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June 16, 2020

Bernadette Juarez
APHIS Deputy Administrator
Biotechnology Regulatory Services
4700 River Rd, Unit 98
Riverdale, MD 20737
AIRinquiry@aphis.usda.gov

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Dear Deputy Administrator Juarez:

Pairwise requests formal confirmation from USDA APHIS Biotechnology Regulatory Services (BRS) that *Brassica juncea* (L.) with improved flavor developed using gene-editing plant breeding tools is not a "regulated article" subject to APHIS oversight under 7 C.F.R. part 340 because it will not contain any inserted genetic material from a plant pest and changes to the genome consist only of site-directed double-strand breaks followed by non-homologous end joining (NHEJ) using the plant's endogenous repair mechanisms. No introduced DNA remains. In addition, there is no basis to believe that CRISPR edited *Brassica juncea* (L.) with improved flavor will become a plant pest within the meaning of the Plant Protection Act.

Developer (Responsible Party) name and contact information:
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Taxonomic description of the organism:

Brassica juncea (L.) belongs to the family Brassicaceae or Cruciferae. This species is believed to have arisen about 10,000 years ago as the result of multiple *natural hybridizations* between *Brassica rapa* and *Brassica nigra* (Olson 1960, Prakash *et al,* 2009). *B. juncea* (AABB, 2n=36) is a natural amphidiploid. This means that *B. juncea* retained the diploid set of chromosomes from the progenitor species: *B. rapa* (AA, 2n=20) and *B. nigra* (BB, 2n=16). Axelsson et al. (2000) have shown by molecular analysis that *B. juncea* contains conserved genomes of the progenitors. No species of *Brassica* is listed as Federal Noxious Weeds (https://plants.usda.gov/java/noxious) and we know of no mechanisms by which the mutations for improving flavor would make *B. juncea* into a weed.

Description of the intended phenotype:

Brassica juncea (L.) are among the most nutrient dense leafy green options available for consumers and are characterized by intraspecific diversity with variation of leaf traits such as color, size, texture, and heading morphology etc. If eaten fresh, they are pungent due to the presence of compounds involved in the plant's natural defenses which are produced in response to herbivory. This plant response is known as the 'mustard bomb'. Consequently, mustard greens are typically cooked in order to minimize pungency and/or consumed fresh in smaller quantities or as baby greens. Thus, prevention of the 'mustard bomb' reaction presents an opportunity to encourage broader consumption of fresh healthy leafy greens by consumers who would otherwise be averse to doing so. Using a gene editing approach, we have disarmed this reaction by knocking out multiple homologues of the gene responsible for the 'mustard bomb.' Therefore, the intended phenotype is an improved flavor and highly nutritious mustard green for domestic fresh-market consumption.

Description of intended activity:

Field trials followed by commercial product development and release

Description of intended genetic change in final product:

Similar to what has occurred naturally in *Arabidopsis thaliana* ecotypes [ ], we have created lines of *Brassica juncea* with deletions in [ ]. CBI-deleted CBI-deleted

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Description of vector or vector agent used to induce genetic change in the organism:
Disarmed *Agrobacterium tumefaciens* strain [ ] was used to introduce a T-DNA cassette from the binary plasmid [ ]. The T-DNA cassette expresses a [ ]. The T-DNA further expresses [ ]. The T-DNA further expresses [ ]

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Name of construct:
[ ]

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Description of the elements of the construct in order in which they occur:

Genetic Element Type	Genetic Element Name	Source Organism	Genetic Element's function	
Left Border	LFTBorder1	*Agrobacterium tumefaciens*	Octopine left border sequence for transfer of T-DNA from *Agrobacterium tumefaciens*.	
Promoter	[ ]	[ ]	[ ]	CBI-deleted, CBI-deleted, CBI-deleted
Gene	[ ]	[ ]	[ ]	CBI-deleted, CBI-deleted, CBI-deleted

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Terminator	[ ]	[ ]	[ ]	CBI-deleted, CBI-deleted, CBI-deleted
Promoter	[ ]	[ ]	[ ]	CBI-deleted, CBI-deleted, CBI-deleted
Gene	[ ]	[ ]	[ ]	CBI-deleted, CBI-deleted, CBI-deleted
Terminator	[ ]	[ ]	[ ]	CBI-deleted, CBI-deleted, CBI-deleted

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Promoter	[ ]	[ ]	[ ]	CBI-deleted, CBI-deleted, CBI-deleted
Gene	[ ]	[ ]	[ ]	CBI-deleted, CBI-deleted, CBI-deleted
Promoter	[ ]	[ ]	[ ]	CBI-deleted, CBI-deleted, CBI-deleted
Gene	[ ]	[ ]	[ ]	CBI-deleted, CBI-deleted, CBI-deleted
Right Border	RGTBorder1	*Agrobacterium tumefaciens*	Octopine right border sequence for transfer of T-DNA from *Agrobacterium tumefaciens*.	

Description of Method used or intended to be used to Confirm Intended Genetic Changes were Achieved and the Absence of Foreign DNA:

PCR and next generation sequencing are being used to confirm that intended genetic changes were achieved. Genomic DNA is isolated from leaf tissue and used as a template in PCR reactions using primers specific to the genes targeted. The amplified products are subsequently sequenced and characterized to confirm the genetic changes. Primary transformants were created using *Agrobacterium* and selection. Events of interest were advanced and non-transgenic progeny selected from the segregating population. qPCR was then used to confirm the absence of foreign DNA. Genomic DNA isolated from plants was used as a template in three qPCR reactions with primers specific to elements of the constructs introduced (i.e. the nuclease gene sequence). Only the plants shown to be absent of the construct elements were maintained.

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Table 1 Results of qPCR assay detecting the absence of the nuclease sequence

Sample Name	Assay 1	Assay 2	Assay 3	Final Call
[ ]	[ ]	[ ]	[ ]	[ ]

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CONCLUSION

Confirmation by BRS that these *B. juncea* lines are not "regulated articles" subject to regulations at 7 C.F.R. part 340 would be consistent with the Agency's responses to numerous other AIR inquiries in which disarmed *Agrobacterium* was used to introduce CRISPR editing elements into the plant to target multiple DNA sequences. Some recent examples, listed by developer and APHIS posting date, are: CoverCress Inc., 5/7/2020; Yield 10 Bioscience, 4/23/2020; Max Planck Institute, 5/25/2019; Yield 10 Bioscience, 9/27/2018; and USDA ARS, 10/16/2017. As with those previous cases, the edits here involve directed cuts, but then rely on non-homologous end joining (NHEJ) using the plant's endogenous repair mechanisms to produce the mutations. Also, as with previous cases, only plants shown to be free of the inserted DNA, including plant pest sequences, will be commercially advanced by the company.

We look forward to your response. Do not hesitate to contact us if you have questions or need additional information.

Cited References:

Axelsson, T., Bowman, C., Sharpe, A., Lydiate, D., Lagercrantz, U. (2000). Amphidiploid Brassica juncea contains conserved progenitor genomes Genome 43(4), 679-688.

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Olsson G (1960) Species crosses within the genus Brassica II. Artificial Brassica napus L. Hereditas 46:171–222, 351–386

Prakash, S., Bhat, S., Quiros, C., Kirti, P., Chopra, V., "Brassica and Its Close Allies: Cytogenetics and Evolution" in Plant Breeding Reviews Vol 31 ed. Jules Janick (2009)