NEPA Decision Summary for Permit # 09-344-105r

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

Based on a review of Permit #09-344-105r, the following determinations were made:

1. The gene constructs proposed for the confined field releases are expected to result in the production of two recombinant proteins in tobacco. This is accomplished using tobacco mosaic virus-based expression vectors in which the gene of interest is under the control of the TMV (U1) coat protein subgenomic promoter. The expression vector has been previously used to produce aprotinin. This construct containing the aprotinin gene has been previously used under permits 09-090-101r, 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. For more information on aprotinin, see the Environmental Assessment prepared by APHIS http://www.aphis.usda.gov/brs/aphisdocs/04_12101r_ea.pdf). The gene constructs contains sequences derived from a plant pest (tobacco mosaic virus). This construct has been safely used before in field releases. The genes encoding the recombinant proteins do not have any inherent plant pest characteristics and are not likely to pose plant pest risks.

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 genes of interest will be spread to other susceptible plants.

4. The recombinant protein genes are 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 mechanical transplanter and the plants will be prepped for inoculation and sprayed with genetically engineered TMV. There will be multiple plantings (less than 5). 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 the 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 protein expression in different *Nicotiana excelsiana* lines, extract and purify the proteins from the plants, and to test the effects of agronomic management practices on the yield of TMV-produced recombinant proteins in tobacco plants. Recombinant protein 1, claimed as CBI, a lectin-like protein, has antiviral properties, is 121aa in length, and has a molecular weight of ~ 12.7kDa. It is naturally produced by algae. There are a few differences in amino acids and glycosylation between the recombinant and native protein; the recombinant protein is N-acetylated, and amino acid 31 is replaced with alanine (this is because this amino acid could not be identified in the original protein). However, these changes result in no discernable differences in the activity of the recombinant protein in comparison with the native protein. Allergy and toxicology screens were performed using the FARRP Allergen Database and NCBI BLAST searches; the results indicated that the recombinant protein with its signal peptide showed 18 hits. However, none of these hits show 35% identity or greater over an 80 amino acid window and no hit showed 100% homology in any 6 amino acid window. Thus, according to the criteria established by the WHO, recombinant protein 1 is not considered cross-reactive with any known or putative allergen. Recombinant protein 2 is an inactive precursor of a serine protease and is derived from a porcine source. It is activated by removal of an N-terminal hexapeptide. The protein is claimed as CBI, but is a widely occurring protein produced by numerous animals, fungi, and bacteria. The production of the protein by numerous animals (mammals, fish, birds, frogs, insects, mites, and jellyfish), bacteria (Streptomyces erythreaus), and fungi (Fusarium oxysporum, Metarhizium anisopliae) is chronicled in the MEROPS Peptidase; it has 6 disulfide bridges. There are no differences in amino acids between the recombinant and native protein. Therefore, no biological properties are expected to change. Allergy and toxicology screens were performed using the FARRP Allergen Database and NCBI BLAST searches; the results indicated that the recombinant protein with its signal peptide yielded 10 hits when compared with the FARRP Allergen Database showing 35% identity or greater over an 80 amino acid window and 7 hits showing 100% homology in any 8 amino acid window. All of the hits were to known dust mite allergens (European House dust mite; Dermatophagoides pteronyssinus, American House dust mite; Dermatophagoides farinae, or Storage mite; Blomia tropicallis). BLAST searches showed no homology to known toxins or allergens. Given the information on the two recombinant proteins, it is unlikely that they would display either toxic or allergenic properties. Since tobacco is not a food or feed crop, data on foraging animals is not available. Applicant intends to test the toxicological consequences

on foraging animals and insects in the current year permit along with non-target organism monitoring if needed.

7. The proposed field site is located in rural Kentucky in Daviess County. The test site is isolated by at least 100 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/pub/stateListingIndividual.jsp?state=KY&status=listed; Accessed on February 22nd, 2010) there are 34 federally listed threatened and endangered animals and 8 threatened and endangered plant species in the state of Kentucky. Of the 34 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 proteins produced during these field trials are non-toxic and are not expected to harm animals feeding on this plant. Therefore, these field trials should have no effect on threatened or endangered species.

10. There is no designated critical habitat or proposed designated critical habitat found in this county.

11. The gene products used in this field trial are not known to be toxic by oral or dermal exposure. 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 **09-344-105r** involves confined field trials of genetically engineered organisms or products that do NOT involve new species or organisms or novel modifications 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/

John Cordts
Branch Chief, Plant Pests and Protectants Branch
Biotechnology Regulatory Services

Date: 2-22-2010
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