IN THE UNITED STATES DISTRICT COURT
FOR THE NORTHERN DISTRICT OF ILLINOIS
WESTERN DIVISION

DEKALB GENETICS CORPORATION, Plaintiff, v. SYNGENTA SEEDS, INC., SYNGENTA BIOTECHNOLOGY, INC., GOLDEN HARVEST SEEDS, INC., GARWOOD SEED CO., INC., GOLDEN SEED CO., L.L.C., SOMMER BROTHERS SEED CO., INC., THORP SEED CO., INC., AND J.C. ROBINSON SEED COMPANY Defendants.	C.A. NO. 04 C 50323 JURY TRIAL DEMANDED Judge Philip G. Reinhard Magistrate Judge P. Michael Mahoney

FIRST AMENDED COMPLAINT

Plaintiff, DEKALB Genetics Corporation ("DEKALB"), files this first amended complaint against Defendants Syngenta Seeds, Inc., Syngenta Biotechnology, Inc., Golden Harvest Seeds, Inc., Garwood Seed Co., Inc., Golden Seed Co., L.L.C., Sommer Brothers Seed Co., Inc., Thorp Seed Co., Inc., and J.C. Robinson Seed Company (collectively "Defendants"), prior to any responsive pleadings by Defendants and alleges as follows:

THE PARTIES

1. Plaintiff DEKALB Genetics Corporation is a corporation organized and existing under the laws of the State of Delaware with a principal place of business at 3100 Sycamore Road, DeKalb, Illinois 60115. DEKALB is a wholly owned subsidiary of Monsanto Company.

2. On information and belief, Defendant Syngenta Seeds, Inc. ("Syngenta Seeds") is a corporation organized and existing under the laws of the State of Delaware, with offices located at 7500 Olson Memorial Highway, Golden Valley, Minnesota 55427.

3. On information and belief, Defendant Syngenta Biotechnology, Inc. ("Syngenta Biotechnology") is a corporation organized and existing under the laws of the State of Delaware, with offices located at 3054 Cornwallis Road, Research Triangle Park, North Carolina 27709-2257.

4. On information and belief, Defendant Golden Harvest Seeds, Inc. is a corporation organized and existing under the laws of the Delaware, with its principle place of business in Bloomington, Illinois.

5. On information and belief, Golden Harvest is an organization consisting of five (5) companies ("Golden Harvest Companies") related through common ownership of, and financial interest in, Golden Harvest Seeds, Inc. The Golden Harvest Companies are identified in the following paragraphs 6-10.

6. On information and belief, Defendant Garwood Seed Co., Inc. is a corporation organized and existing under the laws of the State of Illinois, with its principle place of business in Stonington, Illinois.

7. On information and belief, Defendant Golden Seed Co., L.L.C. is a corporation organized and existing under the laws of the State of Illinois, with its principle place of business in Cordova, Illinois.

8. On information and belief, Defendant Sommer Brothers, Seed Co. is a corporation organized and existing under the laws of the State of Illinois, with its principle place of business in Pekin, Illinois.

9. On information and belief, Defendant Thorp Seed Co., Inc. is a corporation organized and existing under the laws of the State of Illinois, with its principle place of business in Clinton, Illinois.

10. On information and belief, Defendant J.C. Robinson Seed Company is a corporation organized and existing under the laws of the State of Nebraska, with its principle place of business in Waterloo, Nebraska.

JURISDICTION AND VENUE

11. This is an action for patent infringement arising under the Patent Laws of the United States, Title 35, United States Code § 1 et seq. Subject matter jurisdiction is proper under 28 U.S.C. §§ 1331 and 1338.

12. Venue is proper in this Court under 28 U.S.C. §§ 1391 and 1400(b).

BACKGROUND

13. Plaintiff DEKALB, a leader in the development of crops that have been genetically engineered to express new traits of value to farmers, such as herbicide resistance, has developed corn containing genes that confer resistance to the herbicide glyphosate. Glyphosate is a commercial herbicide that kills plants by binding to a critical enzyme in the plant called "EPSPS." Monsanto Company sells a glyphosate herbicide under the trademark Roundup®. Genetically engineered products made by Monsanto Company that have the glyphosate resistance trait, including DEKALB branded corn, are sold under the trademark Roundup Ready®. Roundup Ready® products have been a recognized commercial success. Since the introduction of Roundup Ready® crops in 1996, farmers have consistently increased the number of acres they plant in the United States with Roundup Ready® products.

14. Recognizing the value of DEKALB's glyphosate resistance technology, Defendants have attempted to make, import into the United States, offer to sell, sell, or use within the United States, glyphosate resistant genes and corn and have conspired with others to make, import into the United States, offer to sell, sell, or use within the United States, glyphosate resistant corn under the name Agrisure GT in violation of DEKALB's patent rights.

THE INFRINGEMENT

15. Plaintiff realleges and incorporates by reference each of paragraphs 1-14 above as if set forth herein.

16. DEKALB has been the owner of all right, title and interest to and under United States Patent No. 5,538,880 entitled "Method for Preparing Fertile Transgenic Corn Plants" and naming Ronald C. Lundquist and David A. Walters as inventors. U.S. Patent No. 5,538,880 ("the '880 Patent") was duly and legally issued to DEKALB on July 23, 1996. A copy of the patent is attached as Exhibit 1 to this Complaint.

17. DEKALB previously brought actions in 1996 in this Court against two Syngenta predecessor companies, Northrup King Co. (Civil Action No. 96 C 50169) and Ciba-Geigy Corporation (Civil Action No. 96 C 50241), for infringement of the '880 patent. Both actions were resolved and dismissed in 1999, after three years of litigation, including extensive fact and expert discovery and construction of the claims by the Court. That litigation did not involve the Defendants' products that are the subject of this action.

18. Defendants do not have any license or other right to practice the claims of U.S. Patent No. 5,538,880 for the products that are the subject of this action.

19. Upon information and belief, Defendants have infringed and continue to infringe one or more of claims 1-9 of the '880 Patent by at least making corn containing genes that confer

resistance to the herbicide glyphosate and importing into the United States, or offering to sell, selling, or using within the United States, corn containing genes that confer resistance to the herbicide glyphosate made by the process of one or more claims 1-9 of the '880 Patent; and will continue to do so unless enjoined by this Court.

20. Upon information and belief, Defendants have infringed one or more of claims 1-9 of the '880 Patent by at least inducing others and contributing to the infringement by others.

21. Defendants' acts of infringement of the '880 Patent, upon information and belief, have been carried out in deliberate and willful disregard of DEKALB's patent rights.

22. DEKALB has been the owner of all right, title and interest to and under United States Patent No. 6,013,863 entitled "Fertile Transgenic Corn Plants" and naming Ronald C. Lundquist and David A. Walters as inventors. U.S. Patent No. 6,013,863 ("the '863 Patent") was duly and legally issued to DEKALB on January 11, 2000 from a series of continuation applications relating back to the parent application that led to the '880 patent. A copy of the patent is attached as Exhibit 2 to this Complaint.

23. Defendants do not have any license or other right to practice the claims of U.S. Patent No. 6,013,863 for the products that are the subject of this action.

24. Upon information and belief, Defendants have infringed and continue to infringe one or more claims of the '863 Patent by at least making corn containing genes that confer resistance to the herbicide glyphosate and importing into the United States, or offering for sale, selling, or using within the United States, corn containing genes that confer resistance to the herbicide glyphosate made by the process of the '863 Patent; and will continue to do so unless enjoined by this Court.

25. Upon information and belief, Defendants have infringed one or more of the claims of the '863 Patent by at least inducing others and contributing to the infringement by others.

26. Defendants' acts of infringement of the '863 Patent, upon information and belief, have been carried out in deliberate and willful disregard of DEKALB's patent rights.

JURY DEMAND

27. Pursuant to Rule 38(b), Fed. R. Civ. P., Plaintiff requests a trial by jury.

PRAYER FOR RELIEF

WHEREBY PLAINTIFF PRAYS FOR THE FOLLOWING RELIEF:

A. A judgment that Defendants have infringed claims 1-9 of the '880 Patent and the claims of the '863 Patent;

B. A judgment that Defendants have willfully and deliberately infringed claims 1-9 of the '880 Patent and the claims of the '863 Patent;

C. A declaration by the Court that any making, using, selling or offering for sale by Defendants of glyphosate resistant corn products that are within the scope of claims 1-9 of the '880 Patent and the claims of the '863 Patent would constitute an act of infringement of the '880 and '863 Patents;

D. A declaration by the Court that the importation into the United States, or offering for sale, selling or using within the United States, of corn containing genes that confer resistance to the herbicide glyphosate made by the process and/or processes that are within the scope of claims 1-9 of the '880 Patent and the claims of the '863 Patent, would constitute an act of infringement of the '880 and '863 Patents;

E. A preliminary and final injunction enjoining Defendants and all those in privy with them from infringing, from inducing infringement, and from contributing to the infringement of claims 1-9 of the '880 Patent and the claims of the '863 Patent;

F. An award of compensatory and exemplary damages, but not less than a reasonable royalty, resulting from Defendants' infringement, including allowance of multiplied damages based on Defendants' willful and deliberate infringement;

G. An award of interest, costs, and attorneys' fees; and

H. Such other and further relief as this Court shall deem just and proper.

Dated: 1/27/05

Respectfully submitted,

By _____

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CERTIFICATE OF SERVICE

It is hereby certified that a true and correct copy of **DEKALB'S FIRST AMENDED COMPLAINT** was served this 27 day of January, 2005, via facsimile and overnight delivery to the following:

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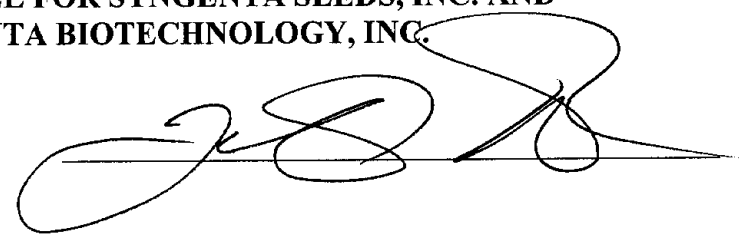

CASE NO. 04 c 50323

ATTACHMENT NO. _____

TAB DESCRIPTION #1

EXHIBIT _____

US005538880A

United States Patent [19]

Lundquist et al.

[11] **Patent Number: 5,538,880**

[45] **Date of Patent: * Jul. 23, 1996**

[54] **METHOD FOR PREPARING FERTILE TRANSGENIC CORN PLANTS**

[75] Inventors: **Ronald C. Lundquist**, Minnetonka; **David A. Walters**, Bloomington, both of Minn.

[73] Assignee: **DeKalb Genetics Corporation**, St. Paul, Minn.

[*] Notice: The term of this patent shall not extend beyond the expiration date of Pat. No. 5,538,877.

[21] Appl. No.: **249,458**

[22] Filed: **May 26, 1994**

Related U.S. Application Data

[63] Continuation of Ser. No. 974,379, Nov. 10, 1992, which is a continuation of Ser. No. 467,983, Jan. 22, 1990, abandoned.

[51] **Int. Cl.**[6] **C12N 15/00**; C12N 15/05; A01H 1/06; A01H 4/00

[52] **U.S. Cl.** **435/172.3**; 435/172.1; 435/240.48; 435/240.49; 935/52; 935/55; 935/67; 935/85; 800/205; 800/235; 800/DIG. 56

[58] **Field of Search** 435/172.3, 172.1, 435/240.4, 240.45, 240.49, 287; 800/205, 235; 935/52, 53, 55, 67, 85

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(List continued on next page.)

Primary Examiner—Gary Benzion
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[57] **ABSTRACT**

Fertile transgenic *Zea mays* (corn) plants which stably express heterologous DNA which is heritable are disclosed along with a process for producing said plants. The process comprises the microjectile bombardment of friable embryogenic callus from the plant to be transformed. The process may be applicable to other graminaceous cereal plants which have not proven stably transformable by other techniques.

18 Claims, 10 Drawing Sheets

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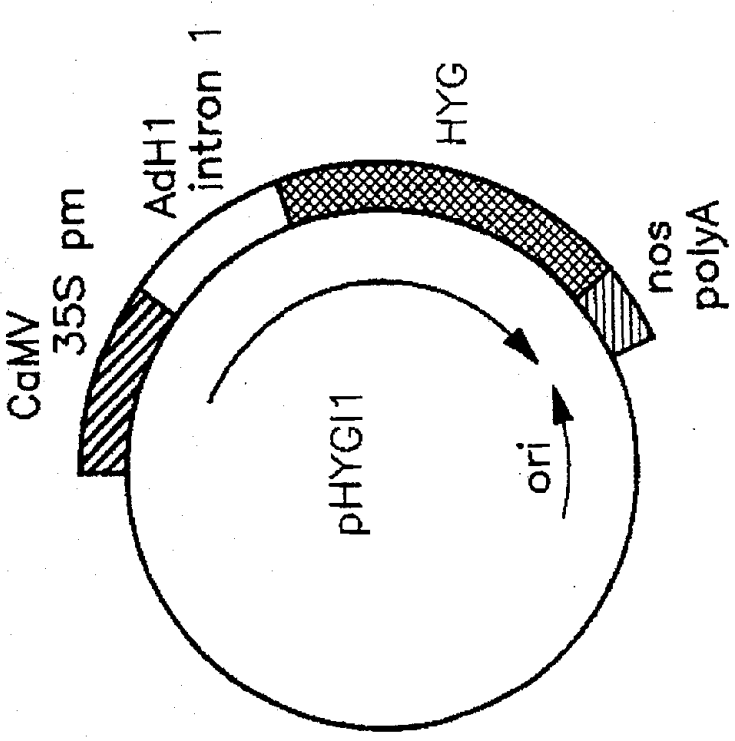

CaMV
35S pm
AdH1
intron 1
HYG
nos
polyA
pHYG11
ori

FIG. 1A

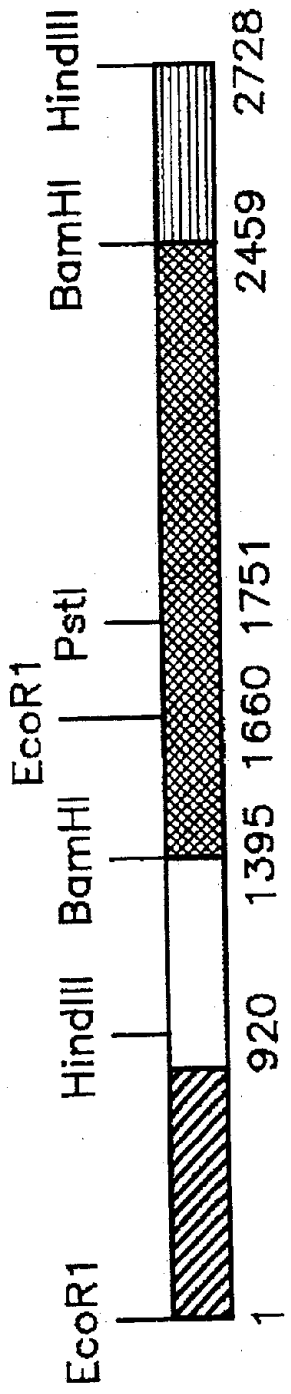
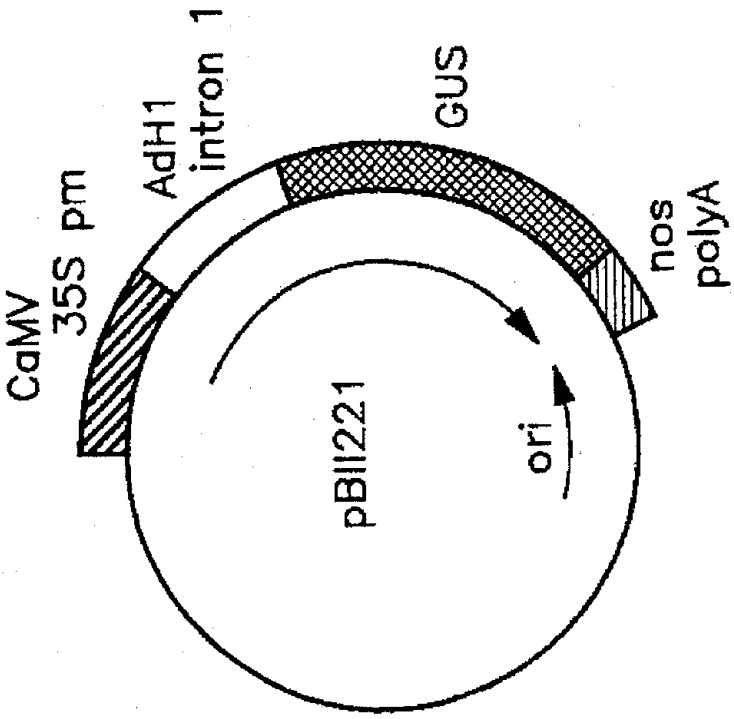

HindIII
BamHI
2728
2459
EcoR1
PstI
1751
1660
BamHI
1395
HindIII
920
EcoR1
1

FIG. 1B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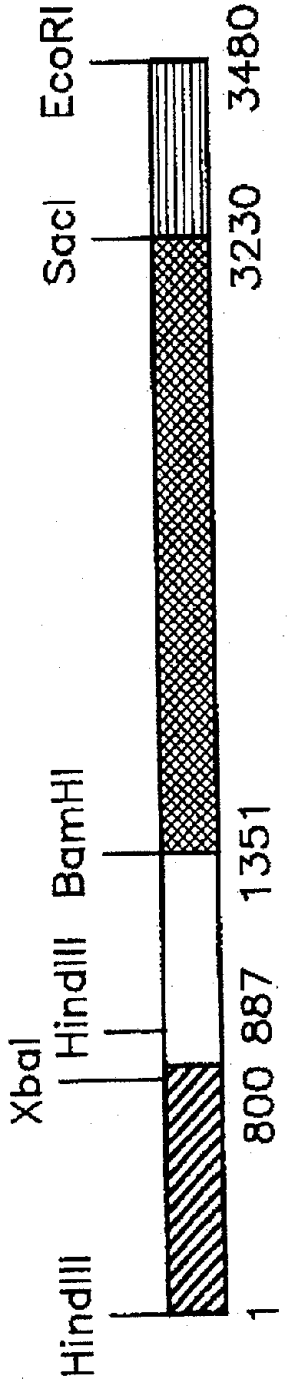
AdH1
intron 1
GUS
nos
polyA
pBII221
ori

FIG. 2A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HindIII
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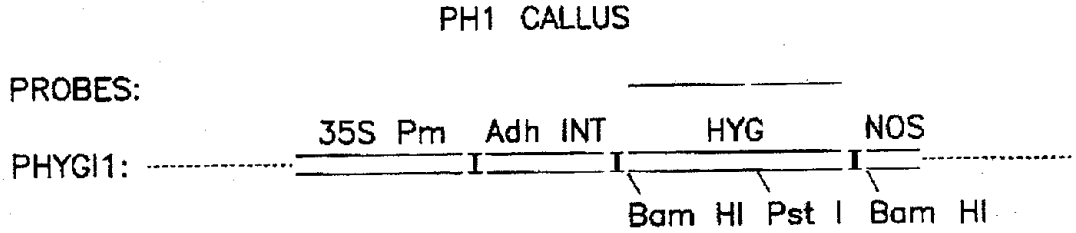
HindIII
1

FIG. 2B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PROBES:
PHYGI1:
35S Pm
Adh INT
HYG
NOS
Bam HI
Pst I
Bam HI

FIG. 3A

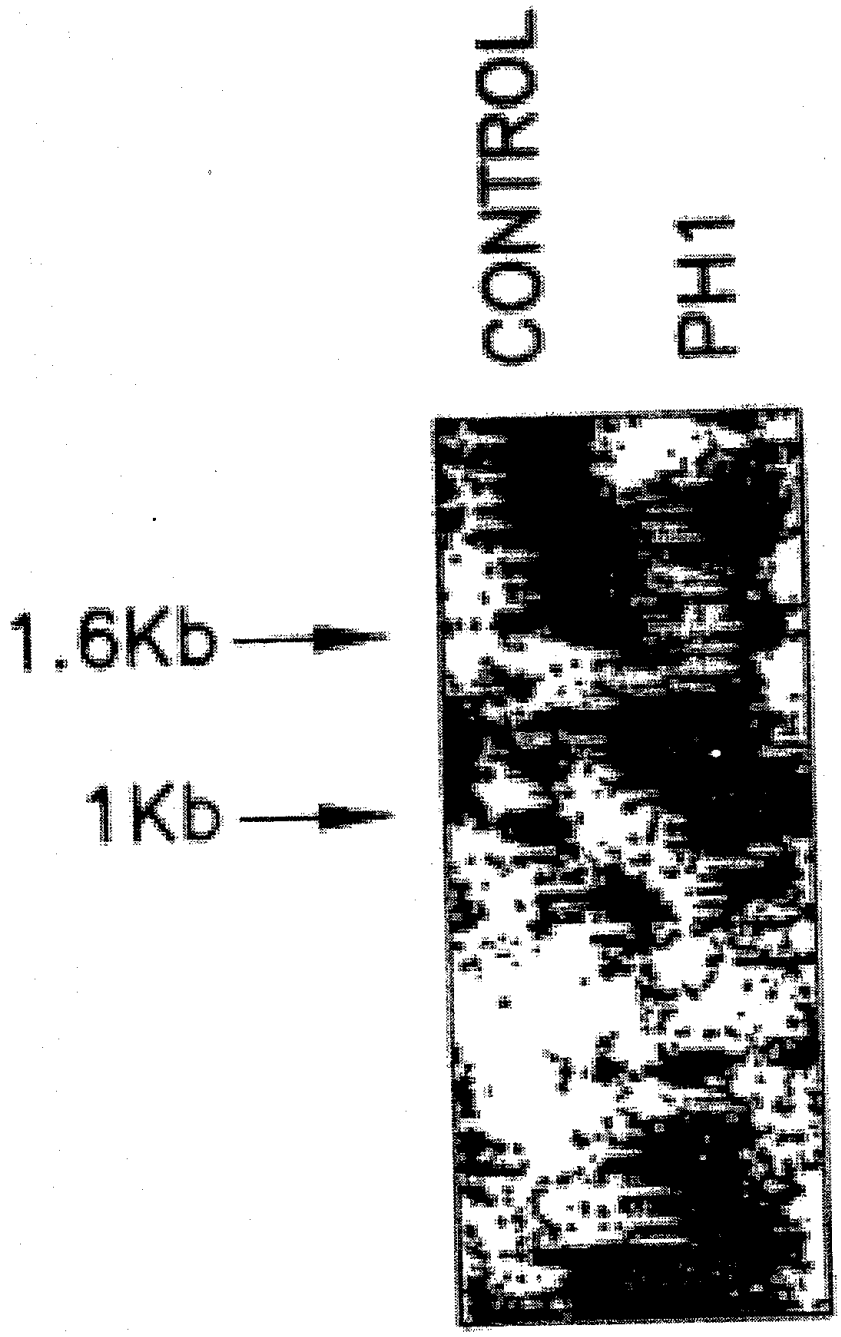


FIG. 3B

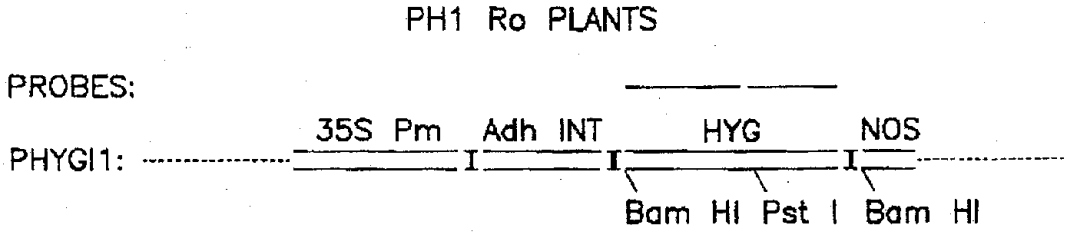

PH1 Ro PLANTS
PROBES:
PHYGI1:
35S Pm
Adh INT
HYG
NOS
Bam HI
Pst I
Bam HI

FIG. 4A

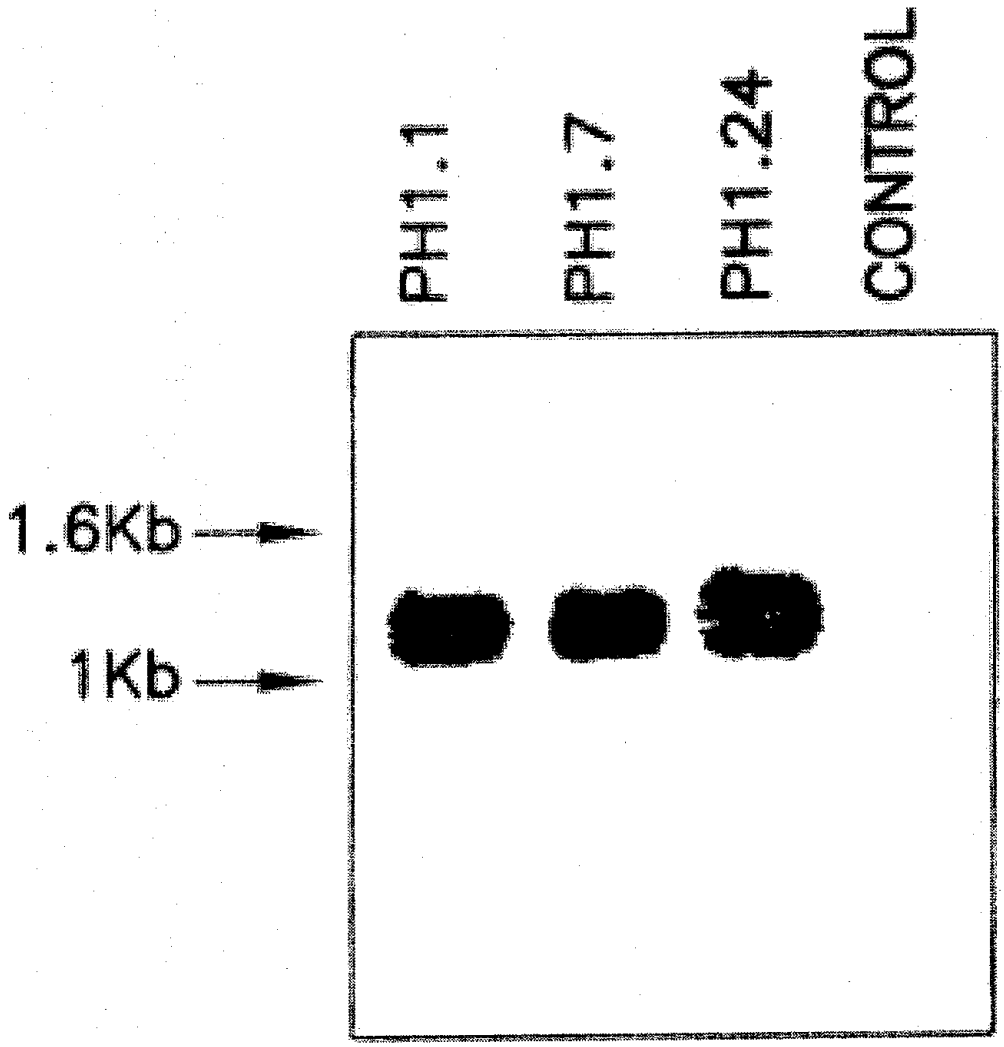


FIG. 4B

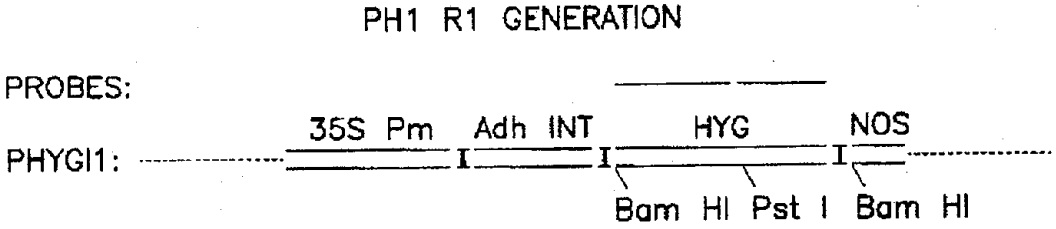
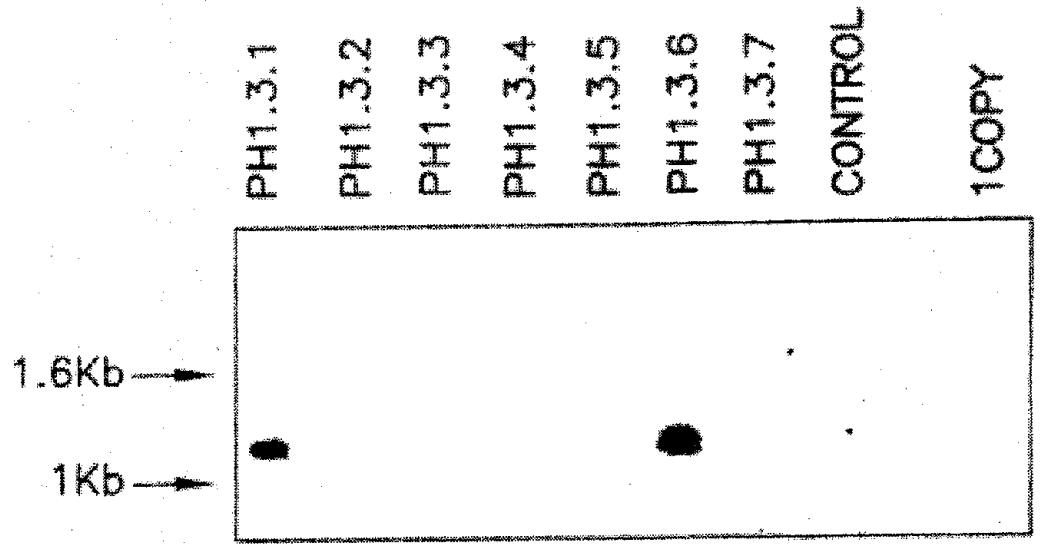

PH1 R1 GENERATION
PROBES:
PHYGI1:
35S Pm
Adh INT
HYG
NOS
Bam HI
Pst I
Bam HI

FIG. 5A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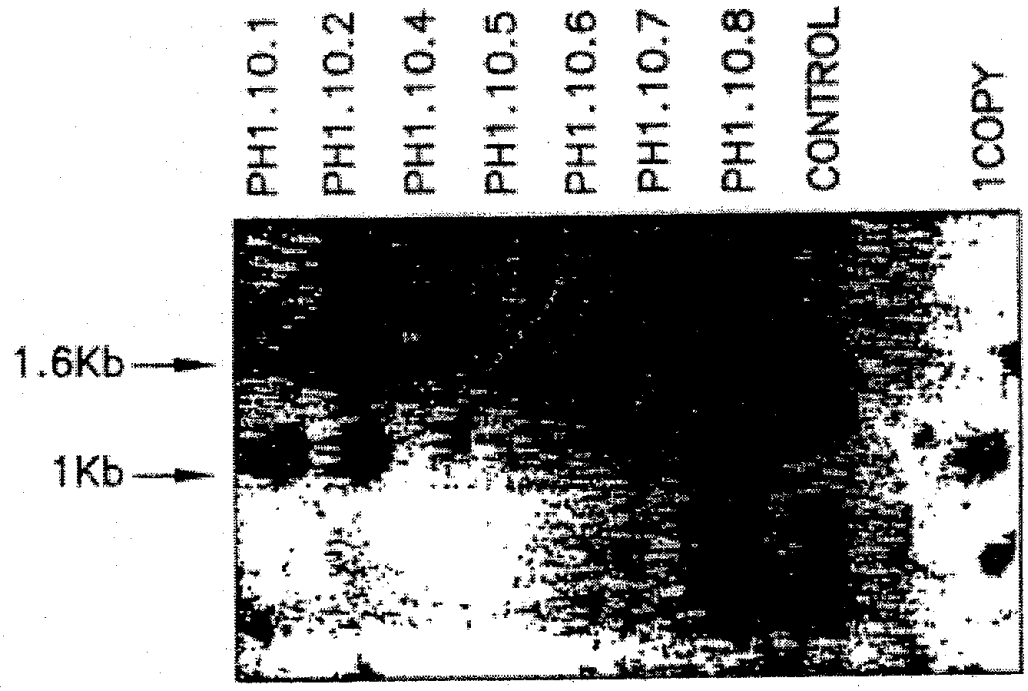
PH1.3.2
PH1.3.3
PH1.3.4
PH1.3.5
PH1.3.6
PH1.3.7
CONTROL
1COPY
1.6Kb
1Kb

FIG. 5B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PH1.10.8
CONTROL
1COPY
1.6Kb
1Kb

FIG. 5C

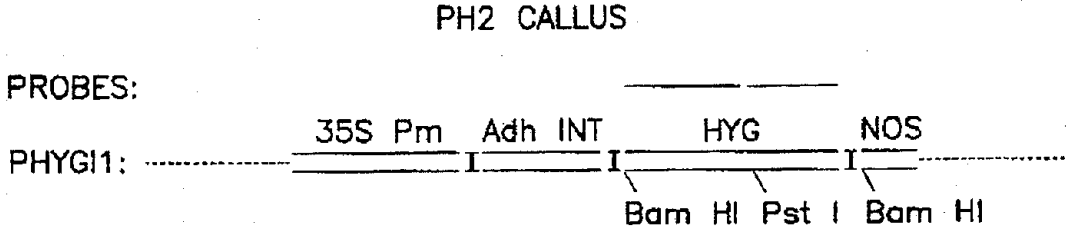
PH2 CALLUS
PROBES:
PHYGI1:
35S Pm
Adh INT
HYG
NOS
Bam HI
Pst I
Bam HI

FIG. 6A

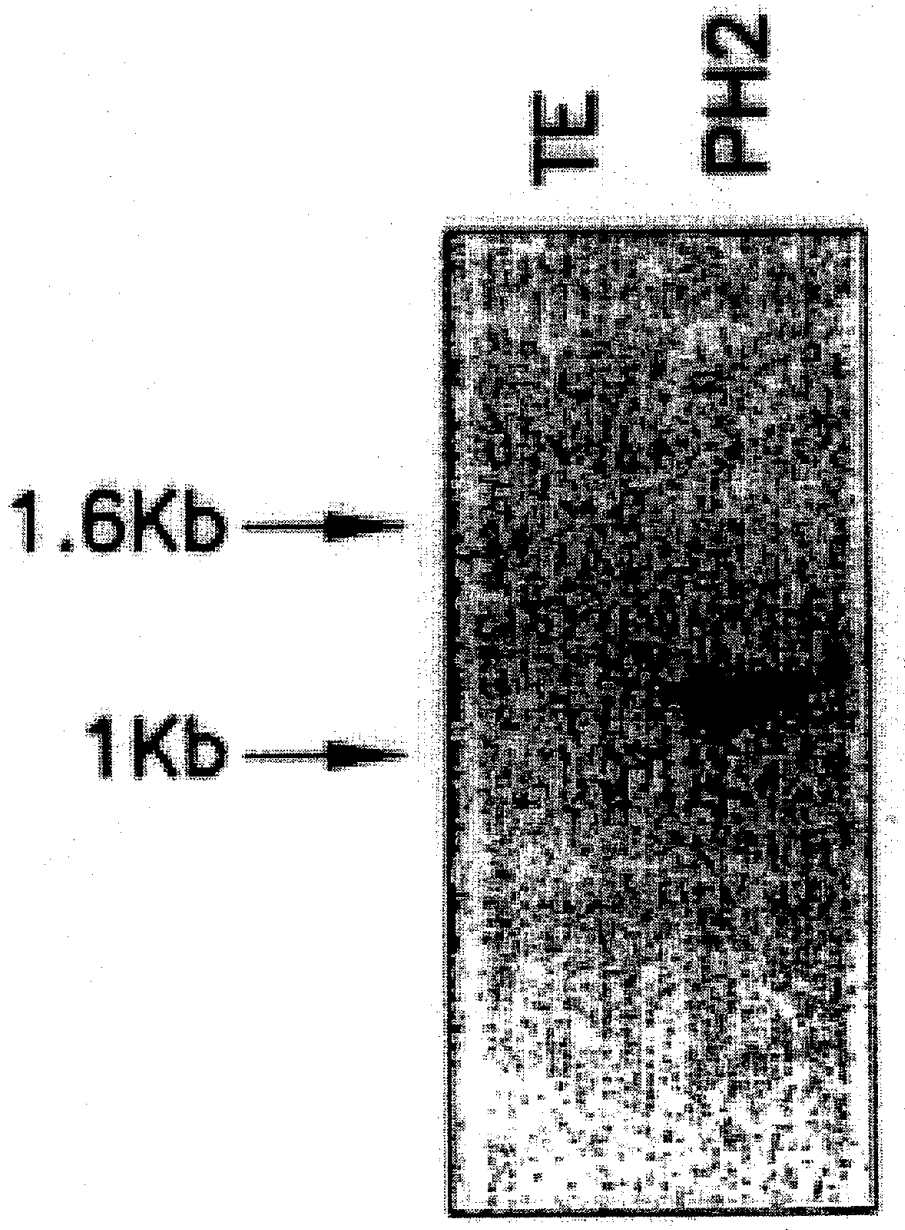

FIG. 6B

METHOD FOR PREPARING FERTILE TRANSGENIC CORN PLANTS

This is a division of application Ser. No. 07/974,379, filed Nov. 10, 1992, which is a continuation of application Ser. No. 07/467,983, filed Jan. 22, 1990, abandoned.

BACKGROUND OF THE INVENTION

This invention relates to fertile transgenic plants of the species *Zea mays* (oftentimes referred to herein as maize or corn). The invention further relates to producing transgenic plants via particle bombardment and subsequent selection techniques which have been found to produce fertile transgenic plants.

Genetic engineering of plants, which entails the isolation and manipulation of genetic material (usually in the form of DNA or RNA) and the subsequent introduction of that genetic material into a plant or plant cells, offers considerable promise to modern agriculture and plant breeding. Increased crop food values, higher yields, feed value, reduced production costs, pest resistance, stress tolerance, drought resistance, the production of pharmaceuticals, chemicals and biological molecules as well as other beneficial traits are all potentially achievable through genetic engineering techniques. Once a gene has been identified, cloned, and engineered, it is still necessary to introduce it into a plant of interest in such a manner that the resulting plant is both fertile and capable of passing the gene on to its progeny.

A variety of methods have been developed and are currently available for the transformation of various plants and plant cells with DNA. Generally these plants have been dicotyledonous, and some success has been reported with certain of the monocotyledonous cereals. However, some species have heretofore proven untransformable by any method. Thus, previous to this discovery, no technology had been developed which would permit the production of stably transformed *Zea mays* plants in which the transforming DNA is heritable thereof. This failure in the art is well documented in the literature and has been discussed in a number of recent reviews (Potrykus, 1989; Weising et al., 1988; Cocking et al., 1987).

European Patent Publns. 270,356 (McCabe et al.) and 275,069 (Arntzen et al.) describe the introduction of DNA into maize pollen followed by pollination of maize ears and formation of seeds. The plants germinated from these seeds are alleged to contain the introduced DNA, but there is no suggestion that the introduced DNA was heritable, as has been accomplished in the present invention. Only if the DNA introduced into the corn is heritable can the corn be used in breeding programs as required for successful commercialization of transgenic corn.

Graves et al. (1986) claims *Agrobacterium*-mediated transformation of *Zea mays* seedlings. The alleged evidence was based upon assays known to produce incorrect results.

Despite extensive efforts to produce fertile transformed corn plants which transmit the transforming DNA to progeny, there have been no reported successes. Many previous failures have been based upon gene transfer to maize protoplasts, oftentimes derived from callus, liquid suspension culture cells, or other maize cells using a variety of transformation techniques. Although several of the techniques have resulted in successful transformation of corn cells, the resulting cells either could not be regenerated into corn plants or the corn plants produced were sterile (Rhodes et al. 1988). Thus, while maize protoplasts and some other cells have previously been transformed, the resulting transformants could not be regenerated into fertile transgenic plants.

On the other hand, it has been known that at least certain corn callus can be regenerated to form mature plants in a rather straightforward fashion and that the resulting plants were often fertile. However, no stable transformation of maize callus was ever achieved, i.e. there were no techniques developed which would permit a successful stable transformation of a regenerable callus. An example of a maize callus transformation technique which has been tried is the use of Agrobacterium mediated transfer.

The art was thus faced with a dilemma. While it was known that corn protoplast and suspension culture cells could be transformed, no techniques were available which would regenerate the transformed protoplast into a fertile plant. While it was known that corn callus could be regenerated into a fertile plant, there were no techniques known which could transform the callus, particularly while not destroying the ability of the callus both to regenerate and to form fertile plants.

Recently, a new transformation technique has been created based upon the bombardment of intact cells and tissues with DNA-coated microprojectiles. The technique, disclosed in Sanford et al. (1987) as well as in EPO Patent Publication 331,855 of J. C. Sanford et al. based upon U.S. Ser. No. 161,807, filed Feb. 29, 1988, has been shown effective at producing transient gene expression in some plant cells and tissues including those from onion, maize (Klein et al. 1988a), tobacco, rice, wheat, and soybean, and stable expression has been obtained in tobacco and soybeans. In fact, stable expression has been obtained by bombardment of suspension cultures of *Zea mays* Black Mexican Sweet (Klein et al. 1989) which cultures are, however, non-regenerable suspension culture cells, not the callus culture cells used in the process of the present invention.

No protocols have been published describing the introduction of DNA by a bombardment technique into cultures of regenerable maize cells of any type. No stable expression of a gene has been reported by means of bombardment of corn callus followed by regeneration of fertile plants and no regenerable fertile corn has resulted from DNA-coated microprojectile bombardment of the suspension cultures. Thus, the art has failed to produce fertile transformed corn plants heretofore.

A further stumbling block to the successful production of fertile transgenic maize plants has been in selecting those few transformants in such a manner that neither the regeneration capacity nor the fertility of the regenerated transformant are destroyed. Due to the generally low level of transformants produced by a transformation technique, the need for selection of the transformants is self-evident. However, selection generally entails the use of some toxic agent, e.g. herbicide or antibiotic, which can effect either the regenerability or the resultant plant fertility.

It is thus an object of the present invention to produce fertile, stably transgenic, *Zea mays* plants and seeds which transmit the introduced gene to progeny. It is a further object to produce such stably transgenic plants and seeds by a particle bombardment and selection process which results in a high level of viability for a few transformed cells. It is a further object to produce fertile stably transgenic plants of other graminaceous cereals besides maize.

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SUMMARY OF THE INVENTION

The present invention relates to fertile transgenic *Zea mays* plants containing heterologous DNA, preferably chromosomally integrated heterologous DNA, which is heritable by progeny thereof.

The invention further relates to all products derived from transgenic *Zea mays* plants, plant cells, plant parts, and seeds.

The invention further relates to transgenic *Zea mays* seeds stably containing heterologous DNA and progeny which inherit the heterologous DNA.

The invention further relates to a process for producing fertile transgenic *Zea mays* plants containing heterologous DNA. The process is based upon microprojectile bombardment, selection, and plant regeneration techniques.

The invention further relates to a process for producing fertile transformed plants of graminaceous plants other than *Zea mays* which have not been reliably transformed by traditional methods such as electroporation, *Agrobacterium*, injection, and previous ballistic techniques.

The invention further relates to regenerated fertile mature maize plants from transformed embryogenic tissue, transgenic seeds produced therefrom, and R1 and subsequent generations.

In preferred embodiments, this invention produces the fertile transgenic plants by means of a DNA-coated microprojectile bombardment of clumps of friable embryogenic callus, followed by a controlled regimen for selection of the transformed callus lines.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1A** shows a map of plasmid vector pHYGI1 utilized in Example I. FIG. **1B** shows the relevant part of pHYGI1 encompassing the HPT coding sequence and associated regulatory elements. The base pair numbers start from the 5' nucleotide in the recognition sequence for the indicated restriction enzymes, beginning with the EcoRI site at the 5' end of the CaMV 35S promoter.

FIG. **2A** shows a map of plasmid vector pBII221 utilized in Example I.

FIG. **2B** shows the relevant part of pBII221 encompassing the GUS coding sequence and associated regulatory elements.

FIG. **3A** depicts the pHYG1-containing fragments employed as probes in a Southern blot analysis of PH1 callus.

FIG. **3B** is a Southern blot of DNA isolated from the PH1 callus line and an untransformed control callus line.

FIG. **4A** depicts the pHYG1-containing fragments employed as probes in a Southern blot analysis of PH1 RO plants.

FIG. **4B** is a Southern blot of leaf DNA isolated from Ro plants regenerated from PH1 and untransformed callus.

FIG. **5A** depicts the pHYG1-containing fragments employed as probes in a Southern blot analysis of PHI R1 plants.

FIG. **5B** and **5C** are Southern blots of leaf DNA isolated from R1 progeny of PH1 Ro plants and untransformed Ro plants.

FIG. **6A** depicts the pHYG1-containing fragments employed as probes in a Southern blot analysis of PH2 callus.

FIG. **6B** is a Southern blot of DNA isolated from the PH2 callus line and an untransformed control callus line.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is directed to the production of fertile transgenic plants and seeds of the species *Zea mays* and to the plants, plant tissues, and seeds derived from such transgenic plants, as well as the subsequent progeny and products derived therefrom. The transgenic plants produced herein include all plants of this species, including field corn, popcorn, sweet corn, flint corn and dent corn.

"Transgenic" is used herein to include any cell, cell line, callus, tissue, plant part or plant which contains heterologous DNA that was introduced into plant material by a process of genetic engineering, or which was initially introduced into a plant species by such a process and was subsequently transferred to later generations by sexual or asexual cell crosses or cell divisions.

By "heritable" is meant that the DNA is capable of transmission through a complete sexual cycle of a plant, i.e. passed from one plant through its gametes to its progeny plants in the same manner as occurs in normal corn.

The transgenic plants of this invention may be produced by (i) establishing friable embryogenic callus from the plant to be transformed, (ii) transforming said cell line by a microprojectile bombardment technique, (iii) controllably identifying or selecting transformed cells, and (iv) regenerating fertile transgenic plants from the transformed cells. Some of the plants of this invention may be produced from the transgenic seed produced from the fertile transgenic plants using conventional crossbreeding techniques to develop commercial hybrid seed containing heterologous DNA.

I. Plant Lines and Tissue Cultures

The cells which have been found useful to produce the fertile transgenic maize plants herein are those callus cells which are regenerable, both before and after undergoing a selection regimen as detailed further below. Generally, these cells will be derived from meristematic tissue which contain cells which have not yet terminally differentiated. Such tissue in graminaceous cereals in general and in maize, in particular, comprise tissues found in juvenile leaf basal regions, immature tassels, immature embryos, and coleoptilar nodes. Preferably, immature embryos are used. Methods of preparing and maintaining callus from such tissue and plant types are well known in the art and details on so doing are available in the literature, c.f. Phillips et al. (1988), the disclosure of which is hereby incorporated by reference.

The specific callus used must be able to regenerate into a fertile plant. The specific regeneration capacity of particular callus is important to the success of the bombardment/selection process used herein because during and following selection, regeneration capacity may decrease significantly. It is therefore important to start with cultures that have as high a degree of regeneration capacity as possible. Callus which is more than about 3 months and up to about 36 months of age has been found to have a sufficiently high level of regenerability and thus is currently preferred. The regenerative capacity of a particular culture may be readily determined by transferring samples thereof to regeneration medium and monitoring the formation of shoots, roots, and plantlets. The relative number of plantlets arising per Petri dish or per gram fresh weight of tissue may be used as a rough quantitative estimate of regeneration capacity. Generally, a culture which will produce at least one plant per gram of callus tissue will be preferred.

While maize callus cultures can be initiated from a number of different plant tissues, the cultures useful herein are preferably derived from immature maize embryos which are removed from the kernels of an ear when the embryos are about 1–3 mm in length. This length generally occurs about 9–14 days after pollination. Under aseptic conditions, the embryos are placed on conventional solid media with the embryo axis down (scutellum up). Callus tissue appears from the scutellum after several days to a few weeks. After the callus has grown sufficiently, the cell proliferations from the scutellum may be evaluated for friable consistency and the presence of well-defined embryos. By "friable consistency" is meant that the tissue is easily dispersed without causing injury to the cells. Tissue with this morphology is then transferred to fresh media and subcultured on a routine basis about every two weeks.

The callus initiation media is solid because callus cannot be readily initiated in liquid medium. The initiation/maintainence media is typically based on the N6 salts of Chu et al. (1975) as described in Armstrong et al. (1985) or the MS salts of Murashige et al. (1962). The basal medium is supplemented with sucrose and 2,4-dichlorophenoxyacetic acid (2,4-D). Supplements such as L-proline and casein hydrolysate have been found to improve the frequency of initiation of callus cultures, morphology, and growth. The cultures are generally maintained in the dark, though low light levels may also be used. The level of synthetic hormone 2,4-D, necessary for maintainence and propagation, should be generally about 0.3 to 3.0 mg/l.

Although successful transformation and regeneration has been accomplished herein with friable embryogenic callus, this is not meant to imply that other transformable regenerable cells, tissue, or organs cannot be employed to produce the fertile transgenic plants of this invention. The only actual requirement for the cells which are transformed is that after transformation they must be capable of regeneration of a plant containing the heterologous DNA following the particular selection or screening procedure actually used.

II. DNA Used for Transformation

The heterologous DNA used for transformation herein may be circular or linear, double-stranded or single-stranded. Generally, the DNA is in the form of a plasmid and contains coding regions of beneficial heterologous DNA with flanking regulatory sequences which serve to promote the expression of the heterologous DNA present in the resultant corn plant. "Heterologous DNA" is used herein to include all synthetically engineered or biologically derived DNA which is introduced into a plant by man by genetic engineering, including but not limited to, nonplant genes, modified genes, synthetic genes, portions of genes, as well as DNA and genes from maize and other plant species.

The compositions of and methods for constructing heterologous DNA for successful transformations of plants is well known to those skilled in the art, and the same compositions and methods of construction may be utilized to produce the heterologous DNA useful herein. The specific composition of the DNA is not central to the present invention and the invention is not dependent upon the composition of the specific transforming DNA used. Weising et al. (1988), the subject matter of which is incorporated herein by reference, describes suitable DNA components thereof which include promoters, polyadenylation sequences, selectable marker genes, reporter genes, enhancers, introns, and the like, as well as provides suitable references for compositions thereof. Sambrook et al. (1989) provides suitable methods of construction.

Generally the heterologous DNA will be relatively small, i.e. less than about 30 kb to minimize any susceptibility to physical, chemical, or enzymatic degradation which is known to increase as the size of the DNA increases.

Suitable heterologous DNA for use herein includes all DNA which will provide for, or enhance, a beneficial feature of the resultant transgenic corn plant. For example, the DNA may encode proteins or antisense RNA transcripts in order to promote increased food values, higher yields, pest resistance, disease resistance, and the like. For example, a bacterial dap A gene for increased lysine; Bt-endotoxin gene or protease inhibitor for insect resistance; bacterial ESPS synthase for resistance to glyphosate herbicide; chitinase or glucan endo-1,3-B-glucosidase for fungicidal properties. Also, the DNA may be introduced to act as a genetic tool to generate mutants and/or assist in the identification, genetic tagging, or isolation of segments of corn DNA. Additional examples may be found in Weising, supra.

The heterologous DNA to be introduced into the plant further will generally contain either a selectable marker or a reporter gene or both to facilitate identification and selection of transformed cells. Alternatively, the selectable marker may be carried on a separate piece of DNA and used in a cotransformation procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in plants. Useful selectable markers are well known in the art and include, for example, antibiotic and herbicide resistance genes. Specific examples of such genes are disclosed in Weising et al, supra. A preferred selectable marker gene is the hygromycin B phosphotransferase (HPT) coding sequence, which may be derived from *E. coli*. Other selectable markers known in the art include aminoglycoside phosphotransferase gene of transposon Tn5 (AphII) which encodes resistance to the antibiotics kanamycin, neomycin, and G418, as well as those genes which code for resistance or tolerance to glyphosate, methotrexate, imidazolinones, sulfonylureas, bromoxynil, dalapon, and the like. Those selectable marker genes which confer herbicide resistance or tolerance are also of commercial utility in the resulting transformed plants.

Reporter genes which encode for easily assayable marker proteins are well known in the art. In general, a reporter gene is a gene which is not present or expressed by the recipient organism or tissue and which encodes a protein whose expression is manifested by some easily detectable property, e.g. phenotypic change or enzymatic activity. Examples of such genes are provided in Weising et al, supra. Preferred genes include the chloramphenicol acetyl transferase gene from Tn9 of *E. coli*, the beta-glucuronidase gene of the uidA locus of *E. coli*, and the luciferase genes from firefly *Photinus pyralis*.

The regulatory sequences useful herein include any constitutive, inducible, tissue or organ specific, or developmental stage specific promoter which can be expressed in the particular plant cell. Suitable such promoters are disclosed in Weising et al, supra. The following is a partial representative list of promoters suitable for use herein: regulatory sequences from the T-DNA of *Agrobacterium tumefaciens*, including mannopine synthase, nopaline synthase, and octopine synthase; alcohol dehydrogenase promoter from corn; light inducible promoters such as, ribulose-biphosphate-carboxylase small subunit gene from a variety of species; and the major chlorophyll a/b binding protein gene promoter; 35S and 19S promoters of cauliflower mosaic virus; developmentally regulated promoters such as the waxy, zein, or bronze promoters from maize; as well as synthetic or other natural promoters which are either inducible or constitutive, including those promoters exhibiting organ specific expression or expression at specific development stage(s) of the plant.

Other elements such as introns, enhancers, polyadenylation sequences and the like, may also be present on the DNA. Such elements may or may not be necessary for the function of the DNA, although they can provide a better expression or functioning of the DNA by affecting transcription, stability of the mRNA, or the like. Such elements may be included in the DNA as desired to obtain the optimal performance of the transforming DNA in the plant. For example, the maize AdhlS first intron may be placed between the promoter and the coding sequence of a particular heterologous DNA. This intron, when included in a DNA construction, is known to generally increase expression in maize cells of a protein. (Callis et al. 1987) However, sufficient expression for a selectable marker to perform satisfactorily can often be obtained without an intron. (Klein et al. 1989) An example of an alternative suitable intron is the shrunken-1 first intron of Zea mays. These other elements must be compatible with the remainder of the DNA constructions.

To determine whether a particular combination of DNA and recipient plant cells are suitable for use herein, the DNA may include a reporter gene. An assay for expression of the reporter gene may then be performed at a suitable time after the DNA has been introduced into the recipient cells. A preferred such assay entails the use of the *E. coli* beta-glucuronidase (GUS) gene (Jefferson et al. 1987). In the case of the microprojectile bombardment transformation process of the present invention, a suitable time for conducting the assay is about 2–3 days after bombardment. The use of transient assays is particularly important when using DNA components which have not previously been demonstrated or confirmed as compatible with the desired recipient cells.

III. DNA Delivery Process

The DNA can be introduced into the regenerable maize callus cultures via a particle bombardment process. A general description of a suitable particle bombardment instrument is provided in Sanford et al. (1987), the disclosure of which is incorporated herein by reference. While protocols for the use of the instrument in the bombardment of maize non-regenerable suspension culture cells are described in Klein et al. (1988a, 1988b, and 1989), no protocols have been published for the bombardment of callus cultures or regenerable maize cells.

In a microprojectile bombardment process, also referred to as a biolistic process, the transport of the DNA into the callus is mediated by very small particles of a biologically inert material. When the inert particles are coated with DNA and accelerated to a suitable velocity, one or more of the particles is able to enter into one or more of the cells where the DNA is released from the particle and expressed within the cell. While some of the cells are fatally damaged by the bombardment process, some of the recipient cells do survive, stably retain the introduced DNA, and express it.

The particles, called microprojectiles, are generally of a high density material such as tungsten or gold. They are coated with the DNA of interest. The microprojectiles are then placed onto the surface of a macroprojectile which serves to transfer the motive force from a suitable energy source to the microprojectiles. After the macroprojectile and the microprojectiles are accelerated to the proper velocity, they contact a blocking device which prevents the macroprojectile from continuing its forward path but allows the DNA-coated microprojectiles to continue on and impact the recipient callus cells. Suitable such instruments may use a variety of motive forces such as gunpowder or shock waves from an electric arc discharge J. C. Sanford et al., *J. Particle Science and Technology*, 5,27 (1987). An instrument in which gunpowder is the motive force is currently preferred and such is described and further explained in Sanford et al. (1987), the disclosure of which is incorporated herein by reference.

A protocol for the use of the gunpowder instrument is provided in Klein et al. (1988a, b) and involves two major steps. First, tungsten microprojectiles are mixed with the DNA, calcium chloride, and spermidine free base in a specified order in an aqueous solution. The concentrations of the various components may be varied as taught. The currently preferred procedure entails exactly the procedure of Klein et al. (1988b) except for doubling the stated

optimum DNA concentration. Secondly, in the actual bombardment, the distance of the recipient cells from the end of the barrel as well as the vacuum in the sample chamber. The currently preferred procedure for bombarding the callus entails exactly the procedure of Klein et al. (1988b) with the recipient tissue positioned 5 cm below the stopping plate tray.

The callus cultures useful herein for generation of transgenic plants should generally be about 3 months to 3 years old, preferably about 3 to 18 months old. Callus used for bombardment should generally be about midway between transfer periods and thus past any "lag" phase that might be associated with a transfer to a new media, but also before reaching any "stationary" phase associated with a long time on the same plate.

The specific tissue subjected to the bombardment process is preferably taken about 7–10 days after subculture, though this is not believed critical. The tissue should generally be used in the form of pieces of about 30 to 80, preferably about 40 to 60, mg. The clumps are placed on a petri dish or other surface and arranged in essentially any manner, recognizing that (i) the space in the center of the dish will receive the heaviest concentration of metal-DNA particles and the tissue located there is likely to suffer damage during bombardment and (ii) the number of particles reaching a cell will decrease (probably exponentially) with increasing distance of the cell from the center of the blast so that cells far from the center of the dish are not likely to be bombarded and transformed. A mesh screen, preferably of metal, may be laid on the dish to prevent splashing or ejection of the tissue. The tissue may be bombarded one or more times with the DNA-coated metal particles.

IV. Selection Process

Once the calli have been bombarded with the DNA and the DNA has penetrated some of the cells, it is necessary to identify and select those cells which both contain the heterologous DNA and still retain sufficient regenerative capacity. There are two general approaches which have been found useful for accomplishing this. First, the transformed calli or plants regenerated therefrom can be screened for the presence of the heterologous DNA by various standard methods which could include assays for the expression of reporter genes or assessment of phenotypic effects of the heterologous DNA, if any. Alternatively and preferably, when a selectable marker gene has been transmitted along with or as part of the heterologous DNA, those cells of the callus which have been transformed can be identified by the use of a selective agent to detect expression of the selectable marker gene.

Selection of the putative transformants is a critical part of the successful transformation process since selection conditions must be chosen so as to allow growth and accumulation of the transformed cells while simultaneously inhibiting the growth of the non-transformed cells. The situation is complicated by the fact that the vitality of individual cells in a population is often highly dependent on the vitality of neighboring cells. Also, the selection conditions must not be so severe that the plant regeneration capacity of the callus cells and the fertility of the resulting plant are precluded. Thus the effects of the selection agent on cell viability and morphology should be evaluated. This may be accomplished by experimentally producing a growth inhibition curve for the given selective agent and tissue being transformed beforehand. This will establish the concentration range which will inhibit growth.

When a selectable marker gene has been used, the callus clumps may be either allowed to recover from the bombardment on non-selective media or, preferably, directly transferred to media containing that agent.

Selection procedures involve exposure to a toxic agent and may employ sequential changes in the concentration of the agent and multiple rounds of selection. The particular concentrations and cycle lengths are likely to need to be varied for each particular agent. A currently preferred selection procedure entails using an initial selection round at a relatively low toxic agent concentration and then later round(s) at higher concentration(s). This allows the selective agent to exert its toxic effect slowly over a longer period of time. Preferably the concentration of the agent is initially such that about a 5–40% level of growth inhibition will occur, as determined from a growth inhibition curve. The effect may be to allow the transformed cells to preferentially grow and divide while inhibiting untransformed cells, but not to the extent that growth of the transformed cells is prevented. Once the few individual transformed cells have grown sufficiently the tissue may be shifted to media containing a higher concentration of the toxic agent to kill essentially all untransformed cells. The shift to the higher concentration also reduces the possibility of nontransformed cells habituating to the agent. The higher level is preferably in the range of about 30 to 100% growth inhibition. The length of the first selection cycle may be from about 1 to 4 weeks, preferably about 2 weeks. Later selection cycles may be from about 1 to about 12 weeks, preferably about 2 to about 10 weeks. Putative maize transformants can generally be identified as proliferating sectors of tissue among a background of non-proliferating cells. The callus may also be cultured on non-selective media at various times during the overall selection procedure.

Once a callus sector is identified as a putative transformant, transformation can be confirmed by phenotypic and/or genotypic analysis. If a selection agent is used, an example of phenotypic analysis is to measure the increase in fresh weight of the putative transformant as compared to a control on various levels of the selective agent. Other analyses that may be employed will depend on the function of the heterologous DNA. For example, if an enzyme or protein is encoded by the DNA, enzymatic or immunological assays specific for the particular enzyme or protein may be used. Other gene products may be assayed by using a suitable bioassay or chemical assay. Other such techniques are well known in the art and are not repeated here. The presence of the gene can also be confirmed by conventional procedures, i.e. Southern blot or polymerase chain reaction (PCR) or the like.

V. Regeneration of Plants and Production of Seed

Cell lines which have been shown to be transformed must then be regenerated into plants and the fertility of the resultant plants determined. Transformed lines which test positive by genotypic and/or phenotypic analysis are then placed on a media which promotes tissue differentiation and plant regeneration. Regeneration may be carried out in accordance with standard procedures well known in the art. The procedures commonly entail reducing the level of auxin which discontinues proliferation of a callus and promotes somatic embryo development or other tissue differentiation. One example of such a regeneration procedure is described in Green et al. (1981). The plants are grown to maturity in a growth room or greenhouse and appropriate sexual crosses and selfs are made as described by Neuffer (1981).

Regeneration, while important to the present invention, may be performed in any conventional manner. If a selectable marker has been transformed into the cells, the selection agent may be incorporated into the regeneration media to further confirm that the regenerated plantlets are transformed. Since regeneration techniques are well known and not critical to the present invention, any technique which accomplishes the regeneration and produces fertile plants may be used.

VI. Analysis of R1 Progeny

The plants regenerated from the transformed callus are referred to as the R0 generation or R0 plants. The seeds produced by various sexual crosses of the R0 generation plants are referred to as R1 progeny or the R1 generation. When R1 seeds are germinated, the resulting plants are also referred to as the R1 generation.

To confirm the successful transmission and inheritance of the heterologous DNA in the sexual crosses described above, the R1 generation should be analyzed to confirm the presence of the transforming DNA. The analysis may be performed in any of the manners such as were disclosed above for analyzing the bombarded callus for evidence of transformation, taking into account the fact that plants and plant parts are being used in place of the callus.

VII. Breeding of Genetically Engineered Commercial Hybrid Seed

Generally, the commercial value of the transformed corn produced herein will be greatest if the heterologous DNA can be incorporated into many different hybrid combinations. A farmer typically grows several varieties of hybrids based on differences in maturity, standability, and other agronomic traits. Also, the farmer must select a hybrid based upon his physical location since hybrids adapted to one part of the corn belt are generally not adapted to another part because of differences in such traits as maturity, disease, and insect resistance. As such, it is necessary to incorporate the heterologous DNA into a large number of parental lines so that many hybrid combinations can be produced containing the desirable heterologous DNA. This may conveniently be done by breeding programs in which a conversion process (backcrossing) is performed by crossing the initial transgeniC fertile plant to normal elite inbred lines and then crossing the progeny back to the normal parent. The progeny from this cross will segregate such that some of the plants will carry the heterologous DNA whereas some will not. The plants that do carry the DNA are then crossed again to the normal plant resulting in progeny which segregate once more. This crossing is repeated until the original normal parent has been converted to a genetically engineered line containing the heterologous DNA and also possessing all other important attributes originally found in the parent. A separate backcrossing program will be used for every elite line that is to be converted to a genetically engineered elite line. It may be necessary for both parents of a hybrid seed corn to be homozygous for the heterologous DNA. Corn breeding and the techniques and skills required to transfer genes from one line or variety to another are well-known to those skilled in the art. Thus introducing heterologous DNA into lines or varieties which do not generate the appropriate calli can be readily accomplished by these breeding procedures.

VIII. Uses of Transgenic Plants

The transgenic plants produced herein are expected to be useful for a variety of commercial and research purposes. Transgenic plants can be created for use in traditional agriculture to possess traits beneficial to the grower (e.g. agronomic traits such as pest resistance or increased yield), beneficial to the consumer of the grain harvested from the plant (e.g. improved nutritive content in human food or animal feed) or beneficial to the food processor (e.g. improved processing traits). In such uses, the plants are generally grown for the use of their grain in human or animal foods, however, other parts of the plants, including stalks, husks, vegetative parts, and the like, may also have utility, including use as part of animal silage or for ornamental purposes (e.g. Indian corn). Often chemical constituents (e.g. oils or starches) of corn and other crops are extracted for food or industrial use and transgenic plants may be created which have enhanced or modified levels of such components. The plants may also be used for seed production for a variety of purposes.

Transgenic plants may also find use in the commercial manufacture of proteins or other molecules encoded by the heterologous DNA contained therein, where the molecule of interest is extracted or purified from plant parts, seeds, and the like. Cells or tissue from the plants may also be cultured, grown in vitro, or fermented to manufacture such molecules, or for other purposes (e.g. for research).

The transgenic plants may also be used in commercial breeding programs, or may be crossed or bred to plants of related crop species. Improvements encoded by the heterologous DNA may be transferred, e.g. from corn cells to cells of other species e.g. by protoplast fusion.

The transgenic plants may have many uses in research or breeding, including creation of new mutant plants through insertional mutagenesis, in order to identify beneficial mutants that might later be created by traditional mutation and selection. The methods of the invention may also be used to create plants having unique "signature sequences" or other marker sequences which can be used to identify proprietary lines or varieties.

The following non-limiting examples are illustrative of the present invention. They are presented to better explain the general procedures which were used to prepare the fertile *Zea mays* plants of this invention which stably express the heterologous DNA and which transmit that DNA to progeny. All parts and percents are by weight unless otherwise specified. It must be recognized that a specific transformation event is a function of the amount of material subjected to the transfermation procedure. Thus when individual situations arise in which the procedures described herein do not produce a transformed product, repetition of the procedures will be required.

EXAMPLE I

Fertile transgenic *Zea mays* plants which contain heterologous DNA which is heritable were prepared as follows:

I. Initiation and Maintenance of Maize Cell Cultures Which Retain Plant Regeneration Capacity

Friable, embryogenic maize callus cultures were initiated from hybrid immature embryos produced by pollination of inbred line A188 plants (University of Minnesota, Crop Improvement Association) with pollen of inbred line B73 plants (Iowa State University). Ears were harvested when

the embryos had reached a length of 1.5 to 2.0 mm. The whole ear was surface sterilized in 50% v/v commercial bleach (2.63% w/v sodium hypochlorite) for 20 min. at room temperature. The ears were then washed with sterile distilled, deionized water. Immature embryos were aseptically isolated and placed on nutrient agar initiation/maintenance media with the root/shoot axis exposed to the medium. Initiation/maintenance media (hereinafter refered to as F medium) consisted of N6 basal media (Chu 1975) with 2% (w/v) sucrose, 1.5 mg per liter 2,4-dichlorophenoxyacetic acid (2,4-D), 6 mM proline, and 0.25% Gelrite (Kelco, Inc. San Diego). The pH was adjusted to 5.8 prior to autoclaving. Unless otherwise stated, all tissue culture manipulations were carried out under sterile conditions.

The immature embryos were incubated at 26° C. in the dark. Cell proliferations from the scutellum of the immature embryos were evaluated for friable consistency and the presence of well defined somatic embryos. Tissue with this morphology was transferred to fresh media 10 to 14 days after the initial plating of the immature embryos. The tissue was then subcultured on a routine basis every 14 to 21 days. Sixty to eighty milligram quantities of tissue were removed from pieces of tissue that had reached a size of approximately one gram and transferred to fresh media. Subculturing always involved careful visual monitoring to be sure that only tissue of the correct morphology was maintained. The presence of somatic embryos ensured that the cultures would give rise to plants under the proper conditions. The cell culture named AB12 used in this example was such a culture and had been initiated about 1 year before bombardment.

II. Plasmids—pCHNI-1, pHYGI1, pBII2.21, and pLUC-1

The plasmids pCHN1-1, pHYGI1, and pLUC-1 were constructed in the vector pBS+ (Stratagene, Inc., San Diego, Calif.), a 3.2 Kb circular plasmid, using standard recombinant DNA techniques. pCHN1-1 contains the hygromycin B phosphotransferase (HPT) coding sequence from *E. coli* (Gritz et al. 1983) flanked at the 3' end by the nopaline synthase (nos) polyadenylation sequence of *Agrobacterium tumefaciens* M. Bevan et al., *Nuc. Acids Res.*, 11, 369, 1983). Expression is driven by the cauliflower mosaic virus (CaMV) 35S promoter (Guilley et al. 1982), located upstream from the hygromycin coding sequence. The plasmid pHYGI1 was constructed by inserting the 553 bp Bcl-BamHI fragment containing the maize AdhIS first intron (Callis et al. 1987) between the CaMV 35S promoter and the hygromycin coding sequence of pCHN1-1. A map of pHYGI1 is provided as FIG. 1A.

pBII221 contains the *E. Coli* B-glucuronidase coding sequence flanked at the 5' end by the CaMV 35S promoter and at the 3' end by the nos polyadenylation sequence. The plasmid was constructed by inserting the maize AdhIS first intron between the 35S promoter and the coding sequence of pBI221 (Jefferson et al. 1987). A map of pBII221 is provided as FIG. 2A.

pLUC-1 contains the firefly luciferase coding sequence (DeWet et al. 1987) flanked at the 5' end by the CaMV 35S promoter and at the 3' end by the nos polyadenylation sequence. This plasmid was used solely as a negative control.

Plasmids were introduced into the embryogenic callus culture AB12 by microprojectile bombardment.

III. DNA Delivery Process

The embryogenic maize callus line AB12 was subcultured 7 to 12 d prior to microprojectile bombardment. AB12 was prepared for bombardment as follows. Five clumps of callus, each approximately 50 mg in wet weight were arranged in a cross pattern in the center of a sterile 60 x 15 mm petri plate (Falcon 1007). Plates were stored in a closed container with moist paper towels throughout the bombardment process. Twenty six plates were prepared.

Plasmids were coated onto M-10 tungsten particles (Biolistics) exactly as described by Klein, et al (1988b) except that, (i) twice the recommended quantity of DNA was used, (ii) the DNA precipitation onto the particles was performed at 0° C., and (iii) the tubes containing the DNA-coated tungsten particles were stored on ice throughout the bombardment process.

All of the tubes contained 25 ul 50 mg/ml M-10 tungsten in water, 25 ul 2.5M $CaCl_2$, and 10 ul 100 mM spermidine free base along with a total of 5 ul 1 mg/ml total plasmid content. When two plasmids were used simultaneously, each was present in an amount of 2.5 ul. One tube contained only plasmid pBII221; two tubes contained both plasmids pHYGI1 and pBII221; two tubes contained both plasmids pCHNI-1 and pBII221; and one tube contained only plasmid pLUC-1.

All tubes were incubated on ice for 10 min., pelletized by centrifugation in an Eppendorf centrifuge at room temperature for 5 seconds, and 25 ul of the supernatant was discarded. The tubes were stored on ice throughout the bombardment process. Each preparation was used for no more than 5 bombardments.

Macroprojectiles and stopping plates were obtained from Biolistics, Inc. (Ithaca, N.Y.). They were sterilized as described by the supplier. The microprojectile bombardment instrument was obtained from Biolistics, Inc.

The sample plate tray was positioned at the position 5 cm below the bottom of the stopping plate tray of the microprojectile instrument, with the stopping plate in the slot below the barrel. Plates of callus tissue prepared as described above were centered on the sample plate tray and the petri dish lid removed. A 7×7 cm square rigid wire mesh with 3×3 mm mesh and made of galvanized steel was placed over the open dish in order to retain the tissue during the bombardment. Tungsten/DNA preparations were sonicated as described by Biolistics, Inc. and 2.5 ul was pipetted onto the top of the macroprojectiles. The instrument was operated as described by the manufacturer. The following bombardments were performed:

2×pBII221 prep To determine transient expression frequency

10×pHYGI1/pBII221 As a potential positive treatment for transformation

1×pCHN1-1/pBII221 As a potential positive treatment for transformation

4×pLUC-1 Negative control treatment

The two plates of callus bombarded with pBII221 were transferred plate for plate to F medium (with no hygromycin) and the callus cultured at 26° C. in the dark. After 2 d this callus was then transferred plate for plate into 35×10 mm petri plates (Falcon 1008) containing 2 ml of GUS assay buffer which consists of 1 mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide (Research Organics), 100 mM sodium phosphate pH 7.0, 5 mM each of potassium ferricyanide and potassium ferrocyanide, 10 mM EDTA, and 0.06% Triton X-100. These were incubated at 37° C. for 3 d after which the number of blue cells was counted giving 291 and 477 transient GUS expressing cells in the two plates, suggesting that the DNA delivery process had also occurred with the other bombarded plates. These plates were discarded after counting since the GUS assay is destructive.

IV. Selection Process

Hygromycin B (Calbiochem) was incorporated into the medium by addition of the appropriate volume of filter sterilized 100 mg/ml Hygromycin B in water when the media had cooled to 45° C. prior to pouring plates.

Immediately after all samples had been bombarded, callus from all of the plates treated with pHYGI1/pBII221, pCHN1-1/pBII221 and three of the plates treated with pLUC-1 were transferred plate for plate onto F medium containing 15 mg/l hygromycin B, (five pieces of callus per plate). These are referred to as round 1 selection plates. Callus from the fourth plate treated with pLUC-1 was transferred to F medium without hygromycin. This tissue was subcultured every 2–3 weeks onto nonselective medium and is referred to as unselected control callus.

After two weeks of selection, tissue appeared essentially identical on both selective and nonselective media. All callus from eight plates from each of the pHYGI1/pBII221 and pCHN1-1/pBII-221 treatments and two plates of the control callus on selective media were transferred from round 1 selection plates to round 2 selection plates that contained 60 mg/l hygromycin. The round 2 selection plates each contained ten 30 mg pieces of callus per plate, resulting in an expansion of the total number of plates.

The remaining tissue on selective media, two plates each of pHYGI1/pBII221 and pCHN1-1/pBII221 treated tissue and one of control callus, were placed in GUS assay buffer at 37° C. to determine whether blue clusters of cells were observable at two weeks post-bombardment. After 6 d in assay buffer this tissue was scored for GUS expression.

TREATMENT	REPLICATE	OBSERVATIONS
pLUC-1		no blue cells
pHYGI1/pBII221	plate 1	11 single cells 1 four cell cluster
	plate 2	5 single cells
pCHN1-1/pBII221	plate 1	1 single cell 2 two cell clusters
	plate 2	5 single cells 1 two cell cluster 2 clusters of 8–10 cells

After 21 d on the round 2 selection plates, all viable portions of the material were transferred to round 3 selection plates containing 60 mg/l hygromycin. The round 2 selection plates, containing only tissue that was apparently dead, were reserved. Both round 2 and 3 selection plates were observed periodically for viable proliferating sectors.

After 35 d on round 3 selection plates both the round 2 and round 3 sets of selection plates were checked for viable sectors of callus. Two such sectors were observed proliferating from a background of dead tissue on plates treated with pHYGI1/pBII221. The first sector named 3AA was from the round 3 group of plates and the second sector named 6L was from the round 2 group of plates. Both lines were then transferred to F medium without hygromycin.

After 19 d on F medium without hygromycin the line 3AA grew very little whereas the line 6L grew rapidly. Both were transferred again to F medium for 9 d. The lines 3AA and 6L were then transfered to F medium containing 15 mg/l hygromycin for 14 d. At this point, line 3AA was observed to be of very poor quality and slow growing. The line 6L however grew rapidly on F medium with 15 mg.l hygromycin. In preparation for an inhibition study of the line 6L on hygromycin, the line was then subcultured to F medium without hygromycin.

After 10 d on F medium an inhibition study of the line 6L was initiated. Callus of 6L was transfered onto F medium containing 0, 10, 30, 100, and 250 mg/l hygromycin B. Five plates of callus were prepared for each concentration and each plate contained ten approximately 50 mg pieces of callus. One plate of unselected control tissue was prepared for each concentration of hygromycin.

It was found that the line 6L was capable of sustained growth over 9 subcultures on 0, 10, 30, 190, and 250 mg/l hygromycin. The name of the line 6L was changed at this time from 6L to PH1 (Positive Eygromycin transformant 1)

Additional sectors were recovered at various time points from the round 2 and 3 selection plates. None of these were able to grow in the presence of hygromycin for multiple rounds, i.e. two or three subcultures.

V. Confirmation of Transformed Callus

To show that the PH1 callus had acquired the hygromycin resistance gene, a Southern blot of PH1 callus was prepared as follows: DNA was isolated from PH1 and unselected control calli by freezing 2 g of callus in liquid nitrogen and grinding it to a fine powder which was transferred to a 30 ml Oak Ridge tube containing 6 ml extraction buffer (7M urea, 250 mM NaCl, 50 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1% sarcosine). To this was added 7 ml of phenol:chloroform 1:1, the tubes shaken and incubated at 37° C. 15 min. Samples were centrifuged at 8K for 10 min. at 4° C. The supernatant was pipetted through miracloth (Calbiochem 475855) into a disposable 15 ml tube (American Scientific Products, C3920–15A) containing 1 ml 4.4M ammonium acetate, pH 5.2. Isopropanol, 6 ml, was added, the tubes shaken, and the samples incubated at –20° C. for 15 min. The DNA was pelleted in a Beckman TJ-6 centrifuge at the maximum speed for 5 min. at 4° C. The supernatant was discarded and the pellet was dissolved in 500 ul TE-10 (10 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0) 15 min. at room temperature. The samples were transferred to a 1.5 ml Eppendorf tube and 100 ul 4.4M ammonium acetate, pH 5.2 and 700 ul isopropanol were added. This was incubated at –20° C. for 15 min. and the DNA pelleted 5 min. in an Eppendorf microcentrifuge (12,000 rpm). The pellet was washed with 70% ethanol, dried, and resuspended in TE-1 (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

The isolated DNA (10 ug) was digested with BamHI (NEB) and electrophoresed in a 0.8% w/v agarose gel at 15V for 16 hrs in TAE buffer (40 mM Tris-acetate, i mM EDTA). The DNA within the gel was then depurinated by soaking the gel twice in 0.25 M HCl for 15 min., denatured and cleaved by soaking the gel twice in 0.5M NaOH/1.0M NaCl 15 min., and neutralized by soaking the gel twice in 0.5M Tris pH 7.4/3M NaCl 30 min. DNA was then blotted onto a Nytran membrane (Shleicher & Shuell) by capillary transfer overnight in 6X SSC (20X SSC, 3M NaCl, 0.3M sodium citrate pH 7.0). The membrane was baked at 80° C. for 2 hrs under vacuum. Prehybridization treatment of the membrane was done in 6X SSC, 10X Denhardt's solution, 1% SDS, 50 ug/ml denatured salmon sperm DNA using 0.25 ml prehybridization solution per cm^2 of membrane. Prehybridization was carried out at 42° C. overnight.

A 32P labelled probe was prepared by random primer labelling with an Oligo Labelling Kit (Pharmacia) as per the suppliers instructions with 32P-dCTP (ICN Radiochemicals). The template DNA used was the 1055 bp BamHI fragment of pHYGI1, which is the HPT coding sequence. The fragment was gel purified and cut again with PsI (NEB) before labelling.

The hybridization was performed in 50% formamide, 6X SSC, 1% SDS, 50 ug/ml denatured salmon sperm DNA (Sigma), 0.05% sodium pyrophosphate and all of the isopropanol precipitated heat denatured probe (107 CPM/50ug template). The hybridization was carried out at 42° C. overnight.

The membrane was washed twice in 50 ml 6X SSC, 0.1% SDS 5 min. at room temperature with shaking, then twice in 500 ml 6X SSC, 0.1% SDS 15 min. at room temperature, then twice in 500 ml 1X SSC, 1% SDS 30 min. at 42° C., and finally in 500 ml 0.1X SSC 1% SDS 60 min. at 65° C. Membranes were exposed to Kodak X-OMAT AR film in an X-OMATIC cassette with intensifying screens. As shown in FIG. 3B, a band was observed for PH1 callus at the expected position of 1.05 Kb, indicating that the HPT coding sequence was present. No band was observed for control callus.

VI. Plant Regeneration and Production of Seed

PH1 callus was transferred directly from all of the concentrations of hygromycin used in the inhibition study to RM5 medium which consists of MS basal salts (Murashige et al. 1962) supplemented with thiamine HCl 0.5 mg/l, 2,4-D 0.75 mg/l, sucrose 50 g/l, asparagine 150 mg/l, and Gelrite 2.5 g/l (Kelco Inc. San Diego).

After 14 d on RM5 medium the majority of PH1 and negative control callus was transferred to R5 medium which is the same as RM5 medium, except that 2,4-D is omitted. These were cultured in the dark for 7 d at 26° C. and transferred to a light regime of 14 hours light and 10 hours dark for 14 d at 26° C. At this point, plantlets that had formed were transferred to one quart canning jars (Ball) containing 100 ml of R5 medium. Plants were transferred from jars to vermiculite after 14 and 21 d. Plants were grown in vermiculite for 7 or 8 d before transplanting into soil and grown to maturity. A total of 65 plants were produced from PH1 and a total of 30 plants were produced from control callus.

To demonstrate that the introduced DNA had been retained in the Ro tissue, a Southern blot was performed as previously described on leaf DNA from three randomly chosen Ro plants of PH1. As shown in FIG. 4B, a 1.05 Kb band was observed with all three plants indicating that the HPT coding sequence was present. No band was observed for DNA from a control plant.

Controlled pollinations of mature PH1 plants were conducted by standard techniques with inbred lines A188, B73 and Oh43. Seed was harvested 45 days post-pollination and allowed to dry further 1–2 weeks. Seed set varied from 0 to 40 seeds per ear when PH1 was the female parent and from 0 to 32 seeds per ear when PH1 was the male parent.

VII. Analysis of the R1 Progeny

The presence of the hygromycin resistance trait was evaluated by a root elongation bioassay, an etiolated leaf bioassay, and by Southern blotting. Two ears each from regenerated PH1 and control plants were selected for analysis. The pollen donor was inbred line A188 for all ears.

(A) Root Elongation Bioassay

Seed was sterilized in a 1:1 dilution of commercial bleach in water plus alconox 0.1% for 20 min. in 125 ml Erlenmeyer flasks and rinsed 3 times in sterile water and imbibed overnight in sterile water containing 50 mg/ml captan by shaking at 150 rpm.

After imbibition, the solution was decanted from the flasks and the seed transferred to flow boxes (Flow Laboratories) containing 3 sheets of H_2O saturated germination paper. A fourth sheet of water saturated germination paper was placed on top of the seed. Seed was allowed to germinate 4 d.

After the seed had germinated, approximately 1 cm of the primary root tip was excised from each seedling and plated on MS salts, 20 g/l sucrose, 50 mg/l hygromycin, 0.25% Gelrite, and incubated in the dark at 26° C. for 4 d.

Roots were evaluated for the presence or absence of abundant root hairs and root branches. Roots were classified as transgenic (hygromycin resistant) if they had root hairs and root branches, and untransformed (hygromycin sensitive) if they had limited numbers of branches. The results are shown in Table 1.

(B) Etiolated leaf bioassay

After the root tips were excised as described above, the seedlings of one PH1 ear and one control ear were transferred to moist vermiculite and grown in the dark for 5 d. At this point 1 mm sections were cut from the tip of the coleoptile, surface sterilized 10 seconds, and plated on MS basal salts, 20 g/l sucrose, 2.5 g/l Gelrite with either 0 (control) or 100 mg/l hygromycin and incubated in the dark at 26° C. for 18 hr. Each plate contained duplicate sections of each shoot. They were then incubated in a light regimen of 14 hours light 10 hours dark at 26° C. for 48 hr, and rated on a scale of from 0 (all brown) to 6 (all green) for the percent of green color in the leaf tissue. Shoots were classified as untransformed (hygromycin sensitive) if they had a rating of zero and classified as transformed (hygromycin resistant) if they had a rating of 3 or greater. The results are shown in Table 1.

(C) Southern Blots

Seedling form the bioassays were transplanted to soil and are growing to sexual maturity. DNA was isolated from 0.8 g of leaf tissue after about 3 weeks and probed with the HPT coding sequence as described previously. Plants with a 1.05 Kb band present in the Southern blot were classified as transgenic. As shown in FIG. 5B, two out of seven progeny of PH1 plant 3 were transgenic as were three out of eight progeny of PH1 plant 10. The blot results correlated precisely with data from the bioassays, confirming that the heterologous DNA was transmitted through one complete sexual life cycle. All data are summarized in Table 1.

TABLE 1

	ANALYSIS OF PH1 R1 PLANTS						
PH1 PLANT	ROOT ASSAY	LEAF ASSAY	BLOT	CONT. PLANT	ROOT ASSAY	LEAF ASSAY	BLOT
3.1	+	ND	+	4.1	–	ND	ND
3.2	–	ND	–	4.2	–	ND	ND

TABLE 1-continued

	ANALYSIS OF PH1 R1 PLANTS						
PH1 PLANT	ROOT ASSAY	LEAF ASSAY	BLOT	CONT. PLANT	ROOT ASSAY	LEAF ASSAY	BLOT
3.3	–	ND	–	4.3	–	ND	ND
3.4	–	ND	–	4.4	–	ND	ND
3.5	–	ND	–	4.5	–	ND	ND
3.6	+	ND	+	4.6	–	ND	ND
3.7	–	ND	–	4.7	–	ND	ND
				2.1	–	ND	–
10.1	+	+	+	1.1	–	–	–
10.2	+	+	+	1.2	–	–	ND
10.3	–	–	ND	1.3	–	–	ND
10.4	–	–	–	1.4	–	–	ND
10.5	–	–	–	1.5	–	–	ND
10.6	–	–	–	1.6	–	–	ND
10.7	–	–	–	1.7	–	–	ND
10.8	ND	+	+	1.8	–	–	ND

KEY: + = transgenic; – = nontransgenic; ND = not done

EXAMPLE II

The procedure of Example I was repeated with minor modifications.

I. Plant lines and tissue cultures

The embryogenic maize callus line, AB12, was used as in Example I. The line had been initiated about 18 months before the actual bombardment occurred.

II. Plasmids

The plasmids pBII221 and pHYGI1 described in Example I were used.

III. DNA delivery process

Callus was bombarded exactly as in Example I except that the DNA used in the tungsten/DNA preparations differed. All of the tubes contained 25 ul 50 mg/ml M-10 tungsten in water, 25 ul 2.5M $CaCl_2$, and 10 ul 100 mM spermidine free base along with a total of 5 ul 1 mg/ml total plasmid content. One tube contained only plasmid pBII221; two tubes contained only plasmid pHYGI1; and one tube contained no plasmid but 5 ul TE-1 (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0).

The following bombardments were done:

2 × pBII221 prep	For transient expression
7 × pHYGI1 prep	Potential positive treatment
3 × TE prep	Negative control treatment

After all the bombardments were performed, the callus from the pBII221 treatments was transferred plate for plate to F medium as five 50 mg pieces. After 2 d the callus was placed into GUS assay buffer as per Example I. Numbers of transiently expressing cells were counted and found to be 686 and 845 GUS positive cells, suggesting that the particle delivery process had occurred in the other bombarded plates.

IV. Selection of Transformed Callus

After bombardment the callus from the pHYGI1 treatments was placed onto round 1 selection plates, F medium containing 15 mg/l hygromycin, as ten 25 mg pieces per plate (different from Example I). The same was done for two of the plates bombarded with the TE preparation (selected control callus). One plate of callus bombarded with the TE preparation was placed onto F medium with no hygromycin; this callus was maintained throughout the ongoing experiment as a source of control tissue and was referred to as unselected control callus.

After 13 d the callus on round 1 selection plates was indistinguishable from unselected control callus. All of the callus was transferred from round 1 selection plates to round 2 selection plates containing 60 mg/l hygromycin. An approximate five fold expansion of the numbers of plates occurred.

The callus on round 2 selection plates had increased substantially in weight after 23 d, but at this time appeared close to dead. All of the callus was transfered from round 2 selection plates to round 3 selection plates containing 60 mg/l hygromycin. This transfer of all material from round 2 to round 3 selection differs from Example I in which only viable sectors were transferred and the round 2 plates reserved.

At 58 d post-bombardment three live sectors were observed proliferating from the surrounding dead tissue. All three lines were from pHYGI1 treatments and were designated 24C, 56A, and 55A.

After 15 d on maintainance medium, growth of the lines was observed. The line 24C grew well whereas lines 55A and 56A grew more slowly. All three lines were transferred to F medium containing 60 mg/l hygromycin. Unselected control callus from maintenance was plated to F medium having 60 mg/l hygromycin.

After 19 d on 60 mg/l hygromycin the growth of line 24C appeared to be entirely uninhibited, with the control showing approximately 80% of the weight gain of 24C. The line 56A was completely dead, and the line 55A was very close. The lines 24C and 55A were transferred again to F 60 mg/l hygromycin as was the control tissue.

After 23 d on 60 mg/l hygromycin the line 24C again appeared entirely uninhibited. The line 55A was completely dead, as was the negative control callus on its second exposure to to F 60 mg/l hygromycin.

At 88 d post-bombardment, a sector was observed proliferating among the surrounding dead tissue on the round 3 selection plates. The callus was from a plate bombarded with pHYGI1 and was designated 13E. The callus was transferred to F medium and cultured for 19 d. Portions of the callus were then transferred to (i) F media containing 15 mg/l hygromycin and (ii) F media containing 60 mg/l hygromycin. Control callus was plated on F media with 15 mg/l hygromycin. After 14 d of culture, the callus line 13E

appeared uninhibited on both levels of hygromycin. The control callus appeared to have about 80% of the weight gain of 13E. The callus lines were transferred to fresh media at the same respective levels of hygromycin.

V. Confirmation of Transformed Callus

A Southern blot was prepared from DNA from the line 24C. As shown in FIG. 6B, a band was observed for the line 24C at the expected size of 1.05 Kb showing that the line 24C contained the HPT coding sequence. No band was observed for DNA from control tissue. The name of the callus line 24C was changed to PH2.

VI. Plant Regeneration and Production of Seed

The line 24C along with unselected control callus were placed onto RM5 medium to regenerate plants as in Example I. After 16 d the callus was transferred to R5 medium as in Example I.

EXAMPLE III

The procedure of Example II was repeated exactly except that different plasmids were used.

The plasmids pBII221 and pHYGI1 described in Example I were used as well as pMS533 which is a plasmid that contains the insecticidal Bacillus thuringiensis endotoxin (BT) gene fused in frame with the neomycin phosphotransferase (NPTII) gene. 5' of the fusion gene are located segments of DNA from the CaMV 35S and nopaline synthase promoters. 3' from the fusion gene are segments of DNA derived from the .tomato protease inhibitor I gene and the poly A region of the nopaline synthase gene.

Callus was bombarded exactly as in Example I except that the DNA used in the tungsten/DNA preparations differed. Two tubes contained plasmids pHYGI1 and pMS533 and one tube contained no plasmid but 5 ul TE-1 (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0).

The following bombardments were done:

9 × pHYGI1/pMS533	Potential positive treatment
2 × TE prep	Control treatment

After bombardment the callus from the pHYGI1/pMS533 treatments was placed onto round 1 selection plates, F medium containing 15 mg/l hygromycin, as ten 25 mg pieces per plate. The same was done for one of the plates bombarded with the TE preparation (selected control callus). One plate of callus bombarded with the TE preparation was placed onto F medium with no hygromycin; this callus was maintained throughout the ongoing experiment as a source of control tissue and was referred to as unselected control callus.

After 12 d the callus on round 1 selection plates appeared to show about 90% of the weight gain of the unselected control callus. All of the callus was transferred from round 1 selection plates to round 2 selection plates containing 60 mg/l hygromycin as ten 30 mg pieces per plate.

After 22 d of selection on round 2 selection plates, the callus appeared completely uninhibited. All of the callus was transferred from round 2 selection plates to round 3 selection plates containing 60 mg/l hygromycin.

At 74 d post-bombardment a single viable sector was observed proliferating from the surrounding necrotic tissue. The callus line was from pHYGI1/pMS533 treated material and was designated 86R. The callus line 86R was transferred to F medium.

After 24 d the callus line 86R had grown substantially. Portions of the callus were then transferred to (i) F media containing 15 mg/l hygromycin and (ii) F media containing 60 mg/l hygromycin. Control callus was plated on F media with 15 mg/l hygromycin.

After 19 d of culture, the callus line 86R appeared to grow rapidly and was uninhibited on both levels of hygromycin. The control callus appeared to have only about 50% of the weight gain of 86R. The callus lines were transferred to fresh media at the same respective levels of hygromycin to further test the resistance of the callus line 86R to hygromycin.

COMPARATIVE EXAMPLE A

The basic procedures of Examples i-III have been attempted except varying the selection regime or the form of the callus. These other attempts, which are detailed in Table 2 below, were not successful. Since they were not repeated several times, it is not known whether they can be made to work. In all of the procedures, no viable sectors were observed. In the Table, "Sieved" indicates that the callus was passed through an 860 micron sieve before bombardment; the selective agent was hygromycin for each case except when pMXTI1 was the plasmid and methotrexate the selection agent.

TABLE 2

Summary of Comparative Example A

Recip. Tissue	Plasmids	Recov. Period	Round 1 Level	Round 1 Period	Round 2 Level	Round 2 Period
Clumps	pCHN1-1 pBII221	13	60	21	60	81
Clumps	pCHN1-1 pBII221	14	100	22	—	—
Clumps	pHYGI1 pBII221	8	60	19	30	132
Clumps	pCHN1-1 pBII221	0	30	22	60	109
Clumps	pMTXI1 pBII221	8	3	103	—	—
Sieved	pCHN1-1 pBII221	13	—	—	—	—

What is claimed is:

1. A process for producing a fertile transgenic *Zea mays* plant comprising the steps of (i) bombarding intact regenerable *Zea mays* cells with DNA-coated microprojectiles, (ii) identifying or selecting a population of transformed cells, and (iii) regenerating a fertile transgenic plant therefrom, wherein said DNA is transmitted through a complete sexual cycle of said transgenic plant to its progeny, and imparts herbicide resistance thereto.

2. The process of claim 1 wherein the fertile transgenic *Zea mays* plant is regenerated from transformed embryogenic tissue.

3. The process of claim 1 wherein the cells are derived from immature embryos.

4. A process comprising obtaining progeny from a fertile transgenic plant obtained by the process of claim 1 which comprise said DNA.

5. The process of claim 4 wherein said progeny are obtained by crossing said fertile transgenic plant with an inbred line.

6. The process of claim 4 comprising obtaining seed from

said progeny and obtaining further progeny plants comprising said DNA from said seed.

7. The process of claim **5** wherein the progeny obtained are crossed back to the inbred line, to obtain further progeny which comprise said DNA.

8. The process of claim **6** wherein seeds are obtained from said further progeny plants and plants comprising said DNA are recovered from said seed.

9. The process of claim **7** wherein said further progeny are crossed back to the inbred line to obtain progeny which comprise said DNA.

10. A process for producing a fertile transgenic *Zea mays* plant comprising the steps of (i) bombarding intact regenerable *Zea mays* cells with DNA-coated microprojectiles, (ii) identifying or selecting a population of transformed cells, and (iii) regenerating a fertile transgenic plant therefrom, wherein said DNA is transmitted through a complete sexual cycle of said transgenic plant to its progeny, wherein the DNA imparts insect resistance thereto.

11. The process of claim **10** wherein the fertile transgenic *Zea mays* plant is regenerated from transformed embryogenic tissue.

12. The process of claim **10** wherein the cells are derived from immature embryos.

13. A process comprising obtaining progeny from a fertile transgenic plant obtained by the process of claim **10**, which comprise said DNA.

14. The process of claim **13** wherein said progeny are obtained by crossing said fertile transgenic plant with an inbred line.

15. The process of claim **13** comprising obtaining seed from said progeny and obtaining further progeny plants comprising said DNA from said seed.

16. The process of claim **14** wherein the progeny obtained are crossed back to the inbred line, to obtain further progeny which comprise said DNA.

17. The process of claim **15** wherein seeds are obtained from said further progeny plants and plants comprising said DNA are recovered from said seed.

18. The process of claim **16** wherein said further progeny are crossed back to the inbred line to obtain progeny which comprise said DNA.

* * * * *

CASE NO. 04 C 50323

ATTACHMENT NO. _____

TAB DESCRIPTION #2

EXHIBIT _____

US006013863A

United States Patent [19]

Lundquist et al.

[11] Patent Number: 6,013,863

[45] Date of Patent: Jan. 11, 2000

[54] **FERTILE TRANSGENIC CORN PLANTS**

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[73] Assignee: **Dekalb Genetics Corporation**, Dekalb, Ill.

[21] Appl. No.: **08/844,555**

[22] Filed: **Apr. 21, 1997**

Related U.S. Application Data

[62] Division of application No. 08/677,695, Jul. 10, 1996, which is a continuation of application No. 07/974,379, Nov. 10, 1992, Pat. No. 5,538,877, which is a continuation of application No. 07/467,983, Jan. 22, 1990, abandoned.

[51] **Int. Cl.**[7] **C12N 15/00**; C12N 15/82; A01H 1/06; A01H 4/00

[52] **U.S. Cl.** **800/293**; 800/278; 800/288; 800/300; 435/430; 435/285.3

[58] **Field of Search** ..... 800/205, 200, 800/250, DIG. 56, 293, 278, 288; 47/58, DIG. 1; 435/172.3, 172.1, 412, 424, 430, 430.1, 287, 285, 285.3

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[57] **ABSTRACT**

A process of preparing fertile *Zea mays* plants is provided wherein said plants are resistant to the herbicide glyphosate, as well as processes for preparing seed, human food or animal feed therefrom.

8 Claims, 6 Drawing Sheets

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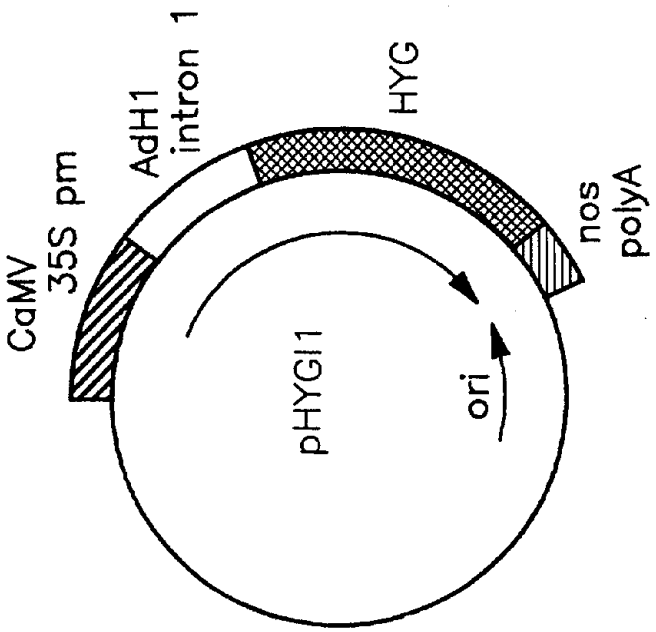

CaMV
35S pm
AdH1
intron 1
HYG
nos
polyA
pHYGI1
ori

FIG. 1A

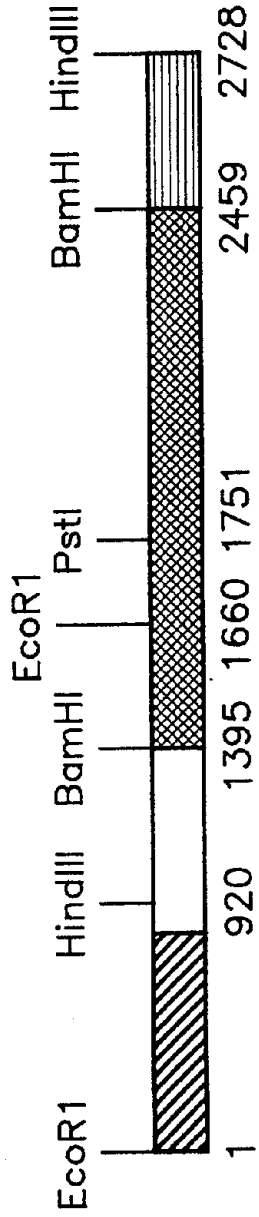

EcoR1
HindIII
BamHI
PstI
EcoR1
BamHI
HindIII
1
920
1395
1660
1751
2459
2728

FIG. 1B

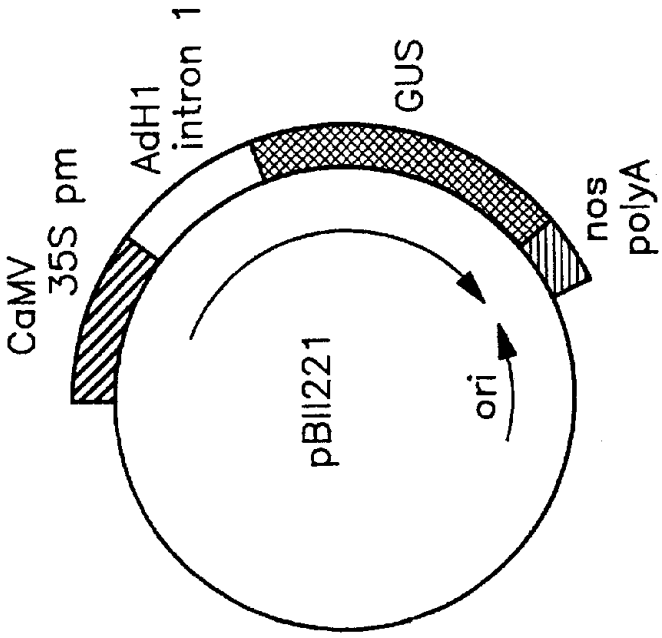

CaMV
35S pm
AdH1
intron 1
GUS
nos
polyA
pBII221
ori

FIG. 2A

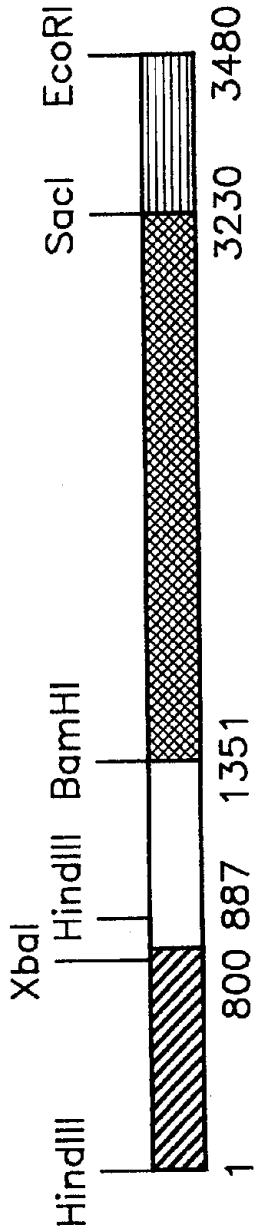

EcoRI
3480
SacI
3230
BamHI
1351
XbaI
HindIII
887
800
HindIII
1

FIG. 2B

FIG. 3

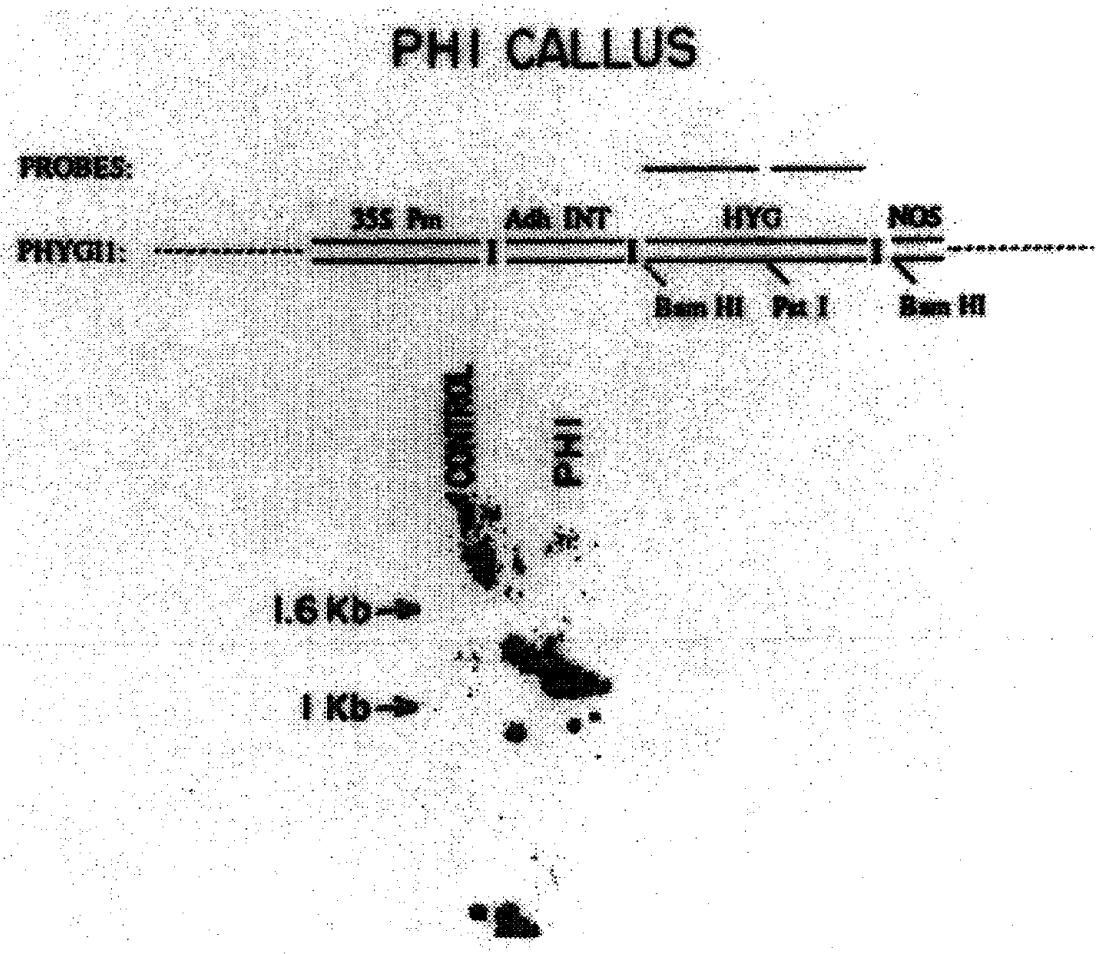

PHI CALLUS
PROBES:
PHYGH1:
35S Pm
Adh INT
HYG
NOS
Bam HI
Pst I
Bam HI
CONTROL
PHI
1.6 Kb
1 Kb

FIG. 4

PHI R_o PLANTS

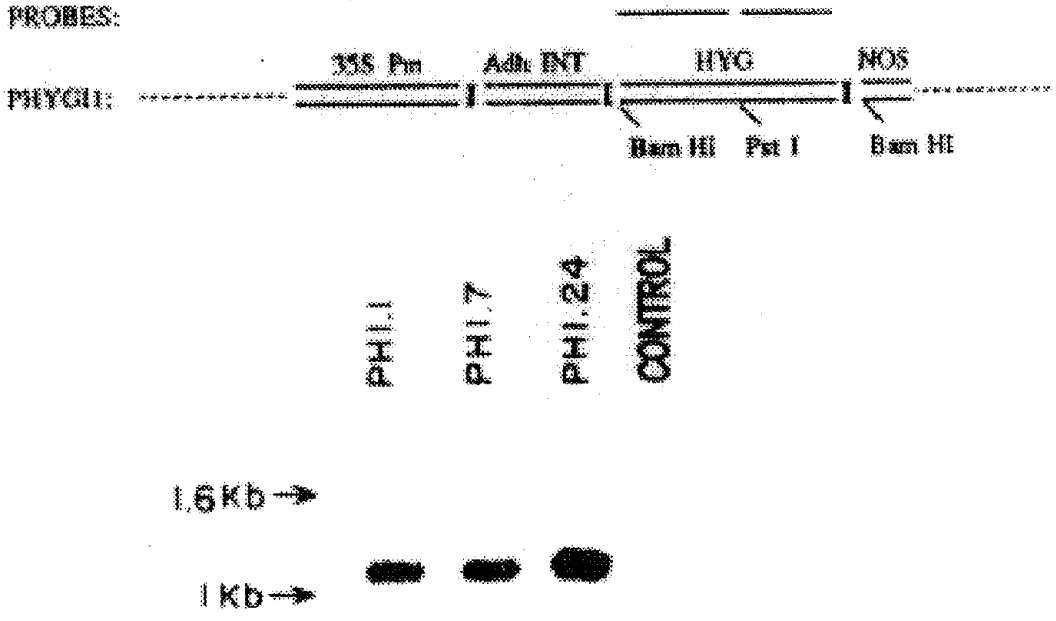


FIG. 5

PHI R_1 GENERATION

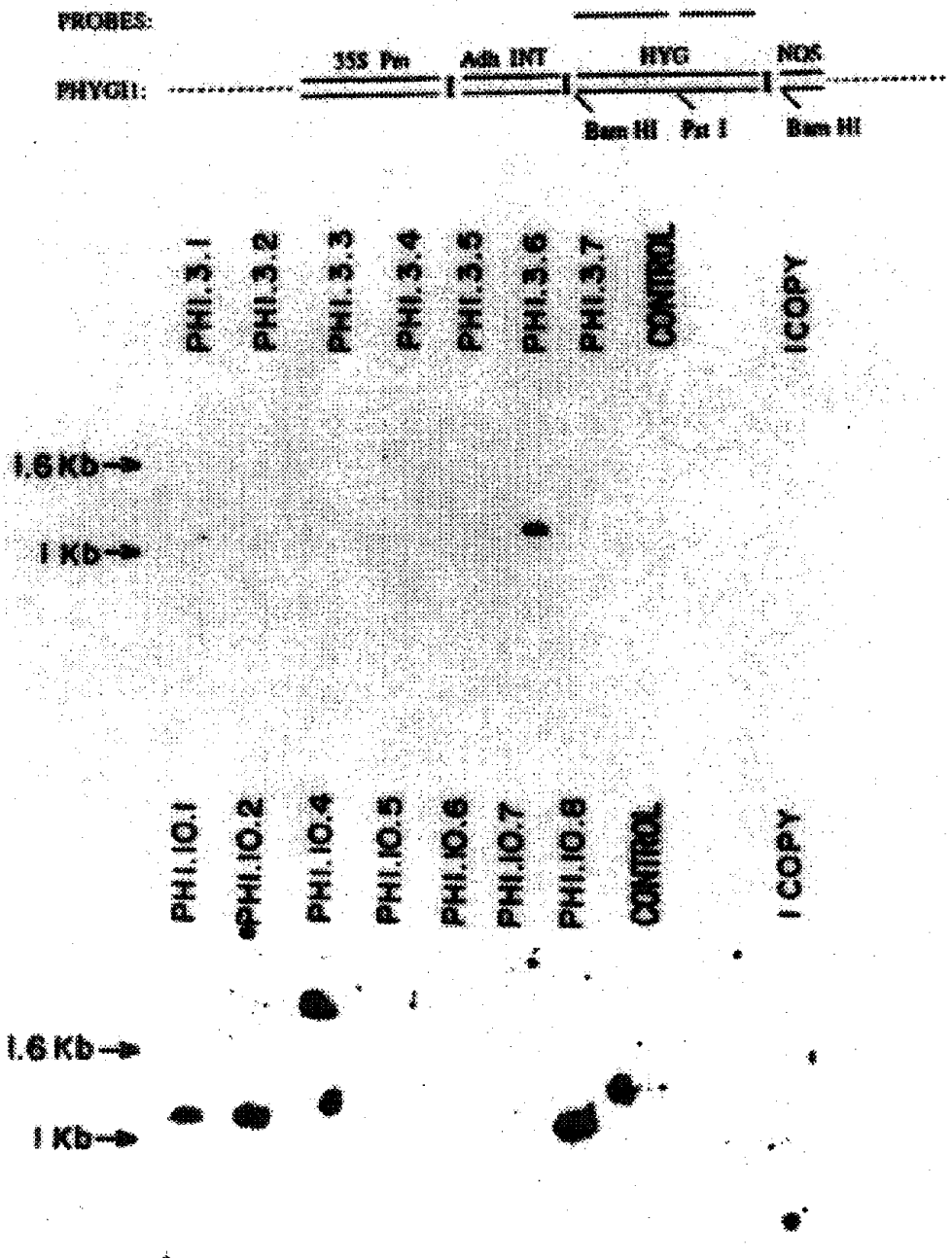
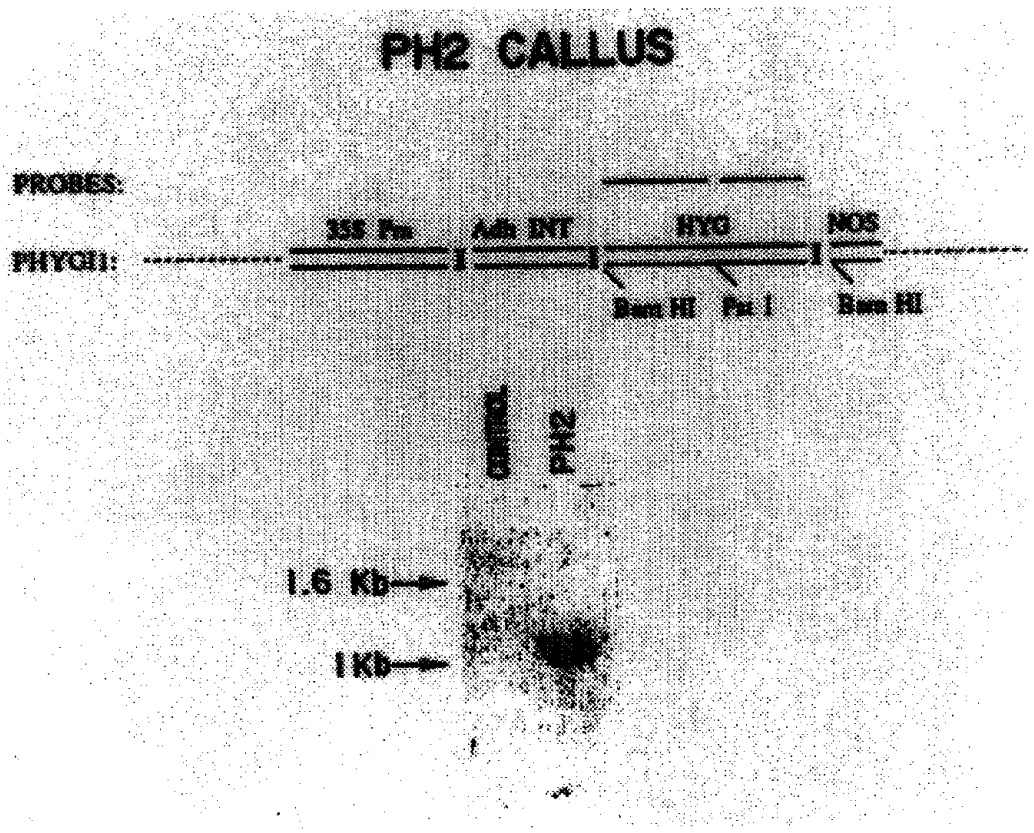


FIG. 6


PH2 CALLUS
PROBES:
PHYGI:
35S Pro
Adh INT
HYG
NOS
Bam HI
Pst I
Bam HI
PH2
1.6 Kb
1 Kb

FERTILE TRANSGENIC CORN PLANTS

This application is a division of U.S. patent application Ser. No. 08/677,695, filed Jul. 10, 1996, which is a continuation of U.S. patent application Ser. No. 07/974,379, filed Nov. 10, 1992, now U.S. Pat. No. 5,538,877, which in turn is a continuation of U.S. patent application Ser. No. 07/467,983, filed Jan. 22, 1990, now abandoned.

BACKGROUND OF THE INVENTION

This invention relates to fertile transgenic plants of the species *Zea mays* (oftentimes referred to herein as maize or corn). The invention further relates to producing transgenic plants via particle bombardment and subsequent selection techniques which have been found to produce fertile transgenic plants.

Genetic engineering of plants, which entails the isolation and manipulation of genetic material (usually in the form of DNA or RNA) and the subsequent introduction of that genetic material into a plant or plant cells, offers considerable promise to modern agriculture and plant breeding. Increased crop food values, higher yields, feed value, reduced production costs, pest resistance, stress tolerance, drought resistance, the production of pharmaceuticals, chemicals and biological molecules as well as other beneficial traits are all potentially achievable through genetic engineering techniques. Once a gene has been identified, cloned, and engineered, it is still necessary to introduce it into a plant of interest in such a manner that the resulting plant is both fertile and capable of passing the gene on to its progeny.

A variety of methods have been developed and are currently available for the transformation of various plants and plant cells with DNA. Generally these plants have been dicotyledonous, and some success has been reported with certain of the monocotyledonous cereals. However, some species have heretofore proven untransformable by any method. Thus, previous to this discovery, no technology had been developed which would permit the production of stably transformed *Zea mays* plants in which the transforming DNA is heritable thereof. This failure in the art is well documented in the literature and has been discussed in a number of recent reviews (Potrykus, 1989; Weising et al., 1988; Cocking et al., 1987).

European Patent Publns. 270,356 (McCabe et al.) and 275,069 (Arntzen et al.) describe the introduction of DNA into maize pollen followed by pollination of maize ears and formation of seeds. The plants germinated from these seeds are alleged to contain the introduced DNA, but there is no suggestion that the introduced DNA was heritable, as has been accomplished in the present invention. Only if the DNA introduced into the corn is heritable can the corn be used in breeding programs as required for successful commercialization of transgenic corn.

Graves et al. (1986) claims Acrobacterium-mediated transformation of *Zea mays* seedlings. The alleged evidence was based upon assays known to produce incorrect results.

Despite extensive efforts to produce fertile transformed corn plants which transmit the transforming DNA to progeny, there have been no reported successes. Many previous failures have been based upon gene transfer to maize protoplasts, oftentimes derived from callus, liquid suspension culture cells, or other maize cells using a variety of transformation techniques. Although several of the techniques have resulted in successful transformation of corn cells, the resulting cells either could not be regenerated into

corn plants or the corn plants produced were sterile (Rhodes et al. 1988) or, in some cases, it even turned out that the plants were in fact not transformed. Thus, while maize protoplasts and some other cells have previously been transformed, the resulting transformants could not be regenerated into fertile transgenic plants.

On the other hand, it has been known that at least certain corn callus can be regenerated to form mature plants in a rather straightforward fashion and that the resulting plants were often fertile. However, no stable transformation of maize callus was ever achieved, i.e. there were no techniques developed which would permit a successful stable transformation of a regenerable callus. An example of a maize callus transformation technique which has been tried is the use of Agrobacterium mediated transfer.

The art was thus faced with a dilemma. While it was known that corn protoplast and suspension culture cells could be transformed, no techniques were available which would regenerate the transformed protoplast into a fertile plant. While it was known that corn callus could be regenerated into a fertile plant, there were no techniques known which could transform the callus, particularly while not destroying the ability of the callus both to regenerate and to form fertile plants.

Recently, a new transformation technique has been created based upon-the bombardment of intact cells and tissues with DNA-coated microprojectiles. The technique, disclosed in Sanford et al. (1987) as well as in EPO Patent Publication 331,855 of J. C. Sanford et al. based upon U.S. Ser. No. 161,807, filed Feb. 29, 1988, has been shown effective at producing transient gene expression in some plant cells and tissues including those from onion, maize (Klein et al. 1988a), tobacco, rice, wheat, and soybean, and stable expression has been obtained in tobacco and soybeans. In fact, stable expression has been obtained by bombardment of suspension cultures of *Zea mays* Black Mexican Sweet (Klein et al. 1989) which cultures are, however, non-regenerable suspension culture cells, not the callus culture cells used in the process of the present invention.

No protocols have been published describing the introduction of DNA by a bombardment technique into cultures of regenerable maize cells of any type. No stable expression of a gene has been reported by means of bombardment of corn callus followed by regeneration of fertile plants and no regenerable fertile corn has resulted from DNA-coated microprojectile bombardment of the suspension cultures. Thus, the art has failed to produce fertile transformed corn plants heretofore.

A further stumbling block to the successful production of fertile transgenic maize plants has been in selecting those few transformants in such a manner that neither the regeneration capacity nor the fertility of the regenerated transformant are destroyed. Due to the generally low level of transformants produced by a transformation technique, the need for selection of the transformants is self-evident. However, selection generally entails the use of some toxic agent, e.g. herbicide or antibiotic, which can effect either the regenerability or the resultant plant fertility.

It is thus an object of the present invention to produce fertile, stably transgenic, *Zea mays* plants and seeds which transmit the introduced gene to progeny. It is a further object to produce such stably transgenic plants and seeds by a particle bombardment and selection process which results in a high level of viability for a few transformed cells. It is a further object to produce fertile stably transgenic plants of other graminaceous cereals besides maize.

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SUMMARY OF THE INVENTION

The present invention relates to fertile transgenic *Zea mays* plants containing heterologous DNA, preferably chromosomally integrated heterologous DNA, which is heritable by progeny thereof.

The invention further relates to all products derived from transgenic *Zea mays* plants, plant cells, plant parts, and seeds.

The invention further relates to transgenic *Zea mays* seeds stably containing heterologous DNA and progeny which inherit the heterologous DNA.

The invention further relates to a process for producing fertile transgenic *Zea mays* plants containing heterologous DNA. The process is based upon microprojectile bombardment, selection, and plant regeneration techniques.

The invention further relates to a process for producing fertile transformed plants of graminaceous plants other than *Zea mays* which have not been reliably transformed by traditional methods such as electroporation, Afrobacterium, injection, and previous ballistic techniques.

The invention further relates to regenerated fertile mature maize plants from transformed embryogenic tissue, transgenic seeds produced therefrom, and R1 and subsequent generations.

In preferred embodiments, this invention produces the fertile transgenic plants by means of a DNA-coated microprojectile bombardment of clumps of friable embryogenic callus, followed by a controlled regimen for selection of the transformed callus lines.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A shows a map of plasmid vector pHYGI1 utilized in Example I.

FIG. 1B shows the relevant part of pHYGI1 encompassing the HPT coding sequence and associated regulatory elements. The base pair numbers start from the 5' nucleotide in the recognition sequence for the indicated restriction enzymes, beginning with the EcoRI site at the 5' end of the CaMV 35S promoter.

FIG. 2 shows a map of plasmid vector pBII221 utilized in Example I.

FIG. 3 is a Southern blot of DNA isolated from the PH1 callus line and an untransformed control callus line.

FIG. 4 is a Southern blot of leaf DNA isolated from Ro plants regenerated from PH1 and untransformed callus.

FIG. 5 is a Southern blot of leaf DNA isolated from R1 progeny of PH1 Ro plants and untransformed Ro plants.

FIG. 6 is a Southern blot of DNA isolated from the PH2 callus line and an untransformed control callus line.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is directed to the production of fertile transgenic plants and seeds of the species *Zea mays* and to the plants, plant tissues, and seeds derived from such transgenic plants, as well as the subsequent progeny and products derived therefrom. The transgenic plants produced herein include all plants of this species, including field corn, popcorn, sweet corn, flint corn and dent corn.

"Transgenic" is used herein to include any cell, cell line, callus, tissue, plant part or plant which contains heterologous DNA that was introduced into plant material by a process of genetic engineering, or which was initially introduced into a plant species by such a process and was subsequently transferred to later generations by sexual or asexual cell crosses or cell divisions.

By "heritable" is meant that the DNA is capable of transmission through a complete sexual cycle of a plant, i.e. passed from one plant through its gametes to its progeny plants in the same manner as occurs in normal corn.

The transgenic plants of this invention may be produced by (i) establishing friable embryogenic callus from the plant to be transformed, (ii) transforming said cell line by a microprojectile bombardment technique, (iii) controllably identifying or selecting transformed cells, and (iv) regenerating fertile transgenic plants from the transformed cells. Some of the plants of this invention may be produced from the transgenic seed produced from the fertile transgenic plants using conventional crossbreeding techniques to develop commercial hybrid seed containing heterologous DNA.

I. Plant Lines and Tissue Cultures

The cells which have been found useful to produce the fertile transgenic maize plants herein are those callus cells which are regenerable, both before and after undergoing a selection regimen as detailed further below. Generally, these cells will be derived from meristematic tissue which contain cells which have not yet terminally differentiated. Such tissue in graminaceous cereals in general and in maize, in

particular, comprise tissues found in juvenile leaf basal regions, immature tassels, immature embryos, and coleoptilar nodes. Preferably, immature embryos are used. Methods of preparing and maintaining callus from such tissue and plant types are well known in the art and details on so doing are available in the literature, c.f. Phillips et al. (1988), the disclosure of which is hereby incorporated by reference.

The specific callus used must be able to regenerate into a fertile plant. The specific regeneration capacity of particular callus is important to the success of the bombardment/ selection process used herein because during and following selection, regeneration capacity may decrease significantly. It is therefore important to start with cultures that have as high a degree of regeneration capacity as possible. Callus which is more than about 3 months and up to about 36 months of age has been found to have a sufficiently high level of regenerability and thus is currently preferred. The regenerative capacity of a particular culture may be readily determined by transferring samples thereof to regeneration medium and monitoring the formation of shoots, roots, and plantlets. The relative number of plantlets arising per Petri dish or per gram fresh weight of tissue may be used as a rough quantitative estimate of reqeneration capacity. Generally, a culture which will produce at least one plant per gram of callus tissue will be preferred.

While maize callus cultures can be initiated from a number of different plant tissues, the cultures useful herein are preferably derived from immature maize embryos which are removed from the kernels of an ear when the embryos are about 1–3 mm in length. This length generally occurs about 9–14 days after pollination. Under aseptic conditions, the embryos are placed on conventional solid media with the embryo axis down (scutellum up). Callus tissue appears from the scutellum after several days to a few weeks. After the callus has grown sufficiently, the cell proliferations from the scutellum may be evaluated for friable consistency and the presence of well-defined embryos. By "friable consistency" is meant that the tissue is easily dispersed without causing injury to the cells. Tissue with this morphology is then transferred to fresh media and subcultured on a routine basis about every two weeks.

The callus initiation media is solid because callus cannot be readily initiated in liquid medium. The initiation/ maintainence media is typically based on the N6 salts of Chu et al. (1975) as described in Armstrong et al. (1985) or the MS salts of Murashige et al. (1962). The basal medium is supplemented with sucrose and 2,4-dichlorophenoxyacetic acid (2,4-D). Supplements such as L-proline and casein hydrolysate have been found to improve the frequency of initiation of callus cultures, morphology, and growth. The cultures are generally maintained in the dark, though low light levels may also be used. The level of synthetic hormone 2,4-D, necessary for maintainence and propagation, should be generally about 0.3 to 3.0 mg/l.

Although successful transformation and regeneration has been accomplished herein with friable embryogenic callus, this is not meant to imply that other transformable regenerable cells, tissue, or organs cannot be employed to produce the fertile transgenic plants of this invention. The only actual requirement for the cells which are transformed is that after transformation they must be capable of regeneration of a plant containing the heterologous DNA following the particular selection or screening procedure actually used.

II. DNA Used for Transformation

The heterologous DNA used for transformation herein may be circular or linear, double-stranded or single-stranded. Generally, the DNA is in the form of a plasmid and contains coding regions of beneficial heterologous DNA with flanking regulatory sequences which serve to promote the expression of the heterologous DNA present in the resultant corn plant. "Heterologous DNA" is used herein to include all synthetically engineered or biologically derived DNA which is introduced into a plant by man by genetic engineering, including but not limited to, non-plant genes, modified genes, synthetic genes, portions of genes, as well as DNA and genes from maize and other plant species.

The compositions of and methods for constructing heterologous DNA for successful transformations of plants is well known to those skilled in the art, and the same compositions and methods of construction may be utilized to produce the heterologous DNA useful herein. The specific composition of the DNA is not central to the present invention and the invention is not dependent upon the composition of the specific transforming DNA used. weising et al. (1988), the subject matter of which is incorporated herein by reference, describes suitable DNA components thereof which include promoters, polyadenylation sequences, selectable marker genes, reporter genes, enhancers, introns, and the like, as well as provides suitable references for compositions thereof. Sambrook et al. (1989) provides suitable methods of construction.

Generally the heterologous DNA will be relatively small, i.e. less than about 30 kb to minimize any susceptibility to physical, chemical, or enzymatic degradation which is known to increase as the size of the DNA increases.

Suitable heterologous DNA for use herein includes all DNA which will provide for, or enhance, a beneficial feature of the resultant transgenic corn plant. For example, the DNA may encode proteins or antisense RNA transcripts in order to promote increased food values, higher yields, pest resistance, disease resistance, and the like. For example, a bacterial dap A gene for increased lysine; Bt-endotoxin gene or protease inhibitor for insect resistance; bacterial ESPS synthase for resistance to glyphosate herbicide; chitinase or glucan endo-1,3-B-glucosidase for fungicidal properties. Also, the DNA may be introduced to act as a genetic tool to generate mutants and/or assist in the identification, genetic tagging, or isolation of segments of corn DNA. Additional examples may be found in Table 1:

TABLE 1

Eukaryotic genes transferred to higher plants

Origin of gene	Transferred constructs	Transformed species	Mode of foreign gene expression
Animals			
Drosophila heat shock gene hsp 70	5' hsp70/nptII/3' ocs	tobacco (plants, tumors)	+ temperature-dependent and organ-specific
Drosophila copia element LTR	5' copia/cat	rice, wheat and sorghum (protoplasts)	(–) transient, but strong

TABLE 1-continued

Eukaryotic genes transferred to higher plants

Origin of gene	Transferred constructs	Transformed species	Mode of foreign gene expression
firefly luciferase gene	5' CaMV 35S/luciferase cDNA/3' nos 5' CaMV 19S/luciferase cDNA/3' nos 5' deletion series	tobacco (plants) carrot (protoplasts)	- (+) transient
Rabbit β-globin gene	genomic	tobacco (tumors)	(-) not expressed
Human α-globin gene	5' nos/α-globin	tobacco (plants)	(+) incorrect transcript processing
Chicken α-actin gene	genomic	tobacco (tumors)	(-) not expressed
Chicken oyalbumin gene	genomic	tobacco (tumors)	(+) incorrect transcript processing
Mouse metallothionein gene (mmt)	5' mmt/cat	tobacco (tumors)	(-) not expressed
Mouse dihydrofolate reductase gene (DHFR)	5' CaMV 35S/DHFR-cDNA/ 3' nos	Petunia (plants)	+ expression confers methotrexate resistance
Human growth hormone gene (hgh)	5' CaMV 35S/hgh	tobacco (plants, tumors)	(+) incorrect transcript processing
	5' nos/hgh/3' nos	tobacco and sunflower (tumors)	(+) transcription, but neither processing nor translation
	5' CaMV 35S/hgh/hgh 3'	tobacco (plants)	(+) incorrect transcript polyadenylation
SV40 early genes	5' SV40/cat	tobacco (tumors)	(-) not expressed
	5' CaMV 35S/cat/3' SV40	tobacco (plants)	(+) incorrect transcript polyadenylation
HSV thymidine kinase gene (tk)	5' HSV/tk/cat	tobacco (tumors)	(-) not expressed
Adenovirus type 5 EIA gene	5' CaMV 35S/EIA/ 3' EIA/3' rbcS	tobacco (plants)	(+) termination within rbcS, EIA polyadenylation site not used
Yeast			
Yeast ADH	genomic	tobacco (plants)	(-) not expressed
Plant virus			
cDNA encoding TMV coat protein (tobacco mosaic virus)	5' CaMV 35S/TMVcDNA/ 3' nos	tobacco (plants)	+ expression confers enhanced resistance to TMV infection
cDNA encoding AMV coat protein (alfalfa mosaic virus)	5' CaMV 19S/AMVcDNA 5' CaMV 35S/AMVcDNA/ 3' nos.	tobacco and tomato (plants)	+ expression confers enhanced resistance to AMV infection
cDNAs encoding A- and B-component of TGMV (tomato golden mosaic virus)	separate transformation with either A- or B-component via agroinfection	Petunia (plants)	+ only A-components in tandem are able to replicate
cDNA encoding CMV (cucumber mosaic virus) satellite RNA	5' CaMV 35S/CMVcDNA/ 3' nos	tobacco (plants)	+ expression confers enhanced resistance to CMV infection
Plants			
Bean phaseolin gene	5' ocs/phaseolin genomic genomic	sunflower (tumors) tobacco (plants)	+ expressed and processed correctly + development-specific expression in seeds: targeting to protein bodies in endosperm and embryos
	5' phaseolin/phaseolin-cDNA/3' phaseolin	tobacco (tumors)	+ higher expression than using a genomic clone
	5' phaseolin/maize zein/ 3' phaseolin	tobacco (plants)	+ development-specific zein gene expression in tobacco seeds. Zein accumulation
Bean phytohemagglutinin-L gene (PHA-L)	genomic	tobacco (plants)	+ development-specific expression in tobacco seeds
Soybean β-conglycinin gene (α subunit)	genomic 5' deletion series	Petunia (plants)	+ development-specific expression in Petunia seeds depending on 5' sequences
	5' CaMV 35S or 19S/ conglycinin/3' nos	Petunia (plants)	+ constitutive expression: 35S> 19S; 20 fold clonal variation
Soybean β-conglycinin gene (β subunit)	genomic	tobacco and Petunia (plants)	+ development-specific expression in seeds
Potato patatin gene	5' patatin/cat/3' nos 5' ST-LS.1/patatin/3' patatin	potato (plants) tobacco (plants)	+ organ-specific expression in tubers + light-regulated and organ-specific expression depending on the ST-LS.1 promoter. Correct splicing of patatin mRNA.
Maize zein gene	genomic	sunflower (tumors)	(+) transcription, but no detectable protein
	5' phaseolin/zein/3' phaseolin	tobacco (plants)	+ development-specific expression in tobacco seeds
Wheat glutenin genes	5' glutenin/cat/3' nos	tobacco (plants)	+ development-specific expression

TABLE 1-continued

Eukaryotic genes transferred to higher plants

Origin of gene	Transferred constructs	Transformed species	Mode of foreign gene expression
			in tobacco seeds
Wheat chlorophyll a/b-binding protein (cab) gene	genomic	Petunia and tobacco plants	+ light-regulated and organ-specific expression in leaves
	genomic 5' cab/cat 5' cab/5' CaMV 35S/cat/ 3' rbcS 5' deletion series	tobacco (plants)	+ phytochrome-regulated expression in leaves depending on 5' sequences
Pea cab gene	5' cab/nptII 5' cab/5' nos/nptII	tobacco (plants)	+ light-regulated, organ-and cell-specific expression; depending on enhancer/silencer-like 5, sequences. Involvement of phytochrome. Correlation to the presence of chloroplasts.
Petunia cab gene	5' cab/ocs 5' cab/nos	Petunia and tobacco (plants)	+ clonal variation of expression (200 fold) independent of copy number and homo-/ heterologous host genome
Arabidopsis cab gene	5' cab/cat	tobacco (plants)	+ light-regulated and organ-specific expression
Pea ribulose 1.5-Bisphosphate carboxylase small subunit gene (rbcS) E9	genomic 5' rbcS/cat 5' deletion series	Petunia (tumors)	+ light-regulated expression dependent on 5' sequences
	genomic 5' deletion series	Petunia and tobacco (plants)	+ light-regulated and organ-specific expression dependent on 5' sequences: 25-fold clonal variation
Pea rbcS 3.6	5' rbcS/cat/3' nos 5' deletion series	tobacco (tumors)	+ light-regulated expression dependent on enhancer-like 5' sequences
	5' rbcS/rbcS transit sequence/ nptII	tobacco (tumors and plants)	+ light-regulated expression and targeting of neomycin phosphotransferase into chloroplasts; analysis of signal sequences
	5' rbcS/nptII	tobacco (plants)	+ light-regulated, organ- and cell-specific expression. Involvement of a blue-light receptor
Pea rbcS 3A, 3C	genomic	Petunia (plants)	+ regulation of transcription by phytochrome- and/or blue-light receptor depending on the developmental state
Pea rbcS 3A, E9	5' rbcS/5' CaMV 35S/cat 5' deletion series	Petunia and tobacco (plants)	+ light-regulated and organ-specific expression depending on enhancer- and silencer-like 5' sequences
Soybean rbcS	5' rbcS/nptII/3' ocs	soybean (tumors)	+ light-regulated expression
	5' rbcS/nos	Kalanchoe (tumors)	+ light-regulated expression
	5' rbcS/nptII/3' nos	Petunia (plants)	+ light-regulated expression mediated by phytochrome
Soybean, pea and Petunia rbcS	5' rbcS/nptII/3' nos	tomato (plants)	+ expression stronger than directed by nos-promoter
Nicotiana plumbaginifolia rbcS 8B	5' rbcS/cat	tobacco and Petunia (plants)	+ light-regulated and organ-specific expression; 3-fold clonal variation independent of homo-/ heterologous host genome
Wheat rbcS	genomic 5' CaMV 35S/rbcS/3' rbcS	tobacco (plants)	(+) no expression under the control of wheat promoter; CaMV 35S promoter is necessary
Potato ST-LS.1 gene	genomic; modified by exon tagging	potato and tobacco (plants)	+ light-regulated and organ-specific expression depending on the presence of chloroplasts and 5' sequences. Clonal variation parallels copy number, but is independent of homo-/ heterologous host.
	5' ST-LS.1/patatin/3' patatin	tobacco (plants)	+ light-regulated and organ-specific expression depending on the ST-L.1 promoter sequences
Petroselinum and Antirrhinum chalcone synthase genes (chs)	5' chs(A)/nptII/3' chs(P) 5' deletion series	tobacco (plants)	+ UV-B-light-regulated expression dependent on enhancer-like 5' sequences

TABLE 1-continued

Eukaryotic genes transferred to higher plants

Origin of gene	Transferred constructs	Transformed species	Mode of foreign gene expression
potato proteinase inhibitor II gene (PI II)	genomic 5' PI II/cat/3' T-DNA gene 6b 5' PI II/cat/3' PI II	tobacco (plants)	+ wound-inducible expression depending on 5' and 3' sequences; systemic spreading by transacting factors
Soybean heat shock gene hs 6871	genomic 5' deletion series	sunflower (tumors) and tobacco (plants)	+ temperature-regulated expression depending on 5' sequences
Soybean heat shock gene Gmhsp 17.SE	genomic 5' deletion series	sunflower (tumors)	+ expression regulated by temperature and presence of cadmium and arsenite depending on 5' sequences
Maize heat shock gene hsp70	genomic	Petunia (plants)	+ temperature-regulated expression
Maize alcohol dehydrogenase I gene (AdhI)	5' CaMV 35S/cat/AdhI intron/3' rbcS	tobacco (plants)	(+) maize AdhI intron is not removed from the transcript
	5' AdhI/cat 5' ocs/5' AdhI/cat 5' CaMV 35S/5' AdhI/cat 5' AdhI deletion series	tobacco (plants) and maize (protoplasts)	(+) anaerobically inducible cat-expression dependent on 5' AdhI sequences only if additional CaMV- or ocs-promoter/enhancer sequences are present.
Maize sucrose synthase gene (ss)	5' ss/nptII/3' ocs	wheat (protoplasts)	(+) transient expression
	5' ss/nptII	maize (protoplasts)	(+) transient expression suspension-culture derived but not in leaf-derived protoplasts
Soybean β-tubulin gene	genomic	tobacco (plants)	+
cowpea trypsin inhibitor gene (CpTI)	5' CaMV 35S/CpTI/3' nos	tobacco (plants)	+ expression enhances resistance to insect pests
Petunia EPSP synthase gene	5' CaMV 35S/EPSP/3' nos	Petunia (plants)	+ 35S-directed EPSP overproduction confers glyphosate tolerance
	5' T7-T-DNA/Petunia EPSP transit sequence/bacterial EPSP (aroA) coding region	tobacco (plants)	+ expression of bacterial EPSP; targeting into chloroplasts; glyphosate tolerance
Soybean leghemoglobin gene lbc3	genomic	tobacco (plants)	n.d.
	5' lbc3/cat/3' lbc3	Lotus corniculatus (plants)	+ development-specific and inducible expression only in nodules and only after infection by Rhizobium; dependence on 5' regulatory sequences
Nicotiana plumbaginifolia "insert 7" enhancer-like sequence	5' insert 7/5' nos/nptII	tobacco (plants)	+ transient protoplast-specific overexpression of nptII
Nicotiana plumbaginifolia ATP synthase gene (atp2-1); β subunit	5' CaMV 35S/atp2-1 signal sequence/cat	tobacco (plants)	+ cat enzyme is targeted into mitochondria
Maize transposable elements Ac and Ds	Ac or Ds within borders of waxy locus	tobacco (tumors and shoots)	+ Ac is capable of self-catalyzed transposition

Abbreviations:
copia LTR - long terminal repeat of copia taansposable element
HSV - Herpes simplex virus
Adh - alcohol dehydrogenase gene
ST-LS 1 - Solanum ruberosum leaf/stem-specific gene
EPSP - 5 -enolpyruvylshikimate-3-phosphate (gene)
lbc3 - member of the leghemoglobin gene family
Ac, Ds - maize transposable elements (activator, dissociation)
rbcS - ribulose-1,5-bisphosphate carboxylase small subunit gene
cab - chlorophyl a/b binding protein gene
nptII - neomycin phosphotransferase II gene
ocs - octopine synthase gene
nos - nopaline synthase gene
cat - chloramphenicol acetyltransferase gene
CaMV 35S, 19S - cauliflower mosaic virus genes encoding 35S and 19S-transcript, respectively
genomic - transferred construct contains the entire gene including 5' and 3' regions
Mode of expression:
+ - correct expression of stably integrated gene
(+) - transient expression, or transcription followed by incorrect processing and/or translation
(-) - gene is not transcribed

The heterologous DNA to be introduced into the plant further will generally contain either a selectable marker or a reporter gene or both to facilitate identification and selection of transformed cells. Alternatively, the selectable marker may be carried on a separate piece of DNA and used in a cotransformation procedure. Both selectable markers and

mental stage specific promoter which can be expressed in the particular plant cell. Suitable such promoters are disclosed in Weising et al, supra. The following is a partial representative list of promoters suitable for use herein: regulatory sequences from the T-DNA of *Agrobacterium tumefaciens*, including mannopine synthase, nopaline synthase, and octopine synthase; alcohol dehydrogenase promoter from corn; light inducible promoters such as, ribulose-biphosphate-carboxylase small subunit gene from a variety of species; and the major chlorophyll a/b binding protein gene promoter; 35s and 19S promoters of cauliflower mosaic virus; developmentally regulated promoters such as the waxy, zein, or bronze promoters from maize; as well as synthetic or other natural promoters which are either inducible or constitutive, including those promoters exhibiting organ specific expression or expression at specific development stage(s) of the plant.

Other elements such as introns, enhancers, polyadenylation sequences and the like, may also be present on the DNA. Such elements may or may not be necessary for the function of the DNA, although they can provide a better expression or functioning of the DNA by affecting transcription, stability of the mRNA, or the like. Such elements may be included in the DNA as desired to obtain the optimal performance of the transforming DNA in the plant. For example, the maize Adh1S first intron may be placed between the promoter and the coding sequence of a particular heterologous DNA. This intron, when included in a DNA construction, is known to generally increase expression in maize cells of a protein. (Callis et al. 1987) However, sufficient expression for a selectable marker to perform satisfactorily can often be obtained without an intron. (Klein et al. 1989) An example of an alternative suitable intron is the shrunken-1 first intron of *Zea mays*. These other elements must be compatible with the remainder of the DNA constructions.

To determine whether a particular combination of DNA and recipient plant cells are suitable for use herein, the DNA may include a reporter gene. An assay for expression of the reporter gene may then be performed at a suitable time after the DNA has been introduced into the recipient cells. A preferred such assay entails the use of the *E. coli* beta-glucuronidase (GUS) gene (Jefferson et al. 1987). In the case of the microprojectile bombardment transformation process of the present invention, a suitable time for conducting the assay is about 2–3 days after bombardment. The use of transient assays is particularly important when using DNA components which have not previously been demonstrated or confirmed as compatible with the desired recipient cells.

III. DNA Delivery Process

The DNA can be introduced into the regenerable maize callus cultures via a particle bombardment process. A general description of a suitable particle bombardment instrument is provided in Sanford et al. (1987), the disclosure of which is incorporated herein by reference. While protocols for the use of the instrument in the bombardment of maize non-regenerable suspension culture cells are described in Klein et al. (1988a, 1988b, and 1989), no protocols have been published for the bombardment of callus cultures or regenerable maize cells.

In a microprojectile bombardment process, also referred to as a biolistic process, the transport of the DNA into the callus is mediated by very small particles of a biologically inert material. When the inert particles are coated with DNA and accelerated to a suitable velocity, one or more of the particles is able to enter into one or more of the cells where the DNA is released from the particle and expressed within the cell. While some of the cells are fatally damaged by the bombardment process, some of the recipient cells do survive, stably retain the introduced DNA, and express it.

The particles, called microprojectiles, are generally of a high density material such as tungsten or gold. They are coated with the DNA of interest. The microprojectiles are then placed onto the surface of a macroprojectile which serves to transfer the motive force from a suitable energy source to the microprojectiles. After the macroprojectile and the microprojectiles are accelerated to the proper velocity, they contact a blocking device which prevents the macroprojectile from continuing its forward path but allows the DNA-coated microprojectiles to continue on and impact the recipient callus cells. Suitable such instruments may use a variety of motive forces such as gunpowder or shock waves from an electric arc discharge (Swain et al. 1988). An instrument in which gunpowder is the motive force is currently preferred and such is described and further explained in Sanford et al. (1987), the disclosure of which is incorporated herein by reference.

A protocol for the use of the gunpowder instrument is provided in Klein et al. (1988a, b) and involves two major steps. First, tungsten microprojectiles are mixed with the DNA, calcium chloride, and spermidine free base in a specified order in an aqueous solution. The concentrations of the various componenets may be varied as taught. The currently preferred procedure entails exactly the procedure of Klein et al. (1988b) except for doubling the stated optimum DNA concentration. Secondly, in the actual bombardment, the distance of the recipient cells from the end of the barrel as well as the vacuum in the sample chamber. The currently preferred procedure for bombarding the callus entails exactly the procedure of Klein et al. (1988b) with the recipient tissue positioned 5 cm below the stopping plate tray.

The callus cultures useful herein for generation of transgenic plants should generally be about 3 months to 3 years old, preferably about 3 to 18 months old. Callus used for bombardment should generally be about midway between transfer periods and thus past any "lag" phase that might be associated with a transfer to a new media, but also before reaching any "stationary" phase associated with a long time on the same plate.

The specific tissue subjected to the bombardment process is preferably taken about 7–10 days after subculture, though this is not believed critical. The tissue should generally be used in the form of pieces of about 30 to 80, preferably about 40 to 60, mg. The clumps are placed on a petri dish or other surface and arranged in essentially any manner, recognizing that (i) the space in the center of the dish will receive the heaviest concentration of metal-DNA particles and the tissue located there is likely to suffer damage during bombardment and (ii) the number of particles reaching a cell will decrease (probably exponentially) with increasing distance of the cell from the center of the blast so that cells far from the center of the dish are not likely to be bombarded and transformed. A mesh screen, preferably of metal, may be laid on the dish to prevent splashing or ejection of the tissue. The tissue may be bombarded one or more times with the DNA-coated metal particles.

IV. Selection Process

Once the calli have been bombarded with the DNA and the DNA has penetrated some of the cells, it is necessary to identify and select those cells which both contain the heterologous DNA and still retain sufficient regenerative capacity. There are two general approaches which have been found useful for accomplishing this. First, the transformed

calli or plants regenerated therefrom can be screened for the presence of the heterologous DNA by various standard methods which could include assays for the expression of reporter genes or assessment of phenotypic effects of the heterologous DNA, if any. Alternatively and preferably, when a selectable marker gene has been transmitted along with or as part of the heterologous DNA, those cells of the callus which have been transformed can be identified by the use of a selective agent to detect expression of the selectable marker gene.

Selection of the putative transformants is a critical part of the successful transformation process since selection conditions must be chosen so as to allow growth and accumulation of the transformed cells while simultaneously inhibiting the growth of the non-transformed cells. The situation is complicated by the fact that the vitality of individual cells in a population is often highly dependent on the vitality of neighboring cells. Also, the selection conditions must not be so severe that the plant regeneration capacity of the callus cells and the fertility of the resulting plant are precluded. Thus the effects of the selection agent on cell viability and morphology should be evaluated. This may be accomplished by experimentally producing a growth inhibition curve for the given selective agent and tissue being transformed beforehand. This will establish the concentration range which will inhibit growth.

When a selectable marker gene has been used, the callus clumps may be either allowed to recover from the bombardment on non-selective media or, preferably, directly transferred to media containing that agent.

Selection procedures involve exposure to a toxic agent and may employ sequential changes in the concentration of the agent and multiple rounds of selection. The particular concentrations and cycle lengths are likely to need to be varied for each particular agent. A currently preferred selection procedure entails using an initial selection round at a relatively low toxic agent concentration and then later round(s) at higher concentration(s). This allows the selective agent to exert its toxic effect slowly over a longer period of time. Preferably the concentration of the agent is initially such that about a 5–40% level of growth inhibition will occur, as determined from a growth inhibition curve. The effect may be to allow the transformed cells to preferentially grow and divide while inhibiting untransformed cells, but not to the extent that growth of the transformed cells is prevented. Once the few individual transformed cells have grown sufficiently the tissue may be shifted to media containing a higher concentration of the toxic agent to kill essentially all untransformed cells. The shift to the higher concentration also reduces the possibility of non-transformed cells habituating to the agent. The higher level is preferably in the range of about 30 to 100% growth inhibition. The length of the first selection cycle may be from about 1 to 4 weeks, preferably about 2 weeks. Later selection cycles may be from about 1 to about 12 weeks, preferably about 2 to about 10 weeks. Putative maize transformants can generally be identified as proliferating sectors of tissue among a background of non-proliferating cells. The callus may also be cultured on non-selective media at various times during the overall selection procedure.

Once a callus sector is identified as a putative transformant, transformation can be confirmed by phenotypic and/or genotypic analysis. If a selection agent is used, an example of phenotypic analysis is to measure the increase in fresh weight of the putative transformant as compared to a control on various levels of the selective agent. Other analyses that may be employed will depend on the function of the heterologous DNA. For example, if an enzyme or protein is encoded by the DNA, enzymatic or immunological assays specific for the particular enzyme or protein may be used. Other gene products may be assayed by using a suitable bioassay or chemical assay. Other such techniques are well known in the art and are not repeated here. The presence of the gene can also be confirmed by conventional procedures, i.e. Southern blot or polymerase chain reaction (PCR) or the like.

V. Regeneration of Plants and Production of Seed

Cell lines which have been shown to be transformed must then be regenerated into plants and the fertility of the resultant plants determined. Transformed lines which test positive by genotypic and/or phenotypic analysis are then placed on a media which promotes tissue differentiation and plant regeneration. Regeneration may be carried out in accordance with standard procedures well known in the art. The procedures commonly entail reducing the level of auxin which discontinues proliferation of a callus and promotes somatic embryo development or other tissue differentiation. One example of such a regeneration procedure is described in Green et al. (1981). The plants are grown to maturity in a growth room or greenhouse and appropriate sexual crosses and selfs are made as described by Neuffer (1981).

Regeneration, while important to the present invention, may be performed in any conventional manner. If a selectable marker has been transformed into the cells, the selection agent may be incorporated into the regeneration media to further confirm that the regenerated plantlets are transformed. Since regeneration techniques are well known and not critical to the present invention, any technique which accomplishes the regeneration and produces fertile plants may be used.

VI. Analysis of R1 Progeny

The plants regenerated from the transformed callus are referred to as the R0 generation or R0 plants. The seeds produced by various sexual crosses of the R0 generation plants are referred to as R1 progeny or the R1 generation. When R1 seeds are germinated, the resulting plants are also referred to as the R1 generation.

To confirm the successful transmission and inheritance of the heterologous DNA in the sexual crosses described above, the R1 generation should be analyzed to confirm the presence of the transforming DNA. The analysis may be performed in any of the manners such as were disclosed above for analyzing the bombarded callus for evidence of transformation, taking into account the fact that plants and plant parts are being used in place of the callus.

VII. Breeding of Genetically Engineered Commercial Hybrid Seed

Generally, the commercial value of the transformed corn produced herein will be greatest if the heterologous DNA can be incorporated into many different hybrid combinations. A farmer typically grows several varieties of hybrids based on differences in maturity, standability, and other agronomic traits. Also, the farmer must select a hybrid based upon his physical location since hybrids adapted to one part of the corn belt are generally not adapted to another part because of differences in such traits as maturity, disease, and insect resistance. As such, it is necessary to incorporate the heterologous DNA into a large number of parental lines so that many hybrid combinations can be produced containing the desirable heterologous DNA. This may conveniently be done by breeding programs in which a conversion process (backcrossing) is performed by crossing the initial transgenic fertile plant to normal elite inbred lines and then

crossing the progeny back to the normal parent. The progeny from this cross will segregate such that some of the plants will carry the heterologous DNA whereas some will not. The plants that do carry the DNA are then crossed again to the normal plant resulting in progeny which segregate once more. This crossing is repeated until the original normal parent has been converted to a genetically engineered line containing the heterologous DNA and also possessing all other important attributes originally found in the parent. A separate back-crossing program will be used for every elite line that is to be converted to a genetically engineered elite line. It may be necessary for both parents of a hybrid seed corn to be homozygous for the heterologous DNA. Corn breeding and the techniques and skills required to transfer genes from one line or variety to another are well-known to those skilled in the art. Thus introducing heterologous DNA into lines or varieties which do not generate the appropriate calli can be readily accomplished by these breeding procedures.

VIII. Uses of Transgenic Plants

The transgenic plants produced herein are expected to be useful for a variety of commercial and research purposes.

Transgenic plants can be created for use in traditional agriculture to possess traits beneficial to the grower (e.g. agronomic traits such as pest resistance or increased yield), beneficial to the consumer of the grain harvested from the plant (e.g. improved nutritive content in human food or animal feed), or beneficial to the food processor (e.g. improved processing traits). In such uses, the plants are generally grown for the use of their grain in human or animal foods. however, other parts of the plants, including stalks, husks, vegetative parts, and the like, may also have utility, including use as part of animal silaage or for ornamental purposes (e.g. Indian corn). often chemical constituents (e.g. oils or starches) of corn and other crops are extracted for food or in dustrial use and transgenic plants may be created which have enhanced or modified levels of such components. The plants may also be used for seed production for a variety of purposes.

Transgenic plants may also find use in the commercial manufacture of proteins or othermolecules encoded by the heterologous DNA contained therein, where the molecule of interest is extracted or purified from plant parts, seeds, and the like. Cells or tissue from the plants may also be cultured, grown in vitro, or fermented to manufacture such molecules, or for other purposes (e.g. for research).

The transgenic plants may also be used in commercial breeding programs, or may be crossed or bred to plants of related crop species. Improvements encoded by the heterologous DNA may be transferred, e.g. from corn cells to cells of other species e.g. by protoplast fusion.

The transgenic plants may have many use s in research or breeding, including creation of new mutant plants through insertional mutagenesis, in order to identify beneficial mutants that might later be created by traditional mutation and selection. The methods of the invention may also be used to create plants having unique "signature sequences" or other marker sequences which can be used to identify proprietary lines or varieties.

The following non-limiting examples are illustrative of the present invention. They are presented to better explain the general procedures which were used to prepare the fertile *Zea mays* plants of this invention which stably express the heterologous DNA and which transmit that DNA to progeny. All parts and percents are by weight unless otherwise specified. It must be recognized that a specific transformation event is a function of the amount of material subjected to the transformation procedure. Thus when individual situations arise in which the procedures described herein do not produce a transformed product, repetition of the procedures will be required.

EXAMPLE I

Fertile transgenic *Zea mays* plants which contain heterologous DNA which is heritable were prepared as follows:

I. Initiation and Maintenance of Maize Cell Cultures which Retain Plant Regeneration Capacity

Friable, embryogenic maize callus cultures were initiated from hybrid immature embryos produced by pollination of inbred line A188 plants (University of Minnesota, Crop Improvement Association) with pollen of inbred line B73 plants (Iowa State University). Ears were harvested when the embryos had reached a length of 1.5 to 2.0 mm. The whole ear was surface sterilized in 50% v/v commercial bleach (2.63% w/v sodium hypochlorite) for 20 min. at room temperature. The ears were then washed with sterile distilled, deionized water. Immature embryos were aseptically isolated and placed on nutrient agar initiation/maintenance media with the root/shoot axis exposed to the medium. Initiation/maintenance media (hereinafter refered to as F medium) consisted of N6 basal media (Chu 1975) with 2% (w/v) sucrose, 1.5 mg per liter 2,4-dichlorophenoxyacetic acid (2,4-D), 6 mM proline, and 0.25% Gelrite (Kelco, Inc. San Diego). The pH was adjusted to 5.8 prior to autoclaving. Unless otherwise stated, all tissue culture manipulations were carried out under sterile conditions.

The immature embryos were incubated at 26° C. in the dark. Cell proliferations from the scutellum of the immature embryos were evaluated for friable consistency and the presence of well defined somatic embryos. Tissue with this morphology was transferred to fresh media 10 to 14 days after the initial plating of the immature embryos. The tissue was then subcultured on a routine basis every 14 to 21 days. Sixty to eighty milligram quantities of tissue were removed from pieces of tissue that had reached a size of approximately one gram and transferred to fresh media. Subculturing always involved careful visual monitoring to be sure that only tissue of the correct morphology was maintained. The presence of somatic embryos ensured that the cultures would give rise to plants under the proper conditions. The cell culture named AB12 used in this example was such a culture and had been initiated about 1 year before bombardment.

II. Plasmids—pCHN1-1, pHYGI1, pBII221, and pLUC-1

The plasmids pCHN1-1, pHYGI1, and pLUC-1 were constructed in the vector pBS+ (Stratagene, Inc., San Diego, Calif.), a 3.2 Kb circular plasmid, using standard recombinant DNA techniques. pCHN1-1 contains the hygromycin B phosphotransferase (HPT) coding sequence from *E. coli* (Gritz et al. 1983) flanked at the 3' end by the nopaline synthase (nos) polyadenylation sequence of Agrobacterium tumefaciens (Chilton and Barnes 1983). Expression is driven by the cauliflower mosaic virus (CaMV) 35S promoter (Guilley et al. 1982), located upstream from the hygromycin coding sequence. The plasmid pHYGI1 was constructed by inserting the 553 bp Bcl-BamHI fragment containing the maize Adh1S first intron (Callis et al. 1987) between the CaMV 35S promoter and the hygromycin coding sequence of pCHN1-1. A map of pHYGI1 is provided as FIG. 1.

pBII221 contains the *E. Coli* B-glucuronidase coding sequence flanked at the 5' end by the CaMV 35S promoter and at the 3' end by the nos polyadenylation sequence. The plasmid was constructed by inserting the maize AdhIS first

intron between the 35S promoter and the coding sequence of pBI221 (Jefferson et al. 1987). A map of pBII221 is provided as FIG. 2.

pLUC-1 contains the firefly luciferase coding sequence (DeWet et al. 1987) flanked at the 5' end by the CaMV 35S promoter and at the 3' end by the nos polyadenylation sequence. This plasmid was used solely as a negative control.

Plasmids were introduced into the embryogenic callus culture AB12 by microprojectile bombardment.

III. DNA Delivery Process

The embryogenic maize callus line AB12 was subcultured 7 to 12 d prior to microprojectile bombardment. AB12 was prepared for bombardment as follows. Five clumps of callus, each approximately 50 mg in wet weight were arranged in a cross pattern in the center of a sterile 60×15 mm petri plate (Falcon 1007). Plates were stored in a closed container with moist paper towels throughout the bombardment process. Twenty six plates were prepared.

Plasmids were coated onto M-10 tungsten particles (Biolistics) exactly as described by Klein, et al (1988b) except that, (i) twice the recommended quantity of DNA was used, (ii) the DNA precipitation onto the particles was performed at 0° C., and (iii) the tubes containing the DNA-coated tungsten particles were stored on ice throughout the bombardment process.

All of the tubes contained 25 ul 50 mg/ml M-10 tungsten in water, 25 ul 2.5 M $CaCl_2$, and 10 ul 100 mM spermidine free base along with a total of 5 ul 1 mg/ml total plasmid content. When two plasmids were used simultaneously, each was present in an amount of 2.5 ul. One tube contained only plasmid pBII221; two tubes contained both plasmids pHYGI1 and pBII221; two tubes contained both plasmids pCHNl-1 and pBII221; and one tube contained only plasmid pLUC-1.

All tubes were incubated on ice for 10 min., pelletized by centrifugation in an Eppendorf centrifuge at room temperature for 5 seconds, and 25 ul of the supernatant was discarded. The tubes were stored on ice throughout the bombardment process. Each preparation was used for no more than 5 bombardments.

Macroprojectiles and stopping plates were obtained from Biolistics, Inc. (Ithaca, N.Y.). They were sterilized as described by the supplier. The microprojectile bombardment instrument was obtained from Biolistics, Inc.

The sample plate tray was positioned at the position 5 cm below the bottom of the stopping plate tray of the microprojectile instrument, with the stopping plate in the slot below the barrel. Plates of callus tissue prepared as described above were centered on the sample plate tray and the petri dish lid removed. A 7×7 cm square rigid wire mesh with 3×3 mm mesh and made of galvanized steel was placed over the open dish in order to retain the tissue during the bombardment. Tungsten/DNA preparations were sonicated as described by Biolistics, Inc. and 2.5 ul was pipetted onto the top of the macroprojectiles. The instrument was operated as described by the manufacturer. The following bombardments were performed:

2×pBII221 prep To determine transient expression frequency

10×pHYGI1/pBII221 As a potential positive treatment for transformation

10×pCHN1-1/pBII221 As a potential positive treatment for transformation

4×pLUC-1 Negative control treatment

The two plates of callus bombarded with pBII221 were transferred plate for plate to F medium (with no hygromycin) and the callus cultured at 26° C. in the dark. After 2 d this callus was then transferred plate for plate into 35×10 mm petri plates (Falcon 1008) containing 2 ml of GUS assay buffer which consists of 1 mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide (Research Organics), 100 mM sodium phosphate pH 7.0, 5 mM each of potassium ferricyanide and potassium ferrocyanide, 10 mM EDTA, and 0.06% Triton X-100. These were incubated at 37° C. for 3 d after which the number of blue cells was counted giving 291 and 477 transient GUS expressing cells in the two plates, suggesting that the DNA delivery process had also occurred with the other bombarded plates. These plates were discarded after counting since the GUS assay is destructive.

IV. Selection Process

Hygromycin B (Calbiochem) was incorporated into the medium by addition of the appropriate volume of filter sterilized 100 mg/ml Hygromycin B in water when the media had cooled to 45° C. prior to pouring plates.

Immediately after all samples had been bombarded, callus from all of the plates treated with pHYGI1/pBII221, pCHN1-1/pBII221 and three of the plates treated with pLUC-1 were transferred plate for plate onto F medium containing 15 mg/l hygromycin B, (five pieces of callus per plate). These are referred to as round 1 selection plates. Callus from the fourth plate treated with pLUC-1 was transferred to F medium without hygromycin. This tissue was subcultured every 2–3 weeks onto nonselective medium and is referred to as unselected control callus.

After two weeks of selection, tissue appeared essentially identical on both selective and nonselective media. All callus from eight plates from each of the pHYGI1/pBII221 and pCHN1-1/pBII221 treatments and two plates of the control callus on selective media were transferred from round 1 selection plates to round 2 selection plates that contained 60 mg/l hygromycin. The round 2 selection plates each contained ten 30 mg pieces of callus per plate, resulting in an expansion of the total number of plates.

The remaining tissue on selective media, two plates each of pHYGI1/pBII221 and pCHN1-1/pBII221 treated tissue and one of control callus, were placed in GUS assay buffer at 37° C. to determine whether blue clusters of cells were observable at two weeks post-bombardment. After 6 d in assay buffer this tissue was scored for GUS expression.

TREATMENT	REPLICATE	OBSERVATIONS
pLUC-1		no blue cells
pHYGI1/pBII221	plate 1	11 single cells
		1 four cell cluster
	plate 2	5 single cells
pCHN1-1/pBII221	plate 1	1 single cell
		2 two cell clusters
	plate 2	5 single cells
		1 two cell cluster
		2 clusters of 8–10 cells

After 21 d on the round 2 selection plates, all viable portions of the material were transferred to round 3 selection plates containing 60 mg/l hygromycin. The round 2 selection plates, containing only tissue that was apparently dead, were reserved. Both round 2 and 3 selection plates were observed periodically for viable proliferating sectors.

After 35 d on round 3 selection plates both the round 2 and round 3 sets of selection plates were checked for viable sectors of callus. Two such sectors were observed proliferating from a background of dead tissue on plates treated with pHYGI1/pBII221. The first sector named 3AA was from the round 3 group of plates and the second sector named 6L was

from the round 2 group of plates. Both lines were then transferred to F medium without hygromycin.

After 19 d on F medium without hygromycin the line 3AA grew very little whereas the line 6L grew rapidly. Both were transferred again to F medium for 9 d. The lines 3AA and 6L were then transfered to F medium containing 15 mg/l hygromycin for 14 d. At this point, line 3AA was observed to be of very poor quality and slow growing. The line 6L however grew rapidly on F medium with 15 mg.l hygromycin. In preparation for an inhibition study of the line 6L on hygromycin, the line was then subcultured to F medium without hygromycin.

After 10 d on F medium an inhibition study of the line 6L was initiated. Callus of 6L was transfered onto F medium containing 0, 10, 30, 100, and 250 mg/l hygromycin B. Five plates of callus were prepared f or each concentration and each plate contained ten approximately 50 mg pieces of callus. one plate of unselected control ti ssue was prepared for each concentration of hygromycin.

It was found that the line 6L was capable of sustained growth over 9 subcultures on 0, 10, 30, 100, and 250 mg/l hygromycin. The name of the line 6L was changed at this time from 6L to PH1 (Positive Hlygromycin transformant 1).

Additional sectors were recovered at various time points from the round 2 and 3 selection plates. None of these were able to grow in the presence of hygromycin for multiple rounds, i.e. two or three subcultures.

V. Confirmation of transformed callus

To show that the PHi callus had acquired the hygromycin resistance gene, a Southern blot of PH1 callus was prepared as follows: DNA was isolated from PH1 and unselected control calli by freezing 2 g of callus in liquid nitrogen and grinding it to a fine powder which was transferred to a 30 ml Oak Ridge tube containing 6 ml extraction buffer (7M urea, 250 mM NaCl, 50 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1% sarcosine). To this was added 7 ml of phenol:chloroform 1:1, the tubes shaken and incubated at 37° C. 15 min. Samples were centrifuged at 8K for 10 min. at 4° C. The supernatant was pipetted through miracloth (Calbiochem 475855) into a disposable 15 ml tube (American Scientific Products, C3920-15A) containing 1 ml 4.4 M ammonium acetate, pH 5.2. Isopropanol, 6 ml, was added, the tubes shaken, and the samples incubated at −20° C. for 15 min. The DNA was pelleted in a Beckman TJ-6 centrifuge at the maximum speed for 5 min. at 4° C. The supernatant was discarded and the pellet was dissolved in 500 ul TE-10 (10 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0) 15 min. at room temperature. The samples were transferred to a 1.5 ml Eppendorf tube and 100 ul 4.4 M ammonium acetate, pH 5.2 and 700 ul isopropanol were added. This was incubated at −20° C. for 15 min. and the DNA pelleted 5 min. in an Eppendorf microcentrifuge (12,000 rpm). The pellet was washed with 70% ethanol, dried, and resuspended in TE-1 (10 mM Tris-HCl pH 8.0, 1 EM EDTA).

The isolated DNA (10 ug) was digested with BamHI (NEB) and electrophoresed in a 0.8% w/v agarose gel at 15 V for 16 hrs in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). The DNA within the gel was then depurinated by soaking the gel twice in 0.25 M HCl for 15 min., denatured and cleaved by soaking the gel twice in 0.5 M NaOH/1.0 M NaCl 15 min., and neutralized by soaking the gel twice in 0.5M Tris pH 7.4/3M NaCl 30 min. DNA was then blotted onto a Nytran membrane (Shleicher & Shuell) by capillary transfer overnight in 6×SSC (20×SSC, 3M NaCl, 0.3M sodium citrate pH 7.0). The membrane was baked at 80° C. for 2 hrs under vacuum. Prehybridization treatment of the membrane was done in 6×SSC, 10×Denhardt's solution, 1% SDS, 50 ug/ml denatured salmon sperm DNA using 0.25 ml prehybridization solution per cm^2 of membrane. Prehybridization was carried out at 42° C. overnight.

A 32P labelled probe was prepared by random primer labelling with an Oligo Labelling Kit (Pharmacia) as per the suppliers instructions with 32P-dCTP (ICN Radiochemicals). The template DNA used was the 1055 bp BamHI fragment of pHYGI1, which is the HPT coding sequence. The fragment was gel purified and cut again with PstI (NEB) before labelling.

The hybridization was performed in 50% formamide, 6×SSC, 1% SDS, 50 ug/ml denatured salmon sperm DNA (Sigma), 0.05% sodium pyrophosphate and all of the isopropanol precipitated heat denatured probe (10^7 CPM/50ug template). The hybridization was carried out at 42° C. overnight.

The membrane was washed twice in 50 ml 6×SSC, 0.1% SDS 5 min. at room temperature with shaking, then twice in 500 ml 6×SSC, 0.1% SDS 15 min. at room temperature, then twice in 500 ml 1×SSC, 1% SDS 30 min. at 42° C., and finally in 500 ml 0.1×SSC 1% SDS 60 min. at 65° C. Membranes were exposed to Kodak X-OMAT AR film in an X-OMATIC cassette with intensifying screens. As shown in FIG. 3, a band was observed for PH1 callus at the expected position of 1.05 Kb, indicating that the HPT coding sequence was present. No band was observed for control callus.

VI. Plant Regeneration and Production of Seed

PH1 callus was transferred directly from all of the concentrations of hygromycin used in tne inhibition study to RM5 medium which consists of MS basal salts (Murashige et al. 1962) supplemented with thiamine HCl 0.5 mg/l, 2,4-D 0.75 mg/l, sucrose 50 g/l, asparagine 150 mg/l, and Gelrite 2.5 g/l (Kelco Inc. San Diego).

After 14 d on RM5 medium the majority of PH1 and negative control callus was transferred to R5 medium which is the same as RMT medium, except that 2,4-D is omitted. These were cultured in the dark for 7 d at 26° C. and transferred to a liaht regime of 14 hours light and 10 hours dark for 14 d at 26° C. At this point, plantlets that had formed were transferred to one quart canning jars (Ball) containing 100 ml of R5 medium. Plants were transferred from jars to vermiculite after 14 and 21 d. Plants were grown in vermiculite for 7 or 8 d before transplanting into soil and grown to maturity. A total of 65 plants were produced from PH1 and a total of 30 plants were produced from control callus.

To demonstrate that the introduced DNA had been retained in the Ro tissue, a Southern blot was performed as previously described on leaf DNA from three randomly chosen Ro plants of PH1. As shown in FIG. 4, a 1.05 Kb band was observed with all three plants indicating that the HPT coding sequence was present. No band was observed for DNA from a control plant.

Controlled pollinations of mature PH1 plants were conducted by standard techniques with inbred lines A188, B73 and Oh43. Seed was harvested 45 days post-pollination and allowed to dry further 1–2 weeks. Seed set varied from 0 to 40 seeds per ear when PH1 was the female parent and from 0 to 32 seeds per ear when PH1 was the male parent.

VII. Analysis of the R1 Progeny

The presence of the hygromycin resistance trait was evaluated by a root elongation bioassay, an etiolated leaf bioassay, and by Southern blotting. Two ears each from regenerated PH1 and control plants were selected for analysis. The pollen donor was inbred line A188 for all ears.

(A) Root Elongation Bioassay

Seed was sterilized in a 1:1 dilution of commercial bleach in water plus alconox 0.1% for 20 min. in 125 ml Erlenmeyer flasks and rinsed 3 times in sterile water and imbibed overnight in sterile water containing 50 mg/ml captan by shaking at 150 rpm.

After imbibition, the solution was decanted from the flasks and the seed transferred to flow boxes (Flow Laboratories) containing 3 sheets of H_2O saturated germination paper. A fourth sheet of water saturated germination paper was placed on top of the seed. Seed was allowed to germinate 4 d.

After the seed had germinated, approximately 1 cm of the primary root tip was excised from each seedling and plated on MS salts, 20 g/l sucrose, 50 mg/l hygromycin, 0.25% Gelrite, and incubated in the dark at 26° C. for 4 d.

Roots were evaluated for the presence or absence of abundant root hairs and root branches. Roots were classified as transgenic (hygromycin resistant) if they had root hairs and root branches, and untransformed (hygromycin sensitive) if they had limited numbers of branches. The results are shown in Table 1.

(B) Etiolated leaf bioassay

After the root tips were excised as described above, the seedlings of one PH1 ear and one control ear were transferred to moist vermiculite and grown in the dark for 5 d. At this point 1 mm sections were cut from the tip of the coleoptile, surface sterilized 10 seconds, and plated on MS basal salts, 20 g/l sucrose, 2.5 g/l Gelrite with either 0 (control) or 100 mg/l hygromycin and incubated in the dark at 26° C. for 18 hr. Each plate contained duplicate sections of each shoot. They were then incubated in a light regimen of 14 hours light 10 hours dark at 26° C. for 48 hr, and rated on a scale of from 0 (all brown) to 6 (all green) for the percent of green color in the leaf tissue. Shoots were classified as untransformed (hygromycin sensitive) if they had a rating of zero and classified as transformed (hygromycin resistant) if they had a rating of 3 or greater. The results are shown in Table 3.

(C) Southern Blots

Seedling form the bioassays were transplanted to sail and are growing to sexual maturity. DNA was isolated from 0.8 g of leaf tissue after about 3 weeks and probed with the HPT coding sequence as described previously. Plants with a 1. 05 Kb band present in the Southern blot were classified as transgenic. As shown in FIG. 5, two out of seven progeny of PH1 plant 3 were transgeflic as were three out of eight progeny of PHI plant 10. The blot results correlated precisely with data from the bioassays, confirming that the heterologous DNA was transmitted through one complete sexual life cycle. All data are summarized in Table 3.

TABLE 3

ANALYSIS OF PH1 R1 PLANTS

PH1 PLANT	ROOT ASSAY	LEAF ASSAY	BLOT	CONT. PLANT	ROOT ASSAY	LEAF ASSAY	BLOT
3.1	+	ND	+	4.1	–	ND	ND
3.2	–	ND	–	4.2	–	ND	ND
3.3	–	ND	–	4.3	–	ND	ND
3.4	–	ND	–	4.4	–	ND	ND
3.5	–	ND	–	4.5	–	ND	ND
3.6	+	ND	+	4.6	–	ND	ND
3.7	–	ND	–	4.7	–	ND	ND
				2.1	–	ND	–
10.1	+	+	+	1.1	–	–	–
10.2	+	+	+	1.2	–	–	ND
10.3	–	–	ND	1.3	–	–	ND
10.4	–	–	–	1.4	–	–	ND
10.5	–	–	–	1.5	–	–	ND
10.6	–	–	–	1.6	–	–	ND
10.7	–	–	–	1.7	–	–	ND
10.8	ND	+	+	1.8	–	–	ND

KEY: + = transgenic; – = nontransgenic; ND = not done

EXAMPLE II

The procedure of Example I was repeated with minor modifications.

I. Plant Lines and Tissue Cultures

The embryogenic maize callus line, AB12, was used as in Example I. The line had been initiated about 18 months before the actual bombardment occurred.

II. Plasmids

The plasmids pBII221 and pHYGI1 described in Example I were used.

III. DNA delivery process

Callus was bombarded exactly as in Example I except that the DNA used in the tungsten/DNA preparations differed. All of the tubes contained 25 ul 50 mg/ml M-10 tungsten in water, 25, ul 2.5 M $CaCl_2$, and 10 ul 100 mM spermidine free base along with a total of 5 ul 1 mg/ml total plasmid content. One tube contained only plasmid pBII221; two tubes contained only plasmid pHYGI1; and one tube contained no plasmid but 5 ul TE-1 (10 mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0).

The following bombardments were done:

2 × pBII221 prep	For transient expression
7 × pHYGI1 prep	Potential positive treatment
3 × TE prep	Negative control treatment

After all the bombardments were performed, the callus from the pBII221 treatments was transferred plate for plate to F medium as five 50 mg pieces. After 2 d the callus was placed into GUS assay buffer as per Example I. Numbers of transiently expressing cells were counted and found to be 686 and 845 GUS positive cells, suggesting that the particle delivery process had occurred in the other bombarded plates.

work. In all of the procedures, no viable sectors were observed. In the Table, "Sieved" indicates that the callus was passed through an 860 micron sieve before bombardment; the selective agent was hygromycin for each case except when pMXTI1 was the plasmid and methotrexate the selection agent.

TABLE 4

Summary of Comparative Example A

Recip. Tissue	Plasmids	Recov. Period	Round 1 Level	Round 1 Period	Round 2 Level	Round 2 Period
Clumps	pCHN1-1 pBII221	13	60	21	60	81
Clumps	pCHN1-1 pBII221	14	100	22	—	—
Clumps	pHYGI1 pBII221	8	60	19	30	132
Clumps	pCHN1-1 pBII221	0	30	22	60	109
Clumps	pMTXI1 pBII221	8	3	103	—	—
Sieved	PCHN1-1 pBII221	13	—	—	—	—

What is claimed is:

1. A process for producing a fertile transgenic *Zea mays* plant comprising the steps of (i) bombarding intact regenerable *Zea mays* cells with DNA-coated microprojectiles, wherein said DNA comprises at least a screenable marker gene; (ii) selecting a population of transformed cells expressing the selectable marker gene; and (iii) regenerating a fertile transgenic plant therefrom, wherein said DNA is expressed so as to impart glyphosate resistance to said transgenic plant and is transmitted through a normal sexual cycle of said transgenic plant to progeny plants.

2. The process of claim 1 wherein the fertile transgenic *Zea mays* plant is regenerated from transformed embryogenic tissue.

3. The process of claim 1 wherein the cells are derived from immature embryos.

4. The process of claim 1 wherein said DNA encodes an EPSP synthase.

5. The process of claim 1 further comprising obtaining transgenic glyphosate resistant progeny plants of subsequent generations from said fertile transgenic plant.

6. The process of claim 5 further comprising obtaining seed from one of said progeny plants.

7. A process for producing seed comprising:

(a) providing a fertile transgenic *Zea mays* produced by the process of claim 1, plant comprising heterologous heritable DNA that is expressed so as to impart glyphosate resistance to said transgenic *Zea mays* plant;

(b) cultivating said transgenic *Zea mays* plant;

(c) obtaining seed from said cultivated *Zea mays* plant.

8. The process of claim 7 wherein the DNA encodes an EPSP synthase.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,013,863
DATED : January 11, 2000
INVENTOR(S) : Lundquist et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 10,
Line 12, delete the comma and insert a prime sign, therefor.
Line 73, delete "ST.L.1" and insert -- ST.LS.1 --, therefor.

Column 11,
Line 44, delete "Nicatiana" and insert -- Nicotiana --, therefor.

Column 13-14,
In the first two rows of Table 2, delete all four occurences of "ncomycin" and insert -- neomycin --, therefor.
In the second to last row and first column of Table 2, delete "luciterase" and insert -- luciferase --, therefor.
Footnote 1, add a space between "EMBO" and "J", both occurrences.
Footnote 2, add a space between "PNAS" and "USA".
Footnote 2, underline "Nucl. Acids Res., 14,".
Footnote 3, underline "EMBO J., 3," and "Nature, 303,".
Footnote 4, underline "Nature, 310,", "ibid.," and "EMBO J., 2,".
Footnote 5, delete "Somat. Cell, Mol. Genet." and replace with -- Somat. Cell. Mol. Genet., 13, --therefore.
Footnote 6, underline "Theor, Appl. Genet., 66", Nature, 300,", "Plant Mol. Biol., 5," and "J. Mol. Appl. Genet., 2".
Footnote 7, underline "EMBO J., 4,", "Nuc. Acids Res., 14," and "EMBO J., 2,".
Footnote 8, underline "Science, 284" and "Plant Mol. Biol., 5," both occurrences.
Footnote 9, underline "Plant Mol. Biol., 2,".
Footnote 10, underline "Mol. Gen. Genet., 210,".
Footnote 11, underline "Nature, 317" and "Biotechnology, 5,".
Footnote 12, underline "EMBO J., 6,", both occurrences.
Footnote 13, underline "Biotechnology, 2,".
Footnote 14, underline "Nuc. Acids Res., 15" and "EMBO J., 6,".
Footnote 15, underline "Mol. Gen. Genet., 204,".
Footnote 16, underline "Science, 234,", "PNAS USA", and "Nucl. Acids Res., 15,".

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,013,863
DATED : January 11, 2000
INVENTOR(S) : Lundquist et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 29,
Line 29, delete "screenable" and insert -- selectable --, therefor.

Column 30,
Line 21, insert "plant" before -- produced --.
Line 22, insert "said" before -- plant --.

Signed and Sealed this

Twenty-third Day of October, 2001

Attest:

Nicholas P. Godici

Attesting Officer

NICHOLAS P. GODICI
Acting Director of the United States Patent and Trademark Office

IN THE UNITED STATES DISTRICT COURT FOR
THE NORTHERN DISTRICT OF ILLINOIS
WESTERN DIVISION

DEKALB GENETICS CORPORATION, Plaintiff, vs. SYNGENTA SEEDS, INC., SYNGENTA BIOTECHNOLOGY, INC., GOLDEN HARVEST SEEDS, INC., GARWOOD SEED CO., INC., GOLDEN SEED CO., L.L.C., SOMMER BROTHERS SEED CO., INC., THORP SEED CO., INC., and J.C. ROBINSON SEED COMPANY, Defendants.	CIVIL ACTION NO.: 04 C 50323 JURY TRIAL DEMANDED Judge Philip G. Reinhard Magistrate Judge P. Michael Mahoney

NOTICE OF FILING

TO: Mr. David C. Van Dyke
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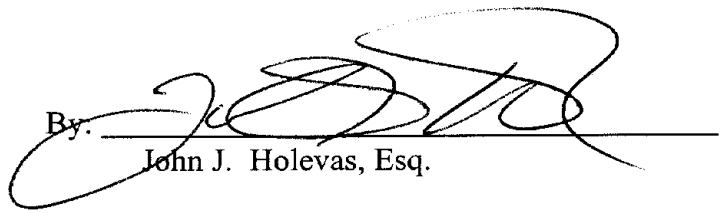
PLEASE TAKE NOTICE that Plaintiff, DEKALB Genetics Corporation, has on the 27 day of January, 2005, filed a First Amended Complaint in the above-captioned matter.

A copy of which is served upon you herewith.

DEKALB Genetics Corporation, Plaintiff
By: Williams & McCarthy, One of Its Attorneys



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CERTIFICATE OF SERVICE

The undersigned hereby certifies that at 5:00 o'clock p.m. on January 27, 2005, at 321 West State Street, Rockford, Illinois, a copy of the foregoing instrument was served upon the following party:

VIA FACSIMILE AND FIRST-CLASS MAIL

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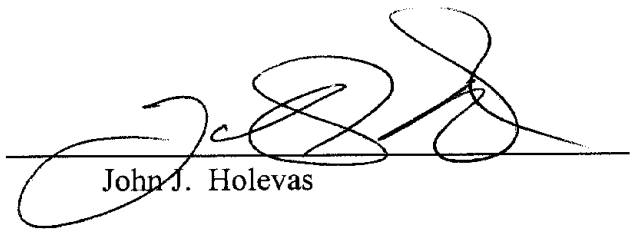

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Not of Filing.03.wpd