

Preliminary Extended Determination¹ of Nonregulated Status for Okanagan Specialty Fruits Non-Browning Arctic[®] Apple PG451

In response to a request from Okanagan Specialty Fruits Inc. (hereinafter referred to as OSF) to extend a determination of nonregulated status to Okanagan non-browning Arctic[®] apple event PG451 (PG451 apple) with non-browning phenotype via suppression of four genes for polyphenol oxidase (Extension No. 20-213-01.ext), the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) has determined, based on similarity to its antecedent organisms, that PG451 apple and progeny derived from it are unlikely to pose a greater plant pest risk than the unmodified organism from which it was derived, and are no longer to be considered regulated under APHIS' Biotechnology Regulations in 7 CFR 340². This extension request is based upon APHIS' determination of nonregulated status of the OSF antecedent organisms: Arctic[®] Golden (Event GD743) and Arctic[®] Granny (Event GS784), with non-browning phenotype. OSF antecedent GD743 and GS784 described in petition number 10-161-01p received a determination of non-regulated status on February 18, 2015. APHIS-approved permits or acknowledged notifications that were previously required for environmental release, interstate movement, or importation will no longer be required for PG451 apple and its progeny. Importation of PG451, other propagative material, and bulk or table stock, will still be subject to APHIS foreign quarantine notices at 7 CFR part 319 and the Federal Seed Act regulations at 7 CFR parts 201 and 361.

The same genetic construct GEN-03, used to transform the OSF antecedents GD743 and GS784 with non-browning characteristics was also used to transform and generate PG451 apple. APHIS evaluated the plant pest risk of PG451 apple by assessing its similarity to the deregulated OSF GD743 and GS784 apples.

APHIS previously conducted a Plant Pest Risk Assessment on the antecedent organisms and concluded that they are unlikely to pose a greater plant pest risk than the unmodified organisms from which they were derived. Based on the plant pest risk similarity assessment (see Appendix A) of PG451 apple to the antecedents, APHIS concludes that PG451 apple is unlikely to pose a greater plant pest risk than the unmodified organism from which it was derived and should no longer be regulated under 7 CFR part 340. From the similarity assessment, APHIS concludes the following with respect to PG451 apple and its progeny:

- (1) No plant pest risk was identified from the transformation process, the insertion and/or expression of new genetic material, or from changes in metabolism in PG451 apple.
- (2) Disease and pest incidence and/or damage are not expected to be increased or atypical in PG451 apple. No plant pest effects are expected on these or other agricultural products and no impacts are expected to APHIS pest control programs.

¹ This extended determination is not effective until officially signed and published.

² The extension for nonregulated status described in this notice is being evaluated under the version of the regulations effective at the time that it was received. The Animal and Plant Health Inspection Service (APHIS) issued a final rule, published in the Federal Register on May 18, 2020 (85 FR 29790-29838, Docket No. APHIS-2018-0034), revising 7 CFR part 340; however, the final rule is being implemented in phases. This extension of a determination of nonregulated status is being evaluated in accordance with the regulations at 7 CFR 340.6 (2020) as it was received by APHIS on July 31, 2020..

- (3) Based on an evaluation of the gene products, and their similarity to the antecedents GD743 and GS784, and on data submitted in the extension request, PG451 apple is unlikely to adversely impact nontarget organisms beneficial to agriculture.
- (4) PG451 apple is no more likely to become weedier or more difficult to control as a weed than the antecedents GD743 and GS784, which are not weedy.
- (5) PG451 apple is not likely to increase the weed risk potential of other species with which it can interbreed in the U.S. or its territories. Gene flow, hybridization and/or introgression of inserted genes from PG451 to other sexually compatible relatives with which it can interbreed is not likely to occur.
- (6) Significant changes to agricultural or cultivation practices (e.g. pesticide applications, tillage, irrigation, harvesting, etc.) from adoption of PG451 are not expected.
- (7) Horizontal gene transfer of the new genetic material inserted into the PG451 apple to other organisms is highly unlikely and is not expected to lead directly or indirectly to disease, damage, injury or harm to plants, including the creation of new or more virulent pests, pathogens, or parasitic plants.

In addition to our finding that PG451 apple is unlikely to pose a greater plant pest risk than the organism from which it was derived, APHIS prepared a Record of Categorical Exclusion Determination for this action based on an Environmental Assessment completed for the antecedent GD743 and GS784 apples in 2014. PG451 will have no significant impacts, individually or collectively, on the quality of the human environment and will have no effect on federally listed threatened or endangered species, species proposed for listing, or their designated or proposed critical habitats (http://www.aphis.usda.gov/biotechnology/not_reg.html).

Based on my review and consideration of all of the scientific and environmental data, analyses, information, and previous conclusions regarding the plant pest risk assessment for the antecedent organisms, the plant pest risk similarity assessment, and record of categorical exclusion determination, and my knowledge and experience as APHIS' Deputy Administrator for APHIS Biotechnology Regulatory Services, I have determined and decided that this determination of nonregulated status of PG451 apple is the most scientifically sound and appropriate regulatory decision.

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

Date

Okanagan Specialty Fruit Inc. Request (20-213-01ext) for Extension of Determination of Nonregulated Status for Non-Browning Arctic[®] Apple Event PG451.

OECD Unique Identifier: OKA-NB004-2

Plant Pest Risk Similarity Assessment

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A. Introduction

Okanagan Specialty Fruits Inc. (OSF) has submitted a request that the Animal and Plant Health Inspection Service (APHIS) of the United States Department Agriculture (USDA) to extend a determination of nonregulated status to Non-Browning Arctic® Apple Event PG451 (OECD Unique Identifier: OKA-NB004-2), which was developed using genetic engineering, based on its similarity to the antecedent organisms GD743 and GS784 apple lines in accordance with 7 CFR 340. This extension was assigned the number 20-213-01.ext, hereafter referenced as OSF 2020. The antecedent GD743 and GS784 apple lines were developed using genetic engineering for non-browning phenotype. USDA announced its determination of nonregulated status for two non-browning apples: GD743, Golden Delicious variety marketed as Arctic® Golden, and GS784 variety Granny Smith marketed as Arctic® Granny (petition number 10-161-01p), on February 18, 2015.

Under the authority of the plant pest provisions of the Plant Protection Act (7 U.S.C. 7701 et seq.), the regulations in 7 CFR part 340, "Movement of Organisms Modified or Produced Through Genetic Engineering," regulate, among other things, the importation, interstate movement, or release into the environment of organisms modified or produced through genetic engineering that are plant pests or pose a plausible plant pest risk. This plant pest risk similarity assessment (PPRSA) was conducted to determine if PG451 apple is unlikely to pose a plant pest risk.

The extension for nonregulated status described in this PPRSA is being evaluated under the version of the regulations effective at the time that it was received. Animal and Plant Health Inspection Service (APHIS) issued a final rule, published in the Federal Register on May 18, 2020 (85 FR 29790-29838, Docket No. APHIS-2018-0034)³, revising 7 CFR part 340; however, the final rule is being implemented in phases. This extension of a determination of nonregulated status is being evaluated in accordance with the regulations at 7 CFR 340.6 (2020) as it was received by APHIS on September 30, 2020.

PG451 apple was produced by *Agrobacterium tumefaciens* mediated transformation of apple leaf tissue (OSF 2020) and some of the introduced border and regulatory sequences come from plant pest organisms listed in 7 CFR 340.2 (OSF 2020). Therefore, the PG451 apple is considered regulated under APHIS regulations at 7 CFR part 340.

Potential impacts analyzed in this Plant Pest Risk Similarity Assessment are those that pertain to plant pest risk associated with PG451 apple and its progeny and their use in the absence of confinement relative to the antecedents GD743 and GS784. APHIS utilizes data and information submitted by the applicant, in addition to current literature, to determine if PG451 apple is any more likely than the antecedents GD743 and GS784 to pose a plant pest risk. APHIS specifies in 7 CFR 340.6(e) that an extension request for nonregulated status shall include information to establish the similarity of the antecedent organism to the regulated organism in question.

³ To view the final rule, go to www.regulations.gov and enter APHIS-2018-0034 in the Search field.

APHIS may also consider information relevant to reviews conducted by other agencies that are part of the ‘Coordinated Framework for the Regulation of Biotechnology’ (51 FR 23302 1982; 57 FR 22984 1992; 80 FR 60414 2015). Under the Coordinated Framework, the oversight of biotechnology-derived plants rests with USDA APHIS, the Food and Drug Administration (FDA), and the Office of Pesticide Programs of the U.S. Environmental Protection Agency (EPA). Depending on its characteristics, certain biotechnology-derived products are subjected to review by one or more of these agencies.

B. Development of PG451 Apple.

The domesticated apple, is one of the most important fruit crops of temperate regions of the world and is cultivated widely in temperate latitudes or at high elevations in the tropics on all continents except Antarctica (Harris et al. 2002; Lubby 2003; Velasco et al. 2010; Cornille et al. 2012). It is also one of top 20 most productive crops in the world in terms of tonnage (Gross et al. 2014).

As described in the extension request (OSF 2020) Okanagan Specialty Fruits developed PG451 apple (*Malus domestica*, *syn Malus pumila*), from the commercially grown apple cultivar Gala using genetic engineering, to exhibit a non-browning phenotype.

Browning of apple flesh due to damage, cutting or bruising, is caused by an enzymatic reaction catalyzed by polyphenol oxidases (PPO). The phenolic substrates for this reaction and the PPOs are separately compartmentalized in the cell, with PPO in plastids and phenolic substrates in the vacuole. Loss of compartmentalization occurs when cells are damaged; if little to no PPO is present in the cells, cell disruption does not lead to browning. These non-browning apples are intended to benefit the fresh cut and dehydrated apple markets by reducing browning associated with bruising and cutting, eliminating the need for chemical treatments to reduce browning of fresh cut apple slices (OSF 2020). Browning also impacts the market for fresh fruit and juice. If the presence of PPO in cells is reduced or eliminated cell disruption does not lead to browning (OSF 2020).

PG451 apple was developed through *Agrobacterium*-mediated transformation of apple leaf tissue using the binary plasmid vector pGEN-03. The antecedents GD743 and GS784 were developed using the same plasmid vector and the same *Agrobacterium*-mediated transformation method. The T-DNA in pGEN-03 comprises a PPO suppression transgene (PGAS), the *nptII* gene for expression of a selection marker, and regulatory sequences flanked by *A. tumefaciens* T-DNA borders.

The PGAS gene is a chimeric polyphenol oxidase (PPO) suppression transgene consisting of partial coding sequences of four members of the apple PPO genes (PPO2, GPO3, APO5 and pSR7). It was designed to suppress the apple PPO gene family and to reduce expression of four PPO genes to induce the non-browning phenotype in apple fruit. The genes donor organism for the genes used to develop PGAS is the domestic apple, *M. domestica* (OSF 2020).

PG451 apple meets the criteria for extension of determination of nonregulated status from GD743 and GS784 apples (Petition 10-161-01p). This is based on these antecedent products possessing a similar PPO suppression mechanism of action and a non-browning apple trait phenotype, respectively. The antecedents GD743 and GS784 are agronomically and phenotypically comparable to the non-transformed controls.

APHIS completed a plant pest risk assessment (PPRA) and an environmental assessment (EA) for the antecedent GD743 and GS784 apples (USDA-APHIS 2014b, a) (<https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/permits-notifications-petitions/petitions/petition-status>)

The EA fully addressed all resource areas of potential concern. For the antecedent petition, 10-161-01p, APHIS concluded on the basis of the EA that the impacts would not be significant. The agency issued a Finding of No Significant Impacts (FONSI) and made determinations of nonregulated status for each of the antecedent events GD743 and GS784 (USDA-APHIS 2014b).

C. Description of Inserted Genetic Material, Its Inheritance and Expression, Gene Products, and Changes to Plant Metabolism

To inform the potential hazards resulting from the genetic modification and potential routes of exposure related to the inserted DNA and its expression products, APHIS assessed data and information presented in the extension request related to the similarity of the PG451 to the antecedent GD743 and GS784 apple events, including the transformation process; the source of the inserted genetic material and its function in both the donor organism and the PG451; and the integrity and number of loci inserted. The stability and mode of inheritance of the inserted genetic material through sexual or asexual reproduction have been determined for the GD743 and GS784.

APHIS also assessed data presented in the extension request on whether the genetic modification results in the expression of new genes, proteins, or enzymes, or is likely to result in changes in plant metabolism or composition in PG451, comparatively to the antecedents GD743 and GS784.

The similarity assessment encompassed a consideration of the expression of the PPO suppression transgene and any observed or anticipated effects on plant metabolism including any potential relevant changes in levels of metabolites, anti-nutrients, or nutrients in apples derived from PG451, based on comparisons with the antecedents GD743 and GS784, for which comparisons were made to published nutritional data for apple (NDB09003) provided by the USDA National Nutrient Database for Standard Reference – Release 22 (2009) as referenced in the petition submitted by OSF (OSF 2012).

Description of the genetic modification and inheritance of inserted DNA

According to the request for extension of nonregulated status, PG451 apple was generated through *Agrobacterium*-mediated transformation of leaf explants from apple cultivar Gala with the binary vector GEN-03. The genetic elements contained in the

vector T-DNA are the RB and LB sequences derived from *Agrobacterium tumefaciens* pTIT37 (Depicker et al. 1982), sequences for the transgene PGAS and the coding sequence for *nptII*, as well as regulatory sequences. PGAS is a chimeric PPO suppression sequence comprised of fragments of four apple PPO genes (PPO2, GPO3, APO5 and pSR7), designed to suppress the entire apple PPO gene family via RNA silencing (OSF 2020). Transcription of PGAS is directed by the duplicated-enhancer CaMV 35S promoter from *Cauliflower Mosaic Virus* with the untranslated leader sequence from *Alfalfa mosaic virus* RNA4 (P70) (Datla et al. 1992). A 3' untranslated region (UTR) from the *A. tumefaciens* nopaline synthase gene (T_{NOS}) involved in transcription termination and polyadenylation, is used to terminate transcription of the transgene (Depicker et al. 1982; Bevan et al. 1983a). The neomycin phosphotransferase type II gene (*nptII*) from *Escherichia coli* transposon Tn5 was used for expression of a selectable marker for resistance to kanamycin (Rothstein et al. 1981). The *nptII* gene is under the control of the nopaline synthase promoter (P_{NOS}) (Bevan et al. 1983b), and the terminator (T_{NOS}), (Depicker et al. 1982) both are derived from *A. tumefaciens* (OSF 2020, p. 14 Table 2: Components of the GEN-03 Vector DNA Used to Develop PG451).

This same vector pGEN-03 was used in the transformation of the parental varieties Golden Delicious and Granny Smith to develop the antecedent apple events GD743 and GS784. The same pGEN-03 was also used to develop NF872, a non-browning Fuji variety described in a previous request for extension (16-004-01p) of non-regulated status from GD743 and GS784 (OSF 2016). A Plant Pest Risk Similarity Assessment (PPRSA) and a Finding Of No Significant Impact (FONSI) were prepared for the non-browning NF872, and it was granted non-regulated status in 2016 (USDA-APHIS 2016b, a).

Molecular characterization of PG451 was performed using low coverage (25X) short read Illumina HiSeq2500 whole genome sequencing of genomic DNA from PG451, and a mapping strategy based on the whole genome sequencing data. Reads were sorted into three read pools that matched one of the following:

1. The GEN-03 vector;
2. Apple reference genomes HFTH1, GDDH13, v3.0.a1 and v1.0 combined haplotypes available at the Genome Database for Rosaceae;
3. Junction sites, either vector to genome insertion site or vector to vector junctions associated with structural rearrangements.

The reads mapping to junction sites were used to develop insertion maps and identify insertion sites in PG451 (OSF 2020, p 16, 18, 19 Figure 3: Workflow for Detection of T-DNA Insertions, and pp 20-21).

Whole genome sequencing data shows evidence of two complex GEN-03 insertion sites within the PG450 genome, one in chromosome10 and one in chromosome 17. There are five full-length copies of the T-DNA and a structural rearrangement which results in two copies of the PGAS suppression gene forming an inverted repeat, necessary to induce RNA interference. There is also readthrough at the LB of the insertion assigned to chromosome 10 resulting in one full length and one partial length copy of the vector backbone (OSF 2020, pp 13-15, p 27 Figure 14: Chromosome 10 Insertion).

Consequently, in addition to the sequences of interest for expression of the non-browning phenotype and for kanamycin resistance a full copy of the vector backbone is present in the genome of PG451.

The vector backbone sequence in PG451 comprises sequences of prokaryotic elements:

- *trfA*, gene with two protein products that bind *oriV* to promote replication from pRK2 (Frisch et al. 1995).
- *nptIII*, neomycin phosphotransferase type III from *Streptococcus faecalis* R plasmid providing resistance to kanamycin (Scutt et al. 2002).
- ColE1, RNA origin of replication used to increase the plasmid copy number, obtained from pBR322 (van Engelen et al. 1995).
- *nptIII* 5', upstream elements (Frisch et al. 1995).
- *oriV*, origin of replication that functions in *A. tumefaciens* and *Escherichia coli* from pRK2 (Frisch et al. 1995).

The remaining vector backbone sequences incorporated in PG451 genome were derived from vectors used in the development of the binary transformation vector GEN-03. These were fragmented in the cloning process for development of GEN-03 and are considered to be non-functional. The binary vector GEN-03 does not contain a functional tetracycline resistance determinant, and therefore a functional gene for resistance to tetracycline was not transferred into the parental variety Pacific Gala during the development of PG451 (OSF 2020).

The presence of the entire vector backbone sequence in PG451 is a relevant difference between PG451 and the antecedents GD743 and GS784 described in the petition 10-161-01p. No backbone sequences were identified in the genomes of either GD743 or GS784 using the Southern blot method (OSF 2020).

NF872 non-browning apple, subject of the previous extension request (16-004-01p) included an insertion of approximately 1,400 bp of vector backbone sequence with no functional sequences in chromosome 3 (OSF 2016).

Expression of inserted DNA and changes in gene expression, new proteins or metabolism

PG451 has been developed using genetic engineering with a PPO suppression construct designed to reduce the expression of four apple genes coding for PPO proteins: PPO2, GPO3, APO5, and pSR7 (OSF 2020). The product of this construct is a chimeric, sense-silencing RNA rather than a functional protein or new enzyme. This is the same construct used in the antecedents GD743 AND GS784 (OSF 2012).

Based on whole genome sequence analysis, the insertions identified in PG451 are not located within any predicted endogenous coding or regulatory regions of the PG451 genome (OSF 2020, p 22 Figure 8: PG451 - Chromosome 10 - Genomic Location, p 23 Figure 9: PG451 - Chromosome 17 - Genomic Location). No additional open reading

frames were identified as having been introduced in the inserted sequences or generated as a consequence of structural rearrangements that align with known allergens. Assessments of the potential toxicity of inserted sequences were performed by sequence analysis using blastp searches and alignments of open reading frames against the National Center for Biotechnology Information (NCBI) non-redundant protein databases. Manual searches of alignments did not indicate the presence of proteins described as toxic or as toxins. The entire backbone sequence was included in the ORF analysis.

The *nptII* gene was used for expression of nopaline synthase as a selectable marker for selection of apple transformants. The FDA has previously identified the safe use of the *nptII* gene for the development of genetically modified cotton, oilseed rape and tomatoes for food and feed purposes (21 CFR 173.170 1994; 21 CFR 573.130 1994; OSF 2020).

The polyphenol oxidase suppression gene is under the control of a constitutive promoter designed to suppress PPO activity in all parts of the plant. PG451 was identified as PPO suppressed through a repeated screening process at various developmental stages by OSF. Polyphenol oxidase (PPO) enzyme activity studies were conducted to determine the level of enzymatic activity in PG451 relative to the Pacific Gala untransformed control. Samples of tissue culture leaves and leaves from greenhouse plants were used for these studies (OSF, 2020). For all tissues tested there was reduced PPO activity in PG451 compared to the untransformed control. In tissue culture leaves, the PPO activity was reduced by 90% (OSF 2020, p 32 Table 5: PPO Activity in PG451 - Tissue Culture Leaves). However, in tests of PPO activity in leaves collected from grafted PG451 grown in greenhouse facilities, PPO activity was observed to be 74% in PG451 relative to its control when grafted apple trees were grown in the greenhouse. (OSF 2020, p 32, Table 6: PPO Activity in PG451 - Greenhouse Leaves).

Controlled experiments using an impact device were used to further verify reduction of PPO activity and enzymatic browning in response to mechanical bruising of mature fruit of PG451 relative to the untransformed control. The level of bruising response is reported as “Total Change in Color” between bruised and non-bruised tissue as measured using a Minolta Chroma Meter CR-400, following bruising of mature fruit using an impact device. A reduced bruising response was observed on PG451 apples relative to apples from the untransformed parent cultivar PG, further demonstrating success of the genetic transformation resulting non browning phenotype (OSF 2020, p 33 Table 7: Controlled Bruising of PG451 - Mature Fruit).

No compositional and nutritional evaluations were performed to determine whether PG451 apples are equivalent to apples of the untransformed control and to the published nutritional data for raw apple with skin (NDB09003 reference standard) provided by the USDA (2009). However, the data resulting from compositional and nutritional evaluations have been previously provided for the antecedents GD743 and GS784 (OSF 2012). The results of compositional and nutritional studies in the petition 10-161-01p indicated that apple cultivars GD743 and GS784 are in all aspects (proximates, phenolics antioxidants and vitamin C) compositionally and nutritionally equivalent to the published norms for apple (OSF 2012).

Additionally, apples from event NF872 (subject of a previous extension request) and the control NF were subjected to nutritional and proximate analysis and measured for total phenolic content. The composition of NF872 and of the NF control fall within the range of published data (OSF 2016).

Based on data provided for three Arctic[®] apple cultivars that previously received the determination of nonregulated status by APHIS, and the molecular data provided in the extension request, the cultivar PG451 is likely to be nutritionally equivalent to the untransformed parental cultivar.

APHIS reviewed the information provided by OSF in the extension request and determined the following:

- There are two complex T-DNA insertions with multiple GEN-03 fragments in two chromosomes, chromosomes 10 and 17 in PG451 (OSF, 2020).
- The insertion assigned to chromosome 10 includes the entire vector backbone sequence, with multiple elements that are expressed in bacteria and are unlikely to be expressed in plants. No backbone sequences were reported in the antecedent events GD743 and GS784.
- A significant reduction in PPO activity was observed in PG451 over the control Pacific Gala, in leaves from tissue culture and in leaves collected from plants growing in the greenhouse. PPO suppression was demonstrated in controlled bruising experiments using mature apple fruit of PG451 and the Pacific Gala control. These observations were similar to those of the antecedent events.

D. Potential Plant Pest and Disease Impacts

APHIS assessed data and information presented in the extension request related to the similarity of PG451 to the antecedents GD743 and GS784 to determine whether potential plant pest or disease impacts are likely to result from the transformation process, from DNA sequences from plant pests, or from any other expression products, new enzymes, proteins or changes in plant metabolism or composition in PG451 that are known or anticipated to cause disease symptoms, or to affect plant pests or diseases or plant defense responses. APHIS also assessed whether PG451 is more likely to have significantly increased disease and pest susceptibility as compared to the antecedents GD743 and GS784. Impacts or changes in similarity to GD743 and GS784 were assessed to determine if they would (1) affect and/or result in significant introduction or spread of a damaging pest or disease to other plants; (2) result in the introduction, spread, and/or creation of a new disease; and/or (3) result in a significant exacerbation of a pest or disease for which APHIS has a control program.

Plant Protection and Quarantine (PPQ) is an APHIS program that safeguards agriculture and natural resources from the entry, establishment, and spread of animal and plant pests and noxious weeds into the United States; and supports trade and exports of U.S. agricultural products. PPQ responds to new introductions of plant pests to eradicate, suppress, or contain them through various programs in cooperation with state

departments of agriculture and other government agencies. These may be emergency or longer term domestic programs that target a specific pest, and there are a variety of insect, plant disease, mollusk, nematode and weed programs in PPQ (USDA-APHIS 2020). However, none specifically target pests of PG451 apple.

Because PG451 was obtained using the same transformation vector pGEN-03 carrying the PPO suppression transgene that was used for transformation of the antecedent events GD743 and GS784, and also because no significant changes in composition were detected from the expression of the PPO suppression transgene in GD743 and GS784, no significant changes in composition are expected from the expression of pGEN-03 in the PG451.

The petition 10-161-01p describing the antecedent events, provided plant pest and disease data for them. Based on extensive monitoring and pest and disease incidents, the petition concluded that in a commercial orchard setting GD743 and GS784 events were not systematically more susceptible to plant pests and diseases (OSF 2020). The PPRA prepared by APHIS on the same antecedent events GD743 and GS784 Arctic[®] apples concluded that they are unlikely to pose a plant pest risk (USDA-APHIS 2014b). PG451 is not expected to differ from the antecedents or the untransformed Pacific Gala in its ability to harbor or transmit plant pathogens or pests and cause indirect plant pest effects on other agricultural products. Particularly when cultivated in highly managed orchards with the standard stewardship conditions under which it is intended to be cultivated.

E. Potential Impacts on Non-Target Organisms Beneficial to Agriculture

APHIS has previously evaluated the potential impacts on non-target organisms beneficial to agriculture that could result from the deregulation of the OSF antecedents GD743 and GS784. The OSF antecedents GD743 and GS784 were determined by APHIS to be unlikely to have an adverse effect on non-target organisms in the environment (USDA-APHIS 2014b). The genetic construct pGEN-03 used to transform the deregulated varieties contains the transgene (PGAS) designed to simultaneously reduce expression of four polyphenol oxidase genes for PPO2, GPO3, APO5 and pSR7 to induce a non-browning phenotype in apple fruit.

Therefore, based on the high similarity of the PG451 event to the OSF antecedents GD743 and GS784, the unlikely impacts of non-target effects due to RNAi, and on the finding that the GD743 and GS784 were unlikely to harm non-target organisms. APHIS concludes that it is unlikely that PG451 event will have an adverse effect on non-target organisms, including those beneficial to agriculture.

F. Potential for Enhanced Weediness of PG451 Apple

As documented in the PPRA prepared for the OSF antecedents GD743 and GS784, they are no more likely to be a weed when compared to the conventional apple. Cultivated apple is not regarded as a weedy species although seedlings can be persistent and the

species has escaped cultivation and naturalized in the U.S. (CFIA 2014). Apple is a highly domesticated fruit tree species and cultivated varieties in the U.S are not listed as weeds or as Federal Noxious Weeds (7 CFR 360 2010; USDA-NRCS 2020).

Although *M. domestica* trees are occasionally found growing outside of cultivation, and naturalized growing in abandoned pastures, clearings, roadsides and borders of woods, the domestic apple possesses few of the characteristics of plants that are notably successful as weeds (Baker 1974). Apple seeds have persistent dormancy and require specific environmental conditions for germination, chilling and humidity, which are expected to happen in Spring. Apple seed dormancy results from embryonic dormancy that is only completely removed by chilling (cryogenic dormancy), and coat imposed inhibition (testa paradormancy) which limits the availability of water for germination (Lewak 2010). Symptoms of embryonic dormancy are eliminated by several weeks of stratification, treatment with moisture and cold (Maciejewska and Lewak 2006). Seeds from discarded fruit, or from fruit moved by animals produce seedling trees that have a long juvenile growth phase before producing flowers that can be pollinated and produce fruit and seeds. Apple trees have long generation time, from three to eight years and sometimes longer (Ignatov and Bodishevskaya 2011). Environmental conditions outside of highly managed orchards are not the ideal conditions for establishment of apple trees. In commercial cultivation *M. domestica* is propagated by grafting a scion with desirable fruit characteristics onto a rootstock that is selected for specific properties such as dwarfing and disease resistance. It is a labor intensive, highly managed crop, and volunteer plants are rare due to the perennial nature of the crop and orchard management practices that include treatment with herbicide and mowing of the alley between rows. (CFIA 2014).

In addition to considerations of the known biology of apple, APHIS analyzed information submitted in the petition for the antecedent organisms on a suite of agronomic characteristics and plant-disease and plant-insect interactions. This agronomic data from the field showed that the antecedents were not different from their non-transgenic comparators. The assessments concluded that the antecedents GD743 and GS784 were unlikely to become weeds. Based on the biology of the plant and the high similarity of the PG451 event to the GD743 and GS784 modified with the same construct and expressing similar proteins, and on the finding that GD743 and GS784 were unlikely to become weeds, APHIS concludes that it is unlikely that PG451 will become a weed.

In addition to the above, the OSF petition 10-161-01p for the deregulation of GD743 and GS784 mentions that Arctic[®] Apple cultivars will be royalty bearing and the propagation, planting area, cultivation, packing and selling of these cultivars and their trees will be carefully monitored and tracked. OSF intends to license these cultivars to a very limited number of integrated producer/packer/sellers, to create a managed marketing environment, and that the trees will not be available for widespread commercial distribution, nor for backyard and small-scale plantings (OSF 2012). As PG451 is another variety of Arctic[®] Apple, the same management practices intended for GD743 and GS784 will be applicable to PG451.

G. Potential Impacts on the Weediness of Any Other Plants with which the PG451 Apple Can Interbreed

APHIS evaluated the potential for gene introgression to occur from the antecedents GD743 and GS784 to sexually compatible wild relatives and considered whether such introgression would result in increased weediness.

The domestic apple, *Malus domestica*, is considered an interspecific hybrid complex (Lubby 2003), with well-known capacity to hybridize and many named hybrids (Kron and Husband 2009). Apple cultivars are highly heterozygous hybrids from intra or interspecific hybridizations and are propagated vegetatively. Most apple cultivars are diploid, $2n=34$ such as Gala, Golden Delicious, Granny Smith and Fuji. Some cultivars are triploid ($2n=51$) and a few are tetraploids (Janick et al. 1996). Severe inbreeding depression in self-pollinated progenies is attributed to the hybrid nature of the plants. Fertile hybrids are obtained from most pollination combinations between different *Malus* species (Korban 1986) and interspecific hybrids were widely used for the improvement of commercial apple cultivars. Some wild apples are suggested to be natural hybrids and can be successfully propagated by seeds (Ignatov and Bodishevskaya 2011).

The Plants Database included 36 species in the genus *Malus* (USDA-NRCS 2020), including the cultivated and crab apples. Many species and hybrids have been introduced and are used as ornamentals, and some have escaped and become naturalized. The following are present in the United States: *Malus angustifolia*, *Malus x arnoldiana* (*baccata x floribunda*), *M. baccata*, *M. coronaria*, *Malus x dawsoniana* (*fusca x pumila*), *M. floribunda*, *M. fusca*, *M. halliana*, *M. hupehensis*, *M. ioensis*, *Malus x magdeburgensis* (*pumila x spectabilis*), *Malus x platycarpa* (*coronaria x pumila*), *Malus x purpurea* (*atrosanguinea x niedzwetzkyana*), *M. prunifolia*, *M. pumila* (*syn M. domestica*), *Malus x robusta*, *M. sargentii*, *M. spectabilis*, *Malus x soulardii* (*ioensis x pumila*), *M. sylvestris*, *M. toringo*, *M. zumi*. The *Malus* species native to the United States are: *M. angustifolia* (southern crab apple), *M. coronaria* (sweet crab apple), *M. diversifolia* (*syn M. fusca*) (Oregon crab apple), *M. ioensis* (prairie crab apple) and *M. glabrata* (Biltmore crab apple). Although *Malus x zumi* and *M. floribunda* have been described to be weedy under trees where starlings are known to roost in Ohio (Vincent and Cusick 1989), none of the species and hybrids above is listed as a noxious weed on Federal or State weed lists (7 CFR 360 2010; USDA-NRCS 2020).

There is the potential for gene flow, hybridization and/or introgression of the introduced genetic material from cultivated PG451 to native *Malus* species with synchronous flowering in North America. In a natural population of *M. coronaria* in which feral *M. domestica* trees were prevalent, hand pollination of *M. coronaria* with *M. domestica* pollen, and collection of seeds from *M. coronaria* demonstrated the presence of triploid seeds with markers from both species. Seeds of other ploidies (pentaploid and tetraploid) were also observed. The high proportion of hybrid seeds suggests that there is potential for gene flow from domestic apples to native *M. coronaria* populations. However no studies were performed to establish the fitness of plants from the hybrid seeds (Kron and Husband 2009).

Studies of apple pollination have shown that cross pollination occurred mostly between trees that are in close proximity, with a median observed pollination distance of

approximately 23 m (75 ft). A few studies noted that long distance pollinations increased the average pollination distance to approximately 60 m (approx. 197 ft). In natural populations, pollination at distances above 50 m (164 ft) is likely and above 300 m (984 ft) may occur at low frequency. Pollination at low frequency for a pollination distance of more than 500 m (1,640 ft) must be expected (Larsen and Kjær 2008). In another study pollen was dispersed at least 104m (341 ft) (Reim et al. 2006).

M. domestica can escape cultivation and be spread by animals, including people, and may become naturalized under certain conditions. Although hybridization between cultivated apples and crab apples species can occur when they are grown together, APHIS concluded that even if such introgression were to occur, *Malus* species and hybrids are not considered to be weeds.

According to information provided in this extension request and in the antecedent OSF petition 10-161-01p, the risk of trait out-crossing and pollen gene flow from Arctic[®] Apple is low and can be readily managed. Arctic[®] Apple growers will be provided with stewardship guidelines as part of licensing requirements and these will be monitored for compliance by OSF. Stewardship obligations will include aspects related to beehives used for pollination and the use of suitable isolation distances. In addition Arctic[®] Apples can be identified at all steps along the value chain, using a bioassay, PCR test or by DNA fingerprinting (OSF 2012, p.104).

APHIS concluded that the genes inserted into PG451, which are the same as in the deregulated GD743 and GS784, are unlikely to impact the weediness of wild *Malus* species, since the antecedent GD743 and GS784 do not exhibit characteristics that cause them to be any weedier than other cultivated apples. Therefore, PG451 is not expected to increase the weed risk potential of other species with which it can interbreed in the U.S. and its territories based on apple biology and the similarity to GD743 and GS784.

H. Potential Changes to Agriculture or Cultivation Practices

APHIS assessed whether significant changes to agricultural or cultivation practices from the antecedent GD743 and GS784 apples are likely to impact plant diseases or pests or their management, including any APHIS control programs. This included consideration of any changes in pesticide applications, tillage, irrigation, harvesting, etc. as they relate to plant pests and diseases.

APHIS did not identify any significant changes to agricultural or cultivation practices (e.g. pesticide applications, tillage, irrigation, harvesting, rotations, management of volunteers, etc.) caused by the cultivation of GD743 and GS784 apples and concluded that no impact on plant diseases or pests or their management is likely to occur.

Based on the similarity of PG451 to the antecedents GD743 and GS784, APHIS concludes that it is unlikely that any significant changes to agriculture or cultivation practices would be associated with PG451 and therefore no impact on plant diseases or pests of their management is likely to occur.

I. Potential Impacts from Transfer of Genetic Information to Organisms with which the PG451 Apple Cannot Interbreed

APHIS has previously examined the potential for the genetic material inserted into GD743 and GS784 to be horizontally transferred without sexual reproduction to other organisms and whether such an event could lead directly or indirectly to disease, damage, injury or harm to plants, including the creation of new or more virulent pests, pathogens, or parasitic plants. The horizontal gene transfer (HGT) between unrelated organisms is one of the most intensively studied fields in the biosciences since 1940, and the issue gained extra attention with the release of transgenic plants into the environment (Droge et al. 1998). Potential risks from stable horizontal gene transfer (HGT) from organisms developed using genetic engineering to another organism without reproduction or human intervention were recently reviewed (Keese 2008). Mechanisms of HGT include conjugation, transformation and transduction, and other diverse mechanisms of DNA and RNA uptake and recombination and rearrangement, most notably through viruses and mobile genetic elements. APHIS has previously reviewed the potential for horizontal gene transfer from GE apple to bacteria, fungi, invertebrates, viruses, and parasitic plants (USDA-APHIS 2014b).

APHIS previously concluded that HGT of the inserted genetic material from the OSF antecedent GD743 and GS784 apples to other organisms is highly unlikely, and is not expected to lead directly or indirectly to disease, damage, injury or harm to plants, including the creation of new or more virulent pests, pathogens, or parasitic plants.

A relevant difference between the antecedents GD743 and GS784, and PG451, is the presence of a full copy of the vector backbone sequence in PG451, which is not present in GD743 or in GS784 (OSF 2020). According to the petition 10-161-01p submitted by OSF, Southern blotting data, combined with Mendelian Inheritance data indicated that there are two unlinked insertions of the transfer T-DNA in GD743, while GS784 has four unlinked insertions of the T-DNA. Southern blots also confirmed that there was no evidence for the inclusion of a complete copy of the vector backbone in either GD743 or GS784 (OSF 2012). However, the analysis of inserted sequences in PG451 was performed using high throughput sequencing, and a full copy of the vector backbone was found to have been transferred to the PG451 genome, unlike the antecedent organisms. The vector backbone comprises a number of prokaryotic elements that will not be expressed in planta (OSF 2020).

Therefore, APHIS concludes based on the similarity of these events, the data presented in the petition and review of the pertinent literature that HGT from the PG451 to other organisms is highly unlikely.

J. Conclusion

APHIS has reviewed the information submitted in the extension request, supporting documents, and other relevant information to assess the similarity of plant pest risk of

PG451 compared to the OSF antecedents GD743 or GS784 (OSF 2012). APHIS concludes that PG451 is unlikely to pose a greater plant pest risk than the previously deregulated antecedent events Arctic[®] apple GD748 and GS784.

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L. Similarity Table

Description	Extension Request PG451 20-213-01ext	Antecedent GS784 and GD743 Petition 10-161-01p	Comments
Organism	Apple	Apple	
Phenotype	Reduced Browning	Reduced Browning	Same phenotype
Genotype	<p><u>PPO inhibitor cassette:</u></p> <p><i>P70</i> - the duplicated-enhancer cauliflower mosaic virus (CaMV 35S) promoter with an untranslated leader sequence from alfalfa mosaic virus RNA4 that directs transcription of the PGAS transgene. From Cauliflower mosaic virus and Alfalfa mosaic virus</p> <p>PGAS - a chimeric suppression transgene comprising fragments of four apple polyphenol oxidase (PPO) genes (PPO2, GPO3, APO5 and pSR7).</p>	<p><u>PPO inhibitor cassette:</u></p> <p><i>P70</i> - the duplicated-enhancer cauliflower mosaic virus (CaMV 35S) promoter with an untranslated leader sequence from alfalfa mosaic virus RNA4 that directs transcription of the PGAS transgene. From Cauliflower mosaic virus and Alfalfa mosaic virus</p> <p>PGAS - a chimeric suppression transgene comprising fragments of four apple polyphenol oxidase (PPO) genes (PPO2, GPO3, APO5 and pSR7).</p>	<p>Same genes and regulatory elements,</p> <p>PGAS consists of 394 to 457 bp regions of four apple polyphenol oxidase (PPO) genes (PPO2, GPO3, APO5, and pSR7) in tandem that upon transcription the transgene is designed to suppress the expression of these four members of the apple PPO gene family. The genes are derived from <i>Malus domestica</i></p>

	<p>From <i>Malus domestica</i></p> <p><i>Tnos</i> - a terminator consisting of the 3'untranslated region (3'UTR) from the nopaline synthase gene involved in transcription, termination and polyadenylation. From <i>Agrobacterium tumefaciens</i>.</p>	<p>From <i>Malus domestica</i></p> <p><i>Tnos</i> - a terminator consisting of the 3'untranslated region (3'UTR) from the nopaline synthase gene involved in transcription, termination and polyadenylation. From <i>Agrobacterium tumefaciens</i>.</p>	
	<p><u>Selectable marker cassette</u></p> <p><i>Pnos</i> - a nopaline synthase promoter that directs transcription of the <i>nptII</i> selection marker. From <i>Agrobacterium tumefaciens</i></p> <p><i>nptII</i> - gene for neomycin phosphotransferase type II from Tn5 providing resistance to kanamycin. From <i>Escherichia coli</i></p> <p><i>Tnos</i> - a terminator consisting of the 3'untranslated region</p>	<p><u>Selectable marker cassette</u></p> <p><i>Pnos</i> - a nopaline synthase promoter that directs transcription of the <i>nptII</i> selection marker. From <i>Agrobacterium tumefaciens</i></p> <p><i>nptII</i> - gene for neomycin phosphotransferase type II from Tn5 providing resistance to kanamycin. From <i>Escherichia coli</i></p> <p><i>Tnos</i> - a terminator consisting of the 3'untranslated region</p>	Same genes and regulatory elements

	(3'UTR) from the nopaline synthase gene involved in transcription, termination and polyadenylation. From <i>Agrobacterium tumefaciens</i> .	(3'UTR) from the nopaline synthase gene involved in transcription, termination and polyadenylation. From <i>Agrobacterium tumefaciens</i> .	
	<u>Backbone Sequences</u> <i>trfA</i> - Gene with two protein products that bind oriV to promote replication from pRK2. <i>nptII</i> - Neomycin phosphotransferase type III from <i>Streptococcus faecalis</i> R plasmid providing resistance to kanamycin. From <i>S. faecalis</i> ColE1 - RNA origin of replication used to increase the plasmid copy number, obtained from pBR322. <i>nptIII</i> – <i>nptIII</i> 5' upstream elements. oriV – Origin of replication that functions in	<u>Backbone Sequence</u> No backbone sequences detected using Southern blot	The elements in the backbone sequences are prokaryotic elements that support plasmid replication and selection during the transformation process.

	<p><i>Agrobacterium tumefaciens</i> and <i>Escherichia coli</i> from pRK2.</p> <p>Sequences from non-functional elements that were fragmented during the cloning process.</p>		
Transformation Method	<i>Agrobacterium tumefaciens</i> -mediated	<i>Agrobacterium tumefaciens</i> -mediated	Same
Insert and Copy Number	<p>Short read Illumina (HiSeq 2500) whole genome sequencing data and junction mapping analysis revealed evidence of two insertions, one in chromosome 10 and one insertion in chromosome 17. There are five full length copies of the T-DNA</p>	<p>Southern data, combined with Mendelian Inheritance data indicated that GD743 arises from two unlinked insertions, with two copies of the T-DNA.</p> <p>The same data indicated that GS784 arises from four unlinked insertions with multiple copies of the T-DNA</p>	<p>Proposed insertional positions and placement speculative from data obtained from Southern and PCR, as described in the antecedent petition.</p> <p>Low coverage (25X), short read Illumina (HiSeq 2500) whole genome sequencing data and junction mapping analysis were used to map and describe the insertions in PG451 genome, as described in the extension request.</p>
Compositional analysis	No data was provided,	Compositionally equivalent to the untransformed parental varieties and to composition data of conventional apple.	

Backbone Absent	No.	Yes.	<p>Vector backbone sequences were not detected using Southern Blot technique on genomic DNA from the antecedents GS784 and GD743.</p> <p>The entire pGEN-03 vector including backbone sequence, is present in PG451 according to data obtained using short read Illumina (HiSeq 2500) whole genome sequencing data and junction mapping analysis.</p>
Mechanism of Action	RNA interference via insertion of the construct PGAS, a chimeric PPO suppression transgene comprising fragments of four apple polyphenol oxidase (PPO) genes (PPO2, GPO3, APO5 and pSR7).	RNA interference via insertion of the construct PGAS, a chimeric PPO suppression transgene comprising fragments of four apple polyphenol oxidase (PPO) genes (PPO2, GPO3, APO5 and pSR7).	<p>The same vector pGEN-03 for suppression of polyphenol oxidases (PPO) and non-browning upon injury via RNA interference was used.</p> <p>The sequences and sources of the four PPO gene sequences in <i>PGAS</i> are described in the Patent application “Genetically modified reduced-browning fruit-producing plants and produced fruit thereof, and method of obtaining such” (Armstrong and Lane, 2009)</p>
Date of antecedent EA/ EIS	N/A	EA on February 2015	
Plant Pest Risk			
Disease and pest susceptibilities	Based on the similarity assessment PG451 is unlikely to pose a greater plant pest risk than the antecedents.	Unlikely to change disease and pest susceptibilities	

Impacts on beneficial non-targets	Based on the similarity assessment PG451 is unlikely to have greater impact on beneficial non-target organisms than the antecedents.	Unlikely to impact beneficial non-target organisms	
Enhanced weediness	Based on the similarity assessment PG451 is unlikely to have enhanced weediness as the antecedents.	Unlikely to enhance weediness	
Enhanced weediness of relatives	Based on the similarity assessment PG451 is unlikely to enhance weediness of relatives compared as the antecedents.	Unlikely to enhance weediness of relatives	
Changes to agriculture or cultivation practices	Based on the similarity assessment, and on established practices for apple orchards, PG451 is unlikely to cause changes to agriculture or cultivation. OSF intends to use same practices and stewardship for Arctic® apple varieties.	Unlikely to change agriculture or cultivation practices	
Horizontal Gene Transfer	Based on the similarity assessment PG451 is unlikely to increase the risk of horizontal gene transfer.	Unlikely to affect the probability of horizontal gene transfer	

Plant Pest Risk	Based on the similarity assessment PG451 is unlikely to pose a greater plant pest risk than the antecedents or the untransformed variety.	Unlikely to pose a greater plant pest risk than the untransformed varieties.	
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