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January 26, 1998

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PETITION FOR DETERMINATION OF NON-REGULATED STATUS
MALE STERILE CORN LINES 676, 678 AND 680 WITH RESISTANCE TO GLUFOSINATE

Pioneer Hi-Bred International, Inc. is submitting the enclosed revised petition for determination of the regulatory status of male sterile corn lines 676, 678 and 680. An original petition on these lines was submitted on December 4, 1997. During the review for completeness, USDA/APHIS requested a number of clarifications including the title of the document. We were advised to provide these clarifications in a new draft of the petition, in particular because the title on the signature page had changed.

This petition contains data and information that we believe demonstrates there is no longer "reason to believe" that these male sterile corn events should be considered a regulated article under APHIS regulations at 7 CFR Part 340. The male sterile corn lines do not present a plant pest risk and are not a risk to the environment. The enclosed petition does not contain confidential business information.

Sincerely,

Lawrence Zeph

Lawrence Zeph, Ph.D.
Regulatory Affairs Manager

cc: R. Townsend

1/27/98
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PETITION FOR DETERMINATION OF NON-REGULATED STATUS

MALE STERILE CORN LINES 676, 678 AND 680 WITH RESISTANCE TO GLUFOSINATE

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

Submitted by

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January 26, 1998

This petition contains no confidential business information

Summary

Pioneer Hi-Bred International, Inc. (Pioneer) has developed maize lines with controlled fertility that reduces the need to detassel large acreages of maize inbreds during seed production. The strategy is based on the introduction of genes that prevent pollen production in corn. Male sterile corn lines 676, 678, and 680, derived from initial transformation events TC676, TC678, and TC680, are the subject of this petition.

Pioneer identifies its maize male sterility system as the Tissue Specific Sterility (TSS) system. The functional gene in the TSS system is the DNA adenine methylase gene or *dam*. The TSS corn lines containing *dam* are characterized phenotypically by their inability to produce anthers and pollen. This phenotype most likely results from methylation of DNA in anther cells of the corn plant and disruption of normal cell function.

In maize, the male (tassel) and female (ear) reproductive organs occur on the same plant, although physically separated. Plants that contain *dam* are used as female parents during hybrid seed production as they will be male sterile and will not need to be detasseled. In addition, male sterile corn lines 676,678, and 680 are tolerant to the herbicide glufosinate, encoded by the *pat* gene, which facilitates the process of seed production.

The male sterility system developed by Pioneer will be utilized for corn seed production purposes only. Seed production fields are spatially isolated to prevent introgression of genetic material from unwanted sources of maize pollen. When seed produced utilizing the TSS system is planted by growers, 50% of the plants will exhibit the male sterile phenotype because they contain *dam*. However, the male sterile plants cannot produce pollen and therefore cannot transfer *dam* to other corn plants in the vicinity through cross-pollination. These male sterile plants will themselves be readily pollinated through cross-pollination by tassel-bearing plants (one maize plant can produce in excess of six million pollen granules). Extensive experience with cytoplasmic male sterility confirms that normal pollination and seedset will occur in commercial fields.

The donor organism for *dam* is *Escherichia coli*. In *E. coli*, *dam* plays a role in DNA repair processes through its methylation function. Methylation of the adenine moiety in specific GATC sequences determines which strand in the *E. coli* DNA double helix is repaired by cellular repair mechanisms. In TSS corn lines, *dam* is linked to a tissue specific promoter to enable expression in anthers, rather than grain, forage, or other plant tissues. Anther cell destruction (cell ablation) is observed in TSS corn lines. This phenotype is consistent with the known role of DAM methylase in the modification of DNA in *E. coli* through methylation of GATC sequences. Methylation of DNA in TSS corn lines should result in disruption of normal cell function in anther tissue and subsequent cell ablation. DAM methylase activity in male sterile corn lines 676, 678, and 680 is therefore limited by two significant means: first, to specific maize tissues (anthers) and, second, to certain stages in the development of the maize plant (anthesis). Other than the inability to produce anthers and pollen, TSS corn lines exhibit normal plant development and agronomic characteristics. DAM methylase is not considered a microbial toxin. There is no significant plant pest or environmental risk from the use of these transgenic plants in seed production.

Glufosinate tolerance is conferred by the *pat* gene that encodes the protein phosphinothricin acetyltransferase. The *pat* gene and protein in corn have been the subject of a previous determination of non-regulated status by the USDA (USDA, 1995). Male sterile corn lines 676, 678, and 680 expressing the identical PAT protein are therefore not expected to present any significant plant pest or environmental risk.

Certification

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

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PETITION FOR DETERMINATION OF NON-REGULATED STATUS OF MALE STERILE CORN LINES 676, 678, AND 680 WITH RESISTANCE TO GLUFOSINATE

Table of Contents

Tables

Appendix 1: Figures

I. Rationale for the development of Tissue Specific Sterility (TSS) for male sterility.

Pioneer Hi-Bred International, Inc. (Pioneer) has developed maize lines with controlled fertility that reduces the need to detassel large acreages of maize inbreds during seed production. The mechanism controlling fertility is based on the introduction of a gene that prevents pollen production. As this gene is introduced into the genetic material contained in the plant cell nucleus, the resulting phenotype is referred to as nuclear male sterility.

Pioneer identifies its maize male sterility system as the Tissue Specific Sterility (TSS) system. A tissue specific promoter, active only in maize anther cells, is linked to a gene encoding a protein that inhibits pollen development. In maize, the male (tassel) and female (ear) reproductive organs are formed on the same plant, although physically separated. Plants that express the male sterile phenotype will be used as female parents during hybrid seed production and will not need to be detasseled. The male sterile phenotype is closely linked genetically with the *pat* gene conferring glufosinate tolerance, which allows for easier selection of male sterile plants during hybrid seed production.

Three male sterile corn lines, derived from separate transformation events and designated lines 676, 678, and 680, are the subject of this petition.

I.A. Hybrid seed production

The development of maize hybrids begins with the development of homozygous inbred lines that exhibit desirable traits. A maize hybrid is the progeny of two parent inbred maize lines whose genes combine to exhibit heterosis, or "hybrid vigor," which is the enhancement of beneficial characteristics of the parent inbreds. Hybrid vigor can be expressed in the plants in a variety of ways such as increased vegetative growth and increased yield. This has been the basis for continual improvements in the characteristics of maize since the 1920's. Seed obtained from hybrid crops is not suitable for replanting as much of the hybrid vigor is lost in succeeding generations. Instead new hybrid seed must be planted each year.

The production of commercial hybrid seed from two inbreds involves optimizing conditions for cross-pollination between the designated male pollen donor and the female inbred. One row of the male inbred is planted for every four to six rows of the female inbred. The pollen-bearing tassel is removed from the inbred designated as the female (detasseled) before anthesis (pollen shed) so that the female inbred will only be fertilized with pollen from the male inbred. The resulting seed is therefore hybrid and will form hybrid plants.

The detasseling process is a critical step in successful hybrid seed production. Detasseling can be accomplished in several ways. The most common method has been manual removal of the pollen bearing tassels of the female inbreds. Manual detasseling is labor intensive and costly and some self-pollination can occur if plants are not correctly detasseled. However, hybrid maize seed has been produced by this method for decades. Mechanical detasseling, through the use of detasseling machines, is currently the primary means of tassel removal. As with manual detasseling, mechanical detasseling may be incomplete and allow a degree of self-pollination. In present practice, both methods are used to detassel maize hybrid production fields with manual detasseling primarily used as a final "clean-up" step. Both techniques result in loss of leaf tissue, causing some reduction in seed yield. This is an important factor for seed producers because hybrid seed is far more valuable, weight for weight, than the grain the farmer harvests from it.

An alternative approach is the development of "male sterile" plants through the use of naturally occurring cytoplasmic male sterility (CMS). CMS female inbreds are deficient in their ability to produce pollen through the action of genes located in the cytoplasm (mitochondria) of plant cells. In the 1970's one of the most widely used and reliable CMS genotypes ("Texas cytoplasm") was found to be susceptible to a potentially serious fungal disease, *Helminthosporium maydis*. Other CMS systems are still used for seed production, but the CMS trait is sometimes associated with reduced agronomic performance, such as lower yields, in resulting hybrids. The male sterile phenotype may also be unstable in certain environments. Furthermore, not all types of maize germplasm can be converted to CMS. Although CMS genotypes are still widely used in seed production, Pioneer researchers have continued to look for improvements in the reliability and utility of male sterility systems in maize and thus limit the need for detasseling and improving product quality.

The Tissue Specific Sterility (TSS) system developed by Pioneer allows the use of corn lines that do not produce pollen and are phenotypically male sterile. TSS corn lines contain the gene designated *dam* which encodes the DNA adenine methylase (DAM methylase) in *E. coli*. Plants containing *dam* will be utilized as female parents during hybrid seed production, as their inability to produce pollen will ensure cross pollination as shown below in Table 1. These female parents, however, will not need to be detasseled.

Table 1. Production of hybrid seed using TSS corn lines

Female parent (contains *dam*, phenotype is male sterile)	**X**	**Male parent (male fertile)**
⇓		
Hybrid seed		
- 50% of the hybrid seed will contain *dam* and be male sterile		
- 50% will <u>not</u> contain *dam* and remain fertile		

Utilizing the TSS maize lines in hybrid seed production means that 50% of the hybrid seed will express the male sterile phenotype when grown in commercial fields. However, these plants will be readily pollinated through cross-pollination as it is estimated that one maize plant can produce in excess of six million pollen granules. The experience with CMS sterility systems confirms that normal pollination and seedset will occur in commercial fields. Agronomic testing of all lines using the TSS male sterility system will be conducted in order to ensure that hybrids will perform as expected.

Seed increase of TSS female inbreds is accomplished by crossing to fertile inbreds with equivalent genotypes (Table 2). Crossing of TSS inbreds to fertile inbreds results in a 1:1 segregation of the male sterility gene. However, because the male sterility gene is closely linked with a gene for tolerance to glufosinate ammonium herbicide, the application of this herbicide will allow only male sterile plants to grow in the inbred seed increase fields (Songstad et al., 1997). Glufosinate herbicide is registered for use on corn in the US.

Table 2. Inbred production using TSS corn lines

Female parent contains *dam*	**X**	**Male parent**
phenotype: male sterile (Ms); glufosinate tolerant (G)		phenotype: male fertile (ms); glufosinate sensitive (g)
genotype: Ms (*dam*) /ms (normal) G (*pat*)/g		genotype: ms/ms g/g

⇓

50% of plants are male sterile and herbicide tolerant (Ms/ms and G/g)

50% of plants are male fertile and herbicide sensitive (ms/ms and g/g)

⇓

Apply glufosinate to allow growth of only male sterile inbreds

⇓

Harvest inbred seed

I.B. Implications for Pioneer customers of TSS for improved seed quality

The rationale for developing genetically engineered nuclear male sterility is that it will markedly improve the control of the genetic purity of hybrid seed during seed production compared to detasseling. Pioneer's quality control standards require that over 99.7% of the female inbreds in a hybrid seed production field must be detasseled. Because one maize plant can produce in excess of six million pollen granules, even a limited amount of detasseling failure can result in an unacceptable level of self-pollination. The use of the TSS system will allow Pioneer to meet these high quality control standards without detasseling.

I.C. Economic importance of TSS to Pioneer

The use of the TSS system allows high quality seed production without the requirement for detasseling. Cost savings from the introduction of this seed production technology are estimated in the millions of dollars annually. Cost savings are largely due to the decreased labor costs for those seed production fields that utilize the TSS system.

I.D. Regulatory status of TSS corn

Initial consultations have been held with the U.S. Food and Drug Administration (FDA) on transgenic male sterile corn utilizing the TSS system of male sterility. A notification to the FDA willed be filed in 1998. The proteins introduced into TSS male sterile corn lines are not considered plant-pesticides under U.S. Environmental Protection Agency policy (EPA). This has been determined through consultation with the EPA Office of Pesticide Programs.

II. The Corn Family (*Zea mays* L.)

II.A. Corn as a crop

Maize has been cultivated in North America for thousands of years (Agriculture and Agri-Food Canada, 1994). Maize grain has been produced primarily from hybrid-derived varieties since the 1930's and 1940's. Hybrid varieties are superior to open-pollinated varieties in a variety of agronomic characteristics, for example, in grain yield.

The primary use of maize is for animal feed. The yellow dent type of maize is used for feed and this is grown as a commodity crop in North America. Maize is also processed into valuable food and industrial products, such as ethyl alcohol by fermentation, cornmeal by dry milling, and highly refined starch by the wet milling process. The greatest volume is processed by wet milling to produce starch and sweetener products for foods. Non-food products such as industrial starches, corn gluten feed, and corn gluten meal are also manufactured (White and Pollak, 1995). The primary products derived from the dry milling process are corn grits, cornmeals, and corn flours. The largest food/feed product volume of the dry-milling industry is animal feed followed by brewing and food uses.

II.B. Taxonomy of corn

Family name:	Gramineae
Genus:	*Zea*
Species:	*mays* (2n = 20)
Subspecies:	none
Cultivar/line:	"High Type-II" germplasm (Hi-II)

A summary description of the taxonomy of corn can be found in Agriculture and Agri-Food Canada (1994). *Zea mays* is a member of the Gramineae (Poaceae) family, commonly known as the grass family. Maize is a monoecious annual, with overlapping sheaths and broad, conspicuously distichous blades; with numerous staminate spikelets in long spikelike racemes, forming large spreading terminal panicles (tassels). Pistillate (silk) inflorescence is in the axis of the leaves on a thickened, almost woody axis (cob). The silks are composed of numerous large foliaceous bracts or spathes, with long styles protruding from the summit as a mass of silky threads.

II.C. Genetics of corn

Maize is a monoecious species with separate staminate (tassels) and pistillate (silk) flowers which encourages the natural outcrossing between maize plants (Agriculture and Agri-Food Canada, 1994). Repeated cycles of self-pollination lead to homogeneity of the genetic characteristics within a single plant line (inbred). Controlled cross-pollination of inbred lines from chosen genetic pools combines desired genetic traits in a hybrid and results in a yield increase. This inbred-hybrid concept and resulting yield response is the basis of the modern maize seed industry. Maize varieties planted by U.S. farmers are almost entirely hybrids. Open pollination of hybrids in the field leads to the production of grain with the properties of many different lines and is no longer "true-breeding."

II.D. Pollination of corn

Maize is a wind-pollinated plant. The separate tassels and silk flowers encourage the natural outcrossing between maize plants. Typical of wind-pollinated plants, a large amount of redundant maize pollen is produced for each successful fertilization of an ovule on the ear (Kiesselbach, 1949). Wind movements across the maize field cause pollen from the tassel to fall on the silks of the same or adjoining plants. Maize pollen measures about 0.1 mm in diameter and is the largest of any pollen normally disseminated by wind from a comparably low level of elevation. Dispersal of maize pollen is influenced by its comparatively large size and rapid settling rate (Raynor *et al.*, 1972). Pollen survival is highly dependent on relative humidity. Maize pollen remains viable for about 30 min under optimal conditions of temperature and humidity.

II.E. Weediness of corn

Corn does not exhibit any weedy tendencies and is non-invasive in natural environments (Agriculture and Agri-Food Canada, 1994). Corn hybrids have been domesticated for such a long period of time that the seeds cannot be separated from the cob and disseminated without human intervention. Corn

plants are annuals that will not survive in the U.S. from one growing season to the next because of the poor dormancy. Corn seed is non-dormant, but can persist from one growing season to the next under favorable climatic conditions and, when the temperature and moisture are adequate, the seed will germinate. These volunteers are easily identified and controlled through manual or chemical means. Some *Zea* species are successful wild plants in Central America, but they have no pronounced weedy tendencies.

II.F. Modes of gene escape in corn

Z. mays crosses readily with other cultivated maize. However, sexually compatible wild relatives of maize are not found in the U.S. For example, one of the related species of teosinte (*Zea mexicana*) is a wild grass found in Mexico and Guatemala. The genus most closely related to *Zea* is *Tripsicum*, a genus of eleven species, three species of which occur in the U.S. Only one species, *Tripsacum dactyloides* (Eastern Gamagrass) is found in the corn growing regions of the Midwestern U.S.

Crosses can be made between *Z. mays* and *T. dactyloides*, but these require human intervention and even then are very difficult plants (Agriculture and Agri-Food Canada, 1994). Progeny are frequently sterile or genetically unstable. Recent studies have failed to show any evidence of recent introgression between maize and teosinte (Smith *et al.*, 1985). *Z. mays* does not appear to be a hybrid of the wild and cultivated forms of *Zea* as had been previously postulated. Therefore, *Z. mays* probably does not serve as a genetic bridge between wild and cultivated forms (Doebley, 1984).

The male sterile maize inbreds described in this petition will be used in seed production fields only. Seed production fields are isolated from other sources of corn pollen in order to ensure the genetic purity of the hybrid corn seed. For all these reasons, genetic introgression from TSS inbreds into wild relatives is extremely unlikely.

II.G. Characteristics of nontransformed cultivar

The "High Type-II" germplasm (Hi-II), the initial recipient of the added genes, is a cross between A188 and B73 inbred lines of maize. These are publicly available inbred lines developed by the University of Minnesota and Iowa State University, respectively. The Hi-II material was developed to have a higher regeneration potential during the tissue culture stages, but does not have acceptable commercial performance (Armstrong *et al.*, 1991). It is, therefore, necessary to conduct further breeding with transformed Hi-II germplasm to produce varieties for commercial sale.

III. Description of the Transformation System

The TSS maize lines were produced through particle gun transformation processes. Micro-projectile bombardment was used to carry purified insert DNA into plant cells, essentially as described by Klein *et al.*, 1989. The particles are accelerated and penetrate the plant cells where the insert DNA is deposited and incorporated into the cell chromosome. The plant cells were grown on selectable media that supports callus growth. The shoots that develop from the transformed plant cells express the phenotype encoded by the genes on the delivered DNA.

A linear DNA insert derived from plasmid PHP6710 was used to transform the TSS maize lines. Plasmid PHP6710 is based on the *E. coli* pUC18 plasmid (Yannish-Perron *et al.*, 1985). The coding and non-coding sequences in the pUC18 plasmid is well characterized. The sequence for this plasmid backbone is contained in the Genbank accession number 52325. Minor sequence changes have been made to facilitate cloning. The components of plasmid PHP6710 are described in Table 3. A map of plasmid PHP6710 is shown in Figure 1 in Appendix 1.

The plasmid PHP6710 was digested with the restriction enzyme *Rca*I, which cuts the DNA outside of the *bla* gene that confers resistance to ampicillin. A 4.5 kb fragment (see plasmid map in Figure 1), containing the coding regions for male sterility and glufosinate tolerance (5126del::*dam* and 35S::*pat*, respectively) was isolated and used for maize transformation. The linear insert is described in Figure 2 (see Appendix 1). The absence of *bla* gene in these DNA fragment preparations was confirmed by Southern analysis of the purified fragment as well as Southern analysis performed to characterize the inserted DNA in each of the male sterile corn lines as described in Section V.A.III.

Table 3. Genetic elements of the vector PHP 6710

Genetic element	Size (kb)	Function
5126del	0.42	A modified *Z. mays* anther specific promoter (unpublished data; promoter isolated by Pioneer Hi-Bred International, Inc.)..
dam	0.95	*E. coli* gene encoding DNA adenine methylase or DAM methylase (Brooks et al., 1983). This enzyme methylates *E. coli* DNA within the sequence GATC.
pinII	0.27	Terminator sequence from *Solanum tuberosum* proteinase inhibitor II (An et al., 1989).
CaMV35S	0.55	Cauliflower Mosaic Virus (CaMV) 35S promoter (Odell *et al.*, 1985).
pat	0.53	The synthetic glufosinate resistance gene (Eckes et al., 1989). The pat gene acetylates phosphinothricin or its precursor demethylphosphinothricin conferring tolerance to a chemically synthesized phosphinothricin, glufosinate-ammonium herbicide.
CaMV35S terminator	0.21	Cauliflower Mosaic Virus (CaMV) 35S transcription terminator (Odell *et al.*, 1985).
bla	0.86	Ampicillin resistance gene of pUC18 expresses a β-lactamase only in bacteria. (Yanisch-Perron et al., 1985)

IV. The Donor Genes and Regulatory Sequences

IV.A. The DNA adenine methylase gene.

The male sterility system is based on a bacterial gene designated *dam* that codes for DNA adenine methylase (DAM methylase) enzyme. The donor organism for *dam* is *Escherichia coli* (Brooks et al., 1983). The gene plays a role in microbial DNA mismatch and loop repair processes through its methylation function (Carraway and Marinus, 1993). Methylation of the adenine moiety in specific GATC sequences determines which strand in the DNA double helix is repaired by cellular repair mechanisms.

DAM methylase is classified as a methyltransferase enzyme (E.C.# 2.1.1.72; International Union of Biochemistry and Molecular Biology, 1992). Methylases are a class of enzymes found in both procaryotic and eucaryotic organisms, including plants and animals. The adenine and cytosine residues of DNA can be methylated in procaryotes and eucaryotes in a species-specific manner (Voet and Voet, 1995). The methyl groups on the DNA strand can interact with DNA-binding proteins because they project into the major groove of the DNA double helix. Only a few percent of adenine and cytosine residues are methylated in most organisms, however, in certain plants over 30% of the cytosine residues are modified. Although the primary function of DNA methylation in procaryotes appears to be DNA mismatch repair, for plants the evidence suggests that methylation plays a role in gene regulation and plant development (Richards, 1997).

The activity of DAM methylase in *E. coli* has been recently described (Carraway and Marinus, 1993; Bergerat and Guschlbauer, 1990). DAM methylase catalyzes the following methylation reaction with the adenine moiety of DNA.

S-ADENOSYL-L-METHIONINE + DNA ADENINE
= S-ADENOSYL-L-HOMOCYSTEINE + DNA 6-METHYLAMINOPURINE

Thus, expression of the DAM methylase in *E. coli* results in the transfer of one methyl group to a GATC sequence. Methylation of this sequence signals the cell to initiate a number of possible cellular processes including mismatch repair, DNA replication, transcription of certain genes, among other processes. The *E. coli* DAM methylase has a molecular weight of 32 kilodaltons.

In the Pioneer TSS male sterility system *dam* is under the control of the maize promoter 5126del, which is an anther-specific promoter, and the *Solanum tuberosum* proteinase inhibitor II transcription terminator (pinII). The male sterile phenotype of the TSS corn lines is consistent with the expression of DAM methylase in the anther tissue, resulting in methylation of cellular DNA, disruption of normal cell functions, and finally cell destruction (termed "cell ablation"). The observed phenotype is incomplete development of the pollen grains and male sterility.

A comparison of pollen development in normal corn lines and male sterile TSS lines is illustrated in Figures 3 and 4. Figure 3 shows a light micrograph of a cross section of anther tissue from a normal corn line. The dark staining, circular cell layer shows healthy tapetum cells from which pollen spores are generated. The stain accumulates in the tapetum cell nuclei. Immature pollen cells are shown

developing inside the tapetal cell layer. The immature pollen cells often appear paired as they are undergoing meiosis. Figure 4 shows the complete inhibition of pollen cell development in TSS corn lines. This cross section of anther tissue shows that the destruction of the tapetal cell layer. Large vacuoles form in the tapetal cells. The immature pollen grains also develop vacuoles and lose their normal round shape.

The 5126 promoter was isolated from corn tassel tissue. It was determined that the gene corresponding to the 5126 cDNA was low or single copy, mapping on chromosome 7S near a gene for male sterility designated *ms7*. Therefore, the 5126 gene most likely has a role in maize fertility. Northern analysis demonstrated that the mRNA transcript from the 5126 gene was active specifically in anthers at the quartet to early uninucleate microspore stage of pollen development. Deletions were made from the 5' end of 5126 promoter to an *Nde*I site approximately 415 base pairs from the putative start of transcription. When this promoter, designated 5126del, was used to express a luciferase gene the resulting levels of enzyme expression were higher based on assays of luciferase activity. Therefore, the 5126del promoter was utilized in generating male sterile corn events TC676, TC678, and TC680.

In summary, the phenotypic evidence suggests that DAM methylase production is limited to maize anther tissue by use of a tissue specific promoter that functions only in anther cells that either support pollen development or that become pollen. Neither *dam* nor the DAM methylase have known plant pest characteristics. A variety of endogenous DNA methylating enzymes are normally expressed in maize plants and are widely found in other plants. Therefore, DNA methylating enzymes are a naturally occurring component of all plants, including the maize plant. Moreover, the *pinII* terminator sequence from potato does not impart plant pest characteristics to corn plants.

IV.B. The selectable marker gene: phosphinothricin acetyltransferase

The phosphinothricin acetyltransferase gene (*pat*) was isolated from the bacterium *Streptomyces viridochromogenes*. The PAT protein acetylates phosphinothricin, or its precursor demethylphosphinothrycin, conferring tolerance to a chemically synthesized phosphinothricin, the herbicide glufosinate-ammonium.

The *pat* gene has been the subject of a previous petition for a determination of non-regulated status for genetically modified maize (Van Wert, 1994) and was granted non-regulated status in 1995 (USDA, 1995). It is a synthetic version of the native bacterial *pat* gene. The synthetic version was produced in order to modify the guanine and cytosine codon content to a level more typical for plant DNA, thus improving expression of the PAT protein in maize plants. The amino acid sequence of the PAT protein is unchanged by this modification in codon preference (Van Wert, 1994). The native and synthetic *pat* genes are 70% homologous in DNA sequence.

In the TSS maize lines the *pat* gene is expressed from the CaMV 35S promoter derived from the cauliflower mosaic virus. The 35S promoter is widely used as a promoter in transgenic plants because of the relatively high expression of genes driven by this promoter (Van Wert, 1994). In addition, CaMV terminator sequences are used to control expression of the *pat* gene in TSS lines. The CaMV sequences do not impart plant pest characteristics to corn plants.

The *pat* and *dam* genes are closely linked in the TSS male sterile corn lines 676, 678, and 680. Expression of the PAT protein therefore allows selection of those plants that will express the male sterile phenotype. During seed production, glufosinate ammonium herbicide will be used to eliminate those plants that are male fertile, due to Mendelian segregation of the closely linked *dam* and *pat* genes.

V. Genetic Analysis and Agronomic Performance

V.A. Southern gel analysis of the DNA insert

V.A.I. Number of DNA inserts

Analysis of the inserted DNA was conducted for male sterile corn events TC676, TC678, and TC680. Genomic DNA was isolated from corn leaf tissue. An aliquot of 5 µg of genomic DNA was separately digested with *Spe*I and *Eco*RI restriction enzymes, electrophoresed on 0.8% agarose gels, transferred to nylon membranes, and hybridized with random primed ^{32}P labeled DNA probes consisting of:

- ◊ A 635 bp *Bam*HI DNA restriction fragment containing *dam*.
- ◊ A 315 bp *Bam*HI DNA restriction fragment containing *pat*.
- ◊ A 1008 bp *Rca*I DNA restriction fragment containing *bla*.

The same filter was consecutively hybridized with the *dam*, *pat*, and *bla* probes.

The *Spe*I restriction enzyme site is located in the pinII terminator for *dam*. Digestion of DNA from male sterile corn events TC676, TC678, and TC680 with *Spe*I allows analysis of the insert copy number for *dam* and *pat* (See Fig. 1 for a map of restriction enzyme sites). *Eco*RI digestion will release the intact 3.1 kb fragment containing both *dam* and *pat* genes, which identifies those inserts that contain both *dam* and *pat*. Double digestion with *Eco*RI and *Spe*I will release *dam* and *pat* separately, which allows a determination of whether full or partial copies of these genes are present. A summary of the results is shown in Table 4.

Table 4. Summary of the number of DNA inserts in male sterile corn events TC676, TC678, and TC680

	TC676	TC678	TC680	Nonmodified Corn Line
dam	1	3	4	0
pat	2	2	1	0
bla	0	0	0	0

Table 5. Hybridizing fragments observed in *Spe*I digestion for insert number analysis of male sterile corn events TC676, TC678, and TC680.

Gene probe and Event	Expected Fragment (kb)	Observed Fragment
dam - 676	>2.6	1 band, >6.5 kb
pat - 676	>2.1	2 bands, >4.4 kb
dam - 678	>2.6	3 bands, >6.5 kb
pat - 678	>2.1	1 band, <1.0 kb; 1 band of ~ 2kb
dam - 680	>2.6	4 bands, >2 kb
pat - 680	>2.1	1 band, > 6.5 kb

Detailed analysis of male sterile corn event TC676

The number of *dam* and *pat* DNA inserts in male sterile corn event TC676 is summarized in Table 5. *Spe*I digestion of DNA from male sterile corn line 676 indicates the presence of one *dam* insert (Figure 5) and two *pat* inserts (Figure 6).

One of the *pat* inserts co-migrates with *dam*, indicating that one of the inserts is comprised of both a *pat* and a *dam* gene. This is confirmed by the results of *Eco*RI digestion of event TC676 DNA as a band of the expected 3.1 kb size was observed when the DNA was probed for either *dam* (Figure 7) or *pat* (Figure 8). A second *pat* band was observed that corresponds to the second *pat* insert. The *Eco*RI and *Spe*I double digest demonstrates that a full copy of *dam* is present as a band of the expected 1.6 kb size is seen (Figure 9).

DNA from the control inbred line exhibited no hybridization when probed with the *pat* and *dam* probes (Table 4).

Detailed analysis of male sterile corn event TC678

The number of *dam* and *pat* DNA inserts in male sterile corn event TC678 is summarized in Table 5. The results indicate three *dam* inserts and two *pat* inserts based on *Spe*I digestion. Three bands of >6.5 kb were observed with the probe for *dam* (Figure 5) and two smaller bands were observed with the probe for *pat* (Figure 6).

*Eco*RI digestion confirms that one of the inserts is comprised of both a *pat* and a *dam* gene. This is based on the observation that one band, corresponding to the 3.1 kb *Eco*RI fragment containing *dam* and *pat*, is observed when the *Eco*RI-digested DNA was probed for either *dam* or *pat* (Figures 7 and 8). The other

pat insert appears to be a partial copy as it observed on a <1.0 kb *Eco*RI/*Spe*I fragment (Figure 10). The results of *Eco*RI/*Spe*I digestion also show that there is at least one full copy of *dam* present in event 678 (Figure 9). However one band, larger that the expected 1.6 kb size, is also observed. This data is consistent with a conclusion that a rearrangement has occurred at the 3' end of one of the *dam* inserts resulting in the loss of the *Spe*I restriction enzyme site internal to the PinII promoter. The presence of a *Spe*I site in the maize chromosomal DNA would then explain the large (>6.5 kb) *Eco*RI/*Spe*I fragment containing a *dam* insert.

Detailed analysis of male sterile corn event TC680

The number of *dam* and *pat* DNA inserts in male sterile corn event TC680 is summarized in Table 5. Based on the results of *Spe*I digestion, male sterile corn line 680 contains four *dam* inserts and a single *pat* insert (Figures 5 and 6, respectively).

*Eco*RI digestion results are consistent with the conclusion that one of the inserts is comprised of both a *pat* and a *dam* gene. This is based on the observation that one band of the same size is present when the *Eco*RI digested DNA was probed for either *dam* or *pat* (Figures 7 and 8, respectively). However, this band is slightly larger than the 3.1 kb expected size for an *Eco*RI fragment containing *dam* and *pat*, suggesting that one of the *Eco*RI restriction sites has been removed from the linear DNA insert during transformation. This can occur through the activity of endogenous maize nucleases that remove nucleotides from the free ends of a linear DNA strand. *Eco*RI/*Spe*I digestion resulted in a *dam* fragment of the expected size of 1.6 kb (Figure 9), and a *pat* fragment of slightly larger than the expected size of 1.5 kb (Figure 10), suggesting that the *Eco*RI site in the CaMV terminator at the 3' end of the *pat* gene has been modified through nuclease activity.

The other three *dam* inserts appear to contain partial copies of *dam*. Although *Spe*I digestion indicates four *dam* inserts (Figure 5), only the *pat/dam* insert appears to contain an intact *Eco*RI site internal to the 5126 promoter for *dam* (Figure 7). Results of *Eco*RI/*Spe*I digestion are consistent with one *pat/dam* insert and a second *dam* insert with an intact *Spe*I site in the PinII terminator (Figure 9). These results suggest that one intact *dam* and one intact *pat* gene are present in male sterile corn event TC680. This is also supported by evidence that the Mendelian inheritance of these genes is as expected (Section V.B).

V.A.II. Confirmation of the absence of the *bla* Gene

No DNA sequences homologous with the *bla* gene for resistance to ampicillin were detected in DNA from lines 676, 678, and 680 (Figure 11). This is expected as the linear insert DNA used in the transformation of events TC676, TC678, and TC680 contained no promoter or coding sequences for the *E. coli bla* gene.

DNA from the control inbred line exhibited no hybridization when probed with the *bla* gene probe (Table 4).

V.B. Mendelian inheritance

The Mendelian segregation of male sterile corn lines 676, 678, and 680 has been monitored for three generations of backcross breeding (Table 6). Plants were sprayed with the recommended rate of glufosinate ammonium to distinguish those plants that inherited the closely linked *dam* and *pat* genes from those that did not. The 5125del::*dam* and 35S::*pat* gene construct shows no evidence of instability and Mendelian inheritance is normal.

Table 6. Segregation data and analysis of progeny of male sterile maize lines 676, 678, and 680.

Line 676:

Generation[1]	Actual[2]	Expected	ChiSq
BC1	11:12	11.5:11.5	0.043*
BC2	18:13	15.5:15.5	0.610*
BC3	18:13	15.5:15.5	0.610*

Line 678:

Generation	Actual	Expected	ChiSq
BC1	90:106	98:98	1.306*
BC2	20:9	14.5:14.5	4.172
BC3	14:16	15:15	0.133*

Line 680:

Generation	Actual	Expected	ChiSq
BC1	53:41	47:47	1.532*
BC2	18:17	17.5:17.5	0.029*
BC3	12:16	14:14	0.571*

1 Backcross generation

2 Data expressed as number of susceptible plants: number of sterile-resistant plants based on tolerance to glufosinate and plant phenotype observed at flowering.

* Not significant at p= 0.05 (chi square = 3.84, 1 df)

Southern analysis provides further confirmation of the genetic stability of a transformation. DNA hybridization studies were conducted on different backcross generations of male sterile corn lines 676, 678, and 680 in order to show that *dam* and *pat* are inherited in a stable manner. EcoRI digestion of DNA was performed in order to demonstrate that an insert containing both *dam* and *pat* was present in early and late backcross generations. The results are shown in Figure 7 for *dam* and Figure 8 for *pat*. No evidence of genetic instability was observed in any of the three male sterile corn lines.

V.C. Expression of Inserted Genes

V.C.I. Expression of DAM methylase

V.C.I.A. Phenotypic evidence of DAM methylase expression

Regulation of DAM methylase expression is controlled by the 5126del promoter isolated from corn. The 5126del promoter is believed to be normally active only during pollen development and only in anther tissue where pollen develops. DAM methylase has not been detected to date in anther tissue of TSS corn lines by standard ELISA techniques. The disruption of normal cell function in anther tissue of TSS corn lines, and the resulting cell ablation, prevents immunological detection of plant cell proteins.

Expression of *dam* is most readily demonstrated phenotypically by quantifying the number of observed male sterile plants versus the number of expected sterile plants. Data was collected on the fertility phenotype of 23 inbreds containing event TC676 over several generations of the breeding process (backcross generations). Between two to five backcross generations were analyzed per inbred. Fertile plants can be readily detected based on normal tassel development as opposed to sterile plants that develop tassels but not anthers. Any plant found to be both male fertile and resistant to glufosinate (indicating the presence of the *pat* gene) was considered an "offtype" that resulted from inheritance of *pat*, but not *dam*. The compilation of all data shows that the male sterile trait is observed as expected in over 99.7% of the plants (2289 observed male sterile plants out of 2295 expected male sterile plants). This result demonstrates that *dam* is inherited in a stable manner and results in a male sterile phenotype.

V.C.I.B. Molecular evidence of DAM methylase expression

Molecular evidence of DAM methylase expression in anther tissue was obtained by northern analysis of messenger RNA extracted from leaf tissue and developing anther tissues. Separate experiments were conducted on two different sets of transgenic corn lines in order to provide confirmation of the phenotypic evidence of male sterility observed in the field. First, male sterile corn lines 676, 678, and 680 were analyzed by northern analysis for the presence of *dam*-specific mRNA in anther tissue. However, it was not known whether this mRNA could be detected in tissues from lines 676, 678, and 680 because the anther cell ablation observed in TSS corn lines may prevent detection of any cellular mRNA. A second line of evidence is available from experiments performed with tissues from corn lines FS5, FS6, and FS7 that contain a frameshift mutation in 5126del::*dam*. The frameshift mutation should allow the detection of mRNA expressed from *dam* because the resulting DAM methylase protein is nonfunctional and therefore will not result in cell ablation in developing anther tissue.

Leaf and tassel tissue were utilized in these experiments. Probes were designed to detect mRNA from the *dam* and *pat* genes, along with two controls: the maize actin gene and the native maize 5126 gene

that is believed to function in maize male fertility in conventional corn hybrids. The actin probe was used to control for sample variability and RNA integrity in both the leaf and tassel tissue assays. Tassel tissue was sampled at various stages in the process of pollen cell development as indicated in the figure legend. A schematic diagram of the stages involved in the normal development of maize pollen is shown in Figure 12. During this process the sporogenous cell proceeds through meiosis, followed by mitosis, and maturation of the pollen cell.

Total RNA was prepared from young leaf tissue or developing tassels following a modification of the guanidinium thiocyanate method in Chomczynski and Sacchi (1987). PolyA+ RNA was selected from 600 μg of total RNA using oligo-dT chromotography. PolyA+ mRNA was separated by gel electrophoresis, transferred to a nylon membrane, and hybridized sequentially with the following probes:

dam:	A 635 bp *Bam*HI DNA restriction fragment internal to the coding region of *dam* from *E. coli*.
pat:	A 315 bp *Bam*HI DNA restriction fragment containing *pat* from *Streptomyces viridochromogenes*.
Actin:	A 900 bp *Eco*RI/XhoI DNA restriction fragment containing a nearly full-length maize actin cDNA.
5126:	A 570 bp *Alw*NI DNA restriction fragment from the endogenous, anther-specific maize 5126 gene. This probe was used in northern analysis of tassel tissue only.

Northern analysis of DAM methylase mRNA in male sterile corn lines 676, 678, and 680

Northern analysis of mRNA transcripts from *dam* shows no detectable *dam*-specific mRNA in tassel tissue from male sterile corn lines 676 and 680 as expected (Figure 13, Panel 1-B). It is thought that the expression of the DAM methylase in TSS corn lines inhibits normal anther cell function and thus naturally limits gene transcription, including transcription of *dam*, in anther cells. However, tissue from the tetrad release stage of male sterile corn line 678 contained detectable *dam*-specific mRNA (Figure 13, Panel 1-B). In addition, mRNA from the endogenous maize 5126 gene was detected in the same sample, albeit at lower levels compared to wild type (Figure 13, Panel 1-D). Detection of mRNA from *dam* and the endogenous 5126 gene in line 678 was possible most likely because the tissue was sampled at a point after initiation of *dam* expression, but before the occurrence of cell ablation.

DAM methylase is not expected to be present in other tissues of the corn plant because of the tissue specificity of the 5126del promoter used with *dam*. The results demonstrated that no *dam*-specific mRNA was detected in leaf tissue from male sterile corn lines 676, 678 and 680 (Figure 13, Panel 2).

Northern analysis of DAM mRNA in corn lines FS5, FS6, FS7 containing a frameshift mutation in *dam*

To further investigate the expression of DAM methylase from the tissue-specific promoter, experiments were conducted utilizing a version of *dam* that contained a frameshift mutation. The three corn lines containing the frameshift mutation, designated lines FS5, FS6, and FS7, were generated by adding a two base pair insertion at the *Bam*HI site of *dam* (see Figure 1 for restriction enzyme sites). The frameshift mutation allows expression of a truncated DAM methylase enzyme that is no longer functional. Corn plants containing the frameshift mutation exhibit normal anther development and are fertile. Northern analysis of these plants should detect mRNA from *dam* in tissue from tassels at similar stages of development (see Figure 12 for stages of pollen development in corn).

Northern analysis of mRNA levels in tassel tissue from FS5, FS6, and FS7 showed that *dam*-specific mRNA transcript could be detected in three of the four tissue samples from the tetrad release and early vacuolate stage of pollen development (Figure 13, Panel 1-B, Lanes 13, 15, and 18). Tissue from other stages of pollen development, such as dyad, midvacuolate, and meiosis, did not contain *dam*-specific mRNA transcript. Similarly, hybridization of the maize 5126 probe with the endogenous 5126 mRNA transcript was detected in all fertile tassels from FS5, FS6, and FS7. Endogenous 5126 mRNA was detected in the nontransgenic control inbred A, however, as expected, no mRNA transcript was detected that hybridized with the *dam*-specific probe. It was not expected that mRNA transcript from *dam* would be detected in tassel tissue due to the activity of DAM methylase in the prevention of normal anther development. However, *dam*-specific transcript was detected in one sample of tetrad release tissue from male sterile corn line 678. Apparently this tissue was sampled when cell ablation had not occurred to such an extent that *dam*-specific transcript could not be detected.

In conclusion, these results are consistent with the phenotypic evidence that 5126del::*dam* functions in maize in a tissue specific manner during the tetrad release/early vacuolate stages of pollen development, and preventing the production of pollen in male sterile corn lines 676, 678, and 680.

V.C.II. Expression of PAT protein

In order to readily identify and select TSS male sterile plants during breeding and seed production, the DNA insert used to transform male sterile corn lines 676, 678, and 680 contained the *pat* gene closely linked to *dam*. The *pat* gene, driven by the CaMV35S constitutive promoter, confers tolerance to the herbicide glufosinate ammonium. As a result, all TSS male sterile plants are also resistant to glufosinate. Conversely, whenever a plant is male fertile due to genetic segregation of the linked *dam* and *pat* genes, it is susceptible to glufosinate application. Eliminating male fertile corn plants during hybrid seed production can therefore be accomplished by applying glufosinate to the seed production fields (Songstad et al., 1997).

PAT protein expression in leaf tissue of male sterile corn lines 676, 678, and 680 was analyzed during the 1997 growing season. Three field sites were planted in the Midwestern corn belt at Johnston, Iowa, Algona, Iowa, and Princeton, Illinois. Hybrid seed was planted in three replications at each site. Leaf samples were analyzed by ELISA methods.

The expression of the PAT protein in leaf tissues of male sterile corn lines 676, 678, and 680 is shown in Table 7. Levels in male sterile corn lines 676 and 678 were 601 to 717 μg/g total protein and 204 to 278 μg/g total protein, respectively. The lower expression levels in male sterile corn line 678 may reflect the presence of only one complete copy of *pat*, compared to two copies on the DNA inserts observed in male sterile corn line 676. The PAT levels in lines 676 and 678 are comparable to those reported in other studies on PAT expression from a constitutive promoter. PAT proteins levels in corn leaf tissue reported by Flick (1995) were 1000 to 2800 μg/g total protein. In this study, the *bar* gene from *Streptomyces hygroscopicus* was utilized to express PAT. The structural and functional equivalence of the PAT protein from the *pat* and *bar* genes has been established (Wehrman et al., 1996).

PAT expression in male sterile corn line 680 was below the limit of detection of the ELISA assay (<20 μg/g total protein). However, this amount of PAT protein was sufficient to confer tolerance to the application of glufosinate ammonium herbicide. Similar results have been observed in other corn lines transformed with the *pat* gene as a selectable marker (data not shown). Data from northern analyses of PAT mRNA levels in leaf tissue support the conclusion that male sterile corn line 680 expresses relatively low levels of PAT protein (Figure 13, Panel 2). Levels of mRNA transcribed from *pat* were below detection in male sterile corn line 680 as no bands are observed on the northern analysis. However, mRNA from *pat* was evident as expected for male sterile corn lines 676 and 678. The same results were observed in tassel tissue (Figure 13, Panel 1).

Table 7. Expression of PAT protein in leaf tissue of male sterile corn lines 676, 678, 680.

MALE STERILE CORN LINE	PAT CONCENTRATION µg/g total protein	LITERATURE VALUE
676	601 to 717	1000 to 2800 (Flick, 1995)
678	204 to 278	1000 to 2800 (Flick, 1995)
680	< 20	1000 to 2800 (Flick, 1995)

V.D. Disease and Pest Resistance Characteristics

Male sterile corn lines have been tested in field trials in the US since November 1995 in Hawaii and the Midwestern U.S (See Appendix 2). Field tests conducted in Iowa (planted in May 1996) and other states in the Midwestern U.S. (planted in May 1997) examined the disease and pest characteristics of corn lines 676, 678, and 680. The breeder visually inspects corn plants for evidence of disease or insect pest damage in both transgenic and non-transgenic lines. There were no differences reported in severity of disease symptoms or insect damage between the plants from male sterile corn lines 676, 678, and 680 and non-transgenic corn lines.

VI. Environmental Consequences of Introduction of the Transformed Cultivar

VI.A. The TSS male sterility system in corn.

The male sterility system developed by Pioneer will be utilized for corn seed production purposes only. Therefore, exposure of the environment to male sterile corn expressing DAM methylase is limited in a number of ways. First, exposure is limited geographically during seed production by the relatively limited number of acres planted for seed production purposes. Not all of these seed production acres will be planted to TSS male sterile corn lines, as other types of sterility systems will still be used with certain maize hybrids. Second, the seed production areas are physically isolated in order to maintain Pioneer's standards of seed purity. Seed production fields are isolated from other sources of maize pollen to limit the ingress of wind borne pollen from nearby corn fields. Finally, exposure is limited biologically because normal anther development does not occur in TSS corn lines. Thus, the mature corn plant should not contain tissues which are capable of expressing *dam*.

The agronomic characteristics of male sterile corn lines 676, 678 and 680 are comparable to other corn hybrids.

VI.B. Weediness of male sterile corn lines 676, 678, and 680

Weediness traits have been generally described by Baker (1974) as (1) the ability to germinate in many different environments; (2) discontinuous germination and great longevity of seed; (3) rapid growth through vegetative phase to flowering; (4) continuous seed production for as long as growing conditions permit; (5) self-compatibility but partially autogamous and apometic; (6) ability to be cross-pollinated by unspecialized visitors or wind-pollinated; (7) high seed output in favorable environments and some seed production in a wide range of environments; (8) adaptation for short and long-distance dispersal; (9) vegetative production or regeneration from fragments and brittleness (hard to remove from the ground); and (10) ability to compete interspecifically by special means.

Corn does not exhibit any significant weedy tendencies and is non-invasive in natural environments (AAFC, 1994). Corn hybrids have been domesticated for such a long period of time that the seeds cannot be disseminated without human intervention, nor can corn readily survive in the U.S. from one growing season to the next because of the poor dormancy. Volunteer corn plants are, in any case, easily identified and controlled through manual or chemical means.

The introduction of the trait for male sterility does not confer a weediness trait to corn as a reduction in fertility would not increase the fitness capabilities of these corn varieties. Similarly, the trait for resistance to glufosinate-ammonium herbicides has been used widely in plant breeding as a selective marker. The addition of this phenotypic trait to the male sterile varieties described in this petition is not expected to increase fitness. A fitness advantage would only occur in managed agricultural environments where glufosinate ammonium herbicide is applied to corn plants. As noted above, the survival of volunteer corn plants in the agricultural fields is easily controlled by manual or chemical means.

VI.C. Vertical transfer of the new genes

Non-cultivated *Zea mays* species are not found in the United States. The genus most closely related to *Zea* is *Tripsicum*, a genus of eleven species, three species of which occur in the U.S. Crosses can be made between *Z. mays* and *T. dactyloides*, but these require human intervention and progeny are frequently sterile or genetically unstable. Therefore, cross-pollination between *Z. mays* and *T. dactyloides* in the natural environment is not expected to occur.

The male sterility system developed by Pioneer will be utilized for corn seed production purposes only. Seed production fields are located in isolation to prevent introgression of genetic material from unwanted sources of maize pollen. When seed produced utilizing the TSS system is planted by growers, 50% of the plants will exhibit the male sterile phenotype because they contain *dam*. However as the male sterile plants cannot produce pollen, there will be no transfer of *dam* to other corn plants in the vicinity through cross-pollination. These male sterile plants will themselves be readily pollinated through cross-pollination by tassel-bearing plants (one maize plant can produce in excess of six million pollen granules). Extensive experience with cytoplasmic male sterility confirms that normal pollination and seedset will occur in commercial fields.

VI.D. Horizontal transfer of the new genes

There is no known mechanism for, or definitive demonstration of, DNA transfer from plants to microbes (Nap *et al.*, 1992; FDA, 1994; Redenbaugh *et al.*, 1994). Even if such a transfer were to take place, transfer of *pat* or *dam* from corn lines 676, 678, or 680 would not present a plant pest risk. Genes encoding the PAT enzyme and similar acetyl transferases are found in nature. Similarly, *dam* was isolated from the microorganism *E. coli* and a variety of DNA methylating enzymes are found in organisms. The *bla* gene coding for resistance to the antibiotic ampicillin was demonstrated to not be present in male sterile corn lines 676, 678, and 680.

VII. Adverse Consequences of New Cultivar Introduction

The male sterility system developed by Pioneer will be utilized for corn seed production purposes only. As discussed above, exposure of the environment to the DAM methylase produced in the male sterile corn varieties is limited geographically, physically, temporally, and biologically. The tissue specific promoter for *dam* ensures that expression occurs only in anthers, not in grain, forage, or other plant tissues. There is no reason to expect that DAM methylase is toxic to humans or animals. DNA sequences homologous to *dam* have been detected in a variety of bacterial species, including *Enterobacteriaceae*, *Agrobacterium*, *Xanthomonas*, *Pseudomonas*, *Rhizobium*, and *Haemophilus* species (Brooks et al., 1983).

Methylase enzymes capable of methylating DNA are found in both procaryotes and eucaryotes, including those produced naturally in plants as part of the plant cell's normal metabolism. Therefore, methylase enzymes have been widely consumed by humans and animals in foods and feeds. Given the low concentration of DAM methylase expressed in corn lines 676, 678, and 680, and the history of dietary consumption of this protein, there is no significant human health environmental risk from the expression of DAM methylase in these male sterile lines.

The *pat* gene and PAT protein in corn have been the subject of a previous determination of non-regulated status by the USDA (USDA, 1995). The subject corn events T14 and T25, developed by AgrEvo USA, contain the same synthetic version of the *pat* gene as contained in male sterile corn lines 676, 678, and 680. USDA determined that events T14 and T25: (1) exhibit no plant pathogenic properties; (2) are no more likely to become weeds than other corn developed by traditional breeding techniques; (3) are unlikely to increase the weediness potential for any other cultivated or wild species with which they can interbreed; (4) will not harm other organisms, such as bees, that are beneficial to agriculture; and (5) should not cause damage to processed agricultural commodities. Similarly, male sterile corn lines 676, 678, and 680 expressing the identical PAT protein are not expected to present any significant plant pest or environmental risk. The PAT protein has been shown to present no significant human health environmental risk based on acute oral toxicity studies and in vitro digestibility studies (EPA, 1997; EPA, 1995). In addition, glufosinate herbicide is registered for use on corn in the US.

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Appendix 1 - Figures

Figure 1. Plasmid Map of PHP6710

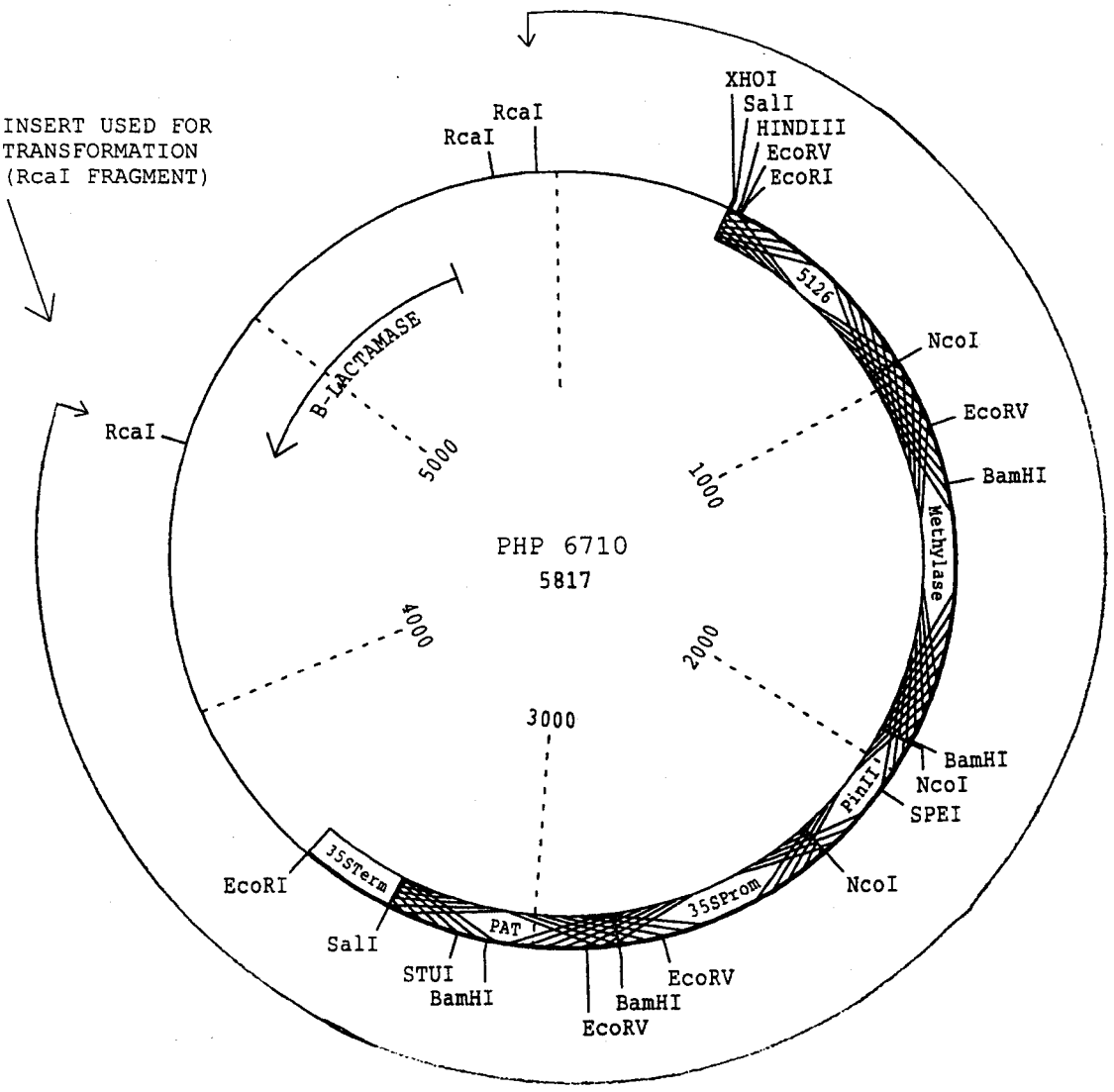


Figure 2. Insert from plasmid PHP6710 (not drawn to exact scale).

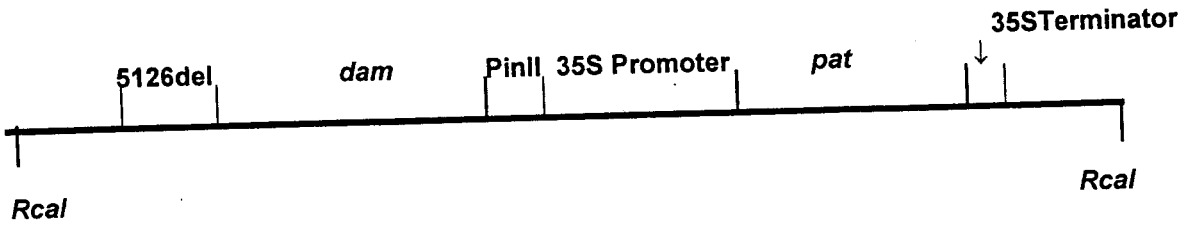


Figure 3. Pollen development in corn hybrids (non-transgenic). Light micrograph of thin section of developing anther tissue.

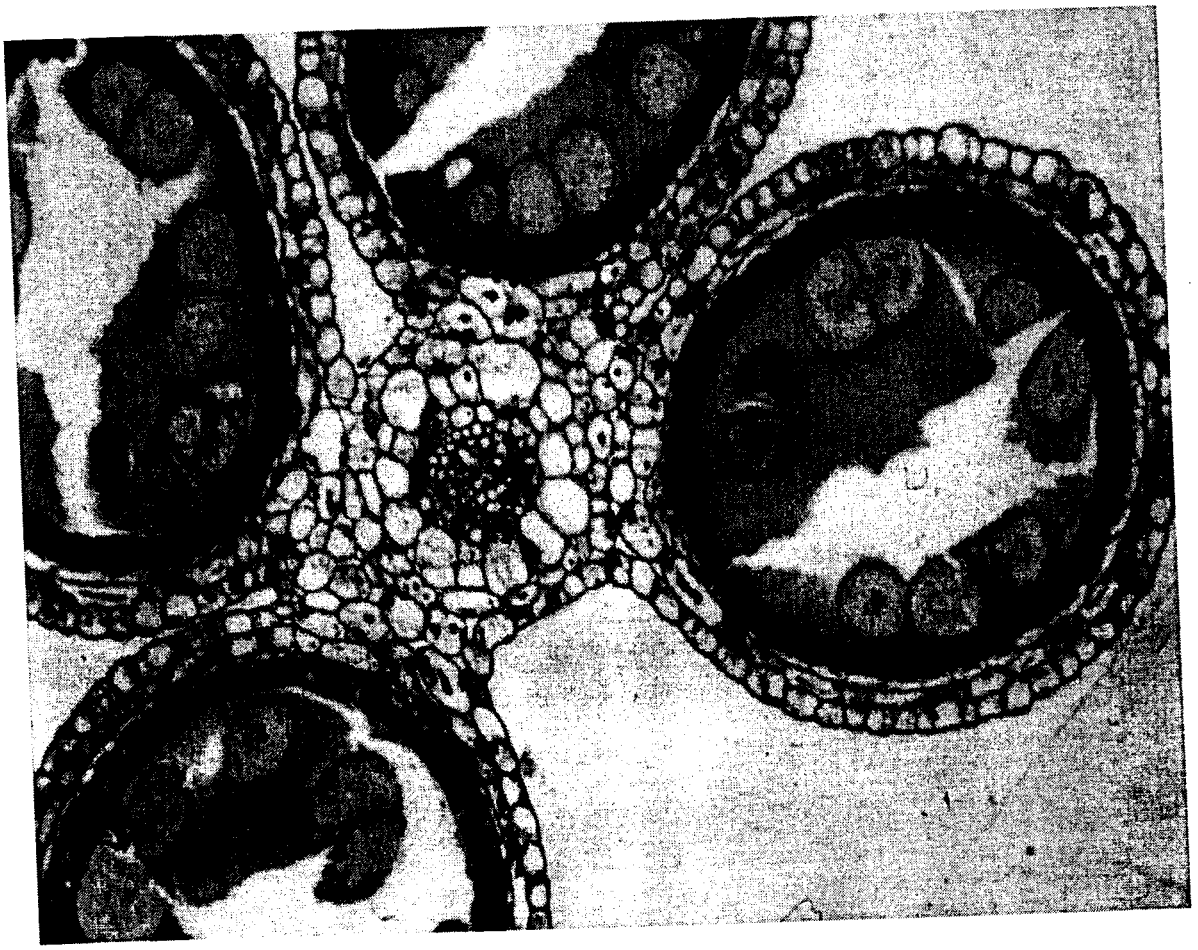
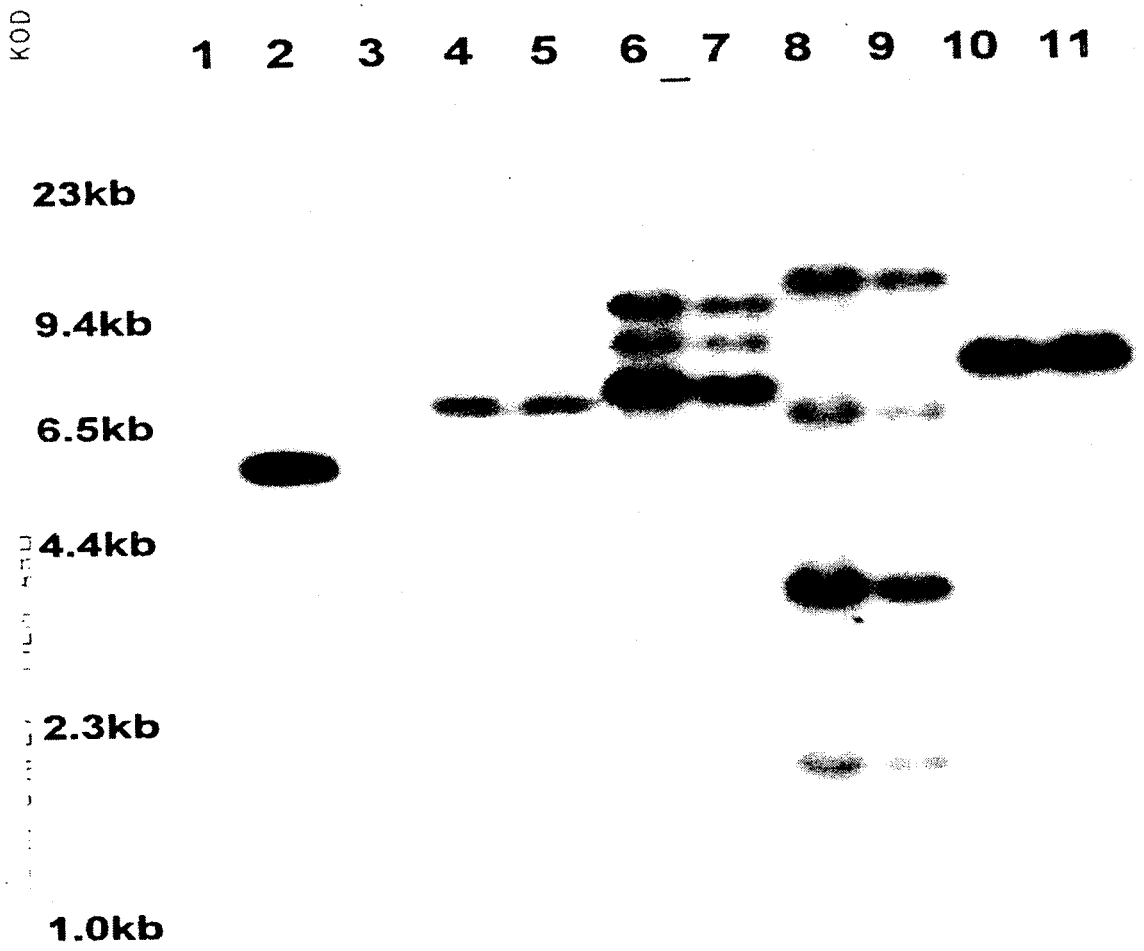


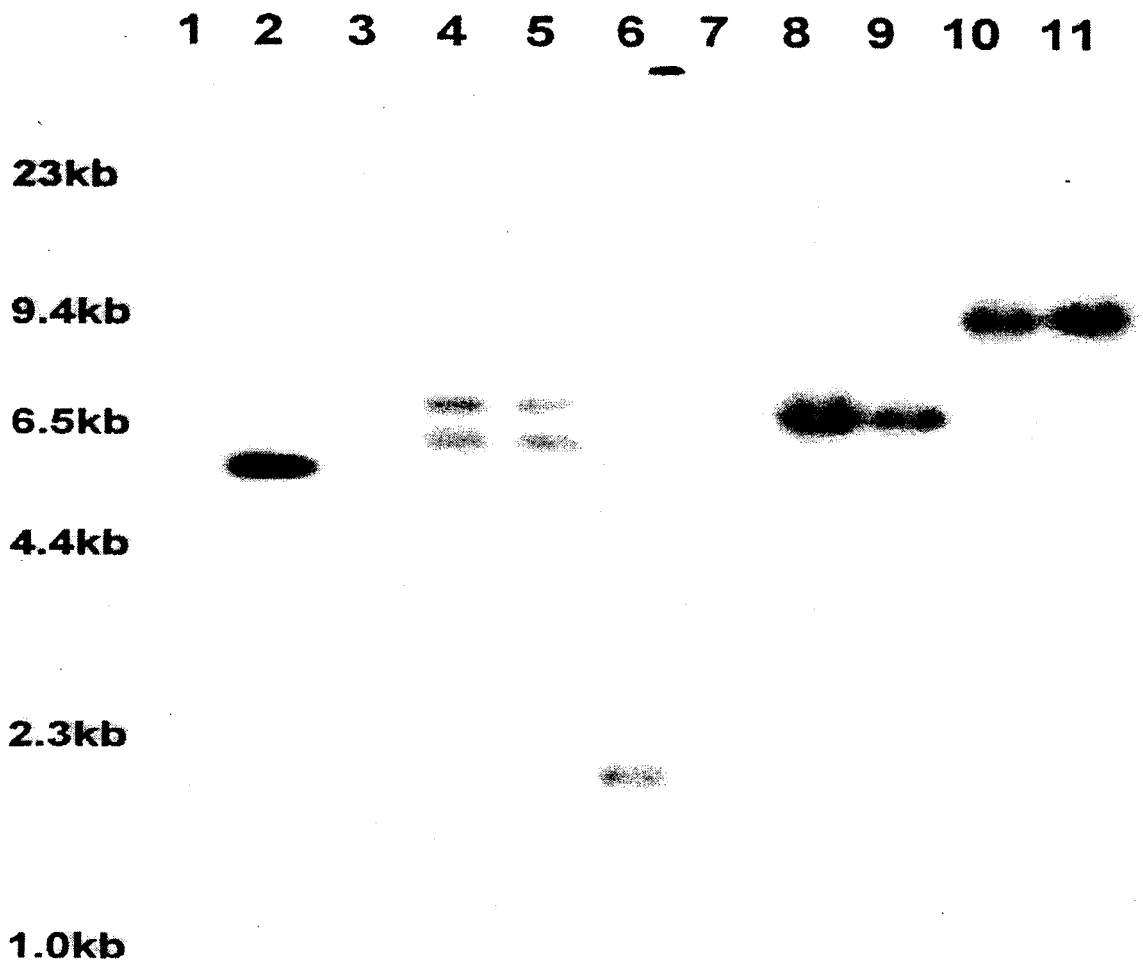
Figure 4. Inhibition of pollen cell Development in TSS corn

Figure 5. Insert Number Analysis Using a *dam* Probe and *Spe*I Digestion



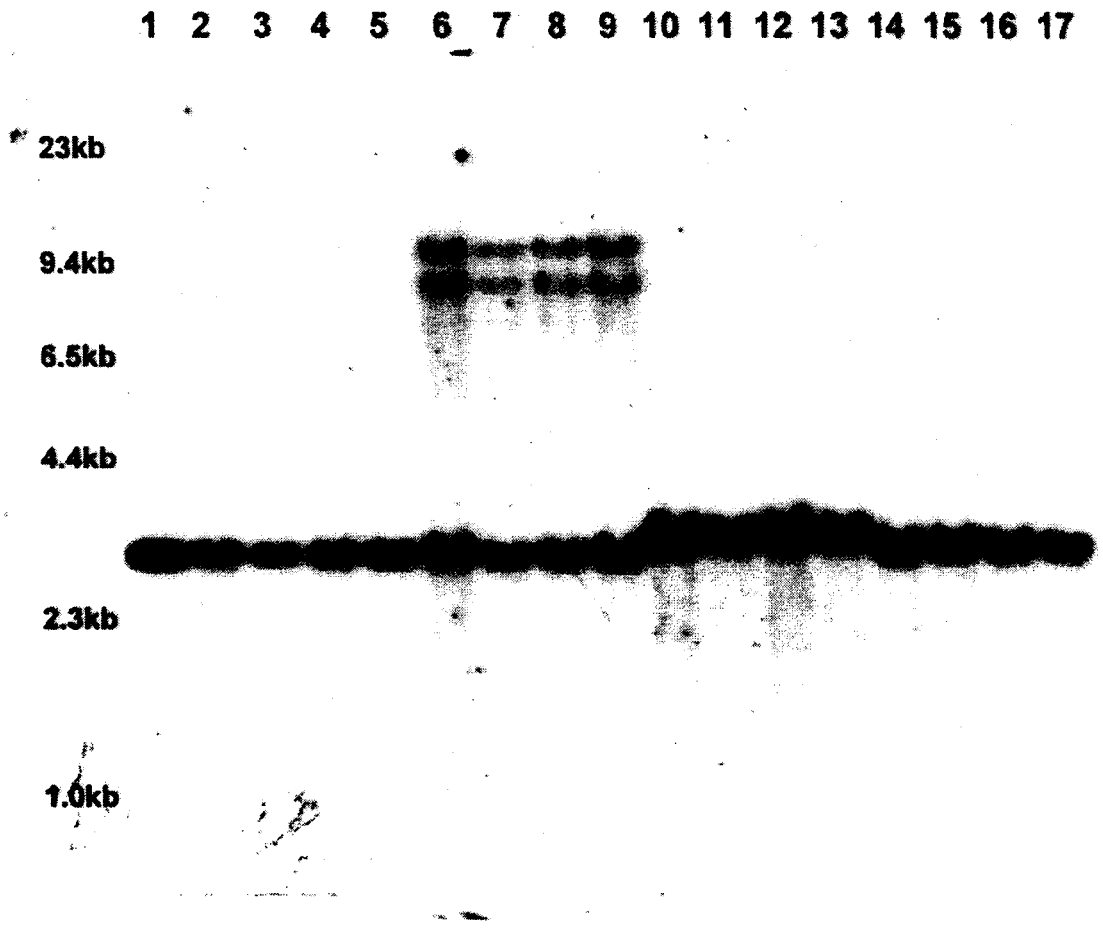
Lane 1:	Lambda HindIII molecular weight markers
Lane 2:	Plasmid PHP6710 (15picograms)
Lane 3:	Inbred A
Lane 4:	676
Lane 5:	676
Lane 6:	678
Lane 7:	678
Lane 8:	680
Lane 9:	680
Lane 10:	1922 (not subject of this petition)
Lane 11:	1992 (not subject of this petition)

Figure 6. Insert Number Analysis Using a *pat* Probe and *Spe*I Digestion



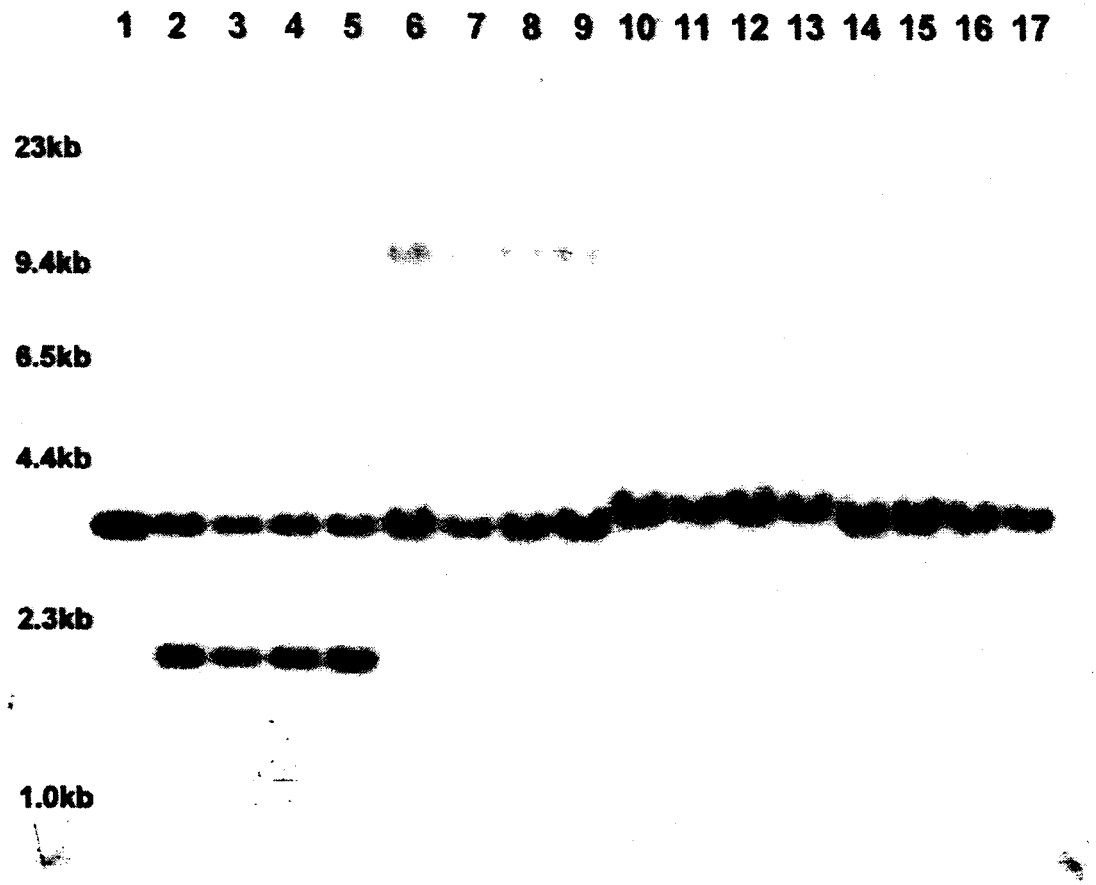
Lane 2:	Plasmid PHP6710 (15picograms)
Lane 3:	Inbred A
Lane 4:	676
Lane 5:	676
Lane 6:	678
Lane 7:	678
Lane 8:	680
Lane 9:	680
Lane 10:	1922 (not subject of this petition)
Lane 11:	1992 (not subject of this petition)

Figure 7. Southern Analysis Using a Probe for *dam* Probe and *Eco*RI Digestion



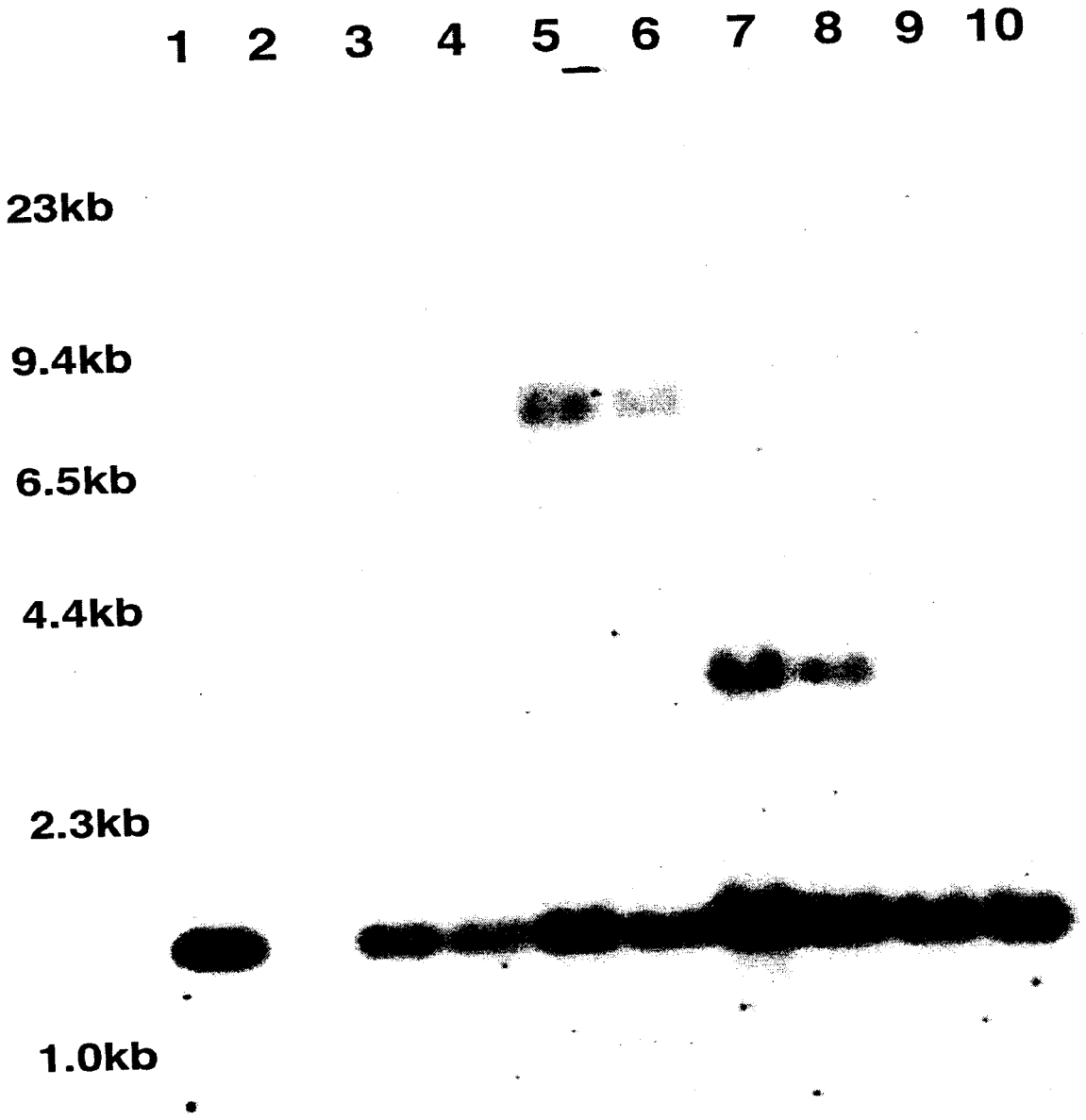
Lane 1: Plasmid PHP6710 (15picograms)
Lane 2: 676 Backcross generation two (BC2)
Lane 3: 676 BC2
Lane 4: 676 BC5
Lane 5: 676 BC5
Lane 6: 678 BC2
Lane 7: 678 BC2
Lane 8: 678 BC6
Lane 9: 678 BC6
Lane 10: 680 BC2
Lane 11: 680 BC2
Lane 12: 680 BC6
Lane 13: 680 BC6
Lane 14: 1922 BC2
Lane 15: 1922 BC2
Lane 16: 1922 BC3
Lane 17: 1922 BC3

Figure 8. Southern Analysis Using a Probe for *pat* and *Eco*RI Digestion



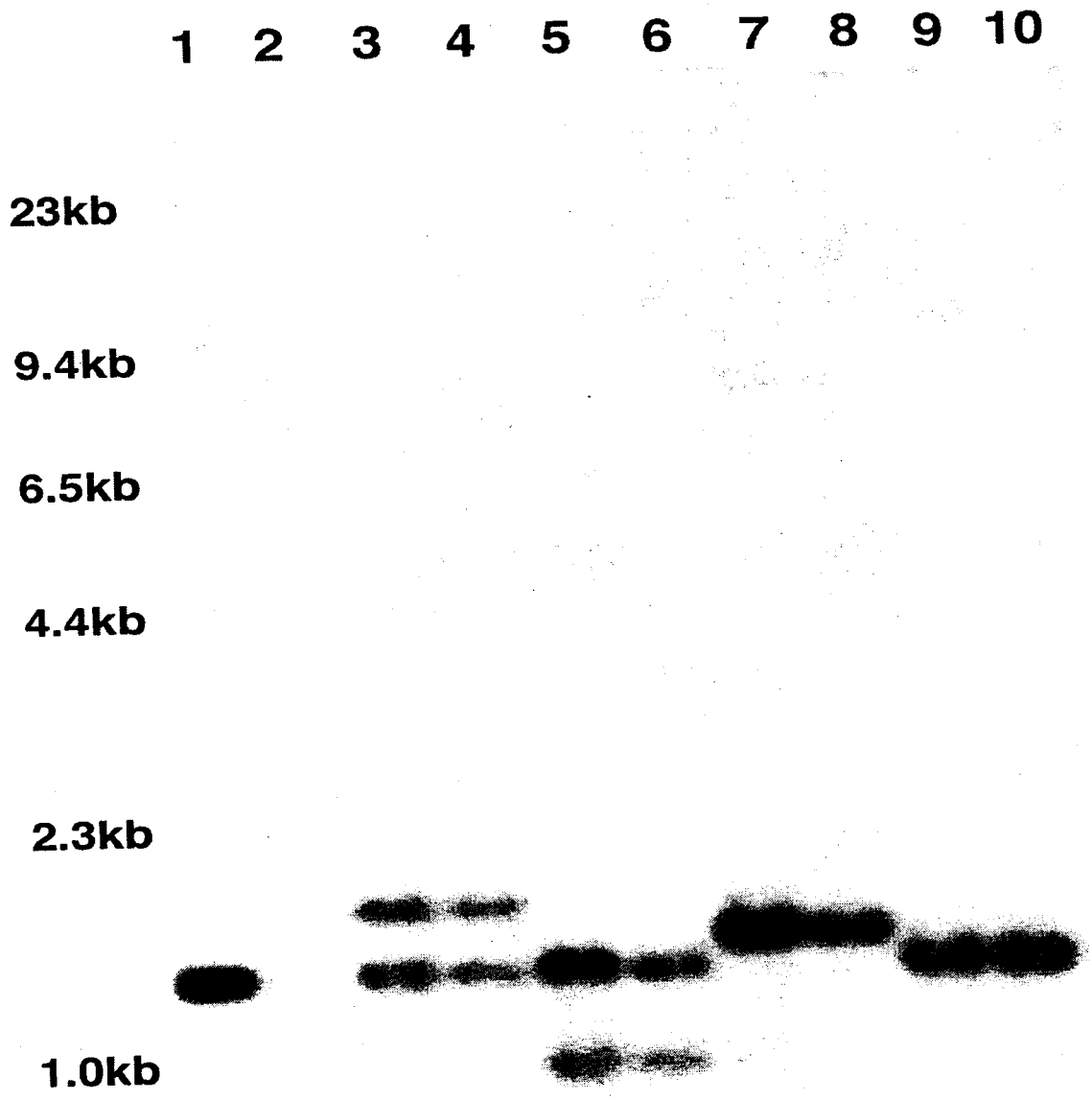
Lane 1: Plasmid PHP6710 (15picograms)
Lane 2: 676 Backcross generation two (BC2)
Lane 3: 676 BC2
Lane 4: 676 BC5
Lane 5: 676 BC5
Lane 6: 678 BC2
Lane 7: 678 BC2
Lane 8: 678 BC6
Lane 9: 678 BC6
Lane 10: 680 BC2
Lane 11: 680 BC2
Lane 12: 680 BC6
Lane 13: 680 BC6
Lane 14: 1922 BC2
Lane 15: 1922 BC2
Lane 16: 1922 BC3
Lane 17: 1922 BC3

Figure 9. Southern Analysis Using a Probe for *dam* and *Eco*RI/*Spe*I Digestion



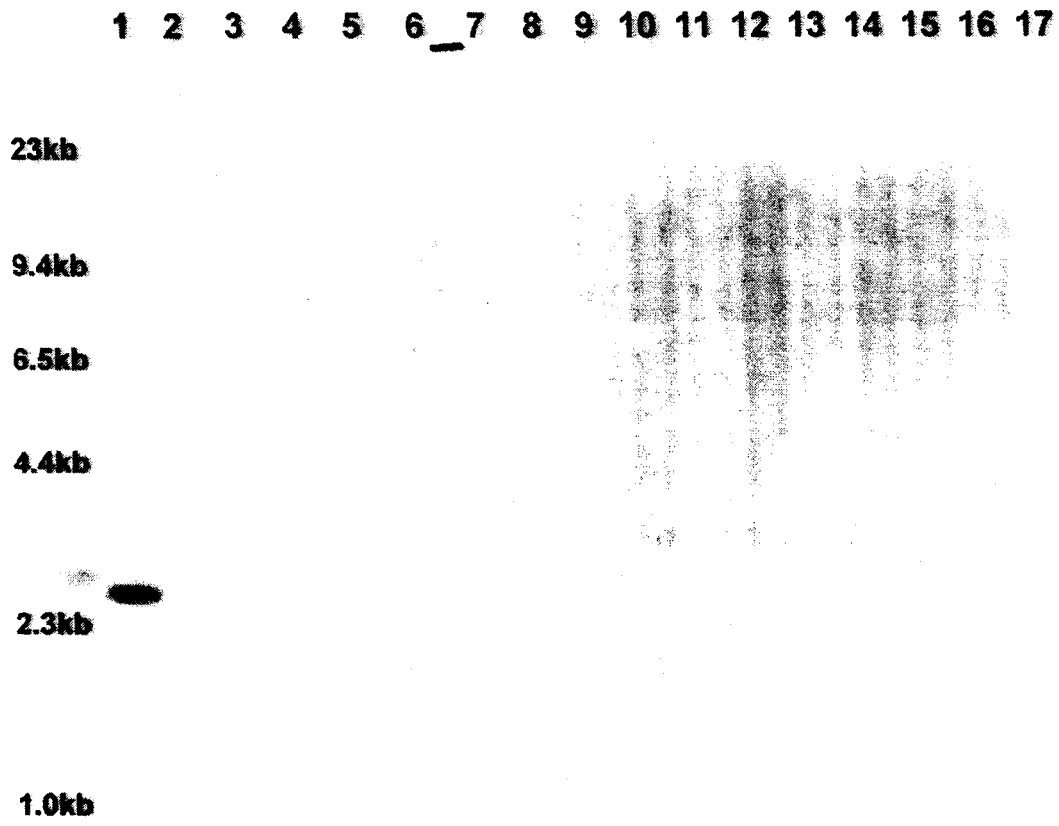
Lane 1:	Plasmid PHP6710 (15picograms)
Lane 2:	Inbred A
Lane 3:	676
Lane 4:	676
Lane 5:	678
Lane 6:	678
Lane 7:	680
Lane 8:	680
Lane 9:	1922 (not subject of this petition)
Lane 10:	1992 (not subject of this petition)

Figure 10. Southern Analysis Using a Probe for *pat* and *Eco*RI/*Spe*I Digestion



Lane 1:	Plasmid PHP6710 (15picograms)
Lane 2:	Inbred A
Lane 3:	676
Lane 4:	676
Lane 5:	678
Lane 6:	678
Lane 7:	680
Lane 8:	680
Lane 9:	1922 (not subject of this petition)
Lane 10:	1992 (not subject of this petition)

Figure 11. Southern Analysis Using an *bla* Probe and *Eco*RI Digestion



Lane 1: Plasmid PHP6710 (15picograms)
Lane 2: 676 Backcross generation two (BC2)
Lane 3: 676 BC2
Lane 4: 676 BC5
Lane 5: 676 BC5
Lane 6: 678 BC2
Lane 7: 678 BC2
Lane 8: 678 BC6
Lane 9: 678 BC6
Lane 10: 680 BC2
Lane 11: 680 BC2
Lane 12: 680 BC6
Lane 13: 680 BC6
Lane 14: 1922 BC2
Lane 15: 1922 BC2
Lane 16: 1922 BC3
Lane 17: 1922 BC3

Figure 12. Stages of Pollen Cell Development

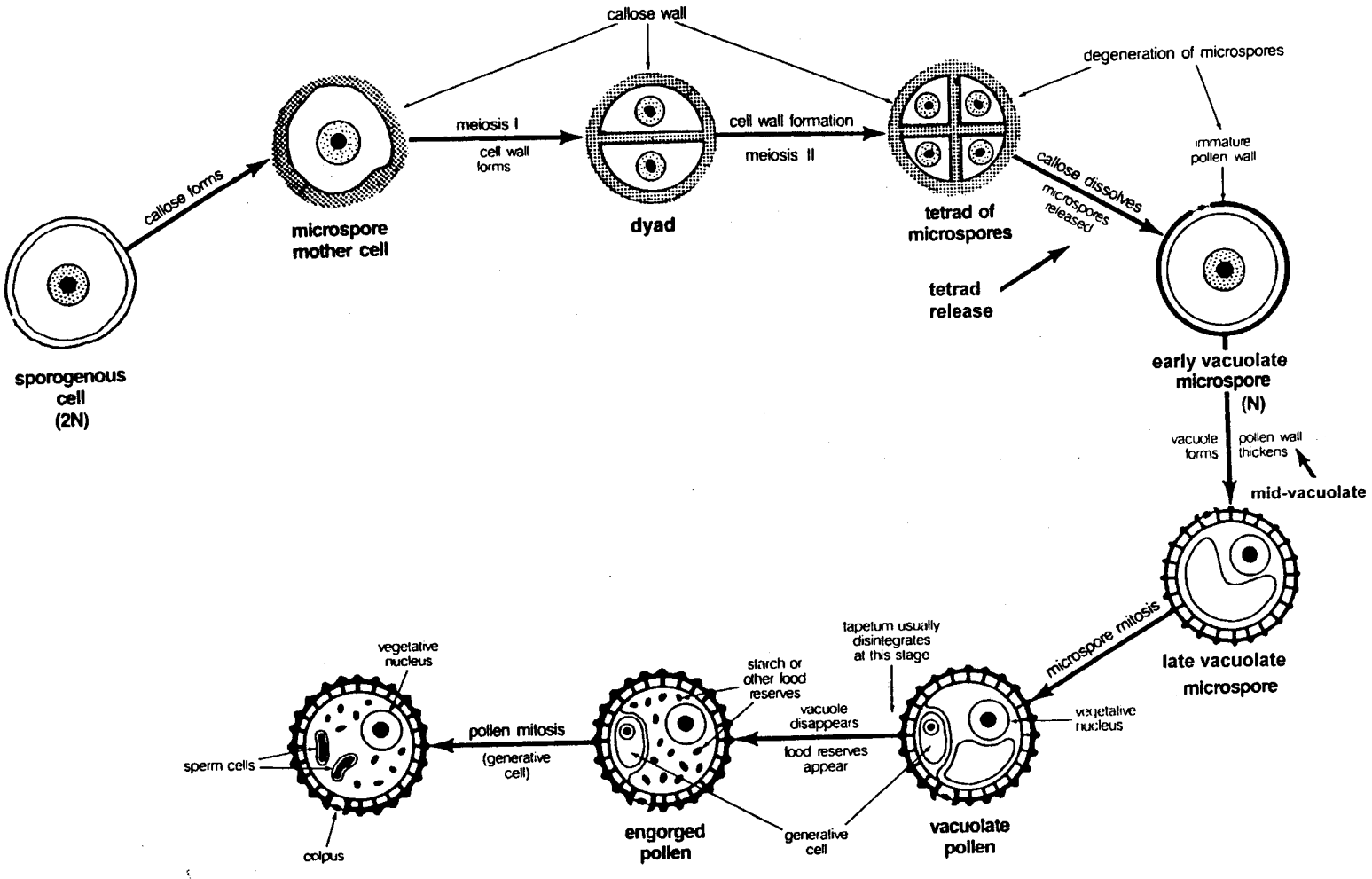


Figure 13. Northern analysis of tissues from male sterile corn lines.

PANEL 1: TASSEL SAMPLES

Lane	Corn line	Anther tissue sampled
1.	TC676	TETRAD RELEASE
2.	TC676	MEIOSIS I
3.	TC676	MEIOSIS II
4.	TC678	TETRAD RELEASE
5.	TC678	MEIOSIS II
6.	TC680	DYAD
7.	TC680	MEIOSIS I
8.	TC680	TETRAD RELEASE
9.	TC1922	DYAD/TETRAD
10.	TC1922	MEIOSIS I
11.	TC1922	TETRAD RELEASE
12.	FS5	DYAD/TETRAD
13.	FS5	EARLY VACUOLATE
14.	FS6	EARLY VACUOLATE
15.	FS6	TETRAD RELEASE/EARLY VACUOLATE
16.	FS6	MID VACUOLATE
17.	FS7	TETRAD
18.	FS7	TETRAD RELEASE/EARLY VACUOLATE
19.	FS7	MEIOSIS II
20.	Inbred A	MEIOSIS II/MID VACUOLATE

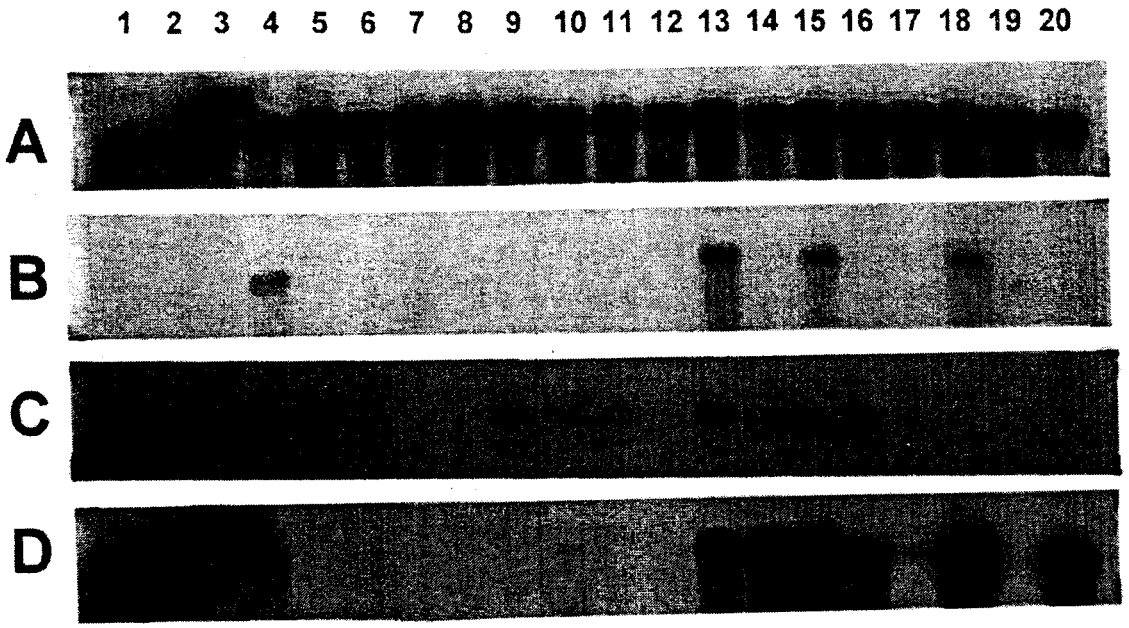
PANEL 2: LEAF SAMPLES

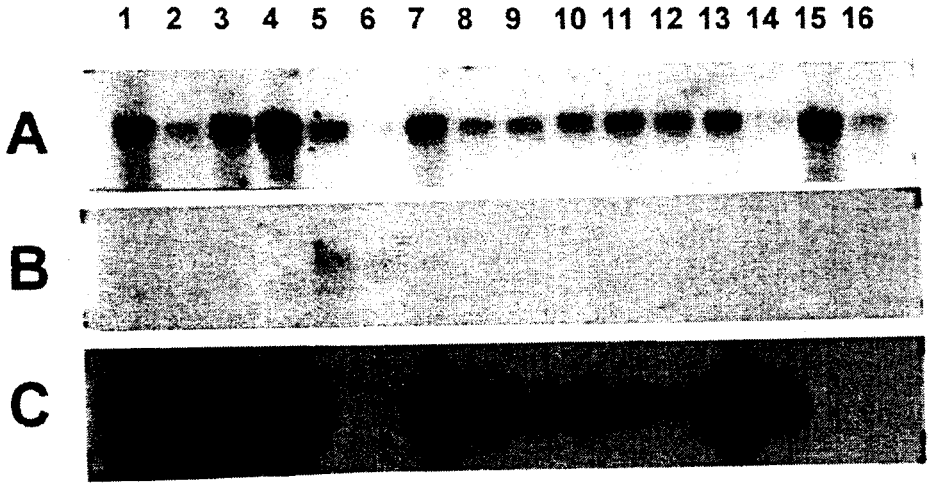
Lane	Corn line	Lane	Corn line
1.	TC676	9.	FS7
2.	TC676	10.	FS7
3.	TC678	11.	FS5
4.	TC678	12.	FS5
5.	TC680	13.	FS6
6.	TC680	14.	FS6
7.	TC1922	15.	Inbred A
8.	TC1922	16.	Inbred A

DNA PROBES USED:

A. Maize actin
B. *dam*
C. *pat*
D. Maize 5126

Figure 13
Panel 1





Panel 2

Appendix 2: USDA Field Trial Approvals for Male Sterile Corn Lines 676, 678, and 680

PHI Number	USDA Number	Planting Date
CORN-HI-95-60	95-269-02N	November, 1995
CORN-NE-96-37	96-080-14N	May, 1996
CORN-IL-96-39	96-080-16N	May, 1996
CORN-IA-96-52	96-094-02N	May, 1996
CORN-PR-96-79	96-247-09N	October, 1996
CORN-HI-96-76	96-247-06N	November, 1996
CORN-IL-97-64	97-063-02N	May, 1997
CORN-IN-97-65	97-063-03N	May, 1997
CORN-MI-97-66	97-063-04N	May, 1997
CORN-MN-97-67	97-063-05N	May, 1997
CORN-MO-97-68	97-063-06N	May, 1997
CORN-NE-97-69	97-063-07N	May, 1997
CORN-PA-97-70	97-063-08N	May, 1997
CORN-SD-97-71	97-063-09N	May, 1997
CORN-WI-97-72	97-063-10N	May, 1997
CORN-TN-97-73	97-063-11N	May, 1997
CORN-IA-97-63	97-097-14N	May, 1997
CORN-IA-97-80	97-083-07N	May, 1997
CORN-IA-97-82	97-083-09N	May, 1997

SUMMARY REPORT OF FIELD TEST DATA

Approved Permit Number:	96-080-14N
Pioneer Number:	CORN-IA-96-37N
Name:	Pioneer Hi-Bred International, Inc.
Institute Address:	c/o Jeffrey Rowe
	7100 N.W. 62nd Avenue
	P.O. Box 1000
	Johnston, IA 50131
Telephone Number:	515-270-3499
Fax Number:	515-334-4478
Date Of This Report:	October 10, 1997

PURPOSE

Gene efficacy trial and breeding/observation nursery

SUMMARY OF EXPERIMENTAL RESULTS

This permit covered one planting site at our research/breeding station located in York, NE. The trial was planted on May 19, 1996 and comprised of approximately 0.2 acres. The trial was isolated by a distance of at least 660 feet from any other corn. Events planted were: TC258, TC259, TC677, TC678, TC679 and TC680. Some of the ears were harvested on October 11, 1996 and saved for future breeding efforts. The remaining ears were destroyed by tilling into the ground with all vegetative material. In the spring of 1997 the site was planted to a crop other than corn.

GENERAL FIELD OBSERVATIONS

The plants were frequently observed by personnel experienced in corn breeding and plant pathology during the growing season. The resulting observations were recorded and the results are listed below.

There were no unanticipated morphological differences between the transformed organisms and appropriate unmodified controls.

The only observation of altered fertility and seed set was male sterility in the transgenic plants; this was the trait the plants were modified to exhibit.

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

The transgenic plants deviated from the parent/control plants by their lack of ability to outcross. No pollen was produced by the transgenics; therefore, no outcrossing could occur.

There was no differences reported in severity of disease symptoms or insect damage between the transgenic plants and the non-transgenic controls.

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

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

FINAL DISPOSITION

All remaining vegetative material was returned to the plot for soil composting.

POST-TRIAL MONITORING

The site was examined several times the following season for volunteer corn. The volunteer plants that were observed were destroyed by hand weeding.

SUMMARY REPORT OF FIELD TEST DATA

Approved Permit Number:	96-080-16N
Pioneer Number:	CORN-IL-96-39N
Name:	Pioneer Hi-Bred International, Inc.
Institute Address:	c/o Jeffrey Rowe 7100 N.W. 62nd Avenue P.O. Box 1000 Johnston, IA 50131
Telephone Number:	515-270-3499
Fax Number:	515-334-4478
Date Of This Report:	October 10, 1997

PURPOSE

Gene efficacy trial and breeding/observation nursery

SUMMARY OF EXPERIMENTAL RESULTS

This permit covered four planting sites near two of our research/breeding stations. The four Illinois sites were: Champaign, Seymour County, Macomb, McDonough County, Long Point, Livingston County, Princeton, Bureau County. Planting began around 5/14/96 and was completed by 5/24/96. The total planted acres did not exceed 1.5 acres. All of the trials were isolated by a distance of at least 660 feet from any other corn. The events planted were: TC258, TC259, TC677, TC678, TC679 and TC680. All of the trials were harvested by 10/12/96. Some of the ears were harvested and saved for future breeding efforts. Any remaining ears were destroyed by tilling into the ground with all vegetative material. In the spring of 1997, all of the sites were planted to a crop other than corn

GENERAL FIELD OBSERVATIONS

The plants were frequently observed by personnel experienced in corn breeding and plant pathology during the growing season. The resulting observations were recorded and the results are listed below.

There were no unanticipated morphological differences between the transformed organisms and appropriate unmodified controls.

The only observation of altered fertility and seed set was male sterility in the transgenic plants; this was the trait the plants were modified to exhibit.

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

The transgenic plants deviated from the parent/control plants by their lack of ability to outcross. No pollen was produced by the transgenics; therefore, no outcrossing could occur.

There was no differences reported in severity of disease symptoms or insect damage between the transgenic plants and the non-transgenic controls.

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

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

FINAL DISPOSITION

All remaining vegetative material was returned to the plot for soil composting.

POST-TRIAL MONITORING

The site was examined several times the following season for volunteer corn. The volunteer plants that were observed were destroyed by hand weeding.

SUMMARY REPORT OF FIELD TEST DATA

Approved Permit Number:	96-094-02N
Pioneer Number:	CORN-IA-96-52N
Name:	Pioneer Hi-Bred International, Inc.
Institute Address:	c/o Jeffrey Rowe
	7100 N.W. 62nd Avenue
	P.O. Box 1000
	Johnston, IA 50131
Telephone Number:	515-270-3499
Fax Number:	515-334-4478
Date Of This Report:	October 10, 1997

PURPOSE

Gene efficacy trial and breeding/observation nursery

SUMMARY OF EXPERIMENTAL RESULTS

This permit covered six planting site near three of our research/breeding stations. The six Iowa sites were: Marion, Linn County, Algona, Kossuth County, Callendar, Webster County, Vinton, Benton County, Sheldahl, Polk County, and Johnston, Polk County. Planting began around 5/07/96 and was completed by 5/20/96. The total planted acres did not exceed 2.5 acres. The trials were isolated by distance of at least 660 feet from any other corn. Events planted were: TC258, TC259, TC676, TC677, TC678, TC679 and TC680. Harvest began around 10/7/96 and was completed by 10/25/96. Some of the ears were harvested and saved for future breeding efforts. The remaining ears were destroyed by tilling into the ground with all vegetative material. In the spring of 1997, all of the sites were planted to a crop other than corn.

GENERAL FIELD OBSERVATIONS

The plants were frequently observed by personnel experienced in corn breeding and plant pathology during the growing season. The resulting observations were recorded and the results are listed below.

There were no unanticipated morphological differences between the transformed organisms and appropriate unmodified controls.

The only observation of altered fertility and seed set was male sterility in the transgenic plants; this was the trait the plants were modified to exhibit.

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

The transgenic plants deviated from the parent/control plants by their lack of ability to outcross. No pollen was produced by the transgenics; therefore, no outcrossing could occur.

There was no differences reported in severity of disease symptoms or insect damage between the transgenic plants and the non-transgenic controls.

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

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

FINAL DISPOSITION

All remaining vegetative material was returned to the plot for soil composting.

POST-TRIAL MONITORING

The site was examined several times the following season for volunteer corn. The volunteer plants that were observed were destroyed by hand weeding.

INTERIM REPORT OF FIELD TEST DATA

Approved Permit Number:	96-247-06N
Pioneer Number:	CORN-HI-96-76N
Name:	Pioneer Hi-Bred International, Inc.
Institute Address:	c/o Jeffrey Rowe 7100 N.W. 62nd Avenue P.O. Box 1000 Johnston, IA 50131
Telephone Number:	515-270-3499
Fax Number:	515-334-4478
Date Of This Report:	October 10, 1997

PURPOSE

Gene efficacy trial and breeding/observation nursery

SUMMARY OF EXPERIMENTAL RESULTS

This permit covered three planting sites at our research/breeding station located in Kekeha, Kauai, HW. The trials were completed with planting by November 22, 1996, April 3, 1997, and July 24, 1997. The total acreage for the all of the plantings was approximately 1.5 acres. The trials were all isolated by distance of at least 660 feet from any other corn. The following events were planted: TC676, TC677, TC678, TC680, TC1922, TC1167, TC1168, TC139, 203-17-28-27, TC404, and TC2612. The grain from all rows will be harvested and retained at our facility for future breeding efforts or shipped to our facility in Johnston, IA. Post harvest, the sites will remain fallow for at least 30 days with cycles of irrigation and cultivation.

GENERAL FIELD OBSERVATIONS

The plants are frequently observed by personnel experienced in corn breeding and plant pathology during the growing season. The resulting observations were recorded and the results are listed below.

There were no unanticipated morphological differences between the transformed organisms and appropriate unmodified controls.

The only observation of altered fertility and seed set was male sterility in the transgenic plants; this was the trait the plants were modified to exhibit.

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

The transgenic plants deviated from the parent/control plants by their lack of ability to outcross. No pollen was produced by the transgenics; therefore, no outcrossing could occur.

Northern leaf blight and common rust was observed on 2/20/97. There was no apparent differences in disease or insect susceptibility between the transgenic plants and the non-transgenic controls.

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

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

FINAL DISPOSITION
All remaining vegetative material will be returned to the plot for soil composting.

POST-TRIAL MONITORING
The site will be examined several times the following season for volunteer corn. Any volunteer plants that are observed will be destroyed by hand weeding.