May 15, 2023

Bernadette Juarez
APHIS Deputy Administrator
Biotechnology Regulatory Services
4700 River Rd, Unit 98
Riverdale, MD 20737
Re: Request for Confirmation of Exemption

Delivered via email:
RSRrequests@usda.gov

Confidential Business Information Deleted

Dear Ms. Juarez,

Ohalo Genetics Inc. ("Ohalo") respectfully requests a Regulatory Status Review for tetraploid potato plants modified to reduce the expression of potato Vacuolar Invertase ("VINV"). The plants are referred to here as "Reduced VINV" or "RedVin" potatoes for short.

1. Requestor's information

Judson Ward
President and Chief Technology Officer
Ohalo Genetics, Inc.
ops@ohalogenetics.com

2. Confidential Business Information ("CBI")

This request contains claimed CBI. The claimed CBI is justified because it is covered by trade secrets that are currently held as highly confidential by Ohalo. Ohalo's trade secrets are the result of detailed proprietary and confidential internal analysis and experimentation. Publishing the claimed CBI would undermine those trade secrets and the competitive advantages that they protect.

3. Description of plant's genus, species

Kingdom: Plantae
Phylum: Tracheophytes
Class: Angiosperms
Order: Solanales
Family: Solanaceae
Genus: Solanum
Species: *S. tuberosum*

Common name: Potato

Breeding lines: Russet Burbank, Ranger Russet, Atlantic, Peter Wilcox & Desiree. Other tetraploid potato lines will also be used.

4. Genotype of the modified plants

Ohalo's proprietary plant breeding technology results in ***non-transgenic plants***, with modifications that are indistinguishable from the types of modifications obtained by conventional plant breeders for centuries. Our technology involves the use of DNA-free, marker free techniques for modifications, wherein [CBI-Deleted
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CBI-Deleted
] thereby reducing VINV expression. CBI-Deleted

Sequence of *S. tuberosum VINV*

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ATGGCCACCCAGTACCATTCCAGTTATGACCTGGAAAACTCCGCCTCCCATTACACAT
TCCTCCCGGATCAACCCGATTCCGGCCACCGGAAGTCCCTTAAAATCATCTCCGGCAT
TTTCCTCTCCTCTTTCCTTTTGCTTTCTGTAGCCTTCTTTCCGATCCTCAACAACCAA
TCACCGGACTTGCAGAGTAACTCCCGTTCGCCGGCGCCGCCGTCAAGAGGTGTTTCTC
AGGGAGTCTCCGATAAGACTTTTCGAGATGTCGTCAATGCTAGTCACGTTTCTTATGC
GTGGTCCAATGCTATGCTTAGCTGGCAAAGAACTGCTTACCATTTTCAACCTCAAAAA
AATTGGATGAACGGTAATTAACTTTCTTATTTTGACTTTTCTGTAATTTCCTATTTAT
TTGATCTTAGAATTGAAAAAAATTATAAATACTTATACCGTTTTTTTTTTTCTTAATG
ATATTTATGGCTATTGATCTGTTGGGGTATCTTTTGGATTCTGATTGGATGCTATTCT
GCAGATCCTAATGGTGAGTTCAAAGTTAATTATCATCACTATTTTCTGTTAGTATTTA
ATTAATTATATTCTTAAACCATGAATTAAAACTTTAAAGGCAGTAAAATTCTCTCATG
AGGTAATTATGGTTTTATTTGATTTAAGCCTATAAGTGCCAACCAATCCATGTATGAG
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AACAGGTCCATTGTACCACAAGGGATGGTATCATCTTTTTTATCAATACAATCCAGAT
TCAGCTATTTGGGGAAATATCACATGGGGCCATGCCGTATCCAAGGACTTGATCCACT
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GACTGGGTCCGCTACCATCCTACCCGATGGTCAGATCATGATGCTTTATACCGGTGAC
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CTTCCAACTTCACAAGCTTTAAGCTATTGGATGAAGTGCTGCATGCGGTTCCGGGTAC
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GGGAAACTGATAGTGAATCTGCTGACCTGCAGAAGGGATGGGCATCTGTACAGGTATG
GACTTCGATTAACACATTGTTTTGTTGTTTTAGTTTGCACCATACACAATAATCGTGC
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AAAATGGAGATGGATAAAAGTAGCGCGTTATGATCTGATATATGCAGATCCTCAGAGG
CTCCGGGAGTTGCTAAACAAGTTTATGGTAGTTCAGTACCCGTGTTGGACGGTGAAAA
ACATTCGATGAGATTATTGGTAAGTGATGATGATTCCCTTATTTACCTTTGTTTATAT
CAAGCTTATATTCAGTTCTTGTAGTCTAGTTGGTTCACTATAAAAAAAGTACTTGGCA
GTTGCATTTGAGTAAAAGTTTTATAAACTGAATTTTAGGTGGACCACTCAATTGTGGA
GAGCTTTGCTCAAGGAGGAAGAACAGTCATAACATCGCGAATTTACCCAACAAAGGCA
GTGAATGGAGCAGCACGACTCTTCGTTTTCAACAATGCCACAGGGTCTAGCGTGACTG
CCTCCGTCAAGATTTGGTCACTTGAGTCGGCTAATATTCAATCCTTCCCCTTGCAAGA
CTTGTAA

Nature of the modifications

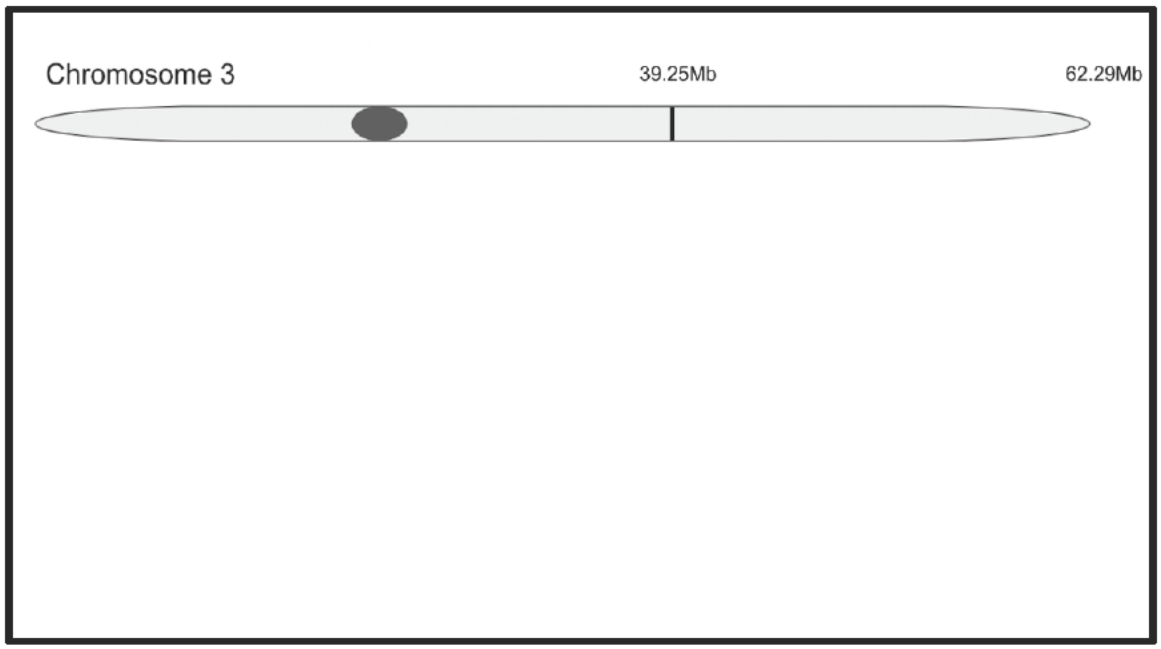
This request encompasses tetraploid potato plants with the following modification: [CBI-Deleted]. CBI-Deleted

To date, Ohalo is evaluating nine (9) unique RedVin potato plants as described below in **Table 1**. We have also developed a large inventory of additional RedVin potato plants which will be evaluated in the next 6 months. We note that the USDA Guide for Requesting a Regulatory Status Review under 7 CFR part 340, at page 12, states that "if a large number of RSR requests are pending requiring APHIS responses, **APHIS will prioritize products that have been developed** over products that are conceptual in nature." To the extent that situation applies here, we respectfully request a prioritized review since RedVin potato plants have been developed.

Plant ID	Modification
E-PED060-8966	[ ] CBI-Deleted
E-PED060-8916	[ ] CBI-Deleted
E-PED060-8904	[ ] CBI-Deleted
E-PED060-8903	[ ] CBI-Deleted
E-PED060-8917	[ ] CBI-Deleted
E-PED060-8985	[ ] CBI-Deleted
E-PED060-8951	[ ] CBI-Deleted
E-PED060-8981	[ ] CBI-Deleted
E-PED060-8982	[ ] CBI-Deleted

Table 1. Nine (9) RedVin potato plants and their respective modifications.

The RedVin potato plants listed in **Table 1** have [CBI-Deleted
] as shown in **Figure 1**. CBI-Deleted

[

] CBI-Deleted

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Figure 1. Illustration of the *S. tuberosum VINV* gene [CBI-Deleted
]. The top portion of CBI-Deleted
Figure 1 illustrates a zoomed-out view of the *VINV* gene located on potato chromosome 3. The middle portion of Figure 1 illustrates the *VINV* gene, denoting exon and intron positions [CBI-Deleted
CBI-Deleted
]. CBI-Deleted

Ohalo

Sequence comparisons

Sequences of the modifications from the RedVin potato plants from **Table 1** were compared to the respective parental sequence. These sequences comparisons are shown in **Figure 2**.

[ ] CBI-Deleted

Figure 2. Alignment illustrating sequences of the modifications from the RedVin potato plants from **Table 1**.

No Unintended Modifications

To demonstrate that no unintended modifications are present in RedVin potato plants, [ ]. CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted

5. Description of the Plant-Trait-MOA ("PTMOA")

Intended trait: Altered tuber sugar profile.

Intended phenotype: Reduced glucose and fructose content in tubers.

MOA: Modification of the *VINV* gene, leading to reduced VINV expression, resulting in less hydrolysis of sucrose to glucose and fructose.

The potato (*Solanum tuberosum*) is the third most important food crop in the world after rice and wheat, in terms of human consumption. More than a billion people worldwide eat potato, and global total crop production exceeds 300 million metric tons. (Clasen et al. 2016).

Most potatoes grown by U.S. farmers are used by food processors for potato chips, French fries, and other processed products. However, because potatoes are only harvested once a year, it is necessary to cold store the tubers to ensure a year-round supply of high-quality potatoes for processing. Without cold storage, potatoes have a shelf life of about 6 months, after which they rapidly deteriorate in quality. (Sowokinos 2001). In addition to prolonged storage, cold temperatures also reduce sprouting, losses due to shrinkage and the spread of disease.

Unfortunately, cold storage also has undesirable side-effects on potato quality. One of those undesirable side-effects is referred to in the industry as "cold-induced sweetening" ("CIS"). CIS involves the accumulation of the reducing sugars glucose and fructose from the breakdown of starch. See **Figure 3** below. When processed at high temperatures, those reducing sugars form dark-pigmented products that are bitter and unacceptable to consumers. (Sowokinos 2001). In the U.S., CIS causes up to 15% of potatoes being rejected at processing plants every year. (Bhaskar et al. 2010).

In addition to producing bitter-tasting products, heat processing also causes reducing sugars to react with free amino acids (for example, asparagine) to form the potential cancer-causing agent, acrylamide. Acrylamide formation in products like potato chips and French fries has been shown to occur via the nonenzymatic Maillard reaction. (Mottram et al. 2002). Acrylamide is particularly prevalent in heat-processed potatoes that have undergone CIS, due to their high levels of reducing sugars. Thus, methods that reduce acrylamide are being sought by U.S. potato growers.

At least one way to reduce CIS and therefore acrylamide content in heat-processed potatoes is to decrease the accumulation of reducing sugars formed during cold storage. Accumulation of reducing sugars during cold storage is influenced by several metabolic processes including starch synthesis and starch degradation. See **Figure 3** below. In

particular, potato VINV plays a particularly important role in the conversion of starch into reducing sugars during storage. (Sowokinos 2001).

VINV is an invertase enzyme localized to the vacuole, and genetic approaches have confirmed that reducing VINV expression reduces the accumulation of reducing sugars during storage. (See, for example, Bhaskar et al. 2010; Clasen et al. 2016).

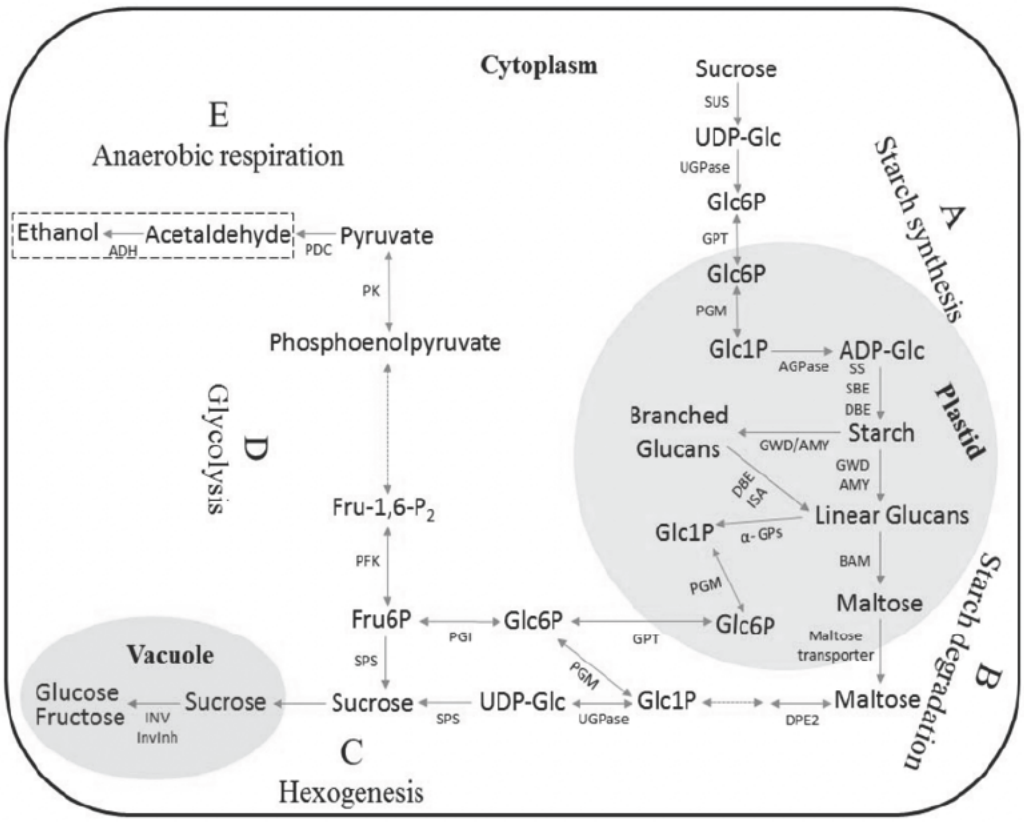

Figure 3. Vacuolar invertase (here, referred to as "INV") is illustrated in the gray oval labeled "Vacuole." During prolonged periods of cold storage, VINV converts sucrose from starch into the reducing sugars glucose and fructose, otherwise referred to as "cold-induced sweetening" or "CIS.

Of potential relevance here, we note that a similar PTMOA combination has previously been reviewed and approved by USDA pursuant to § 340.6 as that section was set forth prior to August 17, 2020 (Ref. No. 14-093-01p, 16-064-01p, 19-099-02p) and in accordance with 7 CFR § 340.4 (Ref. No. 21-270-01rsr). See **Table 2** below.

Table 2. Description of previously approved PTMAO combination.

Plant	Potato
Scientific name	*Solanum tuberosum*
Trait	Altered tuber sugar profile
Phenotype	Reduced glucose and fructose content in tubers
MOA	Tuber-specific RNAi mediated silencing of *vinv* gene decreases Vinv protein expression, resulting in less hydrolysis of sucrose to glucose and fructose.
Reference Number	14-093-01p, 16-064-01p, 19-099-02p; 21-270-01rsr

The previously approved PTMOA above is tuber-specific and uses RNAi (i.e., double-stranded RNA) to reduce VINV expression. Here, the PTMOA requested for review is similar but differs in at least a couple of potentially relevant ways.

First, the PTMOA requested for review here involves modification of the *VINV* gene, rather than the use of RNAi. In that context then, it is notable that the previously approved PTMOA for "tuber-specific RNAi" resulted in a plant that would not be reasonably possible to produce through traditional, conventional breeding. On the other hand, the PTMOA requested here for the RedVin potato results in a plant that is essentially indistinguishable from a plant produced through conventional breeding.

Second, in the PTMOA requested for review here, reduction of VINV expression is not tuber-specific but instead occurs throughout the plant. For potentially relevant background on the effect of reduced VINV occurring throughout the plant, using a mechanism other than RNAi, we would point to, for example, Greiner 1999. In that study, a protein inhibitor reduced VINV activity throughout the plant without any noted deleterious effect on plant growth or yield.

Confirmation of PTMOA

To demonstrate that RedVin potato plants have decreased VINV protein expression, resulting in less hydrolysis of sucrose to glucose and fructose, RNA will be isolated from RedVin tubers and control tubers (i.e., tubers from the respective parent lines). Expression analysis will be performed according to standard next generation sequencing or qPCR methods to confirm down-regulation of expression. Invertase activity of protein isolated from RedVin and control tubers will also be assessed using standard colorimetric assays or by direct measurement of sucrose, glucose, and fructose concentrations.

Nutritional analysis will also be conducted to evaluate the levels of key nutrients (*e.g.*, proximates, vitamins, amino acids, and minerals) and anti-nutrients (*e.g.*, glycoalkaloids) compared to control parent lines. FDA will review the details of the compositional analyses as a component of the food safety assessment for RedVin potatoes.

6. Conclusion

Based on the above, Ohalo respectfully requests review and confirmation that Redvin potato plants do not present a plausible pathway to increased plant pest risk.

Sincerely,

Judson Ward
President & Chief Technology Officer
Ohalo Genetics, Inc.

7. References

Bhaskar, P; Wu, L; Busse, J; Whitty, B; Hamernik, A; Jansky, S; Buell, C; Bethke, P; and Jiang, J, **Suppression of the Vacuolar Invertase Gene Prevents Cold-Induced Sweetening in Potato**. PLANT PHYSIOLOGY 154:939-48 (2010).

Clasen BM, Stoddard TJ, Luo S, Demorest ZL, Li J, Cedrone F, Tibebu R, Davison S, Ray EE, Daulhac A, Coffman A, Yabandith A, Retterath A, Haun W, Baltes NJ, Mathis L, Voytas DF, Zhang F. **Improving cold storage and processing traits in potato through targeted gene knockout**. PLANT BIOTECHNOL J. 14(1):169-76 (2016).

Greiner S, Rausch T, Sonnewald U, Herbers K. **Ectopic expression of a tobacco invertase inhibitor homolog prevents cold-induced sweetening of potato tubers**. NAT BIOTECNOL. 17(7):708-11 (1999).

Hang, H., Hou, J., Liu, J., Zhang, J., Song, B. and Xie, C., **The roles of starch metabolic pathways in the cold-induced sweetening process in potatoes**. STARCH – STÄRKE 69:1600194 (2017).

Mottram DS, Wedzicha BL, Dodson AT. **Acrylamide is formed in the Maillard reaction**. NATURE 419(6906):448-9 (2002).

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Wiberley-Bradford, A.E., Busse J.S. & Bethke, P.C., **Temperature-dependent regulation of sugar metabolism in wild-type and low-invertase transgenic chipping potatoes during and after cooling for low-temperature storage**. POSTHARVEST BIOLOGY AND TECHNOLOGY 115:60-71 (2016).

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