pairwise

October 26, 2023

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Bernadette Juarez
Deputy Administrator
Biotechnology Regulatory Services
Animal and Plant Health Inspection Service
U.S. Department of Agriculture

Confidential Business Information Deleted

Subject: Request for Regulatory Status Review of Gene Edited *Brassica juncea* with Altered Pungency and Altered Texture – Revised Submission 22-159-01rsr

Dear Ms. Juarez,

Pairwise Plants Services, Inc, a food technology company based in Durham, North Carolina, USA, respectfully requests a Regulatory Status Review under 7 CFR § 340.4(a)(1) of a Pairwise product produced using CRISPR/Cas gene editing technology and described in detail below. No genetic material has been inserted in the plant as a result of the modifications.

The following description has been provided to assist in your review. Please do not hesitate to contact us if you have questions or need additional information.

Thank you in advance for your attention to this request.

Sincerely,

Nicole Juba

Nicole Juba, Ph.D.
Associate Director, Regulatory
Pairwise

Part I: Requestor

Pairwise Plants Services, Inc
Attn: Nicole Juba, Ph.D., Regulatory Manager
807 East Main Street, Suite 4-100
Durham, NC 27701
NJuba@pairwise.com
+1 (919) 321-0820

Part II: Confidential Business Information (CBI) Statement

Pairwise Plants Services, Inc. (hereafter the "Company") has marked certain information in this Regulatory Status Review as confidential business information (CBI). Information marked by the Company as CBI is exempt from release and/or public disclosure under Freedom of Information Act (FOIA) Exemption 4, 5 U.S.C. Section 552(b)(4), and is prohibited from release pursuant to the Trade Secrets Act, 18 U.S.C. Section 1905. Release of information marked by the Company as CBI would provide competitive information about the nature of the research, development, and commercialization plans of the Company and could jeopardize protection of its intellectual property rights. Access to this information would cause competitive and/or financial harm to the Company.

Information claimed as CBI is consistent with the Guidance for Claiming Confidential Business Information (CBI) in Submission to USDA APHIS BRS (Document ID BRS-GD-2020-0004) v. December 20, 2021, found at https://www.aphis.usda.gov/brs/pdf/CBI_Submission_Guidance.pdf and include, but are not necessarily limited to the following:

Gene name, sequence information, including DNA and amino acid sequences, whole or partial, of the target sequence, including the wild type and the modified sequences, and supporting citations that disclose or allude to such information have been marked as CBI.

Information in each of these categories constitutes trade secrets related to the design, production, and benefit of edited *B. juncea* which was developed at the expense of the Company. This information is commercially valuable, is used in the Company business, and is held by the Company as strictly secret. The release of this information would cause harm to the Company and provide a competitive advantage to others.

Part III: Description of the Plant

Family: Brassicaceae
Genus: *Brassica*
Species: *Brassica juncea*
Common names: brown mustard, India(n) mustard, Chinese mustard, leaf mustard, oriental mustard, vegetable mustard (CFIA, 2017; USDA-ARS, 2022)

Part IV: Genotype of the Modified Plant

No genetic material has been inserted in the plant as a result of the modifications.

Nature of the Modifications

Two modifications (Table 1) have been made to *Brassica juncea* to create the traits for altered pungency and altered texture leading to the phenotypes of reduced pungency to improve flavor and reduced trichome development on leaves and stems, respectively. The genetic differences between the modified *B. juncea* relative to a comparator plant are alterations to existing endogenous genetic sequences that could introduce non-templated insertions, deletions, inversions, and/or base pair substitution(s) resulting in reduced or complete loss of function of the native protein.

Table 1. Summary of modified *B. juncea*.

	Modification 1	Modification 2	
Intended Trait	Altered pungency	Altered texture	
Intended Phenotype	Reduced pungency to improve flavor	Reduced trichome development on leaves and stems	
Genetic Component	Dioxygenase	Transcription factor	
Target Sequence	[ ]	[ ]	CBI-deleted
Number of Target Sequences	[ ]	[ ]	CBI-deleted
Function of Native Sequence	Formation of alkenyl glucosinolates	Epidermal cell differentiation	
Function of Modified Sequence	Loss of function	Loss of function	
Mechanism of Action	Loss of function of a dioxygenase [ ] preventing the hydrolysis of glucoiberin (3-methylsulfinylpropyl glucosinolate) to sinigrin (2-propenyl glucosinolate). Absence of sinigrin prevents the downstream formation of allyl isothiocyanate, the compound responsible for the pungency of *B. juncea*.	Loss of function of a [ ] transcription factor [ ] preventing cell expansion, branching, and maturation of the trichome cell wall resulting in reduced or absent trichomes on leaves and stems.	CBI-deleted

The trait for altered pungency leading to the phenotype of reduced pungency to improve flavor in *B. juncea* was achieved by creating loss of function mutations in the native [ ] sequences. [ ], the gene encoding [ ] dioxygenase, is responsible for the formation of alkenyl glucosinolates by catalyzing the hydrolysis of glucoiberin (3-methylsulfinylpropyl glucosinolate) and glucoraphanin (4-methylsulfinylbutyl glucosinolate) to sinigrin (2-propenyl glucosinolate) and gluconapin (3-butenyl glucosinolate), respectively [ ] loss of function would halt the conversion of glucoiberin to sinigrin thereby reducing or completely eliminating sinigrin for use in downstream reactions to produce allyl isothiocyanate, the compound responsible for the pungency of *B. juncea*. Naturally occurring loss of function mutations in [ ] sequences have been identified in other non-pungent *Brassica* species, such as broccoli (*B. oleracea*), which has three [ ] genes, only one of which is functional as the other two copies have premature stop codons [ ]. See Part V for a mechanism of action discussion.

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The trait for altered texture leading to the phenotype of reduced trichome development on leaves and stems was achieved by creating loss of function mutations in the native [ ] sequences. [ ], also called [ ], is part of the family of [ ] transcription factors that play a role in epidermal cell differentiation, specifically the development of trichomes [ ]. Lower expression of [ ] has been shown to correlate with lower trichome incidence in *Brassica* species [ ]. See Part V for a mechanism of action discussion. CBI-deleted

B. juncea is an allotetraploid (AABB genome, 2n=36) (Jabeen, 2020) and as such there are multiple copies of the [ ] sequences within the genome. All known copies of the [ ] sequences were targeted for loss of function mutations in the same plant. In *B. juncea* it was determined that there are [ ] copies of the [ ] sequence and [ ] copies of the [ ] sequence (Figure 1). The [ ] sequences in the *B. juncea* genome were identified using the Basic Local Alignment Search Tool (BLAST) to query the genome for similar sequences, followed by ortholog inference using phylogenetic gene tree construction. For [ ] sequences, *Brassica rapa* genes [ ] were used as the query sequence. For [ ] sequences, *Arabidopsis thaliana* gene [ ] was used as the query sequence [ ]. CBI-deleted

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Figure 1. Genomic location and distribution of [ ] sequences and [ ] sequences across the A and B genomes in *B. juncea*. Red bars represent the [ ] sequences. Blue bars represent the [ ] sequences. CBI-deleted

Sequence of the Modifications

All [ ] of the [ ] sequence and [ ] of the [ ] sequences were targeted for modification in the same plant. The nucleotide sequences of the entire edited region in FASTA format can be found in Appendix A [ ]. CBI-deleted

Sequence Comparison

The final product was confirmed to have modifications in [ ] copies of the [ ] sequence and [ ] copies of the [ ] sequence by amplicon sequencing (SASi). Figure 2 shows the modified [ ] sequence compared with the unmodified [ ] sequence for each of the [ ] copies. Figure 3 shows the modified [ ] sequence compared with the unmodified [ ] sequence for [ ] copies. A summary of the modifications is described in Table 2 [ ]. CBI-deleted

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Figure 2. Nucleotide sequence alignment of wildtype (WT) and modified [ ] sequences [CBI-deleted] in *B. juncea*. *B. juncea* has [ ] numbered [ ]. CBI-deleted The deletion observed in each modified [ ] sequence is shown in the figure. CBI-deleted

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Figure 3. Nucleotide sequence alignment of wildtype (WT) and modified [ ] sequences [CBI-deleted] in *B. juncea*. *B. juncea* has [ ] numbered [ ]. The CBI-deleted deletion observed in each modified [ ] sequence is shown in the figure. CBI-deleted

Table 2. Characterization of modified sequences in *B. juncea*

Target	Modification	Effect
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Part V: Description of the New Traits

Intended Trait: Altered Pungency

Intended Phenotype: Reduced pungency to improve flavor
Glucosinolates are a class of secondary metabolites produced by plant species in the Brassicaceae family, which include crops such as broccoli, cabbage, brussels sprouts, mustard, and oilseed rape These compounds are believed to be produced by the plant's natural defenses in response to herbivory. The type and concentration of glucosinolates vary within the different Brassicaceae species and can fluctuate in response to internal and external conditions such as growth stage or drought (Brown et al., 2003; Frazie et al., 2017; Rask et al., 2000). Over 100 glucosinolates have been identified in brassicaceous plants, however, an individual species typically has only a few predominate glucosinolates. *B. juncea* has 11 major glucosinolates with sinigrin accounting for greater than 97% of the glucosinolate concentration in both baby/immature and mature leaves (Frazie et al., 2017).

When consumed as fresh leaves, *B. juncea* have a distinct flavor and are sometimes pungent due to the presence of certain compounds. This reaction is often referred to as the 'mustard bomb' (Figure 4) and requires two components be present simultaneously; the glucosinolate substrate (e.g., sinigrin) and the myrosinase enzyme that hydrolyzes the substrate, resulting in the breakdown product (i.e., allyl isothiocyanate), which is responsible for the pungent flavor. Consequently, mustard greens are typically cooked, causing inactivation of the myrosinase enzyme, 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.

Description of the Mechanism of Action:
Using a gene editing approach, we have disarmed the 'mustard bomb' reaction in *B. juncea* by reducing or preventing the biosynthesis of the glucosinolate sinigrin. Without the sinigrin glucosinolate substrate, the 'mustard bomb' reaction cannot be carried out, meaning no allyl isothiocyanate production, resulting in *B. juncea* with reduced pungency when consumed as fresh leafy greens. This was achieved through creation of loss of function mutations in multiple copies of the [ ] sequence. CBI-deleted [ ] in the biosynthesis of the glucosinolate sinigrin. [CBI-deleted] dioxygenase responsible for the formation of alkenyl glucosinolates by CBI-deleted catalyzing the conversion of glucoiberin (3-methylsulfinylpropyl glucosinolate) and glucoraphanin (4-methylsulfinylbutyl glucosinolate) to sinigrin (2-propenyl glucosinolate) and gluconapin (3-butenyl glucosinolate), respectively [ ]. Naturally occurring genetic modifications in CBI-deleted [ ] sequences have been identified in other non-pungent *Brassica* species, such as broccoli (*B. oleracea*), which has only one functional copy of the [ ] gene. The other two copies have CBI-deleted premature stop codons [ ]. CBI-deleted

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Figure 4. The 'mustard bomb' reaction. Biosynthesis of sinigrin from glucoiberin. Hydrolysis of sinigrin to form allyl isothiocyanate.

Intended Trait: Altered Texture

Intended Phenotype: Reduced trichome development on leaves and stems
A wide variety of plant species have trichomes, or epidermal 'hairs', which can vary in size, location, and number and are often associated with leaves and stems. Trichome function can also vary from species to species serving roles to increase surface area allowing for increased transpiration and photosynthetic rates, as well as acting as a feeding deterrent. When plants with trichomes, such as *B. juncea*, are consumed as fresh leafy greens, the trichomes can create an unpleasant oral sensation. Thus, reducing trichome development on leaves and stems is a way to improve the eating experience and encourage broader consumption of fresh, healthy leafy greens by consumers who would otherwise be averse to doing so.

Description of the Mechanism of Action:
The genetic drivers for trichome development have been studied in the model plant *Arabidopsis thaliana* and have led to the identification of genes involved in trichome production and suppression [ ]. One of the earliest acting genes involved in trichome morphogenesis is [ ] protein part of the family of [ ] transcription factors. [ ] is believed to enhance the expression of genes already transcribed in epidermal cells that respond to developmental signals and stimulate cell expansion, branching, and maturation of cell wall for trichome development [ ]. Using a gene editing approach, we have reduced trichome development on leaves and stems. This was achieved through creation of loss of function mutations in multiple copies of the [ ] sequence in *B. juncea*. Downregulation of developmental transcription factors, like [ ], have been shown to correlate with reduced trichome development [ ]. For example, in *B. napus*, the lower expression of [ ] correlates with reduced or lack of trichomes [ ] and *Arabidopsis* with a non-functional [ ] do not produce trichomes [ ].

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Appendix A

The nucleotide sequences of the entire edited region of the [ ] sequences and [ ] sequences in modified Brassica juncea in FASTA format.

Start codon has been indicated with bold text with yellow highlight.
Premature stop codon has been indicated with underlined text with grey highlight.
Region where the deletion occurred in the modified sequence relative to the native sequence has been noted as an asterisk (*) with blue highlight.

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