USDA/APHIS Environmental Assessment

In response to permit application (04-309-01r)
received from Ventria Bioscience
for field-testing of rice, *Oryza sativa*, genetically engineered to express human lysozyme

U.S. Department of Agriculture

Animal and Plant Health Inspection Service

Biotechnology Regulatory Services

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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 04-309-01r) received from Ventria Bioscience, Sacramento, California, to grow genetically engineered rice (*Oryza sativa* L.) plants in Scott County, Missouri. These transgenic plants have been modified to express the human (*Homo sapiens*) enzyme lysozyme. These plants have also been engineered with the selectable marker gene *hpt*[1] which encodes for the enzyme hygromycin B phosphotransferase, Hpt. Hpt inactivates the antibiotic hygromycin. This gene is not expressed in the mature plant due to the nature of the promoter that drives this gene. Some of the details of the field site have been claimed as confidential business information (CBI) by the applicant (FR 50 38561-63). The transgenic line to be planted for the production of lysozyme has been designated by the company as LZ159-53.

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

These field tests are scheduled to begin in March/April 2005, on a site in Scott County, Missouri. These tests should be completed in the fall of 2005. Similar plantings are planned in future years with an increase in acreage over time.

The bases of confinement for these field plantings are:

- The field test site is located on private land in Scott County. Scott County is not a major producer of rice.

- In nature, chromosomal genetic material of rice can only be transferred to other sexually compatible plants by cross-pollination. Rice is highly inbred and outcrosses at a very low frequency. The field test plot will be at least 1/4 mile from any other rice plant with which it might cross-pollinate.

- To prevent intermingling of seeds with other crop plants Ventria will use dedicated equipment, storage and processing facilities on site. The harvested seeds will be milled on site and will not be shipped to any outside milling facilities.

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

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[1] By convention, the gene is designated by small italic letters, and the protein produced by that gene is designated by non-italicized letters, first letter capitalized.

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

The proposed field planting is a controlled release of the regulated article into the environment. The experimental protocols and field plot design as well as the procedures for termination of the field tests have been deemed sufficient to ensure that none of the modified rice plants persist in the environment beyond the termination of the experiments. The proposed field tests do not present a significant impact on populations of non-target animal species, including any threatened or endangered species in the County of the proposed test site. The APHIS review and analyses of the data package presented by the applicant indicate that the proposed field planting does not present a risk of introduction and dissemination of a plant pest and should not have a significant impact, either individually or cumulatively, on the quality of the human environment.

II. Purpose and Need

USDA/APHIS is considering the issuance a permit for confined field release of genetically engineered rice (*Oryza sativa* L.) plants in Scott County, Missouri. The purpose of this proposed introduction is to produce grain to be milled into flour from which lysozyme will be extracted.

A permit application was submitted by Ventria Bioscience to USDA/APHIS pursuant to regulations codified in 7 CFR § 340 which are titled "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 United States. A genetically engineered organism is considered 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 plants have been genetically engineered using recombinant DNA techniques, and *Agrobacterium tumefaciens* is the donor of the *nos* DNA regulatory sequence that facilitates the expression of the introduced gene in the engineered plants. The *nos* sequence is from the soil-inhabiting bacterial plant pathogen, *Agrobacterium*, which is one of the listed taxa in the 7 CFR § 340. Thus, the genetically engineered organism in this Ventria submission is deemed a regulated article.

Generally issuance of a permit 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 Scott County, Missouri, 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 are anticipated to increase in size and will be required to 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

APHIS has considered the following three alternatives in response to the applicant's request for a permit:

- **Alternative 1**: Deny the permit. Release of the regulated organism would not be authorized.

- **Alternative 2:** Issue the permit. The planting and growing conditions proposed by the applicant would be authorized.

- **Alternative 3:** Issue the permit with additional conditions required by APHIS for conducting the field planting.

APHIS field test permits typically include supplemental permit conditions that may reflect input from the relevant State regulatory officials. The supplemental permit conditions also include additional post-planting and post-harvest volunteer monitoring reports following completion of the field tests. These post-planting and monitoring reports assist APHIS in evaluating the specific field test under permit and also provide guidance for evaluating future proposed field tests.

IV. Discussion of the Alternatives

- **Alternative 1:** No Action/denial of the permit application. Under this alternative, field release of the genetically engineered rice plants would not be authorized.

- **Alternative 2:** Issue the permit for growing under the conditions proposed by the applicant. Under this alternative, field release of the genetically engineered rice plants would be authorized at the specified locations with no additional conditions outside of those the applicant provided in the request. Standard permit conditions under 7 CFR § 340.4 would be required (see Appendix I). Standard management practices, including use of some pesticidal and herbicidal sprays, will be included as part of the planting design.

- **Alternative 3:** Issue the permit with additional conditions for carrying out the field planting. Supplemental permit conditions, based on APHIS analysis, comments from U.S. Fish and Wildlife Service, the State of Missouri and public comment from this environmental assessment, would be required. If warranted, based on environmental risk of escape of the engineered organism, APHIS will require mitigating measures to prevent spread of the organism outside the field production area.

V. Description of the Regulated Article – Rice Biology

In this section of the environmental assessment, the biology of rice and plants related to rice are considered along with potential routes of gene escape. Because the mechanism by which genes are moved from one flowering plant to another is through cross-pollination of sexually compatible plants, the plants with which rice can cross-pollinate are described. Below is an

analysis of the biology of rice. This review focuses solely on rice in the United States. Other sources of information include a review prepared by the Organization for Economic Cooperation and Development (OECD), "Consensus Document on the Biology of Oryza sativa (Rice)" found at: http://www.olis.oecd.org/olis/1999doc.nsf/LinkTo/env-jm-mono(99)26 and the "Biology and Ecology of Rice (*Orzya sativa* L.) In Australia" found at http://www.ogtr.gov.au/pdf/ir/biologyrice.pdf

Systematics of Rice

Cultivated rice is included in the genus *Oryza* of the grass family (Poaceae). The genus *Oryza* contains twenty two species distributed through the tropical and subtropical regions of Asia, Africa, Central and South America, and Australia. Two species are cultivated and twenty are wild (Morishima, 1984; Vaughan et al., 2003). *O. sativa* is commonly referred to as Asian rice and is cultivated worldwide. The word "rice" generally indicates a plant and a crop of this species. *O. glaberrima* is commonly referred to as African rice and is cultivated in West and Central Africa. The genus *Oryza* is not native to the continental United States. One species, *Oryza latifolia* Desv., Broadleaf rice, is native to Puerto Rico. Only the single species, *Oryza sativa* is cultivated in the United States. The only recorded instance of the introduction of wild rice in the United States was the introduction of *O. rufipogon*, which was introduced to a single area within the Everglades of Florida. It was removed, and monitored without reoccurrence (Vandriver et al., 1992). No other species of *Orzya* are known to occur wild in the United States.

Red rice (*O. sativa*) is a weedy rice associated with cultivated rice grown in the southern United States. It is a weedy biotype of the crop plant. Red rice has a red pericarp or seed coat, pubescent light-green leaves, pubescent seeds that are shed easily (shatter) and a dormancy mechanism that enables seed survival for extended periods under unfavorable soil and environmental conditions (Eastin, 1979; Diarra et al., 1985; Ladinsky, 1985). These characteristics are different from most cultivated rice which has a tan pericarp, does not shatter readily and has little if any seed dormancy. Red rice is also taller at maturity than most of the cultivars grown today. It can be a troublesome weed in rice growing operations in the southern U.S.

Rice genetic improvement

Rice is a highly inbred crop and most rice growers in the United States use pure-line cultivars. Hybrid rice is currently under experimental evaluation in the United States but a large majority of the rice grown is from pure lines (McKenzie et al., 1987). There is no hybrid rice under development in Scott County where this rice will be grown.

Weediness of Rice

Rice plants (*Oryza sativa*) growing unintentionally around rice growing areas are regarded as weeds (Vaughan and Morishima, 2003). Weedy rice can result from the escape of cultivated varieties into surrounding areas if conditions are suitable for establishment. It appears that weedy rice commonly evolves through the degeneration of domesticated rice (Vaughan et al., 2003). Weedy rice may be derived from hybridization between different cultivars, selection of weedy traits present in cultivars, relics of abandoned cultivars, or may have been brought into the growing region through contaminated seed stocks (Vaughan and Morishima, 2003). Weedy rice

typically grows only as a component of agro-ecosystems where rice is grown or has been grown. It does not persist in environments inhospitable to rice cultivation.

Weedy red rice can be a major economic problem when it occurs in rice fields because it can lead to a loss in yield through competition with the desired cultivar as well as decreasing the value of the harvested grain. It is for this reason that many seed certification standards have a zero tolerance for red rice contamination in fields established for certified seed increases. For example see www.moseed.org/rice.htm.

Modes of Gene Escape in Rice

Genes of rice may escape from the test plot in two ways. The first pathway of escape is by pollen transfer. The second is by movement of propagative material, *i.e.*, the whole seeds.

Movement by outcrossing

Rice is not sexually compatible with plant species outside of the *Oryza* genus. There are no sexually compatible species of *Oryza* other than *Oryza sativa* growing in the United States. Rice is primarily self-pollinating and outcrossing rates usually occur at a very low rate (generally less than 1%) (OECD, 1999). The floral structure of *O. sativa* and the short viability of its pollen present biological barriers to cross-pollination (Gealy et al., 2003). A rice floret opens only once for a short period of time, usually for a little over an hour or less, during which fertilization can occur. Pollen viability is for no longer than five to ten minutes, but the stigma can remain viable for two to four days and can be fertilized by foreign pollen if for some reason it is not fertilized by its own pollen (Gealy et al., 2003). Due to the high selfing characteristic of rice, the Association of Official Seed Certifying Agencies (AOSCA) certified seed regulations for foundation seed require a minimum isolation distance from other rice varieties of at least ten feet when ground drilled and 50 feet if ground broadcast (AOSCA, 2003). With proper isolation distances maintained between Ventria's rice and other cultivars of rice, gene escape via cross-pollination would be highly unlikely. Temporal isolation can further reduce the likelihood of effective pollination and fertilization.

In addition, another mechanism for gene escape would be outcrossing with weedy/red rice. The establishment of a weedy rice population next to the field site could offer a means of escape of the gene from the production area. Since red rice seeds often have dormancy and shatter easily, the gene could be harbored in a weedy population for a number of years.

Movement by animals

A certain percentage of rice seeds (approximately 1 to 5 %) fall to the ground during harvesting. Although rice seeds that remain on the ground after harvesting can be consumed by animals, the lack of rice volunteers observed outside the proximity of the fields strongly supports that rice seeds are not widely dispersed by animals or birds. Rice seeds that are plowed under after fields are disked and burned are quickly decomposed under anaerobic conditions.

Movement of seeds by water

Rice seeds are first planted in dry fields after which the fields are flooded. The time of year when rice seeds or seedlings are most likely to move about are in the first 2 weeks of planting,

when the field is first flushed. After that period, seeds or seedlings will have sunk to the soil surface or be desiccated.

Movement by human error

In a recent workshop hosted by APHIS dealing with gene confinement issues in genetically engineered crops (http://www.aphis.usda.gov/brs/confine_workshop_2004.html), one of the more likely mechanisms contributing to the breakdown of confinement and movement of seed was identified as human error, and the most reliable means of preventing this is to maintain and reinforce stringent standard operating procedures

VI. Description of the Regulated Rice Plant

Ventria has engineered the rice plant to produce human lysozyme in the seeds. The starting background of this engineered plant was Taipei 309, which is a *Japonica* type variety. This variety is not grown commercially in the United States. The company has designated the transgenic line to be planted for the production of lysozyme as LZ159-53. Gene expression is targeted to the developing seed so lysozyme is not produced in other parts of the plant.

Lysozyme is ubiquitous in the human body where it acts as a protective barrier against environmental agents and, in doing so, helps prevent infection. Lysozyme is a small enzyme that attacks the protective cell walls of bacteria. It breaks the carbohydrate chains in bacterial cell walls, destroying the structural integrity and causes the bacteria to burst under their own internal pressure. Lysozyme plays a role in antibacterial disease defence, particularly against gram-positive bacteria. Both antiviral (O'Neil et al., 2001) and antifungal (Samaranayake et al., 2001) activity has been reported. Lysozyme in cattle plays a role in gastric digestion, and in chicken egg whites functions as an antibiotic. Lysozyme occurs in tears, nasal mucus, milk, saliva, blood serum, many types of tissues and secretions of different animals including, vertebrates and invertebrates and in plant latex (O'Neil et al., 2001).

The Vectors

The genes were transferred into rice plants via a two vector system using microprojectile bombardment. This is a well characterized transformation system which integrates the donor genes into the chromosome of the recipient plant cell (Batty and Evans, 1992). The system does not require the use of the plant pathogen, *Agrobacterium tumefaciens*, or other transformation vectors. The donor DNA sequences are stably and irreversibly integrated into the plant's chromosomal or organellar DNA, where they are maintained and inherited as any other genes of the plant cell. Ventria estimated by Southern blots that there are 2 copies of the expression cassette for these genes in line LZ159-53.

Agrobacterium tumefaciens and *Orzya sativa* (rice) are donors for non-coding DNA regulatory sequences that are associated with the introduced genes to facilitate expression in plants. The *nos* sequence is from the soil-inhabiting bacterial plant pathogen, *Agrobacterium* sp. and does not encode a protein. It does not cause plant disease and has a history of safe use in a number of genetically engineered plants (e.g., rice, corn, cotton and soybean varieties). The regulatory sequences from rice are the Gns9 promoter Gt1 promoter, gt1 signal peptide, and the RAmyl 1A terminator (see details below). None of the DNA regulatory sequences can cause plant disease

by themselves or in conjunction with the genes that were introduced into the transgenic rice lines.

The Selectable Marker

To facilitate the selection of transformed plants, the rice plants were engineered with the *hpt* gene which encodes for hygromycin phosphotransferase, an enzyme which confers tolerance to the antibiotic hygromycin. The selectable marker gene expression cassette consists of the rice glucanase 9 (Gns 9) promoter, fused to the *hpt* gene coding region, terminated by the Rice Alpha Amylase 1A (RAmy1A) terminator.

The *hpt gene* (Kaster et al., 1983; Waldron, 1997) was isolated from the donor organism *E. coli*, and encodes the 341-amino acid enzyme, hygromycin B phosphotransferase (Hpt). Hpt inactivates the antibiotic hygromycin by adding a phosphate, which allows cells containing this gene to grow on medium containing hygromycin. The *hpt* gene is devoid of inherent plant pest characteristics. The gene is driven by the Glucanase 9 (Gns9) promoter derived from rice, a tissue specific promoter that is only expressed during the plant cell culture phase of the transformation process(Huang et al., 2001). Since the promoter is only expressed during the cell culture regeneration process, it is not active in any tissue of the mature plant and therefore no *hpt* is expressed in the tissues of the plant. The *hpt* gene is terminated by the rice Alpha amylase 1A (RAmyl 1A) terminator. Because both the promoter and terminator are regulatory sequences from rice, they should pose no environmental risks.

The Gene of Interest

The rice plants were engineered to express the gene for human lysozyme. The gene of interest consists of the Glutelin 1 (Gt1) promoter and glutelin 1 (gt1) signal peptide, the coding sequence for human lysozyme, and is terminated by the nopaline synthase (nos) 3' terminator from *Agrobacterium tumefaciens*.

The Glutelin 1 (Gt1) promoter from rice is used to drive the production of the human lysozyme gene, but the promoter sequence itself does not encode a protein. This promoter normally drives expression of the glutelin seed storage protein, and in this gene expression cassette it preferentially directs expression of lysozyme in seeds and is active in the endosperm cells of the seed while the seeds are maturing on the plant. It is active between seven to thirty days after pollination (Okita et al., 1989). The gt1 signal peptide, also from the rice Gt1 gene, is used to target the lysozyme protein to Type II protein bodies within the cells of the endosperm (Nandi et al., 2002). Both of these regulatory elements ensure that the lysozyme protein is produced only in the mature seeds and not in other parts of the plant. The *nos* sequence from *Agrobacterium* sp. terminates the gene and does not encode a protein.

Characterization of the Engineered Plant and the Expressed Lysozyme

The applicant submitted a Safety Assessment consisting of a detailed analysis addressing all of APHIS's requested permit application information. The applicants studies conclude: 1) no sequence homology of the recombinant lysozyme to known toxicants, allergens, or proteins likely to harm non-target organisms, 2) there are approximately 2 copies of the lysozyme gene in the engineered rice lines and they are stably inherited over multiple generations, 3) the physical

and molecular properties of the recombinant lysozyme are similar to those of human lysozyme, 4) like most proteins, the recombinant lysozyme is destroyed by cooking and its digestive properties are similar to human lysozyme, 6) there are no anticipated impacts on threatened and endangered species by the transformed rice plants. The information the applicant supplied is summarized below.

Sequence homology to known allergens or toxicants

Ventria's results demonstrate that the human lysozyme shares no nucleotide sequence homology with known allergens or toxins based on a computerized search of public databases. The SWISS-PROT and TrEMBL databases were utilized and no amino acid sequence homology was found between human lysozyme and known allergens. Ventria indicated that the search algorithms were consistent with CODEX Alimentarius guidelines for such homology searches. Lysozyme is a protein found in most mammal breast milk, including humans, as well as in most epithelial surface secretions including tears, nasogastric, saliva, and bronchial secretions. Human milk contains between 0.2 and 0.9 g/l of lysozyme (Jolles and Jolles, 1984; Lonnerdal, 1985; Montagne et al., 2001). It is highly unlikely that this molecule is either an allergen or toxin.

Molecular characterization

Ventria submitted Southern blot analysis data demonstrating that there are approximately 2 copies of the lysozyme coding sequence integrated into the rice genome for line LZ159-53. This line (LZ159-53) to be planted is in the 7^{th} generation and the Southern blot analysis showed that all the bands from the R_0 generation have been inherited as a single linkage unit. Thus stable inheritance of the lysozyme gene has been observed over multiple generations.

The human lysozyme gene sequence was based on the DNA sequence from GenBank accession number J03810 but the coding sequence was re-synthesized with rice preferred codons to enhance its expression in the rice plants. Ventria submitted data demonstrating that the recombinant and non-recombinant human forms of lysozyme have essentially the same amino acid composition and that all values were within an acceptable analytical variation (< 1%).

Protein characterization

Ventria analyzed the chemical and physical properties of the recombinant lysozyme (rhLZ) compared to purified human lysozyme (hLZ). Molecular mass, isoelectric focusing point, pH stability range, thermal stability, specific activity, bactericidal activity, solubility, reaction to antibody in ELISA and Western bolts, were all found to be identical. There are no-glycosylation sites on lysozyme (Lerouge et al., 1998). These data indicate that the lysozyme produced in the rice seeds is biochemically equivalent to human lysozyme.

Potential environmental and animal exposure levels

The gene intended for protein expression in these field plantings codes for human (*Homo sapiens*) lysozyme. Lysozymes constitute a family of compounds which occur in a wide variety of organisms (Düring, 1996), and are known to destroy bacteria by digesting their cell wall (Cunningham et al., 1991). In humans, lysozyme is found in tears (~1.3 mg/l) (Jolles and Jolles, 1984), saliva, white blood cells and milk (0.24-0.89 g/l) (Montagne et al., 2001).

Ventria submitted and APHIS reviewed lysozyme protein expression data from roots, stems, leaves, pollen, booting panicle, peduncle, immature seed, mature seed, and husks for LZ159-53. There was no detectable expression in any tissues except immature seed and mature seed. The levels of expression were 62 µg/g in immature seed, 3410 µg/g in mature seed, and < 25 µg/g in husks. The expression level in mature seeds is approximately 4 mg/g on a fresh weight basis in whole seeds with husks. When the seeds are dehusked (moisture level of about 12-14%) and the material is ground into flour, the expression level is approximately 5 mg/g. This is about 4-5 x that expressed in white blood cells and milk. The expression from isolated husks is due to the carryover of the endosperm tissues during the dehusking process. It is very difficult to eliminate all of the endosperm tissues when the seeds are mechanically dehusked, thus there is a slight carryover that is detectable (<1%). When the husk is removed carefully by hand there is no detectable amount of lysozyme found.

Thermal stability and sensitivity to gastric digestion conditions

It is the intention of Ventria to purify the lysozyme from the rice grain to use in various products, not to sell the rice grain itself. Any potential consumption of the lysozyme rice grain is unlikely given the conditions of the test.

Rice is normally consumed cooked by humans, so potential consumption of lysozyme from the transgenic rice would normally involve cooking the rice grain. According to (Juliano, 1985) when rice is parboiled (10 min at 121°C) its starch, protein and fat are disrupted. Ventria conducted laboratory tests on the thermal stability of plant derived lysozyme. To determine the thermal stability, hLZ and rhLZ were compared at heat treatments of 65, 72, 85 and 100^{o} C. At 60 and 72^{o}C both proteins survive 15 minutes incubation time. The proteins start losing their activity after 5 minutes at 85^{o}C and almost immediately at 100^{o}C. The thermal stability of both proteins is the same. The heat treated protein does not remain active or detectable in the cooked rice product under typical cooking conditions; using commercial rice cookers which boil rice for ~ 20 minutes. After cooking for 20 minutes, lysozyme protein could no longer be detected by Western blots.

Ventria has supplied data demonstrating the similarity between the recombinant lysozyme and purified human lysozyme so no unanticipated effects due to digestion of the recombinant lysozyme are anticipated. In gastric digestion studies, Ventria examined susceptibility of lysozyme to pepsin digestion using the ILSI Health and Environmental Sciences Institute protocol (Thomas et al., 2004). This method monitors the digestion of a protein in simulated gastric fluid at 37^{o}C over time. Using this *in vitro* protocol, Ventria's rice-derived lysozyme is equivalent to native human lysozyme which is degraded within 5 minutes in gastric fluid. This study demonstrated that the plant-derived form of human lysozyme degrades with the same kinetics as human-derived lysozyme.

The effects of Ventria's recombinant lysozyme on the health and intestinal flora of chicks were analyzed (Humphrey et al., 2002). In this study the growth rate and intestinal structure of broiler chicks fed varying amounts of Ventria rice for 21 days after hatching were analyzed. The results showed that lysozyme improved the health of the chicks and growth rates were improved over the control. These results indicate that the rice containing the recombinant lysozyme should not be harmful to the health of avian species if ingested.

Egg white lysozyme is listed on the Food and Drug Administration's Summary of All GRAS (Generally Recognized as Safe) Notices(FDA, 2001) which includes the use of egg white lysozyme as an antimicrobial agent in casings for frankfurters and on cooked meat and poultry products.

When added to foods, lysozyme extends the shelf life of a variety of processed foods, including pickles, dairy foods, and meats (Cunningham et al., 1991). Oral administration of egg white lysozyme is used clinically in human medicine for the therapy of inflammatory diseases of respiratory and digestive epithelia (Seno and Inoue, 1998). Lysozyme is also used as an additive to toothpaste and mouthwash products (http://www.biotene.com/). Lysozyme supplements are widely available over the counter. Over the counter lysozyme supplements typically contain 10 mg lysozyme/tablet (http://www.gaines.com/store/Atrium/ATR4030info.html). Lysozyme tablets containing 50 or 150 grams lysozyme are also available commercially for use in home winemaking and brewing (http://www.morebeer.com/product.html?product_id=15498).

In these field production sites, lysozyme will be expressed exclusively in the seed by the use of a seed-specific promoter. No expression of the gene will occur in other plant tissues. Since lysozyme in rice grains is quickly denatured by cooking, humans or other animals would have to consume raw uncooked rice to ingest the lysozyme from these plantings. Given the history of safe use of lysozyme supplements in food and oral hygiene products, and the unlikely event that large quantities of uncooked rice would be consumed from these field sites, APHIS concludes that humans are unlikely to be significantly affected if accidental ingestion of raw rice containing lysozyme were to occur.

VII.Description of the Field Test/Affected Environment

Purpose

The purpose of this proposed introduction is for grain production for product development. The regulated introduction is proposed for planting between March and April 2005.

APHIS has reviewed protocols that were proposed by Ventria to prevent the escape and dissemination of these plants submitted on APHIS Form 2000. In addition, Standard Operating Procedures (SOPs) submitted by Ventria identify more detailed instructions and provide additional guidance.

Field Plot Design, Breeding Procedures and Agricultural Practices

Plot Design and Location

Field test plots will be separated by a distance of at least ¼ mile from other rice fields in order to maintain confinement. Ventria will monitor for commercial rice production and scout for red rice within this ¼ mile distance. These plots will be surrounded by a fallow zone of 50 feet. The fallow zone may be planted with a low-growing crop that will not be used for food or feed to prevent erosion. The rice will be grown in flooded fields and in contiguous paddies.

The location of the field planting chosen by Ventria is free of weedy red rice (Bryan, 2005). The area has not had a history of rice farming; soybean and corn have been grown in the area for

many years. Therefore there has not been the opportunity for a weedy rice population to develop.

Agricultural Practices

The rice will be allowed to self pollinate to produce seed. No breeding operations will occur under this permit. Agricultural practices consistent with growing healthy rice plants will be used. Weeds will be controlled by herbicide applications. If necessary, pesticides such as insecticides and/or fungicides will be used to control insect pests and disease 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 field will be monitored for noxious weeds and other plant pests during the growing season. Three times during the growing season the plants will be inspected for traits such as weediness, resistance/susceptibility to insects or disease, or unusual differences in plant growth or morphology. The plot will be inspected weekly while conducting agricultural practices. The areas nearby will be growing other crops such as corn and soybeans beyond the 50 foot fallow zone. EPA registered chemical pesticides are likely to be used to control insect pests on these crops.

Field Observation and Monitoring

The applicant has thoroughly described field site monitoring and management practices that should provide the necessary degree of biological and physical confinement. Confinement practices under the permit include the following:

- The test site will be located more than ¼ mile from the nearest non-engineered rice planting;

- The applicant will provide APHIS and State regulatory officials information on the location of the nearest rice 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 coordinates;

- The applicant will use screens on irrigation outlets to prevent movement of seeds/seedlings out of the field with water used to flood the field. They will also employ flooding methods which create a closed system so that ungerminated seeds can not leave the field site;

- 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 fields, the production site and the 50 foot fallow zone may not be planted with rice unless transgenic rice is repeated. If the same crop does not follow in subsequent years, the site will be monitored for volunteer rice plants throughout the next year. Any volunteer rice plants will be destroyed before flowering.

- Ventria will use equipment dedicated to this field test as outlined in their SOPs. This equipment will not be used for any other purposes during the course of the field test, and after the field test is completed, all equipment will be thoroughly cleaned and inspected to ensure that all genetically-engineered seed and other plant material has been removed and destroyed.

- Even though weedy red rice is not currently present in the area, Ventria will scout for red rice during the entire growing season both within the growing plots and for ¼ mile from the production fields. Any red rice found will be destroyed and will not be allowed to flower. Ventria will also inform APHIS if any red rice is found within the ¼ mile zone or in their production plots.

Alteration in susceptibility to disease or insects

There has been no intentional genetic change in these plants to affect their susceptibility to disease or insect damage. Neither the selectable marker gene, *hpt*, nor the lysozyme gene is expected to alter the susceptibility of the transgenic rice plants to disease or insect damage. Execution of the prescribed periodic monitoring of the field plots will allow the detection of any unexpected infestation by plant disease organisms or animal pests. Ventria is required to report any such unanticipated effects to APHIS under the terms of the permit. See 7 CFR § 340.4(f)(10)(ii).

Termination of the field test and final disposition of the test plants

At harvest, the seed will be machine harvested using a dedicated combine, dried and cleaned in a designated staging area adjacent to the field location using a dedicated dryer and cleaner. The seeds will be stored in dedicated storage bins on site and will be processed as desired. During the process the seed will be dehusked using a dedicated dehusker on site and will be milled on site in a dedicated staging area at the field site using a dedicated mill. Milled rice flour will be shipped to designated locations for subsequent processing. Any devitalized waste material from the milling operation will be returned to the field test site and incorporated into the soil. All the operation up to milling will be performed in an APHIS inspected dedicated staging area and dedicated equipment. Any material will be shipped only after milling. There will be no shipping of the viable seeds from the dedicated processing site to any other site.

After harvest, as soon as possible as the weather allows, Ventria will burn and disk the fields to degrade all plant material in the field to remove and decompose any remaining seed. Off-season flushing with water may also be used to accelerate the germination of any remaining viable seed prior to winter.

Ventria plans to grow the lysozyme rice in the same location in subsequent years. However, if a change to a different crop is anticipated, the field will be fallowed for one cropping season after the harvest of the transgenic lines. During this 18 month period (the period between harvest year 1 to planting year 3), the field will be monitored and managed to germinate and destroy any live seed. Ventria employs a “pureland” procedure to control volunteers. This includes flushing the field with water during the growing season to germinate weed and rice seed, drying the field to kill seedlings, and then disking to dry and kill seedlings. This process is repeated three times.

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. All the surrounding fields outside the food/feed crop fallow zone will all be planted to soybean or corn. The site is not prone to flooding. The closest body of water is Lake Tywappity which is located about 4 miles to the east The Mississippi River is located about 8-10 miles west of the planting site.

VIII. Potential Environmental Impacts

Alternative 1: No action/denial of the permit request.

Field release and research would not be allowed and no environmental impact would result.

Alternative 2: Issue the permit with APHIS standard permit conditions.

The proposed field test is a controlled release of the regulated article into the environment. The risks associated with the introduction of genetically engineered organisms are the same kind as those associated with the introduction into the environment of unmodified organisms or organisms modified by other genetic techniques.

APHIS BRS has considered the safety assessment information presented by the applicant and independently assessed the risk of this protein 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. The product of hazard times the exposure is the actual risk. Data regarding exposure has several components: (1) where in the plant are the proteins produced; (2) how much protein is produced; (3) which organisms are likely to consume these tissues; and (4) how likely is consumption to cause harm. After careful analysis, we found no identifiable hazards and determined that environmental exposure and exposure to non-target organisms would be extremely low or none. There is little potential for environmental exposure, since the seed will be harvested and experience shows that only a small portion (< 5%) of the seed is dropped during harvesting operations. Seed husks and other post-harvest waste remaining on the field will be burned.

Therefore the actual risk is low or none.

Potential for Persistence of the Modified Plants in the Environment

Rice is a highly domesticated aquatic crop species, which grows exclusively in highly managed aquatic ecosystems. It is non-competitive with weed species and is self-pollinated; errant seed does not pose a threat to wild or managed, non-flooded ecosystems. Taipei 309 is a *Japonica* type rice which germinates very quickly and has no dormancy period (FAO, Accessed 2005). Japonica rice seed looses viability quickly under ambient conditions. Therefore the likelihood of persistence of seed in the environment is minimal. The applicant has also described methods that will be employed to minimize the persistence of rice seed in the environment (see “Termination of the field test and final disposition of the test plants” section above). The proposed procedures for confinement of the plant material and for termination of the field test, as proposed by Ventria

and described in this document, should be sufficient to ensure that none of the genetically engineered plants persist in the environment.

Previous field data reports for small scale trials of lysozyme-producing rice have not reported differences in weediness, resistance/susceptibility to insects or disease, or unusual differences in plant growth or morphology in 5 generations of field tests. No change in general agronomic traits (leaf color, shape, growth habit, days to pollen shed, days to maturity and seed germination rates) have been observed in the genetically-engineered plants that might affect the plant's ability to persist in the environment. The presence of human lysozyme in the rice seeds has not altered seed germination rates.

Potential for Gene Transfer

Movement by outcrossing

As outlined in Section V, rice is not sexually compatible with plant species outside of the *Oryza* genus, there are no sexually compatible species of *Oryza* other than *Oryza sativa* growing in the United States, the pollen is very short-lived, and pollen does not travel for long distances. With proper isolation distances maintained between Ventria's rice and other cultivars of rice, gene escape would be highly unlikely. Temporal isolation can further reduce the likelihood of effective pollination and fertilization. Ventria 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. Effects of dissemination of pollen will be mitigated in this test *via* reproductive isolation distances of greater than ¼ mile.

Another mechanism for gene escape would be outcrossing with weedy/red rice. The location of the field planting chosen by Ventria is free of weedy red rice but Ventria will be required to monitor for and rogue any red/weedy rice that might appear in or near the field site. Monitoring for red/weedy rice will be required within the ¼ mile isolation distance. Careful monitoring and rouging of red/weedy rice populations within or adjacent to a field test site before flowering or seed set will preclude movement of transgenes to red rice via cross pollination and/or the persistence of the gene in the seed bank of red rice.

Because this EA is being written months before planting, it is impossible to know how close the nearest rice fields will be. Based on past plantings it is estimated that closest rice fields will be more than 10 miles away (Bryan, 2005). Ventria will be required to maintain an isolation distance of at least ¼ mile, but whether there will greater than ¼ mile isolation, as well as, temporal isolation cannot be firmly assessed at this time. Because rice pollen viability declines within a few minutes, a distance of at least ¼ mile between any rice plantings is an effective means to mitigate gene flow. Given the small percentage of rice production in Scott County, APHIS concludes that pollination of any rice plant outside the ¼ mile isolation distance, would be at *de minimis* 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).

Movement by animals

To protect against unintentional consumption and movement of seeds by animals, Ventria will monitor the field sites during the growing season for any animal pests that consume rice and measures will be taken to discourage their presence. Flooded rice fields, while the crop is in the field, are not attractive to land-based mammals so this discourages the movement of seeds by rodents and other small mammals. Measures will be taken to inhibit waterfowl and animal predation and movement of seed. These will include burning stubble and straw as soon as possible after harvest, leaving the fields dry in the fall and winter to discourage waterfowl from using the area as a flooded habitat, maintaining an open 50 foot fallow area to reduce rodent movement, and the use of bait stations for rodents in warrens.

Rice seed is a highly digestible grain that does not pass in a viable form through waterfowl, the most likely animals to consume grain in rice fields (Powers et al., 1978; Smith and Sullivan, 1980; Drobney, 2005). In a Louisiana study conducted in Vermillion parish, a rice growing area, Powers et al. (1978) checked the gut of 51 hunter killed waterfowl, and identified, counted and germinated the seed found. They found that the seeds of red rice, which are large and have a thin seed coat did not pass intact. Drobney (2005) has observed "several miles" of bird intestines and has never found intact rice seed. Smith and Sullivan (1980) used the information from the Power's study to design chemical-free, weed-reducing strategies in Arkansas rice producing areas by flooding rice fields to attract waterfowl and reduce weed populations. Therefore it is highly unlikely that rice seeds can be dispersed in bird feces.

Movement of seeds by water

Since rice seeds are most likely to move about during the first 2 weeks of planting in a flooded field, Ventria will use screens on the rice boxes or exit pipes to catch any seed that might float to the surface to prevent escape. In addition Ventria will use flooding methods which will create a closed system so that ungerminated seeds can not leave the field site.

Movement by human error

As outlined in Section V, one mechanism that could contribute to the breakdown of confinement and movement of seed is human error. The most reliable means of preventing this is to maintain and reinforce stringent standard operating procedures. Ventria has submitted Standard Operating Procedures (SOPs) as a part of the permit submission and these have been reviewed by APHIS. In addition to having stringent SOPs, all the harvested seeds will be stored in dedicated storage bins on site and seeds will be processed on site. During the processing, the seed will be dehusked using a dedicated dehusker on site and will be milled on site in a dedicated staging area at the field site using a dedicated mill owned by Ventria. Only Ventria personnel or employees assigned and trained by Ventria will be allowed to handle any seeds. Employing these methods along with following their SOPs will minimize the possibility of human error moving seed into other fields.

Impacts from the use of the marker gene

The selectable marker gene hygromycin B phosphotransferase, *hpt*, is also present in these plants, but Ventria has provided data which show that it is not expressed in mature tissues or seeds for the transformed rice plants. This is because it is driven by the Glucanase 9 promoter which is only expressed during the cell culture phase of the transformation system. Therefore

APHIS has determined that in this case the presence of the *hpt* gene will have no significant environmental impacts.

Impact on native floral and faunal communities

Ventria has assayed for lysozyme in the soil surrounding the roots and germinating seeds of the engineered plants, and has supplied data indicating that none has been detected. Based on the lack of toxicity of the proteins that will be produced and the prescribed permit conditions to minimize any seed remaining on the soil surface, APHIS concludes that there will be no significant effect on any native floral or faunal species listed by U.S. Fish and Wildlife for Scott County, Missouri http://midwest.fws.gov/endangered/lists/missouri-cty.html.

Vertebrate Animals

In these proposed field tests lysozyme is preferentially expressed in the seeds of the genetically engineered rice plants. The most likely vertebrate animals that would be exposed to lysozyme in rice seed are the seed eaters, e.g. rodents and birds. APHIS believes that consumption of these seeds would pose no significant risks for the following reasons:

1. If any seeds containing lysozyme were to be ingested, the lysozyme would be digested in the digestive tract of these animals. Egg-white lysozyme is listed for specific uses in the Food and Drug Administration's Summary of All GRAS Notices. Recombinant human lysozyme has been shown to be safe if ingested by avian species. Carnivores that might consume the vertebrate animals that had eaten the rice seeds are not expected to be exposed to lysozyme as the protein should have already been digested.

2. In order to minimize exposure by seed consumption, Ventria will be required under the permit conditions to monitor the test fields after harvest to incorporate any remaining plant material into the soil.

3. APHIS will inspect the site during planting, harvesting and seed processing to ensure all permit conditions are met. APHIS concludes there would be no significant effect on any vertebrate animal.

Invertebrate Animals

The most likely invertebrate animals would be exposed to the lysozyme in the rice seed would be seed-feeding invertebrates and soil-dwelling organisms. Arthropods such as insects and crayfish that consume seeds and snails that consume all plant parts are considered plant pests and will likely be controlled by pesticides applied during the time course of the field tests.

Since lysozyme production is limited to the seeds, and the seeds will harvested from the field and processed on site by Ventria, it is unlikely that the any soil organisms will be exposed to lysozyme resulting from the proposed field tests. Although lysozyme is not produced in roots, seeds that remain in the field would contain lysozyme. Based on a worst-case analysis, where 5% of the seeds would remain, the amount of lysozyme would be about 400 grams per acre (assuming a yield 4500 lbs per acre and 4 mg lysozyme per gram of seed). This is equivalent to approximately 100 mg per square meter. Ventria has submitted data demonstrating that

lysozyme disappears as seed germination and seedling growth proceeds. But as a precaution, APHIS will require Ventria to monitor lysozyme levels in the soil (see Appendix II).

Earthworms constitute approximately 90% of the invertebrate soil biomass (Ville et al., 1995) but since Ventria grows the rice crop under flooded conditions it is unlikely that earthworms will colonize these fields and would thus not be exposed to lysozyme containing seeds.

APHIS therefore concludes there would be no significant effect on any invertebrate species.

Aquatic Organisms

It is unlikely that any aquatic organism would be exposed to rice seed expressing lysozyme because the closest major body of water is Lake Tywappity which is approximately 4 miles from the field test site. It is unlikely that aquatic organisms will establish themselves in the flooded rice fields due to the ephemeral nature of the flooding. The farm Ventria is proposing as its production site has not grown rice in the past. For this reason, it is not expected to be inhabited by fish or typical aquatic habitat arthropods such as crayfish http://www.conservation.state.mo.us/nathis. APHIS therefore concludes there would be no significant effect on any aquatic organisms.

Native Floral Communities

The proposed field test sites are located on land that has been under constant agricultural use for the past 40 years. APHIS concludes there would be no significant effect on any native floral species.

Alteration in susceptibility to disease or insects

There has been no intentional genetic change in these plants to affect their susceptibility to disease or insect damage. Neither the selectable marker gene, *hpt*, nor the lysozyme gene is expected to change any plant pest characteristics. There is no reason to believe that these or similar characteristics are different between the genetically engineered and non-engineered plants. The selectable marker gene designed to provide tolerance to the hygromycin is not expected to alter the susceptibility of the transgenic rice plants to disease or insect damage. Execution of the prescribed periodic monitoring of the field plots will allow the detection of any unexpected infestation by plant disease organisms or animal pests. Ventria is required to report any such unanticipated effects to APHIS under the terms of the permit. See 7 CFR § 340.4(f)(10)(ii).

Impact on Existing Agricultural Practices

No impact on existing agricultural practices is expected. Ventria will employ agricultural practices consistent with growing healthy rice plants. Weeds will be controlled using herbicide applications. If necessary, insecticides and/or fungicides will be used to control pests such as rice water weevil, armyworm, rice stalk borer, rice blast, rice sheath blight, and other rice diseases that would diminish the health of the plant and reduce grain yield. Any approved pesticides will be applied by trained personnel in their use and application. The plot will be inspected at least weekly during the growing season. In 2004, Scott County produced only about 0.5% (or less than 1%) of all the rice produced in Missouri

(http://agebb.missouri.edu/rice/ricenews.htm). Thus, Scott County, Missouri, is not a major producer of rice.

Impact on adjacent row crops

Two row crops (soybean and corn) will be grown on the same farm and on adjacent farms. Pesticides used to control pests on the transgenic rice plants will be similar to those used on the adjacent crops. The permit conditions will stipulate a fallow zone of 50 feet be maintained between Ventria's rice and soybean and corn grown on the adjacent fields. Should any seed be picked up by birds or other animals and dropped into these fields it would not be likely to grow due to the lack of flood irrigation. No environmental impacts on nearby crops are expected.

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, (ISAAA) 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. The EPA has exempted from a tolerance DNA that encodes currently registered plant incorporated protectants because of a lack of toxicity (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; Duggan et al., 2000; Duggan et al., 2003; Chambers et al., 2002; Netherwood et al., 2002; Einspanier et al., 2001). The majority of DNA consumed is degraded in the gastro-intestinal tract although this degradation is not 100% efficient. There is evidence that both transgenic and plant DNA can move from the GI tract lumen to other areas of the body and that this is a normal occurrence. No risks have been identified as a result of this movement.

Transfer and expression of DNA from the plant to bacteria is unlikely to occur due to several known 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 (1999) and Schluter *et al.* (1995) have studied transgenic DNA movement to bacteria and although theoretically possible, it occurs at extremely low rates (approximately 1 in 10^{-14}). Many genomes (or parts thereof) have been sequenced from bacteria that are closely associated with plants including *Agrobacterium* and *Rhizobium* (Kaneko et al., 2000; Galibert et al., 2001; Wood et al., 2001; Kaneko et al., 2002). There is no evidence that these organisms contain genes derived from plants. Koonin *et al.* (2001) and Brown (2003) presented reviews based on sequencing data that revealed horizontal gene transfer occurs occasionally on an evolutionary time scale of millions of years.

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 lysozyme is absent from the pollen. The seeds are unlikely to be mixed with seeds intended for human or

animal consumption because of numerous measures (described in Appendix II) and APHIS inspections during harvesting and processing. All the harvested seeds will be stored in dedicated storage bins on site and seeds will be processed on site. During the processing the seed will be dehusked using a dedicated dehusker on site and will be milled on site in a dedicated staging area at the field site using a dedicated mill owned by Ventria. There are no rice driers, mills or rice seed processing facilities present in Scott and nearby Cape Girardeau County.

Ventria proposes to use recombinant human lysozyme for the general population as supplements in yogurts, meal replacement and performance beverages, bars (for example granola bars), and in nutritional supplement drinks. Ventria also proposes to use lysozyme in the preparation of medical foods such as oral rehydration solutions.

The use of the purified product is not regulated by APHIS. The safety of such use is regulated by the FDA. Any food or feed uses of transgenic plants must comply with the requirements of the Federal Food Drug and Cosmetic Act.

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 Center for Drug Evaluation and Research (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 (FD & C 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).

Effects of field test on Threatened and Endangered Species

The proposed field tests are controlled releases of the regulated article into the environment in Scott County, Missouri. The field site is also close to adjacent Cape Girardeau County. Ventria's future plans may include growing in Mississippi County. Neither the engineered rice plants nor the lysozyme and *hpt* genes will affect any non-target organism including any threatened and endangered species (TES) listed in Scott, Cape Girardeau, and Mississippi Counties, Missouri. An analysis of TES distribution in these Counties using the U.S. Fish and Wildlife databases (http://ecos.fws.gov/ecos/index.do) and (http://midwest.fws.gov/endangered/lists/missouri-cty.html) lists a bat species *Myotis sodalis* (Indiana bat), two bird species; *Haliaeetus leucocephaslus* (Bald eagle) and *Sterna antillarum* (Least tern,) two fish; *Scaphirhynus albus* (Pallid sturgeon) and *Potamilus capax* (Fat pocketbook); and one endangered plant *Boltonia decurrens* (Decurrent false aster) as existing or once existing in these Counties.

As part of its on-going discussion with FWS on genetically engineered organisms, APHIS BRS met with the agency (2003) to discuss potential impacts of the field testing of plants producing products that would require approval from FDA's Center for Biologics Evaluation and Research (human biologics), Center for Drug Evaluation and Research (human drugs), Center for Veterinary Medicine (animal drugs) or USDA's Center for Veterinary Biologics (animal

biologics) before commercial use. A worksheet was developed by the parties for these types of products (see Appendix III). Ventria and APHIS used the developed worksheet to review the procedures proposed for this test. Ventria's assessment is included in Appendix IV (TES worksheet). APHIS concludes a "no harm" decision can be reached for these proposed field tests.

Cumulative Environmental Effects

This is the first field test of the engineered rice plants at this location. There is little likelihood that lysozyme will accumulate in the soil. Ventria has assayed for lysozyme in the soil surrounding the roots and germinating seeds of the engineered plants, and has supplied data indicating that none has been detected. However, any as yet unidentified cumulative effects should be found in the subsequent monitoring periods required by APHIS in the same field sites in following years.

Special Considerations: Other Environmental Statutes and 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 significant 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 significant 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 are expected to have disproportionately high or significant 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. Rice is not invasive and 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.

Alternative 3: Issue the permit with additional conditions.

The potential environmental impacts under this alternative include all those noted under Alternative 2.

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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X. CONSULTATIONS

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XI. APPENDICES

Appendix I. 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, non-target 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 II: Proposed Supplemental Permit Conditions

FOR RELEASE OF TRANSGENIC RICE
USDA-APHIS-BRS Permit: 04-309-01r

1) Compliance with Regulations

Any regulated article introduced not in compliance with the requirements of 7 CFR Part 340 or supplemental permit conditions, shall be subject to the immediate application of such remedial measures or safeguards as an inspector determines necessary, to prevent the introduction of such plant pests. The responsible party may be subject to fines or penalties as authorized by the Plant Protection Act.

This 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 chemicals; 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.

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

The procedures, processes, and safeguards used to prevent escape, dissemination, and persistence of the transgenic virus as described in the permit application, in APHIS-approved Standard Operating Procedures (SOPs) and, in the supplemental permit conditions must be strictly followed. The permittee must maintain records sufficient to verify compliance with these procedures, including information regarding who performed the activity. Persons performing such activities shall have received training as described in a training program submitted to and approved by APHIS. These records are subject to examination by APHIS. APHIS, BRS must be notified of any proposed changes to the protocol referenced in the permit application.

2) Distance to other rice

To prevent cross-pollination of the transgenic rice with other rice, there must be at least ¼ mile between the transgenic rice and any other rice not included under this permit. This ¼ mile buffer includes a 50 foot fallow zone.

3) Weeds

Weeds in the field test plot will be controlled by herbicide treatment or by hand rouging.

4) Perimeter Fallow Zone

To ensure that transgenic plants are not inadvertently commingled with plants to be used for food or feed, a perimeter fallow zone of at least 50 ft. must be maintained around the transgenic test site in which no crops are grown that will be harvested or used for food or feed. The perimeter fallow zone must start outside of any permitted border rows of non-transgenic plants that are the same as, or sexually-compatible with, the regulated article, and it shall be managed in such a way as to allow detection and destruction of volunteer plants that are the same as or sexually compatible with the transgenic plants.

5) Dedicated Planting and Harvesting Equipment

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) from the time of planting through the end of harvesting. After this time, APHIS authorization will not be required for this equipment to be used on APHIS-permitted sites planted to the same types of transgenic crops as authorized under this permit (e.g. the same or different sites planted to the same crop with the same target protein(s) in subsequent growing seasons under an extension of this permit or a different permit), but authorization will be required from APHIS before this planting and harvesting equipment can be used on sites planted to crops not included under this permit. In the latter case, the permittee must notify APHIS, BRS and the PPQ Regional Biotechnologist and State Regulatory Official at least 21 calendar days in advance of cleaning this equipment for this purpose so that APHIS may schedule an inspection to ensure that the equipment has been cleaned appropriately.

6) Cleaning of Equipment

To minimize the risk of seed movement and commingling, equipment used for planting and harvesting, as well as other field equipment (e.g. tractors and tillage attachments, such as disks, plows, harrows, and subsoilers) used at any time from the time of planting through the post-harvest monitoring period must be cleaned in accordance with procedures submitted to and approved by APHIS before they are moved off of the test site. Equipment used to transport harvested material must also be cleaned prior to loading and after transportation to the authorized site in accordance with procedures submitted to and approved by APHIS. Seed cleaning and drying must also be performed in accordance with the procedures submitted to and approved by APHIS so as to confine the plant material and minimize the risk of seed loss, spillage, or commingling.

7) Use of Dedicated Storage Facilities

Dedicated facilities (locked or secured buildings, bins, or areas, posted as restricted to authorized personnel only) must be used for storage of equipment and regulated articles for the duration of the field test. Before these facilities are returned to general use, they must be cleaned in accordance with procedures submitted to and approved by APHIS. In this case, the permittee must notify APHIS, BRS, the PPQ Regional Biotechnologist and State Regulatory Official at least 21 calendar days in advance of cleaning facilities for return to general use so that APHIS may schedule an inspection to ensure that the facilities have been cleaned appropriately.

8) Post Harvest

As soon as physically possible following the fall harvest, the field must be burned and disked, and may be flooded during the off-season, to degrade the plant material. If weather does not permit burning, then the field must be disked as soon as possible. An attempt should be made to schedule the harvests so that rainy conditions do not prevent disking under the unharvested plant material.

9) Post Harvest Monitoring

For the cropping season following harvest of the transgenic lines, unless the fields will be planted back into transgenic lines of the same target molecule (with the appropriate APHIS permit), the field test site may be reflooded to promote growth of any rice seed which may have escaped harvest the previous year. The field test site and perimeter fallow zone must be monitored for volunteer rice plants monthly whenever weather conditions are favorable for seed germination **at least until October 31, 2006**, and volunteers must be eradicated by mechanical destruction or with a herbicide prior to flowering.

10) Post Harvest Land Use Restrictions

Production of food and feed crops at the field test site and the perimeter fallow zone is restricted during the growing season that follows harvest or termination of the field test. Permission must be obtained from APHIS, BRS prior to planting any food or feed crop at the field test site and perimeter fallow zone during the post-harvest monitoring period. Requests for such permission are not encouraged and will not be granted in cases where there is a reasonable potential for plant material derived from or originating from the regulated articles to become mixed with the proposed food or feed crop during harvesting.

11) Reports and Confidential Business Information

Confidential Business Information (CBI) will be handled according to the APHIS policy statement at 50 F.R. 38561-63.

12) Pre-Planting Notification

The permittee is required to notify the APHIS, BRS Permits office and the appropriate PPQ Regional Biotechnologist and State Regulatory Official(s) at least 7 calendar days before the anticipated planting date.

13) Planting Report

Within 28 calendar days after planting, submit a planting report that includes the following information for each field test site:

A. A detailed map of the planted site (inclusive of the border rows of any sexually compatible plants); and
B. The location and the approximate number and/or acres of transgenic plants which were actually planted at the test site for the target protein.
C. The total acreage of the test plot (exclude border rows, if any).
D. 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.

Fax the planting report to the following APHIS personnel:

A. The Chief, Biotechnology Risk Assessment Staff at Area Code (301) 734-8669
B. The PPQ Regional Biotechnologist (fax number enclosed)
C. The State Regulatory Official (CBI-Deleted copy only)

Provide APHIS with the contact information for each field test site, and indicate if planting and harvesting equipment will be moved between authorized field test sites.

Contact information for the APHIS PPQ Regional Biotechnologists is included on the attached map http://www.aphis.usda.gov/brs/regbiot.html and for the State Regulatory officials at http://www.aphis.usda.gov/brs/lt_sta.html.

14) Termination Report

At least 21 calendar days before the anticipated harvest/termination of the field test the permittee is required to notify the APHIS, BRS Permits office and the appropriate PPQ Regional Biotechnologist and State Regulatory Official(s) (http://www.aphis.usda.gov/brs/regbiot.html)(http://www.aphis.usda.gov/brs/lt_sta.html).

15) Field Test Data Report

Within 6 months after the end of the field test (final harvest or crop destruct), the permittee is required to submit a field test data report to the BRS Permits office. Field test reports shall include: methods of observation, resulting data, and analysis regarding all deleterious effects on plants, nontarget organisms, or the environment.

16) Monitoring Report

Post-harvest/post-season monitoring report must be submitted within 3 months after the end of the monitoring period that includes the dates the field site and perimeter fallow zone were inspected for volunteers, the number of volunteers observed, and the actions taken.

17) Unauthorized Release

APHIS shall be notified orally immediately upon discovery and in writing within 24 hours in the event of any accidental or unauthorized release of the regulated article.

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

APHIS BRS Deputy Administrator's office [phone numbers: (301) 734-7324; (301) 734-6331; (301)734-0029]. Indicate that you wish to report an unauthorized or accidental release of a regulated article to the BRS Regulatory Division Director; or in that person's absence, to the BRS Chief of Permits or BRS Chief of Risk Assessment, or the permit reviewer. In the event that one of these persons cannot be reached, contact:

The appropriate APHIS PPQ Regional Biotechnologist.

The appropriate APHIS State Plant Health Director.

Contact information is maintained at the APHIS Biotechnology Regulatory Services website at http://www.aphis.usda.gov/brs/regulatory.html.

Unless otherwise directed, written notification should be sent to:

Animal and Plant Health Inspection Service (APHIS)
BRS Regulatory Division (2) Director, Rm. 5B54
4700 River Rd. Unit 147
Riverdale, MD 20737.

18) Inspections

APHIS's Biotechnology Regulatory Services (BRS) and/or an APHIS PPQ Regional Biotechnologist or APHIS State Plant Health Director may conduct inspections of the test site, facilities, and/or records at any time. APHIS may invite the FDA or State Regulatory Officials to participate in these inspections. Inspections will likely correspond to the beginning of the field test, mid-season or during flowering, at and/or following harvest, and during the post-harvest monitoring period. Inspections will include examination of records that verify compliance with regulations and SOPs.

19) Additional Data Requirements

A. Permittee must monitor for lysozyme in the soil surrounding the plants mid way through the growing season and after the crop is harvested. These data must be submitted with the field data report.

B. Permittee must quantify the amount of lysozyme in stem, leaves, roots and flower parts at flowering and the amount of lysozyme in stem, leaves, roots and seeds at harvest.

Appendix III. 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 often claimed as confidential business information by the applicants, the United States Government 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[2].

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[2]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 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.

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 non-target organism that "feeds" on tobacco that is not a plant pest except possibly skunks. Because of nicotine production, earthworms are killed even by the non-engineered 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[3].

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 non-engineered 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.

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[3] 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 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 non-target 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 confinement, 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[4]. Depending on the plant species,

[4] 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

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, disking into the soil, burning, treatment with appropriately labeled herbicides 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:

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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."

This is not a complete list of all information provided but focuses on elements associated with risks to non-target 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 non-target 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 non-targets.

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 non-target 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 it will initiate consultation. To document our decision making process APHIS will complete an TES assessment sheet for every gene-site combination.

Appendix IV. Applicant supplied TES Worksheet

Ventria Bioscience
TES WORKSHEET

Table of Contents

Ventria Bioscience
TES WORKSHEET
For Lysozyme Rice
Trials and Production
Cape Girardeau, Mississippi, Scott Counties, Missouri

RECIPIENT ORGANISM: Rice, *Oryza sativa*

PRODUCTS:
Recombinant human lysozyme expressed in the endosperm of rice grains is the product.

Lysozyme is ubiquitous in the human body where it acts as a protective barrier against environmental agents and, in doing so, helps prevent infection. The following are some examples of how lysozyme could be used to enhance human health:

1) Improvement of gastrointestinal health, via inclusion in infant formula.
2) For the management of acute watery diarrhea by the inclusion of rhLZ, in oral re-hydration solution (ORS).
3) In the management and prevention of topical infections.

LOCATION OF THE FIELD TEST:

The trial and scale-up locations are planned for Cape Girardeau, Mississippi, and Scott Counties in the southeast corner of Missouri, adjacent to the Mississippi River. These counties are primarily agricultural and planted to cotton, corn, sorghum, soy and wheat. Although rice is grown in this regional area of Missouri, less than 500 acres per year has been grown in Mississippi and Scott County and none in Cape Girardeau County. No rice driers, mills or rice seed processing facilities are present in these counties. Ventria is working with the sole rice producer in Mississippi County.

In all cases, a rice-free 50 foot fallow area, which may include roads, borders the transgenic rice. In addition, Ventria performance standards call for a ¼ mile distance to any commercial rice production.

The nearest non-agricultural water is more than one mile from the field site.

The land to be used is currently in agricultural production. No practices are performed which would affect TES any more severely than any other local agricultural practice or commercial rice field. Routine, appropriate, and registered for Missouri agricultural practices will be performed. Ventria-dedicated equipment will be used under USDA-approved SOP's for equipment and cleanout practices. In addition, our rice area is within a much larger agricultural zone.

LEVELS PRODUCED AND TISSUE:

The product is expressed only in the endosperm of rice seed and in no other plant part. Approximately five mg of lysozyme protein per gram of dried brown rice flour is the expression level (5mg/g). Levels detected in the field may be lower due to environmental interactions.

Ventria uses a targeted system for production of its recombinant molecules in which the molecules are manufactured exclusively in the grain of the plant. All other plant tissues: roots, stems, leaves, and pollen do not contain our target molecules. Biochemically, these tissues are identical to their counterparts from non-transgenic plants, as shown by proximate analysis of these tissues to non-transgenic controls. We have performed western blots on tissues from our plants to verify the controlled expression of target molecules in our expression system.

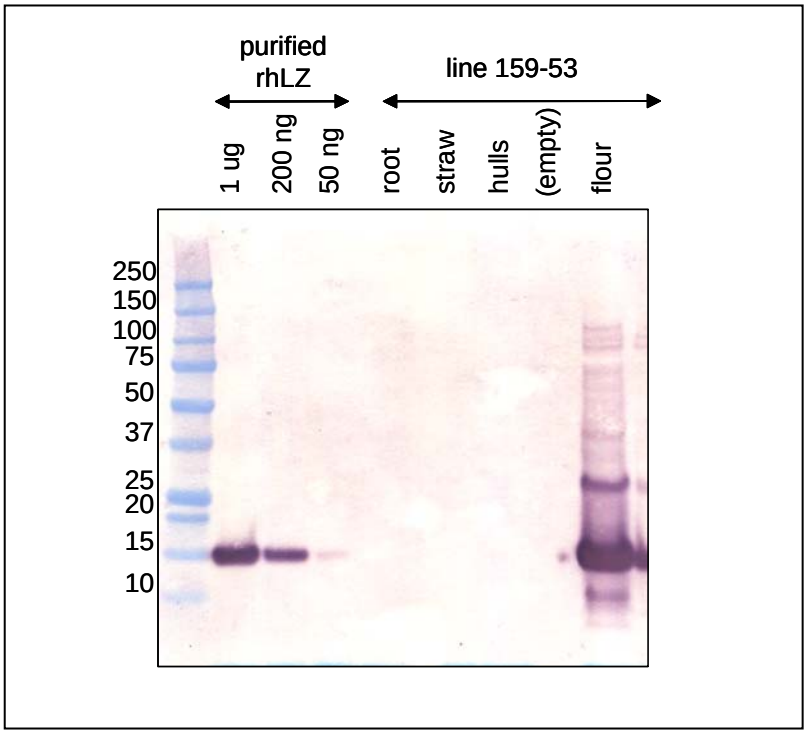

Figure 1. Western blot of 2003 field samples of LZ159-53 mature plant organs. Only the mature grain (brown rice flour, in this case) contains rhLZ.

ASSESSMENT:

Lysozyme is a protein found in most mammal breast milk, including humans, as well as in most epithelial surface secretions including tears, nasogastric, saliva, and bronchial (See Table 1 for lysozyme levels in humans). Human milk contains up to 0.250 mg/mL of lysozyme (Lonnerdal, 1985)... It is highly unlikely that this molecule is either an allergen or toxin. In addition, amino acid sequences have been determined for many

diverse species including: humans, cows, chickens, quail, pheasant, guinea fowl, turkey, duck, chachalaca, baboon, rat, and tortoise (Jolles, 1984).

Not surprisingly, it isn't listed as a toxin in the SWISS-PROT and TrEMBL databases. Nor does it share any amino acid sequence homology with known toxins, as determined by FASTA search against known toxins listed in the SWISS-PROT and TrEMBL databases. The criteria for the FASTA search are described by Gendel (Gendel 1998). The wordsize used initially was set to two, and the regions of amino acid sequence identity greater than 6 were scored. None were found.

Various forms of the lysozyme protein are found and characterized in numerous mammals including humans, cows, chickens, quail, pheasant, guinea fowl, turkey, duck, chachalaca, baboon, rat, and tortoise (Jolles, 1984). Chicken lysozyme is a widely used commercial product in the U.S... Although the digestive system anatomy of birds is very different from humans and other mammals, the composition of the gastric fluid is similar. Both mammal and bird gastric fluids contain HCl and pepsin and both operate at about a pH of 2.0 (Sturkie, 1976 and Klasig, 1998). In addition the pancreatic system of birds and mammals is also similar. Therefore, human digestive studies with lysozyme should apply to avian species as well.
Humphrey et al. have analyzed the effects of ingestion of Ventria's lysozyme (with lactoferrin) rice on the health and intestinal flora of chickens. (Humphrey. 2002) In their study, they measured growth rate and intestinal structure of Cobb broiler chicks fed varying amounts of Ventria rice for 21 days after hatching. As predicted, the results show that lysozyme improved the health of the chicks. Growth rates of the chicks were improved relative to controls. Also histological indices of intestinal health showed improvement relative to controls. These data support the hypothesis that Ventria rice is not harmful to avian species.

Chicken egg white is currently the major commercial source of lysozyme. Sequence analysis shows that human lysozyme is about 60% homologous to its chick counterpart, a known allergen, but the human version is not an allergen. The fact that antibodies against human and chicken egg white lysozyme do not cross react indicates significant structural differences (Faure and Jolles, 1970).

In contrast to reports of allergenicity, lysozyme has shown efficacy at combating infections in experimental animals and humans following administration by oral, intravenous, intraperitoneal and topical routes. In rainbow trout, lysozyme injections decrease mortality from a challenge with *Aeromonas salmonicida* by more than 3-fold and oral administration decreased mortality from infectious pancreatic necrosis virus by 2-fold. (Siwicki et al. 1998). Oral administration of egg white lysozyme is now used clinically in human medicine for the therapy of inflammatory diseases of respiratory and digestive epithelia (Seno, et al. 1998).

Finally, mineral and vitamin analysis and proximate analysis on paddy, straw and rice flour of the transgenic rice and the non transgenic parent show either no differences or

differences within the standard range (Table 1 and 2). That is, the differences between the two can be accounted for by varietal differences in rice.

Table 1: Mineral and vitamin composition of paddy rice (rough rice) from LZ159 53 rice and Taipei 309 non-transgenic rice.

Analysis mg/100 gram	LZ159-53		Taipei 309		Standard Value
	Mean	SD	Mean	SD	
Minerals					
Phosphorus	26.61	2.19	21.47	0.71	26.00 -36.00
Iron	2.89	0.36	3.37	0.51	2.80 - 5.70
Calcium	23.58	1.57	21.55	1.61	15.00– 70.00
Vitamins					
Niacin	5.85	0.57	4.21	0.59	1.50 - 6.50
B1	0.53	0.09	0.39	0.08	0.14 - 0.38
B2	0.22	0.09	0.20	0.02	0.04 - 0.13

Table 2. 2003 Field-grown rice. Proximate analysis on paddy rice (rough rice) from LZ159-53 rice and Taipei 309 non-transgenic rice.

Analysis % Dry weight	LZ159-53		Taipei 309		Standard Value
	Mean	SD	Mean	SD	
Moisture (as received)	9.80	0.77	9.94	0.75	11.00- 13.70
Crude fat	1.67	0.41	1.91	0.53	1.80 - 2.70
Crude protein	9.00	1.35	6.73	0.75	6.70 - 9.00
Ash	5.52	0.56	5.09	0.25	3.40 - 6.00
Carbohydrate	83.81	1.04	86.24	0.79	78.70 - 95.60
Total dietary fiber	16.74	0.67	15.22	1.37	19.10
Fiber, acid detergent	12.65		12.97		
Fiber, neutral detergent	13.88		13.19		16.40 - 19.20

Humphrey et al. have analyzed the effects of ingestion of Ventria's lysozyme on the health and intestinal flora of chicks. (Humphrey, Huang et al. 2002). In their study, they measured growth rate and intestinal structure of Cobb broiler chicks fed varying amounts of lysozyme rice for 21 days after hatching. As predicted, the results show that lysozyme improved the health of the chicks. Growth rates of the chicks were improved relative to controls. Also histological indices of intestinal health showed improvement relative to controls. These data support the hypothesis that the lysozyme reduced the pathogen load in the intestines of the chicks.

There are no known beneficial invertebrates in rice agriculture, and only a handful of insects that inhabit a rice habitat. Since Ventria's expression system involves only expression of the target protein in grains, only insects that consume grain would be exposed to the target molecules. Any such insect is considered a pest.

There are several rice-field coincidental pests that are known to feed on rice grains in Missouri, as opposed to other plants parts. Rice Stink Bugs feed on the developing rice

grain from the milk stage through grain maturity; grasshoppers may feed on the very young immature head of the rice plant; crayfish, if present, often feed on young, germinating rice grains. None of these insects are classified as a beneficial: they are considered pests, and are eradicated using normal agricultural practices. Any invertebrates (beneficial or not) that consume other parts of a rice plant will be unaffected by the transgenic nature of our plants, since the expression of our target proteins is limited exclusively to the grain.

We conducted an analysis of which Missouri threatened and endangered species (TES) are present in Cape, Mississippi and Scott Counties and would be most likely to consume Ventria's mature grain or germinating seedlings, if present. We did this by examining the known most recent location and diet of each of the animals listed in the Federal TES list. There were some animals that were clearly identifiable as non-seed or seedling eaters, such as bats, eagles, and mollusks. Two species listed for these counties, the pallid sturgeon and Fat Pocketbook (mollusk), are known to potentially inhabit the Mississippi River which borders the eastern edge of Cape Girardeau, Mississippi and Scott Counties, not agricultural areas. The Interior Least Tern, listed as a TES by the US Fish and Wildlife Service for the counties of interest, feeds almost entirely on small fish and utilizes islands and beaches of the Mississippi River.

The one plant species on the Federal TES list found in Ventria's proposed counties is the Decurrent False Aster. Ventria's field data to date shows no movement or leaching of lyzozyme into the soil and we believe there are no exposure routes which could negatively affect plant species of any kind. To date we have seen no depression of weed species in our fields relative to commercial fields.

We have reviewed the current literature, including the data in sections above, with Dr. Kirk Klasing, a professor in the animal science department at the University of California, Davis; and discussed with him the potential for lysozyme contained within rice grains, to have deleterious effects on non-target organisms inadvertently exposed to them either topically or through digestion. Based on the current literature, and the data generated to date by Ventria, we do not believe there is any reason to think that these molecules will harm non-target organisms in the environment.

Finally, all seed is harvested and stored in Ventria's dedicated storage. The remaining straw is burned as soon as possible and the field disked. No appreciable transgenic rice or lysozyme remains in the field for consumption by wildlife. In-season and post-harvest management practices are also designed to deter wildlife form moving into our fields.

CONCLUSION:

There are no Threatened or Endangered Species which are expected to inhabit Ventria's rice fields, none which eat rice, and no expose routes from our fields to likely TES habitats, such as the Mississippi River.

MISSOURI TES LINKS
Endangered and Threatened State and Federal TES:

http://www.conservation.state.mo.us/nathis/endangered/checklst/endspec.htm

Accessed October, 2004;

Endangered Species Guidesheets:

http://www.conservation.state.mo.us/nathis/endangered/endanger/guide.htm

Accessed October, 2004

FEDERAL SUMMARY LINKS:

http://ecos.fws.gov/ecos/apps.doc

Accessed October, 2004;

U.S. Fish and Wildlife Service, Region 3:

http://midwest.fws.gov/endangered/lists/missouri-cty.html

Accessed October, 2004;

Table 1. Federal Threatened and Endangered Species in Missouri, follows.

Table 1. Federal Threatened and Endangered Species in Missouri.

Animals -- 17

Status	*Listing*	*Category*	*Eat Rice ?*	Cape Girardeau County Y/N?	*Mississippi County Y/N?*	*Scott County Y/N?*	Location Notes
E	Bat, gray (Myotis grisescens)	Mammal	N	No	No	No	Taney , Boone, Dent , Hickory, Renyolds and Wright
E	Bat, Indiana (Myotis sodalis)	Mammal	N	No	Listed by US.FWS for this County	No	Indiana bats hibernate in only eight specific locations, three of which are located in Shannon, Washington, and Iron counties of Missouri. Summer roosting Indiana bats have been recorded in northern Missouri
E	Bat, Ozark big-eared (Corynorhinus (=Plecotus) townsendii ingens)	Mammal	N	No	No	No	McDonald, Barry, Stone, Christian, Torey, Ozark, Douglas, Howell
T	Cavefish, Ozark (Amblyopsis rosae)	Fish	N	No	No	No	Thirteen Ozark cavefish sites are known to exist in Missouri. Presently, populations are known from Newton, Jasper, Lawrence, Greene, Stone, and Barry counties.

T	Darter, Niangua (Etheostoma nianguae)	Fish	N	No	No	No	Niangua darters occur only in Missouri and are located in counties in the Osage River basin including: Osage, Maries, Miller, Camden, Hickory, Dallas, Benton, Greene, Webster, Cedar, Polk, and St. Clair counties.
T	Eagle, bald (lower 48 States) (Haliaeetus leucocephalus)	Bird	N	Listed by US.FWS for this County	Listed by US.FWS for this County	Listed by US.FWS for this County	Pike, Lincoln, St. Charles, Henry Pulaski and Stoddard are most recent sightings
E	Higgins eye (pearlymussel) (Lampsilis higginsii)	Mollusks	N			No	
T	Madtom, Neosho (Noturus placidus)	Fish	N	No	No	No	This species is peripheral to Missouri; the entire distribution of the Neosho madtom is 250-300 stream miles of the Arkansas River basin,
E	Mucket, pink (pearlymussel) (Lampsilis abrupta)	Mollusks	N	No	No	No	In Missouri, the pink mucket lives primarily in the Meramec, Gasconade, and Black rivers, and stretches of the Osage River. Pink mucket shells, but not live pink muckets have been found in the Sac, Big, St. Francis, and Little Black rivers.

E	Mussel, scaleshell (Leptodea leptodon)	Mollusks	N			No	
E	Pearlymussel, Curtis (Epioblasma florentina curtisii)	Mollusks	N	No	No	No	
T	Plover, piping (except Great Lakes watershed) (Charadrius melodus)	Bird				No	
E	Pocketbook, fat (Potamilus capax)	Mollusks	N	No	Listed by US.FWS for this County / Adjacent Mississippi River areas	No	
E	Shiner, Topeka (Notropis topeka (=tristis))	Fish	N	No	No	No	Topeka shiner inhabits streams in Boone, Cooper, and Moniteau counties.
E	Sturgeon, pallid (Scaphirhynchus albus)	Fish	N	Listed by US.FWS for this County / Adjacent Mississippi River Area	Listed by US.FWS for this County / Adjacent Mississippi River Area	No	
E	Tern, least (interior pop.) (Sterna antillarum)	Bird		Listed by US.FWS for this County / Adjacent Mississippi River Area	Listed by US.FWS for this County / Adjacent Mississippi River	No	Mississippi and Missouri River, islands, beaches and sandbars

					Area		
T	Wolf, gray Eastern Distinct Population Segment (Canis lupus)	Mammal	N			No	
						No	
Plants -- 8						No	
Status	*Listing*					No	
T	Milkweed, Mead's (Asclepias meadii)	Flora	-	No	No	No	It is presently found in the Osage Plains region and the St. Francois mountains region of the Ozarks.
T	Aster, decurrent false (Boltonia decurrens)	Flora	-	Formerly, not currently	Listed by US.FWS for this County	No	Former distribution of this plant included Lincoln, St. Charles, St. Louis, and Cape Girardeau counties. Presently it is only known to occur in St. Charles County.
T	Geocarpon minimum (No common name)	Flora	-	No	No	No	Geocarpon populations in Missouri are restricted to Dade, Polk, Greene, Cedar, Jasper, Lawrence, and St. Clair Counties in the Ozark and Osage Plains Natural Divisions

T	Sneezeweed, Virginia (Helenium virginicum)	Flora	-			No	
T	Bladderpod, Missouri (Lesquerella filiformis)	Flora	-	No	No	No	Missouri bladderpod is presently found in the following Missouri counties: Dade, Greene, Christian, and Lawrence.
E	Pondberry (Lindera melissifolia)	Flora	-	No	No	No	In Missouri, pondberry is found only in Ripley County of the Missouri Lowlands Region.
T E	Orchid, western prairie fringed (Platanthera praeclara)	Flora	-	No	No	No	Presently, this orchid has only been recorded from Atchinson, Holt, and Harrison counties.
	Clover, running buffalo (Trifolium stoloniferum)	Flora	-	No	No	No	May be extirpated. One natural site for running buffalo clover was discovered in Madison county in 1994 and another was discovered in Maries county in 1998.

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