

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

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U.S. DISTRICT COURT

PIONEER HI-BRED INTERNATIONAL,
INC.,

Plaintiff

v.

THE MONSANTO COMPANY, and DEKALB
GENETICS CORPORATION,

Defendants.

Case No. **01C50219**

Hon.

Magistrate Judge

JURY TRIAL DEMANDED

COMPLAINT FOR PATENT INFRINGEMENT

Plaintiff Pioneer Hi-Bred International, Inc. ("Pioneer"), by and through its attorneys, alleges as and for its complaint against Defendants The Monsanto Company ("Monsanto"), and DeKalb Genetics Corporation ("DeKalb"), as follows:

Nature of the Action

1. This is an action for patent infringement under the Patent Laws of the United States, Title 35 U.S.C., § 271 *et seq.* In this action, Pioneer seeks monetary damages and preliminary and permanent injunctive relief for Monsanto and DeKalb's infringement, or active inducement or contributory infringement, of Pioneer's United States Patent No. 6,258,999.

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Parties

2. Plaintiff Pioneer is a corporation organized and existing under the laws of the State of Iowa. Pioneer's principal place of business is located at 400 Locust Street, Des Moines, Iowa 50306. Pioneer is the leading producer and seller of corn seed in the United States.

3. On information and belief, defendant Monsanto is a corporation organized and existing under the laws of the State of Delaware, with its principal place of business at 800 North Lindbergh Boulevard, St. Louis, Missouri. Monsanto, alone and in conjunction with its affiliates and subsidiaries, produces and sells a diversified line of agricultural products, including seed products, chemicals and pharmaceuticals throughout the world. Monsanto is also involved in developing technology used in the genetic engineering of agricultural products, including corn.

4. On information and belief, defendant DeKalb was and/or is a corporation organized and existing under the laws of the State of Delaware. DeKalb publicizes that it maintains an office at 3100 Sycamore Road, DeKalb, Illinois 60115. On information and belief, DeKalb currently has no employees, no business operations, and is merely a brandname for Monsanto. Since December of 1998, Monsanto has been the sole and complete owner of Defendant DeKalb.

5. On information and belief, for all relevant times, DeKalb has been the alter-ego of, and wholly controlled by, Defendant Monsanto. Monsanto, through DeKalb, manufactures and sells corn seed throughout the United States including in this judicial district.

Jurisdiction and Venture

6. In this action for patent infringement, this Court has subject matter jurisdiction pursuant to 28 U.S.C. § 1331 and 28 U.S.C. § 1338(a).

7. This Court has personal jurisdiction over the Defendants because Monsanto and DeKalb each have extensive contacts and substantial ongoing business activities in Illinois, including in this judicial district.

8. Venue is proper in this Court pursuant to 28 U.S.C. §§ 1391(c) and 1400(b).

Count I – Patent Infringement

9. Plaintiff Pioneer realleges and incorporates by reference Paragraphs 1-8 of this Complaint as if fully set forth herein.

10. Pioneer is the owner of United States Patent No. 6,258,999 ("the '999 patent"), issued on July 10, 2001 to Tomes, *et al.*, entitled "Stable Transformation of Plant Cells." (A copy of which is attached hereto as Exhibit A). Pioneer has the right to maintain this action. The '999 patent claims, inter alia, fertile transgenic *Zea mays* plants comprised of stably incorporated foreign DNA, wherein in one or more of the claims of the patent, that foreign DNA imparts an agronomic trait.

11. On information and belief, defendants Monsanto and DeKalb, alone or in conjunction with affiliates and/or subsidiaries, are making, using, selling or offering for sale one or more corn product or corn hybrid which infringes one or more claims of the '999 patent.

12. On information and belief, defendants Monsanto and DeKalb, alone or in conjunction with affiliates and/or subsidiaries, are making, using, selling or offering for sale one or more corn product or corn hybrid which infringes one or more claims of the '999 patent, directly or indirectly through as sales distribution network to farmers who plant those corn products or corn hybrids to grow corn.

13. On information and belief, defendants Monsanto and DeKalb have directly or indirectly infringed, and have or are contributorily infringing, or actively inducing others to infringe, one or more claims of the '999 patent through the activities complained of herein.

14. Pioneer has suffered and continues to suffer damages because of all of the above-described acts of infringement of the '999 patent in an amount to be proven at a trial.

15. Unless enjoined, the Defendants will continue to infringe the '999 patent, causing irreparable harm to Pioneer, and to its patent rights, for which there is no adequate remedy at law.

Prayer for Relief

WHEREFORE, Pioneer respectfully requests the following relief:

A. A preliminary injunction enjoining Defendants and all those affiliated, or in active concert, with them from infringing, directly or indirectly, inducing others to infringe, and from contributing to the infringement of the '999 patent during pendency of this action;

B. A judgment that Defendants have infringed the '999 patent;

C. A judgment that Defendants have willfully infringed, induced infringement, or contributorily infringed the '999 patent;

D. A injunction permanently enjoining Defendants and all those affiliated, or in active concert, with them from infringing, directly or indirectly, from inducing others to infringe, or from contributing to the infringement of, the '999 patent;

E. An accounting to determine an award of damages, not less than a reasonable royalty, adequate to compensate Pioneer for Defendants' infringement of the '999 patent;

F. A finding that this is an exceptional case under 35 U.S.C. § 285 and an award to Pioneer of its attorneys' fees, costs and expenses in this action, together with such further relief as allowed by the Patent Laws;

G. Any other relief the Court deems just and proper.

Jury Demand

Pursuant to Rule 38(b) of the Federal Rules of Civil Procedure, and the Local Rules of this Court, Pioneer requests a trial by jury on all claims in this Complaint.

Respectfully submitted,

**PIONEER HI-BRED INTERNATIONAL,
INC.**

Dated: July 10, 2001

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US006258999B1

(12) **United States Patent**
Tomes et al.

(10) Patent No.: **US 6,258,999 B1**
(45) Date of Patent: ***Jul. 10, 2001**

(54) **STABLE TRANSFORMATION OF PLANT CELLS**

(75) Inventors: **Dwight T. Tomes, Cumming, IA (US); Arthur Watsinger, Raleigh, NC (US); John C. Sanford, Geneva, NY (US); Theodore M. Klein, Wilmington, DE (US)**

(73) Assignee: **Pioneer Hi-Bred International, Inc., Des Moines, IA (US)**

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **08/442,522**

(22) Filed: **May 16, 1995**

Related U.S. Application Data

(62) Division of application No. 08/319,352, filed on Oct. 6, 1994, now Pat. No. 5,990,387, which is a continuation of application No. 07/906,018, filed on Jun. 26, 1992, now abandoned, which is a continuation of application No. 07/711,583, filed on Jun. 6, 1991, now abandoned, which is a continuation of application No. 07/581,154, filed on Sep. 7, 1990, now abandoned, which is a continuation of application No. 07/205,155, filed on Jun. 10, 1988, now abandoned.

(51) Int. Cl.⁷ **A01H 5/00; A01H 1/00; C12N 15/09**

(52) U.S. Cl. **800/300.1; 800/320.1; 435/468; 435/470**

(58) Field of Search **800/205, 300.1, 800/320.1, 278, 279, 293; 435/172.3, 240.4, 320.1, 468, 470**

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(57)

ABSTRACT

Fertile, stably transformed plants regenerated from culture, produced by one or more bombardments of target cells or tissues with microprojectiles coated with foreign DNA comprising one or more genes coding for a desired trait, and selection of transformed cells in culture, are provided.

40 Claims, 1 Drawing Sheet

EXHIBIT

A

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FIG. 1

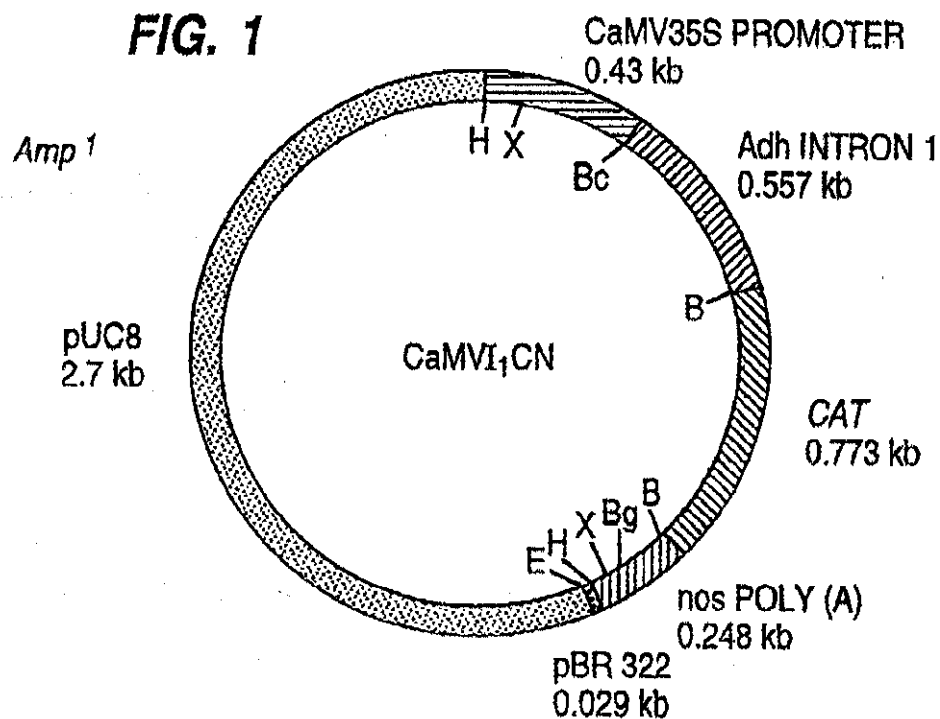
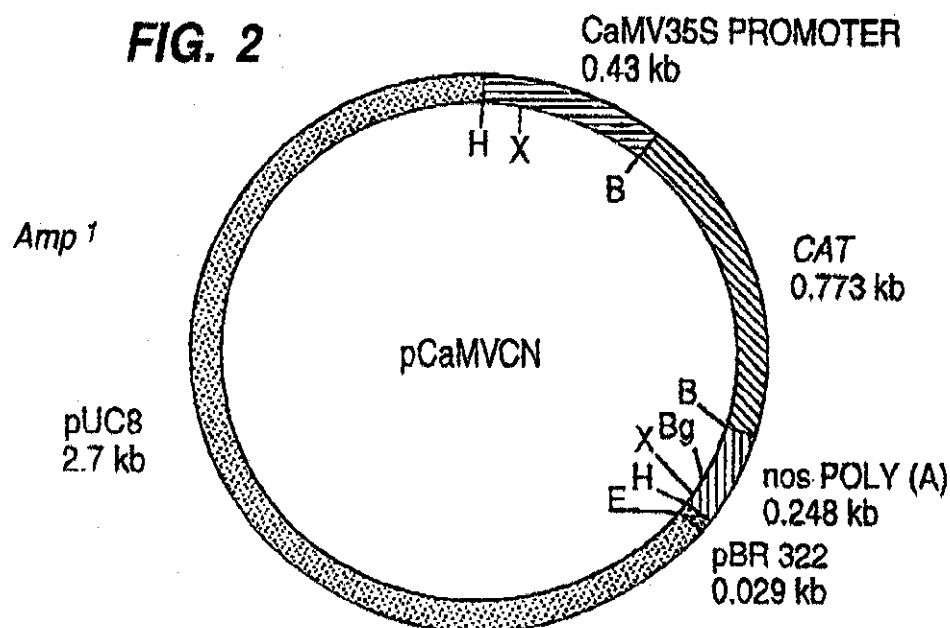


FIG. 2



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STABLE TRANSFORMATION OF PLANT CELLS

This is a divisional application of prior application Ser. No. 08/319,352, filed Oct. 6, 1994, now U.S. Pat. No. 5,990,387, which is a continuation of prior application Ser. No. 07/906,018, filed Jun. 26, 1992, now abandoned, which was a continuation of application Ser. No. 07/711,585, filed Jun. 6, 1991, now abandoned, which was a continuation of application Ser. No. 07/581,154, filed Sep. 7, 1990, now abandoned, which was a continuation of application Ser. No. 07/205,155, filed Jun. 10, 1988, now abandoned.

TECHNICAL FIELD

This invention relates to the use of recombinant DNA technology for the transformation of plants. More specifically, this invention concerns the techniques and materials necessary for stable transformation of plant cells and the regeneration of fertile whole plants therefrom.

BACKGROUND ART

Much research in plant molecular biology is now directed to the improvement of plant varieties via use of recombinant DNA techniques. Historically, plant breeders used classical genetic techniques to identify, preserve and crossbreed varietal lines having desirable traits. More recently, new plant varieties were induced by chemicals or by radiation treatment to mutate plant cells which were then regenerated using tissue culture techniques. These random and unpredictable approaches have obvious drawbacks. By the use of recombinant DNA technology, specific genes producing specific proteins, such as those imparting insect resistance, may be introduced into a plant to produce a desired variety with a particular trait. It is thus desirable to provide a method for regeneration of plant cells which have been transformed with recombinant DNA.

DISCLOSURE OF THE INVENTION

This invention provides methods for the construction and use of expression vectors which include at least one gene associated with the expression of a desired agronomic trait or the production of a desired protein. It further provides methods for the transformation of plant cells with the expression vector, and methods for regeneration of whole, fertile transformed plants from transformed cells in tissue culture. Finally, this invention provides transformed plants which have been produced according to the foregoing methods.

DESCRIPTION OF THE DRAWINGS

FIG. 1 is a diagram of the vector CaMV1, CN showing the DNA fragments used in the plasmid's construction.

FIG. 2 is a diagram of vector pCaMVCN showing the DNA fragments used in the plasmid's construction. In the Figures the following symbols are used:

B	BamHI
Bc	PcII
Bg	BglII
E	EcoRI
H	HindIII
X	XbaI

DESCRIPTION OF THE PREFERRED EMBODIMENTS

This invention provides a method for transforming plant cells and regenerating the transformed cells into a whole, fertile plant, comprising the steps of

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inserting at least one structural gene coding for a desired trait into an expression vector;

preparing host plant cells for transformation by subculturing the cells from a parent culture of germ line cells; introducing the vector containing the structural gene into the plant cells by one or more microparticle bombardments within a period of from about 12 hours to about 1 week after the subculture;

culturing the bombarded cells under conditions which select for survival of transformed cells; and regenerating the surviving transformed cells into a whole plant from tissue culture.

By the term "vector" herein is meant a DNA sequence which is able to replicate and express a foreign gene in a host cell. Typically, the vector has one or more endonuclease recognition sites which may be cut in a predictable fashion by use of the appropriate enzyme. Such vectors are preferably constructed to include structural gene sequences imparting antibiotic or herbicide resistance, which then serve as markers to identify and separate transformed cells. Preferred markers/selection agents include kanamycin, chlorosulfuron, hygromycin and methotrexate. A cell in which the foreign genetic material in a vector is functionally expressed has been "transformed" by the vector and is referred to as a "transformant."

A particularly preferred vector is a plasmid, by which is meant a circular double-stranded DNA molecule which is not a part of the chromosomes of the cell.

Both genomic and cDNA encoding the gene of interest may be used in this invention. The vector of interest may also be constructed partially from a cDNA clone and partially from a genomic clone. When the gene of interest has been isolated, genetic constructs are made which contain the necessary regulatory sequences to provide for efficient expression of the gene in the host cell. According to this invention, the genetic construct will contain (a) a first genetic sequence coding for the protein or trait of interest and (b) one or more regulatory sequences operably linked on either side of the structural gene of interest. Typically, the regulatory sequences will be selected from the group comprising of promoters and terminators. The regulatory sequences may be from autologous or heterologous sources.

Promoters that may be used in the genetic sequence include nos, ocs and CaMV promoters.

An efficient plant promoter that may be used as an overproducing plant promoter. Overproducing plant promoters that may be used in this invention include the promoter of the small sub-unit (ss) of the ribulose-1,5-bisphosphate carboxylase from soybean (Berry-Lowe et al., *J. Molecular and App. Gen.*, 1:483-498 (1982)), and the promoter of the chlorophyll a-b binding protein. These two promoters are known to be light-induced, in eukaryotic plant cells (see, for example, *genetic engineering of plants, an agricultural perspective*, A. Cashmore, Pelham, N.Y., 1983, pp. 29-38, G. Coruzzi et al., *J. Biol. Chem.*, 258:1399 (1983), and P. Dunsmuir, et al., *J. Molecular and App. Gen.*, 2:285 (1983)).

The genetic sequence comprising the gene of interest operably linked to the desired control sequences can be ligated into a suitable cloning vector. In general, plasmid or viral (bacteriophage) vectors containing replication and control sequences derived from species compatible with the host cell are used. The cloning vector will typically carry a replication origin, as well as specific genes that are capable of providing phenotypic selection markers in transformed host cells. Typically, genes conferring resistance to antibiotics or selected herbicides are used. After the genetic material is introduced into the target cells, as described

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Preferably, germ line cells will be used in the practice of this invention rather than somatic cells. The term "germ line cells" refers to cells in the plant organism which can trace their eventual cell lineage to either the male or female reproductive cell of the plant. Other cells, referred to as "somatic cells" are cells which give rise to leaves, roots and vascular elements which, although important to the plant, do not directly give rise to gamete cells. With regard to callus and suspension cells which have somatic embryogenesis, many or most of the cells in the culture have the potential capacity to give rise to an adult plant. If the plant originates from single cells or a small number of cells from the embryogenic callus or suspension culture, the cells in the callus and suspension can therefore be referred to as germ cells. This classification is made because these individual cells have the potential to form an entire plant, including the gamete cells. In the case of immature embryos which are prepared for treatment by microparticle bombardment, certain cells in the apical meristem region of corn have been shown to produce a cell lineage which eventually gives rise to the female and male reproductive organs. With many or most species, the apical meristem is generally regarded as giving rise to the lineage that eventually will give rise to the gamete cells. An example of a non-gamete cell in an embryo would be the first leaf primordia in corn which is destined to give rise only to the first leaf and none of the reproductive structures. Although both somatic and germ line cells can be effectively transformed for brief periods of time, up to 72 hours following microparticle bombardment, germ line cells are preferred for stable transformation which in turn results in transformed seed and their progeny.

All tissues which can be cultured to give whole regenerated plants can be transformed by the present inventions so that whole plants are recovered which contain the foreign gene. When maize or sunflower are to be modified, it is

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In the overall method of this invention, a suspension of transformed plant cells containing the foreign gene is created by microparticle bombardment. Embryo formation is then induced from the cell suspension, to the stage of maturing and germination into plants. Efficient regeneration depends upon the medium, the genotype and the history of the culture. Regeneration is performed as follows:

A two-culture medium sequence is used to germinate somatic embryos observed on a callus maintenance medium. Callus is transferred first to a culture medium (maturation medium) which, instead of 0.75 mg/L, 2,4-D has 5.0 mg/L indoleacetic acid (IAA). The callus culture remains on this medium for 10 to 14 days while callus proliferation continues at a slower rate. At this culture stage, it is important that the amount of callus started on the culture medium not be too large, or fewer plants will be recovered per unit mass of material. Especially preferred is an amount of 50 milligrams of callus per plate.

Toward the end of this culture phase, observation under a dissecting microscope often indicates somatic embryos that have begun germinating although they are white in color because this culture phase is done in darkness.

Following this first culture phase, callus is transferred from "maturation" medium to a second culture medium which further promotes germination of the somatic embryos into a plantlet. This culture medium has a reduced level of indoleacetic acid versus the first culture medium, preferably a concentration of 1.0 mg/L. At this point, the cultures are placed into the light. Germinating somatic embryos are characterized by a green shoot which elongates, often with a connecting root axis. Plants are subcultured at approximately 14-day intervals until plantlet size necessitates the transfer into a culture tube instead of a petri plate. Although plants are identified rapidly, it usually takes approximately six to eight weeks to produce a plant of sufficient size for transfer to a growth chamber or greenhouse. Preferably, the plantlets will be approximately five centimeters tall with a healthy root system at the point they are transferred to a growth chamber.

Plants to be transferred to the growth chamber are removed from sterile containers and the solidified agar medium is rinsed off the roots. The plantlets are placed in a commercial potting mix in a growth chamber equipped with a misting device which maintains the relative humidity near 100% without excessively wetting the plant roots. Approximately three to four weeks are required in the misting chamber before the plants are robust enough for transplantation into pots or into field conditions. At this point, many plantlets, especially those regenerated from short-term callus cultures will grow at a rate and to a size similar to seed-derived plants. Plants regenerated from long-term callus, from suspension cultures, and from in vitro-selected callus will sometimes show phenotypic abnormalities, such as reduced plant size, leaf striping and delayed maturation. Care must be taken to assure controlled pollination with such plants. Ten to fourteen days after pollination, the plants are checked for seed set. If there is seed, the plants are then placed in a holding area in the greenhouse to mature and dry down. Harvesting is typically performed six to eight weeks after pollination.

The culture medium for initiating callus consists of a mineral salt base including major and micronutrients and vitamins (CT base) with two important additions. The first is 2,4-D at about 2 mg/L and two specific amino acids, proline at 1400 mg/L and asparagine at 900 mg/L. The preferred nutrient media employed in the regeneration of the mature plant from the transformed plant cell are described in Table

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A. Variations and omissions of various constituents, well known to those of ordinary skill in the plant cell culture arts, may be included or modified to accommodate species other than corn.

TABLE A

Constituents and Concentrations of Culture Medium		
Macronutrients	mM	mg/l
KNO ₃	28.0	2,800
CaCl ₂ · 2H ₂ O	2.0	300
MgSO ₄ · 7H ₂ O	1.0	280
KH ₂ PO ₄	2.2	300
(NH ₄) ₂ SO ₄	4.0	530
FeSO ₄ · 7H ₂ O	0.11	37.3
Na ₂ · EDTA	0.10	27.8
Micronutrients		
KI	0.00045	0.75
H ₃ BO ₃	0.049	3.0
MnSO ₄ · H ₂ O	0.060	10.0
2NaSO ₄ · 7H ₂ O	0.007	2.0
Na ₂ MoO ₄ · H ₂ O	0.001	0.25
CuSO ₄ · 5H ₂ O	0.0001	0.025
CuCl ₂ · 6H ₂ O	0.0001	0.025
Vitamins		
Nicotinic acid	0.004	0.5
Pyridoxine HCl	0.002	0.5
Thiamine HCl	0.003	1.0
Glycine	0.027	2.0
Myo-inositol		1,000
Amino Acids		
Proline	12	1,400
Asparagine	6	900
Sucrose		20,000
Gelrite		3,000
2,4-D	0.038	0.75

Having now generally described this invention, the same will be better understood by reference to specific examples, which are included herein for purposes of illustration only, and are not intended to be limiting unless otherwise specified.

EXAMPLE 1

Plasmids were prepared having the structures illustrated in the drawings. pCaMVN was derived from pCaMVneo obtained according to the method of Fromm et al., by replacement of the neomycin phosphotransferase coding region with the CAT coding region. Addition of the Adh1 intron fragment (Bc to B) to the BamHI site at the 5' end of the CAT coding region yielded pCaMVI, CN.

The plasmid pCaMVI, CN consists of the 35s promoter from cauliflower mosaic virus, a fragment (Bc to B) from the Adh1 intron 1, a CAT coding region, and the nopaline synthase polyadenylation region. This plasmid is identical to pCaMVCN with the addition of the Adh1 intron fragment.

Separately, embryogenic suspension cultures 3-86-17 and 13-217 were derived from Type II embryogenic culture according to the method of Green et al., *Molecular Genetics of Plants and Animals*, eds. Downey et al. (Academic Press, New York, N.Y.), 20, 147 (1983). The callus was initiated from maize inbreds designated R21 (for 3-86-17) and B73 xG35 (for 13-217). Inbred R21 was derived from a regenerated plant from a long-term callus culture of public inbred B73, and is very similar to B73. Both R21 and G35 are proprietary inbred lines developed by Pioneer Hi-Bred International, Inc., Des Moines, Iowa. Suspension cultures of the cultivar "Black Mexican Sweet" ("BMS") were

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obtained from Stanford University. The cultures were maintained in Murashige and Skoog ("MS") medium, as described in Murashige, T. and Skoog, F., *Physiol. Plant.* 15:453-497 (1962), supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) at 2 mg/L and sucrose at 30 g/L. The suspension cultures were passed through a 710 micron sieve 7 days prior to the experiment, and the filtrate was maintained in MS medium. (Modified?) In preparation for microparticle bombardment, cells were harvested from the suspension culture by vacuum filtration on a Buchner funnel (Whatman #614). The same cell batch from each genotype was used within each experiment.

Prior to microparticle bombardment, 100 mg (fresh weight) of cells were placed in a 3.3 cm petri plate. The cells were dispersed in 0.5 ml. fresh culture medium to form a thin layer of cells covered by a film of medium. The uncovered petri plate was placed in the sample chamber of a particle gun device manufactured by Biolistics, Inc., Geneva, N.Y. A vacuum pump was used to reduce the pressure in the chamber to 0.1 atmosphere to reduce deceleration of the microprojectiles by air friction. The cells were bombarded with tungsten particles having an average diameter of about 1.2 microns, obtained from GTE Sylvania, Precision Materials Group, Towanda, Pa. In some trials, as indicated below, cells were bombarded with microprojectiles having an average diameter of 0.6 microns (GTE Sylvania) or 2.4 microns (General Electric Corp., Refractory Metals Division, Cleveland, Ohio). The microprojectiles had a DNA loading applied by adding 5 μ L of a 0.1 g-% solution of DNA in TE buffer at pH 7.7 to 25 μ L of a suspension of 50 mg tungsten particles per mL distilled water in a 1.5 mL Eppendorf tube. In one trial, DNA from two plasmids (pCaMV1, CN and pCaMV1, HygN) were combined for a total of 10 μ L DNA in 25 μ L of tungsten particle suspension. CaCl_2 (25 μ L of a 2.5 M solution) and spermidine free base (10 μ L of a 0.1 M solution) were then added to the suspension. Particles became agglomerated and settled to the bottom of the Eppendorf tube after about 10 minutes after the addition of the CaCl_2 and spermidine. Most of the supernatant was removed and the particles were deagglomerated by briefly touching the outside of the tube to the probe of a sonicator (Heat Systems-Ultrasonics, Inc.) Five microliters of the resulting suspension were then placed on the front surface of a cylindrical polyethylene macroprojectile. The macroprojectile was then placed into the barrel of the particle gun and a powder charge (#1 gray extra light, Speed Fasteners, Inc., St. Louis, Mo.) was loaded into the barrel behind the macroprojectile. A firing pin mechanism was used to detonate the powder charge and accelerate the macroprojectile down the barrel of the device to impact with a stopping plate. Upon impact, the momentum of the microprojectiles carried them from the surface of the macroprojectile, through a small aperture in the stopping plate and into the cells, which were positioned in the sample chamber 15 cm from the end of the barrel of the gun. After bombardment, the petri dish was removed from the apparatus and the cells were transferred to 5 mL fresh medium in a 15 mL polypropylene tube. The cells were then maintained in the tube with agitation at 27° C. until harvested. Control specimens were treated identically except that no DNA was applied to the tungsten particles.

In one series of trials, the plant cells were treated either with a medium of high osmotic potential or with a mixture of mineral oil and water. 100 mg of BMS cells were dispersed in 0.5 mL MS medium supplemented with mannitol (0.1 M) 30 minutes prior to bombardment. After microparticle bombardment, the cells were left in the

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mannitol-containing medium for an additional 30 minutes, after which the cells were resuspended in 5 mL of standard MS medium. Another sample of cells was dispersed in an emulsion of either 0.1 mL sterile mineral oil and 0.5 MS medium, 0.2 mL mineral oil and 0.4 mL MS medium, or in 0.6 mL MS medium lacking mineral oil. After bombardment the cells were suspended in 5 mL of MS medium.

Analyses of CAT activity were performed 12 or 96 hours after bombardment. Significantly higher levels of activity were taken to be evidence of plant cell synthesis of this foreign protein, and thus to indicate transformation of the cell. BMS, 3-86-17 and 13-217 suspension cells were collected for the assay following growth against either 10 mg/mL (BMS) or 25 mg/mL (3-86-17, 13-217) hygromycin. Tissue extracts were prepared by sedimenting the cells at approximately 13,000 \times G for 10 minutes in a 1.5 mL microfuge tube. The supernatant was removed and 100 μ L of buffer (0.25 M Tris HCl, pH 7.8) were added to the pellet. The sample was homogenized on ice for approx. 2 minutes with a disposable polypropylene pestle driven at 300 rpm by an electric motor. After grinding, the sample was vortexed briefly to complete the extraction of soluble protein. Cell debris was removed by centrifugation at 13,000 \times G in a microfuge at 4° C. for 10 minutes. The supernatant was decanted and normalized to a volume of 200 μ L with Tris HCl buffer.

The CAT activity in the cell extracts was determined by the procedure of Gorman et al., *Mol. Cell Biol.* 2:1044-1051 (1982) with the exception that the samples were heated at 60° C. for 12 minutes prior to the addition of substrates. The reaction mixture was incubated for 1.5 hours at 37° C., reaction products were extracted from the mixture with 300 μ L of cold ethyl acetate, were air dried, and were resuspended in 20 μ L of ethyl acetate for spotting on TLC plates (Bakerflex). After TLC resolution of chloramphenicol and its acetylated derivatives with chloroform:methanol (95:5), autoradiograms of the TLC plates were made (60 hr., exposure at 22° C., DuPont Cronex film).

Quantitative results were obtained by scintillation counting of separated spots of chloramphenicol and its acetylated derivatives, and the percent conversion to acetylated products was calculated. CAT activity was determined by comparison with a standard curve of acetylation conversions obtained with purified bacterial CAT. Protein in the cell extracts was determined according to Bradford, M. *Analyt. Biochem.* 72:248-254 (1976). CAT activity was standardized on the basis of units of CAT activity per gram of soluble protein. (1 unit of CAT catalyzes acetylation of 1 ng chloramphenicol per minute at 37° C.).

CAT activity measured was compared in cell cultures of BMS following bombardment with naked microprojectiles and microprojectiles loaded with pCaMV1, CN. As seen from Table 1, increases in CAT activity were consistently observed in the treatments involving the plasmid vector. The induced levels of CAT were typically 20- to 200-fold greater than the CAT levels in the controls. CAT activity in BMS was monitored over a 4-day period following bombardment. Expression of the CAT gene was detectable 24 hours after bombardment and was still high after 96 hours of incubation. CAT activity was detectable after 12 days in two embryogenic maize cell lines (3-86-17, 13-217), but not in BMS cells (Table 2). CAT activity was also measured after 96 hours in BMS cells bombarded with pCaMV CN (no intron).

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Expression in these cells was not greater than background levels in cells treated with uncoated microprojectiles. Consequently, all subsequent trials were performed with the pCaMV1₁CN plasmid.

TABLE 1

Introduction of DNA into BMS Madre Suspension Cells Using High Velocity Microprojectiles MEAN CAT ACTIVITY					
Experiment	N	Treatment	Control	Fold Increase	F Test
1	10	57 (36.1)	01 (18)	20.1	0.00 (0.00)
2	4	18 (19.3)	00 (09)	21.4	0.17 (0.11)
3	3	43 (34.7)	00 (02)	173.5	0.10 (0.06)
4	2	15 (17.1)	00 (02)	85.5	0.10 (0.26)

Treatment and control values represent units of enzymic activity per gram of protein. Figures in parentheses indicate % conversion of chloramphenicol to acetylated derivatives. F Test values indicate the probability that the difference between treatment and control are due only to chance variation.

TABLE 2

Delivery of DNA into Cell Suspensions of Embryogenic (13-217, 3-86-17) and Non-embryogenic (BMS) Cell Lines MEAN CAT ACTIVITY					
Cell Line	N	Treatment	N	Control	Fold Increase
BMS	2	00 (0.25)	1	00 (0.23)	—
13-217	2	02 (0.9)	—	lost	—
3-86-17	2	03 (2.4)	1	00 (0.44)	5.5

The post-bombardment CAT activity after 96 hours of culture in embryogenic cell suspensions clearly indicated that functional DNA was being delivered into the cells, although at lower rates than observed in BMS (Table 3). Expression after 12 days tissue culture in the embryogenic cell lines was as great as at 96 hours.

TABLE 3

Delivery of DNA into Cell Suspensions of Embryogenic (13-217, 3-86-17) and Non-embryogenic (BMS) Cell Lines MEAN CAT ACTIVITY					
Cell Line	N	Treatment	N	Control	Fold Increase
BMS	3	16 (7.7)	1	01 (0.5)	15.4
13-217	2	01 (0.7)	1	00 (0.3)	2.3
3-86-17	2	02 (1.7)	1	00 (0.4)	4.3

EXAMPLE 2

Some samples of BMS cells were subjected to repeated bombardments with plasmid-loaded microparticles. Multiple bombardments of the same cells clearly produced higher levels of CAT activity than did single bombardments (Table 4). Triple bombardment was found to increase CAT activity in a similar manner in embryogenic line 3-86-17.

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TABLE 4

Effect of Multiple Bombardments on BMS Using 1.2 Micron Particles Loaded with pCaMV1 ₁ CN or Non-loaded MEAN CAT ACTIVITY					
Bombardments	N	Treatment	N	Control	Fold Increase
1	4	35 (28.3)	1	00 (0.2)	141.5
2	2	110 (57.0)	1	00 (0.2)	285.0
3	2	135 (70.0)	1	00 (0.2)	350.0

EXAMPLE 3

The effect of microprojectile diameter was also determined by monitoring CAT expression after bombardment with particles of varying diameters. Significant levels of CAT activity were found only in cells bombarded with microparticles having a mean diameter of 1.2 microns; however, some clumping has been observed in particles within successfully cells, so that larger particles or clumps may be used, up to about 20 microns.

TABLE 5

Effect of Microparticle Diameter on DNA Delivery into BMS Cells, Comparing Loaded and Unloaded Particles MEAN CAT ACTIVITY			
Size (microns)	Treatment	Control	Fold Increase
0.6	00 (0.2)	00 (0.2)	1.0
1.2	15 (17.1)	00 (0.2)	85.8
2.4	00 (0.3)	00 (0.2)	2.5

EXAMPLE 4

Transformation of Regenerable Cell Line 3-86-17

An embryonic suspension culture capable of plant regeneration was obtained from the regenerative tissue culture inbred R21. This suspension was then transformed by micro-particle bombardment according to the general method of Example 1. The general techniques described by Klein et al. in *Proc. Nat. Acad. Sci. USA* 85:4305-4309 (June 1988) were employed. The cells were treated approximately one day after subculture. Clumps of suspension culture cells not greater than 710 microns were divided into 100 mg samples for bombardment. Two different vectors were used. One was the pCaMV1₁CN plasmid vector containing the structural gene for chloramphenicol acetyl transferase. The second vector was a plasmid having identical regulatory sequences but containing the structural gene for beta glucuronidase. Each sample was bombarded twice with particles having a diameter of 1.2 microns and a DNA loading of 5 ug/mL.

After the bombardments, each sample was diluted with 5 mL of liquid culture medium containing 2,4-D at a concentration of 2 mg/L. Liquid medium containing the cells was transferred to 15 ml sterile centrifuge tubes where the cells were allowed to settle. Excess culture medium was removed to leave a final volume of 3 mL. Tubes with cells were incubated at 27° C. in light on a shaker at approximately 100 rpm. Cells were sampled for gene expression after 3 days. Both CAT and beta glucuronidase expression were approximately two orders of magnitude greater in the cells treated with loaded microparticles versus non-loaded controls.

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EXAMPLE 5

Leaves of sterile, in vitro propagated *Nicotiana tabacum* Var. KY 17 having a length of 5 to 7 cm and a width of 2 to 3 cm were removed under aseptic conditions and prepared for microprojectile bombardment by cutting them into thin strips 1 cm long x1 mm wide in order to maximize surface area exposed to bombardment. Leaf strips were placed directly onto a culture medium consisting of MS salts and 20 mg/L naphthaleneacetic acid (NAA) and 0.5 mg/L benzylaminopurine (BAP). 200 mg/L kanamycin was added as the selection agent. Microprojectiles were prepared by precipitating 10 ug of plasmid DNA on about 1.25 mg of tungsten particles having an average diameter of 1.2 microns. Precipitation was carried out using 0.89 M CaCl₂ and about 0.014 M spermidine. DNA coated particles were incorporated into cells by two consecutive bombardments in the manner previously described herein. Each bombardment delivered about 89 ug of microprojectiles.

After treatment, the strips were incubated in low light (about 350 lux) for 7 days and then transferred to fresh culture medium. Leaf pieces were transferred weekly regardless whether they showed visible growth or not. The first two subcultures were to culture medium which allowed callusing (2.0 mg/L NAA plus 0.5 mg/L BAP) followed by six successive transfers to shoot regeneration medium, which consisted of the same composition without the NAA.

Strips which were placed on culture medium without kanamycin showed rapid callus proliferation. Shoot regeneration was obvious after 5 weeks in culture. Control leaf strips placed on kanamycin-containing medium showed expansion during the first week of culture and then lost color and died over the next few weeks. Strips which had been bombarded with loaded microparticles providing the gene for kanamycin resistance at first showed symptoms of kanamycin injury. However, after two weeks in culture, very small areas of growth with differing morphology were observed. These first appeared as small protuberances at several different areas of leaf tissue. Some strips which atrophied and died left small surviving pieces of tissue. Visible callus was evident after 8 weeks' culture on medium containing 200 mg kanamycin per liter. Plant regeneration, both in the presence and absence of kanamycin, began to occur after about 10 weeks in culture.

After visible shoot initiation, plantlets were transferred to a shoot elongation/rooting medium consisting of MS salts with 4% sucrose and 50 mg/L kanamycin. About half of the plants from one of the growing calli showed shoot elongation, maintenance of green color and root elongation into the culture medium while the remainder showed poor growth characteristics, loss of greening and failure to root in the medium.

Analysis of the green healthy growing shoots showed both enzyme activity in the leaves and positive incorporation of the foreign gene into the plant cell genome as determined by both slot blot and Southern blot analysis.

The plantlets were grown to maturity and self-pollinated. Upon germination on culture medium containing 50 mg kanamycin per liter, Mendelian inheritance of the gene was established.

EXAMPLE 6

Data on Inbred Corn Line R21

The following example provides data on regeneration of inbred corn line R21, which was used in Example 4. This

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inbred line was obtained by recovering plants capable of seed production from a long-term callus culture (older than 12 months) of inbred B73.

TABLE 6

Tissue Culture Response—Percent Embryos Giving Rise to Type I and Type II Embryogenic Callus from TC2 Plants Regenerated from Inbred B73			
Pedigree	28-Day Response		
Identity	N	% Type I	% Type II
710#1**11	42	0	21.4
	50	2.0	28.0
B73	75	0	0
	170	0	0

**Progenitor of R21.

TABLE 7

Percent Embryos Giving Rise to Type I and Type II Embryogenic Callus after 14 and 28 Days in Culture from Seed Derived B73 and Several Tissue Culture Derived Plants from Inbred B73					
Pedigree	N	14-Day Response		28-Day Response	
		Type I	Type II	Type I	Type II
B73-1C710#1-B73-TC	711	1.0	31.4	1.8	25.7A
694#3-X*					
B73**	279	0.0	.1	0.0	.6D

*Progenitor of R21.

**Plants from seed derived B73.

TABLE 8

Percent Embryos Giving Rise to Type I and Type II Embryogenic Callus after 14 and 28 Days in Culture from Inbred R21 Derived from B73 Callus and from Seed Derived from B73					
Pedigree	N	14-Day Response		28-Day Response	
		Type I	Type II	Type I	Type II
R21*	80	3.8 B	3.8 D	0.0	6.3
B73-TC710#1-B73-TC694#5)ZX					
B73	277	0.0 B	0.0 B	0.0	0.0

*Data shown from same ears used for per se and top cross yield data.

TABLE 9

Plant Regeneration from R21 and B73 Embryogenic Cultures Approximately 28 Days after Culture Initiation		
Genotype	Number Embryos	X Plants/Embryo
R21	14	2.85
B73	2	2.50
B73 Regenerants	1	1.00

What is claimed is:

1. A fertile transgenic *Zea mays* plant comprised of stably incorporated foreign DNA, wherein said foreign DNA consists of DNA that is not from a corn plant and that is not comprised of a T-DNA border.

2. A fertile transgenic *Zea mays* plant according to claim 1, wherein said foreign DNA imparts herbicide resistance to said plant.

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3. A fertile transgenic *Zea mays* plant according to claim 2, wherein said foreign DNA imparts resistance to chloro-sulfuron.

4. A fertile transgenic *Zea mays* plant according to claim 1, wherein said transgenic *Zea mays* plant is made by a method comprising the steps of:

- (a) preparing a foreign DNA comprising an expression vector carrying a gene encoding an agronomic trait;
- (b) preparing a maize embryogenic callus or suspension culture;
- (c) introducing said foreign DNA into said embryogenic callus or suspension culture by one or more microparticle bombardments; and
- (d) regenerating said fertile transgenic *Zea mays* plant.

5. A fertile transgenic *Zea mays* plant according to claim 1, wherein said transgenic *Zea mays* plant is made by a method comprising the steps of:

- (a) preparing a foreign DNA comprising an expression vector carrying a gene encoding an agronomic trait;
- (b) preparing a maize immature embryo;
- (c) introducing said foreign DNA into said immature embryo by one or more microparticle bombardments; and
- (d) regenerating said fertile transgenic *Zea mays* plant.

6. The fertile transgenic *Zea mays* plant according to claim 4, wherein said maize embryogenic callus or suspension culture of step (b) is passed through a sieve prior to step (c).

7. The fertile transgenic *Zea mays* plant according to claim 6, wherein the sieve has an opening size of about 700 microns.

8. The fertile transgenic *Zea mays* plant according to claim 4, wherein prior to step (c), said maize embryogenic callus or suspension culture is plated to a density of from about 2.5 mg (fresh weight)/cm² to about 10 mg (fresh weight)/cm².

9. The fertile transgenic *Zea mays* plant according to claim 8, wherein said plated maize embryogenic callus or suspension culture has a fresh weight of from about 50 mg to about 300 mg.

10. The fertile transgenic *Zea mays* plant according to claim 4, wherein step (b) further comprises providing said maize embryogenic callus or suspension culture on a solid support.

11. The fertile transgenic *Zea mays* plant according to claim 4, wherein step (b) further comprises providing said maize embryogenic callus or suspension culture on a liquid medium.

12. The fertile transgenic *Zea mays* plant according to claim 1, wherein said foreign DNA imparts hygromycin resistance to said plant.

13. A fertile transgenic *Zea mays* plant comprising a foreign DNA that encodes an agronomic trait, wherein the plant is obtainable by:

- introducing foreign DNA into a maize embryogenic callus cell by microprojectile bombardment to obtain a transformed callus cell;
- generating the fertile transgenic *Zea mays* plant from the transformed callus cell.

14. The fertile transgenic *Zea mays* plant according to claim 13, wherein the agronomic trait is resistance to a phytotoxic agent.

15. The fertile transgenic *Zea mays* plant according to claim 14, wherein the phytotoxic agent is an antibiotic.

16. The fertile transgenic *Zea mays* plant according to claim 15, wherein the antibiotic is kanamycin.

17. The fertile transgenic *Zea mays* plant according to claim 15, wherein the antibiotic is hygromycin.

18. The fertile transgenic *Zea mays* plant according to claim 14, wherein the phytotoxic agent is an herbicide.

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19. The fertile transgenic *Zea mays* plant according to claim 13, wherein the foreign DNA is operably linked to an over-producing plant promoter.

20. The fertile transgenic *Zea mays* plant according to claim 13, wherein the foreign DNA is operably linked to a NOS promoter.

21. The fertile transgenic *Zea mays* plant according to claim 13, wherein the foreign DNA is operably linked to an OCS promoter.

22. The fertile transgenic *Zea mays* plant according to claim 13, wherein the foreign DNA is operably linked to a light-inducible promoter.

23. The fertile transgenic *Zea mays* plant according to claim 13, wherein the light-inducible promoter is a promoter for the gene encoding a small sub-unit of ribulose-1,5-bisphosphate carboxylase.

24. The fertile transgenic *Zea mays* plant according to claim 13, wherein the light-inducible promoter is a promoter for the gene encoding a chlorophyll a-b binding protein.

25. The fertile transgenic *Zea mays* plant according to claim 13, wherein the foreign DNA further comprises a polyadenylation region.

26. The fertile transgenic *Zea mays* plant according to claim 13, wherein the maize embryogenic callus cell is in a suspension culture.

27. A fertile transgenic *Zea mays* plant comprising a foreign DNA that encodes an agronomic trait, wherein the plant is obtainable by:

- introducing foreign DNA into a maize immature embryo by microprojectile bombardment to obtain a transformed immature embryo;

generating the fertile transgenic *Zea mays* plant using the transformed immature embryo.

28. The fertile transgenic *Zea mays* plant according to claim 27, wherein the agronomic trait is resistance to a phytotoxic agent.

29. The fertile transgenic *Zea mays* plant according to claim 28, wherein the phytotoxic agent is an antibiotic.

30. The fertile transgenic *Zea mays* plant according to claim 29, wherein the antibiotic is kanamycin.

31. The fertile transgenic *Zea mays* plant according to claim 29, wherein the antibiotic is hygromycin.

32. The fertile transgenic *Zea mays* plant according to claim 28, wherein the phytotoxic agent is an herbicide.

33. The fertile transgenic *Zea mays* plant according to claim 28, wherein the foreign DNA is operably linked to an over-producing plant promoter.

34. The fertile transgenic *Zea mays* plant according to claim 27, wherein the foreign DNA is operably linked to a NOS promoter.

35. The fertile transgenic *Zea mays* plant according to claim 27, wherein the foreign DNA is operably linked to an OCS promoter.

36. The fertile transgenic *Zea mays* plant according to claim 27, wherein the foreign DNA is operably linked to a light-inducible promoter.

37. The fertile transgenic *Zea mays* plant according to claim 36, wherein the light-inducible promoter is a promoter for the gene encoding a small sub-unit of ribulose-1,5-bisphosphate carboxylase.

38. The fertile transgenic *Zea mays* plant according to claim 36, wherein the light-inducible promoter is a promoter for the gene encoding a chlorophyll a-b binding protein.

39. The fertile transgenic *Zea mays* plant according to claim 27, wherein the foreign DNA further comprises a polyadenylation region.

40. The fertile transgenic *Zea mays* plant according to claim 27, wherein the immature embryo comprises a meristem cell.

* * * * *

Civil Cover Sheet

Page 1 of 2

**UNITED STATES DISTRICT COURT
NORTHERN DISTRICT OF ILLINOIS**

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Civil Cover Sheet

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U.S. DISTRICT COURT

This automated JS-44 conforms generally to the manual JS-44 approved by the Judicial Conference of the United States in September 1974. The data is required for the use of the Clerk of Court for the purpose of initiating the civil docket sheet. The information contained herein neither replaces nor supplements the filing and service of pleadings or other papers as required by law. This form is authorized for use only in the Northern District of Illinois.

**Plaintiff(s): PIONEER HI-BRED
INTERNATIONAL, INC.**

**Defendant(s): MONSANTO COMPANY and
DEKALB GENETIS CORPORATION**

County of Residence: Polk County

County of Residence:

Plaintiff's Atty: Leora Ben-Ami
Clifford, Chance, Rogers &
Wells
200 Park Avenue, New York,
NY 10166
(212) 878-8000

Defendant's Atty:

II. Basis of Jurisdiction: 3. Federal Question (U.S. not a party)

**III. Citizenship of Principle
Parties (Diversity Cases Only)**

Plaintiff: - N/A
Defendant: - N/A

IV. Origin : 1. Original Proceeding

V. Nature of Suit: 830 Patent

**VI. Cause of Action: This is an action for patent infringement under the Patent Laws of
the United States, Title 35 U.S.C. sec. 271 et. seq.**

VII. Requested in Complaint

Class Action: No
Dollar Demand:
Jury Demand: Yes

VIII. This case Is NOT a refiling of a previously dismissed case. (If yes case number ___ by Judge ___)

Signature:

Date:

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JUL 10 2001

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**UNITED STATES DISTRICT COURT
NORTHERN DISTRICT OF ILLINOIS**

In the Matter of

Western Division

FILED-WD

Pioneer Hi-Bred International, Inc.,
Plaintiff,

01 JUL 10 PM 12:03

vs.

Case Number:

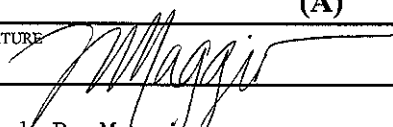
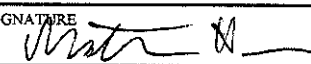
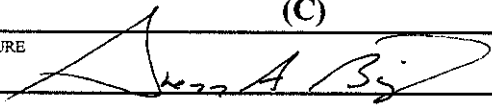
01C50219

Monsanto Company and DeKalb Genetics Corporation,
Defendants.

CLERK
U.S. DISTRICT COURT

APPEARANCES ARE HEREBY FILED BY THE UNDERSIGNED AS ATTORNEY(S) FOR:

Pioneer Hi-Bred International, Inc., Plaintiff

(A)		(B)	
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		DESIGNATED AS LOCAL COUNSEL? YES <input checked="" type="checkbox"/> NO <input type="checkbox"/>	
(C)		(D)	
SIGNATURE 		SIGNATURE	
NAME Gregory A. Biegel		NAME	
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E-MAIL ADDRESS GABIEGEL@AOL.COM		E-MAIL ADDRESS	
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