November 1, 2022

Bernadette Juarez
APHIS Deputy Administrator
Biotechnology Regulatory Services
4700 River Rd, Unit 98
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Requestor

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Confidential Business Information (CBI) Statement

This Regulatory Status Review request contains CBI.

Re: **Request for a Regulatory Status Review (RSR)** of Reduced Browning Banana with Altered Fruit Quality due to Reduced Polyphenol Oxidase (PPO) Enzyme and [CBI-Deleted] Selectable Marker CBI-Deleted

Dear Ms. Juarez,

Tropic Biosciences respectfully requests a Regulatory Status Review (RSR) for reduced browning banana plants based on the provisions in 7 CFR part 340 pursuant to § 340.4. The request for an RSR is for reduced browning banana (*Musa acuminata*, Cavendish subgroup, Grande Naine cultivar) with altered fruit quality. The bananas were developed using Cas9 base editing, in which precise nucleotide substitutions are created by Cas9-directed base deamination, involving the banana plant's endogenous mechanisms. The targeted gene for the reduced browning phenotype was a polyphenol oxidase (*PPO*) gene, which contributes to enzymatic browning in banana fruit. In addition, [CBI-Deleted]. CBI-Deleted

A [CBI-Deleted] Cas9 base editor was used to introduce targeted, [CBI-Deleted] nucleotide substitutions [CBI-Deleted] directed to the banana *PPO*[CBI-Deleted] gene and [CBI-Deleted] to the banana [CBI-Deleted] gene. [CBI-Deleted] substitutions were identified [CBI-Deleted] at the target regions, [CBI-Deleted]. [CBI-Deleted] substitutions were identified [CBI-Deleted] at the target regions in the *PPO*[CBI-Deleted] gene. One [CBI-Deleted]

[ ] substitution in a low-likelihood potential secondary target for [ ] gene was identified. CBI-Deleted CBI-Deleted

The base substitutions in the *PPO*[ ] gene, [ ], created premature stop codons in the coding sequence [ ], resulting in truncated non-functional PPO[ ] protein being produced from the edited alleles. The base substitutions in the [ ], created [ ], which confer resistance to the [ ]. The base substitution in the [ ], which also confers resistance to the [ ]. CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted

A. Description of Comparator Plant

Scientific Name: *Musa acuminata*, Cavendish subgroup, Grande Naine cultivar
Common name: Banana

B. Genotype of Modified Plant

1. **<u>Name of the altered genetic components and nature of the modifications.</u>**
Cavendish bananas have a triploid genome (AAA) and as such there are three alleles per gene. When sequence differences are present, the homologs can be distinguished into three alleles based on single nucleotide polymorphisms (SNPs).

   a) <u>Polyphenol oxidase gene (*PPO*).</u> In bananas, PPO enzymes are released from plastids upon mechanical damage of the fruit, including peeling, bruising and slicing (Taranto et al., 2017; Escalante-Minakata et al., 2018). The released PPO enzyme oxidizes phenolic compounds in fruit tissues, resulting in discoloration known as enzymatic browning and ultimately lowering the quality of the bananas (Palmer, 1963; Galeazzi et al., 1981; Sojo et al., 1998; Yang et al., 2000; Yang et al., 2004; Ünal, 2007; Chaisakdanugull and Theerakulkait, 2009). As shown in Figures 1 and 2, the reduced browning banana contains [ ] nucleotide substitutions in the *PPO*[ ] gene. [ ]. These substitutions [ ], resulting in [ ] non-functional PPO[ ] protein being produced during protein synthesis. [ ] *PPO*[ ] gene, PPO[ ] enzyme activity is decreased in the reduced browning bananas. Gene editing-induced truncations of PPO proteins have been previously used to reduce enzymatic browning in potatoes (González *et al.*, 2020). CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted

b) [ ]. In plants including banana plants, [ ]. As shown in Figures 1, 3 and 4, the reduced browning banana contains [ ]. These substitutions create [ ]. CBI-Deleted

2. **Sequence of the modification.** Appendix A includes the FASTA format for the full-length genomic sequence of non-edited and edited *PPO*[ ] alleles. Consensus sequences were used to create Figures 2 - 4. CBI-Deleted

3. **Sequence comparison.** Partial genomic sequence of non-edited *PPO*[ ] genes and the targeted nucleotide substitutions that are present in reduced browning bananas are indicated in Figure 2A, Figure 3A, and Figure 4A, respectively. Alignments of full-length non-edited and edited PPO[ ] proteins are given in Figure 2B, Figure 3B, and Figure 4B, respectively. CBI-Deleted

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Figure 1: Gene editing observed in the reduced browning banana. (**A**) Edits were observed [CBI-Deleted]. The three alleles of each gene are CBI-Deleted indicated by solid vertical lines, edits across these alleles are indicated by green asterisks, corresponding to sgRNA target sites listed to the left of the gene/allele representations. (**B**) [CBI-Deleted]. Edited sequences are indicated in green, with the corresponding change CBI-Deleted in the amino acid sequence of the associated protein.

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Figure 2: (A) Partial consensus genomic sequence from the banana *PPO*[ ] gene. Black text indicates nucleotides from the coding sequence [ ] are highlighted in green, and their protospacer adjacent motif (PAM) sequences are highlighted in grey. For each sgRNA, the base editing window is indicated by underlined nucleotides, and the nicking site (NS) is indicated with a dotted line. Red shading indicates the nucleotides that are mutated in reduced browning banana plants [ ]. Yellow shading indicates primers used for PCR to amplify the target regions for sequencing and confirmation of edits. (**B**) Alignment of full-length PPO[ ] protein sequences produced from non-edited and edited *PPO*[ ]. Red shading indicates the [ ] induced by sgRNA [ ]- and sgRNA [ ]-guided Cas9 base editor substitutions, which result in [ ] non-functional protein. [ ]. Green shading indicates allele-specific differences in amino acids arising from SNPs in the coding sequence of the *PPO*[ ] gene. CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted

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Figure 3: (**A**) Partial consensus genomic sequence from the banana [ ] gene. Black text indicates nucleotides from the coding sequence [ ]. The sgRNA [ ] is highlighted in light green, and its protospacer adjacent motif (PAM) sequence is highlighted in grey. The base editing window is indicated by underlined nucleotides, and the nicking site (NS) is indicated with a dotted line. Red shading indicates the nucleotides that are mutated in reduced browning banana plants [ ]. Yellow shading indicates primers used for PCR to amplify the target regions for sequencing and confirmation of edits. (**B**) Alignment of full-length [ ] protein sequences produced from non-edited and edited [ ]. Red shading indicates the [ ] mutations induced by sgRNA [ ]-guided Cas9 base editor substitutions, which enables [ ] function in the presence of [ ]. Green shading indicates allele-specific differences in amino acids arising from SNPs in the coding sequence of the [ ] gene.

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Figure 4: (**A**) Partial consensus genomic sequence from the banana [ ] gene. Black text indicates nucleotides from the coding sequence [ ]. The sgRNA [ ] is highlighted in light green, and its protospacer adjacent motif (PAM) sequence is highlighted in grey. The base editing window is indicated by underlined nucleotides, and the nicking site (NS) is indicated with a dotted line. Dark green shading indicates the nucleotide mismatches between sgRNA [ ] and the complementary [ ] region. Red shading indicates the nucleotide that is mutated in reduced browning banana plants [ ]. Yellow shading indicates primers used for PCR to amplify the target regions for sequencing and confirmation of edits. (**B**) Alignment of full-length [ ] protein sequences produced from non-edited and edited [ ]. Red shading indicates the [ ] mutation induced by sgRNA [ ]-guided Cas9 base editor substitution, which enables [ ] function in the presence of [ ]. CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted

C. Method Used to Produce the Modification

1. Cas9 base editing

The CRISPR system is part of the adaptive immune system of prokaryotes, in which CRISPR-*associated* (Cas) nucleases cleave nucleic acids as a way of protecting the cells from invading viruses. Cas proteins are targeted to specific loci by small RNAs, which in prokaryotes are transcribed from CRISPR loci, sites at which bacteriophages have integrated into the genome. This system has been developed into a widely-used gene editing technique in eukaryotic organisms, whereby a Cas protein, such as Cas9 from *Streptococcus pyogenes*, can be targeted to a specific sequence in the genome using a synthetic single guide RNA (sgRNA) with a complementary sequence. The Cas9 protein creates a double-stranded break at the precise target location, which is repaired by the host organism either via the error-prone non-homologous end joining (NHEJ) pathway creating indels or the sequence-specific homology-directed recombination (HDR) pathway (Wang et al., 2016; Jaganathan et al., 2018).

'Cas9 base editors' are fusion proteins, comprised of a Cas9 protein with inactivated nuclease activity and a DNA deaminase. The Cas9 protein has been mutated to inactivate one of two nuclease domains, reducing its double-stranded DNA break activity to single-stranded nicking. This enables the Cas9 nickase to bind and generate single-stranded nicks at specific genomic regions without creating indels. The fusion of nucleotide-modifying enzymes [ ] to a Cas9 nickase, enables targeted nucleotide substitution at specific sequences in the genome using sgRNAs, enabling the creation of programmable DNA mutations (Zhu et al., 2020). Additional fusion of proteins that direct endogenous DNA repair machinery [ ], reduce the frequency of undesired outcomes at the base editor target site, such as reversion to the original sequence (Anzalone et al., 2020). CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted

[ ]. Such base substitutions can be used to create [ ] mutations, to knock out the expression of functional gene products. CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted

CRISPR/Cas9 gene editing of *PPO* genes has been previously used to reduce enzymatic browning in mushrooms and potatoes (Waltz, 2016; González *et al.*, 2020), and since the development of Cas9 base editors this precise gene editing technology has been established in a variety of plant species (Zong et al., 2017; Lu and Zhu, 2017; Shimatani et al., 2017; Qin et al., 2019).

2. Banana transformation and regeneration

Banana embryogenic cell suspension cultures were generated from immature male flowers (e.g. Escalant et al., 1994; Côte et al., 1996; Navarro et al., 1997). Embryogenic cells were transformed by co-cultivation with *Agrobacterium tumefaciens*, and regenerated into somatic embryos on embryo development media, [ ]. The plasmid used for transformation contains a T-DNA region with the [ ] CBI-Deleted CBI-Deleted CBI-Deleted

[ ], the Cas9 base editor expressed from a strong constitutive promoter and sgRNAs [CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted CBI-Deleted]. Plants were genotyped by sampling a small piece of leaf tissue, as described below, and confirmed edited non-transgenic plants were propagated clonally in shoot multiplication media (e.g., Dagnew et al., 2012).

3. **Molecular characterization – PCR amplification and sequencing of targeted *PPO*[*2*] gene modifications** CBI-Deleted CBI-Deleted

In the initial screen, genomic DNA was extracted from a single leaf of banana plants regenerated from *Agrobacterium*-transformed embryogenic cells [CBI-Deleted] and selected for [ ]. CBI-Deleted The regions of the banana *PPO*[ ] genes targeted by sgRNAs were CBI-Deleted amplified by PCR and analyzed using Sanger sequencing. To fully characterize the genetic modifications in *PPO*[ ] genes and assess the numbers of edited CBI-Deleted alleles, genomic DNA was extracted from leaves from at least two distinct regions of the plants, and *PPO*[ ] target regions were amplified by PCR and analyzed using CBI-Deleted next-generation sequencing. These analyses confirmed the presence of [ ] CBI-Deleted substitutions in the *PPO*[CBI-Deleted] and [ ] substitutions in the [CBI-Deleted CBI-Deleted]. CBI-Deleted

4. **Molecular characterization – quantitative PCR analyses for absence of plasmid DNA**

Genomic DNA was extracted from leaves from at least two distinct regions of the banana plants regenerated from *Agrobacterium*-transformed embryogenic cells and selected for [ ]. Absence of T-DNA in the banana plants was CBI-Deleted assessed using quantitative PCR (qPCR) with primers designed to amplify thirteen regions of the T-DNA and plasmid backbone, including Cas9, sgRNA cassettes, bacterial and plant resistance markers, and left and right T-DNA borders. Plasmid-specific primers failed to amplify target sequences from genomic DNA extracted from reduced browning banana plants. This was also the case for DNA from negative control wild-type plants, whereas these primers did amplify plasmid sequences from genomic DNA extracted from positive control transgenic plants. As an internal control, an endogenous banana genomic region amplified in all samples. These analyses, therefore, confirm that plasmid sequences are absent from the genome of reduced browning banana plants.

5. **Molecular characterization – analyses of potential sgRNA secondary targets**

Two low-likelihood potential secondary targets were identified via the Cas-OFFinder and Breaking-Cas tools, one for sgRNA [ ] and another for sgRNA [ ]. These CBI-Deleted identified potential secondary targets were examined for the absence of CRISPR/Cas9 edits compared to a negative control wild-type banana sequence, using specifically designed primer sets to selectively amplify the regions that might be targeted by sgRNA [ ] and sgRNA [ ] in these genes, followed by Sanger sequencing of the PCR CBI-Deleted

amplicons. The sequence results confirmed the absence of edits in the potential secondary target of sgRNA [ ] in *PPO*[ ]. However, secondary targeting of sgRNA [ ] was observed. To fully characterize the genetic modification in [ ] and assess the numbers of edited alleles, genomic DNA was extracted from leaves from at least two distinct regions of the plants, and the [ ] secondary target region was amplified by PCR and analyzed using next-generation sequencing. This analysis confirmed the presence of [ ]. Although predicted to be a low-likelihood potential secondary target, the [ ] will be observed in reduced browning banana plants. CBI-Deleted

D. Description of New Trait

1. Intended Trait

Altered banana fruit quality

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2. Intended Phenotype

Reduced browning of the banana fruit due to nucleotide substitutions that create [ ] in the coding sequence of [ ] the *PPO*[ ] gene. The mRNA from the edited gene creates a [ ] non-functional PPO[ ] protein during protein synthesis, and as a consequence the PPO enzyme content in the banana fruit is expected to be reduced. CBI-Deleted

[ ], as a result of nucleotide substitutions in [ ]. CBI-Deleted

3. Description of the Mechanism of Action (MOA)

a) PPO MOA: In bananas, PPO enzymes are released from plastids upon mechanical damage of the fruit, including peeling, bruising and slicing (Taranto et al., 2017; Escalante-Minakata et al., 2018). The released PPO enzyme oxidizes phenolic compounds in fruit tissues, resulting in discoloration known as enzymatic browning and ultimately lowering the quality of the bananas (Palmer, 1963; Galeazzi et al., 1981; Sojo et al., 1998; Yang et al., 2000; Yang et al., 2004; Ünal, 2007; Chaisakdanugull and Theerakulkait, 2009). *PPO*[ ] is one of [ ] *PPO* genes expressed in banana [ ]. Among these [ ] *PPO* genes, *PPO*[ ] accounts for [ ] of mRNA abundance in [ ]. It is predominantly expressed in [ ], with low levels of expression in [ ] (Tropic Biosciences RNA-seq expression data). CBI-Deleted

The reduced browning banana fruit will have reduced levels of PPO enzyme as a result of the loss of function of [ ] the *PPO*[ ] gene. **CBI-Deleted**

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4. <u>Previously evaluated plant with same Trait or MOA</u>

Both the reduced PPO enzyme activity and [ ] MOAs in different **CBI-Deleted** plant taxon have been reviewed and cleared (no longer considered regulated articles under 7 CFR part 340) by USDA/APHIS/BRS in submission petitions as listed in Table 1. Requests for Confirmation of Exemption (CR) have also been granted (exempt from regulation under 7 CFR part 340), as listed in Table 2, for reduced PPO enzyme activity and [ ] MOAs in different plant taxon, including a gene edited **CBI-Deleted** altered fruit quality banana. Apple (*Malus x domestica*) and potato (*Solanum tuberosum*) plants with reduction of active PPO enzyme have been previously assessed in multiple applications as listed. In these petitions, reduction of PPO enzyme is achieved through RNAi mediated silencing and in the case of this RSR the PPO enzyme reduction is achieved through a gene edit [ ]. The **CBI-Deleted** expressed RNA encodes a non-functional PPO enzyme in a similar manner as that of the gene edited altered fruit quality banana granted exemption in the CR process. In all cases listed in Table 1, USDA has fully assessed and determined that plants across different taxa, with either altered fruit quality due to reduction in PPO enzyme activity, [**CBI-Deleted**
] are not likely to pose a plant pest risk and are no longer considered regulated **CBI-Deleted** articles under Title 7 of the Code of Federal Regulations (CFR), part 340.

Table 1: List of previously reviewed and cleared petition submissions with PPO and [ ] MOAs[1] CBI-Deleted

Plant	Scientific Name	Trait	Phenotype	Mechanism of Action	Reference Number(s)
Apple	*Malus x domestica*	Altered fruit quality	Reduced browning	RNAi mediated silencing of PPO (polyphenol oxidase) encoding genes of apple isoenzymes PPO2, GPO3, APO5, and pSR7.	10-161-01p, 16-004-01p, 20-213-01ext
Potato	*Solanum tuberosum*	Altered tuber quality	Reduced black spot	Tuber specific RNAi mediated silencing of Ppo5 (polyphenol oxidase-5) gene.	13-022-01p, 14-093-01p, 15-140-01p, 16-064-01p, 19-099-02p
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[1]https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/permits-notifications-petitions/confirmations/moa/moa-table

Table 2: List of previously reviewed and cleared Confirmation of Exemption Requests with PPO and [ ] MOAs[1] CBI-Deleted

CR Number	Requestor	Plant	Scientific Name	Trait	Exemption Category
21-356-01cr	Tropic Biosciences UK LTD	Banana	*Musa acuminata*	Altered Fruit Quality	(b)(1)
21-141-01cr	Tropic Biosciences UK LTD	Potato	*Solanum tuberosum L.*	Altered Tuber Quality	(c)
21-105-01cr	Okanagan Specialty Fruits Inc.	Apple	Malus x domestica	Altered Fruit Quality	(c)

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[1]https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/permits-notifications-petitions/confirmations/responses/cr-table

Conclusion and Request for Regulatory Status Review

As described within this RSR request, the reduced browning banana plants (*Musa acuminata*, Cavendish subgroup, Grande Naine cultivar) with altered fruit quality were developed using Cas9 base editing. Loss-of-function [ ] *PPO*[ ] gene in banana is expected to result in lower levels of enzymatic browning due to reduced abundance of polyphenol oxidase (PPO) enzymes released from plastids during damage of banana fruit. [ ] of edited plants, *in vitro.* The presence of the intended genetic modifications, induced by sgRNA-guided Cas9 base editor base substitutions involving endogenous banana DNA repair pathways, and the absence of plasmid DNA sequences in the reduced browning banana plants were confirmed by next-generation sequencing of target PCR fragments and quantitative PCR analyses, respectively.

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The two traits and MOAs in the reduced browning bananas have been fully assessed in different plant taxa by USDA/APHIS and determined to be exempt from regulation under 7 CFR part 340 and are not likely to pose a plant pest risk. The banana plant used for incorporating the gene edits (*Musa acuminata*, Cavendish subgroup, Grande Naine cultivar) are cultivated in tropical and subtropical climates, propagate vegetatively, produce sterile seedless fruit, are considered male-sterile and are not known as an invasive weedy species.

Based on the information provided herein, Tropic Biosciences respectfully requests an RSR for reduced browning banana under 7 CFR part 340 pursuant to § 340.4.

Sincerely,

DocuSigned by:
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CONFIDENTIAL APPENDIX A

Allelic sequences in FASTA format for the *PPO*[ ] genes, non-edited and edited, with base edits indicated in red text CBI-Deleted

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