AgrEvo

94-357-01p

A company of Hoechst and NOR-AM

December 28, 1994

Ms. Dianne Hatmaker
Chief, Biotechnology Program Operations
USDA, APHIS, BBEP, BP
6505 Belcrest Road
Federal Building, Suite 7
Hyattsville, MD 20782

Re: **Petition for Determination of Nonregulated Status for Glufosinate Resistant Corn Transformation Events T14 and T25**

Dear Ms Hatmaker:

AgrEvo USA Company is providing two sets of eight correction pages for their Petition for Determination of Nonregulated Status for Glufosinate Resistant Corn Transformation Events **T14 and T25** submitted December 23, 1994. These replacement pages correct errors in reference to Figures or Tables which were incorrectly referred to as Figure or Table VI instead of IV. Please exchange these pages with the ones found in the December 23 submission, and return the ones from the December 23 submission to me.

Please contact me at (302) 892-3155 if you have any questions concerning our petition, or this request.

Best Regards,

Sally Van Wert, Ph.D.
Manager, Regulatory Affairs - Biotechnology

Enclosures (2)

AgrEvo USA Company
Little Falls Centre One, 2711 Centerville Road, Wilmington, DE 19808, Telephone: (302) 892-3000, Fax: (302) 892-3013

January 23, 1995

Ms. Diane Hatmaker
Chief, Biotechnology Program Operations
USDA, APHIS, BBEP, BP
6505 Belcrest Road
Federal Building, Suite 7
Hyattsville, MD 20782

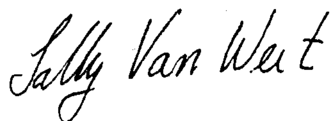
Re: **Petition for Determination of Nonregulated Status for Glufosinate Resistant Corn Transformation Events T14 and T25**

Dear Ms. Hatmaker:

AgrEvo USA Company is providing two original copies of our Petition for Determination of Nonregulated Status for Glufosinate Resistant Corn Transformation Events **T14 and T25**, 94-357-01P, originally submitted December 23, 1994. These copies contain the corrections requested by the reviewers. It is my understanding that the submission date will remain unchanged and that the petition will be accepted as is with the original cover letter.

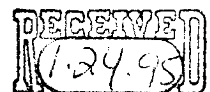
Please contact me at (302) 892-3155 if you have any questions concerning our petition.

Best Regards,



Sally Van Wert, Ph.D.
Manager, Regulatory Affairs - Biotechnology

Enclosures (2)



#1 KP

94-357-01P

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:

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Manager, Regulatory Affairs - Biotechnology

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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.

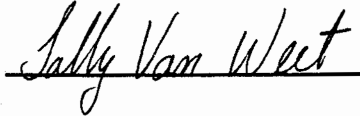
Events T14 and T25 have been field tested by AgrEvo USA Company, formerly Hoechst-Roussel Agri-Vet Company, since 1992 in the primary corn growing regions of the United States. These tests have occurred at approximately 78 sites under field release authorizations granted by APHIS (USDA authorizations: 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.



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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 luxarians: 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 *35*S 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 *Sal*1 site, between the CaMV derived *35*S gene promoter and terminator sequences, of the pUC derived plasmid pDH51 (Pietrzak et al., 1986). The chimeric *pat* gene cassette (*35*S promoter::*pat*::*35*S 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. <u>CaMV *35S* promoter and terminator</u> 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. <u>*pat*</u> 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 *E.coli* plasmid pUC18 used for cloning of DNA sequences. (Yanisch-Perron et al., 1985)
ampR	3783-2923	0.86	Ampicillin resistance gene of pUC18 expresses a ß-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)
pat	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-alany-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 at el. 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* (*b*ialaphos-*r*esistance) 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

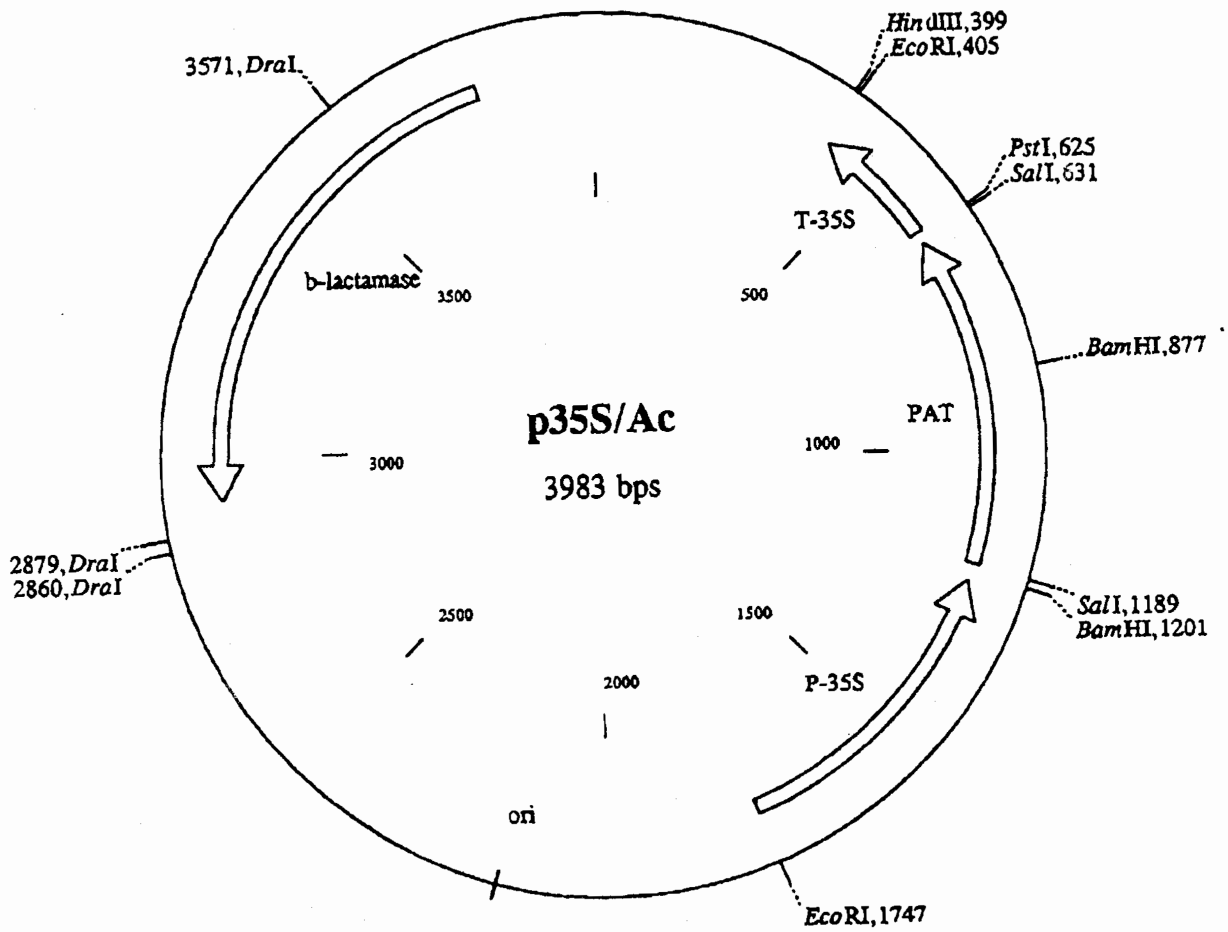

Figure III.2. Comparison of the synthetic *pat* nucleotide sequence (capital letters) with that of the native sequence (small letters)

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              .         .         .         .         .
   1 ATGTCTCCGGAGAGGAGACCAGTTGAGATTAGGCCAGCTACAGCAGCTGA  50
      ||   || ||  |  | || || |||||  | || || || || || ||
   1 gtgagcccagaacgacgcccggtcgagatccgtcccgccaccgccgccga  50
              .         .         .         .         .
  51 TATGGCCGCGGTTTGTGATATCGTTAACCATTACATTGAGACGTCTACAG 100
      ||||| |||||| || || |||||| || || ||||| ||||||   || |
  51 catggcggcggtctgcgacatcgtcaatcactacatcgagacgagcacgg 100
              .         .         .         .         .
 101 TGAACTTTAGGACAGAGCCACAAACACCACAAGAGTGGATTGATGATCTA 150
     | |||||  | || ||||| || || || || |||||||| || || || 
 101 tcaacttccgtacggagccgcagactccgcaggagtggatcgacgacctg 150
              .         .         .         .         .
 151 GAGAGGTTGCAAGATAGATACCCTTGGTTGGTTGCTGAGGTTGAGGGTGT 200
     ||| |  | || ||  | ||||| ||| | || || ||||| ||||| ||
 151 gagcgcctccaggaccgctacccctggctcgtcgccgaggtggagggcgt 200
              .         .         .         .         .
 201 TGTGGCTGGTATTGCTTACGCTGGGCCCTGGAAGGCTAGGAACGCTTACG 250
      || || || || || ||||| || |||||||||||  | ||||| ||||
 201 cgtcgccggcatcgcctacgccggcccctggaaggcccgcaacgcctacg 250
              .         .         .         .         .
 251 ATTGGACAGTTGAGAGTACTGTTTACGTGTCACATAGGCATCAAAGGTTG 300
     | ||||| || |||   || || ||||| || ||  |||| ||  || | 
 251 actggaccgtcgagtcgacggtgtacgtctcccaccggcaccagcggctc 300
              .         .         .         .         .
 301 GGCCTAGGATCCACATTGTACACACATTTGCTTAAGTCTATGGAGGCGCA 350
     || || || |||||  | ||||| ||  |||| ||||| |||||||| ||
 301 ggactgggctccaccctctacacccacctgctgaagtccatggaggccca 350
              .         .         .         .         .
 351 AGGTTTTAAGTCTGTGGTTGCTGTTATAGGCCTTCCAAACGATCCATCTG 400
      || || |||   ||||| || || || || || ||||| ||    |
 351 gggcttcaagagcgtggtcgccgtcatcggactgcccaacgacccgagcg 400
              .         .         .         .         .
 401 TTAGGTTGCATGAGGCTTTGGGATACACAGCCCGGGGTACATTGCGCGCA 450
     |  |  |||| |||||  | |||||||| || || || ||  |||| |||
 401 tgcgcctgcacgaggcgctcggatacaccgcgcgcgggacgctgcgggca 450
              .         .         .         .         .
 451 GCTGGATACAAGCATGGTGGATGGCATGATGTTGGTTTTTGGCAAAGGGA 500
     || || |||||||| || || ||||| || || || || ||||  | ||
 451 gccggctacaagcacgggggctggcacgacgtggggttctggcagcgcga 500
              .         .         .         .         .
 501 TTTTGAGTTGCCAGCTCCTCCAAGGCCAGTTAGGCCAGTTACCCAGATCT 550
      || ||| |||| || || ||  | || ||   |||| || || |||||||
 501 cttcgagctgccggccccgccccgccccgtccggcccgtcacacagatct 550

 551 GA 552
     ||
 551 ga 552
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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^{2b}
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 p35S\AC 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 p35S\AC. 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 p35S\AC 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. <u>Verification of Insert Integrity</u>

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 p35S\AC; 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 p35S\AC 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.

<u>Transformation event T25</u>. 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 Dra1 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 it's 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 EcoR1 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 Dra1 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 p35S\AC 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 reprobed 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.3. Interpretation of the Southern results for event T25. The sizes (kb) of observed hybridizing fragments are provided for event T25 DNA digested with specific restriction enzymes and probed with the synthetic *pat* gene. See Table IV.2. for expected fragment sizes.

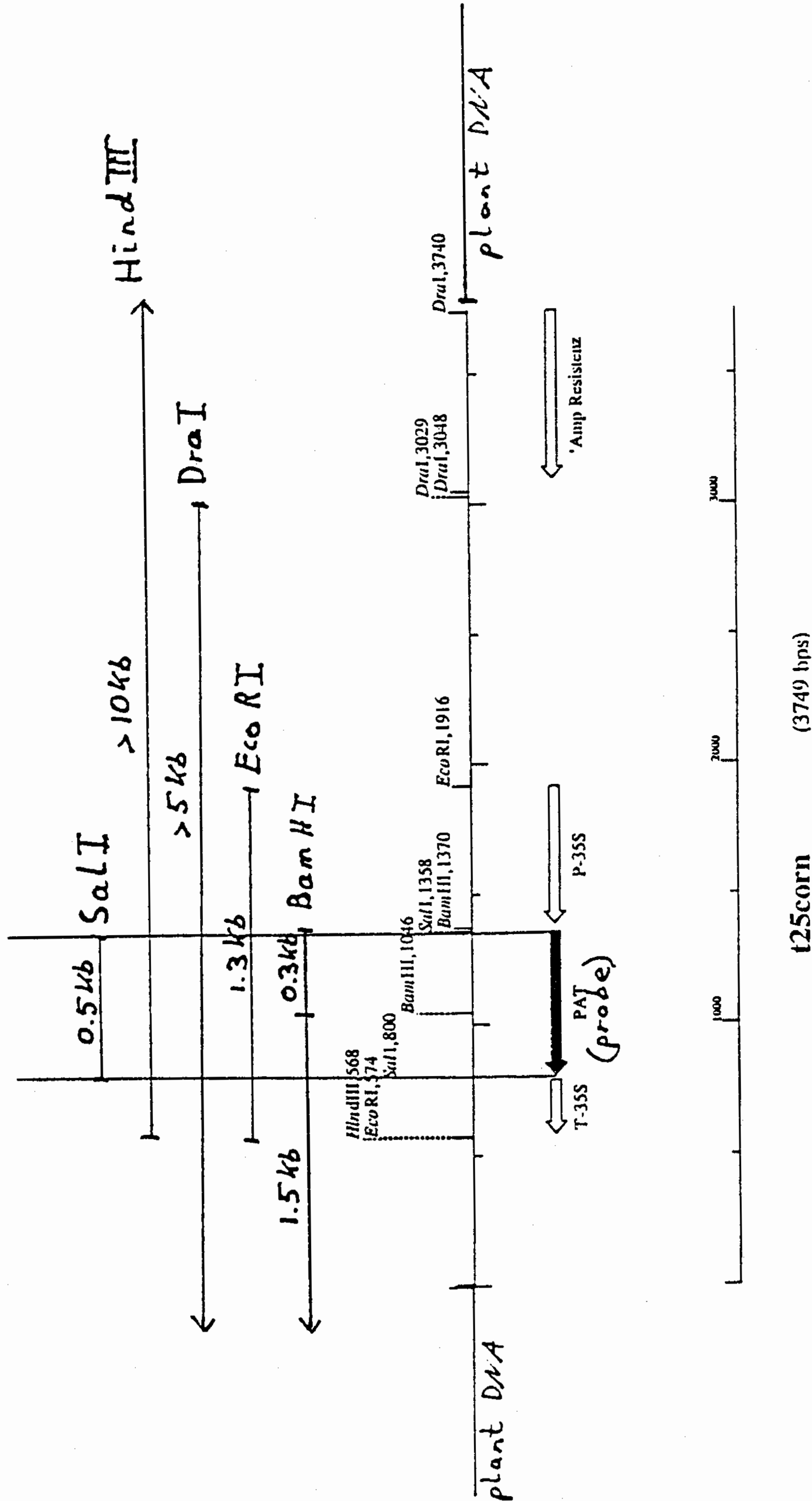


Figure IV.4. Location of primers for PCR analysis of event T25. Locations are indicated with small tailed and tailless arrows.

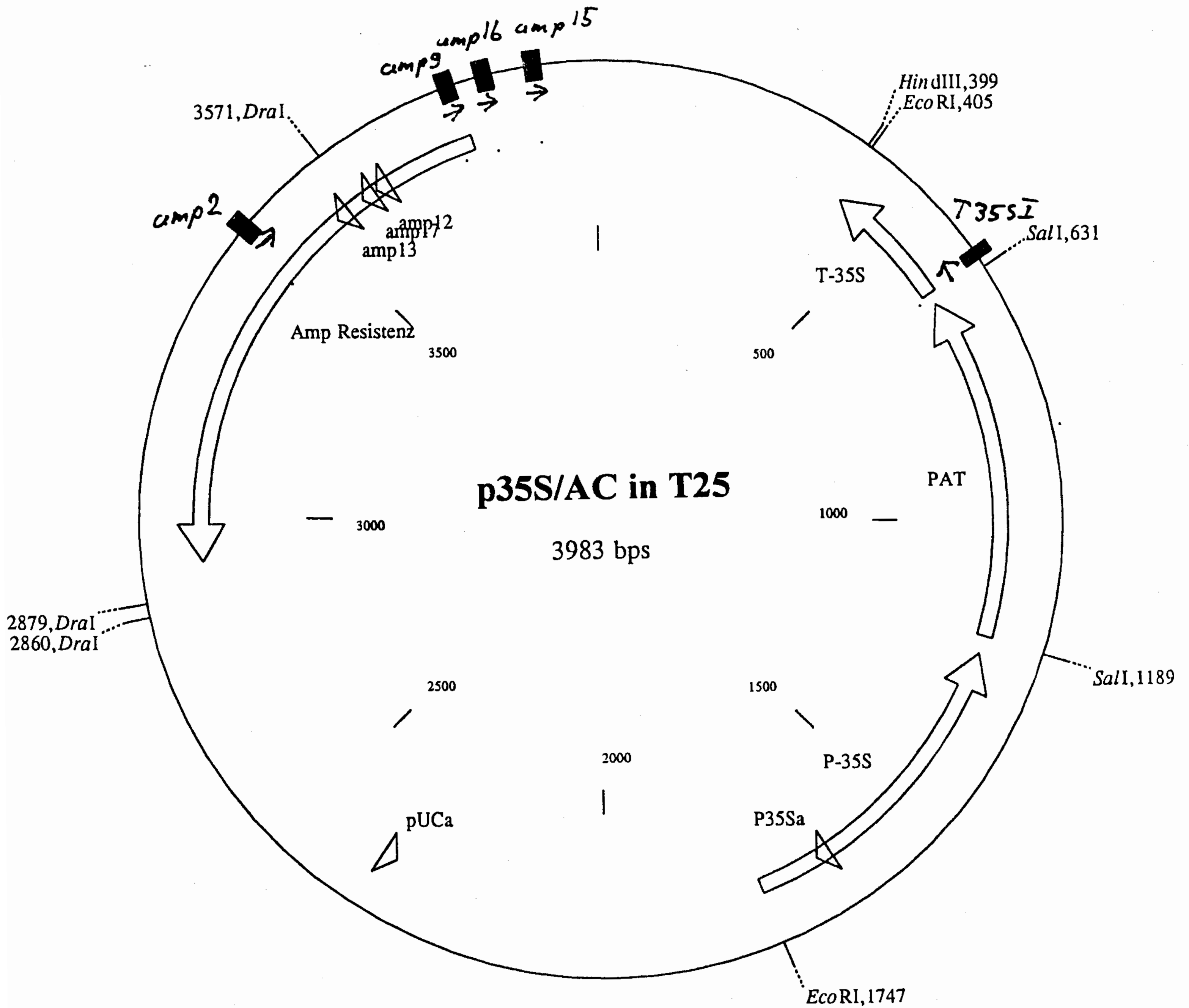


Figure IV.6. Location of primers for PCR analysis of event T14. Locations are indicated with small tailed and tailless arrows.

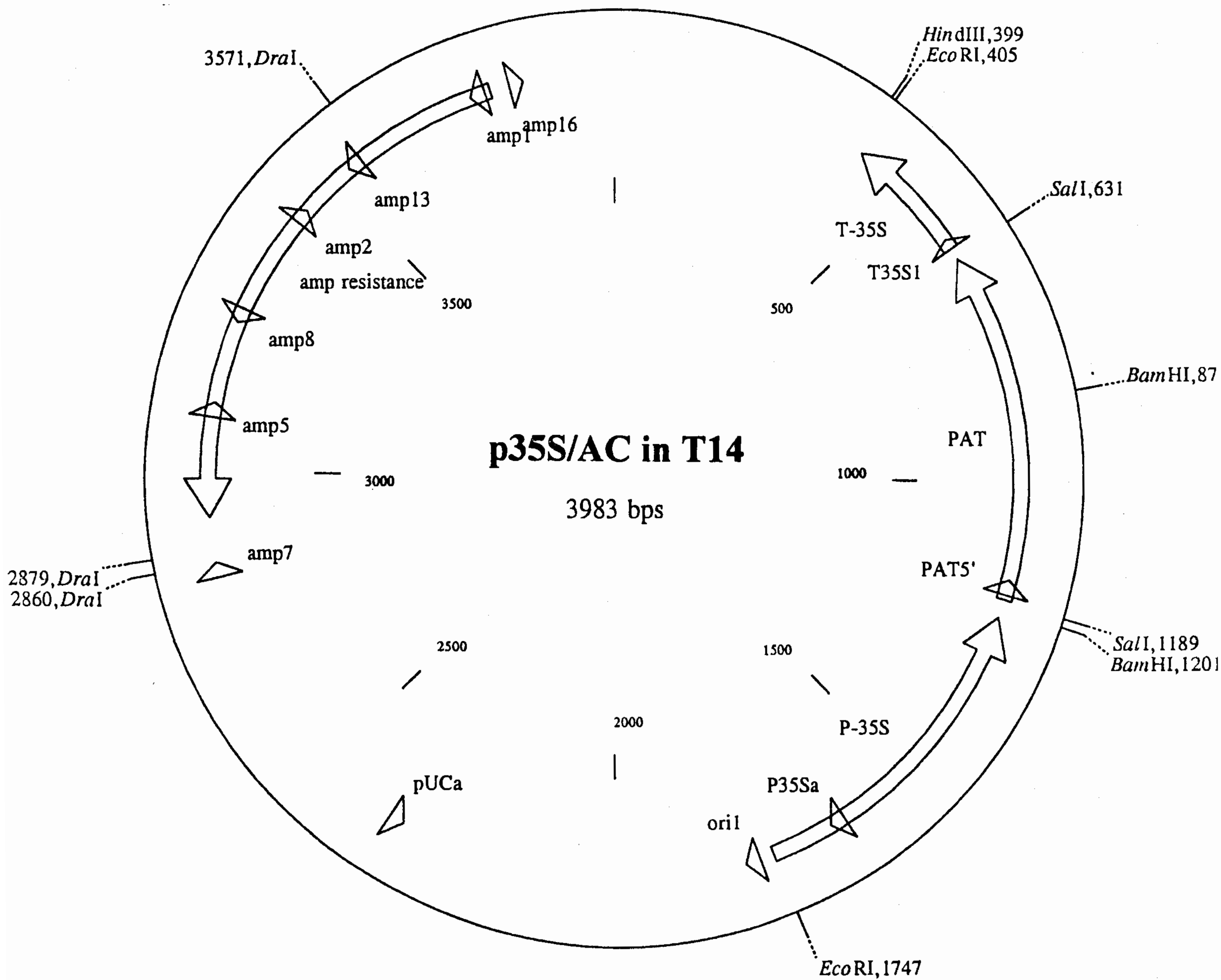


Figure IV.8. Interpretation of the PCR results obtained with event T14 DNA and primer pairs 2, 4, 5, 6, and 7. The three copies of the disrupted *ampR* gene are labeled 1, 2, or 3 copy. See Table IV.3. for description of primer pairs.

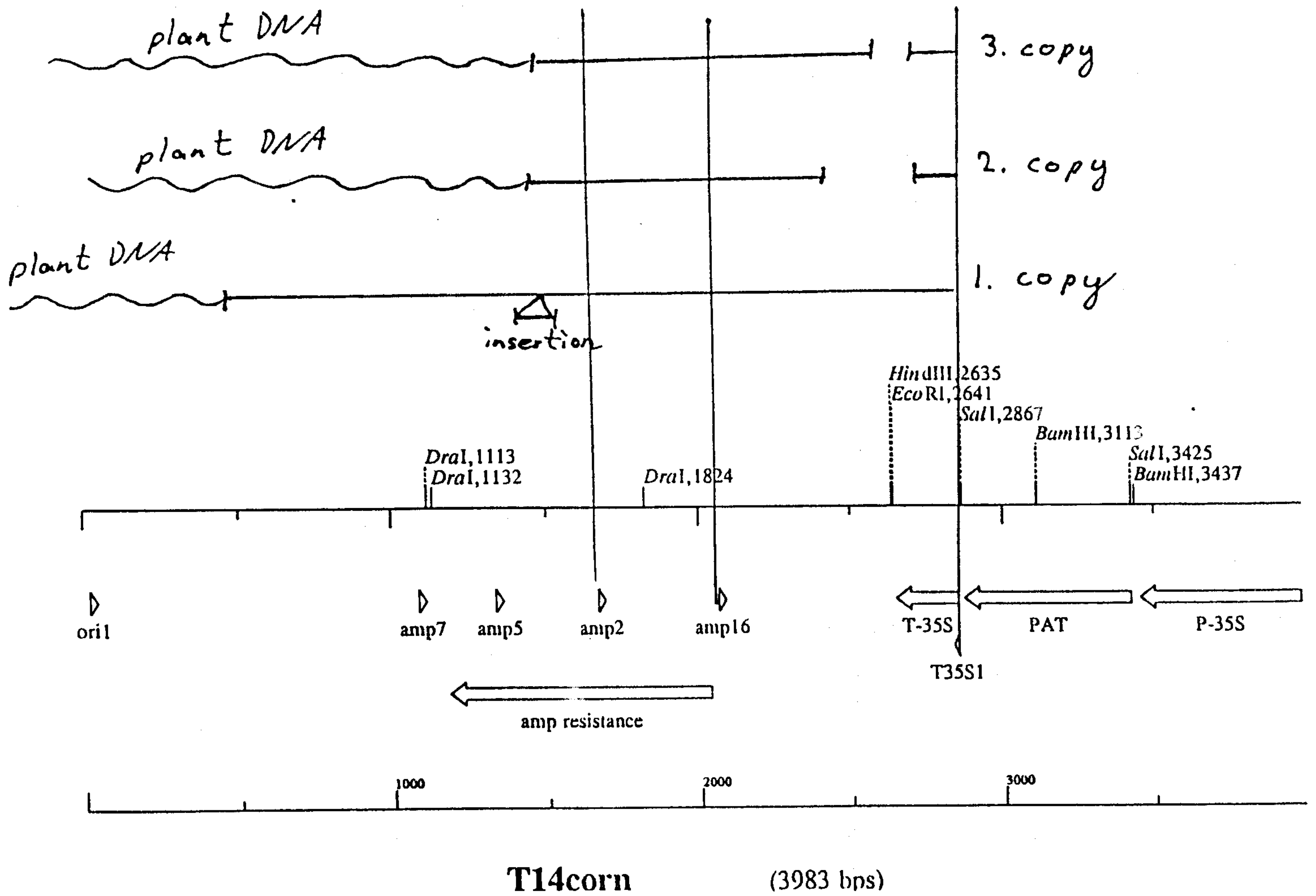


Figure IV.10. HPLC analysis of β-lactamase activity in event T25. Crude protein extracts were prepared from nontransgenic counterpart (left panel) and event T25 (right panel) leaves and incubated with ^{14}C-labeled penicillin for 5 minutes (top row) or 60 minutes (second row). Samples were analyzed 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.

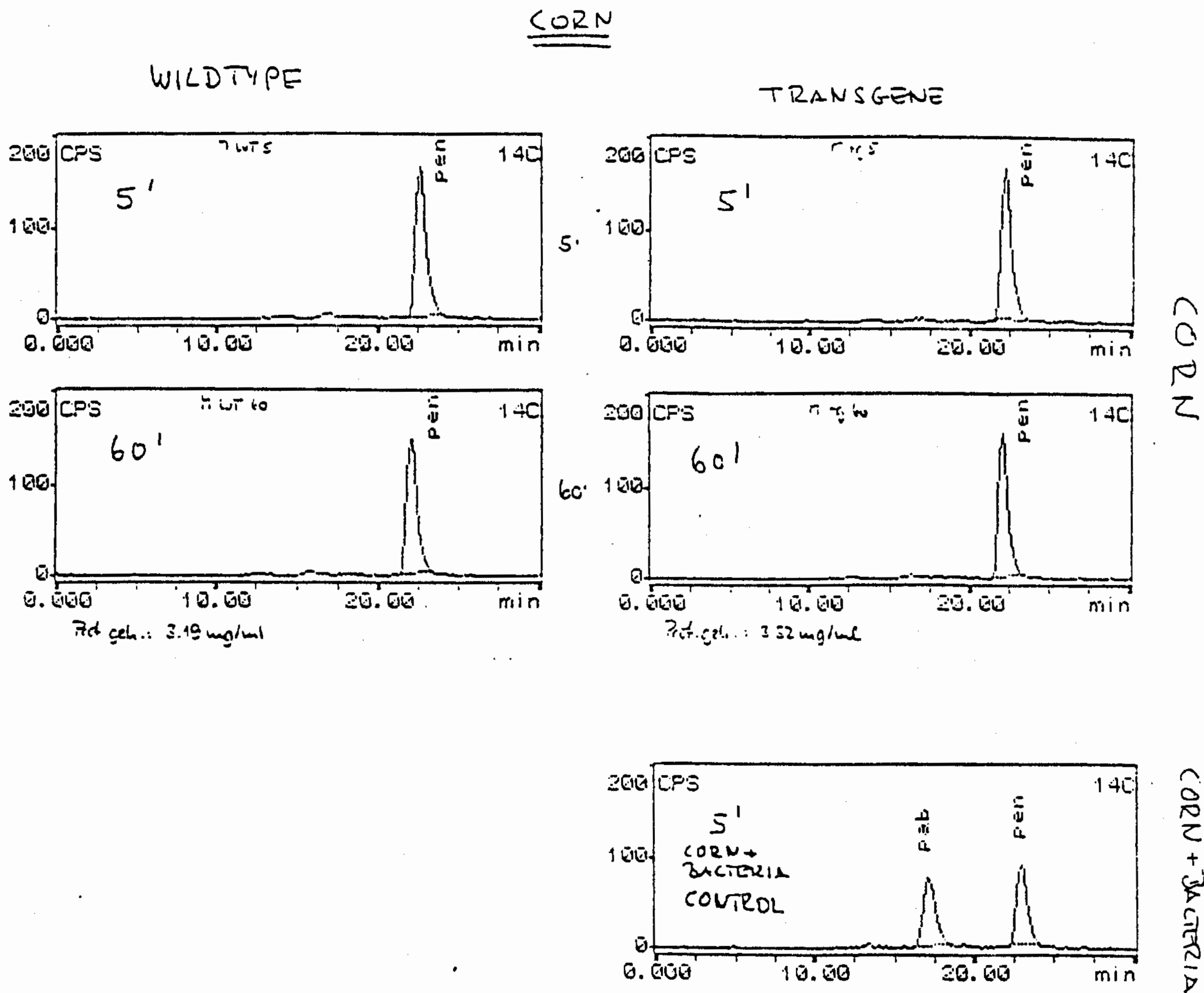
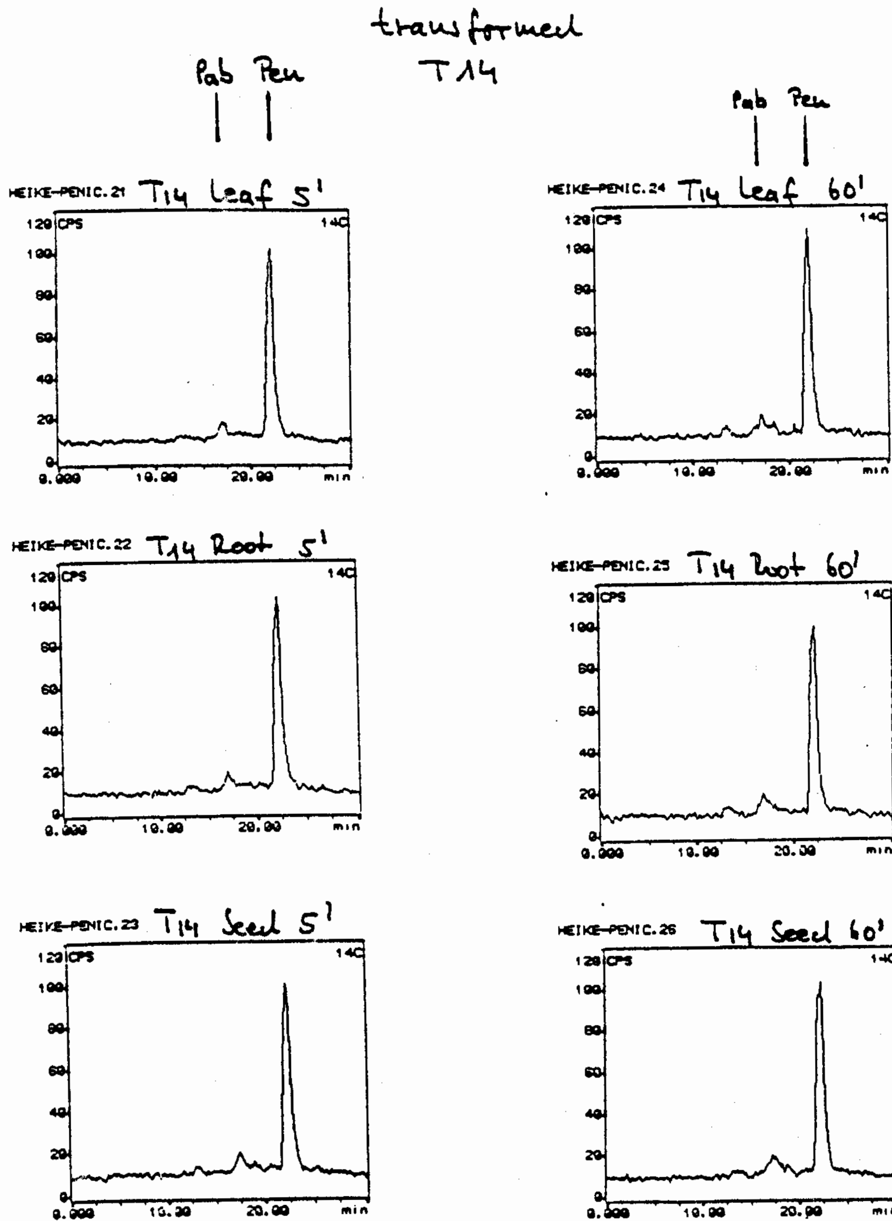


Figure IV.11. HPLC analysis of β-lactamase activity in event T14. Crude protein extracts were prepared from event T14 leaves (upper row), roots (middle row), and seeds (lower row) and incubated with ^{14}C-labeled penicillin for 5 minutes (left column) or 60 minutes (right column). Samples were analyzed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



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 (*Diabritica 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 backgounds 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 then 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 backgounds 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]	Nontrangenic 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 backgounds 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 anitbiotic 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 (*Abulilon 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; sulfonylurea.
    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 postemerge
   - Apply 2,4-D to broadleaf weeds
   - Cultivate two times

2. <u>Possible Effect of Glufosinate Resistant Corn on Current Practices</u>

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. <u>Likelihood of Appearance of Glufosinate-resistant Weeds</u>

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