NEPA Decision Summary for Permit 12-334-118r

Kentucky Bioprocessing, LLC (KTB) has requested a permit for a small-scale confined field release of genetically engineered tobacco mosaic virus (TMV) that will be used to inoculate *Nicotiana* species at one site in Daviess County, Kentucky.

Based on a review of Permit 12-334-118r, the following determinations were made:

1. The gene construct proposed for the confined field release is expected to result in the production of bovine lung aprotinin in tobacco. This is accomplished using a tobacco mosaic virus-based expression vector in which the gene of interest, aprotinin, is under the control of the TMV (U1) coat protein subgenomic promoter. This construct containing the aprotinin gene has been previously used under permits 12-032-107r , 11-041-107r , 10-011-101r , 09-090-101r, 08-051-101r and 07-131-101r. The gene construct contains sequences derived from a plant pest (TMV). The gene encoding the recombinant protein, aprotinin, does not have any inherent plant pest characteristics and is not likely to pose a plant pest risk.

2. The recombinant aprotinin gene is incorporated into the viral genome. Tobacco plants used in the field trial are not transgenic. TMV has been the subject of extensive research and its epidemiology is very well understood. The virus enters the cell and replicates, then moves from cell to cell via plasmodesmata. Plant symptoms from TMV usually take the form of molting or mosaic patterns in the leaves, necrosis, stunting, leaf curling, or yellowing of tissues. One of the key reasons why TMV is used for the expression of recombinant proteins is because it is one of the most studied viruses, is only spread by mechanical transmission and it is not transmitted by insect vectors. Proper sanitation of field equipment will prevent the spread of TMV. APHIS has previously prepared four EAs (91-007-08r, 94081-01r, 95041-01r, 96-051-01r) for the environmental release of transgenic TMV.

3. Genetically modified TMV (as an expression vector) is very efficient at producing high levels of heterologous proteins in plants infected with the modified virus, but only for a short predictable time. The inserted gene is recognized as nonessential by the TMV, and is deleted from the viral genome over time; the virus only preserves the sequences needed for optimal replication and movement. Furthermore, the modified TMV has a lower replicative capability than the wild type virus. A comparative challenge study in tobacco, between recombinant and wild-type TMV, indicated that the wild-type virus was more competitive, vigorous and pathogenic than the modified virus. Therefore, the lower replicative capability of the genetically modified TMV, along with the high frequency of excision of the inserted gene, and lack of transmission by an insect vector, reduces the likelihood that the gene of interest will be spread to other susceptible plants.

4. Tobacco seedlings will be transplanted to the field location using a mechanical transplanter and the plants will be prepped for inoculation and sprayed with genetically engineered TMV. Once the plants are inoculated, they will remain in the field for 10-28 days. On average, the plants will be harvested within two weeks of inoculation. The

plants will be allowed to flower; because TMV is not seed-borne or transmitted through pollen, there is no potential for dissemination of the virus. Bulk lots of infected plant material will be transported to KBP extraction facility either using a dedicated mechanical harvester attached to a leak-proof sealed wagon or sealed in a plastic bag, and placed in a cooler for transport from the field trial to the extraction facility. All transport of infected material to and from the field will be performed under requirements of 7CFR § 340.8.

5. The intent of this field release is to test the level of recombinant aprotinin expression in *Nicotiana* species, extract and purify aprotinin from the plants for use a reagent component in cell culture and as a research grade protease inhibitor. An Environmental Assessment was prepared by APHIS for the field release of corn expressing aprotinin, http://www.aphis.usda.gov/brs/aphisdocs/04_12101r_ea.pdf. Aprotinin is naturally produced in bovine lung tissue. It is a naturally occurring Kunitz-type serine protease inhibitor consisting of 58 amino acid residues in a single chain, cross-linked by 3 disulphide bridges, and with a total molecular weight of 6,512 daltons. The amino acid sequence of recombinant aprotinin is identical to naturally occurring aprotinin; the properties of both molecules are essentially the same as confirmed using a number of activity and characterization assays. A BLAST search was performed using rAprotinin sequence, and the sequence was identical to bovine aprotinin. Aprotinin has been studied in humans since the early 1960s and has a very good safety profile. Because it is present in bovine tissue, most individuals who consume meat have been exposed to this protein due to oral consumption of beef. Similarity to known allergens was determined by using a FARRP Allergen Database (not all proteins identified in FARRP are confirmed clinical allergens) and NCBI BLAST search. The results indicated that the aprotinin protein and signal peptide shows some similarity to 9 sequences in the databases. However, none of the identified sequences showed 35% or greater homology over the 80 amino acid (aa) window, and no hit showed 100% homology in any 6 aa window. Thus, according to criteria established by the WHO, recombinant aprotinin is not considered cross-reactive with any known or putative allergen. Naturally occurring aprotinin is used in humans as an FDA-approved product (Bayer Pharmaceuticals Traylol), and has a notable safety record. Given that the recombinant aprotinin has the identical amino acid sequence as the native aprotinin, as well as its lack of similarity to known proteins and allergens, it is unlikely that the recombinant aprotinin would display either toxic or allergenic properties.

6. The proposed field site is located in rural Kentucky in Daviess County. The test site is isolated by 20 foot fallow zone to reduce physical contact and minimize unintended transmission of the virus. The field site will be monitored at least three times between inoculation and harvest at each planting for potential weed hosts; any plants showing TMV-like symptoms will be harvested and assayed for the presence in TMV. On completion of the field testing all plant material will be chopped up and root systems destroyed with a tractor-mounted disk harrow. The test plots will be redisked to ensure destruction of all TMV material. Vigorous weed control by herbicide treatment or hand rouging is used in the field test plot to eliminate any TMV compatible weeds in the area.

7. Equipment and employee protective wear will be cleaned before leaving the field trial site. Tools and equipment used in the release site will be treated with a bleach solution to inactivate the recombinant TMV, and rinsed with fresh water at the field site after each use before storage or transport. Employees entering and working in the field during inoculation and harvest will wear disposable gloves that will be autoclaved and discarded. Following inoculation, footwear (boots) will be cleaned with bleach to inactivate the virus.

8. The gene product used in this field trial is not known to be toxic to humans by oral or dermal exposure and has been used previously in clinical trials (see "Aprotinin Biology Information" attached to the permit file). Also, there is no significant absorption of aprotinin in the blood stream of vertebrates. Insecticidal activity of aprotinin toward European corn borer (*Ostrinia nubilalis*) and corn rootworm larvae (*Diabrotica undecimpunctata howardii*) has been documented at higher concentrations of aprotinin than present recombinant TMV-infected plants. However, European corn borer and corn rootworm larvae are non-TES organisms and not known to feed on *Nicotiana* species. During the 2010 and 2011 field trial, the field site was monitored for honey bee mortality and no honey bee mortality was observed.

9. According to the Fish and Wildlife Service (http://ecos.fws.gov/tess_public/countySearch!speciesByCountyReport.action?fips=21059 ; accessed on 1/3/2013) there is one listed or proposed federally listed threatened and endangered animal in Daviess County, Kentucky (Indiana bat (*Myotis sodalist*). The only known animal that forages on tobacco is skunk. Indiana bats hibernate during winter in caves or occasionally in abandoned mines. During summer they roost under the peeling bark of dead and dying trees. Indiana bats eat a variety of flying insects found along rivers or lakes and in uplands. In the unlikely event of accidental consumption, the pharmaceutical protein produced during this field trial is non-toxic to mammals and is not expected to harm animals feeding on this plant. Therefore, this field trial should have no effect on threatened or endangered species.

10. There is no designated critical habitat or proposed designated critical habitat found in Daviess County, KY (http://crithab.fws.gov/, accessed 1/3/2013).

11. Regulated materials in this field trial are not intended for food and/or feed. Any use of these products for food or feed must be in compliance with the guidelines published in the Federal Register by the United States Food and Drug Administration (57 FR 22984, May 29, 1992).

For the above reasons, and those documented on the NEPA/ESA decision document, APHIS has determined that permit application 12-334-118r involves confined field trials of genetically engineered organisms or products that do NOT involve a new species or organism or novel modification that raises new issues. APHIS has determined that the actions authorized under this permit do NOT have the potential to significantly affect the quality of the human environment. Therefore, approval of this permit is properly categorically excluded from the need to prepare an

EA (or EIS) pursuant to 7 CFR § 372.5(d) and none of the exceptions to this categorical exclusion apply.