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Dr. Michael J. Firko
APHIS Deputy Administrator
Biotechnology Regulatory Services
USDA-APHIS
4700 River Rd, Unit 98
Riverdale, MD 20737

Re: Confirmation that transgene-free, CRISPR-edited mushroom is not a regulated article

Dear Dr. Firko,

My laboratory is developing and applying the CRISPR/Cas9 mediated genome editing technology in precision breeding of agricultural crops for trait improvement. One of the products recently developed by my group is the transgene-free, anti-browning white button mushroom (*Agaricus bisporus*). The anti-browning property significantly improves the appearance and shelf life of white button mushroom, and is expected to facilitate automated mechanical harvesting. As shown in my presentation at APHIS Biotechnology Regulatory Services in Riverdale, MD on Oct. 7, 2015 and in the technical information below, the genome-edited mushroom has small deletions (1 to 14 bp) in a specific polyphenol oxidase gene, but contains no foreign DNA integration in its genome as verified by PCR and Southern blot analyses.

Relevant technical information:

- Transformation method: The plasmid constructs carrying gRNA and Cas9 were introduced into and transiently expressed in mushroom protoplasts using the PEG-mediated transformation method. In the absence of any antibiotics selection, mushroom mycelia were regenerated from protoplasts and characterized by PCR and Southern blot analyses. Genome-edited mushroom strains containing no foreign DNA integration were selected for further studies and potential commercialization.

- Construct: The pUC19-based plasmid construct contains the following genetic elements:
  Ori (*E. coli*): Origin of replication for plasmid DNA replication in *E. coli*;
  Amp (*E. coli*): The promoter and gene encoding a beta-lactamase;
  U6 promoter (*A. bisporus*): Driving the expression of guide RNA;

Guide RNA (synthetic): Directing Cas9 nuclease to specific genomic target site;
Pol III terminator (synthetic): Terminating guide RNA transcription;
gpd promoter (*A. bisporus*): Driving the expression of Cas9 nuclease;
Cas9 (synthetic): *Streptococcus pyogenes* Cas9 nuclease sequence with eukaryotic codon optimization;
Nos terminator (*A. tumefaciens*): Terminating Cas9 gene transcription;
BsaI cloning site (synthetic): Facilitating insertion of 20 bp guide RNA spacer.

- Recipient organism: White button mushroom (*A. bisporus*).

- Donor organisms: No foreign DNA from any donor organisms was actually inserted into the mushroom genome.
- Trait: Reducing the formation of brown pigment (melanin), improving the appearance and shelf life of mushroom, and facilitating automated mechanical harvesting.

Because white button mushroom is not a plant pest or federal noxious weed, and the CRISPR-edited mushroom regenerated from transiently transformed protoplasts contains no foreign plasmid DNA sequences, there is no scientifically valid basis to conclude that the CRISPR-edited mushroom is, or will become, a plant pest as defined by the Plant Protection Act. Therefore, I assert that the CRISPR-edited mushroom is not a regulated article based on the definition described in 7 CFR § 340.1. Before proceeding with further product development, however, I would like to seek confirmation from APHIS that the anti-browning mushroom, which has small deletions but no integration of plant pest elements or foreign DNA sequences, is not considered a regulated article under current regulations.

Thank you for your consideration, and I look forward to your response.

Sincerely,

Yinong Yang
Associate Professor

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