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[ ] **CBI-delete**

Dr. Firko,

With this letter we are requesting confirmation from USDA-APHIS's Biotechnology Regulatory Services (BRS) that our *Thlaspi arvense* L. (pennycress; field pennycress) [ ] mutant plant lines having [ ] seed oil phenotype, which were **2x CBI-delete** developed using CRISPR/Cas9 genome editing technology, are not themselves a plant pest and do not meet the definition of a regulated article under 7 CFR Part 340 since the final lines do not contain any foreign DNA including that from a "plant pest". Upon confirmation, we plan to field test these lines at [ ]. **CBI-delete**

Introduction into pennycress cultivar [ ] of a CRISPR/Cas9 DNA construct **CBI-delete** designed to target genome edits in [ ] was carried out using a disarmed **CBI-delete** *Agrobacterium tumefaciens* strain [ ] and [ ] method. When integrated **2x CBI-delete** into the plant genome, the expressed [ ] CRISPR-associated protein 9 (Cas9) **CBI-delete** endonuclease was guided [ ]. At that location, the Cas9 endonuclease catalyzed **CBI-delete** DNA double-strand breaks, which were then repaired by the plant's error-prone endogenous DNA repair mechanisms resulting in a low frequency of mutations. Two of the independent plant lines we isolated [ ] were found to have mutations at the **CBI-delete** expected location adjacent to the Cas9 [ ] PAM site [ ] gene. [ ] **3x CBI-delete** plants harbor a four base-pair deletion whereas [ ] plants harbor a single base- **CBI-delete** pair insertion. The seed oil of homozygous [ ] was found to have the expected **CBI-delete** [ ] compared to seed oil of wild-type pennycress plants. **CBI-delete**

[ ] null-segregant mutant lines containing no traces of the CRISPR/Cas9 DNA **CBI-delete** construct were generated by back-crossing the originally-transformed lines to wild-type plants and then screening of parent and progeny lines by negative antibiotic plant selection and by PCR analysis to confirm null-segregant progeny (please see figures 1 and 2). The segregation of integrated transgenic material was possible since the CRISPR/Cas9 DNA construct had inserted into a chromosomal location not linked to the pennycress [ ]. **CBI-delete**

The CRISPR/Cas9 DNA construct used to deliver the mutagenesis reagent was comprised of the following four components:

1) A [ ] driving expression of the [ ] *Cas9* gene. The Cas9 protein is a RNA-guided DNA endonuclease enzyme used to mutate the gene of interest. The [ ] terminator from [ ] was used to end transcription of the *Cas9* cassette. **2x CBI-delete** **CBI-delete** **CBI-delete**

2) The [ ] from [ ] driving expression of the *Cas9* guide RNA sequences. The guide RNA contained [ ] adjacent to a [ ] PAM site [ ]. **2x CBI-delete** **2x CBI-delete** **CBI-delete**

3) The [ ] driving expression of the [ ] and terminated with [ ]. **3x CBI-delete**

4) T-DNA Left Border (LB) and Right Border (RB) sequences from *Agrobacterium tumefaciens* for integration into the plant genome.

Although the original transgenic lines were generated using a construct containing plant pest sequences, the resulting [ ] null-segregant lines do not contain these sequences. Therefore, we are writing to obtain confirmation that these [ ] null-segregant lines are not considered regulated articles under 7 CFR Part 340, before moving forward with planting in field test plots. **CBI-delete** **CBI-delete**

We sincerely thank you for your time and consideration.

Best,

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Figure 1
[ ] **CBI-delete**

[ ] agar-containing plates containing [ ]. **2x CBI-delete**
Left: CRISPR/Cas9 construct-containing pennycress [ ] seedlings [ ]. **2x CBI-delete**
Right: Null-segregant pennycress [ ]. **CBI-delete**

Figure 2
[ ] **CBI-delete**

PCR analysis performed on [ ] genomic DNA from transgene CRISPR/Cas9 **CBI-delete**
construct-containing and null-segregant siblings, using primers specific to [ ]. **CBI-delete**
Lane 1: 1kb GeneRuler ladder.
Lanes 2-5: CRISPR/Cas9 construct-containing pennycress [ ]. **CBI-delete**
Lanes 6-7: Null-segregant pennycress [ ]. **CBI-delete**