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May 22, 2020

Bernadette Juarez
APHIS Deputy Administrator
Biotechnology Regulatory Services
4700 River Road, Unit 98
Riverdale, MD 20737

Re: Confirmation of Regulatory Status of CRISPR-Cas Rice with Broad-Spectrum Resistance to Bacterial Blight

Dear Ms. Juarez,

With this letter, we respectfully request confirmation from USDA-APHIS' Biotechnology Regulatory Services (BRS) of the regulatory status of rice (*Oryza sativa* L.) with broad-spectrum resistance to bacterial blight disease developed using CRISPR (clustered regularly interspaced short palindromic repeats)-Cas gene editing technology. As described below, we do not consider that CRISPR-Cas rice with broad-spectrum resistance to bacterial blight meets the definition of a regulated article under 7 CFR Part 340 as the final product does not contain any genetic material from a plant pest, nor is there a basis to believe that CRISPR-Cas rice with resistance to bacterial blight is or will become a plant pest.

Intended Genetic Change in the Final Product: Bacterial blight of rice is an important disease in Asia and Africa. The pathogen, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), secretes one or more of six known transcription-activator-like effectors (TALes) that bind specific promoter sequences and induce, at minimum, one of the three host sucrose transporter genes *SWEET11*, *SWEET13*, and *SWEET14*, the expression of which is required for disease susceptibility. We used multiplex CRISPR-Cas gene editing technology to introduce mutations via non-homologous end joining DNA repair of targeted double-strand breaks within the promoter regions of the three sugar transporter genes.[1] The promoter changes resulted in loss of binding of *SWEET*-inducing TALes, thus conferring resistance to *Xoo*-mediated bacterial blight disease.

Vector Construct: The CRISPR-Cas gene editing reagents were introduced via transformation with disarmed *Agrobacterium tumefaciens* harboring plasmid vector IRS1132. As described in Table 1, the transfer-DNA (T-DNA) region of plasmid IRS1132 contained four guide RNA (gRNA) cassettes, each containing a rice U6 promoter, a target sequence, a gRNA scaffold, and a rice U6 terminator. Expression of Cas9 endonuclease was under control of the ubiquitin 1 promoter from maize (*Zm*Ubi1) and the 3'

[1] Oliva, R., Ji, C., Atienza-Grande, G., Huguet-Tapia, J. C., Perez-Quintero, A., Li, T., Eom, J. S., Li, C., Nguyen, H., Liu, B., Auguy, F., Sciallano, C., Luu, V. T., Dossa, G. S., Cunnac, S., Schmidt, S. M., Slamet-Loedin, I. H., Vera Cruz, C., Szurek, B., … Yang, B. (2019). Broad-spectrum resistance to bacterial blight in rice using genome editing. *Nature Biotechnology*, **37**(11), 1344–1350. https://doi.org/10.1038/s41587-019-0267-z

untranslated region from the *A. tumefaciens* nopaline synthase gene (NOS 3'). The final cassette contained the hygromycin phosphotransferase (HPT) encoding gene from *Escherichia coli* under control of the 35S promoter and termination sequences from cauliflower mosaic virus (CaMV).

Table 1: Genetic elements within the T-DNA region of plasmid IRS1132

Cassette	Type	Name	Source	Description
gSWEET11, gSWEET13, gSWEET14_1, gSWEET14_2	Promoter	U6	*Oryza sativa*	Polymerase III promoter of the U6 small nuclear RNA gene to drive transcription
	gRNA			Targeting sequences specific for *SWEET11*, *SWEET13*, and *SWEET14* gene promoter regions
	Scaffold		*S. pyogenes*	Guide RNA scaffold
	Terminator	U6	*Oryza sativa*	Terminator of U6 RNA polymerase III
Cas9	Promoter	*Zm*Ubi1	*Zea mays*	Polyubiquitin gene promoter which controls expression of the Cas9 coding sequence
	Coding sequence	Cas9		Cas9 endonuclease introduces a double-strand break in the target endogenous DNA sequence
	Terminator	NOS 3'	*Agrobacterium tumefaciens*	Termination sequences of nopaline synthase (*nos*) gene from *A. tumefaciens*
HPT	Promoter	CaMV 35S	Cauliflower mosaic virus	35S promoter from CaMV with duplicated enhancer region to control expression of the *hpt* gene
	Coding sequence	*hpt*	*Escherichia coli*	Hygromycin phosphotransferase (HPT) encoding gene
	Terminator	CaMV 35S poly-A	Cauliflower mosaic virus	Polyadenylation signal from CaMV 35S

Confirmation of Intended Change: The identity of the introduced mutations in each of the three *SWEET* gene promoter regions was confirmed by nucleotide sequencing of polymerase chain reaction (PCR) amplicons.

Absence of DNA Insertions: Conventional breeding was used to generate null-segregant progeny that contained the intended mutations without introduced exogenous DNA. The absence of DNA insertions was confirmed by PCR amplification using ten different primer pairs corresponding to different components of the CRISPR-Cas construct.

Conclusion: In summary, CRISPR-Cas rice with broad-spectrum resistance to bacterial blight disease caused by *Xanthomonas oryzae* pv. *oryzae* is not a plant pest, nor does it incorporate any plant pest material. We respectfully ask for APHIS' confirmation that rice with broad-spectrum resistance to bacterial blight disease conferred by CRISPR-Cas gene editing, as described herein, is not a regulated article subject to APHIS oversight under 7 CFR Part 340.

Thank you in advance for your consideration of this request and we look forward to answering any questions you may have.

Sincerely,

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