95-228-01P

PLANT GENETIC SYSTEMS

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Mr Michael A. Lidsky
Deputy Director, BBEP, APHIS, USDA
Att. BCPA
4700 River Road
Unit 146
Riverdale, MD 20737-1237 (USA)

Ref. PGS/95/PRIH:hb/2666 Date: August 10, 1995

Re : **Petition for Determination of Nonregulated Status for Male Sterile, Glufosinate Tolerant Corn Transformation Event MS3**

Dear Mr. Lidsky:

Plant Genetic Systems (America) Inc. is submitting a Petion for Determination of Nonregulated Status to the Animal and Plant Health Inspection Service (APHIS) regarding Male Sterile, Glufosinate Tolerant Corn Transformation Event MS3. This petition requests a determination from APHIS that transformation event MS3 and any progeny derived from crosses between event MS3 and traditional corn varieties, and any progeny derived from crosses of event MS3 with transgenic corn varieties that have also received a determination of nonregulated status, no longer be considered regulated article under regulations in 7 CFR part 340. Event MS3 has been field tested by several partners of Plant Genetic Systems since 1992 in the primary corn growing regions of the United States. The copies of the final reports for these field trials are included in this petition.

We appreciate your attention to this matter. Should you have any questions, please feel free to contact us either at 515-276-6642 (K. Newhouse) or at 32-9-235-8461 (P. Rüdelsheim).

Yours Sincerely,

Keith Newhouse

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SeedLink™ :

POLLINATION CONTROL IN CORN

95-228-01P

Petition for Determination of Nonregulated Status

Male Sterile, Glufosinate Tolerant Corn Transformation Event MS3

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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Director Business Development.

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August 10, 1995

This document contains no Confidential Business Information

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.

Keith Newhouse, PhD
Director Business Development.

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Summary

Plant Genetic Systems (America) Inc. is submitting a Petition for Determination of Nonregulated Status to the Animal and Plant Health Inspection Service (APHIS) for Male Sterile, Glufosinate Tolerant Corn Transformation Event MS3. Plant Genetic Systems (America) Inc. requests a determination from APHIS that corn transformation event MS3, and any progeny derived from crosses of event MS3 with traditional corn varieties, and any progeny derived from crosses of event MS3 with transgenic corn varieties that have also received a determination of nonregulated status, no longer be considered regulated articles under 7 CFR Part 340. Event MS3 is considered regulated article because it contains sequences from the plant pests, Cauliflower Mosaic Virus and *Agrobacterium tumefaciens*.

To provide a more reliable pollination control system, Plant Genetic Systems N.V. (PGS) has developed a new hybridization system, designated SeedLink™. The new type of male sterility linked to an efficient field selection system, has been introduced via immature embryo electroporation in yellow dent corn material, resulting in transformation event MS3.

The chimeric *barnase* gene construct induces male sterility of the plants. The *barnase* gene, isolated from *Bacillus amyloliquefaciens*, encodes the barnase enzyme, a ribonuclease that degrades RNA. Under the control of the TA29 promoter, cloned from *Nicotiana tabacum*, the *barnase* gene is expressed in the tapetal cell layer of the anther, a cell layer that plays a vital nutritive role during pollen formation. Introduction of the chimeric *barnase* gene construct therefore inhibits pollen formation and results in male sterility of the transformed plants. The protein does not contain pesticidal activity and does not have any adverse environmental or toxicological effect.

Linkage of the *barnase* gene to a marker gene - a glufosinate tolerance gene, called *bar* - provides a useful means for integration of the system in breeding schemes and for seed production. The chimeric *bar* gene encodes the enzyme phosphinothricin acetyltransferase. The *bar* gene was isolated from *Streptomyces hygroscopicus*, a non-pathogenic bacterium. The integration of the *bar* gene enables the selection of the male sterile line independent of the plant stage, which is a prerequisite for efficient rogueing of fertile plants in a segregating population, the basis of quality assurance in the hybrid seed production. The protein product does not confer any pesticidal activity and does not have any adverse environmental or toxicological effects.

Event MS3 has been field tested by several partners of PGS since 1992 in the primary corn growing regions of the United States. These tests have occurred under field release authorizations granted by APHIS (USDA authorizations : permits 92-105-02, 92-244-03, 93-076-02, 93-076-03, 92-245-02, 92-080-05, 93-043-02; USDA Notification Numbers 94-080-10N, 94-080-11N, 94-076-23N). Corn transformation event MS3 has also been field tested in Belgium, France, Chile and Argentina

Data collected from these trials, laboratory analyses, two expert letters, reports, and literature references presented herein demonstrate that transformation event MS3 : 1) exhibits no plant pathogenic properties; 2) is no more likely to become a weed than non-transgenic corn; 3) is unlikely to increase the weediness potential of any other cultivated plant or native wild species; 4) does not cause damage to processed agricultural commodities; and 5) is unlikely to harm other organisms that are beneficial to agriculture.

Transformation event MS3 was selected for commercial development. It has been crossed with both public inbred lines and proprietary inbred lines.

Plant Genetic Systems (America) Inc. requests a determination from APHIS that the corn transformation event MS3, and any progeny derived from crosses of event MS3 with traditional corn varieties, and any progeny derived from crosses of event MS3 with transgenic corn varieties that have also received a determination of nonregulated status, no longer be considered regulated article under CFR Part 340.

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1. RATIONALE

Today, virtually one hundred percent of the corn (*Zea mays*) grown in the United States for food and feed is hybrid corn. A number of hybrid corn seed production systems are in use to ensure hybridization by forced cross pollination between the female and the male parental corn lines. Pollen control of the female parent in the hybrid seed production is extremely critical. Various methods of pollen control in corn seed fields have been utilized or investigated, primarily aiming at improving efficiency while still maintaining the desired genetic purity of the seeds. In corn, artificial emasculation (detasselling) and male sterility systems (especially cytoplasmic male sterility) are currently the most widely used methods of pollen control.

To provide a more reliable pollination control system for hybrid seed production in corn, PGS has developed a novel genetically engineered hybridization system. A new type of male sterility has been successfully introduced in a *Zea mays* public line (H99) by molecular biology techniques. The male sterility of the corn plants is caused by the expression of an RNase (barnase) at a specific stage early during anther development and in a specific cell layer of the anther. Linkage of the *barnase* gene to a marker gene - a glufosinate tolerance gene -provides a useful means for integration of the system in breeding schemes and for seed production.

2. RECIPIENT ORGANISM : CORN (*ZEA MAYS* L.)

2.1. Production and usage of *Zea mays*

Corn (*Zea mays* L.) is one of the major cereal grains grown in the world, being exceeded only by rice and wheat in terms of quantity produced. World corn production now normally exceeds 400 million tonnes, with US total production accounting for more than half of that for the entire world. Corn is the major crop on the cultivated land of the United States. It is produced on 70 to 80 million acres annually and plays an important role in the economy of the country. Although corn is produced throughout the United States, the bulk of US production occurs in the region known as the Corn Belt : two states, Iowa and Illinois, produce about 40 percent of the corn crop in the US (Jugenheimer, 1976; Hallauer et al., 1988; Olson and Sander, 1988; Hallauer, 1994).

Very little corn is consumed directly as human food. When processed into meat, milk, eggs and other animal products, as three-fourths of it is, corn becomes the basic food plant of the modern American civilization. As indicated in Table 2.1., more than 75% of domestic corn is used for the feeding of livestock. Since corn generally has a low quantity and quality of protein for animal feed, it is usually complemented in feed rations with high quality-quantity protein sources such as soybean meal. Industrial utilization of corn accounts for about 20% of domestic maize consumption, either as starch *per se* or converted into products such as High Fructose Corn Syrup (HFCS), alcohol or glucose/dextrose. HFCS is currently used in most sweet drinks and snack foods. In the United States, direct human consumption of whole kernel or processed corn is limited (2-3%) and primarily derived from specialty corns such as white, pop and sweet corn (Mangelsdorf, 1974; Watson, 1988; Duvick, 1993; Rhoades, 1993; Hallauer, 1994).

Table 2.1. Corn usage in the United States (1991-1992) (Source : Duvick, 1993)

	Million tonnes	**(% domestic use)**	**(% industrial use)**
Feed	**124.39**	**77**	
Cattle and other	63.01	39	
Hogs	38.14	24	
Poultry	23.24	14	
Food, seed, industrial	**36.42**	**23**	
Corn syrup (HFCS)	9.96	6	30
Fuel alcohol	9.60	6	29
Starch	6.02	4	18
Glucose, dextrose	5.33	3	16
Beverage alcohol	2.08	1	6
Cereal, other products	2.95	2	
Seed	0.51	0.3	
Exports	**40.23**		
Total use	**201.03**		

2.2. Biology of *Zea mays*

2.2.1. Taxonomy of *Zea mays* and its close relatives

Zea mays L. (2n=20) is a member of the family *Poaceae*, commonly known as the grass family, tribe Maydeae (Kiesselbach, 1980). Although consensus does no exist on the origin and early evolution of corn, reasonably complete agreement exists among experts that corn was first domesticated in 8000 to 5000 B.C. in tropical south-central or south-western Mexico (Troyer, 1994). Corn is only known as a domesticated species. It can not reproduce itself successfully without the aid of man (Jugenheimer, 1976).

Teosinte is the closest relative of corn. There are three taxa of teosinte : *Zea mexicana* (Schrader) O. Knutze 2n=20, the annual diploid of wide distribution in Mexico and Guatemala; *Zea perennis* (Hitchcock) Reaves and Mangelsdorf 2n=40, the tetraploid perennial form now extinct in the wild; and *Zea diploperennis* Iltis, Doebley & Guzman 2n=20, the diploid perennial form found in a single locality, El Chante in Jalisco. Alternative taxonomy to that used here can be found in the literature (Wilkes, 1982). All three taxa can hybridize with corn; the F_1 hybrid from diploid parents is both robust and fertile. Teosinte is not native to the US. Though it is known to have survived as an escape from cultivation in Florida and Texas, teosinte is not considered a serious weed. It has been argued that the survival characteristics of teosinte as a wild plant are damaged by introgression from corn (Galinat, see letter in Annex 1.).

The genus *Tripsacum* is the second closest relative of corn. Most species (13 to 16 different species are recognized) are native to Mexico, Central and South America. *T. floridanum* Porter ex Vassy 2n=36, however, is native to Southern Florida. *T. dactyloides* (Eastern gamagrass) 2n=36 is native to the central and western US, and the tetraploid form of *T. dactyloides* 2n=72 extends along the Eastern seaboard from Massachusetts to Florida and along the Gulf Coast from Florida to Texas. *T. lanceolatum* 2n=72 is a tetraploid that occurs in the Southwestern US. There is no evidence for natural hybridization between corn and *Tripsacum* in North America. F_1 hybrids (male sterile) have been obtained with varying degrees of difficulty under experimental conditions only. *Tripsacum* is not considered an aggressive weed. Recently, there has been a growing demand for *T. dactyloides* seed for planting as a new forage or haylage crop in the Great Plains area of the US (Galinat, see letter in Annex 1.; Kindiger, see letter in Annex 2.).

2.2.2. Morphology of *Zea mays* with special reference to its unique floral characteristics

As a member of the *Poaceae*, corn has many characteristics common to other grasses, such as conspicuous nodes in the stem, a single leaf at each node, the leaves in two opposite ranks, each leaf consisting of a sheath surrounding the stem and an expanded blade connected to the sheath by a blade joint. As in other grasses, there is a tendency to form branches at the nodes, and adventitious roots at the base of the internodes. Corn's uniqueness among the important cereal grasses lies in the nature of its inflorescences : corn bears male and female flowers on different positions on the same plant, a system that structurally promotes cross-pollination.

The female flowers are found as the familiar ear in a lateral position half way down the plant (Figure 2.1.). The male (pollen - producing) flowers are born terminally in the tassel (Figure 2.1.), facilitating pollen dispersal by wind. Pollen grains are produced in large numbers : one estimate for an average sized tassel was 25 million. Pollen grains of corn are small (about 1/250 inch in diameter), light in weight, and easily carried by the wind, sometimes for considerable distances. For example, seed production fields are planned with isolation of 125 to 201 meters as the base distance from other corn pollen (Mangelsdorf, 1974; Kiesselbach, 1980).

Corn has been selected for thousands of years for characteristics which are beneficial in agriculture. The plant is superbly constructed for producing grain under man's protection, as the kernels are firmly attached to a rigid axis, the "cob". Additionally, while in other cereals the kernels are protected individually, in maize they are covered en mass. The entire ear is enclosed, often quite tightly, by modified leaf sheaths, the husks. Consequently, the crop has a low survival value in nature for it lacks a mechanism for seed dispersal. When an ear of corn drops to the ground and finds conditions for germination, scores of seedlings emerge, creating such fierce competition among themselves for moisture and soil nutrients that usually all plantlets die and none reaches the reproductive stage (Mangelsdorf, 1974).

2.2.3. Cultivation of *Zea mays*

As with all commercially grown crops, optimal growth conditions are essential to assure a high yielding corn crop. Although corn can be grown under a wide range of conditions, particular attention is required to optimum planting procedures, a balanced fertilization of the plants, seed selection, weed control and pest and disease control.

General methods

Corn is a summer row crop, appreciating a warm but not excessively hot environment for growth. Most agronomists agree that the optimum time for planting corn is as soon as the soil temperature (at 5 cm depth) reaches a minimum of 10°C(50°F) for a relatively sustained period of time. Since the mid-1970s, there has been a major trend away from extensive conventional primary and secondary tillage towards reduced or no tillage. A combination of several factors has been responsible for this evolution, most importantly the advent of effective herbicides for weed control, the benefits from a residue cover in erosion control and the obvious economic advantages of reduced implement usage. Other trends include increasing plant densities and narrower row spacing. Corn has a high nitrogen demand and substantially more nitrogen is used for its production than any other primary fertilizer nutrients such as phosphorous, potassium and sulfur. With the exception of a few unique conditions and/or localized areas, addition of other elements are considered unnecessary (Hallauer et al., 1988; Olson and Sander, 1988; Wych, 1988).

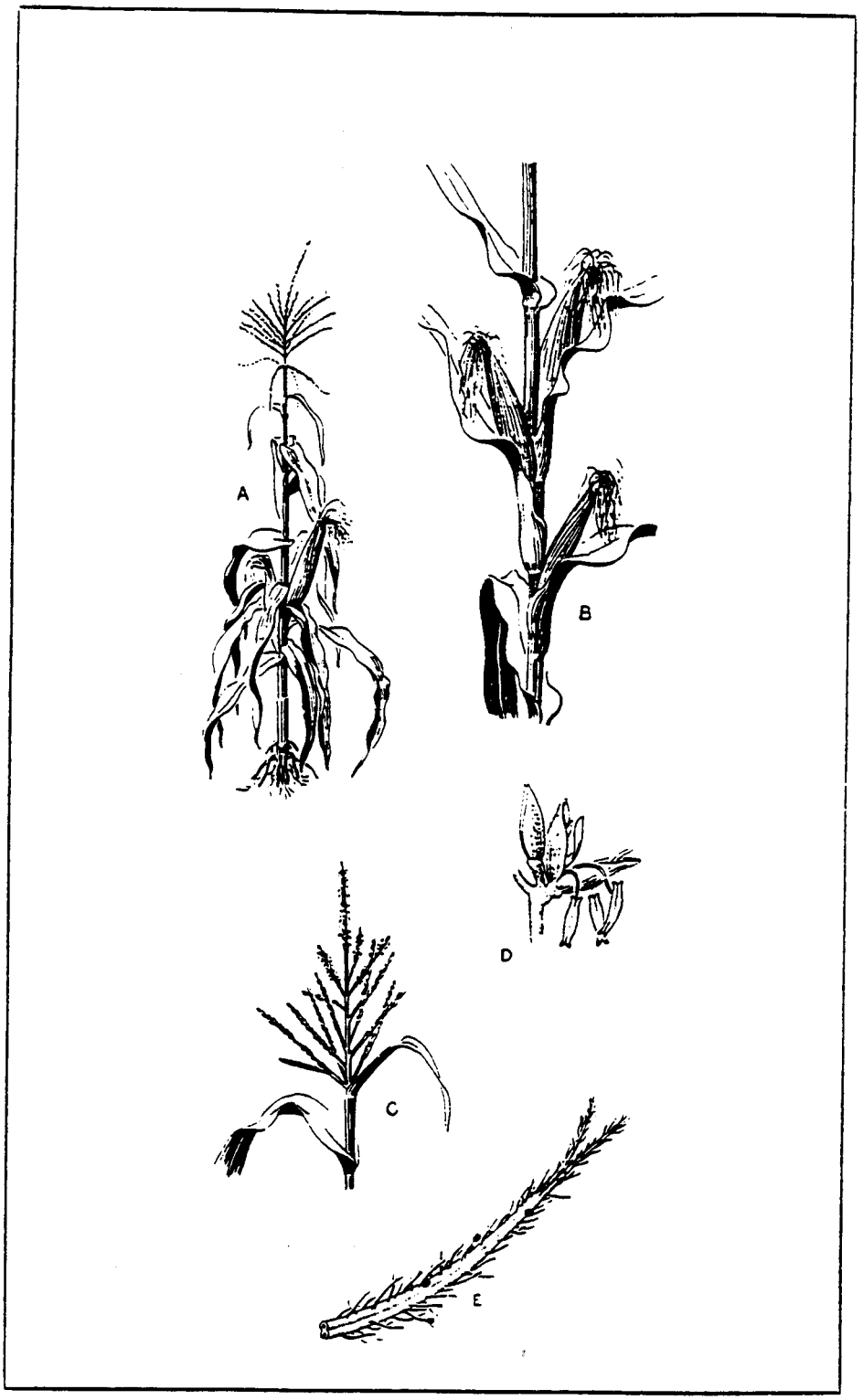

Figure 2.1. **Botanical characteristics of the modern corn plant.** A. The entire plant (a short-stalked variety) showing the male inflorescence, the tassel terminating the stalk, and the female inflorescences, the ears, in the middle region. B. Young ears enclosed in husks with the pollen receptive organs, the silks, protruding from the ends. C. Typical tassel. D. Typical male flower with three anthers containing pollen. E. A single silk magnified to show hairs and adhering pollen grains (Source : Mangelsdorf, 1974)

Weed control practices

Corn is not considered to be a strong competitor (Keeler, 1989). Uncontrolled weeds can easily cause a complete loss of corn yield and even small numbers of weeds can substantially reduce the yield of the crop (Olson and Sander, 1988). The major weeds of corn are listed in Table 2.2..

Table 2.2. Major corn weeds (FAO, 1982)

Grasses	Broadleafs
Giant foxtail	Cocklebur
Green foxtail	Lambsquarter
Yellow foxtail	Mustard
Barnyard-grass	Pigweed
Crabgrass	Ragweed
Panicum	Smartweed
Wooly cupgrass	Velvetleaf
Wild proso millet	Wild sunflower
Nutsedge	Canada thistle
Quackgrass	

It is essential that early weed control is achieved after emergence either through tillage (rotary hoe or harrow) or via chemical treatment. Later weeds are normally controlled by cultivation, most commonly with shovel cultivators.

Though a combination of physical, mechanical and chemical methods is available to control weeds in corn, herbicides are an important means of weed control. Without herbicides, some reduced and no-till systems of corn production would be doomed to failure. In recent years, more environmental friendly herbicides have been especially developed for specific weed problems and specific cropping and tillage systems. Since new chemicals and new combinations are constantly being developed, we refer to the most recent Agricultural Extension publications for a list of currently used herbicides in *Zea mays* (Olson and Sander, 1988).

Insect control practices

The corn crop is subject to attack by a complex of insects from the time it is planted until it is utilized as a food or feed (see Dicke and Guthrie, 1988 for an extensive review). Two of the most important insect pests in the U.S. are the corn rootworms (*Diabrotica* spp.) and the European corn borer (*Ostrinia nubilalis* (Hübner)), which are described in more detail in Table 2.3..

Table 2.3. General description of two of the most important insect pests in the U.S., the severity of their attack and some control practices

The two most important *Diabrotica* pest are *D. virgifera virgifera*, the western corn rootworm (WCR) and *D. barberi*, the northern corn rootworm (NCR). These pests have caused an estimated 10-13% yield loss per year. WCR and NCR are mainly found in the Northern U.S., east of the Rocky Mountains. The WCR has become the dominant rootworm pest in a large area of the Corn Belt. WCR and NCR are particularly a serious problem where continuous corn is grown. Consequently, the practice of short rotation, particularly with an intervening crop of soybean, has become common in the Corn Belt. Where continuous corn is practiced, several effective insecticides are available to ensure against appreciable yield losses; approximately 50-60% of the corn acreage is treated with soil insecticides. Treatment costs and crop losses are in the range of $1 billion per year (Metcalf, 1986).

European corn borer (ECB) is also a major pest of maize in North America. Yield loss of 3-7% per borer per plant can result from ECB feeding at various stages of plant growth. Evaluating germplasm for corn borer resistance by manual infestation of artificially-reared ECB eggs is a typical part of a commercial corn breeding program. Increased levels of ECB resistance contributes to improved plant health and has been one factor that has contributed to the genetic gains experienced in newer hybrids. Resistance is not absolute, however, and hybrids can vary widely in their degree of resistance. Thus, insecticides are still a common control measure for ECB. Treatment costs and crop losses are estimated at $50 and $400 million respectively (Lynch, 1980; Lewis, 1991).

Certain cultural practices and a number of insecticides can be used to minimize or control insect damage. As the list of registered insecticides may change every year, publications have to be checked annually for changed recommendations (Dicke and Guthrie, 1988).

Disease control practices

Corn is rarely grown in the absence of diseases. Estimates made of disease losses for corn in the U.S. caused by all pathogens have ranged from 2-7% to 7-17% yearly. A wide range of organisms, including certain fungi, bacteria, viruses, nematodes, at least one mycoplasma, one spiroplasma and one parasitic seed plant are pathogens of corn in the U.S. (see Smith and White, 1988 for an extensive review). These pathogens vary in their adaptability to specific environments and geographic area of recognized occurrence, which may shift over time (Smith and White, 1988).

Protection of the corn crop from diseases is accomplished largely through the use of chemical control measures, resistant cultivars and cultural practices. The seed of commercial hybrid corn is treated with fungicides to help prevent losses from seed rots and seedling blights (e.g. *Pythium* spp.) that can occur if adverse environmental conditions occur shortly after planting. Although foliar fungicides are used on high-value corn crops such as seed production fields, commercial popcorn and sweet corn fields, currently no routine chemical control of diseases

on grain or silage crops is used. Currently, most corn germplasm used in hybrids in the U.S. is being selected in regular corn breeding nurseries for acceptable levels of resistance to the common diseases to which the resulting hybrids would be most likely exposed. The most common cultural practice to control diseases in corn is crop rotation to reduce the perpetuation and intensification of many corn pathogens which can occur with continuous cropping of corn (Smith and White, 1988).

3. HYBRID CORN (*ZEA MAYS*) DEVELOPMENT AND PRODUCTION SYSTEMS

3.1. Development of hybrid *Zea mays*

Before the advent of hybrid corn, farmers grew open-pollinated, highly heterogeneous corn varieties. These open pollinated cultivars were developed by a type of mass selection that was based on plant, ear and grain type. The early corn breeding work, which was primarily done by farmers and seedsmen, provided the germplasm sources from which were developed the inbred parental lines, used to produce the first double-cross corn hybrids in the United States. Unlike their inbred parents, hybrids between pure lines were vigorous, uniform and productive, a phenomenon known as heterosis. Some hybrids were definitely superior to the original open-pollinated varieties from which they had been derived. Furthermore, in contrast to open-pollinated corn lines, hybrids can be reproduced continously and in any quantity from the inbred lines (Jugenheimer, 1976; FAO, 1982; Hallauer et al., 1988).

The superiority and enormous economic importance of hybrid corn varieties was demonstrated by the rapid acceptance of them in the US and especially in the primary corn-producing areas. It was the 1930s before farmer use of hybrid seed became an acceptable practice (Figure 3.1.). The acreage in the country planted with hybrid corn was approximately 0.1 percent in 1933. By 1943, hybrid corn already occupied approximately 100 percent of the corn area in Iowa, 90 percent of the corn area in the US Corn Belt and 60 percent of the corn area for the entire USA. Today, virtually 100 percent of the corn grown in the US is hybrid corn. Conservative estimates indicate that hybrid seed has increased corn production in the US from 25 to 50 percent (Jugenheimer, 1976; Hallauer et al., 1988).

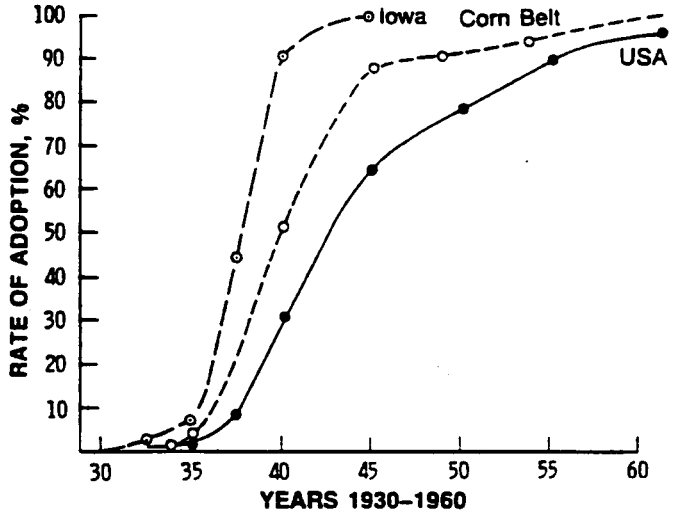

Figure 3.1. **Rate of acceptance of double-cross hybrids in Iowa, the US Corn Belt and the USA** (Source Hallauer et al., 1988)

The cost of hybrid seed is a small input (about 4 to 6%) relative to other costs such as fuel, labor, fertilizer, pesticides, loan interest and depreciation. However, since productivity of the seed is critical to a farmer's yields and profits, the farmer receives a high return on investment for hybrid seed corn purchases (Harvard Business School, 1985; Troyer, 1994).

3.2. Hybrid corn production systems

According to Fehr (1987), the commercial production of hybrid seeds must meet four requirements :
- heterosis has to be exhibited by the progeny of the crosses between the parents;
- the fertile pollen from the female parent should easily be eliminated;
- pollen from the male parent should effectively be transported to the female parent; and
- the hybrid seeds have to be produced in a reliable and economical manner.

Today, a number of hybrid corn seed production systems are in use to ensure hybridization by forced cross pollination between the female and male parents. Pollen control of the female parent in the hybrid seed production field is extremely critical. Various methods of pollen control in corn seed fields have been utilized or investigated, primarily aiming at improving efficiency while still maintaining the desired genetic purity of the seeds. In corn, artificial emasculation (detasseling) and male sterility systems (especially cytoplasmic male sterility) are the currently most widely used methods of pollen control (Wych, 1988).

3.2.1. Manual and mechanical detasseling

Detasseling involves the physical removal of the tassel from the female plant, either as manual operation or in combination with mechanical devices. The detasseling period for the seed producer is probably the most difficult to manage of any of the steps involved in hybrid corn seed production (Mangelsdorf, 1974; Jugenheimer, 1976; Wych, 1988).

The detasseling period is short as the tassels from the female parent rows need to be removed before they shed pollen and/or before the silks emerge on the ear shoots of the female parent. Manual detasseling is a labor-intensive and expensive operation : it requires locating, training, supervising and transporting (to the various seed production fields) a large number of people (>100,000 at peak period) who are needed for as little as one week to not more than five weeks. Mechanical detasselers have been developed to reduce the need for labor. A primary disadvantage of mechanical detasselers is however that they remove the top leaves of the plant together with the tassel. Labor cost savings attained through mechanical detasseling may therefore be offset by seed yield reductions if the operation is not carefully managed to minimize leaf damage. Additionally, poor weather may make it difficult to get the machines through the seed fields (Mangelsdorf, 1974; Jugenheimer, 1976; Wych, 1988).

With most female parents, the combination of mechanical and hand detasseling resulted in a cost savings when compared with hand detasseling alone. Detasseling costs have been estimated to range from $198 to $247 per ha with a combination of mechanical and hand detasseling, compared to $ 269 to $321 per ha for all hand detasseling alone (Wych, 1988).

3.2.2. Male sterility

Due to the failure to produce functional pollen, male sterile plants can be used to facilitate cross-pollination. Several natural sources of male sterility in plants are available. The use of both Genic Male Sterility and Genic-Cytoplasmic Male Sterility (CMS) systems have been investigated in hybrid corn seed production (Kaul, 1988; Wych, 1988).

One type of male sterility that has been observed in corn, is Nuclear or Genic Male Sterility (NMS), i.e. male sterility resulting from mutation in the nuclear plant genome. More than fifty different genic male sterile corn loci have been reported. In general, their genetic control is by recessive nuclear alleles, although nuclear encoded genic male sterile corn plants controlled by nuclear dominant alleles have also been found. None of these types of male sterility has a selectable marker linked to the male sterility gene which would allow their early identification in hybrid seed breeding programs. Moreover, the absence of a selectable marker does not allow the design of an efficient hybrid seed production scheme, which needs rogueing of the male fertile segregants before flowering (Kaul, 1988).

Several corn CMS types (e.g. *cms*-T, *cms*-S, *cms*-C) have been reported and used for commercial production of hybrid corn seeds. CMS results from a specific interaction of the nuclear and mitochondrial plant genomes. The so-called *cms*-T type, has been exploited in hybrid corn seed production for about two decades prior to the epidemic of southern corn leaf blight that swept the USA in 1970. At that time, it was estimated that 70 to 90% of the hybrid corn grown in the US carried the T-type of cytoplasm, being more susceptible to the southern blight than normal corn cytoplasm. The 1970 epidemic prompted a retreat from the extensive use of cms in preference to detasselling (Mangelsdorf, 1974; Jugenheimer, 1976; Kaul, 1988; Wych, 1988).

It has now become a general practice to produce corn hybrids by detasseling and blend 25 to 50 % of these fertile seeds with 50 to 75 % of hybrid seeds produced by CMS. The hybrids produced by detasseling then provide adequate pollen for the entire field. In 1987, blends accounted for 33 percent of seed sales (20.2% with *cms*-C and 4.5% with *cms*-S) (Wych, 1988).

3.3. SeedLink™

To provide a cheaper, more reliable pollination control system, PGS has developed a new hybridization system, SeedLink™ (see Mariani et al., 1990 and Mariani et al., 1992 in Annex 3 and Annex 4.). In corn, the SeedLink™ system is based on a dominant nuclear male sterility (NMS) gene that is linked to a convenient field selection marker.

3.3.1. Application of SeedLink™ in corn

In corn, SeedLink™ comprises two linked components : the male sterility function and an efficient field selection system. The dominant male sterility function is based on a disruption of the tapetal development in the anthers. The linked field selection system, based on

glufosinate ammonium tolerance, allows selection of the male sterile plants in a segregating population.
As is the case with naturally-occurring male sterile mutants, maintenance and multiplication of the male sterile line is accomplished by crossing the male sterile plants with a fertile counterpart. A 1:1 segregation of male sterile and fertile plants is obtained in the offspring. In the hybrid seed production field, the male sterile female parent is therefore planted at double density and the herbicide is sprayed on the female rows to selectively rogue the fertile plants at the seedling stage (Table 3.1.). Using SeedLink™, 50% of the hybrid seed carries the male sterility and herbicide tolerance genes (Table 3.1.). Since corn is an efficient cross-pollinator and produces large quantities of pollen, fertility in all hybrid plants is not required for good pollination and seedset in commercial fields, as experience with CMS-normal blends has demonstrated.

Table 3.1. Development and maintenance of the male sterile female parent and production of an F_1 hybrid

Development of the female parent line
Transform corn with the foreign DNA sequence including the *barnase* gene (Ms) and the herbicide resistance marker gene *bar* (H), giving rise to the female parent phenotype : $A^{MsH/msh}$.
Maintenance of the female parent line
Cross $A^{MsH/msh}$ x $A^{msh/msh}$ giving rise to : 50% $A^{MsH/msh}$ and 50% $A^{msh/msh}$, of which the latter phenotype can be eliminated by spraying glufosinate-ammonium on the plantlets in seed production fields.
Production of a F_1hybrid
$A^{MsH/msh}$ x $B^{msh/msh}$ ↓ giving rise to 100% hybrids 50% $AB^{MsH/msh}$ and 50% $AB^{msh/msh}$

3.3.2. Description of the male sterility function

The chimeric PTA29-*barnase*-3'nos gene construct induces male sterility of plants. The *barnase* gene, isolated from *Bacillus amyloliquefaciens*, encodes the barnase enzyme, a ribonuclease that degrades RNA (Hartley, 1989). Under the control of the TA29 promoter, cloned from *Nicotiana tabacum*, the *barnase* gene is expressed in the tapetal cell layer of the

anther, a cell layer that plays a vital nutritive role during pollen formation. Introduction in a tissue-specific way and expression of the chimeric *barnase* gene construct therefore inhibits pollen formation, resulting in male sterility of the transformed plants (Mariani et al., 1990).

The tapetum plays an essential role in pollen development

In flowering plants, the male gamete formation is a highly regulated developmental process that occurs in the anther. The beginning of anther differentiation is indicated by the fact that the anther takes on a four-cornered shape. From the first cell divisions, one row of inner archespore cells and one row of outer cells arise. While the cells of the archespore continue to grow and multiply, the outer cells divide by their anticlinal walls. At the same time, the outer cells divide by periclinal walls, usually so that three cell layers are created. The innermost layer of the wall of the microsporangium becomes the tapetum (Figure 3.2.). The tapetal cells serve for the nourishment of the archespore cells from which under normal circumstances, the pollen mother cells and finally the pollen grains arise (Weberling, 1989; Kaul, 1988). Since the tapetum cell layer is the tissue adjacent to the developing pollen grains, this cell layer was assumed to play an essential nutritive role in pollen grain formation, as the food material, growth substances, water and other essential supplies have to pass through the tapetal cells or are synthesized by it (Kaul, 1988). As an example, the tapetum seems to be responsible for the production of a β-1,3-glucanase (callase) which liberates the young microspores from the thick callose walls of the meiotic tetrads. As demonstrated by Mariani et al. (1990), the tapetum plays an important role in pollen development. The essential role of the tapetum in pollen development is evidenced by the examples found in nature, where abnormal nuclear behavior and/or malfunctioning of the tapetum layer lead to male sterility (Kaul, 1988).

Tapetal gene expression

Plant organ systems contain many different cell types, which may be morphologically similar but which express genes (encoding cell-specific transcripts and proteins) specific for that particular cell type and that organ. Moreover, *in situ* hybridization experiments have demonstrated that many organ-specific mRNAs are present within specific cell or tissue types (Drews et al., 1989). Several scientific groups have localized anther-specific mRNAs that are only expressed in the tapetum during gametogenesis (Goldberg, 1988; Drews et al., 1989; McCormick, 1991). These tapetal cell specific mRNAs accumulate early in anther development at the moment when the tapetum is active, and decay when the pollen is mature and the tapetum is destroyed (Drews et al., 1989).

Hybridization experiments with the tobacco (*Nicotiana tabacum*) floral organ system mRNA populations (petal, anther, ovary) have shown that each of these organs expresses approximately 25,000 diverse genes. Most genes expressed in the flowers encode rare mRNAs that represent approximately 0.001% of the mRNA mass when averaged over the entire mRNA population. Both the anther and the ovary contain approximately 10,000 diverse mRNAs that are not detectable in mRNA of heterologous organ system or nuclear RNA populations (Drews et al., 1989).

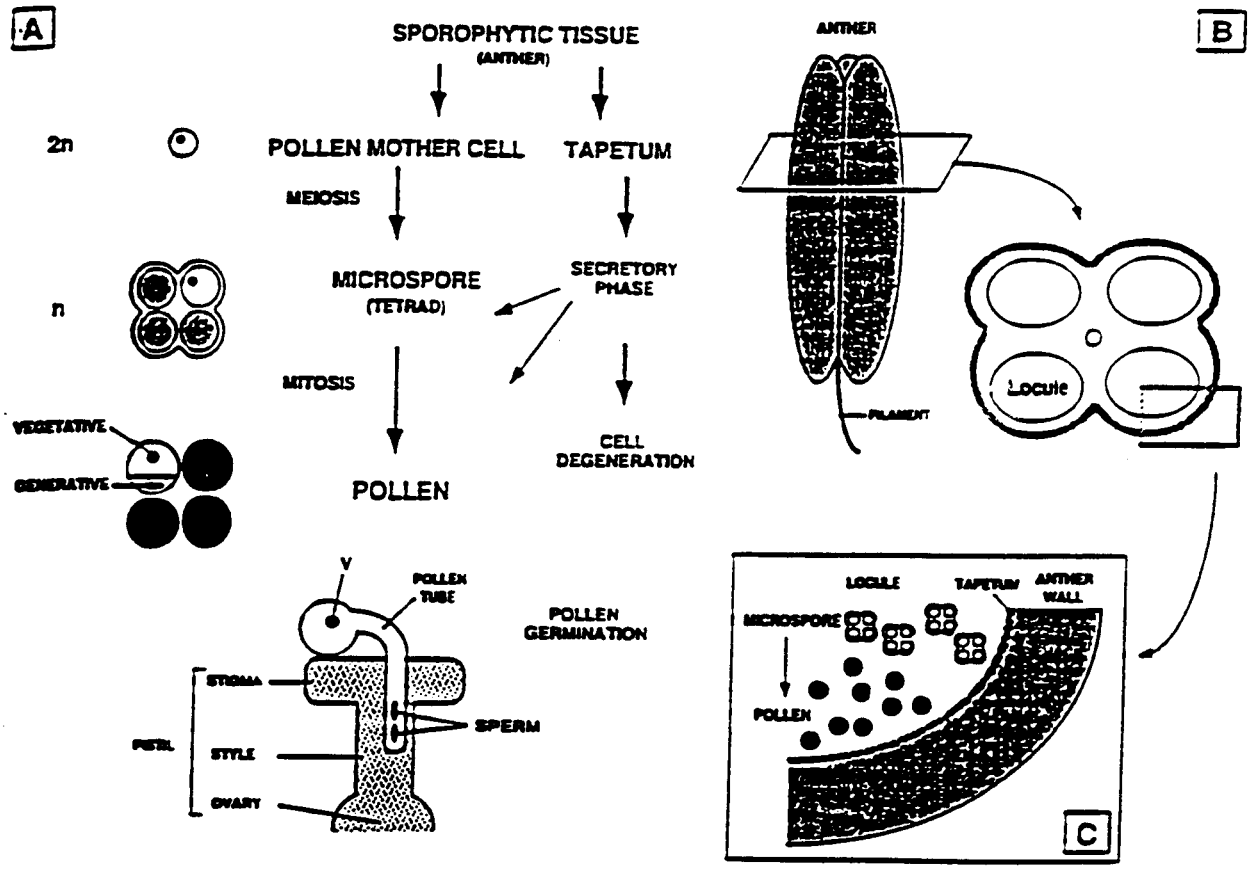

Figure 3.2. **Generalized outline of male gametogenesis and pollen germination.** (A). The meiotic products (microspores) develop into pollen grains. The tapetum deposits components onto the pollen wall. Pollen grains land on the stigmatic surface and germinate in order to deliver sperm to the ovules. (v : vegetative nucleus) (B). Cross-section of the anther. (C). Detail showing relationship of anther wall, tapetum and locule (McCormick, 1991).

One of these organ-specific tapetal mRNAs is the tobacco TA29 mRNA. TA29 mRNA has been shown to accumulate early during flower development and to disappear as the tapetum degenerates at later stages of anther development. The TA29 gene is a member of a small gene family which seems to be well conserved in distantly related species. It has been shown that genes under the control of the PTA29 promoter are specifically expressed in the tapetal tissues of several other crops (see Mariani et al., 1990 in Annex 3.).

Mode of action of the PTA29-barnase gene in transgenic corn

The intensive interaction of the tapetal cell layer with the developing pollen indicates that a well functioning tapetum is essential for microspore development (Mariani et al., 1990). By selectively destroying tapetal cells, naturally occurring male sterile plants can be mimicked. Therefore, the *barnase* gene, under the control of the PTA29 promoter, was inserted into the plant genome. The activity of the barnase enzyme, a ribonuclease that catalyzes the hydrolysis of single stranded RNA- molecules, has been demonstrated to be detrimental for tapetal RNA and thus for its cell functioning (Mariani et al., 1990). As a result of the destruction of the corn tapetum, the anthers become incapable of producing viable pollen grains.

3.3.3. Description of the field selection system : glufosinate ammonium tolerance

The chimeric P35S-*bar*-3'nos gene construct induces tolerance to the herbicide glufosinate ammonium (active ingredient phosphinothricin). The *bar* gene, isolated from the bacterium *Streptomyces hygroscopicus* (Thompson et al., 1987), codes for a phosphinothricin acetyl transferase (PAT), which detoxifies the herbicidal compound phosphinothricin (PPT). The *bar* gene gives a selective advantage to the transformed plants when they are sprayed with glufosinate-ammonium (De Block et al., 1987). By linking the chimeric *bar* gene to the chimeric *barnase* gene, the phosphinothricin acetyl transferase becomes an integrated tool in the new PGS hybrid seed production scheme.

Mode of action of the bar *gene product*

The *bar* gene encodes tolerance to herbicides with phosphinothricin as active ingredient (e.g. Basta® , Buster®, Finale® , Ignite®, Challenge®, Harvest®, Liberty®; tradenames of AgrEvo). As an analogue of glutamate, phosphinothricin (PPT) inhibits glutamine synthetase (GS) in plants. The inhibition of GS by PPT results in an accumulation of ammonium. In addition, a process in connection with photorespiration plays a central role on photosynthesis inhibition by PPT (Wild et al., 1984; Manderscheid et al., 1985; Wild et al., 1987; Sauer et al., 1987; Wendler et al., 1990). To protect the *Zea mays* plant against the toxic effects of the phosphinothricin compound, the *bar* gene incorporated into the plant genome, can be expressed leading to the production of the enzyme acetyl transferase. This enzyme acetylates PPT and inactivates the molecule, thereby preventing the death of the plant cell (De Block et al., 1987). When linked to the 35S-promoter from the Cauliflower Mosaic Virus, the *bar* gene is expressed in a constitutive manner (Odell et al., 1985; Fromm et al., 1990; Gordon-Kamm et al., 1990).

The bar *gene : an efficient selectable marker*

The integration of the *bar* gene enables the use of glufosinate as a selective agent at the *in vitro* stage. Since the *barnase* gene construct is physically linked with the *bar* gene, these genes will cosegregate as a single locus. Therefore, the male sterile line can be maintained through crossing with wild type plants followed by the application of the herbicide (Mariani et al., 1990). Furthermore, the integration of the *bar* gene enables identification of the male sterile line independent of the stage of the plant, which is a prerequisite for efficient rogueing of fertile plants in a segregating population as such efficient rogueing is the basis of quality assurance in the hybrid seed production.

4. TRANSFORMATION METHODOLOGY

4.1. Plasmids

Two plasmids have been used to introduce the genes of interest into corn. The plasmid carrying the gene construct conferring male sterility, is pVE108. The helper plasmid is pMc5barstar.

4.1.1. Plasmid pVE108 confers male sterility

The plasmid pVE108 is shown in Figure 4.1.. The plasmid pVE108 contains two chimeric gene constructs, designed to be functional in plants, i.e. :

- the PTA29-*barnase*-3'nos gene construct, conferring male sterility (see 3.3.2.), and
- the P35S-*bar*-3'nos gene construct, conferring tolerance to herbicides with phosphinothricin as active ingredient (see 3.3.3.).

Both gene constructs are cloned on a small plasmid, containing an origin of replication (*ori*) required for replication of the plasmid in *Escherichia coli,* and the β-lactamase gene (*bla*). Both these sequences have a function in the bacterial host only, i.e. :

- *ori* as a sequence at which plasmid replication in the bacterial host is initiated and controlled;
- *bla* to confer resistance to β-lactam antibiotics (such as ampicillin and carbenicillin); this resistance is used to selectively grow bacterial cells containing a plasmid.

An overview of the pVE108 plasmid construct and a detailed description of its different genetic elements and their origin is outlined in Annex 5..

4.1.2. Helper plasmid pMc5barstar

The presence of the plasmid pVE108 in the *E.coli* strain might result in the synthesis of active barnase enzyme in *E. coli*, that could affect the viability of the host cell. To that end, we also introduced an additional plasmid in the strain that directs expression of *barstar*. Barstar is a specific inhibitor of barnase (Hartley, 1988). The plasmid pVE108 was constructed and propagated in *E.coli* WK6 (Zell and Fritz, 1987) that also carried the plasmid pMc5barstar, which carries a Ptac-*barstar* gene construct directing the expression of *barstar* in the bacterial host. The pMc5barstar helper plasmid is shown in Figure 4.2.. A detailed description of the construction of plasmid pMc5barstar is outlined in Annex 5..

The pMc5barstar plasmid contains the following components (for a detailed description see Annex 5.) that are only functional in the bacterial host cells :

- f1 *ori* is an origin for DNA replication as a circular stranded molecule; the activity of this replication origin in *E.coli* requires the presence of additional gene products from bacteriophage f1;
- *ori* is an origin for plasmid replication in the bacterial host as a circular double stranded molecule; it is required for propagation of the plasmid;
- *bla* is a modified β-lactamase gene, that confers resistance to β-lactam antibiotics only in an *E.coli* host that carries a suppressor tRNA gene for the TAG (amber) codon;

<3nos barnase PTA29< > P35S3 bar 3nos>
5616 Base Pairs

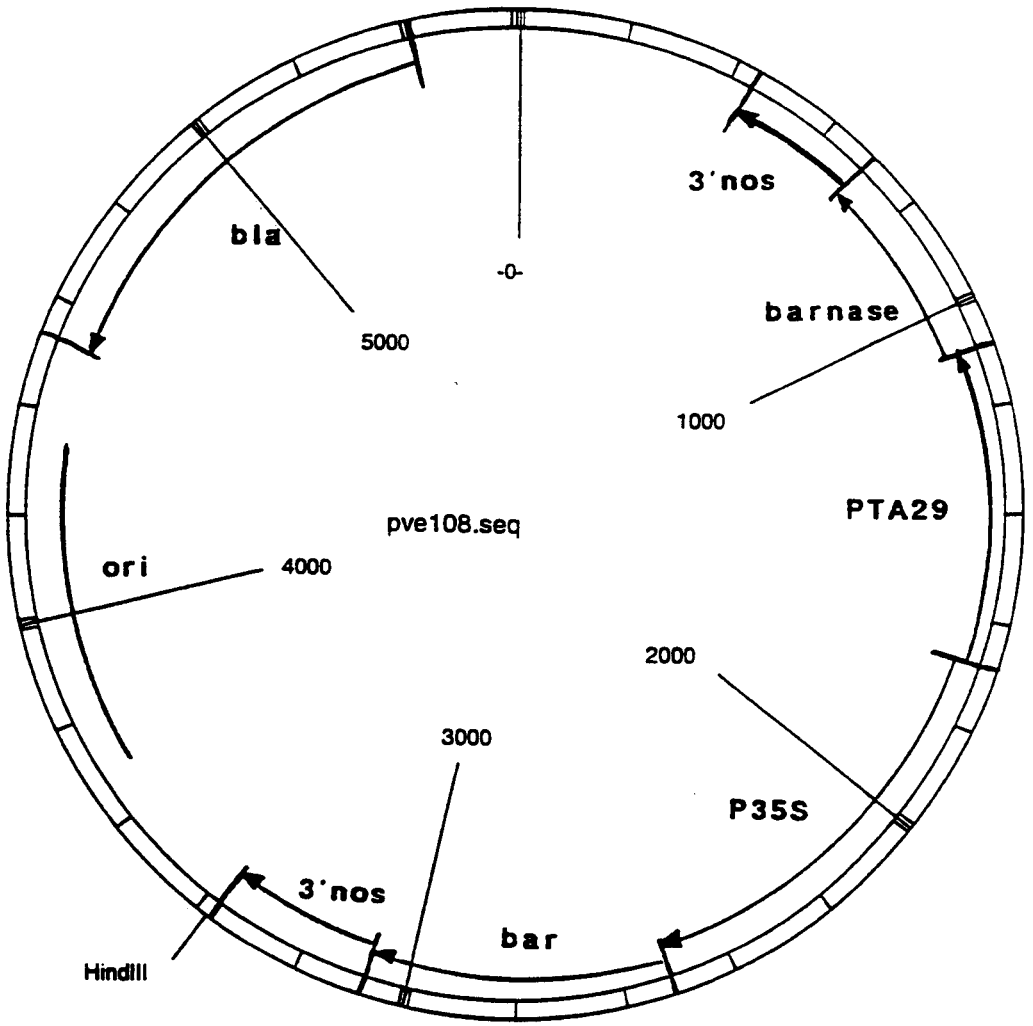

Figure 4.1. Plasmid map of pVE108

pMc5barstar. Ptac-barstar in pMc5-8.
4219 Base Pairs

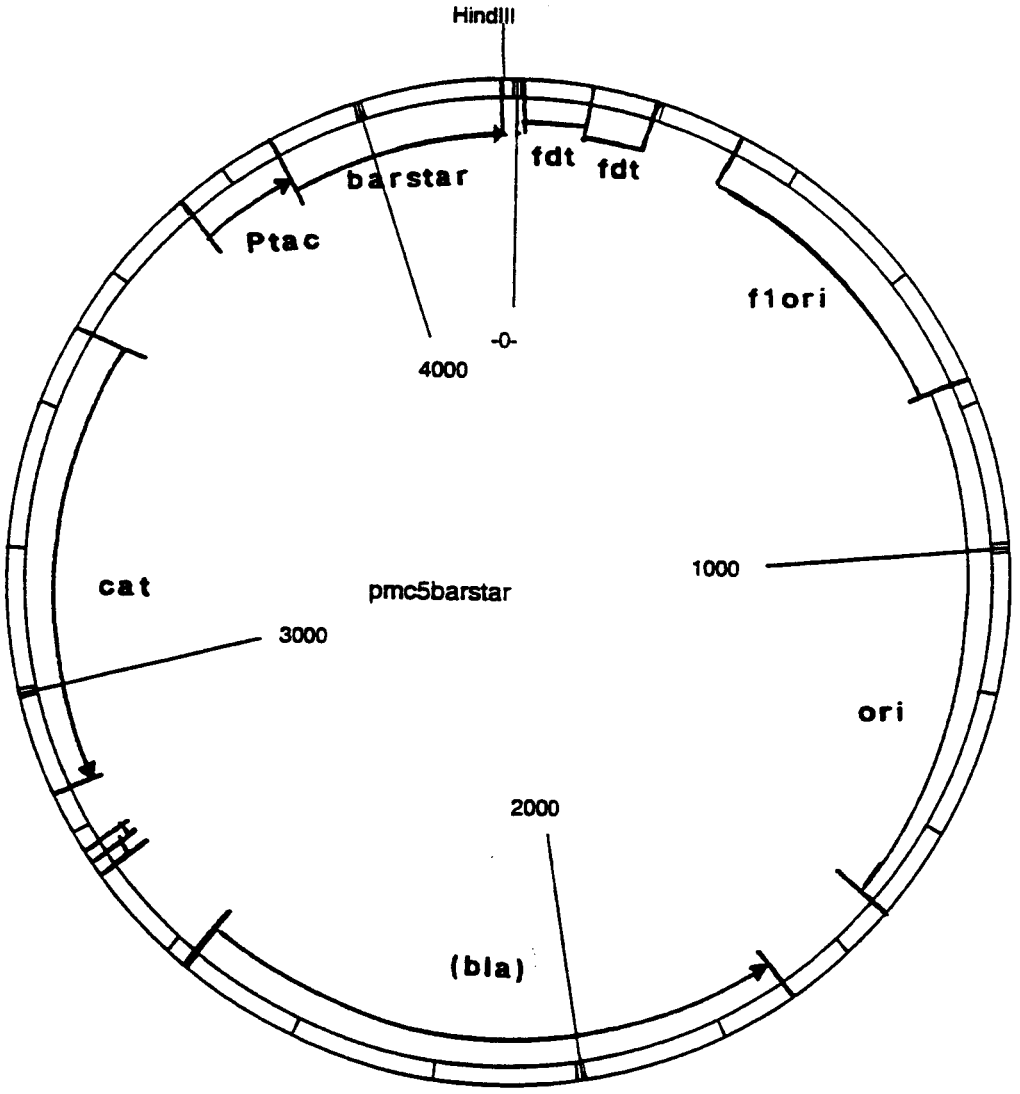

Figure 4.2. Plasmid map of pMc5barstar

- *cat* encodes a chloramphenicol acetyl transferase enzyme that confers resistance to chloramphenicol antibiotics in the bacterial host; this resistance is used to selectively grow bacteria that contain the plasmid;
- the *barstar* gene produces barstar protein.

4.2. Transformation procedure

Plant transformation was performed using electroporation of corn tissue. Introduction of DNA into plant protoplasts via electroporation is a well-known procedure. By giving electrical pulses of high field strength, the cell membrane is reversibly permeabilized so that DNA molecules can be introduced into the cell (Fromm et al., 1985).

Electroporation of protoplasts has been used to produce stable corn transformants. However, these plants were not fertile (Rhodes et al., 1988). Dekeyser et al. (1990) demonstrated that intact tissues were susceptible to electroporation-mediated DNA uptake as well : transient expression of reporter genes was obtained. D'Halluin et al. (1992) reported stable, fertile transformants of corn. While the procedure is described in Annex 6., a summary is presented below.

The immature embryos are first preplasmolyzed for a few hours and rinsed several times in fresh substrate to remove nucleases excreted by damaged cells. The preplasmolysed tissue is then coincubated with linearized plasmid DNA for about one hour prior to application of the electrical pulse. Efficient DNA delivery into tissues requires a pulse duration that is 5 to 10 times higher than the pulses used to electroporate protoplasts. The electroporated tissue is then cultured on a plant tissue culture medium containing a selective agent, e.g. glufosinate ammonium.

Molecular analysis of transgenic lines produced by tissue electroporation has shown that the insertion pattern can vary from very simple (one intact copy) to very complex (>50 copies with fragments and rearrangements of the original plasmid). Since for the majority of transformants the transgenes segregated as a single dominant unit, they contain only one insertion site or different closely linked copies (D'Halluin et al., 1992). Tissue electroporation has been used for the co-delivery of two different plasmid DNAs. Transgenes on both plasmids were expressed and co-segregated as a single, dominant unit indicating that the two DNAs were integrated at the same loci.

4.3. Transformation event MS3

4.3.1. Recipient inbred line H99

The corn inbred line H99 was used as the recipient line for transformation. H99 is an inbred line developed at Purdue University, West Lafayette, Indiana (USA), where it was released in 1974. It was produced by self-pollination in the population called Illinois Synthetic 60C, which in turn was developed by crossing the USDA Blight Resistant Double population with the inbred lines B8, Ia55:473, M14, Oh43, Oh45, Oh51A, R160 and R168 (Personal

communication with Dr B. Zehr, Purdue University). H99 was chosen as the recipient line because of its superior qualities in tissue culture, particularly its high frequency of type I callus formation (Duncan et al., 1985; Hodges et al., 1986).

4.3.2. Transformation and regeneration

The transformation event MS3, harboring an insertion of the male sterility gene construct in a single locus, was obtained via a transformation experiment started in April, 1991.

Plasmid pVE108 was grown together with the helper plasmid pMc5barstar in *E.coli* WK6. In order to reduce the amount of 'helper' plasmid (pMc5barstar) in the pVE108 DNA preparation used for plant transformation, chloramphenicol (the selectable marker for pMc5barstar) is omitted from the culture medium at the final growth step. In the absence of chloramphenicol, pMc5barstar is gradually reduced in the culture through plasmid incompatibility with pVE108 (Sambrook et al., 1989).

The transformation event MS3 was obtained by electroporation of enzymatically-treated immature embryos of the inbred line H99 in the presence of 10µg DNA from a pVE108 plasmid preparation linearized with the restriction endonuclease *Hin*dIII. Immediately after electroporation the embryos were cultured on Mh1VII medium supplemented with 0.2M mannitol and 2mg/l glufosinate ammonium (Mh1VII: N6 macronutrients, N6 micronutrients, N6 vitamins (Chu et al., 1975), 0.5g/l 2-(N-morpholino)ethanesulfonic acid (Mes), 1 mg/l 2,4-D, 2% sucrose, and solidified with 1.6g/l Phytagel (Sigma) supplemented with 0.75g/l $MgCl_4$, pH5.8) in the dark. Ten days later the embryos were transferred to Mh1VII substrate without mannitol and 10 mg/l glufosinate ammonium and further cultered in the dark. Three weeks later the developing embryogenic tissue was isolated and transferred to MS medium (Murashige and Skoog, 1962) supplemented with 5mg/l 6-benzylaminopurine and 2mg/l glufosinate ammonium and cultured with a daylength of 16 hours. After two weeks, the embryogenic tissue was transferred to MS medium with 6% sucrose, 2mg/l glufosinate ammonium and without hormones. Developing shoots were transferred to half-strength MS medium with 1.5% sucrose for further development into plantlets. Plantlets, approximately 10-15cm in length, were sprayed *in vitro* with a 1% glufosinate ammonium solution. Regenerated plant #RZM34-1, subsequently designated transformation event MS3, was transferred to the greenhouse on July 31, 1991.

5. CHARACTERIZATION OF THE MALE STERILE CORN TRANSFORMATION EVENT MS3

The particular DNA insert to be considered for non-regulated status has been designated MS3. The commercialization strategy for SeedLink™ in corn will be to use traditional backcrossing to transfer the MS3 allele, resulting from one specific transformation event, into elite commercial inbreds to be used as female parents in F_1 hybrid seed production. Consequently, this petition requests non-regulated status for the MS3 event, not only in the recipient inbred line H99 but in any corn genotype, traditional corn lines and other corn lines having received the non-regulated status.

Transformation event MS3 has been field tested since 1992 in different areas of the United States (Iowa, Illinois, Hawaii), in Belgium, France, Chile and Argentina.

The event MS3 trials included tests of the stability of male sterility, F_1 hybrid seed production trials, F_1 hybrid yield trials, efficacy trials in order to determine the tolerance level to glufosinate ammonium and backcrossing programs involving a large variety of corn elite inbred lines. In general, comparable male sterile and male fertile versions of the test material were cultivated. Observations were made by corn breeders and by company researchers on agronomic performance and characteristics, and also on disease and pest characteristics of the test material. Also, plant material was sampled for biochemical analyses.

Annex 10 includes termination reports submitted to the USDA for the environmental releases that have been completed in the United States. Annex 9 includes selected reports of the environmental releases that have been completed in Europe.

5.1. The structure of the insert in male sterile corn transformation event MS3 in a H99 background

Molecular analyses of the transgenic plants were carried out to determine the number of insertion sites and the structure of the MS3 event. A summary is presented below. A detailed description of the analyses is outlined in Annex 7.

The transgenic DNA in male sterile corn transformation event MS3 is inserted at a single locus

The number of loci containing pVE108 transgenic DNA has been determined by Southern blot analysis. This laboratory analysis showed that the DNA derived from the plasmid pVE108 has been inserted at a single site in the corn genome.

The structure of the insert has been characterized in detail (Figure 5.1.)

Molecular analyses of the genomic DNA of event MS3 have demonstrated that three copies of the pVE108 plasmid and parts of the pMc5barstar plasmid have been inserted at one site

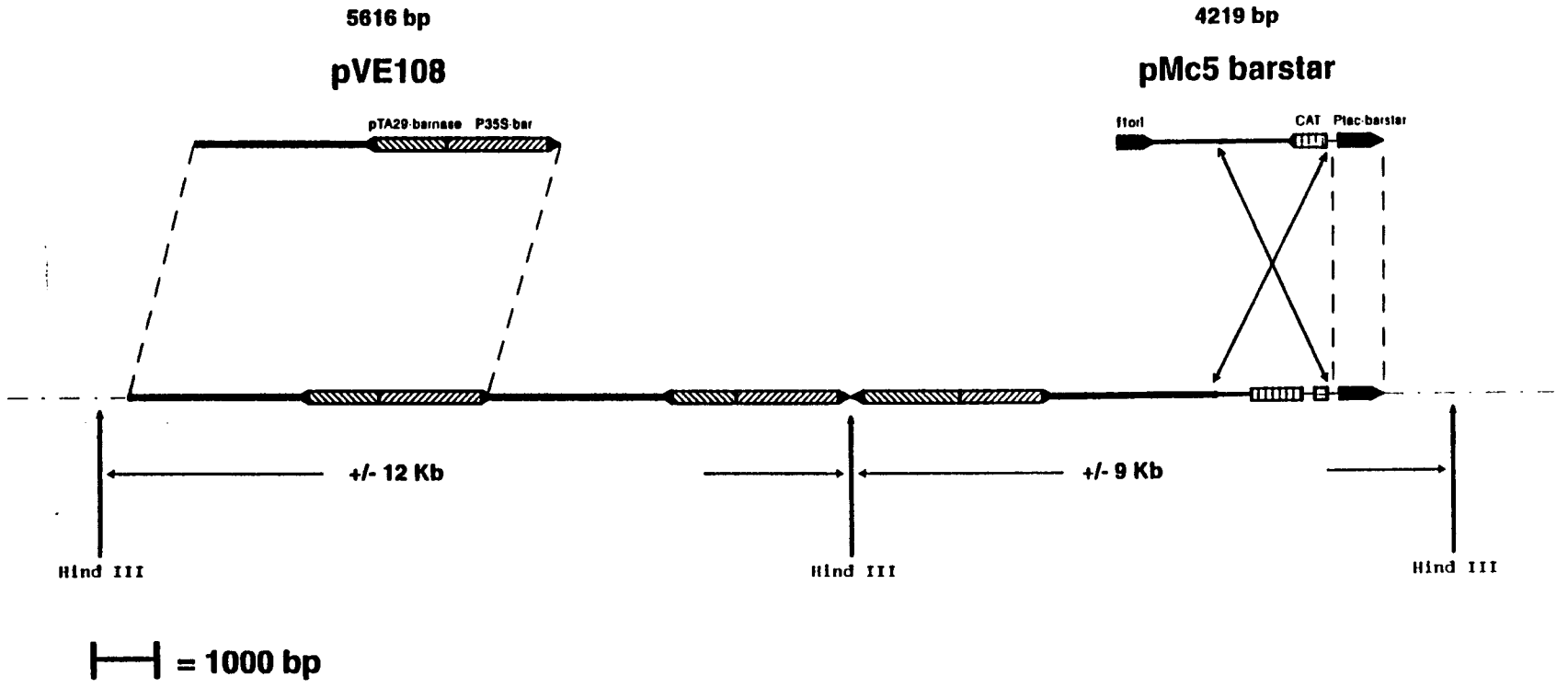

Figure 5.1. Schematic presentation of the structure of the MS3 insert

of the corn genome. The inserted DNA resides on two adjacent HindIII fragments :

- a ±12kb HindIII fragment consists of a head-to-tail dimer of pVE108,
- a ±9kb HindIII fragment consists of one pVE108 copy and a rearranged piece of pMc5barstar.

The pVE108 copy on the ±9kb HindIII fragment forms a tail-to-tail configuration (with the *bar* genes oriented towards each other) with the dimer on the ±12kb HindIII fragment. The pMc5barstar copy has lost the f1*ori* and contains rearranged *ori*, *bla* and *cat* units and *barstar* gene. For a detailed description, we refer to Annex 7.1..

5.2. The pattern of expression of the introduced transgenes in male sterile corn transformation event MS3 in a H99 background

The spatial and temporal expression pattern of the *bar* and *barnase* gene was determined :

- the expression of the *bar* gene was investigated by Northern blot analysis, while PAT activity was determined via a specific PAT activity assay;
  mRNA from *bar* was detected in leaves and immature kernels, but not in roots, dry seeds, and germinating seeds (see Annex 7.2.); no PAT activity could be determined above detection level in seeds carrying event MS3 (see Annex 7.5.);
- the expression of the *barnase* gene was deduced from the plant phenotype; any expression of the *barnase* gene would lead to disruption of the normal cell function; with the exception of the male sterility trait, transgenic plants containing event MS3 developed in a way comparable to non-transgenic corn (see Annex 8 and Annex 9.); therefore, it was concluded that the expression of the *barnase* gene is limited to the tapetal cell layer.

No other coding sequences are expressed as determined by the absence of mRNAs corresponding to any of the coding regions of pVE108 and pMc5barstar that are present in MS3 (see Annex 7.3.). No Bla activity could be determined above detection level in seeds carrying the event MS3 using a specific Bla activity assay (see Annex 7.6.).

5.3. The stability of event MS3 in H99 background and in other genetic backgrounds

The procedures for maintenance and multiplication of a SeedLink™ male sterile line are described in Chapter 3.

The primary transformant carrying event MS3 was pollinated with pollen of corn inbred line H99 for multiplication of this genetic material. The transgenic plants of the first - and further -progeny generations were used as females, on the one hand for continuous multiplication of event MS3 in H99, and on the other hand for experimental F_1 hybrid production and for conversion of other corn inbred lines with event MS3 (backcrossing programs).

Since the *barnase* gene and the *bar* gene are physically linked, these genes will segregate in the offspring as a single locus in a 1:1 segregation ratio (dominant functions): 50% of the progeny plants are herbicide tolerant and male sterile, the other 50% of the progeny plants are herbicide susceptible and male fertile. The latter phenotype is eliminated in each generation of a multiplication or of a backcrossing program by treating the plants with

glufosinate-ammonium. Thus, at each generation the segregation of the transgenes can be monitored and the linkage between the two genes can be confirmed.

In the following text several examples of segregation of the *bar* and *barnase* genes in maintenance, multiplication and backcrossing procedures are given. The examples demonstrate the genetic stability of event MS3 in these processes.

The results of segregation studies carried out in the greenhouse in the first and the six subsequent progenies (all H99) of the primary transformant carrying the event MS3 are presented in Table 5.1. Herbicide tolerance was evaluated in these experiments either by spraying the plants at the 3-4 leaf growth stage or by "brushing" a 0.5% glufosinate ammonium solution on the 4th or 5th leaf (dot-test). Herbicide susceptible plants can survive the latter test and can be grown to maturity.

Table 5.1. Segregation data for event MS3 (H99; greenhouse studies)

Generation	Number of glufosinate-ammonium tolerant and male sterile plants	Number of glufosinate-ammonium sensitive and male fertile plants	X^2 *
M1	32	35	0.06 n.s..
M2	13	18	0.52 n.s.
	23	22	0.00 n.s.
M3	74	69	0.11 n.s.
	63	57	0.30 n.s.
M4	35	53	3.68 n.s.
	5	5	0.00 n.s.
M5	24	16	1.60 n.s.
	42	33	0.85 n.s.
M6	41	63	4.66* p<0.05
	48	51	0.04 n.s.
M7	56	43	1.98 n.s.
Pooled	456	465	0.07 n.s.

* : uncorrected X^2 goodness-of-fit test for hypothesis of 1:1 segregation
n.s. : not significantly different at the 0.05 level

The data (Table 5.1.) indicate that MS3 is a stable insertion event and is transmitted to the progeny as a Mendelian dominant gene. The linkage between herbicide tolerance and male sterility is absolute : all glufosinate-ammonium tolerant plants are male sterile and all glufosinate-ammonium sensitive plants are male fertile.

An example for the segregation of the *bar* gene in four subsequent generations of a backcrossing program is given in Table 5.2. The backcrossing program was carried out in the greenhouse, and embryo rescue was used in the process. The immature embryos were placed on PPT containing agar medium for germination. The segregation ratio presents the number of embryos that survived this selection and gave rise to plantlets compared to the number of non-developing embryos, eliminated through this selection. The recurrent parent is an elite inbred line (designation U03). Subsets of tolerant plants of each backcrossing generation were grown to maturity. These plants were male sterile.

Table 5.2. Segregation data for event MS3 in a backcrossing program (based on embryo rescue; greenhouse study)

Generation	Number of embryos cultivated on selective medium	Number of glufosinate-ammonium tolerant embryos	Number of glufosinate -ammonium sensitive embryos	X^2 *
BC_2	255	126	129	0.02 n.s.
BC_3	240	110	130	1.67 n.s.
BC_4	127	63	64	0.00 n.s.
BC_5	727	364	363	0.00 n.s.

* : uncorrected X^2 goodness-of-fit test for hypothesis of 1:1 segregation
n.s. : not significantly different at the 0.05 level

Table 5.3. presents an example of the segregation of the *bar* and *barnase* genes in a field experiment (see also Annex 9., page 37). Plant emergence and segregation for glufosinate-ammonium tolerant plants were determined in subplots (4 x 15 m) of each of the three replicates of the trial. The entire trial included approximately 3290 glufosinate tolerant plants (event MS3 in H99). Except for four plants, all glufosinate-ammonium tolerant plants were completely male sterile. The four plants were male fertile and are thought to be "escapes" from the herbicide treatment.

The inheritance of the transgenes has been monitored in a large number of greenhouse and field experiments. A selection of these experiments is presented in Annex 8., Annex 9. and Annex 10. It can be concluded from these experiments that, in general, the segregation ratio for the linked *barnase* and *bar* genes did not differ significantly from the 1:1 ratio that is expected in accordance with Mendelian inheritance of dominant genes.

Table 5.3. Segregation data for event MS3 in a field experiment (H99 background)

Material	Number of emerged plants (subplots)	Number of glufosinate-ammonium tolerant plants (subplots)	X^2 *
Event MS3 (in H99, 6th maintained generation)	671	329	0.21 n.s.

* : uncorrected X^2 goodness-of-fit test for hypothesis of 1:1 segregation
n.s. : not significantly different at the 0.05 level

Molecular analyses of event MS3 in various generations, including maintenance products, F_1 hybrids, and backcross generations have shown that the structure of the transgenic DNA is indistinguishable for the primary transformant and its progeny (see Annex 7.4.).

Event MS3 induces male sterility in corn plants by inhibiting the production of functional pollen grains in the anthers. The male sterile anthers are shriveled and non-dehiscent, and the spikelets containing such anthers are thin and flattened. The shriveled anthers usually do not contain starch-filled pollen grains. In some instances the squashes from male sterile anthers showed a few shrunken cell walls of microspores or pollen grains. Usually, the male sterile anthers do not exsert from the spikelets. In some instances it was observed that the spikelets dried out and popped open, and the shriveled male sterile anthers emerged. The tassel of male sterile plants carrying event MS3 can appear more slender than the tassel of the non-transgenic male fertile counterpart.

A stable male sterile phenotype in the inbred seed-parent is crucial for the field production of F_1 corn hybrids. With respect to stability of the male sterility trait, the following features have been determined for event MS3.

Event MS3 induces in an inbred genetic background, such as line H99, complete male sterility. Sterility fluctuation with the environment has never been detected. Distinct environments (the corn belt and Hawaii in the United States, Flanders in Belgium, 'les Pyrénées Atlantiques' in France, the Region Metropolitana in Chile and Miramar in Argentina) have been selected for field tests, and H99 plants containing event MS3 were completely male sterile in all environments, while their non-transgenic counterparts were male fertile (see Annex 8., Annex 9. and Annex 10.).

Some variation in the degree of male sterility has been noticed in F_1 hybrid plants : tassels were not completely but partially sterile; non-dehiscent and/or dehiscent anthers emerged from a few or from many spikelets of both the main axis and the lateral branches of the tassel. An example for this observation is presented in Annex 8, page 7. In this experiment the tassel phenotype of plants from 6 different F_1 hybrids has been carefully examined; depending on

the genetic constitution of the F_1 hybrid, only a very few (5 to 10) and pollenless anthers per tassel exserted, or anthers emerged from up to 50% of the spikelets and shed pollen. However, in the subsequent BC generation the degree of male sterility increased again : the majority of BC_1 combinations and BC_1 plants containing event MS3 were completely male sterile (see Annex 8, page 11). Therefore, the occurrence of partial sterility in the F_1 generation can be explained by variable expressivity, which means that the extent to which event MS3 is expressed phenotypically in different individuals varies. This lack of full expression may be due to genotypic factors.

Conversion programs involving event MS3 and approximately 30 elite inbred lines have been carefully examined over several backcross generations; in general, the transgenic plants of the BC_3 or BC_4 generation were again completely male sterile, also in cases where the corresponding F_1 plants have shown a certain degree of partial sterility (for examples see Annex 9., page 51 and Annex 10., page 19 and page 22).

Molecular analysis (Southern blot) of the plants that contained event MS3 and were partially sterile confirmed that the insert pattern of event MS3 was not changed (see also Annex 7.4.).

Since partially sterile event MS3 plants shed functional pollen, these plants can be used as pollinators. Consequently, cytoplasmic uniformity in the various inbred seed-parent lines that will be used in future F_1 hybrid seed production can be avoided.

5.4. The agronomic performance of event MS3

In a series of field trials visual observations of several agronomic traits of event MS3 were made, qualitative evaluations were made and some quantitative data were taken.
All evaluations included the non-transformed male fertile genetic counterpart and/or non-transgenic standard corn inbred lines. Reference is usually made to this material (see also Annex 9. and Annex 10.).

Event MS3 confers stable male sterility

Event MS3 induces in inbred line H99 complete male sterility (as already mentioned in the previous chapter, page 27). Backcrossing programs in which event MS3 is being crossed into inbred lines that represent very diverse germplasm types, are at present continuing. Complete male sterility was found in all these genotypes from BC_3/BC_4 generation onwards. Sterility fluctuation with the environment has not been detected.

It can be concluded from the currently available data that neither the environment nor the genetic background in which event MS3 is placed through backcrossing appear to have adverse effects on the stability of male sterility. The exception is the trend for partial sterility in the very vigorous F_1 plants, in BC_1 and possibly BC_2 plants which may be due to variable expressivity of the *barnase* gene (see Annex 8., Annex 9. and Annex 10.).

Event MS3 confers tolerance to glufosinate ammonium

The use of SeedLink™ in seed production includes the application of glufosinate ammonium in female rows of seed production fields in order to selectively rogue the non-transgenic fertile plants (see also Chapter 3.3.).

A rate of glufosinate ammonium has been determined that can eliminate non-transgenic corn. After a one time application of the herbicide at a rate of 300 g a.i./ha on non-transgenic corn seedlings, some plants survived but were stunted, were delayed in their further development and did not reach the flowering stage. A rate of 500 g a.i./ha, sprayed on non-transgenic seedlings, destroyed all corn plantlets (see Annex 9, page 23).

Transgenic plants containing event MS3 exhibit tolerance to glufosinate ammonium at the concentration that efficiently eliminates fertile non-transgenic plants. In a number of field trials the plants with event MS3 were sprayed with rates between 450 and 600 g a.i./ha. These concentrations did not cause adverse effects on yield in seed production (Annex 9, page 37) or on yield of F_1 hybrids that were produced on sprayed seed-parents.

Phytotoxic effects, such as reduced plant vigor, reduced plant height and tillering were observed on plants treated with 1000 g a.i./ha and 2000 g a.i./ha (see Annex 9, page 28).

Event MS3 does not induce differences in general plant features

The germination of seeds containing event MS3 was comparable to the germination of control seeds (for examples see Annex 8, page 19 and Annex 10, page 12 and page 22).

In field tests, no differences were observed between event MS3 and the non-transgenic counterpart or a non-transgenic standard line in seedling emergence or in plant vigor, under favorable and under unfavorable weather conditions (e.g. excessive rainfall, US, spring 1993; cool temperatures, Belgium, spring 1994).

In some instances a reduction in plant height has been observed among plants containing event MS3. A shorter uppermost internode seems to contribute to the reduced plant height. The phenomenon that male sterile plants can be shorter than their male fertile versions has also been described for pollen sterile plants of the CMS type (Duvick, 1965) and for male sterile mutants (Kaul, 1988).

Time to tassel emergence appeared similar in male sterile event MS3 plants and in male fertile plants.

No differences were detected in time to silk extrusion and/or in the process of silk extrusion between event MS3 plants and male fertile plants.

The general appearance of the female inflorescences was not different in plants carrying event MS3 and their non-transgenic counterparts or non-transgenic standard lines. The husk leaves of both the transgenic male sterile and the non-transgenic male fertile plants usually formed a continuous and persistent cover to the caryopses and remained in position around the mature ear.

Female fertility was not adversely affected in event MS3 male sterile plants (see also below, yield). No differences were observed in seed set and in cob size between male sterile and male fertile plants. In both types of plants seed size and seed shape was similar, and the grains were arranged regularly in an even number of rows, as usual for corn.

Event MS3 does not adversely effect yield

Yield parameters such as number of cobs per plant, cob weight, filling of cobs, or 1000 kernel weight have been observed in a number of small-scale trials between 1992 and 1994. No obvious differences between transgenic plants carrying event MS3 and non-transgenic plants were found.

Grain yield has been determined in a hybrid seed production experiment (see Annex 9, page 37). The female (line H99) containing event MS3, was planted at double density and sprayed with glufosinate ammonium in order to rogue the fertile segregants. The production field was planted in a pattern of 4 female to 2 male rows. The non-transgenic control was line H99 which was sown at normal density and detasseled by hand. The yield data (in quintals per hectare) are presented in Table 5.4. No difference in yield was found between the transgenic (event MS3) and the non-transgenic seed-parent.

Table 5.4. Event MS3 : yield in hybrid seed production

Female line	Yield (qx/ha, at 15% GM)	GM(%)
Event MS3 (in H99)	26.7	13.0
H99 (non-transgenic)	25.8	13.1

note: qx/ha: quintals per hectare; GM: Grain Moisture

This experiment demonstrates the successful application of SeedLink™ in corn. The treatment of the seed-parent with glufosinate ammonium and the less even stand in the female rows (caused by double planting and chemical rogueing of fertile segregants) did not adversely effect the female plants and did not lead to a reduction in seed yield.

The grain yield from F_1 hybrids has also been determined. The hybrids were produced on event MS3 containing seed-parent plants (H99). Fifty percent of the hybrid seed carried the male sterility and the herbicide tolerance genes. The non-transgenic control F_1 hybrids were produced on hand-detasseled non-transgenic H99 seed-parent plants.

Table 5.5. summarizes the results of a test with 5 hybrid combinations in France in summer 1994 (see also Annex 9, page 44).

Table 5.5. Yield of transgenic (event MS3) and non-transgenic F_1 hybrids

F_1 hybrid	Yield (qx/ha, at 15% GM)	GM (%)
Event MS3 x C115	108.8	31.6
H99 x C115	102.7	32.4
Event MS3 x C108	102.3	28.5
H99 x C108	103.7	28.8
Event MS3 x C118	97.5	28.4
H99 x C118	102.6	29.3
Event MS3 x C110	88.0	29.8
H99 x C110	94.6	30.2
Event MS3 x C109	97.0	33.9
H99 x C109	88.6	33.4

note: qx/ha: quintals per hectare; GM: Grain Moisture

No significant difference was detected between the yield of the F_1 hybrids produced on event MS3 (in H99) females and the F_1 hybrids produced on non-transgenic H99 females. Different F_1 hybrid combinations (different pollinator lines used in F_1 production) varied in yield. The same hybrid combinations were also tested in trials in the United States (data not shown); the results are comparable to the results presented above.

Event MS3 does not change the composition profile of kernels

The composition profiles of kernels produced on male sterile plants containing event MS3 and produced on male fertile non-transgenic plants were determined. The seed samples were harvested from 6 male sterile and from 6 male fertile plants. The analyzed seeds represent a BC_5 generation in a backcrossing program and were produced under greenhouse conditions.

The starch composition, total protein and total oil were determined using procedures adapted from the Corn Refiners Association Manual (Standard Analytical Methods for Proteins - June 1980, Starch - April 1986 and Crude Fat - April 1989). The amino acid distribution was determined using procedures adapted from the USDA Chemistry Laboratory Guidebook. The fatty acid distribution was determined using protocols according to Bannon et al. (1982).

The results of the analyses are presented in Table 5.6., Figure 5.2. and Figure 5.3. (additional information can be found in Annex 10.). All data were statistically analyzed by a one-way analysis of variance. No significant differences in content of total protein, starch and total oil and in the distribution of amino acids and fatty acids were detected between seed samples containing 50% seeds with event MS3 and non-transgenic seed samples.

Table 5.6. Composition of kernels (mean values and standard deviation of 6 individual samples)

Seeds harvested from	Moisture %	Protein % As Is Leco	Protein % D.B. Leco	Oil % As Is Spex Mill	Oil % D.B. Spex Mill	Starch % As Is Starch	Starch % D.B. Starch
Male sterile parent (event MS3)	11.60 ± 0.10	9.96 ± 0.64	11.27 ± 0.72	4.31 ± 0.13	4.87 ± 0.15	61.10 ± 1.21	69.11 ± 1.42
Male fertile parent	11.55 ± 0.16	10.25 ± 1.01	11.29 ± 0.84	4.22 ± 0.24	4.77 ± 0.26	59.83 ± 2.16	66.19 ± 6.01

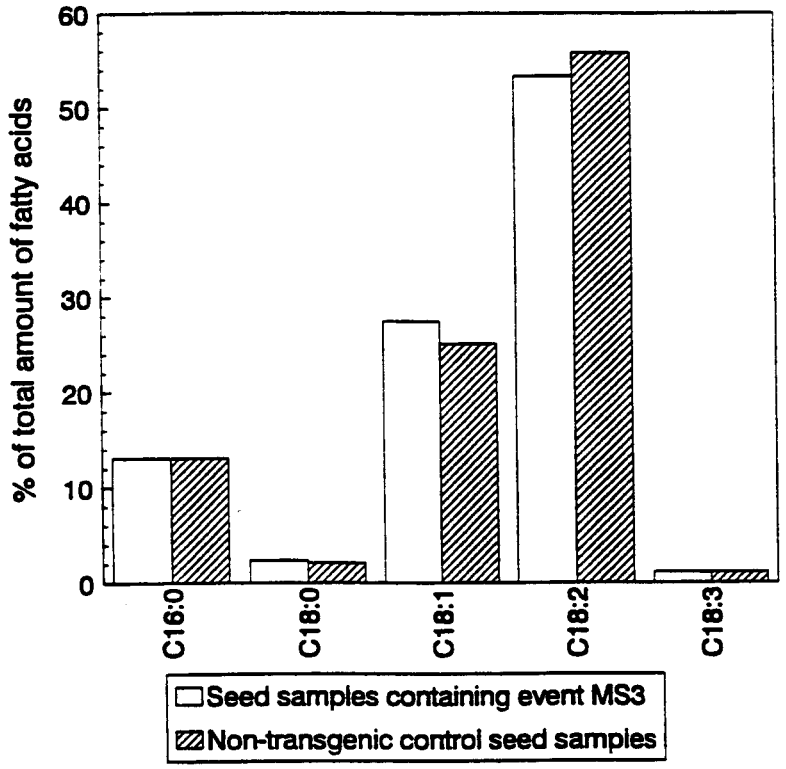

Figure 5.2. The distribution of main fatty acids in seed samples containing MS3 and in non-transgenic control seed samples (average over 6 samples)

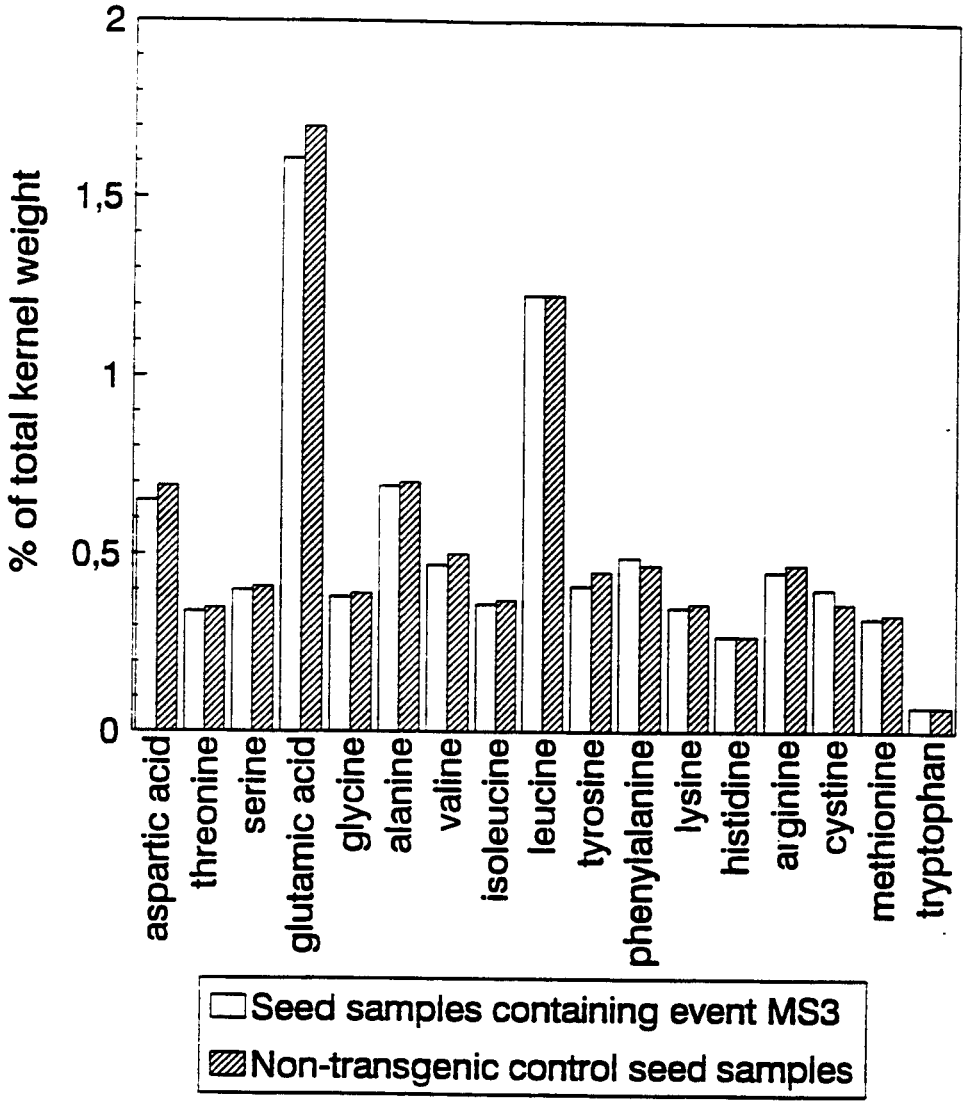

Figure 5.3. Amino acid profile of seed samples containing event MS3 and in non-transgenic control seed samples (average over 6 samples)

Conclusion

It can be concluded from the greenhouse and field observations and evaluations that there were no significant differences in agronomic characteristics between event MS3 and the non-transgenic counterpart with the exception that the non-transgenic material was not tolerant to glufosinate ammonium and was male fertile.

6. ENVIRONMENTAL IMPACT ASSESSMENT

In this part, we document that the introduction of the male sterility gene construct in corn does not cause adverse effects on the environment. Based on the absence of such effects, we will conclude that the impact of hybrid corn harboring the MS3 allele is identical to the impact of non-transgenic hybrid corn cultivated today. The data to support our conclusions were gathered via literature studies, laboratory analyses and via small and large scale field trials; the latter have been especially designed to assess the performance of transgenic corn in the environment. We document that there are no indications to anticipate :

- the introduction of plant pest characteristics,
- significant exposure to new proteins,
- altered weediness and/or invasiveness of the transgenic versus the non-transgenic corn in natural and agricultural environments,
- the cultivation of the transgenic plants to influence agricultural practices or biotic organisms in another way than non-transgenic corn.
- horizontal and vertical gene transfer between the transgenic corn and respectively microorganisms and corn relatives native to the United States.

6.1. No plant pest characteristics have been introduced

The newly introduced coding sequences have been isolated from microorganisms (i.e. *Streptomyces hygroscopicus* and *Bacillus amyloliquefaciens*) that are not known to be plant pests.

Two sequences, i.e. P35S and 3'nos, were isolated from known plant pests, Cauliflower Mosaic Virus and *Agrobacterium tumefaciens* respectively. These sequences were thoroughly characterized and identified to be non-coding regulatory sequences, not related to the pathogenic status of the donor organisms from which they were isolated.

- The P35S promoter of the Cauliflower Mosaic Virus is one of the most frequently-used promoters for plant transformation. This promotor sequence is widely used to obtain a high and constitutive expression level of desirable traits in transgenic plants. Although derived from a plant virus, it is considered by APHIS to be a well-characterized non-coding regulatory sequence that does not present a risk of the introduction and dissemination of a plant pest (Federal Register Vol. 57, No. 216).
- The function of 3'nos is to provide 3' plant functional polyadenylation control signals. The 3'nos sequence is a well-characterized non-coding DNA regulatory sequence. Although derived from *Agrobacterium tumefaciens*, a pathogen of dicotyledonous plants, the 3'nos non-coding sequence is not considered to present a risk of the introduction and dissemination of a plant pest.

The newly expressed traits (male sterility and tolerance to glufosinate-ammonium) are well characterized and fit completely within today's overall breeding objectives for corn improvement. There are no indications that their expression could lead to a new type of plant pest.

6.2. No significant exposure to new products are anticipated

Growing and consuming transgenic corn seeds derived from transformation event MS3, no significant exposure to new products are envisaged for livestock and for human beings.

Based on the expression patterns, exposure to new proteins is highly unlikely.

According to the observed expression patterns, expression of the genes *bar* and *barnase* is limited :

- the *bar* gene is linked to the nearly constitutive P35S promoter; while the *bar* mRNA level was low in most tissues, no *bar* mRNA has been detected above the detection level in seeds of the transgenic corn (see Northern blot analyses in Annex 7.3.)
- the *barnase* gene linked to the specific PTA29 promoter is only expressed in the tapetum cells of the pollen sac during a limited period of time (i.e. during anther development); no *barnase* mRNA levels have been detected in transgenic corn seeds as confirmed by Northern blot analysis (Annex 7.3.).

Corn is the most important feed grain produced in the United States, of which a major part is fed to livestock directly as grain (Perry, 1988). Giving the fact that we were not able to detect mRNA levels of the transgenes in seeds of the transgenic corn, the presence of the whole proteins and/or activity of the enzymes in corn seeds is highly unlikely.
Analysis of PAT activity in seeds confirmed that no such activity could be established above detection limit in seeds (see Annex 7.6.). Detection of barnase has revealed not to be feasible, as even a low detection level results in the deterioration of the expressing cells. Since the seeds are well developed, this fact can be used for concluding that barnase activity is not present at a significant level in seeds.

Processing of corn derived products is an additional guarantee for the absence of exposure to new proteins.

The major corn derived products for humans (i.e. corn oil, High Fructose Corn Syrup (HFCS) and alcohol) are processed before consumption (Watson, 1988). These processing conditions are expected to completely degrade the minute amount of the eventually remaining proteins:

- quality requirements for commercial oil do not allow the presence of any protein upon oil extraction and purification; since proteins are generally water soluble, they are not expected to be a component of refined corn oil; additionally, as part of the oil refining process, crude corn oil is high-vacuum distilled at 225-260°C, degrading any remaining PAT protein which is rapidly degraded at temperatures above 35°C (Watson, 1988; Botterman et al., 1991);
- industrial corn starch contains only about 0.25% protein; HFCS is made from a fully converted dextrose syrup, which is made by treating a corn starch slurry with 0.15N hydrochloric acid and heated under pressure to 140°C (Watson, 1988);
- beer and distilled liquors are the leading products with respect to volume production and utilization of corn in the United States; in beer making, corn grits, which are degerminated broken bits from the hard portion of the endosperm, and corn syrup

(described above), are used as ingredients; during the brewing process, the extract is boiled to concentrate the solids, to sterilize and to precipitate excess proteins (Watson, 1988); consequently, it is extremely unlikely that the new enzymes would be present, and if so, they would be inactivated by boiling. Liquors, such as whiskey and bourbon are distilled solutions and therefore do not contain any protein (Watson, 1988).

The new proteins do not raise safety concerns.

None of the proteins has ever been associated with any pathogenic reaction towards animals or human beings. Thus even under the unlikely event that the newly expressed proteins would be present in the corn seeds, no negative responses are expected after the direct consumption of the seeds by livestock :

- the PAT enzyme is not expected to provoke negative effects after uptake as (Botterman et al., 1991):
  - the PAT enzyme is very sensitive to proteases and therefore it will be rapidly degraded in the intestinal tube;
  - the PAT enzyme is completely inactivated at a pH lower than 5.0 and a pH above 9.5; the stomach of animals and humans has an average pH lower than 3.0;
- the enzyme barnase presents no particular health risk. Other RNases showing the same specificity have been handled for many years in laboratories without any toxicity being reported. RNases are naturally expressed in all plant tissues; each cell contains a certain amount of RNase required by its cellular metabolism. This means that RNases are daily consumed by people and animals.

The new proteins do not raise specific concerns in relation to allergenicity

A computer search for polypeptides homologous to the polypeptides that are encoded in event MS3 (PAT and barnase), has been carried out. The homology of the PAT and barnase polypeptides to other polypeptides in the HIVAA7, PIR42 and Swiss-Prot30 databases was very low and scattered over the polypeptides. Therefore this homology is deemed highly unlikely to be significant.

The changes in plant metabolism do not lead to exposure to new products.

Two types of changes to plant metabolism can be especially envisaged when evaluating the transgenic corn line :

- a localized disturbance of the transcription mechanisms by the RNase :

  The expression of the particular RNase can be considered as the localized enhancement of an existing metabolic pathway. The substrate or the breakdown products are not specific for the barnase. The disturbance confined to the tapetum cell layer, leads to the destruction of the cells in which this change occurred. The absence of major changes in agronomic and developmental characteristics of the selected corn transformation event MS3 (see Annex 9.)

provided extensive evidence that no such disturbance occurs in other parts of the plant.

- addition of an acetylase activity :

  Acetylases are indigenous present in plants. The newly introduced specific acetylase has a high affinity for its substrate phosphinothricin. Phosphinothricin is normally not present in plant cells as it inhibits the activity of the glutamine synthase enzyme, a key enzyme in the nitrogen metabolism and the only known enzyme in plants that can detoxify ammonia in a sufficient way (Wedler et al., 1976; Miflin et al., 1977; Wild et al., 1984; Wild et al., 1987). Therefore, the only new products to be formed would be the metabolites of phosphinothricin upon application on the modified plants (Dröge-Laser et al., 1994).

  AgrEvo USA is actively pursuing the registration of glufosinate-ammonium for use on glufosinate tolerant corn (see USDA petition). Relevant information on residue and metabolite studies is being compiled in that process. In case of the MS3 event, a similar glufosinate ammonium application is considered. However, this application will be targeted at eliminating segregating plants in the hybrid seed production fields. Therefore, the level of residues and metabolites are considered to be negligible in the final product, i.e., the seeds harvested on the F_1 hybrid cultivar, which will not be sprayed with glufosinate-ammonium.

No other changes to plant metabolism have been intended, nor were observed. Based on the detailed plant observations, there are no indications that any other metabolic change has occurred.

6.3. No changes of the agronomic performance or susceptibility to disease and pest infestations have been observed for corn plants derived from transformation event MS3

Performance of corn plants derived from transformation event MS3

The agronomic performance of corn plant derived from transformation event MS3 has been studied in detail via multiple-site multiple-year field trials (see Annex 9.). During the process of selecting and developing the male sterile line, following conclusions were formulated :

- The inserted traits are expressed, resulting in the intended phenotype. These traits are not expected to have any effect on the agronomic performance of the plants as such.
- The breeding and development observations on the transgenic corn plants are identical to those made on the non-transformed control lines in tests performed in a range of environments and when applying distinct agricultural practices.
- There was no record of any unintended change either because of the transgenic nature of the plants, or because of the particular genes expressed.

No altered disease and pest susceptibility

The introduced traits aim at a combination of male sterility and the specific tolerance to glufosinate. Therefore, no change in the susceptibility or (for the same reason) tolerance of the transgenic corn line to diseases and pests was expected to result from the introduction of these traits. In chapter 2. an overview of some of the diseases and pests has been included. To a large extent, they can be controlled by proper management practices. Based on the specifics of the SeedLink™ system, it is not foreseeable that any of these practices would be rendered inapplicable.

These expectations were further supported by sporadic disease and pest observations in the field. Combined with the agronomic and breeding observations, attention was given in field trials to the effect of diseases and pests on the transgenic corn. Since all trials basically represented comparisons between transgenic material and their non-transgenic controls, or involved segregating populations, they offered a realistic approach to the possible identification of unintended changes in the susceptibility. From 1992 on, event MS3 has been field tested in Belgium, France, United States, Chile and Argentina. No differences in disease susceptibility or insect infestation or differences in severity of the attack have been detected between event MS3 and the control line H99 (see Table 6.1.).
To control the development of initial diseases and pest populations or to slow down their rates of increase, general control farming practices have been applied. The transgenic as well as the non-transgenic control corn plants were successfully treated with appropriate pesticides to protect the transgenic and non-transgenic corn plants.

Table 6.1. Disease and pest observations on event MS3(H99 background) and control line H99

Locations	Examples of observed disease infestations	Examples of observed pest infestations	Remarks
Belgium	*Ustilago maydis*	-	No differences in susceptibility observed
France	-	*Ostrinia nubilalis* *Sesamia nonagrioides*	No differences in susceptibility observed
USA	Stalk rotting diseases Ear molds and smuts Leaf anthracnose Leaf rust	*Ostrinia nubilalis* Aphids, Leafhoppers Thrips, Spider mites Earworms	No differences in susceptibility observed
Argentina	*Puccinia sorghi*	-	No differences in susceptibility observed

6.4. Insertion of the male sterility gene construct did not alter the weediness characteristics of transformation event MS3 compared to its non-transgenic control line

Non-transgenic corn is not regarded as a weedy species

Whether corn plants can establish, colonize and invade new habitats (i.e. have weedy characteristics), depends on the relationship of these plants with their environment. In general, weeds have been specified as plants interfering with the objectives and requirements of people as they may be unwanted and undesirable in some human environments during particular periods of time. As reported by Baker (1974), weedy species possess 'general purpose genotypes' in which adaptability and plasticity form critical components of the adaptive strategy. As described by Baker (1965, 1974), an ideal weed would germinate readily under a variety of conditions, have hardy seeds, not all of them germinating at once, and grow fast from germination to flowering. Such a plant would continuously produce seeds : a few under very adverse conditions but an enormous number under favorable conditions. In addition, an ideal weed would possess adaptations for both short and long distance dispersal and compete effectively interspecifically (Keeler, 1985). In these respects, corn has not been regarded to posses weedy characteristics, nor has been reported as a weed (Keeler, 1985). In order to yield some offspring, the crop needs agricultural management (Gould, 1968; Jugenheimer, 1976).

In managed areas, some of the most troublesome weed problems are the growth of volunteer economic plants with a planted economic crop, e.g. volunteer corn in soybeans (*Glycine max L.*) (Scott and Aldrich, 1970). However, given the domesticated characteristics of the corn crop, it is generally known that corn plants do not survive long. In addition, currently used managing practices for corn volunteer management are sufficient to control the corn volunteers (Gould, 1968; Olson and Sander, 1988). In this respect, it has to be highlighted that none of the products used in today's practices for volunteer management, are based on glufosinate-ammonium. Glufosinate is also not used in any of the crops prone to be found in normal crop rotation cycles.

The insertion of the glufosinate tolerance trait and the male sterility trait did not alter the weediness characteristics of corn

Based on the nature of the introduced traits, it was not anticipated that the introduction of the transgenes would change the competitive performance of the event MS3 and/or its progeny, since :

- with reference to the *bar* gene :
  incorporation of this gene leads to glufosinate tolerance; consequently the transgenic corn plants will only get a competitive advantage over their non-transgenic wild-type plants when some selective pressure (i.e. glufosinate-ammonium application) is present; due to characteristics of the herbicide (contact herbicide, with rapid decay), a competitive advantage can only be anticipated when such herbicides are directly applied on the crop (see Annex 9. and Annex 10.);

- in unmanaged and semi-managed environments, no glufosinate-ammonium is used; however, in unmanaged and semi-managed areas, some selective pressure might be created through unintended drift from glufosinate-ammonium; this indirect pressure level will be at a lower level than the normal field dose; since it was shown that corn plants not having the PAT function can survive a dose of 200 g active ingredient per hectare, one can envisage that non-transgenic plants are able to survive such influx and will continue to compete for the available resources;
- there are no reasons to anticipate that the glufosinate tolerant corn will turn into unmanageable weeds in managed areas, since glufosinate-ammonium is not used to control volunteer corn in crop rotations of e.g. soybean; if in the future, a crop rotation crop would be designed to contain the same tolerance (e.g. glufosinate tolerant soybean), then glufosinate-ammonium may be used to control all weeds except volunteer glufosinate tolerant corn; these volunteers would therefore be controlled in a way compared to what is known today (see Annex 9.) and would not result in a net loss of options to the farmer relative to the current situation;
- in industrial areas and hedges, glufosinate-ammonium can still be used as non-specific broad-spectrum herbicides considering the fact that if the modified corn plants would survive a glufosinate application, this will be observed at an early stage and other treatments (mechanical or chemical) (see 95GZM005 in Annex 8.) can be chosen;

- with reference to the *barnase* gene :
  incorporation of this gene leads to male sterility, which will not give the plants any selective advantage over the non-transgenic wild-type plants.

Detailed experimental observations (see different field trial reports in Annex 9.) confirmed that the insertion of the *bar* gene and the *barnase* gene did not alter the colonization capacity or weedy character of event MS3 :

- the shape and the size of the transgenic seeds were identical to that of the original variety; there were no developmental structures (such as hair and needles) facilitating transport;
- the germination ability and dormancy of the seeds of the transgenic line did not differ from the control variety;
- plant development, growth ability and vegetative vigor of non-transgenic and transgenic plants were comparable; additionally, event MS3 did not differ from their non-transgenic counterpart in its response to light, water and nutrients;
- no differences in seed dispersal were observed between the transgenic and non-transgenic plants : all kernels were firmly attached to the cob; no transgenic neither non-transgenic corn volunteers have been observed in the Belgian (suboptimal growing conditions) and French field trials; though event MS3 may volunteer (see United States growing conditions), the range in number of volunteers will not be different from their non-transgenic counterparts;
- the transgenic and non-transgenic corn lines responded identically to the different growing conditions; normal agronomic cultivation and managing practices were needed to yield some offspring; even under suboptimal conditions no significant differences

in growth ability and development rate between the transgenic and non-transgenic corn were observed.

Hybridization between the transgenic corn and wild corn relatives is highly unlikely to result into glufosinate tolerant wild corn relatives

As stated in 2.2.1., hybridization between *Tripsacum* spp., the only relatives of corn native to the United States, and corn does not naturally occur. The introduced traits do not facilitate the spread of genes from corn to any wild relative. Consequently, transfer of the new traits to wild corn relatives is highly unlikely.

6.5. Impact on agricultural practices

SeedLink™ will improve corn hybrid seed production schemes.

In seed production fields, the use of the SeedLink™ male-sterility system broadens the scope of procedures available for corn hybrid seed production. Though the use of the system will require proper adaptation of the production field design, this is not anticipated to introduce any major change. In fact, the overall production scheme is expected to be implemented easily and be more cost-effective.

SeedLink™ does not introduce any change in the cultivation of the F_1 hybrid.

Since this particular application aims at the hybrid seed production stage, there will be no impact on the agricultural or cultivation practices of the farmer. The farmer will acquire F_1 hybrid seed of the same superior quality.

The use of glufosinate-ammonium is limited to the F_1 hybrid seed production fields.

Glufosinate-ammonium is only applied on the female rows of the hybrid seed production fields. This represents approximately 0.5% of the total corn acreage. Consequently, the relative increase in the use of glufosinate-ammonium due to SeedLink™ is minute. Because event MS3 would be present in only 50% of the F_1 hybrid seed, SeedLink™ does not provide a method to allow the use of glufosinate-ammonium by the farmer.

6.6. Effect on non-target organisms

The male sterility system has been designed to work in plant material only. Since 1992, event MS3 has been field tested at numerous sites across the world. No negative interactions between transgenic corn and non-target organisms such as beneficial insects, birds, animals, have ever been observed. Under the different circumstances, the transgenic plants and their hybrid progeny did not show toxicity effects toward insects, birds or animal species that frequent corn field trials.

6.7. Indirect plant pest effects on other agricultural products

Above it was documented that the functions of the introduced genes are well defined. Phenotypic evaluations confirm the proper expression of the genes (glufosinate ammonium tolerance and male sterility). Genetic stability was documented. None of the functions is targeted at changing the use of the agricultural product. Furthermore, detailed agronomic analyses confirm the absence of any change, warranting a special treatment of these products. No direct plant pest effects on other agricultural products are anticipated.

6.8. Other transfers

The USDA's Interpretive Ruling on Calgene, Inc., Petition for Determination of Regulatory Status (FR 57, No. 202, pp 47608-47616, October 19, 1992) states that : "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. Whether genes can be transferred from plants to micro-organisms such as bacteria remains a controversial issue. Since no additional indications are available for such hypothetical transfers, we can only comment on the consequences of such a transfer.

In order for any horizontal gene transfer to lead to a new type of micro-organisms, some of the following conditions need to be fulfilled :

- the uptake should result in the incorporation of the intact coding DNA sequence;
- the expression should represent a significant increase over the background level; it has to be taken into account, that the genes inserted in the plants have originally been isolated out of bacteria; it can be anticipated that in most environments there will be a background level of expression in wild type bacterial populations;
- the traits should convey a competitive advantage to the strain in which they are incorporated :
  - the *bar* gene has been isolated from *Streptomyces*; other micro-organisms containing the gene could have an advantage during the application with glufosinate-ammonium; since the application and the soil residue is limited both in doses and in duration, this advantage is very limited;
  - expression of the *barnase* would be deleterious to the micro-organism;
  - the host range of the *ori* replicon seems to be limited to *E. coli* and a few other organisms such as *Serratia marcescens* (Balbás et al., 1986);
  - human bacteria already contain extended spectrum *bla* resistances; the particular *bla* of pUC19 is not effective against the newer β-lactam antibiotics; the extended spectrum *bla* genes are present on highly transmissible multi-drug resistance elements, infinitely more capable of horizontal movement than corn DNA; consequently, the presence of the *bla* of pUC19 in event MS3 does not incur a significant risk of contributing to the spread of antibiotic resistance.

Since none of these conditions seem to be fulfilled, we conclude that the impact of any remote potential for horizontal transfer is negligible.

7. STATEMENT OF GROUNDS UNFAVORABLE

Plant Genetic Systems (America) Inc. is unaware at this time of any conditions that are unfavorable to this request for nonregulated status of SeedLink™ hybrid corn seeds.

8. LITERATURE

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CORN MS3 - ANNEX

Annex 1. Letter of Professor Emeritus Walton C. Galinat (Univeristy of Massachusetts, Cooperative extension system)

Annex 2. Letter of Bryan Kindiger (USDA-ARS, Southern Plains Range Research Staion, Oklahoma)

Annex 3. Mariani, C., De Beuckeleer, M., Truettner, J., Leemans, J., Goldberg, R.B. (1990). Induction of male sterility in plants by a chimaeric ribonuclease gene. Nature, **347**, 737-741.

Annex 4. Mariani, C., Gossele, V., De Beuckeleer, M., De Block, M., Goldberg, R.B., De Greef, W., Leemans, J. (1992). A chimearic ribonuclease-inhibitor gene restores fertility to male sterile plants. Nature, **357**, 384-387

Annex 5. Detailed description of the DNA used in transformation : pVE108 and pMc5barstar

Annex 6. D'Halluin, K., Bonne, E., Bossut, M., De Beuckeleer, M., Leemans, J. (1992). Transgenic Maize Plants by Tissue Electroporation. The Plant Cell, **4**, 1495-1505.

Annex 7. Molecular characterization of transformation event MS3

Annex 8. Greenhouse data of event MS3

Annex 9. Field data of event MS3 (Europe)

Annex 10. USDA field trial termination reports

Annex 11. Description of glufosinate ammonium

Annex 1. Letter of Professor Emeritus Walton C. Galinat (Univeristy of Massachusetts, Cooperative extension system)

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September 13, 1994

Dr. Mark Williams and
Dr. Suri Sehgal
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Dear Sirs,

As a neutral expert on the relatives of corn in the U.S., I am glad to assist you.

The answers to your several questions are summarized here from several of my papers with copies enclosed for greater details about the relatives of corn from wild habitats in the U.S.

I. The genus Tripsacum is the second closest relative of corn. It is based on 18 pairs of chromosomes rather than the 10 pairs of corn and teosinte. All 16 species of Tripsacum are perennials. Only three of these are adapted to continental U.S. area. (T. dactyloides (2n & 4n), T. floridanum (2n), and T. lanceolatum (4n)).
Tripsacum dactyloides grows throughout the eastern half of the U.S. with the tetraploids near the east coast, sometimes in the salty soils where a river meets the ocean. The diploids are more in the mid-west of the U.S. Resistance to corn rootworm comes from T. dactyloides (Branson 1971).
T. floridanum is a small narrow-leafed diploid confined to open or the edge of pine lands in Florida.
It has been a source of genes for resistance to Helminthosporium turcicum (Hooker and Perkins, 1980) and corn leaf aphis (Branson, 1972).
T. lanceolatum is a tetraploid that occurs in the Southwest.

There is no evidence for natural hybridization between corn and Tripsacum in North America let alone just United States. There is controversial evidence for such introgression in South America.

Experimental hybridization and introgression between corn and Tripsacum is difficult but possible, usually requiring embryo culture. Some of the chromosomes have been cross-mapped by using old-fashioned marker genes (Galinat, 1973). Molecular markers are now being used for this purpose at CIMMTT in Mexico.

II. Teosinte is not only the closest relative of corn, it is the wild form of corn. It crosses freely with corn and is a good

source for corn improvement. Introgression from teosinte may be used to increase the hybrid productivity of corn and serve as a source of genes for disease and insect resistance. The perennial teosintes (2n & 4n) are a good source of resistance to virus diseases while the annual teosintes provide resistance to fungus diseases.

The habitat and distribution of teosinte is normally confined to Mexico, Guatemala and Honduras. It is know to have survived as an escape from cultivation in Florida and Texas. A day-neutral cultivar of teosinte occasionally grows as an escape in the corn breeder's nursery, but it is not considered as a serious weed and is easily killed with "Round-up" herbicide.

In parts of Mexico, teosinte is deliberately planted in corn fields because of its known beneficial effect on the corn. But the survival of the teosinte as a wild plant is damaged by introgression from corn and so natural selection in teosinte favors crossing barriers to corn such as different flowering times and gametophyte factors excluding corn pollen.

Details that I haven't reported here, you can find in my reprints enclosed.

If I can be of further assistance, do not hesitate to request it.

Sincerely,

Walton C. Galinat

Walton C. Galinat
Professor Emeritus

cc: Dr. Suri Sehgal
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Annex 2. Letter of Bryan Kindiger (USDA-ARS, Southern Plains Range Research Staion, Oklahoma)

United States Department of Agriculture
Agricultural Research Service
Southern Plains Area

September 26, 1994

Dr. Mark Williams
Research and Development
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Dear Dr. Williams:

I congratulate you and your company on the development of new male-sterile system for maize. If transgenic plants can ever be readily be entered into the U.S. commercial market, this system should be well received.

Following are responses to your questions which should help in your patent process. I have provided a rather indepth review of the topic so that you can better address your patent concerns.

1) Three species of *Tripsacum* are native to North Amnerica. They are *Tripsacum floridanum*, *T. lanceolatum* and *T. dactyloides*. *T. floridanum* is found in S. Florida and is often used as a ornamental grass for landscaping yards. Other than that, *T. floridanum* is difficult to find. It is fairly rare and not extremely vigorous or aggressive. *T. lanceolatum* is found in S.W. Texas and S. Arizona. *T. dactyloides* is indigenous to most of the southern, central and northeast U.S. **No Tripsacum species cross readily with maize outside the laboratory**. Your research on statements found in the literature that *T. floridanum* crosses readily with corn applies only under laboratory, greenhouse or controlled field conditions. In fact, most *Tripsacum* species can cross readily with corn under laboratory conditions. To make such hybrids, pollen must be applied near the base of the maize silk. This requires husking back the maize ear and deliberately applying the *Tripsacum* pollen near the developing cob. **Tripsacum pollen tubes do not grow long enough to allow natural fertilization to occur between these species.** This is one barrier which prevents cross hybridization between the two species.

2) Hybrids made in the laboratory are either generated from small, nearly aborted hybrid seeds or from various embryo rescue techniques. If such seed were produced in nature, they would not survive. This goes for both maize x 2x (diploid) and 4x (tetraploid) *Tripsacum* crosses. The only known case of a naturally occurring "Zea"-*Tripsacum* hybrid is *Tripsacum andersoni.* It is native to Guatemala and is 100% male and nearly 99% female sterile. The very few seed this plant produces are products of apomixis. The plant itself has been propogated vegetatively by the indians for thousands of years. This cross is actually a 3x *Tripsacum* x maize hybrid where *Tripsacum* is the female parent. This cross is extremely difficult to make since *T. andersoni* is the only known representative of such a hybridization.

Very few *Tripsacum* x maize crosses have been successfully developed in any laboratory. I have not been able to generate one but several years ago, Harlan & deWet apparently developed a single hybrid which was completely male and female sterile.

3) All maize-*Tripsacum* hybrids are completely male sterile. Many are completely female sterile. About 10-20% of all maize-*Tripsacum* hybrids will set seed when backcrossed by maize. Some hybrids are vigorous, but none are able to withstand even the mildest winters and all eventually flower themselves to death. They can only be maintained indefinately in the greenhouse with human intervention. We have attempted backcrossing the hybrids with *Tripsacum* and have obtained some seed. However, generation of seed via this backcross pathway is even more difficult than a backcross by maize since it requires embryo rescue techniques. In addition, no one, even ourselves, has been able to take a maize-*Tripsacum* hybrid, backcross it by *Tripsacum* and successfully recreate a *Tripsacum* plant. We are trying this cross and the genetics just don't work in that direction. Once again, these are laboratory generated materials and as such could not be derived in nature.

4) *Tripsacum* in itself could be considered a non-aggressive weed. However, their is a growing demand for *Tripsacum* (eastern gamagrass) seed for planting. A few small companies in Nebraska, Missouri, Kansas and Oklahoma are commercially cultivating *Tripsacum* to be utilized as a new forage or haylage crop. We are in the middle of this research providing new genetic and germplasm materials. Sales are growing each year and the continued expansion of this market appears likely. Therefore, their population or levels of *Tripsacum dactyloides* found in the U.S. will be growing.

5) Crossability between all species of *Tripsacum* is excellent. This also goes for inter-ploidy crosses. This probably explains why we have no fewer than 11-16 taxonomic species of *Tripsacum*. Generally, I consider *Tripsacum* a germplasm swarm with multiple ploidy forms. If by some near miracle your gene jumps or escapes into *T. floridanum*, it is remotely possible that it could be eventually transferred into *T. dactyloides*. Again, I do not consider this a problem you should be concerned with.

6) I do not consider the probability of your genes escaping into *Tripsacum* a situation you should be concerned with. It is non-reality. However, in southern Florida, their does exist a sparsely dispersed, fairly rare, "native" teosinte called "Florida teosinte" . I say "native" with some hesitance since some believe it was introduced as a experimental forage crop many years ago and merely escaped. In any case, as with all teosintes, it can cross readily with maize. This occurs quite often in Central Mexico with *Zea diploperennis* and I suspect it could happen in Florida if their native teosinte were more widespread. You should check with the USDA in Florida to determine if they consider this species a true native. If so, this may present you with a minor problem considering the aggressive nature of anti-transgenic plant groups in the U.S. If they know their species, I suspect you will probably have to address the situation. As a geneticist, I do not foresee any problems.

To conclude, any concerns about your "transgenes" escaping into *Tripsacum* are not warranted. Their may however be some concern about their potential for escaping into the teosinte native to Florida. Good luck with your efforts. If you require any further assistance

with *Tripsacum* or the generation of haploid maize, let me know.

I also include a few manuscripts which may be relevant or of interest to you.

Sincerely,

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Annex 3. Mariani, C., De Beuckeleer, M., Truettner, J., Leemans, J., Goldberg, R.B. (1990). Induction of male sterility in plants by a chimaeric ribonuclease gene. Nature, **347**, 737-741.

Induction of male sterility in plants by a chimaeric ribonuclease gene

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Chimaeric ribonuclease genes that are expressed in the anthers of transformed tobacco and oilseed rape plants were constructed. Chimaeric ribonuclease gene expression within the anther selectively destroys the tapetal cell layer that surrounds the pollen sac, prevents pollen formation, and leads to male sterility. These nuclear male sterility genes should facilitate the production of hybrid seed in various crop plants.

MALE reproductive processes in flowering plants occur in the anther[1]. This organ is composed of several tissues and cell types, contains several thousand anther-specific messenger RNAs[2,3], and is responsible for producing pollen grains that contain the sperm cells. A specialized anther tissue, the tapetum, plays an important part in pollen formation[1,4-6]. The tapetum surrounds the pollen sac early in anther development, degenerates during the later stages of development, and is not present as an organized tissue in the mature anther[1]. The tapetum produces a number of proteins and other substances that either aid in pollen development or become components of the pollen outer wall[1,4,6].

Cytoplasmic and nuclear mutations have been identified that prevent normal pollen development and result in male sterility[7]. Many male sterility mutations interfere with tapetal cell differentiation and/or function, indicating that this tissue is essential for the production of functional pollen grains[7]. Male sterility mutations have proved useful for producing hybrids beneficial in increasing crop productivity[7]. Hybrid production, however, has been limited to those plants in which male sterile lines that can be restored to fertility have been identified, and/or those in which mechanical removal of anthers from flowers is both possible and practical[7]. The ability to produce hybrid plants in various crops would be greatly facilitated by the availability of a dominant male sterility gene that could be introduced into plant cells by genetic engineering.

Here we report that the 5′ region of a tobacco tapetum-specific gene[8,9] can activate the expression of a β-glucuronidase (GUS) marker gene and two different ribonuclease (RNase) genes within the tapetal cells of transgenic tobacco and oilseed rape plants. Expression of the chimaeric RNase genes selectively destroys the tapetum during anther development, prevents pollen formation, and leads to the production of male sterile plants.

Tobacco gene expression in anther tapetum

We previously described the identification of two tobacco anther complementary DNA clones, designated as TA29 and TA13[8]. These cDNA clones are 85% similar at the nucleotide level (J. Seurinck, C.M. and R.B.G., unpublished data), and are both complementary to 1.1- and 1.2-kilobase (kb) anther mRNAs that are undetectable in other floral and vegetative organ systems (Fig. 1). *In situ* hybridization studies with anther sections showed that the TA29 and TA13 mRNAs are both localized within tapetal cells[8].

DNA gel blot studies indicated that there are less than five TA29- and TA13-like genes in the tobacco genome, and that related genes exist in many other plants, such as tomato, oilseed rape, lettuce and alfalfa (data not shown). We isolated a clone containing the *TA29* gene by screening a tobacco genome library with the *TA29* cDNA[9]. DNA sequencing studies showed that the *TA29* gene does not contain introns, and that it encodes a glycine-rich (20%) protein of relative molecular mass 33,000 (M_r 33K) with potential glycosylation sites[9]. Together, our results indicate that the *TA29* gene is expressed specifically in anther tapetal cells, is present in distantly related plant species, and encodes a protein that has properties similar to some plant cell wall proteins[10-12].

Control of tapetal-specific expression

Transcription studies with RNAs synthesized in isolated anther and leaf nuclei demonstrated that the *TA29* gene is regulated primarily at the transcriptional level (A. Koltunow and R.B.G., unpublished results). To demonstrate that 5′ sequences control *TA29* gene developmental specificity, we fused the *Escherichia coli GUS* gene[13] with a 1.5-kb *TA29* gene upstream fragment (nucleotides −1,477 to +51; ref. 9) containing the start codon,

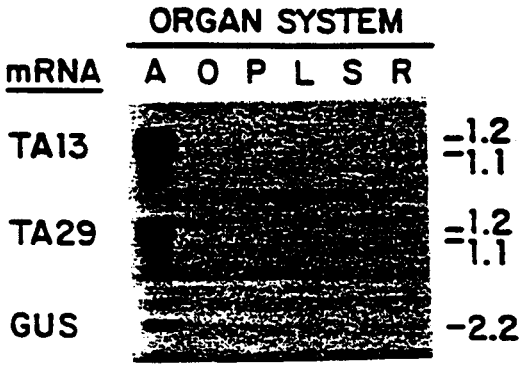

FIG. 1 Organ-specific *TA29* and *TA29–GUS* gene expression patterns. Tobacco anther (A), ovary (O), petal (P), leaf (L), stem (S), and root (R) RNA gel blots were hybridized with either labelled cDNA plasmids (TA13 and TA29) or with a labelled anti-mRNA probe (*GUS*). Tobacco plants from which vegetative organ systems were collected, as well as flower developmental stages, were described previously[2,3,8,21]. Polysomal poly(A)$^+$ mRNAs (1 μg) from untransformed plants were used for the TA13 and TA29 gel blots. Total RNAs (10 μg) from a plant transformed with the chimaeric *TA29–GUS* gene were used for the *GUS* gel blot. Autoradiograms were exposed for ~2 h. DNA size markers (kb) to the right.
METHODS. RNAs were isolated as described[2,3,21]. RNA gel blot experiments and labelling of DNA and RNA probes were as described[21,22]. The *TA29–GUS* gene was introduced into a cointegration vector that contains the bialaphos resistance gene (*bar*) as a selectable marker[18,19]. The *TA29–GUS* gene was transferred to tobacco (*Nicotiana tabacum* cv. 'SR-1') using standard *Agrobacterium* transformation procedures[18,19,23,24].

‡ To whom correspondence should be addressed.

and then transformed tobacco plants with the chimaeric *TA29-GUS* gene. We generated 13 independent transformants that contained from one to three unrearranged copies of the *TA29-GUS* gene (data not shown). Although the levels varied, the anthers of each transformant contained both *GUS* mRNA and enzyme activity, suggesting that the chimaeric *TA29-GUS* gene was regulated correctly (data not shown).

We hybridized a *GUS* gene probe with different RNAs from one transformant, designated as N102-2, to determine the *TA29-GUS* gene organ specificity. This plant expressed the *TA29-GUS* gene at a level that was about average for all our transformants. *GUS* mRNA was observed only in the anther, and was undetectable in other organ systems (Fig. 1). Experiments with anthers at different developmental stages showed that both *GUS* mRNA and enzyme activity accumulated and decayed in parallel with tapetal cell appearance and disintegration, and were coordinated with changes in endogenous *TA29* mRNA levels (data not shown).

We hybridized *TA29* and *GUS* anti-mRNA probes with adjacent N102-2 anther sections *in situ* to compare the cell-specific expression pattern of the *TA29-GUS* gene with that of the endogenous *TA29* gene. Figure 2*a* and *b* shows bright field photographs of anther sections at two developmental stages[8]. Intense hybridization grains were produced by the *GUS* anti-mRNA probe exclusively within the tapetum at stage 2 (Fig. 2*g*). By contrast, no *GUS* hybridization grains above background were observed at stage 8 when the tapetum had degenerated (Fig. 2*h*). These hybridization patterns were identical to those produced with the *TA29* anti-mRNA probe (Fig. 2*d*, *e*). *GUS* enzyme activity was also detected within the tapetum at stage 2 (Fig. 2*i*), and was not detectable in other anther tissues. Together, these data show that the chimaeric *TA29-GUS* gene is regulated exactly like the endogenous *TA29* gene, and that the 1.5-kb *TA29* gene 5′ fragment contains all the information required to programme the tapetal-specific expression pattern.

TA29-RNase gene causes male sterility

We fused the 1.5-kb *TA29* gene regulatory fragment with two different ribonuclease genes to selectively destroy the tapetal cell lineage during anther development. One was a chemically synthesized *Aspergillus oryzae* gene encoding RNase-T1[14]. The other was a natural ribonuclease gene from *Bacillus amyloliquefaciens* called *barnase*[15,16]. Both chimaeric *TA29-RNase* genes were introduced individually into tobacco plants that were either untransformed previously, or that contained a chimaeric *TA29-GUS* gene (N102-2).

We obtained 20 *TA29-RNase T1* and 115 *TA29-barnase* transformants. Sixty per cent of these transformants contained a single *TA29-RNase* gene. The rest had several *TA29-RNase* inserts ranging from two to six copies, depending on the transformant (data not shown). *TA29-RNase T1* and *TA29-barnase* transformants were identical to each other, and to untransformed control plants, with respect to growth rate, height, morphology of vegetative and floral organ systems, time of flowering, and flower coloration pattern. However, 10% of *TA29-RNase T1* transformants (2/20), and 92% of *TA29-barnase* transformants (106/115) failed to shed pollen (Fig. 3). In contrast to mature untransformed anthers (Fig. 3*a*), anthers on flowers of these plants were shrivelled, greyish-brown in colour, and devoid of pollen grains (Fig. 3*b*).

The pollenless *TA29-RNase* plants failed to produce fruit capsules and seeds; flowers simply senesced and fell off. By contrast, when these plants were cross-pollinated with pollen from untransformed anthers (Fig. 3*a*) fruit capsules developed and normal seed set was obtained. These results indicated that the pollenless *TA29-RNase* plants were male sterile, that their pistils were able to recognize and transmit pollen normally, and that female fertility was unaffected. Progeny from cross-pollinated plants with single-copy *TA29-RNase* inserts segregated 1:1 for male sterility and male fertility, and these phenotypes correlated directly with the presence or absence of a chimaeric *TA29-RNase* gene (data not shown). Together, these data indicate that the presence of either a chimaeric *TA29-RNase T1* gene or a *TA29-barnase* gene leads to the production of male sterile tobacco plants.

We analysed the male sterile anthers of *TA29-RNase T1* plants for the presence of both *RNase T1* and *TA29* mRNAs. In addition, the anthers of male sterile N102-2 plants (Fig. 2) that contained both the *TA29-RNase T1* and *TA29-GUS* genes were analysed for *GUS* enzyme activity. Hybridization of a *TA29* anti-mRNA probe with stage 2 anther sections *in situ* revealed only residual amounts of endogenous *TA29* mRNA (Fig. 2*f*). This result contrasted with the intense tapetal-specific signals obtained with anthers containing the *TA29-GUS* gene (Fig. 2*d*, *g*). Hybridization of adjacent sections with a *RNase T1* anti-mRNA probe failed to produce hybridization grains above background (data not shown). RNA dot blot experiments showed that *RNase T1* mRNA was present in male sterile anthers, but only at a period before the time of maximum *TA29* mRNA accumulation in untransformed plants (stage 1 versus stage 3). In addition, the *RNase T1* mRNA level was at least 100-fold lower than that of either *TA29* mRNA or *GUS* mRNA in anthers containing only the *TA29-GUS* gene (data not shown). In contrast to the high tapetal-specific GUS enzyme activity in N102-2 anthers (Fig. 2*i*), GUS enzyme activity was undetectable in male sterile anthers that contained both *TA29-RNase* and *TA29-GUS* genes (data not shown). Together these data show that the male sterility phenotype is associated with a dramatic decrease in tapetal-specific mRNA levels, and that this decrease is correlated with the presence of a chimaeric *TA29-RNase* gene.

Selective destruction of the tapetum

We compared anther development in male sterile tobacco plants containing either the *TA29-RNase T1* or *TA29-barnase* genes to that of untransformed control plants. No differences were observed from stage 1 (0.8 cm flower bud) to stage 12 (open flower) with respect to timing, colour, changes in size and weight, external morphology and filament length. In addition, the male sterile anthers dehisced correctly at flower opening. The single difference between male sterile and male fertile anthers was the apparent absence of pollen grains (Fig. 3*a*, *b*).

We prepared transverse tobacco anther sections at each stage of development to compare the tissue differentiation patterns of male sterile and male fertile anthers. Figures 2 and 4 show bright field photographs of stage 2 anthers from male fertile plants (Figs 2*a* and 4*a*), and male sterile plants containing a *TA29-RNase* gene (Figs 2*c* and 4*b*). Male fertile anthers contained a prominent tapetum (T) surrounding a well-formed pollen sac (PS) that was packed with developing pollen grains (Figs 2*a* and 4*a*). By contrast, male sterile anthers lacked a detectable tapetum, and had a collapsed pollen sac without visible microspores or pollen grains (Figs 2*c* and 4*b*). All other tissues and cell types were identical in male sterile and male fertile anthers (Fig. 4). Together these data indicate that the presence of a chimaeric *TA29-RNase* gene selectively destroys the tapetum during anther development.

Male sterile anthers do not produce pollen

No pollen grains were observed in any of the 106 male sterile tobacco plants containing a chimaeric *TA29-barnase* gene. By contrast, we obtained a small number of pollen-like structures from dehiscent male sterile tobacco anthers containing the *TA29-RNase T1* gene. These pollen-like structures either failed to germinate or produced abnormal pollen tubes, could not successfully pollinate the pistils of either male sterile or male fertile plants, and were 100-fold less prevalent than pollen grains produced by male fertile anthers (data not shown). We visualized these pollen-like structures in the scanning electron microscope. The abnormal pollen (MS) from male sterile anthers is ~50-fold

smaller in size than normal tobacco pollen grains (WT) (Fig. 5*a*). Higher magnification of these abnormal pollen grains shows that they do not have a normal exine, lack a groove or sulcus, and are irregular in shape (Fig. 5*b*). Together, these results indicate that the selective destruction of the tapetum by the expression of the chimaeric *TA29–RNase T1* gene or the *TA29–barnase* gene blocks tobacco pollen grain development.

TA29–RNase expression in other plants

We transformed oilseed rape plants (*Brassica napus* cv. 'Drakkar') with the chimaeric *TA29–RNase T1* and *TA29–barnase*

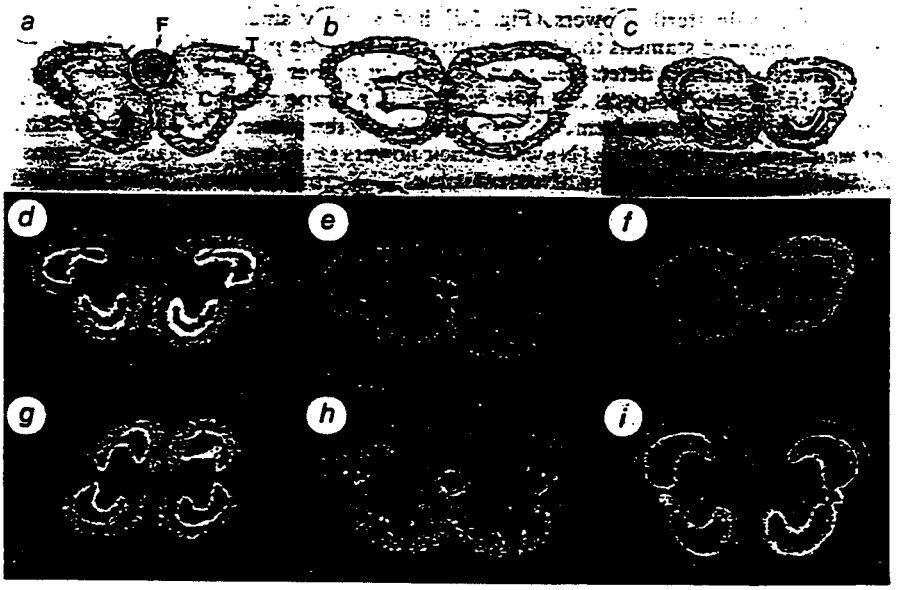

FIG. 2 Localization of TA29 and *TA29–GUS* gene expression patterns in tobacco anthers. *a* and *b*, Bright field photographs of anthers containing the *TA29–GUS* gene at stage 2 (*a*) and stage 8 (*b*) of flower development[8]. C, E, F, PS and T refer to connective, epidermis, filament, pollen sac and tapetum, respectively. *c*, Bright field photograph of an anther containing the *TA29–RNase T1* gene at flower development stage 2 (ref. 8). *d* and *e*, *In situ* hybridization of a *TA29* anti-mRNA probe with anthers containing the *TA29–GUS* gene at flower development stage 2 (*d*) and stage 8 (*e*). Photographs taken by dark field microscopy. White grains represent regions containing RNA–RNA hybrids. Hybridization grains produced with stage 8 anthers were not detectably greater than those produced with a *TA29* mRNA control probe (data not shown). *f*, *In situ* hybridization of a *TA29* anti-mRNA probe with anthers containing a *TA29–RNase* gene at flower development stage 2 (ref. 8). Anthers from a *TA29–RNase T1* transformant were used for this experiment. Photograph taken by dark field microscopy. White regions along the anther wall represent dark-field light scattering through the stained anther section. Identical results were obtained with a *TA29* mRNA control probe (data not shown). White grains outlining the residual tapetum represent RNA–RNA hybrids, and were only 10-fold greater in density than background grains in adjacent connective tissue. *g* and *h*, *In situ* hybridization of a *GUS* anti-mRNA probe with anthers containing the *TA29–GUS* gene at flower development stage 2 (*g*) and stage 8 (*h*). Sections were taken from the same fixed anthers used for the experiments shown in Fig. 2*d*, *e*. Photographs taken with dark field microscopy. White grains, regions of RNA–RNA hybridization. Grains produced with stage 8 anthers (*h*) represent background hybridization, and were not significantly greater than those produced with a *GUS* mRNA control probe (data not shown). *i*, Localization of *GUS* enzyme activity in stage 2 anthers containing the *TA29–GUS* gene. Pink areas, regions with enzyme activity. Photograph taken with dark field microscopy.

METHODS. Anthers at the relevant stage were collected, and their ends sliced off with a razor blade to facilitate fixative penetration. Paraffin-embedded anther sections were hybridized *in situ* with single-stranded ^{35}S-labelled-anti-mRNA and ^{35}S-labelled mRNA (control) probes exactly as described[21]. Autoradiography and photography techniques used for the hybridized sections were published previously[21,25]. All photographs were taken with the same magnification. *GUS* enzyme activity was localized by incubating unfixed, tipless anthers in 50 mM phosphate buffer, pH 7 containing 1 mM X-Glu (ref. 13) for several hours at 37 °C. After a visible histochemical reaction occurred, the anthers were fixed in glutaraldehyde[21], embedded in LR-white resin (Polysciences), and sectioned with a glass knife. The *TA29–RNase T1* and *TA29–barnase* genes were recloned into cointegration and binary vectors, respectively[18,26], and introduced into tobacco plants as outlined in the legend to Fig. 1.

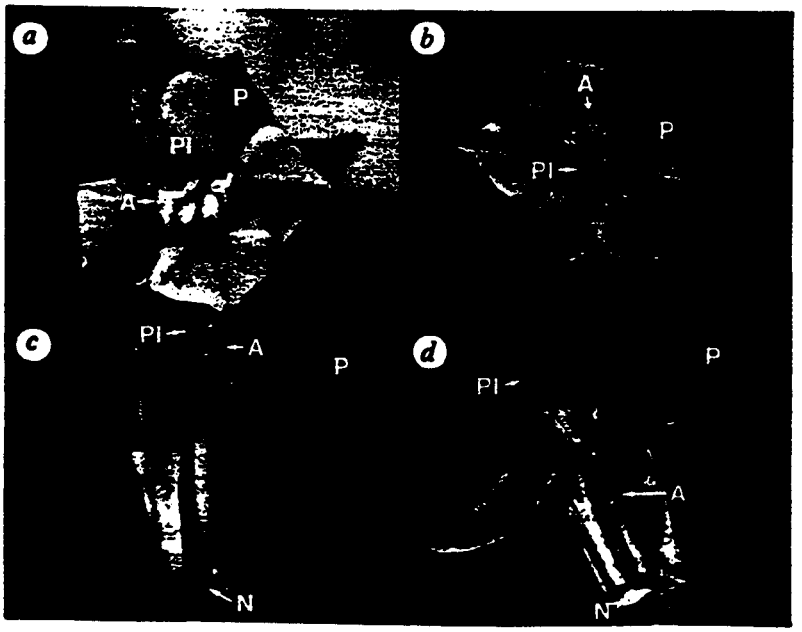

FIG. 3 Male sterile tobacco and oilseed rape flowers. *a* and *b*, Tobacco flowers from untransformed plants (*a*), and plants transformed with *TA29–RNase* gene (*b*). A, P and Pl, anther, petal and pistil, respectively. *c* and *d*, Oilseed rape flowers from untransformed plants (*c*), and plants transformed with a *TA29–RNase* gene (*d*). A, P, Pl and N, anther, petal, pistil and nectar, respectively.

METHODS. The *TA29–RNase T1* and *TA29–barnase* genes were transferred to a binary vector[26] containing the *bar* gene[18]. Oilseed rape hypocotyls (*Brassica napus* cv. 'Drakkar') were transformed with *Agrobacterium* according to the procedure of De Block *et al.*[27] using bialaphos resistance (*bar*) as a selectable marker.

genes to determine whether the tobacco *TA29* gene 5 regulatory region could function in a distantly related plant and lead to male sterility. We obtained 24 *TA29-RNase T1* and 13 *TA29-barnase* transformants that contained one or two intact copies of the relevant *TA29-RNase* gene (data not shown). A male sterile phenotype that cosegregated with the *TA29-RNase* gene was observed in 71% (17/24) and 77% (10/13) of the *TA29-RNase T1* and *TA29-barnase* transformants, respectively. Compared with the flowers of untransformed oilseed rape plants (Fig. 3*c*), male sterile flowers (Fig. 3*d*) had slightly smaller petals, contained stamens that did not extend above the petals, and did not contain detectable pollen grains at anther dehiscence. In all other respects the male sterile oilseed rape plants were identical to untransformed controls, including the presence of well-developed nectaries (N) within their flowers (Fig. 3*c*, *d*).

We examined the anatomy of male sterile oilseed rape anthers present in 5-mm-long immature flower buds. Figure 4*c* shows a bright field photograph of a male fertile oilseed rape anther containing a well-formed tapetum (T) surrounding a pollen sac (PS) with developing pollen grains. By contrast, Fig. 4*d* shows that male sterile oilseed rape anthers containing a chimaeric *TA29-RNase* gene lack a detectable tapetum, and have irregularly shaped pollen sacs that do not contain visible microspores or pollen grains. Scanning electron micrographs of a rare pollen-like body present in only 2 of the 27 dehiscent male sterile anthers (Fig. 5*d*) showed that this structure was abnormal in size, and lacked a regular exine pattern compared with a normal oilseed rape pollen grain (Fig. 5*c*). Together, these data show that the tobacco *TA29* gene 5′ region functions in oilseed rape anthers, that *TA29-RNase* gene expression selectively destroys the tapetum, and that the absence of the tapetum leads to male sterile oilseed rape plants.

Discussion

The experiments presented here show that the *TA29* gene 5′ region programmes the expression of *GUS*, *RNase T1* and *barnase* genes specifically to anther tapetal cells, indicating that the *TA29* gene is regulated primarily at the transcriptional level. This finding is consistent with *TA29* gene run-off transcription studies (A. Koltunow and R.B.G., unpublished results), and with earlier population studies that showed that most anther-specific genes are under transcriptional control[3]. Recent studies have shown that only 0.3 kb of 5′ sequence is required to programme both the temporal and cell-specific *TA29* gene transcription patterns during anther development (A. Koltunow and R.B.G., unpublished results). These studies suggest that transcriptional events occur during anther development to activate unique gene sets within the tapetum.

The phenotype of plants containing the chimaeric *TA29-RNase* gene strengthens our conclusion that the *TA29* gene is expressed exclusively in the tapetum in both tobacco and oilseed rape plants. If this gene were expressed at other times of the life cycle, the presence of RNase would have disrupted the normal course of vegetative and floral development. Destruction of the tapetum by *TA29-RNase* gene expression does not interfere detectably with anther development, suggesting that the tapetal cell lineage can be eliminated with no effect on subsequent stages of anther development. This result indicates that tapetal cells function autonomously, and that their continued presence is not required for the differentiation and/or function

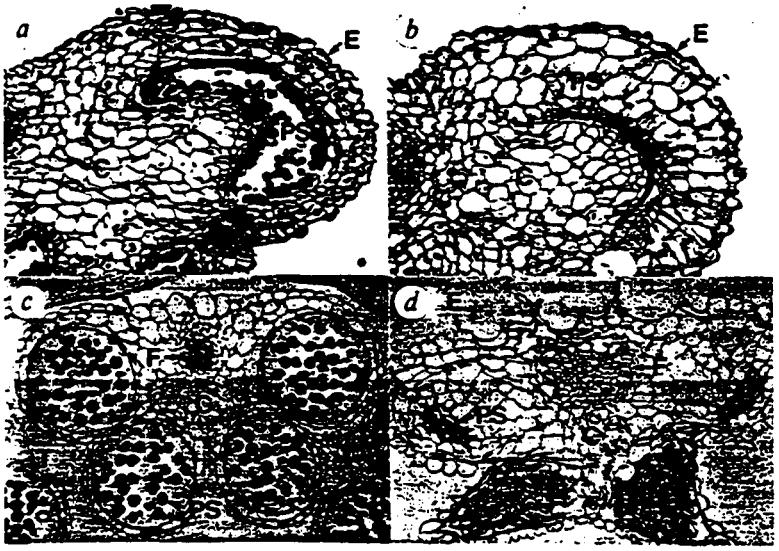

FIG. 4 Tissue abnormalities in male sterile tobacco and oilseed rape anthers. *a* and *b*, Bright field photographs of an untransformed tobacco anther (*a*), and a male sterile anther from a tobacco plant containing a *TA29-RNase* gene (*b*). C, E, F, PS and T, refer to connective, epidermis, filament, pollen sac and tapetum, respectively. *c* and *d*, Bright field photographs of an untransformed oilseed rape anther (*c*), and a male sterile oilseed rape anther from a plant containing a *TA29-RNase* gene (*d*).
METHODS. Stage 1 tobacco anthers were fixed and sectioned in a transverse orientation as outlined in the legend to Fig. 3. Oilseed rape anthers were harvested from 2.5-mm flower buds and fixed in glutaraldehyde[21]. Fixed anthers were embedded in LR-white, sliced into 1.5 μM transverse sections with a glass knife, and stained with 0.05% toluidine blue.

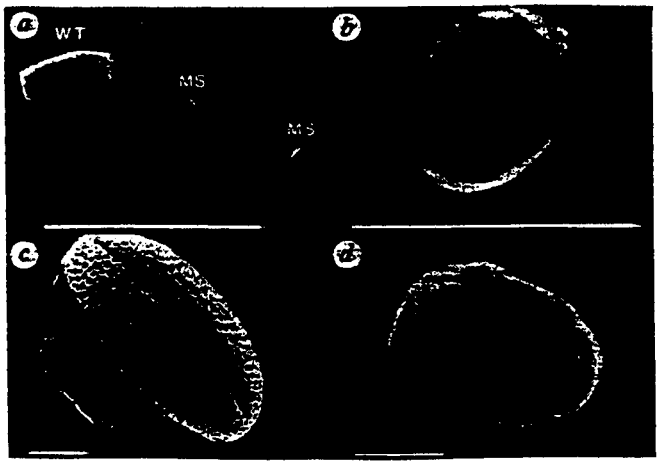

FIG. 5 Scanning electron micrographs of pollen grains produced by male sterile tobacco and oilseed rape anthers. *a*, Tobacco pollen grains from untransformed anthers (WT), and anthers transformed with a *TA29-RNase* gene (MS). White bar, 100 μm. Magnification, ×710. *b*, Higher magnification of male sterile tobacco pollen grains shown in (*a*). White bar, 10 μm. Magnification factor ×9,150. *c* and *d*, Oilseed rape pollen grains from untransformed anthers (*c*) and anthers transformed with a *TA29-RNase* gene (*d*). White bars, 10 μm. Magnification factors, ×1,930 and ×2,980 for the wild-type and male sterile pollen grains, respectively.
METHODS. Pollen grains were collected from dehisced anthers in open flowers (Fig. 4) and photographed in a scanning electron microscope[28].

of anther cell types later in development.

The expression of either the *TA29-RNase T1* gene or the *TA29-barnase* gene leads to the production of male sterile plants. These plants are normal in all respects except failure to produce functional pollen. The tapetum is therefore essential for normal pollen development. Expression of both classes of RNase selectively destroyed tapetal cells, presumably by hydrolysing tapetal cell RNAs. An analogous process may occur naturally in the reproductive structures of self-incompatible plants[17]. Barnase seems to be more effective in tobacco than RNase T1. Genetic crosses with male sterile tobacco plants indicated that at least four *TA29-RNase T1* gene copies are required to produce male sterile anthers (C.M. and J.L., unpublished results). By contrast, only one *TA29-barnase* gene copy is required to produce male sterile plants in tobacco, and one copy of either the *TA29-RNase T1* or *TA29-barnase* gene is sufficient to produce male sterile oilseed rape plants. Because the same *TA29* gene 5′ fragment was used in both chimaeric *TA29-RNase* genes, these results suggest that RNase T1 is less active than barnase in tobacco tapetal cells.

The ability of the *TA29-RNase* gene to induce male sterilit provides a new strategy for the production of hybrid crop plants Transferring this dominant male sterility gene to plants such as corn should enable hybrids to be produced without mechanical removal of the anthers. By coupling the chimaeric *TA29-RNas* gene to a dominant herbicide gene (for example, *bar*; refs 18, 19) breeding systems can be devised to select for uniform populations of male sterile plants. In crop plants where fruit is not the harvested product (for example, lettuce, carrot, cabbage) male sterile plants can be crossed with any pollinator line t produce hybrid seeds. By contrast, in other crops such as tomato, wheat, rice and corn it will be necessary to restore full male fertility in the offspring. Antisense RNA technology[20], and th existence of barstar, a protein inhibitor of barnase[15,16], shoul facilitate the development of strategies for male fertility restoration. □

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Annex 4. Mariani, C., Gosselé, V., De Beuckeleer, M., De Block, M., Goldberg, R.B., De Greef, W., Leemans, J. (1992). A chimearic ribonuclease-inhibitor gene restores fertility to male sterile plants. Nature, **357**, 384-387

A chimaeric ribonuclease-inhibitor gene restores fertility to male sterile plants

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Male fertility was restored to genetically engineered male sterile oilseed rape plants. Male sterile plants that express a chimaeric ribonuclease gene in the anther tapetal cell layer were crossed with male fertile plants that were transformed with a chimaeric tapetal-cell-specific ribonuclease-inhibitor gene. F_1 progeny expressing both genes are restored to male fertility by the suppression of cytotoxic ribonuclease activity in the anther by the formation of cell-specific RNase/RNase inhibitor complexes. Genetically engineered male sterility and fertility restorer genes should facilitate hybrid seed production in crop plants.

THE improvement of crop plants through the production of hybrid varieties is a major goal of plant breeding[1]. Crosses between inbred plant lines often result in progeny with higher yield, increased resistance to disease, and enhanced performance in different environments compared with the parental lines[1]. The molecular basis of this hybrid vigour is not understood.

The production of hybrid seed on a large scale is challenging because many crops have both male and female reproductive organs (stamen and pistil) on the same plant, either within a single flower (for example oilseed rape, tomato) or in separate

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flowers (for example corn). This arrangement results in a high level of self-pollination and makes large-scale directed crosses between inbred lines difficult to accomplish. To guarantee that outcrossing will occur to produce hybrid seed, breeders have either manually or mechanically removed stamens from one parental line, used natural self-incompatibility systems that prevent self-pollination, or exploited male sterility mutations that disrupt pollen development[1-4]. Each of these strategies presents its own set of problems. Manual emasculation is labour intensive and impractical for plants with small bisexual flowers (such as oilseed rape), many crop plants do not have self-incompatibility and/or male sterility genes, and use of male sterility requires a fertility restorer system[4].

Recently, we established a genetic engineering strategy for hybrid seed production by demonstrating that chimaeric RNase T1 and barnase genes containing the tobacco *TA29* gene promoter[5,6] can induce male sterility in tobacco and oilseed rape plants[7]. The *TA29* gene is highly regulated and is transcribed specifically in tapetal cells that surround the pollen sacs in the anther[6,7]. Expression of the cytotoxic *TA29*–RNase genes selectively destroys the tapetal cell layer, prevents pollen formation, and results in male sterility[7]. The *TA29*-barnase gene contains the coding sequence for the extracellular RNase of *Bacillus amyloliquefaciens*[7-9], which has a corresponding inhibitor protein, called barstar[8,9]. Barstar is produced intracellularly and protects the bacteria from the lethal effects of barnase by forming a stable complex with barnase in the cytoplasm[3,9].

Here we show that crosses between male fertile oilseed rape plants containing a *TA29*-barstar gene and male sterile plants containing a *TA29*-barnase gene produce progeny with both genes that are male fertile. The *TA29*-barstar and *TA29*-barnase genes are co-expressed in anthers of the male fertile progeny, indicating that the *TA29*-barstar gene is a dominant suppressor of cytotoxic *TA29*-barnase gene activity, and that fertility restoration is due to the formation of tapetal-cell-specific barnase/barstar complexes. The availability of these genetically engineered nuclear male sterility and fertility restorer genes should facilitate the development of new breeding and production systems for hybrid crops.

Chimaeric *TA29*-barstar expression

We fused the barstar gene coding sequence[9] with a 1.5-kilobase (kb) *TA29* gene upstream fragment that contains all regulatory elements necessary for tapetal-specific expression[5-7]. We introduced the *TA29*-barstar gene into oilseed rape plants by Ti-plasmid-mediated transformation using the *bar* gene (bialaphos resistance) as a selectable marker[10,11] and regenerated 41 transformants that contained 1–5 copies of the chimaeric gene. The *TA29*-barstar transformants were male fertile, produced normal flowers (Fig. 1*a*), and had well developed anther tapetal cell layers (Fig. 1*b*). Anther messenger RNA gel blots from several different transformants demonstrated the presence of a prevalent 0.5-kb barstar mRNA, indicating that the *TA29*-barstar gene was expressed correctly (data not shown).

Plants with both genes are male fertile

To determine whether *TA29*-barstar gene expression could inhibit barnase activity in tapetal cells and lead to male fertility restoration, we selected four single-copy *TA29*-barstar gene transformants and used these as male parents in crosses with four male sterile *TA29*-barnase single-copy gene lines[7] (Fig. 2*a*). If co-expression of these genes in the anther leads to the formation of stable barnase–barstar complexes, then there should be a 2:1 ratio of fertile to sterile plants in the F_1 progeny after removal of bialaphos-sensitive segregants (Fig. 2*b*). By contrast, if *TA29*-barstar gene expression does not lead to male fertility restoration, there should be a 1:2 F_1 ratio of fertile to sterile plants (Fig. 2*b*).

All crosses produced F_1 progeny that segregated with the 3:1 bialaphos-resistant (*HR*) to bialaphos-sensitive (*hs*) ratio expected for parents that contained a single copy of each chimaeric gene (Fig. 2*b*). The genotypes of the bialaphos-resistant survivors were checked using the polymerase chain reaction (PCR) and male fertility phenotypes were scored by examining pollen release at anther dehiscence (Fig. 2*b*, *c*). Six of the nine crosses produced F_1 progeny consistent with the expected 2:1 ratio of male-fertile to male-sterile plants, indicating that male fertility restoration had occurred (shaded boxes, Fig. 2*c*). All F_1 progeny from these crosses that contained both chimaeric genes were male fertile (numbers in parentheses, Fig. 2*c*), indicating that the *TA29*-barstar gene functioned as a dominant suppressor of *TA29*-barnase gene activity. Selfing these *TA29*-barstar/*TA29*-barnase plants (progeny of 88–18 × 94–10 cross; Fig. 2*b*, *c*) and removing bialaphos-sensitive segregants produced a 12:3 F_2 ratio of male-fertile to male-sterile plants, indicating that male fertility restoration was co-inherited with the *TA29*-barstar gene and was passed on to the next generation (data not shown).

Two of the initial crosses (88–11 × 93–101 and 88–11 × 94–3; Fig. 2*b*, *c*) failed to show fertility restoration and produced a 1:2 F_1 ratio of male fertile to male sterile plants (Fig. 2*c*). PCR analysis of the fertile progeny in these crosses indicated that they were segregants that contained only the *TA29*-barstar gene (Fig. 2*b*, *c*). None of the plants containing both chimaeric genes were male fertile (Fig. 2*c*). In addition, one cross (91–4 × 94–10; Fig. 2*a*) produced an intermediate male fertile to male sterile

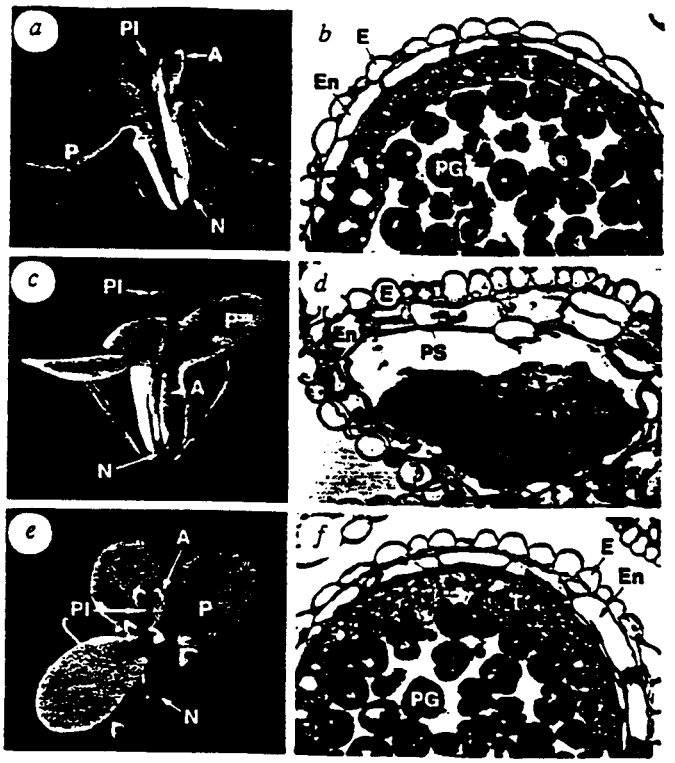

FIG. 1 Oilseed rape flowers and anther cross-sections. *a*, *c* and *e*, Flowers from male-fertile plants containing the *TA29*-barstar gene (*a*), male-sterile plants containing the *TA29*-barnase gene (*c*), and male-fertile plants restored to fertility containing both the *TA29*-barstar and *TA29*-barnase genes (*e*). A, P, Pl and N refer to anther, petal, pistil and nectary, respectively. *b*, *d*, *f*, Bright-field photographs of anther cross-sections from male-fertile plants containing the *TA29*-barstar gene (*b*), male-sterile plants containing the *TA29*-barnase gene (*d*), and plants restored to male fertility that contain both the *TA29*-barnase and *TA29*-barstar genes (*f*). E, En, PG, PS and T refer to epidermis, endothecium, pollen grain, pollen sac and tapetum, respectively.

METHODS. The *TA29*-barstar and *TA29*-barnase genes were placed independently in an *Agrobacterium* vector system containing the *neo* and *bar* genes as selectable markers[7,10] and used to transform oilseed rape (*Brassica napus* cv. 'Drakkar') hypocotyls by the procedure of De Block *et al.*[11]. Anthers from 4-mm flower buds were collected, embedded in Historesin, sliced into 1–2 μm sections and stained with 0.05% toluidine blue[12].

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F_1 ratio (Fig. 2c). We do not know the reason for the absence of full male fertility restoration in these three crosses. However, to eliminate the cytotoxic effects of barnase in the tapetum, the amount of barstar must be equal to or greater than that of barnase[5]. Thus it is likely that the *TA29*-barstar gene in the non-restored plants was expressed at a lower level than the *TA29*-barnase gene.

Restored fertility anthers develop normally

We compared anthers restored to male ferility (MS/RF) with those produced by wild-type plants (WT), male sterile *TA29*-barnase plants (MS), and male fertile *TA29*-barstar plants (RF). Wild-type and *TA29*-barstar plants had anthers that were above the petals (Fig. 1a), contained well developed tapetal cell layers (Fig. 1b), and produced functional pollen grains (Figs 1b and 3a)[7]. Male sterile anthers remained below the petals (Fig. 1c), lacked a tapetum (Fig. 1d), and did not contain viable pollen grains (Figs 1d and 3b)[7]. By contrast, *TA29*-barstar/*TA29*-barnase anthers restored to male fertility were indisinguishable from those of wild-type and *TA29*-barstar plants (Fig. 1e), had a normal tapetal cell layer (Fig. 1f), and produced large anounts of functional pollen grains that were identical in structure to those produced by either wild-type or *TA29*-barstar plants (Figs 1f and 3c, d, e).

a

Male Sterile ♀ — *TA29-barnase* — $\frac{MS\ HR}{ms\ hr}$ $\frac{rf\ hr}{rf\ hr}$ × Male Fertile ♂ — *TA29-barstar* — $\frac{ms\ hr}{ms\ hr}$ $\frac{RF\ HR}{rf\ hr}$

b

♀ Gametes \ ♂ Gametes	ms hr	RF HR	ms hr	rf hr
MS HR / rf hr	MS HR / ms hr	RF HR / rf hr	MS HR / ms hr	rf hr / rf hr
	RESTORED MALE FERTILE		MALE STERILE	
ms hr / rf hr	ms hr / ms hr	RF HR / rf hr	ms hr / ms hr	rf hr / rf hr
	MALE FERTILE		HERBICIDE-SENSITIVE (DIE)	

c

Barnase ♀ \ Barstar ♂	93-101	94-1	94-10	94-3
88-18	18:6 (10/10)		16:6 (11/11)	
91-4	31:11 (14/14)		17:16 (1/4)	
91-5	20:7 (3/3)	19:4 (9/9)	9:4 (5/5)	
88-11	3:6 (0/3)			12:23 (0/5)

FIG. 2 Restoration of male fertility by crossing oilseed rape plants containing the *TA29*-barstar and *TA29*-barnase genes. a, Genotypes of male sterile plants (*TA29*-barnase) and male fertile restorer plants (*TA29*-barstar). *MS*, *RF* and *HR* designate the *TA29*-barnase, *TA29*-barstar, and *bar* herbicide-resistance genes, respectively. *ms*, *rf* and *hr* refer to hemizygous chromosomal loci that lack the *TA29*-barnase, *TA29*-barstar and *bar* genes, respectively. b, Genotypes and phenotypes of progeny expected from a cross between male sterile *TA29*-barnase and male fertile restorer *TA29*-barstar lines containing linked herbicide-resistance genes. c, Progeny obtained from a cross between male sterile *TA29*-barnase and male fertile *TA29*-barstar plants. Ratio in each box refers to the actual number of male fertile to male sterile plants scored in the progeny. Number in parenthesis below each ratio refers to the number of progeny containing both the *TA29*-barnase and the *TA29*-barstar genes that were male fertile. Boxes shaded in grey highlight crosses in which the *TA29*-barstar gene suppressed *TA29*-barnase gene activity and restored fertility to male sterile plants.
METHODS. Oilseed rape plants containing a single-copy of either the *TA29*-barnase[7] or the *TA29*-barstar genes were identified by DNA gel blot analysis. These plants were crossed as diagrammed in a and seeds were collected from mature siliques. Seeds from each cross were planted and young seedlings were sprayed with bialaphos to kill herbicide-sensitive plants that did not contain either the *TA29*-barnase or the *TA29*-barstar genes as shown in b. The genotypes of herbicide-resistant progeny were determined by PCR analysis and the phenotypes were scored by the presence or absence of viable pollen on the anthers at dehiscence.

Restored anthers express both chimaeric genes

We analysed anthers of *TA29*-barstar/*TA29*-barnase plants restored to male fertility (Fig. 2c) for the presence of barstar, barnase and their mRNAs. The RNA gel blots shown in Fig. 4 demonstrate that both barnase and barstar mRNAs were present in anthers of one plant restored to fertility (MS/RF) by the *TA29*-barstar gene. Similar results were obtained with six independent *TA29*-barstar/*TA29*-barnase plants restored to fertility (data not shown). By contrast, neither of these mRNAs were detected in wild-type or male sterile anthers (Fig. 4). The absence of detectable barnase mRNA in male sterile anthers is due to tapetal cell RNA hydrolysis by barnase activity[7].

A two-dimensional gel of wild-type anther proteins shows the presence of at least 100 distinct protein spots (Fig. 5a). Proteins from *TA29*-barnase anthers, by contrast, were much fewer in number, suggesting that the missing spots represented tapetal cell and/or pollen grain proteins that were absent in male sterile anthers (Fig. 5b). The two-dimensional protein pattern from *TA29*-barstar/*TA29*-barnase anthers restored to fertility (MS/RF), however, was indistinguishable from that obtained

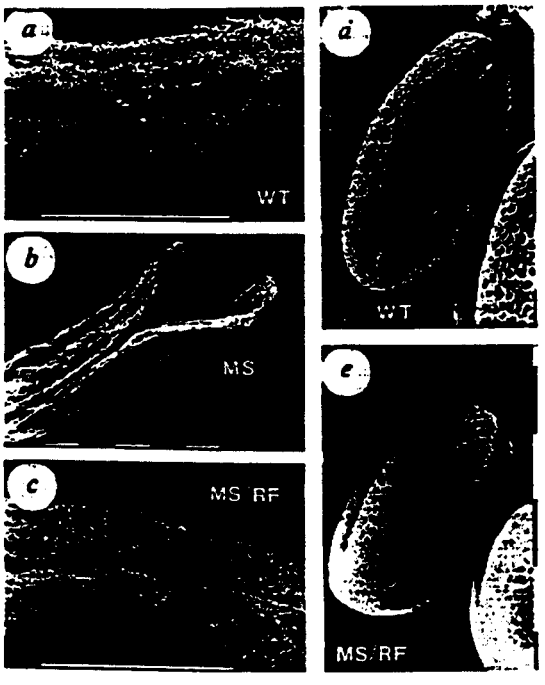

FIG. 3 Scanning electron micrographs of oilseed rape pollen grains and dehiscing anthers. a, Dehiscing anthers from an untransformed plant. Scale bar, 1 mm; magnification factor, ×69. b, Dehiscing male sterile anther from a plant containing the *TA29*-barnase gene. Scale bar, 0.1 mm; magnification factor, ×131. c, Dehiscing anther from a plant restored to male fertility containing both the *TA29*-barstar and *TA29*-barnase genes. Scale bar, 1 mm; magnification factor, ×69. d, e, Pollen grains from untransformed anthers (d), and anthers containing both the *TA29*-barstar and *TA29*-barnase genes that were restored to fertility (e). Scale bars, 10 μm; magnification factors, ×2,400.
METHODS. Pollen grains and dehiscing anthers were collected from open oilseed rape flowers and photographed by scanning electron microscopy as outlined previously[7]. Dehiscing anthers were removed from 7-mm flower buds and fixed before scanning electron microscopy as described[6,7].

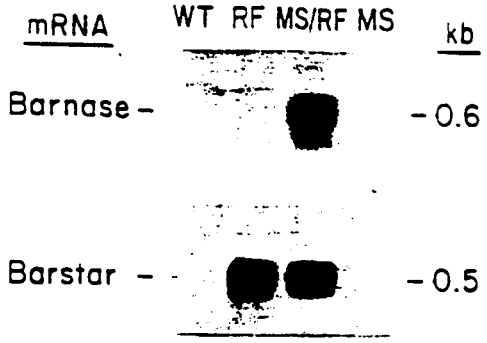

FIG. 4 Presence of barstar and barnase mRNAs in anthers of oilseed rape plants restored to male fertility. Gel blots containing 1 μg anther poly(A)⁻ mRNAs from untransformed plants (WT), *TA29*-barstar plants (RF), male fertile plants containing both the *TA29*-barnase and *TA29*-barstar genes (MS/RF), and male sterile *TA29*-barnase plants (MS) were hybridized sequentially with *TA29*-barnase and *TA29*-barstar gene probes.
METHODS. Anthers were collected from 4-mm flower buds, total RNAs were isolated as described[13], and poly(A)⁻ mRNAs were selected according to the protocol supplied with the Pharmacia-LKB poly(A)⁻ RNA purification kit.

with wild-type anther proteins (Fig. 5*a*, *c*). Immunoblots of these proteins using antibodies raised against the barnase-barstar complex demonstrated the presence both of barnase and of barstar (Fig. 5*d*, *e*), indicating that fertility restoration was due to the inhibition of barnase activity by the formation of tapetal-cell-specific barnase-barstar protein complexes.

Discussion

We have shown previously that the *TA29*-barnase gene acts as a dominant male sterility gene both in tobacco and in oilseed rape plants[7]. *TA29*-barnase gene expression leads to the selective destruction of tapetal cells during anther development and prevents the formation of functional pollen grains[7]. The results presented here show that the *TA29*-barstar gene suppresses *TA29*-barnase gene expression in oilseed rape anthers by protein-protein interactions, protects tapetal cells from barnase cytotoxic activity, and restores male fertility. The *TA29*-barstar gene also acts as a dominant restorer of male ferility in tobacco, indicating that barstar is able to work effectively in different plants (C.M. *et al.*, unpublished results).

Barnase and barstar are single-chain proteins that can function under a variety of conditions[3]. Barnase inhibition is due to the formation of a diffusion-dependent, one-to-one complex between barnase and barstar[8]. This complex is extremely stable and has a dissociation constant of about 10^{-14} M, indicating that once it forms it rarely dissociates. The *TA29*-barstar and *TA29*-barnase genes are both controlled by the same tapetal-specific regulatory sequences[6,7]. We adopted this strategy to ensure that each gene would be activated in tapetal cells at the same time and to maximize the chance that barstar molecules would accumulate in equal or greater amounts than barnase in the tapetal cell layers.

The majority of crosses between *TA29*-barstar and *TA29*-barnase plants produced progeny with both chimaeric genes that are male fertile (Fig. 2*c*), indicating that this strategy was successful. Anthers restored to male fertility are indistinguishable from those of wild-type plants, develop and dehisce normally (Figs 1 and 3), have well-differentiated tapetal cell layers (Fig. 1*f*), and produce large amounts of functional pollen grains (Figs 1 and 3). These results show that barstar is able to complex efficiently with barnase in anther tapetal cells and that use of the *TA29*-barstar gene as a dominant restorer of male fertility only requires that it is expressed equally or to a greater extent than the *TA29*-barnase gene.

In addition to oilseed rape[7], we have shown that the tobacco *TA29* gene promoter is active in other crop plants, including lettuce, chicory, cauliflower, tomato, cotton and corn (A. Reynaerts, K. D'Halluin and C.M., unpublished results). In

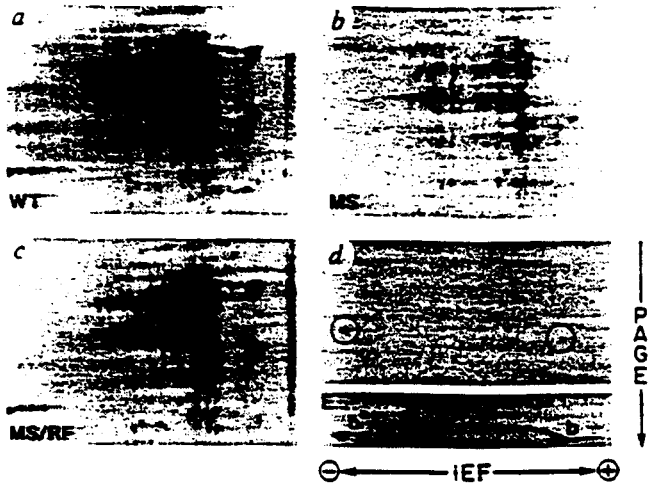

FIG. 5 Presence of barstar and barnase proteins in oilseed rape anthers restored to male ferility. *a*, *b*, *c*, *d*, Anther proteins from wild-type (WT), untransformed plants (*a*), male sterile (MS) plants containing the *TA29*-barnase gene (*b*), and plants restored to male fertility (MS/RF) that contain both the *TA29*-barnase and *TA29*-barstar genes (*c*, *d*) were fractionated by two-dimensional gel electrophoresis and either stained with silver (*a*, *b*, *c*) or blotted and reacted with antibodies specific for the barstar–barnase complex (*d*). Barstar and barnase proteins present in anthers restored to male fertility are circled in the immunoblot (*d*). *e*, Purified barstar–barnase complexes (1 μg) were denatured, fractionated by two-dimensional gel electrophoresis, and stained with silver. *b** and *b* refer to barstar and barnase, respectively.
METHODS. Oilseed rape anthers were collected from 4-mm flower buds and proteins were extracted by grinding anthers (50 mg) in 500 μl 50 mM phosphate buffer (pH 7), 10 mM EDTA, 10 mM β-mercaptoethanol, 0.1% Triton X-100, 0.1% sarcosyl, 0.6% polyvinylpolypyrrolidone, and 25 μg ml^{-1} PMSF. Proteins were precipitated with 10% TCA, washed with 90% acetone, dried, and resuspended in Pharmacia lysis buffer according to the protocol supplied by Pharmacia-LKB. Protein fractionation by two-dimensional gel electrophoresis was done using the Pharmacia-LKB Multiphor II 2-D System following the protocol supplied by the manufacturer. Total anther protein (5 μg) was used for the silver-stained gels (*a*, *b*, *c*) and 200 μg protein for the immunoblot (*d*). The immunoblot analysis was done according to the Pharmacia-LKB protocol.

each crop, the expression of the *TA29*-barnase gene leads to the production of male sterile plants (A. Reynaerts, K. D'Halluin and C.M., unpublished results). Use of the *TA29*-barnase gene alone in plants such as lettuce and chicory should permit the efficient production of hybrid plants because leaves are the harvested product. By contrast, both the *TA29*-barstar and *TA29*-barnase genes will be required to produce hybrid seeds and fruit in plants such as tomato and oilseed rape. The effectiveness of the chimaeric *TA29*-barnase and *TA29*-barstar genes in male sterility induction and male fertility restoration should permit the breeding of genetically engineered hybrid crop plants in the near future. □

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Annex 5. Detailed description of the DNA used in transformation : pVE108 and pMc5barstar

Annex 5. Description of the plasmids pVE108 and pMc5barstar

Annex 5.1. Construction of the plasmids

The plasmid pVE108 contains 2 gene constructs : the male sterility gene construct (PTA29-*barnase*-3'nos) and the linked marker gene construct (P35S-*bar*-3'nos). Both gene constructs are cloned on a small *E. coli* plasmid.

pVE108 is produced by excising the EcoRV-EcoRI fragment containing PTA29-*barnase*-3'nos from pTTM8 (Mariani et al., 1989), and inserting it in the large EcoRI-StuI fragment of pDE110 (D'Halluin and Göbel, 1992). pDE110 was obtained by inserting the chimeric P35S-*bar*-3'nos construct in pUC19 (Yanish-Perron et al., 1985; Denecke et al., 1989). pVE108 and several other plasmids containing a *barnase* gene are transformed and propagated in an *E. coli* host that also expresses *barstar*. Low expression of the *barnase* gene in the bacterial host could affect the viability of the *E. coli* cells. Expression of the *barstar* gene encoding a specific inhibitor protein of barnase activity, can counter any negative effects on the host cells (Hartley, 1988). Plasmid pVE108 was constructed and propagated in *E. coli* strain WK6 (Zell and Fritz, 1987) that carries the plasmid pMc5barstar.

pMc5barstar is obtained by inserting the *Bacillus amyloliquefaciens* genes *barnase* and *barstar* from pMT416 (Hartley 1988) in the EcoRI and HindIII sites of pMc5-8 (Stanssens et al., 1989). In the resulting plasmid, the sequence starting at the initiation codon of the phoA signal sequence and ending with the last nucleotide before the translation initiation codon of the *barstar* coding region was deleted by looping-out mutagenesis (Sallazo et al., 1985). The resulting plasmid is pMc5barstar. *E. coli* strains carrying pMc5barstar direct increased levels produce of barstar protein when the bacterial *tac* promoter is induced.

DNA of pVE108 was prepared from *E. coli* strain WK6 (Zell and Fritz, 1987) containing plasmids pMc5barstar and pVE108 as follows : a 5 mL culture was grown in Luria-Bertani medium (LB) (Sambrook et al., 1989) with 100 mg/L ampicillin (selection for the presence of pVE108), 25 mg/L chloramphenicol (selection for the presence of pMc5barstar), and 0.1 mM isopropyl-ß-D-galactopyranoside (IPTG; inducer of the bacterial *tac* promoter to express *barstar*). This culture was used to inoculate 500 mL of LB containing ampicillin and IPTG. In the absence of chloramphenicol, pMc5barstar is gradually lost from the culture through plasmid incompatibility (Sambrook et al., 1989). However, it produces sufficient barstar to allow the culture of WK6(pVE108) to grow to saturation.

pVE108 DNA was prepared from the saturated culture, and the integrity of the plasmid and the presence of pMc5barstar was checked on an ethidium bromide stained agarose gel. Before transformation, the DNA was linearized with restriction enzyme HindIII. This enzyme cleaves at one position downstream of the *bar* gene.

Annex 5.2. The elements in the plasmids

This description lists the functional elements present on the plasmids that were used in transformation. It also specifies the position and the orientation of these elements in the plasmids. When reference is made to other plasmids, the sequence coordinates are as in the EMBL and NIH sequence databases.

Table 1. Elements of plasmid pVE108 (length of the plasmid : 5616 basepairs)

nt 0001 - 0421	**Sequence derived from plasmid pUC19 (Yanisch-Perron et al., 1985)**
nt 0001 - 0235	DNA sequence derived from the replication and mobilization region of plasmid pMB1 (reviewed in Balbas et al., 1986). The mobilization elements (*mob* and *nic/bom*) are not functionally intact on this sequence element (Sambrook et al., 1989). The sequence is partially homologous to nt 1646 - 1371 of plasmid pColE1 (pColE1 is closely related to pMB1).
nt 0399 - 0236	The coding sequence for the amino-terminal part of the *Escherichia coli* beta-galactosidase gene (lacZ), that can complement lacZ genes carrying a amino-terminal deletion, such as lacZdeltaM15.
nt 0400 - 0421	Polylinker sequence of pUC19.
nt 0422 - 1679	**The male sterility gene construct PTA29 - *barnase* - 3'nos**
nt 0422 - 0425	Synthetic polylinker derived sequence
nt 0426 - 0686	A 261 bp TaqI fragment containing polyadenylation signals obtained from the 3' untranslated end of the nopaline synthase gene (3'nos) from the T-DNA of pTiT37 (Depicker et al., 1982). The 3'untranslated end of the nopaline synthase gene from the T-DNA of *Agrobacterium tumefaciens* has been characterized at different laboratories : Laboratory of Genetics, University Gent (Belgium), Max Planck Institut für Züchtungforschung, Koln (Germany).
nt 0687 - 0702	Synthetic polylinker derived sequence.
nt 0798 - 0703	*Bacillus amyloliquefaciens* sequence originating from the region downstream of the *barnase* gene (Hartley, 1988).
nt 1134 - 0799	The coding region of the mature part of the *barnase* gene from *Bacillus amyloliquefaciens.* (Hartley, 1988). The ribonuclease gene has been isolated by Dr Hartley at the Department of Health and Human Services (NIH), Bethesda, Maryland USA.
nt 1679 - 1137	The promoter region (PTA29) of the anther-specific gene TA29 from tobacco (*Nicotiana tabacum*). (Seurinck et al., 1990, Koltunow et al., 1990). The promoter was derived from a genomic clone which was obtained from Dr R. Goldberg at the Department of Biology, Univeristy of California, Los Angeles, California USA.
nt 1680 - 3343	**The selectable marker gene construct P35S - *bar* - 3'nos**
nt 1680 - 2512	The promoter region encoded on the Cauliflower mosaic virus 35S RNA molecule. Several variants of the CaMV35S promoter have been described (see e.g. Odell et al., 1985, and references therein).The 35S promoter has been obtained from the Laboratory of Genetics, University of Gent (Belgium).
nt 2513 - 3064	The coding region of the phosphinothricin acetyl transferase gene (bar) from *Streptomyces hygroscopicus* (Thompson et al., 1987). The *bar* gene has been isolated by Dr C. Thompson at Biogen SA, Geneva, Switserland. The *Streptomyces hygroscopicus* strain ATTC21705 was provided by Meiji Seika Kaisha Ltd (Japan).
nt 3065 - 3082	Synthetic polylinker derived sequence.
nt 3083 - 3343	A 261 bp TaqI fragment containing polyadenylation signals obtained from the 3' untranslated end of the nopaline synthase gene (3'nos) from the T-DNA of pTiT37 (Depicker et al., 1982).
nt 3344 - 3347	Synthetic polylinker derived sequence.
nt 3348 - 5616	**Sequence derived from plasmid pUC19**
nt 3384 - 3348	Polylinker sequence of pUC19.
nt 3377	The HindIII restriction site used to linearize the DNA before transformation.
nt 3614 - 3385	A DNA segment from the *Escherichia coli* lac operon, comprising the C-terminal part of the lacI coding sequence (30 codons), and the promoter and first five codons of the lacZ gene.
nt 3617 - 4408	A DNA fragment containing the DNA replication functions of plasmid pMB1. The sequence is partially homologous to nt 1364 - 582 of plasmid pColE1.
nt 4409 - 5616	The ß-lactamase gene conferring resistance to ß-lactam antibiotics (ampicillin, penicillin) onto the bacterial host. The gene was originally isolated from *Escherichia coli* transposon Tn3, carried on the plasmid pRSF2124 (reviewed in Balbas et al., 1986). nt 4555 - 4409 contains sequences downstream of ß-lactamase. nt 5416 - 4556 contains the ß-lactamase coding sequence nt 5616 - 5417 contains the promoter of ß-lactamase, as it is found on transposon Tn3. The fragment also contains the last five codons of the Tn3 repressor gene.

PVE108.SEQ
Circular sequence, 5616 nucleotides

```
        10          20          30          40          50
TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG

        60          70          80          90         100
GAGACGGTCA CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG

       110         120         130         140         150
TCAGGGCGCG TCAGCGGGTG TTGGCGGGTG TCGGGGCTGG CTTAACTATG

       160         170         180         190         200
CGGCATCAGA GCAGATTGTA CTGAGAGTGC ACCATATGCG GTGTGAAATA

       210         220         230         240         250
CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGGCGCC ATTCGCCATT

       260         270         280         290         300
CAGGCTGCGC AACTGTTGGG AAGGGCGATC GGTGCGGGCC TCTTCGCTAT

       310         320         330         340         350
TACGCCAGCT GGCGAAAGGG GGATGTGCTG CAAGGCGATT AAGTTGGGTA

       360         370         380         390         400
ACGCCAGGGT TTTCCCAGTC ACGACGTTGT AAAACGACGG CCAGTGAATT

       410         420         430         440         450
CGAGCTCGGT ACCCGGGGAT CTTCCCGATC TAGTAACATA GATGACACCG

       460         470         480         490         500
CGCGCGATAA TTTATCCTAG TTTGCGCGCT ATATTTTGTT TTCTATCGCG

       510         520         530         540         550
TATTAAATGT ATAATTGCGG GACTCTAATC ATAAAAACCC ATCTCATAAA

       560         570         580         590         600
TAACGTCATG CATTACATGT TAATTATTAC ATGCTTAACG TAATTCAACA

       610         620         630         640         650
GAAATTATAT GATAATCATC GCAAGACCGG CAACAGGATT CAATCTTAAG

       660         670         680         690         700
AAACTTTATT GCCAAATGTT TGAACGATCT GCTTCGGATC CTCTAGAGCC

       710         720         730         740         750
GGAAAGTGAA ATTGACCGAT CAGAGTTTGA AGAAAAATTT ATTACACACT

       760         770         780         790         800
TTATGTAAAG CTGAAAAAAA CGGCCTCCGC AGGAAGCCGT TTTTTTCGTT

       810         820         830         840         850
ATCTGATTTT TGTAAAGGTC TGATAATGGT CCGTTGTTTT GTAAATCAGC
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       860         870         880         890         900
CAGTCGCTTG AGTAAAGAAT CCGGTCTGAA TTTCTGAAGC CTGATGTATA

       910         920         930         940         950
GTTAATATCC GCTTCACGCC ATGTTCGTCC GCTTTTGCCC GGGAGTTTGC

       960         970         980         990        1000
CTTCCCTGTT TGAGAAGATG TCTCCGCCGA TGCTTTTCCC CGGAGCGACG

      1010        1020        1030        1040        1050
TCTGCAAGGT TCCCTTTTGA TGCCACCCAG CCGAGGGCTT GTGCTTCTGA

      1060        1070        1080        1090        1100
TTTTGTAATG TAATTATCAG GTAGCTTATG ATATGTCTGA AGATAATCCG

      1110        1120        1130        1140        1150
CAACCCCGTC AAACGTGTTG ATAACCGGTA CCATGGTAGC TAATTTCTTT

      1160        1170        1180        1190        1200
AAGTAAAAAC TTTGATTTGA GTGATGATGT TGTACTGTTA CACTTGCACC

      1210        1220        1230        1240        1250
ACAAGGGCAT ATATAGAGCA CAAGACATAC ACAACAACTT GCAAAACTAA

      1260        1270        1280        1290        1300
CTTTTGTTGG AGCATTTCGA GGAAAATGGG GAGTAGCAGG CTAATCTGAG

      1310        1320        1330        1340        1350
GGTAACATTA AGGTTTCATG TATTAATTTG TTGCAAACAT GGACTTAGTG

      1360        1370        1380        1390        1400
TGAGGAAAAA GTACCAAAAT TTTGTCTCAC CCTGATTTCA GTTATGGAAA

      1410        1420        1430        1440        1450
TTACATTATG AAGCTGTGCT AGAGAAGATG TTTATTCTAG TCCAGCCACC

      1460        1470        1480        1490        1500
CACCTTATGC AAGTCTGCTT TTAGCTTGAT TCAAAAACTG ATTTAATTTA

      1510        1520        1530        1540        1550
CATTGCTAAA TGTGCATACT TCGAGCCTAT GTCGCTTTAA TTCGAGTAGG

      1560        1570        1580        1590        1600
ATGTATATAT TAGTACATAA AAAATCATGT TTGAATCATC TTTCATAAAG

      1610        1620        1630        1640        1650
TGACAAGTCA ATTGTCCCTT CTTGTTTGGC ACTATATTCA ATCTGTTAAT

      1660        1670        1680        1690        1700
GCAAATTATC CAGTTATACT TAGCTAGATC CTACGCAGCA GGTCTCATCA

      1710        1720        1730        1740        1750
AGACGATCTA CCCGAGTAAC AATCTCCAGG AGATCAAATA CCTTCCCAAG
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      1760        1770        1780        1790        1800
AAGGTTAAAG ATGCAGTCAA AAGATTCAGG ACTAATTGCA TCAAGAACAC

      1810        1820        1830        1840        1850
AGAGAAAGAC ATATTTCTCA AGATCAGAAG TACTATTCCA GTATGGACGA

      1860        1870        1880        1890        1900
TTCAAGGCTT GCTTCATAAA CCAAGGCAAG TAATAGAGAT TGGAGTCTCT

      1910        1920        1930        1940        1950
AAAAAGGTAG TTCCTACTGA ATCTAAGGCC ATGCATGGAG TCTAAGATTC

      1960        1970        1980        1990        2000
AAATCGAGGA TCTAACAGAA CTCGCCGTGA AGACTGGCGA ACAGTTCATA

      2010        2020        2030        2040        2050
CAGAGTCTTT TACGACTCAA TGACAAGAAG AAAATCTTCG TCAACATGGT

      2060        2070        2080        2090        2100
GGAGCACGAC ACTCTGGTCT ACTCCAAAAA TGTCAAAGAT ACAGTCTCAG

      2110        2120        2130        2140        2150
AAGACCAAAG GGCTATTGAG ACTTTTCAAC AAAGGATAAT TTCGGGAAAC

      2160        2170        2180        2190        2200
CTCCTCGGAT TCCATTGCCC AGCTATCTGT CACTTCATCG AAAGGACAGT

      2210        2220        2230        2240        2250
AGAAAAGGAA GGTGGCTCCT ACAAATGCCA TCATTGCGAT AAAGGAAAGG

      2260        2270        2280        2290        2300
CTATCATTCA AGATGCCTCT GCCGACAGTG GTCCCAAAGA TGGACCCCCA

      2310        2320        2330        2340        2350
CCCACGAGGA GCATCGTGGA AAAAGAAGAC GTTCCAACCA CGTCTTCAAA

      2360        2370        2380        2390        2400
GCAAGTGGAT TGATGTGACA TCTCCACTGA CGTAAGGGAT GACGCACAAT

      2410        2420        2430        2440        2450
CCCACTATCC TTCGCAAGAC CCTTCCTCTA TATAAGGAAG TTCATTTCAT

      2460        2470        2480        2490        2500
TTGGAGAGGA CACGCTGAAA TCACCAGTCT CTCTCTATAA ATCTATCTCT

      2510        2520        2530        2540        2550
CTCTCTATAA CCATGGACCC AGAACGACGC CCGGCCGACA TCCGCCGTGC

      2560        2570        2580        2590        2600
CACCGAGGCG GACATGCCGG CGGTCTGCAC CATCGTCAAC CACTACATCG

      2610        2620        2630        2640        2650
AGACAAGCAC GGTCAACTTC CGTACCGAGC CGCAGGAACC GCAGGAGTGG
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      2660        2670        2680        2690        2700
ACGGACGACC TCGTCCGTCT GCGGGAGCGC TATCCCTGGC TCGTCGCCGA

      2710        2720        2730        2740        2750
GGTGGACGGC GAGGTCGCCG GCATCGCCTA CGCGGGCCCC TGGAAGGCAC

      2760        2770        2780        2790        2800
GCAACGCCTA CGACTGGACG GCCGAGTCGA CCGTGTACGT CTCCCCCCGC

      2810        2820        2830        2840        2850
CACCAGCGGA CGGGACTGGG CTCCACGCTC TACACCCACC TGCTGAAGTC

      2860        2870        2880        2890        2900
CCTGGAGGCA CAGGGCTTCA AGAGCGTGGT CGCTGTCATC GGGCTGCCCA

      2910        2920        2930        2940        2950
ACGACCCGAG CGTGCGCATG CACGAGGCGC TCGGATATGC CCCCCGCGGC

      2960        2970        2980        2990        3000
ATGCTGCGGG CGGCCGGCTT CAAGCACGGG AACTGGCATG ACGTGGGTTT

      3010        3020        3030        3040        3050
CTGGCAGCTG GACTTCAGCC TGCCGGTACC GCCCCGTCCG GTCCTGCCCG

      3060        3070        3080        3090        3100
TCACCGAGAT CTGATCTCAC GCGTCTAGGA TCCGAAGCAG ATCGTTCAAA

      3110        3120        3130        3140        3150
CATTTGGCAA TAAAGTTTCT TAAGATTGAA TCCTGTTGCC GGTCTTGCGA

      3160        3170        3180        3190        3200
TGATTATCAT ATAATTTCTG TTGAATTACG TTAAGCATGT AATAATTAAC

      3210        3220        3230        3240        3250
ATGTAATGCA TGACGTTATT TATGAGATGG GTTTTTATGA TTAGAGTCCC

      3260        3270        3280        3290        3300
GCAATTATAC ATTTAATACG CGATAGAAAA CAAAATATAG CGCGCAAACT

      3310        3320        3330        3340        3350
AGGATAAATT ATCGCGCGCG GTGTCATCTA TGTTACTAGA TCGGGAAGAT

      3360        3370        3380        3390        3400
CCTCTAGAGT CGACCTGCAG GCATGCAAGC TTGGCGTAAT CATGGTCATA

      3410        3420        3430        3440        3450
GCTGTTTCCT GTGTGAAATT GTTATCCGCT CACAATTCCA CACAACATAC

      3460        3470        3480        3490        3500
GAGCCGGAAG CATAAAGTGT AAAGCCTGGG GTGCCTAATG AGTGAGCTAA

      3510        3520        3530        3540        3550
CTCACATTAA TTGCGTTGCG CTCACTGCCC GCTTTCCAGT CGGGAAACCT
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      3560        3570        3580        3590        3600
GTCGTGCCAG CTGCATTAAT GAATCGGCCA ACGCGCGGGG AGAGGCGGTT

      3610        3620        3630        3640        3650
TGCGTATTGG GCGCTCTTCC GCTTCCTCGC TCACTGACTC GCTGCGCTCG

      3660        3670        3680        3690        3700
GTCGTTCGGC TGCGGCGAGC GGTATCAGCT CACTCAAAGG CGGTAATACG

      3710        3720        3730        3740        3750
GTTATCCACA GAATCAGGGG ATAACGCAGG AAAGAACATG TGAGCAAAAG

      3760        3770        3780        3790        3800
GCCAGCAAAA GGCCAGGAAC CGTAAAAAGG CCGCGTTGCT GGCGTTTTTC

      3810        3820        3830        3840        3850
CATAGGCTCC GCCCCCCTGA CGAGCATCAC AAAAATCGAC GCTCAAGTCA

      3860        3870        3880        3890        3900
GAGGTGGCGA AACCCGACAG GACTATAAAG ATACCAGGCG TTTCCCCCTG

      3910        3920        3930        3940        3950
GAAGCTCCCT CGTGCGCTCT CCTGTTCCGA CCCTGCCGCT TACCGGATAC

      3960        3970        3980        3990        4000
CTGTCCGCCT TTCTCCCTTC GGGAAGCGTG GCGCTTTCTC AATGCTCACG

      4010        4020        4030        4040        4050
CTGTAGGTAT CTCAGTTCGG TGTAGGTCGT TCGCTCCAAG CTGGGCTGTG

      4060        4070        4080        4090        4100
TGCACGAACC CCCCGTTCAG CCCGACCGCT GCGCCTTATC CGGTAACTAT

      4110        4120        4130        4140        4150
CGTCTTGAGT CCAACCCGGT AAGACACGAC TTATCGCCAC TGGCAGCAGC

      4160        4170        4180        4190        4200
CACTGGTAAC AGGATTAGCA GAGCGAGGTA TGTAGGCGGT GCTACAGAGT

      4210        4220        4230        4240        4250
TCTTGAAGTG GTGGCCTAAC TACGGCTACA CTAGAAGGAC AGTATTTGGT

      4260        4270        4280        4290        4300
ATCTGCGCTC TGCTGAAGCC AGTTACCTTC GGAAAAAGAG TTGGTAGCTC

      4310        4320        4330        4340        4350
TTGATCCGGC AAACAAACCA CCGCTGGTAG CGGTGGTTTT TTTGTTTGCA

      4360        4370        4380        4390        4400
AGCAGCAGAT TACGCGCAGA AAAAAAGGAT CTCAAGAAGA TCCTTTGATC

      4410        4420        4430        4440        4450
TTTTCTACGG GGTCTGACGC TCAGTGGAAC GAAAACTCAC GTTAAGGGAT
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      4460        4470        4480        4490        4500
TTTGGTCATG AGATTATCAA AAAGGATCTT CACCTAGATC CTTTTAAATT

      4510        4520        4530        4540        4550
AAAAATGAAG TTTTAAATCA ATCTAAAGTA TATATGAGTA AACTTGGTCT

      4560        4570        4580        4590        4600
GACAGTTACC AATGCTTAAT CAGTGAGGCA CCTATCTCAG CGATCTGTCT

      4610        4620        4630        4640        4650
ATTTCGTTCA TCCATAGTTG CCTGACTCCC CGTCGTGTAG ATAACTACGA

      4660        4670        4680        4690        4700
TACGGGAGGG CTTACCATCT GGCCCCAGTG CTGCAATGAT ACCGCGAGAC

      4710        4720        4730        4740        4750
CCACGCTCAC CGGCTCCAGA TTTATCAGCA ATAAACCAGC CAGCCGGAAG

      4760        4770        4780        4790        4800
GGCCGAGCGC AGAAGTGGTC CTGCAACTTT ATCCGCCTCC ATCCAGTCTA

      4810        4820        4830        4840        4850
TTAATTGTTG CCGGGAAGCT AGAGTAAGTA GTTCGCCAGT TAATAGTTTG

      4860        4870        4880        4890        4900
CGCAACGTTG TTGCCATTGC TACAGGCATC GTGGTGTCAC GCTCGTCGTT

      4910        4920        4930        4940        4950
TGGTATGGCT TCATTCAGCT CCGGTTCCCA ACGATCAAGG CGAGTTACAT

      4960        4970        4980        4990        5000
GATCCCCCAT GTTGTGCAAA AAAGCGGTTA GCTCCTTCGG TCCTCCGATC

      5010        5020        5030        5040        5050
GTTGTCAGAA GTAAGTTGGC CGCAGTGTTA TCACTCATGG TTATGGCAGC

      5060        5070        5080        5090        5100
ACTGCATAAT TCTCTTACTG TCATGCCATC CGTAAGATGC TTTTCTGTGA

      5110        5120        5130        5140        5150
CTGGTGAGTA CTCAACCAAG TCATTCTGAG AATAGTGTAT GCGGCGACCG

      5160        5170        5180        5190        5200
AGTTGCTCTT GCCCGGCGTC AATACGGGAT AATACCGCGC CACATAGCAG

      5210        5220        5230        5240        5250
AACTTTAAAA GTGCTCATCA TTGGAAAACG TTCTTCGGGG CGAAAACTCT

      5260        5270        5280        5290        5300
CAAGGATCTT ACCGCTGTTG AGATCCAGTT CGATGTAACC CACTCGTGCA

      5310        5320        5330        5340        5350
CCCAACTGAT CTTCAGCATC TTTTACTTTC ACCAGCGTTT CTGGGTGAGC
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      5360        5370        5380        5390        5400
AAAAACAGGA AGGCAAAATG CCGCAAAAAA GGGAATAAGG GCGACACGGA

      5410        5420        5430        5440        5450
AATGTTGAAT ACTCATACTC TTCCTTTTTC AATATTATTG AAGCATTTAT

      5460        5470        5480        5490        5500
CAGGGTTATT GTCTCATGAG CGGATACATA TTTGAATGTA TTTAGAAAAA

      5510        5520        5530        5540        5550
TAAACAAATA GGGGTTCCGC GCACATTTCC CCGAAAAGTG CCACCTGACG

      5560        5570        5580        5590        5600
TCTAAGAAAC CATTATTATC ATGACATTAA CCTATAAAAA TAGGCGTATC

      5610
ACGAGGCCCT TTCGTC
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Table 2. Elements of plasmid pMc5barstar (length of the plasmid : 4219 basepairs)

nt 0001 - 3759	**Sequence derived from pMc5-8 (Stanssens et al., 1989).**
nt 0001 - 0105	Transcription terminator from bacteriophage fd (Beck et al., 1978).
nt 0106 - 0110	Synthetic polylinker derived sequence.
nt 0111 - 0215	Second identical copy of bacteriophage fd transcription terminator.
nt 0216 - 0220	Synthetic polylinker derived sequence.
nt 0221 - 0307	Sequence derived from plasmid pBR325, nt 2069 - 2155.
nt 0308 - 0312	Synthetic polylinker derived sequence.
nt 0313 - 0768	Intergenic region of bacteriophage f1 (Beck and Zink, 1981), carrying the sequences required in cis for replication as a single stranded circular DNA molecule and for morphogenesis of bacteriophage particles. When complemented in *trans* with functions of bacteriophage f1, bacteriophage particles with single stranded DNA can be produced (Sambrook et al., 1989)
nt 0769 - 0771	Synthetic polylinker derived sequence.
nt 0772 - 1538	Replication functions of plasmid pMB1, as they are used in pBR325 (reviewed in Balbas et al., 1986). The sequence is partially homologous to nt 1339 - 582 of plasmid pColE1.
nt 2575 - 1539	The ß-lactamase gene encoding resistance to ß-lactam antibiotics in bacteria. The gene was originally isolated from transposon Tn3, carried on the plasmid pRSF2124 (reviewed in Balbas et al., 1986). nt 1685 - 1539 contains sequence downstream of ß-lactamase. nt 2546 - 1686 contains the ß-lactamase coding sequence. The coding region is interrupted by changing nt 2238 from G to C (codon 103 is changed from TAC encoding tyr -> TAG encoding a stop codon). nt 2575 - 2547 contains the promoter of ß-lactamase.
nt 2576 - 2684	Sequence corresponding to nt 1018 - 1130 of *Escherichia coli* transposon Tn903. It contains an inverted repeat of the transposon iS element, but no known coding information.
nt 2685 - 2717	Synthetic polylinker derived sequence.
nt 2745 - 2718	Transcription terminator from the *Escherichia coli* trpA gene (Yanofsky et al., 1981). (This sequence element was produced synthetically by Pharmacia Biotech).
nt 2773 - 2746	Second identical copy of the trpA transcription terminator.
nt 3741 - 2774	Chloramphenicol acetyl transferase gene, conferring resistance to chloramphenicol onto bacteria expressing the gene. The gene was originally isolated from the bacteriophage P1Cm (reviewed in Balbas et al., 1986). It is highly homologous to the chloramphenicol acetyl transferase gene from *Escherichia coli* transposon Tn9 and Tn981. nt 2774 - 2860 contains sequences located downstream of the chloramphenicol acetyl transferase coding sequence. nt 3520 - 2861 corresponds to the chloramphenicol acetyl transferase coding sequence. nt 3741 - 3521 corresponds to the promoter region of the chloramphenicol acetyl transferase gene.
nt 3742 - 3754	Synthetic polylinker derived sequence.
nt 3755 - 4199	***barstar* gene with bacterial expression signals.**
nt 3755 - 3884	Nucleotide sequence derived from pMT416 (Hartley, 1988), essentially containing the bacterial Ptac promoter (De Boer et al., 1983).
nt 3885 - 4157	Coding sequence of *barstar*, as described in Hartley (1988).
nt 4158 - 4199	*Bacillus amyloliquefaciens* sequences following *barstar*, as described in Hartley (1988).
nt 4200 - 4219	**pMc5-8 sequence (synthetic polylinker DNA).**
nt 4201	The HindIII site where the DNA was linearized before transformation.

PMC5BARSTAR
Circular sequence of 4219 nucleotides.

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        10         20         30         40         50
AATTCACCTC GAAAGCAAGC TGATAAACCG ATACAATTAA AGGCTCCTTT

        60         70         80         90        100
TGGAGCCTTT TTTTTTGGAG ATTTTCAACG TGAAAAAATT ATTATTCGCA

       110        120        130        140        150
ATTCCAAGCT AATTCACCTC GAAAGCAAGC TGATAAACCG ATACAATTAA

       160        170        180        190        200
AGGCTCCTTT TGGAGCCTTT TTTTTTGGAG ATTTTCAACG TGAAAAAATT

       210        220        230        240        250
ATTATTCGCA ATTCCAAGCT CTGCCTCGCG CGTTTCGGTG ATGACGGTGA

       260        270        280        290        300
AAACCTCTGA CACATGCAGC TCCCGGAGAC GGTCACAGCT TGTCTGTAAG

       310        320        330        340        350
CGGATGCAGA TCACGCGCCC TGTAGCGGCG CATTAAGCGC GGCGGGTGTG

       360        370        380        390        400
GTGGTTACGC GCAGCGTGAC CGCTACACTT GCCAGCGCCC TAGCGCCCGC

       410        420        430        440        450
TCCTTTCGCT TTCTTCCCTT CCTTTCTCGC CACGTTCGCC GGCTTTCCCC

       460        470        480        490        500
GTCAAGCTCT AAATCGGGGG CTCCCTTTAG GGTTCCGATT TAGTGCTTTA

       510        520        530        540        550
CGGCACCTCG ACCCCAAAAA ACTTGATTAG GGTGATGGTT CACGTAGTGG

       560        570        580        590        600
GCCATCGCCC TGATAGACGG TTTTTCGCCC TTTGACGTTG GAGTCCACGT

       610        620        630        640        650
TCTTTAATAG TGGACTCTTG TTCCAAACTG GAACAACACT CAACCCTATC

       660        670        680        690        700
TCGGTCTATT CTTTTGATTT ATAAGGGATT TTGCCGATTT CGGCCTATTG

       710        720        730        740        750
GTTAAAAAAT GAGCTGATTT AACAAAAATT TAACGCGAAT TTTAACAAAA

       760        770        780        790        800
TATTAACGTT TACAATTTGA TCTGCGCTCG GTCGTTCGGC TGCGGCGAGC

       810        820        830        840        850
GGTATCAGCT CACTCAAAGG CGGTAATACG GTTATCCACA GAATCAGGGG
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       860         870         880         890         900
ATAACGCAGG AAAGAACATG TGAGCAAAAG GCCAGCAAAA GGCCAGGAAC

       910         920         930         940         950
CGTAAAAAGG CCGCGTTGCT GGCGTTTTTC CATAGGCTCC GCCCCCCTGA

       960         970         980         990        1000
CGAGCATCAC AAAAATCGAC GCTCAAGTCA GAGGTGGCGA AACCCGACAG

      1010        1020        1030        1040        1050
GACTATAAAG ATACCAGGCG TTTCCCCCTG GAAGCTCCCT CGTGCGCTCT

      1060        1070        1080        1090        1100
CCTGTTCCGA CCCTGCCGCT TACCGGATAC CTGTCCGCCT TTCTCCCTTC

      1110        1120        1130        1140        1150
GGGAAGCGTG GCGCTTTCTC AATGCTCACG CTGTAGGTAT CTCAGTTCGG

      1160        1170        1180        1190        1200
TGTAGGTCGT TCGCTCCAAG CTGGGCTGTG TGCACGAACC CCCCGTTCAG

      1210        1220        1230        1240        1250
CCCGACCGCT GCGCCTTATC CGGTAACTAT CGTCTTGAGT CCAACCCGGT

      1260        1270        1280        1290        1300
AAGACACGAC TTATCGCCAC TGGCAGCAGC CACTGGTAAC AGGATTAGCA

      1310        1320        1330        1340        1350
GAGCGAGGTA TGTAGGCGGT GCTACAGAGT TCTTGAAGTG GTGGCCTAAC

      1360        1370        1380        1390        1400
TACGGCTACA CTAGAAGGAC AGTATTTGGT ATCTGCGCTC TGCTGAAGCC

      1410        1420        1430        1440        1450
AGTTACCTTC GGAAAAAGAG TTGGTAGCTC TTGATCCGGC AAACAAACCA

      1460        1470        1480        1490        1500
CCGCTGGTAG CGGTGGTTTT TTTGTTTGCA AGCAGCAGAT TACGCGCAGA

      1510        1520        1530        1540        1550
AAAAAAGGAT CTCAAGAAGA TCCTTTGATC TTTTCTACGG GGTCTGACGC

      1560        1570        1580        1590        1600
TCAGTGGAAC GAAAACTCAC GTTAAGGGAT TTTGGTCATG AGATTATCAA

      1610        1620        1630        1640        1650
AAAGGATCTT CACCTAGATC CTTTTAAATT AAAAATGAAG TTTTAAATCA

      1660        1670        1680        1690        1700
ATCTAAAGTA TATATGAGTA AACTTGGTCT GACAGTTACC AATGCTTAAT

      1710        1720        1730        1740        1750
CAGTGAGGCA CCTATCTCAG CGATCTGTCT ATTTCGTTCA TCCATAGTTG
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      1760        1770        1780        1790        1800
CCTGACTCCC CGTCGTGTAG ATAACTACGA TACGGGAGGG CTTACCATCT

      1810        1820        1830        1840        1850
GGCCCCAGTG CTGCAATGAT ACCGCGAGAC CCACGCTCAC CGGCTCCAGA

      1860        1870        1880        1890        1900
TTTATCAGCA ATAAACCAGC CAGCCGGAAG GGCCGAGCGC AGAAGTGGTC

      1910        1920        1930        1940        1950
CTGCAACTTT ATCCGCCTCC ATCCAGTCTA TTAATTGTTG CCGGGAAGCT

      1960        1970        1980        1990        2000
AGAGTAAGTA GTTCGCCAGT TAATAGTTTG CGCAACGTTG TTGCCATTGC

      2010        2020        2030        2040        2050
TGCAGGCATC GTGGTGTCAC GCTCGTCGTT TGGTATGGCT TCATTCAGCT

      2060        2070        2080        2090        2100
CCGGTTCCCA ACGATCAAGG CGAGTTACAT GATCCCCCAT GTTGTGCAAA

      2110        2120        2130        2140        2150
AAAGCGGTTA GCTCCTTCGG TCCTCCGATC GTTGTCAGAA GTAAGTTGGC

      2160        2170        2180        2190        2200
CGCAGTGTTA TCACTCATGG TTATGGCAGC ACTGCATAAT TCTCTTACTG

      2210        2220        2230        2240        2250
TCATGCCATC CGTAAGATGC TTTTCTGTGA CTGGTGACTA CTCAACCAAG

      2260        2270        2280        2290        2300
TCATTCTGAG AATAGTGTAT GCGGCGACCG AGTTGCTCTT GCCCGGCGTC

      2310        2320        2330        2340        2350
AACACGGGAT AATACCGCGC CACATAGCAG AACTTTAAAA GTGCTCATCA

      2360        2370        2380        2390        2400
TTGGAAAACG TTCTTCGGGG CGAAAACTCT CAAGGATCTT ACCGCTGTTG

      2410        2420        2430        2440        2450
AGATCCAGTT CGATGTAACC CACTCGTGCA CCCAACTGAT CTTCAGCATC

      2460        2470        2480        2490        2500
TTTTACTTTC ACCAGCGTTT CTGGGTGAGC AAAAACAGGA AGGCAAAATG

      2510        2520        2530        2540        2550
CCGCAAAAAA GGGAATAAGG GCGACACGGA AATGTTGAAT ACTCATACTC

      2560        2570        2580        2590        2600
TTCCTTTTTC AATATTATTG AAGCAGACAG TTTTATTGTT CATGATGATA

      2610        2620        2630        2640        2650
TATTTTTATC TTGTGCAATG TAACATCAGA GATTTTGAGA CACAACGTGG
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2660 2670 2680 2690 2700
CTTTGTTGAA TAAATCGAAC TTTTGCTGAG TTGACTCCCC GCGCGCGATG

2710 2720 2730 2740 2750
GGTCGAATTT GCTTTCGAAA AAAAAGCCCG CTCATTAGGC GGGCTAAAAA

2760 2770 2780 2790 2800
AAAGCCCGCT CATTAGGCGG GCTCGAATTT CTGCCATTCA TCCGCTTATT

2810 2820 2830 2840 2850
ATCACTTATT CAGGCGTAGC AACCAGGCGT TTAAGGGCAC CAATAACTGC

2860 2870 2880 2890 2900
CTTAAAAAAA TTACGCCCCG CCCTGCCACT CATCGCAGTA CTGTTGTAAT

2910 2920 2930 2940 2950
TCATTAAGCA TTCTGCCGAC ATGGAAGCCA TCACAGACGG CATGATGAAC

2960 2970 2980 2990 3000
CTGAATCGCC AGCGGCATCA GCACCTTGTC GCCTTGCGTA TAATATTTGC

3010 3020 3030 3040 3050
CCATAGTGAA AACGGGGGCG AAGAAGTTGT CCATATTCGC CACGTTTAAA

3060 3070 3080 3090 3100
TCAAAACTGG TGAAACTCAC CCAGGGATTG GCTGAGACGA AAAACATATT

3110 3120 3130 3140 3150
CTCAATAAAC CCTTTAGGGA AATAGGCCAG GTTTTCACCG TAACACGCCA

3160 3170 3180 3190 3200
CATCTTGCGA ATATATGTGT AGAAACTGCC GGAAATCGTC GTGGTATTCA

3210 3220 3230 3240 3250
CTCCAGAGCG ATGAAAACGT TTCAGTTTGC TCATGGAAAA CGGTGTAACA

3260 3270 3280 3290 3300
AGGGTGAACA CTATCCCATA TCACCAGCTC ACCGTCTTTC ATTGCCATAC

3310 3320 3330 3340 3350
GAAATTCCGG ATGAGCATTC ATCAGGCGGG CAAGAATGTG AATAAAGGCC

3360 3370 3380 3390 3400
GGATAAAACT TGTGCTTATT TTTCTTTACG GTCTTTAAAA AGGCCGTAAT

3410 3420 3430 3440 3450
ATCCAGCTGA ACGGTCTGGT TATAGGTACA TTGAGCAACT GACTGAAATG

3460 3470 3480 3490 3500
CCTCAAAATG TTCTTTACGA TGCCATTGGG ATATATCAAC GGTGGTATAT

3510 3520 3530 3540 3550
CCAGTGATTT TTTTCTCCAT TTTAGCTTCC TTAGCTCCTG AAAATCTCGA

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      3560        3570        3580        3590        3600
TAACTCAAAA AATACGCCCG GTAGTGATCT TATTTCATTA TGGTGAAAGT

      3610        3620        3630        3640        3650
TGGAACCTCT TACGTGCCGA TCAACGTCTC ATTTTCGCCA AAAGTTGGCC

      3660        3670        3680        3690        3700
CAGGGCTTCC CGGTATCAAC AGGGACACCA GGATTTATTT ATTCTGCGAA

      3710        3720        3730        3740        3750
GTGATCTTCC GTCACAGGTA TTTATTCGAA GACGAAAGGG CATCGCGCGC

      3760        3770        3780        3790        3800
GGGGAATTCG AGCTCGAGCT TACTCCCCAT CCCCCTGTTG ACAATTAATC

      3810        3820        3830        3840        3850
ATCGGCTCGT ATAATGTGTG GAATTGTGAG CGGATAACAA TTTCACACAG

      3860        3870        3880        3890        3900
GAAACAGGAT CCGCGGATCC GTGGAGAAAA TAAAATGAAA AAAGCAGTCA

      3910        3920        3930        3940        3950
TTAACGGGGA ACAAATCAGA AGTATCAGCG ACCTCCACCA GACATTGAAA

      3960        3970        3980        3990        4000
AAGGAGCTTG CCCTTCCGGA ATACTACGGT GAAAACCTGG ACGCTTTATG

      4010        4020        4030        4040        4050
GGATTGTCTG ACCGGATGGG TGGAGTACCC GCTCGTTTTG GAATGGAGGC

      4060        4070        4080        4090        4100
AGTTTGAACA AAGCAAGCAG CTGACTGAAA ATGGCGCCGA GAGTGTGCTT

      4110        4120        4130        4140        4150
CAGGTTTTCC GTGAAGCGAA AGCGGAAGGC TGCGACATCA CCATCATACT

      4160        4170        4180        4190        4200
TTCTTAATAC GATCAATGGG AGATGAACAA TATGGAAACA CAAACCCGCA

      4210
AGCTTGGTCT AGAGGTCGA
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Transgenic Maize Plants by Tissue Electroporation

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In this paper, we describe the transformation of regenerable maize tissues by electroporation. In many maize lines, immature zygotic embryos can give rise to embryogenic callus cultures from which plants can be regenerated. Immature zygotic embryos or embryogenic type I calli were wounded either enzymatically or mechanically and subsequently electroporated with a chimeric gene encoding neomycin phosphotransferase (*neo*). Transformed embryogenic calli were selected from electroporated tissues on kanamycin-containing media and fertile transgenic maize plants were regenerated. The *neo* gene was transmitted to the progeny of kanamycin-resistant transformants in a Mendelian fashion. This showed that all transformants were nonchimeric, suggesting that transformation and regeneration are a single-cell event. The maize transformation procedure presented here does not require the establishment of genotype-dependent embryogenic type II callus or cell suspension cultures and facilitates the engineering of new traits into agronomically relevant maize inbred lines.

INTRODUCTION

Genetic transformation has become an important tool in the study of basic plant processes and in crop improvement. The development of genetic transformation techniques for the major cereal crops has been relatively slow, mainly as a consequence of their limited susceptibility to Agrobacterium and their poor capacity to regenerate fertile plants from protoplasts (Rhodes et al., 1988).

Recently, microprojectile bombardment using DNA-coated particles has been used to transform embryogenic maize cultures, which have subsequently been regenerated into fertile transgenic plants (Fromm et al., 1990; Gordon-Kamm et al., 1990; Walters et al., 1992). These authors used derivatives of a particular maize inbred line, A188. This inbred has no agronomical value but is superior to most other maize inbreds in its capacity to regenerate plants at high frequency from embryogenic callus or cell suspension cultures. Particular callus cultures, the so-called type II callus, were a prerequisite for the initiation of cell cultures suitable for transformation (Fromm et al., 1990; Gordon-Kamm et al., 1990; Walters et al., 1992). Type II callus is highly embryogenic, white or pale yellow, friable, and rapidly growing. Its establishment is very genotype dependent and is only achieved at low frequency (Vasil et al., 1984, 1985; Armstrong and Green, 1985). The cell culture properties of A188 can be transmitted through genetic crosses to recalcitrant inbreds (Hodges et al., 1986). Backcrossing, combined with selection in tissue culture in each generation, can lead to the development of agronomically relevant inbreds with tissue culture properties amenable to genetic transformation using microprojectile bombardment.

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It would be advantageous if fertile transgenic plants could be generated directly from elite maize inbred lines. Our goal was to develop a transformation technique that is less genotype dependent and which would eliminate the difficulty of establishing type II cell cultures. Therefore, we investigated whether immature zygotic embryos or type I callus could be used as target material in transformation experiments. Type I embryogenic callus is compact, nodular, and organized; it can be obtained readily and at high frequency from cultured immature zygotic embryos in a wide variety of maize inbreds and hybrids (Lu et al., 1982, 1983; Novak et al., 1983; Duncan et al., 1985; Tomes and Smith, 1985; Hodges et al., 1986).

In this paper, we describe DNA delivery by electroporation into maize immature zygotic embryos and into type I callus cultures. Transgenic embryogenic calli were obtained using the neomycin phosphotransferase (*neo*) gene as selectable marker. Transgenic maize plants were regenerated from these cultures and the inheritance of the introduced gene was studied over several generations.

RESULTS

Transient NPTII Expression in Electroporated Maize Tissues

Intact immature zygotic embryos of maize inbred lines H99 or Pa91 were electroporated in maize electroporation buffer (EPM) with plasmid pDE108 DNA, schematically shown in Figure 1, containing a chimeric cauliflower mosaic virus (CaMV) 35S–*neo* gene, using electroporation conditions as described

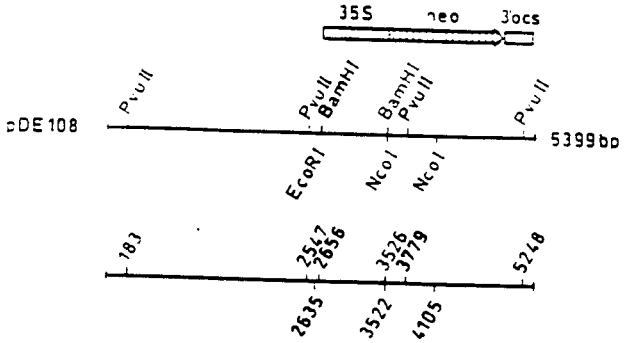

Figure 1. Plasmid pDE108.

Schematic representation of plasmid pDE108 linearized at the HindIII site. The chimeric *neo* gene is indicated. The coding region of the *neo* gene is represented by a stippled bar. The fragment containing the 3' untranslated region of the octopine synthase gene (3' ocs) is represented by an open bar. The arrow indicates the direction of transcription of the CaMV 35S promoter.

for leaf bases of rice seedlings (Dekeyser et al., 1990). Figure 2 shows that no detectable NPTII activity was observed when the embryos were assayed 4 to 6 days after electroporation. We reasoned that DNA delivery would require wounding of the embryos. This was achieved by an enzymatic treatment of the embryos in a 0.3% solution of macerozyme, an enzyme that degrades pectic substances for a short period varying from 1 to 3 min prior to electroporation. With this technique, NPTII activity could be detected reproducibly 4 to 6 days after electroporation with plasmid pDE108 DNA (Figure 2). NPTII activity was not significantly influenced either by increasing the DNA concentration from 10 to 20 μg per cuvette or by using linearized instead of covalently closed circular plasmid DNA. We also investigated whether transient expression was detectable in finely chopped embryogenic sectors of type I callus cultures, preplasmolyzed for 3 hr, and subsequently electroporated in the presence of plasmid pDE108 DNA. NPTII activity was detected in extracts of these tissues 4 to 6 days after electroporation (data not shown). Together, these data show that DNA can be electroporated into enzymatically wounded immature embryos and into mechanically wounded type I callus, and that the introduced gene is transiently expressed.

Influence of Wounding and Electroporation on Tissue Culture and Plant Regeneration

We analyzed the influence of enzymatical or mechanical wounding and electroporation on the capacity of immature embryos, as shown in Figure 3, and type I callus, as shown in Figure 4, respectively, to proliferate into embryonic callus and to regenerate plants. Routinely, untreated immature zygotic embryos of line Pa91 or H99 formed embryogenic type I calli at a frequency of ~100% upon in vitro culture. A short (1 to 3 min) enzymatic treatment and subsequent electroporation reduced this frequency to 50 to 90% (Figures 3B and 3C). An enzymatic treatment longer than 3 min drastically reduced type I callus formation.

The initial quality of immature embryos was a critical factor in the establishment of embryogenic type I callus. We observed that maize plants grown under suboptimal conditions produced cobs whose embryos were very poor in type I callus formation. Enzymatic wounding of the embryos reduced their capacity to form type I callus even further.

Mechanical wounding and subsequent electroporation did not significantly affect the growth of treated type I calli when plated on proliferation medium (Figure 4B). Although a slimy type of tissue proliferated frequently from the plated aggregates, subculturing of embryogenic sectors readily led to embryogenic callus cultures.

Selection of Stably Transformed Calli and Regeneration of Kanamycin-Resistant Plants

The above data indicate that DNA can be delivered into enzymatically wounded immature embryos and into mechanically wounded type I calli, and that the capacity of both tissues to proliferate into embryogenic type I calli is not significantly affected. We then investigated whether this procedure allowed the selection of stably transformed cell lines from which fertile transgenic plants could be regenerated.

Figure 3A shows immature embryos that were electroporated with 10 μg of linearized pDE108 DNA per cuvette and transferred immediately to selective medium. Embryos electroporated without DNA showed only some swelling and did not proliferate into callus on substrate containing 200 mg/L kanamycin (Figures 3D and 3E). Embryos electroporated in the presence of linearized pDE108 plasmid started forming type I callus within 2 weeks after transfer to selective medium (Figures 3F and 3G). Figure 4A shows type I callus that was

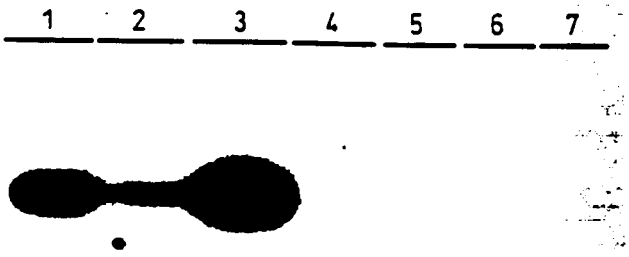

Figure 2. Transient Expression in Immature Zygotic Embryos.

NPTII assay on 50 μg of protein extract of different samples of ~30 immature embryos 5 days after electroporation with linearized pDE108 plasmid DNA. Lanes 1, 2, and 3, enzymatically treated immature embryos; lanes 4, 5, 6, and 7, nonenzymatically treated immature embryos. Exposure time, 20 hr.

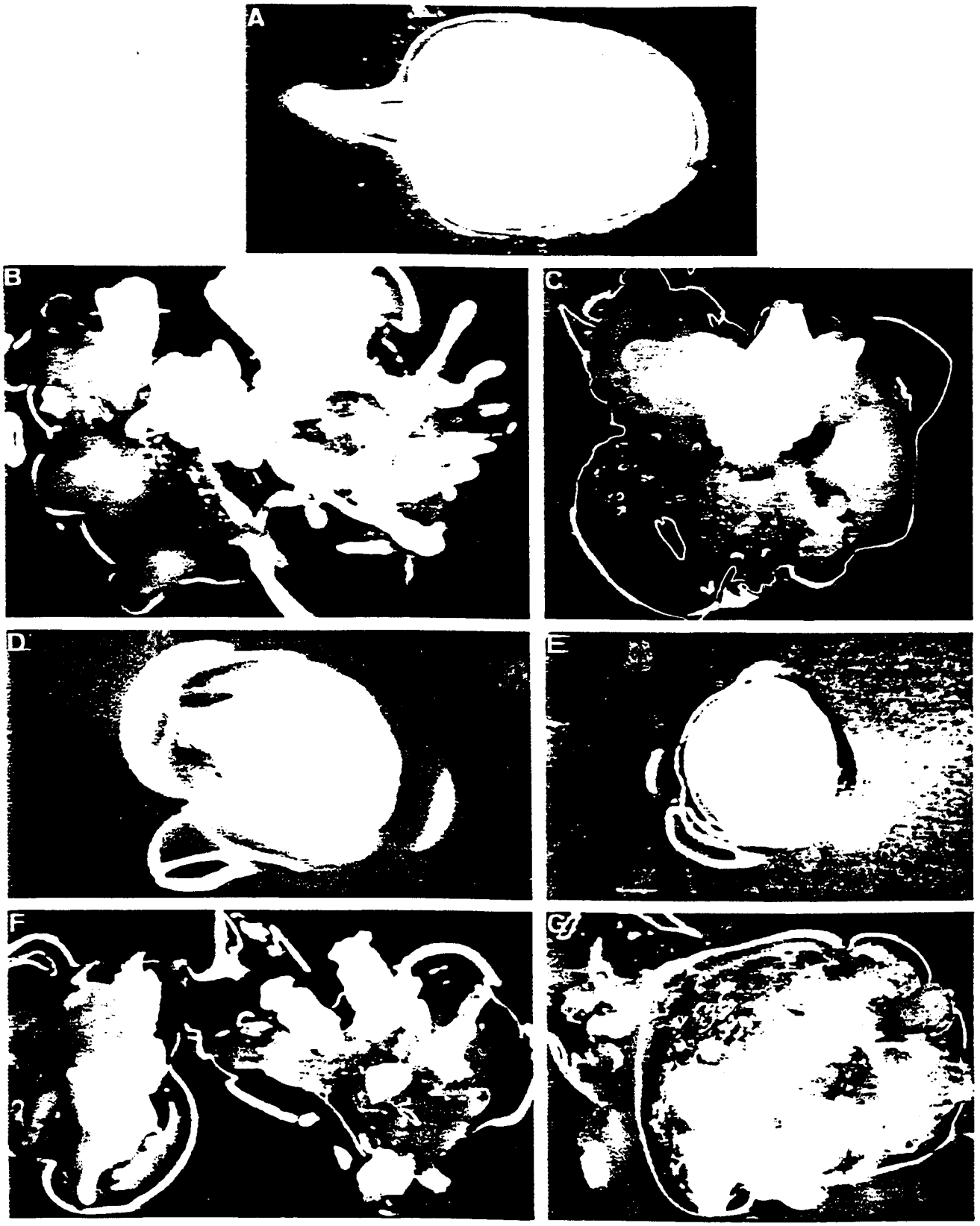

Figure 3. Electroporation of Enzymatically Treated Immature Embryos of Line H99.

(A) Enzymatically treated immature embryo after electroporation.
(B) and (C) Embryos electroporated with pDE108 DNA, cultured for 3 weeks on nonselective substrate.
(D) and (E) Embryos electroporated without DNA, cultured for 3 weeks on substrate containing 200 mg/L kanamycin.
(F) and (G) Embryos electroporated with DNA, cultured for 3 weeks on substrate containing 200 mg/L kanamycin.

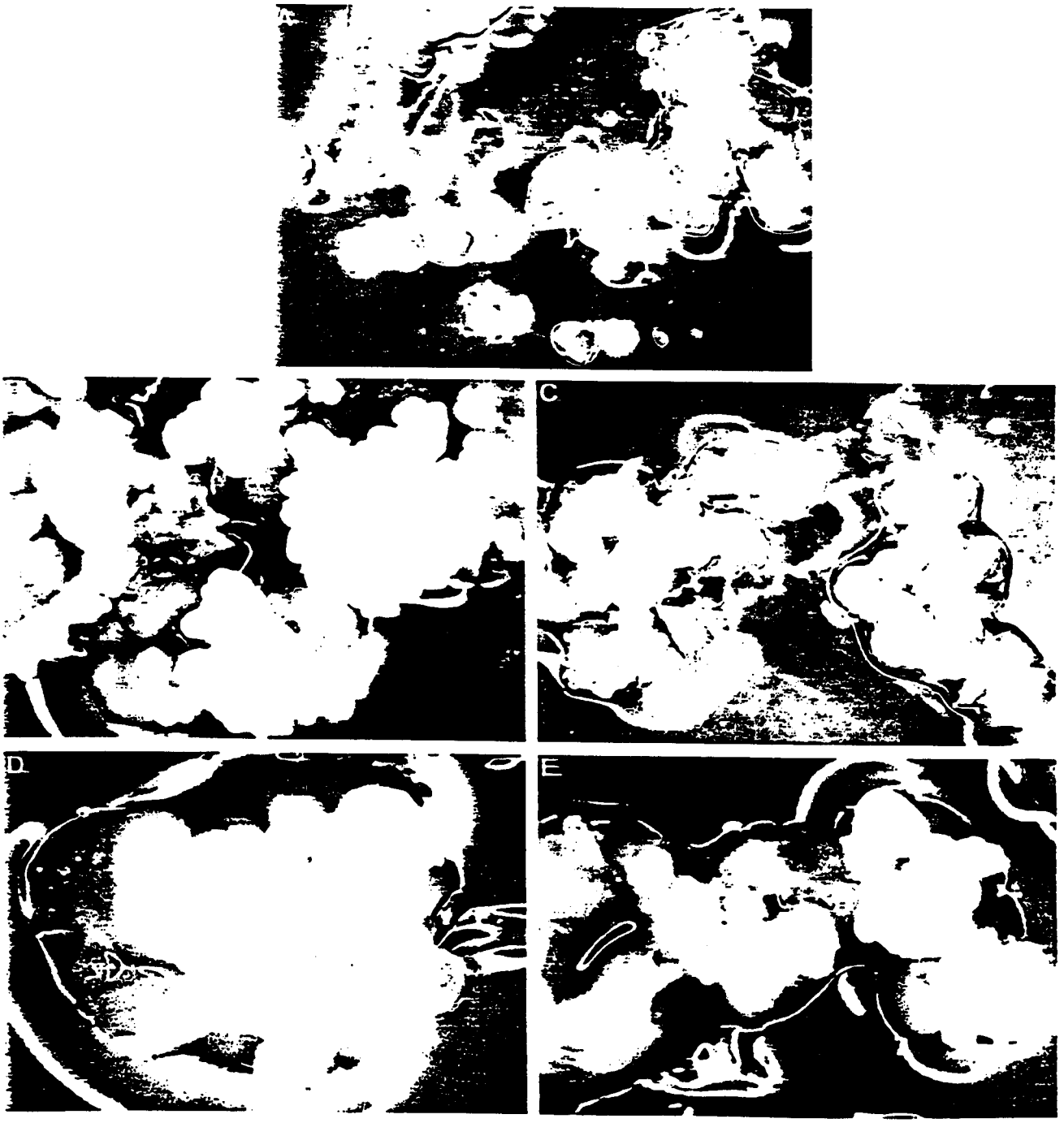

Figure 4. Electroporation of Mechanically Wounded Type I Callus of Line Pa91.

(A) Finely chopped type I callus after electroporation.
(B) Type I callus electroporated with pDE108 DNA, cultured for 5 weeks on nonselective substrate.
(C) Type I callus electroporated without DNA, cultured for 5 weeks on substrate containing 200 mg/L kanamycin.
(D) and (E) Type I callus electroporated with pDE108 DNA, cultured for 5 weeks on substrate containing 200 mg/L kanamycin.

electroporated with pDE108 DNA, as described for immature embryos, and immediately plated as small aggregates onto selective substrate. Type I calli electroporated without DNA did not proliferate into embryogenic tissue (Figure 4C), whereas proliferation from type I callus electroporated in the presence of DNA became apparent 4 weeks after electroporation (Figures 4D and 4E).

Proliferating calli from both immature embryos and type I callus were subcultured on selective medium for 6 to 8 weeks. Subsequently, kanamycin-resistant calli were transferred to nonselective medium containing a high concentration of cytokinin for 10 to 14 days to induce germination of somatic embryos. The embryogenic tissues were then transferred to a hormone-free medium to allow development into green plantlets, which were transferred to soil 2 to 4 weeks later.

Table 1 summarizes the results from 10 independent transformation experiments. The number of immature embryos that showed proliferation of embryogenic callus on selective substrate varied from 4 to 28%. The frequency at which finely chopped type I calli showed proliferation is expressed as the number of embryogenic calli obtained from the total amount of finely chopped tissue plated as small aggregates. Each selected callus was derived from either a separate embryo or a single aggregate of type I calli. Thus, each selected callus line represents an independent transformation event. The number of selected callus lines from which plants could be regenerated varied dramatically from one experiment to the other. Approximately 90% of plants recovered from kanamycin-resistant calli expressed the *neo* gene, as evidenced by NPTII gel assays (data not shown). The number of plants that regenerated from a selected callus line varied from one to 30. Optimally, 12 weeks were required to obtain transgenic plants from the start of the experiment to their transfer to the greenhouse.

Table 1. Summary of 10 Transformation Experiments

Inbred	No. of Explants[a]	Selected Embryogenic Calli (%)[b]	Shoot Regenerating Calli[c]	Regenerated Plants[d]	Transformed Plants[e]
Immature Embryos					
H99	675	186 (28)	8	78	70
H99	125	17 (14)	2	3	3
Pa91	75	4 (5)	2	10	9
H99	90	4 (4)	1	3	2
H99	150	23 (15)	2	6	6
H99	325	85 (26)	0	0	0
Type I Callus					
Pa91	25	41	7	31	27
Pa91	10	25	1	1	1
Pa91	10	13	2	3	3
Pa91	10	9	0	0	0

[a] The number of immature embryos or the number of cuvettes containing ~150 mg finely chopped type I callus.
[b] The number of embryos or type I calli aggregates showing proliferation after ~2 months on substrate with 200 mg/L kanamycin. Numbers in parentheses represent percent of total explants that showed proliferation of embryogenic callus.
[c] The number of kanamycin-resistant callus lines from which plants could be regenerated.
[d] The number of plants obtained from the regenerating callus lines.
[e] The number of regenerated plants that expressed the *neo* gene, assessed by kanamycin dot assays or by NPTII gel assays.

Phenotype of R_0 Plants and Inheritance of the *neo* Gene

Over a 4-month period, 148 R_0 plants were regenerated from 31 independent kanamycin-resistant calli and transferred to the greenhouse. The majority (>95%) of kanamycin-resistant plants survived transfer to soil, flowered, and produced viable pollen. As shown in Figure 5, most (>90%) plants developed normally and formed a normal tassel and ear. Seed set was obtained either by selfing or cross-pollination. Selfed R_0 plants produced 20 to 100 seeds per ear, whereas cross-pollination to wild-type plants yielded 100 to 200 seeds per ear. Sometimes R_0 plants showed characteristics typical for tissue culture–induced stress, such as reduced stature and pistillate flowers on the tassel. In such cases, 10 to 100 viable seeds were produced on the tassel.

Progeny from independently transformed R_0 plants were analyzed to determine the inheritance of the *neo* gene. NPTII activity was assessed by a localized application of a kanamycin solution (dot assay). Figure 5D shows that plants expressing the *neo* gene had no symptoms, whereas the newly formed leaves of nontransformed plants bleached and turned white. The dot assay allowed us to monitor the segregation of NPTII activity in large numbers of progeny. Table 2 presents segregation data of R_1 progeny from nine transformed R_0 plants. The segregation data obtained were compared with the expected frequencies in a chi-square test. The results are not significantly different from a 1-to-1 segregation in crosses and a 3-to-1 segregation in selfings. These data indicate that the NPTII activity was encoded by, and transmitted as, a single, dominant allele. In addition, progeny from 36 other primary regenerants were analyzed. The *neo* gene segregated in 34 of 36 progeny as a single, dominant allele. Two progeny revealed a higher number of sensitive plants than expected. NPTII enzyme assays, performed on some of the progeny, confirmed the segregation data obtained by the kanamycin dot assay.

To further test the inheritance of NPTII activity in the R_2 and R_3 generations, R_1 and R_2 plants expressing the *neo* gene were selfed and crossed to nontransformed plants. Transgenic plants were used as either the female or male parent. The data in Table 2 show that the *neo* gene was stably transmitted to the R_2 and R_3 generations in a Mendelian manner through both male and female gametes.

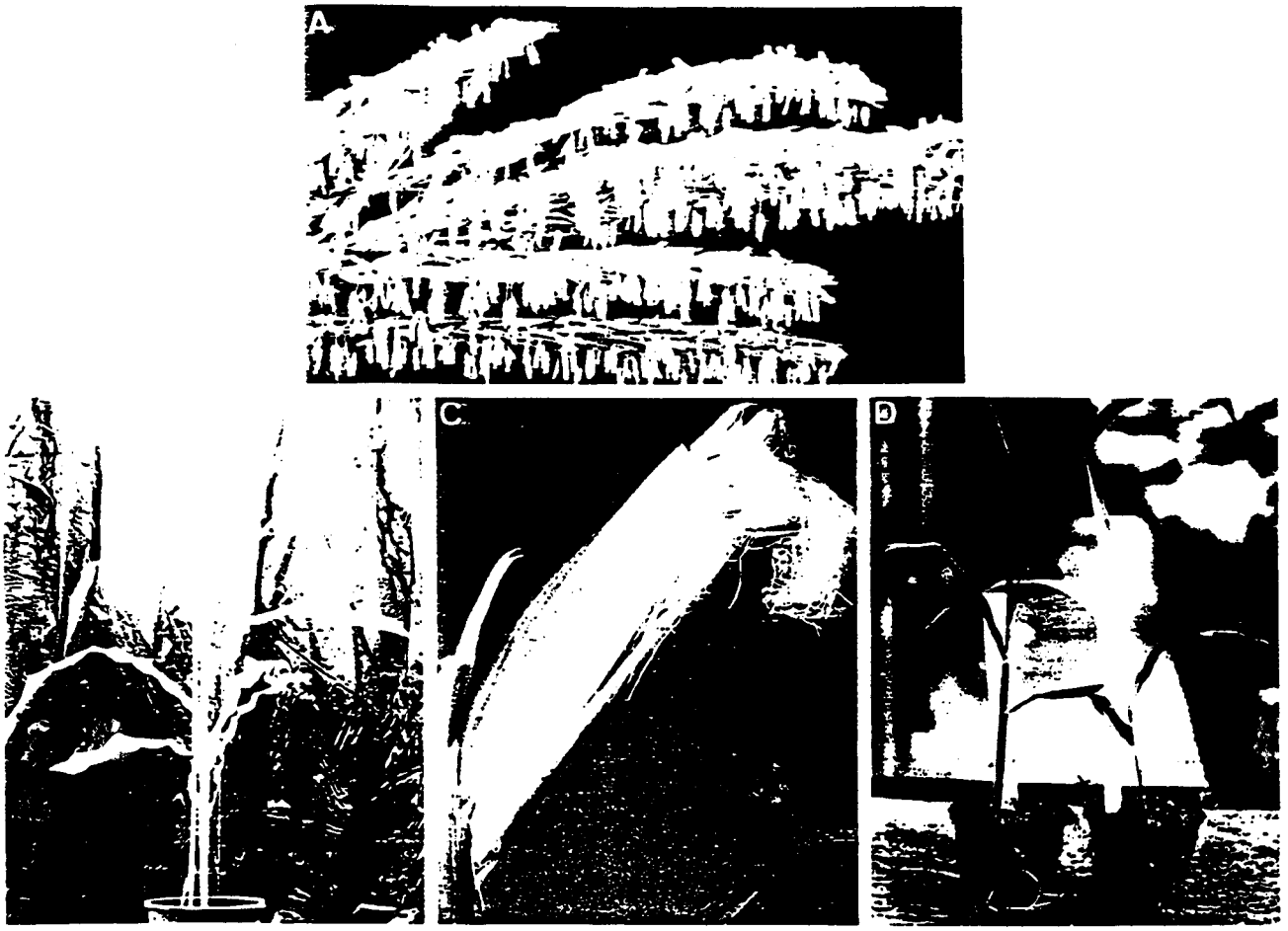

Figure 5. Phenotype of R_0 Plants.

(A) Tassel.
(B) Flowering plant.
(C) Ear.
(D) Kanamycin dot assay of a transgenic plant expressing the *neo* gene (left) and a nontransformed plant (right), 8 days after kanamycin application.

Molecular Analysis of Transgenic Plants

Figure 6 presents a DNA gel blot hybridization analysis of genomic DNA of several primary transformants digested with BamHI and hybridized with a 583-bp NcoI fragment from the *neo* gene. Both relatively simple and complex integration patterns were observed. One copy of the *neo* gene was present in transformants P4, H4, and H3. Transformants P1, H6, and H1 revealed two hybridizing bands and thus contain two copies of the *neo* gene. A 4.5-kb hybridizing fragment is present in all three plant DNAs. This 4.5-kb fragment is most likely generated from two pDE108 plasmid copies inserted in a head-to-tail configuration. The other hybridizing fragment constitutes plasmid–plant junction DNA which is of a different size in each transformant.

The inserted DNA in transformant P1 was analyzed in more detail. Figure 7 shows a DNA gel blot hybridization of BamHI- and EcoRI-digested P1 DNA using the complete pDE108 plasmid as a probe. The BamHI lane now shows three hybridizing bands: a 4.5-kb fragment, which agrees with a head-to-tail junction fragment, and two plasmid–plant junction fragments of 17 and 2.6 kb. The 870-bp fragment that is internal to pDE108 (Figure 1) and which is only visible after long exposure time is not shown on Figure 7. Because EcoRI cleaves pDE108 only once, a head-to-tail configuration would reveal a band with the exact size of pDE108. Such a 5.4-kb fragment is indeed present, together with two plasmid–plant junction fragments of 7.9 and 7.7 kb. The exact linkage of the plasmid copies that had been linearized at the HindIII site prior to DNA delivery was determined. A polymerase chain reaction (PCR) was performed

Table 2. Segregation of NPTII Activity in R_1, R_2, and R_3 Progeny of Transgenic Maize Plants[a]

Plant[b]	NPTII + [c]	NPTII − [c]	P[d]	χ^2[e]
R_1				
Selfing				
P1	2	1		
P2	26	19	0.18	1.77
P3	28	12	0.77	0.06
P4	29	23	0.06	3.44
P5	26	7	0.77	0.08
H5	13	2	0.65	0.21
P6	27	21	0.08	2.95
Male				
P1	47	32	0.26	1.25
P2	20	28	0.54	0.38
P3	31	19	0.31	1.01
P4	32	30	0.99	0.00
P5	9	8	0.99	0.00
H5	19	17	0.99	0.00
P6	22	27	0.84	0.04
H6	36	35	0.86	0.28
Female				
H4	27	15	0.27	1.21
R_2				
Selfing				
HP99 × H6	66	11	0.30	1.09
P1 × P1	32	11	0.99	0.00
H99 × P1	37	9	0.62	0.24
H99 × P2	38	10	0.81	0.06
Ms71 × P4	33	15	0.64	0.20
Male				
Pa91 × H6	23	25	0.99	0.00
H99 × P2	20	26	0.67	1.75
Ms71 × P4	23	25	0.99	0.00
Female				
H99 × H6	20	21	0.83	0.05
Pa91 × H6	26	22	0.83	0.04
H99 × P1	38	42	0.87	0.02
H99 × P2	31	17	0.21	1.53
Ms71 × P4	22	26	0.84	0.04
R_3				
Selfing				
(H99 × H6) × (H99 × H6)	30	4	0.24	1.39
Male				
(H99 × H6) × H99	32	32	0.86	0.03
(H99 × P1) × H99	22	25	0.99	0.00

[a] Transgenic R_0 plants or kanamycin-resistant R_1 or R_2 plants were either selfed or used as male or female parent in crosses with wild-type plants of H99, Pa91, or Ms71.
[b] Nomenclature: H and P, R_0 plants, see Figure 6; (H99 × H6), R_1 plant from the cross using H99 as female and H6 as male; (H99 × H6) × H99, R_2 plant from the cross using (H99 × H6) as female and H99 as male.
[c] With or without NPTII activity.
[d] P = χ^2 probability with 1 degree of freedom.
[e] χ^2 = chi-square values with Yates (continuity) correction.

to amplify the junction fragment carrying the linearization site. Sequence analysis of the amplified fragment proved the head-to-tail configuration. The DNA sequence at the junction revealed that the 5′ protruding nucleotides at the HindIII cleavage site plus one base pair were absent (data not shown).

BamHI-digested DNA of transformant H5 revealed four hybridizing bands when probed with the *neo* gene (Figure 6). The 4.5-kb fragment is again indicative for two plasmid copies in a head-to-tail configuration. The 3.8-kb fragment could be derived from two plasmid copies inserted in a tail-to-tail configuration, because it is twice the size of the 1873-bp BamHI–HindIII fragment of pDE108, which contains the *neo* gene. The two remaining hybridizing fragments probably constitute plasmid–plant junction DNA.

The other transformants P5, P3, P2, P6, and H2 have a complex integration pattern and the copy number could not be precisely ascertained. Intense bands of 4.5 and 3.8 kb are clearly visible, indicating that plasmid concatamers exist in head-to-tail and tail-to-tail configurations. Some of the hybridizing bands are smaller than what would be expected if only intact copies of pDE108 were present. These fragments thus clearly indicate that some plasmid DNA has undergone deletions and/or rearrangements. Transformants P2 and P5 have a nearly identical integration pattern, indicating that they originated from the same transformation event.

The fact that for the majority of transformants the *neo* gene segregated as a single, dominant allele suggests that they contain only one active copy of the *neo* gene, or that copies of the *neo* gene are closely linked. From four transformants with complex integration patterns (P2, P3, P5, and P6), four NPTII-positive and four NPTII-negative R_1 plants were analyzed by DNA gel blot hybridization. NPTII-negative R_1 plants did not hybridize to plasmid pDE108, whereas the complex integration patterns were stably inherited in NPTII-positive R_1 plants (data not shown). These data indicate that the inserted DNA sequences are integrated at the same or closely linked loci. The inheritance of the inserted DNA sequences in transformant P1 was followed over three generations. Figure 7 shows that the kanamycin-resistant progeny of three generations had integration patterns that were indistinguishable from that of the R_0 plant.

DISCUSSION

Cell Competence for Transformation and Regeneration

The establishment of embryogenic, friable, type II cell cultures is no longer a prerequisite for the genetic transformation of maize. All published data suggested that such cell lines were the only source of totipotent cells for genetic transformation. We have shown that DNA can be delivered by electroporation into preconditioned immature zygotic embryos or into type I callus. The preconditioning involves a mild enzymatic treatment of immature embryos or cutting and preplasmolysis of

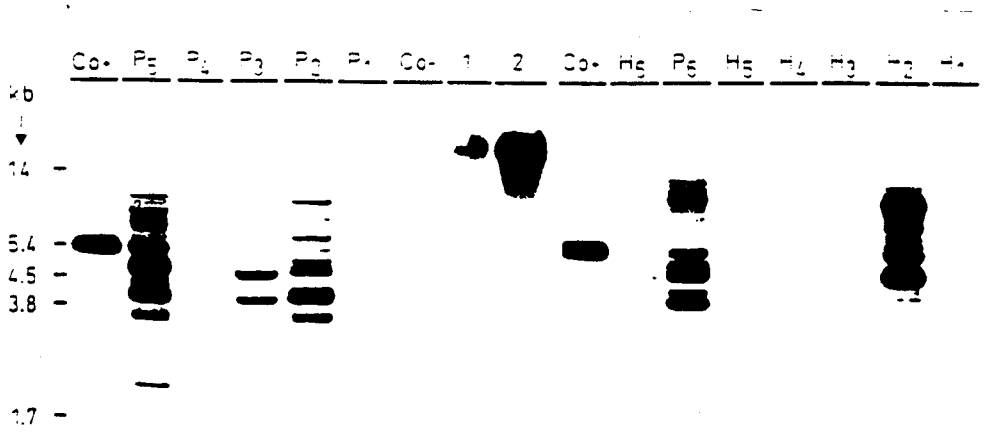

Figure 6. DNA Hybridization Analysis of Maize Transformants.

Genomic DNA was digested with BamHI and hybridized with a 583-bp ^{32}P-labeled NcoI fragment of the *neo* gene. P and H refer to transformants of lines Pa91 and H99, respectively. P5, P4, P3, P2, and P1, transformants from type I callus electroporation; H6, P6, H5, H4, H3, H2, and H1, transformants from immature embryo electroporation; lanes 1 and 2, undigested genomic DNA of H6 and P2, respectively; Co−, nontransformed control of Pa91; Co+, 95 pg (10-copy reconstruction) of plasmid pDE108. DNA linearized with HindIII.

type I callus. It is unclear whether this preconditioning makes transformation-competent cells accessible to the DNA and/or whether it induces competence for DNA uptake in the target cells.

We have shown that stably transformed callus lines can be established upon culturing DNA electroporated tissues on selective media. Stably transformed lines were subcultured as embryogenic calli and plants were regenerated under standard conditions. The transgene was inherited by the progeny as a single, dominant allele. The inserted DNA sequences segregated as single units in the transgenic lines, indicating that these sequences were integrated at the same or closely linked loci.

The number of kanamycin-resistant callus lines from which plants could be regenerated varied considerably from one experiment to the other. The quality of immature embryos was best in spring, probably as a consequence of high light and moderate temperature, whereas high temperature in summer or low-intensity light in winter dramatically reduces the response of immature embryos in transformation experiments.

Do Transgenic Maize Plants Originate from Single Cells?

Histological and ultrastructural examination has shown that the scutellum of maize immature zygotic embryos cultured for 3 days contain a broad subepidermal region on the abaxial surface with meristematic cells, whereas the adaxial scutellum cells enlarge and degenerate (Vasil et al., 1985; Fransz and Schel, 1990). It is not known whether regenerating shoots originate from a single cell within the scutellum. Similarly, it is unclear whether plantlets regenerated from type I callus are derived from a single cell. If regeneration occurs from multicellular structures composed of transformed and nontransformed

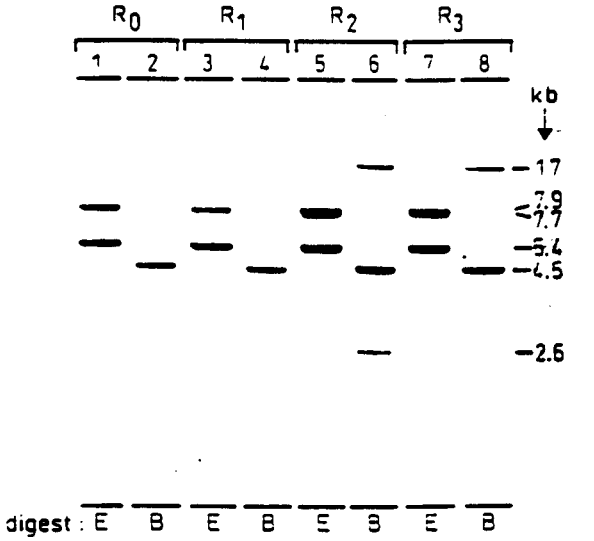

Figure 7. DNA Hybridization Analysis of Primary Transformant P1 (R_0) and Kanamycin-Resistant Plants from the R_1, R_2, and R_3 Generations.

Genomic DNA was digested with BamHI (B) and EcoRI (E) and hybridized with ^{32}P-labeled pDE108 plasmid.

cells, chimeric plants would occur at high frequency. The fact that the *neo* gene was inherited in a Mendelian fashion in progeny of transgenic plants indicates that the primary transformants were not chimeric for the transgene. Thus, our data strongly suggest that single cells in immature embryos or type I callus can be transformed and regenerated into plants. Histological analysis of enzymatically treated immature embryos or mechanically wounded type I callus electroporated with reporter genes, such as the β-glucuronidase gene or regulatory genes controlling anthocyanin biosynthesis, should provide more precise information on which cells from the scutellum of the embryos or from type I callus are susceptible to gene transfer by electroporation.

Advantages of Tissue Electroporation

Electroporation of organized and easily regenerable tissue, such as type I callus or immature embryos, allowed us to generate transgenic maize plants. In rice, immature embryos have also been successfully used as starting material for transformation by electric-discharge particle bombardment (Christou et al., 1991, 1992). Prior to this report, transgenic maize plants had only been obtained by using type II callus–derived cell cultures. These cultures were initiated from immature embryos at least 6 to 12 months prior to the transformation experiment, and transformants often showed phenotypic abnormalities and reduced fertility, most likely as a consequence of the long tissue culture period (Fromm et al., 1990; Gordon-Kamm et al., 1990; Walters et al., 1992). The method presented here only requires a short tissue culture period; under optimal conditions, rooted transformed plants could be transferred to the greenhouse 3 months after transformation experiments with immature embryos. Fertility problems were not observed and embryo rescue on R_0 plants was not required to recover progeny.

Previous reports on maize transformation have indicated that the tissue culture characteristics of A188, a maize inbred line of no agronomic value, were transferred to important breeding lines by crossing and backcrossing. Many backcrosses were required to obtain agronomically relevant lines with adequate tissue culture capabilities. We focused our transformation experiments on two public inbred lines, H99 and Pa91, both of which are highly regenerable from type I callus cultures (Hodges et al., 1986). As type I callus derived from cultured immature zygotic embryos can be obtained in a wide variety of maize lines, it should be possible to apply the tissue electroporation procedure to a wide variety of maize lines.

METHODS

Plant Material

Maize plants of the public inbred lines H99 and Pa91 were grown in the greenhouse in 20-L pots containing slow-release fertilizer. Growth conditions were at 25°C and 16-hr light of ~20,000 lux (daylight supplemented by sodium vapor and mercury halide lamps); temperature was reduced to 15 to 20°C at night. Immature zygotic embryos (1 to 1.5 mm in length) were excised from ears 10 to 14 days after pollination and plated with their embryonic axis in contact with the substrate. Type I callus was initiated from immature embryos in the dark at 23°C on Mah1VII substrate: N6 medium (Chu et al., 1975) supplemented with 100 mg/L casein hydrolysate, 6 mM L-proline, 0.5 g/L 2-(*N*-morpholino)ethanesulfonic acid (Mes), 1 mg/L 2, 4-D, and 2% sucrose solidified with 1.6 g/L Phytagel (Sigma), and supplemented with 0.75 g/L $MgCl_2$, pH 5.8.

Plasmids

Plasmid pDE108 carries a chimeric cauliflower mosaic virus (CaMV) 35S-*neo*-3′ *ocs* gene (Figure 1) (Denecke et al., 1989). Plasmid DNA was purified on Qiagen (Qiagen Inc.) columns and resuspended in 10 mM Tris-HCl, pH 7.9, and 0.1 mM EDTA at a concentration of 1 mg/mL. The plasmid DNA was linearized at the unique HindIII site prior to electroporation.

Electroporation

Immature Embryos

Excised immature embryos of H99 or Pa91 were enzymatically treated for 1 to 3 min with an enzyme solution containing 0.3% macerozyme (Kinki Yakult, Nishinomiya, Japan) in CPW salts (Frearson et al., 1973) supplemented with 10% mannitol and 5 mM Mes, pH 5.6. The embryos were then carefully washed with a N6adh solution (macro- and microelements of N6 medium supplemented with 6 mM asparagine, 12 mM proline, 1 mg/L thiamin-HCl, 0.5 mg/L nicotinic acid, 100 mg/L casein hydrolysate, 100 mg/L inositol, 30 g/L sucrose, and 54 g/L mannitol). After washing, 100 to 150 embryos were transferred into a disposable microcuvette (1938 PS microcuvettes; Kartell, Binasco, Italy) containing 200 μL maize electroporation buffer (EPM [80 mM KCl, 5 mM $CaCl_2$, 10 mM Hepes, and 0.425 M mannitol, pH 7.2]). Ten or 20 μg of plasmid DNA was added per cuvette and coincubated with the enzyme-treated embryos. After 1 hr, the cuvettes were transferred to an ice bath. After a 10-min incubation on ice, the electroporation was carried out by discharging one pulse with a field strength of 375 V/cm from a 900 μF capacitor. The pulse strength, capacitance, and electroporation apparatus are as described by Dekeyser et al. (1990). Immediately thereafter, 200 to 400 μL of fresh liquid N6adh substrate was added and the cuvettes were incubated for 10 min on ice prior to transfer of the embryos onto selective medium.

Type I Callus

Embryogenic tissue was dissected from developing type I callus of Pa91 that had been cultured on Mah1VII substrate for a period of ~2 months with subculture intervals of 14 to 20 days. The embryogenic tissue was cut in pieces ~1.5-mm thick in EPM buffer without KCl. After ~3 hr of preplasmolysis in this buffer, the callus pieces were transferred to cuvettes containing 200 μL of EPM supplemented with 80 mM KCl. Approximately 150 mg of callus fragments was transferred to each cuvette. Subsequent conditions were as for electroporation of immature embryos.

Selection and Regeneration of Transformants

Immature Embryos

The embryos were transferred immediately after electroporation to selective Mahi1VII substrate (Mahi1VII supplemented with 0.2 M mannitol and 200 mg/L kanamycin) and cultured in the dark at 23°C. After ~14 days, the embryos were transferred to Mahi1VII substrate (without mannitol) supplemented with 200 mg/L kanamycin. The embryos were further subcultured in the dark on this substrate for 6 to 8 weeks with subculturing intervals of ~3 weeks. For regeneration, the developing embryogenic tissue was isolated and transferred to MS medium (Murashige and Skoog, 1962) supplemented with 5 mg/L 6-benzylaminopurine for line H99 and 5 mg/L zeatin for line Pa91 and cultured at 23°C with a daylength of 16 hr. Fluorescent lamps ("lumilux white" and "natural"; Osram, Munich, Germany) were used with a light intensity of 2000 lux. The embryogenic tissue was maintained on this medium for 10 to 14 days and subsequently transferred to MS medium without hormones and 6% sucrose. Developing shoots were transferred to half-strength MS medium with 1.5% sucrose for further development into plantlets. These plantlets were transferred to soil and grown to maturity in the greenhouse.

Type I Callus

Immediately after electroporation the callus pieces were transferred to Mahi1VII substrate with 200 mg/L kanamycin and cultured in the dark at 23°C. The tissue of one cuvette was plated in random orientation onto one Petri dish of 9-cm diameter. Fourteen days later, the callus pieces were subcultured on the same selective substrate but without mannitol (Mahi1VII). The further regeneration protocol was as described for immature embryos.

Neomycin Phosphotransferase II Assays

Gel Assay

Neomycin phosphotransferase II (NPTII) activity was detected by the in situ gel assay according to the method of Reiss et al. (1984).

Dot Assay

An incision was made with a pair of scissors up to the midvein in leaves of ~4-week-old plants, and a 2% kanamycin solution containing 0.2% SDS was applied with a cotton wrap. Plants were assessed 8 to 10 days after kanamycin application.

DNA Gel Blot Hybridization

Total plant DNA was isolated as described by Dellaporta et al. (1983). The DNA was digested, separated by electrophoresis on a 1% agarose gel, transferred to nylon Hybond-N+ membranes (Amersham), and hybridized with ^{32}P-radioactive probes that were labeled as described by Amersham (Megaprime) or Pharmacia (T7 Quick Prime).

Polymerase Chain Reaction

DNA was prepared according to Dellaporta et al. (1983). For polymerase chain reaction (PCR) analysis, 500 ng of DNA was heat denatured at 95°C for 5 min prior to the start of the PCR cycles. The complete PCR mixture contained 5 μL DNA (500 ng); 15 μL H_2O and 30 μL Mastermix (5 μL amplification buffer, 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin); 1 μL 10 mM each dATP, dCTP, dGTP, and dTTP; 0.2 μL *Thermus aquaticus* DNA polymerase (5 units per μL); 0.5 μL each primer (10 pmol/μL); and H_2O (added to 30 μL). The primers used were 5′-CAGTGACGACAAATCGTTGGGC-3′ (position on pDE108, 2916–2937) and 5′-AATACGCAAACCGCCTCTCC-3′ (position on pDE108, 3372–3391). The PCR cycle was 1 min at 95°C, 1 min at 53°C, and 2 min at 72°C, for a total of 35 cycles. Sequencing according to Maxam and Gilbert (1980) was performed on an agarose gel–purified 476-bp fragment.

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Annex 7. Molecular characterization of transformation event MS3

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C. Vanderstraeten, PGS Technician

Annex 7.1. Molecular analysis techniques

Annex 7.2. Characterization of the insert of event MS3

Annex 7.3 Expression of the introduced transgenes and analysis of the possible occurrence of cryptic gene expression

Annex 7.4 Stability of the insert of event MS3

Responsible : A. Van Vliet, PGS Researcher

Annex 7.5. Quantification of phosphinothricin acetyl transferase (PAT) levels in H99 and MS3 corn seeds

Annex 7.6. Quantification of β-lactamase in H99 and MS3 corn seeds

Annex 7.1. · Molecular analysis techniques

1. Southern hybridization procedure

2. Polymerase chain reaction (PCR)

MOLECULAR ANALYSIS TECHNIQUES

1. Southern hybridization procedure

Introduction

Total genomic DNA is isolated from plant tissue according to Dellaporta et al. (1983, Plant Molecular Biology Reporter, 1, vol.3, p.19-21). Localization of particular sequences within genomic DNA is accomplished by the transfer technique described by Southern (J.Mol.Biol., 98, pp.503-517, 1975). Genomic DNA is digested with one, or more, restriction enzyme(s), and the resulting fragments are separated according to size by electrophoresis through an agarose gel. The DNA is then denaturated and transferred from the gel to a solid support (nylon membrane). The DNA attached to the membrane is hybridized to a radiolabeled DNA probe, and autoradiography is used to locate the positions of bands complementary to the probe. Based on the mobility of the respective fragments, results are interpreted.

Preparation of total genomic DNA

- Collect between 0.5 and 1g of leaf tissue, freeze in liquid nitrogen, grind to a fine powder in a mortar with a pestle, and transfer the powder into a 30ml Oak Ridge tube containing 15ml extraction buffer (100mM Tris.HCl pH 8, 50mM EDTA, 500mM NaCl, 10mM β mercaptoethanol).
- Add 1ml 20% SDS, mix thoroughly by vigorous shaking and incubate the tubes at 65°C for 10 min.
- Add 5ml 5M potassium acetate, shake tubes vigorously and incubate at 0°C for about 20 min.
- Spin tubes at 25000xg for 20 min (13000 rpm in Sorvall SA 600 rotor). Pour supernatant trough a Miracloth filter (Calbiochem) into a clean 30ml tube, containing 10ml isopropanol. Mix and incubate at -20°C for 30 min .
- Pellet the DNA at 20000xg for 15 min. Gently pour off the supernatant and dry pellets by inverting the tubes on paper towels for 10 min.
- Redissolve DNA pellets with 700 µl of TE20 buffer (50mM Tris.HCl pH 8, 20mM EDTA), and transfer to a microfuge tube.
- Add 5 µl RNase (10 mg/ml) and incubate for 10 min at 37°C.
- Spin tubes for 10 min in a microfuge to remove insoluble debris.
- Transfer the supernatant to a new eppendorf tube and add 75 µl 3M sodium acetate and 500µl isopropanol. Mix well and pellet the DNA for 30 seconds in a microfuge.
- Wash pellets with 80% ethanol, dry and redissolve DNA in 100 µl TE buffer (10mM Tris.HCl pH 8, 1mM EDTA).
- Determine the concentration of the DNA by measuring the UV absorbance at 260 nm. An OD of 1 corresponds to 50µg/ml DNA.

Restriction digests of total genomic DNA

- 5 or 10µg genomic DNA are digested in a total volume of 50µl.

- Mix in a microfuge tube : 10µg of genomic DNA
  5µl 10xRE buffer (*)
  10 to 20 units of restriction enzyme
  H_2O up to 50 µl
- Incubate the digest overnight at the recommended temperature.
- Add 5µl of gel-loading buffer.

(*): composition of 10 x RE buffer
- 100mM Tris.HCl pH 8
- 50mM $MgCl_2$
- 60mM β mercaptoethanol
- 1mM EDTA
- 1mg/ml BSA

Supplemented with 0.5M NaCl (RE50), 1M NaCl (RE100) or 1.5M NaCl (RE150). Or alternatively, the buffer recommended by the manufacturer was used.

Separation of the restriction fragments on agarose gels

- Prepare 1% agarose gel in TAE buffer (40mM Tris, 5mM sodium acetate, 1mM EDTA, pH 7.8 with acetic acid), containing 0.3 µg/ml Ethidium Bromide.
- Pour the gel into a, preferably, horizontal gel support and let solidify.
- Load the DNA samples into the wells of the gel. Include a MW marker (λ-DNA digested with PstI, or commercial available MW ladder, such as the 1Kb ladder from BRL-Life technologies).
- Run the gel slowly (1V/cm) overnight .
- Cover the gel with Saran-wrap after the samples have migrated about 1 cm into the gel.

Blotting of the restriction fragments to nylon membranes

- After electrophoresis is completed, cut the gel from the support. Place a fluorescent ruler alongside the gel and document the fractionation of the DNA. The image is acquired, processed and copied to thermal paper using the Foto/Analyst™ Visionary imaging system from FOTODYNE (CCD camera: charge-coupled device).
- Blot the separated DNA fragments to Hybond-N+ (Amersham). Hybond-N+ is a positively charged nylon membrane which yields excellent sensitivity in both alkali blotting and conventional Southern blotting.

Southern blotting

- Depurinate the gel in 0.25M HCl until the bromophenol blue changes colour.
- Rinse the gel with water. Place the gel in denaturation solution (1.5M NaCl, 0.5M NaOH) for 30 to 45 min.
- Rinse the gel with water. Place the gel in neutralization solution (1.5M NaCl, 0.5M

Tris-HCl, pH7.2, 0.001M EDTA) for 30 to 45 min.
- Rinse the gel with water and set up the capillary blot using 20xSSC (3M NaCl, 0.3M Na_3citrate) as blotting buffer.

Alkali blotting

- Depurinate the gel in 0.25M HCl until the bromophenol blue changes colour.
- Rinse the gel with water and set up the capillary blot using 0.4M NaOH as blotting buffer.

Capillary blotting

- Fill a glass dish with blotting buffer (Either 20xSSC or 0.4M NaOH). Make a platform and cover it with a Whatman 3MM filter paper wick, saturated with buffer.
- Place the gel on the wick and avoid trapping air bubbles beneath it. A sheet of Hybond-N membrane, cut to the exact size of the gel, is placed on top of the gel. Avoid trapping bubbles beneath the membrane.
- Place a sheet of Whatman 3MM cut to size and wetted with blotting buffer, on top of the Hybond-N membrane.
- Surround the gel with SaranWrap foil to prevent the blotting buffer being absorbed directly into the paper towels above.
- Place a stack of absorbent paper towels on top of the 3MM paper.
- Place a glass plate on top of the paper towels and a 0.5 - 1Kg weight on top. Allow the transfer to proceed for 8 to 16 hours.
- After blotting carefully, dismantle the setup. Before removing from the gel, mark the membrane with a pencil to allow later identification of the tracks.
- Rinse the membrane in 2xSSC. Air dry the membrane.
- For capillary blotting using 20xSSC: fix the DNA to the membrane by baking in an oven at 80°C for 2 hours. For alkali blots: there is no need to fix DNA after alkali blotting.

Purification of fragments for probe preparation

- Digest ±20µg of the plasmid DNA with the appropriate restriction enzyme.
- Separate the DNA fragments on a 1% Low Melting Agarose gel, prepared in TAE buffer, containing 0.3 µg/ml Ethidium bromide.
- After electrophoresis is completed, cut the desired fragment from the gel with a scalpel. Put the gel slice in an Eppendorf tube.
- Add an equal volume of TE buffer (10mM Tris.HCl pH 8 , 1mM EDTA).
- Melt the gel slice in a 65°C waterbath for 10 min.
- Preheat an equal volume of phenol (equilibrated with TE buffer) 30 sec. at 65°C.
- Add the phenol to the melted gel slice and shake the mixture for 15 min.
- Centrifuge for 10 min in a microfuge to separate the two phases.
- Transfer the water phase to a new Eppendorf tube and extract for a second time with an equal volume of phenol.
- Precipitate the DNA from the water phase with 0.1 volume of 5M Sodium perchlorate and 1 volume of isopropanol.
- Pellet the precipitated DNA by spinning for 15 min in a microfuge.

- Dry pellets and redissolve in 50µl of TE.
- Measure the concentration of the DNA solution.

DNA Labelling

Feinberg and Vogelstein (Analyt.Biochem., 132, pp.6-13, 1983 and Analyt. Biochem., 137, P:266, 1984) introduced the use of random sequence hexanucleotides to prime DNA synthesis on denaturated template DNA at numerous sites along its length. Amersham International (Buckinghamshire, UK) has developed the Rediprime DNA labelling system, using nonamer primers, for extra convenience and performance. The system provides individually dispensed reaction mixes which are dried in the presence of a stabilizer and a dye. This makes labelling probes easier and more reproducible.

- Dilute the DNA to be labelled to a concentration of 2.5 - 25ng in 45µl of sterile water.
- Denature the DNA sample by heating to 95 - 100°C for 5 minutes in a boiling water bath.
- Centrifuge briefly and add the denaturated DNA to the labelling mix and reconstitute the mix by gently flicking the tube until the blue colour is evenly distributed.
- Centrifuge briefly.
- Add 5µl of Redivue [^{32}P]dCTP and mix by gently pipetting up and down.
- Centrifuge briefly and incubate the tube at 37°C for 10 minutes.
- Removal of unincorporated nucleotides is sometimes desirable to reduce background during hybridization. Probes can be purified by Sephadex™ chromatography or selective precipitation.

Hybridization and autoradiography

- The hybridization and washing steps are carried out in an hybridization oven with rotating bottle rack.
- Prehybridize the filters for 1 - 2 hours in 6xSSC, 5x Denhardt's, 0.5% SDS and 100µg/ml carrier DNA at 65°C.

  20xSSC: 3M NaCl, 0.3M Sodium citrate
  100xDenhardt's solution: 2%(w/v) BSA, 2%(w/v) ficoll and 2%(w/v) Polyvinylpyrrolidone

- Denature the labelled probe by heating for 5 min. at 95°C.
- Remove the hybridization solution from the bottle. Add new hybridization solution together with the denatured radiolabeled probe to the tube and continue the incubation over night (use 5 to 10ml per 200cm^2 of membrane).
- Wash the filters for 5 min. in 6xSSC, followed by 2 washes of 20 to 40 minutes each in 2xSSC, 0.1%SDS. A high stringency wash can be done when the background signal is still unacceptably high: wash the membrane between 5 and 10 minutes in 0.1xSSC, 0.1%SDS solution.
- Remove excess washing solution from the membrane and wrap in Saran-wrap.
- Establish an autoradiograph by exposing the filter for an appropriate time period (usually between 12 and 24 hours) to X-Ray film (Kodak-Xomat) at -70°C with an intensifying screen.

2. Polymerase chain reaction (PCR)

Preparation of Plant Genomic DNA

The rapid extraction of small amounts of plant genomic DNA suitable for PCR analysis is done according to the method described by Edwards *et al.* (K. Edwards *et al.*, NAR vol 19, No 6, page 1349, 1991).

- Collect samples for PCR analysis (usually leaf tissue) by using the lid of a Eppendorf tube to pinch out a disc of material into the tube.
- Macerate the tissue with a plastic pestle at room temperature, without buffer for 5 to 15 sec.
- Add 400 µl extraction buffer. (EB: 200 mM Tris HCl pH 7.5, 250 mM NaCl, 25mM EDTA, 0.5% SDS). The mixture can be left at room temperature until all samples have been extracted (> 1 hour).
- Centrifuge the extracts for 1 minute at max. speed and transfer 300 µl of the supernatant to a fresh Eppendorf tube.
- Mix with 300 µl isopropanol and leave at room temperature for 2 minutes.
- Centrifuge at max. speed for 5 minutes.
- Dry pellet and dissolve in 100 µl water.
- Centrifuge for 2 minutes and transfer supernatant to a new Eppendorf tube.
- Use 5 µl of this sample in a 50 µl PCR reaction.

Polymerase chain reaction

Standard procedure

5µl of the isolated DNA is used in a 50µl PCR reaction containing 10 mM Tris-HCl (pH8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 200µM of each dNTP; 0.001% (w/v) gelatin; 1 unit *Taq* DNA polymerase (Boehringer Mannheim); 10 pmole each of the downstream and upstream oligonucleotide primers.
A master mix of reagents (water, buffer, dNTP's, primers and enzyme) for all samples is prepared first and then aliquoted to the individual samples. The reaction mixtures are overlayed with 50µl mineral oil and thermocycling is started.

Thermocycling profile:

4 min. at 95°C
Followed by: 1 min. at 95°C
1 min. at 57°C
2 min. at 72°C
For 5 cycles
Followed by: 30 sec. at 92°C
30 sec. at 57°C
1 min. at 72°C
For 22 cycles
Followed by: 10 min. at 72°C

15µl of each PCR sample is separated on a 1.5% agarose gel. The BRL 123bp ladder or the Pharmacia 100bp ladder is used as a MW marker. Results are documented by Polaroid photography.

XL-PCR procedure

The GeneAmp®XL PCR kit (Perkin Elmer, California, USA) is a total system optimized to produce high yields of long (or XL) PCR product. The specially-designed XL buffer greatly enhances this long-target PCR process as does the rTth DNA polymerase XL (a mixture of the rTth DNA Polymerase and $Vent_R$® DNA polymerase).
The DNA template is mixed with 3.3x XL buffer (containing tricine, potassium acetate, glycerol and DMSO), dNTP's (200µM each, final concentration), 20 to 40 pmoles downstream and upstream primers, $Mg(OAc)_2$ (1.1mM final concentration).
Overlay this mixture with mineral oil and raise the temperature to 80°C (Hotstart principle). Add a mixture containing 3.3x XL buffer and 2units/50µl reaction volume of the DNA ploymerase to each tube. Start thermocycling.

Thermocycling profile:

4 min. at 94°C
Followed by: 15 sec. at 94°C
3 min. at 60°C
For 16 cycles
Followed by: 15 sec. at 94°C
4 min. at 60°C
For 7 cycles
Followed by: 15 sec. at 94°C
5 min. at 60°C
For 7 cycles

Oligonucleotide primers used

Barnase:

MDB6 5' CTG.GGT.GGC.ATC.AAA.AGG.GAA.CC 3'

MDB7 5' TCC.GGT.CTG.AAT.TTC.TGA.AGC.CTG 3'

Amplified fragment length: 160bp

Barstar

MDB8 5' TCA.GAA.GTA.TCA.GCG.ACC.TCC.ACC 3'

MDB9 5' AAG.TAT.GAT.GGT.GAT.GTC.GCA.GCC 3'

Amplified fragment length: 235bp

pVE108 primers

MDB54 5' AGT.CAG.TGA.GCG.AGG.AAG.CG 3'

MDB55 5' AGA.TTG.AAT.CCT.GTT.GCC.G 3'

MDB56 5' GAG.TTA.GCT.CAC.TCA.TTA.GGC 3'

MDB185 5' GTC.AGG.TAT.TAT.AGT.CCA.AGC 3'

pMc5barstar primers

VDS13 5' ATC.ACT.GGA.TAT.ACC.ACC.G 3'

VDS14 5' AGG.TTT.TCA.CCG.TAA.CAC.GCC 3'

VDS15 5' ATC.ACA.GAC.GGA.ATG.ATG.AAC.C 3'

VDS16 5' AGC.TCA.CCG.TCT.TTC.ATT.GCC 3'

MDB225 5' CTG.TGA.CGG.AAG.ATC.ACT.TCG.C 3'

Annex 7.2. . Characterization of the insert of event MS3

1. Introduction

2. Molecular analysis of the insert in corn transformation event MS3

CHARACTERIZATION OF THE INSERT OF EVENT MS3

1. Introduction

As described in detail earlier, transformation event MS3 was generated by electroporating immature zygotic embryos of the public corn line H99 in the presence of DNA of the plasmid pVE108. The plasmid DNA was linearized with the restriction enzyme HindIII prior to transformation (Figure 1.). The elements and the sequence of pVE108 have been described (Annex 5.).
The pVE108 plasmid, used to generate event MS3, was isolated from *Escherichia coli* strain WK6 containing the plasmid pMc5barstar, also denoted as "helper plasmid". This helper plasmid expresses the *barstar* gene in *E.coli*, countering possible adverse effects of expressed barnase in *E.coli*. A diagram of plasmid pMc5barstar, linearized with HindIII, is shown in Figure 2.. The elements and the sequence of pMc5barstar have been described (Annex 5.).
Prior to plant transformation, the linearized pVE108 plasmid batches were checked for completeness of the digest and for the presence of pMc5barstar by gel electrophoresis. Molecules of pMc5barstar may be present in the pVE108 plasmid preparation in concentrations below the detection limit of the method used to monitor the purity of the pVE108 plasmid preparation. Results of the in depth molecular analysis of the insert of event MS3 revealed that the transgenic insert contains part of pMc5barstar.

2. Molecular analysis of the insert in corn transformation event MS3

2.1. Summary

The inserted DNA resides on two adjacent HindIII fragments. A ±12Kb HindIII fragment consisting of a Head-to-Tail dimer of pVE108 and a ±9Kb HindIII fragment consisting of one pVE108 copy and a rearranged piece of pMc5barstar. The orientation of the pVE108 copy on this ±9Kb HindIII fragment forms a Tail-to-Tail configuration (with the *bar* genes orientated towards each other) with the dimer on the ±12Kb HindIII fragment. These elements are integrated at one site in the corn genome and are inherited as a single locus.
For clarity, we will describe the physical arrangement of the two HindIII fragments separately. The link between the two HindIII fragments will be demonstrated.

2.2. Remark

Because of the complexity of the insert, a number of digests (> 30) were hybridized with a number of probes (8).

Probes used: pVE108 total plasmid
barnase
bar
PTA29-P35S or P35S
ori & *bla*
cat : 800bp NruI-HindIII fragment of pFM136
barstar
F1 *ori* : 460bp Sau3A fragment of pMc5barstar (from bp309 to bp769)

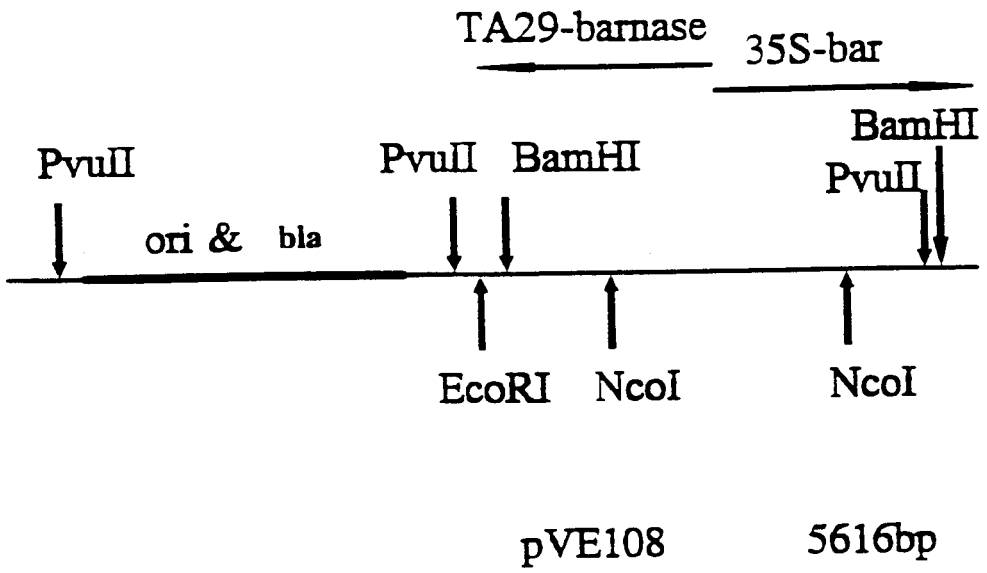


Figure 1. Diagram of plasmid pVE108 linearized with HindIII. The elements of pVE108 are *ori* and *bla*, PTA29-*barnase*-3'nos, P35S-*bar*-3'nos

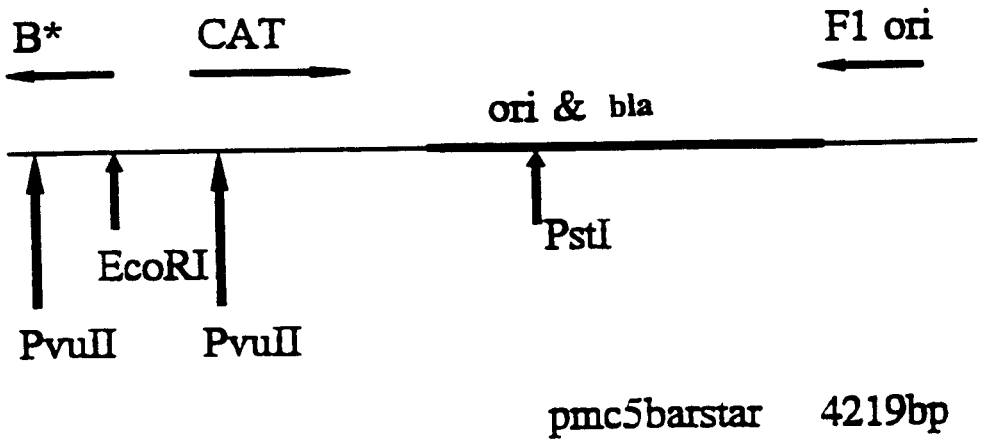


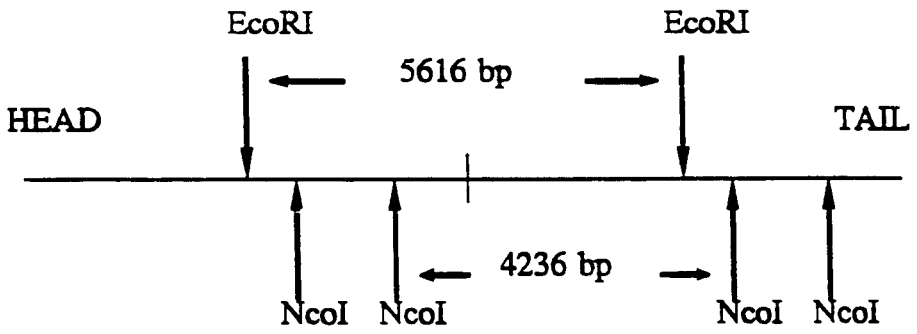
Figure 2. Diagram of plasmid pMc5barstar linearized with HindIII. The elements of this plasmid are Ptac-*barstar*, *ori* and *bla*, F1 *ori*.

2.3. Analysis of the Head-to-Tail pVE108 dimer (±12Kb HindIII fragment)

Upon the integration of the transforming plasmid, the recognition site for the restriction enzyme used to linearize the plasmid, is usually lost. Two distinct fragments of ±12Kb and ±9Kb are observed when HindIII digested plant DNA is hybridized with pVE108 and elements thereof. *Barstar* and *cat* probes (elements of pMc5barstar) hybridize only with the ±9Kb HindIII fragment
From the hybridization data obtained we can conclude the presence of a Head-to-Tail concatemer of pVE108 on the ±12 Kb HindIII fragment and a third pVE108 copy, linked to a rearranged piece of pMc5barstar, on the ±9 Kb HindIII fragment.

Southern Blot analysis:

For clarity, the Head-to-Tail concatemer formation is shown in Figure 3.



Head - to - Tail
Concatemer

Figure 3 Diagram of the pVE108 Head-to-Tail dimer in event MS3

The Southern blot data relevant to the pVE108 Head-to-Tail dimer are listed in Table 1. and schematically presented in Figure 4..

- In the EcoRI digest (single cut in pVE108), the 5600bp band is the concatemer fragment and consequently hybridizes with every element of pVE108. Because the 5800bp band hybridizes with the *barnase*, *bar* and P35S probes, it can be designated to be the "Tail" fragment of the dimer. The 4300bp band hybridizes only with *ori&bla* and thus it can be designated to be the "Head" fragment of the dimer. The 4800bp fragment specifically hybridizes with *cat*, *bar* and *ori&bla* sequences. This EcoRI fragment is part of a pVE108/helper-plasmid complex of the ±9Kb HindIII fragment.
- The EcoRI - PstI double digest basically confirms the previous data and conclusions. The 5600bp EcoRI concatemer fragment is cut by PstI to give a 3000bp fragment hybridizing to *barnase*, P35S and *bar* probes; and a 2600bp fragment that hybridizes to *ori&bla*. The faint 5600bp fragment reflects partial digestion of the EcoRI concatemer fragment. The 5800bp EcoRI fragment (Tail-to-Tail fragment) is not cut by PstI: either the PstI sites are lost or they are methylated.

Table 1. (Figures between brackets: weak hybridization signals) (d: doublet; t : triplet)

Probes	Digests								
	HindIII	EcoRI	NcoI	EcoRI HindIII	EcoRI HindIII NcoI	EcoRI NcoI	HindIII NcoI	PvuII	EcoRI PstI
pVE108	12 Kb 9 Kb	5800 5600 4800 4300	15 Kb 13 Kb (5600) 4200 1600 1380	5600 4800 2900(t)	4800 3500 2900 1380 800(d) 735	4800 4300 3500 1600 1380(t) 735	6800 4200 3650 1380(t) 800(d)	6500 (3400) 3200 (2700) 2400 2140	5800 (5600) 4800 4300 3000 2600
ori &bla	12 Kb 9 Kb	5600 4800 4300	15 Kb 13 Kb 4200	5600 4800 2900	4800 3500 2900	4800 4300 3500	6800 4200 3650	2400 2140	(5600) (4800) 4300 4000 2650
barnase	12 Kb 9 Kb	5800 5600	15 Kb 13 Kb 4200	5600 2900(d)	735	735	6800 4200 3650	6500 3400 3200 2700	5800 5600 2950
PTA29-P35S	12 Kb 9 Kb	5800 5600	1380(t)			1380(t)	1380(t)	6500 3400 3200 2700	5800 3000
bar	12 Kb 9 Kb	5800 5600 (4800)	4200 1600	5600 2900(d)	3500 800(d)	(4800) 3500 1600	4200 800	6500 3400 3200 2700 2140	5800 (4800) 3000
cat	9 Kb	4800	15 Kb	4800	4800	4800	6800	2140	(4800) 800
barstar	9 Kb	11 Kb	15 Kb	1200	1200	9 Kb	6800	2300 664	11 Kb
F1 *ori*	-	-	-	-		-	-	-	-

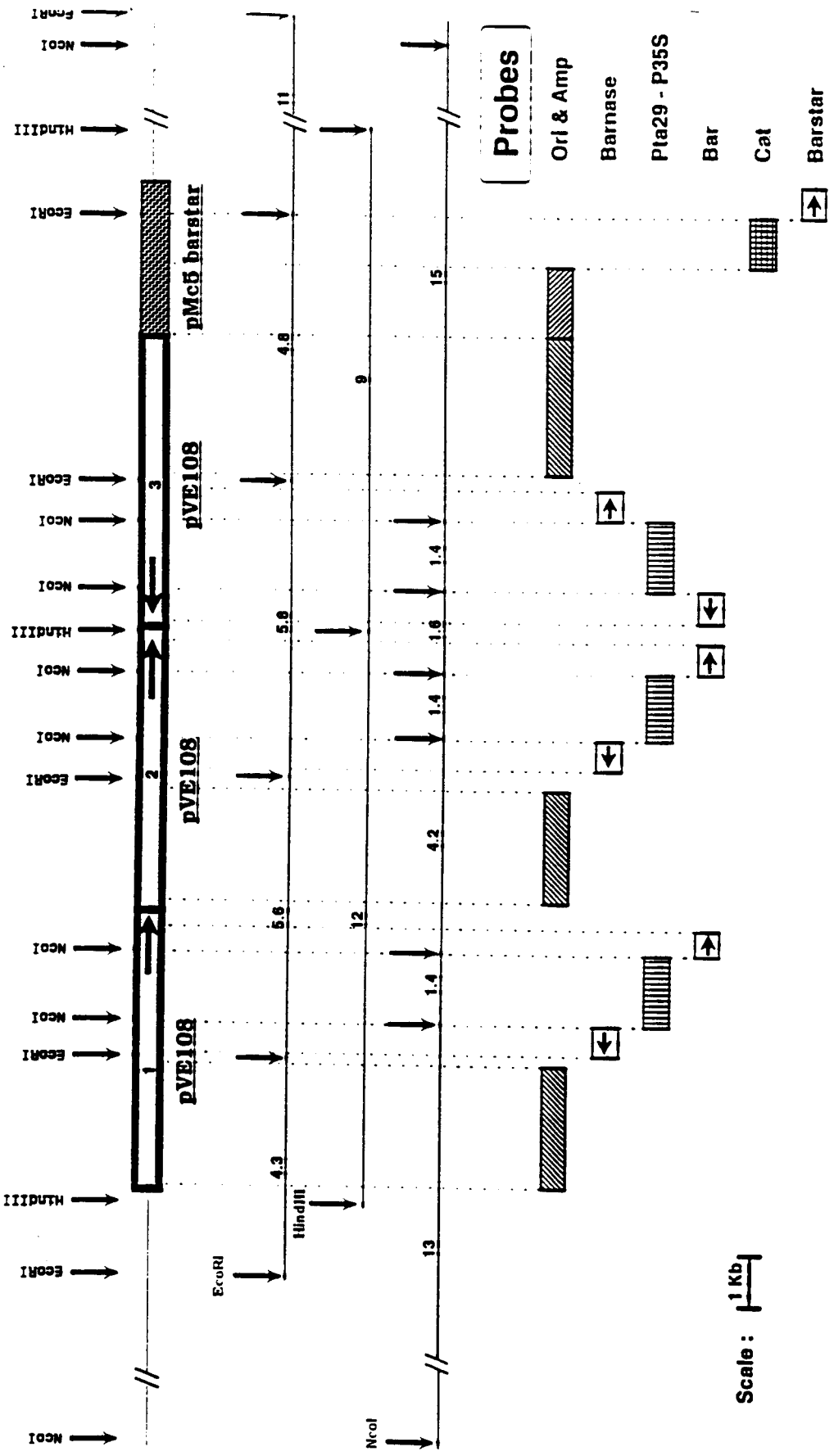

Probes
Ori & Amp
Barnase
Pta29 - P35S
Bar
Cat
Barstar
pMc5 barstar
pVE108
pVE108
pVE108
EcoRI
NcoI
HindIII
NcoI
Scale : 1 Kb

Figure 4.

- In the NcoI digest, the concatemer fragment has a size of 4200bp. This fragment harbors *barnase*, *bar* and *ori&bla* to which it hybridizes. The 1380bp P35S- PTA29 fragment is specifically detected with the P35S probe. Here we can designate the 13Kb NcoI fragment to be the Head fragment because it hybridizes to *barnase* and *ori&bla*. The 1600bp fragment that hybridizes to *bar* is cut into two 800bp fragments with HindIII: it is the tail fragment of the dimer.
  The 15Kb fragment hybridizes with *barnase*, *bar* and *bla&ori*. It also hybridizes to *barstar* and *cat* probes (features from the helper-plasmid). This 15Kb fragment is in fact the 3'end of the complete insert.

- The EcoRI-HindIII, EcoRI-NcoI and EcoRI-HindIII-NcoI digests further confirm the transgene organization on the 12Kb and 9Kb HindIII fragments. Hybridizing the EcoRI-HindIII double digest with the pVE108 plasmid, gives rise to a 5600bp and a 4800bp fragment and a triplet with a MW of about 2900bp. One of these 2900bp fragments is the 5'end of the insert with the HindIII recognition site located in the plant DNA. This fragment hybridizes also to the *ori&bla* probe. The two other 2900bp fragments are the result of the HindIII cleavage of the 5800bp EcoRI fragment that forms the tail of the dimer. Both fragments hybridize to the *barnase*, *bar* and P35S-PTA29 probes.

- In the PvuII digest, we are unable to denote the Head fragment. This is not unexpected: to detect this specific PvuII fragment we would have to probe the PvuII digested plant DNA with the small HindIII-PvuII fragment (position in pVE108: 3378 up to 3561).
  The PvuII concatemer fragment should have a length of 553bp (PvuII sites at position 3561 and 3008 in pVE108). We didn't observe this fragment because we never used the specific 553bp fragment as a probe, and when using the total plasmid as a probe, this concatemer fragment would in the best circumstances give a weak signal. Moreover we observed the following: when hybridizing with *barnase*, *bar* or P35S probes, we expect to find a 2700bp fragment. We do find this fragment but it hybridizes weakly to these probes. Instead we always observe a more prominent 3200bp fragment. We believe that the PvuII site (at position 3008) is partially digested (possibly due to methylation) and that hence we obtain a 2699 + 553 = 3252bp fragment.
  We can designate the 6500bp to be the Tail fragment of the concatemer: it hybridizes with PTA29-P35S and *bar*. Normally we should observe a 2700bp fragment because of the presence of a PvuII site at bp 3008 on pVE108. This 6500bp PvuII fragment is cut by HindIII and is actually the Tail-to-Tail fragment. Either the PvuII sites near the HindIII site are lost or they are methylated.
  The 2140bp PvuII fragment hybridizes with *bar* and *bla&ori* probes. It also hybridizes to *cat* sequences from the helper-plasmid. This fragment resides on the 9Kb HindIII fragment.

PCR analysis of the Head-To-Tail junction of the dimer

The loss of the HindIII restriction site was further confirmed by PCR. We could amplify a 380bp fragment using oligonucleotide primers located upstream (MDB56, position in pVE108: 3503--->3483) and downstream (MDB55, position in pVE108: 3123--->3142) of the HindIII site at which the plasmid was linearized. The DNA sequence of the amplified fragment proved the Head-to-Tail configuration. The sequence at the junction also revealed that the 5' protruding nucleotides plus one base pair were absent :

pVE108-HindIII digested

G A C C T G C A G G C A T G C A **A G C T T** G G C G T A
C T G G A C G T C C G T A C G T **T C G A** **A** C C G C A T

MS3 Head-to-Tail Junction

G A C C T G C A G G C A T G C A G G C G T A

2.4. Analysis of pVE108-pMc5barstar complex (±9Kb HindIII fragment)

Southern Blot analysis

The general structure can be derived from the data in Table 1.

On the ±9Kb HindIII fragment we localized a pVE108 copy with the 3'nos-*bar*-P35S cassette at the 5'end and part of pMc5barstar at the 3'end.

As already mentioned, a 15Kb NcoI fragment hybridized with the *barnase*, 3'nos, *ori&bla*, *cat* and *barstar* probes. Very weak hybridization was observed with the *bar* probe. A double digest (NcoI-HindIII) resulted in a 6800bp NcoI-HindIII fragment that hybridized to the same probes.

In the EcoRI digest, we identified a 4800bp fragment hybridizing to *ori&bla*, 3'nos and the *cat* probes (very weak hybridization signals with *bar* were also observed), and a 11Kb EcoRI fragment hybridizing solely to the *barstar* probe. The *barstar* probe hybridized to a 1200bp fragment in a EcoRI-HindIII double digest.
With these data we could position the different EcoRI and EcoRI-HindIII fragments on the 15Kb NcoI fragment. The 5800bp EcoRI fragment (Tail-to-Tail) has the *barnase* gene at its 3'end. The adjacent 4800bp EcoRI fragment hybridizes to the *ori&bla*, 3'nos and *cat* probes. It also hybridizes very weakly to the *bar* probe. Adjacent to this fragment we localized the 1200bp EcoRI-HindIII fragment harbouring the *barstar* and the plant DNA sequences at the 3'end of the insert.

To analyze the structure of the transgene on the ±9Kb HindIII fragment, additional digests were made (Table 2.).

The analysis of the 4800bp EcoRI fragment (EcoRI-PstI, EcoRI-PvuII, PstI-PvuII double digests) revealed the intactness of the *ori&bla* originating from the pVE108 plasmid. A rearranged 2140bp PvuII fragment hybridizes to *ori&bla*, 3'nos, *cat* and weakly to *bar*. If the rearranged part contains *ori&bla* sequences derived from the pMc5barstar plasmid, then this *ori&bla* would be integrated in a Tail-to-Tail configuration with the *ori&bla* of the pVE108 plasmid copy.

Finally, it was analyzed which known functional elements on the transforming DNA were present on the ±9Kb HindIII fragment.

Table 2

Probes	Digests									
	NcoI PvuII	**EcoRI PvuII**	**PvuII**	**PstI**	**PstI EcoRI**	**PstI NcoI**	**PstI PvuII**	**PstI HindIII**	**MunI HindIII**	**MunI**
barnase	735	5800 5600 3300 3100 2600	6500 3400 3200 2700	> 12 Kb	5800 (5600) 2950	11 Kb 4600 3500	6500 3400 3200 3000 2700	7000 5800 5600	7500 5600 3800	> 14 Kb 5600
PTA29-P35S			6500 3400 3200 2700		5800 3000				5600 3800 1700	
bar			6500 3400 3200 2700 2140		5800 (4800) 3000				7500 5600 1700	14 Kb 5600 3400
ori & bla	2400 2140	2400 2140	2400 2140	> 12 Kb	4800 4300 4000 2650	11 Kb 4600 3300	2400 1700	7000 5800 5600	7500 5600 3800	> 14 Kb 5600
barstar	2300 664	300	2300 664	>	11 Kb	10 Kb	2300 664	2200	7500	14 Kb
cat	2140	2140	2140	>	4800 800	10 Kb	664 500	2200	7500	

>: due to the limitations of the DNA extraction procedure and the gel system used, a smear instead of a fragment with a defined MW is observed.

Barstar : from position 3885 ---> 4157 in the helper plasmid

The following scheme summarizes the hybridization data with a *barstar* probe and event MS3 DNA

Digest	Relevant pos. in pMc5barstar	Expected fragment length	Observed fragment length
PvuII	3407, 4071	664	664 2300 (weak) 6000 (weak)
Sau3A	3866, 4161	295	1090 900 (weak)
HindIII	4201	-	± 10Kb
EcoRI	3755	-	> 10Kb
NcoI	no site	-	> 15Kb
BamHI	3866	-	> 10Kb

- The typical 664bp PvuII fragment is observed. A second (and sometimes a third) weak band is also observed. The second weak band (2300bp) is due to the very small piece of homology between the *barstar* probe and this PvuII fragment: PvuII site at position 4071 and end of *barstar* at position 4157 (length of homology: 86bp). The third weak fragment of about 6000bp is not always observed and probably reflects incomplete digestion of the template.
- The typical 295bp Sau3A fragment is not observed as such. This might be due to the transfer method used for the Southern blots. We performed an alkali blot and it's our experience that hybridization signals of very small fragments are usually very weak compared to the 20xSSC transfer method.
  But since we detect a 1090bp fragment, a more likely explanation is the following. The Sau3A site (position 4161) is only 40bp upstream of the HindIII site (position 4201), which was lost upon electroporation.
  Using *barstar* primers MDB8 (position in pMc5barstar: 3916 ---> 3939) and MDB 9 (position in pMc5barstar: 4128 <---4151), we can amplify the typical 235bp *barstar* fragment in a PCR reaction. This proves that *barstar* sequences up to base-pair 4151 are present. To prove the absence/presence of the Sau3A site at position 4161 a PCR reaction using primer MDB8 and MDB185 (position in pMc5barstar: 4160 <---4178) was carried out. This primer pair failed to amplify the specific *barstar* fragment.

Conclusion: *Barstar* is present and not rearranged. From the PCR data we can conclude that upon the integration of the pMc5barstar sequences, sequences between bp 4152 (±10bp) up to the HindIII restriction site are lost.

Cat : from position 3520 --->2861 in pMc5barstar

The following scheme summarizes the hybridization data with a *cat* probe and event MS3 DNA

Digest	Relevant pos. in pMc5barstar	Expected fragment length	Observed fragment length
PstI EcoRI	2005 3755	1750	4800 800
Sau3A	2438, 3576	1138	900 700 1500(weak)
EcoRI	3755	-	4800
PvuII	3407, 4071	664 > 3407	2000 850 664(weak)
HindIII	4201	-	10Kb
BamHI	3858	-	5100 1200 8Kb(weak)

- The *cat* probe hybridizes to a 4800bp fragment in EcoRI digested DNA. In the EcoRI-PstI double digest, we observe two fragments: 4800bp and 800bp. The 4800bp fragment in the EcoRI-PstI digest is less intense than the hybridization signal in the EcoRI digest: it reflects partial digestion of the genomic DNA. The characteristic 1750bp EcoRI - PstI fragment is not observed. From data obtained with the barstar probe, we know that the EcoRI site at position 3755 in pMc5barstar is present in the transgene (it resides on the 664bp PvuII fragment). The rearrangement in the transforming DNA has to occur upstream of this site.
  The 664bp PvuII fragment is detected but very weakly. This is expected because the homology between the *cat* probe and the PvuII fragment is only 113bp.
- The hybridization data of the other digests indicate that the *cat* region of the helper-plasmid is not complete and that it is very heavily rearranged. The expected Sau3A fragment is for instance not observed: instead 2 smaller fragments and third weaker fragment are found.
- We designed some primers homologous to the *cat* gene to see if we could amplify specific *cat* fragments in a PCR reaction.

Primer-pairs (position in pMc5barstar)	Expected fragment length
VDS13 (3508 ---> 3490) + VDS15 (2930 ---> 2951)	578bp
VDS13 (3508 ---> 3490) + VDS14 (3129 ---> 3149)	379bp
VDS13 (3508 ---> 3490) + VDS16 (3276 ---> 3296)	232bp
VDS14 (3129 ---> 3149) + MDB9 (4151 ---> 4128)	1022bp
VDS15 (2930 ---> 2951) + MDB9 (4151 ---> 4128)	1221bp

All PCR's yielded the expected products in reconstruction experiments, but failed to amplify the expected fragments in event MS3 DNA

- Since we demonstrated the presence of helper-plasmid sequences from position 4151 (5'end of MDB9) up to position 3407 (PvuII site: 664bp fragment observed with barstar probe), the start of rearrangements of the *cat* gene must occur between position 3296 (3'end of VDS16) and the PvuII site.

Conclusion: Sequences homologous to the *cat* gene are present in a heavily rearranged form.

Ori & bla

This part of the helper-plasmid is more difficult to analyze since it is homologous to pVE108. We unequivocally demonstrated with available Southern blot data the presence of the intact *ori&bla* piece of pVE108. We also demonstrated the presence of a rearranged *ori&bla* piece next to the intact one.

F1 ori : from position 314 ---> 768

We did not observe hybridization signals with the F1*ori* probe.

2.5. Analysis of the link between the two HindIII fragments

The analysis of the link between the pVE108 copy at the 3'end of the 12Kb HindIII fragment and the pVE108 copy at the 5'end of the 9kb HindIII fragment is hindered by the inverted repeat configuration. PCR analysis of this region is in progress but it is seriously hindered by the Tail-to-Tail configuration.
Analysis of the Southern blot data resulted in the determination of a number of restriction fragments that could be denoted as being the 3'ends of the dimer and most probably the 5'ends of the pVE108-helper plasmid complex. Subsequently we analyzed a number of double digests with HindIII. Results are listed in Table 3 .
As can be noticed, all tail fragments are cut with HindIII into two restriction fragments that have exactly half of the MW of the tail fragment. This confirms the Tail-to-Tail configuration and the linkage between the two HindIII fragments.

Table 3. Probe bar

Digest (pos.of relevant sites in pVE108)	**Fragments**	**Fragments in the double digest (+ HindIII)**
NdeI (185)	6200 6100 5600	 6100 5600 3100(d)
NarI (237)	6200 5600 5000	 5600 5000 3100(d)
ApaI (2739)	> 7000 5600 1200	8500 5600 5400 600(d)
SspI (5434)	7000 5600 1400	 5600 3500(d) 1400

(d): doublet

Annex 7.3 Expression of the introduced transgenes and analysis of the possible occurrence of cryptic gene expression

1. Goals of the experiment

2. Plant material

3. Methods for the analysis of messenger RNA

    3.1. Extraction and purification of total RNA
    3.2. *In vitro* synthesis of control RNA transcripts
    3.3. Fractionation of RNA
    3.4. Transfer of denatured RNA to nylon membranes
    3.5. *In vitro* synthesis of RNA probes
    3.6. Hybridization and autoradiography

4. Results and conclusions

    4.1. Transgene expression
    4.2. Cryptic gene expression

EXPRESSION OF THE INTRODUCED TRANSGENES AND ANALYSIS OF THE POSSIBLE OCCURRENCE OF CRYPTIC GENE EXPRESSION

1. Goals of the experiment

To demonstrate the expression of introduced transgenes in the male sterile progenies and to analyze the possible occurrence of cryptic gene expression.

2. Plant material

Molecular analysis has been performed on plants carrying the male sterility gene. Non-transgenic *Zea mays* (H99) plants have been used as negative control.

NMS lines used:
- M5917: 6th generation of event MS3
- M5918: 6th generation of event MS3

Material:

- leaf	A:	M5917-41
	B:	M5917-40
- roots	A:	M5917-41
	B:	M5917-40
- immature kernel	A:	M5917-41
	B:	M5917-40
- dry seeds	M5918	
- germinating seeds	A:	M5918
	B:	M5918

Seedlot M5917 and M5918 are derived from M4989-7 and M4989-8 respectively, which have the identical integration pattern as the original transformant.

3. Methods for the analysis of messenger RNA

The following procedure has been used to demonstrate the expression of the introduced transgenes in the male sterile progenies. The same procedure was used to analyze the possible occurrence of cryptic gene expression.

3.1. Extraction and purification of total RNA

Total RNAs are isolated according to Jones *et al.*(Jones D., Dunsmuir P & Bedbrook J., The EMBO Journal, 4, 2411-2418,1985).

- Grind 1 to 2 grams of tissue to a fine powder in liquid nitrogen.
- Add 9 ml of NTES buffer (0.1M NaCl, 0.01M Tris-HCl pH 7.5, 1mM EDTA, 1% SDS) and 6 ml of phenol/chloroform/isoamylalcohol (24:24:1).
- Vortex intensively (approximately 10 min.) in 50 ml Falcon tubes.

- Transfer to a DEPC-treated 30 ml Corex tube and centrifuge in the HB4 Sorvall rotor at 8000 rpm for 10 min.
- Take the aqueous phase, add 1/10 volume of 2M NaOAc and add 2 volumes ethanol.
- Mix well and keep at least 1 hour at -20°C.
- Pellet the precipitate at 8000 rpm for 10 min. (HB4, Corex tubes).
- Rinse the pellet with 70% ethanol.
- Dissolve the pellet in 2 ml water. Spin 5 min. at 5000 rpm (HB4 rotor) to sediment impurities.
- Transfer supernatant to a 15 ml Corex tube and add 2 ml 4M Lithium Acetate or 4M Lithium Chloride.
- Leave on ice for at least 3 hours (preferable over night).
- Pellet the precipitate as above and dissolve the pellet in 1.8ml water. Add 0.2ml 2M NaOAc pH 4.8 and add 2 volumes ethanol.
- Mix well and keep at least 1 hour at -20°C.
- Pellet the precipitate as above and rinse pellet with 70% ethanol and invert the tubes to dry the pellet.
- Finally dissolve the pellet in 100 to 500µl water.

This method is scaled down for the extraction of RNA from dry seeds and germinating seeds. For quantitating the amount of RNA, spectrophotometric readings are taken at a wavelength of 260 nm. An OD of 1 corresponds to 40µg/ml RNA.

3.2. *In vitro* synthesis of control RNA transcripts

Templates

A. Plasmids for preparing RNA probes

pVE113: *barnase-barstar* in pGEM1 vector (see Figure 1)

- HindIII digested pVE113 DNA transcribed with T7 DNA polymerase produces sense *barstar/barnase* RNA transcripts.
- EcoRI digested pVE113 DNA transcribed with SP6 DNA polymerase gives anti-sense *barstar/barnase* RNA transcripts.

pGemBar: *bar* in pGEM2 vector (see Figure 2)

- EcorI digested pGemBar DNA transcribed with T7 DNA polymerase produces sense *bar* RNA transcripts.
- HindIII digested pGemBar DNA transcribed with SP6 DNA polymerase produces anti-sense *bar* RNA transcripts.

pFM136: *cat* in pGEM3z vector (see Figure 3)

- HindIII digested pFM136 DNA transcribed with T7 DNA polymerase produces sense *cat* RNA transcripts.
- PvuII digested pFM136 DNA transcribed with SP6 DNA polymerase produces anti-sense *cat* RNA transcripts.

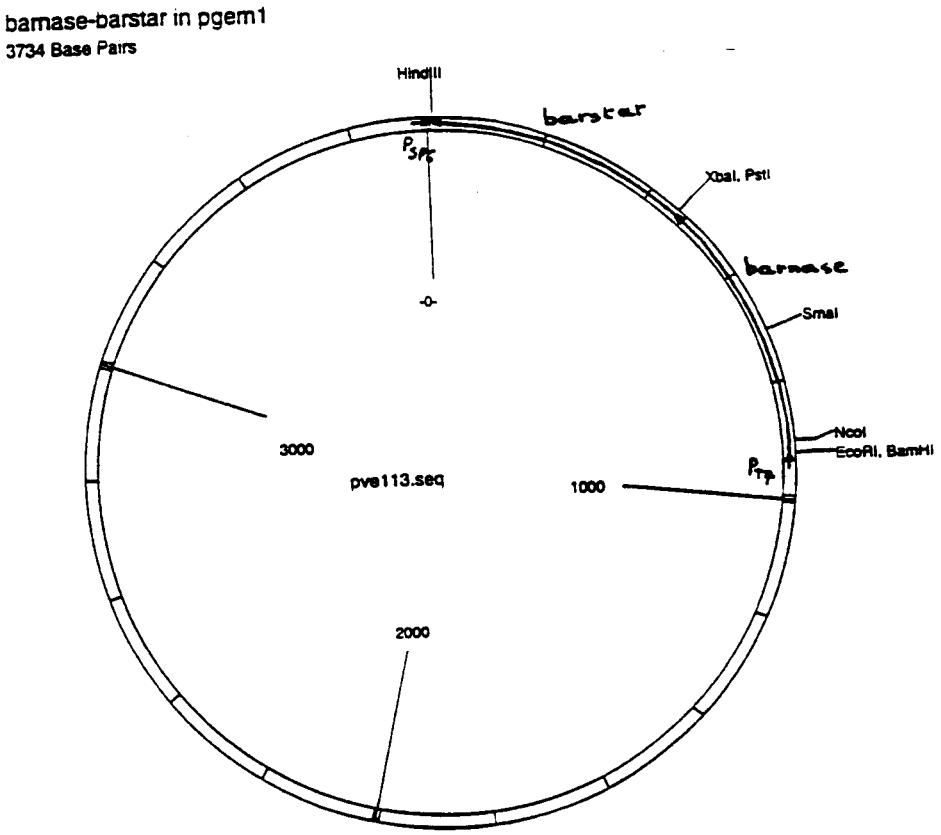


Figure 1. pVE113 : *barnase* and *barstar* reading frames in pGEM1 vector

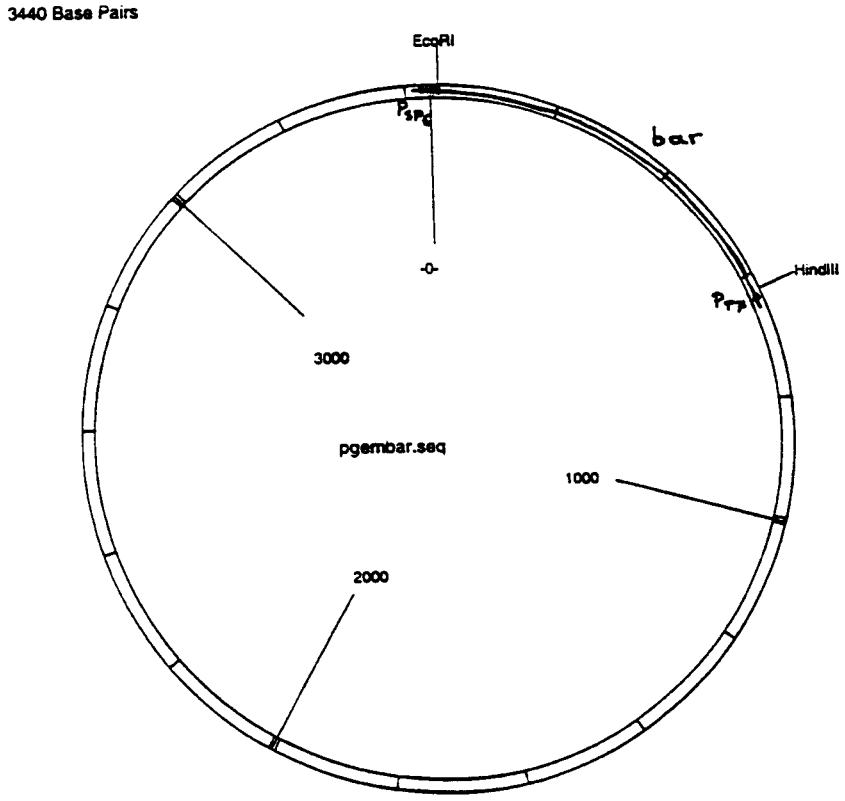


Figure 2. pGEMBar : *bar* reading frames in pGEM2 vector

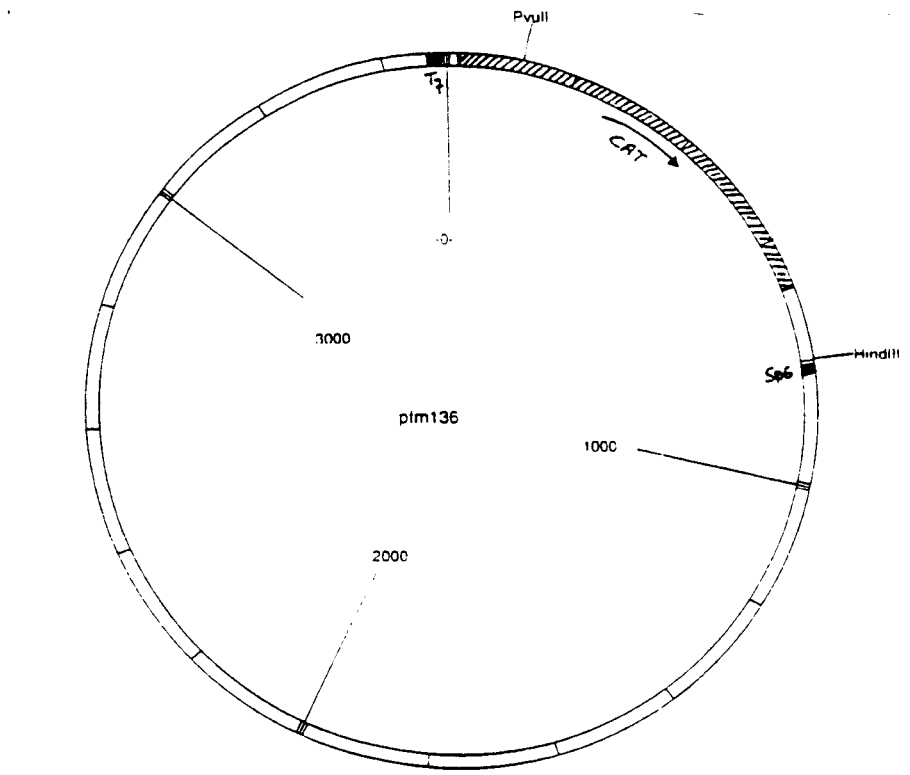


Figure 3. pFM136 : *cat* in pGem3z vector

B. PCR amplification of DNA templates for in vitro RNA synthesis

For the analysis of occurrence of cryptic gene expression we amplified specific T-DNA fragments, by means of PCR, to serve as templates for in vitro RNA synthesis.

For every template, two primers are designed: an upstream primer which comprises the T7 promoter (including the 6 nucleotides GGGAGA that are present at the 5' end of transcripts) adjacent to specific insert sequences and a downstream primer which comprises the SP6 promoter (including the 6 nucleotides GAATAC that are present at the 5' end of transcripts) adjacent to specific insert sequences (see Figure 4).

The sequences of the different synthesized primers can be found in Table 1. Amplified fragment lengths and the region of the insert they cover, can be found in Table 2.

PCR is carried out by using the thermostable Vent DNA polymerase (New England Biolabs, Inc.). This polymerase contains a 3' ---> 5' proofreading exonuclease activity, resulting in much higher fidelity of base incorporation compared to Taq DNA polymerase.

100ng of HindIII linearized pVE108 DNA and 30pmoles of upstream primer and downstream primer were mixed in a 50µl PCR reaction containing 10mM KCl, 10mM $(NH_4)_2SO_4$, 20mM Tris-HCl (pH8.8 at 25°C), 2mM $MgSO_4$, 0.1% Triton-X-100,

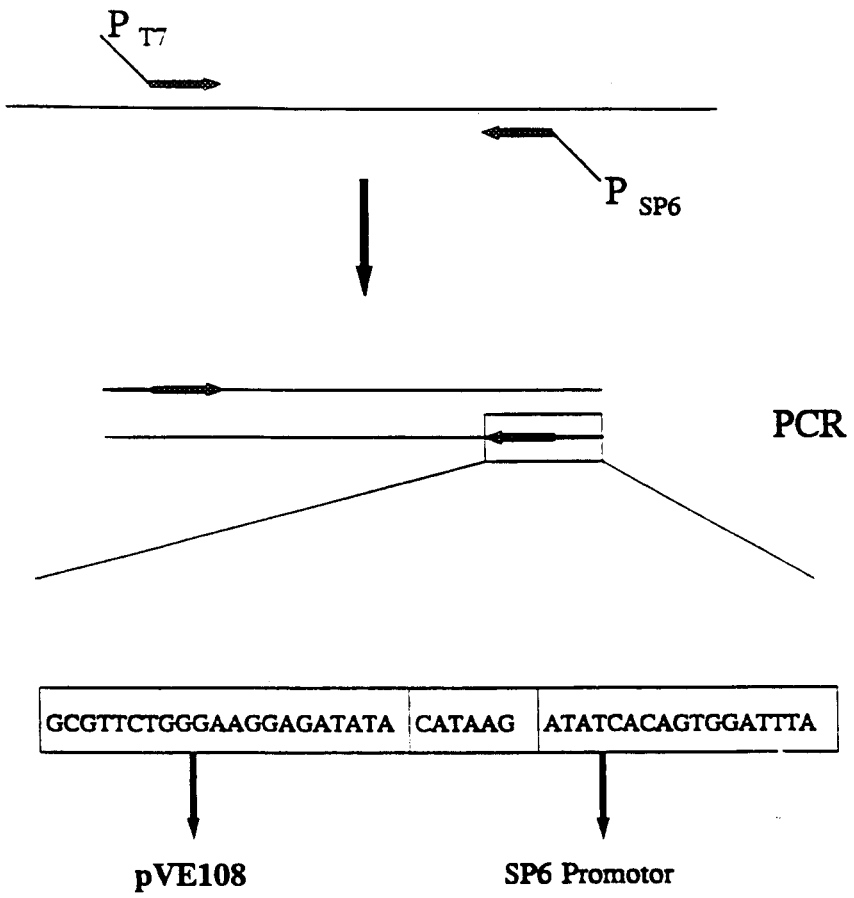


Figure 4. Outline for the generation of specific fragments for use in the in vitro transcription of RNA probes (The oligonucleotide sequence shown is VDS39.

200µM of each deoxyribonucleoside triphosphate and 1 unit of Vent DNA polymerase. DNA amplification occurred during 25 cycles.

Thermocycling profile:	4 min. at 95°C
Followed by:	30 sec. at 95°C
	30 sec. at 57°C
	45 sec. at 75°C
	For 5 cycles
Followed by:	5 sec. at 92°C
	30 sec. at 60°C
	45 sec. at 75°C
	For 20 cycles
Followed by:	10 min. at 75°C

The synthesized fragments were checked on agarose gels.
After phenol-chloroform extractions, the fragments were precipitated, washed and subsequently dissolved in water.
The concentration of the DNAs was measured spectrophotometrically.

In vitro synthesis

- Mix the following components in the given order in a microfuge tube at room temperature:

DEPC-treated water	up to 50µl volume
Template DNA	4µg
10x Transcription buffer	5µl
0.5M DTT	1µl
RNAse inhibitor (25 units/µl)	2µl
NTP mix (2.5 mM each)	10µl
DNA- dependent RNA polymerase	1µl

(10x Transcription buffer: 400mM Tris-HCl pH 7.5 at 37°C, 60mM $MgCl_2$, 20mM spermidine and 50mM NaCl)

- Incubate at 37°C for 120 minutes.
- Add 1µl 10x Transcription buffer, 8µl NTP mix and 1µl polymerase. Incubate of another 120 minutes at 37°C.
- The template DNA is removed by treatment with DNAse I for 10 minutes at 37°C.
- The synthesized RNA transcripts are extracted with phenol-chloroform and purified from unincorporated nucleotides on a Bio-Spin® 30 chromatography column (Bio-Gel P-30 polyacrylamide gel, Bio-Rad), equilibrated with DEPC-treated water.
- The concentration is measured.spectrophotometrically.
- 1µg of the synthesized RNA transcripts are checked on a 1.5% agarose-formaldehyde gel.

3.3. Fractionation of RNA

The RNA is separated according to size by electrophoresis through a denaturing agarose gel containing formaldehyde.

The gels are prepared by melting agarose (1.5% final concentration) in water, cooling it to 60°C, adding 10x formaldehyde gel-running buffer (0.2M MOPS, 0.05M NaOAc pH7.0 and 0.01M EDTA) and formaldehyde to give a final concentration of 1x and 2.2M respectively. Cast the gels in a chemical hood and allow the gel to set at least for 30 min. at room temperature.

Samples are prepared by mixing the following in a sterile microfuge tube:

- RNA (5µg or 10µg) x µl
- 10x formaldehyde gel-running buffer 2 µl
- formaldehyde 3.5 µl
- formamide 10 µl
- Ethidium bromide (1mg/ml) 1 µl
- H_2O up to 20µl

Note: The control RNA dilutions are complemented with 5µg or 10µg control leaf RNA

Incubate the samples for 15 minutes at 55°C and then chill them on ice. Add 2 µl of sterile DEPC-treated dye (50% glycerol, 0.5% bromophenol blue and 0.5% xylene cyanol FF). Run the gel submerged in 1x formaldehyde gel-running buffer at ±5 V/cm.

Loading sequence of the gels:

Gel A:	**Line**	**Plant N°**	**Tissue**	**µg RNA loaded**
1.	MW (0.16 - 1.77 kb RNA ladder, Life Technologies Inc.)			
2.	event MS3-A	M5917-41	leaf	5µg
3.	event MS3-B	M5917-40	leaf	5µg
4.	control	H99	leaf	5µg
5.	event MS3-A	M5917-41	roots	5µg
6.	event MS3-B	M5917-40	roots	5µg
7.	control	H99	roots	5µg
8.	event MS3-A	M5917-41	immature kernel	5µg
9.	event MS3-B	M5917-40	immature kernel	5µg
10.	control	H99	immature kernel	5µg
11.	event MS3	M5918	dry seed	5µg
12.	control	H99	dry seed	5µg
13.	event MS3	M5918	germinating seed	5µg
14.	event MS3	M5918	germinating seed	5µg
15.	control	H99	germinating seed	5µg

Gel B:

1. MW (0.16 - 1.77 kb RNA ladder, Life Technologies Inc.)
2.-7. Control RNA dilution series (*in vitro* synthesized RNA complementary to the probe used): 0.5pg - 1pg - 2pg - 4pg - 8pg and 16pg. In the case of the *cat* - **T7** transcript: 32pg - 16pg - 8pg - 4pg - 2pg - 1 pg and 0.5pg.
In the case of the *ori&bla*-**T7** transcript: 0.5pg was not loaded.
These control RNA samples are complemented with 5µg control leaf RNA.

Gel C:

	line	**Plant N°**	**Tissue**	**µg RNA loaded**
1.	MW (0.16 - 1.77 kb RNA ladder, Life Technologies Inc.)			
2.	event MS3-A	M5917-41	leaf	10µg
3.	event MS3-B	M5917-40	leaf	10µg
4.	control	H99	leaf	10µg
5.	event MS3-A	M5917-41	roots	10µg
6.	event MS3-B	M5917-40	roots	10µg
7.	control	H99	roots	10µg
8.-14.	Control RNA dilution series (*in vitro* synthesized RNA complementary to the *bar*-**SP6** probe): 0.5pg - 1pg - 2pg - 4pg - 8pg - 16pg and 32pg. These control RNA samples are complemented with 10µg control leaf RNA.			

Gel D:

	line	**Plant N°**	**Tissue**	**µg RNA loaded**
1.	MW (0.16 - 1.77 kb RNA ladder, Life Technologies Inc.)			
2.	event MS3-A	M5917-41	immature kernel	10µg
3.	event MS3-B	M5917-40	immature kernel	10µg
4.	control	H99	immature kernel	10µg
5.	event MS3	M5918	dry seeds	10µg
6.	control	H99	dry seeds	10µg
7.	event MS3	M5918	germinating seeds	10µg
8.	event MS3	M5918	germinating seeds	10µg
9.	control	H99	germinating seeds	10µg
10.-16.	Control RNA dilution serie (*in vitro* synthesized RNA complementary to the *bar*-**SP6** probe): 0.5pg - 1pg - 2pg - 4pg - 8pg - 16pg and 32pg. These control RNA samples are complemented with 10µg control leaf RNA.			

3.4. Transfer of denatured RNA to nylon membranes

The RNAs are transferred immediately after electrophoresis from the agarose to nylon membranes (Hybond-N, Amersham) by capillary elution.

- Fill a glass dish with blotting buffer (20x SSPE = 3.6M NaCl, 0.2M Sodium phosphate, 0.02M EDTA pH 7.7). Make a platform and cover with a Whatman 3MM filter paper wick, saturated with buffer.
- Place the gel on the wick and avoid trapping air bubbles beneath it. A sheet of Hybond-N membrane, cut to the exact size of the gel, is placed on top of the gel. Avoid trapping bubbles beneath the membrane.
- Place a sheet of Whatman 3MM cut to size and wetted with blotting buffer, on top of the Hybond-N membrane.
- Surround the gel with Saran Wrap foil to prevent the blotting buffer being absorbed directly into the paper towels above.
- Place a stack of absorbent paper towels on top of the 3MM paper.
- Place a glass plate on top of the paper towels and a 0.5 - 1 Kg weight on top. Allow the transfer to proceed for 12 to 20 hours.
- After blotting carefully dismantle the setup. Before removing from the gel, mark the membrane with a pencil to allow later identification of the tracks.
- The samples are fixed to the membrane by baking in an oven at 80°C for 2 hours.

Documentation of the fractionation of the RNA is done at this stage. The image is acquired, processed and copied to thermal paper using the Foto/Analyst ™ Visionary imaging system from FOTODYNE (CCD camera: charge-coupled device) (see figure 5).

3.5. *In vitro* synthesis of RNA probes

Single-stranded RNA probes of high specific activity are prepared by using as template DNA either plasmid vectors containing polycloning sites downstream from powerful promoters derived from the *Salmonella typhimurium* bacteriophage SP6 or from the *E. coli* bacteriophage T7 or by either using PCR generated templates with 5' extensions containing the sequences from the before mentioned promoters.

In vitro labeling

- Mix the following components in the order given in a microfuge tube at room temperature:

DEPC-treated water	up to 20 µl total volume
Template DNA	500 ng
10x Transcription buffer	2 µl
NTP mix (-UTP), 2.5mM each	3 µl
1mM UTP	1 µl
0.2M DTT	1 µl
RNAse inhibitor (25 units/µl)	1 µl
[α-^{32}P]UTP (20mCi/ml)	5 µl
Bacteriophage DNA-dependent RNA polymerase (7-12 units/µl)	1 µl

(10x Transcription Buffer: 400mM Tris-HCl pH7.5 at 37°C, 60mM $MgCl_2$, 20mM spermidine and 50mM NaCl).

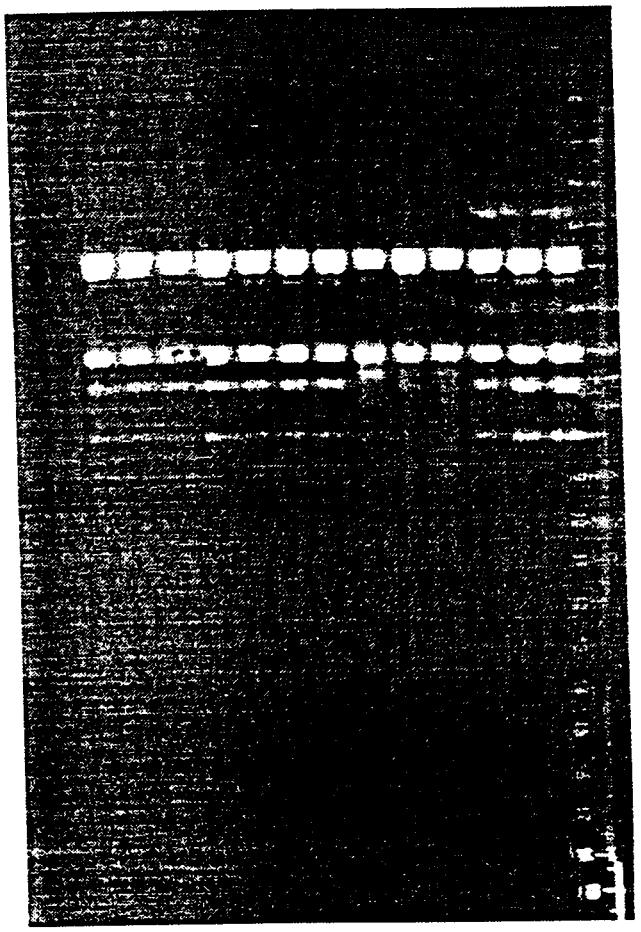


Figure 5: Image of a Nylon membrane after RNA transfer from a type C gel.
From right to left: lanes 1 to 14

- Mix the reagents by gentle tapping.
- Incubate the reaction for 1 hour at 40°C (SP6 RNA polymerase) or 37°C (T7 RNA polymerase).
- Add 1 µl RNAse inhibitor and 1 µl of RNAse-free pancreatic DNAseI (20 units/µl). Mix and incubate for 15 min. at 37°C.
- Analyze 0.5 µl on a 6% denaturing acrylamide gel.
- The rapid removal of unincorporated nucleotides from the labeling reaction is done by using Bio-spin® 30 chromatography columns (Bio-Gel P-30 polyacrylamide gel, Bio-Rad).

3.6. Hybridization and autoradiography

- The filters are prehybridized for 1-2 hours in a hybridization oven using 10ml prehybridization buffer (for 3 filters of 14cm x 19cm) at 65°C.
  Prehybridization buffer: 50% formamide, 5x SSPE, 5x Denhardt's, 0.1% SDS and 100µg/ml carrier DNA at 65°C.

  (20x SSPE: 3.6M NaCl, 0.2M Sodium phosphate, 0.02M EDTA pH7.7)

  (100x Denhardt's solution: 2% (w/v) BSA, 2% (w/v) ficoll and 2% (w/v) Polyvinylpyrrolidone)

- Remove the prehybridization buffer.
- Add fresh prehybridization buffer supplemented with the denaturated radiolabeled probe to the hybridization tube and continue the incubation overnight.
- Wash the filters for 5 min. in 5x SSPE, followed by 2-3 washes of 20-30 minutes each in 2x SSPE, 0.1% SDS and 1 wash of 10-20 minutes in 0.1x SSPE, 0.1% SDS.
- Establish an autoradiography by exposing the filter for 3 up to 96 hours to X-ray film at -70°C with an intensifying screen. The shorter exposures are performed for accurate quantification and for reproduction of the results. The longer exposures are performed to assure the absence of any signals in control samples or in the analysis of occurrence of cryptic gene expression.

- Reproduction of the results in this document is done by using the iphoto deluxe software (U-lead Systems, Taipei, Taiwan, ROC) and the Harvard Graphics Software.
- After the exposure, the membranes are stripped to remove the probes. For this purpose a 0.5% SDS solution is boiled. Membranes are submerged in this solution and allowed to cool to room temperature.
- To check that the probe was removed completely, an autoradiograph for the normal exposure time was established.
- Subsequently, the filters can be prehybridized and hybridized with a new probe.

4. Results and conclusions

4.1. Transgene expression

Bar

The detected *bar* mRNA levels in the leaves and immature kernels are approximately 0.05pg/µg total RNA.
For the other samples (roots, dry seeds, germinating seeds), we didn't detect any *bar* mRNA hybridization signals (detection limit is 0.05pg/µg total RNA, see Table 3. and Figure 6.).
There are no differences visible between the hybridization signals of the roots samples from the transformed plant and those from the control plant. The sample of the dry seeds (lane 5, gel type D, figure 6) also shows some background hybridization.
One germinating seed sample (lane 7, gel type D, figure 6) shows some degradation of the mRNA sample.

Barnase

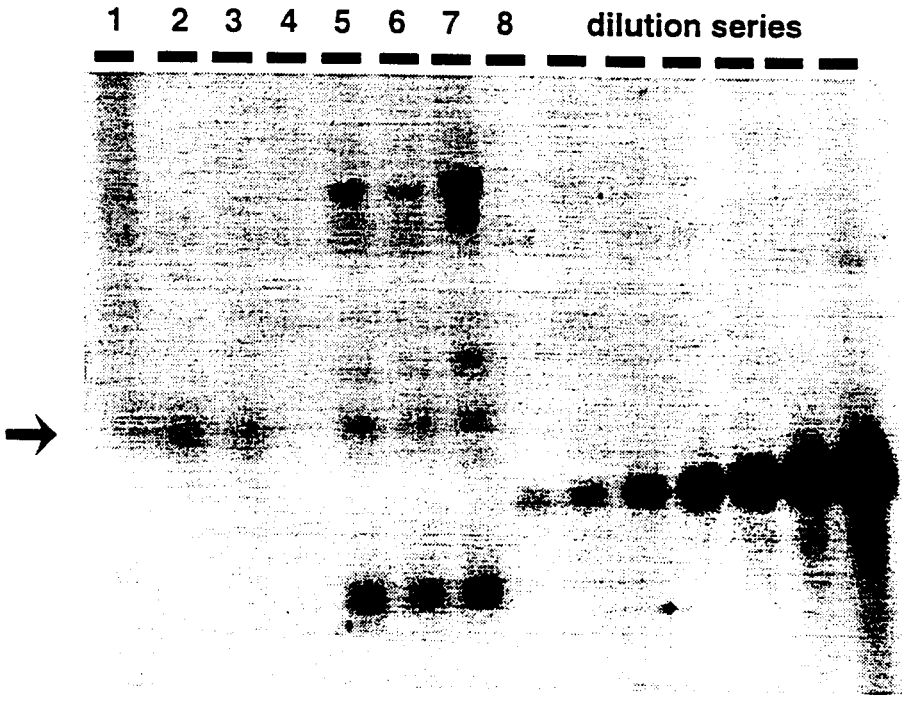
We couldn't detect any *barnase* mRNA signals whatsoever (detection limit 0.1pg/µg total RNA, see Table 3.) This is expected since the *barnase* is driven by the tapetum specific PTA29 promoter.

4.2. Cryptic gene expression

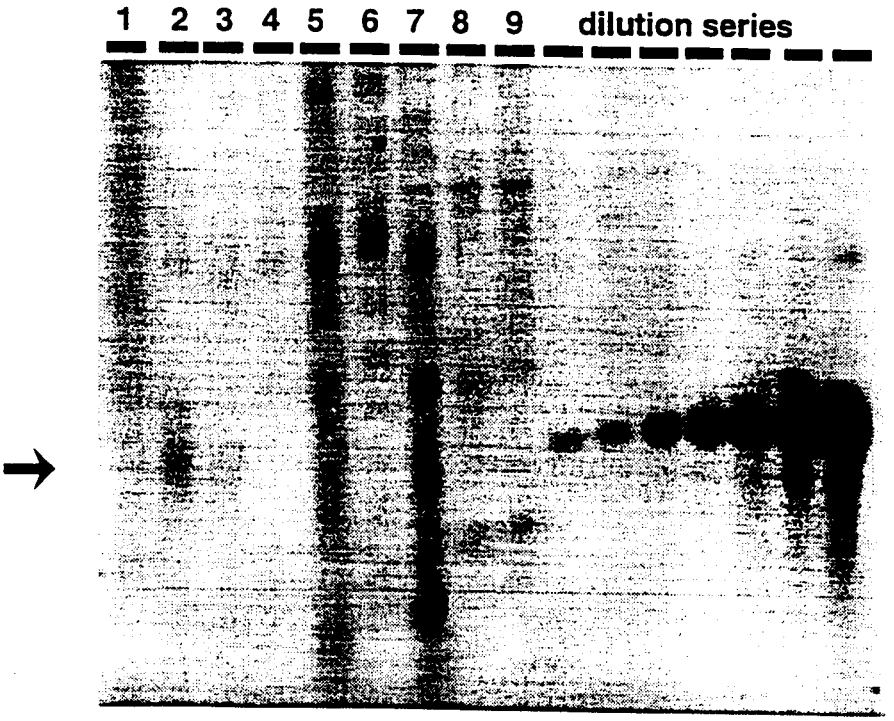
Bar **;** ***Barnase*** **;** ***Barstar*** **; PTA29-P35S;** ***ori*** **&** ***bla*****;** ***cat***

Using sense RNA probes of the specific transgenes, and using anti-sense and sense RNA probes of the other specified regions of the insert, we were unable to detect any hybridization signals whatsoever (Figure 7, Figure 8 and Figure 9. and data summarized in Table 3.).

Transgene expression



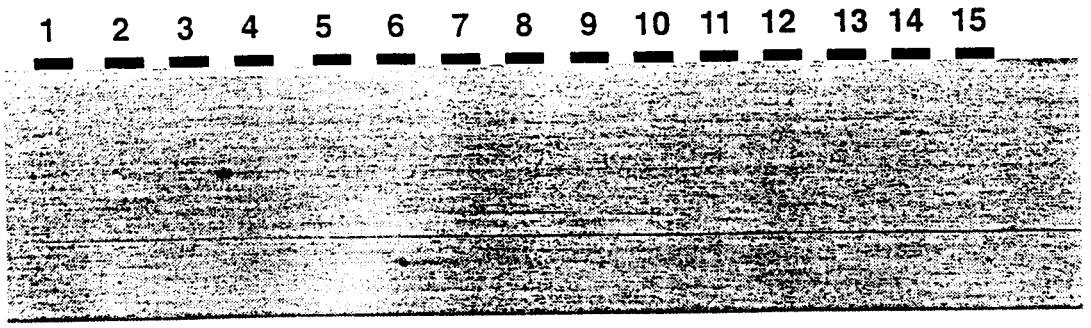
loading sequence: see gel C



loading sequence: see gel D

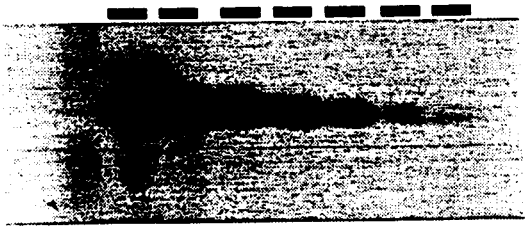
FIGURE 6 : BAR mRNA HYBRIDIZATION RESULTS

Cryptic gene expression



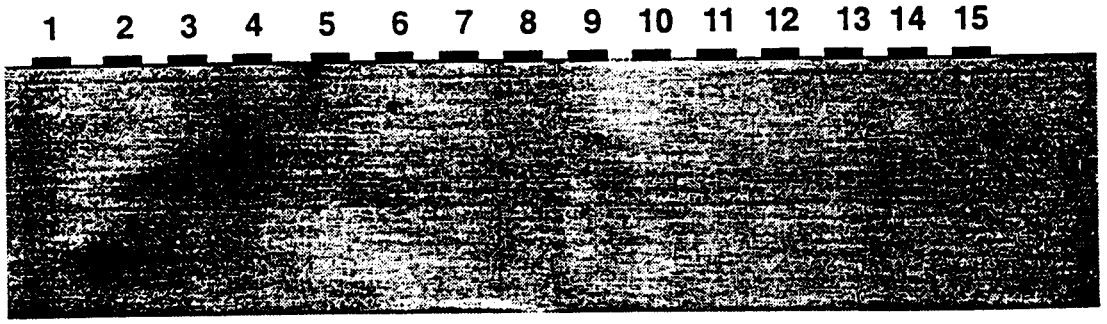
loading sequence: see gel A

dilution series



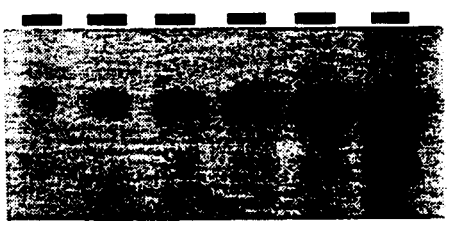
loading sequence: see gel B

FIGURE 7 : CAT /SP6 HYBRIDIZATION RESULTS



loading sequence: see gel A

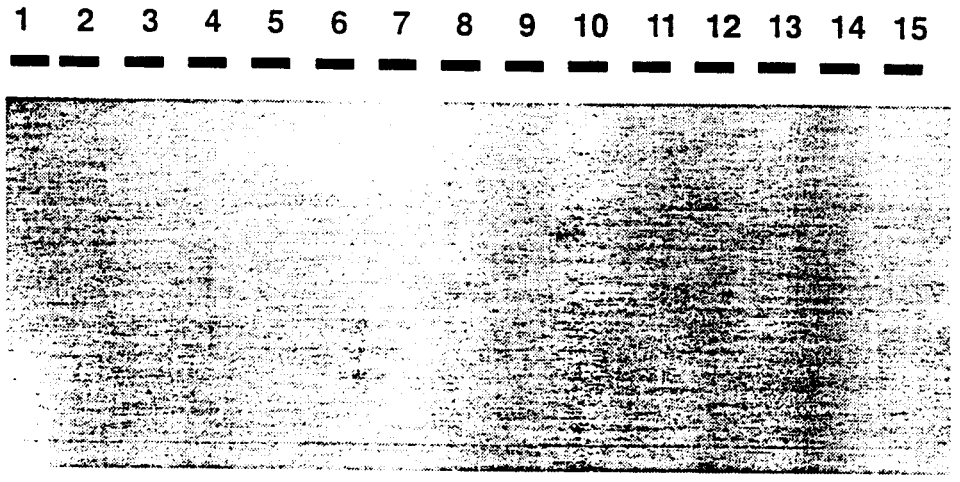
dilution series



loading sequence: see gel B

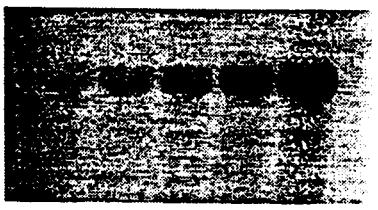
FIGURE 8 : PTA29-P35S/SP6 HYBRIDIZATION RESULTS

Cryptic gene expression



loading sequence: see gel A

dilution series



loading sequence: see gel B

FIGURE 9 : ori & bla /SP6 HYBRIDIZATION RESULTS

Table 1: Oligonucleotide sequences for PCR amplification of DNA templates suited for *in vitro* RNA synthesis (5' end: the 6 nucleotides that are present at the 5' end of transcripts)

Oligo	Promoter	5' end	DNA sequence	Position in pVE108
MDB172	**T7** 5' TAA.TAC.GAC.TCA.CTA.TA	G.GGA.GA	C.TGT.TAC.ACT.TGC.ACC.ACA.AGG 3'	1185 ---> 1206
VDS39	**SP6** 5' ATT.TAG.GTG.ACA.CTA.TA	G.AAT.AC	A.TAT.AGA.GGA.AGG.GTC.TTG.CG 3'	2433 ---> 2413
VDS40	**T7** 5' TAA.TAC.GAC.TCA.CTA.TA	G.GGA.GA	C.GGT.ATC.AGC.TCA.CTC.AAA.GG 3'	3670 ---> 3690
VDS41	**SP6** 5' ATT.TAG.GTG.ACA.CTA.TA	G.AAT.AC	T.TCA.ACA.TTT.CCG.TGT.CGC 3'	5409 ---> 5391

Table 2: Primer-pair for the analysis of occurence of cryptic gene expression

Primer-pair	Amplified fragment	Position in pVE108	Features
MDB172 - VDS39	1248bp	1185 ---> 2433	P_{TA29} - P_{35S}
VDS40 - VDS41	1739bp	3670 ---> 5409	ori & bla

Table 3: Summary

Total RNA	Transgene expression pg/µg total RNA		Cryptic gene expression									
	bar pGemBar/SP6	barnase pVE113/SP6	bar pGemBar/T7	barnase pVE113/T7	barstar		MDB172-VDS39		VDS40-VDS41		cat	
					pVE113/ T7	pVE113/ SP6	SP6	T7	SP6	T7	pFM136/ SP6	pFM136/ T7
1. MS3 leaves A	±0.05	-	-	-	-	-	-	-	-	-	-	-
2. MS3 leaves B	±0.05	-	-	-	-	-	-	-	-	-	-	-
3. MS3 roots A	-̇	-	-	-	-	-	-	-	-	-	-	-
4. MS3 roots B	-	-	-	-	-	-	-	-	-	-	-	-
5. MS3 immature kernel A	±0.05	-	-	-	-	-	-	-	-	-	-	-
6. MS3 immature kernel B	±0.05	-	-	-	-	-	-	-	-	-	-	-
7. MS3 dry seeds	-	-	-	-	-	-	-	-	-	-	-	-
8. MS3 germinating seeds A	-̇	-	-	-	-	-	-	-	-	-	-	-
9. MS3 germinating seeds B	-̇	-	-	-	-	-	-	-	-	-	-	-
Detection limit (pg/µg total RNA)	0.05	0.1	0.2	0.1	0.1	0.1	0.1	0.2	0.2	0.1	0.1	0.1

-: no signal detectable
-̇: difficult to interprete because of non-specific background hybridization

ANNEX 7/11 PAGE 44/56

Annex 7.4 Stability of the insert of event MS3

1. Stability of the insert in a maintained background, determined via PCR analysis

    1.1. Method and material
    1.2. Results
    1.3. Conclusion

2. Stability of the insert in maintained and F_1 hybrid backgrounds, determined via Southern blot

    2.1. Method and material
    2.2. Results
    2.3. Conclusion

3. Stability of the insert in a F_1 hybrid background, determined via Southern blot analysis

    3.1. Method and material
    3.2. Results and conclusion

4. Stability of the insert in a backcrossing program, determined via Southern blot analysis

    4.1. Method and material
    4.2. Results
    4.3. Conclusion

STABILITY OF THE INSERT OF EVENT MS3

1. Stability of the insert in a maintained background, determined by PCR analysis

1.1. Method and material

PCR analyses were performed on 292 plants of the 6the maintained generation of event MS3. Plant material was obtained from two field experiments in Belgium (FZM9411 and FZM9413). The primer-pair MDB6 - MDB7 (*barnase*) and the primer-pair MDB8-MDB9 (*barstar*) were used in all tests.

1.2. Results

All plants were positive for *barnase* and *barstar*, with the exception of 1 plant which was negative for *barnase* and *barstar*, and consequently considered to be an escape of the glufosinate-ammonium application.

1.3. Conclusion

It was demonstrated by PCR that the insert of event MS3 was stably inherited in the tested generations.

2. Stability of the insert in maintained and F_1 hybrid backgrounds, determined by Southern blot analysis

2.1. Method and material

The DNA isolation and Southern blot procedure were carried out as earlier described.
Probes used:
- pVE108 total plasmid
- *barstar*

Leaf material of the following plant populations was used :
- M4 (event MS3) : the 4th maintained generation of event MS3
- M6 (event MS3) : the 6th maintained generation of event MS3
- F1 of (event MS3 x Inbred line C119)
- F1 of (event MS3 x Inbred line C115)

All sampled plants were male sterile.

2.2. Results

Southern blot 1 : Plant A = plant DNA of the 4th maintained generation of event MS3
Plant B = plant DNA of the 6th maintained generation of event MS3

Southern blot 2 : Plant A = F1 (MS3 x Inbred line C119) plant DNA
Plant B = F1 (MS3 x Inbred line C115) plant DNA

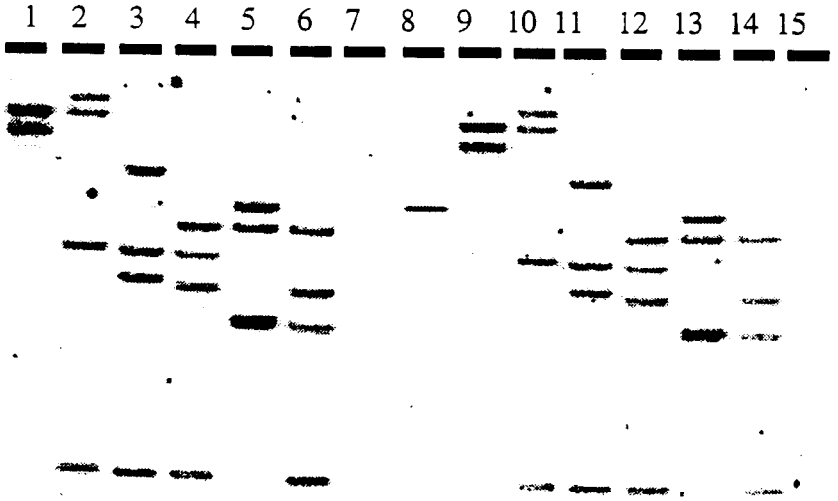
The typical loading sequence of the blots (see Figure 1 and Figure 2):

1. Plant A - HindIII
2. Plant A - NcoI
3. Plant A - HindIII/NcoI
4. Plant A - EcoRI/NcoI
5. Plant A - HindIII/EcoRI
6. Plant A - HindIII/EcoRI/NcoI
7. H99 control - HindIII
8. λ - PstI MW marker + 1 copy pVE108 - HindIII
9. Plant B - HindIII
10. Plant B - NcoI
11. Plant B - HindIII/NcoI
12. Plant B - EcoRI/NcoI
13. Plant B - HindIII/EcoRI
14. Plant B - HindIII/EcoRI/NcoI
15. H99 control - HindIII

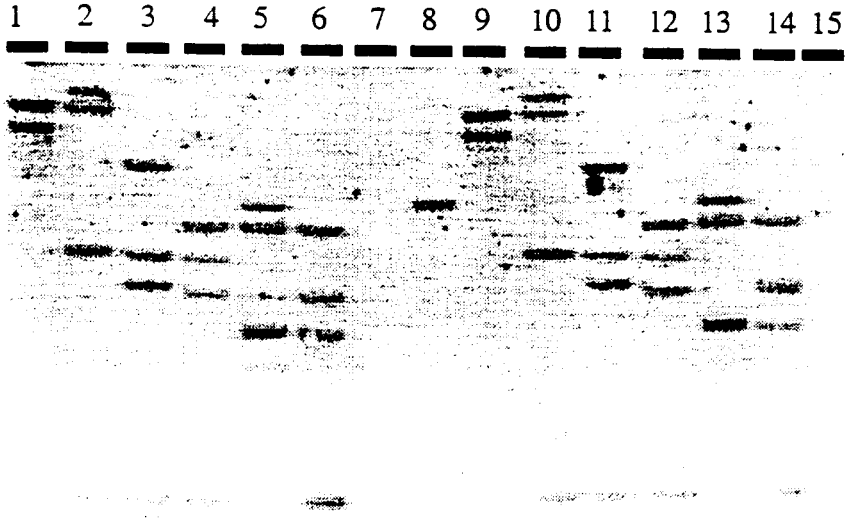
2.3. Conclusion

We demonstrated by Southern blot hybridization that the insert of event MS3 is stably integrated in the tested 4th and 6th maintained generation of event MS3 and in F_1 hybrids produced on female plants containing event MS3 (see Figure 1 and Figure 2).

STABILITY OF THE INSERT



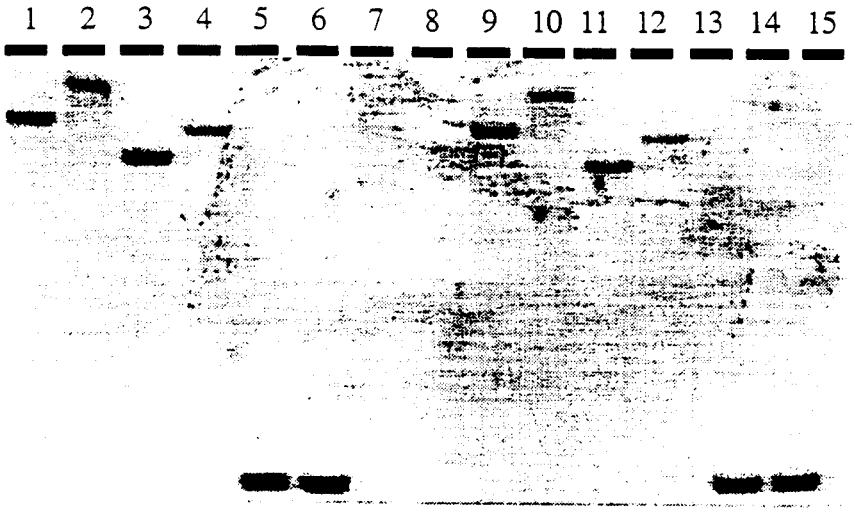
Blot 1. Lanes 1 - 6 : Plant DNA of the 4th maintained generation of event MS3
Lanes 9 - 14: Plant DNA of the 6th maintained generation of event MS3
Lanes 7 & 15: H99 control DNA
Lane 8: MW marker + 1 copy pVE108-HindIII



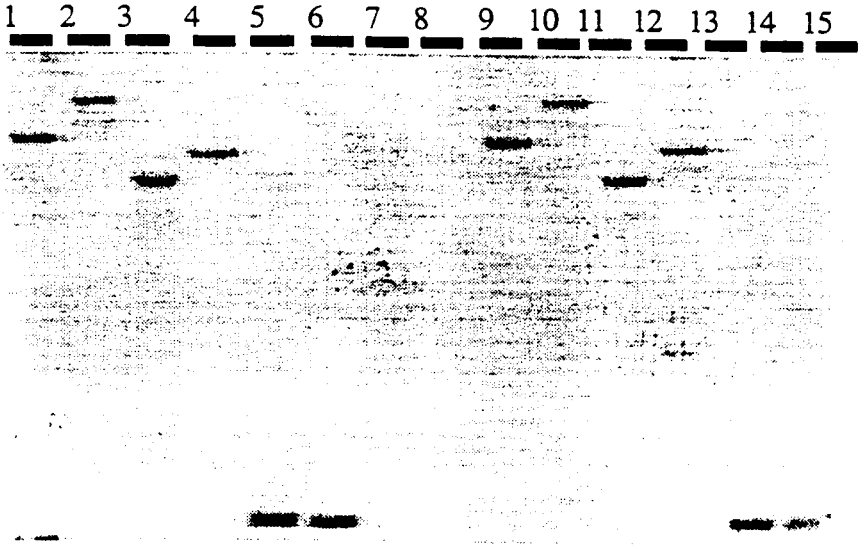
Blot 2. Lanes 1 - 6 : F1 (MS3 x Inbred line C119) plant DNA
Lanes 9 - 14: F1 (MS3 x Inbred line C115) plant DNA
Lanes 7 & 15: H99 control DNA
Lane 8: MW marker + 1 copy pVE108-HindIII

Figure 1: pVE108 hybridization results

STABILITY OF THE INSERT



Blot 1. Lanes 1 - 6 : Plant DNA of the 4th maintained generation of event MS3
Lanes 9 - 14: Plant DNA of the 6th maintained generation of event MS3
Lanes 7 & 15: H99 control DNA
Lane 8: MW marker + 1 copy pVE108-HindIII



Blot 2. Lanes 1 - 6 : F1 (MS3x Inbred line C119) plant DNA
Lanes 9 - 14: F1 (MS3x Inbred line C115) plant DNA
Lanes 7 & 15: H99 control DNA
Lane 8: MW marker + 1 copy pVE108-HindIII

Figure 2: barstar hybridization results

3. Stability of the insert in a F_1 hybrid background, determined by Southern blot analysis

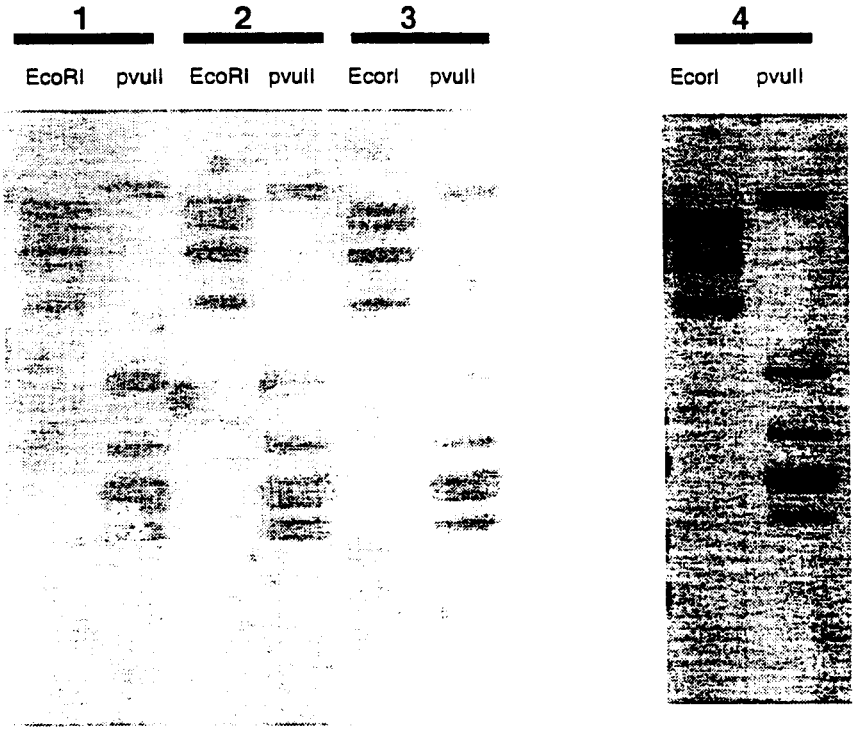
3.1. Method and material

Some F_1 hybrid plants derived from event MS3 extruded a few anthers which contained starch-filled pollen grains. These hybrid plants were analyzed by Southern blot analysis.

DNA was isolated according to Dellaporta et al. (1983). The DNA of the hybrid plants was digested by the enzymes EcoRI and PvuII. The whole plasmid pVE108, linearized with HindIII, was used as probe.

3.2. Results and conclusion

The hybridization data revealed that all the analyzed F_1 hybrid plants had an identical integration pattern as transformation event MS3 (see Figure 3). Consequently, it was shown that the insert of event MS3 is stably inherited in a F_1 hybrid background.



1 =(MS3 X Inbred line) - 2
2 = (MS3x Inbred line) - 3
3 = (MS3X Inbred line) - 4
4 = M3171 - 19 = (MS3 X H99) - 19

Figure 3. pVE108 hybridization results

4. Stability of the insert in a backcrossing program, determined by Southern blot analysis

4.1. Method and material

Male sterile plants containing the event MS3 from generations BC_1 and BC_3 of a backcrossing program, including 3 elite inbred lines, were analyzed in Southern hybridization. Five plants per BC generation were analyzed. Event MS3 (6th maintained generation) and non-transgenic H99 plants were included as controls.

DNA was isolated according to Dellaporta et al. (1983). The DNA of the hybrid plants was digested by the enzymes EcoRI and NcoI. The whole plasmid pVE108, linearized with HindIII, was used as probe.

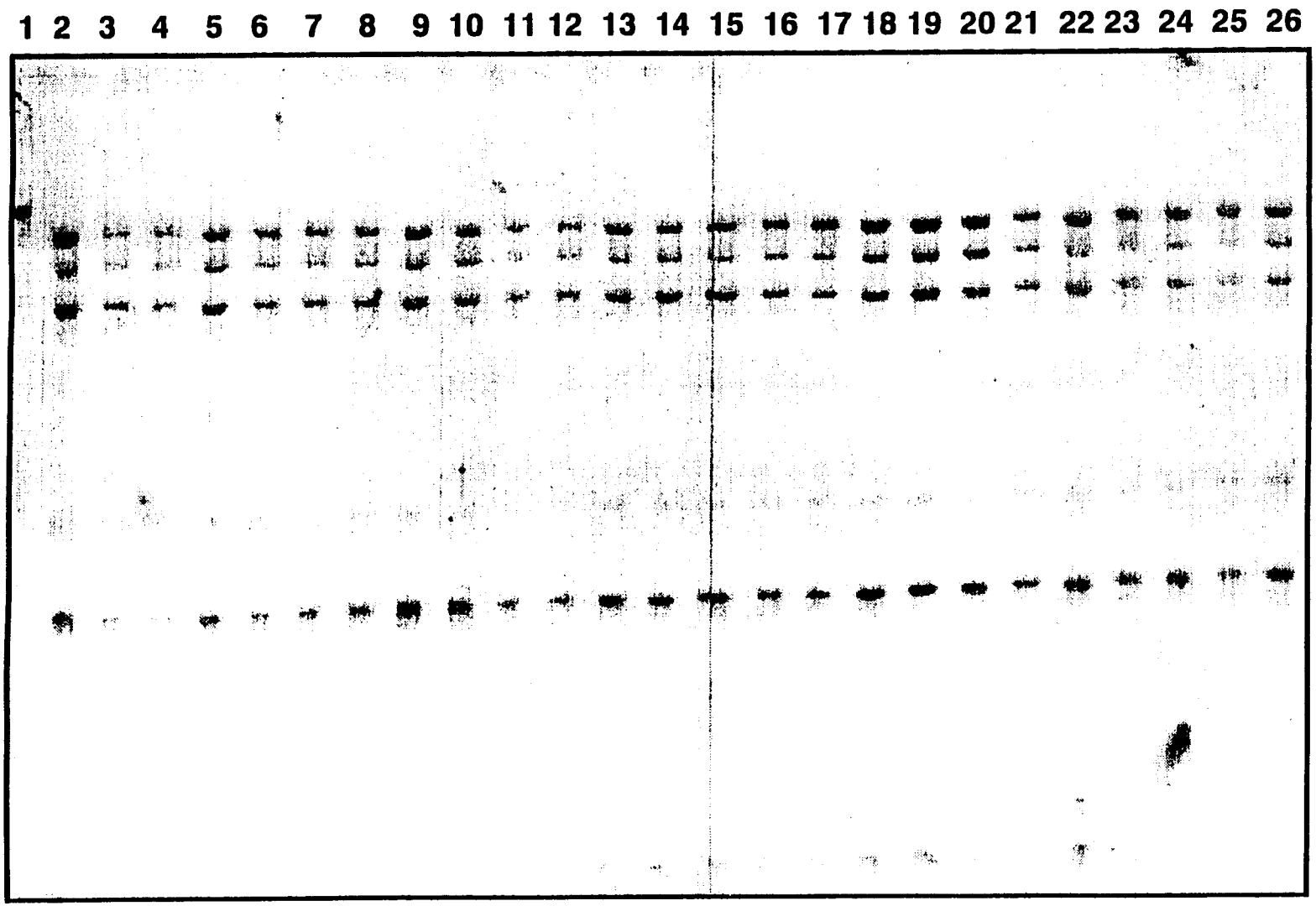
4.2. Results

The different lane numbers in Figure 4. correspond to :
1. Marker lambda/PstI
2. Event MS3
3. BC3 (event MS3 x Inbred line D) -7
4. BC3 (event MS3 x Inbred line D) -13
5. BC3 (event MS3 x Inbred line D) -15
6. BC3 (event MS3 x inbred line D) -16
7. BC3 (event MS3 x Inbred line D) -18
8. BC1 (event MS3 x Inbred line D) -3
9. BC1 (event MS3 x Inbred line D) -4
10. BC1 (event MS3 x Inbred line D) -8
11. BC1 (event MS3 x Inbred line D) -11
12. BC1 (event MS3 x Inbred line D) -14
13. BC3 (event MS3 x Inbred line C) -1
14. BC3 (event MS3 x Inbred line C) -2
15. BC3 (event MS3 x Inbred line C) -3
16. BC3 (event MS3 x Inbred line C) -5
17. BC3 (event MS3 x Inbred line C) -7
18. BC1 (event MS3 x Inbred line C) -1
19. BC1 (event MS3 x Inbred line C) -3
20. BC1 (event MS3 x Inbred line C) -8
21. BC1 (event MS3 x Inbred line C) -10
22. BC1 (event MS3 x Inbred line C) -13
23. BC3 (event MS3 x Inbred line A) -1
24. BC3 (event MS3 x Inbred line A) -2
25. BC3 (event MS3 x Inbred line A) - 3
26. BC3 (event MS3 x Inbred line A) - 8

4.3. Conclusion

The hybridization data revealed that all the analyzed hybrid plants had an identical integration pattern as transformation event MS3 (Figure 4.). Consequently, it was shown that the insert of event MS3 is stably inherited in backcrossing programs.

Figure 4. HindIII hybridization results



Annex 7.5. . Quantification of phosphinothricin acetyl transferase (PAT) levels in H99 and MS3 corn seeds

1. Material and methods

    1.1. Preparation of corn kernel extracts
    1.2. Spectrophotometric assay for PAT

2. Results

3. Conclusion

QUANTIFICATION OF PHOSPHINOTHRICIN ACETYL TRANSFERASE (PAT) LEVELS IN H99 AND MS3 CORN SEEDS

1. Material and methods

1.1. Preparation of corn kernel extracts

- H99 seeds, stock number M7048 (harvested November 1994 and stored until April 1995).
- MS3 seeds, stock number M6970, 7th maintained generation (harvested August 1994 and stored until April 1995).

Fifty control seeds (H99) and fifty transgenic seeds (MS3) were milled in a Waring Blendor apparatus at high speed for 3 times 30 seconds with 1 minute intervals to cool. This was done at 4°C. Flour of the kernels was collected and the weight was determined to be 0.2 grams of flour per kernel.

1.2. Spectrophotometric assay for PAT

After PAT catalyzed acetylation of phosphinothricin (PPT) at the expense of acetyl coenzyme A (AcCoA) the free sulfhydryl group of coenzyme A can react with the Ellman's reagent (5,5' Dithiobis(2-nitrobenzoic acid) (DTNB)). During this reaction a yellow colored product is formed which can be followed in time and is a measure for the PAT activity.

Solutions:
1. 0.4 mg DTNB/mL 100 mM TRIS/Cl pH 7.5
2. 9.75 mg PPT/ mL H_2O
3. 20.2 mg AcCoA/ mL H_2O

Measurement:
968 µL DTNB
2 µL PPT
20 µL AcCoA
10 µL sample

Optical density (OD) is measured at 412 nm, 37°C against reference cuvette containing all solutions except sample. 1 Unit is defined as the increase of 1 OD at 412 nm/min at 37°C.

The experiment was designed to demonstrate any effect of the flour matrix on the extracted and/or recovered PAT activity. Internal references (spiked PAT purified from a bacterial production system) allow the determination of the detection limit of PAT activity. By comparing the results of the control and the transgenic MS3 samples it can be clarified whether the genetic modification has resulted in a significant change in PAT activity.

In an amount of flour equivalent to that from one kernel (0.2 grams) from control and transgenic seeds, different amounts of PAT were spiked ranging from 1.4 to 138 µg PAT. The flour was mixed well and stored over night at 4°C. The other day 500 µL of extraction buffer (50 mM sodium phosphate buffer pH 7.0; 10 mM EGTA; 10 mM EDTA) was added to each of the samples and they were shaken for 30 minutes at 4°C. After centrifugation for 15 minutes in an Eppendorf centrifuge at maximum speed, supernatants were collected and tested for PAT activity.

2. Results:

Results are summarized in Table 1.

Table 1. PAT activity (U/mL) recovered after extraction of flour samples of control and transgenic MS3 corn kernels. As a reference the quantity of spiked PAT protein and the expected activity in the extract is indicated.

µg PAT spiked	U/mL PAT in extract	U/mL PAT recovered	
		Control H99 flour	Transgenic MS3 flour
0.0	0.0	0.3	0.1
0.0	0.0	0.3	0.1
1.4	0.5	0.7	0.6
4.2	1.4	1.8	1.5
8.3	2.8	3.2	3.5
16.6	5.6	6.4	7.4
34	11.8	14.9	14.5
69	23.5	30.9	35.0
138	47.0	63.8	84.9

3. Conclusion

By adding purified PAT to the samples, it was shown that the extraction and detection procedures are adequate for detection of PAT in corn seeds.

The detection limit for PAT in corn kernels is about 1 µg per kernel (5 mg/kg).
In flour from H99 kernels and MS3 kernels some activity can be detected. Since there is no difference between the control and transgenic corn this can be a non-specific reaction of the samples with the Ellman's reagent

We have not found any evidence or indication for the presence of PAT in MS3 corn seeds.

Annex 7.6. Quantification of β-lactamase in H99 and MS3 corn seeds

1. Material and methods

    1.1. Preparation of corn kernel extracts
    1.2. Spectrophotometric assay for β-lactamase

2. Results

3. Conclusion

QUANTIFICATION OF β-LACTAMASE IN H99 AND MS3 CORN SEEDS

1. Material and methods

1.1. Preparation of corn kernel extracts

- H99 seeds, stock number M7048 (harvested November 1994 and stored until April 1995).
- MS3 seeds, stock number M6970, 7th maintained generation (harvested August 1994 and stored until April 1995).

Fifty control seeds (H99) and fifty transgenic seeds (MS3) were milled in a Waring Blendor apparatus at high speed for 3 times 30 seconds with 1 minute intervals to cool. This was done at 4°C. Flour of the kernels was collected and the weight was determined to be 0.2 grams of flour per kernel.

1.2.Spectrophotometric assay for β-Lactamase

The method of Bush and Sykes (1984; Methods of Enzymatic analysis, 3d ed., vol.IV: 280-285, Verlag Chemie) was used to detect β-Lactamase.
Upon hydrolysis of nitrocefin an increase in absorbance at 495 nm can be measured which is directly correlated with β-Lactamase activity.

The experiment was designed to demonstrate any effect of the flour matrix on the extracted and/or recovered β-Lactamase activity. Internal references (commercially available β-Lactamase (Boehringer no.663441)) allow the determination of the detection limit of β-Lactamase activity. By comparing the results of the control and the transgenic MS3 samples it can be clarified whether the genetic modification has resulted in a significant change in β-Lactamase activity.

In an amount of flour equivalent to that from one kernel (0.2 grams) from control and transgenic seeds, different amounts of a commercially available β-Lactamase were spiked ranging from 20 to 10,000 ng in 10 µL. The flour was mixed well and stored overnight at 4°C. The other day 500 µL of extraction buffer (50 mM sodium phosphate buffer pH 7.0; 10 mM EGTA; 10 mM EDTA) was added to each of the samples and they were shaken for 30 minutes at 4°C. After centrifugation for 15 minutes in an Eppendorf centrifuge at maximum speed, supernatants were collected and tested for β-Lactamase activity.

2. Results

The results are summarized in Table 1.

Table 1. **β-Lactamase activity (U/mL) recovered after extraction of flour samples of control and transgenic MS3 corn kernels. As a reference the quantity of spiked β-Lactamase protein and the expected activity in the extract is indicated.**

µg β-lactamase. spiked in flour	U/L β-lactamase in extract	Control H99 flour		Transgenic MS3 flour	
		Δ495/ min	U/L β-Lact. reoverd	Δ495/ min	U/L β-lact. recovered
0.00	0.0	0.0006	2.6	-0.0005	-2.2
0.00	0.0	0.0010	4.3	-0.0005	-2.2
0.02	2.7	0.0008	3.5	0.0004	1.7
0.04	5.4	0.0010	4.3	0.0004	1.7
0.08	10.8	0.0010	4.3	0.0006	2.6
0.20	27	0.0034	14.8	0.0018	7.8
0.40	54	0.0035	15.2	0.0027	11.7
0.80	108	0.0083	36.0	0.0070	30.4
2.00	270	0.0380	165	0.0400	174
4.00	540	0.0880	382	0.0800	347
8.00	1080	0.1840	798	0.1660	720
10.00	1350	0.2300	998	0.2300	998

3. Conclusion

The detection limit for β-Lactamase activity in corn kernel extracts is about 10 U/L which is equivalent to 0.15 µg β-Lactamase per kernel (750 µg/kg).
Recovery of spiked β-Lactamase activity from the kernels is about 75%.

In flour from H99 kernels and MS3 kernels no detectable amounts of β-Lactamase activity were found.

Annex 8. . Greenhouse data of event MS3

PGS has carried out experiments in the greenhouse in order to evaluate the male sterile *Zea mays* plants derived from transformation event MS3. These greenhouse trials were carried out under contained use procedures. On the following pages, reports of selected greenhouse trials are presented. Commercially available public inbred lines and proprietary inbred lines that were used, were coded from C101 to C119.

Annex 8.

*** A selection of greenhouse experiments in which corn plants containing event MS3 were tested**

Greenhouse trial	Subject
93/GZM007	Event MS3 in H99 background : segregation study and stability of expression
93/GZM008	Event MS3 in F_1 hybrid background : segregation study and stability of expression
GZM091	Event MS3 in F_1 hybrid background : conversion of inbred lines
93/GZM012	Event MS3 in BC_1 background : segregation study and stability of expression
95/GZM005	Event MS3 : treatment with Round up® and Gramoxone®

*** Germination test of a seedlot containing event MS3**

MEMO

To : Elke Göbel
From : Catherine Dickburt
Date : December 17th, 1993
Ref. :

Re : ***Glasshouse experiment 93/GZM007 - Final report***
Event MS3 in H99 background : segregation study and stability check
(seedlots from Chile Winter Nursery 92-93)

Objectives:

1. Check on segregation ratios
2. Check on phenotype stability
3. Check on genotype stability

Material and Methods:

Material:

NMS ENTRIES:
M4637 : NMS RZM35/1
M4636 : NMS RZM34/1 (further designated as event MS3), 4th maintained generation

Glufosinate treatment:
Basta dot-test (0.5%) at the 3-4 leaf growth stage.

Molecular analysis:
PCR on 50 glufosinate tolerant plants per seedbatch

Observations:
Emergence, segregation, glufosinate tolerance, flower phenotype (fertility/sterility)

Results:

Seeds were sown on 19/04/93. Two hundred seeds of each entry (RZM35/1 and event MS3) were sown. The Basta treatment was done on 30/04/93 at the 3-4 leaf growth stage. Assessment of the test was done 6 days after treatment. PCR analysis was carried out on 50 resistant plants per entry to check the presence of the transgenes. Emergence and segregation results are given in Table 1. Flowering data are given in Table 2. PCR revealed that all analyzed glufosinate tolerant plants from event MS3 and RZM35/1 were positive for both *barnase* and 35S.

Twenty glufosinate tolerant plants of each transformation event were transplanted and grown to maturity.

- All plants from the maintained progeny of event MS3 were completely male sterile. On 3 plants some very small anthers were observed extruding out of the glumes. The shrivelled anthers did not contain pollen grains. Male sterility was stable during the flowering period.
- In the maintained progeny of RZM35/1 many plants showed extruding anthers; male flower phenotype of this progeny ranged from full sterility (4/20 plants) or a few anthers (9/20 plants) up

to 50% anther extrusion (1/20 plants). Pollen viability was checked (Alexander's staining and pollen germination test in a Pfahler medium) on plants presenting anther extrusion; result of this test was positive.

Conclusions:

Segregation data for both lines did not differ significantly from the 1:1 ratio expected under normal Mendelian segregation assuming the female parent had one active copy of the construct *bar* + *barnase* integrated. Plants carrying event MS3 contain the *barnase* gene construct and are male sterile

Table 1 - 93/GZM007- Emergence and segregation data

Plant material	Number of seedlings/Total number of seeds	Glufosinate segregation ratio	
		Number of tolerant/sensitive plants	
Event MS3	174/200	76R/98S	n.s.
RZM35/1	148/200	59R/88S	n.s.

n.s. stands for not significantly different from a 1:1 ratio in a Chi-square test at the 0.05 level.

Table 2 - 93/GZM007- Flower observations

Plant material	Flower Phenotype	Number of plants (out of 20)		
		21/06/93	24/06/93	28/06/93
Event MS3	Male Sterile few very small anthers (no pollen)	20	20	17 3
RZM35/1	Male Sterile few anthers (viable pollen) 5% anther extrusion (viable pollen) 25% anther extrusion (viable pollen) 50% anther extrusion (viable pollen)	20	14 4 1 1	4 9 3 3 1

'% anther extrusion' descibes the percentage of spikelets per tassel from which anthers emerge

MEMO

To : Elke Göbel
From : Catherine Dickburt
Date : December 17th, 1993
Ref. :

Re : ***Glasshouse experiment 93/GZM008 - Final report***
Event MS3 in F_1 hybrid background : segregation study and stability check

Objectives:

1. Check on segregation ratios
2. Check on phenotype stability
3. Check on genotype stability

Material and Methods:

Material:

NMS HYBRIDS:
M4691: M4224 F1 (Event MS3 x C101)
M4692: M4224 F1 (Event MS3 x C102)

CONTROL HYBRIDS:
M4696: H99 x C101
M4695: H99 x C102

Glufosinate treatments:
Basta dot-test (0.5%) at the 3-4 leaf growth stage.

Molecular analysis:
PCR on 50 glufosinate tolerant plants per hybrid

Observations:
Emergence, segregation, glufosinate tolerance, flower phenotype (fertility/sterility)

Results:

The test was sown on 12/05/93. Two hundred seeds of each hybrid were sown. The glufosinate treatments were done on 24/05/93 at the 3-4 leaf growth stage. Assessment of the test was done 4 days after treatment. PCR analysis was carried out on 50 glufosinate tolerant plants per seedbatch to check the presence of the MS3 event. Emergence and segregation results are given in Table 1. Flowering data are given in Table 2.

All PCR analysed Basta tolerant hybrid plants (Event MS3 x C101, Event MS3 x C102) contained the *barnase* gene.

Twenty resistant plants of each hybrid combination and five plants of the control hybrid were transplanted and grown to maturity.

The F_1 hybrids presented partial sterility. Male flower phenotype of F1 (Event MS3 x C102) ranged from a few anthers on the tassel to 5% of the spikelets extruding anthers. Most of the F1 (Event MS3 x C101) plants also presented a few to 5% of the spikelets extruding anthers although a few plants presented 25 and 50% anther extrusion.

Conclusions :

Glufosinate segregation data for the hybrids did not differ significantly from the 1:1 ratio expected under normal Mendelian segregation assuming the female parent (Event MS3) had one active copy of the construct *bar* + *barnase* integrated. PCR analyses confirmed the presence of the transgenes. The F_1 hybrids presented partial sterility.

Table 1 - 93/GZM008- Emergence and segregation data

Plant material	Emergence No.emerged/Total	Basta segregation ratio
		No. tolerant/sensitives
F1(Event MS3 x C101)	200/200	98R/102S n.s.
F1(Event MS3 x C102)	200/200	106R/94S n.s.

n.s. stands for not significantly different from a 1:1 ratio in a Chi-square test at the 0.05 level.

Table 2 - 93/GZM008- Flower observations

Plant material	Flower Phenotype	No. of plants (out of 20)		
		12/07/93	14/07/93	20/07/93
F1 (Event MS3 x C101)	Sterile	13	1	0
	Few anthers (viable pollen)	7	9	4
	5% anther extrusion (viable pollen)	0	6	12
	25% anther extrusion (viable pollen)	0	3	3
	50% anther extrusion (viable pollen)	0	1	1
F1 (Event MS3 x C102)	Sterile	12	6	0
	Few anthers (viable pollen)	8	13	12
	5% anther extrusion (viable pollen)	0	1	8

'% anther extrusion' descibes the percentage of spikelets per tassel from which anthers emerged

MEMO

To : Elke Göbel
From : Catherine Dickburt
Date : August 24th, 1993
Ref. :

Re : ***Glasshouse experiment GZM091 - Final report***
Event MS3 in F1 hybrid background
Conversion of inbred lines (coded C102 to C107) with event MS3

Objectives:

1. Production of F1 hybrids for field evaluation in 1993.
2. Production of BC1 seeds for back-crossing program of event MS3 into elite lines.
3. Glasshouse observation of the male sterility trait in F1 hybrids produced in the field in 1992 (event MS3 x public or elite lines).

Material and Methods:

Material:

Male sterile material:	Inbred lines:
M4224: 4th maintained generation of event MS3	H99
F1 Event MS3 x C103	C101
F1 Event MS3 x C104	C103
F1 Event MS3 x C105	C104
F1 Event MS3 x C106	C105
F1 Event MS3 x C102	C106
F1 Event MS3 x C107	C102
	C107

Glufosinate treatment:
Basta dot-test (0.5%) at the 3-4 leaves growth stage.

Molecular analysis:
PCR and Southern Blot on all resistant F1 plants.

Observations:
Emergence, segregation, glufosinate tolerance, flower phenotype (fertility/sterility)

Crosses:
Production of BC1 seeds on all glufosinate tolerant F1 (event MS3 x inbred line) plants.
Production of following F1 seeds: H99xC101, event MS3xC101, H99xC102, event MS3xC102.

Results:

The first lines of the test were sown on 16/12/92. Fifteen seeds were sown of the 6 F1 hybrids (event MS3 x inbred line) produced in a 1992 field trial. Basta treatments on transgenic entries were carried out on 30/12/92 at the 3-4 leaf growth stage. Assessment of the Basta dot-tests was done 7 days after treatment on 06/01/93. Emergence and segregation results are given in Table 1.

All glufosinate tolerant plants from the maintained progeny of event MS3 were completely male sterile.

All tolerant F1 plants were analysed by PCR (for the presence of 35S and *barnase*) and by Southern Blot.The hybridization data revealed that all the analysed hybrid plants had an identical integration pattern to event MS3.

On all F1 hybrids, an intermediate flower phenotype was observed. Individual data on flowering date and flower phenotype are given in Table 1 and Table 2, respectively. Male flower phenotype ranged from complete male sterility (or a few anthers) (F1 event MS3xC106 and F1 event MS3xC102) to 50% fertility (F1 event MS3xC103, 50% anther extrusion on the tassel).

Conclusions :

In general, glufosinate tolerance segregation data were as expected. Glufosinate tolerant plants from the maintained progeny of event MS3 were male sterile. By Southern blot hybridization, it was demonstrated that the insert was stably integrated in the analyzed glufosinate tolerant F1 plants and that no differences in the integration pattern were observed compared to the transgenic male sterile plants from which they were derived. Flower phenotype of the transgenic F1 plants ranged from completely male sterile to 50% fertility, the latter in one particular F1 hybrid.

Table 1 - GZM091- Emergence and segregation data

Plant material	Sowing date	Emergence No.emerged/ Total (30/12/92)	Basta tolerance segregation ratio		Flowering date
			No. tolerant/Total	%	50% silking
H99	16/12	50/50	N.A.	N.A.	01/03
C104	16/12 21/12	6/11 9/10	N.A.	N.A.	N.D.
C103	16/12 21/12	11/11 11/11	N.A.	N.A.	N.D.
C105	16/12 21/12	10/11 10/10	N.A.	N.A.	N.D.
C106	16/12 21/12	11/11 10/10	N.A.	N.A.	N.D.
C102	16/12 21/12	25/25 27/27	N.A.	N.A.	N.D.
C107	16/12 22/12	11/12 9/10	N.A.	N.A.	N.D.
Event MS3 (M4224)	16/12	89/90	35R/88 n.s.	40	01/03
Event MS3xC104	16/12	15/15	6R/15 n.s.	40	19/02
Event MS3XC103	16/12	16/16	7R/16 n.s.	44	22/02
Event MS3xC105	21/12	15/15	5R/15 n.s.	33	01/03
Event MS3xC106	21/12	15/15	11R/15 n.s.	73	04/03
Event MS3xC102	21/12	15/15	3R/15 s.	20	03/03
Event MS3xC107	26/12	14/15	6R/14 n.s.	43	08/03

N.A. Not Applicable
N.D. Not Determined
n.s. stands for not significantly different from a 1:1 ratio in a Chi-square test at the 0.05 level.
s. stands for significantly different from a 1:1 ratio in a Chi-square test at the 0.05 level.

Table 2. GZM091. Flower phenotype results:

Plant no.		Flower phenotype (% anther extrusion)	Pollen viability test	
			Alexander staining	Germination test
Event MS3 x C104	-1	25	OK	OK
	-2	25	OK	OK
	-3	25	OK	OK
	-4	25	OK	OK
	-5	7 anthers	OK	OK
	-6	25	OK	OK
Event MS3 x C103	-1	50	OK	OK
	-2	50	OK	OK
	-3	50	OK	OK
	-4	50	OK	OK
	-5	50	OK	OK
	-6	50	OK	OK
	-7	50	OK	OK
Event MS3 x C105	-1	5	OK	OK
	-2	25	OK	OK
	-3	25	OK	OK
	-4	25	OK	OK
	-5	25	OK	OK
Event MS3 x C102	-1	5-10 anth	OK	OK
	-2	Male sterile		
	-3	5-10 anth	OK	OK
Event MS3 x C106	-1	5-10 anth	NO	NO
	-2	5-10 anth	NO	
	-3	5-10 anth	NO	
	-4	5-10 anth	NO	
	-5	Male sterile		
	-6	5-10 anth	OK	
	-7	5-10 anth	NO	
	-8	5-10 anth		
	-9	5-10 anth	OK	
	-10	5-10 anth	NO	OK
	-11	5-10 anth	NO	
Event MS3 x C107	-1	5	OK	OK
	-2	5	OK	OK
	-3	5	OK	OK
	-4	5	OK	OK
	-5	5	OK	OK
	-6	5	OK	OK

- The Alexander staining was carried out on one or more anthers. Starch-filling of the pollen grains was examined under the microscope. If red stained starch filled grains (viable pollen) were observed, they were counted out of 20. OK stands for at least one red coloured, starch filled grain observed. NO stands for none observed on the slide glass.
- For Germination test, OK: at least one grain germinating on a Phahler medium, NO: none.
- '% anther extrusion' describes the percentage of spikelets per tassel from which anthers emerge

MEMO

To : EG/MW/LH/HVM/KDH
From : Catherine Dickburt
Date : September 14th, 1993
Ref. :

Re : ***Glasshouse experiment 93/GZM012 - Final report***
Event MS3 in a BC1 background : segregation study and stability of expression
Backcrossing Program

Objectives:

1. Continuation of the backcross program of event MS3 into elite material (Follow up of experiment GZM091).
2. Observation of flower phenotype to check male sterility trait stability in BC1 plants.

Material and Methods:

Material:

INBRED LINES
M4803: C102
M4807: C106
M4810: C105
M4809: C107
M4805: C104
M4804: C103

BC1 ENTRIES			MALE FLOWER PHENOTYPE OF F1 PLANTS
M4658:	BC1	(EVENT MS3 x C104)-4 x C104	25% ANTHER EXTRUSION
M4659:	BC1	(EVENT MS3 x C104)-5 x C104	FEW ANTHERS
M4661:	BC1	(EVENT MS3 x C103)-1 x C103	50% ANTHER EXTRUSION
M4668:	BC1	(EVENT MS3 x C105)-1 x C105	5% ANTHER EXTRUSION
M4669:	BC1	(EVENT MS3 x C105)-2 x C105	25% ANTHER EXTRUSION
M4684:	BC1	(EVENT MS3 x C102)-1 x C102	5-10 ANTHERS
M4685:	BC1	(EVENT MS3 x C102)-2 x C102	MALE STERILE
M4673:	BC1	(EVENT MS3 x C106)-1 x C106	5-10 ANTHERS
M4677:	BC1	(EVENT MS3 x C106)-5 x C106	MALE STERILE
M4688:	BC1	(EVENT MS3 x C107)-3 x C107	5% ANTHER EXTRUSION

NMS LINES
M4365: EVENT MS3 (4TH MAINTAINED GENERATION OF EVENT MS3)

CONTROL LINE
M4473: H99

Glufosinate treatment:
Basta dot-test (0.5%) at the 4-5 leaf growth stage.

Observations:
Emergence, segregation, glufosinate tolerance, flowering date (50% silking) and phenotype (fertility/sterility), plant phenotype, cob filling (number of seeds produced)

Crosses:
Crosses of the BC1 glufosinate tolerant plants with either the parental line or with H99.

Results:

Seeds were sown between 08/06/93 and 14/06/93. Fifteen seeds were sown of 10 BC1 lines [(event MS3 x inbred line) x inbred line] produced in glasshouse experiment GZM091. Glufosinate treatment on transgenic entries was carried out on 21/06/93 at the 4-5 leaf growth stage. Assessment of the glufosinate dot-test was done 4 days after treatment on 25/06/93. Emergence and segregation results are given in Table 1.

All plants from the maintained progeny of event MS3 were completely male sterile .

Five glusosinate tolerant plants and two sensitive plants per BC1 line were transplanted into big pots and grown to maturity.
Completely male sterile plants were observed in all BC1 lines tested. In some lines partially sterile plants were observed. The degree of male sterility was in general improved in the BC1s in comparison to the F1 hybrids (Table 2.).

- Full male sterility on all plants was observed for both [(Event MS3 x C104) x **C104** and [(Event MS3 x C106) x **C106**] BC1 lines. The degree of sterility was improved for these lines in comparison to the F1 hybrids: up to 25% anther extrusion on F1(Event MS3 x C104) plants and a few anthers on F1(Event MS3 x C106) hybrid plants.
- In [(Event MS3 x C102) x **C102** and [(Event MS3 x C107) x **C107**] BC1 lines, male flower phenotype ranged from full sterility to a few anthers. Flower phenotype for F1 (Event MS3 x C102) plants had ranged from full sterility to a few anthers while 5% anther extrusion had been observed on all F1 (Event MS3 x C107) plants. In these cases, the sterility level was only slightly improved in the BC1 line in comparison to the F1 hybrids.
- Full male sterility to 25% anther extrusion was observed in [(event MS3 x C103) x **C103** and [(Event MS3 x C105) x **C105**] BC1 lines. F1 (Event MS3 x C103) plants had presented 50% anthers development and 5%-25% anther extrusion had been observed on F1 (Event MS3 x C105) plants.

In general, partially sterile phenotypes were observed on plants that were still quite distinct in plant phenotype from the inbred lines which are used in the backcrossing program (see Table 2.).

BC1 plants were crossed to either the parental line or to H99, as indicated in the test protocol.

Conclusions:

Segregation data for the male sterile material did not differ significantly from the 1:1 ratio expected under normal Mendelian segregation for a dominant gene.

The degree of male sterility of the BC1 generation was improved compared to the level of male sterility of the F1 hybrid generation.

Table 1 - 93/GZM012- Emergence and segregation data

Plant material	Sowing date	Emergence No.emerged /Total	Basta tolerance segregation ratio	Flowering date
			No. resistant/Total	50% silking (D.A.S)
Event MS3	10/06	10/10	5/10 n.s.	
C104	08/06 13/06	Not determined	Not applicable	Not determined
C103	08/06 13/06	Not determined	Not applicable	Not determined
C105	08/06 14/06	Not determined	Not applicable	Not determined
C106	08/06 13/06	Not determined	Not applicable	Not determined
C102	08/06 14/06	Not determined	Not applicable	Not determined
C107	08/06 14/06	Not determined	Not applicable	Not determined
BC1 (Event MS3xC104)-4xC104	08/06	15/15	7R/8S n.s.	55
BC1 (Event MS3xC104)-5xC104	08/06	15/15	8R/7S n.s.	55
BC1 (Event MS3XC103)-1xC103	08/06	15/15	8R/7S n.s.	56
BC1 (Event MS3xC105)-1xC105	11/06	15/15	9R/6S n.s.	57
BC1 (Event MS3xC105)-2xC105	11/06	15/15	8R/7S n.s.	57
BC1 (Event MS3xC106)-1xC106	13/06	15/15	7R/7S n.s.	59
BC1 (Event MS3xC106)-5xC106	13/06	15/15	7R/8S n.s.	59
BC1 (Event MS3xC102)-1xC102	11/06	15/15	8R/7S n.s.	62
BC1 (Event MS3xC102)-2xC102	11/06	15/15	8R/6S n.s.	59
BC1 (Event MS3xC107)-3xC107	13/06	15/15	5R/10S n.s.	62

n.s. stands for not significantly different from a 1:1 ratio in a Chi-square test at the 0.05 level.
Flowering date (50% silking) is given in D.A.S (number of days after sowing).

Table 2. 3. ZM012 Flower phenotype results.

BC1 seedlot	Female parent	Male parent	Plant no.	Flower phenotype (% anther extrusion)*1	Plant phenotype (scale 0 to-3)*2		
					Height	Leaves/ stem	Cob/ Tassel
M4658	F1 (Event MS3 x C104)-4 25% anther extrusion	C104	1	Male sterile	3	1	2
			2	Male sterile	1	1	1
			3	Male sterile	1	3	3
			4	Male sterile	2	2	1
			5	Male sterile	1	2	2
M4659	F1 (Event MS3 x C104)-5 few anthers	C104	1	Male sterile	2	1	2
			2	Male sterile	2	1	1
			3	Male sterile	3	1	2
			4	Male sterile	2	1	2
			5	Male sterile	3	2	3
M4661	F1 (Event MS3 x C103)-1 50% anther extrusion	C103	1	few anthers (pollen not viable)	3	1	3
			2	few anthers (pollen not viable)	2	2	2
			3	few anthers (viable pollen)	3	2	2
			4	25%	2	2	3
			5	Male sterile	0	2	2
M4668	F1 (Event MS3 x C105)-1 5% anther extrusion	C105	1	5% (viable pollen)	1	2	1
			2	Male sterile	1	2	1
			3	Male sterile	1	2	1
			4	5% (viable pollen)	2	3	1
			5	5% (viable pollen)	1	2	1
M4669	F1 (Event MS3 x C105)-2 25% anther extrusion	C105	1	5% (viable pollen)	3	3	3
			2	5% (viable pollen)	2	2	2
			3	Male sterile	2	2	2
			4	Male sterile	2	2	3
			5	25%	2	2	3
M4673	F1 (Event MS3 x C106)-1 few anthers	C106	1	Male sterile	1	2	2
			2	Male sterile	3	2	2
			3	Male sterile	2	2	2
			4	Male sterile	3	1	2
			5	Male sterile	1	1	2
M4677	F1 (Event M3 x C106)-5 Male sterile	C106	1	Male sterile	1	1	2
			2	Male sterile	2	2	1
			3	Male sterile	1	1	2
			4	Male sterile	2	2	2
			5	Male sterile	0	1	1
M4684	F1 (Event MS3 x C102)-1 few anthers	C102	1	Male sterile	1	1	1
			2	Male sterile	3	2	1
			3	Male sterile	3	2	1
			4	Male sterile	2	1	1
			5	few anthers (viable pollen)	2	1	1
M4685	F1 (Event MS3 x C102)-2 Male sterile	C102	1	Male sterile	1	2	1
			2	Male sterile	1	1	1
			3	Male sterile	2	2	1
			4	Male sterile	2	1	1
			5	5% (pollen not viable)	2	1	1
M4688	F1 (Event MS3 x C107)-3 5% anther extrusion	C107	1	Male sterile	1	2	2
			2	few anthers (viable pollen)	1	2	2
			3	Male sterile	1	3	2
			4	Male sterile	1	1	2
			5	Male sterile	2	2	1

*1 : An Alexander staining was carried out to check pollen viability when up to 5% anther extrusion was observed.
*2 : Scale 0 to 3 : 0=similar characteristics as the male inbred parent, 3=very different from the male inbred parent
'% anther extrusion' describes the percentage of spikelets per tassel from which anthers emerge

Memo:

To : Elke Göbel, Patrick Rüdelsheim
From : Catherine Dickburt
Date : May 11th, 1995

Re : ***Glasshouse experiment 95/GZM005 - Final report***
Event MS3 : treatment with Roundup® and Gramoxone®

Objectives

Two herbicides (glyphosate and paraquat) were sprayed at different rates on seedlings (3-4 leaves) of event MS3 in H99 and of non-transgenic H99 in order to confirm the susceptibility of the corn material to these chemicals.

Detailed objectives:

1. Confirm that event MS3 and H99 corn plants are destroyed by the recommended rate of glyphosate and paraquat.
2. Determine the breaking rates of the two products (highest rate of the product not giving total elimination of the H99 corn seedlings).
3. Determine whether there is any competitive advantage of event MS3 versus its non-transgenic control H99.

Material and Methods:

Material:

Event MS3:	M6025= M6	:	2000 seeds
Control:	M6026= H99:	:	1000 seeds

Basta treatment:

Basta sprayment (glufosinate ammonium 200g/L), 0.5% solution at the 3-4 leaves growth stage on MS3 only to select transgenic plants.

Other herbicides sprayed on event MS3 and on H99:

Round up (glyphosate 360g/L) sprayment rates: 0, 0.625, 1.25, 2.5 and 5 L/Ha
(or 0, 0.0625, 0.125, 0.25 and 0.5%).

Gramoxone (paraquat 200 g/L) sprayment rates: 0, 0.625, 1.25, 2.5 and 5L/Ha
(or 0, 0.0625, 0.125, 0.25 and 0.5%).

Volume of application of 100 ml/m2 (or 1000 L/Ha). Usually, volume for field application is of 500L/Ha; in the greenhouse, however, a good coverage of the plants can only be obtained using a volume of 100ml/m2 because of the spraying equipment used.

Observations:

% Emergence. Basta segregation (event MS3), number of dead plants 2 weeks after sprayment with glyphosate and paraquat, plant height of surviving plants (10 plants/tray).

Results and conclusion

Seeds were sown on 14/03/95. Emergence was good (mean of 90%) for both event MS3 and H99.

Event MS3 was sprayed on 30/03/95 with Basta to select the transgenic plants.

Basta tolerant plants of event MS3 and the H99 plants were sprayed with Round up or Gramoxone at rates ranging from 0 up to 0.5% (5 L/ha, recommended rate for field use). Two weeks after the treatment, the plants were assessed to determine the number of plants destroyed by the different treatments. Plant height of surviving plants was measured (Table 1. and Table 2.). Event MS3 and H99 plants were both destroyed by the recommended rates of Round up and Gramoxone.

No differences in breaking rates of the two total herbicides used were observed between event MS3 and H99. A rate of 0.125% (1.25 L/ha or 1/4 of the recommended rate) Round up, or a rate of 0.25% (2.5 L/ha or 1/2 of the recommended rate) Gramoxone was needed to eliminate event MS3 and H99 plants under the experimental conditions (Table 1. and Table 2.).

Applying 1/8 of the recommended rate of Round up on the plants drastically reduced the height of both event MS3 and H99 (12 cm and 13 cm respectively in comparison to 24 cm for unsprayed plants). The application of 1/8 of the recommended rate of Gramoxone led to a similar plant height reduction (16 cm for both event MS3 and H99 in comparison to the unsprayed controls). Plant height was also reduced with 1/4 of the recommended rate of Gramoxone (12 cm and 15 cm for event MS3 and H99 respectively compared to 24 cm for the controls) (Table 1. and Table 2.).

This greenhouse experiment did not identify any competitive advantage of event MS3 over its non-transgenic control (H99) when sprayed with widely used total herbicides such as Round up or Gramoxone. Event MS3, though Basta tolerant, can be destroyed by other herbicides that also destroy the non-transgenic H99 counterpart.

Table 1 - 95/GZM005- Emergence, Basta tolerance segregation and Round up damage on event MS3 versus H99

Treatment		Emergence (%)	Basta segregation (N° tolerant/ Total)	Plants destroyed by Round up 14 days after sprayment (% plants)	Plant height of surviving plants 14 days after sprayment Mean over 10 plants (cm)
Plant material	Round up rate (%)				
MS3	0	93	35R/77	0	24 cm
	0.0625	94	38R/82	77	12 cm
	0.125	90	33R/76	100	-
	0.25	91	44R/83	100	-
	0.5	90	35R/76	100	-
	Mean	92	47%		
H99	0	86	-	0	24 cm
	0.0625	92	-	54	13 cm
	0.125	90	-	100	-
	0.25	96	-	100	-
	0.5	86	-	100	-
	Mean	90			

Notes:
1. Test was sown on 14/03/95.
2. Basta sprayment was carried out on MS3 plants with a 0.5% Basta solution at the 3-4 leaves growth stage (30/03/95). Assessment of the test was done on 03/04/95 by counting the remaining plants in the sprayed trays.
3. s. stands for significantly different from a 1:1 ratio in a Chi-square test.
4. Round up sprayments were carried out 4 days after the Basta sprayment on 03/04/95. Assessment of the test was done 14 days after treatment on 17/04/95 by counting the surviving plants in the trays.
5. Plant height was assessed by measuring 10 plants per treatment from the soil surface up to the last formed node.

Table 2 - 95/GZM005- Emergence, Basta tolerance segregation and Gramoxone damage on MS3 versus H99

Treatment		Emergence (%)	Basta segregation (N° tolerant/ Total)	Plants destroyed by Gramoxone 14 days after sprayment (% plants)	Plant height of surviving plants 14 days after sprayment Mean over 10 plants (cm)
Plant material	Gramoxone rate (%)				
MS3	0	87	33R/75	0	24 cm
	0.0625	95	44R/81	52	16 cm
	0.125	96	42R/88	83	12 cm
	0.25	94	51R/89	100	-
	0.5	89	45R/82	100	-
	Mean	92	52%		
H99	0	96	-	0	24 cm
	0.0625	90	-	78	16 cm
	0.125	92	-	97	15 cm
	0.25	94	-	100	-
	0.5	90	-	100	-
	Mean	92			

Notes:
1. Test was sown on 14/03/95.
2. Basta sprayment was carried out on MS3 plants with a 0.5% Basta solution at the 3-4 leaves growth stage (30/03/95). Assessment of the test was done on 03/04/95 by counting the remaining plants in the sprayed trays.
3. s. stands for significantly different from a 1:1 ratio in a Chi-square test.
4. Gramoxone sprayments were carried out 4 days after the Basta sprayment on 03/04/95. Assessment of the test was done 14 days after treatment on 17/04/95 by counting the surviving plants in the trays.
5. Plant height was assessed by measuring 10 plants per treatment from the soil surface up to the last formed node.

Testing of seed quality

One seedlot containing event MS3 in corn background H99 and one control H99 seedlot were used in a test that determines :
- seed germination at warm temperature,
- seed germination at cold temperature,
- 1000 kernel weight, and
- hectoliter weight.

The seeds were produced in the winter nursery in Chile. The seed-parent of event MS3 was tolerant to glufosinate-ammonium and male sterile.

For the seed germination at warm temperature, 400 seeds were sown in sand and incubated at 20°C. After 7 days, the material was monitored and the number of normal seedlings, abnormal seedlings and non-germinating seeds was determined.

For the seed germination at cold temperature, the seeds were sown in soil, kept for 7 days at 5°C and were then incubated for 5 days at 25°C. The number of normal seedlings, abnormal seedlings and non-germinating seeds was determined.

The results are summarized in Table S1.

Table S1. Seed quality analyses of event MS3

Test parameters	H99 control	Event MS3
Seed purity (%)	99.9	100
Germination at 20°C, after 7 days		
- normal seedlings (%)	90	86
- abnormal seedlings (%)	7	9
- dead seeds (%)	3	5
Germination at 5°C:20°C,after 12 days		
- normal seedlings (%)	89	87
- abnormal seedlings (%)	6	8
- dead seeds (%)	5	5
1000 kernel weight (g)	249.3	239.4
Hectoliter weight (kg)	78.8	79.2

It can be concluded from this test that no major differences exist in the parameters tested between a seedlot containing event MS3 and a non-transgenic control seedlot.

Annex 9. . Field data of event MS3 (Europe)

PGS has carried out several experiments in order to evaluate the male sterile *Zea mays* plants derived from transformation event MS3 over generations and under different environmental conditions. Field trials were carried out under the inspection of national governments, federal and/or provincial authorities of each country in which an experiment was performed. On the following pages, reports and summaries of selected European field trials are presented. These documents have been adapted to focus on event MS3 for this petition. If commercially available public inbred lines and proprietary inbred lines were used, these were coded from C101 to C118.

Annex 9. A selection of European field trials in which corn plants containing event MS3 were tested

Field trial	Authorization code	Country	Subject
Field evaluation of event MS3			
FZM9211-3202	BIOT/92/M18	Belgium	Primary field evaluation of male sterile corn lines RZM19/3, RZM35/1, RZM34/1 (event MS3) and RZM34/14
FZM9413-3208	BIOT/94/W4	Belgium	Field evaluation of eleven NMS transformants (Level 2 evaluation)
Evaluation of glufosinate tolerance			
FZM9291-3202	-	Belgium	Observation of the effect of different glufosinate rates on (non-transgenic) corn
FZM9311-3202	BIOT/93/M10	Belgium	A comparison of the effect of the formulations Basta® and Ignite® on event MS3
Application of SeedLink™ in F_1 hybrid seed production			
FZM9402-3310	94.02.10	France	Application of SeedLink™ in F_1 hybrid seed production
Detailed agronomic evaluation of event MS3			
FZM9421-3309	94.02.09	France	Agronomic evaluation of F_1 hybrids produced on event MS3 and event RZM19-1 containing seed-parent plants
Stability of the male sterility trait in a backcrossing program			
FZM9403-3209	B/B/94/V10WB	Belgium	Study of the male sterility trait in a backcrossing program

Field evaluation of event MS3 -

FIELD TRIAL SUMMARY

CODE	CROP	TRAIT	LOCATION
FZM9211-3202	Corn	NMS	Belgium (Gent)

TITLE: Primary field evaluation of male sterile corn lines RZM19/3, RZM35/1, RZM34/1 (event MS3) and RZM34/14

AUTHOR: Catherine Dickburt

DATE: 01/10/92

MATERIAL: M2 and F1 progenies of RZM19/3, RZM35/1, RZM34/1 (event MS3) and RZM34/14

CONCLUSIONS:

Four NMS corn transformation events, RZM19/3, RZM35/1, event MS3 and RZM34/14, were tested in a primary evaluation of the male sterility in the field. The seeds used in the field for each of these lines were either M2 seeds ((Transformation event x H99) x H99) or F1 seeds ((Transformation event x H99) x C101).

Segregation ratio for glufosinate tolerance and the male sterility trait did not differ from the 1:1 ratio expected under normal Mendelian segregation, assuming that the female parent had one active copy (or several copies but integrated at one locus) of the construct integrated.

Plant growth and flowering date of the male sterile plants were similar to those of the fertile plants for lines RZM19/3, event MS3 and RZM34/14. In the line RZM35/1, a plant growth delay (due to leaf whitening symptoms) was observed for the male sterile plants in comparison to the fertile plants.

Male sterility was observed during the flowering period. Although a few anthers were seen on a few plants in the F1 RZM19/3 line, these did not contain any viable pollen.

Apart from a yield reduction observed for the male sterile plants of the line M2 RZM35/1 compared to the fertile plants, no other noticeable differences in yield components were observed between male sterile and fertile plants.

1. METHODS AND MATERIAL

1.1. Trial design

Plot: row of 50 plants
length 7.5 m
Distance between rows 1 m
Distance in row 0.15 m
Replicates: 2
Border: 2m (non transgenic, variety Sanora) - 3 rows

1.2. Objects

A	M2 RZM19/ 3	=(RZM19/ 3xH99)pos x H99
B	F1 RZM19/ 3	=(RZM19/ 3xH99)pos x C101
C	M2 RZM35/ 1	=(RZM35/ 1xH99)pos x H99
D	F1 RZM35/ 1	=(RZM35/ 1xH99)pos x C101
E	M2 Event MS3	=(Event MS3xH99)pos x H99
F	M2 RZM34/14	=(RZM34/14xH99)pos x H99
G	F1 RZM34/14	=(RZM34/14xH99)pos x C101
H	F1 RZM19/ 3 neg	=(RZM19/ 3xH99)neg x C101
I	F1 RZM35/ 1 neg	=(RZM35/ 1xH99)neg x C101
J	M2 RZM34/14 neg	=(RZM34/14xH99)neg x H99
K	H99	
L	C101	
M	H99 x C101	

Seeds were sown in Jiffy pots in the glasshouse on 27-04-92. Seedlings were transferred to the field at the 3-4 true leaf growth stage on 26/05/92.
Two qualities of seedlots were present: tassel seeds produced during the winter period [RZM35/1 and event MS3 progenies] and cob seeds [RZM19/3 and RZM34/14 progenies].
The seeds for the negative controls H, I and J were produced in the greenhouse under the same conditions and timing as the seeds for the lines B, D and F respectively: they can thus be considered as true controls. The controls K, L and M (H99, C101 and H99xC101) were produced separately.

1.3. Observations and tests

Observations:

* % Emergence
* Glufosinate segregation ratios if applicable
* Leaf whitening symptoms (RZM35/1 progenies)
* Plant vigor (1-9)
* Plant height
* Flowering date
* Segregation male sterility
* Yield components (No. cobs/plant, Yield/cob, 1000 kernels weight)

Basta dot-test:

The Basta dot-test was carried out on 11-06-92 with a 1% Basta solution. It was carried out on two leaves per plant. Young plant leaves were brushed with the Basta solution. Plants were assessed 6 days after application.

Crosses:

Crosses were done in the field to produce M3 seeds of all 4 NMS lines. In parallel, upscaling of control seeds was done (H99, C101 and H99xC101).

1.4. Agronomy:

1.4.1. Fertilization

Date	Product	Quantity
30/03/92	Lime	1000 kg/ha
11/05/92	NH4NO3	888 kg/ha
11/05/92	Superphosphate	722 kg/ha
14/05/92	Patentkali	357 kg/ha

1.4.2. Treatments

Date	Type	Product	Quantity
09/06/92	Herbicide	Laddok + paraffin oil	3.5 L/ha

1.4.3. Operations

Date	Variables	Activity
27/04/92	All	Sowing in glasshouse
09/05/92	Border rows	Sowing in field
26/05/92	All	Transplanting to the field
26/05/92	Nonusedseedlings	Steaming
22/10/92	Inbred lines	Harvesting
04/11/92	Hybrid lines	Harvesting
04/11/92	All	Steaming of remaining transgenic cobs
		Ploughing

2. RESULTS & CONCLUSIONS

Assessments dates

% Emergence	18/05/92 and 25/05/92
Basta segregation	17/06/92
Leaf whitening symptoms (RZM35/1 progenies)	24/06/92
Plant vigor	30/06/92
Plant height	03/07/92
Flowering date	17/07/92 to 21/08/92
Segregation male sterility	31/07/92 to 19/08/92
Yield assessments	03/12/92

2.1. **% Emergence** in the glasshouse (See Table 1)

Two emergence assessments were carried out at one week interval to determine eventual emergence delays.
All lines germinated well (ranging from 75% to 100% emergence).
A delay in emergence was observed for the line M2 RZM34/14 as well as for its negative control (36% and 32% at 20 days after sowing [20DAS] increasing to 100 and 97% at 27DAS respectively).
H99 germination was reduced in comparison to the other control lines C101 and H99xC101 (84% compared to 96% and 100% respectively).
No clear difference was seen in emergence between tassel and cob seed although cob seed germinated generally slightly better than tassel seeds.

2.2 **Basta segregation** in the field (See Table 1)

Basta segregation for the transgenic plants (event MS3, RZM35/1 and RZM34/14) were analyzed in a χ^2 test. Values obtained, either for each plot either totalized over the two replicates, were not significantly different from the expected value for a 1:1 segregation. This confirms the theory that the transformants have the transgenes (different copies, if more than 1) integrated at the same locus.

2.3 **Leaf whitening symptoms**

Leaf whitening symptoms were observed in the lines RZM35/1 (M2 and F1 seeds) short after transplanting the seedlings into the field (8 days after). Molecular analysis (PCR) was done on all plants.
All the plants affected by the leaf whitening were shown to contain the *barnase* gene, to be tolerant to Basta and to be male sterile. The Basta sensitive plants were not affected by leaf whitening, were fertile and did not contain the male sterility gene.

2.4. **Plant vigor** (See Table 2.)

Plant vigor was assessed on a 1-9 scale (1=poor, 9=very good) at a 9-12 leaf growth stage. Both transgenic and non-transgenic populations in a segregating plot were given a score. The transgenic plants in a plot were identified by the use of pegs.

The highest score 9 was given to the control hybrid H99xC101.
The F1 lines were compared to the H99xC101 control whilst the M2 lines were compared to the H99 control (score 6).

Plant vigor of RZM19/3, event MS3 and RZM34/14 male sterile plants was comparable to the non-transgenic plants in the plots. This was true for both the M2 inbred lines and for the F1 hybrid lines.
Plant vigor of the RZM35/1 male sterile plants was decreased in comparison to the internal negative plants. A plant growth delay was observed for these plants that were affected by leaf whitening.

2.5. **Plant height** (See Table 2.)

Plant height was measured on 10 plants/population, 2 populations/plot (transgenic and non-transgenic plants). Plant height of RZM19/3, event MS3 and RZM34/14 sterile populations did not differ significantly from that of the fertile populations in the same plots. This was true both for the M2 and the F1 lines.

For RZM35/1, plant height of the sterile plants was reduced in comparison to the fertile plants although the difference was only significant for the F1 line and not for the M2 line (growth delay caused by the leaf whitening symptoms described previously).

In the line M2 RZM34/14, some heterogeneity was observed in the rows. Most of the plants presented an inbred phenotype while some of the plants had an hybrid phenotype (as tall as the hybrid plants). Three M2 RZM34/14 seedlots were mixed for this test : M3765, M3766 and M3767. Unevenness observed in the rows may be due to a contamination at the time of pollinating in the glasshouse.

2.6. **Flowering dates** (See Table 3)

The control lines H99 and H99xC101 started flowering at about the same time. The 50% tassel emergence growth stage was reached 87 days after sowing and the 50% silk emergence stage was reached after 92 and 91 days after sowing respectively for H99 and the hybrid H99xC101. C101 was more tardive and started flowering (50% tassel emergence) 95 days after sowing.

RZM19/3, event MS3 and RZM34/14 M2 and F1 lines started flowering at about the same time as their respective controls H99 and H99xC101. Within the populations of these lines, sterile and fertile populations behaved similarly.

For the RZM35/1 lines, flowering of the sterile populations was delayed in comparison to the fertile populations within the plots and in comparison to the controls H99 and H99xC101. The delay was of 9 and 21 days at the 50% tassel emergence growth stage for the M2 and the F1 lines of RZM35/1 respectively. Silk emergence was delayed by 16 and 21 days for the M2 and F1 lines respectively.

2.7. **Flower phenotype** (See Table 4)

Individual plant data on sterility/fertility were collected 4 times at one week interval from the beginning of flowering on. Male sterility was monitored carefully.
A few anthers were extruded in lines F1 RZM19/3 (9 out of 49 sterile plants). For those plants, anther squashes were examined under the microscope. Only some aborted pollen grains were seen. Pollen viability-tests conducted with Alexander staining for all those plants with anthers were negative.

Segregation ratio for the male steriliy trait was in all cases not significantly different from a 1/1 ratio expected for a one locus insertion.

2.8. . **Yield components** (See Table 5)

A yield assessment was carried out on 20 plants/population, 2 populations per plot (sterile and fertile) for a first indication of female fertility of male sterile plants in comparison to fertile controls. The figures have to be taken with caution since the number of plants sampled is too small to lead to fair conclusions on yield. After removing the husks, the cobs were dried and threshed. The 1000 kernels weight was calculated for a 15%RH.

No noticeable difference was seen in the number of cobs produced per plant between sterile and fertile populations of the lines.

Cobs weight was usually similar or slightly higher in the male sterile population than in the fertile population within a line.
The only severe reduction in yield in the sterile population compared to the fertile population was observed in line M2 RZM35/1 (no figure available for F1 RZM35/1). Cobs pollination for this line may have been difficult (delayed in comparison to the other lines, and not much pollen available any more). In the 1000 kernels weight figures, we can see however that seeds produced on the sterile plants of line M2 and F1 RZM35/1 are of the same size as those of the fertile plants.

TABLE 1: FZM9211-3202 - Primary field evaluation

Emergence assessment - 18/05/92 & 25/05/92 (20 and 27 days after sowing [20DAS, 27DAS]
Basta tolerance assessment - 17/06/92 (6 days after application [6DAA])

Code	Emergence 20DAS	%	Emergence 27DAS	%	Basta tolerance Segregation ratio Rep 1	Rep 2	Segregation ratio Total (Rep1+Rep2)	%**
M2 RZM19/3	77/98	79	77/98	79	Not applicable	Not applicable	Not applicable	Not applicable
1 RZM19/3	98/98	100	98/98	100	Not applicable	Not applicable	Not applicable	Not applicable
M2 RZM35/1	85/104	82	85/104	82	24/50 ns	21/36 ns	45/86 ns	52
F1 RZM35/1	73/100	73	75/100	75	25/50 ns	11/25 ns	36/75 ns	48
M2 Event MS3	96/101	95	96/101	95	28/46 ns	26/48 ns	54/94 ns	57
M2 RZM34/14	36/100	36	100/100	100	26/50 ns	27/50 ns	53/100 ns	53
F1 RZM34/14	98/100	98	100/100	100	23/50 ns	22/50 ns	45/100 ns	45
F1 RZM19/3 negative control	101/102	99	101/102	99	-	-	-	-
F1 RZM35/1 negative control	100/100	100	100/100	100	-	-	-	-
M2 RZM34/14 negative control	30/94	32	91/94	97	-	-	-	-
H99	276/336	82	283/336	84	-	-	-	-
C101	324/336	96	324/336	96	-	-	-	-
H99 x C101	335/336	100	335/336	100	-	-	-	-

ns : Not significantly different in a X^2 test at the 0.05 level for hypothesis of 1:1 segregation
** : % Basta tolerant plants

TABLE 2: FZM9211-3202 - Primary field evaluation

Plant vigor assessment - 30/06/92 (64 days after sowing [64DAS])
Plant height assessment - 03/07/92 [67 DAS], 9-12 leaf growth stage

Code	Plant vigor (1-9) Male Sterile	Fertile	Plant height (cm) Male Sterile	Fertile
M2 RZM19/3	6	4.5	74.9 FGH	65.9 FGHI
F1 RZM19/3	7.5	7.5	99.7 BC	103.5 AB
M2 RZM35/1	3	5.5	54.7 I	61.4 HI
F1 RZM35/1	4	7	63.5 GHI	90.1 CD
M2 Event MS3	6	5	73.7 FGH	72.3 FGH
M2 RZM34/14	6.5	5	74.9 FGH	79.5 DEF
F1 RZM34/14	8	6.5	104.9 AB	103.7 AB
F1 RZM19/3 negative control	-	8	-	104.2 AB
F1 RZM35/1 negative control	-	9	-	104.5 AB
M2 RZM34/14 negative control	-	6	-	76 EFG
H99	-	6	-	88.6 CDE
C101	-	7	-	96.5 BC
H99 x C101	-	9	-	116.6 A
Fobs Fth5%, Fth1% St.err. (single plot) Coeff. Var.	-	-	-	34.95 2.17, 3.03 10.49 12.28%

Notes:
1. Plant vigor assessment was done by giving a score from 1-9 to the 2 populations (transgenic and non transgenic in the segregating plots, 2 replicates).
2. Plant height was assessed on 10 plants per population, 2 populations per segregating plot, 2 replicates.
3. A two-way ANOVA was carried out on these results. Data was analysed untransformed.
   Treatments with no letter in common are significantly different at the 1% level in the Duncan's Multiple Range test for the Comparison of Means.

TABLE 3: FZM9211-3202 - Primary field evaluation

Flowering date assessment (in days after sowing, DAS)

Code	50% tassel emergence		50% silk emergence	
	Male Sterile	Fertile	Male Sterile	Fertile
M2 RZM19/3	87	88	95	94
F1 RZM19/3	88	88	95	95
M2 RZM35/1	96	87	108	92
F1 RZM35/1	108	87	116	95
M2 Event MS3	87	87	90	93
M2 RZM34/14	90	90	95	95
F1 RZM34/14	90	87	95	95
F1 RZM19/3 negative control	-	88	-	95
F1 RZM35/1 negative control	-	87	-	95
M2 RZM34/14 negative control	-	88	-	96
H99	-	87	-	92
C101	-	95	-	95
H99 x C101	-	87	-	91

Notes: 1. Flowering has been observed every 2-3 days.

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TABLE 4: FZM9211-3202 - Primary field evaluation

Flower phenotype assessment

Code	Male sterility Segregation ratio Rep 1	Rep 2	Segregation ratio Total (Rep1+Rep2)	%**
M2 RZM19/3	24/45 ns	14/22 ns	38/67 ns	57
F1 RZM19/3	25/49 ns	24/48 ns	49/97 ns	51
M2 RZM35/1	23/44 ns	21/35 ns	44/79 ns	56
F1 RZM35/1	23/47 ns	10/24 ns	33/71 ns	46
M2 Event MS3	29/43 ns	25/48 ns	54/91 ns	59
M2 RZM34/14	22/47 ns	27/50 ns	49/97 ns	51
F1 RZM34/14	23/50 ns	21/50 ns	44/100 ns	44

ns : Not significantly different in a X^2 test at the 0.05 level for hypothesis of 1:1 segregation
** : % male sterile plants

TABLE 5: FZM9211-3202 - Primary field evaluation

Yield components

Code	Number of cobs/plant		Cobs weight, 15%RH (g)		1000 kernels weight, 15% RH (g)	
	Male Sterile	Fertile	Male Sterile	Fertile	Male Sterile	Fertile
M2 RZM19/3	1.25	1.15	62.88	59.50	232.80	238.80
F1 RZM19/3	1.48	1.38	164.91	122.61	319.43	243.82
M2 RZM35/1	1.10	1.05	17.96	54.17	249.90	206.90
F1 RZM35/1	1.00	1.00	*	99.18	315.89	243.87
M2 Event MS3	1.20	1.25	55.92	58.82	260.19	262.69
M2 RZM34/14	1.12	1.00	69.23	49.33	277.50	272.40
F1 RZM34/14	1.03	1.10	157.26	137.10	276.33	321.58
F1 RZM19/3 negative control	-	1.08	-	141.20	-	244.64
F1 RZM35/1 negative control	-	1.00	-	180.16	-	286.01
M2 RZM34/14 negative control	-	1.10	-	62.63	-	251.44
H99	-	1.05	-	75.22	-	274.43
C101	-	1.20	-	31.55	-	145.34
H99 x C101	-	1.00	-	200.38	-	309.44

Notes:
1. Yield assessment was carried out on 20 plants/plot when possible.
2. The presented figures are means over 2 replicates for the F1 lines. For the M2 lines, only one replicate could be assessed since the other replicate was used for material upscaling.
3. No statistical analysis were carried out (Variability of the data too high)
*. Part of the cobs sample missing

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FIELD TRIAL SUMMARY

CODE	CROP	TRAIT	LOCATION
FZM9413-3208	Corn	NMS	Belgium (Ophain)

TITLE: Field evaluation of eleven NMS transformants (Level 2 evaluation)[1]

AUTHOR: Catherine Dickburt

DATE: 15/01/95

MATERIAL: Event MS3 (in H99) in comparison with non-transgenic H99.

CONCLUSIONS: Basta segregation of the male sterile event MS3 was as expected. No differences in plant growth, plant vigor, flowering dates, lodging and cob filling were observed when comparing event MS3 and non-transgenic H99. Basta sprayed plants were male sterile. Male sterility was complete and stable. Common smut (*Ustilago maydis*) was sporadically observed in the field trial. No differences in common smut sensitivity were observed between the transgenic entry and the non transgenic control line H99.

[1]This summary is adapted to the petition document and focusses on the results for event MS3.

1. METHODS AND MATERIAL

1.1. Trial design

Strip plots design:

Main plot:	2 rows of 50 plants Length 7 m Distance between rows 1 m Distance in row 0.15 m
Sub plot:	2 rows of 25 plants length 3m
Replicates:	2

Border: 2m (non transgenic variety Anthony) - 3 rows

1.2. Objects

Code	Line	Background	Generation
A	H99	-	
B	Event MS3	H99	M6
C to L	other male sterile entries in a H99 background		

The plots were split in two parts, one part was sprayed with Basta (A1, B1), the other was not sprayed (A2, B2).
The seeds of entries A and B were produced in the winter nursery 1993-1994 in Chile.
The seeds of the other entries were produced in the greenhouse.

1.3. Observations and tests

Observations:

- % Emergence
- Basta segregation ratio
- Plant vigor (1-9)
- Plant height (only on MS3 and H99)
- Flowering dates
- Segregation male sterility
- Lodging
- Disease observation
- Cobs filling observation

Basta sprayment:

Basta sprayment was carried out on 28-06-94 at the 4-5 leaf growth stage at the 3L/ha rate. Plants were assessed 20 days after application.

1.4. Agronomy:

1.4.1. Fertilization

Date	Product	Quantity
21/04/94	NH_4NO_3	600 kg/ha
21/04/94	Superphosphate	555 kg/ha
22/04/94	K_2SO_4	260 kg/ha

1.4.2. Treatments

Date	Type	Product	Quantity
27/06/94	Herbicide	Mikado	1.5 L/ha
		+ Atrazine	1.5 L/ha

1.4.3. Operations

Date	Variables	Activity
19/05/94	-	Seedbed preparation
19/05/94	ALL but entries J&K	Sowing in field by hand
28/10/94	ALL	Chopping + Ploughing in

2. RESULTS & CONCLUSIONS

Assessments dates

% Emergence	27/06/94
Basta segregation	18/07/94
Disease observations	
Plant vigor	24/07/94
Plant height	30/08/94
Flowering date	16/08/94 to 29/09/94
Segregation male sterility	16/08/94 to 29/09/94
Lodging observation	13/10/94
Cob filling assessment	24-25/10/94

2.1. **% Emergence** (see Table 1)

Emergence was poor and uneven due to cold weather conditions after sowing. Emergence was only observed in mid-June.

2.2 **Basta segregation** (see Table 1)

Basta segregation for the transgenic plants were analyzed in a χ^2 test. Values obtained, totalized over the two replicates, were not significantly different from the expected 1:1 ratio.

2.3. **Plant vigor** (see Table 2)

Plant vigor was assessed on a 1-9 scale (1=poor, 9=very good) at a 8-9 leaf growth stage. Both transgenic and non-transgenic populations in the unsprayed sub-plots were given a score.

None of the entries were found to be significant different from each other.

2.4. **Plant height** (see Table 2)

Plant height was measured on 10 plants/population, 2 populations/plot (transgenic and non-transgenic plants in the unsprayed sub-plots). This assessment was carried out after flowering of the plots up to the tassel top on event MS3 and H99.

When comparing transgenic and non-transgenic plants of the same unsprayed subplot of event MS3, no significant difference was observed for the sterile plants in comparison to the fertile plants. When comparing event MS3 plants from an unsprayed and a sprayed plot, a reduction in plant height of about 10 cm was observed. This height reduction could be partly attributed to the difference in plant density.

2.5. **Flowering dates** (see Table 3)

The control line H99 started silking at 91 DAS. Event MS3 started silking within two days of the control line in the unsprayed subplots. For those subplots, both fertile and sterile plants started flowering at the same time.

2.6. **Flower phenotype** (see Table 3)

Flowering was observed every 2-3 days (mid-August 94). In the unsprayed event MS3 subplots, a male sterile/male fertile segregation ratio of 1:1 (single locus) was observed.

Event MS3 was completely male sterile over the entire flowering period.

2.7. **Lodging** (see Table 4)

In late August, a storm hit the field trial. A lodging assessment was carried out 15 days before harvest to determine eventual differences between entries. No striking differences were observed between the transgenic plants and the control (H99).

2.8. **Cob filling observation** (see Table 4)

Cob filling observation was carried out on 10 cobs per population by giving a score from 1-5 to each cob (1=28 seeds, 2 = 100 seeds, 3 = 169 seeds, 4 = 525 seeds, 5 = 420 seeds). No significant differences were observed between event MS3 and the control entry.

2.9. **Disease assessment**

Common smut (*Ustilago maydis*) was sporadically observed in the trial area. No differences in disease development were seen between the transgenic plots and the non-transgenic control plot (H99 control).

TABLE 1: FZM9413-3208 - Field evaluation of event MS3 (1994)

Emergence assessment - 27/06/94 (39 days after sowing [39DAS]
Basta resistance assessment - 18/07/94 (20 days after application [20DAA])

Plant material		Emergence 39DAS	% Mean	Basta resistance Segregation ratios (Resistant/Total) Rep 1	Rep 2	Segregation ratios Totals (Rep1+Rep2)	%
H99	non sprayed	75/100					
	sprayed	60/100	67.5	0/33	0/27	0/60	00.0
Event MS3	non sprayed	71/100					
	sprayed	50/100	60.5	13/26	9/24	22/50	44.0

TABLE 2: FZM9413-3208 - Field evaluation of event MS3 (1994)

Plant vigor assessment - 24/07/94 (65 days after sowing [65DAS]), 8-9 leaf growth stage
Plant height assessment - 30/08/94

Plant material		Plant vigor (1-9) Mean (rep1+rep2)	Plant height (cm) Sterile Mean	Fertile Mean
H99	non sprayed	9	-	127.2
	sprayed	-	-	-
Event MS3	non sprayed	9	121.15	125.25
	sprayed	8.5	110.65	-

- Plant vigor assessment was done by giving a plot score from 1-9, 2 replicates.
- Plant height was assessed on 10 plants per population, 2 populations per segregating plot, 2 replicates

TABLE 3: FZM9413-3208 - Field evaluation of event MS3 (1994)

Flowering date assessment (in days after sowing, DAS)

Plant material		50% silk emergence		Male flower phenotype		Flower phenotype of the male sterile plants
		Sterile	Fertile	Sterile/Total	%	
H99	non sprayed		91	-	0	Fertile
	sprayed		-			-
Event MS3	non sprayed	89	89	35/67 n.s.	52	Sterile
	sprayed	92	-	10/10	100	Sterile

n.s. : not significantly different in a X^2 test at the 0.05 level for hypothesis of 1:1 segregation

TABLE 4: FZM9413-3208 - Field evaluation of event MS3 (1994)

Lodging assessment - 13/10/94 (147 days after sowing [147DAS]
Cob filling assessment - 24-25/10/94 (158-159 DAS)

Plant material		Lodging (1-5) 1= poor stand 5= good stand	Cob filling observation (1-5)	
			Sterile	Fertile
H99	non sprayed	4.5		2.7
	sprayed	-		-
Event MS3	non sprayed	4.5	2.9	2.3
	sprayed	4	2.3	-

Evaluation of glufosinate tolerance

FIELD TRIAL REPORT

CODE	CROP	TRAIT	LOCATION
FZM9291-3202	Corn	Non-transgenic	Belgium (Gent)

TITLE: Observation of the effect of different glufosinate rates on (non-transgenic) corn

AUTHOR: Catherine Dickburt

DATE: 25/11/92

MATERIAL: Non-transgenic corn cultivar SANORA

CONCLUSIONS:

The product Basta was applied at rates of 0 to 2.5 L/ha and at 2 different growth stages (3-4 leaf and 6-8 leaf growth stage) on the corn cv. Sanora to identify the lowest application rate to destroy non-transgenic plants.

A rate of 2.5 L Basta/ha eliminated all non-transgenic plants when applied at the 3-4 leaf growth stage. The 2.5 L/ha Basta rate was not sufficient to achieve total elimination when applied at the later growth stage of 6-8 leaves, however, surviving plants did not grow further after the sprayment.

1. METHODS AND MATERIAL

1.1. Trial design

Plot: 2 rows * 3m (20 plants)
Distance between rows 0.8 m
Replicates: 2
Path: 2 m

1.2. Objects

A	Control	0 L/ha	3-4 leaf growth stage
B	Basta sprayment	0.5 L/ha	3-4 leaf growth stage
C	Basta sprayment	1.0 L/ha	3-4 leaf growth stage
D	Basta sprayment	1.5 L/ha	3-4 leaf growth stage
E	Basta sprayment	2.0 L/ha	3-4 leaf growth stage
F	Basta sprayment	2.5 L/ha	3-4 leaf growth stage
G	Control	0 L/ha	6-8 leaf growth stage
H	Basta sprayment	0.5 L/ha	6-8 leaf growth stage
I	Basta sprayment	1.0 L/ha	6-8 leaf growth stage
J	Basta sprayment	1.5 L/ha	6-8 leaf growth stage
K	Basta sprayment	2.0 L/ha	6-8 leaf growth stage
L	Basta sprayment	2.5 L/ha	6-8 leaf growth stage

1.3. Observations

* Number of plants emerged per plot
* Number of plants surviving the Basta application
* Plant height

1.4. Agronomy:

1.4.1. Fertilization: None

1.4.2. Treatments

Date	Type	Product	Quantity
30/07/92	Herbicide	Basta	0-2.5L/ha (3-4 leaf growth stage)
06/08/92	Herbicide	Basta	0-2.5L/ha (6-8 leaf growth stage)

Basta sprayments were applied in a 500L/ha volume.

1.4.3. Operations

Date	Variables	Activity
16/07/92	All	Sowing
30/09/92	All	Harvest

2. RESULTS & CONCLUSIONS

Assessment dates

No. of plants emerged	27/07/92
No. of plants surviving after Basta sprayment	13/08/92
	27/08/92
	08/09/92
Plant Height	08/09/92

1. **No. of plants surviving after Basta sprayment** (See Table 1)

First count was carried out 11 days after sowing just before the first Basta sprayment was applied. Total number of plants were counted in all plots.
Emergence was good (overall mean of 91%).
The second count was carried out 2 weeks after the first sprayment (3-4 leaf growth stage, treatments A-F). 2.5 L/ha was needed to destroy all plants in the plot.

The third count was carried out 3 weeks after the second sprayment (6-8 leaf growth stage, treatments G-L). Plots sprayed at this second timing with the highest rate, 2.5 L/ha were severely damaged by Basta application but total elimination was not achieved (33% of the plants still alive, 3 weeks after spraying).

A last count of the plants was carried out on the 08/09, 40 days and 33 days after the first and the second sprayment respectively. Plots sprayed with 2.5 L/ha Basta at the 3-4 leaf growth stage were completely bare at this stage whilst a few very small plants were still surviving when Basta had been applied at the 6-8 leaf growth stage (still 3% of the plants alive).

2. **Plant height** (See Table 2)

A plant height assessment was carried out on 10 plants per plot on 08/09 (same date as our last count) to provide more data on Basta damage to the plants.

It was observed that even though some plants are still alive at the 2.5 L/ha (6-8 leaf growth stage application only), these plants are very small (a few cms) and will never reach the flowering stage.

TABLE 1: FZM9291-3202 - The effect of different glufosinate rates on corn

No. plants emerged/plot - before Basta sprayment: 27/07/92 (11 days after sowing [11DAS])
No. plants surviving - after Basta sprayment: 13/08/92, 27/08/92 and 08/09/92

	No. pl. emerged/plot		No pl. surviving after Basta (13/08)			No pl. surviving after Basta (27/08)			No pl. surviving after Basta (08/09)		
	Rep1	Rep2	Rep1	Rep2	Means(%)	Rep1	Rep2	Means(%)	Rep1	Rep2	Means(%)
Control -	45.0	45.0	45.0	45.0	100% A	-	-	-	44	43	97% A
Basta 0.5L/ha 3-4 leaf GS	18.0	47.0	16.0	44.0	91% B	-	-	-	16	43	90% AB
Basta 1.0L/ha 3-4 leaf GS	46.0	43.0	17.0	11.0	31% C	-	-	-	16	10	29% CD
Basta 1.5L/ha 3-4 leaf GS	44.0	46.0	8.0	12.0	22% CD	-	-	-	6	12	20% CD
Basta 2.0L/ha 3-4 leaf GS	41.0	44.0	1.0	1.0	2% D	-	-	-	0	1	1% D
Basta 2.5L/ha 3-4 leaf GS	42.0	44.0	0.0	0.0	0% D	-	-	-	0	0	0% D
Control -	44.0	46.0	-	-	-	44.0	46.0	100% A	44	44	98% A
Basta 0.5L/ha 6-8 leaf GS	46.0	48.0	-	-	-	45.0	44.0	95% AB	44	44	94% AB
Basta 1.0L/ha 6-8 leaf GS	44.0	47.0	-	-	-	41.0	37.0	86% BC	16	40	61% BC
Basta 1.5L/ha 6-8 leaf GS	46.0	47.0	-	-	-	31.0	18.0	53% CD	16	7	24% CD
Basta 2.0L/ha 6-8 leaf GS	44.0	41.0	-	-	-	23.0	7.0	35% D	16	4	23% CD
Basta 2.5L/ha 6-8 leaf GS	44.0	46.0	-	-	-	25.0	4.0	33% D	3	0	3% D
Fobs Fth (5%), Fth (1%)	-	-	-	-	255.58 5.05, 10.97	-	-	33.74 5.05,10.97	-	-	20.01 2.82, 4.46

Note: 1. Basta application was done at a 3-4 leaf growth stage (GS) (30/07/92) or at a 6-8 leaf GS (06/08/92).
2. *: only one of the 2 rows sprayed properly with Basta 0.5 L/ha.
3. A one-way ANOVA was carried out on these results. Data were analysed arcsin transformed.
Treatments with no letter in common are significantly different at the 1 % level in the Duncan's Multiple Range test for the Comparison of Means.

TABLE 2: FZM9291-3202 - The effect of different glufosinate rates on corn

Plant height (cm) - 10 plants/plot - 08/09/92

	Plant height, 10 plants/plot Rep1	Rep2	Means	
Control -	145.4	151.6	148.5	A
Basta 0.5L/ha 3-4 leaf GS	105.6	107.0	106.3	B
Basta 1.0L/ha 3-4 leaf GS	61.6	47.4	54.5	CD
Basta 1.5L/ha 3-4 leaf GS	35.2	42.8	39.0	DE
Basta 2.0L/ha 3-4 leaf GS	0.0	2.7	1.4	F
Basta 2.5L/ha 3-4 leaf GS	0.0	0.0	0.0	F
Control -	150.3	143.6	147.0	A
Basta 0.5L/ha 6-8 leaf GS	83.9	62.0	73.0	C
Basta 1.0L/ha 6-8 leaf GS	54.1	39.7	46.9	CD
Basta 1.5L/ha 6-8 leaf GS	44.8	13.1	29.0	DE
Basta 2.0L/ha 6-8 leaf GS	29.0	4.9	17.0	EF
Basta 2.5L/ha 6-8 leaf GS	7.5	0.0	3.8	F
Fobs Fth (5%), Fth (1%) Standard error Coeff. var.	-	-	69.03 2.82, 13.88 12.51%	 4.46

Notes: 1. Basta application was done at a 3-4 leaf growth stage (GS) (30/07/92) or at a 6-8 leaf GS (06/08/92).
2. A two-way analysis was carried out on these data. Data were analysed untransformed.
Treatments with no letter in common are significantly different at the 1 % level in the Duncan's Multiple Range test for the Comparison of Means.

FIELD TRIAL SUMMARY

CODE	CROP	TRAIT	LOCATION
FZM9311-3202	Corn	NMS	Belgium (Gent)

TITLE:
a) Segregation trial for RZM35-1[1]
b) A comparison of the effect of the formulations Basta® and Ignite® on event MS3

AUTHOR: Catherine Dickburt

DATE: 01/07/94

MATERIAL: Event MS3, in H99, 4th maintained generation.

CONCLUSIONS:

Basta (200 g/L glufosinate) and Ignite (150 g/L glufosinate) were sprayed at the 4-5 leaf stage on event MS3 at rates of 0, 250, 500, 1000 and 2000 g.a.i./ha.

Segregation data for Basta/Ignite tolerance for all entries generally did not differ from the 1:1 ratio expected for Mendelian inheritance of a dominant gene.

Similar effects were observed on sensitive and tolerant plants (high rates) with both Basta and Ignite. Based on visual observation of the plots, Ignite might have a slightly stronger action than Basta, .

A rate of 5 L/ha of Basta completely eliminated the fertile subpopulation. A rate of 2.5 L/ha Basta (2.33 L/ha Ignite) allowed a few fertile plants to grow although these plants were much delayed in growth and flowering. The optimal rate must therefore lay at 3-4 L/ha Basta (4-5L/ha Ignite).
Some phytotoxicity was seen at the 10L/ha rate of Basta (13.33 L/ha Ignite). At this rate, some plants were stunted and reshooting.

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[1] The results of title a), the segregation trial for RZM35/1, are not considered in this version of the field trial summary

1. METHODS AND MATERIAL

1.1. Trial design

Plot: 2 rows of 50 plants
Length 7.5 m
Distance between rows 1 m
Distance in row 0.15 m
Replicates: 2 (Basta trial)
Border: 2m (non-transgenic hybrid Sanora) - 3 rows

1.2. Objects

K	M4636: M4 Event MS3	Basta 0L/ha
L	M4636: M4 Event MS3	Basta 1.25L/ha (250g active ingredient (ai)/ha)
M	M4636: M4 Event MS3	Basta 2.5L/ha (500g active ingredient/ha)
N	M4636: M4 Event MS3	Basta 5L/ha (1000g active ingredient/ha)
O	M4636: M4 Event MS3	Basta 10L/ha (2000g active ingredient/ha)
P	M4636: M4 Event MS3	Ignite 0L/ha
Q	M4636: M4 Event MS3	Ignite 1.67L/ha (250g active ingredient/ha)
R	M4636: M4 Event MS3	Ignite 3.33L/ha (500g active ingredient/ha)
S	M4636: M4 Event MS3	Ignite 6.66L/ha (1000g active ingredient/ha)
T	M4636: M4 Event MS3	Ignite 13.32L/ha (2000g active ingredient/ha)

The seeds for this trial were produced in the Chile winter nursery 1992-1993.

1.3. Observations and tests

Observations:

* % Emergence
* Basta segregation ratio
* Plant vigor
* Plant height (at flowering)
* Flowering dates
* Flower phenotype: male sterility or fertility

Basta/Ignite sprayment:

The Basta and Ignite sprayments were carried out on 2-7-1993 at the 4-5 leaf growth stage with a small plots experimental sprayer. The weather was windy at time of spraying and screens were used to avoid drift. Plants were assessed 6 to 7 days after the application.

1.4. Agronomy:

1.4.1. Fertilization

Date	Product	Quantity
21/04/93	Lime	1000 kg/ha
25/05/93	NH_4NO_3	750 kg/ha
25/05/93	Superphosphate	666 kg/ha
25/05/93	Potassiumsulphate	360 kg/ha

1.4.2. Treatments

Date	Type	Product	Quantity
22/06/93	Herbicide	Laddok + mixtop	3.5 l/ha 1l/ha

1.4.3. Operations

Date	Variables	Activity
26/03/93	All	Ploughing
26/05/93	All	Sowing in the field

2. RESULTS & CONCLUSIONS

Assessments dates

% Emergence	25/06/93
Basta/Ignite segregation	08/07/93
Plant vigor	29/07/93
Plant height	02/09/93
Flowering date	01/09/93 to 20/09/93
Segregation male sterility/fertility	01/09/93 to 20/09/93

2.1. Crop emergence and Basta segregation (See Table 1)

End May, the trial was sown by hand. Crop emergence was relatively good (overall mean of 87.5%).
Basta/Ignite sprayments were carried out at the 4-5 leaf growth stage. Since the weather was windy at the time of spraying, protective screens were used to avoid drift. Basta segregation observed in the sprayed plots was in most cases not different from the expected 1:1 ratio. For the plots sprayed with Ignite at 2000g a.i./ha the ratio of tolerant plants (61%) was different from the expected value (at the 5% level in a Chi-square test).

2.2. Plant vigor (See Table 2)

Plant vigor was assessed on a 1-5 scale (1=poor, 5=very good) at a 8-9 leaf growth stage. The highest score 5 was given to the non-treated event MS3 control.
Vigor of event MS3 plants started to be affected at the 1000 g.ai/ha rate. More severe symptoms were seen at the 2000 g. ai/ha rate. At this rate, some plants presented a plant height reduction and were reshooting. On these plants, silk formation on the tassels was often observed (more often than on unsprayed event MS3 plants). Damage seen on Ignite sprayed plots was usually slightly more severe than that observed on the Basta sprayed plots (same concentration).

A rate of 1000 g. ai/ha was needed to eliminate all sensitive non-transgenic plants in the event MS3 plots. A few sensitive plants survived the rate of 500 g.ai/ha, although

these plants were very badly affected in growth and usually died thereafter.

2.3. Plant height (See Table 2)

A plant height assessment was carried out at flowering on 10 plants/population. If fertile plants were present in the sprayed plots, these were also measured, and a mean was calculated.

A statistical analysis for split-plots designs was carried out with Agrobase on the means for the tolerant plants (sterile plants) at the different growth stages.
Results thereof are presented below:

ANOVA SPLIT-PLOTS - plant height event MS3 (cm) - Agrobase 4:

Main factor: glufosinate ammonium concentration
Sub factor: formulation type

Glufosinate ammonium rate	Mean plant height (cm)[a]		
	Basta	Ignite	Average[b]
0 g.ai/ha	152.3	153.9	153.1 a
250 g.ai/ha	148.0	143.3	146.7 b
500 g.ai/ha	139.7	143.0	141.3 bc
1000 g.ai/ha	138.6	137.7	138.2 c
2000 g.ai/ha	131.7	124.5	128.1 d
Average[b]	142.5 a	140.5 a	

[a]Average of two replicates
[b]In a row (or column), means followed by a common letter are not significantly different at the 5% level. Comparison of means was done using the LSD method.
(LSD for main treatments comparison: 5.9296, LSD subplots: 3.7502).

Statistical analysis showed no block effect neither significant interaction between the two factors (main factor: rate and sub-factor: formulation type).
The ANOVA found some significant differences in plant height between the rates of product used but not between the two formulations used, Basta and Ignite.

Plant height of event MS3 was gradually decreased by a higher sprayment rate of glufosinate ammonium. Sprayment of event MS3 with a 2000 g.ai/ha concentration lead to a plant height reduction of 16% in comparison to the unsprayed control.

In the unsprayed plots of event MS3, no significant differences were seen between sterile and fertile plants. At the 250 g.ai/ha, some sensitive plants that escaped from the glufosinate ammonium sprayment were seen and showed a plant height reduction of 14% and 21% in the Basta and the Ignite sprayed plots respectively.
At the higher concentrations of product, only very few sensitive plants escaped from the glufosinate ammonium sprayments. These plants were usually very severely affected in growth.

It is to be noticed that all plots (sprayed and unsprayed) were sown at single density. In sprayed plots, plant density was reduced to approximately 50% of the plants after spraying. This lower plant density -introducing less competition for light- could lead to height reduction in these plots in comparison to the unsprayed plots.

2.4 **Flowering dates** (See Table 3)

The trial started flowering very late (end of august) due to the late sowing date and the cold summer climate. The flowering period extended over a 3-4 weeks period.

Nearly all sprayed plots started silking a few days before the control plots. Noticeable delay in flowering was observed for the fertile plants escaped from the glufosinate ammonium treatment. One plant that had escaped from the 6.66 L/ha Ignite treatment flowered at the same time as the sterile plants of the same plot. This might be due to a suboptimal cover of the plot plants due to drift during sprayment.

2.5. **Flower phenotype** (See Table 3)

Flowering was observed regularly every 2-3 days.

A sprayment rate of at least 1000 g.ai/ha was needed for clear elimination of the fertile population. The optimal rate should be 3-4 L/ha Basta (4-5 L/ha Ignite) since a rate of 500 g.ai/ha was not sufficient to eliminate all fertile plants.

TABLE 1: FZM9311-3202 - Comparison Basta/Ignite on event MS3

Emergence assessment - 25/06/93 (29 days after sowing [29DAS])
Basta test result - 08/07/93, 6-8 leaf growth stage (GS) (6 days after spray [6DAA])

Treatment	Emergence No pl/plot			Basta/Ignite tolerance No Resis/total				
	Rep 1	Rep 2	Mean (%)	Rep 1	Rep 2	Tot		%
K: Control	85	92	88.5	-	-	-		-
P: Control	78	92	85.0	-	-	-		-
L: Basta 250 gal/ha	93	87	90.0	47/93	43/87	90/180	n.s.	50.0
Q: Ignite 250 gal/ha	92	91	91.5	55/92	58/91	103/183	n.s.	56.3
M: Basta 500 gal/ha	88	82	85.0	46/88	48/82	94/170	n.s.	55.3
R: Ignite 500 gal/ha	90	91	90.5	39/90	44/91	83/181	n.s.	45.9
N: Basta 1000 gal/ha	86	91	88.5	43/86	44/91	87/177	n.s.	49.1
S: Ignite 1000 gal/ha	85	88	86.5	50/85	48/88	98/173	n.s.	56.6
O: Basta 2000 gal/ha	86	88	87.0	43/86	41/88	84/174	n.s.	48.3
T: Ignite 2000 gal/ha	84	82	83.0	52/84	49/82	101/166	s.	60.8

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Notes: 1. Trial was sown on 26/05/93.
2. Emergence assessment was done on 25/06/93 by counting the total number of plants/plot.
3. Plots were sprayed with Basta 3L/ha at the 4-5 leaf growth stage on 02/07/93.
ns. and s. : Not significantly different and significantly different in a X^2 test at the 0.05 level for hypothesis of 1:1 segregation
% : Tolerant plants

TABLE 2. FZM9311-3202 - Comparison Basta/Ignite on event MS3

Vigor score - 29/07/93, 8-9 leaf GS (27 DAA)
Damage from spraymentt - 29/07/93, 8-9 leaf GS (27DAA)
Plant height - 02/09/93
Flower observations - 01/09 till 20/09/93

Treatment	Plant vigor and Damage on sensitive plants		Plant vigor and Damage on tolerant plants		Plant height of tolerant plants (cm)			Plant height of sensitive plants (cm)		
	Plant vigor	Damage	Plant vigor	Damage	Rep 1	Rep 2	Means	Rep 1	Rep 2	Means
K: Control P: Control	5 5	None (not sprayed) None (not sprayed)	5 5	None (Not sprayed) None (Not sprayed)	148.5 155.4	156.1 152.3	152.3 153.9	153.4 156.1	160.0 151.5	156.7 153.8
L: Basta 250 gai/ha Q: Ignite 250 gai/ha	2 2	Pl. surviving (1/3 height of the resist plants	5 5	None None	151.5 145.5	144.6 141.2	148.0 143.3	137.3 117.7	131.8 126.8	134.5 122.2
M: Basta 500 gai/ha R: Ignite 500 gai/ha	0.5 0.5	A few plants surviving (but very small)	5 5	None None	139.8 144.5	139.6 141.5	139.7 143.0	- -	- 134.0 (1 pl.)	- 134.0 (1 pl.)
N: Basta 1000 gai/ha S: Ignite 1000 gai/ha	0 0	All dead	4.5 4.5	Effect on plant vigor Effect on plant vigor	141.0 135.2	136.3 140.2	138.6 137.7	- -	- 134.0 (1 pl.)	- 134.0 (1 pl.)
O: Basta 2000 gai/ha T: Ignite 2000 gai/ha	0 0	All dead	4 3.5	Reduced plant height and tillering	134.0 122.1	129.4 127.0	131.7 124.5	- -	- -	- -

Notes:
1. Trial was sown on 26/05/93.
2. Plant vigor was assessed by giving a 0-5 score to each plant population (0=dead, 5=good vigor).
3. Plant height assessment was done on 10 plants/plot (population if two populations present). Data were analysed on the means using Agrobase 4 for split-plots designs. Statistical analysis is presented in the text.

TABLE3: FZM9311-3202 - Comparison Basta/Ignite on event MS3

Flowering date - 01/09 ->20/09/93
Flower phenotype - 01/09 ->20/09/93

Spray rate	Flowering date (DAS)		Flower phenotype					
	50% silking		No sterile/Total				No fertile plants/Total	
	Sterile pl.	Fertile pl.	Rep1	Rep2	Total	%	Total	%
K: Control	104	104	42/84	52/95	94/179	52	85/179	48
P: Control	104	104	46/88	46/89	92/177	52	85/177	48
L: Basta 250 gai/ha	100	111	52/75	47/76	99/151	66	52/151	34
Q: Ignite 250 gai/ha	102	115	43/65	38/58	81/123	66	42/123	34
M: Basta 500 gai/ha	100	110	44/44	40/42	84/86	98	2/86	2
R: Ignite 500 gai/ha	102	110	50/50	48/51	98/101	97	3/101	3
N: Basta 1000 gai/ha	102	-	40/40	50/50	90/90	100	0/90	0
S: Ignite 1000 gai/ha	100	100	37/37	38/40	75/77	97	2/77	3
O: Basta 2000 gai/ha	102	-	46/46	52/52	98/98	100	0/98	0
T: Ignite 2000 gai/ha	104	-	39/39	43/43	82/82	100	0/82	0

Notes:
1. Trial was sown on 26/05/93.
2. Flowering date and phenotype was observed every two to three days in the test plots. H99 used as a guard for the trial was silking (50% silking) at 104 DAS.
3. A PCR analysis was done on all presumed escapes from Basta above the 250 g.ai/ha rate. Molecular analysis on the plants confirmed the absence of the *barnase* gene in all cases.

Application of SeedLink™ in F_1 hybrid seed production

FIELD TRIAL SUMMARY

CODE	CROP	TRAIT	LOCATION
FZM9402-3310	Corn	NMS	France (L'Isle Jourdain)

TITLE: Application of SeedLink™ in F1 hybrid seed production[1]

AUTHOR: Catherine Dickburt

DATE: 23/11/94

MATERIAL: Event MS3 in H99 as seed-parent, C108 as male

CONCLUSIONS: The use of SeedLink™ in F1 hybrid seed production (event MS3 in the seed-parent) was successful.

- There was no difference in emergence, plant vigor and plant growth between the non-transgenic line H99 and event MS3;
- Rogueing of the male fertile segregants was efficiently achieved by Basta F1 application (3L/ha); Basta resistance segregation did not differ from the 1:1 ratio expected under normal Mendelian segregation;
- No significant differences were observed between event MS3 and H99 in the yield parameters that were measured.

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[1]Event MS3 in female parent

1. METHODS AND MATERIAL

1.1. Trial design

Plot: 4 rows of 300 plants
Length 50 m
Distance between rows 0.8 m
Distance in row 0.16 m
Replicates: 3
Male rows planted as guard

1.2. Objects

Two entries were tested in the 4/2 seed production design (4 female rows alternating with 2 male rows)

A	H99	single density	Hand detasseling
B	Event MS3	double density	Basta sprayment

Seeds used for this trial were produced in Chile winter nursery 93-94 .
M6026: H99
M6025: M6 Event MS3
M6027 + M6028 : C108 (pollinator)

1.3. Observations and tests

Observations:

* % Emergence (on 4 rows of 15m per plot)
* Basta segregation ratios (on 4 rows x 15m per plot)
* Plant vigor at the 5-6 leaf growth stage
* Flowering date and flower phenotype
* Lodging (in vegetation and at harvest)
* Yield components (No. cobs/plant, yield/cob, 1000 kernel weight)
* Seeds size study

Basta treatment:

The Basta sprayment was carried out on 16/06/94 with Basta F1 at the 3 L/ha application rate with a small plot sprayer.

2. RESULTS & CONCLUSIONS

Assessments dates

% Emergence
Basta segregation
Plant vigor
Flowering date
Segregation male sterility/fertility
Yield

2.1. **% Emergence**, **Basta segregation** and **Plant vigor** (see Table 1)

The trial was sown on 22/05/94. The pollinator line was planted twice (22/05/94 and 01/06/94). Emergence (assessed on 4 rows of 15m per plot) and Basta segregation data (assessed on 4 x 15m subplots) were as expected. Basta tolerance segregation did not differ significantly from the 1:1 ratio expected under normal Mendelian segregation.

No differences in plant vigor between the transgenic and non-transgenic entry were observed.

2.2. **Flowering dates and flower phenotype**

The trial started to flower at the beginning of August. Neither uneven stand nor the presence of the transgenes in the plants influenced plant growth or flowering time.

The male fertile H99 plants were detasseled. Just before detasseling a control of evenness was carried out in all plots. Only a few off-type plants were observed. These plants were removed as is the normal procedure in French production fields.

In general, Basta sprayed entries were male sterile. Four fertile plants were found in the entire event MS3 part of the trial, and these plants are considered 'escapes' from the Basta treatment. Relating these 4 plants to the approximate number of 3290 Basta tolerant plants in all three replicates means 0.12% 'escapes' for this trial (stand 68 540 plants per hectare).

2.3. **Yield results** (see Table 2, Table 3)

At the end of October, the trial was harvested. No significant differences were observed between the transgenic and non-transgenic entry for yield, 1000 kernel weight and seed size.

Table 1 - FZM9402-3310 - SeedLink™ in F1 seed production (event MS3 in female parent)

Emergence assessment
Basta segregation data
Plant vigor

Line	Replicate	Emergence (Nb pl./4x15m before sprayment)	Basta tolerance (Nb pl./4x15m after sprayment)	Plant vigor (1-9)
Non-transgenic H99	Replicate 1	331	*(314)*	8
	Replicate 2	335	*(312)*	7
	Replicate 3	328	*(320)*	8
	Mean	331	*(315)*	8
Event MS3	Replicate 1	678	325 n.s.	8
	Replicate 2	681	367 n.s.	8
	Replicate 3	653	295 s.	8
	Mean	671	329 n.s.	8

- n.s. and s. : not significantly or significantly different in a X^3 test at the 0.05 level for hypothesis of 1:1 segregation
- Plant vigor was assessed on a 1-9 scale (1=poor, 9=maximal)
- Basta tolerance : no sprayment in H99 plots, but another count was done as well [*(...)* figures]

Table 2 - FZM9402-3310 - SeedLink™ in F1 seed production (event MS3 in female parent)

Yield data

Line	Replicate	Weight kernels/plot [1 plot = 160m²]	% RH	Yield in qx/ha at 15% RH
Non-transgenic H99 Regular stand	Replicate 1	42.00	12.8	26.93
	Replicate 2	38.80	13.6	24.65
	Replicate 3	40.20	13.0	25.72
	Mean	40.33 A	13.1 A	25.77 A
Event MS3 Uneven stand	Replicate 1	38.40	12.5	24.71
	Replicate 2	43.00	12.9	27.54
	Replicate 3	43.80	13.5	27.86
	Mean	41.73 A	13.0 A	26.70 A
Standard Deviation Coeff of Var.		3.07 7.50%	0.43 3.30%	1.95 7.40%

- Statistical analysis (ANOVA and means comparison) was performed using the Newman-Keuls test (5% level). No significant differences were seen in Yield or RH between H99 and MS3, or between replicates.

- Treatments with a common letter in the table hereabove are not significantly different.

Table 3- FZM9402-3310 - SeedLink™ in F1 seed production (event MS3 in female parent)

Seed size study

Line	Size	1000 kernel weight (g)	% /total kernels
Non-transgenic H99 Regular stand	Little flat	191.99	37.71
	Little round	206.45	38.53
	Middle round	277.96	26.45
	Mean	225.47	
Event MS3 Uneven stand	Little flat	185.80	35.49
	Little round	204.32	37.91
	Middle round	285.98	28.66
	Mean	225.37	

- The seed size study was carried out on a 20 kg sample per line (taken out of the 3 reps batch).
- Little flat (5x10mm), Little round (6x11mm), Middle round (7x11mm)

Detailed agronomic evaluation of event MS3

FIELD TRIAL SUMMARY

CODE	CROP	TRAIT	LOCATION
FZM9421-3309	Corn	NMS	France (Segoufielle)

TITLE: Agronomic evaluation of F1 hybrids produced on event MS3 and event RZM91-1 containing seed-parent plants

AUTHOR: Catherine Dickburt

DATE: 15/01/95

MATERIAL: F1 hybrids produced using:

- female parents with the transgenic male sterile transformation event MS3 and the transformation event RZM91-1, and
- the lines C109, C110, C115, C118 and C119 as male parents.

CONCLUSIONS: With respect to event MS3 :

- no differences in vigor and flowering date were observed between the transgenic and the control hybrids;
- no significant difference was detected between the yield of the F1 hybrids produced on event MS3 (in H99) females and the F1 hybrids produced on non-transgenic H99 females;
- different F1 hybrid combinations (different pollinator lines used in F1 production) varied in yield.

1. METHODS AND MATERIAL

1.1. Trial design

Plot: 4 rows x 48 plants
Length: 7 m
Distance between rows : 0.8 m
Sowing density: 105.000 seeds/ha
Adjustment at 85.000 plants/ha
Replicates: 3
Border: 2m (non transgenic hybrid DK250) - 3 rows

1.2. Objects

A	F1 (H99 x C115) control
B	F1 (H99 x C119) control
C	F1 (H99 x C118) control
D	F1 (H99 x C110) control
E	F1 (H99 x C109) control
F	F1 (Event MS3 x C115)
G	F1 (Event MS3 x C119)
H	F1 (Event MS3 x C118)
I	F1 (Event MS3 x C110)
J	F1 (Event MS3 x C109)
K	F1 (RZM91-1 x C115)
L	F1 (RZM91-1 x C119)
M	F1 (RZM91-1 x C118)
N	F1 (RZM91-1 x C110)
O	F1 (RZM91-1 x C109)

Seeds for this field trial were produced in Chile Winter nursery 1993-1994.

1.3. Observations and tests

Observations:

* % Emergence
* Plant vigor (1-9)
* Flowering dates
* Flower phenotype: male fertility or sterility
* Lodging
* Yield components

RESULTS & CONCLUSIONS

Assessments dates

% Emergence	
Plant vigor (1-9)	17/06/94
Flowering date	22/07/94 to 30/07/94
Segregation male sterility/fertility	05/08/94
Lodging (in vegetation)	29/08/94
Lodging (at harvest), common smut, and *Fusarium*	12/10/94
Yield determination	20/10/94

2.1. % Emergence

Emergence conditions were good and all entries germinated evenly after 7-8 days. A stand adjustment to 85.000 pl/ha was carried out on June 17th.

2.2. **Plant vigor** (1-9) (See Table 1)

A plant vigor assessment was carried out at the 6th leaf growth stage. Plant vigor of the transgenic hybrids was generally good and similar to that of the control hybrids.

2.3. **Flowering dates and flower phenotype** (See Table 1)

The trial started flowering on 22/07/94. The flowering period lasted about a week. At the end of this flowering period, a male flower phenotype assessment was done.

The transgenic hybrids flowered within the same timeframe as the control hybrids. Flower phenotype segregation ratio was recorded in the transgenic plots. Segregation ratios did not significantly differ from the expected 1:1 ratio.

The male flower phenotype of the hybrids carrying event MS3 ranged from male sterility (few anthers) to 25% anthers (F1 :MS3xC115). The F1 hybrid (MS3xC115) extruded the most anthers. The F1 hybrid (MS3xC118) only exerted a few anthers.

2.4 **Lodging data** (See Table 2)

Lodging in vegetation was very rare.

Lodging at harvest was mostly due to European Corn Borer and *Sesamia* infestation. F1(NMSxC118) hybrids seemed to be more sensitive to lodging.
No differences in lodging were seen between the transgenic and control hybrids.

2.5. **Yield results** (See Table 3)

Yield results are summarized in Table 3. Because of the importance of lodging, the trial was harvested early. The 2 internal rows of each plot were harvested for yield determination.

No differences were found in yield/ha between the transgenic F_1 hybrids and their respective controls. A statistical analysis was carried out on the yield/ha data using the Newman-Keuls test at the 5% level. There were no significant differences between any

of the hybrids. The block effect was significant. Plots of the 1st replicate yielded less seeds than the other 2 replicates.

A factorial analysis was performed on the same data (Factor A=female parent, Factor B= male parent). When yield/ha was averaged over the 5 different male parental lines, no difference was observed between H99 based hybrids, MS3 based hybrids and MS4 based hybrids.Differences were observed between the different types of hybrids (5 different male lines).

There were no differences in yield between any of the NMS based hybrid and its non-transgenic control. Yield differences in this trial were seen between the different types of hybrids in test (depending on the male line used).

TABLE 1: FZM9421-3309 - Agronomic evaluation

Plant vigor - 6 leaf growth stage - 17/06/94 (35 days after sowing)
Flowering date - 22 to 30/07/94
Flower phenotype - 05/08/94 (84 days after sowing)

F1 hybrid	Plant vigor (1-9)	Flowering date	Flower phenotype segregation			Flower phenotype of the male sterile population
			N° Fertile /plot	N° Male Sterile /plot	% Male Sterile	
	Mean	Mean	Mean	Mean	Mean	
H99 x C115	8.0	209.7	189.0	0.0	0.0	-
H99 x C119	8.3	207.3	191.3	0.0	0.0	-
H99 x C118	8.7	203.3	187.7	0.0	0.0	-
H99 x C110	8.0	206.7	189.7	0.0	0.0	-
H99 x C109	7.3	207.0	183.0	0.0	0.0	-
MS3 x C115	7.3	209.0	99.0	85.3	46.3	15-25% anth.
MS3 x C119	8.3	205.3	97.0	88.7	47.8	5-10% anth.
MS3 x C118	8.0	203.3	104.0	81.3	43.9	Ster - Few anth.
MS3 x C110	7.7	207.0	98.7	83.3	45.8	Ster - 5% anth.
MS3 x C109	7.7	206.3	97.3	90.7	48.2	5-10% anth.
MS4 x C115	7.3	209.3	90.3	94.7	51.2	Sterile
MS4 x C119	6.3	207.7	104.0	78.0	42.9	Ster-Few anth.*
MS4 x C118	6.7	205.7	98.3	80.7	45.1	Ster-Few anth.*
MS4 x C110	7.0	207.3	107.3	79.0	42.4	5-10% anth.
MS4 x C109	6.0	206.3	102.0	80.3	44.0	Ster-Few anth.

Notes:
1. Plant vigor assessment was done by giving a score from 1-9 to each plot (where 1=poor, 9=good).
2. Mean : mean value of plants in the 3 replicates
3. Flowering date assessment : in days of the year (sowing date was day 133)

* Pollen on one plant.

FIELD OBSERVATIONS MADE:

- Visually response to glufosinate in genetically modified lines

- Morphology traits of engineered lines versus non-engineered control plants.

- Visual monitoring of the male sterile phenotype in engineered lines.

SEGREGATION DATA: Normal 1:1 segregation was observed in the field. No breakage of the sterility trait was observed.

MALE STERILE PHENOTYPE: The male sterility trait appears to be tightly linked to glufosinate resistance. No break in the sterility trait was observed.

VOLUNTEER INFORMATION: The field was monitored routinely for volunteers, none were detected.

MORPHOLOGICAL DATE: No differences were noted between the transgenic and non-transgenic controls with respect to weediness, insect or disease susceptibility.

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TABLE 2: FZM9421-3309 - Agronomic evaluation

Lodging in vegetation - 29/08/94
Lodging at harvest (caused by *Fusarium*, European Corn Borer (ECB), wind) - 12/10/94
Common smut observation - (Total of plant values: Smut on the stem: 1, Smut on the cob: 2) - 12/10/94

F1 hybrid	Lodging in vegetation (1-9)	Lodging at harvest		Common smut observation	Number plants affected by *Fusarium*/plot
		(1-9)	% plants lodged (ECB+ *Sesamia*)		
	Mean	Mean	Mean	Mean	Mean
H99 x C115	9.0	6.3	45.0	1.0	32.0
H99 x C119	8.7	6.7	43.6	4.3	28.0
H99 x C118	8.7	7.0	45.9	1.0	21.0
H99 x C110	9.0	5.7	45.8	11.3	41.3
H99 x C109	8.7	5.3	47.7	3.7	41.0
MS3 x C115	9.0	7.3	36.3	2.3	15.3
MS3 x C119	8.3	7.0	30.0	3.3	17.7
MS3 x C118	8.3	5.3	54.7	0.7	20.7
MS3 x C110	9.0	5.7	54.4	5.7	41.3
MS3 x Oh 43	9.0	5.0	56.5	3.0	34.0
MS4 x C115	8.3	6.0	58.5	1.0	28.0
MS4 x C119	8.7	7.0	39.2	4.0	21.7
MS4 x C118	9.0	4.0	62.7	2.0	34.0
MS4 x C110	8.7	6.7	38.7	8.7	37.3
MS4 x C109	8.7	6.0	47.0	2.0	34.7

Notes: 1. Lodging observations were done by giving a score from 1-9 to each plot (where 1=poor, 9=good).

2. Common smut observation was as follows. A score was given to each plant attacked by smut in the plot (smut on stem=1, smut on cob=2). The plant values were then added to calculate the plot value.

3. Mean : mean value of plants in the 3 replicates

Table 3: FZM9421-3309 - Agronomic evaluation

Yield data - 20/10/94

F1 hybrid	Number pl. harvested/ 2 rows	Yield in kg/plot (1 plot = 11.2 m2)	RH (%)	Yield in qx/Ha at 15% RH	1000 kernel weight
	Means	Means	Means	Means	Means
H99 x C115	94.0	14.47	32.4	102.72	376.0
H99 x C119	94.7	13.88	28.8	103.74	400.0
H99 x C118	94.0	13.33	29.3	102.65	366.0
H99 x C110	92.3	12.90	30.2	94.59	393.4
H99 x C109	91.7	12.67	33.4	88.63	367.4
MS3 x C115	94.3	15.13	31.6	108.81	371.3
MS3 x C119	94.7	13.62	28.5	102.32	391.6
MS3 x C118	89.3	12.97	28.4	97.52	360.5
MS3 x C110	90.0	11.93	29.8	87.97	398.4
MS3 x C109	93.0	13.97	33.9	96.97	372.1
MS4 x C115	91.3	14.52	32.3	103.22	388.1
MS4 x C119	88.3	12.60	29.1	93.75	403.9
MS4 x C118	92.7	12.57	29.7	92.73	367.8
MS4 x C110	94.0	13.33	29.5	98.70	405.4
MS4 x C109	89.3	12.85	32.9	90.51	373.5
Standard Deviation Coeff. of Variation				7.51 qx 7.70%	

Notes: 1. A statistical analysis (ANOVA+Means comparison) was carried out on the yield/ha (15%RH) data using the Newman-Keuls test at the 5% level. There was no significant differences between the hybrids in test. The Block effect was significant.

A factorial analysis was then carried out on the same data (Factor A = female, factor B = male), results are presented below:

Factor A: Female parent

Female parent used for F1 production	Yield in qx/Ha at 15% RH Means (5 hybrids)
H99	98.47
MS3	98.72
MS4	95.78
L.S.D. for factor A	4.67 qx

Factor B: Male parent

Male parent used for F1 production	Yield in qx/Ha at 15% RH Means (3 hybrids)
C115	104.92 A
C119	99.94 AB
C118	97.63 BC
C110	93.75 C
C109	92.03 C
L.S.D for factor B	6.02 qx

No significant difference seen at the 5% level between female parents used.
Significant differences seen at the 1% level between male parents used.

1. METHODS AND MATERIAL

1.1. Trial design

Randomized blocks design:

Plot: 1 row of 60 plants
Length 7.5 m
Distance between rows 1 m
Distance in row 0.15 m

Replicates: 2

Border: 2m (non transgenic, variety Anthony) - 3 rows

1.2. Objects

Trt code	Plot description	M seedlot
1	H99	M6026
2	C101	M6033
3	C115	M6034
4	C116	M5231
5	C108	M6028
6	C110	M6031
7	C109	M6032
8	C114	M6037
9	Event MS3	M6025
10	F1(Event MS3XC101)	M6040
11	F1(Event MS3XC115)	M6010
12	F1(Event MS3XC116)	M6042
13	F1(Event MS3XC108)	M6011
14	F1(Event MS3XC110)	M6013
15	F1(Event MS3XC109)	M6014
16	F1(Event MS3XC114)	M6029
17	BC1(Event MS3x*2C101)	M5176
18	BC1(Event MS3x*2C115)	M5193
19	BC1(Event MS3x*2C116)	M5228
20	BC1(Event MS3x*2C108)	M6077
21	BC1(Event MS3x*2C110)	M6090
22	BC1(Event MS3x*2C109)	M6083
23	BC1(Event MS3x*2C114)	M6094
24	F1[(Event MS3xC101)xH99]	M5173
25	F1[(Event MS3xC115)xH99]	M5190
26	F1[(Event MS3xC116)xH99]	M5227
27	F1[(Event MS3xC108)xH99]	M6080
28	F1[(Event MS3xC110)xH99]	M6093
29	F1[(Event MS3xC109)xH99]	M6087
30	F1[(Event MS3xC114)xH99]	M6097
31	BC2(Event MS3x*3C101)	M6110
32	BC2(Event MS3x*3C115)	M6100
33	BC2(Event MS3x*3C116)	M6106
34	F1[(Event MS3x*2C101)xH99]	M6113
35	F1[(Event MS3x*2C115)xH99]	M6103
36	F1[(Event MS3x*2C116)xH99]	M6109

1.3. Observations and tests

Observations:

* % Emergence
* Basta segregation ratio
* Plant vigor (1-9)
* Flowering dates
* Male flower phenotype
* Lodging

Basta sprayment:

The Basta sprayment was carried out on 28-06-94 at the 4-5 leaf growth stage at the 3L/Ha rate. Plants were assessed 20 days after application.

1.4. Agronomy:

1.4.1. Fertilization

Date	Product	Quantity
21/04/94	NH4NO3	600 kg/ha
21/04/94	Superphosphate	600 kg/ha
22/04/94	Kaliumsulphate	400 kg/ha

1.4.2. Treatments

Date	Type	Product	Quantity
27/06/94	Herbicide	Mikado	1.5 l/ha
		+ Atrazine	1.5 l/ha

1.4.3. Operations

Date	Variables	Activity
19/05/94	-	Seedbed preparation
18/05/94	All	Sowing in field with precision drill
07/11/94	All	Chopping + Ploughing in

2. RESULTS & CONCLUSIONS

Assessments dates

% Emergence	28/06/94
Basta segregation	18/07/94
Plant vigor	24/07/94
Flowering date	16/08/94 to 29/09/94
Lodging	13/10/94

2.1. **% Emergence** (See Table 1)

Emergence of the inbred lines ranged from 53% up to 92%. The percentage of emergence of event MS3 was 70%. F1 crosses of event MS3 with a public line emerged from 87% to 93%. Except for a few combinations, emergence of the other crosses was good.

2.2 **Basta segregation** (See Table 1)

Basta segregation for the transgenic plants was analyzed in a χ^2 test. Values obtained were in all but a few cases not significantly different from the expected 1:1 ratio.

2.3. **Plant vigor** (See Table 1)

Plant vigor was assessed on a 1-9 scale (1=poor, 9=very good) at the 8-9 leaf growth stage.

Plant vigor of inbred lines ranged from 4.5 to 6. Plant vigor of event MS3 was similar to that of the parental line H99 (6 compared to 5.5).
Highest scores were given to the hybrid combinations (7 to 8.5).
Plant vigor of BC1 lines were between those of the inbred and hybrid lines.
At the BC2 level, plant vigor figures are similar to those of the inbred lines.

2.4. **Flowering dates and flower phenotype** (See Table 2)

Flowering was observed every 2-3 days from mid-August 1994. F1 hybrid plants were either completely male sterile or displayed a partially male sterile phenotype (anthers extruded from up to 30% of the spikelets). In a subsequent backcross generation (BC2) fully male sterile plants were observed in several genetic combinations.

2.5. **Lodging** (See Table 3)

A storm hit the field trial. A lodging assessment was carried out 15 days before harvest to look at eventual differences between entries. As outlined in Table 4, no striking differences were observed between the different entries.

TABLE 1: FZM9403-3209 - Stability of event MS3 in a back-crossing program

Emergence assessment - 28/06/94 (41 days after sowing [41DAS]
Basta tolerance assessment - 18/07/94 (20 days after application [20DAA])
Plant vigor assessment - 24/07/94 (67 days after sowing [67DAS]), 8-9 leaf growth stage

Line	Emg 39DAS	Emg % Mean	Basta tolerance Segregation ratio (Resistant/Total) Rep 1	Basta tolerance Segregation ratio (Resistant/Total) Rep 2	Segregation ratio Totals (Rep1+Rep2)	Segregation ratio %	Plant vigour (1-9) Means
H99	67/120	55.8	-	-	-	-	5.5
C101	96/120	80.0	-	-	-	-	6
C115	82/120	68.3	-	-	-	-	4.5
C116	110/120	91.6	-	-	-	-	5.5
C108	100/120	83.3	-	-	-	-	4.5
C110	64/120	53.3	-	-	-	-	5.5
C109	90/120	75.0	-	-	-	-	4.5
C114	100/120	83.3	-	-	-	-	4.5
Event MS3	84/120	70.0	23/41	?	23/41	56.1	6
F1(Event MS3XC101)	105/120	87.5	31/54	33/51	64/105 *	61.0	8.5
F1(Event MS3XC115)	104/120	86.7	24/53	14/51	38/104 *	36.5	7.5
F1(Event MS3XC116)	106/120	88.3	32/50	30/56	62/106	58.5	7
F1(Event MS3XC108)	106/120	88.3	26/49	29/57	55/106	51.2	7.5
F1(Event MS3XC110)	112/120	93.3	35/56	28/56	63/112	56.2	8
F1(Event MS3XC109)	112/120	93.3	31/54	29/58	60/112	53.6	8
F1(Event MS3XC114)	107/120	89.2	33/52	28/55	61/107	57.0	8.5
BC1(Event MS3x*2C101)	116/120	96.7	26/57	26/59	52/116	44.8	7
BC1(Event MS3x*2C115)	106/120	88.3	29/53	29/53	58/106	54.7	7
BC1(Event MS3x*2C116)	114/120	95.0	25/57	32/57	57/114	50.0	6.5
BC1(Event MS3x*2C108)	106/120	88.3	20/55	27/51	47/106	44.3	7
BC1(Event MS3x*2C110)	68/120	56.7	9/31	17/37	26/68	38.2	7.5
BC1(Event MS3x*2C109)	76/120	63.3	22/47	12/29	34/76	44.7	6.5
BC1(Event MS3x*2C114)	104/120	86.7	14/49	15/55	29/104 *	27.9	7
F1[(Event MS3xC101)xH99]	105/120	87.5	27/52	25/53	52/105	49.5	6.5
F1[(Event MS3xC115)xH99]	104/120	86.7	29/53	20/51	49/104	47.1	7
F1[(Event MS3xC116)xH99]	115/120	95.8	35/57	35/58	70/115 *	60.9	6
F1[(Event MS3xC108)xH99]	105/120	87.5	27/49	30/56	57/105	54.3	6
F1[(Event MS3xC110)xH99]	10/44	22.7	1/4	6/6	7/10	70.0	5.5
F1[(Event MS3xC109)xH99]	79/120	65.8	17/38	22/41	39/79	49.3	6
F1[(Event MS3xC114)xH99]	97/120	80.8	26/49	18/48	44/97	45.4	7
BC2(Event MS3x*3C101)	100/120	83.3	14/50	18/50	32/100 *	32.0	6
BC2(Event MS3x*3C115)	106/120	88.3	17/49	29/57	46/106	43.4	5
BC2(Event MS3x*3C116)	110/120	91.6	14/51	20/59	34/110 *	30.9	5.5
F1[(EventMS3x*2C101)xH99]	83/120	69.2	17/41	17/42	34/83	41.0	6.5
F1[(EventMS3x*2C115)xH99]	96/120	80.0	17/45	20/51	37/96 *	38.5	6.5
F1[(EventMS3x*2C116)xH99]	89/120	74.2	18/42	22/47	40/89	44.9	6.5

*: Significantly different in a X^2 test at the 0.05 level for hypothesis of 1:1 segregation.

TABLE 2: FZM9403-3209 - Stability of event MS3 in a backcrossing program

Description	50% Silking (DAS)	Number of 'escapes' (male fertile)	Male Flower phenotype
H99	92	-	Fertile
Event MS3	91		Sterile
F1 (Event MS3xC101)	95		Few anthers to 10% anthers
BC1(EventMS3x*2C101)	103		Sterile to 10% anthers
BC2 (Event MS3x*3C101)	110	1	**Sterile**
F1 [(EventMS3x*2C101)xH99]	95		Sterile to 5% anthers
F1 [(Event MS3xC101)xH99]	94		Sterile to 20% anthers
C101	123		Fertile
Event MS3	91		Sterile
F1 (Event MS3xC115)	94		5 to 30% anthers
BC1 (Event MS3x*2C115)	102		Sterile to 30% anthers
BC2 (Event MS3x*3C115)	102		**Sterile to few anthers**
F1 [(Event MS3x*2C115)xH99]	91		Sterile to 20% anthers
F1 [(Event MS3xC115)xH99]	92	1	Sterile to 40% anthers
C115	116		Fertile
Event MS3	91		Sterile
F1 (Event MS3xC116)	99		Sterile to few anthers
BC1 (Event MS3x*2C116)	104		Sterile to few anthers
BC2 (Event MS3x*3C116)	104		**Sterile to few anthers**
F1 [(EventMS3x*2C116)xH99]	94		Sterile to 5% anthers
F1 [(Event MS3xC116)xH99]	104	1	Sterile to 5% anthers
C116	95		Fertile
Event MS3	91		Sterile
F1 (Event MS3xC108)	90		Few anthers to 10% anthers
BC1 (Event MS3x*2C108)	93		**Sterile to few anthers**
F1 [(Event MS3xC108)xH99]	94	1	Sterile to 10% anthers
C108	105		Fertile
Event MS3	91		Sterile
F1 (Event MS3xC110)	90		Sterile to 5% anthers
BC1 (Event MS3x*2C110)	94		**Sterile to 5% anthers**
F1 [(Event MS3xC110)xH99]	90		Sterile to few anthers
C110	115		Fertile
Event MS3	91		Sterile
F1 (Event MS3xC109)	90		Few anthers to 25% anthers
BC1 (Event MS3x*2C109)	91		**Sterile to 25% anthers**
F1 [(Event MS3xC109)xH99]	90		Sterile to 20% anthers
C109	100		Fertile
Event MS3	91		Sterile
F1 (Event MS3xC114)	85		Sterile to 5% anthers
BC1 (Event MS3x*2C114)	88		**Sterile to 5% anthers**
F1 [(Event MS3xC114)xH99]	86		Sterile to 5% anthers
C114	96		Fertile

Notes: 1. Flowering observations from 11/08 till 29/09
2. Storm on 23/08 and all anthers fallen on the ground consequently. Therefore late entries info should be taken with care (specially, C101, C115 and C116 hybrids)

TABLE 3: FZM9403-3209 - Stability of event MS3 in a back-crossing program

Lodging assessment - 13/10/94 (149 days after sowing [147DAS]

Line	Lodging (1-5) 1= poor stand 5= good stand
H99	4.5
C101	5.0
C115	4.5
C116	5.0
C108	5.0
C110	5.0
C109	5.0
C114	4.0
Event MS3	4.0
F1(Event MS3XC101)	5.0
F1(Event MS3XC115)	5.0
F1(Event MS3XC116)	5.0
F1(Event MS3XC108)	5.0
F1(Event MS3XC110)	5.0
F1(Event MS3XC109)	5.0
F1(Event MS3XC114)	5.0
BC1(Event MS3x*2C101)	5.0
BC1(Event MS3x*2C115)	5.0
BC1(Event MS3x*2C116)	4.5
BC1(Event MS3x*2C108)	3.5
BC1(Event MS3x*2C110)	3.5
BC1(Event MS3x*2C109)	5.0
BC1(Event MS3x*2C114)	4.5
F1[(Event MS3xC101)xH99)]	5.0
F1[(Event MS3xC115)xH99)]	5.0
F1[(Event MS3xC116)xH99)]	4.5
F1[(Event MS3xC108)xH99)]	5.0
F1[(Event MS3xC110)xH99)]	5.0
F1[(Event MS3xC109)xH99)]	5.0
F1[(Event MS3xC114)xH99)]	5.0
BC2(Event MS3x*3C101)	4.5
BC2(Event MS3x*3C115)	5.0
BC2(Event MS3x*3C116)	4.5
F1[(Event MS3x*2C101)xH99)]	5.0
F1[(Event MS3x*2C115)xH99)]	5.0
F1[(Event MS3x*2C116)xH99)]	5.0

Termination Report
Approved Permit Number: 92-105-02

Name: Lori Marshall

Institutional Address:

Holden's Foundation Seeds, Inc.
201 N. Maplewood, P.O. Box 839
Williamsburg, IA 52361

Telephone Number: 319/668-1100
Facsimile Telephone Number: 319/668-2453

Date Of This Report: 9 March 1993

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

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

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

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

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

Summary of experimental results:

Only a handful of plants survived our first planting, due to adverse weather at the time of planting in the field. Of these, some were male-sterile and some were male-fertile, as predicted for these materials expected to segregate for the male-sterilty gene. All were female-fertile and set seed when pollinated by pollen from nontransgenic plants. A second planting was made, and the seedlings were treated with the herbicide Ignite(TM), active ingredient glufosinate. The segregation was

as expected for a single marker gene conferring tolerance to the herbicide. A killing frost destroyed these plants when they were approximately 1-ft. tall.

Even though the transgenic plants were expected to be male-sterile, because we had not confirmed the expression of male-sterility in the field prior to this experiment, we covered the tassels with paper bags during the flowering period. While this method seemed effective at preventing pollen dispersal, it also seemed to have some effect on pollen fertility because pollen from fertile segregants presumed to be nontransgenic appeared to have a significant percentage of aborted pollen when examined under a microscope.

One transgenic line appeared to be segregating for some gene that resulted in a leaf "blotch". We assume that this is a mutation arising from the tissue culture step used in the transformation procedure. Since other transgenic lines did not have this "blotch" phenotype, we do not think that this represents an effect inherent to the presence of the inserted sequence. We will do further work with the transgenic lines that did not show the "blotch" phenotype.

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

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

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

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

Evidence that the inserted sequence combined with DNA or RNA of other indigenous organisms:
There was no opportunity for transgenic pollen to move to other organisms.

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

MOLECULAR BIOLOGY

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

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

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

RESULTS OF OBSERVATIONS AND MONITORING DURING THE FIELD TEST

One transgenic line was segregating for "blotches" on the first seedling leaves. This phenotype was not observed on later-developing leaves. We assume that this "blotch" phenotype is associated with some mutation occurring during the tissue culture process, and this was not observed in other transgenic lines.

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

The transgenic plants were male-sterile, as expected.

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

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

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

HANDLING AND SHIPPING SAFEGUARDS

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

None of the safeguards were breached.

SITE CONSIDERATIONS

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

EXPERIMENTAL DESIGN

The transgenic seed was planted first indoors, because the seed quality was poor and we wanted to optimize conditions for germination. Young seedlings were then transplanted to the field. One day after transplanting, we got a ferocious thunderstorm with high winds that killed many of the seedlings. Therefore, we requested and received permission to have a second planting, in the same spot as the first seedlings were to have been grown.

PHYSICAL CONFINEMENT

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

BIOLOGICAL/ENVIRONMENTAL CONSIDERATIONS

The experiment was inititated during the normal growing season for corn. Containment was insured by bagging the taseels of plants during the flowering period.

The Iowa site will sometimes have incomplete winter kill. All ears from transgenic plants were hand-harvested, and no pollen was allowed to shed from transgenic plants, so there was no opportunity for transgenic progeny to be present at the test site after the test conclusion. Any volunteers appearing at the test site next Spring will be nontransgenic, but we will destroy all volunteers by standard mechanical or herbicide treatments as a precaution.

Pollen movement was prevented by bagging the tassels, so that even though compatible plants were being grown in the vicinity, the transgenic material did not move from the test site.

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

SCALE OF THE EXPERIMENT
As in application for approval to conduct the field test.

BIOLOGICAL MONITORING
The male-sterility observed indicated that the trasgene was expressed, and the tolerance to Ignite observed indicated that the marker gene linked to the male-sterility gene was also expressed.

Molecular analyses were not performed.

The seeedlings were treated with Ignite to identify transgenic plants by the expression of the marker gene.

The test site will be monitored for volunteers. but all volunteers are expected to be nontransgenic.

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

MAINTENANCE
As in application for approval to conduct the field test.

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

TERMINATION OF EXPERIMENT

The bagging of tassels appeared to be an effective way of preventing pollen movement, but we did not test this on very many plants because most of our plants were destroyed by bad weather as very young seedlings.

Since the male-sterility was expressed in the field, we have increased confidence that the transgenic plants will not shed fertile pollen, even if allowed to flower normally. Our limited observations suggest that destruction of progeny that may have remained at the test site wil be practical and effective.

PUBLIC REACTIONS

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

(end)

HOLDEN'S FOUNDATION SEEDS, INC.
P.O. Box 839, Williamsburg, Iowa 52361
FAX 319 668-2453
319 668-1100

27 June 1995

Field Trial Report
Permit Number: 92-244-03 (renewal of 92-105-02)

Submitted by: Dr. Lori Marshall Lori Marshall
Holden's Foundation Seeds, Inc.
201 N. Maplewood, P.O. Box 839
Williamsburg, Iowa 52361
phone: 319/668-1100
fax: 319/668-2453

No changes from original application for field test were made with respect to:

Test organisms, sources of donor DNA, vectors, and transformation systems. (The transgenic plant source was named MS3-06, and also called MS3-RZM34/1. Subsequently, the name was shortened to MS3.)

Location of field test. (The site was in Maui County, Molokai, Hawaii, near Holden's Foundation Seeds, Inc. office in Kaunakakai.)

Summary of experimental results:

Segregation. The plants segregated as expected for a single nuclear gene when treated with Ignite™ (active ingredient glufosinate) or when observed for male-sterility. The Ignite-tolerance and male-sterility co-segregated.

Observations on phenotype. The plants appeared completely normal in their phenotype. The rows containing transgenic plants were planted adjacent to rows of nontransgenic standard corn inbred lines. During the course of the growing season the following circumstances allowed comparison of transgenic vs non-transgenic corn lines:

Response to standard irrigation and Hawaii's warm soil temperatures during germination period - no differences detected.

Response to standard "fertigation" (fertilizer applied through irrigation system) - no differences detected.

Response to normal heavy insect pressure (varied species, typically including leafhoppers, aphids, thrips, spider mites, and ear worms) - no differences detected.

Flowering - no differences in silk extrusion detected. Male-sterile tassels were often more slender than nontransgenic standards, but appeared to emerge similarly.

Seed set on hand-pollinated ears - no differences detected. Transgenic plants had good seed set and kernel quality.

Late-season plant appearance (susceptibility to stalk-rotting diseases, ear molds and smuts, and general plant vigor) - no differences detected.

The transgenic plants displayed the expected tolerance to glufosinate and male-sterility but no other apparent change in phenotype (including no change in disease status).

There were no unanticipated differences in morphology, weediness, flowering (silk) characteristics, or long-term survivability of progeny observed.

Size and containment for trial

This transgenic material was planted during the fall/winter growing season in Hawaii (planting date: 12-Nov-92.) Isolation from other corn was used to contain the trial, and no compromise to this containment was observed.

Nov-92 planting: Approximately 1,000 seeds segregating for male-sterility+Ignite-tolerance were planted, occupying approximately 0.06 acre.

Stability of the male sterility trait in a backcrossing program

FIELD TRIAL REPORT

CODE	CROP	TRAIT	LOCATION
FZM9403-3209	Corn	NMS	Belgium (Ophain)

TITLE: Study of the male sterility trait in a backcrossing program

AUTHOR: Catherine Dickburt

DATE: 15/01/95

MATERIAL:

Event MS3
Backcrossing program including public inbred lines (C101, C108, C109, C110, C114, C115 and C116)

CONCLUSIONS:

The stability of the male sterile phenotype in different genetic backgrounds was evaluated.

F1 hybrid plants of some hybrid combinations displayed a partially male sterile phenotype. A later backcross generation (BC2) seemed to show the expected completely male sterile phenotype.

Termination of experiment

Hand-pollinated ears were harvested from the trial 1-Mar-93, and held in storage at our Kaunakakai, Molokai station. Remaining plant material and seed from open-pollinated ears was disked into the soil in early March 1993.

After this initial disking into the soil, the sites were left fallow for several months. After the initial disking each site went through 3 cycles of (1) irrigation, (2) fallow for about 3 weeks, (3) disking to destroy any seedlings.

Monitoring for Volunteers

For both plantings: During the first irrigation-fallow-disking cycle, many volunteers were observed, as is typical for our standard corn plantings in Hawaii. During the second irrigation-fallow-disking cycle, a handful of volunteers were observed, as is typical in our standard plantings. During the third cycle, no volunteers were observed, as is typical in our standard plantings.

HOLDEN'S FOUNDATION SEEDS, INC.
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27 June 1995

Field Trial Report
Permit Number: 93-076-02 (renewal of 92-105-02)

Submitted by: Dr. Lori Marshall Lori Marshall
Holden's Foundation Seeds, Inc.
201 N. Maplewood, P.O. Box 839
Williamsburg, Iowa 52361
phone: 319/668-1100
fax: 319/668-2453

No changes from original application for field test were made with respect to:

Test organisms, sources of donor DNA, vectors, and transformation systems. (The transgenic plant source was named MS3-06, and also called MS3-RZM34/1. Subsequently, the name was shortened to MS3.)

Location of field test. (The site was in Iowa County, near Holden's Foundation Seeds, Inc. headquarters in Williamsburg, IA. Standard fertilizers and herbicides were used as is typical for our traditional corn breeding research.)

Summary of experimental results:
Segregation. The plants segregated as expected for a single nuclear gene when treated with Ignite™ (active ingredient glufosinate) or when observed for male-sterility. The Ignite-tolerance and male-sterility co-segregated. A very small number of hybrid plants (less than 1% of the total observed, and all with full hybrid vigor) were clearly expressing the inserted gene as evidenced by Ignite-tolerance and compromised male-fertility but the sterility appeared incomplete and some anthers were

observed that appeared to contain some viable pollen.

Observations on phenotype. The plants appeared completely normal in their phenotype. The rows containing transgenic plants were planted adjacent to rows of nontransgenic standard corn inbred lines. During the course of the growing season the following circumstances allowed comparison of transgenic vs non-transgenic corn lines:

Excessive rainfall during germination period - no differences detected.

Excessive rainfall during seedling growth period - no differences detected.

Unusually heavy infestations of leaf anthracnose and leaf rust (foliar diseases) - no differences detected.

Flowering - no differences in silk extrusion detected. Male-sterile tassels were often more slender than nontransgenic standards, but appeared to emerge similarly.

Seed set on hand-pollinated ears - no differences detected. Transgenic plants had good seed set and kernel quality.

Late-season plant appearance (susceptibility to stalk-rotting diseases, ear molds and smuts, natural infestations of European Corn Borer, and general plant vigor) - no differences detected.

The transgenic plants displayed the expected tolerance to glufosinate and male-sterility but no other apparent change in phenotype (including no change in disease status).

There were no unanticipated differences in morphology, weediness, flowering (silk) characteristics, or long-term survivability of progeny observed.

Size and containment for trial

The experiment was conducted during the normal growing season for corn. Isolation from other corn was used to contain the trial, and no compromise to this containment was observed.

27-May-93 planting: Approximately 7,000 seeds segregating for male-sterility+Ignite-tolerance were planted, occupying approximately 0.29 acres

Termination of experiment

Hand-pollinated ears were harvested from the trial 25 October 1993, and held in storage at our Williamsburg, IA station. Remaining plant material and seed from open-pollinated ears were disked and plowed under 8 November 1993.

Monitoring for Volunteers

Our previous experience with standard corn lines suggested that fall plowing the remaining plants and seed under would greatly reduce the chances that volunteers would survive and grow in the following spring, and no volunteers were observed in April and May 1994 monitoring of the field site.

HOLDEN'S FOUNDATION SEEDS, INC.
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27 June 1995

Field Trial Report
Permit Number: 93-076-03 (renewal of 92-244-03)

Submitted by: Dr. Lori Marshall Lori Marshall
Holden's Foundation Seeds, Inc.
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fax: 319/668-2453

No changes from original application for field test were made with respect to:

Test organisms, sources of donor DNA, vectors, and transformation systems. (The transgenic plant source was named MS3-06, and also called MS3-RZM34/1. Subsequently, the name was shortened to MS3.)

Location of field test. (The 3 plantings were in Maui County, Molokai, Hawaii, near Holden's Foundation Seeds, Inc. office in Kaunakakai.)

Summary of experimental results:

Segregation. The plants segregated as expected for a single nuclear gene when treated with Ignite™ (active ingredient glufosinate) or when observed for male-sterility. The Ignite-tolerance and male-sterility co-segregated. The MS3 transgenic plants are in a backcrossing program in which the MS3 gene is being crossed into a set of Holden's proprietary inbred lines (designated "LH" followed by a number). This set includes inbreds which represent very diverse germplasm types, and the MS3 gene appears to segregate and express as expected in this wide sampling of genetic backgrounds. There were a few cases in which F1 or BC1

generation material did demonstrate anther extrusion, and in one case fertile pollen was recovered from such an anther. We suspect that the occurrence of partial male sterility may be confined to early backcross generation material and may be related to hybrid vigor, and we will make observations on early and later generation materials in future trials.

Observations on phenotype. The plants appeared completely normal in their phenotype. The rows containing transgenic plants were planted adjacent to rows of nontransgenic standard corn inbred lines. During the course of the growing season the following circumstances allowed comparison of transgenic vs non-transgenic corn lines:

Response to standard irrigation and Hawaii's warm soil temperatures during germination period - no differences detected.

Response to standard "fertigation" (fertilizer applied through irrigation system) - no differences detected.

Response to normal heavy insect pressure (varied species, typically including leafhoppers, aphids, thrips, spider mites, ear worms) - no differences detected.

Flowering - no differences in silk extrusion detected. Male-sterile tassels were often more slender than nontransgenic standards, but appeared to emerge similarly.

Seed set on hand-pollinated ears - no differences detected. Transgenic plants had good seed set and kernel quality.

Late-season plant appearance (susceptibility to stalk-rotting diseases, ear molds and smuts, and general plant vigor) - no differences detected.

The transgenic plants displayed the expected tolerance to glufosinate and male-sterility but no other apparent change in phenotype (including no change in disease status). The transgenic plants were completely tolerant to glufosinate applications that were lethal to non-transgenic corn plants (up to 600 g/ha).

There were no unanticipated differences in morphology, weediness, flowering (silk) characteristics, or long-term survivability of progeny

observed..

Size and containment for trial

This transgenic material was planted 3 times during the period from May 1993 to May 1994. For all three plantings, isolation from other corn was used to contain the trial, and no compromise to this containment was observed.

27-May-93 planting: Approximately 2,000 seeds segregating for male-sterility+Ignite-tolerance were planted, occupying approximately 0.1 acre.

10-Sep-93 planting: Approximately 2,000 seeds segregating for male-sterility+Ignite-tolerance were planted, occupying approximately 0.1 acre.

4-Jan-94 planting: Approximately 4,000 seeds segregating for male-sterility+Ignite-tolerance were planted, occupying approximately 0.25 acre.

Termination of experiment

May-93 planting: Hand-pollinated ears were harvested from the trial 1-Sep-93, and held in storage at our Kaunakakai, Molokai station. Remaining plant material and seed from open-pollinated ears was disked into the soil in early September 1993.

Sep-93 planting: Hand-pollinated ears were harvested from the trial 28-Dec-93, and held in storage at our Kaunakakai, Molokai station. Remaining plant material and seed from open-pollinated ears was disked into the soil in early January 1994.

Jan-94 planting: Hand-pollinated ears were harvested from the trial 17-Apr-94, and held in storage at our Kaunakakai, Molokai station. Remaining plant material and seed from open-pollinated ears was disked into the soil in late April 1994.

For all plantings: After this initial disking into the soil, the sites were left fallow for several months. After the initial disking each site went through 3 cycles of (1) irrigation, (2) fallow for about 3 weeks, (3)

disking to destroy any seedlings.

Monitoring for Volunteers

For all plantings: During the first irrigation-fallow-disking cycle, many volunteers were observed, as is typical for our standard corn plantings in Hawaii. During the second irrigation-fallow-disking cycle, a handful of volunteers were observed, as is typical in our standard plantings. During the third cycle, no volunteers were observed, as is typical in our standard plantings.

HOLDEN'S FOUNDATION SEEDS, INC.
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FAX 319 668-2453
319 668-1100

27 June 1995

Field Trial Report
USDA Notification Number: 94-080-11N
(applicant reference: 94 IA/IN MS)

Submitted by: Dr. Lori Marshall Lori Marshall
Holden's Foundation Seeds, Inc.
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Williamsburg, Iowa 52361
phone: 319/668-1100
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Test site information:
One site in Iowa was planted, in Iowa County, near Holden's Foundation Seeds, Inc. headquarters in Williamsburg, IA. (A second site was requested in the notification letter but never used.) Standard fertilizers and herbicides were used as is typical for our traditional corn breeding research.

The Indiana site was cancelled (never planted).

Summary of experimental results:
Segregation. The plants segregated as expected for a single nuclear gene when treated with Ignite™ (active ingredient glufosinate) or when observed for male-sterility. The Ignite-tolerance and male-sterility co-segregated. The MS3 transgenic plants are in a backcrossing program in which the MS3 gene is being crossed into a set of Holden's proprietary inbred lines (designated "LH" followed by a number). This set includes inbreds which represent very diverse germplasm types, and the MS3 gene appears to segregate and express as expected in this wide sampling of genetic backgrounds. There were a few cases in which F1 or BC1 generation material did demonstrate anther extrusion, and in a few cases fertile pollen was observed. We observed this partial male-sterility only

in early backcross generation material. Later backcross generations of the same inbred backgrounds showed the expected tight sterility.

Observations on phenotype. The plants appeared completely normal in their phenotype. The rows containing transgenic plants were planted adjacent to rows of nontransgenic standard corn inbred lines. During the course of the growing season the following circumstances allowed comparision of transgenic vs non-transgenic corn lines:

Response to generally excellent growing conditions - no differences detected.

Flowering - no differences in silk extrusion detected. Male-sterile tassels were often more slender than nontransgenic standards, but appeared to emerge similarly.

Seed set on hand-pollinated ears - no differences detected. Transgenic plants had good seed set and kernel quality.

Late-season plant appearance (susceptibility to stalk-rotting diseases, ear molds and smuts, natural infestations of European Corn Borer, and general plant vigor) - no differences detected.

The transgenic plants displayed the expected tolerance to glufosinate and male-sterility but no other apparent change in phenotype (including no change in disease status).

There were no unanticipated differences in morphology, weediness, flowering (silk) characteristics, or long-term survivability of progeny observed.

Size and containment for trial

This transgenic material was planted during the 1994 growing season in Iowa. Isolation from other corn was used to contain the trial, and no compromise to this containment was observed.

31-May-94 planting: Approximately 15,000 seeds segregating for male-sterility+Ignite-tolerance were planted, occupying approximately 0.9 acre.

HOLDEN'S FOUNDATION SEEDS, INC.
P.O. Box 839, Williamsburg, Iowa 52361
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27 June 1995

Field Trial Report
USDA Notification Number: 94-080-10N
(applicant reference: 94 HI MS)

Submitted by: Dr. Lori Marshall Lori Marshall
Holden's Foundation Seeds, Inc.
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Williamsburg, Iowa 52361
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Test site information:
The 3 plantings were grown from May 1994 to May 1995, in Maui County, Molokai, Hawaii, near Holden's Foundation Seeds, Inc. office in Kaunakakai.

Summary of experimental results:
Segregation. The plants segregated as expected for a single nuclear gene when treated with Ignite™ (active ingredient glufosinate) or when observed for male-sterility. The Ignite-tolerance and male-sterility co-segregated. The MS3 transgenic plants are in a backcrossing program in which the MS3 gene is being crossed into a set of Holden's proprietary inbred lines (designated "LH" followed by a number). This set includes inbreds which represent very diverse germplasm types, and the MS3 gene appears to segregate and express as expected in this wide sampling of genetic backgrounds. There were a few cases in which F1 or BC1 generation material did demonstrate anther extrusion, and in a few cases fertile pollen was observed. We observed this partial male-sterility only in early backcross generation material. Later backcross generations of the same inbred backgrounds showed the expected tight sterility.

Observations on phenotype. The plants appeared completely normal in their phenotype. The rows containing transgenic plants were planted

Termination of exeriment

Hand-pollinated ears were harvested from the trial during the month of October 1994, and held in storage at our Williamsburg, Iowa station. Remaining plant material was disked into the soil in April 1995.

Monitoring for Volunteers

In late April 1995, numerous volunteers were observed at the site, as expected since we were unable to complete the fall plowing in November 1994 due to unfavorable weather. The frequency of volunteers was the same for the transgenic plants as for an area within the transgenic isloation field that was planted to non-transgenic corn, and was consistent with our general experience with similiar situations. Volunteers were mechanically removed during May 1995.

adjacent to rows of nontransgenic standard corn inbred lines. During the course of the growing season the following circumstances allowed comparision of transgenic vs non-transgenic corn lines:

Response to standard irrigation and Hawaii's warm soil temperatures during germination period - no differences detected.

Response to standard "fertigation" (fertilizer applied through irrigation system) - no differences detected.

Response to normal heavy insect pressure (varied species, typically including leafhoppers, aphids, thrips, spider mites, ear worms) - no differences detected.

Flowering - no differences in silk extrusion detected. Male-sterile tassels were often more slender than nontransgenic standards, but appeared to emerge similarly.

Seed set on hand-pollinated ears - no differences detected. Transgenic plants had good seed set and kernel quality.

Late-season plant appearance (susceptibility to stalk-rotting diseases, ear molds and smuts, and general plant vigor) - no differences detected.

The transgenic plants displayed the expected tolerance to glufosinate and male-sterility but no other apparent change in phenotype (including no change in disease status).

There were no unanticipated differences in morphology, weediness, flowering (silk) characteristics, or long-term survivability of progeny observed.

Size and containment for trial

This transgenic material was planted 3 times during the period from May 1993 to May 1994. For all three plantings, isolation from other corn was used to contain the trial, and no compromise to this containment was observed.

4-May-94 planting: Approximately 2,000 seeds segregating for male-sterility+Ignite-tolerance were planted, occupying approximately 0.1

acre.

26-Aug-94 planting: Approximately 4,000 seeds segregating for male-sterility+Ignite-tolerance were planted, occupying approximately 0.25 acre.

16-Dec-94 planting: Approximately 20,000 seeds segregating for male-sterility+Ignite-tolerance were planted, occupying approximately 1.0 acre.

Termination of exeriment

May-94 planting: Hand-pollinated ears were harvested from the trial 10-Aug-94, and held in storage at our Kaunakakai, Molokai station. Remaining plant material and seed from open-pollinated ears was disked into the soil in early September 1994.

Aug-94 planting: Hand-pollinated ears were harvested from the trial 1-Dec-94, and held in storage at our Kaunakakai, Molokai station. Remaining plant material and seed from open-pollinated ears was disked into the soil in early December 1994.

Dec-94 planting: Hand-pollinated ears were harvested from the trial 24-Apr-95, and held in storage at our Kaunakakai, Molokai station. Remaining plant material and seed from open-pollinated ears was disked into the soil in late April 1995.

For all plantings: After this initial disking into the soil, the sites were left fallow for several months. After the initial disking each site went through 3 cycles of (1) irrigation, (2) fallow for about 3 weeks, (3) disking to destroy any seedlings.

Monitoring for Volunteers

For all plantings: During the first irrigation-fallow-disking cycle, many volunteers were observed, as is typical for our standard corn plantings in Hawaii. During the second irrigation-fallow-disking cycle, a handful of volunteers were observed, as is typical in our standard plantings. During the third cycle, no volunteers were observed, as is typical in our standard plantings.

FIELD SUMMARY:

NUCLEAR MALE STERILE CORN

USDA-APHIS FIELD RELEASE PERMIT 92-245-02

REPORT ONE

CARGILL HYBRID SEEDS

SUBMITTED TO:
U.S. DEPARTMENT OF AGRICULTURE
HAWAII DEPARTMENT OF AGRICULTURE

NUCLEAR MALE STERILE CORN

USDA-APHIS FIELD RELEASE PERMIT 92-245-05

HAWAII - 1992

FIELD INFORMATION

REPORT ONE

FIELD RELEASE TEST SITE:

Field OloOlo: Maui County, Molokai Island, Hawaii.

PLANTING DATE:

December 5, 1992.

TREATMENTS:

No treatments were applied, fertile plants were bagged.

HARVEST:

April 7, 1993.

NUCLEAR MALE STERILE CORN

USDA-APHIS FIELD RELEASE PERMIT 92-245-02

HAWAII - 1992

GENERAL INFORMATION

REPORT ONE

PURPOSE:

To backcross male sterile material into Cargill elite germplasm. The F1 is expected to be segregating so that 50% of the plants are expected to carry the gene for male sterility. Plants will be visually evaluated for the sterility trait. Sterile plants will be advanced in our breeding program, fertile plants will be bagged.

OBSERVATIONS MADE:

Morphology traits of engineered lines versus non-engineered lines.

Visual monitoring of the male sterile phenotype in the engineered lines.

MORPHOLOGY:

No morphological differences were observed between the engineered lines and the non-engineered lines.

VOLUNTEERS:

The field release test site was monitored for volunteers, none were found.

NUCLEAR MALE STERILE CORN

USDA-APHIS FIELD RELEASE PERMIT 92-245-02

HAWAII - 1992

GENERAL INFORMATION

REPORT ONE

SEGREGATION DATA: Based on visual observations of the sterility phenotype. The segregation ratio based on the flower phenotype has to be considered with caution due to the appearance of partial sterility in the F1 and early backcross stages. Please note that the data expressed below regards the F1 planting.

line	sterile plants	fertile plants	ratio (S:F)
MS1-01 X U03			
MS1-01 X U09			
MS1-01 X U04			
MS1-01 X U06			
MS1-01 X U05			
TOTAL	**17**	**39**	**1 : 2.3**
MS2-01 X U03			
MS2-01 X U04			
MS2-01 X U05			
MS2-01 X U06			
TOTAL	**18**	**21**	**1 : 1.2**
MS3-01 X U03	59	60	1 : 1
MS3-01 X U09	92	100	1 : 1.1
MS3-01 X U07	12	15	1 : 1.25
MS3-01 X U05	10	18	1 : 1.8
MS3-01 X U06	14	11	1 : 0.8
MS3-01 X U02	49	39	1 : 0.8
TOTAL	**236**	**243**	**1 : 1**

MS3-01 containing lines were advanced into subsequent breeding programs. MS1-01 and MS2-01 were discontinued from future development.

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ANNUAL FIELD RELEASE SUMMARY

REPORT TWO

USDA/APHIS PERMIT 92-245-02

NUCLEAR MALE STERILE CORN

CARGILL HYBRID SEEDS

SUBMITTED TO:
U.S. DEPARTMENT OF AGRICULTURE
HAWAII DEPARTMENT OF AGRICULTURE

ANNUAL FIELD RELEASE SUMMARY

REPORT TWO

USDA/APHIS PERMIT 92-245-02

NUCLEAR MALE STERILE CORN

FIELD INFORMATION

LOCATION: Molokai Island, Maui County, Hawaii
Field 18, Newhart

DATE PLANTED: May 26, 1993

PLOT SIZE: Less than 40 feet X 120 feet

TREATMENT: July 2, 1993, 1% BASTA. Hawaii EUP approved.
Registrant: Tom Hill, Cargill Hybrid Seeds
Applicator license number C30802.
Expiration: 3/28/98.

DATE HARVESTED: September 13, 1993

VOLUNTEER INFORMATION: The field was monitored routinely for volunteers, none were detected.

SEGREGATION DATA: Variances from the normal 1:1 segregation was observed in the field. We expected and confirmed the appearance of partial sterility in the F1 and early backcrossing stages of conversion. Plants expressing partial sterility were bagged or detasseled.

MORPHOLOGICAL DATA: No differences were noted between the transgenic and non-transgenic controls with respect to weediness, insect or disease susceptibility.

FIELD OBSERVATIONS MADE:

- Visually response to glufosinate in genetically modified lines

- Morphology traits of engineered lines versus non-engineered control plants.

- Visual monitoring of the male sterile phenotype in engineered lines.

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ANNUAL FIELD RELEASE SUMMARY

REPORT THREE

USDA/APHIS PERMIT 92-245-02

NUCLEAR MALE STERILE CORN

CARGILL HYBRID SEEDS

SUBMITTED TO:
U.S. DEPARTMENT OF AGRICULTURE
HAWAII DEPARTMENT OF AGRICULTURE

ANNUAL FIELD RELEASE SUMMARY

REPORT THREE

USDA/APHIS PERMIT 92-245-02

NUCLEAR MALE STERILE CORN

FIELD INFORMATION

LOCATION: Molokai Island, Maui County, Hawaii
Field 14, Guiterres

DATE PLANTED: October 18, 1993

PLOT SIZE: 2145 ft^2

TREATMENT: 1% BASTA. Hawaii EUP approved.
Registrant: Tom Hill, Cargill Hybrid Seeds
Applicator license number C30802.
Expiration: 3/28/98.

DATE HARVESTED: February 16, 1994

FIELD OBSERVATIONS MADE:

- Visually response to glufosinate in genetically modified lines

- Morphology traits of engineered lines versus non-engineered control plants.

- Visual monitoring of the male sterile phenotype in engineered lines.

SEGREGATION DATA: Normal 1:1 segregation was observed in the field. No breakage of the sterility trait was observed.

MALE STERILE PHENOTYPE: The male sterility trait appears to be tightly linked to glufosinate resistance. No break in the sterility trait was observed.

VOLUNTEER INFORMATION: The field was monitored routinely for volunteers, none were detected.

MORPHOLOGICAL DATE: No differences were noted between the transgenic and non-transgenic controls with respect to weediness, insect or disease susceptibility.

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ANNUAL FIELD RELEASE SUMMARY

USDA/APHIS NOTIFICATION 94-076-23N

MAINLAND U.S.

NUCLEAR MALE STERILE CORN

FIELD SEGREGATION DATA

LOCATION: KANE COUNTY, ILLINOIS

A. PURPOSE: Segregation of Male Sterility trait.

LINE:	STERILE	NORMAL	RATIO
U03	86	91	1 : 1.1

B. PURPOSE: Stability of Male Sterile trait in different public inbreds.

LINE:	STERILE	NORMAL	RATIO
MS3 x B73	65	92	1 : 1.4
MS3 X A632	74	84	1 : 1.1
MS3 X A239	63	73	1 : 1.2
MS3 X PA91	48	56	1 : 1.2

C. PURPOSE: First year evaluation of hybrids.

LINE:	STERILE	NORMAL	RATIO
MS3	32	42	1 : 1.3
MS4	27	20	1 : 0.7
MS6	45	38	1 : 0.8

D. PURPOSE: Glufosinate tolerance - line MS3.

LINE:	STERILE	NORMAL	RATIO
2.5X	81	77	1 : 1
3X	81	72	1 : 0.9
4X	41	53	1 : 1.3
5X	74	84	1 : 1.1

E. PURPOSE: Glufosinate tolerance - line MS4.

LINE:	STERILE	NORMAL	RATIO
2.5X	72	77	1 : 1.1
3X	79	80	1 : 1
4X	46	39	1 : 0.8
5X	69	76	1 : 1.1

F. PURPOSE: Segregation data - F1s

LINE:	STERILE	NORMAL	RATIO
MS4 X U03	35	27	1 : 0.8
MS6 X U03	19	14	1 : 0.7

G. GREENHOUSE SEGREGATION DATA (1993 - 1995)

LINE:	STERILE	NORMAL	RATIO
MS3 X U03	663	686	1 : 1
MS3 X U04	197	204	1 : 1
MS3 X U06	82	81	1 : 1
MS3 X U02	52	82	1 : 1.6
MS3 X U09	14	6	1 : 0.4
MS4 X U03	72	74	1 : 1
MS6 X U03	50	74	1 : 1.5

H. GERMINATION RESULTS OF GREENHOUSE GROWN U03 MALE STERILE HYBRID SEED VS FIELD GROWN COMMERCIAL HYBRID

- Twenty samples each of greenhouse grown U03 Male Sterile Hybrid Seed, and field grown commercial hybrid seed were germinated in laboratory conditions. This experiment will be repeated with seed grown in the same environment.

	Avg Germination
- U03 Male Sterile Hybrid Seed	93.2%
- Field Grown Commercial Hybrid Seed	97.6%

I. COMPOSITION ANALYSIS OF MALE STERILE VS MALE FERTILE SEED

- The starch, protein and oil composition of line MS3 X U03 was analyzed to evaluate potential differences between this and male fertile seed. No significant differences were found (See Table 1).

Genetically Engineered

MS3, U03

Date Input	R&D Lab #	Hybrid #	Bottom Corner#		Drawer
11/4/94	7000	RA25	229K	Sterile	11.1
11/4/94	7001	RA26	444K	Sterile	11.1
11/4/94	7002	QA45	468K	Fertile	11.1
11/4/94	7003	QA60	391K	Sterile	11.1
11/4/94	7004	QA62	381K	Sterile	11.1
11/4/94	7005	QA63	452K	Sterile	11.1
11/4/94	7006	QA64	420K	Fertile	11.1
11/4/94	7007	QA65	448K	Sterile	11.1
11/4/94	7008	QA66	295K	Fertile	11.1
11/4/94	7009	QA67	388K	Fertile	11.1
11/4/94	7010	QA61	92K	Fertile	11.1

R&D #	% Moisture	Protein %As Is Loco	Protein % D.B. Loco	Oil % As Is Spox Mill	Oil % D.B. Spox Mill	Starch % As Is Starch	Starch % D.B. Starch
7000	11.74	10.04	11.37	4.09	4.63	61.36	69.52
7001	11.61	9.43	10.67	4.39	4.97	61.28	69.33
7002	11.68	9.83	11.13	4.27	4.83	61.16	69.26
7003	11.62	10.76	12.18	4.29	4.86	60.97	68.99
7004	11.60	10.16	11.48	4.23	4.78	60.94	68.93
7005	11.42	10.37	11.70	4.46	5.03	59.13	66.75
7006	11.44	10.35	11.69	4.49	5.07	60.13	67.89
7007	11.60	9.00	10.19	4.37	4.94	62.90	71.16
7008	11.37	10.82	12.21	4.20	4.74	58.38	65.87
7009	11.69	8.80	9.96	3.92	4.44	63.38	71.77
7010	0.00	11.46	11.46	0.00	0.00	56.14	56.14

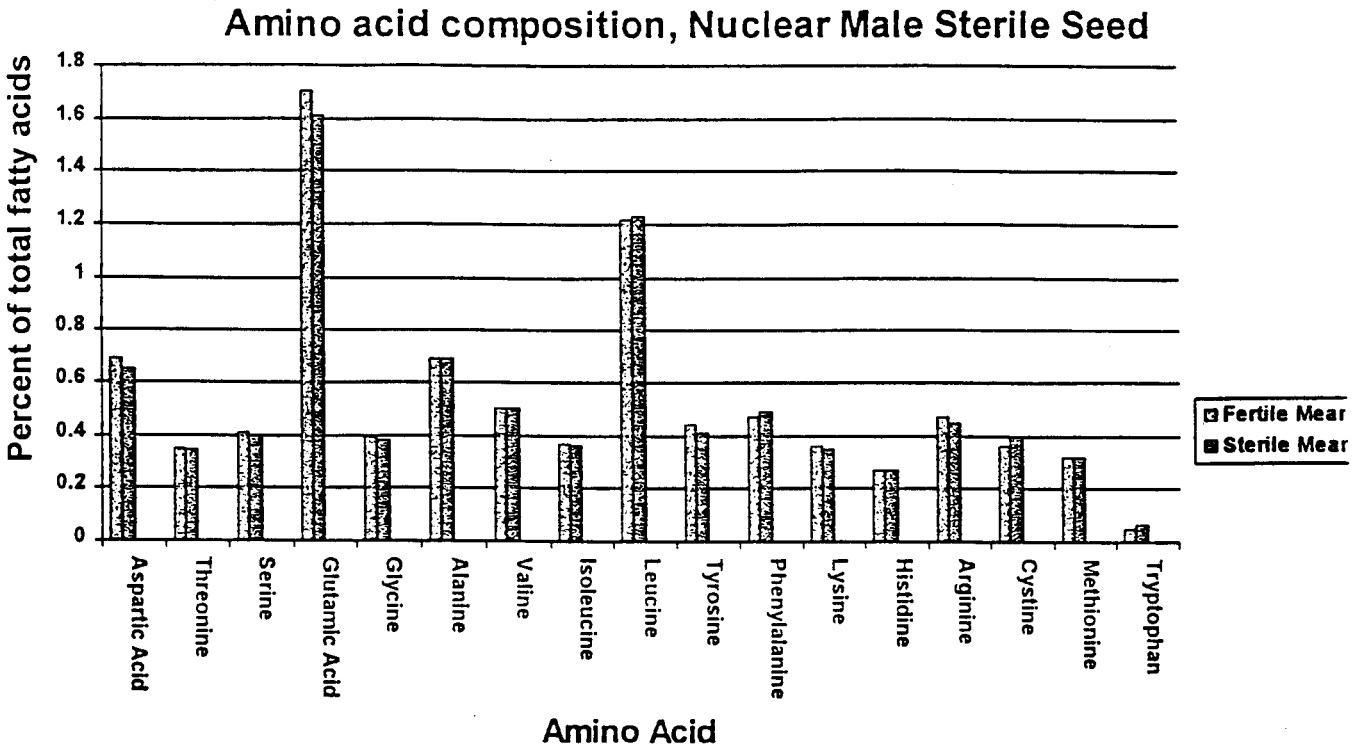
- Fatty Acid Composition analysis:

Male Fertile

Fatty Acid	Mean % total Fatty Acid (N=4)
C16:0	13.11
C18:0	2.15
C18:1	25.16
C18:2	55.83
C18:3	1.16

Male Sterile

Fatty Acid	Mean % total Fatty Acid (N=6)
C16:0	13.08
C18:0	2.37
C18:1	27.48
C18:2	53.37
C18:3	1.15


Amino acid composition, Nuclear Male Sterile Seed
Percent of total fatty acids
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Aspartic Acid
Threonine
Serine
Glutamic Acid
Glycine
Alanine
Valine
Isoleucine
Leucine
Tyrosine
Phenylalanine
Lysine
Histidine
Arginine
Cystine
Methionine
Tryptophan
Amino Acid
Fertile Mear
Sterile Mear

LOCATION: Kane County, IL; Piatt County, IL; Carroll County, MO; Miami County, OH; Poweshiek County, IA.

Purpose: Evaluate Nuclear Male Sterility Trait in a BC3 engineered hybrid versus a commercial hybrid.

Plot Size: 0.010 acres/site

Observations Made:

- No differences were noted between the transgenic and non-transgenic controls with respect to weediness, insect or disease susceptibility

- Visual monitoring of the male sterile phenotype in engineered lines.

- The yield from engineered line advanced in the trial was not significantly different from the commercial hybrid with which it was compared.

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FIELD SUMMARY:

NUCLEAR MALE STERILE CORN

USDA-APHIS FIELD RELEASE PERMIT 92-080-05

AURORA, ILLINOIS - 1992

CARGILL HYBRID SEEDS

SUBMITTED TO:
U.S. DEPARTMENT OF AGRICULTURE
ILLINOIS DEPARTMENT OF AGRICULTURE

NUCLEAR MALE STERILE CORN

USDA-APHIS FIELD RELEASE PERMIT 92-080-05

AURORA, ILLINOIS - 1992

FIELD INFORMATION

PLANTED IN GREENHOUSE: June 10, 1992

TRANSPLANTED TO FIELD TEST SITE: June 22, 1992

TREATED: July 17, 1992

TREATMENTS:

Kanamycin dot test:
A kanamycin solution was applied to leaf tissue. New leaves of kanamycin sensitive plants were yellow/white in appearance.

Glufosinate dot test:
A BASTA solution was applied to leaf tissue. Glufosinate sensitive plants demonstrated a localized necrosis of the treated leaves.

HARVEST: October 7 - October 15, 1992

FIELD GLEANED: October 14 - October 15, 1992

NUCLEAR MALE STERILE CORN

USDA-APHIS FIELD RELEASE PERMIT 92-080-05

AURORA, ILLINOIS - 1992

GENERAL INFORMATION

PURPOSE:

To backcross male sterile material into Cargill elite germplasm. Progeny is expected to be segregating so that 50% of the plants are expected to carry the gene for male sterility and for the marker. Plants will be evaluated with glufosinate or kanamycin.

OBSERVATIONS MADE:

Visual response to glufosinate or kanamycin dot tests in genetically modified lines.

Morphology traits of engineered lines versus non-engineered lines.

Visual monitoring of the male sterile phenotype in the engineered lines.

MORPHOLOGY:

No differences were observed between the engineered lines and the non-engineered lines.

MALE STERILE PHENOTYPE:

The male sterility trait appeared to be tightly linked to either glufosinate or kanamycin resistance. No break in the sterility trait was observed.

VOLUNTEERS:

The field release test site was monitored for volunteers, none were found.

SEGREGATION DATA:

	resistant plants	normal plants	ratio
glufosinate marker:	20	17	1 : 1.2
kanamycin marker:	10	7	1 : 1.4

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FIELD SUMMARY:

NUCLEAR MALE STERILE CORN

USDA-APHIS FIELD RELEASE PERMIT 93-043-02

AURORA, ILLINOIS - 1993

CARGILL HYBRID SEEDS

NUCLEAR MALE STERILE CORN

USDA-APHIS FIELD RELEASE PERMIT 93-043-02

AURORA, ILLINOIS - 1993

FIELD INFORMATION

PLANTED:

May 18, 1993

TREATED:

June 22, 1993

TREATMENTS:

Glufosinate:
A 0.5% BASTA solution (commercial formulation) was sprayed on each plant. Glufosinate sensitive plants exhibited necrosis.

HARVEST:

October 7-15, 1993

FIELD GLEANED:

October 14-15

TRANSGENIC PLOT SIZE:

80 ft X 120 ft

VOLUNTEERS:
The test site was monitored routinely for volunteers, none were detected.

NUCLEAR MALE STERILE CORN

USDA-APHIS FIELD RELEASE PERMIT 93-043-02

AURORA, ILLINOIS - 1993

SEGREGATION DATA

KANAMYCIN MARKER:

line	sterile	normal	ratio
MS2-01 (RZM35) X U07*	STRESSED		
	STRESSED		
MS2-01 (RZM35) X U08*	STRESSED		
	STRESSED		

* line discontinued from further development

BASTA MARKER:

line	sterile	normal	ratio (S:N)
MS3-01 (RZM34) X U02	14	15	1 : 1.1
	18	14	1 : 0.8
	20	11	1 : 0.6
	19	12	1 : 0.6
	18	10	1 : 0.6
TOTAL	89	62	1 : 0.7
MS3-01 (RZM34) X U03	14	15	1 : 1.1
	15	12	1 : 0.8
	1	0	
TOTAL	30	27	1 : 0.9
MS3-01 (RZM34) X U04	15	16	1 : 1.1
	12	17	1 : 1.4
	13	14	1 : 1.1
	13	19	1 : 1.4
	12	17	1 : 1.4
	9	19	1 : 2
TOTAL	74	102	1 : 1.4
MS3-01 (RZM34) X U05	15	13	1 : 0.8
	13	15	1 : 1.1
	17	11	1 : 0.7
	18	14	1 : 0.8
	17	12	1 : 0.7
	8	20	1 : 2.5
TOTAL	88	85	1 : 1

NUCLEAR MALE STERILE CORN

USDA-APHIS FIELD RELEASE PERMIT 93-043-02

AURORA, ILLINOIS - 1993

SEGREGATION DATA

BASTA MARKER (CONT):

line	sterile	normal	ratio (S:N)
MS3-01 (RZM34) X U06	17	15	1 : 0.9
	14	15	1 : 1.1
	12	12	1 : 1
	16	20	1 : 1.3
	15	17	1 : 1.1
	3	0	
TOTAL	77	79	1 : 1

NUCLEAR MALE STERILE CORN

BC2 SEGREGATION DATA

AURORA GREENHOUSE SUMMER 1993

CROSS	FAMILY	BC1 ID	SEG	RATIO (STERILE:NORMAL)
MS3-01 X U07	DA14	BA67-8	19:24	1 : 1.3
MS3-01 X U07	DA04	BA67-17	17:17	1 : 1
MS3-01 X U07	CA96	BA67-19	22:26	1 : 1.2
MS3-01 X U07	DA05	BA67-42	23:25	1 : 1.1
MS3-01 X U07	CA95	BA67-62	13:15	1 : 1.2
MS3-01 X U07	DA06	BA67-93	26:27	1 : 1
MS3-01 X U07	DA03	BA67-66	19:29	1 : 1.5
AVERAGE SEGREGATION:			**139:163**	**1 : 1.2**

CROSS	FAMILY	BC1 ID	SEG	RATIO (STERILE:NORMAL)
MS3-01 X U05	CA94	BA92-5	8:8	1 : 1
AVERAGE SEGREGATION:			**8:8**	**1 : 1**

CROSS	FAMILY	BC1 ID	SEG	RATIO (STERILE:NORMAL)
MS3-01 X U06	DA60	BA81-1	12:8	1 : 0.7
AVERAGE SEGREGATION:			**12:8**	**1 : 0.7**

CROSS	FAMILY	BC1 ID	SEG	RATIO (STERILE:NORMAL)
MS3-01 X U02	CA93	BA87-2	7:14	1 : 2
AVERAGE SEGREGATION:			**7:14**	**1 : 2**

CROSS	FAMILY	BC1 ID	SEG	RATIO (STERILE:NORMAL)
MS3-01 X U08	CA98	BA98-22	31:26	1 : 0.9
MS3-01 X U08	DA08	BA99-7	40:50	1 : 1.25
MS3-01 X U08	CA99	BA99-8	55:53	1 : 1
AVERAGE SEGREGATION:			**126:129**	**1 : 1**

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ANNUAL FIELD RELEASE SUMMARY

REPORT ONE

USDA/APHIS NOTIFICATION 94-076-023N

NUCLEAR MALE STERILE CORN

CARGILL HYBRID SEEDS

SUBMITTED TO:
U.S. DEPARTMENT OF AGRICULTURE
HAWAII DEPARTMENT OF AGRICULTURE

ANNUAL FIELD RELEASE SUMMARY

REPORT ONE

USDA/APHIS NOTIFICATION 94-076-23N

NUCLEAR MALE STERILE CORN

FIELD INFORMATION

LOCATION: Molokai Island, Maui County, Hawaii
Field 14, Guiterres

DATE PLANTED: May 20, 1994

PLOT SIZE: 3729 ft^2

TREATMENT: 1% BASTA. AgrEvo EUP approved.
Registrant: Tom Hill, Cargill Hybrid Seeds
Applicator license number C30802. Expiration: 3/28/98.

DATE HARVESTED: September 7, 1994

FIELD OBSERVATIONS MADE:

- Visually response to glufosinate in genetically modified lines

- Morphology traits of engineered lines versus non-engineered control plants.

- Visual monitoring of the male sterile phenotype in engineered lines.

SEGREGATION DATA: Normal 1:1 segregation was observed in the field. No breakage of the sterility trait was observed.

MALE STERILE PHENOTYPE: The male sterility trait appears to be tightly linked to glufosinate resistance. No break in the sterility trait was observed.

VOLUNTEER INFORMATION: The field was monitored routinely for volunteers, none were detected.

MORPHOLOGICAL DATE: No differences were noted between the transgenic and non-transgenic controls with respect to weediness, insect or disease susceptibility.

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ANNUAL FIELD RELEASE SUMMARY

REPORT TWO

USDA/APHIS NOTIFICATION 94-076-023N

NUCLEAR MALE STERILE CORN

CARGILL HYBRID SEEDS

SUBMITTED TO:
U.S. DEPARTMENT OF AGRICULTURE
HAWAII DEPARTMENT OF AGRICULTURE

ANNUAL FIELD RELEASE SUMMARY

REPORT TWO

USDA/APHIS NOTIFICATION 94-076-23N

NUCLEAR MALE STERILE CORN

FIELD INFORMATION

LOCATION: Molokai Island, Maui County, Hawaii
Field 14, Guiterres

DATE PLANTED: September 26, 1994

PLOT SIZE: 4059 ft^2

TREATMENT: 1% BASTA. AgrEvo EUP approved.
Registrant: Tom Hill, Cargill Hybrid Seeds
Applicator license number C30802. Expiration: 3/28/98.

DATE HARVESTED: January 23, 1995

ANNUAL FIELD RELEASE SUMMARY

REPORT THREE

USDA/APHIS NOTIFICATION 94-076-023N

NUCLEAR MALE STERILE CORN

CARGILL HYBRID SEEDS

SUBMITTED TO:
U.S. DEPARTMENT OF AGRICULTURE
HAWAII DEPARTMENT OF AGRICULTURE

ANNUAL FIELD RELEASE SUMMARY

REPORT THREE

USDA/APHIS NOTIFICATION 94-076-23N

NUCLEAR MALE STERILE CORN

FIELD INFORMATION

LOCATION: Molokai Island, Maui County, Hawaii
Field 14, Guiterres

DATE PLANTED: February 2, 1995

PLOT SIZE: 4719 ft^2

TREATMENT: 1% BASTA. AgrEvo EUP approved.
Registrant: Tom Hill, Cargill Hybrid Seeds
Applicator license number C30802. Expiration: 3/28/98.

DATE HARVESTED: May 26, 1995

FIELD OBSERVATIONS MADE:

- Visually response to glufosinate in genetically modified lines

- Morphology traits of engineered lines versus non-engineered control plants.

- Visual monitoring of the male sterile phenotype in engineered lines.

SEGREGATION DATA: Normal 1:1 segregation was observed in the field. No breakage of the sterility trait was observed.

MALE STERILE PHENOTYPE: The male sterility trait appears to be tightly linked to glufosinate resistance. No break in the sterility trait was observed.

VOLUNTEER INFORMATION: The field was monitored routinely for volunteers, none were detected.

MORPHOLOGICAL DATA: No differences were noted between the transgenic and non-transgenic controls with respect to weediness, insect or disease susceptibility.

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ANNUAL FIELD RELEASE SUMMARY

USDA/APHIS NOTIFICATION 94-076-23N

MAINLAND U.S.

NUCLEAR MALE STERILE CORN

CARGILL HYBRID SEEDS

SUBMITTED TO:
U.S. DEPARTMENT OF AGRICULTURE
ILLINOIS DEPARTMENT OF AGRICULTURE
IOWA DEPARTMENT OF AGRICULTURE
MISSOURI DEPARTMENT OF AGRICULTURE
OHIO DEPARTMENT OF AGRICULTURE

ANNUAL FIELD RELEASE SUMMARY

USDA/APHIS NOTIFICATION 94-076-23N

MAINLAND U.S.

NUCLEAR MALE STERILE CORN

FIELD INFORMATION

LOCATION: Kane County, IL

DATE PLANTED: May 18 - May 21, 1994

PLOT SIZE: less than 0.50 acres

TREATMENT: 243 g a.i. glufosinate/acre (600 g/ha)

DATE HARVESTED: October 25 - October 26, 1994

VOLUNTEER INFORMATION: The field was monitored routinely for volunteers, none were detected.

SEGREGATION DATA: See attached.

MORPHOLOGICAL DATE: No differences were noted between the transgenic and non-transgenic controls with respect to weediness, insect or disease susceptibility.

Annex 10. USDA field trial termination reports

Permit/Notification Number	Test sites
Holden's Foundation Seeds, Inc.	
92-105-02	Iowa
92-244-03	Hawaii
93-076-02	Iowa
93-076-03	Hawaii
94-080-11N	Iowa/Illinois
94-080-10N	Hawai
Cargill Hybrid Seeds	
92-245-02	Hawaii
92-080-05	Illinois
93-043-02	Illinois
94-076-23N	Illinois/Hawaii/Indiana/Missouri/Ohio

Annex 10. USDA field trial termination reports

Annex 11.. Description of glufosinate ammonium

Glufosinate-ammonium

1. Introduction

The use of herbicides to control weeds is an important part of agricultural practices. Research efforts are directed towards the production of herbicides which are selectively toxic to weed species and environmentally safe (Lindsey et al., 1989). Selective toxicity of herbicides to particular plant species is one of the most difficult properties to achieve, as might be expected from the physiological similarities of weeds and crops. Selectivity is a function of the physicochemical properties of a compound, and of the biochemical interactions of the compound with the crop and the weed (Mazur et al., 1989). Selective insensitivity is restricted to only a few plant species, and a number of herbicides are equally toxic to both crop and weed (Botterman et al., 1988; Lindsey et al., 1989; Mazur et al., 1989).

Many herbicides are not selective, while others can be used selectively on certain crops under certain conditions. A number of important classes of herbicides are more toxic to weeds than to specific crops. In these examples, selectivity results from a unique or enhanced metabolic detoxification of the herbicide by the crop plant, but not by the weed. In other cases, herbicide selectivity results from the sequestering of the herbicide within an internal compartment of the crop plant. External barriers such as plant cuticles can prevent penetration of the herbicide. In some cases, it has been possible to achieve selectivity by seed coat applications of a 'safener', which reduces the toxicity of the herbicide to the crop (Botterman et al., 1988; Stalker et al., 1988; Mazur et al., 1989; Bulcke, 1990).

A major effort has been devoted in several laboratories to engineer selective herbicide-tolerant plants. At least three different mechanisms have been used. In the first, a mutant form of the target enzyme is produced which retains activity but is less sensitive to the herbicide (Botterman, 1989). Overproduction of the herbicide-sensitive biochemical target has been a second approach to obtain herbicide-tolerant plants by genetic engineering. Shah et al. (1986) demonstrated that a chimeric gene, designed to overproduce the target enzyme, conferred tolerance to the transformed calli and the regenerated transgenic plants. In a third approach, a gene coding for an enzyme that detoxifies or degrades the herbicide is incorporated into the plant genome (De Block et al., 1987; Stalker et al., 1988; Botterman et al., 1988).

A relatively new class of glufosinate-ammonium based herbicides acts by the inhibition of a specific amino acid biosynthesis pathway in plants (Wild et al., 1984; De Block et al., 1987; Wild et al., 1987). These herbicides are produced by *Streptomyces* species (Bayer et al., 1972; Leason et al., 1982; Sadaaki Mase, 1984; Murakami et al., 1986). They are highly effective against plants, but are safe to humans and animals and are rapidly biodegraded in the environment (Hoechst info brochure).

The herbicides bialaphos and phosphinothricin (PPT) are potent inhibitors of glutamine synthetase (GS), an enzyme that plays a central role in the assimilation of ammonia and in the regulation of the nitrogen metabolism in the plant (Bayer et al., 1972; Miflin et al., 1977; Sadaaki Mase, 1984; Murakami et al., 1986; Wild et al., 1987; Wendler et al., 1990).

2. Characterization of the herbicide

2.1. Main characteristics of the herbicide

Bialaphos, a tripeptide consisting of two L-alanine molecules and an L-glutamic acid analogue called phosphinothricin is produced by fermentation of *Streptomyces hygroscopicus* (Bayer et al., 1972; Sadaaki Mase, 1984; Murakami et al., 1986). Phosphinothricin (PPT) is the chemically synthesized product with the common name 'Glufosinate' or 'Glufosinate-ammonium' (CAS number 77182-82-2) as an ammonium salt. The commercial names BASTA®, BUSTER®, FINALE®, HARVEST®, CHALLENGE®, LIBERTY®, and IGNITE® are used depending on the country where it is commercialized.

The chemical name of bialaphos is N-{4-(hydroxy(methyl)phosphinoyl)-homoalanyl}alanylalanine, while the chemical formula (IUPAC) of glufosinate-ammonium is designated as ammonium-DL-homoalanin-4-yl(methyl)phosphinate. The structure formulation of both described compounds is given in Figures 1 and 2. The molecular formula of the commercially used glufosinate-ammonium is $C_5H_{15}N_2O_4P$, with a molecular weight of 198.2 (Hoechst info brochure). The chemical and physical properties of the technical active ingredient PPT are summarized in Table 1, while the toxicological properties of the compound are given in Table 2.

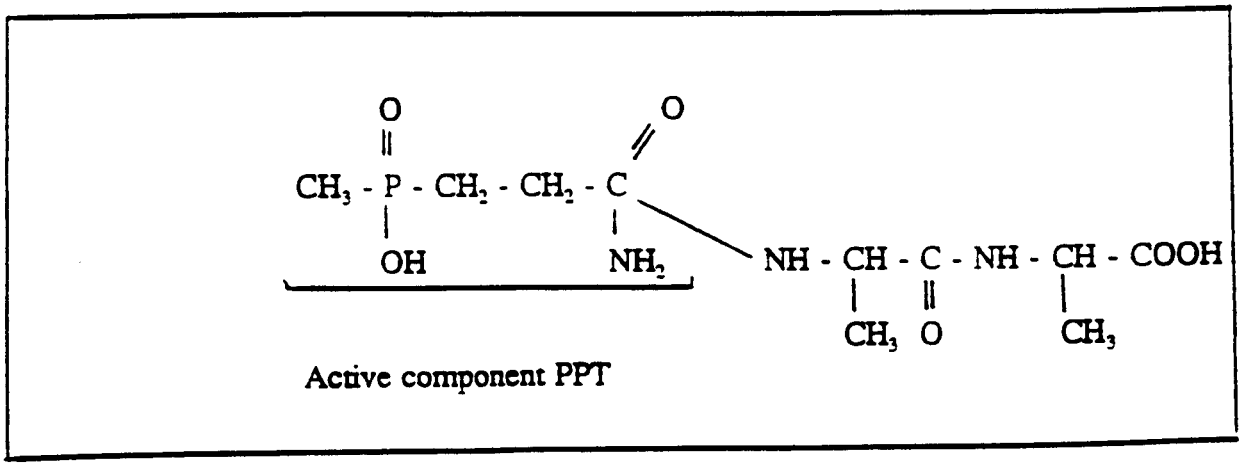

Figure 1. Structural formula of bialaphos

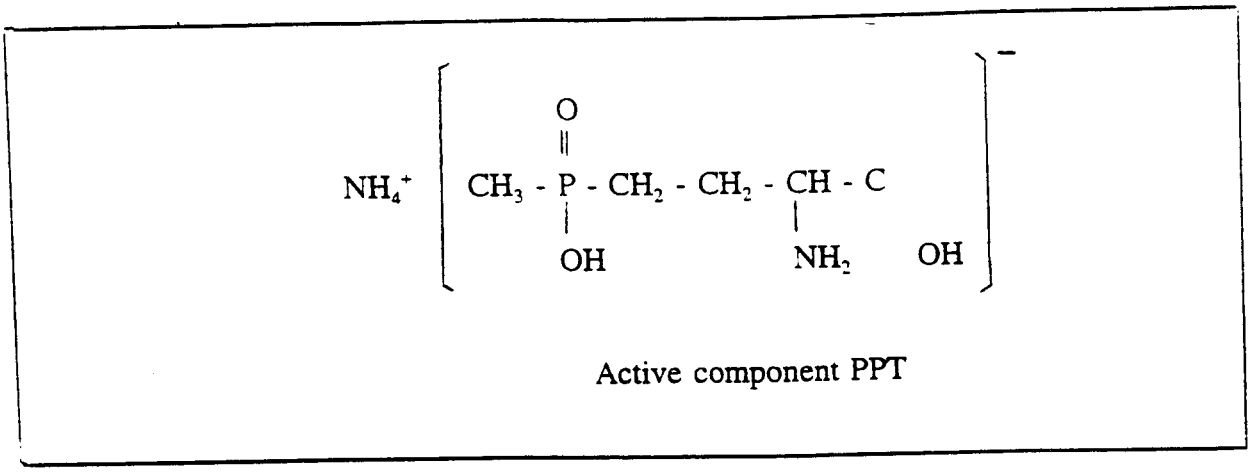

Figure 2. Structural formula of glufosinate ammonium

Table 1. Chemical and physical properties of the technical active ingredient (Hoechst info brochure)

Property	Phosphinothricin
Appearance	Crystalline powder
Colour	White to light yellow
Odour	Slightly pungent
Vapour pressure	Low, but due to composition
Stability	2 years in original sealed containers stored at 25 ±5°C
Solubility	Soluble in water

Table 2. **Toxicological properties of the technical active ingredient** (Hoechst info brochure)

Property	Phosphinothricin
Acute oral toxicity	LD_{50} Rat ♂ 2000mg/kg body weight Rat ♀ 1620mg/kg body weight
Acute dermal toxicity	LD_{50} Rat ♂ >4000mg/kg body weight Rat ♀ approx. 4000mg/kg body weight
Skin and eye irritation	No primary irritation of skin and eye mucosa was observed in rabbits
Inhalation toxicity (4 hours)	LC_{50} Rat ♂,♀ >4170mg/m^3 air product (Basta 20SL)
Subchronic toxicity (90-day feeding trial)	No effect level-rats : 18mg/kg body weight/day No effect level-dogs : 5mg/kg body weight/day
Chronic toxicity	No effect level-dogs : 5mg/kg body weight/day
Embryo toxicity	No observable effect level-rats : 10mg/kg body weight No observable effect level-rabbits : 6.3mg/kg body weight
Mutagenicity	Mutagenic tests in vitro and vivo did not show any mutagenic activity
Neurotoxicity	No signs of neurotoxic effects in hens
Ecological data	Toxicity to birds : *Coturnix* : LD_{50} > 2000mg/kg body weight Toxicity to fish : *Salmo gairdneri* : LC_{50} (96h) > 320 mg/l water Toxicity to beneficial arthropods : Bees : not toxic to bees (Basta 20SL)

2.2. Mode of action of the herbicide

Glufosinate-ammonium is defined as a non-selective and partially systemic contact herbicide. After uptake, the active ingredient phosphinothricin acts via the leaf. No action via the roots could be detected in plants after emergence and no damage is caused to seedlings before emergence. Shortly after the application, the herbicide will disturb the ammonium metabolism of the treated plants. The systemic transport from treated leaves to other parts of the plant is nevertheless limited (Wild et al., 1984; Manderscheid et al., 1985; Wild et al., 1987; Hoechst info brochure; Bulcke, 1990; Wendler et al.,1990).

2.2.1. Mode of action

Ammonia is an important link between catabolic and anabolic processes in the plant metabolism and it is released and reassimilated in large amounts at different processes. Regardless of the origin, however, it is essential that the ammonia is rapidly converted into a form that is not toxic to the organism (Wild et al., 1984). This detoxifying reaction is guided by the glutamine synthetase enzyme (GS).
Under normal conditions, ammonia, produced during various metabolic processes in the plant cell is primarily bound to glutamic acid to form glutamine. This process is catalyzed by the enzyme glutamine synthetase (GS), a key enzyme in the nitrogen metabolism and the only enzyme in plants that can detoxify ammonia in a sufficient way (Wedler et al., 1976; Miflin et al., 1977; Keys et al., 1978; Salisbury et al., 1978; Wild et al., 1984; Gebhardt et al., 1986, Wild et al., 1987; De Block et al., 1987).

Glufosinate-ammonium inhibits the activity of the GS enzyme, from which at least two isoenzymes that differ in their subcellar compartimentation, can occur in the green leaf tissue of higher plants (Miflin et al., 1977; Wild et al., 1984; Ridley et al., 1985). The active herbicidal compound PPT is an analogue of glutamate (Wendler et al., 1990) and is an exceptionally specific inhibitor of the glutamine synthetase enzyme (Wild et al., 1984). It appears to exert its effect as a competitive inhibitor of glutamine synthetase. As a result, the ammonium metabolism in the plant is disturbed shortly after the application of the herbicidal product (Wild et al., 1984; Manderscheid et al., 1986; Lindsey et al., 1989) and ammonia accumulates in the plant tissue (Wild et al., 1987).
Simultaneously, photosynthesis is also severely inhibited (Sauer et al., 1987; Bulcke, 1990; Wendler et al., 1990).

After PPT application, there seem to be three major potential sources for the lethal ammonia accumulation in the plant cells:

- in the photorespiration pathway, the glycine decarboxylase reaction produces not only CO_2, but also an equivalent amount of ammonia (Wild et al., 1987; Bulcke, 1990; Wendler et al., 1990);
- ammonia is formed in the course of nitrate assimilation by reduction of the externally absorbed NO_3^- (Wild et al., 1984; Wild et al., 1987; Bulcke, 1990);
- ammonia also occurs in catabolic and anabolic processes, such as in the breakdown of proteins or nucleotides and in the deamination of phenylalanine or tyrosine (Wild et al., 1984; Wild et al., 1987).

Inhibition of glutamine synthetase as an enzyme of the photorespiratory nitrogen cycle leads on one hand to the described accumulation of ammonia, but on the other hand also to the suppression of glutamine synthesis (Keys et al., 1978; Wendler et al., 1990). Accordingly, the lack of glutamine is essentially responsible for the early damage to photosynthesis by PPT. The disturbance of this amino acid metabolism might act on photosynthesis in different ways, all of them consequences of the lack of transaminations of the glutamine protein (Sauer et al., 1987; Wendler et al., 1990) :

- there is an inhibition of protein biosynthesis; in particular, the biosynthesis of ubiquinon B, a redox component involved in the electron transport during the light dependent turnover reaction, is disturbed, which results in the collapse of the electron transport;
- there is a toxic accumulation of glyoxylate, a reversible inhibitor of ribulose-1,5-diphosphate carboxylase/oxygenase;
- there is a lack of intermediates of the Calvin cycle : the lack of glutamine or any enzyme that prevents regeneration of the carbon channelled into the photorespiration cycle by the oxygenase reaction, finally results in a lack of Ribulose-diphosphate for the Calvin cycle.

Since ammonia is produced mainly during the reaction linked with photosynthetic electron transport, its accumulation is higher in treated plants exposed to light than in those kept in darkness or shade. Exposure to light also accelerates the development of phytotoxic symptoms, which start with the development of pale yellowish discoloration of the green plant parts. After two to five days, the withering followed by plant necrosis appears. Plants die within one or two weeks (Hoechst info brochure; Bulcke, 1990).

The optimum level of performance of the active component PPT can be achieved when local climatic conditions enable excellent growth and when the product is applied on young plants having a sufficient number of leaves to activate the metabolism. The activity is greatly influenced by climatic conditions. Temperatures below 10°C or drought reduce the rate of efficacy of the applied product, though the end effect isn't changed much. Good moisture conditions and higher temperatures improve the speed of action and partly the level of performance (Hoechst info brochure). Rainfall within six hours after the herbicidal application can negatively influence the effect of PPT (Hoechst info brochure; Bulcke, 1990).

2.2.2. Metabolism of phosphinothricin in soil and water

The active ingredient is highly stable as a chemical compound, but its degradation is rapid in an environment with microbial activity. Under natural conditions, there is no translocation in the soil layers deeper than 15 cm, which clearly indicates the rapid biodegradation. Glufosinate-ammonium is rapidly decomposed to 3-methyl phosphinicopropionic acid and finally to CO_2. There is no accumulation in the food chain (Hoechst info brochure; Bulcke, 1990).

2.2.3. Biosynthesis of the compounds

The pathway for the biosynthesis of bialaphos by *Streptomyces hygroscopicus* has been determined. The pathway shown (Figure 3) was investigated by analyzing the products that

were accumulated and converted by a series of nonproducing mutants (Murakami et al., 1986). It was shown that bialaphos is synthesized from three carbon precursors (probably pyruvate or phosphoenolpyruvate) in series of at least thirteen conversions. Many of the genes coding for these enzymes as well as a function which positively regulates their transcription, have been defined by blocked mutants (Thompson et al., 1987). The active ingredient of the commercial product is produced by fermentation (Sadaaki Mase, 1984).
An alternative way to synthesize phosphinothricin, has been published by Bayer et al.(1972) and is indicated in Figure 4.

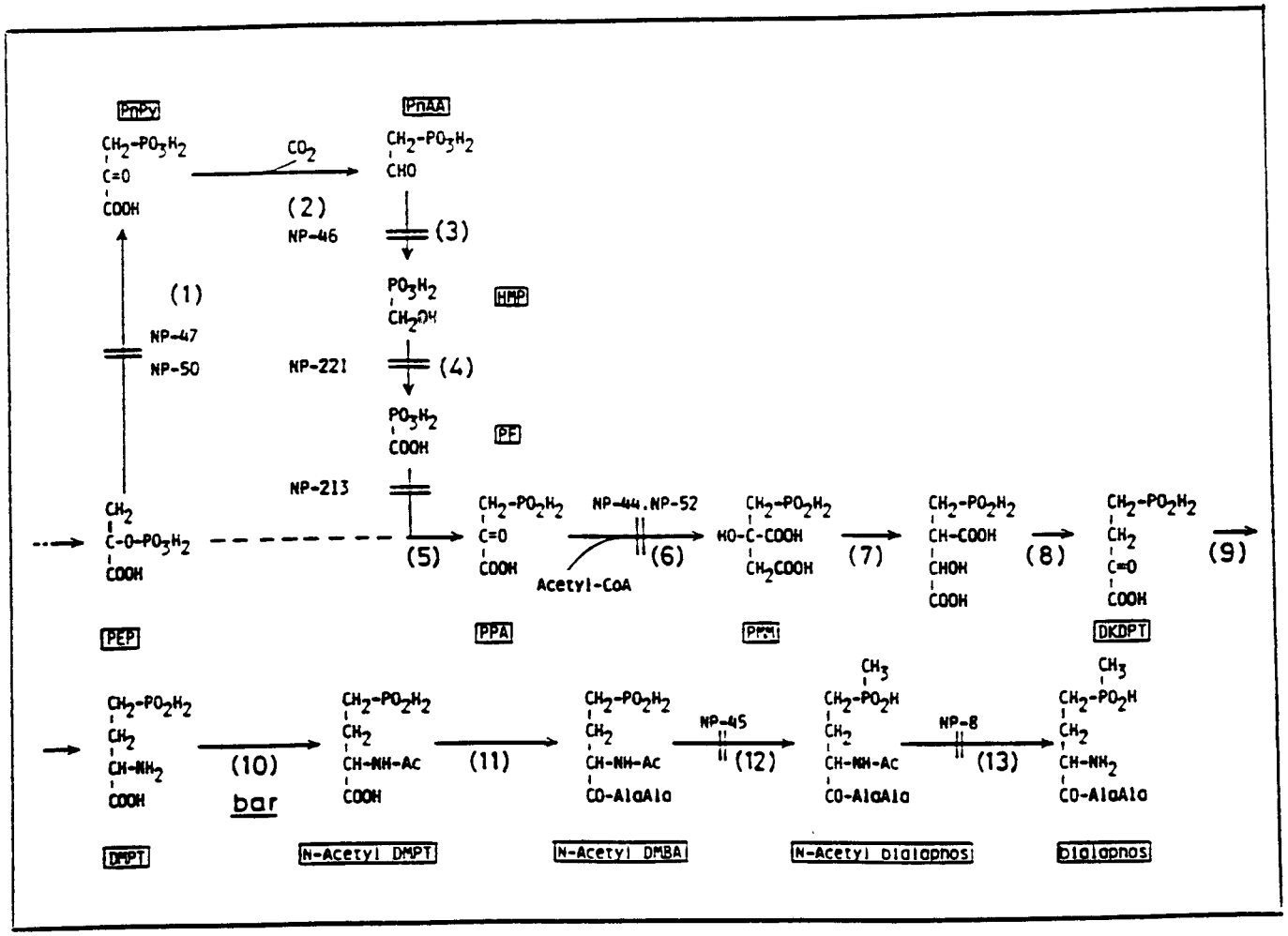

Figure 3. The biosynthesis of bialaphos (Murakami et al., 1986)

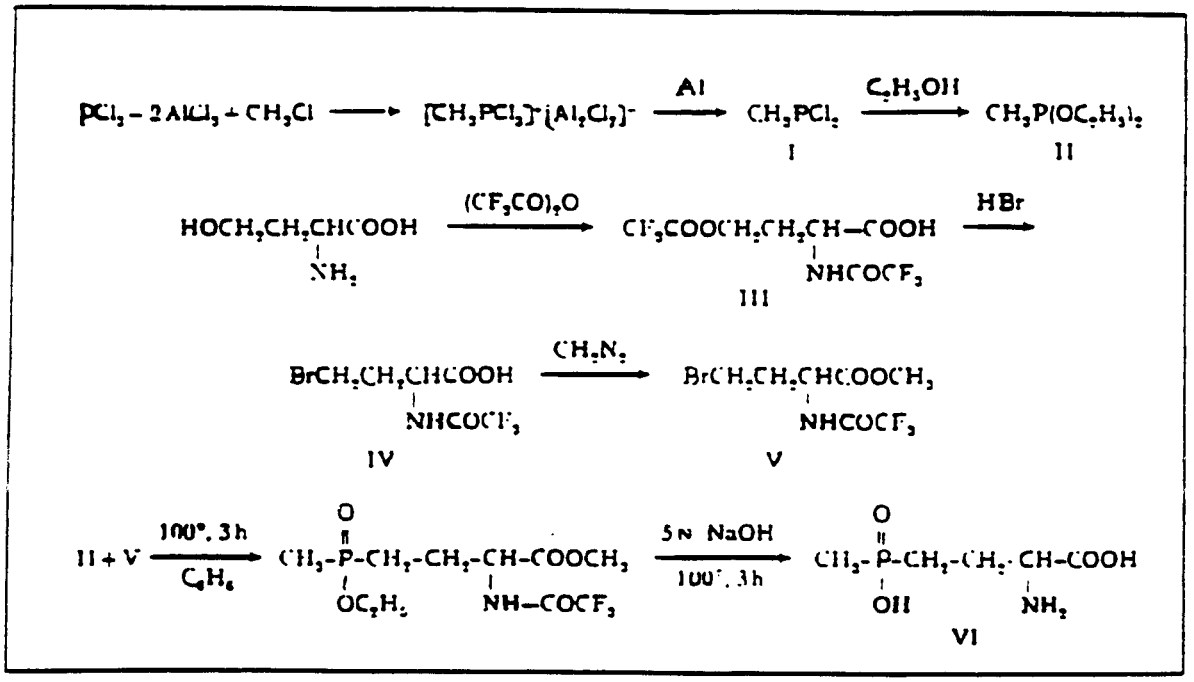

Figure 4. **The synthesis of phosphinothricin (Bayer et al., 1972)**

2.3. Use of the herbicide

Glufosinate-ammonium is considered as a post-emergence, broad-spectrum, non-selective herbicide. The herbicide is effective against a wide range of monocotyledonous and dicotyledonous plants under tropical and subtropical, mediterranean and temperate climatic conditions (Table 3). It acts on the foliage (Sauer et al., 1987; Hoechst info brochure; Wendler et al., 1990; Bulcke, 1990; Logush et al., 1991).

Glufosinate will kill the weeds after application and establish weed-free conditions quickly. It is effective at any stage of the plant growth, although in order to achieve the best results of control, it is necessary to spray the product on actively growing plants. Older weeds under moisture stress will need higher rates of application for effective control.
At harvest time, the chemically synthesized compound can also be used to facilitate collection of low-hanging as well as fallen fruits.
Annual field and vegetable crops can be sown or planted immediately after the application of glufosinate-ammonium. The herbicide is also used in plantation crops (vineyards, pome and stone fruits, citrus, rubber, cacao, banana, oil palm, coffee and tea) for the control of the bottom weeds and the weeds between rows of crops, since it is not absorbed by the root system (Hoechst info brochure; Bulcke, 1990).

Glufosinate-ammonium can be tank mixed with most of the commonly used soil residual herbicides without loss of efficacy.

Table 3. **A list of weeds that can effectively be controlled with glufosinate-ammonium** (Hoechst info brochure)

Type of weed	Some weed species
Annual dicotyledonous species	*Abutilon theophrasti* *Ageratum conycoides* *Chenopodium album* *Datura stramonium* *Erigeron canadensis* *Gallium aparine* *Polygonum* spp *Portulaca oleracea* *Raphanus raphanistrum* *Senecio vulgaris* *Sinapis arvensis* *Solanum nigrum* *Stellaria media*
Perennial dicotyledonous weeds	*Centrosema pubescens* *Euphorbia cyparissias* *Pueraria phaseoloides* *Ranunculus repens* *Rumex* spp *Taraxacum officinale*
Annual monocotyledonous species	*Avena fatua* *Bromus* spp *Echinochloa crusgalli* *Eleusine indica* *Lolium* spp *Panicum maximum* *Poa annua* *Setaria* spp *Sorghum bicolor*
Perennial monocotyledonous species	*Agropyron repens* *Allium canadense* *Cynodon dactylon* *Cyperus esculentus* *Cyperus rotundus* *Imperata cylindrica* *Paspalum* spp *Pennisetum clandestinum* *Sorghum halepense*
Other weeds and ferns	*Equisetum arvense* *Rubus* spp