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Yield10 BIOSCIENCE

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February 26th, 2021

Bernadette Juarez
APHIS Deputy Administrator
Biotechnology Regulatory Services
ConfirmationRequests@usda.gov

Re: Confirmation of Exemption Request for Genome-Edited *Camelina sativa* Null Segregant Lines Developed by CRISPR/Cas Technology

Dear Ms. Juarez,

Yield10 Bioscience respectfully requests confirmation from USDA-APHIS's Biotechnology Regulatory Services (BRS) that our genome-edited *Camelina sativa* (L.) Crantz plant lines developed using the CRISPR/Cas9 genome editing technology fall under Exemption 1 of §340.1(b)(1). The final edited lines contain single changes resulting from cellular repair of a targeted DNA break in the absence of an externally provided repair template. The final plant lines do not contain any DNA from a "plant pest".

Camelina sativa is an oilseed crop in the family Brassicaceae that is not on the USDA federal noxious weed list. [ ] lines, herein referred to as lines [ ], were developed at Metabolix Oilseeds, Inc. (110 Gymnasium Place, Saskatoon, SK, S7N 0W9, Canada), a wholly owned Canadian subsidiary of Yield10 Bioscience. We used disarmed *Agrobacterium tumefaciens* to deliver a gene encoding the endonuclease Cas9, as well as two cassettes coding each for a different guide RNA to direct the Cas9 enzyme to defined sites in the plant genome, into plant cells. The targeted site for genome editing was the [ ] gene in *C. sativa*, [ ], that [ ]. The inactivation of the [ ] gene is thus expected to [ ].

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It has been shown previously by other researchers that the Cas9 enzyme produces double-strand DNA breaks, that when repaired, incorporate small deletions or insertions of DNA. DNA sequence analysis has shown that lines [ ] each contain a [ ] that disrupts the coding sequence of the [ ] gene (Table 1).

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Table 1. Summary of nucleotide insertions in [ ] gene in edited *C. sativa* lines. CBI-Deleted
Abbreviations are as follows: WT, wild type (no change in nucleotide sequence); Chr, chromosome; #i, number of nucleotides inserted in line.
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As described below, lines [ ] are null segregants that were obtained using CBI-Deleted
conventional breeding procedures to remove the genetic sequences that allow the CRISPR/Cas9 editing to take place such that only the genome edits remain. These edits are [CBI-Deleted
]. CBI-Deleted

Since lines [ ] are null segregants that each contain [CBI-Deleted
] in the [ ] gene resulting from cellular repair of a targeted DNA break in the CBI-Deleted
absence of an externally provided repair template, it is our opinion that lines [CBI-Deleted
] meet the definition required for exemption under § 340.1(b)(1). However, we seek CBI-Deleted
confirmation of exemption for lines [ ] from USDA-APHIS BRS. CBI-Deleted

Intended Phenotype

The intended phenotype of lines produced from the editing of the [CBI-Deleted
] via inactivation of the endogenous [ ] gene that [CBI-Deleted
]. Camelina is an allohexaploid and contains three sub-genomes CBI-Deleted
[ ] such that there are three copies, or homeologs, of each gene in the plant. CBI-Deleted
Complete editing of the [ ] gene in *C. sativa* would contain edits in all three CBI-Deleted
homeologs/copies of the [ ] gene which are located on three different chromosomes CBI-Deleted
[ ]. As depicted in detail in Table 1, we generated [ ] lines [CBI-Deleted
] that are edited for [ ]. CBI-Deleted

Intended Activity

Upon confirmation from USDA-APHIS BRS that the [ ] edited *C. sativa* lines meet the CBI-Deleted
definition required for exemption under § 340.1(b)(1), Yield10 Bioscience intends to import the lines from its subsidiary in Saskatoon, Canada. In addition, we plan to conduct field releases and interstate movement of the lines.

Genetic Change in Final Product

The genetic changes are [ ] that disrupt the coding sequence of the [ ] gene. These edits are indistinguishable from changes that could result from natural genome variability, conventional breeding, or chemical or radiation-based mutagenesis. CBI-Deleted CBI-Deleted

Development of Edited *C. sativa* Lines

The components for the CRISPR/Cas9 genome editing technology, namely an expression cassette for the gene encoding the Cas9 endonuclease and two expression cassettes for two guide RNAs to target the Cas9 to the desired sites in the *C. sativa* genome, were delivered into the plant cells with *Agrobacterium*-mediated transformation using a binary vector and the floral dip method. CRISPR/Cas9 is a bacterial endonuclease. It utilizes a combination of protein-DNA and RNA-DNA pairing to direct targeted double-strand breaks in the DNA sequence of interest. The guide RNA targets Cas9 to the intended site of action. The spacers C1-29A and C1-29B of the guide RNAs for [ ] were designed to provide sufficient guide RNA specificity (Table 2), to generate edits in all [ ] of the [ ] gene. The lines described below, [ ], however have edits in only [ ] of the [ ] gene. CBI-Deleted CBI-Deleted CBI-Deleted

A detailed list of genetic elements, their origin, and their function is presented in Table 2. In short, the T-DNA of a binary vector [ ] carries four expression cassettes: CBI-Deleted

- An expression cassette (cassette 3) for the two exons of Cas9 endonuclease from *Streptococcus pyogenes* [ ]. The expression of the Cas9 gene is controlled by the [ ]. CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted
- A cassette (cassette 1) encoding the guide RNA spacer C1-29B (targets [ ]) under the control of the polymerase III promoter of the U6-26 small nuclear RNA gene from [ ]. CBI-Deleted CBI-Deleted
- A cassette (cassette 2) encoding the guide RNA spacer C1-29A (targets [ ]) under the control of the polymerase III promoter of the U6-26 small nuclear RNA gene from [ ]. CBI-Deleted CBI-Deleted CBI-Deleted
- An expression cassette (cassette 4) for the [ ] selection marker. CBI-Deleted

Table 2. Genetic elements of transformation vector [ ] used to create lines [ ]. The vector backbone (region outside of the T-DNA) is identical to standard binary vector [ ].

Cassette	Genetic Element	Source	Function
Cassette 1	U6-26 promoter	*[ ]*	Polymerase III promoter of the U6-26 small nuclear RNA gene to drive transcription of cassette 1 composed of the [ ] C1-29B spacer and the guide RNA scaffold which together encode the functional chimeric guide RNA.
	C1-29B spacer	*Camelina sativa*	Encodes [ ]. This "spacer" directs the Cas9 endonuclease to the [ ] gene for cleavage.
	Guide RNA scaffold	*Streptococcus pyogenes*	Encodes crRNA-tracrRNA fusion transcript that is necessary for Cas9 binding. The [ ] C1-29B spacer and the guide RNA scaffold together constitute a functional chimeric guide RNA.
	U6-26 terminator	*[ ]*	Terminator of U6-26 small nuclear RNA polymerase III to terminate transcription of [ ] C1-29B spacer and guide RNA scaffold.
Cassette 2	U6-26 promoter	*[ ]*	Polymerase III promoter of the U6-26 small nuclear RNA gene to drive transcription of cassette 2 composed of the [ ] C1-29A spacer and the guide RNA scaffold which together encode the functional chimeric guide RNA.
	C1-29A spacer	*Camelina sativa*	Encodes [ ]. This "spacer" directs the Cas9 endonuclease to the [ ] genes for cleavage.
	Guide RNA scaffold	*Streptococcus pyogenes*	Encodes crRNA-tracrRNA fusion transcript that is necessary for Cas9 binding. The [ ] C1-29A spacer and the guide RNA scaffold together constitute a functional chimeric guide RNA.

	U6-26 terminator	*[ ]*	Terminator of U6-26 small nuclear RNA polymerase III to terminate transcription of [ ] C3-29A spacer and guide RNA scaffold.	CBI-Deleted CBI-Deleted CBI-Deleted
Cassette 3	[ ] promoter	*[ ]*	[ ], controls expression of the Cas9 coding sequence.	CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted
	[ ]	artificial design [ ]	[ ]	CBI-Deleted CBI-Deleted CBI-Deleted
	[ ]	*[ ]*	[ ].	CBI-Deleted CBI-Deleted CBI-Deleted
	[ ]	*Streptococcus pyogenes*	Exon 1 of Cas9 endonuclease [ ].	CBI-Deleted CBI-Deleted CBI-Deleted
	[ ]	*Solanum tuberosum*	[ ] to optimize Cas9 expression in plants.	CBI-Deleted CBI-Deleted CBI-Deleted
	[ ]	*Streptococcus pyogenes*	Exon 2 of Cas9 endonuclease [ ].	CBI-Deleted CBI-Deleted CBI-Deleted
	[ ]	*[ ]*	[ ].	CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted
	[ ] terminator	*[ ]*	Terminator of the [ ] to terminate transcription of Cas9.	CBI-Deleted CBI-Deleted
Cassette 4	[ ] promoter	*[ ]*	[ ] promoter, drives expression of the [ ] selection marker.	CBI-Deleted CBI-Deleted

	[ ] selection marker	[ ]	[ ].	CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted
	[ ] terminator	*[ ]*	Terminator of the [ ] gene to terminate transcription of the [ ] selection marker.	CBI-Deleted CBI-Deleted CBI-Deleted
	T-DNA Left Border	*Agrobacterium tumefaciens* Ti plasmid	The left border of the T-DNA, required for transfer of the T- DNA into the plant cell genome.	
	aphAIII	*Escherichia coli* K-12	Bacterial kanamycin resistance marker provides kanamycin resistance for plasmid maintenance in *E. coli*.	
	pBR322 ori	*Escherichia coli* K-12	Bacterial origin of replication from plasmid pMB1, used for plasmid maintenance in *E. coli*.	
	pVS1 rep	*Pseudomonas aeruginosa* pVS1	Replication protein from plasmid pVS1, used for plasmid replication in Agrobacterium.	
	pVS1 sta	*Pseudomonas aeruginosa* pVS1	Stability protein from plasmid pVS1, used for plasmid stability in Agrobacterium.	
	T-DNA Right Border	*Agrobacterium tumefaciens* Ti plasmid	The right border of the T-DNA, required for transfer of the T- DNA into the plant cell genome.	

The binary vector backbone is similar to the standard binary vector [ ] and includes both T-DNA borders. CBI-Deleted CBI-Deleted

The next step comprised [ ], indicating the presence of the T-DNA containing the Cas9 expression and guide RNA expression cassettes. T_1 generation seeds were planted in soil and leaf tissue was screened for genome editing initially by Sanger sequencing and further confirmed by Amplicon sequencing. Plants containing genome edits in the [ ] gene were isolated. T_1 plants were grown and second-generation seed was isolated and screened for [ ]. The [ ] second generation seeds were sown to CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted

obtain E2 plants and edits in all three homeologs of the [ ] gene were confirmed by Amplicon sequencing. CBI-Deleted

A summary of lines with stable edits is provided below with more specific details in Table 1.

a) [ ] an [ ] within the detection limits of Amplicon sequencing. The [ ] contain [ ]. CBI-Deleted

b) [ ] an [ ] within the detection limits of Amplicon sequencing. [ ] contain [ ]. CBI-Deleted

Detailed PCR analysis was also performed using [ ] primer pairs targeting multiple regions of the T-DNA insert and the vector backbone. Two primer pairs were used to detect endogenous *C. sativa* wild type control genes. No evidence of any T-DNA or plant vector backbone was detected in lines [ ]. Please find supporting data at the end of this document. CBI-Deleted

Specificity of Single Guide RNAs

There are [ ] total genes in the [ ] gene family in Camelina, [ ]. In this study, two single guide RNAs were designed to target the specific editing of the [ ] genes. Spacer C1-29B in transformation vector [ ] is specific to the [ ] gene whereas spacer C1-29A is specific to the [ ] and [ ] genes (Table 3). The fusion of the 20 bp spacer DNA to DNA encoding a guide RNA scaffold will form a functional chimeric guide RNA. As described above, despite the targeting of [ ] genes with transformation vector [ ], individual lines [ ] were isolated that contained only one edit in each line. CBI-Deleted

Spacers C1-29B and C1-29A are specific to [ ] and can't target the other [ ] gene family [ ]. This is due to the lack of sequence specificity of the spacers to the [ ] genes and the absence of a functional PAM site adjacent to the spacer sequence necessary for target recognition (Table 3). CBI-Deleted

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No Plant Pest Sequences Remain in the Edited *C. sativa* Lines

The edited *C. sativa* lines were generated through the expression of the Cas9 cassette. The [CBI-Deleted], pVS1, T-DNA borders and [ ] terminator are derived from plant CBI-Deleted pest sequences ([ ], *Pseudomonas aeruginosa*, [CBI-Deleted]) (Table 2) as designated in 7 CFR 340.2. However, these sequences are not CBI-Deleted involved in pathogenicity and do not express proteins that would result in infection or pathogenicity of the edited *C. sativa* line. Importantly, the final edited *C. sativa* lines [CBI-Deleted], are null segregants and do not contain these plant pest sequences but retain the CBI-Deleted desired edits.

Conclusions

Metabolix Oilseeds, a wholly owned Canadian subsidiary of Yield10 Bioscience, generated [ ] CRISPR/Cas9 edited lines of the allohexaploid species *C. sativa*. The lines, [CBI-Deleted] contain a [ ] that disrupts the coding sequences of the [ ] CBI-Deleted gene. Lines [ ] are null segregants (genes enabling CRISPR/Cas9 editing CBI-Deleted were removed through conventional breeding) yet retain the desired genome edits and these lines do not contain any foreign DNA or plant pest sequences.

In order to facilitate further testing at our Woburn, MA facilities and in the field, Yield10 Bioscience requests confirmation from Biotechnology Regulatory Services that our edited *C. sativa* lines [ ] fall under Exemption 1 of §340.1(b)(1). **CBI-Deleted**

We look forward to answering any questions you might have.

Sincerely,

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References

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Supporting Data

1. Map of transformation vector showing primer binding sites.

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2. Description of primers used for screening of null segregants and expected amplicons

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3. Agarose gel electrophoresis analysis of PCR reactions of plant genomic DNA from lines [ ] to demonstrate absence of transformation vector sequence in null segregants. PCR was performed with TopTaq Master Mix kit (Qiagen, Cat No./ID: 200403) with 5 µL of a 20 µL reaction loaded on a 1.0% agarose gel. PCR cycling parameters for amplification of all the primer sets are the same as given in the table below. **CBI-Deleted**

Temp. (°C)	Time	Cycles
95	5 min	1
95	30 sec	35
53	30 sec	
72	60 sec	
72	10 min	1
4	∞	

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