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Docket No. 05-006-1 and 05-007-1 (submitted separately to each, in quadruplicate)
Regulatory Analysis and Development, PPD
APHIS Station 3C71
4700 River Rd., Unit 118
Riverdale, MD 20737-1238

Re: Comments on Two Environmental Assessments on Permit Application Number 04-302-01r: Ventria Rice Expressing Lactoferrin (Docket 05-006-1), and Permit Application Number 04-309-01r: Ventria Rice Expressing Lysozyme (Docket 05-007-1)

Dear Sir/Madam:

The Center for Food Safety (CFS) appreciates the opportunity to comment on the above-referenced APHIS Environmental Assessments (EAs) on Ventria Bioscience's proposal to grow genetically engineered (GE) rice that expresses human lactoferrin and lysozyme, both at the same location in Missouri.

CFS believes that these field tests present potentially significant environmental impacts and associated human health risks that have not been adequately addressed in the two EAs. In general, we agree with the recent National Academy of Sciences report that concludes that, food crops are usually not a good choice for the production of pharmaceutical crops due to the difficulty of ensuring that contamination of food will not occur.[1] Similarly, an extensive review by scientists with expertise in relevant disciplines also concludes that the use of food crops to produce pharmaceuticals is ill advised.[2] The authors of that report conclude that although it would be hypothetically possible to ensure that contamination would not occur, in practice, due to the nature of commodity crop production, the prevention of contamination cannot be

guaranteed in today's agricultural environment. This is especially true when the pharm crop is

not geographically isolated from the food crop.

NATIONAL ENVIRONMENTAL POLICY ACT CONCERNS WITH BOTH EA'S

It is remarkable that both EAs address exactly the same applicant, same affected environment, same crop, and same classes of foreseeable impacts. Yet, neither EA **even mentions** the existence of the other proposed field test. Neither EA addresses the cumulative impacts of the two projects at the same site or the possibility of any synergistic effects between the two proposals. Thus, the "Cumulative Environmental Effects" sections of each are facially inadequate. This defect underscores the suitability of submitting this joint comment on both EAs, to urge APHIS to consider the impacts of the two proposals cumulatively.

Both EAs are inadequate in their descriptions of the "Need" for the proposals, that is, the need that Ventria seeks to meet with these field tests. The existing "Purpose" description for both EAs is incorrectly placed in "VII. Description of the Field Test/Affected Environment". It should be moved to the existing "II. Purpose and Need" section where it belongs, and should be expanded on as it is now too sparse to tell the reader what Ventria's aims are. Further, the Proposed Action that requires analysis here is not your agency granting a permit, as the EAs put forth, rather the action is Ventria undertaking the field tests. This conceptual confusion weakens the analysis in the EAs throughout.

Both EAs fail to adequately describe the features of the Affected Environment for the proposal. A fundamental problem is the excessive claims of Confidential Business Information (CBI) by Ventria and the allowance of these claims by APHIS. Perhaps most important are the withholding of the actual location and acreage of the proposals (p. 4 of both EAs). Knowing the location and size of the field plots is vital for determining where, how, and to whom potential unintended exposures could occur, which are key components in determining risk to the public and the environment.

Further, field test locations are **not** CBI under Federal law. This is the ruling by Federal judges in Hawaii, in a lawsuit involving GE pharmaceutical crop field tests, in the only judicial opinions to date that have considered the question. The attached two Orders, in the case of CFS et al. v. Veneman at al., in which the defendants are the Secretary of Agriculture, the Under Secretary for Marketing and Regulatory Programs, the Administrator of APHIS, and the Deputy Administrator in charge of BRS, bind your agency. (Order of U.S. Magistrate Judge Barry M. Kurren dated June 29, 2004, Denying Defendants' Consolidated Motions for a Protective Order, affirmed by Order of U.S. District Court Judge David A. Ezra dated Aug. 3, 2004, Affirming Magistrate Judge's Order for Discovery etc., U.S. District Court, District of Hawaii, civil case no. 03-00621.) At page 3 of Magistrate Judge Kurren's Order, he unambiguously states: "Field test site locations do not constitute confidential commercial or trade secret information." Those orders directed the USDA defendants to provide the claimed-CBI locations in Hawaii to CFS and others, which they have since done.

To bring your policies into alignment with the law, we urge you to now end your past practice of treating locations as CBI, not just for these two EAs, but for all public documents related to all GE crop field tests. Until then our comments are provisional because any final conclusions

about safety or lack thereof depend on the location and size of the proposed planting. Ventria should not be allowed to grow commercial quantities of GE pharmaceutical compounds for a multi-year span - as it asserts it intends to do - on hundreds or even thousands of acres under APHIS's field test regime, without revealing where. The EAs are inadequate on that basis alone.

Although the permit applications evaluated in these EAs are for a single year, Ventria is proposing to grow rice containing lactoferrin and lysozyme in southeastern Missouri for an indefinite period of time as it seeks to commercialize these products. APHIS cannot reasonably rely solely on the current EAs in assessing the risks from future field tests of Ventria's pharmaceutical rice, as is strongly suggested by statements in the EAs, despite the fact that food rice production has been dramatically increasing in the county where the field tests are proposed for.[a] The 8-9 fold increase in production of food rice over an 8 year period in the area is not acknowledged or recognized in the EAs, but could seriously impact the ability to prevent contamination, especially if the trend continues.

SCIENTIFIC CONCERNS WITH THE LACTOFERRIN EA (DOCKET 05-006-1)

In sum, the EA for lactoferrin rice is inadequate for the following reasons:

- APHIS sets an inadequate 1/4 mile isolation distance to separate Ventria rice from food rice or the serious weedy relative, red rice. APHIS has underestimated gene flow from rice in the past (based on recently published research) and has seriously underestimated the ability of genetically engineered creeping bentgrass to contaminate surrounding wild relatives.[3] These and other cases are symptomatic of inadequate data on the ability for

[a] APHIS remarks on page 5 of both EAs: "This EA is prepared because the applicant intends to have 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." (emphasis added). Therefore, it appears that APHIS intends that the current EAs will be adequate to address both current *and future* cumulative risks. This would contravene NEPA, which requires that an agency's environmental assessments be based on just the project proposal that is before it at the time. Thus, APHIS should issue a revised EA that corrects this suggestion of prejudgment of expected future permit proposals by Ventria.

gene flow from crops to occur. The 1/4 mile isolation distance accepted by APHIS is unlikely to ensure that gene flow will not occur.

- APHIS allows farm equipment used with Ventria's rice to be used with food rice after cleaning, despite the inability to ensure that such cleaning can remove all of Ventria's rice, which could then be transferred to food rice or contaminate fields containing red rice. A recent report by experts on farm practices confirms that complete cleaning cannot be ensured.[4] Also, there is no requirement to clean farm machinery that was previously used on conventional rice farms prior to use on Ventria's rice, which could allow the contamination of Ventria fields by weedy red rice from a conventional farm. Because of its long seed dormancy, once in Ventria's fields, red rice could be very difficult to eradicate and would likely hybridize with Ventria's rice.

- Isolation distance must not be considered alone unless prevention of gene flow can be ensured. However, APHIS admits that minimal amounts of gene flow may occur with Ventria's rice. In conjunction with the amount of gene flow, such as by cross pollination, the ability of the transgene to confer a competitive advantage to wild red rice must be carefully considered because enhanced competitive ability can facilitate permanent escape and spread of transgenes even when very low levels of gene flow occur. APHIS does not consider the substantial possibility that the lactoferrin gene could confer a competitive advantage to wild red rice by reducing disease of red rice grain, despite the fact that lactoferrin has been used in transgenic crops in previous field tests for the expressed purpose of reducing disease in those plants.

- APHIS does not consider important recent data, and thereby may seriously underestimate the possibility of horizontal gene transfer of the lactoferrin gene to bacteria, and possible risk should that occur.

- APHIS as an agency is not qualified to determine the potential human safety risks from lactoferrin rice; further, it has not adequately evaluated those risks in the EA.[b] APHIS has not considered the possible immunological (such as allergy) implications of differences between lactoferrin produced in humans, the source of the gene, and lactoferrin produced in rice, which is chemically different than the human version.

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[b] APHIS has consulted with a representative from FDA to help in its human safety determination, however, FDA has not completed its own human safety assessment, so this assistance is unlikely to be adequate.

- APHIS apparently accepts evidence that lactoferrin is degraded in the stomach, which would reduce its risk, contrary to evidence presented by Ventria that intact lactoferrin can be found in infant stool.

- APHIS accepts that lactoferrin is denatured by cooking despite questionable testing methods. More importantly, denaturation does not assure that a protein will not be an allergen, although APHIS is apparently reassured by these data.

Environmental Safety of Human-Derived Lactoferrin Produced in Rice

Gene Flow from Lactoferrin Rice to the Wild Relative, Red Rice

A critical issue for the environmental safety of pharmaceutical crops is gene flow to wild crop relatives. As the EA notes, there appears to be one wild sexually compatible relative of cultivated rice in the U.S., annual red rice, *Oryza sativa* ssp *sativa*. Cultivated rice can sometimes become weedy as well, and can grow as a volunteer feral plant in rice growing areas. Red rice is a serious weed of rice, and is reported to be found in Missouri.[5] In fact, some sources consider it to be the most serious rice weed in Missouri.[6] Red rice often readily hybridizes with, and produces fertile progeny with, cultivated rice that are often more vigorous than either parent, which could enhance survival after gene flow at low frequencies.[7] Furthermore, APHIS likely underestimates the potential for gene flow because it considers rice to be primarily self-pollinating. Although true, this assessment ignores data by Langevin and colleagues who found hybridization of red rice with a cultivar of commercial rice at 52%, which indicates the potential of a high rate of outcrossing under at least some circumstances.[8]

If the lactoferrin rice (hereafter "rL") gene contaminates red rice, it could spread well beyond Ventria's field test sites. Furthermore, because red rice seed has extended dormancy, and can survive in the soil for at least 6 or 7 years, once red rice is contaminated, it would be very difficult to eradicate or prevent further spread.

The EA discloses several permit conditions intended to reduce gene flow to either red rice or cultivated rice. The primary means of reducing gene flow is to require a separation distance of at least 1/4 mile between the Ventria field test and either cultivated rice or red rice. Ventria further claims that there have been no rice fields within 10 miles of the field test site. This estimated

separation is based on a personal communication from a University of Missouri extension specialist, with no indication of how this estimation was derived. As such, it should not be given much weight. In addition, as noted in the EA, it cannot be ensured that rice will not be grown closer to the field test site, so the 1/4 mile requirement must be the standard used for assessing possible gene flow. Furthermore, as noted in the EA, Ventria intends to expand its acreage over time, and to grow in Mississippi and Cape Girardeau counties as well as the current Scott County (EA, page 44, TES Worksheet). Northwest Missouri State University is collaborating with Ventria and its president, Dean Hubbard, has stated that eventually Ventria expects to increase cultivation of its pharmaceutical crops to 25,000 acres.[9] The latter acreage is 39 square miles, which clearly could make ensuring separation distances of greater than 1/4 mile improbable in these counties.

In addition, Ventria claims that the counties discussed in the field test application grow a total of 500 acres of rice (EA page 44, TES worksheet), all in Scott county. To the contrary, recent preliminary state agricultural data for 2003 for Scott county indicate 1200 acres of rice (data for Scott county alone was not previously recorded, but has been since 2003 due to increasing rice acreage). For the three other counties of Cape Girardeau, Mississippi, and Bolinger, 1400 acres were grown in 2003 (individual county data not available). Therefore, the three counties where Ventria plans on growing rL rice, plus Bolinger, grew about 2,600 acres of rice in 2003, compared to about 300 acres in 1996, or an increase of almost 9 fold in 8 years.[10] [11] In addition, surrounding counties grow large amounts of rice, for example, Stoddard County grows about 58,000 acres. Taken together, these data indicate a trend of increasing food rice cultivation in these counties, which may encroach on Ventria's rice significantly if it continues, and already questions Ventria's isolation assumptions.

On page 17 of the EA, APHIS claims that because rice pollen survives for only a few minutes (5 to 20 minutes is often cited), the 1/4 mile separation from food rice or red rice would reduce gene flow to "*de minimus*" levels. The EA does not define what is meant, in practice, by "*de minimus.*" Unfortunately, APHIS assurances about adequate confinement distance have been repeatedly proven wrong, primarily because data on low levels of gene flow that could be important when considering pharmaceutical rice and pollination of wild relatives have generally not been available. For example, APHIS previously required only a 10 foot separation distance for GE rice, based on seed purity standards for conventional breeding. APHIS then increased this distance to 100 or 200 feet for pharmaceutical rice. However, recent research indicates that gene flow may occur at 110 meters, between a quarter and a third of the 1/4 mile distance required separation distance.[12]

Furthermore, the maximum distance for rice pollen dispersal estimated in this research was based on only a few measurements in calm weather, and was extrapolated from those measurements using regression analysis, and where the largest pollen source was only 72 m^2, compared to the reported 204 acres for the Ventria field tests. This method of distance estimation is unlikely to adequately account for either the leptokurtic distribution often seen in pollen flow measurements (i.e., instead of pollen dispersal diminishing in a regular manner to zero over distance, low levels of pollen can be found at very long distances from the source), nor account for weather conditions that could substantially increase pollen dispersal, for example thunder storms, high winds, or air turbulence. The authors note that their suggestion of a 110 meter isolation distance is based on "normal weather conditions." They also note that: "Undoubtedly, a detailed study is needed to find out what the influences of other weather parameters are on the distribution of rice pollen flow under normal field conditions."[13]

The approach of limited incremental increases in isolation distance compared to previous standards, as proposed by Ventria, does not address the fundamental lack of sufficient data on pollen dispersal. Because few experiments have been carried out that include a range of environmental conditions, current data cannot be considered to represent maximum distances for gene flow. The most recent example of APHIS underestimation of pollen dispersal was an experiment with GE creeping bentgrass where gene flow was found at the greatest distances measured - about 13 miles from a field test - which was miles farther than expected based on the much more limited confinement requirements that APHIS placed on that field test.[14] Similarly, although APHIS typically requires a 1/4 mile isolation distance for genetically engineered (non-pharmaceutical) canola, recent experiments show small amounts of gene flow at 3 kilometers.[15]

All of these distances should be considered preliminary, because various conditions may affect the results by affecting the rate of pollen travel and survival time. Factors such as wind speed, air turbulence, ambient temperature, cloud cover (UV light penetration), and humidity may all affect the distance over which gene flow may occur. In addition, the relative sizes of the pollen source (Ventria rice fields) and red rice stands is likewise important, because a large pollen source and relatively small number of accepting plants may increase gene flow.[16] [17] Although APHIS inappropriately does not reveal the size of the field test, press reports citing Scott Deeter of Ventria indicate that their field test applications (presumably for lactoferrin and lysozyme) are for about 200 acres.[18] It is therefore likely that the source of rL pollen will likely be much greater than the size of red rice stands.

APHIS also does not consider observations of possible pollination of rice by insects. For example a small increase in outcrossing of rice has been observed when honey bees are present.[19]

Clearly rice is primarily wind pollinated, but low levels of insect pollination need to be considered. Honey bees often forage over distances greater than 1/4 mile, sometime over several miles. Therefore, bee or other insect pollination could be important for gene flow to red rice.

Finally, data on gene flow distances typically follow a skewed leptokurtic distribution. Graphs of gene flow distances usually show a very long distribution "tail," whereby low levels of gene flow occur over very long distances without falling to zero (at least, not within the distances observed in the experiments).[20] [c] The skewed distribution means that APHIS cannot simply examine most current, limited, data and extrapolate safe isolation distances.

Another possible means of gene flow could occur from farm machinery. In particular, machines with complex internal compartments not readily accessible from the outside may harbor small amounts of seed, even when cleaned. APHIS requires machinery used on biopharm crops to be "dedicated' to that crop. However, APHIS definition of "dedicated" allows the farm equipment to be used on conventional food crops after cleaning. In the case of Ventria, machinery such as planters, and especially harvesters, may be used with food rice after they are used for rL rice after cleaning. Ventria remarks in the EA that it intended to clean its equipment according to APHIS standards prior to use with food rice. A recent review of biopharm crops by experts knowledgeable about farm equipment concluded that such equipment cannot usually be cleaned carefully enough to ensure that biopharm seed will not be co-mingled with food seed.[21] If that occurs, such seed could either end up in the food supply, or in seed used for planting future rice crops. In the latter case, rL rice would be planted unsuspectingly, and could thereby be perpetuated in the food supply, or contaminate red rice.

There is also no requirement to clean farm equipment *prior* to use on Ventria's fields. This allows the possibility for red rice to be introduced to Ventria's fields from other rice fields. Red rice growing in Ventria's fields would be cross pollinated by Ventria's rice, transferring the rL gene. And because of its very long seed dormancy period, such rL red rice could easily evade volunteer rice control protocols, and emerge years after a particular field is no longer used by Ventria. And rL red rice, once in Ventria's fields, would not only be extremely difficult or impractical to eradicate, it may eventually escape by machinery or other means.

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[c] Recent experiments by Rieger et al. show a more random distribution, but this may have occurred due to the combination of data from several sites. In any case, those data also demonstrate higher than expected gene flow at longer distances than would otherwise be expected.

Further, morphological convergence between red rice and cultivated rice has been reported in some cases. This would make the detection of red rice growing among cultivated rice more difficult.[22] [23] This could make assessment of the proximity of red rice more difficult or inaccurate, and increase the possibility that machinery, such as combines, could unwittingly harvest red rice prior to use in Ventria's fields.

Finally, APHIS does not adequately consider the movement of seed by birds, mammals, or water. As discussed in the following section, low frequency events may be sufficient to cause permanent escape of the rL gene to red rice if the fitness of the wild species is enhanced. Therefore, the evaluation by APHIS on dispersal by birds, mammals, or flooding is inadequate because it does not consider potential impact of low levels of initial gene flow. In particular, due to the relatively short required isolation distance of 1/4 mile, incidental transfer, such as rice grains temporarily lodged in the feathers of waterfowl or the fur of mammals, could occur. Similarly, the screens and grates described by APHIS to prevent transfer by water can become clogged or overflow. Although the EA claims that the nearest "large" body of water is 4 miles from the field test site, elsewhere, page 44 of the EA notes that "The nearest non-agricultural water is more than one mile from the field." Such a location may allow the growth of volunteer rice for long enough to transfer to red rice.

APHIS's unsupported claim that the lack of observed volunteer rice at the edges of rice fields is "strong evidence" that transfer of rice does not occur is not credible without any discussion about how frequently anyone looks for such volunteer rice.

In summary, several routes of possible gene flow to red rice or cultivated food rice exist that have not been adequately considered by APHIS. The assertion that gene flow would occur only at *de minimus* levels cannot be considered credible without better data, and absent consideration of the possible fitness consequences of gene flow to red rice, considered in the following section.

Failure to Evaluate the Potential Contribution of the Lactoferrin Gene to the Fitness and Weediness of Red Rice

Isolation distance must not be considered separately from the ability of the transgene(s) to survive after gene flow occurs. Low levels of gene flow can lead to the permanent escape of the transgene if it confers a selective advantage to a wild relative, in this case, red rice.[d] Put differently, a gene that confers a fitness advantage may persist and spread in a wild relative whereas a gene that is disadvantageous may not survive or spread.[24] Therefore, fitness, or the competitive advantage conferred to a wild relative by a transgene, is of fundamental importance in determining the possibility of transgene escape unless the absence of gene flow can be ensured. As has been noted, APHIS does not ensure that gene flow will not occur, but only that it may occur at *de minimus* levels. However, without properly considering the possible fitness conferred to red rice by the lactoferrin gene, APHIS cannot properly determine what a *de minimus* level of gene flow may be.

Unfortunately, APHIS does not adequately consider the ability of the lactoferrin gene to persist and spread if it escapes to its wild relative, red rice. In fact, APHIS apparently does not even consider the possibility that the lactoferrin gene may confer a selective advantage to red rice. APHIS merely states that the lactoferrin (or hygromycin) "is [not] expected to alter the susceptibility of the transgenic rice plants to disease or insect damage." (EA, page 15) This is a fundamental shortcoming of the APHIS argument for the safety of lactoferrin rice, and in combination with inadequate analysis of gene flow and the 1/4 mile isolation distance, constitutes a major failing of the APHIS risk assessment.

In particular, APHIS does not assess the possibility that the well known antimicrobial properties of lactoferrin may confer a competitive advantage to red rice. Resistance to diseases or insects are properties that the National Academy of Sciences (NAS) and others consider to potentially confer a competitive advantage to wild crop relatives, by increasing survival compared to plants that do not possess the gene.[25] The NAS stated that: "Generally, if an allele confers a fitness advantage - once introduced into a population - it is expected to increase in frequency, even if it is introduced only once."

One of the primary commercial applications of lactoferrin is as an anti-diarrheal for infants. APHIS acknowledges in the EA that lactoferrin has been noted to have antimicrobial properties. It is especially remarkable that APHIS dismisses the possibility that lactoferrin could confer disease resistance to rice or red rice, because two field tests have been approved by APHIS for wheat containing bovine lactoferrin specifically intended to mitigate fungal disease.[26] In

[d] A fitness-neutral transgene may also survive in red rice, but at lower numbers and with less likelihood.

addition, the gene is expressed at a very high level compared to most transgenes, approximately 4-5 mg/g. This is comparable to the levels expressed in milk, where it confers antimicrobial properties. For example, by comparison, Bt Cry proteins are typically expressed at microgram/g levels, typically several hundred to several thousand fold less than lactoferrin in rice.

APHIS repeats in its EA the same absence of rigor in determining the possible contribution of a disease resistance gene to fitness that was strongly criticized by the NAS in 2002, when APHIS failed to adequately analyze the possible consequences of virus-resistance gene flow to wild squash.[27] In that case, APHIS conducted an analysis of possible impacts of gene flow to wild squash, but the NAS found the analysis inadequate. In the case of lactoferrin, not even a cursory analysis has been conducted. APHIS merely mentions that no differences in disease susceptibility of transgenic rL rice were observed in previous field tests. However, such an analysis has little value if relevant pathogens were not present, or without discussion about how rigorous the observations were. Those previous field tests also were not conducted in Missouri, which has a somewhat different spectrum of rice diseases than states in other parts of the country (California or Hawai'i), where previous rL rice field trials have occurred. It is also possible that fungicides were applied to control fungal diseases, which would have made disease observations of little value.

As discussed in the NAS report, a proper analysis should consist of determining the susceptibility of red rice to rice seed diseases, prevalence of such diseases in Missouri, susceptibility of rL rice after inoculation with the pathogen, etc. There is no evidence that any such studies were conducted.

Because APHIS provides no explanation for its apparent lack of concern about possible competitive advantages conferred to red rice by lactoferrin, we can only speculate as to the lack of adequate analysis. One reason may be that lactoferrin is said to be expressed only in the seed of rice, rather than in the entire plant, as is the case with many other transgenes. Such reasoning would be misplaced. For example, the American Phytopathological Society lists several diseases of rice kernels, which may affect fecundity.[28] And fecundity, including survival of seed, is a major fitness component. For example, recent research found that a Bt Cry protein substantially increased survival of wild sunflower seed by protection against several lepidopteran (moth) pests, allowing more progeny to be produced.[29]

Seed often contains proteins that confer resistance to insects or diseases. These proteins include trypsin inhibitors, lectins, and alpha-amylases that inhibit insects, and pathogenesis related (PR) proteins such as chitinases and beta-glucanases that inhibit diseases.30 31 32 If these proteins

did not confer a significant advantage to the plant, they would likely not be produced because of the metabolic costs involved. However, although these endogenous pest protections are effective against many insects and diseases, the ones produced in rice seeds are not effective, by definition, against the pathogens and insects that harm rice. Therefore, it is clear that an antimicrobial protein like lactoferrin, that may confer resistance to diseases that are not controlled by endogenous antimicrobial substances in the rice grain, may confer a substantial competitive, selective, advantage to red rice.

Finally, the likelihood that gene flow will occur from continuing field tests or commercial production is greater than from a single field test. Again, Ventria has stated its intention of continuing these field tests indefinitely and expanding the acreage dramatically. Under such circumstances, contamination of food rice and red rice becomes more likely.

Horizontal Gene Transfer to Soil Bacteria from Lactoferrin Rice

APHIS does not consider horizontal gene transfer (HGT) to soil microorganisms to be a significant risk. APHIS does not evaluate important research or fitness concerns when evaluating the possibility of HGT. APHIS cites experimental data indicating that HGT occurs at exceedingly low frequencies, often undetectable, and sometimes estimated to occur at frequencies of less that 1 in 10^{-14} transformants.

However, as noted by Nielsen and colleagues in a review of horizontal gene transfer that acknowledges the often low frequencies of HGT, fitness must be considered, because (as with gene flow to wild relatives), genes may survive and spread if fitness is enhanced, even if initial transfer frequencies are extremely low.[33] APHIS does not consider possible fitness consequences of the transfer of rL to soil microbes. As with antibiotic resistance, lactoferrin may confer a fitness advantage to bacteria that acquire and produce it, by killing competitors.

Furthermore, recent work has demonstrated that when homologous (same sequence) DNA that is part of the transformation vector is also found in soil bacteria, HGT may occur at much higher frequencies than noted by APHIS.[34] The DNA in common between the plant-inserted vector and the bacteria can allow the transfer of adjacent DNA to the bacteria at relatively high frequencies in some cases. Importantly, although apparently not mentioned in the EA, is the presence in rL rice of multiple copies of the bacterial "backbone" of the transformation vector containing the common ColE1 "origin of replication," a kanamycin gene, and part of a lactose metabolism gene (*lacZ*), at several thousand base pairs per inserted copy.[35] The ColE1 sequences in particular

appear to be common among soil bacteria, and may thereby provide ready homology for HGT.[36] Both the nos terminator sequences and the hygromycin gene (*hpt*) also originated in bacteria. The nos terminator is found on the Ti plasmid of *Agrobacterium tumifaciens*, a common soil bacterium often found at 10^2 to 10^4 cells per gram of soil. Although it is a short DNA sequence, about 166 base pairs, it is long enough to allow HGT .[37]

The EA also ignores recent research that demonstrates HGT in humans.[38] Although not an example of HGT in the soil, it clearly demonstrates the real possibility of HGT. Artifacts were unlikely because the bacterial cells that were the source of the tested DNA were subcultured multiple times. Even though a gene commonly found in bacteria was involved in the HGT, the PCR that detected it used primers that bind to both the CP4-EPSPS gene and the plant viral promoter used to express the gene, and this chimeric gene is not found naturally in bacteria. Finally, although full copies of the target CP4-EPSPS gene were not recovered, this is likely due in part to the relatively small sample size. It is possible the CP4-EPSPS gene provided sufficient homology with the EPSPS likely found in gut bacteria to facilitate the HGT.

Additional factors determining the possibility of HGT include the natural transformation competence (natural ability for intact DNA uptake and chromosomal or plasmid integration) of soil bacteria that may contain the homologous DNA, and the possibility of expression of the gene. These factors are unknown and untested for rL to our knowledge. However, although the promoters for the rL gene are from rice, bacteria often transcribe (produce RNA) from "operons" of several genes linked to a single promoter. Therefore, it is possible, depending on the site of insertion, that a bacterial operon promoter could "read" through the plant promoter and express the gene in bacteria.

A final consideration is how the rL gene may confer a competitive advantage over soil microorganisms that may allow the propagation and spread of the gene. This is difficult to assess because although lactoferrin is antimicrobial, it may also kill the bacteria that acquire and express the gene. Furthermore, rL may need to be exported from the bacterial cell to confer an advantage, and there is no clear mechanism for this to occur. However, it may be possible that a portion of the bacterial population that normally lyse after they die could release enough lactoferrin to inhibit competing bacteria. It is also known that some bacteria are resistance to the antibiotic effects of lactoferrin, and although the known examples are human pathogens rather than soil bacteria, if similarly resistant species are found in soil and acquire the lactoferrin gene, they may develop a competitive advantage over some other microorganisms.[39]

In conclusion, the potential for HGT is likely much higher than APHIS claims, because APHIS did not consider the presence of homologous soil-bacterial DNA that may act as a "bridge" to transfer the rL gene or other plant DNA that would otherwise only be transferred at extremely low frequencies. On the other hand, the means for the rL gene to be expressed in a bacterial species and in a manner that could give that bacterial recipient a competitive advantage may only occur at very low frequencies. The overall risk from HGT is therefore difficult to determine, but APHIS has not adequately considered important factors in its EA.

Human Safety of Human-Derived Lactoferrin Produced in Rice

The EA discusses evidence to support the human safety of rL, and we consider some of these: 1) there are approximately 6 copies of the lactoferrin vector cassette in the GE rice, 2) rL is said to be denatured by cooking, 3) the physical and molecular properties of rL are "similar" to those of human lactoferrin. Under "3" are included the stability to digestion and post-translational modification of the protein. The data for all of these parameters, alone or in combination, suggest possible human health concerns.

Multiple copies of genes are often associated with gene silencing and also potentially with recombination or rearrangement. Although the genes are said to be stable, the data to support this statement are not presented. For example, in some of the earliest examples of gene silencing, pigment genes were silenced in some flowers under some environmental conditions but not others. This was easy to determine for flower color because the silencing was often observed as color sectors in individual flowers. Instability could easily be overlooked in rL rice if data from multiple seeds are combined. Therefore these data need to be carefully determined. Also, gene expression data should be gathered for a number of plants grown under different environments to determine if environment affects stability.

Another concern for multiple inserts as in Ventria's rL rice is the potential for substantial genomic rearrangements that have frequently been observed in plants transformed by biolistics. Gene insertions, especially complex insertions as with Ventria's rice, are often accompanied by thousands or tens of thousands of base pairs of scrambled genomic and vector DNA, which could affect the expression of rice genes, and which in turn could have human health or environmental consequences if ingested after contamination or transfer to weedy red rice. Several studies have demonstrated that these complex genomic DNA insertions are generally not detectable by Southern blots, the method used by Ventria to characterize the rL gene insertions.[40] Such

rearranged DNA can be responsible for unintended effects that may have either human health or environmental consequences, and have not been adequately analyzed by APHIS.

In addition, Ventria provides no data to show that expressed rL is identical in amino acid sequence to human lactoferrin. Gene sequences can be altered during the transformation process, and especially in complex transformants such as the lactoferrin rice. The transgene may be cut or sheared and re-spliced during the insertion process. If the resulting gene does not differ greatly in size from the original, for example if it differs by less than about 5 - 10% compared to the original gene, Southern blots or protein gel electrophoresis as apparently used by Ventria to examine rL will usually not detect these differences. Changes in sequence could effect properties such as digestive stability or immunogenicity. Therefore, rL protein sequences should be determined to ensure that possibly harmful sequence changes have not occurred.

Lactoferrin is said to be expressed in rice at levels comparable to those in human milk, or about 4 - 5 mg/g, and is therefore said to present no greater risk than human expressed lactoferrin. However, rL differs from human lactoferrin in that is is glycosylated differently. Plant glycosylation generally differs from human glycosylation in the absence of sialic acid residues on plant proteins, while containing xylosyl and fucosyl residues that have been associated with allergenicity.[41] Glycosylation of proteins in plants is associated with allergenicity, and differences in glycosylation between humans and other species has been associated with immunogenicity in humans. Therefore, the difference in glycosylation between the human and rL could have immunological implications and should not be assumed to be inconsequential.

Finally, the EA notes that the rL is denatured by cooking and that it is unstable in the *in vitro* digestive stability test. However, Ventria used commercial rice cookers to determine heat stability, and such cookers often have locking lids that allow some pressure to build in the cooking chamber, which cooks the rice more quickly than simple boiling. Therefore, Ventria may be using a cooking method that produces higher temperatures than boiling that may be used during home cooking. More importantly, denaturation is not synonymous with degradation. Denatured protein may in some cases be allergenic, as has been observed with the linear IgE epitopes in some cow's milk allergens or the pea allergen vicilin.[42] [43] [44] Therefore, simply demonstrating than the protein is denatured is not sufficient to ensure that it is not allergenic. The combination of altered glycosylation and lack of demonstrated degradation demonstrates that Ventria's allergenicity assessment is incomplete.

Ventria, in the Threatened and Endangered Species section citing Lonerdal, notes that human lactoferrin can be found intact in infant stool. This contradicts data summarized by Ventria that rL is very unstable in an *in vitro* gastric digestion assay that is typically used to assess potential

allergenicity. The usual explanation for the correlation observed between *in vitro* gastric digestive stability and food allergens is that stability allows the protein to reach immune tissue in the intestines, where it can cause allergy. Although CFS endorses the FAO/WHO protocols for gastric stability, which is similar to Thomas et al. used by Ventria, as an interim procedure, *in vivo* demonstration that the protein survives gastric digestion is likely more relevant. This is further evidence that the rL may be a potential allergen.

In summary, the EA does not sufficiently consider several types of data concerning rL that may have human health consequences. Therefore the EA's conclusion that rL is safe for humans is inadequately supported.

SCIENTIFIC CONCERNS WITH THE LYSOZYME EA (DOCKET 05-007-1)

In sum, the EA for lysozyme rice is inadequate for the following reasons:

- APHIS sets an inadequate 1/4 mile isolation distance to separate Ventria rice from food rice or the serious weedy relative, red rice. APHIS has underestimated gene flow from rice in the past (based on recently published research) and has seriously underestimated the ability of genetically engineered creeping bentgrass to contaminate surrounding wild relatives.[45] These and other cases are symptomatic of inadequate data on gene flow from crops. The 1/4 mile isolation distance accepted by APHIS is unlikely to ensure that gene flow will not occur.

- APHIS allows farm equipment used with Ventria's rice to be used with food rice after cleaning, despite the inability to ensure that such cleaning can remove all of Ventria's rice, which could then be transferred to food rice or contaminate the weedy rice relative, red rice. A recent report by experts on farm practices confirms that complete cleaning cannot be ensured.[46] Also, there is no requirement to clean farm machinery that was previously used on conventional rice farms prior to use on Ventria's rice, which could allow the contamination of Ventria fields by weedy red rice from a conventional farm. Because of its long seed dormancy, once in Ventria's fields, red rice could be very difficult to eradicate.

- Isolation distance must not be considered alone unless prevention of gene flow can be ensured. However, APHIS admits that minimal amounts of gene flow may occur with Ventria's rice. In conjunction with the amount of gene flow, such as by cross pollination,

the ability of the transgene to confer a competitive advantage to wild red rice must be carefully considered because enhanced competitive ability can facilitate permanent escape and spread of transgenes even when very low levels of gene flow occur. APHIS does not consider the substantial possibility that the lysozyme gene could confer a competitive advantage to wild red rice by reducing disease of red rice grain, despite the fact that lysozyme has been used in transgenic crops in previous field tests for the expressed purpose of reducing disease in those plants.

- APHIS does not consider important recent data, and thereby may seriously underestimate the possibility of horizontal gene transfer of the lysozyme gene to bacteria, and possible risk should that occur.

- Although APHIS is not qualified to determine the potential human safety risks from lysozyme rice, it has not adequately evaluated those risks.[e]

- APHIS apparently accepts evidence that lysozyme is broken down in the stomach, which would reduce its risk. However, Ventria determined that lysozyme survives for about 5 minutes in a gastric digestion assay, a similar level of stability to several known food allergens.

- APHIS apparently accepts that lysozyme is destroyed by cooking despite inadequate description of testing methods. It may be that the protein is only denatured, which does not assure that a protein will not be an allergen.

Environmental Safety of Human-Derived Lysozyme Produced in Rice

[e] APHIS has consulted with a representative from FDA to help in its human safety determination, however, FDA has not completed its own human safety assessment, so this assistance is unlikely to be adequate.

Gene Flow from Lysozyme Rice to the Wild Relative, Red Rice

A critical issue for the environmental safety of pharmaceutical crops is gene flow to wild crop relatives. As the EA notes, there appears to be one wild sexually compatible relative of cultivated rice in the U.S., annual red rice, *Oryza sativa* ssp *sativa*. Cultivated rice can sometimes become weedy as well, and can grow as a volunteer feral plant in rice growing areas. Red rice is a serious weed of rice, and is reported to be found in Missouri.[47] In fact, some sources consider it to be the most serious rice weed in Missouri.[48] Red rice often readily hybridizes with, and produces fertile progeny with, cultivated rice that are often more vigorous that either parent, which could enhance survival after gene flow at low frequencies.[49] Furthermore, APHIS likely underestimates the potential for gene flow because it considers rice to be primarily self-pollinating. Although true, this assessment ignores data by Langevin and colleagues who found hybridization of red rice with a cultivar of commercial rice at 52%, which indicates the potential of a high rate of outcrossing under at least some circumstances.[50]

If the rLY gene contaminates red rice, it could spread well beyond Ventria's field test sites. Furthermore, because red rice seed has extended dormancy, and can survive in the soil for at least 6 or 7 years, once red rice is contaminated, it would be very difficult to eradicate or prevent further spread.

The EA discloses several permit conditions intended to reduce gene flow to either red rice or cultivated rice. The primary means of reducing gene flow is to require a separation distance of at least 1/4 mile between the Ventria field test and either cultivated rice or red rice. Ventria further claims that there have been no rice fields within 10 miles of the field test site. This estimated separation is based on a personal communication of a University of Missouri extension specialist, with no indication of how this estimation was derived. As such, it should not be given much weight. In addition, as noted in the EA, it cannot be ensured that rice will not be grown closer to the field test site, so the 1/4 mile requirement must be the standard used for assessing possible gene flow. Furthermore, as noted in the EA, Ventria intends to expand its acreage over time, and to grow in Mississippi and Cape Girardeau counties as well as the current Scott County (EA, page 44, TES Worksheet). Northwest Missouri State University is collaborating with Ventria and its president, Dean Hubbard, has stated that eventually Ventria expects to increase cultivation of its pharmaceutical crops to 25,000 acres.[51] The latter acreage is 39 square miles, which clearly could make ensuring separation distances of greater than 1/4 mile improbable in these counties.

In addition, Ventria claims that the counties discussed in the field test application grow a total of 500 acres of rice (EA page 44, TES worksheet), all in Scott county. To the contrary, recent preliminary state agricultural data for 2003 for Scott county indicate 1200 acres of rice (data for Scott County alone was not previously recorded, but has been since 2003 due to increasing rice acreage). For the three other counties of Cape Girardeau, Mississippi, and Bolinger, 1400 acres were grown in 2003 (individual county data not available). Therefore, the three counties where Ventria plans on growing rLY rice, plus Bolinger, grew about 2,600 acres of rice in 2003, compared to about 300 acres in 1996, or an increase of almost 9 fold in 8 years.[52] [53] Taken together, these data indicate a trend of increasing food rice cultivation in these counties, which may encroach on Ventria's rice significantly if it continues, and already questions Ventria's isolation assumptions. In addition, surrounding counties grow large amounts of rice, for example, Stoddard County grows about 58,000 acres of rice. For all of these reasons, the 1/4 mile separation distance to cultivated and red rice must be used as the *de facto* separation distance.

On page 17 of the EA, APHIS claims that because rice pollen survives for only a few minutes (5 to 20 minutes is often cited), the 1/4 mile separation from food rice or red rice would reduce gene flow to "*de minimus*" levels. The EA does not define what is meant, in practice, by "*de minimus.*" Unfortunately, APHIS assurances about adequate confinement distance have been repeatedly proven wrong, primarily because data on low levels of gene flow that could be important when considering pharmaceutical rice and pollination of wild relatives have generally not been available. For example, APHIS previously required only a 10 foot separation distance for GE rice, based on seed purity standards for conventional breeding. APHIS then increased this distance to 100 or 200 feet for pharmaceutical rice. However, recent research indicates that gene flow may occur at 110 meters, between a quarter and a third of the 1/4 mile distance required separation distance.[54]

Furthermore, the maximum distance for rice pollen dispersal estimated in this research was based on only a few measurements in calm weather, and was extrapolated from those measurements using regression analysis, and where the largest pollen source was only 72 m^2, compared to the reported 204 acres for the Ventria field tests. This method of distance estimation is unlikely to adequately account for either the leptokurtic distribution often seen in pollen flow measurements (i.e., instead of pollen dispersal diminishing in a regular manner to zero over distance, low levels of pollen can be found at very long distances from the source), nor account for weather conditions that could substantially increase pollen dispersal, for example thunder storms, high winds, or air turbulence. The authors note that their suggestion of a 110 meter isolation distance is based on "normal weather conditions." They also note that: "Undoubtedly, a detailed study is

needed to find out what the influences of other weather parameters are on the distribution of rice pollen flow under normal field conditions."[55]

The approach of limited incremental increases in isolation distance compared to previous standards, as proposed by Ventria, does not address the fundamental lack of sufficient data on pollen dispersal. Because few experiments have been carried out that include a range of environmental conditions, current data cannot be considered to represent maximum distances for gene flow. The most recent example of APHIS underestimation of pollen dispersal was an experiment with GE creeping bentgrass where gene flow was found at the greatest distances measured - about 13 miles from a field test - which was miles farther than expected based on the much more limited confinement requirements that APHIS placed on that field test.[56] Similarly, although APHIS typically requires a 1/4 mile isolation distance for genetically engineered (non-pharmaceutical) canola, recent experiments show small amounts of gene flow at 3 kilometers.[57]

All of these distances should be considered preliminary, because various conditions may affect the results by affecting the rate of pollen travel and survival time. Factors such as wind speed, air turbulence, ambient temperature, cloud cover (UV light penetration), and humidity may all affect the distance over which gene flow may occur. In addition, the relative sizes of the pollen source (Ventria rice fields) and red rice stands is likewise important, because a large pollen source and relatively small number of accepting plants may increase gene flow.[58] [59] Although APHIS inappropriately does not reveal the size of the field test, press reports citing Scott Deeter of Ventria indicate that their field test applications (presumably for lactoferrin and lysozyme) are for about 200 acres.[60] It is therefore likely that the source of rLY pollen will likely be much greater than the size of red rice stands.

APHIS also does not consider observations of possible pollination of rice by insects. For example a small increase in outcrossing of rice has been observed when honey bees are present.[61] Clearly rice is primarily wind pollinated, but low levels of insect pollination need to be considered. Honey bees often forage over distances greater than 1/4 mile, sometime over several miles. Therefore, bee or other insect pollination could be important for gene flow to red rice.

Data on gene flow distances typically follow a skewed leptokurtic distribution. Graphs of gene flow distances usually show a very long distribution "tail," whereby low levels of gene flow occur over very long distances without falling to zero (at least, not within the distances observed

in the experiments).[62 f] The skewed distribution means that APHIS cannot simply examine most current, limited, data and extrapolate safe isolation distances.

Another possible means of gene flow could occur from farm machinery. In particular, machines with complex internal compartments not readily accessible from the outside may harbor small amounts of seed, even when cleaned. APHIS requires machinery used on biopharm crops to be "dedicated' to that crop. However, APHIS definition of "dedicated" allows the farm equipment to be used on conventional food crops after cleaning. In the case of Ventria, machinery such as planters, and especially harvesters, may be used with food rice after they are used for rLY rice after cleaning. Ventria remarks in the EA that it will clean its equipment according to APHIS standards prior to use with food rice. A recent review of biopharm crops by experts knowledgeable about farm equipment concluded that such equipment cannot usually be cleaned carefully enough to ensure that biopharm seed will not be co-mingled with food seed.[63] If that occurs, such seed could either end up in the food supply, or in seed used for planting future rice crops. In the latter case, rLY rice would be planted unsuspectingly, and could thereby be perpetuated in the food supply, or contaminate red rice.

There is also no requirement to clean farm equipment *prior* to use on Ventria's fields. This allows the possibility for red rice to be introduced to Ventria's fields from other rice fields. Red rice growing in Ventria's fields would be cross pollinated by Ventria's rice, transferring the rLY gene. And because of its very long seed dormancy period, such rLY red rice could easily evade volunteer rice control protocols, and emerge years after a particular field is no longer used by Ventria. And rLY red rice, once in Ventria's fields, would not only be extremely difficult or impractical to eradicate, it may eventually escape in machinery or by other means.

Also morphological convergence between red rice and cultivated rice has been reported in some cases. This makes the detection of red rice growing among cultivated rice more difficult.[64 65] This could make assessment of the proximity of red rice more difficult or inaccurate, and increase the possibility that machinery, such as combines, could unwittingly harvest red rice prior to use in Ventria's fields.

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[f] Recent experiments by Rieger et al. show a more random distribution, but this may have occurred due to the combination of data from several sites. In any case, those data also demonstrate higher than expected gene flow at longer distances than would otherwise be expected.

Finally, APHIS does not adequately consider the movement of seed by birds, mammals, or water. As discussed in the following section, low frequency events may be sufficient to cause permanent escape of the rLY gene to red rice if the fitness of the wild species is enhanced. Therefore, the evaluation by APHIS on dispersal by birds, mammals or flooding is inadequate because it does not consider potential impact of low levels of initial gene flow. In particular, due to the relatively short required isolation distance of 1/4 mile, incidental transfer, such as rice grains temporarily lodged in the feathers of waterfowl or the fur of mammals, could occur. Similarly, the screens and grates described by APHIS to prevent transfer by water can become clogged or overflow. Although the EA (page 16) claims that the nearest body of water is 4 miles from the field test site, elsewhere, page 44 of the EA notes that "The nearest non-agricultural water is more than one mile from the field." Such a location may allow the growth of volunteer rice for long enough to transfer to red rice.

APHIS's unsupported claim that the lack of observer volunteer rice at the edges of rice fields is "strong evidence" that transfer of rice does not occur is not credible without any discussion about how frequently anyone looks for such volunteer rice.

In summary, several routes of possible gene flow to red rice or cultivated food rice exist that have not been adequately considered by APHIS. The assertion that gene flow would occur only at *de minimus* levels cannot be considered credible without better data, and absent consideration of the possible fitness consequences of gene flow to red rice, considered in the following section.

Failure to Evaluate the Potential Contribution of the Lysozyme Gene to the Fitness and Weediness of Red Rice

Isolation distance must not be considered separately from the ability of the transgene(s) to survive after gene flow occurs. Low levels of gene flow can lead to the permanent escape of the transgene if it confers a selective advantage to a wild relative, in this case, red rice.[g] Put differently, a gene that confers a fitness advantage may persist and spread in a wild relative where a gene that is disadvantageous, may not survive or spread.[66] Therefore, fitness, or the competitive advantage conferred to a wild relative by a transgene, is of fundamental importance in determining the possibility of transgene escape unless the absence of gene flow can be ensured. As has been noted, APHIS does not ensure that gene flow will not occur, but only that

[g] A fitness-neutral transgene may also survive in red rice, but at lower numbers and with less likelihood.

it may occur at *de minimus* levels. However, without properly considering the possible fitness conferred to red rice by the lysozyme gene, APHIS cannot properly determine what a *de minimus* level of gene flow may be.

Unfortunately, APHIS does not adequately consider the ability of the lysozyme gene to persist and spread if it escapes to its wild relative, red rice. In fact, APHIS apparently does not even consider the possibility that the lysozyme gene may confer a selective advantage to red rice. APHIS merely states that lysozyme (or hygromycin) **"**is [not] expected to alter the susceptibility of the transgenic rice plants to disease or insect damage." (EA, page 15) This a fundamental shortcoming of the APHIS argument for the safety of lysozyme rice, and in combination with inadequate analysis of gene flow and the 1/4 mile isolation distance, constitutes a major failing of the APHIS risk assessment.

In particular, APHIS does not assess the possibility that the well known antimicrobial properties of lysozyme may confer a competitive advantage to red rice. Resistance to diseases or insects are properties that the National Academy of Sciences (NAS) and others consider to potentially confer a competitive advantage to wild crop relatives, by increasing survival compared to plants that do not possess the gene.[67] The NAS stated that: "Generally, if an allele confers a fitness advantage - once introduced into a population - it is expected to increase in frequency, even if it is introduced only once."

One of the primary commercial applications of lysozyme is as an anti-microbial. APHIS acknowledges in the EA that lysozyme has been noted to have antimicrobial properties. It is especially remarkable that APHIS dismisses the possibility that lysozyme could confer disease resistance to rice or red rice, because nine field tests have been approved by APHIS for four crops containing lysozyme specifically intended to mitigate four bacterial plant diseases.[68] In addition, the gene is expressed at a very high level compared to most transgenes, approximately 5 mg/g. This is about 20 fold higher than found in human milk (about 0.250 mg/ml, according to Ventria, where 1 ml ~ 1 g) where it confers antimicrobial properties. For example, by comparison, Bt Cry proteins are typically expressed at microgram/g levels, typically several hundred to several thousand fold less than for lysozyme in rice.

APHIS repeats in its EA the same absence of rigor in determining the possible contribution of a disease resistance gene to fitness that was strongly criticized by the NAS in 2002, when APHIS failed to adequately analyze the possible consequences of virus-resistance gene flow to wild squash.[69] In that case, APHIS conducted an analysis of possible impacts of gene flow to wild squash, but the NAS found the analysis inadequate. In the case of lysozyme, not even a cursory

analysis has been conducted. APHIS merely mentions that no differences in disease susceptibility of transgenic rLY rice were observed in previous field tests. However, such an analysis has little value if relevant pathogens were not present, or without discussion about how rigorous the observations were. Those previous field tests also were not conducted in Missouri, which has a somewhat different spectrum of rice diseases than states in other parts of the country (California or Hawai'i), where previous rLY field trials have taken place. It is also possible that pesticides were applied to reduce bacterial diseases, which would have made disease observations of little value.

As discussed in the NAS report, a proper analysis should consist of determining the susceptibility of red rice to rice seed diseases, prevalence of such diseases in Missouri, susceptibility of rLY rice after inoculation with the pathogen, etc. There is no evidence that any such studies were conducted.

Because APHIS provides no explanation for its apparent lack of concern about possible competitive advantages conferred to red rice by lysozyme, we can only speculate as to the lack of adequate analysis. One reason may be that lysozyme is said to be expressed only in the seed of rice, rather than in the entire plant, as is the case with many other transgenes. Such reasoning would be misplaced. For example, the American Phytopathological Society lists several diseases of rice kernels, which may affect fecundity.[70] And fecundity, including survival of seed, is a major fitness component. For example, recent research found that a Bt Cry protein substantially increased survival of wild sunflower seed by protection against several lepidopteran (moth) pests, allowing more progeny to be produced.[71]

Seed often contains proteins that confer resistance to insects or diseases. These proteins include trypsin inhibitors, lectins, and alpha-amylases that inhibit insects, and pathogenesis related (PR) proteins such as chitinases and beta-glucanases that inhibit diseases.[72] [73] [74] If these proteins did not confer a significant advantage to the plant, they would likely not be produced because of the metabolic costs involved. However, although these endogenous pest protections are effective against many insects and diseases, the ones produced in rice seeds are not effective, by definition, against the pathogens and insects that harm rice. Therefore, it is clear that an antimicrobial protein like lysozyme, that may confer resistance to diseases that are not controlled by endogenous antimicrobial substances in the rice grain, may confer a substantial competitive, selective, advantage to red rice.

Finally, the likelihood that gene flow will occur from a single field test is lower than for continuing field tests or commercial production. Ventria has expressed the intention of

continuing these field tests indefinitely and expanding the acreage dramatically. Under such circumstances, contamination of food rice and red rice becomes more likely.

Horizontal Gene Transfer to Soil Bacteria from Lysozyme Rice

APHIS does not consider horizontal gene transfer (HGT) to soil microorganisms to be a significant risk. APHIS does not evaluate important research or fitness concerns when evaluating the possibility of HGT. APHIS cites experimental data indicating that HGT occurs at exceedingly low frequencies, often undetectable, and sometimes estimated to occur at frequencies of less that 1 in 10^{-14} transformants.

However, as noted by Nielsen and colleagues in a review of horizontal gene transfer that acknowledges the often low frequencies of HGT, fitness must be considered, because (as with gene flow to wild relatives), genes may survive and spread if fitness is enhanced, even if initial transfer frequencies are extremely low.[75] APHIS does not consider possible fitness consequences of the transfer of rLY to soil microbes. As with antibiotic resistance, lysozyme may confer a fitness advantage to bacteria that acquire and produce it, by killing competitors.

Furthermore, recent work has demonstrated that when homologous (same sequence) DNA that is part of the transformation vector is also found in soil bacteria, HGT may occur at much higher frequencies than noted by APHIS.[76] The DNA in common between the plant-inserted vector and the bacteria can allow the transfer of adjacent DNA to the bacteria at relatively high frequencies in some cases. rLY rice may have approximately two copies of the bacterial "backbone" of the transformation vector, which may contain the common ColE1 "origin of replication," and other sequences of bacterial origin that may be several thousand base pairs per inserted copy.[77] The ColE1 sequences in particular appear to be common among soil bacteria, and may thereby provide ready homology for HGT.[78] Both the nos terminator sequences and the hygromycin gene (*hpt*) also originated in bacteria. The nos terminator is found on the Ti plasmid of *Agrobacterium tumifaciens*, a common soil bacterium often found at 10^2 to 10^4 cells per gram of soil. Although it is a short DNA sequence, about 166 base pairs, it is long enough to allow HGT .[79]

The EA also ignores recent research that demonstrates HGT in humans.[80] Although not an example of HGT in the soil, it clearly demonstrates the real possibility of HGT. Artifacts were unlikely because the bacterial cells that were the source of the tested DNA were subcultured multiple times. Even though a gene commonly found in bacteria was involved in the HGT, the

PCR that detected it used primers that bind to both the CP4-EPSPS gene and the plant viral promoter used to express the gene, and this chimeric gene is not found naturally in bacteria. Finally, although full copies of the target CP4-EPSPS gene were not recovered, this is likely due in part to the relatively small sample size. It is possible the CP4-EPSPS gene provided sufficient homology with the EPSPS likely found in gut bacteria to facilitate the HGT.

Additional factors determining the possibility of HGT include the natural transformation competence (natural ability for intact DNA uptake and chromosomal or plasmid integration) of soil bacteria that may contain the homologous DNA, and the possibility of expression of the gene. These factors are unknown and untested for the rLY gene to our knowledge. However, although the promoters for the rLY gene are from rice, bacteria often transcribe (produce RNA) from "operons" of several genes linked to a single promoter. Therefore, it is possible, depending on the site of insertion, that a bacterial operon promoter could "read" through the plant promoter and express the gene in bacteria.

A final consideration is how the rLY gene may confer a competitive advantage over soil microorganisms that may allow the propagation and spread of the gene. This is difficult to assess because although lysozyme is antimicrobial, it may also kill the bacteria that acquire and express the gene. Furthermore, it is likely that rLY would have to be exported from the bacterial cell to function, and there is no clear mechanism for this to occur. However, it may be possible that a portion of the bacterial population that normally lyse after they die could release enough lysozyme to inhibit competing bacteria. It is also possible that some soil bacteria are resistance to the antibiotic effects of lysozyme, and if they acquire the lysozyme gene, they may develop a competitive advantage over some other microorganisms.

In conclusion, the potential for HGT is likely much higher than APHIS claims, because APHIS did not consider the presence of homologous soil-bacterial DNA that may act as a "bridge" to transfer the rLY gene or other plant DNA that would otherwise only be transferred at extremely low frequencies. On the other hand, the means for the rLY gene to be expressed in a bacterial species and in a manner that could give that bacterial recipient a competitive advantage may only occur at very low frequencies. The overall risk from HGT is therefore difficult to determine, but APHIS has not adequately considered important factors in its EA.

Human Safety of Human-Derived Lysozyme Produced in Rice

The EA discusses evidence to support the human safety of rLY, and we consider some of these: 1) there are approximately two copies of the lysozyme vector cassette in the GE rice, 2) rLY is said to be destroyed by cooking, 3) the physical and molecular properties of rLY are "similar" to those of human lysozyme. Under "3" are included the stability to digestion and post-translational modification of the protein. The data for all of these parameters, alone or in combination, suggest possible human health concerns.

Multiple copies of genes are often associated with gene silencing and also potentially with recombination or rearrangement. Although the genes are said to be stable, the data to support this statement are not presented. For example, in some of the earliest examples of gene silencing, pigment genes were silenced in some flowers under some environmental conditions but not others. This was easy to determine for flower color because the silencing was often observed as color sectors in individual flowers. Instability could easily be overlooked in rLY rice if data from multiple seeds are combined. Therefore these data need to be carefully determined. Also, gene expression data should be gathered for a number of plants grown under different environments to determine if environment affects stability.

Another concern for multiple inserts as in Ventria's rLY rice is the potential for substantial genomic rearrangements that have frequently been observed in plants transformed by biolistics. Gene insertions, especially complex insertions as with Ventria's rice, are often accompanied by thousands or tens of thousands of base pairs of scrambled genomic and vector DNA, which could affect the expression of rice genes, and which in turn could have human health or environmental consequences if ingested after contamination or transfer to weedy red rice. Several studies have demonstrated that these complex genomic DNA insertions are generally not detectable by Southern blots, the method used by Ventria to characterize the rLY gene insertions.[81] Such rearranged DNA can be responsible for unintended effects that may have either human health or environmental consequences, and have not been adequately analyzed by APHIS.

In addition, Ventria provides no data to show that expressed rLY is identical in amino acid sequence to human lysozyme. Gene sequences can be altered during the transformation process, and especially in complex transformants such as the lysozyme rice. The transgene may be cut or sheared and re-spliced during the insertion process. If the resulting gene does not differ greatly in size from the original, for example if it differs by less than about 5 - 10% compared to the original gene, Southern blots or protein gel electrophoresis as apparently used by Ventria to

examine rLY will usually not detect these differences. Changes in sequence could effect properties such as digestive stability or immunogenicity. Therefore, rLY protein sequences should be determined to ensure that possibly harmful sequence changes have not occurred.

The EA notes that the rLY is destroyed by cooking and that it is unstable in the *in vitro* digestive stability test. However, Ventria used commercial rice cookers to determine heat stability, and such cookers often have locking lids that allow some pressure to build in the cooking chamber, which cooks the rice more quickly than simple boiling. Therefore, Ventria may be using a cooking method that produces higher temperatures than boiling that may be used during home cooking. Ventria also claims that rLY cannot be detected by Western (immuno-) blot after cooking. However, Ventria does not disclose the source of antibodies used to detect rLY in the Western blots. If the antibodies were raised to "native" lysozyme, they may primarily bind to conformational epitopes and may not detect linear epitopes of denatured lysozyme. Therefore, without knowing whether the antibodies used can bind denatured rLY, it cannot be assumed that lack of detection on a Western blot reveals degradation as opposed to denaturation. Denaturation is not synonymous with degradation. Denatured protein may in some cases be allergenic, as has been observed with the linear IgE epitopes in some cow's milk allergens or the pea allergen vicilin.[82] [83] [84] Therefore, simply demonstrating than the protein is denatured is not sufficient to ensure that it is not allergenic. APHIS should require experiments that clearly determine whether rLY is degraded or denatured, and if only denatured, what its allergenic potential may be.

Ventria asserts that rLY is undetectable after five minutes in an *in vitro* gastric digestion assay that is typically used to assess potential allergenicity. Many food allergens are somewhat or very stable in this assay while most non-allergenic food proteins are very unstable. The usual explanation for the correlation observed between *in vitro* gastric digestive stability and food allergens is that stability allows the protein to reach immune tissue in the intestines, where it can cause allergy. Although Ventria cites this data in support of non-allergenicity, several important food allergens have been stable for only between about two and 15 minutes in this assay.[85] Therefore, stability of five minutes, an intermediate value, may indicate potential allergenicity.

In summary, the EA does not sufficiently consider several types of data concerning rLY that may have human health consequences. Therefore the EA's conclusion that rLY is safe for humans is inadequately supported.

CONCLUSIONS FOR BOTH EA'S

In sum, the lactoferrin and lysozyme rice EAs are inadequate under NEPA and should be revised to address the issues we raise herein. Then APHIS should put them out again for further public comment before any decision is made on the Ventria permit applications. Alternatively, full environmental impact statements should be prepared.

We look forward to your written responses to each of these comments individually and to further participating in the NEPA compliance process. For further information on these comments, please contact either of us listed below.

Sincerely,

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Peter T. Jenkins, Attorney/Policy Analyst
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Enclosures (incorporated by reference)

Endnotes

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77. Ventria does not provide an adequate description of all DNA inserted into the rLY plants, but the data submission for lactoferrin rice to FDA by Ventria discloses the bacterial vector backbone and other sequences. It is possible that the same vector was used to make rLY plants, because companies will often use such successful vectors to make many different GE crops, and because the particular bacterial backbone, from the pUC19 plasmid, is perhaps the most commonly used of all for such purposes. For lactoferrin, see: Ventria Bioscience, "Safety, Compositional and Nutritional Aspects of LF164 Rice Transformational Event, voluntary data submission to FDA, Center for Food Safety and Applied Nutrition, designated BNF 082, Nov. 24, 2003

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