USDA

United States Department of Agriculture

Animal and Plant Health Inspection Service

Biotechnology Regulatory Services

4700 River Road
Riverdale, MD
20737

Dr. Yinong Yang
College of Agricultural Sciences
The Pennsylvania State University
211 Buckhout Laboratory
University Park, PA 16802

Re: Request for confirmation that transgene-free, CRISPR-edited mushroom is not a regulated article

Dear Dr. Yang:

Thank you for your letter dated October 30, 2015 inquiring whether or not the white button mushroom product described in your letter is a regulated article. Your letter describes CRISPR/Cas9-edited, white button mushroom (*Agaricus bisporus*) with an anti-browning phenotype. The anti-browning trait reduces the formation of brown pigment (melanin), improving the appearance and shelf life of mushroom, and facilitating automated mechanical harvesting.

The Plant Protection Act (PPA) of 2000 gives USDA the authority to oversee the detection, control, eradication, suppression, prevention, or retardation of the spread of plant pests or noxious weeds to protect the agriculture, environment, and economy of the United States. The APHIS mission is to protect the health and value of American agriculture and natural resources.

APHIS regulates the importation, interstate movement and environmental release (field testing) of certain genetically engineered (GE) organisms that are, or have the potential to be, plant pests. Regulations for GE organisms that are or have the potential to be plant pests, under the PPA, are codified at 7 CFR part 340, "Introduction of Organisms and Products Altered or Produced Through Genetic Engineering Which Are Plant Pests or Which There Is Reason To Believe Are Plant Pests." Under the provisions of these regulations, a GE organism is deemed a regulated article if it has been genetically engineered using a donor organism, recipient organism, or vector or vector agent that is listed in §340.2 and meets the definition of a plant pest, or that is an unclassified organism and/or an organism whose classification is unknown, or if the Administrator determines that the GE organism is a plant pest or has reason to believe it is a plant pest.

In your October 30, 2015 letter, you describe your CRISPR/Cas9-edited mushroom as having small deletions (1-14 bp) in a specific polyphenol oxidase gene but containing no foreign DNA integrated into the mushroom genome. Your letter states that plant pest

sequences were used to induce the small deletions into the final mushroom product, and that 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 antibiotic selection, you regenerated mushroom mycelia from protoplasts. Your letter further states that genome-edited mushroom strains containing no foreign DNA integration as verified by PCR and Southern blot analyses were selected for further studies, and that you confirmed that no genetic material, including plant pest sequences, was inserted into the final mushroom genome.

Based on the information cited in your letter, APHIS has concluded that your CRISPR/Cas9-edited white button mushrooms as described in your letter do not contain any introduced genetic material. APHIS has no reason to believe that CRISPR/Cas9-edited white button mushrooms are plant pests. Therefore, consistent with previous responses to similar letters of inquiry, APHIS does not consider CRISPR/Cas9-edited white button mushrooms as described in your October 30, 2015 letter to be regulated pursuant to 7 CFR part 340. Additionally, white button mushroom is not listed as a Federal noxious weed pursuant to 7 CFR part 360, and APHIS has no reason to believe that the anti-browning phenotype of your white button mushroom would increase the weediness of white button mushroom.

Please be advised that the importation of your CRISPR/Cas9-edited white button mushrooms, like all other mushrooms, will be subject to APHIS Plant Protection and Quarantine (PPQ), permit and/or quarantine requirements. For further information, should you plan to import these CRISPR/Cas9-edited white button mushrooms, you may contact Shailaja Rabindran at 301-851-2167 or contact PPQ general number for such inquiries at (877) 770-5990.

Please be advised that your white button mushroom variety described in your letter may still be subject to other regulatory authorities such as FDA or EPA.

GE white button mushrooms from this transformation that retain inserted genetic material would be considered regulated pursuant to 7 CFR part 340 and will require a notification or permit for importation, interstate movement, or environmental release. Furthermore, should you become aware at any time of any issues that may affect the Agency's conclusion regarding this inquiry; you must immediately notify the Agency in writing of the nature of the issue. We hope you appreciate our commitment to plant health and support for the responsible stewardship for the introduction of GE plants.

Sincerely,

M. J. Firko 4/13/2016

Michael J. Firko, Ph.D. Date
APHIS Deputy Administrator
Biotechnology Regulatory Services
Animal and Plant Health Inspection Service
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Oct. 30, 2015

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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