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Request for an Extension of Determination of Nonregulated Status for Non-Browning Arctic® Apple Event PG451

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Author

John Armstrong, Ph.D.

Contributors

Mathieu Dubeau

CERTIFICATION

The undersigned certifies, that to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes all relevant data and information known to the petitioner which is unfavorable to the petition.

Neal Carter

Neal Carter
President

Okanagan Specialty Fruits Inc.
15304 Prairie Valley Road
Summerland, BC V0H 1Z8
Canada

Submitted by:

Okanagan Specialty Fruits Inc.
15304 Prairie Valley Road
Summerland, BC V0H 1Z8
Canada

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September 29, 2020

The undersigned submits this petition under 7 CFR 340.6 to request that the Administrator, make a determination that the article should not be regulated under 7 CFR part 340.

Neal Carter
President

RELEASE OF INFORMATION

The information in this petition is being submitted by Okanagan Specialty Fruits Inc. (OSF) for review by USDA as part of the regulatory process. By submitting this information, OSF does not authorize its release to any third party except to the extent it is requested under the Freedom of Information Act (FOIA), 5 U.S.C. Section 552, and 7 CFR 1, covering all or some of this information. OSF understands that a copy of this information may be made available to the public, as part of a public comment period. Except in accordance with FOIA, OSF does not authorize the release, publication or other distribution of this information without OSF's prior notice and consent.

SUMMARY

Okanagan Specialty Fruits Inc. (OSF) requests that APHIS extend a determination of nonregulated status to Arctic® apple event PG451 based on similarity to Arctic® apple events GD743 and GS784 described in petition 10-161-01p. The subjects of petition 10-161-01p Arctic® Golden (Event GD743) and Arctic® Granny (Event GS784) received a determination of non-regulated status on February 13, 2015.

For clarity, Arctic® apple event PG451, to be marketed under the name Arctic® Gala will be referred to as PG451 for the remainder of the document. Similarly, deregulated Arctic® apple events GD743 and GS784 currently marketed as Arctic® Golden and Arctic® Granny will be referred to GD743 and GS784, respectively.

PG451 was produced by *Agrobacterium*-mediated transformation of the apple cultivar Gala (PG) with the binary vector GEN-03. The GEN-03 vector comprises two functional eukaryotic genes. The first is a chimeric PPO suppression transgene consisting of partial coding sequences of four members of the apple PPO gene family (PPO2, GPO3, APO5 and pSR7) in the sense orientation under control of the cauliflower mosaic virus promoter and nopaline synthase terminator. This transgene is designed to reduce expression of the entire PPO gene family and to induce a nonbrowning phenotype in apple fruit. The second gene is an *nptII* selection marker used in the development of PG451 apple. The antecedent organisms, GD743 and GS784 were also generated by *Agrobacterium*-mediated transformation with the GEN-03 vector.

In PG451, as in the antecedent organisms GD743 and GS784, both transgenes were transferred to the recipient cultivar in functional form. The PPO suppression transgene resulted in reduced levels of polyphenol oxidase (PPO) and a non-browning phenotype typical of all Arctic® apple cultivars. The *nptII* selection marker permitted the identification of PG451 during the transformation process. The level of NptII protein in PG451 fruit was not measured here. However, in the antecedent organisms GD743 and GS784 and in another cultivar Arctic® Fuji (Event NF872) developed using the GEN-03 vector; we determined that the amount of NptII protein in mature fruit was below the limit of detection by ELISA.

In the development of PG451 a full copy of the vector backbone was transferred into the genome, unlike the antecedent organisms. The vector backbone comprises a number of prokaryotic elements that will not be expressed *in planta* and do not affect the safety assessment.

OSF submits that the PG451 apple, described in this petition, was transformed using the same binary vector as events GD743 and GS784, yielding the same non-browning phenotype. Based on the similarity to these antecedent organisms OSF has concluded that previous analysis of impacts completed for GD743 and GS784 are relevant to APHIS's regulatory action in responding to the OSF extension for PG451 apple.

ACRONYMS AND SCIENTIFIC TERMS

APHIS	Animal and Plant Health Inspection Service
bp	base pairs
CaMV 35S	Cauliflower Mosaic Virus 35s
CFIA	Canadian Food Inspection Agency
CFR	Code of Federal Regulations
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FARRP	Food Allergy Research and Resource Program
GD	Golden Delicious
GD743	Arctic® Golden (Event GD743)
GS	Granny Smith
GS784	Arctic® Granny (Event GS784)
LB	left border
mg	milligram
ml	milliliter
N	number of units in a population
n	number of units in a sample
NCBI	National Center for Biotechnology Information
NF	a non-patented strain of Fuji
NF872	Arctic® Fuji (Event NF872)
nptII	neomycin phosphotransferase II gene
NptII	neomycin phosphotransferase II protein
NOS	nopaline synthase
OSF	Okanagan Specialty Fruits Inc.
PG	a patented strain of Gala
PGAS	a chimeric suppression sequence comprising fragments of four apple PPO genes (PPO2, GPO3, APO5 and pSR7)
PG451	Arctic® Gala (Event PG451)
PPO	polyphenol oxidase
P70	duplicated-enhancer CaMV 35S promoter
RB	right border
RNA	ribonucleic acid
S	standard deviation
T-DNA	transfer DNA
USA	United States of America
USC	United States Code
USDA	United States Department of Agriculture
UTR	untranslated region

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1 RATIONALE FOR DEVELOPMENT OF PG451 APPLE

Okanagan Specialty Fruits Inc. (OSF) has developed PG451 apple (*Malus x domestica)*, a new cultivar that has been genetically engineered to be non-browning. PG451 apple was developed through *Agrobacterium*-mediated transformation to stably incorporate the transgenes PGAS and *nptII* into the apple genome. The PGAS gene is a sense suppression gene designed to suppress the apple polyphenol oxidase (PPO) gene family. The *nptII* gene encodes the enzyme neomycin phosphotransferase (NptII) from *Escherichia coli* Tn5. NptII inactivates kanamycin, conferring resistance to it and was used as a selection marker in the development of PG451 apple.

1.1 Basis for Request for Determination of Nonregulated Status under 7 CFR Part 340.6

The United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) is responsible for the protection of the US agriculture infrastructure against noxious pests and weeds. Under the Plant Protection Act (7 USC Sections 7701-7772) APHIS considers plants altered or produced by genetic engineering as restricted articles under 7 CFR 340 which cannot be released into the environment without appropriate approvals. APHIS provides that petitions may be filed under 7 CFR 340.6 to evaluate data to determine that a particular regulated article does not present a plant pest risk. Should APHIS determine that the submitted article does not present a plant pest risk; the article may be deregulated and released without further restrictions.

Under 7 CFR 340.6(e), APHIS may extend a previous determination of nonregulated status to additional regulated articles, based on an evaluation of the similarity of the regulated article to the antecedent organism(s) (i.e., an organism that has already been the subject of a determination of nonregulated status by APHIS under § 340.6, and that is used as a reference for comparison to the regulated article under consideration under the regulations). Such an extension of nonregulated status amounts to a finding that the additional regulated article does not pose a potential for plant pest risk, and should therefore not be regulated.

The USDA-APHIS reviewed and granted determinations of nonregulated status for the nonbrowning apple cultivars GD743 and GS784 (10-161-01p) finding that they did not pose a plant pest risk. The PG451 apple, described in this petition, was transformed using the same binary vector as events GD743 and GS784, yielding the same non-browning phenotype. Based on the similarity to these antecedent organisms OSF has concluded that previous analysis of impacts completed for GD743 and GS784 are relevant to APHIS's regulatory action in responding to the OSF extension for PG451 apple. A comparison of the antecedent organisms GD743 and GS784 to PG451 apple is provided in (Table 1). The only relevant difference between PG451 and the antecedent organisms GD743 and GS784 is the presence of a full copy of the vector backbone in PG451. The vector backbone comprises a number of prokaryotic elements that will not be expressed *in planta* and do not affect the safety assessment.

The data and information provided in the request for extension of deregulation demonstrate that the conclusions reached for GD743 and GS784 apples also apply to PG451 apple, and likewise PG451 apple does not pose a plant pest risk.

Table 1: Comparison of Events PG451, GD743 and GS784

Characteristic	Extension PG451 Apple	Antecedent GD743 Apple	Antecedent GS784 Apple
Organism	Apple	Apple	Apple
Cultivar	Gala	Golden Delicious	Granny Smith
Benefit	Non-Browning	Non-Browning	Non-Browning
Trait	Reduced PPO	Reduced PPO	Reduced PPO
Gene Function	RNA Interference	RNA Interference	RNA Interference
Transformation Method	*Agrobacterium*-mediated	*Agrobacterium*-mediated	*Agrobacterium*-mediated
Binary Vector	GEN-03	GEN-03	GEN-03

GEN-03: The GEN-03 binary vector comprises the genes PGAS and *nptII*.

1.2 Benefits of Arctic® Apple Event PG451

Arctic® apple event PG451 will offer growers, packers, processors, wholesalers, retailers, foodservice and consumers a nonbrowning variant of the popular Gala cultivar they have become accustom to purchasing.

Key benefits of the Arctic® apple cultivars will include:

- reduced shrinkage caused by finger bruising and scuff marks,
- reduced need for antibrowning agents on fresh sliced and dehydrated apple products,
- new uses of apples in high-quality, prepared-produce items,
- longer shelf-life, and
- promotion of consumption of this healthy snack food.

1.3 Submissions to Other Regulatory Agencies

PG451 is within the scope of the U.S. Food and Drug Administration's (FDA) policy statement concerning regulation of products derived from new plant cultivars, including those developed through biotechnology. That statement, which was published in the *Federal Register* on May 19, 1992 (57 FR 22984), clarified the FDA's interpretation of the Federal Food, Drug, and Cosmetic Act. In compliance with that policy, OSF will submit to the FDA a food and feed safety and nutritional assessment for event PG451. Furthermore, regulatory submissions for product approvals will be made to Health Canada and the Canadian Food Inspection Agency (CFIA).

2 THE APPLE FAMILY

The genus *Malus* belongs to the rose family (Rosaceae).

The CFIA biology document on apple (CFIA, 2014) provides information pertaining to the following aspects of apple biology:

- Identity
- Geographical Distribution
- Biology
- Related Species of *Malus domestica*
- Potential Interactions of *Malus domestica* with Other Life Forms

2.1 Recipient Apple Cultivar

The parental cultivar, Gala (PG), is a commercially grown cultivar of apple.

Neal Carter

3 DEVELOPMENT OF EVENT PG451

3.1 The Transformation System

The events PG451, GD743 and GS784 were all generated through *Agrobacterium*-mediated transformation of apple leaf explants with the binary vector GEN-03 (Section 4). By this method, genetic elements between the right border (RB) and left border (LB) of the binary vector (T-DNA) are transferred into the genome of the target plant. Occasionally, genetic elements outside of the border region are transferred as well.

In brief, leaves of three-week-old apple tissue culture plantlets were excised and cut into segments perpendicular to the mid-rib; they were then inoculated with *Agrobacterium tumefaciens* EHA105 carrying the GEN-03 vector at a density of 3 x 10^8 cells/ml for 5-10 minutes. Leaf segments were blotted on filter paper to remove excess bacterial cells, and then placed onto co-cultivation medium with the adaxial surfaces in contact with the medium for four days (all spent in the dark). Infected leaf segments were washed and placed onto regeneration medium containing 6 µg/ml kanamycin with the adaxial surfaces in contact for four weeks (2 weeks dark, 2 weeks light). Leaf segments were transferred to a regeneration medium containing 50 µg /ml kanamycin for four weeks. Green shoots, considered to be transformed, were transferred to proliferation medium with 50 µg /ml kanamycin for four weeks. Surviving shoots were transferred to fresh proliferation medium. Shoots regenerating on 50 µg /ml kanamycin were selected by polymerase chain reaction (PCR) using primers specific to the transgene or selection marker. Shoots identified to be transgenic by PCR were measured for total PPO activity, after 2-3 successive subcultures. Any shoot that was PCR-positive for the transgene and selection marker and significantly suppressed for PPO activity (defined as more than 80 percent suppressed) was micrografted to M9 rootstock in preparation for field testing (Lane *et al.*, 2003).

The steps involved in the development of PG451, GD743 and GS784 shown (Figure 1).

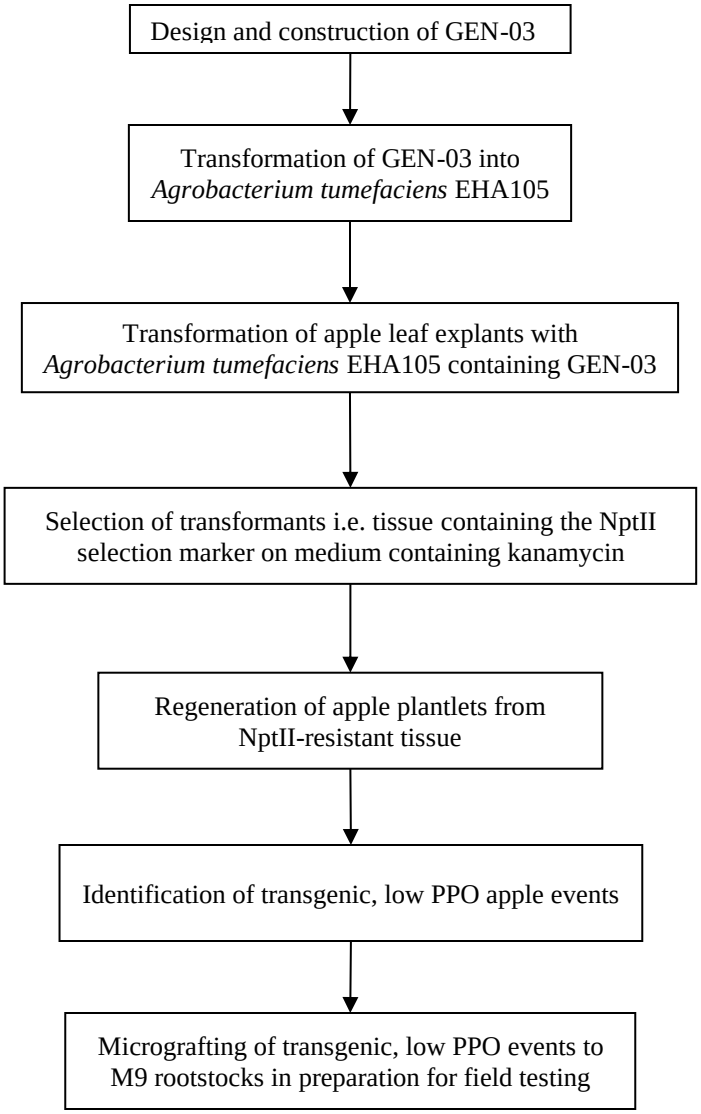


Figure 1: Steps in the Development of Arctic® apple events PG451, GD743 and GS784

4 DONOR GENES AND REGULATORY SEQUENCES

Event PG451 was developed through *Agrobacterium*-mediated transformation of apple leaf tissue using the binary vector GEN-03 (Figure 2) which is based on the binary vector pBINPLUS (van Engelen *et al.*, 1995). Vector pBINPLUS is based on the widely used binary vector BIN19 (Bevan, 1984). The complete sequence of BIN19 (U09365) is available at Genbank. All of the known intact, functional elements of the GEN-03 are described in (Table 2). The T-DNA comprises a PPO suppression transgene (P70:PGAS:T$_{NOS}$) and NptII selection marker (P$_{NOS}$:*nptII*:T$_{NOS}$) flanked by *Agrobacterium tumefaciens* T-DNA borders. The vector backbone includes a series of bacterial elements required to support vector replication and selection (*trfA*, *nptIII*, ColE1, and oriV). The remaining vector backbone sequences that reside between these known functional elements were fragmented in the cloning process that lead to the development of the binary vector and are non-functional. Please note that the entire vector sequence (15,271 bp) was included in the ORF analysis (See Section 5.1.9).

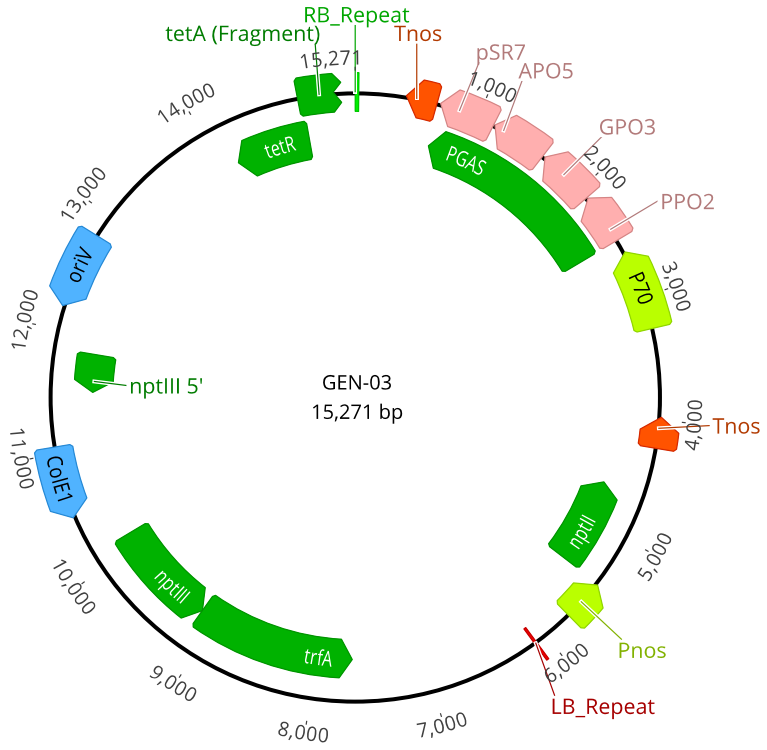


Figure 2: Map of the GEN-03 Vector
The vector pictured here is the GEN-03 vector used to create Arctic® apple events (PG451, GD743 and GS784). The original map provided in 10-161-01p was prepared *in silico* from sequences collected from Genbank. The current map has been corrected by sequencing. The map has been reverse-complemented and renumbered starting at +1 from the 5' end of the right border repeat (RB Repeat). This figure shows the position of a non-function tetracycline resistance determinant (14,157..15,162) that is not included in Table 2. The origin of this non-functional tetracycline-resistance determinant is discussed below.

Table 2: Components of the GEN-03 Vector-DNA Used to Develop PG451

Genetic Element	Size (bp)	Position	Function, Source, Reference
T-DNA			
RB Repeat	25	1 – 25	The right repeat sequence derived from *Agrobacterium tumefaciens* pTIT37 (Depicker *et al.*, 1982) .
T_{NOS}	254	407 – 660	A 3' UTR from the *Agrobacterium tumefaciens* nopaline synthase gene involved in transcription termination and polyadenylation (Depicker *et al.*, 1982) (Bevan, Barnes and Chilton, 1983).
PGAS	1822	685 – 2506	A chimeric suppression sequence comprising fragments of four apple PPO genes (PPO2, GPO3, APO5 and pSR7), designed to suppress the entire apple PPO gene family.
P70	649	2,612 – 3,260	The duplicated-enhancer CaMV 35S promoter with untranslated leader sequence from alfalfa mosaic virus RNA4 (Datla *et al.*, 1992) that directs transcription of the PGAS chimeric suppression sequence.
T_{NOS}	254	3,973 – 4,226	A 3' UTR from the *Agrobacterium tumefaciens* nopaline synthase gene involved in transcription termination and polyadenylation (Depicker *et al.*, 1982) (Bevan, Barnes and Chilton, 1983).
nptII	795	4,616 – 5,410	Neomycin phosphotransferase type II from *Escherichia coli* Tn5 (Rothstein *et al.*, 1981) providing resistance to kanamycin.
P_{NOS}	333	5,423 – 5,755	A nopaline synthase promoter from *Agrobacterium tumefaciens* that directs transcription of the *nptII* selection marker (Bevan, Flavell and Chilton, 1983).
LB Repeat	25	6,087 – 6,111	The left border repeat derived from *Agrobacterium tumefaciens* pTiT37 (Depicker *et al.*, 1982).
Backbone			
trfA	1482	7.664 – 9,145	Gene with two protein products that bind oriV to promote replication from pRK2 (Frisch *et al*., 1995).
nptIII	993	9,146 – 10,138	Neomycin phosphotransferase type III from *Streptococcus faecalis* R plasmid (Scutt, Zubko and Meyer, 2002) providing resistance to kanamycin
ColE1	590	10,472 – 11,061	RNA origin of replication used to increase the plasmid copy number, obtained from pBR322 (van Engelen *et al*., 1995).
nptIII	351	11,503 – 11,853	*nptIII 5*' upstream elements (Frisch *et al*., 1995).
oriV	618	12,201 – 12,818	Origin of replication that functions in *Agrobacterium tumefaciens* and *Escherichia Coli* from pRK2 (Frisch *et al*., 1995).

The sizes of the genetic elements listed here differ slightly from those listed in 10-161-01p. This does not indicate a change in vector but rather a change in our annotations.

Non – Functional Tetracycline Resistance Determinant

The binary vector GEN-03 does not contain a functional tetracycline resistance determinant and therefore no functional tetracycline resistance determinant was transferred into Pacific Gala during the development of PG451. The binary vectors pBIN19 and pBINPLUS, on which GEN-03 is based, do include a residual fragment of a tetracycline resistance determinant that was inactivated during the construction of pBIN19 (Frisch *et al.*, 1995).

There are annotated maps of pBIN19 and pBINPLUS available in public databases which show a *tetR* gene present in the backbone of pBIN19 and pBINPLUS. However, the nomenclature for the tetracycline resistance determinant in somewhat confusing and the *tetR* gene that is annotated on these plasmid maps does not actually confer tetracycline resistance. Tetracycline resistance genes are grouped into several classes, but all confer resistance through the same basic mechanism involving active efflux of tetracycline. The pBIN19 vector on which the GEN-03 is based includes an inactivated tetracycline resistant determinant homologous to *Pseudomonas aeruginosa* plasmid RP4 (Genbank Accession X75761). The tetracycline resistance determinant is comprised of a regulatory gene (*tetR*) encoding a 26 kDa repressor protein and a structural gene (*tetA*) encoding a membrane protein (34 kDa) responsible for resistance. The two genes are adjacent and have divergent transcriptional polarity with their transcriptional signals are located in the short intercistronic region (Waters *et al.*, 1983). During the construction of pBIN19 the tetracycline resistance gene *tetA* was inactivated by the insertion of the T-DNA region (Frisch *et al.*, 1995).

The figure below shows an alignment of the binary plasmids pBIN19 and GEN-03 to the tetracycline determinant (*tetR* / *tetA*) from RP4. The *tetR* repressor sequence is fully intact, but the *tetA* structural gene is mostly missing. It has been shown that mutations within the *tetA* structural gene eliminate tetracycline resistance (Waters *et al.*, 1983).

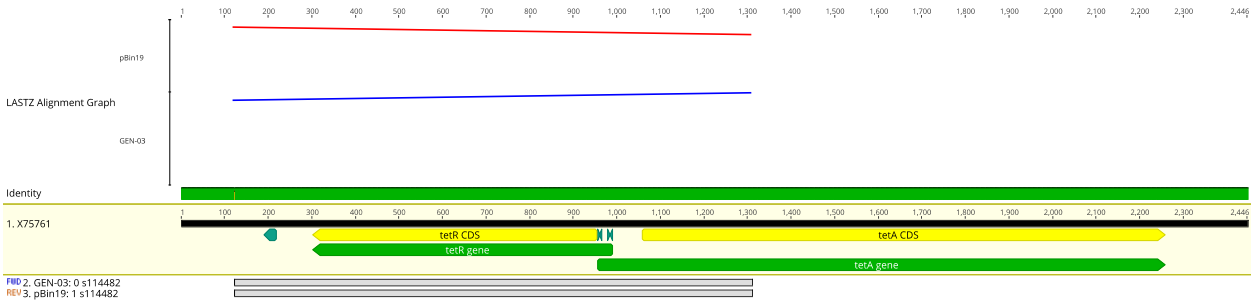


Figure 3: Non-Functional Tetracycline-Resistance Determinant
The figure shows a LASTZ alignment (Geneious 10.2.6) of the backbone region of the binary vectors pBIN19 and GEN-03 to the tetracycline resistance determinant (*tetA* and *tetR* genes) of *Pseudomonas aeruginosa* plasmid RP4 (Genbank Accession X75761). The alignment shows that the structural tetracycline resistance gene (*tetA*) has been largely deleted in pBIN19 and GEN-03. The *tetR* gene that remains in pBIN19 and GEN-03 is a repressor gene that normally suppresses expression of *tetA* and does not itself confer resistance to tetracycline.

5 GENETIC ANALYSIS AND MOLECULAR CHARACTERIZATION

An extensive genetic characterization of the DNA inserted into PG451 was performed. This analysis demonstrated that the GEN-03 was integrated at two locations in the apple genome: in chromosome 10 and in chromosome 17. There are five full-length copies of the T-DNA and structural rearrangement which results in two copies of the PGAS suppression gene forming an inverted repeat, necessary to induce RNA interference. There is read-through at the left border resulting in one full length and one partial copy of the backbone. It was determined that the PG451 inserts did not disrupt the function of any known apple gene. Finally, we show that the PG451 insertion results in the expected non-browning phenotype. These data show that no deleterious changes occurred in PG451 as a result of the transgene insertion.

5.1 Whole Genome Sequencing

5.1.1 Mapping Strategy

To describe the insertions that create PG451 from PG we employ a unique mapping strategy based on short read Illumina whole genome sequencing data. In our approach, the reads pool is systematically sorted into three reads pools: (i) reads that match the GEN-03 vector, (ii) reads that match the apple genome, and (iii) reads that map to junction sites, either vector to genome insertion sites or vector to vector junctions associated with structural rearrangements. The third reads pool is used to develop insertion maps on which the safety assessment is based.

To clarify our mapping approach, we include the following discussion.

Our mapping approach does not attempt to assemble the PG451 genome from next-generation sequencing data. Instead, our mapping approach focuses on identifying reads that match the GEN-03 vector and screening those reads for information, such as vector to genome or vector to vector junctions, presence of vector backbone, left border read though and copy number that can be used to predict insertion structure and genomic location.
We begin by filtering the sequencing reads pool for any read potentially arises from the GEN-03 vector. To do this we use BBduk to identify any read that contains 21 bp of 100% identity to the GEN-03 vector (15,281 bp). A sequence length of 21 bp was chosen because it affords a level of uniqueness that ensures that we are not selecting random sequence while ensuring that all useful mapping reads are identified and selected. This BBDuk filtering process reduces our reads pool from >80,000,000 reads to just over 3,000 reads allowing us to predict the structure and location of the insertions using limited computer resources.

This reads pool is then sequentially filtered to remove reads that match perfectly to the apple reference genome or the GEN-03 vector. The small pool of reads that remain includes chimeric reads (vector to vector or vector to genome) that can be used to predict the structure and location of the insertions that create PG451 from the parent cultivar PG.

Our reads selection process for PG451 ultimately identified 48 reads that were mapped to junctions. The genomic locations of the insertions for 48 reads are shown in Figures 4, 5, 6 and 7, while Figures 10 and 11 display mapping of the internal junctions.

When mapping with 25X overall coverage (12.5X diploid coverage) our genome to vector junctions have an average coverage of 9 reads and a range of 5 to 15 reads per junction. Similar vector to genome junction coverage was seen when mapping insertions in NF872 (USDA 16-004-01p). In order for our mapping process to miss an insertion, we would have to miss two co-located junctions which we predict would be covered by 5 to 15 reads per junction. We find it improbable that we would miss this many chimeric reads in our mapping process. In addition to PG451, we have mapped numerous GEN-03 insertions in a wide variety of apple cultivars and have not observed technical issue in sequencing T-DNA or vector backbone regions. This gives us high confidence in our mapping process.

In the case of one internal junction shown in Figure 11, we found that the junction was covered by only a single read. This internal junction is within a nearly perfect inverted repeat. This type of inverted repeat is not readily amenable to Illumina or Sanger sequencing, so it is not unexpected that this junction is underrepresented in the reads pool. The vector to genome junctions that identify the number and location of insertions are not normally part of an inverted repeat and are far easier to sequence using Illumina technology. As such, we would have expected to identify at least five reads for each additional junction and two such junctions must exist for there to be third insertion site. We have detected no additional chimeric reads that would suggest any additional junction.

5.1.2 Sequencing Library Preparation and Whole Genome Sequencing

Genomic DNA was isolated from tissue culture leaf samples with a DNeasy Plant Mini Kit (Qiagen). Genomic DNA was quantified using the Qubit dsDNA BR Assay Kit (ThermoFisher) and assessed for quality by 1% agarose gel electrophoresis. A TruSeq Nano DNA LT Sample Prep Kit (Illumina) was used to construct a DNA library following the manufacturer's protocol instructions for the 550 bp insert size. Unique adapters were used so that pooled events could be deconvoluted after sequencing[1]. The quality of the library was then checked on a DNA 1000 chip on the 2100 Bioanalyzer (Agilent Technologies Inc.) and the concentration was determined by qPCR using the KAPA SYBR FAST ABI Prism qPCR Kit (Kapa Biosystems) and the StepOnePlus Real-Time PCR System (Applied Biosystems). Equimolar concentrations of the libraries were then pooled and a concentration of 12 pM was used for clustering in one lane of a flowcell on the cBOT (Illumina). The sample was then sequenced (2 x 250 cycles, paired-end reads) on the HiSeq2500 (Illumina) using the HiSeq Rapid SBS Kit v2 (Illumina). Sequence reads were deconvoluted based on their indexed adapter association.

5.1.3 Sequencing Coverage and Quality

The PG451 read library was trimmed for quality below Q20 (99% base call certainty) and a minimum read length of 21 nucleotides using BBDuk[2]. A theoretical sequencing coverage of 25x was calculated using a modification of the Lander/Waterman equation (Table 3). Apple is diploid so our insertions are expected to have a sequencing coverage of 12.5x. The Lander/Waterman equation used to calculate coverage assumes that the reads are distributed evenly about the genome

[1] In this case, three libraries were prepared and sequenced in a single flowcell (PG451 plus two other events that are not related to this submission). This fact is noted here because the number of reads reported in this submission for event PG451 (Table 3) is about one third of what would normally be expected from a single flowcell.

[2] BBDukF [qtrim=rl, trimq=20, minlength=21]
BBMap (Brian Bushnell bbushnell@lbl.gov) is available from (http://sourceforge.net/projects/bbmap/).

and that overlap detection does not vary between reads (Illumina 2014). These assumptions are not fully correct since factors such as fragmentation bias, percent GC content and tandem repeats can lead to sequencing bias.

Table 3: Calculation of Theoretical Sequencing Coverage

Descriptor	Value
Qualified Reads (Q20)	80,292,314
Nucleotides	17,802,375,978
Genome Size (HFTH1)	708,540,000
Coverage	25

Notes: Coverage = Nucleotides / Genome Size (Illumina, 2014) .

The reads were mapped to the most recent and high quality apple reference available, HFTH1 (N50 of 6.99 Mb) (Zhang *et al.*, 2019) available at the Genome Database for Rosaceae (Jung *et al.*, 2019), using Geneious assembler to determine actual sequence coverage (Table 4). Mean coverage ranged from 22.4x to 27.6x with an average of 24.3x. The amount of the reference genome that was covered by at least one sequence read (Percent of RefSeq), ranged from 95.6% to 98.8% with an average of 97%. The predicted gap in our sequencing coverage (3%) corresponds to approximately 21,256, 200 bp.

Table 4: Mapping PG451 Sequencing Library to the HFTH1 Reference Genome using Geneious[1]

Chromosome[2]	Sequence length (bp)	Number of Reads	Nucleotides	Mean Coverage	Pairwise Identity	Percent of RefSeq
1	33,117,969	3,609,121	840,295,430	24.487	97.9%	96.4%
2	38,683,619	3,858,198	898,765,809	22.364	97.7%	95.8%
3	37,347,157	3,905,444	907,648,303	23.462	98.7%	96.3%
4	31,179,315	3,242,334	752,332,659	23.239	98.2%	96.4%
5	48,155,291	5,709,635	1,320,910,454	26.736	99.3%	96.6%
6	35,740,263	3,964,593	920,202,336	24.846	97.9%	98.8%
7	36,106,916	3,994,508	925,993,751	24.740	98.0%	97.6%
8	31,675,850	3,374,333	783,213,239	23.829	97.6%	98.2%
9	34,983,427	3,638,011	845,993,415	23.310	98.4%	96.4%
10	44,062,067	4,479,416	1,041,300,739	22.747	97.9%	95.6%
11	42,707,874	4,375,637	1,017,899,512	22.964	97.7%	96.2%
12	32,462,490	3,484,629	809,525,731	24.133	99.1%	96.4%
13	45,085,373	5,538,398	1,282,646,714	27.563	98.2%	98.4%
14	31,676,889	3,486,964	808,234,555	24.619	98.0%	98.6%
15	56,927,258	5,987,809	1,389,527,344	23.505	97.8%	97.2%
16	41,864,757	5,009,081	1,158,713,287	26.809	98.9%	98.4%
17	34,174,699	3,644,928	846,315,664	23.866	97.9%	96.4%
00	8,056,035	4,695,442	1,030,081,334	138.231	99.4%	80.5%
Unused Reads (Paired)		1,908,222	414,674,602			
Unused Reads (Unpaired)		2,385,629	468,578,320			

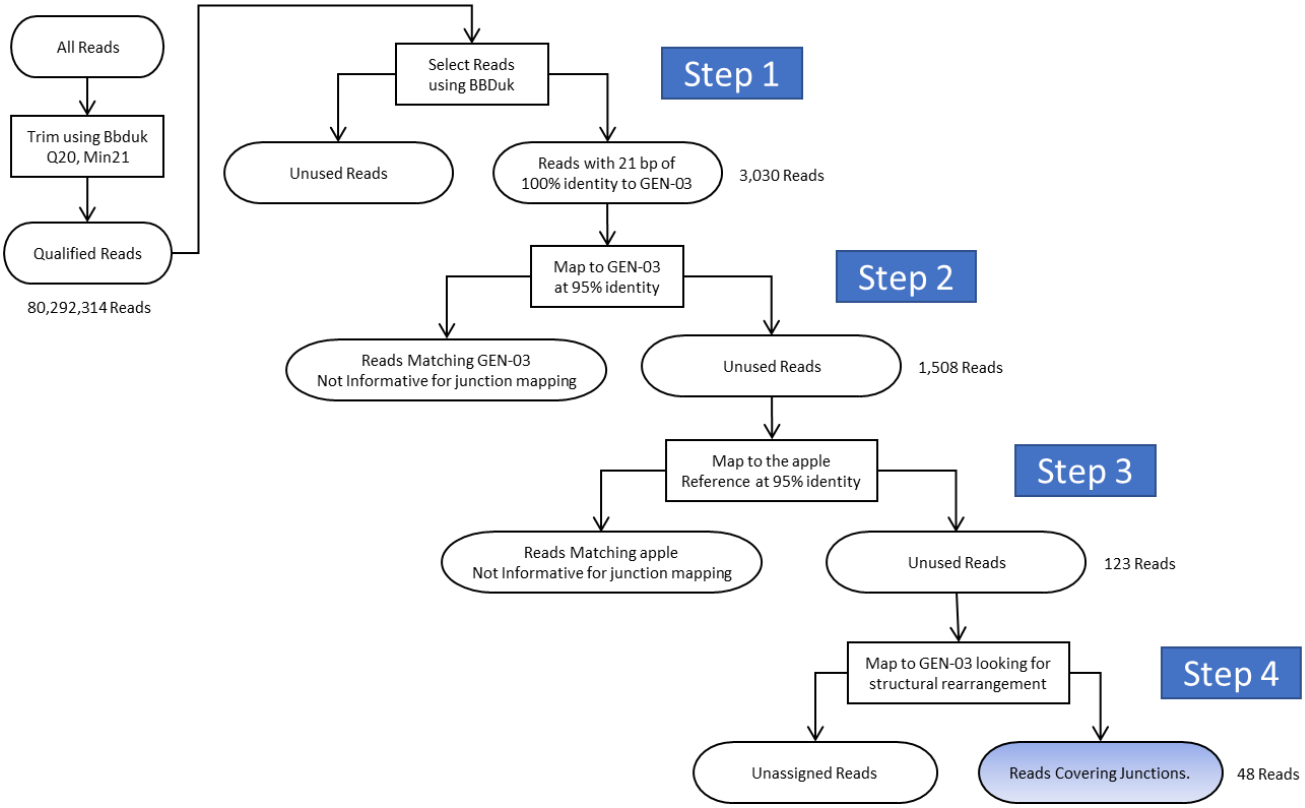
[1] Geneious (10.2.6) Medium-Low Sensitivity / Fast.

[2] Chromosome 00 refers to sequences not mapped to a chromosome in the HFTH1 reference.

5.1.4 Insertion Site Analysis

We have developed a simple method for detecting and characterizing T-DNA junctions using next-generation sequencing data that is similar to the method of (Park *et al.*, 2017). A diagram of the mapping workflow is provided in Figure 4.

Figure 4: Workflow for Detection of T-DNA Insertions



Step 2: The Reads Matching GEN-03 are not informative for creating insertion maps because they do not identify junction sequences used in the mapping process. However, these reads are used to provide information on other features, including copy number and left border read through (See Section 5.1.7).

Step 1: Select Reads using BBDuk

In Step 1, qualified PG451 reads (80,292,314 reads) were selected using BBDuk to identify reads which include 21 bp with 100% identity to the GEN-03 vector[3]. The filtered read pool (3,030 reads) is expected to include three types of reads: (i) reads that map to the GEN-03 vector, (ii) reads that map to endogenous sequences that share short regions of 100% homology to the PPO fragments included in the PPO suppression transgene, and (iii) reads that map to the site of integration and which span the junctions between the plant and the vector. The following steps are designed to classify the reads.

Step 2: Reads Matching the GEN-03 Vector

To identify reads that map entirely to the GEN-03 vector, 3,030 BBDuk selected reads were mapped to the GEN-03 vector using the Geneious assembler with parameters that allow for only a 5% mismatch[4]. A total of 1522 reads matched the GEN-03 and were removed from subsequent mapping steps because they are not informative in the identification of junctions. This reduced the reads pool to 1508 reads.

Step 3: Reads Matching the Apple Genome

To identify reads that map entirely to the apple genome, the 1508 unused reads from Step 2 were mapped to the apple references (HFTH1, GDDH13, v3.0.a1 and v1.0 combined haplotypes) which are available at the Genome Database for Rosaceae (Jung *et al.*, 2019) using the Geneious assembler with parameters that allow for only a 5% mismatch[5] A total of 1,375 reads matched the apple references and were removed from the subsequent mapping because they were not informative in the identification of junctions. This reduced the reads pool to 123 reads. All available references were used in this step as, while the most recent reference is the most complete, older references will have regions of better sequence quality for our event which will map some reads that otherwise would have been left in the pool.

Step 4: Identification of Junctions

To identify junctions, the 123 unused reads from Step 3 were mapped to the GEN-03 vector using Geneious while screening for structural variants[6]. The alignment was examined by hand to identify partially mapped (chimeric) reads. The non-mapping end of chimeric reads was used to blast the apple reference genome or GEN-03 vector to identify its source. Unmapped reads were carefully examined by hand to ensure that no junction sequences were missed. The unused reads were disregarded as non-informative due to one of the following reasons: (i) amplification of a contaminant, (ii) match to reference or vector with high stringency, (iii) represented allelic or varietal variance in the reference, or (iv) being an NGS artifact.

[3] Bbduk.sh in-Trimmed_PG451.fasta out=umatched.fasta outm=readsmatchingGEN-03.fasta ref=GEN-03.fasta k=21 stats=stst.txt

[4] Medium Sensitivity / Fast with Custom Sensitivity, Minimum Overlap Identity 95%, Maximum Mismatches per Read 5%

[5] Medium Sensitivity / Fast with Custom Sensitivity, Minimum Overlap Identity 95%, Maximum Mismatches per Read 5% with the "Search more thoroughly for poor matching reads" checked.

[6] Highest Sensitivity / Slow, Structural Variants

5.1.5 T-DNA Genomic Location

The junction mapping process (Steps 1 – 4, above) identified two apple/vector junctions co-located in chromosome 10 (Figure 5 and Figure 6) and two apple/vector junctions co-located in chromosome 17 (Figure 7 and Figure 8). This is consistent with two insertion sites. No other chimeric (vector to chromosome) reads were identified in the pool of GEN-03 reads.

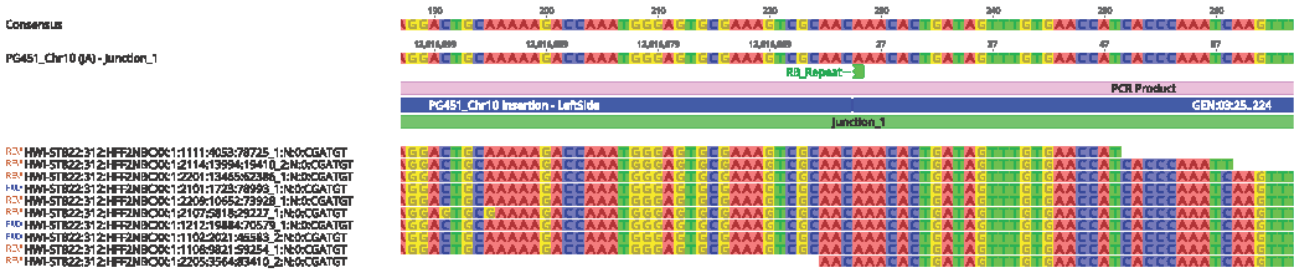


Figure 5: Chromosome 10 Insertion - Left Side
A 60 bp fragment crossing the junction with mapped reads is provided as evidence of the junction. This figure shows chromosome 10 (Chr10:12,616,901..12,616,862) attached to the RB of GEN-03 (GEN-03:25..64). In the Word version of this document the figure can be opened as high-resolution PDF by double-clicking within the figure.

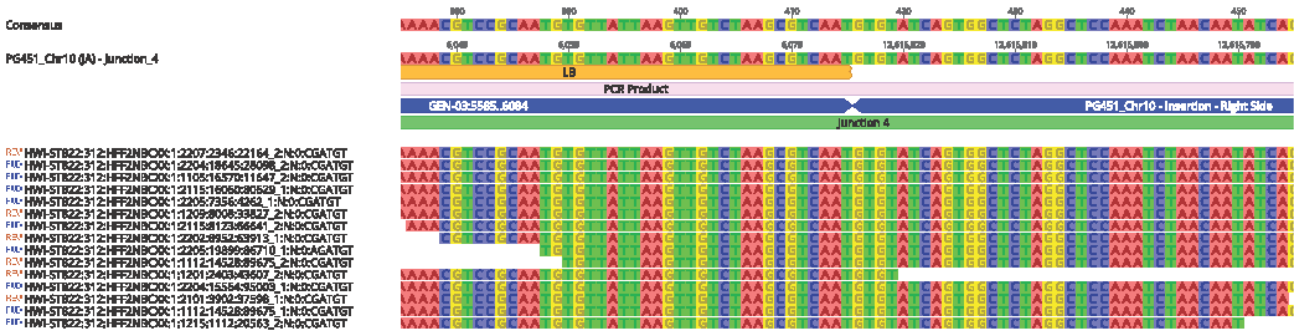


Figure 6: Chromosome 10 Insertion - Right Side
A 60 bp fragment crossing the junction with mapped reads is provided as evidence of the junction. This figure shows the LB of GEN-03 (GEN-03:6084..6045) attached to chromosome 10 (Chr10:12,616,833..12,616,795). In the Word version of this document the figure can be opened as high-resolution PDF by double-clicking within the figure.

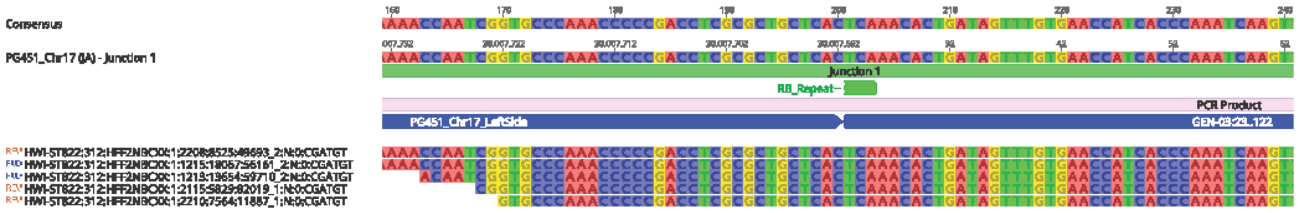


Figure 7: Chromosome 17 Insertion - Left Side
A 60 bp fragment crossing the junction with mapped reads is provided as evidence of the junction. This figure shows chromosome 17 (Chr17:30,007,732..30,007,692) attached to the RB of GEN-03 (GEN-03:23..62). In the Word version of this document the figure can be opened as high-resolution PDF by double-clicking within the figure.

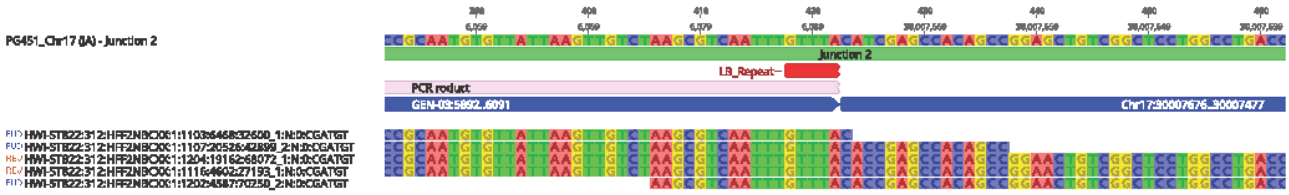


Figure 8: Chromosome 17 Insertion - Right Side
A 60 bp fragment crossing the junction with mapped reads is provided as evidence of the junction. This figure shows the LB of GEN-03 (GEN-03:6091..6052) attached to chromosome 10 (Chr10:30,007,676..30,007,637). In the Word version of this document the figure can be opened as high-resolution PDF by double-clicking within the figure.

The genomic location of the insertions in chromosome 10 (Figure 9) and in chromosome 17 (Figure 10) are shown as evidence that the insertions do not disrupt any known endogenous gene. No predicted genes or transcripts are present in the annotation window for the *M. domestica* HFTH1 v1.0 reference.

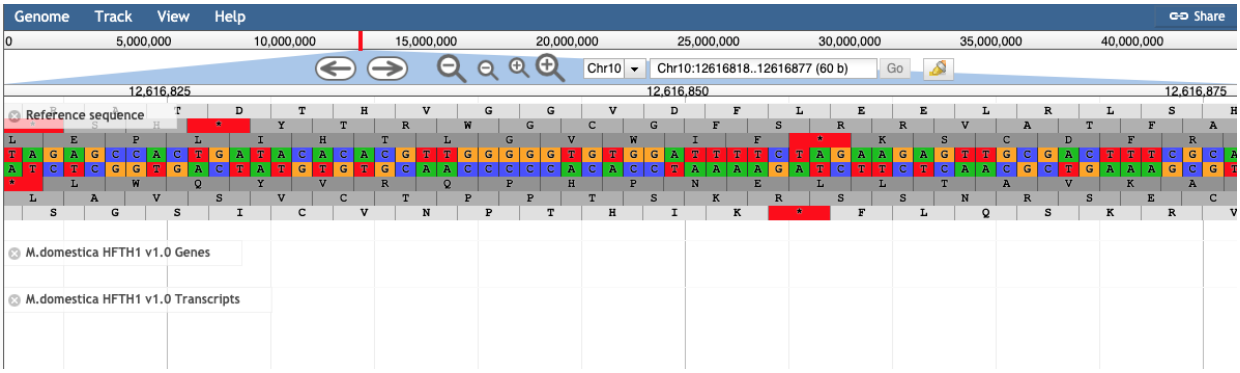


Figure 9: PG451 - Chromosome 10 - Genomic Location
A screenshot covering the insertion site in PG451 Chromosome 10 (Chr10:12,616,862..12,616,833) showing no predicted genes or transcripts present at the insertion site (JBrowse, GDR).

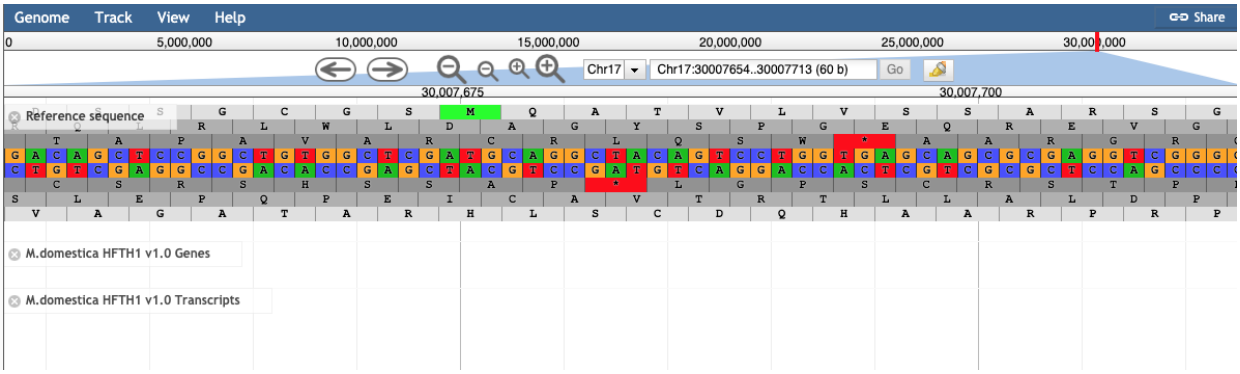


Figure 10: PG451 - Chromosome 17 - Genomic Location
A screenshot covering the insertion site in PG451 Chromosome 17 (Chr17:30,007,692..30,007,676) showing no predicted genes or transcripts present at the insertion site (JBrowse, GDR).

5.1.6 Internal Junctions

The junction mapping process described in Section 5.1.3 also identified two internal junctions which is evidence of transgene rearrangement that occurred upon integration. One internal vector to vector junction connects LB (6,087) to backbone (10,848) (Figure 11). The other internal vector to vector junction connects RB to RB (Figure 12). This latter junction is represented by a single read. The junction that the read overlaps is within an inverted repeat which is traditionally difficult to sequence using standard Illumina or Sanger sequencing. Even though this junction is covered by a single read we have high confidence that the junction is real. The read overlaps the junction by at least 29 bp on either side. The probability of identifying a read overlapping a junction like this that is not real is low.

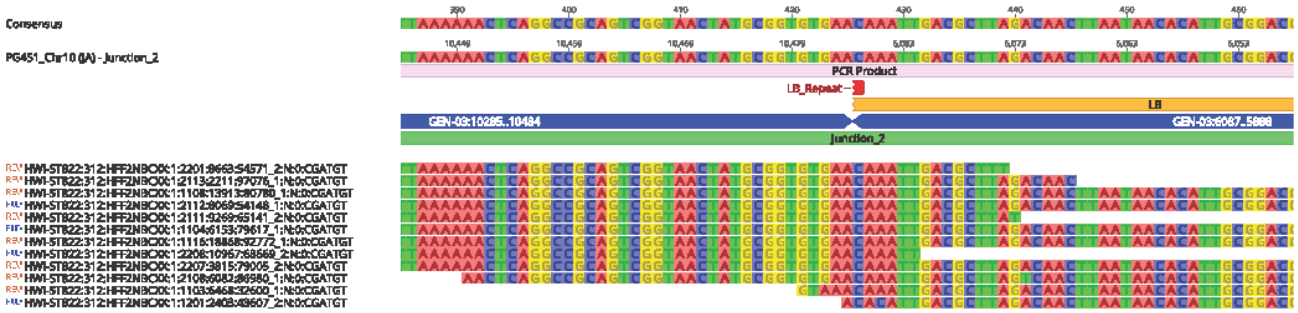


Figure 11: Internal Junction #1
A 60 bp fragment crossing the junction with mapped reads is provided as evidence of the junction. In the Word version of this document the figure can be opened as high-resolution PDF by double-clicking within the figure. The junction coordinates are GEN:03:10484 / GEN-03:6087-.

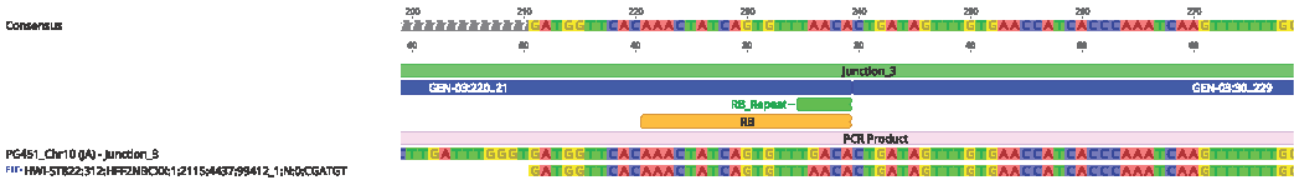


Figure 12: Internal Junction #2
A 60 bp fragment crossing the junction with mapped reads is provided as evidence of the junction. In the Word version of this document the figure can be opened as high-resolution PDF by double-clicking within the figure. The junction coordinates are GEN-03:21- / GEN-03:30.

5.1.7 Evidence of Read Though at the Left Border

In addition to the internal junctions described in Section 5.1.6 the mapping process revealed evidence of read though at the left border. In Section 5.1.4 BBDuk selected reads with 21 bp of 100% homology to the GEN-03 vector were aligned to the GEN-03 vector using the Geneious assembler with parameters that allow for only a 5% mismatch[7]. The alignment is provided in Figure 13. This alignment provides evidence for read through at the left border. It also shows that the entire vector backbone is incorporated in to the apple genome. The unequal coverage of the alignment to the GEN-03 vector also suggests that the copy number of different regions inserted in to the genome varies. The T-DNA region (1 – 6111) has more copies than the region from 6112 to ~10,000, which is higher that the region from~10,000 to 15,273. The PPO containing region of the T-DNA is somewhat over-represented because it will include endogenous reads that match the vector sequence (and are fully contained within one of the four PPO fragments).

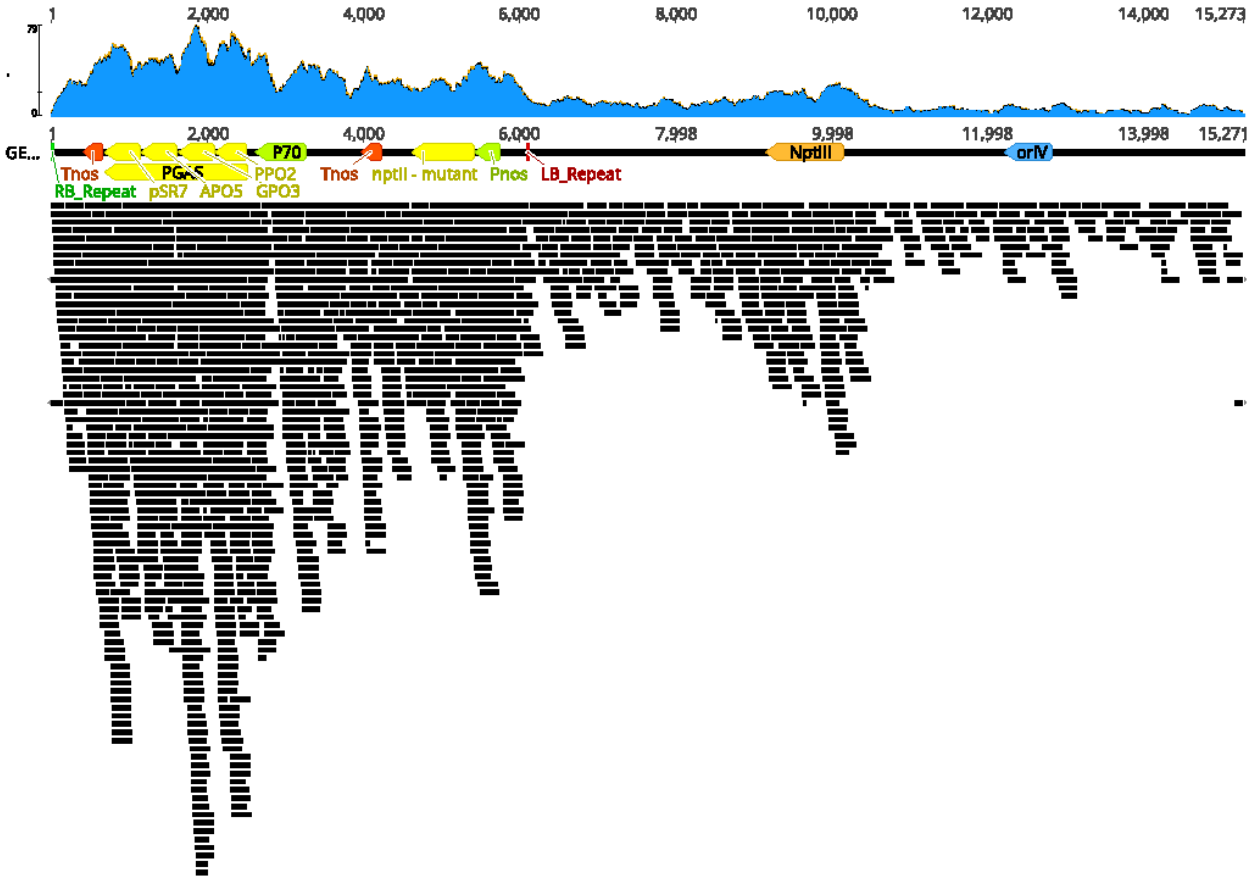


Figure 13: Coverage of the GEN-03 in PG451[8]

A total of 1,522 reads mapped to the GEN-03 binary vector using Geneious reveal which regions of the T-DNA and vector backbone were included in PG451. Sequencing coverage along the vector was variable and decreased from right border (RB) to left border (LB) and in to the vector backbone. The sequencing coverage was used to predict the approximate copy number for each region.

[7] Medium Sensitivity / Fast with Custom Sensitivity, Minimum Overlap Identity 95%, Maximum Mismatches per Read 5%

[8] In the Word version of this document, this figure can be opened as a separate high-resolution PDF by double-clicking within the figure.

5.1.8 Predicted Insertion Maps

Our examination of the whole genome sequencing data for PG451 has revealed evidence of the following: (i) one insertion in chromosome 10 and one insertion in chromosome 17 (Section 5.1.5), (ii) two internal vector to vector junctions (Section 5.1.6) which cannot be definitively assigned to one insertion or another, (iii) evidence of read through at the left border (Section 5.1.7), (iv) incorporation of the entire vector backbone (Section 5.1.7) and (iv) different copy number of different regions of the vector.

Based on the sum of the mapping evidence we have developed insertion maps that account for all junctions and read-through events predicted and are consistent with the estimated copy number for each vector region (Figure 14 and Figure 15). When using short read next-generation sequencing, internal features (internal junctions and left border read through) cannot be definitively assigned to a specific chromosome. However, internal features must be applied in a specific order to account for all of the sequencing evidence. In this case we have assigned the two internal junctions and the left border read through to the chromosome 10 insertion.

There is no evidence that the structural rearrangements of the vector sequence that we have described interrupt the PPO suppression or NptII coding regions. Therefore, the assignment of internal features to one chromosome or the other does not affect the safety assessment of PG451.

There is a theoretical possibility that a third insertion is present in the portion of the genome that was not sequenced. However, we believe that this is unlikely, based on our sequencing approach and analysis. Repetitive sequences, GC content and fragmentation bias are known to be poorly covered by next-generation sequencing. The GEN-03 binary vector, both T-DNA region and vector backbone, do not comprises these factors and are easily sequenced using Illumina technology. Our mapping approach is based on selection of reads which are partially or fully derived from the vector and then mapping any chimeric reads that are identified to a junction site. There is no reason to believe that our vector sequence was not included in the reads pool and every single chimeric read found within our reads pool has been successfully mapped to a junction site. We have found no evidence of an additional insertion or rearrangement.

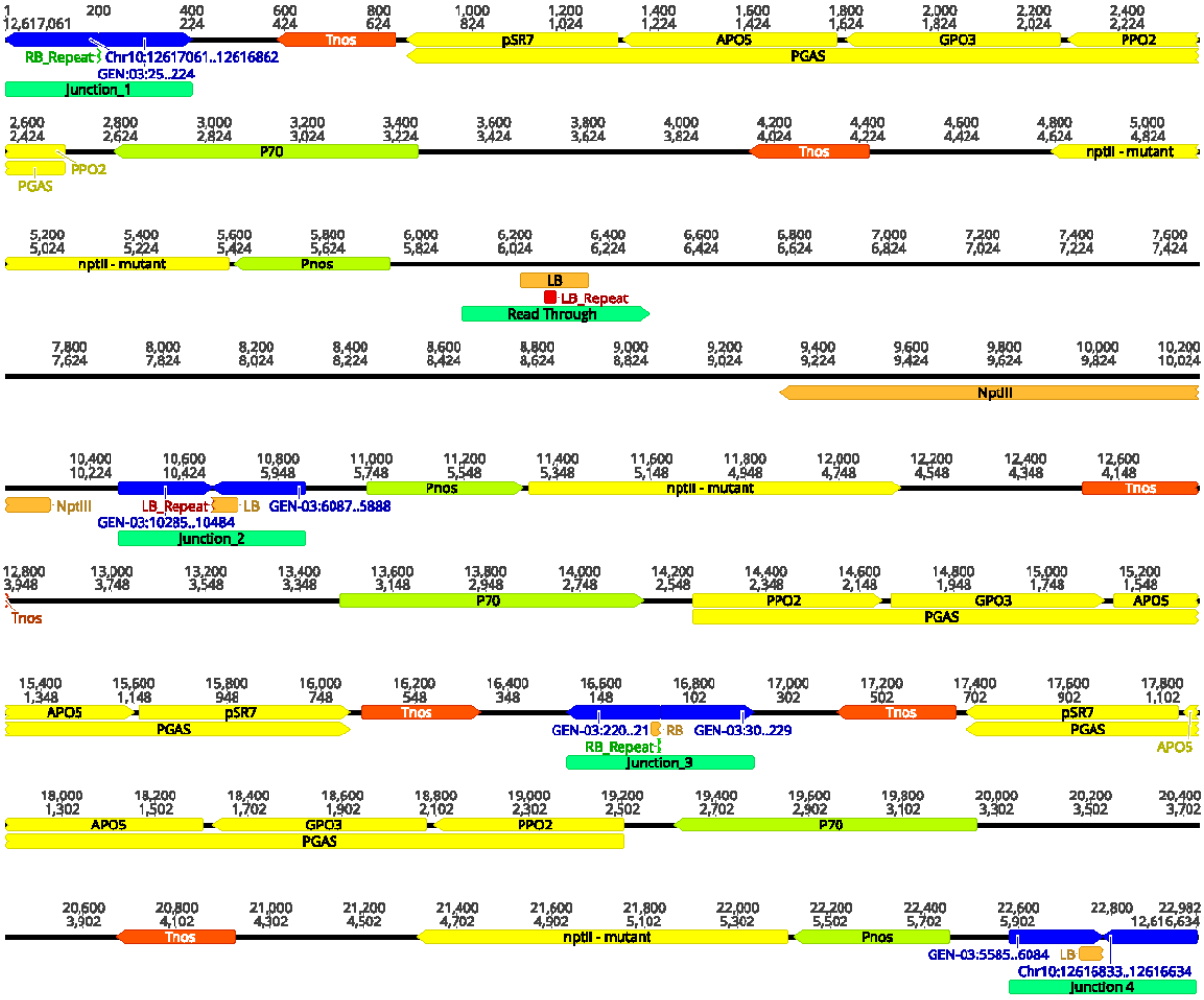


Figure 14: Chromosome 10 Insertion[9]
This insertion comprises a complete intact copy of the T-DNA (RB to LB), followed by read thorough past the LB, followed by a partial copy of the backbone, followed by a structural rearrangement (Junction) from the colE1 origin to the LB, followed by an intact copy of the T-DNA (LB to RB), followed by a structural rearrangement from the RB to RB, followed by an intact copy of the T-DNA (RB to LB).

[9] In the Word version of this document, this figure can be opened as a separate high-resolution PDF by double-clicking within the figure.

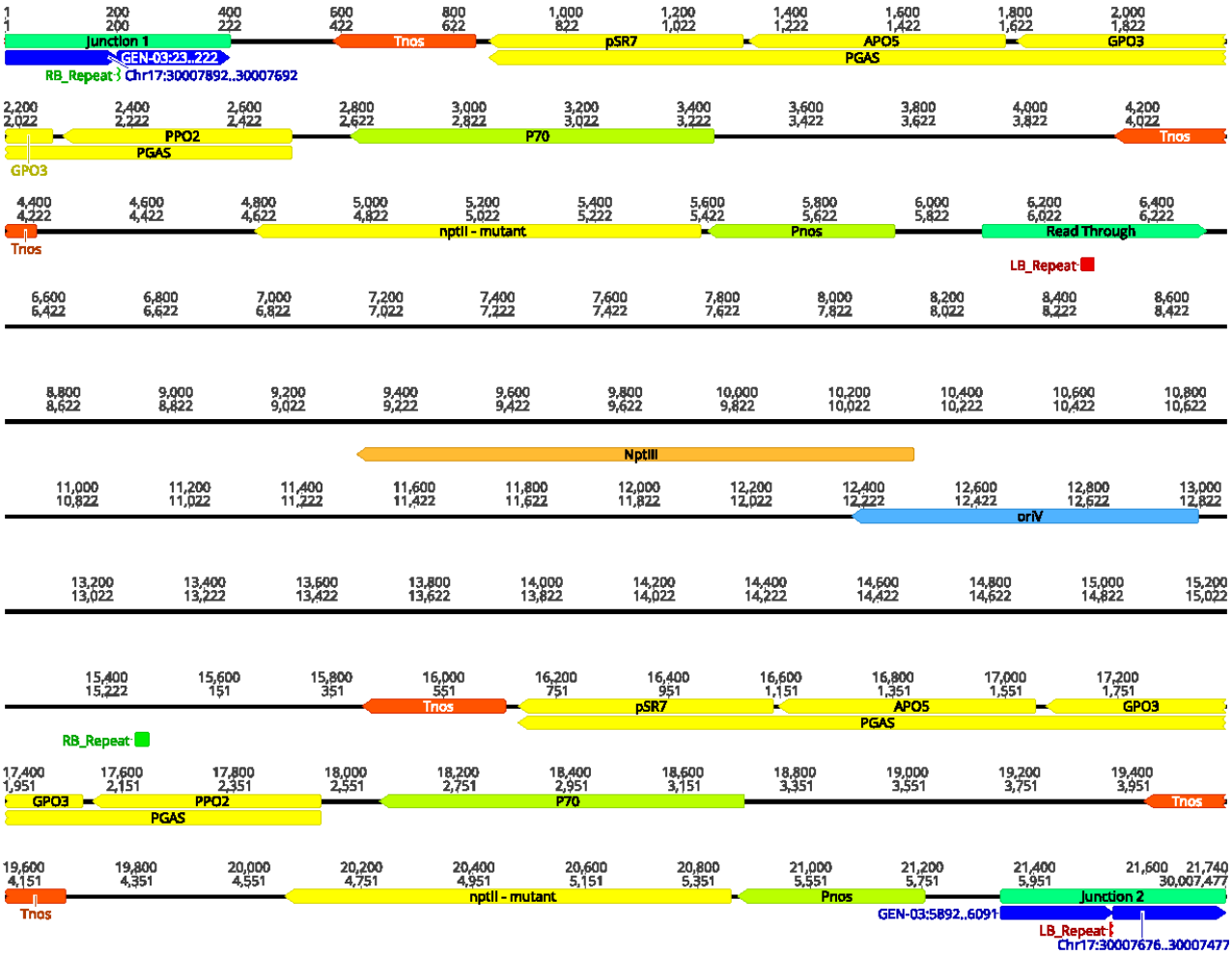


Figure 15: Chromosome 17 Insertion[10]
This insertion comprises a complete intact copy of the T-DNA (RB to LB), followed by read through past the LB, followed by a complete copy of the backbone, followed by a second complete, intact copy of the T-DNA

[10] In the Word version of this document, this figure can be opened as a separate high-resolution PDF by double-clicking within the figure.

5.1.9 Open Reading Frames

The inserted DNA, including the genomic region flanking each insertion, was analyzed for putative open reading frames (ORFs) using Geneious 10.2.6. The deduced amino acid sequences were then compared to a database of known allergens to determine if there was any significant similarity. The putative ORFs are not necessarily associated with any control elements, such as promoters, terminators or translation start codons. Thus, this assessment is purely hypothetical and is designed to identify any ORFs that may yield peptides that are potentially allergenic or toxic, if they were to be placed in a context in which they were expressed.

An ORF was defined as any contiguous nucleic acid sequence beginning at a start codon (ATG) and continuing until the first termination codon (TAA, TGA, TAG). The entire GEN-03 vector sequence plus unique junction sequences created in PG451 were translated in all six reading frames and deduced amino acid sequences of greater than 29 amino acids were chosen for further analysis. A minimum ORF size of 29 amino acids was chosen because this is the minimum polypeptide size that is capable of eliciting the clinical symptoms of an allergic reaction (Bannon *et al.*, 2002).

A total of 106 unique ORFs were identified that are derived from the 15271 bp GEN-03 binary vector, including backbone. An additional 7 ORFs were identified that were generated as a result of insertion and structural rearrangement of vector sequences that generated PG451.

To assess the potential allergenicity of inserted sequences, the deduced amino acid sequences of these 113 ORFs were compared to entries in the Food Allergy Research and Resource Program (FARRP) Allergen Protein Database (Version 20). This version of the database contains 2171 protein (amino acid) sequence entries of proven or putative allergens (food, airway, venom/salivary and contact), but only if there is evidence of elements such as IgE binding. To determine sequence similarity to putative allergens two recommended comparisons were done. The first involved a search for 50% identity over the length of the entire amino acid sequence with an e-value of 10^-4, approximately 1e^-7.[11]. The second involved a search for 35% identity or greater over 80 amino acids in a sliding window (sequences <80 amino acids have a single window). None of the identified ORFs met the thresholds for either search criteria to generate a hit with a known allergen.

To assess the potential toxicity of inserted sequences, all 113 ORFs were first run through blastp against the National Center for Biotechnology Information (NCBI) non-redundant protein database using Geneious. The cutoff e-value used was 10^-2 and this simply divided the ORFs into two categories; those that generated hits and those that didn't. The pool with hits then had each ORF individually aligned with the NCBI online blastp tool[12]) returning up to 20000 results. Alignments were saved as a .txt file that was manually searched for the presence of the words "toxin" or "toxic" that would incite further inquiry. Of the 113 ORFs, 56 derived from GEN-03 and 3 from PG451 produced hits within Geneious. No instances of "toxin" or "toxic" were found in the 59 .txt alignment files generated online using the NCBI tool.

[11] AllergenOnline (http://www.allergenonline.org/databasehelp.shtml, accessed 200515).
[12] NIH-NLM-NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed 200515).

5.1.10 Minimal Potential for Unintended Consequences

Okanagan Specialty Fruits has chosen to use short read next-generation sequencing and a unique mapping approach to describe the insertions that create PG451 from PG apple. The insertion maps generated in this approach are more detailed and accurate than the previous generation molecular biology techniques (i.e. Southern Analysis).

The insertion maps are submitted to regulatory agencies for molecular characterization of PG451 as part of the safety assessment of this enhanced apple cultivar. The whole genome sequencing analysis, based on contiguous reads containing both apple and vector sequence or contiguous reads containing vector to vector rearrangement provides clear evidence to support the predicted insertion maps and their genomic locations. The structure of the inserts was more complicated than the structure of the GEN-03 plasmid, but the coding sequence of the PPO silencing cassette remained intact. Insertions are not located within any predicted endogenous coding or regulatory region. There are no additional open reading frames introduced in the inserted sequence or as a consequence of structural rearrangement that align to known allergens.

The inserted sequence includes a complete copy of the vector backbone. The vector backbone comprises a number of prokaryotic elements (Table 2) to support plasmid replication and selection during the transformation process. These prokaryotic genetic elements do not contain the necessary sequences for expression in plants cells. These sequences are largely from bacterial R plasmids and are ubiquitous in nature. R plasmids are readily transferred from microorganism to microorganisms via conjugal transfer, where conversely, horizontal gene transfer from plants to microorganisms is rare (Nielsen *et al.*, 1998). The presence of these sequences within the plant genome is unlikely to pose a safety concern.

Any additional insertion would potentially create 2 new ORFs at the site of insertion and 1 new ORF for each structural rearrangement that are not discussed in Section 5.1.9. We reiterate that the assessment of open reading frames is purely hypothetical and is designed to identify any ORFs that may yield peptides that are potentially allergenic or toxic, if they were to be placed in a context in which they were expressed.

Our assessment is consistent with a minimum risk for unintended consequences associated with the T-DNA insertions that create PG451 from its parent cultivar PG.

5.2 Basis for Resistance to Enzymatic Browning in PG451

The intended consequence of genetic modification in PG451 is to reduce the expression of the PPO gene family. Reduced gene expression should lead to lower total PPO activity and result in a nonbrowning phenotype. To confirm this result, we show reduced PPO activity (Section 5.2.1) and reduced bruise response (Section 5.2.2) in PG451 relative to its untransformed control (PG).

5.2.1 PPO Enzyme Activity

Okanagan Specialty Fruits has developed a judicious and sensitive process for identifying transgenic events that are PPO suppressed. It is well understood that PPO varies widely in wildtype apple cultivars (Podsedek, Anders and Markowski, 2000; Burke, 2010; Kolodziejczyk *et al.*, 2010). PPO activity also varies widely by tissue source, growth stage and in response to wounding in wildtype apple cultivars (Costeng and Lee, 1986; Boss *et al.*, 1995; Young *et al.*, 2001; Holderbaum *et al.*, 2010). Therefore, it is often difficult to identify lines that are low in PPO activity.

A key trait of PPO activity in our PPO suppressed Arctic® apple cultivars is that PPO is always low and does not vary widely. The application of our PPO assay is designed to identify those events that are always low and do not vary.

Apples are clonally propagated and so samples tested at any stage of development are essentially replications. Additionally, the PPO suppression transgene is under control of a constitutive promoter designed to suppress PPO activity in all parts of the plants, so any tissue selected from an Arctic® apple cultivar, regardless of it source (tissue culture or greenhouse leaves, immature or mature fruit) should be consistently suppressed. As such, tissue samples are taken at various developmental stages and measured for PPO activity. Events are classified as Arctic® when they are low in successive sub-cultures in tissue culture, when grafted on to rootstocks and when planted in the field trial. The apples that are produced from these trees are low in PPO activity and they do not bruise when cut or damaged.

The specific transgenic event PG451 that is the subject of this extension document was identified as PPO suppressed through this repeated PPO activity screening process from a much larger pool of transgenic events that were produced in our transformation process.

Method for Measuring PPO Activity in Apple

PPO activity was measured using a modification of the method of (Broothaerts et al., 2000) in which the assay portion of the procedure was adapted to a microtitre plate. In the modification, tissue samples were ground in a mortar and pestle under liquid nitrogen. Samples of ground tissue (50 mg) were extracted in 1 ml of extraction buffer (0.1 M sodium phosphate, 2% Triton X-100, 1 % PVPP, pH 6.0). PPO activity is measured using 4-methyl catechol as substrate and protein content was measured using bicinchoninic acid (BCA) (Thermo Scientific Pierce). PPO activity was reported as specific activity (U/mg protein). The Unit Definition of enzyme activity is 1 U = 0.001 A400 / min.

Reduced PPO Activity in Tissue Culture Leaves

Tissue culture plants of PG451 and PG control were sub-cultured about every 6 weeks. There were 6 to 7 plants per jar and multiple jars of each cultivar as these were being propagated for micrografting. In order to identify low PPO Arctic® apple events in the context of a potentially widely varying control apple, we sample our tissue culture plants on three different sub-cultures. On each subculture, a leaf sample is taken that comprises leaves from multiple tissue culture plants of PG451 or PG control. Samples were collected, snap frozen in liquid nitrogen and stored at -80°C until processing. The data in Table 5 is a summary of the tissue culture screening for PPO activity in PG451 and the control PG done over three subcultures (n = 3).

We found that PPO activity was reduced by 90% in PG451 relative to its control (Table 5). The standard deviation for PG451 is very small, consistent with the uniformly low PPO activity in Arctic® apple events. The standard deviation for the PG control is very large, as is sometimes seen in wildtype tissue samples that vary widely in PPO activity.

Table 5: PPO Activity in PG451 – Tissue Culture Leaves

Cultivar	Mean Specific Activity	*S*[1]	n[2]	PPO Suppression[3]
PG451	139	5	3	90 %
PG	1457	899	3	

[1] *S* = standard deviation
[2] n = number of leaf samples per cultivar (3 successive subcultures x 1 sample per subculture)
[3] PPO Suppression = ((Mean Specific Activity of PG – Mean Specific Activity of PG451) / Mean Specific Activity of PG)*100

Reduced PPO Activity in Greenhouse Leaves

In preparing trees for field trial assessment, tissue culture shoots of selected transgenic events are grafted onto apple rootstocks and are grown in greenhouse facilities. Leaves of greenhouse plants of PG451 and PG were collected, snap frozen in liquid nitrogen and stored at -80°C until processing. Leaf samples were measured for PPO activity. The data in Table 6 is a summary of our greenhouse screening for PPO activity in PG451.

We found that PPO activity was reduced 74% in PG451 relative to its control (Table 6). Under greenhouse conditions the plants are no longer sterile and we have found that PPO activity introduced from microbial contamination can influence our PPO assay if not controlled. The effect of the microbial contamination is reflected in our higher standard deviation for PG451 under greenhouse conditions.

Table 6: PPO Activity in PG451 – Greenhouse Leaves

Cultivar	Mean Specific Activity	*S*[1]	n[2]	PPO Suppression[3]
PG451	811	439	15	74 %
PG	3173	241	3	

[1] *S* = standard deviation
[2] n = number of samples per cultivar
[3] PPO Suppression = ((Mean Specific Activity of PG – Mean Specific Activity of PG451) / Mean Specific Activity of PG)*100

The event PG451 identified through this PPO screening process and was advanced to field trial for confirmation using a controlled bruising assay (Section 5.2.2).

5.2.2 Controlled Bruising of Apple

Enzymatic browning that occurs in response to mechanical bruising is measured in mature fruit of PG451 relative to its control cultivar PG using Minolta Chroma Meter CR-400.

Mature apple fruit PG451 and PG were randomly harvested and were stored at 4°C until processing. Apples were bruised and left to sit for 2 hours at room temperature to allow bruise development. An apple peeler was used to expose an area that was larger than the bruised area. The peeled area was measured using a colorimeter 'off' and 'on' the bruise. The color of the apple flesh is compared on and off the bruise location. Bruising is reported as Total Change in Color (ΔE*). The ΔE* for PG (27.6) produced visible brown bruising of the apple flesh, while the ΔE* for PG451 (4.9) did not produce visible bruising of the apple flesh (Table 7). The visual effect of PPO suppression is shown in Figure 16.

In this experiment, we have mimicked the effect of minor finger or superficial bruising that occurs as a part of normal handling of apple during picking, sorting, packing and shipping. In control cultivars of apple with normal levels of PPO, this minor and often invisible tissue damage, results in a more progressive tissue damage that is mediated by the activity of PPO. This can lead to a very high shrink (retail and wholesale loss) as these bruises lead to nutrient degradation and loss of visual appeal. Ultimately these progressive bruises become a site of infection leading to microbial loss of the product.

Improved Arctic® apple cultivars, such as PG451, with reduced levels of PPO, are less subject to progressive bruising meaning that more of these apples will reach the marketplace.

Table 7: Controlled Bruising of PG451 – Mature Fruit

Cultivar	ΔE*[1]	*S*[2]	n[3]
PG451	4.9	2.2	20
PG	27.6	4.1	28

[1] ΔE* = Total Change in Color

[2] *S* = standard deviation

[3] n = number of bruises = number of apples x 4 bruises per apple.

Figure 16: Controlled Bruising of Gala (PG) and Arctic® Gala (PG451)
The apples were impacted and left to sit for 2 hours for bruise development. The apple on the left is an untransformed control apple of Gala (PG). The apple on the right is PG451. The lack of bruise response in PG451 is typical of all Arctic® apple cultivars.

Method for Measuring Bruising Response in Apples

An impact device is used to deliver a controlled bruise to the apple with minimal destruction to the tissue. Apples are bruised in a consistent manner using the impact device. Bruise response is reported as Total Change in Color (ΔE*) between bruised and non-bruised tissue as measured using a Minolta Chroma Meter CR-400.

The Impact Device

The impact device consists of a 35 mm diameter glass marble and a 28 cm long cardboard tube, plus a foam support used to stabilize and protect the apple during bruise. The bruise is made by dropping the marble down the tube which is placed on the surface of the apple. The foam support, into which the apple is placed, provides a cushion to prevent damage to the underside of the apple during impact to the top side of the apple.

The impact device is designed to bruise the apple, but with minimum physical damage to the apple tissue. The impact will produce an observable bruise 2 hours after impact but does not produce a significant bruise at time zero.

Procedure

Apples are removed from storage and allowed to come to room temperature for 2 hours. Positions of the bruises are marked with a felt pen on the apple skin. Each apple is bruised 4 times and allowed to sit at room temperature for 2 hours for the bruise to form. The apples are peeled over the bruised areas (careful not to remove the pen marking or to go too deep with the peeling). Each peeled area is measured on the non-bruised area adjacent to the bruise (trt1) and directly on the bruised area (trt2). Total Change in Color (ΔE*) are calculated as:

$$\Delta L^* = L^*_{trt2} - L^*_{trt1}$$

$$\Delta a^* = a^*_{trt2} - a^*_{trt1}$$

$$\Delta b^* = b^*_{trt2} - b^*_{trt1}$$

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

The measured variables are lightness (L* = 0 yields black and L* = 100 indicated diffuse white), its position between red/magenta and green (a*, negative value indicate green while positive values indicate magenta), and its position between yellow and blue (b*, negative values indicate blue and positive values indicate yellow) as described in the CIE 1976 (L*, a*, b*) color space.

The reported variable is change in color (ΔE*), calculated from the measured variables. The change in color (ΔE*) is a positive number and represents the difference (distance) between two colors. A larger ΔE* represents a larger color difference.

5.3 Expression of NptII in Mature Fruit

The GEN-03 vector used to generate GD743, GS784 and PG451 contains the *nptII* gene as a selectable marker. In the original Petition for Determination of Non-Regulated Status: Arctic Apple (*Malus x domestica*) Events GD743 and GS784 (10-161-01p) the molecular characterization was consistent with 3 copies of the transgene in GD743 and 4 copies of the transgene in GS784. A quantitative NptII ELISA was used to determine that the *nptII* gene as expressed by nopaline synthase promoter (Pnos) did not result in detectable amounts of the NptII protein accumulating in mature fruit of either GD743 or GS784. As additional information, we refer to USDA Extension 16-004-01p which describes Arctic® Fuji (NF872). Molecular characterization of NF872 was consistent with five copies of the *nptII* gene in NF872. Consistent with our observations in GD743 and GS784, there was no detectable accumulation of NptII protein in mature fruit of NF872. It seems that irrespective of copy number or genomic location, the *nptII* gene, under control of the Pnos promoter, does not result in significant accumulation of the NptII protein in mature apple fruit. By extension, we have assumed that NptII protein will not accumulate to significant levels in PG451. Please note, that this will be confirmed by NptII ELISA prior to submission of PG451 to the FDA for food and feed approval.

In any event, the *nptII* gene encodes for neomycin phosphotransferase (NptII), which confers resistance to kanamycin in plants (Fraley *et al.*, 1983). NptII is an enzyme which inactivates the antibiotic kanamycin thereby allowing cells containing this gene to grow on medium containing kanamycin. The *nptII* gene is devoid of inherent plant pest characteristics (Fuchs *et al.*, 1993). Previously, APHIS has determined that the presence of the *nptII* gene will have no significant environmental impacts (APHIS 05-294-02r).

6 ENVIRONMENTAL CONSEQUENCES OF INTRODUCTION OF THE TRANSFORMED CULTIVAR

Importance of Apple, Impact of Enzymatic Browning in Fresh and Processed Apple Products, Expected Economic Impact of Arctic® apple, Gene Flow and Stewardship of Arctic® apple Gene Flow were discussed previously.

Gala is the most popular cultivar in the United States (Table 8). It is anticipated Arctic® Gala (Event PG451) covered under this petition, will be a direct replacement for Gala.

Table 8: USA Apple Production, By Cultivar (000 42 lb. Units)

Cultivars	2014	2015	2016	2017	2018	2019 Forecast
Gala	47,138	37,758	46,043	50,465	47,405	50,077
Red Delicious	64,727	50,212	59,193	58,854	50,266	45,819
Granny Smith *	25,554	23,405	28,977	27,216	21,545	24,679
Fuji *	25,341	22,061	27,241	24,485	22,688	25,144
Honeycrisp	14,028	13,192	15,580	19,672	21,013	24,849
Golden Delicious	27,840	22,137	24,377	21,184	15,548	16,547
McIntosh	10,555	11,203	9,962	10,578	8,308	7,615
Rome	8,330	8,188	7,989	8,079	6,533	6,185
Cripps Pink *	5,942	6,981	8,645	7,510	7,852	8,576
Empire	6,137	6,451	6,223	6,189	6,046	5,563
Idared	4,276	4,235	4,524	4,046	4,249	3,973
York	4,359	4,234	3,807	4,485	3,295	3,262
Jonathan	3,033	2,982	3,093	2,546	2,040	2,038
Cortland	2,536	2,714	2,484	2,642	2,409	2,195
Braeburn *	3,239	2,173	2,725	1,583	1,328	1,102
Stayman	1,080	1,046	932	1,077	659	652
Northern Spy	1,137	1,122	1,371	986	850	810
Jonagold *	1,570	974	1,212	643	493	534
Cameo *	995	531	672	117	100	106
All Others	23,463	17,402	18,459	22,730	21,590	23,449
Total	281,280	239,000	273,688	275,088	244,214	253,095

* Includes only western production. Eastern and Midwest production are included in all others. Sum of cultivars may not add up to total due to rounding of individual cultivars. Source: US Apple Association (US_Apple, 2018).

7 ADVERSE CONSEQUENCES OF INTRODUCTION

Given that PG451 was developed using the same binary vector that was used to develop GD743 and GS784 and that the same two functional traits were transferred to all three improved apple cultivars there should be no adverse consequence of the introduction of PG451 beyond what was previously discussed for GD743 and GS784.

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