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DEPARTMENT OF AGRICULTURE

Animal and Plant Health Inspection Service

[Docket No. 04-044-1]

Availability of Environmental Assessment for Field Test of Genetically Engineered Organisms

AGENCY: Animal and Plant Health Inspection Service, USDA.

ACTION: Notice.

SUMMARY: We are advising the public that the Animal and Plant Health Inspection Service has prepared an environmental assessment for a confined field of corn plants genetically engineered to express the protein aprotinin. This environmental assessment is available for public review and comment.

DATES: We will consider all comments we receive on or before [insert date 30 days after date of publication in the Federal Register].

ADDRESSES: You may submit comments by any of the following methods:

- Postal Mail/Commercial Delivery: Please send four copies of your comment (an original and three copies) to Docket No. 04-044-1, Regulatory Analysis and Development, PPD, APHIS, Station 3C71, 4700 River Road Unit 118, Riverdale, MD 20737-1238. Please state that your comment refers to Docket No. 04-044-1.
- E-mail: Address your comment to regulations@aphis.usda.gov. Your comment must be contained in the body of your message; do not send attached files. Please include your name and address in your message and "Docket No. 04-044-1" on the subject line.
- Agency Web Site: Go to http://www.aphis.usda.gov/ppd/rad/cominst.html for a form you can use to submit an e-mail comment through the APHIS Web site.

- Federal eRulemaking Portal: Go to http://www.regulations.gov and follow the instructions for locating this docket and submitting comments.

Reading Room: You may read the environmental assessment and any comments that we receive in our reading room. The reading room is located in room 1141 of the USDA South Building, 14th Street and Independence Avenue SW., Washington, DC. Normal reading room hours are 8 a.m. to 4:30 p.m., Monday through Friday, except holidays. To be sure someone is there to help you, please call (202) 690-2817 before coming.

Other Information: You may view APHIS documents published in the Federal Register and related information, including the names of groups and individuals who have commented on APHIS dockets, on the Internet at http://www.aphis.usda.gov/ppd/rad/webrepor.html.

FOR FURTHER INFORMATION CONTACT: Dr. James White, BRS, APHIS, 4700 River Road Unit 147, Riverdale, MD 20737-1236; (301) 734-5940. To obtain a copy of the environmental assessment, contact Ms. Kay Peterson at (301) 734-4885; e-mail: Kay.Peterson@aphis.usda.gov. The environmental assessment is also available on the Internet at http://www.aphis.usda.gov/brs/aphisdocs/04_12101r_ea.pdf.

SUPPLEMENTARY INFORMATION: The regulations in 7 CFR part 340, "Introduction of Organisms and Products Altered or Produced Through Genetic Engineering Which Are Plant Pests or Which There Is Reason to Believe Are Plant Pests," regulate, among other things, the introduction (importation, interstate movement, or release into the environment) of organisms and products altered or produced through genetic engineering that are plant pests or that there is reason to believe are plant pests. Such genetically engineered organisms and products are considered "regulated articles." A permit must be obtained or a notification acknowledged before a regulated article may be introduced into the United States. The regulations set forth the

permit application requirements and the notification procedures for the importation, interstate movement, and release into the environment of a regulated article.

On April 30, 2004, the Animal and Plant Health Inspection Service (APHIS) received a permit application (APHIS No. 04-121-01r) from ProdiGene, Inc., College Station, TX, for a permit for a confined field test of corn (Zea mays L) plants genetically engineered to express a gene coding for the enzyme (protein) aprotinin. The field test is to be conducted in Frio County, TX. The subject corn plants have been genetically engineered to express an aprotinin protein that is identical to the native bovine (Bos taurus L.) protein. The subject corn plants also express the pat gene from Streptomyces viridochromogenes, a common soil bacterium. The pat gene expresses a phosphinothricin acetytransferase enzyme, which confers tolerance to the herbicide glufosinate, and is useful as a maker gene. The experimental genes were transferred into corn plants through use of the Agrobacterium tumefaciens transformation system, and expression of the added genes is controlled in part by the plant pathogen cauliflower mosaic virus. The genetically engineered corn plants are considered regulated articles under the regulations in 7 CFR part 340 because they contain gene sequences from plant pathogens.

The purpose of the proposed field trial is to produce grain, hybrid seed, and to develop a research line in a nursery. The tests will be conducted through use of a combination of biological and physical containment measures. In addition, the experimental protocols and field plot design, as well as the procedures for termination of the field tests, are designed to ensure that none of the subject corn plants persist in the environment beyond the termination of the experiments.

To provide the public with documentation of APHIS' review and analysis of any potential environmental impacts and plant pest risk associated with the proposed confined field

test of the subject corn plants, an environment assessment (EA) has been prepared. The EA was prepared in accordance with (1) The National Environmental Policy Act of 1969 (NEPA), as amended (42 U.S.C. 4321 et seq.), (2) regulations of the Council on Environmental Quality for implementing the procedural provisions of NEPA (40 CFR parts 1500-1508), (3) USDA regulations implementing NEPA (7 CFR part 1b), and (4) APHIS' NEPA Implementing Procedures (7 CFR part 372).

Authority: 7 U.S.C. 1622n and 7701-7772; 31 U.S.C. 9701; 7 CFR 2.22, 2.80, and 371.3.

Done in Washington, DC, this________day of___________________________.

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Administrator, Animal and Plant Health Inspection Service.

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USDA APHIS Environmental Assessment
In response to permit application (04-121-01r)
Received from ProdiGene Inc.
For field testing of genetically engineered corn, *Zea mays*

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I. SUMMARY

The U.S. Department of Agriculture's (USDA) Animal and Plant Health Inspection Service, Biotechnology Regulatory Services (APHIS BRS) has prepared an environmental assessment (EA) in response to a permit application (APHIS number 04-121-01r) received from ProdiGene Inc., College Station, Texas, to conduct two small scale field tests of genetically engineered corn (*Zea mays* L.) plants in Frio County, Texas. These transgenic plants have been modified to express the aprotinin gene from *Bos taurus* (cow). These plants have also been engineered with the bar gene from *Streptomyces viridochromogenes* that serves as a selectable marker and encodes for phosphinothricin acetyltransferase, an enzyme which confers tolerance to the herbicide glufosinate. Some of the details of the genetic constructs, locations, and procedures have been claimed as confidential business information (CBI) by the applicant (FR 50 38561-63).

This EA was prepared in accordance with: (1) The National Environmental Policy Act of 1969 (NEPA), as amended (42 U.S.C. 4321 *et seq.*); (2) regulations of the Council on Environmental Quality for implementing the procedural provisions of NEPA (40 CFR parts 1500-1508); and (3) USDA regulations and implementing NEPA (7 CFR part 1b) and (4) APHIS' NEPA Implementing Procedures (7 CFR part 372).

These field tests are scheduled to begin in July/August 2004, on an isolated site in Frio County, Texas. These tests should be completed in the fall of 2004. A second planting is requested for March 2005 on the same field site.

The bases of confinement for these field tests are:

The field test site is small and is located on a private land in Frio County. Frio County is not a major producer of corn.

In nature, chromosomal genetic material of corn can only be transferred to other sexually compatible plants by cross-pollination. The field test plot will be at least 1 mile from any other corn plant with which it might cross-pollinate.

Neither of the introduced genes provide the engineered corn plants with any selective advantage over nonengineered corn in the ability to be disseminated or to become established in the environment.

Horizontal movement of the introduced genes is extremely unlikely. The foreign DNA is stably integrated into the plant genome.

II. PURPOSE AND NEED

USDA APHIS is proposing to issue a permit for confined field release of genetically engineered corn (*Zea mays* L.) plants in Frio County, Texas. The

ProdiGene submitted a permit application to USDA APHIS pursuant to regulations codified in 7 CFR Part 340, "Introduction of Organisms and Products Altered or Produced Through Genetic Engineering Which Are Plant Pests or Which There is Reason to Believe Are Plant Pests." The regulations govern the introduction (importation, interstate movement or release into the environment) of certain genetically engineered organisms and products. A permit must be obtained or a notification acknowledged before a regulated article may be introduced into the U.S..

APHIS BRS considers genetically engineered organism a regulated article if it is being introduced and if the donor organism, recipient organism, vector, or vector agent used in engineering the organism belongs to one of the taxa listed in the 7 CFR 340 and is also a plant pest, or if there is reason to believe that it is a plant pest. In this submission, the vector organism is in the genus *Agrobacterium,* which is one of the listed taxa, and it has been genetically engineered using recombinant DNA techniques and the promoter and terminator sequences of the marker gene are from a known plant pest, cauliflower mosaic virus. Thus, APHIS BRS deems the genetically engineered organism in this ProdiGene submission a regulated article.

Generally permitting for field trials of regulated articles is categorically excluded from requirements for an environmental assessment (EA) under APHIS NEPA implementing procedures (7 C.F.R. Section 372.5(c)(3)(i).
However, when APHIS determines that a confined field release of genetically engineered organisms has the potential to affect significantly the quality of the human environment, as those terms are defined in 40 C.F.R. 1508.27 and 1509.14, an environmental assessment or environmental impact statement will be prepared, pursuant to 7 C.F.R. 372.5(d). This EA was prepared because the applicant intends to have repeated plantings of this engineered plant in Frio County, Texas, for the next several years. The potential for cumulative impacts of repeated plantings in the same area raises new issues that this EA addresses. Future plantings will be essentially the same size and meet all the performance and mitigation measures described in this EA, standard and supplemental permit conditions, and the permit application.

III. ALTERNATIVES TO THE PROPOSED ACTION

A. **Alternative 1**: No Action/ denial of permit application:

Under this alternative, the field tests would not be authorized.

B. **Alternative 2**: Issue the permit for the field testing under the conditions proposed by the applicant:

Under this alternative, field release of the genetically engineered corn plants would be authorized at the specified locations with no additional measures outside of what the applicant provided in their request and the standard permit conditions under 7 CFR 340.4 would be required (see Appendix II).

C. **Alternative 3**: Issue the permit with additional conditions for conduction of the field test:

Supplemental permit conditions, based on APHIS analysis, the State of Texas and public comment from this EA, would be required.

IV. DESCRIPTION OF THE REGULATED ARTICLE

A. The Biology of Corn

In this section of the EA, the potential impacts to the environment from the introduction of genetically engineered corn are discussed. The biology of corn and plants related to corn are considered (Coe *et al.*, 1988; Galinat, 1988; Haulauer *et al.*, 1988; Wych, 1988). Because the mechanism by which genes are moved from one flowering plant to another in nature is through cross-pollination of sexually compatible plants, the plants with which corn can cross-pollinate are described. Below is a synopsis of a detailed analysis of the biology of maize that was prepared by the Organization for Economic Cooperation and Development (OECD), "Consensus Document on the Biology of *Zea mays* (Maize)" (see Appendix 1 and the document may be found at: http://www.olis.oecd.org/olis/2003doc.nsf/LinkTo/env-jm-mono(2003)11. Our synopsis focuses solely on the U.S. and references cited in the OECD document are incorporated by reference.

B. Systematics of Corn

Zea is a genus of the family Gramineae (Poaceae), commonly known as the grass family. The genus consists of some five species: *Zea mays*, cultivated corn and teosinte; *Zea diploperennis* Iltis *et al.*, diploperennial teosinte; *Zea luxurians* (Durieu et Asch.) Bird; *Zea nicaraguensis*; and *Zea perennis* (Hitchc.) Reeves et Mangelsd., perennial teosinte.

Of the five species of *Zea*, only *Zea mays* is common in the U.S.. It is known only from cultivation; it occasionally is spontaneous in abandoned fields or roadsides, but is incapable of sustained reproduction outside of cultivation. *Z. perennis* and *Z. mexicana* have been reported occasionally in the U.S. or used as experimental plants at university or experiment stations (Kartesz, 2004). Corn cannot establish itself asexually by roots or stems without human intervention

The closest generic relative to *Zea* is *Tripsacum*, a genus of seventeen species. *Tripsacum* differs from corn in many respects, including chromosome number. All species of *Tripsacum* can cross with *Zea*, but only with difficulty and only resulting in extreme sterility, therefore, gene flow from maize to this species is virtually impossible without human intervention.

C. Hybrid versus Inbred Corn

Almost all corn grown in the U.S. now comes from hybrid seed that is obtained every planting season from private enterprises. The older open-

pollinated varieties are virtually unknown in commerce. Studies of pollination of corn have mostly centered on the needs of hybrid seed production. This production involves the development and maintenance of inbred lines and the subsequent crosses to produce commercial seed. In the former, self-pollination is mandatory. In the latter, cross-pollination is mandatory. Mechanisms have been developed to ensure each kind of pollination. Hybrid seed production fields require isolation. The isolation distance required by USDA's Agricultural Marketing Service (7 CFR part 201.76) for foundation seed is 660 feet.

D. **Modes of Gene Escape in Corn**

Genes of corn may escape from the test plot in two ways. The first pathway of escape is by pollen transfer. The second is by movement of the grain or seed.

Maize, although self-fertile, is typically cross pollinated by the wind because of differences in floral synchrony between male (tassel) and female (silk) flowers on single plant. Usually, tassels begin shedding pollen before the female flowers are receptive to fertilization. The typical tassel may shed pollen for 2 to 14 days depending on genotype of the plant and environmental conditions. Female flower development typically lags behind that of the tassel and anthers with a minimum overlap resulting in about 5% self pollination. Corn pollen is unusually large for a grass species. Plant breeding in the past half-century has not significantly affected pollen mass, thus, past experiments of pollen movements are still valuable (Burris, 2001). Corn inbreds and hybrids differ significantly in the size of the tassel, the number of grains of pollen, longevity of pollen shed and other factors. Most inbreds produce at least 80% less pollen than hybrids (Burris, 2001).

Pollen viability in the field generally lasts no more than 2 hours because of heat and humidity effects (Herrero and Johnson, 1980; Luna *et al.*, 2001). In addition, high temperatures reduce pollen shed (Schoper *et al,* 1987). Pollen can be transferred by wind to any receptive corn stigma within a few hour period of pollen viability. This potential transfer becomes more unlikely as distance increases from the transgenic plants. Numerous studies going back to the 1940's describe the off-source pollen dissemination. These studies vary extensively in experimental design, environmental conditions and quality of data (Jones and Newall, 1946; Jones and Brooks, 1950; Buller, 1951; Haskell and Dow, 1951; Raynor *et al.*, 1970, 1972; Paterniani and Stort, 1974; Du *et al.,* 2000; Narayanaswamy *et al.*, 1997; Das, 1983; Garcia *et al.*, 1998; Jemison and Vyda, 2001; Ministrere de l'Agriculture et de la Peche, 2002). The data, in whole, clearly shows that there is a trend for decreased corn pollen flow with increasing distance. Recent reviews of published research (Eastam and Sweet, 2002; Feil and Schmid, 2002; Burris, 2001; Ingram, 2000) conclude that seed purity levels of 98.5% to 99.5% is achievable for seed production fields when the two fields are from 574 feet to 984 feet apart. To generate this data, scientists maximized gene flow rate by altering the planting dates of the two fields so pollen shed in

one of the fields correspond with maximum receptivity of the silks. APHIS' permit conditions require a minimum of 5,280 feet distance between the engineered corn and the nearest corn field (http://www.aphis.usda.gov/brs/pdf/FR46434.pdf).

Temporal isolation further reduces the likelihood of effective pollination and fertilization. In addition, any physical impediment to this movement, such as 100% effective detasseling or bagging, would completely eliminate the possibility of gene escape by way of pollen. Although corn seeds can remain on the ground after harvesting and are consumed by animals, the lack of corn volunteers observed outside the proximity of the fields strongly support that corn seeds are not dispersed by animals or birds (see Appendix I).

V. THE REGULATED ARTICLE

A. The Vector

The experimental genes were transferred into corn plants *via* a binary *Agrobacterium tumefaciens* transformation system that employs a disarmed Ti-plasmid (*i.e.*, sections of the plasmid DNA essential for plant pathogenicity were deleted). This well-characterized transformation system results in the stable and irreversible integration of the donor genes into the chromosome of the recipient plant cell. These donor sequences are then maintained and inherited as any other gene of the plant cell. This system has been used thousands of times in the past 20 years and many engineered plants have been developed using this system; some commercially grown after review by U.S. governmental agencies (http://usbiotechreg.nbii.gov/).

B. Description of the Aprotinin Gene

Aprotinin is one of the most studied small globular proteins (Kassell, 1970, Fritz and Wunderer 1983, Gebhard *et al*, 1986) that functions as an anti-proteinase and is known under multiple names as Trasylol® (Bayer), Iniprol (Choay Laboratories), Pancreatic Kunitz Inhibitor and Basic Pancreatic Trypsin Inhibitor (Worthington Biochemical).

C. Sources of Aprotinin

Aprotinin is a naturally occurring protein, found in ruminants, such as cow and ox. Since its discovery in 1930 in bovine pancreas, it has now been found in virtually all bovine organs and blood (Fritz and Wunderer, 1983). Until now, the most readily available source for aprotinin was by extraction from cow organs, specifically cow lung. Alternative plant production as a manufacturing platform could reduce the risk of passing on infectious diseases to humans during therapeutic treatment (M. Henney, 2002, former FDA Commissioner).

D. Molecular Characterization

Aprotinin is composed of a single polypeptide chain of 58 amino acids, having a molecular weight of 6,511 Daltons with three disulfide bonds, and is present as a dimer under physiological conditions (Gebhard, *et al.*, 1986; Trautschold *et al.*, 1967). The aprotinin gene was cloned from cow (Anderson and Kingston, 1983). The cloned aprotinin as expressed in the plant is identical to the native bovine protein, as evidenced by N-terminal sequencing of the amino acids revealing that both sources of protein contained identical amino acids (U.S. Patent: 5,824,870 http://164.195.100.11/netacgi/nph-Parser?Sect1=PTO1&Sect2=HITOFF&d=PALL&p=1&u=/netahtml/srchnum.htm&r=1&f=G&l=50&s1=%275,824,870%27.WKU.&OS=PN/5,824,870&RS=PN/5,824,870, accessed May 6, 2004). To optimize plant expression, the ProdiGene aprotinin gene incorporates plant-preferred codons, which increases expression without altering the amino acid acid sequence. Neither the native bovine nor the corn-produced aprotinin is glycosylated.

E. **Physical Properties and Degradation**

Aprotinin is an extremely stable protein under many different physical conditions (Gebhard *et al.*, 1986); it is degraded by certain enzymes. It can be boiled in dilute acid and has an unusual thermostability (Moses and Hinz, 1983). Though fairly resistant to cleavage by certain proteases, aprotinin is degraded by thermolysin in laboratory experiments (Kassell and Wang, 1971). Following therapeutic treatment in humans, the aprotinin polypeptide is broken down into shorter peptides or amino acids by lysosomal activity in the kidney (Trautschold, *et al.*, 1967). During therapeutic delivery, the serum half-life of aprotinin is 70 min., due to lysosomal sequestration in kidney proximal tubule cells (Kaller, *et al.*, 1978, Torok, 1972, Trautschold, *et al.*, 1966), followed by excretion by the kidneys.

F. **Biological Function**

The main usefulness of proteinase inhibitors is derived from their ability to form complexes with proteinases and thereby render them non-functional. A proteinase is an enzyme that degrades proteins into smaller fragments, by hydrolysis of peptide bonds between adjacent amino acids; proteinase-inhibitors block these proteinases. Because proteinase inhibitors are generally isolated by virtue of their ability to inhibit a proteinase, proteinase inhibitors are often by the method of isolation, and not their physiological function in their native cellular environment. In addition, the source of the protein is often included in the name of the proteinase inhibitor (e.g. pancreatic trypsin inhibitor).

Proteinase inhibitors may be classified by their specificity, namely, the types of proteinases that they inhibit in the laboratory. For aprotinin, the binding specificity has been termed both broad and narrow in different contexts. For instance aprotinin binds human pancreatic trypsin I and 2, but not human pancreatic chymotrypsin A or human pancreatic elastase (Belorgey *et al.*, 1996). In the laboratory, numerous enzymes are inhibited by aprotinin including, trypsin and chymotrypsin (Gebhard, *et al.*, 1986, Hewlett, 1990).

Enzymes not inhibited by aprotinin include papain, pepsin, rennin and lysozyme. Proteinases may further be classified by an amino acid residue that is present at the catalytic site. As such, aprotinin inhibits a family of proteinases that contain a serine residue in the active site, known as serine proteases. In plants, it is thought that serine proteinases are involved in the defense response to pest invasion (Filho, 1992), where they discourage feeding by inhibiting the activity of digestive enzymes.

Aprotinin-like proteinase inhibitors have been found in a variety of organisms. Comparison of the inhibitory domain of aprotinin with aprotinin-type inhibitors (for example, bovine serum inhibitor, bovine colostrums inhibitor, snail trypsin inhibitor K, snake venom inhibitor NNV-II, silkworm inhibitor) indicate that the cysteine residues are fully conserved, suggesting that disulphide bridges are critical for correct protein folding (Gebhard *et al*, 1986) and function.

Aprotinin is a member of a class of cationic proteins that possess microbiocidal properties (Lehrer *et al.* 1989). In the broad sense, proteinase inhibitors eliminate unwanted proteolysis (Laskowski and Kato, 1980). Proteinase-inhibitors are ubiquitous, being found in different forms in different tissues types of animals, plants, and microorganisms. In seeds, the concentration of serine proteinase inhibitors may be present in concentrations, ranging from zero to 20% by weight (Filho, 1992). When found in the pancreas, protease inhibitors prevent premature activation of zymogens (protease precursors), and when in the blood, high concentrations serve to reduce blood clotting. In white blood cells, proteinase inhibitors are considered to be a non-oxidative defense mechanism because of bactericidal properties against Gram-positive and Gram-negative bacteria (Pellegrini *et al.*, 1992) and antiviral properties (Zhirnov *et al.*, 1982). As with other proteinase inhibitors and due to their effect on reducing the growth and the survival of insects (Steffens *et al.*, 1978; Brugess *et al.*, 1996), aprotinin has been incorporated into transgenic plants to confer pest resistance (Reeck *et al.*, 1997).

G. **Uses when Purified**

Aprotinin is a non-specific serine protease inhibitor that has been approved for use by the Food and Drug Administration (FDA) since 1991. Uses include laboratory applications, such as cell culture, protein purification, and diagnostic testing. As a therapeutic agent, it is useful in reducing bleeding after surgery, in suture-less wound closures (Hewlet, 1990) and in treatment of acute pancreatitis (Cox, A.G. 1977, Belorgey *et al.*, 1996). Aprotinin has further been shown to decrease blood transfusion requirements in pediatric patients undergoing craniofacial reconstruction (D'Errico, *et al.*, 2003), and spinal surgery (Cole, *et al.*, 2004), and in adult patients during cardiac bypass surgery. This reduction in blood loss, leads to reduce risks

associated with the exposure to banked blood components. Because aprotinin is not absorbed into the blood stream when taken orally (Trautschold, *et al*, 1967), aprotinin is given by intravenous route during drug therapy (http://www.medsafe.govt.nz/DatasheetPage.htm, accessed May 3, 2004 http://www.univgraph.com/bayer/inserts/trasylol.pdf, accessed May 4, 2004).

H. **Toxicity and Mutagenicity**
The results of microbial testing detected no mutagenic response in *Salmonella* and *Bacillus subtilis*. Given that aprotinin is not absorbed upon ingestion (Trautschold, *et al*, 1967), safety data has been generated using intravenous or intraparitoneal administration. The intravenous LD_{50} values obtained were approximately 312 mg aprotinin/kg in mice and greater than 125 mg/kg in dogs (Trautschold *et al.*, 1967).

I. **Routes of Current Exposure to Aprotinin**
What is critical to the assessment and evaluation of exposure to a compound is a determination of the amount of current exposure to a given compound during routine daily processes. Aprotinin is found in numerous organs in cow, sheep, and goat (Fritz and Wunderer, 1983), including cow liver, lung, heart, and blood serum.

The highest concentrations are typically found in the lung (140-210 g aprotinin/kg lung, (Fritz and Wunderer, 1983, Trautschold *et al.*, 1967) with lesser amounts in other organs such as liver (56 g aprotinin/kg liver). Thus, upon ingestion of beef liver, typical human exposure would be approximately 56 g aprotinin/kg liver.

J. **Toxicity to Insects**
Serine proteinase inhibitors, like aprotinin, are widely distributed in plants and may serve in a defensive function against insect infestation. As such, they are targeted for use in the genetic engineering of crop plants to produce pest-resistant transgenic plants (Filho, 1992). However, when the exposure levels of aprotinin is at concentrations of 0.1% or greater (weight of aprotinin: volume of sucrose diet) aprotinin has been shown to be significantly toxic to adult honeybees (*Aphis mellifera*)(Malone *et al.*, 1995, Burgess *et al.*, 1996). It is critical to compare the level at which a proteinase inhibitor is toxic in laboratory experiments to the level that organisms are exposed during field tests (see Section VII. Potential Environmental Impacts).

K. **Exposure to Non-target Organisms**
Aprotinin, because of its anti-protease properties, has been used in laboratory experiments to study proteases involved in frog embryogenesis (Iijima, *et al.*, 1999). At levels above 0.64 μM (= 4.2 mg/l) embryogenesis

was inhibited. Thus, proteinases may play an important role in the developmental processes.

L. **Exposure to Humans**

Aprotinin shares no nucleotide sequence homology with known allergens and toxins based on a search of public databases (see Appendix IV). Allergenic reactions to high intravenous doses of aprotinin during cardio-pulmonary bypass surgery have been reported in a small percentage of patients that have received the drug previously (http://www.medsafe.govt.nz/DatasheetPage.htm, accessed May 3, 2004 http://www.univgraph.com/bayer/inserts/trasylol.pdf, accessed May 4, 2004). The agency notes that if accidental ingestion would occur, similar routes of intravenous exposure would not occur, because aprotinin is not effectively absorbed from the digestive system.

APHIS concludes that aprotinin's potential to negatively impact organisms depends on its route of exposure, the amount of aprotinin present, and the presence of enzymes that degrade aprotinin.

M. **The Selectable Marker**

The donor of the selectable marker gene, *Streptomyces viridochromogenes*, is a common soil bacterium. This marker gene expresses the enzyme, phosphinothricin acetyltransferase. Phosphinothricin acetyltransferase confers tolerance to the herbicide glufosinate. This gene is well-characterized and is widely used as a selectable marker gene in the development of transgenic plants (Wehrmann *et al.*, 1996). This gene poses no known environmental risks and its use has been permitted in food and feed since 1995 by FDA. Decision documents written by the FDA on genetically engineered food additives can be found at: www.cfsan.fda.gov/~lrd/biocon.html.

APHIS has reviewed 21 petitions requesting deregulated status of genetically engineered plants that contain this enzyme and in each case, APHIS reached a finding of no significant impact (FONSI). The majorities were also reviewed by the FDA and/or the Environmental Protection Agency (EPA) (http://usbiotechreg.nbii.gov). In addition, Canada, the European Union, Japan, and other countries also approved food and feed use of plants containing this enzyme. The Organization for Economic Cooperation and Development (OECD) published a detailed risk assessment on this enzyme.
http://www.oecd.org/document/51/0,2340,en_2649_34385_1889395_1_1_1_1,00.html

Cauliflower mosaic virus, *Zea mays*, *Solanum tuberosum*, and *Hordeum vulgare* are donors for non-coding DNA regulatory sequences that are attached to the introduced genes to facilitate expression in plants. Some of these regulatory sequences were derived from plant pests. None of the DNA regulatory sequences can cause plant disease by themselves or in

conjunction with the genes that were introduced into the transgenic corn lines.

The Characterization of the Engineered Plant
The corn plants were engineered to express two genes: aprotinin and a selectable marker phosphinothricin acetyltransferase from *Streptomyces viridochromogenes*. These genes were introduced by a system using a nonpathogenic (disarmed) strain of *Agrobacterium tumefaciens*.

The promoter and terminator sequences to direct the PAT protein are the 35S cauliflower mosaic virus promoter. This promoter has been used extensively since its first discovery in 1985 (Nagy *et al.* and Odell *et al.)*. The phosphinothricin acetyl transferase gene has been well-characterized, reviewed by APHIS and other Federal agencies in the process of reviewing engineered plants (http://usbiotechreg.nbii.gov/).

The plant promoter preferentially expresses aprotinin in the seeds. APHIS reviewed the expression data that ProdiGene submitted. Aprotinin was not detected in pollen. The percent fresh weight limit of quantitation was 0.000471 for pollen tissue (0.0001% equals 1 mg/kg). Prodigene has submitted tissue specific expression data for APHIS review. According to that data, Aprotinin was primarily expressed in the seeds (100-300 mg/kg) which was 50 times higher than any other tissue examined. At flowering time when pollen was assayed, stem, leaf root, ear shoot and tassel tissue were also assayed. The range detection varied from undetectable in the roots (percent fresh weight limit of quantitation was 0.0000002) to a high of 0.000131 for tassel tissue. This data was generated for event APX 12, which will be planted on the largest acreage for production. Two other transformation events with constructs only differing in length of the promoter sequence have similar expression level pattern (see Appendix IV, page 17). The amount of aprotinin in the seed does not alter seed germination rates.

In conclusion, none of the genes or regulatory sequences alone, or in combination, poses a plant pest risk. Since the pathogenicity genes were removed from the vector, the use of disarmed *Agrobacterium tumefaciens* in the development of these plants poses no plant pest risk.

VI. DESCRIPTION OF THE FIELD TEST/AFFECTED ENVIRONMENT

A. Purpose:

The purposes of these proposed introductions are for: (1) grain production; (2) hybrid seed production; and (3) research/line development in a nursery. The introductions are proposed for two consecutive seasons. The first planting will be in late July/early August 2004, and the second in late February/early March 2005, in Frio County, Texas. In 2003, Frio County produced only 0.22% of all the corn produced in Texas. The adjacent counties in District 96 are also small corn producers. The State of Texas produces less than 5% of all the corn produced in the U.S.

(http://www.nass.usda.gov/tx/cecorna0.htm). The cumulative acreage planted under all proposed ProdiGene field tests in Frio County for 2004-2005 will not exceed one-thousandth of the Frio County's land mass. Frio County is not a major producer of corn.

ProdiGene has developed and APHIS has reviewed certain procedures that are designed to prevent the escape and dissemination of these plants. An overview of these procedures is provided on pages 8-12 of Appendix IV. Those items labeled "SOP" (standard operating procedures) identify more detailed work instructions/guidance. These have been approved by APHIS and have been claimed as CBI by ProdiGene.

B. **Plot Design**:
Open pollinated field test plots will be separated by a distance of at least 660 feet in order to maintain seed identity/purity of the various genetic constructs being field-tested. These plots will be surrounded by a fallow zone of 50 feet. The fallow zone may be planted with a low growing non-food, non-feed crop to prevent erosion. The hybrid seed production plot and the nursery will be surrounded by a 50-foot fallow zone, as well as four rows, (10 feet) of a non-transgenic male sterile hybrid corn. Some plots will be pivot irrigated and others flood irrigated.

Uncultivated rangeland is located east, south, and north of the site. To the west, there will be some cabbage, onions, and possibly pickling cucumbers, or the land will be fallow. The closest body of water is located south of the site and is a small contained reservoir for cattle. The Frio River is located 3-4 miles north of the test site.

C. **Breeding procedures**:
Grain production and hybrid production fields will be open pollinated. Controlled hand pollinations will be conducted in the nursery plot. Standard agricultural procedures for hand pollinations of corn will include bagging of the ear shoot prior to silk emergence. Tassels will be bagged only when needed for pollination. Hybrid seed production fields will be planted in a 1to 3, male to female ratio. The female rows will be detasseled before pollen shed and the border rows will be removed prior to viable seed formation. The female rows will be walked every 48 hours to remove tassels.

Agricultural practices consistent with growing healthy corn plants will be used. The plots will be kept reasonably weed free by herbicide applications. If necessary, pesticides such as insecticides and/or fungicides will be used to control pests such as corn rootworm, corn leaf aphids, European corn borer, corn earworms, and various leaf diseases that would diminish the health of the plant and subsequent grain yield. Any pesticides used will be applied by personnel trained in their use and application. The plot will be inspected weekly at first, and then daily during the pollination period. The farm will also be growing other crops such as onions, cucumbers, and

cabbage that are hand harvested. EPA registered chemical pesticides are likely to be used to control insect pest on these crops.

D. **Field Observation and Monitoring**:
The applicant thoroughly described field site monitoring and management practices that should provide the necessary degree of biological and physical confinement. Confinement practices include the following:

- The test site will be located more than one mile from the nearest non-engineered, non-test corn plant;
- The applicant will provide APHIS and State regulatory officials information on the location of the nearest corn plants that are not part of the field test;
- The applicant has provided APHIS and State regulatory officials a map of the proposed test site. One month after planting the applicant will submit a detailed map of the planted test site. Borders of the site will be described with GPS coordinates;
- A zone of 50 feet will be maintained surrounding the field test site. A non-food or non-feed cover crop may be planted in this zone to prevent erosion or may remain fallow; and
- In the subsequent growing season following harvest of the test plants, the test site and the 50-foot fallow zone may not be planted with corn unless the same field test/crop is repeated. The site will be monitored for volunteer corn plants throughout the next season. Any volunteer corn plants will be destroyed before flowering.

E. **Termination of the Field Test/Final Disposition of Test Plants**:
At harvest, the nursery seed and hybrid seed will be hand harvested and dried in a designated staging area at the field location using a dedicated drier. The nursery seed will then be packaged for shipment and hand-carried to a location designated in the permit application. The dried hybrid seed will be shelled and packaged in a designated staging area at the test site. The grain production fields will be machine harvested using a dedicated combine. Seed will be dried in a designated staging area at the field site and then ground to powder using a hammer mill. Milled corn flour will be shipped to designated locations. Any devitalized waste material from the milling operation will be returned to the field test site and incorporated into the soil.

F. **Security of the Field Test Plot**:
The test site is expected to provide adequate physical security. The contract farmer is the owner of the field test site.

VII. POTENTIAL ENVIRONMENTAL IMPACTS

A. **Alternative I**: No Action/ denial of permit request:

Field test would not proceed and no environmental impact would result.

B. **Alternative II**: Issue the permit:

The proposed field test is a controlled release of the regulated article into the environment. The proposed procedures for confinement of the plant material and for termination of the field test, as described in this document, should be sufficient to ensure that none of the genetically modified plants persist in the environment.

BRS has considered the information presented and independently assessed the risk of these products to the environment, to agricultural practices, to non-target organisms and to plant health. Such an assessment of risk considers two different components: hazard and exposure. Hazard is the toxicity or actual potential for harm of an event and exposure is the likelihood that the event will occur. Data that evaluates exposure has several components: (1) in what plant part(s) the proteins are produced; (2) the amount of protein produced; and (3) which organisms are likely to consume these tissues.

1. **Potential for Gene Transfer and Persistence of the Engineered Plant**:
   As described in the Biology of Corn section, corn is the only other plant that is sexually compatible with the experimental plants. Because this EA is being written months before planting, it is impossible to know how close the nearest corn field will be. Based on past plantings, the nearest corn field will be 2.5 miles away. Thus, whether there will greater than one mile isolation, as well as, temporal isolation cannot be firmly assessed at this time. Because corn pollen viability declines within a few hours, a distance of at least one mile between two corn fields is an effective means to mitigate gene flow, and given the small percentage of corn production in Frio County, APHIS concludes that for any corn plant pollinated outside the one mile isolation distance, would be at deminimus levels. APHIS concludes these measures meet the definition of confined field trial as developed by USDA's Agricultural Research Advisory Committee (ABRAC) (http://www.aphis.usda.gov/brs/pdf/abrac%201991.pdf).

   In corn, genes can escape by wind-born pollen or the persistence of corn seed in the environment. The applicant has described factors that will minimize dissemination of pollen to receptive, sexually compatible plants and persistence of the plant material after the conclusion of the field test (see Appendix IV).

   As described above, the nonengineered plant is not a weed. No change in general agronomic traits (leaf color, shape, growth habitat, days to

pollen shed, days to maturity) have been noted including seed germination rates.

2. **Impacts on the Use of the Marker Gene**:
The selectable marker, phosphinothricin acetyl transferase (bar), is also present in these plants. APHIS has reviewed 21 petitions for deregulating engineered plants that contain this enzyme and in each case reached a finding of no significant impact. The majority of these deregulated plants were also reviewed by FDA and/or EPA and are currently being marketed (http://usbiotechreg.nbii.gov/). In addition, Canada, European Union, Japan, and other countries have also approved use of plants containing this enzyme. The OECD published a detail risk assessment on this protein (http://www.oecd.org/document/51/0,2340,en_2649_34385_1889395_1_1_1_1,00.html). APHIS can identify no significant risk to any non-target organism or to the environment by the presence of this enzyme in these plants.

The plants use the 35S promoter from cauliflower mosaic (caulimo) virus to drive the production of bar protein. Although several issues have been raised about using coding sequences derived from plant viruses (OECD: Consensus Document on General Information concerning the Biosafety of Crop Plants Made Virus Resistant through Coat Protein Gene-Mediated Protection, http://www.olis.oecd.org/olis/1996doc.nsf/LinkTo/ocde-gd(96)162), the 35S promoter sequence used does not encode a protein. In addition, no caulimovirus naturally infects corn in Texas (American Phytopathological Society and APHIS' Widely Prevalent Virus by State, 2004-2005 at: http://www.aphis.usda.gov/brs/Stvir02.htm). Ho *et al.* (1999) raised some far fetched concerns about use of the 35S promoter from cauliflower mosaic virus. These hypothetical risks were soundly rebutted by Hull *et al.* (1999). APHIS fully concurs with Hull *et al* conclusions.

3. **Impact on Native Floral and Faunal Communities**:

a. **Vertebrates.**

Aprotinin is preferentially expressed in seeds. Aprotinin expression in stems, leaves, roots, ear shoots, tassels, and pollen is approximately 1/50 of that produced in the seed. Seed production of aprotinin is in the 100- 300 mg/kg range. . Vertebrates most likely exposed to aprotinin are seed eaters, e.g. mice, deer and birds. APHIS believes that consumption of these seeds would pose minimal, if any, risk for the following reasons.

- There is no significant absorption of aprotinin into the blood stream of vertebrates (Trautschold *et al.*, 1966);
- Upon **intravenous** introduction, the majority of aprotinin is rapidly excreted and the remaining is degraded by lysosomal enzymes in the kidneys (Kaller *et al.*, 1978; Torok, 1972; Trautschold *et al.*, 1966); and
  - Birds, rodents, and mice are most likely to consume seeds or cobs that are on the ground. To minimize exposure, harvest procedures and processing of the grain will occur in the field. Seeds will be buried and APHIS will inspect during harvesting, processing, etc. to ensure these conditions are met (see Appendix III). Even if carnivores consume vertebrates that eat the seeds, aprotinin would not be absorbed into the bloodstream. Further, it is already part of the diet of carnivores if they consume bovine liver or lung tissue.

Aprotinin is practically non-toxic by the oral route and moderately toxic by intravenous or intraparitoneal administration. However, non-target vertebrates will not be exposed by intravenous/intrapariteneal administration. Furthermore, expression levels of aprotinin in seeds are sufficiently low, thus vertebrates would need to consume unrealistic levels to reach the LD_{50} levels of aprotinin intravenous administration. Based on 300 mg per kg of aprotinin in seeds, to orally consume the LD_{50} dose at 30-gram mouse would need to consume about three times its body weight and for a 30-pound dog would have to consume its weight in corn. [The **intravenous** LD_{50} values obtained were about 2.5-6.5 million in mice, 2.5-5 million in rats, greater than 1.36 million in dogs, and 500,000 KIU/kg in rabbits (One million KIU units equals about 140 mg aprotinin. http://www.medsafe.govt.nz/DatasheetPage.htm)].

Therefore, APHIS concludes that vertebrates will not be significantly impacted by the field test or intermittent consumption of these corn seeds.

b. **Invertebrates.**

The survival of bees was reduced when fed ad lib in a sugar syrup/aprotinin at 10, 5, or 1 mg/ml but not at 0.1 or 0.01 mg/ml (Malone *et al* 1995; Burgess *et al* 1996). The lack of toxicity of aprotinin at low levels may be due to lysozyme activity that degrades aprotinin (Glinski and Buczek, 2003). Other studies have shown an effect of aprotinin or other serine proteinase inhibitors on bees (Brodsgaard *et al.*, 2003; Picard-Nizou *et al.*, 1997).

However, the engineered plant does not produce aprotinin at detectable levels in pollen. The percent fresh weight limit of quantitation was 0.000471 for pollen tissue (0.0001% equals 1 mg/kg).

At flowering time when pollen was assayed, stem, leaf root, ear shoot, and tassel tissue were also assayed. The amount of aprotinin ranged from undetectable in the roots (percent fresh weight limit of quantitation was 0.0000002) to a high of 0.000131 for tassel tissue. Therefore, the amount of aprotinin produced in pollen tissue is several logs lower than what has been published to have negative impacts on bees. In addition, the common chemical pesticides used to control coleopteran and lepidopertan insects have significant negative impacts on bees and other non-target invertebrates (see Appendix VI and VII). APHIS will require, as a proposed supplemental permit condition, that the applicant monitor for negative impacts on bees (see Appendix III).

APHIS notes that an EPA Science Advisory Panel (http://www.epa.gov/scipoly/sap/2000/june/finbtmamtox.pdf) suggested certain testing for plant-incorporated protectants. However, the focus of this meeting was Section 3 registration data requirements for large scale **commercial** use of plant-incorporated protectants not at the small scale experimental use. This field test is for small scale and the aprotinin is only expressed in the seeds and not at significant levels in roots, leaves, or stems. For use as a commercial plant-incorporated protectant, one would want expression in roots, leaves, or stems because that is where the most significant damage of lepidopteron and coleopteran corn pests occur. Currently, all plants engineered to control insect pests have gene expression in leaves, stems, and/or roots (http://usbiotechreg.nbii.gov/).

Aprotinin is expressed in levels in the seeds that are likely to negatively impact certain insects (e.g. red flour beetles) that consume seeds (Oppert *et al*., 2003). These insects are plant pests and chemical and biological control measures are available to minimize their damage (Herin and Meronuck, 1995; Cuperus and Krischik, 1995). It is generally agreed that products like aprotinin are high value (Freese, 2002) and that ProdiGene will be diligent in controlling pests that consume corn seeds. APHIS acknowledges that the development of resistance to plant-incorporated protectants can occur, but the likelihood of this occurring is extremely low considering the size of the field test versus the total acreage of corn produced in the State. APHIS notes that EPA has not required an insect resistance management plan for plant-incorporated protectants at the experimental use permit stage when the acreage is this small.

Thus, some plant pest insects may be negatively impacted by consuming aprotinin containing seeds. APHIS believes this does not pose a significant risk environment since chemical control and management procedures are routinely used to control plant pests.

Earthworms constitute about 90% of the invertebrate soil biomass (Ville *et al*., 1995). Earthworms produce lysozyme, (Ville *et al*., 1995;

Lassalle *et al.*, 1988) an enzyme that degrades aprotinin. Although aprotinin is not produced in roots, seeds that remain in the field would contain aprotinin. Based on a worst-case analysis, where 5% of the seeds would remain, the amount of aprotinin would be about 200 grams per acre (assuming 140 bushels per acre and 300 mg Aprotinin per kg seed). This is equivalent to approximately 50 mg per square meter. ProdiGene has submitted data demonstrating that aprotinin disappears as seed germination and seedling growth proceeds. As discussed in the review of aprotinin above indicate, serine proteinase inhibitors are common in many seeds, therefore earthworms are routinely exposed to serine proteinase inhibitors. As a precaution, APHIS will require ProdiGene to monitor aprotinin levels in the soil (see Appendix III).

c. **Aquatic organisms.**
The field site is several miles from the nearest natural body of water. It is highly unlikely that any significant amounts of plant debris, seed, or pollen (see section on biology) could be transferred from a field test site to impact aquatic organisms. The amount of aprotinin produced in all tissues except seeds is exceedingly low. Corn seeds do no float so in the unlikely event of a seed reaching a body of water, it would sink and rot. In addition, the enzyme lysozyme that degrades aprotinin is widely prevalent in aquatic species (Ito *et al.*, 1999: Maracano *et al.*, 1997; Sotelo-Mundo *et al.*, 2003).

In conclusion, given that aprotinin is predominantly expressed in the seeds, the only potential non-target organisms likely to be exposed are organisms that consume seeds. Insects and pathogens that consume seeds are clearly plant pests and will likely be controlled by chemical pesticides and management measures to minimize exposure. Vertebrates do not absorb aprotinin and thus consumption of these seeds poses no significant risk. APHIS concludes that these plants pose no significant risk to non-target organisms when the field test is performed under APHIS oversight.

4. **Potential Impacts to Threatened and Endangered species:**
As part of its on-going discussion with Fish and Wildlife Service on engineered organisms, APHIS met with the Fish and Wildlife Services in 2003. The discussion focused on the potential impacts of field testing plant-produced products that would require approval from FDA's Center for Biologics Evaluation and Research (human biologic), Center for Drug Evaluation and Research (human drug), Center for Veterinary Medicine (animal drug), or USDA's Center for Veterinary Biologics (animal biologic) before commercial use. A worksheet was developed for these types of products (see Appendix V).

Aprotinin produced in these transgenic plant seeds is not intended for drug use. As it is also not intended for food or feed, APHIS has **voluntarily** applied the FWS/APHIS/TES review procedures for this field test. See Appendix IV ,for ProdiGene's assessment worksheet. The only two threatened and endangered species identified in the county where the transgenic plants will be grown are carnivores. Based on the lack of effects on vertebrates of both aprotinin and phosphinothricin acetyl transferase, APHIS concludes that a "no harm" decision can be reached for these field tests. As is our policy when EA's are published, concurrent with this public comment period, APHIS is transmitting this EA to the Fish and Wildlife Service.

5. **Impact on Existing Agricultural Practices**:
There has been no intentional genetic change in these plants to affect their susceptibility to disease or insect damage. There is no reason to believe that other such characteristics are different in the transformed and untransformed plants. The selectable marker gene, designed to provide tolerance to the phosphinothricin-based class of broad-spectrum herbicides, is not expected to alter the susceptibility of the transgenic corn plants to disease or insect damage. The periodic monitoring of the test plots will allow the detection of any unexpected infestation by plant disease organisms or animal pests.

No impact on existing agricultural practices is expected. Agricultural practices consistent with growing healthy corn plants will be used. The plots will be kept reasonably weed free by herbicide applications. If necessary, pesticides, such as insecticides and/or fungicides, will be used to control pests such as corn rootworm, corn leaf aphids, European corn borer, corn earworms, and various leaf diseases that would diminish the health of the plant and subsequent grain yield. Any pesticides used will be applied by personnel trained in their use and application. The plot will be inspected weekly at first and then daily during the pollination period.

6. **Fate of Transgenic DNA**:
Transgenic DNA is no different from other DNA consumed as part of the normal diet. Genetically engineered organisms have been used in drug production and microbial fermentation (cheese and yogurt) since the late 1970's. More than 500 million cumulative acres of engineered food and feed crops have been grown and consumed world wide in the past seven years (International Service for the Acquisition of Agri-biotech Applications at: http://www.isaaa.org/kc/CBTNews/press_release/briefs30/es_b30.pdf. The FDA has not reported any significant concerns with bioengineered food and feed currently on the market. Based on lack of toxicity, the EPA has exempted from a pesticide tolerance DNA that are parts of plant-incorporated protectants FR 66 37817-37830).

There have been several studies in humans and animals following the fate of DNA once consumed (Beever and Kemp, 2000; Mercer *et al* 1999, 2001; Duggan *et al*, 2000; Chambers *et al.*, 2002; Netherwood *et al.* 2002; Einspanier *et al.*, 2001; Duggan *et al.*, 2003). The majority of DNA consumed is degraded in the gasto-intestinal tract although this is not 100% efficient. There is evidence that both transgenic and plant DNA can move from the gastro-intestinal tract lumen to other areas of the body and that this is a normal occurrence, but no risk has been identified.

Transfer and subsequent expression of DNA from the plant to bacteria is unlikely to occur due to impediments. First, transgene DNA promoters and coding sequences are optimized for plant expression not prokaryotic bacterial expression and the bacteria must be competent to accept DNA. Gebhard and Smalla (1998) and Schluter *et al* (1995) have studied transgene DNA movement to bacteria, and although possible, DNA transfer would occur at extremely low rates (approximately 1 in 10^{-14}). However, many genomes (or part thereof) have been sequenced from bacterial that are closely associated with plants including *Agrobacterium* and rhizobia (Kaneko *et al.,* 2000; Galibert *et al.,* 2001, Wood *et al.* 2001, Kanekko *et al.* 2002). There is no evidence that these organisms contain genes derived from plants. Syvanen (1994), Kumar and Rzhetsky (1996), Koonin *et al.* (2001), and Brown (2003) reviews of the literature using sequencing data reveals that horizontal gene transfer occurs occasionally on an evolutionary time scale of millions of years.

7. **Impacts on Human Health**:
Since the field test is on an isolated site on privately owned property, the public will not be exposed to the plants nor will they be exposed to the protein through pollen because aprotinin is largely absent from pollen. The seeds are unlikely to be mixed with seeds intended for human or animal consumption because of numerous measures (described in Appendix III) and APHIS inspections during harvesting and processing. The corn seed (or products) will not be sold as food, thus the most likely humans to be exposed are personnel working with the plants. Although allergic reactions in humans to corn pollen, corn seed debris, and powdered enzymes are known, routine precautions will minimize exposure (Colten and Streider, 1980; van Toorenenbergen *et al.*, 1991). The Occupational Safety and Health Administration (OSHA) in its guidance on biotechnology (FR 51:23347-49) states:

> "... Section 8 of the Act [29 U.S.C. 651 *et seq.*] authorizes OSHA to inspect workplaces including laboratories and places of employment relating to biotechnology Section 5(a)(1) of the Act requires that each employer furnish to each of his employees employment and a place of employment which are [sic] free from recognized hazards that are causing or likely to cause death or serious physical harm". APHIS has discussed with ProdiGene its responsibilities with respect to worker exposure.

Registrations of pesticides are under constant review by the U.S. Environmental Protection Agency. APHIS encourages applicants to use only pesticides that bear the EPA registration number and carry the appropriate directions.

Any food or feed uses of transgenic plants must comply with the guidelines published in the Federal Register by FDA (57 *F.R.* 22984, May 29, 1992).

The FDA regulates human biologics, and human and animal drugs derived from bioengineered pharmaceutical plants intended for therapeutic, preventative, or diagnostic purposes. Biological products and drugs for use in humans are regulated by the Center for Biologics Evaluation and Research (CBER) and CDER under authority of the Public Health Service Act (PHS Act) (42 U.S.C. 262 *et seq.*) and the Federal Food, Drug, and Cosmetic Act (FDandC Act) (21 U.S.C. 301 *et seq.*). FDA also regulates animal drugs derived from bioengineered pharmaceutical plants, intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease in animals, or to alter the structure or function of the animal. New animal drugs and animal feeds containing new animal drugs are regulated by the Center for Veterinary Medicine (CVM) under authority of the FD&C Act. The FDA regulations are found at Title 21 of the Code of Federal Regulations (21 CFR).

The USDA regulates veterinary biologics through the Center for Veterinary Biologics 91 (CVB) within Veterinary Services in APHIS under the authority of the Virus, Serum, and Toxins Act (21 U.S.C. 151 *et seq.*). The USDA regulations are found at Title 9 of the Code of Federal Regulations (9 CFR) Parts 101-124.

8. **Cumulative Environmental Effects**:
This is the first field test of the engineered corn plants at this location. As a proposed permit condition, (see Appendix III) ProdiGene will develop an assay for aprotinin in soil prior to any future field permit applications to test whether Aprotinin will accumulate in the soil if repeated plantings occur.

9. **Special Considerations**:
Executive Order (EO)12898, "Federal Actions To Address Environmental Justice in Minority Populations and Low-Income Populations," requires Federal agencies to conduct their programs, policies, and activities that substantially affect human health or the environment in a manner so as not to exclude persons and populations from participation in or benefiting from such programs. It also enforces existing statutes to prevent minority and low-income communities from being subjected to disproportionately high and adverse human health or environmental effects. Each alternative was analyzed in its ability to affect minority and low-income populations. None of the alternatives was found to pose disproportionately high or

adverse human health or environmental effects to any specific minority or low-income group.

EO 13045, "Protection of Children from Environmental Health Risks and Safety Risks," acknowledges that children may suffer disproportionately from environmental health and safety risks because of their developmental stage, greater metabolic activity levels, and behavior patterns, as compared to adults. The EO (to the extent permitted by law and consistent with the agency's mission) requires each Federal agency to identify, assess, and address environmental health risks and safety risks that may disproportionately affect children. None of the alternatives is expected to have disproportionately high or adverse human health or environmental effects to children.

EO 13112, "Invasive Species", states that federal agencies take action to prevent the introduction of invasive species and provide for their control and to minimize the economic, ecological, and human health impacts that invasive species cause. The nonengineered plant is widely prevalent in the U.S. Based on the data submitted by the applicant and reviewed by APHIS, the engineered plant is not significantly different in any fitness characteristics from its parent that might increase its invasive potential.

C. **Alternative III:** Issue the permit with additional conditions:

The potential environmental impacts under this alternative include all those noted under Alternative II.

In accordance with 7 CFR 340.4(b), APHIS has submitted a copy of the CBI deleted permit request for State notification and review. If the State has additional conditions, APHIS will consider making the State conditions part of APHIS' final permit conditions. In addition, if public comments are received regarding certain risks, APHIS will also consider making these comments part of the final decision.

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Appendix I. OECD Consensus Document on the Biology of *Zea mays* subsp. mays (maize)

OECD Environment, Health and Safety Publications

Series on Harmonisation of Regulatory Oversight in Biotechnology

No. 27

Consensus Document on the Biology of *Zea mays* subsp. *mays* (Maize)

Environment Directorate

Organisation for Economic Co-operation and Development

Paris 2003

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ABOUT THE OECD

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation in which representatives of 30 industrialised countries in North America, Europe and the Pacific, as well as the European Commission, meet to co-ordinate and harmonise policies, discuss issues of mutual concern, and work together to respond to international problems. Most of the OECD's work is carried out by more than 200 specialised Committees and subsidiary groups composed of Member country delegates. Observers from several countries with special status at the OECD, and from interested international organisations, attend many of the OECD's Workshops and other meetings. Committees and subsidiary groups are served by the OECD Secretariat, located in Paris, France, which is organised into Directorates and Divisions.

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FOREWORD

The OECD's Working[1] Group on Harmonisation of Regulatory Oversight in Biotechnology decided at its first session, in June 1995, to focus its work on the development of ***consensus documents*** which are mutually acceptable among Member countries. These consensus documents contain information for use during the regulatory assessment of a particular product. In the area of plant biosafety, consensus documents are being published on the biology of certain plant species, on selected traits that may be introduced into plant species, and on biosafety issues arising from certain general types of modifications made to plants.

This document addresses the biology of *Zea mays* subsp. *mays* (Maize). It contains general information as well as more specific information on taxonomy, identification methods, centre of origin/diversity, reproductive biology, crosses and agro-ecology. It is intended for use by regulatory authorities and others who have responsibility for assessments of transgenic plants proposed for commercialisation, and by those who are actively involved with genetic improvement and intensive management of the genus.

Mexico served as lead country (see Appendix E) in the preparation of this document. The document has undergone several rounds of revision based on the input from other member countries.

The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology has recommended that this document be made available to the public. It is published on the authority of the Secretary-General of the OECD.

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[1] In August 1998, following a decision by OECD Council to rationalise the names of Committees and Working Groups across the OECD, the name of the "Expert Group on Harmonisation of Regulatory Oversight in Biotechnology" became the "Working Group on Harmonisation of Regulatory Oversight in Biotechnology."

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PREAMBLE

OECD Member countries are now approving the commercialisation and marketing of agricultural and industrial products of modern biotechnology. They had previously therefore identified the need for harmonisation of regulatory approaches to the biosafety assessment of these products, in order to avoid unnecessary trade barriers.

In 1993, **Commercialisation of Agricultural Products Derived through Modern Biotechnology** was instituted as a joint project of the OECD's Environmental Policy Committee and Committee on Agriculture. The objective of this project is to assist countries in their regulatory oversight of agricultural products derived through modern biotechnology - specifically in their efforts to ensure safety, to make oversight policies more transparent and efficient, and to facilitate trade. The project is focused on the review of national policies, with respect to regulatory oversight that will affect the movement of these products into the marketplace.

The first step in this project was to carry out a survey concentrating on national policies with regard to regulatory oversight of these products. Data requirements for products produced through modern biotechnology, and mechanisms for data assessment, were also surveyed. The results were published in *Commercialisation of Agricultural Products Derived through Modern Biotechnology: Survey Results* (OECD, 1995a).

Subsequently, an OECD Workshop was held in June 1994 in Washington, D.C, with the aims of improving awareness and understanding of the various systems of regulatory oversight developed for agricultural products of biotechnology; identifying similarities and differences in various approaches; and identifying the most appropriate role for the OECD in further work towards harmonisation of these approaches. Approximately 80 experts in the areas of environmental biosafety, food safety and varietal seed certification, representing 16 OECD countries, eight non-member countries, the European Commission and several international organisations, participated in the Workshop. *The Report of the OECD Workshop on the Commercialisation of Agricultural Products Derived through Modern Biotechnology* was also published by the OECD in 1995 (OECD, 1995b).

As a next step towards harmonisation, the Working Group on Harmonisation of Regulatory Oversight in Biotechnology instituted the development of **consensus documents**, which are **mutually acceptable** among Member countries. The goal is to identify common elements in the safety assessment of a new plant variety developed through modern biotechnology, to encourage information sharing and prevent duplication of effort among countries. These common elements fall into two general categories: the first being the biology of the host species, or crop: and the second, the gene product. This document, Biology of *Zea mays* (maize), is the eighth crop plant chosen for review; the first being *Brassica napus* L. (Oilseed Rape), the second being *Solanum tuberosum* subsp. *tuberosum* (Potato), the third being *Triticum aestivum* (Wheat), the fourth being *Oryza sativa* (Rice), the fifth being *Glycine max (*L.) Merr. (Soybean), the sixth being *Beta vulgaris* L. (Sugar Beet) and the seventh being *Prunus* sp. (Stone Fruits).

Safety issues that could give rise to a safety concern are identified in the consensus documents on the biology of a specific crop and include the potential for gene transfer, weediness, trait effects, genetic and phenotypic variability, biological vector effects and genetic material from pathogens (OECD, 1993a). They make no attempt to be definitive in this respect, however, as the many different environments in which the crop species may be grown are not considered individually.

This document is a "snap-shot" of current information that may be relevant in a regulatory risk assessment. It is meant to be useful not only to regulatory officials, as a general guide and reference source, but also to industry, scientists and others carrying out research.

In using this document and others related to the biology of crop plants, reference to two OECD publications which have appeared in recent years will prove particularly useful. *Traditional Crop Breeding Practices: An Historical Review to Serve as a Baseline for Assessing the Role of Modern Biotechnology* (OECD, 1993b) presents information concerning 17 different crop plants. It includes sections on phytosanitary considerations in the movement of germplasm and current end uses of the crop plant. There is also a detailed section on current breeding practices. *Safety Considerations for Biotechnology: Scale Up of Crop Plants* (OECD, 1993a) provides a background on plant breeding, discusses scale dependency effects, and identifies various safety issues related to the release of plants with "novel traits".[2]

To ensure that scientific and technical developments are taken into account, OECD countries have agreed that consensus documents will be updated regularly. Additional areas relevant to the subject of each consensus document will be considered at the time of updating.

Users of this document are therefore invited to provide the OECD with relevant new scientific and technical information, and to make proposals concerning additional areas that might be considered in the future. ***A short, pre-addressed questionnaire is included at the end of this document. The information requested should be sent to the OECD at one of the addresses shown.***

2 For more information on these and other OECD publications, contact the OECD publications Service, 2 rue André-Pascal, 75775 Paris Cedex 16, France, Fax: (33) 01.49.10.42.76; E-mail: PUBSINQ@oecd.org; or consult http://www.oecd.org

SECTION I - GENERAL INFORMATION

1. Maize, or corn, is a member of the *Maydeae* tribe of the grass family, *Poaceae*. It is a robust monoecious annual plant, which requires the help of man to disperse its seeds for propagation and survival. Corn is the most efficient plant for capturing the energy of the sun and converting it into food, it has a great plasticity adapting to extreme and different conditions of humidity, sunlight, altitude, and temperature. It can only be crossed experimentally with the genus *Tripsacum*, however member species of its own genus (teosinte) easily hybridise with it under natural conditions.

2. This document describes the particular condition of maize and its wild relatives, and the interactions between open-pollinated varieties and teosinte. It refers to the importance of preservation of native germplasm and it focuses on the singular conditions in its centre of origin and diversity. Several biological and socio-economic factors are considered important in the cultivation of maize and its diversity; therefore these are described as well.

A. Use as a crop plant

3. In industrialised countries maize is used for two purposes: 1) to feed animals, directly in the form of grain and forage or sold to the feed industry; and 2) as raw material for extractive industries. "In most industrialised countries, maize has little significance as human food" (Morris, 1998; Galinat, 1988; Shaw, 1988). In the European Union (EU) maize is used as feed as well as raw material for industrial products (Tsaftaris, 1995). Thus, maize breeders in the United States and the EU focus on agronomic traits for its use in the animal feed industry, and on a number of industrial traits such as: high fructose corn syrup, fuel alcohol, starch, glucose, and dextrose (Tsaftaris, 1995). It is also noteworthy to understand how corn is used in the rising consumption of sweet corn and popcorn in developed countries (White and Pollak, 1995; Benson and Pearce, 1987).

4. In developing countries use of maize is variable; in countries such as Mexico, one of the main uses of maize is for food. In Africa as in Latin America, the people in the sub-Saharan region consume maize as food, and in Asia it is generally used to feed animals (Morris, 1998).

5. Maize is the basic staple food for the population in many countries of Latin America and an important ingredient in the diet of these people. All parts of the maize plant are used for different purposes: processed grain (dough) to make "tortillas", "tamales" and "tostadas"; grain for "pozole", "pinole" and "pozol"; dry stalks to build fences; a special type of ear cob fungi can be used as food (that is, "corn smut", or *Ustylago maydis*). In general, there are many specific uses of the maize plant depending on the region. Globally, just 21 % of total grain production is consumed as food.

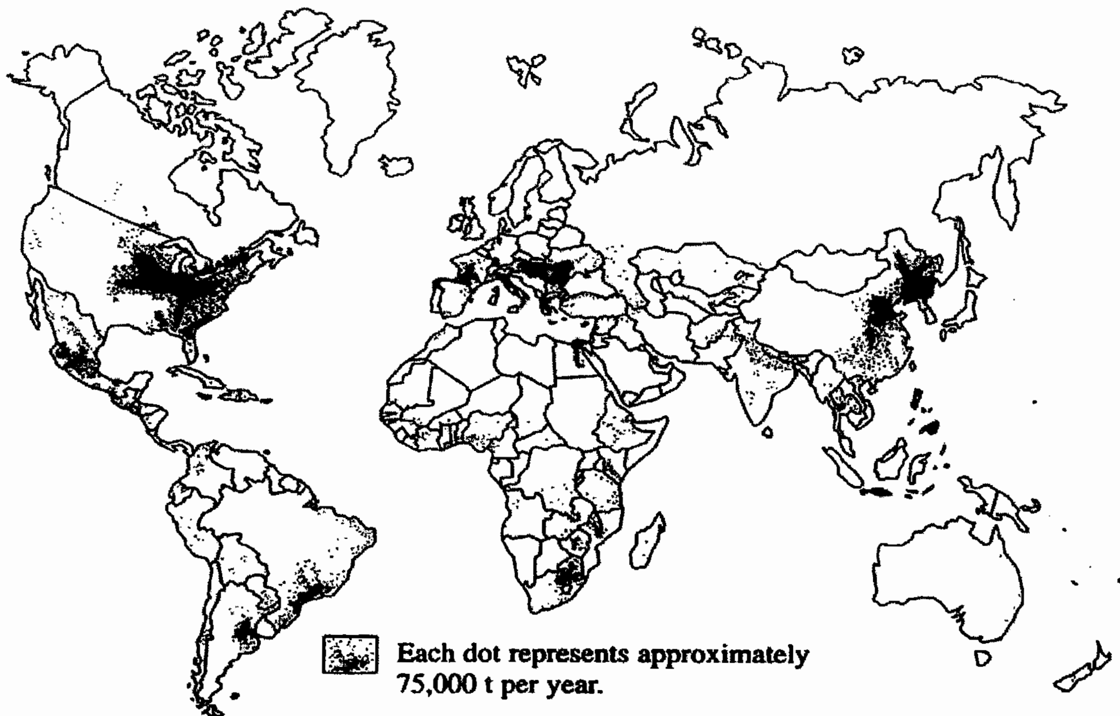
6. The countries, which have the highest annual maize consumption per capita in the world, are listed in Table 1.

Country	Annual consumption of maize per capita (Kg)
Malawi	137
Mexico	127
Zambia	113
Guatemala	103
Honduras	98
South Africa	94
El Salvador	93
Kenya	93
Zimbabwe	89
Lesotho	87
Venezuela	68
Nicaragua	56

Table 1: Consumption of maize *per capita* by country.

Source: (Morris, 1998).

Figure 1. Maize production worldwide.



Source: Morris, 1998.

7. According to Morris (1998), "maize is the world's most widely grown cereal, reflecting its ability to adapt to a wide range of production environments" (Fig. 1).

8. Transgenic maize is already being used as a crop not only with agricultural purposes in several industrialized countries. Industrialised countries have dominant production of maize, because they possess advantageous factors that contribute to generate maize surplus. First, "maize production is generally concentrated in zones of abundant rainfall and fertile soils" (Morris, 1998), and, second, the use of many inputs and technology is extensive (Pollak and White, 1995; Rooney and Serna-Saldivar, 1987; Shaw, 1988; White and Pollak, 1995). By contrast, in developing countries the situation is highly variable. From Mexico to the Northern Andean region in South America, maize is a very important staple food in rural areas and the use of technology together with improved varieties is limited. However, Brazil, Argentina and Chile resemble industrialised countries because in these countries maize is a "cash crop grown by large scale commercial producers using extensive mechanisation" (Morris, 1998).

9. In many countries of Latin America maize is produced on small units of land. For example, in Mexico most of the land planted with maize (77 %) is less than 5 hectares in size, which contributes 67 % of total production (Calva, 1992 in Turrent-Fernández *et al.*, 1997). Only 5 % of the units of land dedicated to the production of maize averaged 12.2 hectares. More recently (Turrent-Fernández *et al.*, 1997), land units of maize production have increased in size but the technology inputs are below average: only 40 % of producers utilised improved seed; 64 % used nitrogen and phosphorous to fertilise the soil; and only 42 % received technical assistance.

10. In Africa, maize is an important crop mainly in the eastern and southern regions where it is "the dominant food crop and the mainstay of rural diets" (Morris, 1998). Also, maize production in Africa is similar to the production in some Latin American countries because the peasants of less developed rural areas grow maize in small plots, using negligible amounts of inputs or technology and no improved varieties.

11. In Asia, China dominates maize production. China is the second largest producer of maize closely behind the United States (Morris, 1998). Asian countries produce maize for livestock feed and likewise Meso-America and most African countries; "farms are small, use of improved germplasm and purchased inputs is modest, and yields are generally low" (Morris, 1998).

SECTION II - TAXONOMIC STATUS OF *ZEA*

12. The Western Hemisphere genera *Zea* and *Tripsacum* are included in the tribe Maydeae (Table 2). The Asian genera of Maydeae are *Coix* (2n = 10, 20), *Polytoca* (2n = 20), *Chionachne* (2n = 20), *Schlerachne* (2n = 20) and *Trilobachne* (2n = 20).

13. Based on the morphology of the glumes of the male spikelets, Iltis and Doebley (1980) and Doebley and Iltis (1980) proposed a new classification system of the genus *Zea*. First, *Zea* was separated into two sections: LUXURIANTES and ZEA. The section LUXURIANTES grouped three species: *Z. luxurians*, *Z. diploperennis* and *Z. perennis*, and very recently it has included *Z. nicaraguensis* (Iltis and Benz, 2000). The section ZEA comprises only one species, *Z. mays*, which in turn is sub-divided into three subspecies: ssp. *mays*, for maize, ssp. *mexicana* for the races Nobogame, Central Plateau, Durango and Chalco (Wilkes, 1967; 1977) and ssp. *parviglumis*. This latter in turn is separated into two varieties, var. parviglumis for the race Balsas of Wilkes (1967) and var. huehuetenangensis for the race Huehuetenango of Wilkes (1967). Later on Doebley (1984, 1990) suggested that the var. huehuetenangensis should be elevated to a subspecies level.

14. Regarding the separation of the genus into sections LUXURIANTES and ZEA there is no controversy since morphological (Doebley, 1983; Smith *et al.*, 1981), isoenzymatic (Doebley *et al.,* 1984; Smith *et al.,* 1984), cytoplasm organelle DNA (Doebley *et al.,* 1987a, b; Sederoff *et al.*, 1981; Timothy *et al.*, 1979), and cytological (Kato, 1984; Kato and Lopez, 1990) evidence supports it.

15. The main controversy resides on the classification system within the section ZEA, particularly the grouping of the annual teosintes and maize into a single species, *Z. mays*. There is evidence showing that annual teosintes and maize are completely isolated from each other based on chromosome knob data (Kato, 1984; Kato and Lopez, 1990), and morphological-ecological data (Doebley, 1984). Although the isoenzymatic data suggest a low level of introgression between populations of these two plant types (Doebley, 1984; 1990), they have mainly the same isozyme alleles and the frequencies of these are distinct between most of the races of teosinte and most of the races of maize (Goodman, 1988). If it is accepted that the annual teosintes and maize are genetically isolated, then according to the biological species concept, the classification of the section ZEA made by Iltis and Doebley (1980) and Doebley and Iltis (1980) would not be acceptable, and would support the one proposed by Wilkes (1967).

16. Wilkes (1967) classified the annual teosintes within six races: Nobogame; Central Plateau; Chalco; Balsas; Huehuetenango; and Guatemala. Bird (1978) raised the race Guatemala into species rank, *Z. luxurians*.

17. The perennial teosintes from Jalisco in Mexico are separated into two more species (Iltis *et al.*, 1979) that have a ploidy difference, *Z. perennis* (2n=40) and *Z. diploperennis* (2n=20).

18. Doebley and Iltis (1980) and Iltis and Doebley (1980) classified teosinte as two subspecies of *Z. mays*: *mexicana* (Chalco, Central Plateau, and Nobogame) and *parviglumis* (var. parviglumis=Balsas and var. huehuetenangensis=Huehuetenango).

Table 2. Classification of the genus *Zea* within the tribe Maydeae of the Western Hemisphere, and the genus *Tripsacum*.

Family: Poaceae
Subfamily: Panicoideae
Tribe: Maydeae

Western Hemisphere:
Genus *Zea*[1]
Section *ZEA*
Zea mays L. (maize)
Zea mays subsp. *mays* (L.) Iltis (maize, 2n[2] = 20)
Zea mays subsp. *mexicana* (Schrader) Iltis (teosinte, 2n = 20))
race Nobogame[3]
race Central Plateau[3]
race Durango[4]
race Chalco[3]
Zea mays subsp. *parviglumis* Iltis and Doebley (teosinte, 2n = 20)
var. parviglumis Iltis and Doebley (=race Balsas)
var. *huehuetenangensis* Doebley (=race Huehuetenango)
Section *LUXURIANTES* Doebley and Iltis
Zea diploperennis Iltis, Doebley and Guzman (perennial teosinte, 2n = 20)
Zea luxurians (Durieu) Bird (teosinte, 2n = 20)
Zea nicaraguensis[5] (2n = 20?)
Zea perennis (Hitchc.) Reeves and Mangelsdorf (2n = 40)

Genus *Tripsacum*
T. andersonii (2n = 64)
T. australe (2n = 36)
T. bravum (2n = 36, 72)
T. cundinamarce (2n = 36)
T. dactyloides (2n = 72)
T. floridanum (2n = 36)
*T. intermedium (2*n = 72)
*T. manisuroides (2*n = 72)
T. latifolium (2n = 36)
*T. pereuvianum (2*n = 72, 90, 108)
T. zopilotense (2n = 36, 72)
T. jalapense (2n = 72)
*T. lanceolatum (2*n = 72)

T. laxum (2n = 36?) *T. maizar (2n =* 36, 72) *T. pilosum (2n =* 72)

[1] Iltis and Doebley, 1980; Doebley, 1990. [2] diploidy number. [3] Wilkes, 1967. [4] Sánchez-González et al., 1998. [5] Iltis and Benz, 2000.

SECTION III - IDENTIFICATION METHODS

A. General description of *Zea mays*

19. *Zea mays* is a tall, monoecious annual grass with overlapping sheaths and broad conspicuously distichous blades. Plants have pistillate inflorescences enclosed in numerous large foliaceous bracts (ears), from 7 to 40 cm long, with spikelets in 8 to 16 rows on a thickened axis (cob) in the leaf axils and staminate spikelets in long spike-like racemes that form large spreading terminal panicles (tassels).

B. Identification among races of *Zea mays*

20. To study and classify this huge variation, a system of racial classification was established (Wellhausen *et al*., 1952; Wellhausen *et al*., 1957; Brown, 1953; Sato and Yoshida, 1956; Hateway, 1957; Roberts *et al*., 1957; Briger *et al*., 1958; Timothy *et al*., 1961, 1963; Grobman *et al*.;1961; Grant *et al*., 1963; Brandolini, 1968; Mochizuki, 1968; Costa-Rodriguez, 1971; Paterniani and Goodman, 1977; Wellhausen, 1988; Avila and Brandolini 1990). Latin American countries, specifically Mexico, possess a great wealth of maize genetic diversity. There have been more than 40 land races of maize in Mexico (Wellhausen *et al*., 1952; Hernández-Xolocotzi and Alanís, 1970; Ortega-Pazcka, 1980; Benz, 1986; Sánchez-González, 1989), and almost 250 land races in the Americas (Goodman and Brown, 1988).

C. Identification among *Zea mays* and wild species

21. The closest known relative of *Zea* is *Tripsacum*. The genus *Tripsacum* comprises two sections: section FASCICULATA with five species; and section TRIPSACUM with twelve species. The chromosome number varies from 2n=36 to 2n=108. All species are perennials (deWet *et al*., 1982, 1983). Twelve of these are native to Mexico and Guatemala with an extension of *T. dactyloides* throughout the eastern half of the United States, the tetraploids being near the East coast and the diploid in the central region. *T. lanceolatum* occurs in the southwest of the United States and *T. floridanum* is native to South Florida and Cuba. Three species of *Tripsacum* are known in South America.

22. Species of the section FASCICULATA are mostly and widely distributed in Meso-America, however, *T. lanceolatum* is found along the North of Sierra Madre Occidental, Mexico, up to Arizona. On the other hand, species of the section TRIPSACUM are distributed more extensively than the section FASCICULATA, although different species are found in relatively restricted territories; for example, *T.*

dactyloides is found from a latitude about 42° North and 24° South. *T. dactyloides* tetraploid forms are also found in Kansas and Illinois in the United States. *T. manisuroides* is known only from Tuxtla Gutierrez, Chiapas, Mexico (deWet *et al.*, 1981, 1982, 1983). *T. andersonii* is of uncertain origin and is mostly sterile, it is an unusual species in that there is cytological (deWet *et al.*, 1983) and molecular evidence showing that its 2n=64 chromosomes comprise 54 *Tripsacum* chromosomes and ten *Zea* chromosomes (Talbert *et al.*, 1990).

D. Genetics and molecular identification

23. Maize has been one of the best studied plants in disciplines ranging from classical genetics to molecular biology. The study of maize has contributed to major breakthroughs in science such as the discovery of transposable elements (McClintock, 1929, 1934, 1944a, 1944b, 1944c, 1945; Fedoroff and Botstein, 1992). McClintock first characterised the ten chromosomes of maize using mitotic studies. Presently cytological research is being conducted on chromosome staining techniques, meiotic mutants, examination of the B chromosomes and better understanding of the events involved during synapsis. Transposable elements are very important in maize genetics. Many different transposable element systems have been described for maize, the best characterised has been the Activator (Ac) and Dissociation (Ds) system. Ac/Ds comprises a family of maize transposable elements. Ac is the autonomous member of the family, capable of producing a transposable factor needed for mobility. Ds elements are not autonomous and capable of transposition only when trans-activated by Ac. Both genes have now been cloned and their mode of action is well characterised (Tsaftaris, 1995). A recent review of transposable elements is found in Federoff (2000).

24. The genetics of mitochondria and chloroplast in maize are of special importance. The mitochondrial genomes (mtDNAs) of higher plants are larger than those of mammalian or fungal mitochondrial genomes. The higher plant mitochondrial genomes are also more variable in their organization and have a larger coding capacity than mitochondrial genomes in mammals and fungi. Five types of mitochondrial genomes have been identified. Their designations are NA and Nb for the normal male fertile phenotypes, and T, S and C for the three different cytoplasmic male sterile (cms) phenotypes. Physical maps for three of the maize cytotypes have been completed. Mitochondrial genomes of higher plants have integrated DNA sequences that originate from other cell compartments (Tsaftaris, 1995). In contrast to plant mitochondria genomes, the chloroplastic genome is smaller and simpler; thus many chloroplastic genomes have been completely sequenced. The similarities between the genomes of chloroplasts and bacteria are striking. The basic regulatory sequences, such as transcription promoters and terminators, are virtually identical in both cases. Protein sequences encoded in chloroplasts are clearly recognisable as bacterial, and several clusters of genes with related functions are organised in the same way in the genomes of chloroplasts, *E. coli*, and cyanobacteria. In about two-thirds of higher plants, including maize, the chloroplast as well as mitochondrial DNA, is maternally inherited (Tsaftaris, 1995).

25. There is an abundant literature on the genetics, physiology, cytogenetics and molecular biology of maize and concise, thorough reviews are available (Coe *et al.*, 1988; Carlson, 1988; Walbot and Messing, 1988; Hageman and Lambert, 1988; Freeling and Walbot, 1994).

E. Maize Genome Maps

26. The first RFLP map of corn was developed by Helentjaris *et al.* (1985, 1986a, 1986b). The corn linkage map encompasses approximately 1200 map units. The RFLP markers are not randomly distributed. The corn genome is about 5 X 10^6 kb, then there would be approximately 4 X 10^3 kb per map unit. It includes highly repeated sequences that constitute about 20% of the genome; these sequences are

present in about ten superabundant sequence types. There are more than 1000 different moderately repetitive sequence families collectively representing 40% of the genome, this leaves approximately 40% single copy sequences, or more than 10^6 approximately gene size pieces.

27. Maize has one of the most well saturated genetic maps of any cultivated plant of this genome size. In principle this offers the possibility of easily locating any transgene and/or identifying any specific genotype (Tsaftaris, 1995). Recent maize genome maps and most of the information on the maize genome can be found in the following web addresses: http://www.agron.missouri.edu; http://www.zmdb.iastate.edu; http://w3.aces.uiuc.edu/maize-coop/. An expressed sequence tag (EST) database can also be found at http://www.zmdb.iastate.edu.

SECTION IV - CENTRE OF ORIGIN / DIVERSITY, MAIZE DIVERSITY

28. There are four main hypotheses on the origin of maize.

1. **The descent from teosinte hypothesis**. This is the oldest proposal and was advanced by Ascherson in 1895 (Mangelsdorf and Reeves, 1939) and proposes that maize was domesticated from teosinte by human selection. This is the most widely accepted hypothesis at present (Beadle, 1986; deWet and Harlan, 1972; Doebley and Stec, 1991; Doebley, 1990; Galinat, 1977; Iltis and Doebley, 1980; Goodman, 1988; Kato, 1984; Kato and López, 1990; Timothy *et al.,* 1979). The main problem with this hypothesis was how the distichous small female spike could have been transformed into the polistichous gigantic maize spike (ear) by human selective domestication. However, Doebley *et al.* (1990) have found five major genes controlling 'key' traits distinguishing maize and teosinte, and more recently Wang *et al.,* (1999) have discussed a gene controlling the inflorescence character in teosinte and maize.

2. **The tripartite hypothesis**. The main assumption of this hypothesis is that there existed a wild maize in the past, which is considered extinct at present. This wild maize gave origin to the annual teosintes by crossing with *Tripsacum*. Further crossing of teosinte with wild maize gave rise to the modern races of maize (Mangelsdorf and Reeves, 1939; and Mangelsdorf, 1974). Later on Mangelsdorf *et al.,* (1981) based on experimental crossing between *Z. diploperennis* and the race Palomero Toluqueño of maize and further observations of its progenies, proposed that the annual teosintes are the products of this crossing. The fact that until now no evidence at all has been found about the existence, in the past or at present, of a wild maize, this hypothesis has lost much credence with time (although see Eubanks, 1995).

3. **The common origin hypothesis**. This hypothesis proposes that maize, teosinte and *Tripsacum* originated by "ordinary divergent evolution" from a common ancestor. Consequently, it is conceived that there existed a wild maize plant that further was transformed into a cultivated plant by the selection and care of man (Weatherwax, 1955; Randolph, 1955; Randolph, 1959). The postulation that wild maize existed in the past makes this hypothesis not acceptable, as in the case of the tripartite hypothesis.

4. **The catastrophic sexual transmutation hypothesis**. This hypothesis proposes that the maize ear evolved from the terminal male inflorescence of teosinte lateral branch by a "... sudden epigenetic sexual transmutation involving condensation of primary branches [and further] genetic assimilation under human selection of an abnormality, perhaps environmentally triggered" (Iltis, 1983). The finding of five mutant genes controlling key characters separating maize from teosinte (Doebley and Stec, 1991; Doebley *et al.,* 1990) seems to make the catastrophic sexual transmutation hypothesis untenable.

Centre of maize domestication

29. The Meso-American region located within middle South Mexico and Central America is recognised as one of the main centres of origin and development of agriculture as well as centre of origin and diversification of more than one hundred crops (Vavilov, 1951; Smith, 1995; Harlan, 1992). At the present time, there is no agreement about where exactly maize was domesticated and there are several proposals in this regard. Based on the findings of archaeological materials from the maize plant (pollen, cobs, husks, and other remnants) in the United States and Mexico, which are older than those found in South America, Randolph (1959) proposed that maize was domesticated, independently, in the southwestern United States, Mexico, and Central America.

30. Mangelsdorf (1974) proposed that "corn had not one origin but several in both Mexico and South America", because the archaeological evidences are found in Mexico and several morphological characteristics in extant population found in the maize races of South America (Andes region) in comparison to those races of Meso-America.

31. The preliminary studies of McClintock (1959, 1960) on the chromosome knob constitutions of several races of maize from South America, Mexico and Central America, led her to conclude "that present-day maize may have derived from several different centres". These chromosome studies were further exploited (Kato, 1976, 1984; McClintock, 1978; McClintock *et al.*, 1981). They confirmed McClintock's previous conclusion and led to the proposal that maize was domesticated, independently, in four centres located in Mexico (two in Oaxaca-Chiapas region, one in the central highlands and one in the mid-highlands of Morelos-northern Guerrero), and one in the highlands of Guatemala. "This conclusion is based on the fact that chromosome knobs are not geographically and racially distributed at random, and that some knobs show restricted distributions following clear-cut pathways through specific territories, dispersion that clearly indicate that they were started in specific regions or centres of distribution. These centres are then considered as the places where original maize germplasm was domesticated from teosinte populations that were already cytogenetically well diversified" (Kato, 1984).

32. Contrary to the above multicentres origin of maize proposals, the isoenzymatic variation studies of maize and teosinte suggested to Doebley *et al.* (1987a) that maize was domesticated once in the Balsas basin region because "... all maize races of Mexico are isoenzymatically closer to var. parviglumis than to other teosintes...". Supporting this hypothesis, further molecular genotyping studies also suggest that maize originated from a single domestication in southern Mexico 9000 years ago (Matsuoka *et al.*, 2002).

Maize Diversity

33. From the time of the discovery of America, Columbus noted the presence of corn on the North coast of Cuba and introduced it to Europe through Spain. At that time, corn was grown from Chile to southeastern Canada. Within two generations, after its introduction in Europe, corn became a cultivated crop throughout the world (Goodman, 1988). Germplasm resources are preserved *ex-situ* in many parts of the world, however, only in the Meso-American region there still exists, *in situ*, the original ancient maize that gave rise to improved varieties that are grown in all regions of the world. Most of the maize variation can be found in the Meso-American region and the northern part of South America. The great diversity of environments and conditions have created the basis for the development of maize varieties well adapted to harsh conditions of soil and climate as well as to biotic stresses. There is a close correlation among community culture, production system and the type of consumption of maize, with the diversification and variation of maize (Aguirre *et al.*, 1998; Louette and Smale, 1998).

34. Maize germplasm diversity is threatened by several factors: improved seed adoption; shift to cultivation of cash crops; and change in land use (Aguirre *et al.*, 1998; Bellon *et al.*, 2000; Louette, 1997). In some areas the adoption of hybrids and improved seed has increased dramatically, which has reduced the production of maize for traditional uses and, consequently, the increase of genetic erosion. Although these factors play an important role in reducing maize germplasm diversity, the persistence of maize land races in the Central American region is evident. Small farmers, peasants and indigenous ethnic groups and communities in many Latin American countries still preserve and select traditional maize.

35. Some arguments to explain the maize land race survival have been advanced (Ortega-Pazcka, 1973). The paramount importance of native maize for small communities, ethnic groups, small farmers and peasants, resides in the fact that land races of maize have very specific qualities for food and special uses as mentioned in Section I, rather than maize yield itself; therefore, many land races of maize have not been displaced by more productive maize types promoted by governmental agencies. For example, in Mexico after 50 years of maize genetic improvement programs, the adoption of hybrids and improved varieties is low. The research of Hernández-Xolocotzi (1972), Ortega-Pazcka (1973), Benz (1986), and Ortega-Pazcka *et al.* (1988), on maize diversity and peasant communities, demonstrates that local maize has been preserved by peasants, using traditional methods, basically intact for decades. As the result of a poll carried out in 1992 (CIMMYT, 1994), it was concluded that open pollinated land races of maize cover 42% of arable land dedicated to maize in less developed countries.

36. The approach for conservation of Latin American maize land races relies on two main criteria: the adaptation to a particular ecological niches and special forms of consumption of specific land races. Native germplasm utilisation has varied depending on the country and the needs of development. In general, the strategy is to identify sources of elite germplasm by means of characterizing and evaluating samples from land race collections, consisting of composite groups, populations and pools. National programs, international institutions, private seed industries and universities use these germplasm materials. Native maize land races have not been widely used for improvement programs and in Mexico, for example, only 10% of Mexican maize land races have been incorporated in specific breeding programs. There are a couple of examples in Mexico where native races of maize were characterised and evaluated for selection to generate improved populations, which were released as new open pollinated varieties: variety V520 (from land race San Luis Potosí-20); and variety Rocamex V7 (from land race Hidalgo-7). However, there is still germplasm in farmers' fields that have not been evaluated for their improvement and utilisation (Márquez-Sánchez, 1993).

37. Examples of maize land races specifically adapted to special conditions are (Hernández-Xolocotzi, 1988): Gaspe, short growing season (early maturity); Guatemalan Big Butt, long growing season (late maturity); Tuxpeño, Celaya, Chalqueño, Cuban Yellow Flint and Cuzco Gigante, high efficiency and productivity under good rainfed conditions; Chococeño, Enano and Piricinco, tolerance to high temperature and humidity; Cónico norteño, tolerance to semi-dry environments; Palomero Toluqueño, Cónico, Cacahuacintle and Sabanero, well adapted to high elevations, low temperature; Nal-tel, adapted to calcareous soil.

SECTION V - REPRODUCTIVE BIOLOGY

A. Sexual reproduction

38. *Zea mays* is an allogamous plant that propagates through seed produced predominantly by cross-pollination and depends mainly on wind borne cross-fertilisation. *Z. mays* is a plant with a protandrous inflorescence; however, decades of conventional selection and improvement have produced varieties of maize with protogynous traits. *Z. mays* has staminate flowers in the tassels and pistillate flowers on the ear shoots.

39. **The tassel.** The structure and development of the stamens are similar to other grasses. The anther develops four chambers or loculi each one containing a central row of archeosporial cells that gives rise to sporogenous tissue. After seven weeks the microspore mother cells are in the meiosis stage. Microspores are organised around four nuclei and become mature pollen grains. The amount of pollen produced by a tassel is estimated at 18 million pollen grains (Kiesselbach, 1980). Probably the best-improved varieties would produce more than this. On average 21,000 pollen grains could be produced for each kernel on an ordinary ear with 1000 kernels. Kiesselbach (1980) calculated that: "With a stand of three stalks in hills 42 inches apart, an area of 588 square inches is available in the field for each stalk. Thus an average of 42,500 pollen grains are provided for each square inch of the field. If the silks of an ear display a total surface of 4 square inches they will intercept about 170,000 pollen grains. Estimating 1,000 silks per ear, this amounts to 170 pollen grains per silk. Considering that corn in the field sheds pollen for 13 days, each silk receives an average of 13 pollen grains per day."

40. **The ear shoot.** At each node of the stem there is an axillary bud enclosed in the prophylum. Only one or two of these axillary buds will develop as ear shoot and reach the fertilisation stage. At first the ear is smooth but protuberances soon form in rows. The basal protuberances are formed first and development advances towards the tip of the ears. Each one becomes two lobed, each lobe developing into a spikelet with two flowers, only one of which commonly persists. The growing point of the upper flower is differentiated to form the functional pistil. The part above the attachment of the carpels develops a single sessile ovule, which consists of a nucellus with two integuments or rudimentary seed coats. The united carpels, which will form the ovary wall or pericarp of the mature kernel, grow upward until they completely enclose the ovule. Where they meet, the functionless so-called stylar canal is formed. The two anterior carpels, which face the ear tip, form outgrowths, which develop into the style or silk. The surface of the silk becomes covered with numerous hairs, which are developed from cells of the epidermis. At the base of the silk is a growth zone where new cells develop, causing continuous elongation of the silk until it is pollinated and fertilisation takes place. The development of the embryo sac is characteristic of the grass family. One of the three nuclei at the micropylar end enlarges and becomes the nucleus of the egg, while the others become the nuclei of the synergids. At this stage the embryo sac is ready for fertilisation but if pollination is prevented it may remain in this condition for some time, perhaps two weeks, after which the embryo sac and nucellus disorganise and fertilisation is no longer possible.

41. Fertilization occurs after the pollen grain is caught by the silk and germinates to create the pollen tube which penetrates up to the micropyle and enters the embryo sac. The pollen is carried mainly by wind, thus it is highlighted that pollination can occur even, although rarely, over long distances measured in kilo-meters.

B. Asexual reproduction

42. There is no asexually reproductive maize. Cell/tissue culture techniques can be used to propagate calli and reproduce tissues or plants asexually; however, with maize cells and tissues these techniques are difficult.

SECTION VI - CROSSES

A. Intra-specific crosses

43 Maize is essentially 100% open-pollinated (cross-fertilising) crop species. Until the 20th century, corn evolved through open pollinated varieties, which are a collection of heterozygous and heterogeneous individuals developed by mass selection of the people from the different civilizations existing in the Americas (Hallauer, 2000). Corn pollen is very promiscuous, lands on any silk, germinates almost immediately after pollination, and within 24 h completes fertilisation. Thus all corns will interpollinate, except for certain popcorn varieties and hybrids that have one of the gametophyte factors of the allelic series *Ga* and *ga* on chromosome four (Kermicle, 1997).

44 There is a great sexual compatibility between maize and annual teosinte and it is known that they produce fertile hybrids (Wilkes, 1977). In areas of Mexico and Guatemala maize and teosinte freely hybridise when in proximity of each other. Wilkes (1977) reported a frequency of one F1 hybrid (corn x teosinte) for every 500 corn plants or 3 to 5 % of the teosinte population for the Chalco region of the Valley of Mexico. Kermicle and Allen (1990) have shown that maize can introgress to teosinte; however, there is incompatibility between some maize populations and certain types of teosinte resulting in low fitness of some hybrids that prevents a high rate of introgression (Evans and Kermicle, 2001).

B. Inter-specific crosses

45 Although it is extremely difficult, *Tripsacum* species (*T. dactyloides*, *T. floridanum*, *T. lanceolatum*, and *T. pilosum*) can be crossed with corn; however, hybrids have a high degree of sterility and are genetically unstable (Mangelsdorf, 1974). Galinat (1988) advanced that since *Tripsacum* and *Zea* have different chromosome numbers, the addition of an extra *Tripsacum* chromosome into the maize genome would occur with a low frequency and consequently the rate of crossing-over would be extremely reduced. Despite these arguments, Eubanks (1995, 1998) developed a method for transferring *Tripsacum* genes into maize. In this method two wild relatives of maize, *Tripsacum* and diploid perennial teosinte (*Zea diploperennis*), are crossed to produce a hybrid, which is called tripsacorn, used to generate maize-tripsacorn hybrids. The use of tripsacorn is intended to confer resistance to pests and disease, drought tolerance and improved uniformity. Recently it has been claimed (Eubanks, 2000) that traits such as apomixis, totipotency, perennialism, adaptation to adverse soil conditions and to carbon dioxide enriched atmosphere can be transmitted to maize via maize x *Tripsacum*-perennial teosinte (and/or its reciprocal).

46 The cross between maize and *Tripsacum* has been studied since long ago (deWet *et al.*, 1973; Bernard and Jewell, 1985), and recently efforts have been made to transfer genes related to traits like apomixis from *Tripsacum* to maize (Burson *et al.*, 1990; Savidan and Berthaud, 1994; Hanna, 1995; Leblanc *et al.*, 1995; Grimanelli *et al.*, 1998; Grossniklaus *et al.*, 1998). Maize x *Tripsacum* hybrids have been produced and consequently several patents on apomictic maize have been published (Kindiger and Sokolov, 1998; Savidan *et al.*, 1998; Eubanks, 2000).

C. Gene flow

47 The interaction between domesticated plants and their wild relatives can lead to hybridisation and in many cases to gene flow of new alleles from a novel crop into the wild population (Ellstrand *et al.*, 1999). While gene flow *per se* is not a concern, theoretically, it can lead to the potential for the evolution of aggressive weeds or the extinction of rare species. There has been preliminary documentation of this in some cases although not for maize (Ellstrand *et al.*, 1999).

48 As mentioned in Section VI-A some teosinte species can produce fertile hybrids with maize. All teosintes, members of the Section LUXURIANTES and subspecies *mexicana* and *parviglumis*, occur only in Mexico and Guatemala (Sánchez-González and Ruiz-Corral, 1997). It has been documented that maize and teosinte often interact, particularly with *Zea mays* ssp. *mexicana* (Wilkes, 1977). Also, the known distribution of teosintes, together with high likelihood of the presence of land races in the maize production areas of Mexico indicates, as shown in Appendix B, that there exist high probabilities of genetic exchange between conventional maize, land races and teosinte (Sánchez-González and Ruiz-Corral, 1997; Serratos-Hernández *et al.*, 1997; Serratos-Hernández *et al.*, 2001). However, there is some evidence of restricted gene flow between *Zea* spp. that occurs predominantly from teosinte into maize (Doebley *et al.*, 1987a). To date, there is no genetic analysis of morphologically intermediate plants that could identify "whether the maize-teosinte intermediates are true hybrids, introgressants or crop mimics" (Ellstrand *et al.*, 1999). Out-crossing of maize with *Tripsacum* species is not known to occur in the wild.

49 Another factor to take into account regarding gene flow is the exchange of seed and traditional maize improvement practised by peasant communities and small farmers. As observed by Louette (1997), rural communities are open systems where "...there is a constant flow of genetic material among communities over large areas." therefore, as in the case of Mexico, "...a land race variety, an improved variety, or a transgenic variety of maize, can reach any zone of the country, even the most isolated ones, such as those where teosinte grows." The human factor together with the changes in policy and strategies in maize production (Nadal, 1999) may increase several fold the chance of gene flow between improved maize, teosinte and landraces.

SECTION VII - AGRO-ECOLOGY

A. Cultivation

50 Although maize was domesticated and diversified mostly in the Meso-American region, at present it is cultivated mainly in warm temperate regions where the conditions are best suited for this crop (Norman *et al.*, 1995).

51 Maize is an annual plant and the duration of the life cycle depends on the variety and on the environments in which the variety is grown (Hanway, 1966). Maize cannot survive temperatures below 0° C for more than 6 to 8 hours after the growing point is above ground (5 to 7 leaf stage); damage from freezing temperatures, however, depends on the extent of temperatures below 0° C, soil condition, residue, length of freezing temperatures, wind movement, relative humidity, and stage of plant development. Light frosts in the late spring in temperate areas can cause leaf burning, but the extent of the injury usually is not great enough to cause permanent damage, although the corn crop will have a ragged appearance because the leaf areas damaged by frost persist until maturity. Maize is typically grown in temperate regions due to the moisture level and number of frost-free days required to reach maturity. The number of frost-free days dictates the latitude at which corn varieties with different life cycle lengths can be grown. Maize having a relative maturity of 100 to 115 days is typically grown in the U.S. corn belt. Maize varieties with different relative maturities do not occur in parallel east-to-west zones because they are also dependent on prevailing weather patterns, topography, large bodies of water, and soil types (Troyer, 1994 in Hallauer, 2000).

52 In tropical regions, maize maturity increases due to altitude effects. Tropical land races of maize in the tropics characteristically show three to five ears and axillary tillering, as opposed to modern cultivars that suppress lower ears and tillers (Norman *et al.*,1995). In the tropics Oxisols, Ultisols, Alfisols and Inceptisols are best suited for maize production; however, maize is adapted to a wide variety of soils in the tropics, from sands to heavy clay. Of particular importance is aluminium toxicity for maize on acid tropical soils. Liming can solve this problem, "Deep lime incorporation in the subsoil of some Oxisols has overcome aluminium toxicity, thereby improving rooting depth in maize and tolerance to dry periods" (Norman *et al.*, 1995).

53 The farmland of Mexico covers a wide range of ecological conditions: from sea level to 2800 meters, from very dry to wet climates, well drained to poorly drained soils, flat to severe slopes, shallow to deep soils, low to high solar radiation; drought, wind and frost damage are common.

54 The poorest farmers are typically Indian farmers that inhabit the Sierras. Dry beans, squash, grain amaranth and several other species were also domesticated by the inhabitants of the region, as complements to their diet. They also developed the typical "milpa cropping system" as a cultivated field that may involve the association, inter-cropping, or relay-cropping of maize, beans, squash, grain amaranth, tree species and several tolerated herbal species. The isolation of these farming communities

has caused the development of a great resource of maize germplasm diversity, which is conserved using *in situ* and *ex situ* (germplasm banks) means. Inter-cropping of maize with other crops is practiced in many areas of less developed countries (Norman *et al.,* 1995). These systems imply changes at the level of cultivation and management of maize production which are important in terms of ecological relationships.

B. Volunteers and weediness

55 Maize has lost the ability to survive in the wild due to its long process of domestication, and needs human intervention to disseminate its seed. Although corn from the previous crop year can over-winter and germinate the following year, it cannot persist as a weed. The presence of corn in soybean fields following the corn crop from the previous year is a common occurrence. Measures are often taken to either eliminate the plants with the hoe or use of herbicides to kill the plants in soybean fields, but the plants that remain and produce seed usually do not persist during the following years. Volunteers are common in many agronomic systems, but they are easily controlled; however, maize is incapable of sustained reproduction outside of domestic cultivation. Maize plants are non-invasive in natural habitats (Gould, 1968). In contrast to weedy plants, maize has a pistillate inflorescence (ear) with a cob enclosed with husks. Consequently seed dispersal of individual kernels does not occur naturally. Individual kernels of corn, however, are distributed in fields and main avenues of travel from the field operations of harvesting the crop and transporting the grain from the harvested fields to storage facilities (Hallauer, 2000).

C. Soil ecology (Microbiology of Maize Rhizosphere)

56 Maize root system acts as a soil modifier due to its association with several microbial groups such as bacteria, fungi, actinomycetes (Vega-Segovia and Ferrera-Cerrato, 1996a), protozoa and mites. The highest microbial population usually is bacteria, followed by fungi and actinomycetes. All these microbial groups play a particular role in the soil ecology, such as nutrimental cycling and the availability of nutrients for plant growth. In addition, these microbial organisms contribute to the protection of the root system against soil pathogens.

57 Some research has been oriented to understand more on microbial activity and its physiology. For instance, the physiology of free nitrogen-fixing bacteria such as Azotobacter, Beijerenckia and Azospirillum which have been found in the rhizosphere of several maize cultivars and teosinte (González-Chávez *et al.*, 1990; González-Chávez and Ferrera-Cerrato, 1995; Vega-Segovia and Ferrera-Cerrato, 1996b).

58 There is information related to symbiosis with arbuscular mycorrhizal fungi (AMF) which shows that these endophytes associate with specific maize genotypes (González-Chávez, and Ferrera-Cerrato, 1989; González-Chávez and Ferrera-Cerrato. 1996). There are reports related to the capability of a single AMF to establish symbiosis with a wide range of maize land races and teosinte (Santamaría and Ferrera-Cerrato, 1996; Benítez *et al.* ;unpublished data). All these materials are used in Mexican agriculture. The role of these symbiosis relationships is to increase root metabolism in order to improve phosphorus uptake.

59 A great deal of life diversity is associated with maize grown in the milpa system of the Sierras. One example is the adaptation developed by a type of maize race in the Mixe Sierra of Oaxaca. The brace roots are overdeveloped and covered by a mucilaginous material that harbours species of nitrogen fixing free bacteria (R. Ferrera-Cerrato, personal comm.).

60 Soil ecology studies are undertaken to identify micro-organisms with agricultural value in places where maize is cultivated (Pérez-Moreno and Ferrera-Cerrato, 1997). Nowadays, these micro-organisms are being studied for the potential to augment corn cultivation. Selective breeding and nutrient management are also being evaluated for enhancing maize production.

D. Maize-insect interactions

61 In Appendix C, a list of common insect pests and pathogens of maize is presented.

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APPENDIX A: MAIZE BIOTECHNOLOGY

A) For practical purposes maize biotechnology could be divided into two fields: genetic engineering and molecular genetics.

B) Molecular genetics refers to the identification and location (genome mapping) of genes within the genome of organisms by means of molecular techniques that make use of the chemical properties of DNA (Hoisington *et al.*, 1998). The marker technologies presently available for genomics work are: 1) Restriction Fragment Length Polymorphisms (RFLPs); 2) Random Amplified Polymorphic DNAs (RAPDs); 3) Sequence Tagged Sites (STSs); 4) Simple Sequence Repeats (SSRs); 5) Amplified Fragment Length Polymorphisms (AFLPs); and 6) Single Nucleotide Polymorphisms (SNPs). These technologies have been applied in maize breeding through fingerprinting for identification of genotypes, monitoring genetic diversity and for the efficient management of genetic resources (Hoisington *et al.*, 1998). Other applications of molecular genetics and molecular markers are 1) Comparative Mapping, and 2) Marker Assisted Selection.

C) Genetic engineering methodologies can make possible the insertion of foreign DNA, from organisms of different species, into another individual organism. In maize, at the commercial level, the introduction of foreign DNA has been successfully accomplished through a technique known as biolistics. In this technique, DNA coated microparticles are shot by means of an air compression device, to cells in plant tissue or callus. In the case of maize, embryogenic callus is used for bombardment with foreign DNA. To identify the cells that have taken up the foreign DNA in maize, a herbicide resistant selectable gene has been used. Fertile transgenic maize plants have also been produced using 1) PEG-mediated protoplast transformation; 2) electroporation of intact or partly degraded cells of immature embryos, callus or embryonic suspensions; 3) 'whiskers' technology; and 4) *Agrobacterium*-mediated transformation.

D) At present there are two types of commercially released transgenic maize produced by means of genetic engineering: 1) Insect pest resistant maize or Bt-maize; and 2) Herbicide resistant maize. However, more research and development in this area is underway. Transgenic maize with elevated (10 KD) zein and methionine has been obtained (Anthony *et al.*, 1997). Antifungal proteins, such as chitinases and beta-1,3-glucanases, have been genetically engineered to attempt expression in the maize kernels with the aim to prevent the growth of *Aspergillus flavus* and the production of aflatoxins (Duncan *et al.*,1985; Wu *et al.*, 1994; Wan *et al.*, 1995). Transgenic maize will serve as bioreactors for producing various biomolecules with applications in food, feed and the pharmaceutical industry (Nikolov, 1999).

E) The complicated and plastic nature of organellar genomes especially those of maize mitochondria, requires special consideration for the stability of the cytoplasmic male sterility genes if they are used for preventing pollen formation. Equally these features of organelle genomes would also apply to any genes cloned into them (since recent developments indicate that organelles could be a better target for generating transgenic plants). Therefore, stable incorporation of a transgene into

the plastid genome guarantees amplification of the transgene, potentially resulting in a very high level of foreign gene expression. Since chloroplast (and mitochondrial) genomes resemble the genomes of other organisms and are most probably evolutionarily related, the possible transfer of genes from these organelles to microorganisms should be studied in the future if more and more transgenes are targeted to these organelles

F) The great similarity between the chloroplastic genome and microbial genomes was one of the reasons for choosing the chloroplast as a target for transferring native microbial genes to plants. For instance since the transcriptional machinery of the plastid is prokaryotic in origin and its genome is relatively A-T rich, it was possible that native Bt toxin genes from *B. thuringiensis* might be efficiently expressed in this organelle without nuclear modification. In addition, plant cells may contain up to 50,000 copies of the circular plastid genome.

G) Transposable elements are not expected to affect transgenes differently from their reported effects on non-modified genes of maize, unless sequences of the transposable element are contained in the inserted genetic material (Tsaftaris, 1995).

H) The potential crossing of landrace maize germplasm with transgenic improved maize, hybrids or inbreds should be considered carefully since, for example in Mexico, it is well known the high incidence of transposable elements in landraces of maize (Gutiérrez-Nava *et al.*, 1998).

I) Several investigations conducted by national and international research instititutions have demonstrated that gene exchange between improved maize and landraces is a continuing process taking place in small farmers' corn fields. The report on the presence of trangenes in peasants' maize fields of Oaxaca (Quist and Chapela, 2001), have been further demontrated by the Mexican government (INE-CONABIO, 2001), confirming that gene movement in traditional agriculture is an open system.

Weediness of transformed corn varieties

J) Gene transformation is the acquisition by a cell of new gene(s) by the uptake of naked DNA, which in the case of maize can be by direct introduction of DNA. As stated before, the more common applications of gene transfer in corn are insect resistance or tolerance to herbicides. Herbicide tolerance is usually conferred by single genes that interact with key enzymes in important metabolic pathways. Insect resistance is conferred by the expression of an insecticidal protein from *B. thuringiensis*. The overall phenotype of transformed plants with these two types of genes is similar to the original phenotype: the reproductive organs (tassels and ears), duration of plant development, methods of propagation, ability to survive as a weed, will not change with these two types of genes.

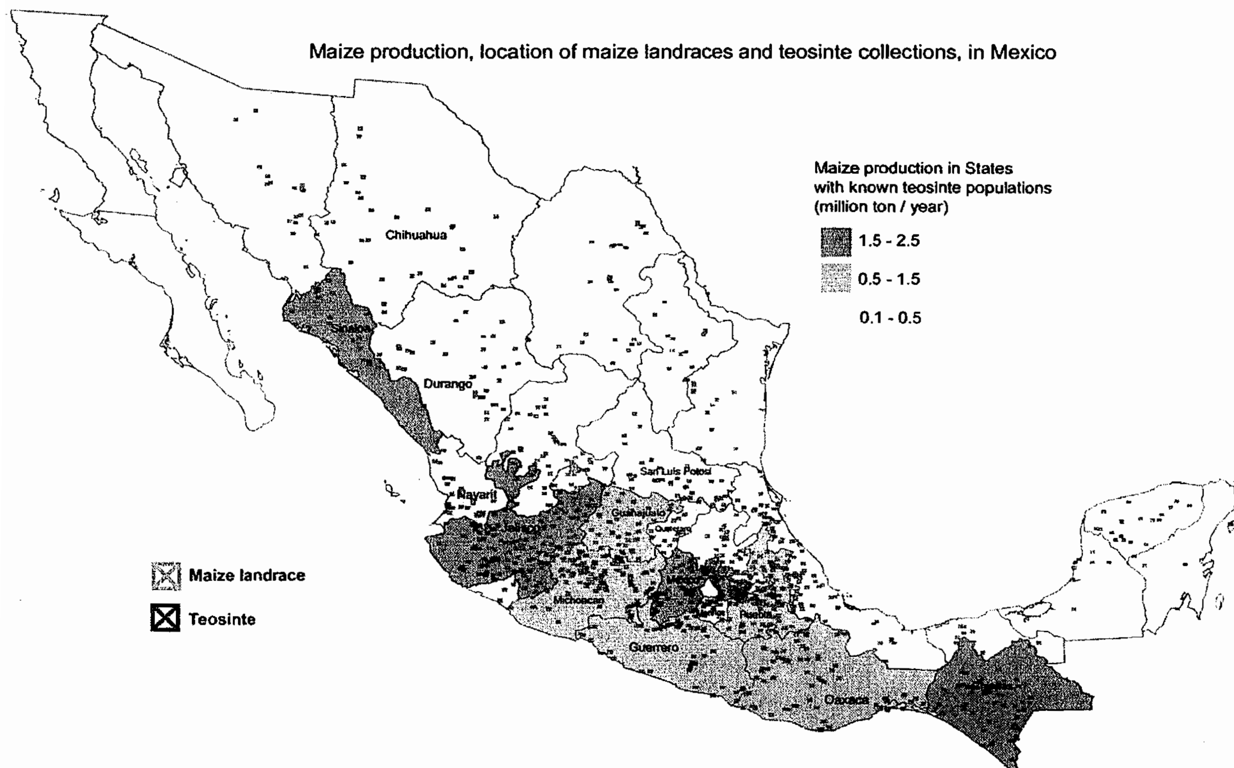
K) Gene exchange between cultivated corn and transformed corn would be similar to that which naturally occurs at the present time. Wind-blown pollen would move about among plants within the same field and among plants in nearby fields. Free flow of genes would be similar to that which occurs in cultivated corn. The transformed plants include individual genes, and depending on the relative expression of the transformed genes (relative levels of dominance for gene expression), plant architecture and reproductive capacities of the inter-crossed plants will be similar to non-transformed corn. With the transgenic maize that is available at this moment in the world, the chance that a weedy type of corn will result from inter-crossing of transgenic maize with cultivated conventional maize is remote.

L) Out-crossing of transformed corn plants with wild relatives of corn will be the same as for non-transformed corn plants. Out-crossing with teosinte species will only occur where teosinte is present in Mexico, Guatemala and probably in some other places of Central America. Out-crossing with *Tripsacum* species is not known to occur in the wild.

Unintended effects

M) The commercial release of transgenic maize expressing delta-endotoxin from *Bacillus thuringiensis* has driven the interest of ecologists concerned with the evolution of pest resistance to pesticide plants (Bergvinson *et al.*, 1997; Willcox and Bergvinson, 1997; Marvier, 2001; Obrycki *et al.*, 2001). The evolution of pest resistance is commonly known in any system where negative selection occurs from the use of traditional chemical pesticides, including plants bred traditionally for pest resistance. Recently, an effect of pollen from transgenic maize on the monarch butterfly larvae, a non-target insect, has preliminarily been described (Losey *et al.*, 1999). However, recent studies in the field have shown a less dramatic effect on non-target organisms (Wraight *et al.*, 2000; Hellmich *et al.*, 2001; Sears *et al.*, 2001; Zangerl *et al.*, 2001).

APPENDIX B: DISTRIBUTION OF MAIZE LANDRACES AND TEOSINTE IN MEXICO



Source: Serratos-Hernández *et al.*, 2001.

APPENDIX C: COMMON DISEASES AND INSECT PESTS OF MAIZE (CIMMYT AND DGSV GUIDES)

Maize	**Insect pests**	**Diseases**
Stalk	**Termites** *(Coptotermes formosanus)*, **Sugarcane borer** (*Diatraea saccharalis*), **Southwestern corn borer** (*Diatraea grandiosella*), **Neotropical corn borer** (*Diatraea lineolata*), **Asian maize borer** (*Ostrinia furnicalis*), **Spotted sorghum stem borer** (*Chilo partellus*), **African maize stem borer** (*Busseola fusca*), **African pink borer** (*Sesamia calamistis*), **African sugarcane borer** (*Eldona saccharina*), **Maize stem weevils** (*Cilindrocopturus adpersus*), **European corn borer** *(Ostrinia nubilaris)*.	**Charcoal rot** (*Macrophomina phaseoli*), **Diplodia stalk rot** (*Diplodia maydis*), **Gibberella stalk rot** and **Fusarium stalk rot** (*Fusarium* spp), **Brown spot** (*Physoderma maydis*), **Black bundle disease** (*Cephalosporium acremonium*), **Late wilt** (*Cephalosporium maydis*), **Maize bushy stunt disease** (MBSD), **Botryodiplodia stalk rot** (*Botryodiplodia theobromae*), **Maize lethal necrosis** (simultaneous infection of maize chlorotic mottle virus and either maize dwarf mosaic virus or wheat streak mosaic virus), **Maize chlorotic mottle virus** (MCMV), **Corn stunt disease** (*Spiroplasma*), **Pythium stalk rot** (*Pythium aphanidermatum*, *Pythium* spp.), **Erwinia stalk rot** (*Erwinia carotovora* f. sp. *zeae*)
Leaf	**Corn stunt leafhoppers** (*Dalbulus maidis*), **Maize streak virus leafhoppers** (*Dalbulus maidis, D. elimatus*), **Fall armyworm** (*Spodoptera frugiperda*), **Armyworm** (*Mythimna unipuncta*), **Spider mites** (*Oligonychus mexicanus*), **Corn leaf aphid** (*Rhopalosiphum maidis, R. padi*), **Maize Whorl Maggots** (*Euxesta* spp.), **Sugarcane Froghoppers** (*Aeneolamia postica, Prosapia simulans*), Chafers, **Grasshoppers** (*Sphenarium* spp., *Melanoplus* spp.).	**Downy mildew** (*Sclerospora* spp., *Sclerophthora* spp), **Curvularia leaf spot** (*Curvularia lunata* and *Curvularia pallescens*), **Cercospora leaf spot** (*Cercospora zeae-maydis*), **Septoria leaf blotch** (*Septoria maydis*), **Turcicum leaf blight** (*Helminthosporium turcicum*), **Diplodia macrospora leaf stripe** (*Diplodia macrospora*), **Phyllosticta leaf spot** (*Phyllosticta maydis*), **Helminthosporium carbonum leaf spot** (*Helminthosporium carbonum*), **Bacterial leaf stripe** (*Pseudomonas rubrilineans*), **Eyespot of maize** (*Kabatiella zeae*), **Leptosphaeria leaf spot** (*Leptosphaeria michotii*), **Maydis leaf blight** (*Helminthosporium maydis*), **Stewart's wilt** (*Erwinia stewartii*), **Maize dwarf mosaic**

	.	(MDMV), **Southern rust** (*Puccinia polysora*), **Common rust** (*Puccinia sorghi*), **Tropical rust** (*Physopella zeae*), **Zonate leaf spot** (*Gloeocercospora sorghi*), **Banded leaf and sheath spot** (*Rhizoctonia solani* f. sp. *sasakii*), **Tar spot** (*Phyllachora maydis*), **Brown spot** (*Physoderma maydis*) **leaf anthracnose** (*Colletotrichum graminicola*), Phaeosphaeria leaf spot, Fine stripe virus, Corn streak virus, Bacterial leaf stripe, Maize chlorotic mottle virus, Fine stripe virus, Fine mosaic virus I, Corn stunt disease, Black bundle disease.
Ear	**Ear maggots, Corn earworms** (*Helicoverpa zea*), **Stink bugs** (*Euschistus servus, Nezara viridula*), **Angoumois grain moth** (*Sitotroga cerealella*), **Indian meal moth** (*Plodia interpunctella*), **Grain weevils** (*Sitophilus granarius, S. zeamais*), **Grain borers** (*Prostephanus truncatus*).	Corn stunt disease, Botrydiplodia, Penicillium ear rot, Cladosporium ear rot, Giberella ear rot, Maydis leaf blight (T strain), Nigrospora ear rot, Tar spot, Black bundle disease, Maize dwarf mosaic, Downy mildew, Giberella ear rot, Helminthosporium carbonum ear rot, Banded leaf and sheath spot, Ergot of maize, Head smut, Aspergillus ear rots, Banded leaf and sheath spot, Maize stripe virus, Comon smut, Gray ear rot, Diploidia ear rot, Charcoal ear rot.
Tassel	**Corn stunt leafhoppers** *(Dalbulus maidis*), **Maize streak virus leafhoppers** (*Dalbulus maidis, D. elimatus*), **Fall armyworm** (*Spodoptera frugiperda*), Armyworm (*Mythimna unipuncta*), **Spider mites** (*Oligonychus mexicanus*), **Corn leaf aphid** (*Rhopalosiphum maidis, R. padi*), **Maize Whorl Maggots, Sugarcane Froghoppers** (*Aeneolamia postica, Prosapia simulans*), **Chafers, Grasshoppers** (*Sphenarium* spp., *Melanoplus* spp.).	Head smut, Downy mildew, Maize chlorotic mottle virus, Bacterial leaf stripe, False head smut, Corn stunt disease, Maize stripe virus.
Seed, Root, and Seedlin g	**Seedcorn maggots** (*Hylemya platura*), **Wireworms** *(Agriotes lineatus*), **Flea beetles** (*Phyllotreta* spp.), **Diabrotica beetles** (*Diabrotica* spp.), **Maize billbugs** (*Sphenophorus maidis*), **White grubs** (*Phyllophaga* spp., *Anomala* spp.), **Cutworms** (*Agrotis* spp.), Thrips	

	(*Frankliniella* spp.), **Lesser cornstalk borer** (*Elasmopalpus lignosellus*).	

APPENDIX D: MAIZE WORLD PRODUCTION

World Maize	Element			
	Seed (Mt)	Area Harvested (Ha)	Yield (Hg/Ha)	Production (Mt)
1961	6,223,099	105,484,151	19,435	205,004,683
1962	6,370,267	103,418,906	19,808	204,856,937
1963	6,193,721	108,384,382	20,319	220,228,333
1964	5,785,022	107,790,032	19,961	215,162,627
1965	5,988,088	106,591,240	21,252	226,524,256
1966	5,944,346	111,157,704	22,096	245,609,160
1967	5,872,917	112,313,038	24,266	272,538,473
1968	5,981,586	111,494,042	22,927	255,620,551
1969	5,838,480	111,242,302	24,226	269,491,068
1970	6,013,828	113,027,431	23,519	265,831,145
1971	6,185,867	118,150,571	26,544	313,622,622
1972	6,137,730	114,910,552	26,875	308,826,290
1973	6,132,362	116,856,034	27,238	318,290,469
1974	6,074,833	119,772,684	25,572	306,287,347
1975	6,429,594	121,442,141	28,133	341,656,971
1976	6,170,127	124,154,181	28,382	352,370,866
1977	6,181,283	125,192,168	29,679	371,561,355
1978	6,235,069	124,664,903	31,570	393,562,091
1979	6,281,256	123,598,634	33,866	418,577,993
1980	6,373,981	125,694,717	31,551	396,573,388
1981	6,440,288	127,816,716	34,950	446,722,107
1982	6,300,922	124,310,829	36,109	448,875,780
1983	6,605,234	117,763,540	29,468	347,024,034
1984	6,711,131	127,703,340	35,269	450,399,992
1985	6,646,135	130,454,042	37,214	485,474,301
1986	6,806,025	131,754,681	36,293	478,178,515
1987	6,623,584	129,888,090	34,880	453,054,894
1988	7,013,976	129,902,556	31,019	402,940,593
1989	7,158,041	131,711,470	36,203	476,833,660
1990	7,090,222	131,315,568	36,801	483,248,513
1991	7,379,181	134,125,220	36,851	494,267,664
1992	5,487,753	136,974,563	38,945	533,443,038
1993	5,497,737	131,500,199	36,242	476,576,466
1994	5,360,864	138,334,591	41,139	569,095,143
1995	5,474,640	136,271,016	37,914	516,655,836
1996	5,691,964	139,856,300	42,127	589,171,299
1997	5,588,723	141,270,173	41,407	584,954,064
1998	5,788,484	138,816,826	44,308	615,063,554
1999	5,765,380	138,460,288	43,786	606,261,782
2000	5,722,092	138,738,942	42,742	592,999,083
2001	5,912,420	137,596,759	44,273	609,181,620

Source: FAOSAT http://apps.fao.org

APPENDIX E: LIST OF CONTRIBUTORS AND PARTICIPATING INSTITUTIONS (FROM THE LEAD COUNTRY) IN THE PREPARATION OF THIS DOCUMENT

Name	Area of Expertise	Institution
J. Antonio Serratos-Hernández*	Biotechnology	*Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias* (National Institute of Research on Forestry Agriculture and Livestock)
Angel Kato	Genetic Resources (maize, teosinte)	*Colegio de Postgraduados* (Post-Graduated College)
Juan Manuel Hernández-Casillas	Maize germplasm bank	*Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias* (National Institute of Research on Forestry Agriculture and Livestock)
María Teresa Fernández-de-Castro	Regulation in biotechnology	*Escuela Nacional de Ciencias Biológicas, IPN.* (National School of Bioscience)
Antonio Turrent-Fernández	Maize program leader	*Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias* (National Institute of Research on Forestry Agriculture and Livestock)
Jorge Nieto	Molecular Biology	*Instituto de Biotecnología, Universidad Nacional Autónoma de México* (Biotechnology Institute, National Autonomous University of Mexico)
José Antonio Garzón-Tiznado	Biotechnology	*Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias* (National Institute of Research on Forestry Agriculture and Livestock)
Irineo Torres-Pacheco	Biotechnology	*Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias* (National Institute of Research on Forestry Agriculture and Livestock)
Alejandro Espinosa	Agronomy, Seed production	*Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias* (National Institute of Research on Forestry Agriculture and Livestock)
Eduardo Casas Díaz	Agronomy	*Universidad Autónoma de Chapingo* (Autonomous University of Chapingo)
Sofia Tinoco (R.I.P)	Regulation in biotechnology	*Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación - Dirección General de Sanidad Vegetal* (Secretariat of Agriculture, Livestock, Rural Development, Fisheries and Food - Phytosanitary General Direction)
Fernando Ortiz Monasterio	Environmental Management	*Comisión Intersecretarial de Bioseguridad y Organismos Genéticamente Modificados - CIBIOGEM* (Interministerial Commission on Biosafety and Genetically Modified Organisms)
Ignacio Ruiz Love	Social Sciences and Agriculture	*Comisión Intersecretarial de Bioseguridad y Organismos Genéticamente Modificados - CIBIOGEM* (Interministerial Commission on Biosafety and Genetically Modified Organisms)

* Document Co-ordinator

We acknowledge comments and support from the following persons:

Sergio Colín from *Instituto Nacional de Ecología* (National Institute of Ecology); Eduardo Morales and Jorge Larson from *Comisión Nacional para el Conocimiento y Uso de la Biodiversidad* (National Commission for the Knowledge and Use of Biodiversity); Ariel Rojo from *Dirección General de Vida Silvestre* (General Direction of Wildlife, Secretariat of Environment and Natural Resources), Veronique Deli and Araceli de la Llave from *Unidad Coordinadora de Asuntos Internacionales* – Secretaría de Medio Ambiente y Recursos Naturales (Foreign Affairs Coordination Unit- Secretariat of Environment and Natural Resources).

Appendix II. Standard Permit Conditions for APHIS Form 2000 (7 CFR 340.4)

(f) *Permit conditions.* A person who is issued a permit and his/her employees or agents shall comply with the following conditions, and any supplemental conditions which shall be listed on the permit, as deemed by the Administrator to be necessary to prevent the dissemination and establishment of plant pests:

(1) The regulated article shall be maintained and disposed of (when necessary) in a manner so as to prevent the dissemination and establishment of plant pests.

(2) All packing material, shipping containers, and any other material accompanying the regulated article shall be treated or disposed of in such a manner so as to prevent the dissemination and establishment of plant pests.

(3) The regulated article shall be kept separate from other organisms, except as specifically allowed in the permit;

(4) The regulated article shall be maintained only in areas and premises specified in the permit;

(5) An inspector shall be allowed access, during regular business hours, to the place where the regulated article is located and to any records relating to the introduction of a regulated article;

(6) The regulated article shall, when possible, be kept identified with a label showing the name of the regulated article, and the date of importation;

(7) The regulated article shall be subject to the application of measures determined by the Administrator to be necessary to prevent the accidental or unauthorized release of the regulated article;

(8) The regulated article shall be subject to the application of remedial measures (including disposal) determined by the Administrator to be necessary to prevent the spread of plant pests;

(9) A person who has been issued a permit shall submit to APHIS a field test report within 6 months after the termination of the field test. A field test report shall include the APHIS reference number, methods of observation, resulting data, and analysis regarding all deleterious effects on plants, nontarget organisms, or the environment;

(10) APHIS shall be notified within the time periods and manner specified below, in the event of the following occurrences:

(i) Orally notified immediately upon discovery and notify in writing within 24 hours in the event of any accidental or unauthorized release of the regulated article;

(ii) In writing as soon as possible but not later than within 5 working days if the regulated article or associated host organism is found to have characteristics substantially different from those listed in the application for a permit or suffers any unusual occurrence (excessive mortality or morbidity, or unanticipated effect on non-target organisms);

(11) A permittee or his/her agent and any person who seeks to import a regulated article into the United States shall:

(i) Import or offer the regulated article for entry only at a port of entry which is designated by an asterisk in 7 CFR 319.37-14(b);

(ii) Notify APHIS promptly upon arrival of any regulated article at a port of entry, of its arrival by such means as a manifest, customs entry document, commercial invoice, waybill, a broker's document, or a notice form provided for such purpose; and

(iii) Mark and identify the regulated article in accordance with 340.5 of this part.

Appendix III. Proposed supplemental permit conditions

SUPPLEMENTAL PERMIT CONDITIONS
Permit: *04-121-01r*

1. APHIS's Biotechnology Regulatory Services (BRS) and/or an APHIS PPQ Regional Biotechnologist or State Plant Health Official may conduct inspections of the test site, facilities, and/or records at the beginning of the test, mid-season or during flowering, and/or following harvest, and during the post-season monitoring period. The permittee is required to notify the appropriate State Regulatory Official(s) and the appropriate APHIS Regional Biotechnologist at least 1-week before the test begins and at least 1 week before the harvest/termination of the field test. Contact information for the APHIS PPQ Regional Biotechnologists are included on the attached map, and for the State Regulatory officials, this information is maintained at http://www.aphis.usda.gov/ppq/biotech/lt_sta.html.

2. The proposed procedures, processes, and safeguards which will be used to prevent escape, dissemination, and persistence of the transgenic plant and its progeny at each of the intended destinations as described in the permit application, in APHIS-approved standard operating procedures, and in these permit conditions must be strictly followed. The permittee must maintain records sufficient to verify compliance with these procedures. These records are subject to audit by APHIS. **APHIS, BRS must be notified of any proposed changes to the protocol referenced in the permit application.**

In addition, the following conditions will apply for field tests with plants expressing pharmaceuticals or biologics, consistent with APHIS' Federal Register Notice on Field Testing of Plants Engineered to Produce Pharmaceutical and Industrial Compounds (Docket No. 03-031-1):

A. **Authorization must be obtained from APHIS, BRS to grow crops for food or feed the following growing season(s) on the field test site and perimeter fallow zone, when there is a potential for volunteer plants to be inadvertently harvested with the crop.**

B. To ensure that regulated articles are not inadvertently removed from the site, planting and harvesting equipment must be dedicated to use in the permitted test site(s) for the duration of the test (the time of planting through the end of harvesting). **APHIS authorization will be required before this equipment is used elsewhere.** In addition, tractors and tillage attachments, such as disks, plows, harrows, and subsoilers, must be cleaned in accordance with the procedures submitted to and approved by APHIS before they are moved off of the test site. Seed cleaning and drying must be performed in accordance with the procedures submitted to and approved by APHIS that are designed to confine the plant material and minimize the risk of seed loss or spillage.

C. Dedicated facilities (locked or secured buildings, bins, or areas, restricted to authorized personnel only) must be used for storage of equipment and regulated articles for the duration of the field test. Facilities must be cleaned in accordance with the procedures submitted to and approved by APHIS before they are returned to general use.

D. **Within 4 weeks after planting, please submit a report that includes the following information for each site:**

1. A map of the site **including the GPS coordinates for each corner of the plot (inclusive of the border rows of any sexually compatible plants).**

2. The number of transgenic plants/seeds which were actually planted at the test site

3. The total acreage of the test plot (exclude border rows, if any)

4. A report which indicates the distance from the genetically engineered plants to the **nearest** plants of the same crop which will be used for food, feed, or seed production. A survey should be done within the distance specified in the chart below for any of these crop plants.

5. A list of the specific containment option(s) selected at **each** site if your permit allows different containment options (e.g. bagging flowers, border rows, or isolation distance).

Fax the report to:
1. The Biotechnologist who reviewed your application at Area Code (301) 734 8669
2. The Regional Biotechnologist (fax number enclosed)
3. The State official where the test is being performed
(see http://www.aphis.usda.gov/biotech/lt_sta.html for fax numbers).

Crop	Scouting Distance
Maize	One mile
Barley	One-eighth mile

Wheat	One-eighth mile
Rice	One-eighth mile
Tobacco	One-half mile

5. This approved Biotechnology Permit (APHIS form 2000) does not eliminate the permittee's legal responsibility to obtain all necessary Federal and State approvals, including: (1) for the use of any non-genetically engineered plant pest or pathogens as challenge inoculum; (2) plants, plant parts or seeds which are under existing Federal or State quarantine or restricted use; (3) experimental use of unregistered chemical; and (4) food, feed, pharmacological, biologic, or industrial use of regulated articles or their products (and co-mingled plant material). In the latter case, depending on the use, reviews by APHIS, the U.S. Food and Drug Administration, or the U.S. Environmental Protection Agency may be necessary.

6. Consistent with standard permit conditions at 7 CFR 340.4(f) (9), field test data reports must be submitted within 6 months after the end of the field test (final harvest or crop destruct). APHIS views these data reports as critical to our assessment of plant pest risk and development of regulatory policies based on the best scientific evidence. Failure by an applicant to provide data reports in a timely manner for a field trial may result in the withholding of permission by APHIS for future field trials. **Confidential Business Information (CBI) will be handled according to the APHIS policy statement at 50 F.R. 38561-63.**

7. Consistent with standard permit conditions at 7 CFR 340.4(f) (10), APHIS shall be notified verbally immediately upon discovery and in writing within 24 hours in the event of any accidental or unauthorized release of the regulated article

For immediate verbal notification, contact the following APHIS staff in the order indicated below.

1. APHIS BRS Deputy Administrator's office [phone numbers: (301) 734-7324; (301) 734-5745; (202) 720-4383)]. Indicate that you wish to report an authorized or accidental release of a regulated article to the BRS Compliance Officer, or alternatively, to the BRS Division 2 Director or permit reviewer. In the event that one of these persons cannot be reached, contact:
2. The appropriate APHIS PPQ Regional Biotechnologist.
3. The appropriate APHIS State Plant Health Official.

Contact information is maintained at the APHIS Biotechnology Regulatory Services website at http://www.aphis.usda.gov/ppq/biotech/.

Unless otherwise directed, written notification should be sent to:

Animal and Plant Health Inspection Service (APHIS)
BRS Compliance Officer, Rm. 5B50
4700 River Rd. Unit 147
Riverdale, MD 20737.

When the regulated article or associated host organism is found to have characteristics substantially different from those listed in the permit application, or suffers an unusual occurrence (excessive mortality or morbidity, or unanticipated effect on non-target organisms), APHIS shall be notified as soon as possible but no later than within 5 working days. In such cases, notice should be sent to

Animal and Plant Health Inspection Service (APHIS)

Tony Roman
Chief, Biotechnology Program Operations , Rm. 5B53
4700 River Rd. Unit 147
Riverdale, MD 20737.

8. ProdiGene must monitor daily for the presence and any mortality of bees during pollen shed. ProdiGene must prior to and during this monitoring period record all pesticide applications including type of pesticide applied and rate applied. This information must be submitted at the time of 6 month field data report

9. In order to arrange inspections, ProdiGene must notify BRS of planting date, expected harvest date, date of pollen shed, harvesting and seed processing.

10. Prior to any additional field tests under new permit at this site, ProdiGene must develop an assay for aprotinin levels in the soil. ProdiGene must collect and preserve soil samples from these site prior to and after harvesting for future assaying.

11. ProdiGene must quantify the amount of aprotinin in stems, leaves, tassels, ear shoot, pollen and seeds at flowering. At harvest, levels in stems, leaves and seeds. This data must be submitted with data report.

Appendix IV. Permit Application and TES Worksheet

PRGP04-04r

This application is authorized by the Federal Plant Pest Act (7 U.S.C. 150aa et seq. and the Plant Quarantine Act (7 U.S.C. 151 et seq)). The information will be used to determine eligibility to receive all types of permits. No permit shall be issued until this application has been approved.

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See reverse side for additional information

FORM APPROVED
OMB NO. -579-0085

U.S. DEPARTMENT OF AGRICULTURE
BIOTECHNOLOGY, BIOLOGICS, AND ENVIRONMENTAL PROTECTION

APPLICATION FOR PERMIT OR COURTESY PERMIT UNDER 7 CFR 340

(Genetically Engineered Organisms or Products)

INSTRUCTIONS: Complete this form and enclose the supporting materials listed on the reverse side. See page 3 for detailed instructions.

1. NAME AND ADDRESS OF APPLICANT

Dr. Donna Delaney
ProdiGene
101 Gateway Blvd., Suite 100
College Station, TX 77845

2. PERMIT REQUESTED ("X" one)

- [ ] Limited - Interstate Movement
- [ ] Limited - Importation
- [x] Release into the Environment
- [ ] Courtesy Permit

3. THIS REQUEST IS ("X" one)

- [x] New
- [ ] Renewal
- [ ] Supplemental

4. TELEPHONE NUMBER

Area Code (979) 690-8537

5. MEANS OF MOVEMENT

- [ ] Mail
- [ ] Common Carrier
- [x] Baggage or Handcarried

By whom trained ProdiGene or [ ] personnel [CBI DELETED]

6. GIVE THE FOLLOWING (IF APPLICABLE) (IF MORE SPACE IS NEEDED, ATTACH ADDITIONAL SHEET)

	Scientific Name	Common Name	Trade Name	Other Designation
a. Donor Organism:	see attached			
b. Recipient Organism:	see attached			
c. Vector or Vector Agent:	see attached			
d. Regulated Organism or Product:	see attached			
e. If product, list names of constituents:	N/A			

7. QUANTITY OF REGULATED ARTICLE TO BE INTRODUCED AND PROPOSED SCHEDULE AND NUMBER OF INTRODUCTIONS

Total acreage: up to [ ] acres over 2 seasons. [CBI DELETED]

8. DATE (or inclusive dates of period) OF IMPORTATION, INTERSTATE MOVEMENT, OR RELEASE

July 15, 2004 to July 14, 2005

9. COUNTRY OR POINT OF ORIGIN OF THE REGULATED ARTICLE

Brazos County, TX

10. PORT OF ARRIVAL, DESTINATION OF MOVEMENT, OR SPECIFIC LOCATION OF RELEASE

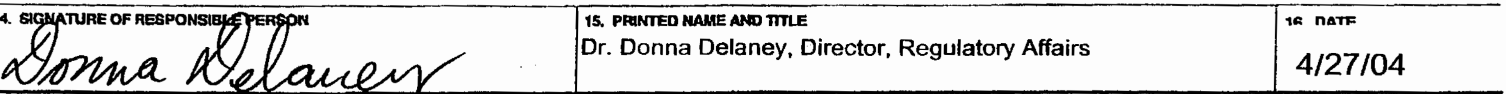
[ ], Frio County, TX [CBI DELETED]

11. ANY BIOLOGICAL MATERIAL *(e.g., culture medium, or host material)* **ACCOMPANYING THE REGULATED ARTICLE DURING MOVEMENT**

None

12. APPLICANTS FOR A COURTESY PERMIT - STATE WHY YOU BELIEVE THE ORGANISM OR PRODUCT DOES NOT COME WITHIN THE DEFINITION OF A REGULATED ARTICL

13. SEE REVERSE SIDE


I hereby certify that the information in the application and all attachments is complete and accurate to the best of my knowledge and belief.

False Statement: Falsification of any item on this application may result in a fine of not more than $10,000 or imprisonment for not more than 5 years or both. (18 U.S.C. 1001

14. SIGNATURE OF RESPONSIBLE PERSON

Donna Delaney

15. PRINTED NAME AND TITLE

Dr. Donna Delaney, Director, Regulatory Affairs

16. DATE

4/27/04

FOR APHIS USE ONLY

State Notification Letter Sent	State Review Received	Permit Issued: [ ] Yes [ ] No
Date of Determination	P 04-121-01r / No. of Permit Labels Issued	Supplemental Conditions Enclosed: [ ] Yes [ ] No
Signature of BBEP Official	Date	Expiration Date

APHIS FORM 2000 (JUL 89) *Replaces PPQ Form 1001 which may be used*

ENCLOSURES	ENCLOSED ("X")	IF PREVIOUSLY SUBMITTED, LIST DATE & PERMIT NO.
a. Names, addresses, and telephone numbers of the persons who developed and/or supplied the regulated article.	X	
b. A description of the anticipated or actual expression of the altered genetic material in the regulated article and how that expression differs from the expression in the nonmodified parental organism (e.g., morphological or structural characteristics, physiological activities and processes, number of copies of inserted genetic material and the physical state of this material inside the recipient organism (integrated or extrachromosomal), products and secretions, growth characteristics).	X	
c. A detailed description of the molecular biology of the system (e.g., donor-recipient-vector) which is or will be used to produce the regulated article.	X	
d. Country and locality where the donor organism, recipient organism, and vector or vector agent were collected, developed and produced.	X	
e. A detailed description of the purpose of the introduction of the regulated article including a detailed description of the proposed experimental and/or production design.	X	
f. A detailed description of the processes, procedures, and safeguards which have been used or will be used in the country of origin and in the United States to prevent contamination, release, and dissemination in the production of the: donor organism; recipient organism; vector or vector agent; constituent of each reglated article which is a product; and regulated article.	X	
g. A detailed description of the intended destination (including final and all intermediate destinations), uses, and/or distribution of the regulated article (e.g., greenhouses, laboratory, or growth chamber location; field trial location, pilot project location; production, propagation, and manufacture location; proposed sale and distribution location).	X	
h. A detailed description of the proposed procedures, processes, and safeguards which will be used to prevent escape and dissemination of the regulated article at each of the intended destinations.	X	
i. A detailed description of the proposed method of final disposition of the regulated article.	X	

Public reporting burden for this collection of information is estimated to average 5 hours per response, including the time for reviewing instructions, searching existing data souces, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Department of Agriculture, Clearance Officer, OIRM, Room 404-W, Washington, D.C. 20250; and to the Office of Information and Regulatory Affairs, Office of Management and Budget, Washington, D.C. 20503.

Section 6. Donor and recipient organisms, vector agent and regulated article

a) Donor organisms:

Bos taurus	cattle
Escherichia coli	
Streptomyces viridochromogenes	
Cauliflower mosaic virus (CaMV)	
Zea mays	corn
Solanum tuberosum	potato
Hordeum vulgare	barley

b) Recipient Organism: *Zea mays* corn

c) Vector or Vector Agent: *Agrobacterium tumefaciens*

d) Regulated Article:
Transformed *Zea mays* expressing proteins with pharmaceutical applications

Section 13. Enclosures

a) Names, addresses, and telephone numbers of the persons who developed and/or supplied the regulated article.

Dr. Michael E. Horn
ProdiGene
101 Gateway Boulevard, Suite 100
College Station, Brazos County, TX 77845
telephone number: (979) 690-8537

b) A description of the anticipated or actual expression of the altered genetic material in the regulated article and how that expression differs from the expression in the nonmodified parental organism:

See attached

c) A detailed description of the molecular biology of the system (e.g. donor-recipient-vector) which is or will be used to produce the regulated article:

The following is a map of the base vector. Vector sequences between the right T-DNA border and the left T-DNA border are transferred to plants during the transformation process. The Col E1 replicon provides an origin for the replication of the vector in *E. coli* and the streptomycin / spectinomycin nucleotydyltransferase gene confers resistance to the antibiotics streptomycin and spectinomycin, thus facilitating selection of *E. coli* cells containing the vector. Extensive DNA sequence outside of the T-DNA borders is identical to sequences in the *A. tumefaciens* superbinary vector and was important to allow the recombination of the vector with the superbinary vector in the *Agrobacterium*. The *Agrobacterium* superbinary vector was disarmed by removal of tumor causing genes.

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1) **Designation of transformed lines:** APX12
Category: OO
Phenotype: Expressing a novel protein
Construct: PGN9048
Genotype:
Promoter: [ ] CBI DELETED
Enhancer: BAASS (=Barley alpha- amylase signal sequence)
Gene: aprotinin – A maize-optimized wild type aprotinin from *Bos taurus*
Terminator: PINII – *Solanum tuberosum* proteinase inhibitor II
Selectable marker:
Promoter: CaMV 35S promoter
Gene: Maize-optimized phosphinothricin acetyl transferase gene (moPAT) from *Streptomyces viridochromogenes*
Terminator: CaMV35s terminator

2) **Designation of transformed lines:** APG02, APG05, APG06, APG09
Category: OO
Phenotype: Expressing a novel protein
Construct: PGN9049
Genotype:
Promoter: [ ] CBI DELETED
Enhancer: BAASS (=Barley alpha- amylase signal sequence)
Gene: aprotinin – A maize-optimized wild type aprotinin from *Bos taurus*.
[ ]. CBI DELETED
Terminator: PINII – *Solanum tuberosum* proteinase inhibitor II
Selectable marker:
Promoter: CaMV 35S promoter
Gene: Maize-optimized phosphinothricin acetyl transferase gene (moPAT) from *Streptomyces viridochromogenes*
Terminator: CaMV35s terminator

Mode of transformation for all lines: *Agrobacterium tumefaciens*

d) Country and locality where the donor organism, recipient organism and vector or vector agent were collected developed and produced:

PRGP04-04r CBI DELETED COPY

All lines of corn were produced by ProdiGene, 101 Gateway Boulevard, College Station, Brazos County, Texas, and at Texas A&M University, West Campus, College Station, Brazos County, Texas

e) A detailed description of the purpose of the introduction of the regulated article including a detailed description of the proposed experimental and/or production design.

The purpose of the introduction is for grain production, hybrid seed production and research/line development in a nursery. The regulated articles will be grown at the following location: [ ], Frio County, Texas. Plantings are proposed for two seasons, the first will be planted in late July/early August 2004 and the second will be planted in late Feb./early March 2005. The 2004 planting will consist of up to [ ], a [ ] site for grain production, a [ ] site for hybrid seed production and [ ] in the nursery. The 2005 planting will consist of up to [ ], a [ ] site for grain production, a [ ] site for hybrid seed production and [ ] in the nursery. Open pollinated sites containing different protein products will be separated by at least 660 feet.
The sites will be surrounded by a fallow area of 50 feet, and the location will be isolated from any other corn by a distance of at least one mile. The hybrid seed production field and the nursery will also be surrounded by 4 rows (10 feet) of border consisting of a non-transgenic male sterile hybrid.
The hybrid seed production fields will be planted in a 1:3 male:female ratio. The female rows will be detasseled before pollen shed and the border rows will be removed prior to viable seed formation. The female rows will be walked at least every 48 hours to remove tassels.
Controlled hand pollinations will be performed in the nursery. Standard procedures for hand pollination of corn will include bagging of the ear shoot prior to silk emergence. Tassels will be bagged only when needed for pollination.
Grain production and hybrid production fields will be open pollinated.
At the end of the season, the nursery and hybrid seed production fields will be hand harvested on the ear and placed in mesh bags. The seed will be dried in a dedicated drier at the field location. Dried nursery seed will be packaged according to SOP #RGP-005 and shipped to [ ], Brazos County, TX where it will be shelled, labeled, packaged, analyzed for protein content, and stored until further use. Dried hybrid seed will be shelled and packaged in a designated staging area at the field location and then stored in [ ] or shipped to [ ], Brazos County, TX for storage.
Grain production fields will be machine harvested using a dedicated combine. The seed will be dried at the field location, and then devitalized by milling, packaged and shipped to a storage or processing location. All milling operations will be performed in a designated staging area at the field location using a mobile equipment skid.
The releases will occur between July 15, 2004 and July 14, 2005.

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f) A detailed description of the processes, procedures, and safeguards which have been used or will be used in the country of origin and in the United States to prevent contamination, release, and dissemination in the production of the: donor organism; recipient organism; vector or vector agent; constituent of each regulated article which is a product; and regulated article.

Donor organisms: all components of donor organisms have are cloned DNA fragments contained in plasmids of *E. coli* or *Agrobacterium tumefaciens*, and maintained in the laboratories of ProdiGene, under BL1 containment conditions.
Recipient organism: non-transformed *Zea mays* has been cultivated in the field and propagated in the laboratory under standard good agricultural or laboratory practices.
Vector and vector agent: *Agrobacterium tumefaciens* has been maintained in the laboratory under BL1 containment conditions.
Regulated article: Transformed *Zea mays* has been maintained in the laboratory and greenhouse under BL1P containment conditions.

g) A detailed description of the intended destination (including final and all intermediate destinations), uses, and/or distribution of the regulated article (e.g. greenhouse, laboratory, or growth chamber location; field trial location, pilot project location, production, propagation, and manufacture location; proposed sale and distribution location).

Prior to planting, the regulated articles will be hand-carried from [ ], Brazos County, TX to the field sites at [ ], Frio County, Texas in an enclosed private vehicle, or in a completely enclosed trailer (according to SOP #RGP-005). If it is necessary to temporarily store the seed before planting it will be enclosed in a locked trailer at the field location. The seed will be under the control of ProdiGene [ ] personnel at all times. CBI DELETED CBI DELETED CBI DELETED

At harvest, the nursery seed and hybrid seed will be hand harvested and dried in a designated staging area at the field location using a dedicated drier. The nursery seed will then be packaged for shipment according to SOP #RGP-005, and then hand-carried from the field location in Frio County, TX to [ ], Brazos County, TX in either an enclosed trailer or dry van by trained [ ] personnel. The vehicle will be equipped with a GPS tracking device. At this site, the nursery seed will then be shelled, packaged, analyzed for recombinant protein content, and stored until further use. CBI DELETED CBI DELETED

The dried hybrid seed will be shelled and packaged in a designated staging area at the field location and then stored in [ ] until it is planted the following season, or shipped to [ ], Brazos County, TX for storage. If it is shipped, the hybrid seed will be packaged for shipment according to SOP #RGP-005, and then hand-carried from the field location in Frio County, TX to [ ], Brazos County, TX in either an enclosed trailer or dry van by trained [ ] personnel. The vehicle will be equipped with a GPS tracking device. CBI DELETED CBI DELETED CBI DELETED

CBI DELETED

The grain production fields will be machine harvested using a dedicated combine. It will be dried in a designated staging area at the field location using dedicated driers. Harvested grain may be temporarily held at the site in covered grain wagons and/or plastic or metal boxes until it can be further processed and devitalized using a mobile milling operation. The rate of grain harvest will be metered to correspond to the rate of milling, as much as possible. This will reduce the need for temporary storage to a minimum. Covers will be sealed with security seals or boxes will be placed in a sealed trailer(s). The milling equipment will be transported to the site in an enclosed trailer. When in operation the sides of the trailer will be opened. The milling operation will be set up and operated in a designated staging at the field location. Milled grain will then be packaged on site and shipped to one or a combination of the following locations:

1. [,] Dodge County, NE for storage — CBI DELETED
2. [,] Sioux County, IA for storage and recombinant protein purification. — CBI DELETED
3. [,] Brazos County, TX for storage and recombinant protein purification. — CBI DELETED

Any devitalized waste material from the milling operation will be returned to the field site and incorporated into the soil.

and aprotinin reincorporated in seeds after aprotinin extraction

h) A detailed description of the proposed procedures, processes, and safeguards which will be used to prevent escape and dissemination of the regulated article at each of the intended destinations.

See attached outline of ProdiGene's Compliance Program.

i) A detailed description of the proposed method of final disposition of the regulated article.

At the end of the first trial (planted in late July/early August 2004), the seed will be harvested by hand or using a dedicated combine and up to [ ] of seed will be shipped to [,] Brazos County, TX for processing, analysis and storage. — CBI DELETED / CBI DELETED
Up to [ ] of seed will [ ] until it is planted the following season. The storage unit will be sealed with serial numbered security seals and regular rodent control measures will be taken. As an alternative, the seed may be shipped to [ ], Brazos County, TX for storage. — CBI DELETED / CBI DELETED / CBI DELETED
The grain produced for processing and protein purification will be milled and devitalized on site in either a de-germing or direct milling process. Up to [ ] of devitalized, milled germ flour or whole seed flour will be shipped to one or a combination of the following locations: — CBI DELETED

1. [,] Dodge County, NE for storage — CBI DELETED
2. [,] Sioux County, IA for storage and recombinant protein purification. — CBI DELETED
3. [,] Brazos County, TX for storage and recombinant protein purification. — CBI DELETED

At the end of the second trial (planted in late Feb./early March), seed will be handled in the same way. Depending on the size of the plot, seed will either be harvested by hand or machine harvested with a dedicated combine. Up to [ ] of seed will be shipped to [ ], Brazos County, TX for processing, analysis and storage. Up to [ ] of seed will [ ] until it is planted the following season. The storage unit will be sealed with serial numbered security seals and regular rodent control measures will be taken. As an alternative, all or part of the seed may be shipped to [ ], Brazos County, TX for storage. The majority of the grain produced will be milled and devitalized on site in either a de-germing or direct milling process. Up to [ ] of devitalized, milled germ flour or whole seed flour will be shipped to one or a combination of the following locations:

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4. [,] Dodge County, NE for storage — CBI DELETED
5. [,] Sioux County, IA for storage and recombinant protein purification. — CBI DELETED
6. [,] Brazos County, TX for storage and recombinant protein purification. — CBI DELETED

Procedures for transport will be as described in Section X of the attached outline of the compliance program. A chain of custody will accompany all shipments and careful records of inventory movements will be kept. Any devitalized waste material from the milling operation will be returned to the field site and incorporated into the soil. Post harvest procedures will be as described in Section XII of the attached outline. After incorporation of the crop residue the field sites will be left fallow, planted to a non-food, non-feed cover crop, or to a low growing vegetable crop (e.g. onions or cabbage) that will be harvested by hand. As much as possible, we will replant field sites to the same recombinant protein product in the following season. Any sites that are not re-used will be monitored for volunteers in the subsequent season as described in Section XIII of the attached outline of the compliance program.

Note: A copy of ProdiGene's 2004 Field Production Notebook and all SOPs covering procedures for seed handling, transport and field testing were submitted earlier with permit application PRGP04-01r (Permit # 04-040-01r). Please consult these documents if there is a question about specific procedures to be followed.

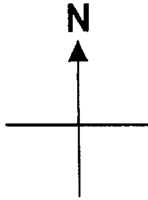

Fig. 1. Attachment to Section 13(e). Map of intended location in Frio Co., TX
(not to scale)

Section 13 (h):
The details of ProdiGene's Compliance Program, which is designed to prevent the escape and dissemination of the regulated articles, are listed in outline form below:

I. Requirements before planting
   A. All involved personnel will be fully trained in all applicable regulations, ProdiGene's SOPs and company policies prior to planting.
   B. Cooperators must be pre-qualified and sign a minimum of a two year contract.

II. Site / Cooperator selection (SOP #'s RGP-008 and RGP-020)
   A. The site must be a minimum of one mile from any other plantings of corn.
   B. The site must not be adjacent to major roads or residential areas to reduce the opportunity for unauthorized access.
   C. The Cooperator must either own the land or have a minimum of a two year lease on the land to cover the full duration of the permit.
   D. The Cooperator must be willing to follow all regulations and ProdiGene SOPs and to participate in a training program.

III. Seed Preparation for Planting
   A. Equipment used for cleaning, sizing and treating the seed will be dedicated for use with only ProdiGene seed, and will be thoroughly cleaned after use.
   B. The facility, or area, the equipment is stored in will also be dedicated.
   C. Seed will be handled only by ProdiGene personnel and trained cooperators.
   D. Seed treatment will be performed at ProdiGene or at the field site prior to planting. (SOP #RGP-031)
   E. Seed will be bagged and then sealed in a second container for shipment to growing location. The bags will be clearly marked with product name and lot number, and a label stating "Not for Food or Feed." (SOP #RGP-005)

IV. Shipment of Planting Seed to the Site
   A. Sealed containers will be transported to the production site in a completely enclosed vehicle.
   B. All doors and ports on the trailer will be locked and sealed with serial numbered security seals to prevent unauthorized entry.
   C. A chain of custody will be maintained from origin to destination.
   D. The driver will be given instructions as to the route to travel, procedures to be followed in the event of an accident in which containment is lost, and emergency contact numbers. (SOP #RGP-017)
   E. The vehicle will be equipped with GPS tracking.
   F. Upon arrival the seed will be stored in a locked trailer prior to planting, with access restricted to authorized personnel, or the seed will be planted immediately. (SOP #RGP-007)

V. Planting (SOP #RGP-009)
   A. USDA will be notified at least one week prior to planting.
   B. At least two individuals trained in ProdiGene's SOPs must be present at planting to verify that the proper procedures were followed.
   C. A pre-planting checklist must be filled out prior to planting to verify the location, size of acreage, isolation from other corn and identity of seed.
   D. A dedicated planter will be used.

E. Seed bags should only be opened in the permitted area.
F. The planter will only be loaded in the permitted area.
G. Seed must not be left unattended unless it is secured within a security sealed and/or locked container.
H. The planter and tractor will be thoroughly cleaned after use and cleanout will be verified before it is transported from the permitted area.

VI. During Growing Season

A. All crop maintenance activities will be performed by trained ProdiGene personnel and/or trained collaborators.
   1. ProdiGene is responsible for:
      a. Herbicide application
      b. Cultivation
      c. Pest and disease control
   2. All equipment brought into the permitted area that comes in contact with the regulated articles must be cleaned before leaving the permitted area. (SOP #RGP-021)

B. Monitoring (SOP #RGP-018)
   1. Sites will be inspected at least every two weeks for the following criteria:
      a. Stage of growth
      b. Overall health of plants
      c. Weed/insect problems
      d. Irrigation requirements
      e. Any unusual characteristics of the plants
      f. No corn can be planted within one mile of the site
      g. All volunteer corn within 0.5 miles of the site should be destroyed before flowering.
      h. Any volunteer corn within the isolation distance from 0.5 mile to 1 mile from the site that may flower at the same time as the regulated articles must be destroyed.
      i. Record all chemical applications
   2. An internal ProdiGene audit will be done monthly
   3. A 3rd party audit will occur once per season prior to pollination

VII. Harvest

A. The USDA will be notified at least 30 days prior to harvest for authorization.
B. At least two individuals trained in ProdiGene's SOPs must be present at harvest to verify that the proper procedures were followed.
C. Dedicated equipment will be used or ears will be harvested by hand.
D. The combine will be off-loaded to a transport vehicle or container within the permitted area.
E. All equipment will be thoroughly cleaned and cleanout will be verified before leaving the permitted area. (SOP #RGP-010)

VIII. Seed Handling and Drying

A. Dedicated equipment will be used for all seed handling and drying operations.
B. Driers will be set up and operated in a designated staging area at the field location. The staging area will be marked and monitored for volunteers in the subsequent season and any volunteer corn will be destroyed prior to flowering.

C. Seed handling operations, such as scalping and cleaning, will also be set up in a designated staging area at the field location. The area will be monitored for volunteers in the subsequent season as above.
D. All equipment will be cleaned for transport after use and will be removed from the location inside a completely enclosed vehicle or trailer. All doors on trailer will be locked and sealed with serial numbered security seals.
E. The equipment will be stored in a dedicated facility, or area, away from any equipment used for food, feed or seed production.
F. The transport vehicle will be cleaned after use according to SOP #RGP-012.

IX. Milling Operations (SOP #RGP-037)
A. Dedicated equipment will be used
B. Milling operations will be carried out using a mobile equipment skid that can be moved from site to site.
C. The equipment will be mounted inside a completely enclosed trailer for secure transport. When in use, the sides of the trailer will be opened up.
D. All milling will be performed in a designated staging area at the field location.
E. All equipment will be cleaned for transport after use, enclosed within the trailer again, and transported to a dedicated facility, or area, for storage.
F. The staging area will be marked and monitored for volunteers in the subsequent season and any volunteer corn will be destroyed prior to flowering.
G. Milled grain will be bagged, placed on pallets, covered with a cardboard box and secured to the pallet with steel strapping. Boxes will be prominently labeled as "Not for Food or Feed".
H. Packaged milled grain or flour will be transported in a completely enclosed vehicle, trailer or dry van to secure storage or processing at a location cited in the permit.
I. Any devitalized waste from the milling process will be returned to the field site and incorporated into the soil.
J. Careful records will be kept at all steps in the process to verify positive control of our inventory.

X. Transport of Grain (seed and flour) to Storage (SOP #RGP-005)
A. Transport will occur via completely enclosed trailers.
B. All doors and/or ports will be sealed with serial numbered security seals to prevent tampering.
C. All transportation equipment must be cleaned of all viable seed before leaving the permitted area.
D. A chain of custody will be maintained from origin to destination.
E. The outside of the trailer will be labeled as "Not for Food or Feed" and emergency contact information will be posted.
F. The vehicle will be equipped with GPS tracking for all interstate movements over 50 miles.
G. The driver will be given instructions as to the route to travel, procedures to be followed in the event of an accident in which containment is lost, and emergency contact numbers. (SOP #RGP-017)
H. All equipment will be thoroughly cleaned after use and cleanout will be verified. (SOP #RGP-012)

XI. Storage (SOP #RGP-015)

A. Upon arrival, the truck driver must present documentation verifying the origin, shipment number and lot number of the seed (chain of custody).
B. The shipment will be inventoried and checked against the shipping documents. (SOP #RGP-033)
C. The truck and the area around the truck will be cleaned of all viable seed. Any viable seed found will be disposed of according to SOP #RGP-014.
D. All grain movements will be documented in a logbook.
E. There will be a complete weight accounting of all grain throughout storage.

XII. Post Harvest Crop Destruction (SOP #RGP-013)

A. As soon as possible after harvest, the site will be inspected and any intact ears, or large pieces of ears, found by the crew will be disposed of by burning or burial (>36" deep)
B. The crop residue will be incorporated into the soil such that most of the remaining plant material is in contact with the soil. This will be accomplished by plowing, multiple rounds of disking, or a combination of disking and some other suitable tillage implement.
C. ProdiGene will conduct a post-harvest inspection of each site to verify that the above procedures were done satisfactorily.

XIII. Subsequent Season (SOP #RGP-013)

A. Each site will be monitored for volunteers for one full year from harvest of the regulated crop.
B. Monitoring will begin as soon as weather conditions are conducive to germination and will occur at least every two weeks throughout the monitoring period.
C. If necessary, sites will be irrigated to promote growth of volunteers.
D. Any volunteers found will be destroyed before flowering.
E. If a large number of volunteers are found at the end of the monitoring period, monitoring will be extended until no volunteers are found.
F. ProdiGene will audit each field monthly during the growing season.
G. 3rd Party audits will be done once per season prior to pollination
H. A crop can be grown in the subsequent season under the following conditions:
   1. If a crop species is grown on the permitted site in the subsequent year, it should be a species that is not used for food or feed (e.g. a cover crop), or a low growing vegetable crop species that will be harvested by hand. The same ProdiGene transgenic corn grown in the previous year is another option, but the grower must have prior authorization from ProdiGene.

   2. The crop, if any, cannot be harvested without prior authorization from ProdiGene or the USDA. ProdiGene will conduct a pre-harvest inspection and will also request a pre-harvest inspection from the USDA and written authorization to harvest.

   3. If the USDA does not respond within 30 days of our request, the crop can be harvested but must be quarantined until release has been authorized. Quarantined storage can be any storage that is secure and isolated from all other commodity crops.

I. If the site is left fallow, or a crop is grown that is not intended for harvest (i.e. a cover crop or green manure crop), the following recommendations apply:

1. If a crop is planted it must not be allowed to reach maturity, in order to provide more options for control of volunteers.
2. Volunteers should be destroyed by one or more of the following methods:
   a. Thorough mowing of the entire site
   b. Cultivation
   c. Herbicide application (must be a product that will kill ProdiGene's transgenic corn)
   d. Plowing down the entire site and incorporation of any plant material into the soil.

XIV. Accidental Release (SOP #RGP-016)

A. All incidents of accidental release, or potential accidental release, will be reported to USDA/APHIS within 24 hours of discovery.

B. Every attempt will be made to assess the situation and regain containment as quickly as possible to protect the food supply.

C. A written report will be submitted to USDA/APHIS within 5 working days of the occurrence.

D. All remedial measures mandated by federal and state regulatory agencies will be carried out.

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Safety Assessment of Aprotinin Produced in Recombinant Maize

Section 13(b) of permit application PRGP04-04r

1. Introduction

Aprotinin is a natural serine protease inhibitor from *Bos taurus* with inhibitory activity against several enzymes with esterolytic and proteolytic activity. It is a small protein (molecular weight 6512 daltons) consisting of 58 amino acid residues arranged in a single polypeptide chain and cross-linked by three disulfide bridges. Aprotinin can be found in several bovine tissues, especially those rich in mast cells, including pancreas, lung, liver, parotid gland and lymph nodes. It is most abundant and most commonly purified from bovine lung.

The biochemistry of aprotinin has been reviewed by Fritz and Wunderer (1983), Gebhard, et.al. (1986) and Laskowski and Kato (1980). Aprotinin exists principally as a dimer aggregate at neutral pH and dissociates to the monomer form at the extremes of the pH range. Aprotinin inhibits the following enzymes: trypsins of cow, pig, human, and turkey; acetyl trypsin; bovine chymotrypsins α and β (the latter only slightly); chicken chymotrypsin; bovine and porcine kallikreins; rabbit, human, and porcine plasmin; and the trypsin-like component of Pronase. The inhibition of trypsin is stoichiometric and pH dependent above pH 11 and below pH 6. Aprotinin binds most strongly to trypsin ($K_I = 2 \times 10^{-11}$ mole/liter) in the neutral pH range. The inhibition of trypsin by aprotinin is considered competitive with complete inhibition accomplished only in the presence of an excess of the inhibitor. In the physiological pH range, 100% inhibition of 0.56 to 0.7μg of trypsin is achieved by 1.2 KIU (Kallikrein inhibition units) of aprotinin. The inhibition of trypsin is not instantaneous however, and occurs most rapidly (1-4 min to equilibrium) at pH 7.8 and 25°C.

Aprotinin has industrial applications in cell culture, diagnostics and protein purification, and pharmaceutical applications in the treatment of patients undergoing cardio-pulmonary bypass surgery, acute pancreatitis and as a component of fibrin sealants. It is used in cell culture as a component of serum free media for inhibiting proteinases and protects product yield during fermentation and downstream purification of recombinant proteins and biopharmaceuticals. It is also used to inhibit trypsin and terminate lysis of animal tissues and cells from incubation flasks, thus maintaining the integrity of the cells. It is used in diagnostics to prevent proteolytic damage to samples in assay systems. Aprotinin's high affinity for serine proteases make it a useful tool in affinity purification protocols for these proteins.

The major pharmaceutical use of aprotinin is in the treatment of patients undergoing cardio-pulmonary bypass surgery where it modulates the systemic inflammatory response resulting in a decreased need for allogenic blood transfusions, reduced bleeding, and decreased mediastinal re-exploration for bleeding. Another use of aprotinin that is gaining prominence is as a component of fibrin sealants for sutureless wound closure where it inhibits fibrinolysis.

ProdiGene has produced recombinant maize plants expressing high levels of native aprotinin in the seed using a seed- and germ-preferred promoter. Purified aprotinin from recombinant maize has the same specific activity as commercially available aprotinin samples derived from bovine lung. ProdiGene will produce seed containing aprotinin and purify it from this seed. Initial markets for this purified aprotinin will be in the cell culture applications. We will be initiating clinical trials to show equivalency in pharmaceutical applications, such as cardio-pulmonary bypass surgery and wound closure kits, as soon as sufficient purified protein can be produced and a commercial partner is secured.

2. Homology to known toxicants, allergens or proteins known or likely to harm non-target organisms

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The amino acid sequence of ProdiGene's aprotinin construct was compared to peptides in the Farrp Allergen Database, which is considered a comprehensive listing of allergens. This database consists of 658 unique known and putative allergens identified by using the key word allergen for searches of Medline and the protein databases Genbank and EMBL version 119, PIR version 56, NRL3D version 56 of RCSD PBD and SwissProt version 38. Note that not all of the proteins in the database have been shown to induce clinical allergic responses. Sequence comparisons of ProdiGene's aprotinin (with the barley alpha amylase signal for cell export fused to the N-terminus) to sequences in the Farrp Allergen Database were made using the FASTA3 algorithm running on a UNIX platform (Pearson, W.R. 2000 Flexible sequence similarity searching with the FASTA3 program package. Methods Mol. Biol. 132, 185-219). Output from searches shows regions of sequence similarities, indicating exact amino acid matches and conservative changes. Values for the percentage identity between the scanned sequence and sequences in the database are shown, both with and without gaps introduced into the sequences. Also, the lengths (in amino acids) over which regions of similarity extend are given.

The World Health Organization (WHO) describes criteria for considering cross-reactivity between a sample protein and a known allergen in a report of a joint FAO/WHO expert consultation on 'Allergenicity of Foods Derived from Biotechnology' conducted from 22-25 January 2001. The limits are set at 35% amino acid sequence identity over an 80 amino acid window or at 100% identity over 6 contiguous amino acids.

ProdiGene's aprotinin sequence has 18 hits against the Farrp Allergen Database. However, none of these hits show a 35% or above sequence identity when extended out over the full length of the aprotinin sequence (58 amino acids) or the WHO defined window of 80 amino acids (the signal sequence plus aprotinin consist of 82 amino acids). Also, none of these hits show 6 or more contiguous amino acids. Thus, according to the WHO criteria, aprotinin is not considered cross-reactive to any allergens.

The amino acid sequence of ProdiGene's aprotinin construct (with the barley alpha amylase signal for cell export fused to the N-terminus) was also subjected to a BLAST search of all non-redundant Genbank CDS translations plus RefSeq proteins, PDB, SwissProt, PIR and PRF. The maximum 1000 limits were set for descriptions of matches and alignments. Output from searches shows regions of sequence similarities, indicating exact amino acid matches and conservative changes. Values for the percentage identity between the scanned sequence and sequences in the database are shown. Also, the lengths (in amino acids) over which regions of similarity extend are indicated.

ProdiGene's aprotinin sequence yields 795 hits in the BLAST search. The aprotinin amino acid sequence is 100% identical to the bovine trypsin inhibitor. No toxic effects of bovine aprotinin have been documented, and in fact, bovine aprotinin is well tolerated even at high intravenous doses, such as the product Trasylol (Santamaria, et. al. 2000).

ProdiGene's aprotinin also has similarity with the following proteins. A 50% identity over a 48 amino acid region to the human amyloid beta protein that is deposited in Alzheimer brains. A 50% identity over a 53 amino acid region to *Araneus ventricosus* toxin 1 (an insecticidal protein of a spider). A 46% identity over a 52 amino acid region to proteinase inhibitor 5 II (a peptide with hemolytic activity) of snake locks sea anemone *Anemonia sulcata*. Similarly, a 46% identity over a 52 amino acid region to the kalicludine 1 toxin of the snake locks sea anemone *Anemonia sulcata*. Also, the same level of match to kalicludine 2 toxin and a 44% identity over 52 amino acids to kalicludine 3 toxin of the same organism. A 41% identity over a 53 amino acid region to the cysteine-rich venom protein 2 of the parasitoid wasp *Pimpla hypochondriaca*. In addition there are many hits on snake venom proteins, some of which are given here. A 49% identity over a 55 amino acid region to the Gaboon viper snake venom Kunitz protease inhibitor 1. A 43% identity over a 53 amino acid region to the Gaboon viper snake venom two-Kunitz protease inhibitor. A 45% identity over a 57 amino acid region to textilinin (a plasmin inhibitor) of the Australian brown snake *Pseudonaja textilis textilis*. A 47% identity over a 57 amino acid region to taicatoxin serine protease inhibitor component from

venom of the Australian taipan snake *Oxyuranus scutellatus scutellatus*. A 50% identity over a 57 amino acid region to basic protease inhibitor II from venom of Ringhal's cobra *Hemachatus haemachatus*. A 50% identity over a 57 amino acid region to protease inhibitor II from venom of Cape cobra *Naja nivea*. A 43% identity over a 57 amino acid region to basic protease inhibitor I from venom of the Western sand viper *Vipera ammodytes ammodytes*. A 45% identity over a 57 amino acid region to the King cobra venom chymotrypsin inhibitor.
Kalicludine is an unusual molecule in that it has function both as a dendrotoxin-like molecule (by blocking voltage-sensitive K^+ channels) and as a proteinase inhibitor (Schweitz, etal. 1995). However, protease inhibitors, such as aprotinin, do not display any dendrotoxin-like activity even at high concentrations (Marshall and Harvey, 1992). Some of the snake venom proteins are also listed as dendrotoxins. Note that snake venoms are a mixture of many different protein components, not all of which have toxic properties. The protease inhibitors in snake venom serve to prevent the proteolytic breakdown of other venom component proteins by the victim. They do not have toxic properties by themselves.

A PubMed/Medline and Medscape literature search was performed with the following keyword search critieria:

Aprotinin AND (allergy OR allergies)
Aprotinin AND anaphylaxia
Aprotinin AND immunogenicity
Aprotinin AND adverse reactions
Aprotinin AND toxicity
Aprotinin AND allergenicity
Aprotinin AND ingestion
Aprotinin AND food allergy
Aprotinin AND oral

Our search revealed no cases of aprotinin food allergy and no toxic effects of aprotinin. There have been reported cases of allergic reactions to high intravenous doses of bovine derived aprotinin used during cardio-pulmonary bypass surgery. These cases mainly occur after re-exposure to the drug (incidence = 1-2%) and range from mild cases requiring no intervention to anaphylactic reactions. Allergic reactions have also been reported after repeated use of aprotinin as a component of fibrin sealants applied to open wounds (5/1 million cases in the US). However, these incidences are not relevant to field production of aprotinin in which the only exposure would be through accidental ingestion of aprotinin containing seeds.
Aprotinin is present in several bovine tissues, including liver, lung and pancreas. Therefore anyone who has eaten beef liver or beef-based hot dogs has been exposed to aprotinin in their diet. The concentration of aprotinin in bovine lung is 100mg per kg tissue, which is the same as the concentration of aprotinin in ProdiGene's transgenic corn seed. Our literature search revealed no cases of toxic effects of aprotinin, nor any adverse reactions from ingestion or any oral application.

3. Molecular Characterization of Aprotinin Corn

a. Southern blot analysis. The event APX12 has been identified as the most promising candidate for further development and commercial production. Southern blot analysis of the event demonstrated that a single functional copy of the aprotinin coding sequence was integrated into the corn genome. Additionally the genomic DNA from the event was probed with the backbone of the PGN9048 plasmid to demonstrate the absence of the *spectinomycin*-coding region. The *spectinomycin* coding sequence was not integrated during transformation of APX12. The aprotinin coding sequence is inherited in the expected Mendelian

The integrity of the insert has been maintained during five generations of breeding. The aprotinin gene was cloned from plants representing event APX12 by PCR and sequenced. Sequence data demonstrates that the aprotinin gene in these plants is 100% identical to the wild type *Bos taurus* gene and contains no errors.

Table 1: Southern blot analysis of event APX12. Southern blot analysis was performed as per standard techniques using the probes indicated on genomic DNA isolated from transgenic seedlings. The aprotinin sequence was also cloned by PCR from transgenic seedlings and multiple clones were sequenced to verify the integrated sequence.

APX12	
Backbone probe	Negative
Sequence of gene in transgenic plant	100% correct
Number of insertions	One

b. Protein Characterization

Aprotinin purified from transgenic corn is indistinguishable from commercially available aprotinin purified from bovine lung. The enzymatic activity of the two proteins is identical. The aprotinin from recombinant corn purifies as a single band on SDS-PAGE with the same molecular weight as bovine aprotinin. Amino acid sequencing of the protein purified from recombinant corn was identical to the native bovine sequence. Western blot analysis of corn seed protein extracts from ProdiGene's aprotinin expressing corn revealed a single immunoreactive band at the same molecular weight as a commercial sample of bovine aprotinin. Native bovine aprotinin is not glycosylated and neither is aprotinin purified from corn.

Table 2: Protein characterization of ProdiGene corn derived aprotinin

Analysis Performed	ProdiGene aprotinin	CalBiochem aprotinin
Amino acid sequencing	Completely sequenced - identical to bovine sequence	Not determined
Molecular Weight	6500 Da	6512 Da
Trypsin Inhibition Activity	5.0 TIU/mg	5.1 TIU/mg
Kallikrein Inhibition Activity	8000 KIU/mg	>7000 KIU/mg
Mass Spec	6572 Da	Not determined
Glycosylation	None	None

4. Analysis of Potential Exposures

a. Environmental

Expression data from stems, roots, leaves, pollen at various stages of development.

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Stage of growth	Tissue type	Ave. % fresh wt.	% fresh wt limit of quantitation
Pre-flowering	Stem		
	Leaf		
	Root		
Flowering	Stem		
	Leaf		
	Root		
	Ear shoot		
	Tassel		
	Pollen		
Harvest	Stem		
	Leaf		
	Root		
	Seed		

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Tissues were also collected from greenhouse grown APG05 and APG06 plants and evaluated for the presence of aprotinin protein. The following table lists the tissues that were analyzed.

Stage of growth	Tissue type	Ave. % fresh wt.	% fresh wt limit of quantitation
Pre-flowering	Stem		
	Leaf		
	Root		
Flowering	Stem		
	Leaf		
	Root		
	Ear shoot		
	Pollen		

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Seeds

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b. Comparison of known exposure levels
The following table lists some of the known exposures of humans and animals to aprotinin.

Organism	Exposure	Dose	mg Aprotinin	Equivalent amt. of transgenic corn seed
Mouse	LD_{50}	2.5 million KIU/kg	312.5mg/kg	93.8g[a]
Dog	Highest tolerated intravenous dose with no complications	1 million KIU/kg	125mg/kg	31.25 pounds[b]
Human	Recommended high dose regime for cardio-pulmonary bypass surgery	6.5 million KIU	812.5mg	17.9 pounds
1 ear of ProdiGene aprotinin corn			15.1 mg	151g or 0.33 pounds

[a]Based on a 30g mouse
[b]Based on a 25 pound (11.3kg) dog

According to the above table, a 30g mouse would have to eat over 3 times its body weight in transgenic corn expressing aprotinin to reach the LD_{50} level. We know from mouse feeding studies conducted here at ProdiGene that mice will only eat 8g of corn per feeding. Aprotinin is not absorbed into the bloodstream when taken orally (even as capsules soluble in the small intestine) (Trautschold, et.al. 1967). The major gastric enzyme in the mammalian gut, pepsin, is neither inhibited by aprotinin nor is aprotinin degraded by pepsin. The high blood levels needed for effective therapeutic use of aprotinin can only be attained by continuous intravenous drip administration because aprotinin is cleared from the bloodstream very quickly. A single intravenous injection of 1000 – 2000 KIU/kg has a half-life in the bloodstream of about 10 minutes.

5. Thermal Stability of aprotinin

Aprotinin is very stable under a wide range of temperature, acid and alkali conditions. It can be heated in dilute acid at 100°C and in 2.5% trichloroacetic acid at 80°C without loss of activity. It may be kept for 18 months at room temperature in 0.14M NaCl without loss of activity. The activity remains constant for 24 hours at room temperature up to pH 12.6, but begins to decrease at pH 12.8.

6. Sensitivity to gastric digestive conditions of mammals and avian species

Aprotinin is not degraded by pepsin and would be expected to be quite stable in gastric fluid. Fu, et.al. (2002) subjected the trypsin inhibitor from bovine pancreas (aprotinin) to digestion using a simulated gastric fluid (SGF) and a simulated intestinal fluid (SIF) and found that it was stable throughout the 120 minutes tested. It is well known that aprotinin is not digested by pepsin (Trautschold, etal 1967). Resistance to digestion however is not necessarily associated with

allergenicity. A number of known food allergens and nonallergens (aprotinin among them) were tested and no clear relationship between digestibility measured in vitro and protein allergenicity was found (Fu, etal 2002; Fu 2002). The majority of known allergenic proteins tested were digested quite rapidly in SGF, whereas several "nonallergenic" proteins tested were resistant.
The composition of avian gastric fluid is very similar to that of mammals (Sturkie 1976; Klasing 1998) in that both contain HCl acid and pepsin and have a pH of about 2.0. The anatomy of their digestive systems is different however. In mammals, mastication occurs in the oral cavity and food moves from there through the esophagus and into the stomach where special glands secrete HCl acid and pepsin. In avian species, food moves from the oral cavity, through the esophagus and crop and into the proventriculus where the HCl and pepsin are secreted and mixed with the food. From there the food moves to the ventriculus, or gizzard, where mastication and initial digestion takes place. The pancreatic enzymes of mammals and avian species are also very similar. The pancreas of birds, like that of mammals, contains amylolytic, proteolytic and lipolytic enzymes which are secreted into the small intestine where the majority of the digestion and absorption takes place. Given the close similarity of the digestive enzymes and pH of gastric fluids in mammals and avian species, assays conducted using simulated mammalian fluid should translate to birds.

7. Environmental Impact of Transformed Maize Expressing Aprotinin

Aprotinin does have some insecticidal properties due to its strong inhibition of trypsin, one of the major digestive enzymes in the insect gut. Studies of the effect of aprotinin on honey bee survival have shown that concentrations at or below 0.01% (w:v) of aprotinin in the diet (as sugar syrup) had no effect on honey bee longevity (Burgess, etal. 1996). Honey bees fed aprotinin as a component of pollen food, at a concentration of 0.25% (w:w) (2.5mg/g) died an average of 3 days sooner than control bees (Malone, etal. 2001). Lower levels were not tested. The level of aprotinin in tissues of ProdiGene's aprotinin expressing corn (APX12) is far below these levels. There was no measurable expression of aprotinin in pollen (although it has been measured at other sites as 0.0001% of fresh weight in pollen) and the expression in leaves and stems is below 0.0002% of fresh weight. At these levels ProdiGene's aprotinin expressing corn will have no impact on insect populations. Therefore the impact of these plants on the environment is minimal and only those species that can potentially feed upon grain need to be considered. The digestive systems of mammals and birds are similar and studies have shown that aprotinin is not absorbed into the bloodstream through oral administration (Trautschold, et.al. 1967). Agronomic practices such as plowing the field immediately after harvest will minimize any potential exposures.
The transformation process used to incorporate the aprotinin sequence into maize would not be expected to alter the levels of any naturally occurring toxicant in the plant. Our characterization of ProdiGene's recombinant maize expressing aprotinin shows the gene to be stably inherited as a single insert that produces a peptide with the same molecular weight and biochemical properties as the native bovine aprotinin. Maize plants expressing aprotinin are indistinguishable from normal maize and show no differences in seed dormancy, seed germination, pollen viability, pollen production or disease and insect susceptibility. No adverse or unusual effects on humans working with the transformed plants have been noted in the eight years we have been working with this gene, and none are expected since expression is mainly targeted to the seed and levels in pollen and other plant tissues is very low.

Conclusion:
In summary:
1. Aprotinin is a natural bovine serine protease inihibitor found in many bovine tissues including lung, liver and pancreas.

2. Recombinant aprotinin purified from ProdiGene's aprotinin expressing corn has 100% amino acid sequence identity to the bovine protein, is identical in molecular weight, activity, and glycosylation.
3. Bovine aprotinin has no documented toxic effects on humans, mammals or birds and is well tolerated even at high doses.
4. Bovine aprotinin is not a food allergen.
5. The levels of expression of aprotinin in pollen, leaves, stems and roots of transgenic plants is so low that it will have no impact on nontarget organisms or the environment.

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TES Worksheet for PRGP04-04r

RECIPIENT ORGANISM: corn, *Zea mays*.

PRODUCTS:

Aprotinin

Bovine aprotinin under the control of a seed preferred promoter and targeted to the cell wall. Aprotinin is a serine protease inhibitor that has a number of applications in research, cell culture and medicine. We are mainly targeting the cell culture market, but may also move into pharmaceutical applications such as the control of blood loss during cardiopulmonary bypass surgery.

LOCATION OF FIELD TEST:

The land has been in cultivation for around 40 years and was probably utilized for grazing before that in an unimproved state. It consists of six sections under center pivot irrigation and another large (150-350 acre) flood irrigated section approximately 1.5-2 miles to the east. Two plantings are proposed for the one year duration of the permit. The sites proposed for the July/Aug. 2004 planting would each be immediately surrounded by the required 50 feet of fallow area. Three separate plots will be planted at the location: one for grain production, one hybrid seed production and one nursery plot. The regulated corn will be open pollinated, with the exception of the nursery where controlled hand pollinations will be made. Beyond the 50 foot fallow area, the sites would be surrounded by plantings of cabbage and onions (both of which are harvested by hand). To the east, south and north is uncultivated rangeland. To the west, there will be some cabbage and pickling cucumbers, or the land will be fallow. The sites proposed for the second planting in Feb./March 2005 are on the flood-irrigated section and will be surrounded by uncultivated pasture and rangeland on all sides. Plots will be surrounded by 50 feet of fallow area and areas between plots will be planted to cabbage or onions. Three separate plots will be planted: one grain production, one hybrid seed production and one nursery. The nearest corn intended for food, feed or seed production (for both plantings) is well over one mile away.
Standard good agricultural practices will be performed.

The closest body of water is a small contained reservoir to the South. The Frio River is 3-4 miles to the North.

LEVELS PRODUCED IN TISSUE:

Aprotinin
The expression of aprotinin in leaves and stems of field grown plants was at or below 0.0002% by weight, and in roots it was undetectable on our assay. Expression in pollen ranges from undetectable to 0.0001% by weight. Expression of aprotinin in seeds ranges from [ ].

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ASSESSMENT

Corn seed planted in this area is occasionally consumed by birds, rodents, deer and feral hogs, with the feral hogs being the most likely to consume seed. Growers familiar

with the area have indicated that plant consumption is not a problem once the stand is up. ProdiGene will also eliminate nearly all waste grain from our sites after harvest by a process of gleaning the fields of ears and large ear pieces followed by multiple rounds of disking to incorporate the crop residue into the soil. This will greatly reduce the amount of waste grain in the field that may be consumed by animals.
There are only two species on the Threatened and Endangered Species List for Frio County and both are carnivores and would not be impacted by ProdiGene's transgenic corn.
None of the gene products produced in ProdiGene's transgenic corn have any known toxic effects and so would not be expected to harm any animal species that consumed them. Aprotinin is produced by all higher vertebrate species and exposure to ProdiGene's recombinant corn would not constitute a new exposure.
ProdiGene's recombinant aprotinin is expressed under the control of a seed preferred promoter. Expression in plant parts other than the seed is so low that it will not cause any harm to pollinating or visiting insect species. Even in the seed, the expression levels are not high enough to cause harm given the innocuous nature of the protein products.
There are no plants on the TES list for this county.
Any unexpected effects from the field test would be minimal by virtue of being confined to the area within the field site and the extreme isolation of the location. Agronomic practices such as plowing or disking soon after harvest will also minimize any potential exposures.

CONCLUSION:
The protein products being expressed in ProdiGene's transgenic corn are not toxic and not known to cause any harm to any wild plant or animal species. Plants will be confined to the field test sites and will be monitored for any impact or contact with wild species. In the absence of any direct identifiable effect of this field test on any wild plants or animals, we must conclude that there will be no adverse effect on any threatened or endangered species.

FRIO COUNTY, TX TES FROM:
http://ifw2es.fws.gov/EndangeredSpecies/lists/ListSpecies.cfm

Animals – 2

Status	
E	Gulf Coast Jaguarundi (*Herpailurus (=Felis) yagouaroundi cacomitli*) – No impact, carnivore, does not feed on corn
E	Ocelot (*Leopardus (=Felis) pardalis*) – No impact, carnivore, does not feed on corn

Appendix V. FWS-APHIS TES Document

[The document below was the basis of APHIS' discussion with the Fish and Wildlife Services discussion on how APHIS' would approach addressing threatened and endangered species issues from field testing].

DECISION TREE ON WHETHER SECTION 7 CONSULTATION WITH FWS IS TRIGGERED FOR TRANSGENIC PLANTS UNDER PERMIT (PHARMACEUTICALS)

BACKGROUND

Some genetically engineered plants and plant viruses are being field tested to produce proteins that may have therapeutic use in human or animal therapy. This document outlines APHIS' evaluation of the risks of these products to threatened and endangered species.

The goal of this research is to produce cheaper and safer therapeutics. Mostly, applicants are **not** developing new therapeutics in plants but are trying to produce existing therapeutics (or close relatives) in plants. Because some of the therapeutics has already been approved by the Food and Drug Administration, a great deal is known about safety and risks of the therapeutic. Although the nature of the therapeutics is oftened claimed as confidential business information by the applicants, USG has access to detailed information about each therapeutic.

REGULATORY AUTHORITY

Human therapeutics are regulated by the Food and Drug Administration (FDA), while veterinary biologics are regulated by the Center for Veterinary Biologics (CVB) of the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA). The plants that are engineered to produce the therapeutics, or infected with a virus engineered to produce the therapeutics, are regulated by Biotechnology Regulatory Services staff (BRS) of APHIS. If they produce a human biologic, they are also regulated in part by FDA as part of its oversight of production of the biologic. FDA is responsible for ensuring that the plant is grown and maintained in a manner that will enable consistent production of a safe, pure, and potent biologic. If plants are engineered to produce a veterinary biologic, the plants are likewise also regulated in part by APHIS CVB as part of its oversight of production of the veterinary biologic[3].

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[3]Beginning in 1999, a working group was established by FDA and APHIS to coordinate efforts on this issue. The group sponsored a public meeting in April 2000 , Transcripts of the Plant-Derived Biologics Seminar and Public Hearing on Plant-Derived Biologics (http://www.fda.gov/cber/minutes/workshop-min.htm#plant); prepares a side bar to case study three in the Office of Science and Technology Policy (OSTP) (http://www.ostp.gov/html/012201.html) and published in the Federal Register for public

An Overview of field testing of pharmaceutical plants.

The first field test of pharmaceutical producing plant was in 1991. Currently, virtually all the field testing is being performed by commercial applicants. Corn, rice, and tobacco are the plants that have the largest acreage.

Researchers are interested in using crop plants to produce pharmaceuticals for a number of reasons. The need for very large quantities of biologics, projected to be 500 to 1000 kilograms per year for some human biologics, is growing rapidly. Production costs may be lower than with traditional fermentation technology, both because of reduced energy costs and reduced cost of raw materials. The energy-expensive process of cleaning and sterilization of large fermentors is not necessary and the need for large volumes of purified culture medium is eliminated. In addition, the use of crop plants removes the potential for contamination of the biologic with animal viruses that potentially can be pathogenic to humans. An inherent risk with biologics produced in animals or animal cells are that the animals or animal cells will become infected with a pathogenic virus that may then contaminate the product. This risk is avoided by producing the biologic in plants, because there are no known plant viruses that can infect people. Because the human pharmaceuticals are costly, producers will take every effort to maximize yields. This will include frequent pesticide applications to ensure maximized plant yields.

The production systems for pharmaceuticals can be divided into two classes - those products that are produced in the seed and those produced in leaves.

For tobacco, the products are being produced in the leaves. To maximize leaf production, tobacco plants are usually "topped" to block flowering. In the absence of flowering, APHIS can identify no nontarget organism that "feeds" on tobacco that is not a plant pest except possibly skunks. Because of nicotine production, earthworms are killed even by the nonengineered tobacco plants. If flowering does occur, bees and other pollinators could be potentially exposed.

There are two systems used in tobacco. The first uses engineered plants. The pharmaceuticals are being produced under wound-inducible promoters. That means, that the engineered plants do not produce detectable amounts of the product until the leaves are wounded[4].

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comment in September 2002, Draft Guidance for Industry: Drugs, Biologics, and Medical Devices Derived from Bioengineered Plants for Use in Humans and Animals - 9/6/2002. The group will continue its work indefinitely.

[4] When insects devour leaves, plants respond by producing a variety of compounds to deter feeding. Using molecular techniques, scientists have identified the DNA sequences (promoters) that trigger the production of compounds by wounding.

The other uses a tobacco mosaic virus which produces products by two systems. The virus is engineered to produce an epitope (the part of the sequence of an antigen that produces an immunological response). The **nonengineered** plants are inoculated with the virus and a few weeks later the leaves are harvested and the virus is extracted and purified. The cut plants are allowed to regenerate and another harvest is performed. The plants are in the field for approximately 2 months.

The other TMV system cause production of the product in the intracellular spaces of the leaves. The leaves are harvested and the product is gently extracted.

For most of the food crops including corn, rice, and barley, the pharmaceutical is being produced in the seed. Production in the seeds offers several advantages: one relatively high level of proteins can be produced, the proteins are generally more stable at room temperature in seeds than as purified products, and the systems to purify proteins from seeds is well developed.

How field tests are performed under APHIS permit.

The goal of APHIS regulations is to establish measures that must be taken to minimize dissemination of the engineered organism into the environment during movement and while in the receiving facility (laboratory, growth chamber, or greenhouse) as specified in 7 CFR 340. A consequence of minimizing dissemination and persistence in the environment, is exposure of any nontarget organism is also minimized.

Permits are required for importation, interstate movement, and field-testing plants engineered to produce pharmaceutical compounds and microorganisms. In the permit the applicant lists the regulated article or product, donor organism, recipient organism, vector or vector agent, dates of the importation, movement or release, quantity of the regulated article and the port of importation or site of release. In addition detailed information is provided as appropriate on the anticipated or actual expression of the altered genetic material in the regulated article and it differs from a non-modified parent organism, the molecular biology of the system, the country or locality where the donor, recipient, and vector were collected and produced, the experimental design at the release site, the facilities at the destination, the measures to insure containment, and the final disposition. This data is required so that a decision can be made to conclude that the transgenic plant is adequately characterized, that no transgenic plant material will persist in the environment, and that any unintentional or unanticipated effects, if any, can be restricted to the confined field site and be managed in such a way that there are no potential plant pest risks after the confined field release is terminated. All field test approvals require that a field data report be filed after the experiment is complete.

For field tests, measures must be taken to confine the transgenic plants to the field site during the defined period of the release and to prevent the transgenic plants or their progeny from persisting in the environment in subsequent growing seasons either within or outside of the site of the confined release. Both the reproductive isolation measures and post harvest land use restrictions are based on the reproductive biology and seed

dormancy characteristics of the species, surrounding land use, proximity of sexually compatible plants and presence of pollinators. Additional mitigation measures may be necessary based on the nature of the introduced trait(s).

During the growing season, measures must be taken to achieve reproductive isolation from plants of the same species and other sexually compatible species that are not part of the confined release, whether they are cultivated, weedy or wild species[5]. Depending on the plant species, this can be achieved by the use of one or a combination of the following: isolation distance, pollen or pollination-proof caging, netting or bagging of plants prior to flowering, guard rows/ border rows of plants to attract pollinators or trap transgenic pollen, flower removal prior to pollination, use of male sterile lines, use of plant growth regulators to block reproductive development, different flowering time, and/or termination of the confined field release prior to flowering. Generally, isolation distances that are used to ensure purity of certified seed (such as breeder seed or foundation classes of certified seed) may be adapted successfully to prevent or minimize out crossing of transgenic pollen to sexually compatible plants that could produce viable progeny capable of persisting outside the confined field release site. When isolation distances are used, these zones are also monitored for the presence of the same species, related species and for proximity of fields of the same species.

Post-harvest land use restrictions may be necessary for a certain number of years following harvest of the transgenic plant material to allow monitoring, removal and destruction of volunteers. Generally, for corn, this would involve monitoring for volunteers either immediately after harvest in warm climates where conditions favorable for germination can be maintained, or in the next growing season in colder climates. Generally, the post-harvest periods used to ensure purity of certified seed may be adapted successfully. For certain plant species, and for certain specific cases, post-harvest land use restrictions may also be necessary for the perimeter of the confined field site itself to monitor for volunteers resulting from potential dissemination of seed, e.g., during mechanical harvesting operations.

Other risk mitigation activities for field tests include: (1) adequate identification, packaging and segregation measures to prevent seed mixing, spillage and dispersal into the environment during transit; (2) adequate cleaning of seeding and transplanting machinery at the confined field site prior to removal to another location to prevent dissemination of viable transgenic plant material into the environment; (3) devitalization/destruction of surplus seed or seedlings, and any viable transgenic plant material remaining after transplantation or after harvesting at the confined field site by suitable means which could include, but are not limited to, dry heat, steam heat, crushing, deep burial, discing into the soil, burning, treatment with appropriately labeled herbicides

[5] APHIS has commented (http://www.aphis.usda.gov/ppq/biotech/pdf/pharma_2000.pdf) on plant species appropriate for field testing. "APHIS believes that some plants are inappropriate for the production of pharmaceuticals. These plants have characteristics like multiple year seed dormancy (e.g. *Brassica rapa*, are bee pollinated, and a sexually compatible with weed species in the locality of the field test."

and/or chemicals (harvested transgenic seed and/or plant material from the confined field site may only be retained in an approved facility if requested at the time of the submission and authorized by the regulatory authority, and should be clearly identified, securely transported, and stored separately from other seed/or plant material to avoid mixing); (4) a contingency plan for destruction of viable transgenic plant material in case of accidental release. The plan should include site marking and monitoring to ensure destruction of viable material and immediate notification of regulatory authorities.

What information applicant provides APHIS for field testing:

This is not a complete list of all information provided but focuses on elements associated with risks to nontarget organisms.

1. Levels of a gene product in roots, stem, leaves, pollen and seeds.

If the desired product is an enzyme, provide quantitative enzyme activity data for the roots, stem, leaves, pollen and seeds of the recipient organism, and for comparison the amount in the organism where the gene was obtained. (The amount of gene products in food or feed may also be supplied).

APHIS will use this information to determine if the nontarget organism is likely to have been exposed to the protein previously and whether the amounts of protein are in the range expected for consumption.

2. Whether the gene product is sensitive to gastric digestive conditions (pH and proteolytic enzymes).

If the product is sensitive to gastric digestion (e.g. many of the new proteins in GMOs are degraded within a minute) then exposure is virtually nil. Being susceptible to protease degradation also is important in disappearance of the protein in plant debris.

3. The thermal stability of the gene product.

The less thermal stable the product the more easily it will be degraded in the environment.

4. Provide APHIS data submitted to the FDA or other regulatory agencies that have been developed as part of a clinical trial.

FDA and CVB reviews for new therapeutics always contain safety data generated in lab animals and occasionally in humans. This data would help address potential impacts on nontargets.

5. Whether there is sequence homology to known toxicants, allergens, or proteins known or likely to harm non-target organisms (pesticidal properties).

The number and functions of proteins being identified have and will continue to increase rapidly. Database searches can quickly determine if a given protein has any sequence homology to known proteins that raise concerns for nontarget organisms. This is an easy screen for all new proteins being field tested under APHIS authorization.
6. If the gene product has some inherent toxic activity, compare levels produced in the transgenic plant with those in the organism of origin (or related organisms). Address possible differences associated with different exposure routes

7. Provide a list of threatened and endangered species for each county that a field test is planned.

ANALYSIS

Considering all the above provided information and literature, APHIS will assess the plants/seeds have damaging or toxic effects directly or indirectly on non-target organisms associated with the plant or its parts, including:

a. beneficial organisms (insect pollinators, earthworms, bees, lady beetles, etc.)
b. foraging birds, rabbits, deer, rodents or other wildlife
c. potential impact on threatened and endangered species (TES)

If APHIS cannot reach a "no harm" decision then will initiate consultation. To document our decision making process APHIS will complete an TES assessment sheet for every gene- site combination. A sample is provide below.

Appendix VI. Environmental and human health safety chemical pesticides used on corn to control lepidopteran pests

	Dimethoate[2] (Cygon) [organophosphate]	Chlorpyrifos[3] (Lorsban) [organophosphate]	Permethrin[4] (Ambush/Pounce) [pyrethroid]	λ-cyhalothrin[5] (Darate) [pyrethroid]
Environmental Fate	Dimethoate is of low persistence in the soil environment. Soil half-lives of 4 to 16 days, or as high as 122 days have been reported, but a representative value may be on the order of 20 days. Because it is rapidly broken down by soil microorganisms, it will be broken down faster in moist soils. Dimethoate is highly soluble in water, and it adsorbs only very weakly to soil particles so it may be subject to considerable leaching. However, it is degraded by hydrolysis, especially in alkaline soils, and evaporates from dry soil surfaces. Losses due to evaporation of 23 to 40% of applied dimethoate have been reported. Biodegradation may be significant, with a 77% loss reported in a nonsterile clay loam soil after 2 weeks. In water, dimethoate is not expected to adsorb to sediments or suspended particles, nor to bioaccumulate in aquatic organisms. The half-life for dimethoate in raw river water was 8 days, with disappearance possibly due to microbial action or chemical degradation.	In soils: Chlorpyrifos is moderately persistent with a half-life of usually 60 and 120 days, and a range from 2 wks - > 1 yr., depending on the soil type, climate, and other conditions . It was less persistent in soils with a higher pH (greater than 7.4). Soil half-life was not affected by soil texture or organic matter content. Adsorbed chlorpyrifos is subject to degradation by UV light, chemical hydrolysis and by soil microbes. When applied to moist soils, the volatility half-life was 45 to 163 hours, with 62 to 89% of the applied chlorpyrifos remaining on the soil after 36 hours. In another study, 2.6 and 9.3% of the chlorpyrifos applied to sand or silt loam soil remained after 30 days . Chlorpyrifos adsorbs strongly to soil particles and it is not readily soluble in water. It is therefore immobile in soils and unlikely to leach or to contaminate groundwater. TCP, the principal metabolite of chlorpyrifos, is moderately mobile and persistent in soils. In water: The concentration and persistence of chlorpyrifos will vary depending on the type of formulation. The increase in the concentration of insecticide is slower for granules and controlled release formulations in the water, but the resulting concentration persists longer . Volatilization is probably the primary route of loss of chlorpyrifos from water. Volatility half-lives of 3.5 and 20 days have been estimated for pond water. The photolysis half-life is 3 to 4 weeks during midsummer in the U.S. Research suggests that in water the rate at which it is hydrolyzed decreases by 2.5- to 3-fold with each 10 C drop in temperature. The rate of hydrolysis increases in alkaline waters. In water at pH 7.0 and 25 C, it had a half-life of 35 to 78 days. In vegetation: Chlorpyrifos may be toxic to some plants. Residues remain on plant surfaces for ~ 10 to 14 days. This insecticide and its soil metabolites can accumulate in certain crops.	Permethrin is of low to moderate persistence in the soil environment, with reported half-lives of 30 to 38 days. Permethrin is readily broken down, or degraded, in most soils except organic types. Soil microorganisms play a large role in the degradation of permethrin in the soil. The addition of nutrients to soil may increase the degradation of permethrin. It has been observed that the availability of sodium and phosphorous decreases when permethrin is added to the soil. Permethrin is tightly bound by soils, especially by organic matter. Very little leaching of permethrin has been reported. It is not very mobile in a wide range of soil types. Because permethrin binds very strongly to soil particles and is nearly insoluble in water, it is not expected to leach or to contaminate groundwater. The results of one study near estuarine areas showed that permethrin had a half-life of less than 2.5 days. When exposed to sunlight, the half-life was 4.6 days. Permethrin degrades rapidly in water, although it can persist in sediments. Breakdown in vegetation: Permethrin is not phytotoxic, or poisonous, to most plants when it is used as directed. No incompatibility has been observed with permethrin on cultivated plants.	λ- cyhalothrin is moderately persistent in the soil environment. Reported field half-lives range from four to 12 weeks. Its field half-life is probably close to 30 days in most soils. It shows a high affinity for soil and so is not expected to be appreciably mobile in most soils. There is little potential for groundwater contamination. Soils with high sand content or with very low organic matter content may tend to retain the compound to a lesser degree. In field studies of Karate, leaching of λ-cyhalothrin and its degradates from the soil were minimal. Breakdown rates of both the technical product and Karate were similar under aerobic and anaerobic conditions. λ- cyhalothrin has extremely low water solubility and is tightly bound to soil, it is therefore not expected to be prevalent in surface waters. One possible source of infiltration into surface waters would be surface runoff. In this event, the compound would most probably remain bound to the solid particle and settle to the bottom.
Avian toxicity	Dimethoate is moderately to very highly toxic to birds. In Japanese quail, a 5-day dietary LC_{50} of 341 ppm is reported. It may be very highly toxic to other birds; reported acute oral LD_{50} values are 41.7 to 63.5 mg/kg in mallards and 20.0 mg/kg in pheasants. Birds are not able to metabolize dimethoate as rapidly as mammals do, which may account for its relatively higher toxicity in these species.	Chlorpyrifos is moderately to very highly toxic to birds. Its oral LD_{50} is 8.41 mg/kg in pheasants, 112 mg/kg in mallard ducks, 21.0 mg/kg in house sparrows, and 32 mg/kg in chickens. The LD_{50} for a granular product (15G) in bobwhite quail is 108 mg/kg. At 125 ppm, mallards laid significantly fewer eggs. There was no evidence of changes in weight gain, or in the number, weight, and quality of eggs produced by hens fed dietary levels of 50 ppm of chlorpyrifos.	Effects on birds: Permethrin is practically non-toxic to birds. The oral LD_{50} for the permethrin formulation, Pramex, is greater than 9900 mg/kg in mallard ducks, greater than 13,500 mg/kg in pheasants, and greater than 15,500 mg/kg in Japanese quail.	λ- cyhalothrin's toxicity to birds ranges from slightly toxic to practically non-toxic. In the mallard duck, the reported oral LD_{50} is greater than 3,950 mg/kg, and the reported dietary LC_{50} is 3,948 ppm. In bobwhite quail the reported dietary LC_{50} is greater than 500 ppm. There is evidence that it does not accumulate in the eggs or tissues of birds.

Aquatic Data	Dimethoate is moderately toxic to fish, with reported LC_{50} values of 6.2 mg/L in rainbow trout, and 6.0 mg/L in bluegill sunfish. It is more toxic to aquatic invertebrate species such as stoneflies and scuds.	Chlorpyrifos is very highly toxic to freshwater fish, aquatic invertebrates and estuarine and marine organisms. Cholinesterase inhibition was observed in acute toxicity tests of fish exposed to very low concentrations of this insecticide. Application of concentrations as low as 0.01 pounds of active ingredient per acre may cause fish and aquatic invertebrate deaths. Chlorpyrifos toxicity to fish may be related to water temperature. The 96-hour LC_{50} for chlorpyrifos is 0.009 mg/L in mature rainbow trout, 0.098 mg/L in lake trout, 0.806 mg/L in goldfish, 0.01 mg/L in bluegill, and 0.331 mg/L in fathead minnow]. Chlorpyrifos accumulates in the tissues of aquatic organisms. Studies involving continuous exposure of fish during the embryonic through fry stages have shown bioconcentration values of 58 to 5100. Due to its high acute toxicity and its persistence in sediments, chlorpyrifos may represent a hazard to sea bottom dwellers. Smaller organisms appear to be more sensitive than larger ones .	Effects on aquatic organisms: Aquatic ecosystems are particularly vulnerable to the impact of permethrin. A fragile balance exists between the quality and quantity of insects and other invertebrates that serve as fish food. The 48-hour LC_{50} for rainbow trout is 0.0125 mg/L for 24 hours, and 0.0054 mg/L for 48 hours. As a group, synthetic pyrethroids were toxic to all estuarine species tested. They had a 96-hour LC_{50} of less than or equal to 0.0078 mg/L for these species. The compound has a low to moderate potential to accumulate in these organisms.	λ- cyhalothrin is very highly toxic to many fish and aquatic invertebrate species. Reported LC_{50} in these species are as follows: bluegill sunfish, 0.21 µg/L; rainbow trout, 0.24 µg/L; Daphnia magna, 0.36 µg/L; mysid shrimp, 4.9 ng/L; sheepshead minnow, 0.807 ng/L. Bioconcentration is possible in aquatic species, but bioaccumulation is not likely. Bioconcentration in channel catfish has been reported as minimal, with rapid depuration (elimination). A bioconcentration factor of 858 has been reported in fish (species unspecified), but concentration was confined to non-edible tissues and rapid depuration was observed.
Nontarget and beneficial insects	Survival of *Microplitis croceipes* (Cresson) adults, parasitoids of the cotton pests *H.zea* and *H.virescens*, exposed to residues of insecticides applied at recommended rates to cotton was measured in 1989. In unsprayed cheek treatments, survival was 91.4% after 24 h. The organophosphates profenofos and acephate and the new-generation pyrethroid bifenthrin were highly toxic to *M. croceipes*. All other compounds tested showed some selectivity, including esfenvalerate, cypermethrin, thiodicarb, oxamyl, dicrotophos, dimethoate, and cyhalothrin in order of decreasing survival. The effectiveness of *M. croceipes* as a biocontrol agent of the bollworm and tobacco budworm might be improved through selective use of insecticides to which the parasitoid is tolerant.	Aquatic and general agricultural uses of chlorpyrifos pose a serious hazard to wildlife and honeybees.	Effects on other organisms: Permethrin is toxic to wildlife. It should not be applied, or allowed to drift, to crops or weeds in which active foraging takes place. The International Organization for Biological Control tested the acute toxicity of permethrin to 13 species of beneficial arthropods and found that permethrin caused 99 percent mortality of 12 of the species, and over 80 percent mortality of the other. Effects were persistent, lasting over 30 days. Sublethal doses also impact beneficial arthropods: permethrin inhibited the emergence of a parasitoid wasp from eggs of the rice moth *Corcyra cephalonica* and disrupted the foraging pattern of another parasitoid wasp as it searched for its aphid prey.	Data not available from sources consulted.
Honeybee toxicity	Dimethoate is highly toxic to honeybees. The 24-hour topical LD_{50} for dimethoate in bees is 0.12 µg per bee	Aquatic and general agricultural uses of chlorpyrifos pose a serious hazard to honeybees.	Permethrin is extremely toxic to bees. Severe losses may be expected if bees are present at treatment time, or within a day thereafter.	λ- cyhalothrin is highly toxic to bees, with a reported oral LD_{50} of 38 ng/bee and reported contact LD_{50} of 909 ng/bee (0.9 µg/bee).
Nontarget soil organisms	A study of the effects of soil moisture and toxicity of dimethoate was conducted with an enchytraeid worm. Laboratory experiments used dimethoate and small Enchytraeus sp. as the test species. Substrate was natural agricultural field soil cultivated without pesticides for several	Data not found in sources consulted.	Data not found in sources consulted.	Data not found in sources consulted

	years. Experimental design consisted of three soil moistures (40, 55, and 70% of water holding capacity) and five pesticide concentrations, plus controls. Measured parameters were survival, size of the parent worms and number and size of juveniles produced. Dimethoate was relatively non-toxic to this species. Dimethoate did not decrease survival, but sublethal effects on adult size and number of juveniles were observed. Adverse conditions in dry soil masked these effects; dimethoate appeared to be less toxic in dry soil than in moist soil.			
EPA toxicity class (Class I -highly toxic to Class IV-relatively nontoxic)	Dimethoate is a moderately toxic compound in EPA toxicity class II. Labels for products containing dimethoate must bear the Signal Word WARNING. Dimethoate is a General Use Pesticide (GUP).	Chlorpyrifos is toxicity class II - moderately toxic. Products containing chlorpyrifos bear the Signal Word WARNING or CAUTION, depending on the toxicity of the formulation. It is classified as a General Use Pesticide (GUP). The EPA has established a 24-hour reentry interval for crop areas treated with emulsifiable concentrate or wettable powder formulations of chlorpyrifos unless workers wear protective clothing.	Permethrin is a moderately to practically non-toxic pesticide in EPA toxicity class II or III, depending on the formulation. Formulations are placed in class II due to their potential to cause eye and skin irritation. Products containing permethrin must bear the Signal Word WARNING or CAUTION, depending on the toxicity of the particular formulation. All products for agricultural uses (except livestock and premises uses) are Restricted Use Pesticides (RUPs) because of their possible adverse effects on aquatic organisms.	λ- cyhalothrin is a Restricted Use Pesticide and so may be purchased and used only by certified applicators. It is in EPA Toxicity Class II, and products containing it must bear the signal word WARNING.
EDF - Integrated Environmental Rankings[6] - Combined human & ecological scores	65 to 100% ranked.on the least to most hazardous scale with 100% being the most hazardous	50 to 75%	0 to 25%	not ranked
Mammalian toxicity	**Acute toxicity:** Dimethoate is moderately toxic by ingestion, inhalation, and dermal absorption. The reported acute oral LD_{50} values for the technical product range from 180 to 330 mg/kg in the rat; although an oral LD_{50} of as low as 28 to 30 mg/kg has been reported. Reported dermal LD_{50} values for dimethoate are 100 to 600 mg/kg in rats, again with a much lower value for an earlier product . Dimethoate is reportedly not irritating to the skin and eyes of lab animals. Severe eye irritation has occurred in workers manufacturing dimethoate, although this may be due to impurities. Via the inhalation route, the reported 4-hour LC_{50} is greater than 2.0 mg/L, indicating slight toxicity. Effects of acute exposure are those typical of organophosphates.	**Acute toxicity:** Chlorpyrifos is moderately toxic to humans. Poisoning may affect the central nervous system, the cardiovascular system, and the respiratory system. It is also a skin and eye irritant. Studies in humans suggest that skin absorption of chlorpyrifos is limited. The oral LD_{50} for chlorpyrifos in rats is 95 to 270mg/kg, 60 mg/kg in mice,1000 mg/kg in rabbits, 32 mg/kg in chickens, 500 to 504 mg/kg in guinea pigs, and 800 mg/kg in sheep. The dermal LD_{50} is greater than 2000 mg/kg in rats, and 1000 to 2000 mg/kg in rabbits. The 4-hour inhalation LC_{50} for chlorpyrifos in rats is greater than 0.2 mg/L. **Chronic toxicity:** Repeated or prolonged exposure to organophosphates may result in the same effects as acute exposure including the delayed symptoms. Human volunteers who ingested for 4 weeks 0.1mg/kg/day of chlorpyrifos showed significant plasma cholinesterase inhibition. **Reproductive effects:** Current evidence indicates that	**Acute toxicity**: Permethrin is moderately to practically non-toxic via the oral route. Via the dermal route, it is slightly toxic, with a reported dermal LD_{50} in rats of over 4000 mg/kg, and in rabbits of greater 2000 mg/kg. Permethrin caused mild irritation of both the intact and abraded skin of rabbits. It also caused conjunctivitis when it was applied to the eyes. The 4-hour inhalation LC_{50} for rats was greater than 23.5 mg/L, indicating practically no inhalation toxicity. **Chronic toxicity**: No adverse effects were observed in dogs fed permethrin at doses of 5 mg/kg/day for 90 days. Rats fed 150 mg/kg/day for 6 months showed a slight increase in liver weights. **Reproductive effects**: The fertility of female rats was affected when they received very high oral doses of 250 mg/kg/day of permethrin during the 6th to 15th day of pregnancy. It is not likely that reproductive effects will be seen in humans under normal circumstances. **Teratogenic effects**: Permethrin is reported to show	λ- cyhalothrin is moderately toxic in the technical form, but may be highly toxic via some routes in formulation (e.g., as Karate). Cyhalothrin is moderately toxic via the oral route in test animals. Data indicate a moderate to high toxicity via the inhalation route for the formulated product Karate. It may cause mild eye irritation in rabbits. **Chronic Toxicity**: The principal toxic effects noted in chronic studies with rates were decreased body weight gain and decreased food consumption. It is unlikely that cyhalothrin would cause chronic effects in humans under normal conditions. **Reproductive Effects**: Cyhalothrin caused reduced numbers of viable offspring at doses of 50 mg/kg/day in the second and third generations in the three-generational rat study noted above. It is unlikely that cyhalothrin

	Chronic toxicity: There was no cholinesterase inhibition in an adult human who ingested dimethoate for 21 days. No toxic effects and no cholinesterase inhibition were observed in individuals who ingested dimethoate for 4 weeks. Repeated or prolonged exposure to organophosphates may result in the same effects as acute exposure, including the delayed symptoms. **Reproductive effects**: When mice were given 9.5 to 10.5 mg/kg/day dimethoate in their drinking water, there was decreased reproduction, pup survival, and growth rates of surviving pups. **Teratogenic effects**: Dimethoate is teratogenic in cats and rats. It is not likely that teratogenic effects will be seen in humans under normal circumstances. **Mutagenic effects**: Mutagenic effects due to dimethoate exposure were seen in mice. Mutagenic effects are unlikely in humans under normal circumstances. **Carcinogenic effects**: An increase in malignant tumors was reported in rats given oral doses of dimethoate for over a year; but the increases were not dose dependent. Thus the evidence of carcinogenicity, even with high-dose, long-term exposure, is inconclusive. This suggests carcinogenic effects in humans are unlikely. **Fate in humans and animals**: Dimethoate is rapidly metabolized by mammals.	chlorpyrifos does not adversely affect reproduction. No effects were seen in 2 studies where animals were tested at doses up to 1.2 mg/kg/day. **Teratogenic effects:** Available evidence suggests that chorpyrifos is not teratogenic. Three studies in pregnant rats or mice indicate that no significant teratogenic effects were seen at doses up to 25 mg/kg/day for 10 days. **Mutagenic effects:** No evidence was found in any of four tests performed that chlorpyrifos is mutagenic. **Carcinogenic effects:** There is no evidence that chlorpyrifos is carcinogenic. There was noincrease in the incidence of tumors when rats were fed 10 mg/kg/day for 104 weeks. **Fate in humans and animals:** Chlorpyrifos is readily absorbed into the bloodstream through the gastro-intestinal tract if it is ingested, through the lungs if it is inhaled, or through the skin if there is dermal exposure. In humans, chlorpyrifos and its principal metabolites are eliminated rapidly. After a single oral dose, the half-life of chlorpyrifos in the blood appears to be about 1 day.	no teratogenic activity. **Mutagenic effects**: Permethrin is reported to show no mutagenic activity. **Carcinogenic effects**: The evidence regarding the carcinogenicity of permethrin is inconclusive. **Organ toxicity**: Permethrin is suspected of causing liver enlargement and nerve damage. **Fate in humans and animals**: Permethrin is efficiently metabolized by mammalian livers. Breakdown products, or "metabolites," of permethrin are quickly excreted and do not persist significantly in body tissues. Permethrin may persist in fatty tissues, with half-lives of 4 to 5 days in brain and body fat.	would cause reproductive effects in humans under normal conditions. **Teratogenic Effects**: No teratogenic or fetotoxic effects were observed in teratology studies of lambda cyhalothrin in rats and rabbits at the highest doses tested in both species (15 mg/kg/day and 30 mg/kg/day, respectively). **Mutagenic Effects**: Cyhalothrin produced negative results in Ames mutagenicity assays and other in-vitro cytogenetic assays and chromosomal structural aberration tests indicated no mutagenic or genotoxic effects. **Carcinogenic Effects**: Evidence is inconclusive, but suggests that it is probably not carcinogenic. **Organ Toxicity**: No specific target organs or organ systems have been identified in the available studies of chronic toxicity. **Fate in Humans & Animals**: In rat studies, lambda cyhalothrin is rapidly metabolized and excreted via the urine and feces.

Dimethoate Data: Pesticide Information Profiles, EXTOXNET Extension Toxicology Network. Revised June 1996. *http://ace.orst.edu/cgi-bin/mfs/01/pips/dimethoa.htm?8#mfs* ; H. M. Puurtinen, E. A. T. Martikainen (1997) Effect of Soil Moisture on Pesticide Toxicity to an Enchytraeid Worm, Enchytraeus sp., Arch. Environ. Contam. Toxicol. 33:34-41. *http://link.springer-ny.com/link/service/journals/00244/bibs/33n1p34.html*; Survival of Microplitis croceipes (Hymenoptera: Braconidae) in contact with residues of insecticides on cotton. Powell, J.E.; Scott, W.P.(1991) Environmental entomology v. 20 (1): p. 346-348; 1991 Feb.

Chlorpyrifos Data: Pesticide Information Profiles, EXTOXNET Extension Toxicology Network. Revised June 1996. http://ace.orst.edu/cgi-bin/mfs/01/pips/chlorpyr.htm. Chemical Fact Sheet for : Chlorpyrifos, Fact Sheet Number: 37, Date Issued: September 30, 1984 available at *http://pmep.cce.cornell.edu/profiles/insect-mite/cadusafos-cyromazine/chlorpyrifos/index.html*.

Permethrin Data: Pesticide Information Profiles, EXTOXNET Extension Toxicology Network. Revised June 1996. *http://ace.orst.edu/cgi-bin/mfs/01/pips/permethr.htm?8#mfs* ; Insecticide Fact Sheet, Coalition for Alternatives to Pesticides/NCAP, P.O.Box 1393, Eugene, Oregon,. J. of Pesticide Reform, Summer, 1998, v. 18, no. 2141. *http://www.safe2use.com/poisons-pesticides/pesticides/permethrin/cox.htm*

Lambda-cyhalothrin Data: Pesticide Information Profiles, EXTOXNET Extension Toxicology Network. *http://ace.orst.edu/cgi-bin/mfs/01/pips/lambdacy.htm?6#mfs*.

For EDF rankings, Environmental Defense Fund. *http://www.scorecard.org/chemical-profiles/*

Appendix VII. Environmental and human health safety chemical pesticides used on corn to control corn rootworm.

	Terbufos (Counter®)	*Tefluthrin (Force®)*	*Fipronil (Combat®)*
Environmental Fate	Terbufos hydrolyzes at pH 5, 7, and 9 with a half-life of 2.2 weeks. Formaldehyde was the major degradate detected in this study. Aerobic soil metabolism study indicate that terbufos degrades in silt loam soil with a half-life of 26.7 days. The major degradates detected in this study included carbon dioxide, terbufos sulfoxide, and terbufos sulfone. Terbufos residues have a half-life of less than 40 days in field plots of loam soil treated with a 15 percent granular formulation at an application rate of 1 lb ai/A. The sampling protocol was inadequate to accurately assess the dissipation of terbufos residues in field soil and a new study is required. The available data reviewed by the Agency are not sufficient to fulfill data requirements nor to assess the environmental fate of terbufos. EPA is concerned about the potential for the two degradates, terbufos sulfoxide and sulfone, to leach to groundwater, and the potential for parent terbufos and the sulfoxide and sulfone degradates to runoff to surface water. Terbufos parent degrades rapidly to the sulfoxide and sulfone metabolites, and is considered moderately mobile. Terbufos sulfoxide and sulfone are more mobile and persistent than parent terbufos. The acute DWLOCs calculated for the general U.S. population is 8.1 Fg/L. The chronic DWLOCs calculated for the general U.S. population is 1.7 Fg/L. Maximum acute and chronic estimated environmental concentrations (EECs) for parent terbufos plus the sulfoxide and sulfone degradates exceed the acute and chronic DWLOCs, respectively, in all cases.(2).	Tefluthrin is immobile in soil and, therefore, will not leach into ground water. Additionally, due to the insolubility and lipophilic nature of tefluthrin, any residues in surface water will rapidly and tightly bind to soil particles and remain with sediment, therefore not contributing to potential Tefluthrin is immobile in soil and, therefore, will not leach into ground water. Additionally, due to the insolubility and lipophilic nature of tefluthrin, any residues in surface water will rapidly and tightly bind to soil particles and remain with sediment, therefore not contributing to potential dietary exposure from drinking water. Plant metabolism studies indicate that tefluthrin per se is not translocated to plants but is degraded in soil to two rincipal metabolites that are capable of being taken up by plants. EPA has decided that Metabolite VI need not be regulated. Based on tefluthrin not being registered for residential non-food sites, EPA concludes that the aggregate short- and intermediate-term risks do not exceed levels of concern (MOE less than 100), and that there is reasonable certainty that no harm will result from aggregate exposure to tefluthrin residues. (3)	Soil: In lab studies, fipronil has a half-life of 122-128 days in oxygenated sandy loam soil. In field studies, dissipation half-life on soil surfaces ranged from 0.7 to 1.7 months. Half-life of fipronil applied by soil incorporation ranged from 3 to 7.3 months. Residues remain mainly in the upper 12 inches of soil. Fipronil has low soil mobility. It binds to the soil and has little potential for groundwater contamination. Anaerobic metabolism: Fipronil degrades slowly in water and sediment that lack oxygen with a half-life ranging from 116-130 days. Hydrolysis: Fipronil is stable to breakdown by water at mildly acid (lower pH) to neutral pH. It degrades with a half-life of 28 days in basic (higher pH) solutions. Photodegradation: In studies where fipronil was exposed to light, fipronil had a half-life of 3.6 hours in water and 34 days in loamy soil. Half-life is the time required for half of the compound to degrade.(6)
Avian toxicity	Seven incidents to nontarget terrestrial organisms have been reported. Up to three of the incidents had some indication of misuse or misapplication. All the mortalities involved bird species (mostly raptors), with the exception of one incident involving red wolves in North Carolina, which is believed to be the result of an intentional poisoning. Calculated RQs for birds and mammals significantly exceed EPA's risk concern for both granular formulations. (2) Dietary Avian Toxicity: 143 and 157 ppm (from two bobwhite studies). - Avian Reproduction: Terbufos was not considered to produce	Low toxicity to birds (4).	Fipronil has been found to be highly toxic to upland game birds, but is practically non-toxic to waterfowl and other bird species. One of the metabolites of fipronil has a higher toxicity to birds than the parent compound itself (6)
Fish toxicity	EPA has concerns about risk to nontarget aquatic organisms from parent terbufos and the terbufos sulfoxide and sulfone degradates based on widespread fish kill incidents involving terbufos use on corn with all application methods. These concerns are further supported by standard LOC criteria, which indicate risk concerns to aquatic fish and invertebrates associated with both the clay-based (15% active ingredient) and polymer-based (20% active ingredient) granular formulations	Highly toxic to fish (4)	Fipronil is highly toxic to fish and aquatic invertebrates. Its tendency to bind to sediments and its low water solubility may reduce the potential hazard to aquatic wildlife (6)

Nontarget and beneficial organisms	Terrestrial Field Study (Level 1): both soil-incorporated (2 lb ai/A) and nonsoil-incorporated (1 lb/A) resulted in nontarget mortalities, with the latter application much more severe in its effects (1,2)	Data not found.	Fipronil is non-toxic to earthworms, soil microorganisms and aquatic plants. Fipronil is moderately toxic to small mammals if ingested (6).
Honeybee toxicity	Not described in available studies.	High toxicity to bees (5)	Fipronil is toxic to bees and should not be applied to vegetation when bees are foraging (6).
Mammalian toxicity	**Acute Oral**: Toxicity Category I (1.6 and 1.3 mg/kg for male and female rats, respectively). - **Acute Dermal**: Toxicity Category I (0.81 and 0.93 mg/kg for male and female rabbits, respectively). - **Acute Inhalation**: Toxicity Category I (< 0.2 mg/L). - **Delayed Neurotoxicity**: No evidence of acute delayed neurotoxicity at the 40 mg/kg dosage level tested in hens. - **Subchronic Feeding**: The NOEL for both systemic effects and cholinesterase inhibition in a rat subchronic study is 0.25 ppm. - **Subchronic Dermal**: The NOEL for systemic effects in a 30-day rabbit study is 0.020 mg/kg. - **Mutagenicity**: Terbufos did not exhibit mutagenic potential in the Ames assay, the in vivo cytogenetic assay, and the dominant lethal test. - **Teratogenicity**: The NOEL for developmental toxicity in a rat teratology study is 0.1 mg/kg/day. - **Reproduction**: The NOEL for reproductive effects in a three-generation rat reproduction study is 0.25 ppm. - **Oncogenicity**: None (1,2)	**Acute toxicity** studies with the technical grade of the active ingredient tefluthrin: oral LD50 in the rat is 21.8 mg/kg for males and 34.6 mg/kg for females; dermal LD50 in the rat is 316 mg/kg in males and 177 mg/kg in females; acute inhalation LC50 in the rat is 0.037 mg/l and 0.049 mg/l in male and female rats, respectively; primary dermal irritation study in the rabbit showed slight irritation; and the acute delayed neurotoxicity study did not show acute delayed neurotoxicity. In an oral toxicity study, the NOEL for female rats is 100 ppm (equivalent to approximately 5 mg/kg/day). The NOEL for skin effects in rats is 1.0 mg/kg). The NOEL for neurological effects (the observed postural effects) may be between 0.025 and 0.1 mg/kg. **Carcinogenicity**: There was no evidence of carcinogenic potential. **Mutagenicity**: There is no mutagenicity concern. **Metabolism**: In both rats and dogs, when given either 1 or 10 mg/kg, most of the radioactivity was found in the feces unchanged and most urinary metabolites were conjugated. In rats, the halflife in the liver is 4.8 days, in the fat is 13.3 days and in the blood is 10.6 days. In a study with rat fat, half of the radioactive residues could be attributed to the parent and the remaining residues consisted of a mixture of fatty acid esters of hydroxylated parent metabolites. **Neurotoxicity**: No acceptable mammalian neurotoxicity studies (3). are available.(3) (((3)	The technical product (96.5% fipronil) has a high order of toxicity with respect to ingestion and inhalation in the rat, but appears to be less toxic via skin absorption. Fipronil may cause mild irritation to the eyes and slight skin irritation. It does not sensitize the skin. Signs of toxicity in rats include reduced feed consumption, anuria (no urination), increased excitability, and seizures. Human toxicity data are not available. The major route of fipronil excretion in rats is via feces. Excretion in the feces ranges from 45-75% of the administered dose, while excretion in urine ranges from 5-25%. Fipronil showed no evidence of causing cancer in mice that were fed large daily amounts in long-term studies. However, there was an increase in thyroid tumors in both sexes of rats in the same type of studies. Human cancer data are not available. Reproduction studies in rats over several generations show that reproductive toxicity occurs at the higher doses tested. Human data on reproductive and developmental toxicity are not available (6).
Nontarget soil organism effects	Not described by present reports.	Not found in these reports	Not described by the present reports.

Toxicity	Classified by EPA as Toxicity Category I	Toxicity class I for dermal, oral, inhalation exposures, and Class IV for skin irritation.	The technical form of fipronil carries the signal word "Warning.", Toxicity Class II. All formulated or end-use fipronil products in the United States have the signal word "Caution." See The Pesticide Label box (above). Formulated products contain diluted amounts of fipronil (6).
EDF's Integrated Environmental Rankings - Combined human & ecological scores (7)	85-100% where 0 is the lowest and 100 is the highest hazard rating (7).	Data lacking; not ranked by any system in Scorecard.	Data lacking; not ranked by any system in Scorecard.

Source of information:

1. EPA Pesticide Fact Sheet http://pmep.cce.cornell.edu/profiles/insect-mite/propetamphos-zetacyperm/terbufos/insect-prof-terbufos.html
2. Overview of Revised Terbufos Risk Assessment, Office of Pesticide Programs-- US EPA
   http://www.epa.gov/pesticides/op/terbufos/terbufosview.htm
3. Tefluthrin; Pesticide Tolerance ENVIRONMENTAL PROTECTION AGENCY (40 CFR Part 180)
   [Federal Register: November 26, 1997 (Volume 62, Number 228)
   http://www.epa.gov/fedrgstr/EPA-PEST/1997/November/Day-26/p30946.htm
4. Farm Chemicals Handbook, p. C374.
5. Ohio State University, Insect Pests of Field Crops Bulletin 545 "Toxicity of Pesticides"
   http://www.ag.ohio-state.edu/b45/b45_48.html
6. National Pesticide Telecommunications Network Fact Sheets. **http://www.ace.orst.edu/info/nptn/factsheets/fipronil.htm**
7. Environmental Defense Fund Scorecard. http://www.scorecard.org/chemical-profiles/
8. Extoxnet: Extension Toxicology Network, Pesticide Information Profiles

http://ace.ace.orst.edu/info/extoxnet/pips/bacillus.htm