the template. The data are not shown for vector DNA.

Transformation event T25. PCR primer pairs 1-4, 8-9, and 11-12 were used to generate the products shown in Figure IV.5. As expected when DNA from untransformed corn was used as template no PCR products were obtained (Figure IV. 5; lanes 9-12; primer pairs 2, 3, 9 and 11). PCR primer pairs 1,2,8, and 9 (Figure IV.5, lanes 1, 2, 5 and 6) produced the same product sizes when T25 DNA was used as template as were obtained with the vector. However, primer pairs 3, 4, 11 and 12 (Figure IV.5, lanes 3, 4, 7, and 8) produced no products. These data show that p35S/AC is integrated into the genome from positions 3814 to 3555 (see Appendix 1 for sequence). At least a portion of the

vector between primer amp17 and amp9 (positions 3583 to 3783) has not been integrated. These data are confirmed by Southern blot analysis of DNA from transformation event T25, where it could be shown that the *Dra*I site at position 3571 is present but not contiguous with the 35S terminator region (data not shown). These results indicate that about 25% of the *ampR* gene at its 5' end are not integrated into the T25 genome. Therefore, transformation event T25 and its progeny do not have an intact copy of the *ampR* gene. An intact ori-pUC is present.

Table IV. 3. PCR Products of p35S/AC DNA

Primer Pairs	PCR Products (bp)
1. T35S1/amp15	717
2. T35S1/amp16	787
3. T35S1/amp9	841
4. T35S1/amp2	1183
5. T35S1/amp5	1518
6. T35S1/amp7	1766
7. T35S1/ori1	2833
8. P35Sa/pUCa	748
9. P35Sa/amp8	1637
10. P35Sa/amp13	1933
11. P35Sa/amp17	1964
12. P35Sa/amp12	2011
13. PAT 5'/amp8	no product
14. PAT 5'/amp13	no product
15. PAT 5'/amp1	no product

Transformation event T14. As was the case with Southern analysis, the PCR data for transformation event T14 are much more complex. PCR primer pairs 2, 4-10, and 13-15 were used to generate the products shown in Figure IV.7. Primer pairs 2 and 4 (Figure IV.7, lanes 1 and 2) produced three different PCR products each, the largest product being the same size as was obtained with vector DNA. The two smaller bands might be derived from deletions in the vector. A deletion of the region around the *Eco*R1 site at the 35S terminator was proposed in the Southern analysis of transformation event T14 (see Section IV.B.2.a). The difference in size of the two smaller products in lanes 1 and 2 corresponds to the difference between the location of primer amp16 and amp2 on the vector, indicating that the products are specific for the primers. The

results support the contention that 3 copies of the vector integrated into the genome of transformation event T14.

Primer pairs 5 and 6 (Figure IV.7, lanes 3 and 4) each produced one product larger than the size obtained when vector DNA was the template. This indicates that one of the vector copies contains the entire *ampR* gene but with a DNA insertion downstream of the *amp2* primer. This result is confirmed by Southern data where it was shown that the internal *DraI* fragment of the *ampR* gene is larger in the T14 material than in vector p35S/AC (see Section IV.B.2.a). The second and third integrated vector copies appear to have their endpoints between *amp2* and *amp5* (see Figure IV. 6) since primers farther away from primer T35S1 yield only one large PCR product. This indicates that the second and third integrated vector copies have a deletion in the *ampR* gene. Primer pair 7 (Figure IV. 7, lane 5) produced no PCR product indicating that the longest integrated copy of the vector ends somewhere between primer *amp7* and primer *ori1*. A product is obtained only when the pUCa primer is paired with primer P35Sa (Figure IV. 7, lane 6), but not with the *amp8* and *amp13* primers (Figure IV. 7, lanes 7 and 8). This indicates that the other end point of the three integrated vectors is near the 35S promoter and located between primers pUCa and *amp8*. A drawing illustrating the results for the *ampR* gene is shown in Figure IV. 8.

Primer pairs 13-15 show unexpected results. Since these primers have the same orientation on the vector (see Figure IV. 6) no fragments can be amplified when p35S/AC is the template (data not shown). However, when DNA from transformation event T14 is used as template PCR products of 900 bp, 1200 bp, and 1400 bp are obtained for each combination respectively (Figure IV. 7, lanes 9-11). The differences in size reflect the distances between the *amp8*, *amp13*, and *amp1* primers on the vector. Combinations of the T35S1 primer with *amp8*, *amp13* or *amp1* do not lead to an amplified product (data not shown). These results indicate that parts of the p35S/AC vector have been integrated into the corn genome in an inverted orientation.

In conclusion, transformation event T14 and its progeny contain 3 disrupted copies of the vector. All of these copies appear to contain an intact *pat* cassette and *ori-pUC*. None of these copies appear to possess an intact *ampR* gene. In one of the copies the *ampR* gene contains an insert. In the other two copies the *ampR* gene is truncated.

3. Stability of Insertions

The Southern and PCR data indicate that there are 1 and 3 disrupted copies of the vector p35S/AC present in the genomes of transformation events T25 and T14, respectively. To confirm that the integrated DNA remains intact in

subsequent generations, the hybridization pattern of progeny from backcrosses to transformation events T14 and T25 was examined. For these analyses genomic DNA was digested with EcoR1 (Figure IV.9.A) or BamH1 (Figure IV.9.B) and separated on an agarose gel. After transfer to a nylon membrane the DNA was hybridized with a ^{32}P -labeled synthetic *pat* gene (552 bp Sal1 fragment). The autoradiographs of the blots show that the integration pattern is unchanged for the number of generations observed (3 for T14.; 5 for T25), thus demonstrating stability of the inserted vector copies. Furthermore, the hybridization patterns indicate the presence of 3 and 1 integration sites for p35SVAC in the genome of T14 and T25, respectively. Segregation data (Section IV.A) further confirm the stability of the inserts, and show that they segregate as one dominant Mendelian locus.

C. Gene Expression in Glufosinate Resistant Corn Events T14 and T25

The levels of PAT protein in the GRC transformation events T14 and T25 and nontransgenic counterparts were determined in whole plants, leaves, roots, seed, and pollen by activity assays and/or Enzyme Linked Immunosorbent Assay (ELISA). Two different activity assays were performed. The Thin Layer Chromatography (TLC) assay is a qualitative assay that shows whether active PAT enzyme is present. With High Pressure Liquid Chromatography (HPLC) the activity of the enzyme can be quantified. In the ELISA assay a polyclonal antibody was used. It detects both degraded and intact PAT enzyme. Therefore, the enzyme detected may not all be functional. To determine whether any of the copies of the *ampR* gene were expressed we performed enzyme activity assays and analysis of the RNA from transformation events T14 and T25. These analysis show that the *ampR* gene is not expressed in the GRC.

1. PAT Expression.

Enzyme assays were performed on crude protein extracts of mature pollen, roots, leaves and stems of flowering GRC and on crude protein extracts of mature seed from plants grown in the greenhouse. For both the TLC and HPLC assays the extracts were added to a reaction mix containing ^{14}C -PPT and acetyl-CoA. PAT catalyzes the conversion of L-PPT to N-acetyl-L-PPT in the presence of acetyl-CoA as a co-substrate. Any activity detected in this reaction mix is due to PAT activity since the substrate is not acetylated by other acetyltransferases. Following incubation the reactions were stopped and analyzed by either TLC or HPLC. In TLC formation of ^{14}C -N-acetyl-L-PPT is visualized by autoradiography. In HPLC the product is detected with a radiodetector. Table IV.4. shows the specific activity of PAT enzyme detected by HPLC.

PAT specific activity was not detected in the pollen from either transformation events T14 or T25, despite the high protein concentration of the pollen samples relative to the other tissue samples. Low PAT specific activity was detected in the seed, with seed derived from event T14 having greater activity than seed derived from event T25. Specific activity was not significantly different between leaves and stems of T14 and T25, but it was significantly different between roots and seed. There was no clear correlation between copy number of the inserted vector p35S/AC and level of PAT activity, except that T14 had significantly higher expression levels in roots and seed.

Table IV. 4. PAT Specific Activity in Tissues of Corn as Detected by HPLC

Tissue^a	Plant^b	Protein Concentration (mg/ml)^c	PAT Specific Activity (mU/mg)^{c, d, e}
Pollen	T14	3.45 (2.39-4.01)	nd ^f
	T25	4.47 (3.87-4.85)	nd
Leaves	T14	1.83 (1.33-2.23)	23.77 (19.38-32.88)
	T25	0.78 (0.57-1.02)	41.32 (33.38-47.39)
Stems	T14	0.38 (0.32-0.45)	38.10 (18.10-49.10)
	T25	0.26 (0.17-0.34)	50.95 (39.39-62.54)
Roots	T14	0.28 (0.22-0.34)	91.16 (72.65-138.5)*
	T25	0.94 (0.74-1.05)	5.36 (1.29-12.0)
Seeds	T14	1.79 (1.62-2.01)	3.85 (2.79-4.46)*
	T25	2.49 (1.56-3.95)	0.68 (0.19-1.29)

a Mature pollen, roots, leaves and stems of flowering GRC; mature seed derived from same plant as seed to grow material for other tissues samples.

b Transformed plants were progeny of transformation events T14 and T25.

c Mean of 4 replicates, (min.-max.).

d One unit (U) of enzyme activity corresponds to 1 uMol/minute.

e * Significantly different at $p=0.05$.

f nd = no PAT activity detected.

In other studies (data not shown) no PAT activity was detected in nontransgenic genetic counterparts of transformation events T14 and T25 even though the protein concentrations were very high. Additionally, no denatured PAT was detected in protein extracts from transgenic pollen or nontransgenic leaf tissue when these extracts were analyzed by western blotting. The antibodies used, however, were able to detect PAT activity in leaf tissue from transgenic corn plants (data not shown).

The PAT ELISA is a sandwich immunoassay in which PAT specific antibodies are used to coat the wells. Samples consisting of transformant extracts, non-transformant extracts as controls, and pure PAT protein as a standard are added to the wells. Following incubation, during which time the PAT in the sample is captured by the bound antibodies, the unbound material is removed. Biotinylated secondary antibody to PAT is then added, which binds to the immobilized primary antibody/PAT complex. After washing, bound biotinylated antibody is quantified colorimetrically after incubation with streptavidin conjugated alkaline phosphatase and substrate. The resultant color development is proportional to the concentration of PAT protein in each microwell. ELISA assays were performed on field grown corn plants harvested at the silage (late milk to early dough) stage, and on grain. The material for silage was harvested from 2 U.S. field sites, while the grain was harvested from 3 U.S. field sites. ELISA analysis of PAT activity in grain from event T25 was not pursued. Results from the ELISA are shown in Table IV. 5.

Table IV. 5. Quantities of PAT in Corn as Detected by ELISA

Matrix	Plant ^a	% Protein ^b	ng PAT/ ug protein ^b	ug PAT/ gm Matrix	% PAT in Matrix
silage	T14	0.19	13.03	36.97	3.70
	T25	0.05	13.54	6.62	0.67
grain	T14	1.59	0.008	0.115	0.0115

^a Transformed plants were progeny of transformation events T14 and T25.

^b Two extracts from each sample (2 each T14 and T25 silage; 6 grain) were analyzed in triplicate. However, means reported are those from all field sites combined.

As was seen when PAT specific activity was measured by HPLC (Table IV.4), PAT activity is much less in grain or seed than in other vegetative portions of the corn plant. The data in Table IV.5. indicate that a small amount of PAT protein is present in the silage and grain, the corn matrices that can constitute a significant part of the livestock diet for cattle, poultry, and swine.

2. ampR Expression.

The GRC from transformation events T14 and T25 contain one or more disrupted copies of the bacterial *ampR* gene (see Section IV.B.2). This gene is under the control of bacterial expression signals and should only be expressed in bacteria. The β -lactamase enzyme confers resistance to β -lactam antibiotics (penicillin, ampicillin, etc.). Although none of the copies of the *ampR* gene

present in transformation events T14 and T25 are intact, β -lactamase assays (Figure IV. 10 and 11) and northern analysis (Figure IV. 12 and 13) were performed to verify that the gene is neither stably transcribed nor translated into active protein.

To detect β -lactamase activity plant extracts from transformed and nontransformed tissues were incubated with ^{14}C -penicillin. After the reaction was stopped the products were analyzed by HPLC. No β -lactamase activity was detected after a 5 or 60 minute reaction time in extracts from leaves of transformation event T25 (Figure IV. 10), T14 (Figure IV. 11), or nontransgenic counterparts (Figures IV. 10). In addition no activity was detected in extracts of roots and seeds from transformation event T14 (Figure IV. 11). Activity was also not detected in roots and seeds from a nontransgenic counterpart (data not shown). The growth medium of *E. coli* cells transformed with the plasmid pUC12 (contains the *ampR* gene) served as a positive control. The β -lactamase enzyme is excreted into the bacterial growth medium. Incubation of penicillin with the bacterial growth medium clearly led to metabolism of penicillin to a degradation product (data not shown). Figure IV. 10 shows that addition of plant extract from transformation event T25 does not inhibit the β -lactamase activity of the bacterial growth medium. From these experiments it is concluded that transformation events T14 and T25 do not produce functional β -lactamase.

To determine that the bacterial expression signals associated with the *ampR* gene are non-functional in GRC we looked for *ampR* positive RNA transcripts. RNA was isolated from the leaf material of transformation events T14 and T25 and from a nontransformed genetic counterpart. The total RNA was separated on a denaturing formaldehyde agarose gel, transferred to a nylon membrane and hybridized. The membrane was first hybridized with a ^{32}P -labeled *ampR* gene probe (Dra1 fragment from pUC18), and subsequently reprobbed with the synthetic *pat* gene (Sal1 fragment from p35S/AC). Figure IV. 12. shows that neither the RNA from transformed corn lines (lanes 1 and 2), nor the RNA from a nontransformed counterpart (lane 3) contain partial or complete *ampR* transcripts. The *ampR* probe was not poorly labeled since it showed a strong hybridization signal when hybridized to *ampR* DNA (Figure IV. 12, lane 4). The integrity of the RNA is good since reprobing the membrane with the *pat* sequence results in detection of a *pat* transcript in RNA from transformed corn (Figure IV. 13, lanes 1 and 2). The strong signal in lane 4 (Figure IV. 13) results from the first probing with the ^{32}P -labeled *ampR* sequence. The filter was not stripped before reprobing with the *pat* sequence. The results from this experiment indicate that none of the *ampR* genes in transformation events T14 or T25 are transcribed. Therefore, it can be concluded that the bacterial expression signals of the gene are either not functional in GRC or the transcripts are unstable.

3. Effect of Transposons on Gene Expression.

Since corn is known to contain transposable elements (Peterson, 1986), AgrEvo USA Company obtained an expert opinion on the possibility of transposition in finished lines and hybrids, and the effect such an event would have on expression of a target gene. The following is a synopsis from the expert letter submitted by Dr. Nina Federoff, Carnegie Institution of Washington (Appendix 2).

The probability of transposition in finished lines and hybrids is so low that it is not a realistic concern. Geneticists who study transposition use lines that are very different from corn cultivated for food production. The genetic regulation of all known corn transposons minimizes both transcription and transposition of the element. Of the three most thoroughly investigated corn elements, transposition is minimized by extensive methylation of the element. Even when transposition functions are supplied by an active element elsewhere in the genome, it is rare that a heavily methylated element responds.

The insertion of a transposable element in or near a target gene is not very likely, as indicated above. However, if an event were to occur, it is highly improbable that it would result in gene activation. Corn transposons show a preference for insertion into the body of the gene (exons or introns), and in most cases investigated, gene expression has been completely, or nearly completely eliminated. In the small number of cases in which an element has inserted in or near a gene's promoter, gene expression has been reduced. There are no known cases to date in which a transposon has enhanced gene expression.

In conclusion, in the unlikely event that transposition were to occur in or near a target gene, the most probable outcome would be to disrupt the structure of the gene, rendering it inactive. Therefore, the chances are extremely remote that a transposition event will occur in GRC and result in extinguishing or enhancing expression of the *pat* gene, or in promoting expression of a disrupted *ampR* gene.

Figure IV.1. Quantification of copy number for the *pat* and *ampR* genes in events T14 and T25. Lanes 1 and 2 contain 5 ug of restricted T14 and T25 DNA, respectively. Lanes 3 through 8 contain amounts of restricted p35SVAC DNA equivalent to 50, 10, 5, 1, 0.5, and 0.1 copies of the *pat* or *ampR* gene, respectively. DNA was restricted with Sal1 (Panel a) or Dra1 (Panel b). A *pat* (552 bp Sal1 fragment)(Panel a) or *ampR* (692 bp Dra1 fragment)(Panel b) gene was used as probe.

Fig.: 1a

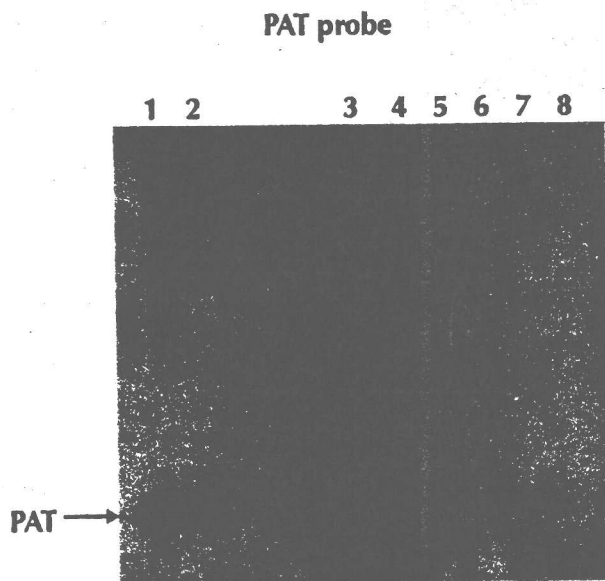


Fig.: 1b

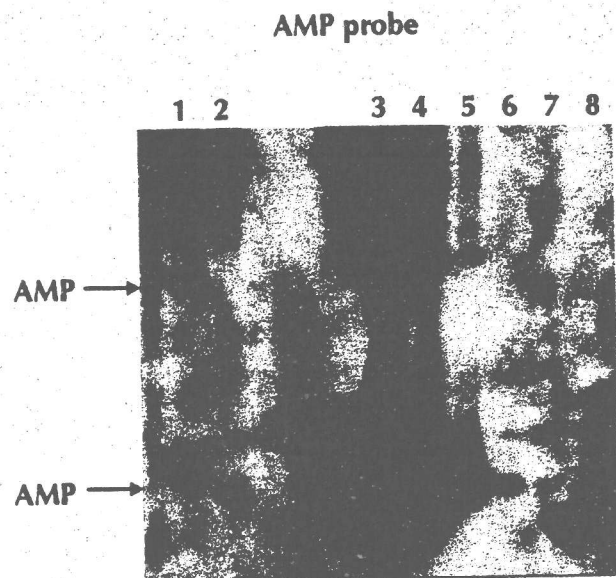


Figure IV.2. Southern blot of events T14 and T25. The following restriction enzymes were used to digest 15 ug of T14 and T25 DNA: BamH1 (lane 1 and 6), Dra1 (lane 2 and 8), EcoR1 (lane 3 and 7), Hind III (lane 4 and 9), Sal1 (lane 5 and 10). Bacteriophage λ DNA digested with Pst1 was used as the size marker. The *pat* gene (552 bp Sal1 fragment) was used as probe.

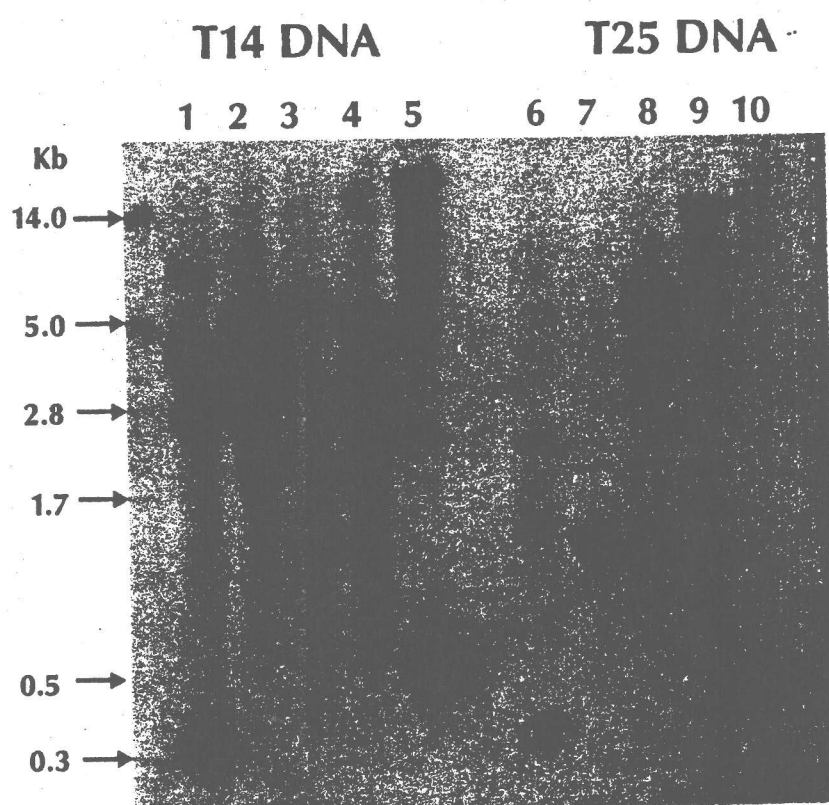


Figure IV.4. Location of primers for PCR analysis of event T25. Locations are indicated with small tailed and tailless arrows.

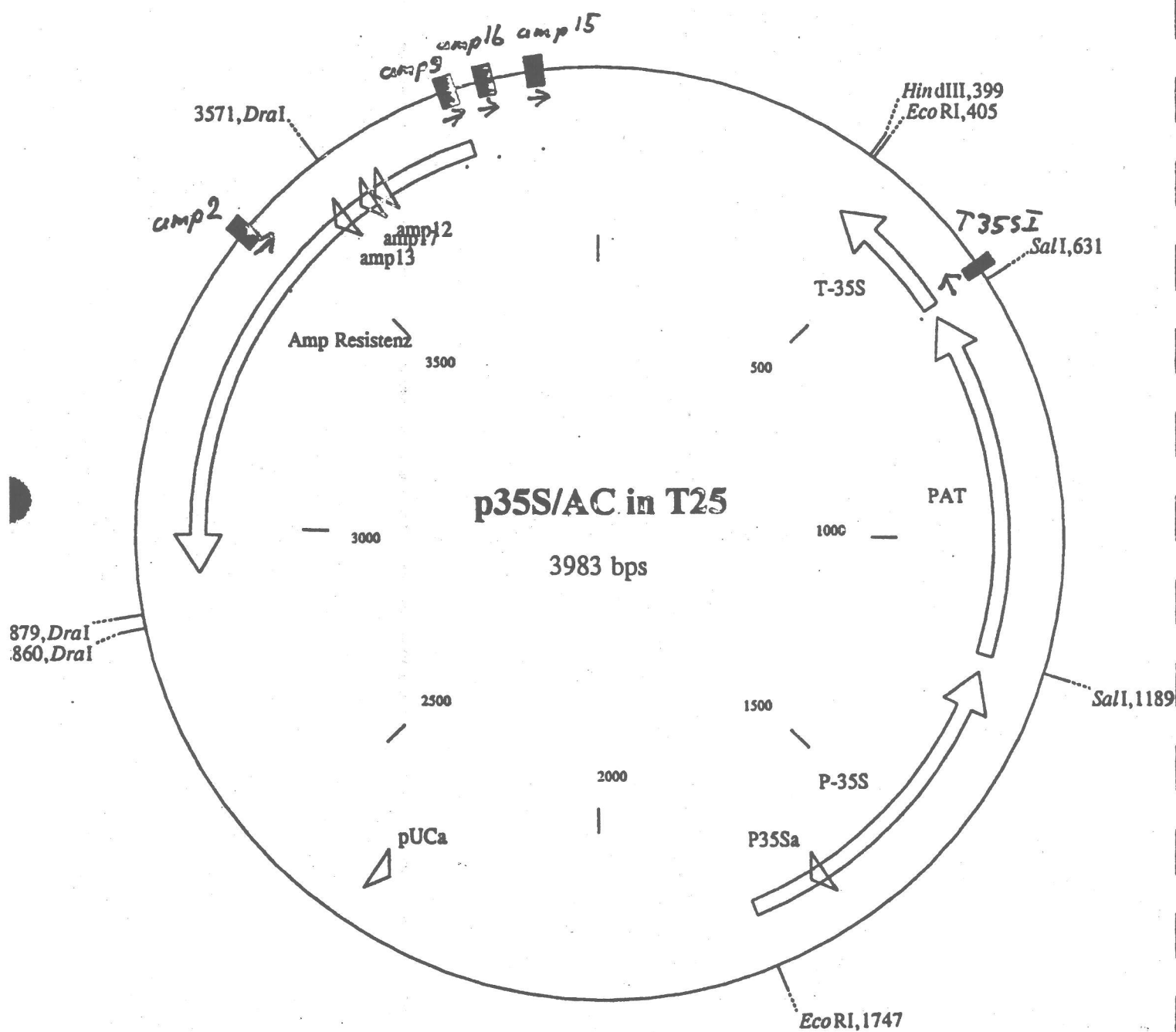


Figure IV.5. PCR analysis of event T25. Primer pairs 1, 2, 3, 4, 8, 9, 11, and 12 (Lanes 1-8, respectively) were used to generate products from T25 DNA. Primer pairs 2, 3, 9, and 11 (Lanes 9-12, respectively) were used to generate products from nontransformed counterpart DNA. See Table IV.3. for description of primer pairs. Bacteriophage λ DNA digested with Pst1 was used as the size marker (Lane L).

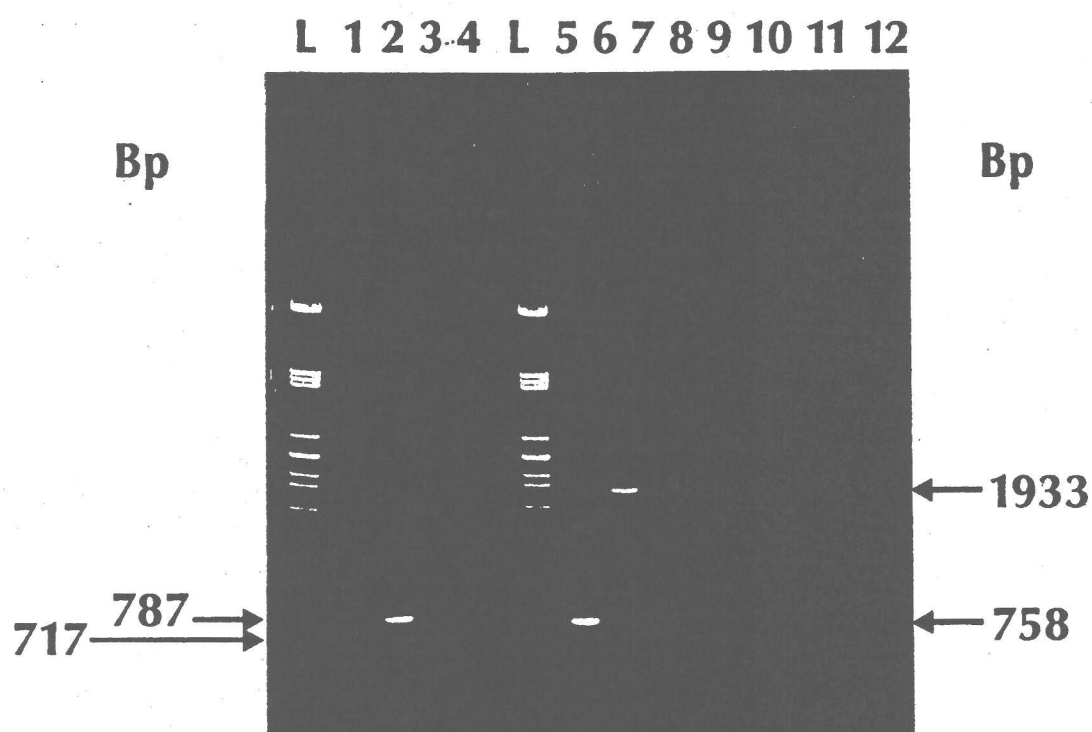


Figure IV.6. Location of primers for PCR analysis of event T14. Locations are indicated with small tailed and tailless arrows.

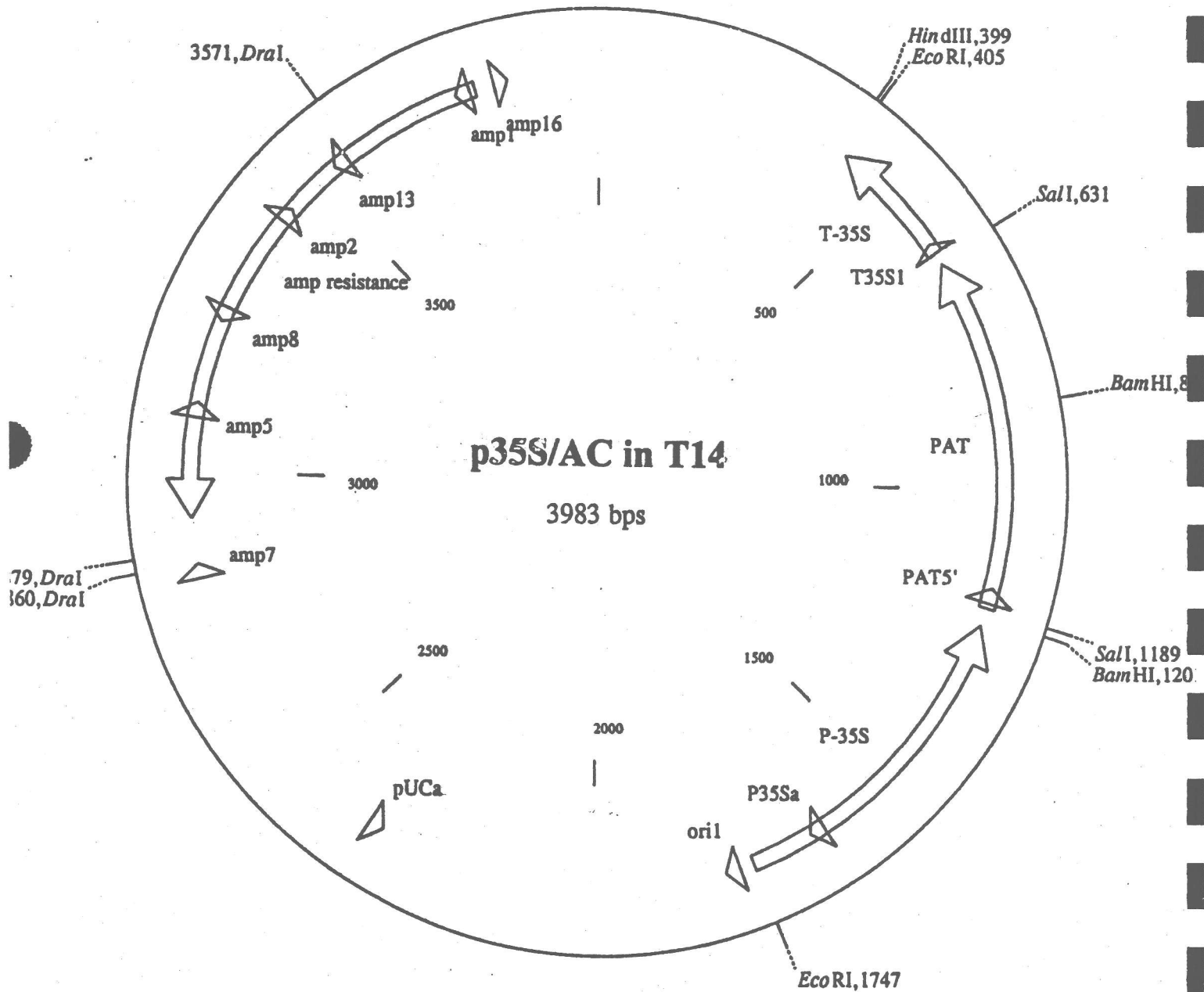


Figure IV.7. PCR analysis of event T14. Primer pairs 2, 4, 5, 6, 7, 8, 9, 10, 13, and 15 (Lanes 1-11, respectively) were used to generate products from T14 DNA. See Table IV.3. for description of primer pairs. Bacteriophage λ DNA digested with Pst1 was used as the size marker (Lane L).

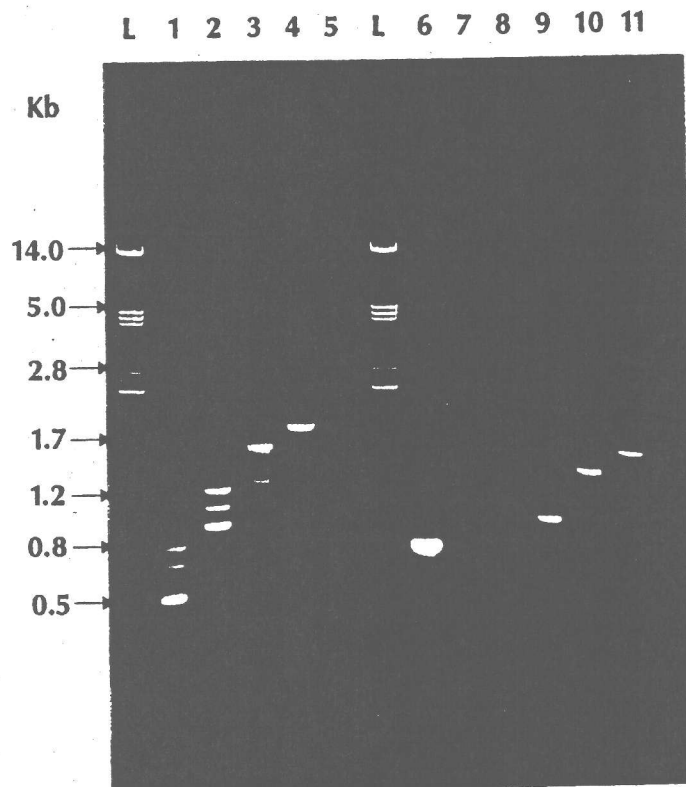


Figure IV.8. Interpretation of the PCR results obtained with event T14 DNA and primer pairs 2, 4, 5, 6, and 7. The three copies of the disrupted *ampR* gene are labeled 1, 2, or 3 copy. See Table IV.3. for description of primer pairs.

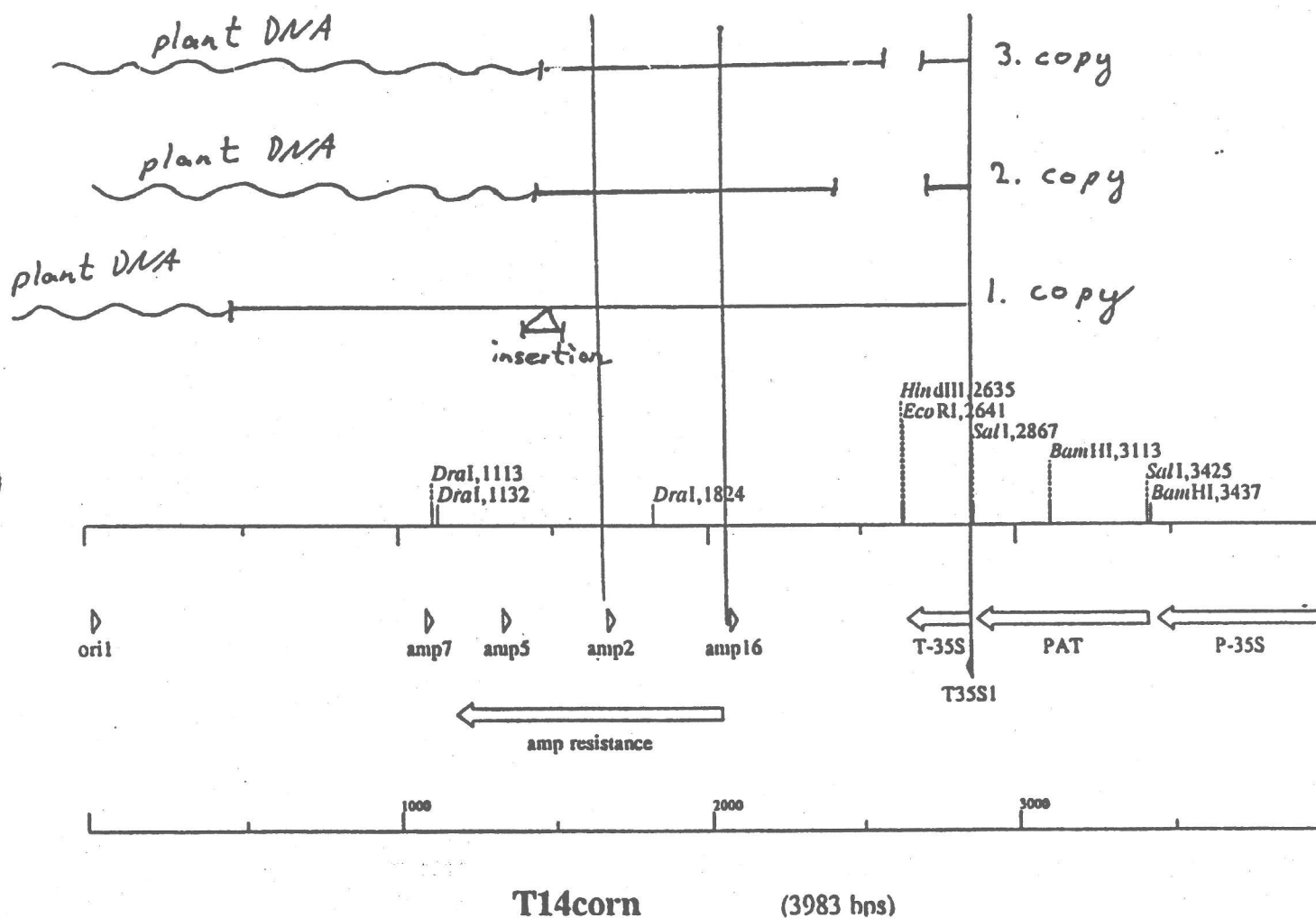


Figure IV.9. Southern blots showing stability of insertions in events T14 and T25. DNA was isolated from a nontransformed proprietary inbred (Lane 1); original event T14 (Lane 2); 3 individuals from a fifth backcrossed generation of event T14 (Lanes 3-5); nontransgenic cell line He/89 (Lane 6); original event T25 (Lane 7); 3 individuals from a third backcrossed generation of event T25 (Lanes 8-10); and a nontransformed public inbred (Lane 11). DNAs were digested with *Eco*RI (Panel A), or *Bam*HI (Panel B). The *pat* gene (552 bp *Sal*I fragment) was used as probe.

A *Eco*RI

1 2 3 4 5 6 7 8 9 10 11 12

0.5 kb →

2 kb →

3 kb →

B *Bam*HI

1 2 3 4 5 6 7 8 9 10 11

← 7

← 4

← 2.5

← 0.3

Figure IV.10. HPLC analysis of β -lactamase activity in event T25. Crude protein extracts were prepared from nontransgenic counterpart (left panel) and event T25 (right panel) leaves and incubated with ^{14}C -labeled penicillin for 5 minutes (top row) or 60 minutes (second row). Samples were analyzed for penicillin (pen) breakdown to a degradation product (pab) by HPLC-radiomonitoring. The control (third row) was 2 ul of *E. coli*/pUC12 growth medium (contains secreted β -lactamase) added to the protein extract from event T25 and incubated for 5 minutes prior to analysis.

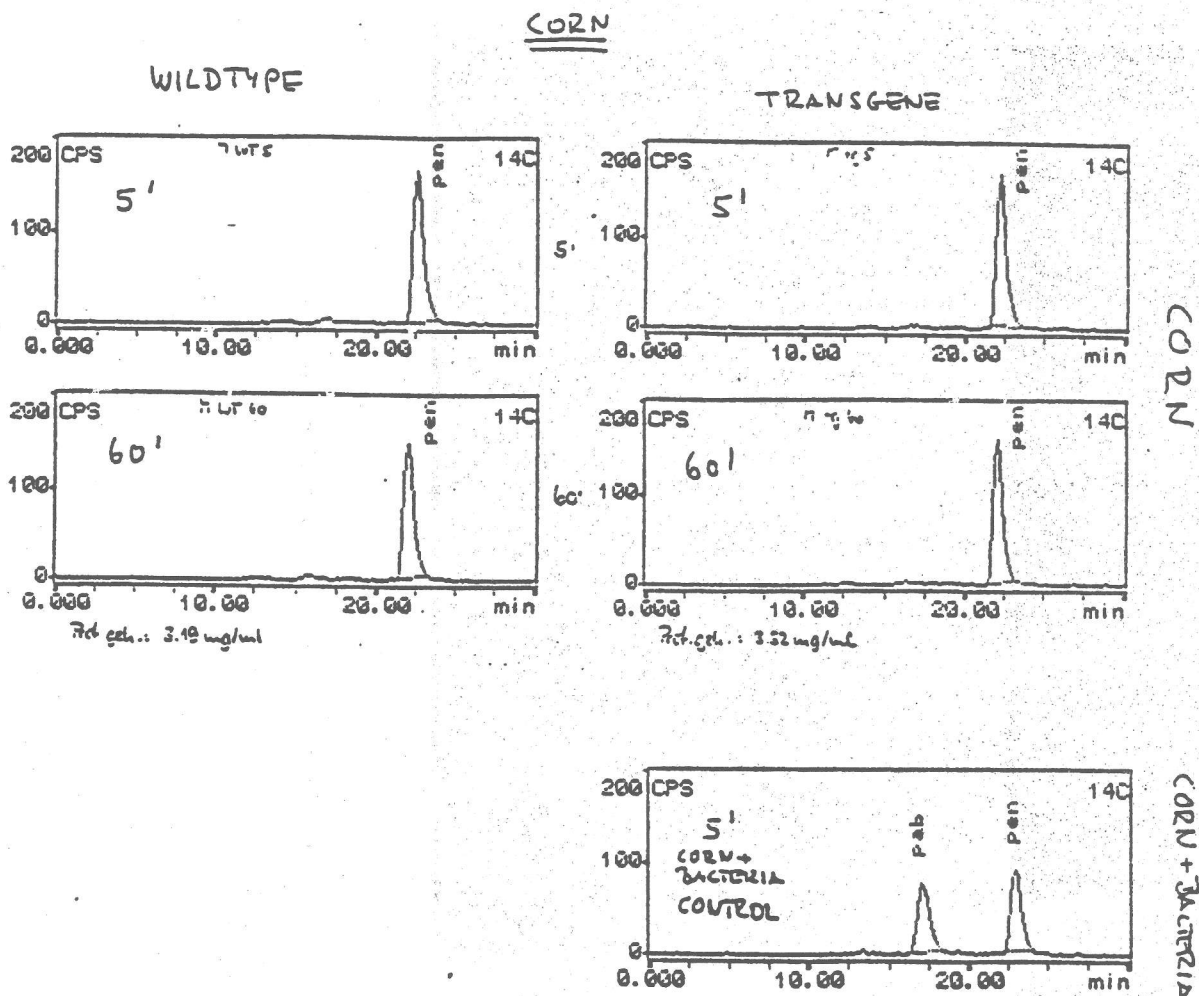


Figure IV.11. HPLC analysis of β -lactamase activity in event T14. Crude protein extracts were prepared from event T14 leaves (upper row), roots (middle row), and seeds (lower row) and incubated with ^{14}C -labeled penicillin for 5 minutes (left column) or 60 minutes (right column). Samples were analyzed for penicillin (pen) breakdown to a degradation product (pab) by HPLC-radiomonitoring.

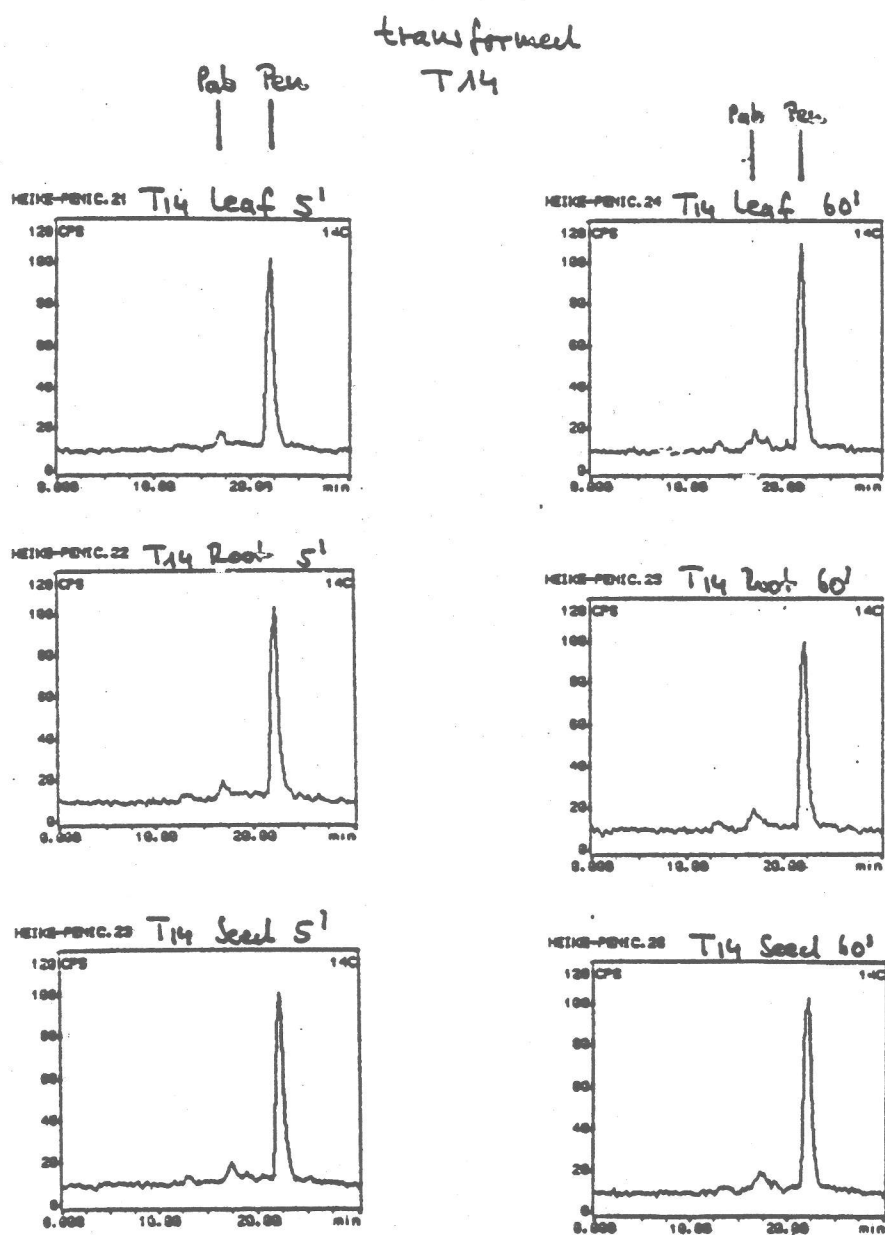


Figure IV.12. Northern analysis of events T14 and T25 probed with *ampR*. Total RNA (30 ug each) from T14 (Lane 1), T25 (lane 2), and a nontransgenic counterpart (Lane 3), and the *ampR* gene DNA (Lane 4) (0.5 ng) (692 bp Dra1 fragment) was separated on a 1.5% denaturing formaldehyde agarose gel. After transfer to a nylon membrane the filter was probed with the *ampR* gene.

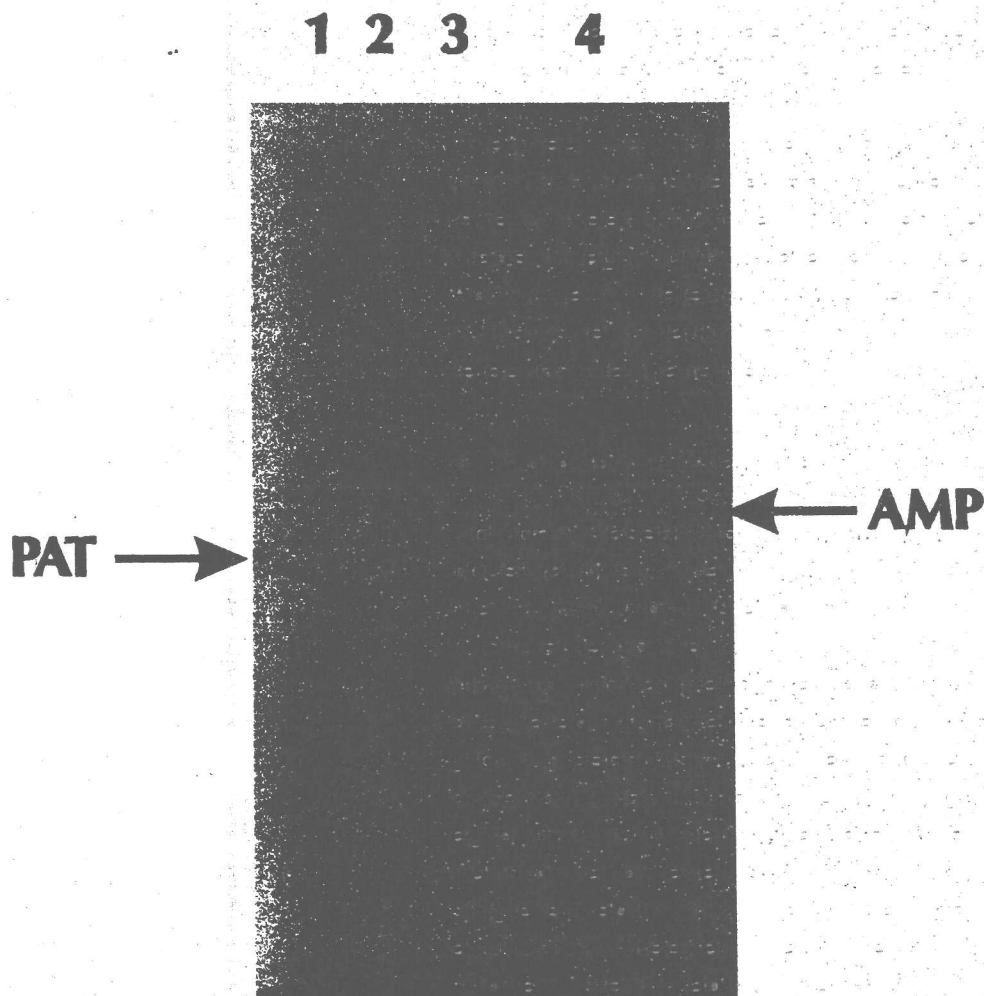
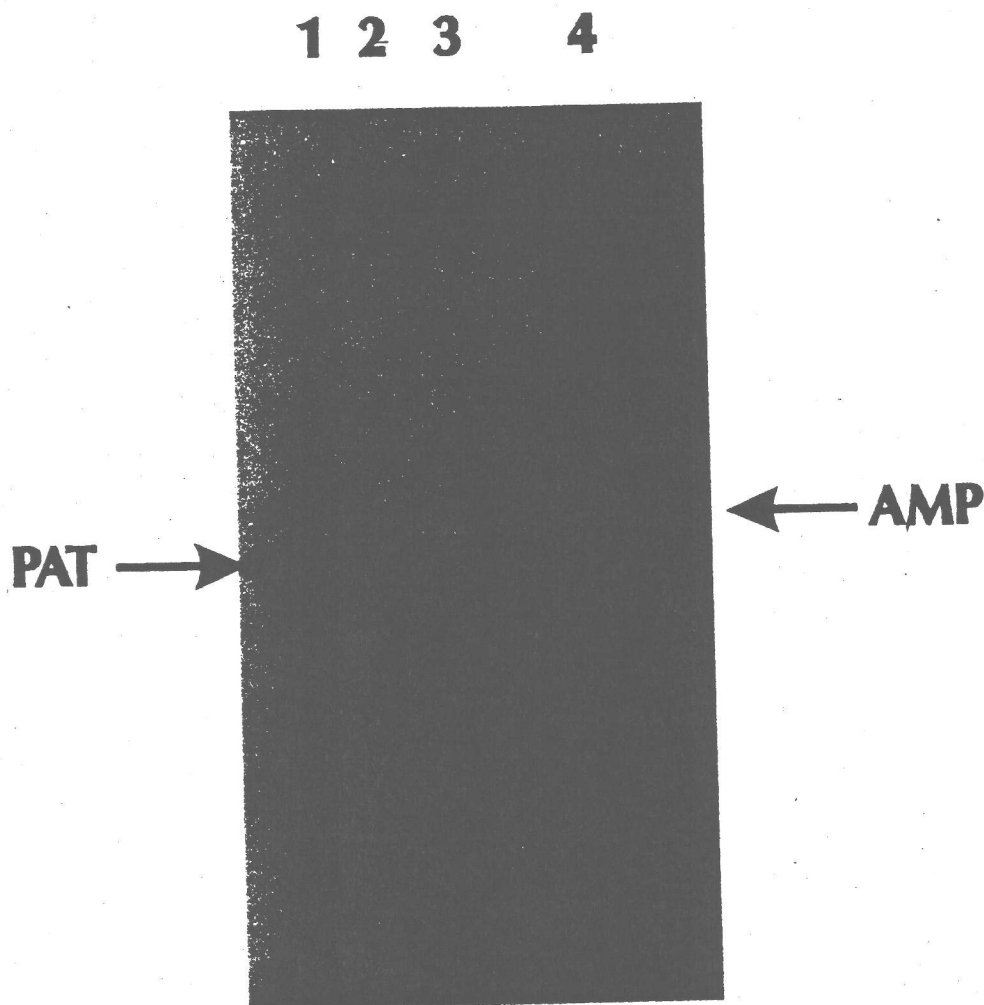


Figure IV.13. Northern analysis of events T14 and T25 probed with *pat*. Total RNA (30 ug each) from T14 (Lane 1), T25 (lane 2), and a nontransgenic counterpart (Lane 3), and the *ampR* gene DNA (Lane 4)(0.5 ng)(692 bp *Dra*I fragment) was separated on a 1.5% denaturing formaldehyde agarose gel. After transfer to a nylon membrane the filter was first probed with the *ampR* gene, and subsequently probed with the *pat* gene (552 bp *Sal*I fragment). The filter was not stripped between hybridizations.



V. Agronomic Performance and Compositional Analysis of Glufosinate Resistant Corn Events T14 and T25

A. Field Tests of Events T14 and T25

Transformation events T14 and T25 have been field tested by AgrEvo USA Company since 1992 in the primary corn growing regions of the United States. These tests have occurred at approximately 78 sites under field release authorizations granted by APHIS (USDA authorizations: permits 92-017-04, 92-043-01, 93-021-10, 93-021-11; notifications 93-120-17, 93-120-27, 94-074-03). A field release is currently in progress under notification 94-272-03. Transformation events T14 and/or T25 have also been field tested in Germany, France, Italy, Canada and Chile. Performance in these countries has been similar to that in the United States.

The great majority of the trials in the United States have been efficacy trials in which the plants have been sprayed with different rates of GA to determine the level of weed control and corn resistance. However, observations were also made on agronomic characteristics and disease and pest characteristics. Additionally, material was harvested for compositional analyses. Appendix 3 contains termination reports submitted to the USDA for the environmental releases that have been completed in the United States.

B. Agronomic Characteristics

Company researchers, university cooperators, and corn breeders made visual observations of many agronomic traits of GRC events T14 and T25 including plant morphology, stand count, plant height, ear height, time to pollen shed, time to silk emergence, crop injury due to chemical application, root lodging, and stalk lodging. For all traits evaluated a nontransgenic genetic counterpart was also evaluated. Qualitative evaluations were made during the 1992 through 1994 growing seasons. Quantitative data were taken during the 1994 growing season at certain sites in the primary corn growing regions of the United States. For all agronomic information gathered, there were no differences between transformation events T14, T25 and the nontransgenic counterparts, with the single exception that the nontransgenic material was not resistant to GA application (See termination reports in Appendix 3). A more thorough discussion of overwintering ability, stand count, germination, and yield is made below.

Although overwintering and germination of GRC events T14 and T25 were not directly tested under field conditions, stand counts were made upon emergence of the plants in the spring, and sites were monitored for volunteers in subsequent seasons. Plots have been observed for volunteers after the 1992/93 and 1993/94 winter months and the number of volunteers ranged from none to the number expected in commercial corn production. Volunteer corn usually

emerges from ears that drop to the ground during harvest the previous season. The number of volunteers can be influenced by tillage type and fall/winter weather. Corn producers usually eliminate volunteers from production fields because they are no longer hybrid types and tend to look scraggly and yield poorly; they do not emerge in line with the newly planted seed; and they compete for resources with the current crop.

During the 1994 growing season stand counts were made throughout the U.S. Corn Belt on up to 30 replications of transformation events T14 and T25 in up to 15 inbred backgrounds. In all cases emergence was close to 100%. Additionally, the percent seed germination for transformation events T14 and T25 and nontransgenic seed harvested from Illinois and Indiana was tested at the Illinois Crop Improvement Association. Seed germination after 10 days was found to be comparable (Table V. 1. and Appendix 2). Treatment of the parent transgenic plant with up to 1500 gm ai/hectare of GA during the growing season did not reduce the germination rate of progeny. These data provide no reason to believe that transformation events T14 and T25 differs from commercially available cultivars in dormancy or ability to survive in the environment.

Table V.1. Germination Rates for Transgenic Events and Nontransgenic Hybrids

Experiment	Treatment ^a	Percent Germination ^b
Illinois Experiment	0 gms, NT	93.0 (91-95)
	0 gms, T14	92.7 (88-96)
	400 gms, T14	92.7 (88-97)
	1500 gms, T14	94.3 (93-96)
Indiana Experiment 1	0 gms, NT	96.0 (95-97)
	0 gms, T14	96.3 (96-97)
	400 gms, T14	95.7 (94-97)
	1500 gms, T14	97.0 (97)
Indiana Experiment 2	0 gms, NT	95.0 (93-97)
	0 gms, T25	97.0 (97)
Indiana Experiment 3	0 gms, NT	96.3 (95-97)
	0 gms, T25	97.0 (97)

^a Growing corn was treated with either 0, 400, or 1500 gm ai/hectare of GA.

NT = nontransgenic hybrid, a genetic counterpart to the transgenic material.

^b Mean of three replications (100 seeds/replication), (min.-max.)

Comparisons were also made to determine the possibility of reduced yield for transformation events T14 and T25. The comparisons were made throughout

the corn belt on up to 30 replications of transformation events T14 and T25 and nontransgenic hybrids in up to 15 inbred backgrounds. In addition, yield was evaluated for plots which had received a one time application of either no (0X rate), 500 (1X), or 2000 (4X rate) gm ai/hectare of GA. Evaluation across genotypes showed no significant differences (95% confidence level) in yield when events T14 and T25 were compared to their nontransgenic counterparts. There was also no significant difference found between events T14 and T25. Comparisons between spray rates showed no significance between the 0X and 1X, but there was a significant difference between 0X and 4X, and 1X and 4X. We believe the difference in yield observed is due to some segregation for the *pat* locus still present in one of the donor lines. AgrEvo prefers that finished lines be resistant to up to 1500 gm ai/hectare of GA even though we are pursuing registration of GA on GRC with a 1 or 2 time application at a rate of 400 gm ai/hectare of GA. Qualitative observation of yield has not identified any reduction when 2 applications of 400 gm ai/hectare of GA are applied to transformation events T14 and T25 material. The standard practice in corn breeding involves evaluating progenies from the initial crosses for several years before selecting commercial lines. This standard practice would remove any slight yield reduction (if any actually exists) associated with these events. Additional field releases to evaluate harvest yield on material closer to commercial release are planned for 1995.

C. Disease and Pest Characteristics

There are many viral, bacterial, fungal, nematode, and insect pests that can damage corn and cause disease (Dicke and Guthrie, 1988; Smith and White, 1988). In any given year one such pest infestation could result in severe damage and yield reduction to the corn crop. However, high disease pressure is rare in corn. Company researchers and cooperators made visual observations for plant pathogenic organisms in trials containing GRC events T14 and T25 during the 1992, 1993, and 1994 growing seasons. Such observations revealed some minor pathogen infections but no infestations (see Appendices 2 and 3). Diseases observed included Stewart's wilt (*Erwinia stewartii*), corn smut (*Ustilago maydis*), gray leaf spot (*Cercospora zeae-maydis*), common rust (*Puccinia sorghi*), northern corn leaf blight (*Exserohilum turcicum*), northern corn leaf spot (*Bipolaris zeicola* = *Cochliobolus carbonum*), eyespot (*Kabatiella zeae*) and stalk rot (can be caused by various fungi). Insect pest infestations of corn rootworm (*Diabrotica* spp.), European corn borer (*Ostrinia nubilalis*), black cutworm (*Agrotis ipsilon*), corn flea beetle (*Chaetocnema pulicaria*) and corn leaf aphid (*Rhopalosiphum maidis*) were observed at release sites. Whenever pests were observed there was no differences in damage or populations found between GRC events T14 and T25 and nontransgenic counterparts. In addition, no differences were observed between plots of GRC treated with no, 400, and 1500

gms GA/hectare (Appendix 2). Events T14 and T25 did not influence susceptibility to disease or pest organisms in diverse genetic backgrounds.

Ear rot diseases and kernel infection in corn are of concern not only because they reduce yield, but more importantly because these fungi produce mycotoxins that are harmful to humans and/or animals that eat infested corn. For this reason GRC events T14 and T25 and nontransgenic counterparts were evaluated for their susceptibility to ear rot infection in Illinois and Indiana during the 1994 season. This evaluation was conducted for AgrEvo USA Company by Dr. Don White, University of Illinois. The following is a synopsis from his report (Appendix 2).

The ear rot diseases included in the study were *Fusarium* ear rot (*Fusarium moniliforme*), *Gibberella* ear rot (*Gibberella zeae*), *Diplodia* ear rot (*Stenocarpella maydis* = *Diplodia maydis*), and *Aspergillus* ear and kernel rot (*Aspergillus flavus*). These four ear rot diseases represent the most common ear rot diseases of corn worldwide. Since natural infection does not necessarily occur every year, plants were inoculated using the most widely accepted methods. Inoculated plants included GRC events T14 and T25 in four genetic backgrounds and nontransgenic counterparts. The effect of GA application at two rates (400 and 1500 gm ai/hectare of GA) on susceptibility of GRC to ear rot disease was evaluated for the T14 event. Three replications of a randomized complete block treatment design were performed at both locations, Indiana and Illinois. Noninoculated plants were also evaluated for natural infection by ear and kernel rot organisms. Ear rot ratings were made after ears were hand harvested and dried. For kernel plating evaluations the grain was surface sterilized, plated on solid medium, and observed after a 10 day incubation period. Kernel plating was not done for those ears inoculated with *S. maydis* because the samples were so badly rotted. Data were analyzed by analyses of variance.

In general, there were no trends in differences between ear rot severity of transformed and nontransformed plants (Appendix 2, Table 1). This was also true with respect to fungi recovered from kernels (Appendix 2, Table 2). When differences did occur most often the nontransformed plants had the higher incidence of infection. As expected, the frequency of isolation of an ear rotting fungus was greater when that fungus had been used for inoculation.

Transformed plants treated with GA at two different rates did not differ in ear rot susceptibility from GRC plants not treated with GA (Appendix 2, Table 3). Events T14 and T25 did not influence susceptibility to ear rot disease in different genetic backgrounds.

In conclusion, transformation events T14 and T25 are no more susceptible to disease or insect infestation or severity than their nontransgenic counterparts. The genetic background in which the *pat* locus was placed does not appear to

influence susceptibility to disease and insect pests. There is no reason to believe that GRC will provide a better substrate for mycotoxin producing fungi than commercially available corn hybrids.

D. Compositional Analysis

Over one-third of the corn grown in the United States is used for the production of silage, whereas only 1% of the crop is utilized for forage. The remainder of the crop is grown for grain, of which only 8-9% is used for seed production, human food products and chemicals. The very large majority of the grain is used as animal feed. The four major end uses of grain are feed, wet milling, dry milling and alkaline cooking (tacos, tortillas, etc.).

Since silage and grain comprise the majority of the harvest endpoints for corn, these were evaluated for their composition. Proximate analyses were performed on both silage and grain from GRC transformation events T14 and T25 and their genetic counterparts grown in the mid-west during the 1994 growing season. Grain grown in Hawaii was also evaluated. The silage was comprised of transformation events in four different genetic backgrounds. The grain was comprised of transformation events in six different genetic backgrounds (4 in the mid-west; 2 in Hawaii). The results of the proximate analyses are shown in Tables V.2 and V.3.

Although some significant differences exist for some of the matrices in the silage proximate analysis when the Fisher's Protected Least Significant Difference test was applied, these differences were not identified using the Dunnett t-test. It is probable that no differences would be revealed with larger sample sizes. There were no significant differences using either statistical test for grain proximate analysis. Further analysis indicated that there was more variation due to geography (Hawaii vs. mid-west) and genetic background than between transgenic and nontransgenic (data not shown). The Iowa Gold Catalog 1993 Grain Quality Tests (1994) provides the following average values compiled over a few years for composition of grain on a percent basis: moisture 15.0%; protein 8.0%, oil 3.6%, and starch 60.0%.

In addition to the proximate analyses, the phytic acid levels in silage, and the amino acid and relative fatty acid profile in grain were determined. For all silage samples the level of phytic acid was less than 0.02%. The remainder of the data will be provided to the FDA in support of AgrEvo's food and feed safety assessment of transformation events T14 and T25, however, there are no apparent differences between the transgenic and nontransgenic counterparts. All the results clearly demonstrate that GRC is substantially equivalent to nontransgenic counterparts.

Table V.2. Average Proximate Analysis for Silage from GRC and Nontransgenic Counterparts in 1994 Field Releases^a

Component %	T14 ^b	Nontransgenic Counterpart	T25 ^b	Nontransgenic Counterpart
Moisture ^c	66.85	67.71	65.09	67.19
Fat ^d	0.88	1.08	0.99	1.11
Protein ^e	2.02*	2.60	2.19*	2.57
Ash ^f	1.11	1.36	1.05	1.08
ADF ^g	8.05	6.90	7.96	7.10
NDF ^h	14.50	12.25	14.55	13.2
Carbohydrate ⁱ	26.49*	28.34	28.59	30.16

^a The silage was harvested from Illinois and Indiana. Each transformation event was in two different genetic backgrounds, the identical or similar background as their nontransgenic counterparts. The values from the two genetic backgrounds were averaged to produce the values given in the table.

^b Values marked with an asterisk (*) are significantly different at the 95% confidence level from their nontransgenic counterparts based on Fisher's Protected Least Significant Difference (LSD) analysis. However, none of the values are significantly different at the 95% confidence level based on the Dunnett t-test.

^c Moisture and Volatile Matter, AOCS Official Method (1989), Ba 2a-38

^d Fat (Crude) or Ether Extract in Animal Feed, AOAC Official Methods of Analysis (1990), 920.39

^e Modified Kjeldahl Method, AOCS Official Method (1991), Ba 4d-90

^f Ash of Animal Feed, AOAC Official Methods of Analysis (1990), 942.05

^g ADF = Acid Detergent Fiber and Lignin in Animal Feed, AOAC Official Methods of Analysis (1990), 973.18

^h NDF = Neutral Detergent Fiber, Journal of the AOAC (1967) 50:50-55.

ⁱ By calculation: % carbohydrate = 100% - (% protein + % moisture + % fat + % ash)

Table V.3. Average Proximate Analysis for Grain from GRC and Nontransgenic Counterparts in 1994 Field Releases^a

Component %	T14 ^b	Nontransgenic Counterpart	T25 ^b	Nontransgenic Counterpart
Moisture ^c	12.93	14.69	13.99	14.76
Fat ^d	3.63	3.34	3.83	3.64
Protein ^e	10.52	9.61	9.03	8.63
Ash ^f	1.21	1.34	1.15	1.18
Fiber ^g	2.2	2.65	2.43	2.5
Carbohydrate ^h	71.72	71.02	71.2	71.78

^a The grain was harvested from Illinois, Indiana, and Hawaii. Transformation events grown in the mid-west (four samples) were in four different genetic backgrounds; those (4 samples) grown in Hawaii were in two different genetic backgrounds. The backgrounds were identical or similar to their nontransgenic counterparts. The values from the genetic backgrounds were averaged to produce the values given in the table.

^b There were no significant differences at the 95% confidence level between the transformation events and their nontransgenic counterparts based on Fisher's Protected Least Significant Difference (LSD) analysis and Dunnett t-test.

^c Moisture and Volatile Matter, AOCS Official Method (1989), Ba 2a-38

^d Fat (Crude) or Ether Extract in Animal Feed, AOAC Official Methods of Analysis (1990), 920.39

^e Modified Kjeldahl Method, AOCS Official Method (1991), Ba 4d-90

^f Ash of Animal Feed, AOAC Official Methods of Analysis (1990), 942.05

^g Crude Fiber, AOCS Official Method (1989), Ba 6-84

^h By calculation: % carbohydrate = 100% - (% protein + % moisture + % fat + % ash)

VI. Potential for Environmental Impact from Noncontained Use of Glufosinate Resistant Corn Events T14 and T25

A. The Herbicide Glufosinate-ammonium and Current Uses

Ammonium-DL-homoalanin-4-yl-(methyl) phosphinate (glufosinate-ammonium, GA) is a non-selective, non-systemic herbicide that controls a broad spectrum of annual and perennial grass and broadleaf weeds. The L-form (L-PPT) is the active component of GA. This form is also the active portion of the naturally occurring antibiotic bialaphos (see Section III.D.). It has a similar structure and shape as glutamic acid, and is therefore called a glutamic acid analog. It inhibits the enzyme GS which converts glutamic acid and ammonia into glutamine (Leason et al., 1982). L-PPT's ability to bind irreversibly with glutamic acid results in the buildup of ammonia that inhibits photophosphorylation in photosynthesis (Weld and Wendler, 1990). Phytotoxic symptoms (chlorosis and wilting) occur rapidly, especially under warm air temperature, high humidity, and bright sunshine conditions. Symptoms usually appear within 48 hours after application. Necrosis occurs in 4-7 days after application.

GA must be absorbed through the leaves to be effective. It has limited stem uptake, and translocation within the plant is dependent upon the application rate, plant species, and stage of plant growth. GA is rapidly degraded in the soil by microorganisms, not only in well aerated soils, but also in soils with stagnant moisture (Anonymous, 1991). Therefore, GA has very low residual soil activity and does not injure seedlings before emergence. Both GA itself and its degradation products are adsorbed to clay particles and humus materials in the soil. This greatly restricts the mobility of these compounds, despite their ready solubility in water, and prevents leaching to deeper soil layers. When used correctly, GA does not affect soil microflora or alter the number or mass of earthworm populations. Application of GA at recommended field application rates presents no hazard to fish or aquatic invertebrates. It is not a contact poison for honeybees. If used in accordance with directions for use, GA is not a hazard to birds or mammals. There were no changes in tumor incidence after lifetime exposure to GA in rats and mice. No mutagenic activity was detected in a battery of mutagenicity tests. When administered to pregnant animals, GA produced no adverse effects on fetuses at doses which were not toxic to the mothers (Anonymous, 1993).

There are presently no registered uses for GA in corn. However, GA is registered for use as a non-selective herbicide on turf (tradename Finale™) and apples, grapes, and tree nuts (tradename Rely®) in the United States. Outside the United States, GA is registered for use on plantation crops, tree nuts, and vines, and for industrial/non-agricultural weed control under a variety of tradenames including Basta® and Ignite®.

B. Effects on Agricultural and Cultivation Practices of Corn

1. Current Practices

In the United States, corn is primarily grown in rotation with soybeans, and most of the corn is grown in twelve midwestern states. About 30 percent of the corn is grown following corn from the previous year. The majority of corn is grown primarily following soybeans. Volunteer corn in corn is not a problem to the farmer and is generally handled by cultivation. The remaining volunteer corn usually does not mature and does not present a problem at harvest. Volunteer corn in soybeans can present a potential problem to farmers. The severity of the problem largely depends on harvest conditions for the corn the previous fall. If corn falls down before or during harvest, there can be a significant amount of corn growing amongst the soybeans in the following year. Volunteer corn is usually treated with a postgrass soybean herbicide such as quizalofop, fluazifop, or sethoxydim. These products are also widely used for post treatments of annual grasses.

Nearly all of the corn acreage in the United States is treated with a herbicide. Products are applied preplant, pre-emergence and post-emergence to the corn crop. Herbicide programs in corn can vary due to the geographic area, weed spectrum, and first-year versus continuous corn. Farmers have traditionally relied upon triazine products in continuous corn where potential for carryover of the residual materials would not be a concern. Several weeds, however, have developed resistance to the triazines (LeBaron, 1991). Adverse weather conditions also reduce the effect of the triazines and other soil applied herbicides. In first year corn triazines are also widely used, however, usually at lower rates and in combination with other soil applied products. These products, such as metolachlor, alachlor, acetochlor, acetamide, and vernolate, are pre-emergence soil applied and used primarily for the residual control of grasses at 1120-3360 gms ai / ha rates. Usually triazines are used with these products in premix formulations. The co-formulation of atrazine and metolachlor (Bicep) is the largest combination product used of a soil residual product. Post-emergence applications of dicamba or 2,4-D are often used for broadleaf control. Recently, sulfonylurea herbicides have been introduced to control grass and broadleaf weeds post-emergence in corn. They are also used for problem weed escapes such as shattercane (*Sorghum bicolor*). In general, corn receives a soil applied herbicide application and a follow-up post-emergence application. Due to potential crop injury, rotational concerns and weed competition, multiple herbicide applications applied post-emergence are not widely used in corn. Also, many products are used in combination as premixes or tankmixes to widen the spectrum of control. The reasons for this are to prevent corn injury, reduce weed pressure on the crop, and reduce rotational restrictions as with soybeans

or other legumes. Harvest aid treatments of 2,4-D, dicamba and other materials, are sometimes used to facilitate harvest.

Problem weeds in corn include shattercane (*Sorghum bicolor*), johnsongrass (*Sorghum halepense*), quackgrass (*Agropyron repens*), fall panicum (*Panicum ciliatum*), foxtails (*Setaria* spp.), wild proso millet (*Panicum miliaceum*) and woolly cupgrass (*Eriochloa villosa*), as these are grassy weeds in a grass crop. Velvetleaf (*Abutilon theophrasti*), pigweeds (*Amaranthus* spp.), wild sunflower (*Helianthus annuus*), ragweeds (*Ambrosia* spp.) and smartweeds (*Polygonum* spp.) are broadleaf concerns. Perennial broadleaf species, such as hemp dogbane (*Apocynum cannabinum*), Canada thistle (*Cirsium arvense*), and dandelion (*Taraxacum officinale*) (weed problem in no-till), are difficult to control in corn. Perennials are difficult to control because they propagate by seed and/or underground plant parts. Control of these diverse species requires the use of multiple herbicide families and multiple applications.

Corn Weed Control Programs

1. Normal Midwest Program

A. Following soybeans

Spring plant by disk or no-till into soybean stubble.

Apply grass residual material pre-emergence (metolachlor [Dual], alachlor [Lasso], acetochlor [Harness Plus]) with atrazine or cyanazine (Bladex).

Apply follow-up broadleaf post-emergent product; 2,4-D or dicamba with grass material; sulfonyleurea.

Cultivate one or two times.

Spot spray as needed for additional perennial weed problems (glyphosate [Roundup])

B. Following corn

Fall tillage, chisel corn stubble

Spring disk ground before planting

Apply PPI or Pre-emergence triazine with residual grass material

Cultivate once or twice

Apply broadleaf and grass post-emergence materials

Apply perennial weed control material to aid harvest

2. No-Till System

Apply burndown (gramoxone or glyphosate)

Apply pre-emergence soil residual herbicides, grass and broadleaf

Apply post-emergence grass and broadleaf combinations (separate applications may be required due to weed stage of growth)

Apply harvest aid treatments if needed

3. Low Input Program

Fall Tillage, chisel
Spring Disk
Apply atrazine + COC early postemergence
Apply 2,4-D to broadleaf weeds
Cultivate two times

2. Possible Effect of Glufosinate Resistant Corn on Current Practices

The use of GA will have no effect on the normal growth patterns of GRC plants. No effect on agronomic traits of GRC will be seen. Positive effects in corn cultivation will come from changes in tillage practices and herbicide use patterns. The broad spectrum, post-emergence activity of GA will help increase the amount of conservation and no-till acres of corn planted in the United States. The use of GA together with GRC will increase the adoption of post-emergence chemistry. Growers have the desire for a broad spectrum, post-emergent herbicide, as is evident in the adoption of post-emergence chemistry on other crops such as soybeans and wheat. Such a herbicide will give growers an opportunity to move away from pre-emergence and residually active compounds.

GRC and GA may positively impact current agronomic practices in corn by 1) offering a broad spectrum, post-emergence weed control system; 2) providing the opportunity to continue to move away from pre-emergent and residually active compounds; 3) providing a new herbicidal mode of action that allows for improved weed resistance management in corn acreage; 4) offering the use of an environmentally sound and naturally occurring herbicide; 5) encouraging herbicide use on an as needed basis; 6) decreasing cultivation needs; and 7) allowing the application of less total pounds of active ingredient than used presently.

3. Likelihood of Appearance of Glufosinate-resistant Weeds

The only foreseeable way by which a weed could develop true resistance to GA is through sexual transmission of the *pat* gene. This can and will occur where the crop and the related wild species are growing together and can exchange genetic material and produce fertile progeny. However, for corn in the United States sexual transfer to weed relatives does not occur (see Section II).

Today there are large numbers of herbicide resistant weed biotypes, with over half of them resistant to triazines (Le Baron, 1991). GA is unrelated to triazines and has a different mode of action, i.e., it inhibits GS. It is unlikely that weeds or any plant species will spontaneously develop resistance to GA under selective pressure, because a plant must either develop mutant forms of GS that do not bind L-PPT, but still recognize glutamic acid, and/or evolve a L-PPT detoxification system. Experimental work to create GA resistant crop plants by

selection has been ongoing for several years with no success. Below is an accounting of attempts to create GA resistant crop plants in the laboratory by selection for mutants that can tolerate L-PPT or overproduce GS.

Over the last 10 years AgrEvo has not succeeded in selecting a glufosinate resistant corn mutant from protoplast cultures. There have been no survivors when wildtype corn protoplasts are placed on medium containing L-PPT. On the other hand, using sulfonylureas as selective agents we have been able to select 44 independent sulfonylurea-resistant mutants within 3 months. Using fenoxaprop-ethyl as a selective agent we have been able to select 2 independent fenoxaprop-resistant mutants during one year. In all cases, there is a correlation with observations in weed populations where glufosinate-resistant weeds have never been observed, but weeds resistant to the other chemicals have been found.

Glutamine synthetase exists in multiple isozymic forms in different plant organs (McNally et al., 1983). These forms can be cytosol or plastid localized, and encoded by a multigene family. Overproduction of the GS isozymes could provide a degree of tolerance to L-PPT. Donn et al. (1984) selected alfalfa suspension cell lines that were more tolerant to L-PPT than wild-type cells. These cell lines have a 3- to 7- fold increase in their GS activity, due to an increase in GS mRNA resulting from amplification of a GS gene. When the amplified GS gene, under the regulation of the CaMV 35S promoter, was integrated into the tobacco plant genome, a 5-fold increase in GS specific activity and a 20-fold increase in resistance to L-PPT was measured *in vitro* (Eckes et al., 1989). Neither the amino acid composition of the plant tissue was altered significantly by GS overproduction; nor were the fertility and growth of the overproducing GS plants affected. Although overproduction of GS in plants has been demonstrated following intensive laboratory manipulation, it is doubtful that weeds will be selected in nature which overproduce GS, thereby conferring commercial levels of resistance to GA.

The likelihood that GS mutants will occur that do not bind L-PPT, but still recognize glutamic acid seems to be extremely low. *In vitro* mutagenesis studies in Dr. Howard Goodman's lab, Massachusetts General Hospital, several years ago showed that GS mutants that could no longer bind L-PPT could be obtained for the alfalfa GS gene (personal communication, Günter Donn, AgrEvo GmbH). However, these mutants were very ineffective in using glutamic acid as a substrate. A plant bearing such a mutation would have difficulties surviving because its ability to detoxify ammonia would be seriously decreased. This theoretical consideration is in accordance with the observations *in vitro* and in the field.

In conclusion, the likelihood of appearance of glufosinate-resistant weeds in the United States is extremely low to none.

C. Effects on Glufosinate Resistant Corn on Non-target Organisms

GRC transformation events T14 and T25 have been field tested at numerous sites across the U.S. since 1992 and no toxicity or alteration of population levels have been observed for beneficial insects, birds or other species that frequent corn fields (see termination reports, Appendix 3). There were no qualitative differences between beneficial species and populations present on transgenic and nontransgenic corn plants. This observation was expected since GRC contain a gene which encodes a protein that is naturally occurring (see Section III. D. 2. and Section VI.A.), and this protein shares no homology with proteins that are known to be toxic (see Section VI. E.).

D. Weediness Potential of Glufosinate Resistant Corn

Corn is generally not regarded as a weed. It is frequently stated that corn is completely dependent upon humans for its survival. Indeed the Union of Concerned Scientists (Rissler and Mellon, 1993) agree that contemporary corn is dependent on human intervention for survival and productivity. Corn is not listed as a noxious weed in the United States (USDA-AMS, 1994), nor is it listed as a weed anywhere else in the world (Holm et al., 1979).

Baker (1994) developed a general consensus list of characteristics common to many weeds. They include: 1) germination requirement fulfilled in many environments; 2) discontinuous germination and great longevity of seed; 3) rapid growth through vegetative phase to flowering; 4) continuous seed production for as long as growing conditions permit; 5) self-compatibility but not completely autogamous and apomictic; 6) when cross-pollinated, pollinated by unspecialized visitors or wind pollinated; 7) high seed output in favorable environments and some seed production in a wide range of environments; 8) adaptation for short- and long-distance dispersal; 9) if perennial, vegetative production or regeneration from fragments and brittleness; and 10) ability to compete by special means (rosette formation and presence of allelochemicals). These characteristics are not shared with all weeds. As is the case for many crop plants, corn does share some of these characteristics.

The introduction of resistance to the herbicide GA has not caused GRC to become a weed. GRC corn retains the same growth rate and growth habit as nontransgenic corn (see Appendix 3, and Section V.B). It continues to be an annual which produces ears that do not shatter and disperse their seed. As shown in Section V.B. GRC events T14 and T25 germinate uniformly and in a short period of time (10 days). In addition, GRC is equally susceptible to ear rot disease and other disease and insect pests as its nontransgenic counterparts (Section V.C. and Appendices 2 and 3). Although GRC events T14 and T25 may volunteer, the range in numbers of volunteers is no different from the number expected in commercial corn production (see Appendix 3, and Section

V.B.). If one chooses to eliminate GRC events T14 and T25, and their progeny by chemical management, they can be removed by treatment with herbicides other than GA (1994 termination report, Appendix 3). Trials where GRC was treated with glyphosate, fenoxaprop or imazethapyr demonstrate that introduction of the PAT enzyme does not impart cross tolerance to chemicals with a mode of action that differs from GA.

E. Indirect Effects of Glufosinate Resistant Corn on other Agricultural Products

As indicated in Section V.D. most of the corn grown in the United States is used for the production of silage and grain. Of the grain production less than 3% is consumed by humans. Corn grain is generally not consumed raw by humans, but is subjected to a number of processing steps during wet- and dry-milling including high temperature drying and oil extraction (temperatures up to 105°C [220°F]). Material harvested for silage is stored under conditions where it undergoes anaerobic fermentation. During the ensiling process temperatures seldom exceed 32°C (90°F), but pH usually reaches 4.0 (Ensminger et al., 1990).

AgrEvo GmbH has conducted studies on purified, synthetic PAT enzyme which show that the enzyme is both heat and acid labile. The enzyme loses 100% of its activity upon incubation at 75°C (103°F) or greater for 30 minutes. At pH values of 4 or less it is inactive after exposure for 30 minutes. Both the ensiling process and the heat treatments used for the processing of grain should eliminate most PAT activity. To confirm this AgrEvo USA Company has submitted silage to ensiling and grain to processing, however, these studies are not yet complete.

Should there be any PAT enzyme remaining after these treatments, the only route of exposure for humans and livestock to PAT in GRC corn would be via oral ingestion. In addition, animals would be exposed orally to PAT present in unprocessed grain, forage, and fodder. AgrEvo GmbH has confirmed experimentally that PAT protein and *pat* DNA in a plant matrix is rapidly degraded *in vitro* by the gastric juices from swine, chicken, and cattle. These animals represent the three primary types of gastric systems among livestock. It has also been experimentally confirmed that PAT is readily degraded in simulated human gastric fluids within minutes.

The PAT enzyme does not have the characteristics of an allergen or a toxin. It is acid and heat labile and contains no glycosylation motifs. The protein has no homology to proteins other than PAT genes from other organisms. The substrate specificity for the PAT enzyme is very strict in that the only substrate is L-PPT. Neither any protein amino acid nor D-PPT is acetylated by PAT. Acetyl transferases are abundant and ubiquitous in nature where they share the

common function of transferring an acetyl group from acetyl CoA to a substrate. Acetyl transferases differ in substrates and the metabolic pathways in which they function (Webb, 1992).

Based on 1) the substrate specificity of PAT; 2) the physicochemical properties of PAT; 3) its rapid degradation upon ingestion; 4) the low levels of PAT in whole tissues (Table IV.5.); and 5) the ubiquitous presence of acetyl transferases in nature, no adverse effects are predicted if the PAT enzyme is a minor constituent of human and animal food.

F. Potential for Gene Transfer to Other Organisms

1. Outcrossing with wild species

As discussed in Section II, hybridization between *Z. mays* and wild *Zea* species is possible. However, wild *Zea* species do not occur widely in the United States. Differences in factors such as flowering time, geographic separation, and developmental factors, for example, make crossing in nature in the United States only speculative. Crossing to the more distant relatives of *Z. mays* in the genus *Tripsicum* is very difficult and produces sterile offspring due to differences in chromosome number between *Zea* and *Tripsicum* species. Accordingly, there is little probability of unaided crosses between GRC events T14 and T25 and wild relatives in the United States, and little potential for loss of biodiversity among wild relatives in the United States.

2. Outcrossing to cultivated corn

Wind pollination is the primary method of pollination in corn. However, outcrossing can be eliminated by several physical methods such as removal of the tassel and covering the silks with bags, or geographic separation. These practices are practical for controlled crossings and the production of inbred corn. A high degree of self-pollination is ensured in the open-pollinated production of foundation and certified seed by planting well isolated blocks. The standard isolation distance for this production is 660 ft (approx. 200 m) from the nearest contaminating source (Wych, 1988). Outcrossing or cross-pollination is the method by which two inbred lines are combined to produce hybrid seed. With hybrid seed production, as with foundation seed, fields must be isolated. Hybrid seed is almost exclusively the type grown for commercial production. Corn is open pollinated during commercial grain production.

When GRC events T14 and T25 are grown for commercial grain production they will participate in unconfined outcrossing with other hybrid corn. Otherwise, the *pat* locus will be maintained in the germplasm just like any other trait. Although GRC or its progeny from commercial grain production may arise as volunteers the following season, volunteer corn is generally removed. In Section V. B. and Section VI. D. we have shown that GRC is no more likely to volunteer or than

nontransgenic corn, and that volunteers can be eliminated by the application of herbicides other than GA.

3. Transfer of genetic information to organisms with which it cannot interbreed

Movement of transgenes from genetically engineered plants to microorganisms has been suggested as a risk if such plants are released into the environment. As initially stated in the USDA's Interpretive Ruling on Calgene, Inc. Petition for Determination of Regulatory Status of FLAVR SAVR™ Tomato (USDA-APHIS, 1992b), and subsequently repeated in other USDA Determination documents, "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. As summarized in these Determination documents, evidence 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 the *pat* gene to a microbe would not pose a plant pest risk. Genes encoding both PAT enzymes and acetyl transferases are found in microbes in nature. Indeed, as described earlier in this document, the synthetic *pat* gene present in GRC events T14 and T25 is derived from a *pat* gene isolated from a naturally occurring soil microbe. Transfer of one of the disrupted *ampR* genes in GRC events T14 and T25 also would not pose a risk, as the disrupted genes would produce nonfunctional enzyme, and β -lactamase genes are common in microbes in nature.

VII. Statement of Grounds Unfavorable

No unfavorable information and data has been demonstrated for GRC Transformation Events T14 and T25.

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IX. Appendices

Appendix 1. DNA Sequence Data

Appendix 2. Expert Letter and Research Reports

Appendix 3. USDA Field Trial Termination Reports

Appendix 4. Literature Reprints

Appendix 1. DNA Sequence Data

Sequence of the plasmid p35S/AC

With 8 enzymes: ECORI HINDIII SALI BAMHI KPNI PVUII ECORV PSTI

November 23, 1992 13:13 ..

```

1  TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCA 60
  -----+-----+-----+-----+-----+-----+-----+
61  CAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTG 120
  -----+-----+-----+-----+-----+-----+-----+
121 TTGGCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGC 180
  -----+-----+-----+-----+-----+-----+-----+
181 ACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCC 240
  -----+-----+-----+-----+-----+-----+-----+
241 ATTCGCCATTTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTAT 300
  -----+-----+-----+-----+-----+-----+-----+
      P
      V
      U
      I
      I
301 TACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGT 360
  -----+-----+-----+-----+-----+-----+-----+
                                     H
                                     i
                                     n
                                     d
                                     I
                                     I
                                     I
                                     I
                                     E
                                     C
                                     O
                                     R
                                     I
361 TTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCCAAGCTTGAATTCGAGCTCGGTA 420
  -----+-----+-----+-----+-----+-----+-----+
      K
      P
      n
      I
421 CCCACTGGATTTTGGTTT TAGGAATTAGAAATTTTATTGATAGAAGTATTTTACAAATAC 480
  -----+-----+-----+-----+-----+-----+-----+
481 AAATACATACTAAGGGTTTCTTATATGCTCAACACATGAGCGAAACCCTATAAGAACCCT 540
  -----+-----+-----+-----+-----+-----+-----+
541 AATTCCTTATCTGGGAAC TACTCACACATTATTATAGAGAGAGATAGATTTGTAGAGAG 600
  -----+-----+-----+-----+-----+-----+-----+
                                     P
                                     S
                                     s
                                     a
                                     t
                                     l
                                     I
                                     I
601 AGACTGGTGATTTTCAGCGGCATGCCTGCAGGTGCACTCAGATCTGGGTAAC TGGCCTAAC 660
  -----+-----+-----+-----+-----+-----+-----+

```


661 TGGCCTTGGAGGAGCTGGCAACTCAAAATCCCTTTGCCAAAAACCAACATCATGCCATCC 720
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+

P
V
U
I
I

721 ACCATGCTTGTATCCAGCTGCGCGCAATGTACCCCGGGCTGTGTATCCCAAAGCCTCATG 780
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+

781 CAACCTAACAGATGGATCGTTTGGGAAGGCCTATAACAGCAACCACAGACTTAAACCTTG 840
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+

B
a
m
H
I

841 CGCCTCCATAGACTTAAGCAAATGTGTGTACAATGTGGATCCTAGGCCCAACCTTTGATG 900
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+

901 CCTATGTGACACGTAAACAGTACTCTCAACTGTCCAATCGTAAGCGTTCCTAGCCTTCCA 960
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+

961 GGGCCCAGCGTAAGCAATACCAGCCACAACACCCTCAACCTCAGCAACCAACCAAGGGTA 1020
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+

1021 TCTATCTTGCAACCTCTCTAGATCATCAATCCACTCTTGTGGTGTGGTGTGGCTCTGTCCT 1080
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+

E
C
O
R
V

1081 AAAGTTCAGTGTAGACGTCTCAATGTAATGGTTAACGATATCACAAACCGCGGCCATATC 1140
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+

P
V
U
I
I

S
a
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I

1141 AGCTGCTGTAGCTGGCCTAATCTCAACTGGTCTCCTCTCCGGAGACATGTCGACTCTAGA 1200
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+

B
a
m
H
I

K
P
n
I

1201 GGATCCCCGGGTACCCTGTCTCTCTCCAAATGAAATGAACTTCCTTATATAGAGGAAGGGT 1260
 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+

E
C
O
R
V

1261 CTTGCGAAGGATAGTGGGATTGTGCGTCATCCCTTACGTGAGTGGAGATATCACATCAAT 1320
 -----+-----+-----+-----+-----+-----+-----+
 1321 CCACTTGCTTTGAAGACGTGGTTGGAACGTCTTCTTTTCCACGATGCTCCTCGTGGGTG 1380
 -----+-----+-----+-----+-----+-----+-----+
 1381 GGGGTCCATCTTTGGGACCACTGTGCGCAGAGGCATCTTCAACGATGGCCTTTCTTTTAT 1440
 -----+-----+-----+-----+-----+-----+-----+
 1441 CGCAATGATGGCATTGTAGGAGCCACCTTCCTTTTCCACTATCTTCACAATAAAGTGAC 1500
 -----+-----+-----+-----+-----+-----+-----+
 1501 AGATAGCTGGGCAATGGAATCCGAGGAGGTTTCCGGATATTACCTTTGTTGAAAAGTCT 1560
 -----+-----+-----+-----+-----+-----+-----+
 1561 CAATTGCCCTTTGGTCTTCTGAGACTGTATCTTTGATATTTTGGAGTAGACAAGCGTGT 1620
 -----+-----+-----+-----+-----+-----+-----+
 1621 CGTGCTCCACCATGTTGACGAAGATTTTCTTCTGTGTCATGACTCGTAAGAGACTCTGTA 1680
 -----+-----+-----+-----+-----+-----+-----+
 1681 TGAACGTTCGCCAGTCTTTACGGCGAGTTCGTTAGGTCCTCTATTGAATCTTTGACT 1740
 -----+-----+-----+-----+-----+-----+-----+
 B
 C
 O
 R
 I
 1741 CCATGGGAATTCGTAATCATGGTCATAGCTGTTTCTGTGTGAAATGTTATCCGCTCAC 1800
 -----+-----+-----+-----+-----+-----+-----+
 1801 AATTCACACAACATACGAGCCGGAAGCATAAAGTGTAAGCCTGGGGTGCTAATGAGT 1860
 -----+-----+-----+-----+-----+-----+-----+
 1861 GAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTC 1920
 -----+-----+-----+-----+-----+-----+-----+
 P
 V
 U
 I
 I
 1921 GTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCG 1980
 -----+-----+-----+-----+-----+-----+-----+
 1981 CTCTTCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCCGGCTGCGGCGAGCGGT 2040
 -----+-----+-----+-----+-----+-----+-----+
 2041 ATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAA 2100
 -----+-----+-----+-----+-----+-----+-----+
 2101 GAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCTTGCTGGC 2160
 -----+-----+-----+-----+-----+-----+-----+
 2161 GTTTTTCATAGGCTCCGCCCCCTGACGAGCATCACAAAATCGACGCTCAAGTCAGAG 2220
 -----+-----+-----+-----+-----+-----+-----+
 2221 GTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGT 2280
 -----+-----+-----+-----+-----+-----+-----+

2281 GCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGG 2340
 2341 AAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTTCG 2400
 2401 CTC CAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGG 2460
 2461 TAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCAC 2520
 2521 TGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTG 2580
 2581 GCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGT 2640
 2641 TACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGG 2700
 2701 TGGTTTTTTTGTGTGCAAGCAGCAGATTACGCGCAGAAAAAAGGATCTCAAGAAGATCC 2760
 2761 TTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAAACGAAACTCACGTTAAGGGATTTT 2820
 2821 GGT CATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTT 2880
 2881 TAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAG 2940
 2941 TGAGGCACCTATCTCAGCGATCTGTCTATTTTCGTTTCATCCATAGTTGCCTGACTCCCCGT 3000
 3001 CGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACC 3060
 3061 GCGAGACCCACGCTCACC GGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGC 3120
 3121 CGAGCGCAGAAGTGGTCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCG 3180
 3181 GGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTTCGCAACGTTGTTGCCATTGCTAC 3240
 3241 AGGCATCGTGGTGTACGCTCGTCTTTGGTATGGCTTCATT CAGCTCCGGTTCCTCAACG 3300
 3301 ATCAAGGCGAGTTACATGATCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCC 3360
 3361 TCCGATCGTTGTCAGAAGTAAGTTGGCCGAGTGTTATCACTCATGGTTATGGCAGCACT 3420
 3421 GCATAATTCTCTTACTGT CATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTC 3480

3481 AACCAAGTCATTCTGAGAATAGTGATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAAT 3540
 -----+-----+-----+-----+-----+-----+
 3541 ACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTT 3600
 -----+-----+-----+-----+-----+-----+
 3601 TTCGGGGCGAAAACCTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCAC 3660
 -----+-----+-----+-----+-----+-----+
 3661 TCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTTACCAGCGTTTCTGGGTGAGCAAA 3720
 -----+-----+-----+-----+-----+-----+
 3721 AACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACT 3780
 -----+-----+-----+-----+-----+-----+
 3781 CATACTCTTCCTTTTCAATATTATTGAAGCATTATCAGGGTTATTGTCTCATGAGCGG 3840
 -----+-----+-----+-----+-----+-----+
 3841 ATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCG 3900
 -----+-----+-----+-----+-----+-----+
 3901 AAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAG 3960
 -----+-----+-----+-----+-----+-----+
 3961 GCGTATCACGAGGCCCTTTTCGTC 3983
 -----+-----+-----+-----+-----+-----+

Cutting enzymes:

BamHI EcoRI EcoRV HindIII KpnI PstI PvuII SalI

Appendix 2. Expert Letter and Research Reports

CARNEGIE INSTITUTION OF WASHINGTON
DEPARTMENT OF EMBRYOLOGY
115 WEST UNIVERSITY PARKWAY
BALTIMORE, MARYLAND 21210
TELEPHONE: 410-554-1200
FAX: 410-243-6311

26 September 1994

██████████
Manager Regulatory Affairs - Biotechnology
AgrEvo USA Company
Little Falls Centre Office
2711 Centerville Road,
Wilmington, De 19808

Dear ██████████

I have had 16 years experience in the genetic and molecular analysis of maize transposable elements and believe I have substantial familiarity with their properties. I have been asked to comment on two questions:

1. What is the probability of insertion of a transposable element in finished lines and hybrids?

Response: I believe that the probability of such an insertion event is so low that it is not a realistic concern. All known maize transposons have robust systems of genetic regulation whose purpose is to minimize both transcription and transposition of the element. The lines used by geneticists interested in transposition are by no means normal lines. They have generally been treated in some extreme way to release the activity of the elements: McClintock initially observed frequent transposition only in plants in which deliberate cycles of chromosome breakage had been initiated genetically (although once activated the elements may remain active). Peterson recovered a single line with an activated transposable element from a substantial volume of maize seed irradiated by an atomic bomb detonation on the Bikini atoll. Others have subjected maize plants to X- or γ -irradiation or other highly abnormal procedures, such as long-term maintenance in tissue culture (which is known to be highly mutagenic in other ways, as well). Thus in plants growing under normal field conditions, transposition occurs extremely rarely.

Although all details of the regulatory systems that minimize transposition of maize transposons are not yet understood, transposition of the three most thoroughly investigated elements is minimized by extensive methylation of element sequences. Our studies show that methylation maintains transposable elements in a form that is not only silent transcriptionally, but also transpositionally inactive. That is, even if transposition functions are supplied by an active element elsewhere in the genome, heavily methylated elements rarely respond. If they do respond, they transpose extremely late in development, and the transposition event is therefore not heritable. Although the spontaneous reactivation frequency for transposable elements has not been investigated for all elements, the Spm element which we have studied in detail, shows a transposition frequency sufficiently low so that no heritable cases of spontaneous reactivation

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were observed among more than 200,000 kernels examined. In sum, there is little or no transposition in normal plants.

2. What is the likelihood that an insertion of a transposable element will result in both transcription and translation of a single target gene in the maize genome?

Response: The insertion of a transposable element in or near a target gene is very improbable, as described above. Even if such an event were to occur, it is highly improbable that it would result in activation of the gene. Although certain transposable elements in other organisms show a preference for insertion into promoter regions, this is not true in maize. In maize, most insertions occur within the body of the gene (in exons or introns) and completely, or almost completely, eliminate expression of the gene. There are just a few known instances in which insertions did not completely abolish gene expression because the transposon was spliced out of the gene together with an intron. Although the transposon itself does not behave like an intron, the ends of certain transposons contain mRNA splice acceptor or donor sites, occasionally permitting an imperfect splice to cut out most of the inserted element sequence from the gene's transcript. If the splice maintains the reading frame, the protein encoded by the altered mRNA can exhibit some enzyme activity. Again, it should be stressed that this is a rare event and even when it does occur, the residual activity of the enzyme encoded by the affected gene is reduced. Thus insertion of the element into the body of the gene has been observed to result in a diminution, not an enhancement of expression.

Most of the transposon insertions that have been analysed in maize are within coding sequences of genes and result in complete or nearly complete gene inactivation (except in restricted tissue areas in which the element has transposed out of the gene, restoring normal or near normal function). There is a small number of cases in which the element has inserted in or near a gene's promoter. Such insertions have also reduced gene expression. There is no known case to date in which a transposon insertion has enhanced expression of a gene, even though there are several cases in which such insertions have not completely eliminated gene expression.

In sum, not only is the likelihood of transposition very low, but even if such an unlikely event were to occur, the most probable outcome would be to disrupt the structure of the gene, effectively guaranteeing its inactivity.

I hope these remarks are of some assistance in your deliberations.

Sincerely,





ILLINOIS CROP IMPROVEMENT ASSOCIATION, INC.

3105 Research Road, P.O. Box 9013, Champaign, IL 61826-9013

Office Hours: 8:00 a.m. - 12 noon 1:00 - 5:00 p.m.

Telephone: 217-359-4053

Fax: 217-359-4075

NOVEMBER 15, 1994

TO: AGREVO USA CO

2711 CENTERVILLE RD
WILMINGTON DE 19808

R.S.T.

FROM: [REDACTED] SEED TECHNOLOGIST

Registered Seed Technologist, Seal No. 046

DEAR CORN PRODUCER:

TESTS HAVE BEEN COMPLETED ON THE FOLLOWING SAMPLES:

GERM. DATE: 11/15/94

TEST NO.	LOT NO.	VARIETY	GRADE	GERM.	HARD SEED	COLD TEST	P&S BLIGH
09277	REP 1	IL CONTROL TRANS		96 %			
09278	REP 2	IL CONTROL TRANS		88 %			
09279	REP 3	IL CONTROL TRANS		94 %			
09280	REP 1	IL CONTROL NON-T		95 %			
09281	REP 2	IL CONTROL NON-T		97 %			
09282	REP 3	IL CONTROL NON-T		94 %			
09283	REP 1	IL 400GMS TRANS		93 %			
09284	REP 2	IL 400GMS TRANS		97 %			
09285	REP 3	IL 400GMS TRANS		88 %			
09286	REP 1	IL 1500GMS TRANS		94 %			
09287	REP 2	IL 1500GMS TRANS		93 %			
09288	REP 3	IL 1500GMS TRANS		96 %			
09289	REP 1	IN EXPL CON TRAN		96 %			
09290	REP 2	IN EXPL CON TRAN		96 %			
09291	REP 3	IN EXPL CON TRAN		97 %			
09292	REP 1	IN EXPL NON-TRAN		97 %			
09293	REP 2	IN EXPL NON-TRAN		96 %			
09294	REP 3	IN EXPL NON-TRAN		95 %			
09295	REP 1	IN EXPL 400 TRAN		94 %			
09296	REP 2	IN EXPL 400 TRAN		96 %			
09297	REP 3	IN EXPL 400 TRAN		97 %			
09298	REP 1	IN EXPL 1500 TRA		97 %			
09299	REP 3	IN EXPL 1500 TRA		97 %			
09300	REP 2	IN EXPL 1500 TRA		97 %			
09301	REP 2	IN EXP2 NON-TRAN		97 %			
09302	REP 3	IN EXP2 NON-TRAN		95 %			

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Appendix 2
Page 4 of 19

NOVEMBER 15, 1994

TO: AGREVO USA CO

2711 CENTERVILLE RD
WILMINGTON DE 19808

FROM: [REDACTED] SEED TECHNOLOGIST

DEAR CORN PRODUCER:

TESTS HAVE BEEN COMPLETED ON THE FOLLOWING SAMPLES:

GERM. DATE: 11/15/94

TEST NO.	LOT NO.	VARIETY	GRADE	GERM.	HARD SEED	COLD TEST	P&S BLIGHT
9303	REP 1	IN EXP2 NON-TRAN		93 %			
9304	REP 3	IN EXP2 TRANS		97 %			
9305	REP 2	IN EXP2 TRANS		97 %			
9306	REP 1	IN EXP2 TRANS		97 %			
9307	REP 3	IN EXP3 NON-TRAN		97 %			
9308	REP 2	IN EXP3 NON-TRAN		97 %			
9309	REP 1	IN EXP3 NON-TRAN		95 %			
9310	REP 1	IN EXP3 TRANS		97 %			
9311	REP 2	IN EXP3 TRANS		97 %			
9312	REP 3	IN EXP3 TRANS		97 %			

**Evaluation of Transgenic and Nontransgenic Corn Hybrids for Susceptibility
to Ear Rot and Other Diseases - 1994**

**[REDACTED], Associate Professor, Department of Plant Pathology,
University of Illinois, Urbana-Champaign**

INTRODUCTION

Ear, kernel, and cob rots occur wherever corn is grown. These diseases rarely cause severe yield losses over wide geographical areas, however, they have been important in localized areas of the United States. Losses result from reduced test weight, poor grain quality and mycotoxins that may contaminate feeds and food. Mycotoxins are important due to the diseases of animals and humans that may result as a result of consumption of contaminated feeds or foods. These diseases vary greatly between years depending upon pre-harvest environment and damage from insects, hail, and frost. Ear rot diseases also may reduce the allowable storage time of corn and grain.

Fusarium kernel or ear rot - Fusarium kernel or ear rot, caused by the fungus *Fusarium moniliforme*, is the most widespread disease of corn ears. It can be found in virtually every field, every year, and is more prevalent in the drier parts of the Corn Belt (2). Symptoms of the disease are scattered or groups of randomly infected kernels over the entire ear. Whitish to pink fungal growth is typical of Fusarium ear rot. Infection is also frequent at the tip of the ear where it is often associated with earworm and other types of damage. The fungus can be found in association with roots, stalks, and ears of the corn plant. The fungus also lives in soil and plant debris. Spores of the fungus are spread by wind and may enter through the silk channel at the tip of the ear and infect immature kernels. Additional infections occur following injury to the ear. The fungus also may be

isolated from symptomless kernels and it is difficult to find grain lots where *F. moniliforme* cannot be found associated with at least a small percentage of the kernels.

Acute toxicity in different animals have been reported with various mycotoxins produced by *F. moniliforme*. Perhaps the most common and most important mycotoxin produced is the toxin Fumonsin which is a problem when consumed by swine and by horses.

Gibberella ear rot - *Gibberella ear rot*, caused by *Gibberella zeae*, occurs throughout the Corn Belt but is more prevalent in northern parts of this region. The disease is favored by cool humid weather particularly with heavy rainfalls following silking of the crop (2). Symptoms of the disease are reddish color that usually begins at the tip of the ear and progresses toward the butt. The rot rarely involves the whole ear. *Gibberella ear rot* is more severe when wet weather occurs 14 to 21 days following flowering. The fungus overwinters in soil and debris and infects silks. It then grows into the ear progressing down the ear during grain fill. The causal agent is the same fungus that is responsible for *Gibberella stalk rot* of corn.

Gibberella zeae produces several mycotoxins. One toxin that is produced is zearalenone, also referred to as F-2. This toxin is responsible for estrogenic mycotoxicoses with symptoms including enlargement of the uteri and mammary glands, vulvar swelling, vaginal prolapse, and atrophy of testes. Another toxin that is produced is deoxynivalenol, which has been responsible for emesis and feed refusal of swine. Other mycotoxins are reported to be produced by the fungus, however, zearalenone and deoxynivalenol are the most common and most studied. Ruminant animals and poultry do not seem to be as affected by these toxins as are swine.

Diplodia ear rot - *Diplodia ear rot*, caused by *Stenocarpella maydis*=*Diplodia maydis*, is widely distributed throughout the Corn Belt but is present most often in fields where reduced tillage is used and corn follows corn. One of the earliest symptoms of ear rot is the bleaching of husks. When husks are open, a white mold is seen on the ear. By harvest, infected ears are completely rotted with

the husks tightly adhering to the ear. *Stenocarpella maydis* is the same fungus that causes Diplodia stalk rot. The fungus may infect the ear at any time, however, ears are most susceptible to infection 15 to 18 days after full silk. Spores produced by the fungus associated with debris on the soil surface are splashed onto silks. Early infection often results in complete rotting of the ear, whereas late infection results in partial rotting of the ear. Diplodia ear rot is usually found close to sources of inoculum and rarely found where inoculum has been buried by tillage or has been reduced following rotation with another crop.

Aspergillus ear and kernel rot - Several species of *Aspergillus* cause ear and kernel rots of corn. However, ear and kernel rots caused by *A. flavus* is the most serious. Symptoms include a greenish or yellowish tan discoloration on and between individual kernels. Often, kernels at the tip of the ear are infected. This is seen particularly on those hybrids where husk coverage does not protect ears from damage due to insects, hail and other factors. Aspergillus ear rot is favored by hot dry environmental conditions. Under hot dry environmental conditions the fungus will increase inoculum on plant debris and spores of the fungus are usually spread by wind to silks that are starting to senesce. In wet environmental conditions *A. flavus* does not compete well with other fungi and bacteria, therefore, inoculum and subsequent infection is reduced.

Aspergillus rot is important due to the production of several mycotoxins generally referred to as aflatoxin. These compounds are a problem when consumed by a number of animals, and are of great concern because of the link to human liver cancer.

Control of ear rot diseases - Generally, ear rot diseases are controlled by avoiding extremely susceptible corn hybrids. Other possible controls include crop rotation and tillage. The use of tillage to control various corn diseases is not utilized as much as it once was because reduced tillage is mandated for control of water and wind erosion of soil.

Other corn diseases present in Illinois and Indiana. Various other leaf and stalk diseases

occur in Indiana and Illinois. Most common diseases in the area in 1994 include Stewart's wilt which is caused by the bacterium *Erwinia stewartii*. Symptoms include death of seedlings, if infection occurs very early, and leaf blight. The bacterium that causes the disease overwinters in the corn flea beetle. The bacterium enters the plant through wounds caused by feeding of the corn flea beetle. Most commercial corn dent hybrids have adequate levels of resistance to this disease to avoid yield loss. Another corn disease that occurred in 1994 in the midwestern United States was gray leaf spot. Gray leaf spot is caused by the fungus *Cercospora acae-maydis*. The disease has become more important in recent years because of the use of reduced tillage. The fungus survives on corn debris, and produces spores which are disseminated by wind and by splashing. Most commercially used corn hybrids are susceptible to the disease, however, the disease requires extended periods of wet weather in order to cause damage, and this environmental condition is usually not present. Other leaf diseases that occurred in 1994 included common rust (*Puccinia sorghii*), northern corn leaf blight (*Exserohilum turcicum*), northern corn leaf spot (*Bipolaris zeicola* = *Cochliobolus carbonum*), and eyespot (*Kabatella zae*) (see ref. 2 for descriptions). All these diseases are caused by fungi and the incident and severity of the disease depends upon the amount of inoculum, environmental conditions, and the susceptibility of the corn hybrid.

Another disease that commonly occurs on corn is corn stalk rot. This is a rotting of the lower stem of the plant following flowering. This disease is caused by a number of different fungi which act either alone or together to cause rotting of the lower stem (2). Plants with symptoms of stalk rot are usually killed prematurely, and may rot to the extent that the plant lodges and the ear cannot be picked up by mechanical harvesters. In 1994 stalk rot diseases were not of any importance in the midwestern United States.

Experiments with transgenic and nontransgenic plants - Transgenic and nontransgenic plants were evaluated for susceptibility to ear rot diseases and kernel infection following inoculation;

susceptibility to ear rot and kernel infection when noninoculated; and susceptibility to other corn diseases existing in the area as a result of natural infection. In this study the ear rot diseases included were: *Fusarium* ear rot, *Gibberella* ear rot, *Diplodia* ear rot, and *Aspergillus* ear and kernel rot. These four ear rot diseases represent the most common ear rot diseases of corn worldwide. Because natural infection with these ear rot diseases does not necessarily occur every year, we elected to inoculate plants. The most widely accepted method of inoculation for *Fusarium* ear ear, *Gibberella* ear rot, and *Diplodia* ear rot is injection of conidia into the silk channel. Inoculations with *Aspergillus* ear and kernel rot must be made by injection of conidia in combination with wounding of kernels to simulate insect damage to ears (1). We evaluated the application of glufosinate-ammonium at two rates to determine the effect of glufosinate-ammonia on ear rot of transgenic hybrids that carried the gene that imparts resistance to glufosinate-ammonium.

METHODS AND MATERIALS

Field Plots - For these studies, field plots maintained by AgrEvo USA Company in Macon County Illinois (located southeast of Decatur near Dalton City) and Johnson County Indiana (south of Indianapolis located near Whiteland) were used to evaluate transgenic and nontransgenic corn hybrids for disease resistance. All plots were treated with Bicep herbicide at a rate of 2850 gms ai/ha preplant. In Illinois, transformation event T14 was in the transformed hybrid, and the nontransformed hybrid was a genetic counterpart. In the plots in Indiana, three separate experiments were conducted using transformed and nontransformed versions of hybrids of event T14 in experiment one and event T25 in experiments two and three. All four transformed hybrids have genetic backgrounds different from one another. At both locations, transformed and nontransformed hybrids were arranged as a

randomized complete block treatment design in three replicates. Ear rot inoculations and control treatments (12-18 plants each) were subplots within each replicate.

In addition, noninoculated corn ears (12-18 ears from each replicate of each treatment) from transformed plants in plots treated with 0, 400, and 1500 gm ai/ha of glufosinate-ammonium herbicide at growth stage V5 were harvested from the hybrid used in Illinois, and from the hybrid used in experiment one in Indiana to compare natural infestation of transformed plants by ear rot organisms in the presence or absence of glufosinate-ammonium herbicide treatment. Only kernel planting evaluations were made for these plots because of the lack of symptoms of naturally occurring ear rot.

Ear rot inoculation - Ear rot inoculations were done on 12-18 plants per subplot in each replicate. Inoculations for *Gibberella* ear rot, *Fusarium* ear rot, and *Diplodia* ear rot were done 1-2 weeks after pollination on 1 August 1994 at both locations. Inoculations were made by injecting 5 ml of the spore suspension containing approximately 200,000 conidia per ml of the fungus into the silk channel. Inoculations for *Aspergillus* ear and kernel rot were done 12 August 1994 at both locations using an inoculation technique developed at the University of Illinois (1). Fungal isolates used in Illinois were originally isolated from diseased plants collected in Illinois, and have been maintained in a culture collection at the University of Illinois. Fungal inocula used in Indiana were obtained from Don Scott, Extension Plant Pathologist, Purdue University, Botany & Plant Pathology Dept., Lilly Hall, West Lafayette, IN 47907.

Ear rot evaluations - Ears (12-18 per subplot) from plots or subplots in Illinois were hand-harvested 22 September 1994, and ears from Indiana plots or subplots were hand-harvested 26 September 1994. Ears were placed into mesh bags and air-dried at the University of Illinois. Ear rot resulting from *Diplodia*, *Gibberella*, and *Fusarium* inoculations was rated on a 1-10 scale (1 = 10% of ear the rotted..., 10 = 100% of the ear rotted). Ear rot ratings (also 1-10 scale) for *Aspergillus*

ear rot were based on percent of rotted kernels in the inoculated area only. Ears from control subplots were also rated even though very little natural ear rot occurred at either location. Ears (12-18 from each replicate) from glufosinate-ammonium treated plots were harvested and dried at the same time but were not rated for ear rot because of the lack of ear rot symptoms.

Following drying and ear rot ratings, ears were shelled and grain from each subplot bulked. Fifty whole randomly selected kernels from each subplot were surface sterilized in a 1.575% sodium hypochlorite (30% commercial bleach) solution for one minute, rinsed in sterile water, plated in standard-sized petri dishes (10 kernels/plate) on malt salt agar (1) and incubated for 10 days. At the end of the incubation period, the percent of kernels from which various organisms grew was recorded. In addition, those kernels from which no fungi grew were recorded as percent clean. Plating was done using kernels from ear rot inoculation subplots, from control subplots, and from glufosinate-ammonium treated plots. Kernel plating was not done from those ears inoculated for *Diplodia* ear rot because samples were so badly rotted. The severe rot noted with *Diplodia* ear rot was expected due to the susceptibility of most commercial corn hybrids following inoculation.

Individual ear rot ratings and plating data (10 plates) for each subplot were averaged and analyses of variance were computed for test of significance between transformed and nontransformed plants. Plating data from the control and glufoninate-ammonium treated plots also were subjected to analysis of variance to test the significance of glufoniate-ammonium application.

RESULTS AND DISCUSSION

Ear rot evaluations: - In general, there were no trends in differences between ear rot severity of transformed and nontransformed plants (Table 1). There was a significant difference ($P=0.05$) with *Gibberella* ear rot inoculation in experiment one in Indiana where the transformed plants had a

lower ear rot rating than the nontransformed plant. The Indiana location did experience drought stress, and some of the nontransformed plant plots were located in a dryer area (lighter soil type) of the field. This probably delayed silking and the nontransformed plants may have been inoculated earlier in relation to silk date than transformed plants. Earlier inoculations usually result in higher amount ear rot due to *G. zeae* (based on personal experience). In general, it is doubtful that there is any real difference between the transformed and nontransformed plants for ear rot susceptibility considering that in all cases, but one, there was no statistical difference between ear rot rating.

Kernel plating evaluations - In general, there was no trend in differences between transformed and nontransformed plants with respect to fungi recovered from kernels. There were no significant differences between transformed and nontransformed plants with respect to isolation of fungi from plated kernels in the Illinois experiment (Table 2). At the Indiana location in experiment one there was significantly higher ($P=0.05$) recovery of *Gibberella* in *Gibberella* inoculated nontransformed plants. This correlates with the ear rot rating which also was higher for nontransformed plants (Table 1). The higher incidence of *G. zeae* in the *Gibberella* inoculated trial with nontransformed plants also resulted in fewer kernels from which no fungi were isolated in the transformed plants (Table 2). There was a significantly higher ($P=0.02$) incidence of kernels from which *F. moniliforme* grew following inoculation with *F. moniliforme* in transformed plants. Here again, this also resulted in differences with respect to kernel number from which no fungi were isolated. In experiment two in Indiana, the nontransformed plants had lower ($P=0.03$) total clean kernels in subplots inoculated with *G. zeae*. There were numerical differences between transformed and nontransformed plants with *G. zeae* isolation, however, the difference was not significant. There was a significant difference ($P=0.04$) in recovery of *A. flavus* from *F. moniliforme* inoculated nontransformed plants. This may have been due to these plants being located in an area of drought stress in the field. In experiment three in Indiana, the only significant differences ($P=0.02$) that

occurred were with *F. moniliforme* inoculated plants where nontransformed plants had higher levels of isolation of *F. moniliforme* and fewer kernels from which no fungi were isolated than transformed plants. Fusarium also is increased by dry environmental conditions (2), and the location of nontransformed plants in a slightly drier soil type may have been responsible for the difference.

In general, inoculation with the three ear rot pathogens increased the frequency of isolation of the fungus used for inoculation but not other ear rotting fungi. The inoculation with *A. flavus* did slightly increase the isolation of *F. moniliforme* compared to other inoculations or the uninoculated control. The increase was small and was expected (personal observation). *Fusarium moniliforme* is commonly isolated from corn silks, and the wounds that were caused by the *A. flavus* inoculation likely favored penetration by *F. moniliforme*.

Glufosinate-ammonium treatments - No differences in fungi recovered from plated kernels were found between transgenic plants treated with 0, 400 and 1500 gm ai/ha glufosinate-ammonium herbicide in either Indiana or Illinois experiments (Table 3). In Indiana, the kernels from the 0 rate had lower isolation of *A. flavus* however the difference was not significant because high levels of isolation of *A. flavus* occurred in only one replicate that was in a drought stressed area (light soil type) of the field. *Aspergillus flavus* is more severe when inoculations are made on drought-stressed plants (personal observation).

Observation of other diseases present - Observations of other diseases that naturally occurred at the locations were also noted at inoculation and at harvest times. At the Illinois location, a trace (less than 5% leaf area affected) of common rust was noted at all three visits to the field. There were, however, no differences observed between transformed and nontransformed plants. Likewise, there was some (trace amounts - one or two lesions per plant) Stewart's wilt at the Illinois location which again did not vary between transformed and nontransformed plants. Observations at harvest indicated no differences in the percent of prematurely dead plants with all plants being healthy

resulting in no differences in stalk rot (caused by various fungi). At the Illinois location, gray leaf spot was present at harvest resulting in about 8-10% blight of leaf tissue. Here again, there was no difference between transformed and nontransformed plants. The Gray leaf spot occurred as a result of wet leaf surfaces in late August and September associated with cool nights and, long lasting frequent dews. At the Indiana location, Stewart's wilt occurred at a very low level (one or two lesions per plant), and there was no difference noted between transformed and nontransformed plants. At harvest, stalk rot was absent in all plots. In addition, no differences in disease was observed in plots of transgenic plants treated with 0, 400 and 1500 gm ai/ha glufosinate-ammonium herbicide.

CONCLUSIONS

In general, there were no general differences in ear rot susceptibility between transformed and nontransformed corn hybrids. When differences did occur most often the nontransformed plants had the higher incidence of infection. Observations of other naturally occurring diseases also indicate no difference in susceptibility to plant disease. Additionally, transformed plants treated with glufosinate-ammonium at two rates did not differ in ear rot susceptibility or in susceptibility to naturally occurring diseases from transformed plants not treated with glufosinate-ammonium. The genetic background in which transformation events T14 and T25 were placed did not influence susceptibility to plant disease.

Literature

1. Campbell, K. W., and D. G. White. 1994. An Inoculation Device to Evaluate Maize for Resistance to Ear Rot and Aflatoxin Production by *Aspergillus flavus*. Plant Dis. 78:778-781.
2. Smith, D.R., and D.G. White. 1987. Diseases of Corn. In: Corn and Corn Improvement. G.F. Sprague ed., Academic Press, New York, N.Y., pp. 701-766.

Table 1. Ear Rot^a Evaluations of Transformed and Nontransformed Corn Hybrids - 1994

Illinois			
Ear Rot Inoculation	Transformed (T14^b)	Nontransformed	Significance
Diplodia	10.0	9.7	NS ^c
Gibberella	7.0	7.5	NS
Fusarium	1.1	1.2	NS
Aspergillus	6.2	5.5	NS
Control (non-inoculated)	0	0	NS
Indiana Experiment 1			
Ear Rot Inoculation	Transformed (T14^b)	Nontransformed	Significance
Diplodia	9.6	10.0	NS
Gibberella	3.0	5.7	@0.05
Fusarium	1.2	1.5	NS
Aspergillus	2.7	2.6	NS
Control (non-inoculated)	0	0	NS
Indiana Experiment 2			
Ear Rot Inoculation	Transformed (T25^b)	Nontransformed	Significance
Diplodia	9.9	10.0	NS
Gibberella	2.4	4.0	NS
Fusarium	1.5	1.5	NS
Aspergillus	2.3	2.6	NS
Control (non-inoculated)	.033	0	NS
Indiana Experiment 3			
Ear Rot Inoculation	Transformed (T25^b)	Nontransformed	Significance
Diplodia	10.0	10.0	NS
Gibberella	4.2	3.1	NS
Fusarium	1.6	1.6	NS
Aspergillus	2.8	3.5	NS
Control (non-inoculated)	0	0	NS

^a Ear rot rating for Diplodia, Gibberella and Fusarium on a 1-10 scale where 1 = 10% of the ear rotted... 10 = 100% of the ear rotted. Aspergillus ear rot rated on the same scale but only in the inoculated area. Rating values based on the average of three replicates with 12-18 plants per replicate.

^b The T14 and T25 hybrids have genetic backgrounds different from one another.

^c NS = not significantly different at P = 0.05 or better.

Table 2. Kernel Plating^a Evaluations of Ear Rot Inoculations and Controls - 1994

Location and Ear Rot Inoculation	Transformed or Nontransformed	% Fus mon	% Diplodia	% Gib	% Asp flav	% Clean ^c
Illinois (T14^b)						
Gibberella	Transformed	2.00	6.00	44.66	3.34	47.34
	Nontransformed	2.66	0.00	66.66	2.00	29.34
	Significance	NS ^d	NS	NS	NS	NS
Fusarium	Transformed	54.66	8.00	1.34	7.34	34.00
	Nontransformed	57.34	6.66	0.00	6.00	32.66
	Significance	NS	NS	NS	NS	NS
Aspergillus	Transformed	11.34	0.00	2.00	54.00	36.00
	Nontransformed	6.66	0.00	4.00	46.66	48.00
	Significance	NS	NS	NS	NS	NS
Control (noninoculated)	Transformed	3.34	0.00	0.66	10.66	85.34
	Nontransformed	4.66	0.00	0.66	29.34	66.66
	Significance	NS	NS	NS	NS	NS
Indiana (T14^b) Experiment 1						
Gibberella	Transformed	6.00	0.00	49.34	4.00	45.34
	Nontransformed	5.34	1.34	74.66	1.34	21.34
	Significance	NS	NS	@0.05	NS	@0.05
Fusarium	Transformed	75.34	0.00	0.00	3.34	22.00
	Nontransformed	58.66	3.34	0.66	2.00	37.34
	Significance	@0.02	NS	NS	NS	@0.03
Aspergillus	Transformed	13.34	0.00	2.66	30.66	55.34
	Nontransformed	16.66	0.66	3.34	39.34	44.66
	Significance	NS	NS	NS	NS	NS
Control	Transformed	2.00	0.00	1.34	2.66	94.00
	Nontransformed	12.00	0.00	0.00	3.34	85.34
	Significance	NS	NS	NS	NS	NS

Location and Ear Rot Inoculation	Transformed or Nontransformed	% Fus mon	% Diplodia	% Gib	% Asp flav	% Clean ^c
Indiana (T25^b) Experiment 2						
Gibberella	Transformed	3.34	0.00	30.66	0.66	65.34
	Nontransformed	4.66	0.00	64.66	2.00	30.66
	Significance	NS	NS	NS	NS	@0.03
Fusarium	Transformed	31.34	2.66	0.66	3.34	63.34
	Nontransformed	31.34	1.34	0.00	12.00	58.00
	Significance	NS	NS	NS	@0.04	NS
Aspergillus	Transformed	22.66	0.00	0.00	24.66	54.66
	Nontransformed	11.34	0.66	2.66	41.34	46.66
	Significance	NS	NS	NS	NS	NS
Control	Transformed	2.66	0.00	0.00	22.66	74.66
	Nontransformed	6.66	0.00	0.00	22.66	72.00
	Significance	NS	NS	NS	NS	NS
Indiana (T25^b) Experiment 3						
Gibberella	Transformed	2.00	0.66	48.66	0.00	49.34
	Nontransformed	0.66	0.00	45.34	0.00	54.66
	Significance	NS	NS	NS	NS	NS
Fusarium	Transformed	44.66	0.00	0.00	4.00	54.00
	Nontransformed	60.00	0.00	0.66	3.34	38.00
	Significance	@0.02	NS	NS	NS	@0.02
Aspergillus	Transformed	10.66	0.00	0.00	32.00	60.00
	Nontransformed	12.66	0.00	0.00	44.00	45.34
	Significance	NS	NS	NS	NS	NS
Control	Transformed	0.66	0.00	0.00	16.66	82.66
	Nontransformed	6.66	0.00	0.00	23.34	70.66
	Significance	NS	NS	NS	NS	NS

^a Kernel plating - percent of kernels (total of 50 kernels per replicate) from which Fus mon (*Fusarium moniliforme*), Diplodia (*Stenocarpella maydis*), Gib (*Gibberella zeae*) and Asp flav (*Aspergillus flavus*) were isolated averaged over three replicates.

^b The T14 and T25 hybrids have genetic backgrounds different from one another.

^c Percent of kernels (total of 50 kernels per replicate) from which no fungi were isolated averaged over three replicates. More than one fungus may be isolated from the same kernel, therefore, % Fus mon, % Diplodia, % Gib, % Asp flav, and % Clean may not add up to 100%.

^d NS = not significantly different at P = 0.05. When differences were detected at levels of significance greater than P=0.05 the P value is given.

Table 3. Kernel Planting^a Evaluation of Noninoculated Glufosinate-Ammonium treated transformed plants-1994

Location	Glufosinate-Ammonium Rate ^b	% Fus mon	% Diplodia	% Gib	% Asp flav	% Clean ^c
Illinois T14 ^d	0	3.34	0.00	0.66	10.66	85.34
	400 gms	6.00	0.00	0.00	10.66	83.34
	1500 gms	3.34	0.00	0.00	20.00	77.34
	significance	NS	NS	NS	NS	NS
Indiana T1 ^d	0	2.00	0.00	1.34	2.66	94.00
	400 gms	6.00	0.00	0.00	18.66	76.00
	1500 gms	4.66	0.00	0.00	16.66	80.66
	significance	NS	NS	NS	NS	NS

^a Kernel plating - percent of kernels (total of 50 kernels per replicate) from which Fus mon (*Fusarium moniliforme*), Diplodia (*Stenocarpella maydis*), Gib (*Gibberella zeae*) and Asp flav (*Aspergillus flavus*) were isolated averaged over three replicates.

^d The T14 hybrids have different genetic backgrounds in Illinois and Indiana

^c Percent of kernels (total of 50 kernels per replicate) from which no fungi were isolated averaged over three replicates. More than one fungus may be isolated from the same kernel, therefore, % Fus mon, % Diplodia, % Gib, % Asp flav, and % Clean may not add up to 100%.

^b Glufosinate-ammonium treatments of 0, 400, and 1500 gm/ha herbicide were applied at growth stage V5.

^e NS=not significantly different at P=0.05 or better

Appendix 3. USDA Field Trial Termination Reports

TERMINATION REPORT**Approved Permit Number:** 92-017-04**Name:** [REDACTED]**Institutional Address:**

Hoechst Roussel Agri-Vet Company

Route 202-206

Somerville, NJ 08876

Telephone Number: [REDACTED]**Facsimile Number:** [REDACTED]**Date Of This Report:** 6 October 1993

There were no changes in the test organisms from those identified in the original application for field testing.

There were no changes in the source(s) of donor DNA from those identified in the original application for field testing.

There were no changes in the vector(s) used from those identified in the original application for field testing.

There were no changes in other genetic sequences used in the test organism expression vector and transformation systems from those identified in the original application for field testing.

There were no changes in the location of the field test then that identified in the original application for field test approval.

SUMMARY OF EXPERIMENTAL RESULTS:

The plants appeared normal in all aspects, and when sprayed with the herbicide glufosinate (Ignite(TM)), exhibited tolerance to the herbicide.

There were no changes in the field test from those identified in the original application for test approval.

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There were no changes in the identity of the nonmodified parental test organism from that identified in the original application for field testing.

The modified organism did not exhibit any reproductive traits which were different from the unmodified parent.

There was no indication that the inserted sequence was capable of surviving independent of the transgenic host.

Evidence that the inserted sequence combined with DNA or RNA of other indigenous organisms:

Observation—A 660 ft. border was maintained between the transgenic and non-transgenic plants not in the experiment. A taller hybrid was planted around the experiment to act as a "pollen trap".

There were no changes in the source and/or function of the DNA sequence from those identified in the original application for field testing.

MOLECULAR BIOLOGY

There were no changes in the methods used for DNA insertion from those identified in the pretest request for approval.

There was no indication that the vector was capable of surviving independent of the transgenic host.

There was no indication the vector altered the disease status of the test organism.

Pretest predictions regarding the stability of inserted DNA remaining in the modified organism were confirmed in the experiment.

RESULTS OF OBSERVATIONS AND MONITORING DURING THE FIELD TEST

There were no unanticipated morphological difference between the transformed organisms and the modified parent.

Observations of the modified plants did not reveal any characteristics associated with weediness.

Observations of flowering characteristics did not disclose any deviations from the parental outcrossing potential.

Observations did not disclose characteristics of the modified organisms which would increase the long-term survival of any progeny that might have escaped the test area.

There was no evidence that the inserted gene was transmitted to any other species.

There were no indications of potential adverse human health effects or impacts on the health of people living in the area of the test.

HANDLING AND SHIPPING SAFEGUARDS

No changes were made in the safeguards identified in the request for approval to conduct the field test.

None of the safeguards were breached.

SITE CONSIDERATIONS

Observations were made which revealed that no commercial varieties were being grown within pollinating range during the conduct of the test.

EXPERIMENTAL DESIGN

As in application for approval to conduct the field test.

PHYSICAL CONFINEMENT

There were no problems with birds, livestock, rodents, or other wildlife invading the test area.

BIOLOGICAL/ENVIRONMENTAL CONSIDERATIONS

Iowa, Illinois, Indiana, and Nebraska sites: Some winter kill is expected, but is not always complete. The test sites will be monitored for surviving volunteers, and any volunteers will be destroyed mechanically or with standard herbicides.

Containment was achieved by planting a taller hybrid as a border around the test site and preventing the survival of volunteers.

The traits transferred to the genetically modified organisms did not result in any adverse environmental consequences.

SCALE OF THE EXPERIMENT

As in application for approval to conduct the field test.

SECURITY

The security measures identified in the application for test approval were successful in preventing potentially serious biosafety breaches.

BIOLOGICAL MONITORING

The plants were treated with the herbicide glufosinate (Ignite(TM)), and the ability of plants to survive indicated that the transgene was expressed. Standard methods for destroying volunteer corn (mechanical, herbicides) insure that volunteers do not persist. Volunteers will be controlled if observed.

EMERGENCY RESPONSE

No emergency occurred which might have adversely affected health or the environment.

MAINTENANCE

As in application for approval to conduct the field test.

TRAINING OF PERSONNEL

The training and supervisory procedures outlined in the request for approval to conduct the field test were adequate to assure health and environmental safety

TERMINATION OF EXPERIMENT

The combination of a 660 ft. buffer and the planting of a taller hybrid to act as a "pollen trap", and destruction of surviving progeny at the test sites, appears to be completely effective at preventing escape of transgenic material.

PUBLIC REACTIONS

There were no public reactions to the test, either positive or negative.

TERMINATION REPORT

Approved Permit Number: 92-043-01

Name: [REDACTED]

Institutional Address:

**Hoechst Roussel Agri-Vet Company
Route 202-206
Somerville, NJ 08876**

Telephone Number: [REDACTED]

Facsimile Number: [REDACTED]

Date Of This Report: 6 October 1993

There were no changes in the test organisms from those identified in the original application for field testing.

There were no changes in the source(s) of donor DNA from those identified in the original application for field testing.

There were no changes in the vector(s) used from those identified in the original application for field testing.

There were no changes in other genetic sequences used in the test organism expression vector and transformation systems from those identified in the original application for field testing.

There were no changes in the location of the field test then that identified in the original application for field test approval.

SUMMARY OF EXPERIMENTAL RESULTS:

The plants appeared normal in all aspects, and when sprayed with the herbicide glufosinate (Ignite(TM), exhibited tolerance to the herbicide.

There were no changes in the field test from those identified in the original application for test approval.

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There were no changes in the identity of the nonmodified parental test organism from that identified in the original application for field testing.

The modified organism did not exhibit any reproductive traits which were different from the unmodified parent.

There was no indication that the inserted sequence was capable of surviving independent of the transgenic host.

Evidence that the inserted sequence combined with DNA or RNA of other indigenous organisms:

Observation—A 660 ft. border was maintained between the transgenic and non-transgenic plants not in the experiment. A taller hybrid was planted around the experiment to act as a "pollen trap".

There were no changes in the source and/or function of the DNA sequence from those identified in the original application for field testing.

MOLECULAR BIOLOGY

There were no changes in the methods used for DNA insertion from those identified in the pretest request for approval.

There was no indication that the vector was capable of surviving independent of the transgenic host.

There was no indication the vector altered the disease status of the test organism.

Pretest predictions regarding the stability of inserted DNA remaining in the modified organism were confirmed in the experiment.

RESULTS OF OBSERVATIONS AND MONITORING DURING THE FIELD TEST

There were no unanticipated morphological difference between the transformed organisms and the modified parent.

Observations of the modified plants did not reveal any characteristics associated with weediness.

Observations of flowering characteristics did not disclose any deviations from the parental outcrossing potential.

Observations did not disclose characteristics of the modified organisms which would increase the long-term survival of any progeny that might have escaped the test area.

There was no evidence that the inserted gene was transmitted to any other species.

There were no indications of potential adverse human health effects or impacts on the health of people living in the area of the test.

HANDLING AND SHIPPING SAFEGUARDS

No changes were made in the safeguards identified in the request for approval to conduct the field test.

None of the safeguards were breached.

SITE CONSIDERATIONS

Observations were made which revealed that no commercial varieties were being grown within pollinating range during the conduct of the test.

EXPERIMENTAL DESIGN

As in application for approval to conduct the field test.

PHYSICAL CONFINEMENT

There were no problems with birds, livestock, rodents, or other wildlife invading the test area.

BIOLOGICAL/ENVIRONMENTAL CONSIDERATIONS

Minnesota: Some winter kill is expected, but is not always complete. The test sites will be monitored for surviving volunteers, and any volunteers will be destroyed mechanically or with standard herbicides.

Containment was achieved by planting a taller hybrid as a border around the test site and preventing the survival of volunteers.

The traits transferred to the genetically modified organisms did not result in any adverse environmental consequences.

SCALE OF THE EXPERIMENT

As in application for approval to conduct the field test.

SECURITY

The security measures identified in the application for test approval were successful in preventing potentially serious biosafety breaches.

BIOLOGICAL MONITORING

The plants were treated with the herbicide glufosinate (Ignite(TM)), and the ability of plants to survive indicated that the transgene was expressed. Standard methods for destroying volunteer corn (mechanical, herbicides) insure that volunteers do not persist. Volunteers will be controlled if observed.

EMERGENCY RESPONSE

No emergency occurred which might have adversely affected health or the environment.

MAINTENANCE

As in application for approval to conduct the field test.

TRAINING OF PERSONNEL

The training and supervisory procedures outlined in the request for approval to conduct the field test were adequate to assure health and environmental safety

TERMINATION OF EXPERIMENT

The combination of a 660 ft. buffer and the planting of a taller hybrid to act as a "pollen trap", and destruction of surviving progeny at the test sites, appears to be completely effective at preventing escape of transgenic material.

PUBLIC REACTIONS

There were no public reactions to the test, either positive or negative.

Hoechst-Roussel Agri-Vet Company

Route 202-206 North • Somerville, New Jersey 08876
Telex 833-449 • Cable Hoechstus, Somerville, N.J.
Telephone (908) 231-2000

Hoechst 
Roussel 

Mississippi Research Farm
Route 1, Box 397
Leland, MS 38756
(601) 686-2327
(601) 686-4906 (Fax)

November 30, 1993


USDA / APHIS / BBEP
Federal Center Building
Hyattsville, MD 20782

Dear 

By way of this letter, I would like to provide you with the information requested in your letter of November 2, 1993 regarding the monitoring of field sites under permit numbers 92-017-04 and 92-043-01.

There were seven field sites in 1992 under the two permits. I have included the information for each site individually as an attachment to this letter. Please contact me if you have questions or desire additional information. I appreciate the cooperation and assistance you have provided Hoechst Roussel as we continue this important project.

Sincerely,

MONITORING REPORT FOR PERMIT NUMBERS 92-017-04 / 92-043-01

Date of Report: November 30, 1993

Author: [REDACTED] **Hoechst Roussel Agri-Vet Company**

LOCATION: Franklin, IN

Spring 1993: No volunteer corn was observed.

May 1993: The field site was cultivated. Soybeans were planted.

June 1993: Post emergence soybeans herbicides were applied. These herbicides would have controlled any volunteers, but none were noted.

LOCATION: Kenyon, MN

September 1992: Field trial destroyed before seed became viable.

Fall 1992: Field site was plowed.

May 1993: Spring tillage.

July 1993: Field site inspected for volunteers, none noted.

LOCATION: Hollandale, MN

September 1992: Corn was killed by hard frost, no viable seed formed.

May 1993: Spring tillage.

June 1993: Field site inspected for volunteers, none noted.

July 1993: Field site inspected for volunteers, none noted.

LOCATION: Geneseo, IL

May 1993: Spring tillage.

May 1993: Field site inspected, no volunteers noted.

This site was planted to soybeans later in the spring, and soybean herbicides were applied which would have controlled any volunteers.

LOCATION: Muscatine, IA

May 1993: Field site inspected, no volunteers noted. Spring tillage.

The field site was kept fallow, with occasional tillage.

LOCATION: Leshara, NE

May 1993 : Spring tillage.

June 1993: Field site inspected, no volunteers noted.

The field site was kept fallow, with occasional tillage.

LOCATION: Williamsburg, IA

April 1993: Field site mowed and tilled.

June 1993: Field site inspected, no volunteers noted. Site was tilled.

August 1993: Field site inspected, no volunteers noted.

**SUMMARY REPORT OF THE FIELD RELEASE OF TRANSGENIC CORN
EXPRESSING TOLERANCE TO THE HERBICIDE GLUFOSINATE**

DATE OF REPORT: October 14, 1994

PERMIT NUMBER(S): 93-021-09 (notification # 93-120-17)
93-021-10
93-021-11
93-040-01 (notification # 93-120-27)

APPLICANT: [REDACTED]
Hoechst Roussel Agri-Vet Company
Route One, Box 397
Leland, MS 38756

([REDACTED] the applicant for permit 93-040-01, is no longer with this company.
Please refer any questions for this permit to [REDACTED].)

DATES OF RELEASE: May through August 1993

DATES OF TERMINATION: July through November 1993

SITES OF RELEASE: (States / Number per State): California/1, Iowa/4, Illinois/4,
Indiana/2, Minnesota/3, Mississippi/1, Missouri/1, North Dakota/1, Nebraska/1, Ohio/1,
South Dakota/1, Virginia/1.

PURPOSE OF RELEASE

To evaluate weed control with glufosinate herbicide when applied to corn plants containing the PAT gene which confers tolerance to glufosinate herbicide. The corn lines utilized in this release were inbred lines from Holden's Foundation Seeds, Williamsburg, IA crossed with the primary transformant line, T14.

RESULTS

Glufosinate herbicide provided control of economically important weeds in corn with no injury to the transgenic corn plants.

OBSERVATIONS

(The frequency of observations differed with each location. Each location was visited an average of five times during the duration of the release. The area planted to the transgenic corn ranged from .10 to 1.50 acres per site. The transgenic corn population was an average of 70 plants per plot or 20,328 plants per acre.)

Herbicide Tolerance: The transgenic corn plants exhibited tolerance to glufosinate herbicide. The transgenic corn plants were also tolerant to other commercially used corn herbicides that were used in the trials as standards. The nontransgenic corn was severely injured by treatment with glufosinate.

Insect Susceptibility: The primary insect pests of corn are corn rootworm, black cutworm, and European corn borer. One trial was treated with insecticides to control European corn borer. This trial was planted later than surrounding plantings and was therefore more attractive to the corn borer. Damaging levels of insect pests were not observed at other locations on either transgenic or nontransgenic corn.

Disease Susceptibility: Diseases in corn production in the Midwest USA are rare. Casual observations throughout the growing season did not note any disease infestations on either transgenic or nontransgenic corn.

Weather Related Conditions: The majority of the sites were located in the midwestern United States which was subjected to excessive amounts of rainfall with associated flooding. Late-season nitrogen deficiency symptoms were observed at sites where the plants were subjected to temporary standing water or water logged soils. The magnitude of the symptoms were similar between the transgenic and non-transgenic plants.

Physical Characteristics: The corn plants were observed from emergence through maturity. No differences were observed between transgenic and nontransgenic corn in emergence, seedling vigor, and stand establishment. Prior to glufosinate application, no morphological differences were observed between the transgenic and non-transgenic plants. After glufosinate application, the transgenic plants continued to grow normally. The nontransgenic corn was severely injured by glufosinate.

Weediness Characteristics: Growth rate and growth habit were identical in both transgenic and nontransgenic plants. Weediness characteristics such as excessive vegetative growth or seed shattering were not present.

MEANS OF PLANT DISPOSITION

The destruction of the plants differed by site and consisted of mechanical mowing, disking, and/or plowing. Ears were hand picked in some sites. Dropped ears were gleaned by hand, and destroyed by incineration or deep burying in the soil.

TIME/METHODS OF MONITORING FOR VOLUNTEERS

Sites were visited one or more times the following spring when soil temperatures reached a level at which corn emergence may be expected. The sites were visually inspected for volunteer corn plants.

NUMBER OF VOLUNTEERS OBSERVED/ACTION TAKEN

The number of volunteers ranged from none, to numbers which would be expected in commercial corn production. The number of volunteers can be influenced by tillage type and fall/winter weather. At some locations, wet weather in the fall did not allow for complete plowing of the corn residue. This may have contributed to the volunteers at these locations. It is important to note that the population makeup of the volunteers may have contained an equal number of nontransgenic and transgenic plants. This can be attributed to the fact that the nontransgenic border rows were allowed to reach maturity and the seed were incorporated into the soil. All volunteer corn plants were

destroyed by mechanical means, removed by hand, or destroyed with herbicides other than glufosinate.

**SUMMARY REPORT TO THE FIELD RELEASE OF TRANSGENIC CORN
EXPRESSING TOLERANCE TO THE HERBICIDE GLUFOSINATE**

DATE OF REPORT: November 18, 1994

NOTIFICATION NUMBER: 94-074-03N

APPLICANT:

██████████
AgrEvo USA Company
2711 Centerville Road
Wilmington, DE 19808

DATES OF RELEASE: April through August 1994

DATES OF TERMINATION: July through November 1994

SITES OF RELEASE: (States/Number per State): Florida/1, Iowa/8, Illinois/6, Indiana/2, Kentucky/2, Michigan/1, Minnesota/3, Missouri/2, New York/1, North Carolina/3, North Dakota/3, Nebraska/4, Ohio/4, Pennsylvania/1, South Dakota/1, Tennessee/1, Texas/1, Washington/1, Wisconsin/1.

PURPOSE OF RELEASE

To evaluate weed control with glufosinate herbicide when applied to corn plants containing the PAT gene which confers tolerance to glufosinate herbicide. The hybrids were derived from backcrossing with transformation events T14 and T25.

RESULTS

Glufosinate herbicide provided control of economically important weeds in corn with no injury to the transgenic corn plants.

OBSERVATIONS

The frequency of observations differed with each location. Each location was visited an average of three times (range of one to eight) during the duration of the release. The area planted to transgenic corn ranged from 0.3 to 2.0 acres per site. The transgenic corn population was an average of 20,328 plants per acre.

Herbicide Tolerance: The transgenic corn plants exhibited tolerance to glufosinate herbicide. The transgenic corn plants were also tolerant to other commercially used corn herbicides that were used in the trials as standards. The nontransgenic corn was severely injured by treatment with glufosinate.

Herbicide Susceptibility: Trials were conducted to demonstrate that there was no cross tolerance between glufosinate resistant plants and other herbicides. The transgenic corn plants were sensitive to glyphosate, fenoxaprop and imazethapyr (herbicides not registered for use on corn). Death of the plants resulted when these herbicides were applied to transgenic corn.

Insect Susceptibility: The primary insect pests of corn are corn rootworm, black cutworm, and European corn borer. Pest infestations of corn rootworm, European corn borer, black cutworms, armyworms, corn earworms, fleabeetles and aphids were observed at release sites. There were no differences between transgenic or non transgenic corn. Lady beetles were a common beneficial insect observed at test sites, but there were no differences in population levels observed on transgenic or nontransgenic corn.

Disease Susceptibility: Diseases in corn production in the Midwest USA are rare. Bacterial infestations were observed at two sites, smut at one site, and Stewart's wilt at one site. There were no differences between transgenic and nontransgenic corn.

Weather Related Conditions: The weather conditions were ideal for corn production. The exception was the Ohio site when below average rainfall occurred early in the year.

Physical Characteristics: The corn plants were observed from emergence through maturity. No differences were observed between transgenic and nontransgenic corn in emergence, seedling vigor, and stand establishment, and other casual observations. Prior to glufosinate application no morphological differences were observed between the transgenic and non-transgenic plants. In an Iowa trial where the T14 and T25 transformations were compared, no significant differences were observed. After glufosinate application, the transgenic plants continued to grow normally. The nontransgenic corn was severely injured by glufosinate.

Weediness Characteristics: Growth rate and growth habit were identical in both transgenic and nontransgenic pants. Weediness characteristics such as excessive vegetative growth or seed shattering were not present.

MEANS OF PLANT DISPOSITION

The destruction of the plants differed by site and consisted of mechanical mowing, disking, land fill, and/or plowing. Ears were hand picked in some sites. Dropped ears were gleaned by hand, and destroyed by incineration or deep burying in the soil.

TIME/METHODS OF MONITORING FOR VOLUNTEERS

Sites will be visited one or more times in the spring of 1995 when soil temperatures reach a level at which corn emergence will be expected. The sites will be visually inspected for volunteer corn plants.

NUMBER OF VOLUNTEERS OBSERVED/ACTION TAKEN

The number of volunteer corn plants will be observed and recorded in 1995. All volunteer corn plants will be destroyed by mechanical means, removed by hand, or destroyed with herbicides other than glufosinate.

Appendix 4. Literature Reprints