CBI-Deleted Copy

Biotechnology Regulatory Services
Animal and Plant Health Inspection Service
United States Department of Agriculture
4700 River Road
Riverdale, MD 20737

RECEIVED
By ajdrummond for BRS Document Control Officer at 1:30 pm, Mar 13, 2023

Confidential Business Information Deleted

ATTN: Bernadette Juarez
APHIS Deputy Administrator
Biotechnology Regulatory Services
Confirmationrequests@usda.gov

March 13, 2023

Subject: Request for Confirmation from USDA-APHIS that a Maize Haploid Inducer line is Exempt from Regulations Under 7 CFR Part 340.1(b)(1)

Dear Bernadette Juarez,

Syngenta Seeds, LLC seeks confirmation from the Biotechnology Regulatory Services (BRS) that a maize haploid inducer line containing a single 19-bp deletion resulting from a targeted DNA break and subsequent repair by the plant's endogenous DNA repair mechanisms in the absence of an externally provided repair template is exempt from regulation pursuant to 7 CFR §340.1(b)(1). Information supporting this request is provided below.

Please contact me with any questions you may have on the information provided.

Scott A Huber
Syngenta Crop Protection, LLC
NA and APAC Regulatory Affairs Head

Requestor's name and contact information, including email address

Scott Huber
Syngenta Crop Protection, LLC
9 Davis Drive
Research Triangle Park, NC 27709
Scott.huber@syngenta.com
Ph: 1-919-279-5001

Plant's genus and species

Zea mays L.

Which regulatory exemption is being claimed for the plant and why it qualifies

The maize haploid inducer line qualifies for exemption under 7 CFR Part 340.1(b)(1). A maize haploid inducer line was developed using CRISPR to introduce a single 19-bp deletion in the *cenh3* gene resulting from a targeted DNA break and subsequent repair by the plant's endogenous DNA repair mechanisms in the absence of an externally provided repair template (Line designation: MZKE01N). This modification was confirmed via amplification of the *cenh3* gene target site and subsequent sequencing (Figure 1).

Description of the trait

The maize haploid inducer plants have a deletion in the *cenh3* gene, which when crossed with non-edited maize plants produce maize haploid plants. The haploid plants produced from this cross will only contain the chromosomes from the non-edited maize plants (Yao *et al.* 2018). The haploid plants will go through a haploid doubling process producing fertile double haploid seed. This double haploid induction process enables rapid fixing of genetic backgrounds advoiding the six generations of breeding that is typically required to produce near-inbred lines. Doubled haploid breeding comprises the most efficient means to improve crop genetics.

Description of the genetic modification and method used to produce the modification

The maize haploid inducer plants were developed through bombardment of a conventional variety with ribonucleoprotein complex containing the Cas12a protein and a guide RNA (gRNA140) targeting the *cenh3* gene. The system was designed to create a double strand break in exon 2 of the *cenh3* gene. The plants were also co-bombarded with a plasmid vector that was designed to produce double-strand breaks in the **[** CBI-Deleted CBI-Deleted **]**. The plasmid vector contained CBI-Deleted Cas12a and guide RNA cassettes and the *pmi* gene cassette used for selection. The transformed maize tissue was transferred to cell culture medium containing mannose. The *pmi* gene in the transformation plasmid was used as a selectable marker during the transformation process (Negrotto et al. 2000). It encodes the enzyme phosphomannose isomerase (PMI), which enables transformed cells to survive on a mannose substrate (Negrotto et al. 2000).

The T0 plants were tested for the presence of the plasmid sequence, the intended edit, and lack of edits to the plasmid vector gene targets using TaqMan polymerase chain reaction (PCR) analysis (Ingham *et al.* 2001). The TaqMan assays used primers and probes specific to all possible edits and to the PMI and Cas12a sequences contained in the plasmid vector. Plants that only had the

edit to the *cenh3* gene were crossed with conventional corn plants. These T0 plants were also positive for the Cas12A sequence. The F1 seed was collected and planted. The F1 plants were tested using the same TaqMan assays used on the T0 plant and the plants that only contained the edit to the *cenh3* gene and were negative for the plasmid vector sequence were selfed. Two more crosses to conventional plants were conducted to produce the seed in the Cytoplasmic Male Sterile (CMS) background. This seed was planted and the plants were tested for absence of the plasmid sequence and presence of only the edit to the *cenh3* gene. The plasmid sequence was confirmed to be absent using DNA capture and Illumina sequencing. For the DNA capture process, the probe panel was designed to cover the entire transformation plasmid sequence. Confirmation of the intended edit to the *cenh3* gene and lack of edits to the plasmid vector gene targets was confirmed by targeted amplification of the edit sites and subsequent Next Generation Sequencing (NGS). The supplier of the ribonucleoprotein confirmed that the CRISPR protein preps were DNase treated and then tested for *E. coli* genomic DNA using qPCR methods. Plants that only contained the edit to the *cenh3* gene and were negative for all the plasmid vector sequences were crossed with conventional plants to produce the MZKE01N seed to be used in Syngenta's breeding program.

Assessment for the potential of unintended modifications

To assess for the potential of unintended modifications in the corn haploid inducer line, off-target prediction was performed for all the guide RNAs using the Cas-OFFinder tool (Bae *et al*. 2014) on the *Zea mays* genome. The off-target analysis confirmed that there is only a single 100% match in the *Zea mays* genome on the *cenh3* gene. The analysis also showed several putative off-target sites: one site with 1 mismatch from the **[ ]** gRNA and two sites with 4 mismatches from the **[ ]** gRNA and **[ ]** gRNA. For the off-target site with 1 mismatch, targeted amplicon sequencing was conducted and confirmed that there was no off-target edit in the corn haploid inducer line MZKE01N. Kim *et al*. (2017) reported that a higher number of mismatches in target sequences triggered a lower indel efficiency (i.e., less off-targets) in CRISPR-Cas system, and 3 or more mismatches in target sequences almost eliminates the formation of off-targets. Similar results from CRISPR-Cas system were observed in genome edited plants, such as rice (Tang *et al*. 2018), Arabidopsis (Bernabe-Orts *et al*. 2019), and maize (Lee *et al*. 2018), and no mutations at the putative off-target sites were identified. Therefore, the two putative off-targets with 4 mismatches will unlikely occur in the corn haploid inducer line MZKE01N.

CBI-Deleted

CBI-Deleted

Figure 1. Sequence alignment between the native *Zea mays* CENH3 gene sequence and the 19-bp deletion conversion line (MZKE01N) sequence.

The 19-bp deletion in the conversion line sequence is denoted with "-". The gRNA140 target is highlighted in grey "C".

```
                                                gRNA140 target
Zea mays CENH3 gene          TTCCTTGTTCCGTCTTTTGCAGGTGGTGCGAGTACCTCGGCGACGCCGGTGAGCGCGTGC  60
19-bp deletion MZKE01N       TTCCTTGTTCCGTCTTTTGCAGGTGGTGCG------------------TGAGCGCGTGC  41

Zea mays CENH3 gene          GTGCG                                                        65
19-bp deletion MZKE01N       GTGCG                                                        46
```

References

Bae S, Park J, Kim JS. 2014. Cas-OFFinder: A fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. *Bioinformatics* 30: 1473-1475.

Bernabe-Orts JM, Casas-Rodrigo I, Minguet EG, Landolfi V, Garcia-Carpintero V, Gianoglio S, Vazquez-Vilar M, Granell A, Orzaez D. 2019. Assessment of Cas12a-mediated gene editing efficiency in plants. *Plant Biotechnol J* doi: 10.1111/pbi.13113.

Ingham DJ, Beer S, Money S, Hansen G. 2001. Quantitative real-time PCR assay for determining transgene copy number in transformed plants. BioTechniques 31:136–140.

Kim HK, Song M, Lee J, Menon AV, Jung S, Kang YM, Choi JW, Woo E, Koh HC, Nam JW, Kim H. 2017. *In vivo* high-throughput profiling of CRISPR-Cpf1 activity. *Nat Methods* 14: 153-159.

Lee K, Zhang Y, Kleinstiver BP, Guo JA, Aryee MJ, Miller J, Malzahn A, Zarecor S, Lawrence-Dill CJ, Joung JK, Qi Y, Wang K. 2018. Activities and specificities of CRISPR/Cas9 and Cas12a nucleases for targeted mutagenesis in maize. *Plant Biotechnol J* doi: 10.1111/pbi.12982.

Negrotto D, Jolley M, Beer S, Wenck AR, Hansen G. 2000. The use of phosphomannose-isomerase as a selectable marker to recover transgenic maize plants (*Zea mays* L.) via *Agrobacterium* transformation. *Plant Cell Rep* 19:798–803.

Tang X, Liu G, Zhou J, Ren Q, You Q, Tian L, Xin X, Zhong Z, Liu B, Zheng X, et al. 2018. A large-scale whole-genome sequencing analysis reveals highly specific genome editing by both Cas9 and Cpf1 (Cas12a) nucleases in rice. *Genome Biol* 19: 84.

Yao L, Zhang Y, Liu C, Liu Y, Wang Y, Liang D, Liu J, Sahoo G, Kelliher T. 2018. *OsMATL* mutation induces haploid seed formation in *indica* rice. Nature Plants: 530-533.