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NEPA Decision Summary for Permit #09-090-101r

Kentucky Bioprocessing, LLC has requested a permit for a confined field release of genetically engineered Tobacco mosaic virus (TMV) that will be used to inoculate [] acres of tobacco plants (*Nicotiana excelsiana*) at a site in Daviess County, Kentucky.

Based on a review of Permit #09-090-101r, 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 08-051-101r, 07-131-101r, 04-309-02r, 04-044-02r, 04-040-01r, 03-147-01r, 01-187-01r and 01-023-03r. The gene construct contains sequences derived from a plant pest (tobacco mosaic virus). This construct has been safely used before in field releases. 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. For more information on aprotinin, see the Environmental Assessment prepared by APHIS http://www.aphis.usda.gov/brs/aphisdocs/04_12101r_ea.pdf .

2. 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, and it is only spread by mechanical transmission; it is not transmitted by insect vectors. Proper sanitation of field equipment will prevent the spread of 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, 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. The recombinant aprotinin gene is incorporated into the viral genome. Tobacco plants used in the field trial are not transgenic.

5. Tobacco seedlings will be transplanted to the field location using a mechanically transplanter and the plants will be prepped for inoculation and sprayed with genetically engineered TMV. 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.

6. The intent of this field release is to test the level of recombinant aprotinin expression in different *Nicotiana excelsiana* lines, extract and purify aprotinin from the plants, and test the effects of agronomic management practices on the yield of TMV-produced aprotinin in tobacco plants. 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.

7. The proposed field site is located in rural Kentucky in Daviess County. The site is surrounded on two sides by corn, a road on one side and a driveway on the fourth side. Corn, is not considered a host plant for TMV and therefore is not at risk of infection nor would recombinant aprotinin be produced in these plants. The test site is isolated by at least 300 feet from fields used for commercial tobacco production. The field site is surrounded by a fifty-foot wide fallow zone to reduce physical contact and minimize unintended transmission of the virus within the field site. The field site will be monitored at least three times between inoculation and harvest at each planting for potential weed hosts, and any such plants showing TMV-like symptoms will be harvested for analysis. Any weeds with TMV-like symptoms, along with random samplings of weeds near the inoculated tobacco, will be analyzed for TMV and the inserted gene using a local lesion

assay and/or PCR. Upon 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. The field will be monitored monthly for 12 months for volunteers. All volunteer tobacco plants found will be removed manually or using an appropriate herbicide. 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.

8. Employees entering and working in the field will wear disposable gloves and protective clothing (boots). Protective wear that comes into contact with the TMV such as gloves or boots will be autoclaved and discarded, or cleaned with bleach to inactivate the virus. Tools and equipment used in TMV fields will be treated with a sodium hypochlorite solution to inactivate the recombinant TMV, and rinsed with fresh water at the field site after each use before storage or transport.

9. According to the Fish and Wildlife Service (http://ecos.fws.gov/tess_public/StateListingAndOccurrence.do?state=KY, accessed 04/20/09) there are 33 federally listed threatened and endangered animals and 8 threatened and endangered plant species in the state of Kentucky. Of the 33 listed animals, none are known to use tobacco as a food plant. None of the threatened and endangered species forage on tobacco pants. The only known animal that forages on tobacco is skunk. In the unlikely event of accidental consumption, the pharmaceutical protein produced during this field trial is non-toxic and is not expected to harm animals feeding on this plant. Therefore, these field trials should have no effect on threatened or endangered species.

10. According to http://crithab.fws.gov/, accessed 04/20/09, there is no designated critical habitat or proposed designated critical habitat found in this county.

11. The gene product used in this field trial is not known to be toxic by oral or dermal exposure. Also there is no significant absorption of aprotinin in the blood stream of vertebrates. Based on the above, these field trials should not harm or have adverse or other significant effects on threatened or endangered species either by direct or indirect exposure.

12. 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 09-090-101r 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., and none of the exceptions to this categorical exclusion apply.

Signed: _____/s/_____
Michael T. Watson, Ph.D.
Branch Chief, Plant Pests and Protectants
Biotechnology Regulatory Services

Date:___4/29/2009_____
SR_/s/_