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February 23, 2023

Bernadette Juarez
APHIS Deputy Administrator
Biotechnology Regulatory Services
4700 River Road, Unit 98
Riverdale, MD 20737

Does Not Contain Confidential Business Information

Dear Bernadette Juarez,

RE: Request for Confirmation of Exemption from Regulations Under 7 CFR Part 340 for Nonbrowning Arctic® Apple Event CPI1013

Okanagan Specialty Fruits Inc. respectfully seeks confirmation from Biotechnology Regulatory Services (BRS) that Nonbrowning Arctic® apple Event CPI1013 developed by supressing PPO (Polyphenol Oxidase) gene expression is exempt from regulations under 7 CFR Part 340.

We are pleased to provide the following information in support of our request, and according to the guidelines provided by USDA-APHIS Biotechnology Regulatory Services document BRS-GD-2020-0001 dated August 31, 2022.

Requester's Information and Contact:

Company	: Okanagan Specialty Fruits Inc.
Contact Person	: Neal Carter, President
Address	: 15304 Prairie Valley Road, Summerland, BC V0H 1Z8, Canada
Phone	: 250-404-0101
Email	: ncarter@okspecialtyfruits.com

Description of the plant:

The recipient of the nonbrowning trait in this application is an apple plant. Specifically, the parental cultivar Cripps Pink *aka* "Pink Lady®" (hereafter referred to as the CPI), is a commercially grown cultivar of apple. Apple belongs to the genus *Malus,* part of the rose family (*Rosaceae*). The CFIA biology document on apple (can be found HERE), "The Biology of *Malus domestica* Borkh.", 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

Type of Claim for regulatory exemption:
We are requesting the exemption of Nonbrowning Arctic® apple Event CPI1013-22 (hereafter referred to as the CPI1013 apple) from regulation under USDA-APHIS article 7 CFR Part 340.1(c)(2) which states "a plant-trait-MOA combination that is the same as that in a plant of the same species APHIS determined to be nonregulated in response to a petition submitted prior to October 1, 2021, pursuant to 340.6 of the previous regulations found at 7 CFR part 340". We believe that our request qualifies under this article since USDA has previously reviewed petitions pertaining to the low PPO trait in the tree crop apple (*Malus* × *domestica*) developed by this applicant (Okanagan Specialty Fruits Inc.) and determined that nonbrowning apples modified to reduce polyphenol oxidase are unlikely to pose a plant pest risk, and such documents are available at the following links.

- https://www.aphis.usda.gov/brs/aphisdocs/10_16101p_fea.pdf
- https://www.aphis.usda.gov/brs/aphisdocs/16_00401p_det_pprsa.pdf
- https://www.aphis.usda.gov/brs/aphisdocs/16_00401p_pdet_pprsa.pdf
- https://www.aphis.usda.gov/brs/aphisdocs/20-213-01ext_det-pprsa.pdf
- https://www.aphis.usda.gov/biotechnology/downloads/confirmation-response/21-105-01cr.pdf

A Description of the Trait : Browning vs Nonbrowning Phenotype in Apple:
Browning of flesh when an apple is cut, sliced, bitten, or bruised is a trait caused by a small gene family coding for an enzyme called polyphenol oxidases (PPO), which is responsible for this "oxidative" browning. Okanagan Specialty Fruits Inc. (OSF) has developed CPI1013 apple (*Malus x domestica)*, a new cultivar that has been genetically engineered to be nonbrowning. In Arctic® apple cultivars, Arctic® being the brand for the nonbrowning trait, the PPO gene(s) driving the browning reaction are essentially switched off.

CPI1013 was developed through Agrobacterium-mediated transformation to stably incorporate the transgenes PGAS2 and nptII into the apple genome. The PGAS2 gene is 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 nonbrowning trait in CPI1013.

Comparison of CPI1013 with antecedent organisms conferring the Nonbrowning Phenotype in Apple:

In CPI1013, as in the antecedent organisms GD743, GS784, NF872, PG451 and HCR835, both transgenes (PPO suppression cassette and NptII) were transferred to the recipient cultivar in functional form. The PPO suppression transgene (PGAS2) resulted in reduced levels of polyphenol oxidase (PPO) and a nonbrowning phenotype in CPI1013 which is typical of all Arctic® apple cultivars. The nptII selection marker permitted the identification of CPI1013 during the transformation process. The level of NptII protein in CPI1013 fruit was not measured here. However, in the antecedent organisms GD743 and GS784 and in another cultivar Arctic® Fuji (Event NF872) developed using the PGAS (GEN-03 vector); we determined that the amount of NptII protein in mature fruit was below the limit of detection by ELISA.

Plant-trait-MOA (Suppression of PPO causing non-browning in apple) is the same in CPI1013 as in all antecedent organisms (GD743, GS784, NF872, PG451, HCR835). An improved gene delivery system was used for the development of CPI1013 (same as for antecedent organism HCR835) by using a binary vector named VOSF compared to binary vector GEN-03 used for the development of GD743, GS784, NF872 and PG451. The vector VOSF, comprises the following improvement to the GEN-03:

1. The binary vector backbone pBINPLUS (GEN-03) has been replaced with pCAMBIA_0390 (VOSF) to increase binary vector copy number and transformation efficiency.
2. The mutant *nptII* gene (GEN-03) has been replaced with the wild-type *nptII* gene (VOSF) which has higher kanamycin-resistance activity to improve recovery and selection of transgenic events.
3. The sense PPO suppression transgene (PGAS-GEN-03) has been replaced with an intron-containing RNA (ihpRNA) transgene (PGAS2-VOSF) which can efficiently induce RNA interference and increase the recovery of PPO suppressed events. The detailed structural differences between VOSF and GEN-03 are provided in Exhibit-4 of the Confirmation of Exemption from Regulations Under 7 CFR Part 340 for Nonbrowning Arctic® Apple Event HCR835 (Link to the retrieve the document).

The following table provides information on the developed nonbrowning trait in relation to genes and events for CPI1013, GD743, GS784, NF872, PG451, HCR835.

Characteristic	Candidate CPI1013 Apple	Antecedent HCR835 Apple	Antecedent GS784, GD743, NF872, PG451 Apple
Organism	Apple	Apple	Apple
Cultivar (s)	Cripps Pink	Honeycrisp	Granny Smith; NagaFu, Golden Delicious; Pacific Gala
Trait-Phenotype	Nonbrowning	Nonbrowning	Nonbrowning

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	VOSF	VOSF	GEN-03

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

VOSF: The VOSF binary vector comprises the genes PGAS2 and *nptII*.

The nonbrowning trait of Arctic® apple event CPI1013 will offer growers, packers, processors, wholesalers, retailers, food service and consumers a nonbrowning variant of the popular Cripps Pink apple cultivar they have become accustomed to purchasing. The key benefits of nonbrowning trait include but limited to:

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

Genetic Modification and Associated MOA(s)

a) Molecular description of the inserted genetic material

Event CPI1013 was developed through Agrobacterium-mediated transformation of apple leaf tissue using the binary vector VOSF (See Exhibit-1, Figure 1) which is based on the binary vector pCAMBIA_0390 a derivative of pPZP200[a]. All the known intact, functional elements of the VOSF are described in Exhibit-1, Table 1. The T-DNA comprises a PPO suppression transgene (P70:PGAS:TNOS) and NptII selection marker (PNOS:nptII:TNOS) flanked by Agrobacterium tumefaciens T-DNA borders. The vector backbone includes bacterial elements required to support vector replication and selection (pVS1 RepA, pVS1 StaA, nptIII, and oriV). The remaining vector backbone sequences that reside between these known functional elements were fragmented in the cloning process that led to the development of the binary vector and are non-functional.

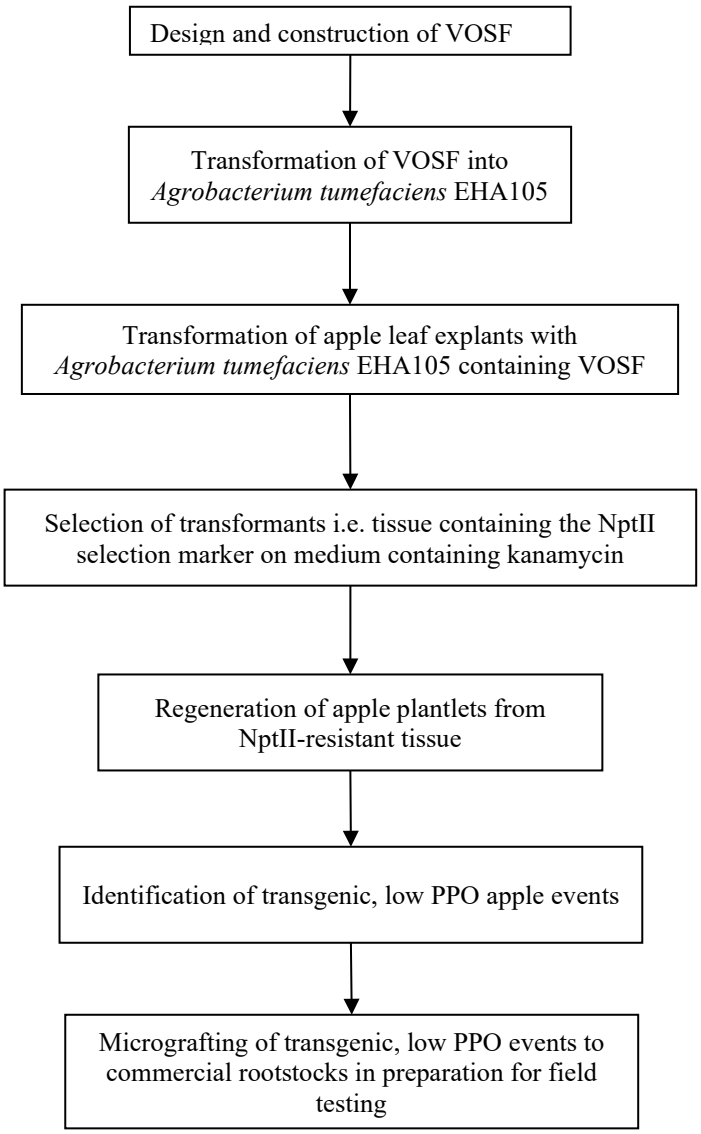
A more in-depth description of the above is given in Exhibit-4.

[a] *Hajdukiewicz, P., Svab, Z. and Maliga, P. (1994) 'The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation', Plant Molecular Biology, 25(6), pp. 989-994.*

b) Method used to produce the modification(s)

The strategy and methodology to produce CPI1013 was the same as used to develop HCR835 (Reference), as well as for antecedent organisms GD743,GS784, NF872 and PG451. The methodology is outlined in Figure 3 below, and details of various steps are provided in Exhibit-2.

Figure 3: Steps in the Development of Arctic® apple events CPI1013, HCR835, PG451, GD743, NF872 and GS784



c) Function of the modified gene or genetic element

CPI1013 was produced by Agrobacterium-mediated transformation of the apple cultivar Cripps Pink (CPI) with the binary vector VOSF. The VOSF 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 and antisense orientation under control of the cauliflower mosaic virus promoter and nopaline synthase terminator. This transgene (PGAS2) is designed to reduce expression of the entire PPO gene family and to induce a nonbrowning phenotype in apple fruit. The second gene is a nptII selection marker used in the development of CPI1013 apple. The antecedent organisms, HCR835 was developed using the exact same vector (VOSF) while GD743,GS784, NF872 and PG451 were generated by Agrobacterium-mediated transformation with a vector (GEN-03) containing the same and/or similar functional elements.

d) Molecular characterization and DNA Sequence Data encompassing the modifications, strategy, and methodology of sequencing

To describe the insertions that create CPI1013 from Cripps Pink we employ a unique mapping strategy based on long read (Oxford Nanopore Technologies) whole genome sequencing data. In our approach, the reads pool is systematically sorted into three reads pools: (i) reads that match the VOSF 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 CPI1013 genome from next-generation sequencing data. Instead, our mapping approach focuses on identifying reads that match the VOSF 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 checking the read quality and filter the reads based on read length (remove less than 1kb) and DNA Sequence Quality (remove less than QC=7). Where, a DNA Sequence Quality Score (QC) is an estimate (q) of the probability of that base being called wrongly: $q = -10 \times \log10(p)$.

This extracted read pool from previous step is then sequentially filtered to remove reads that match perfectly to the apple reference genome or the VOSF 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 CPI1013 from the parent cultivar. For more in-depth narrative and data of this process see Exhibit-3.

Our reads selection process for CPI1013 ultimately identified 18 reads from a total of 2,508,053 reads (~ 165,173 bases) that were used to map the junctions. The genomic locations of the insertions for 18 reads are shown in Exhibit-3 via Figures 4 and 5. We were able to verify the insertions of 18 reads as revealed by Nanopore long read data, with no evidence of any additional insertions.

When mapping with ~ 23X overall coverage (11.5X diploid coverage) our genome to vector junctions has an average coverage of 5.5 reads. 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 approximately ~ 6 reads per junction. We find it improbable that we would miss this many chimeric reads in our mapping process. In addition to CPI1013, we have mapped numerous VOSF 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.

Our examination of the whole genome sequencing data for CPI1013 has revealed evidence of insertion in chromosome 13. (Exhibit-3, 'T-DNA Genomic Location').

Based on the sum of the mapping evidence we have developed an insertion map that accounts for all junctions and is consistent with the estimated copy number for each vector region (Exhibit-3: Figure 7 and 8).

There is a theoretical possibility that a second 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 VOSF binary vector, both T-DNA region and vector backbone, do not comprise these factors and are easily sequenced using Nanopore 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.

Conclusions

Okanagan Specialty Fruits Inc. requests confirmation from Biotechnology Regulatory Services (BRS) that Nonbrowning Arctic® apple Event CPI1013 developed by supressing PPO (Polyphenol Oxidase) gene expression does not meet the definition of a regulated article under 7 CFR Part 340. This request is because Arctic® apple event CPI1013 is same and/or similar to Arctic® apple events HCR-835 , GS784, NF872, GD743 and PG451 and (described in confirmation-response/21-105-01cr and petitions 20-213-01ext, 16-00401p, 10-161-01p) in terms of transgene family, trait and MOA. The subjects of 21-105-01cr received a confirmation

response on May 20, 2021, while petition 20-213-01ext received a determination of non-regulated status on September 22, 2021. Similarly, petition 10-161-01p Arctic® Golden (Event GD743) and Arctic® Granny (Event GS784) received a determination of non-regulated status on February 13, 2015.

CPI1013 was produced by Agrobacterium-mediated transformation of the apple cultivar Cripps Pink (CPI) with the binary vector VOSF. The VOSF 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 and antisense 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 a nptII selection marker used in the development of CPI1013 apple. The antecedent organism, HCR835 was also developed by insertion of VOSF vector in Honey crisp apple genome with agrobacterium, while PG451, NF872, GD743 and GS784 were generated by Agrobacterium-mediated transformation with the GEN-03 vector resulting in suppression of PPO.

In CPI1013, as in the antecedent organisms HCR835, PG451, 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 nonbrowning phenotype typical of all Arctic® apple cultivars. The nptII selection marker permitted the identification of CPI1013 during the transformation process. The level of NptII protein in CPI1013 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.

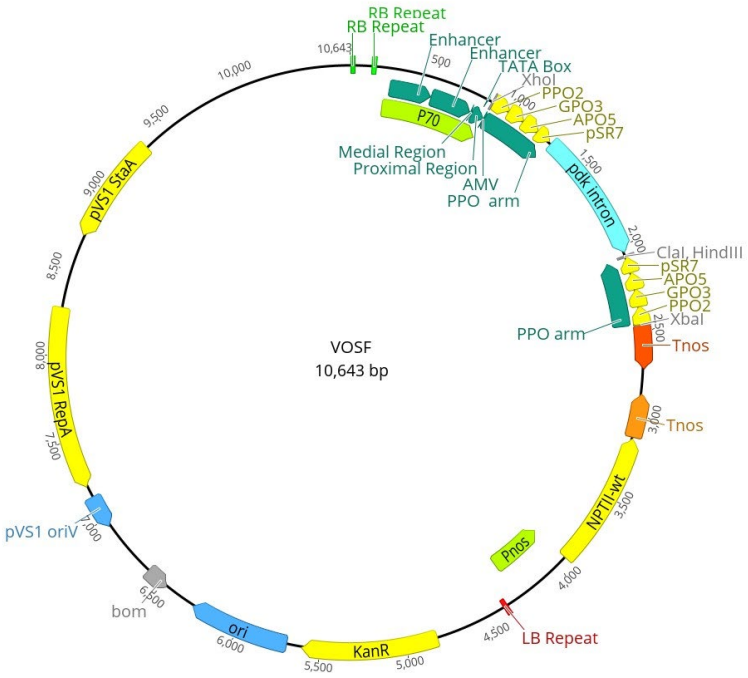
OSF submits that the CPI1013 apple, described in this request for confirmation, was transformed using the same PPO genes and functionally and same (for HCR835) or similar (for PG451, GD743, NF872 and GS784) binary vector as events, yielding the same nonbrowning phenotype, and does not meet the definition of a regulated article under 7 CFR Part 340.

Sincerely,
Okanagan Specialty Fruits Inc.

Neal Carter
President

Exhibit-1: Description of Genetic Material

Figure 1: Map of the VOSF Vector



The vector pictured here is the VOSF vector used to create Arctic® apple event CPI1013. The complete list of structural elements of VOSF is given in the following table (Table 1).

Table 1: Components of the VOSF Vector-DNA Used to Develop CPI1013

Genetic Element	Size (bp)	Position	Function, Source, Reference
T-DNA			
RB Repeat	25	1 – 25, 127-151	The right repeat sequence derived from *Agrobacterium tumefaciens* pTIT37 (Depicker *et al.*, 1982) .
P70	649	210 - 858	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.
PPO arm	396	865 - 1,260	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.
pdk intron	767	1,274 - 2,040	Intron from *Flaveria trinervia* used as a spacer to stabilize the hairpin construct (Smith and Singh, 2000)
PPO arm	396	2,072 - 2,467	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 (reversed).
T_{NOS}	254	2,474 – 2,727	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).
T_{NOS}	254	2,874 – 3,127	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	3,134 – 3,928	Neomycin phosphotransferase type II from *Escherichia coli* Tn5 (Rothstein *et al.*, 1981) providing resistance to kanamycin.
P_{NOS}	347	3,941 – 4,287	A nopaline synthase promoter from *Agrobacterium tumefaciens* that directs transcription of the *nptII* selection marker (Bevan, Flavell and Chilton, 1983).
LB Repeat	25	4,366 – 4,390	The left border repeat derived from *Agrobacterium tumefaciens* pTiT37 (Depicker *et al.*, 1982).
Vector			

KanR	795	3,134 – 3,928	Neomycin phosphotransferase type III from *Streptococcus faecalis* R plasmid (Scutt, Zubko and Meyer, 2002) providing resistance to kanamycin
Ori	589	5,696 – 6,284	RNA origin of replication used to increase the plasmid copy number, obtained from pBR322 (van Engelen *et al*., 1995).
bom	141	6,470 – 6,610	Basis of mobility from pBR322 (Bolivar *et al.*, 1977).
pVS1 oriV	195	6,954 – 7,148	Origin of replication that functions in *Agrobacterium tumefaciens* from the *Pseudomonas* plasmid pVS1 (Heeb *et al*., 2000).
pVS1 RepA	1,068	7,214 – 8,281	Replication protein that functions in *Agrobacterium tumefaciens* from the *Pseudomonas* plasmid pVS1 (Heeb *et al*., 2000).
pVS1 StaA	630	8,715 – 9,344	Stability protein that functions in *Agrobacterium tumefaciens* from the *Pseudomonas* plasmid pVS1 (Heeb *et al*., 2000).

References

Bevan, M., Barnes, W. M. and Chilton, M.-D. (1983) 'Structure and Transcription of the Nopaline Synthase Gene Region of T-DNA', *Nucleic Acids Research*, 11(2), pp. 369–385.

Bevan, M. W., Flavell, R. B. and Chilton, M.-D. (1983) 'A Chimaeric Antibiotic Resistance Gene as a Selectable Marker for Plant Cell Transformation', *Nature*, 304, pp. 184–187.

Bolivar F. *et al.* (1977) 'Construction and characterization of new cloning vehicles. II. A multipurpose cloning system', *Gene* 2(2), pp. 95-113

Datla, R. S. S. *et al.* (1992) 'Modified binary plant transformation vectors with the wild-type gene encoding NPTII*', *Gene*, 211, pp. 383–384.

Depicker, A. *et al.* (1982) 'Nopaline Synthase: Transcript Mapping and DNA Sequence', *Journal of Molecular and Applied Genetics*, 1, pp. 561–573.

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Heeb, S. *et al.* (2000) 'Small, Stable Shuttle Vectors Based on the Minimal pVS1 Replicon for Use in Gram-Negative, Plant-Associated Bacteria', *Molecular Plant-Microbe Interactions* 13(2) pp. 232-237.

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Smith, N.A., Singh, S.P. (2000) ‘Total silencing by intron-spliced hairpin RNAs’, *Nature*, 407, pp. 319-320.

Exhibit-2: Steps in the Development of Arctic® apple event CPI1013

Design and construction of VOSF Vector:
It is previously described in Exhibit-1.

Transformation of VOSF vector into *Agrobacterium tumefaciens strain* EHA105:
VOSF vector was transformed into Agrobacterium by ELECTROPORATION (Wen-jun and Forde, 1989). Later, the culture was grown for 3 hours, plated and colony screening was done by PCR. A positive colony was selected, DNA extracted, integrity verified by DNA sequencing, and used for plant transformation.

Transformation of apple leaf explants with *Agrobacterium tumefaciens* EHA105 containing VOSF vector:

In brief, leaves of four-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 VOSF 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 on delay media (no selection) for 3 days. Leaves were then moved to regeneration medium containing 5 µg/ml kanamycin with the adaxial surfaces in contact for 12 weeks (2 weeks dark, 2 weeks low light, 8 weeks regular), refreshing the media every 3-4 weeks.

Selection of transformants i.e., tissue containing the NptII selection marker on medium containing kanamycin:

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.

Regeneration and propagation of apple Plantlets from NptII-resistant tissue:

Shoots continue to be transferred on fresh proliferation medium at regular intervals (approximately every 6 weeks) with sub-cultures to propagate the tissue into as many viable shoots as is possible.

Identification of transgenic apple events with low PPO activity:

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 CPI1013 and CPI 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 sampled our tissue culture plants on two different sub-cultures. On each subculture, a leaf sample is taken that comprises leaves from multiple tissue culture plants of CPI1013 or CPI control. Samples were collected, snap frozen in liquid nitrogen and stored at -80ºC until processing. The data in Table 2 is a summary of the tissue culture screening for PPO activity in CPI1013 and the control CPI done over three subcultures (n = 9).

We found that PPO activity was reduced by 95% in CPI1013 relative to its control (Table 2). The standard deviation for CPI1013 is very small, consistent with the uniformly low PPO activity in Arctic® apple events. The standard deviation for the CPI control is larger, as is sometimes seen in wildtype tissue samples that vary widely in PPO activity.

Table 2: PPO Activity in CPI1013 – Tissue Culture Leaves

Cultivar	Mean Specific Activity	S[1]	n[2]	PPO Suppression[3]
CPI1013	133	0.63	18	95.2 %
CPI (WILD TYPE)	2773	23	9	

[1] S = standard deviation
[2] n = number of leaf samples per cultivar (2 successive subcultures x 2 or 1 sample per subculture)
[3] PPO Suppression = ((Mean Specific Activity of CPI – Mean Specific Activity of CPI1013) / Mean Specific Activity of CPI)*100

Micrografting of transgenic, low PPO events to M9 rootstocks in preparation for field testing:

Micrografting is routinely done according to the method of (Lane et al., 2003). However, micrografting for this event (CPI1013) is still in process.

References

Broothaerts, W. *et al.* (2000) 'Fast Apple (Malus × domestica) and Tobacco (Nicotiana tobacum) Leaf Polyphenol Oxidase Activity Assay for Screening Transgenic Plants', *Journal of Agricultural and Food Chemistry*, 48(12), pp. 5924–5928.

Lane WD, Bhagwat B, Armstrong JD, Wahlgren S. 2003. Apple micrografting protocol to establish transgenic clones on field ready rootstock. *HortTechnology* 13 pp. 641-646.
es. **17**, 8385

Wen-jun, S. and Forde, B. G. (1989) Efficient transformation of *Agrobacterium* spp. by high voltage electroporation. *Nucleic Acids Res.* **17**, 8385

Exhibit-3: Molecular characterization and DNA Sequence Data encompassing the modifications, strategy, and methodology of sequencing

Sequencing Library Preparation and Whole Genome Sequencing

The genomic DNA library was prepared following the protocol as described by Oxford Nanopore Technologies (Protocol accessed on February 10, 2023) using Ligation Sequencing Kit V14 (Product Code: SQK-LSK114). We used the high accuracy model for base calling (maximum Q20) as described in the above protocol for LSK-114. Briefly, 1,500 ng of high-quality genomic DNA was used for DNA repair and end-prep using NEBNext Companion Module for Oxford Nanopore Technologies Ligation Sequencing kit (New England Biolabs Inc.). The end-prepped DNA was cleaned up using AMPure XP beads with 70 % Ethyl alcohol and then used for adaptor ligation (SQK-LSK114; Oxford Nanopore Technologies). Again, the DNA library was cleaned up using AMPure XP beads with Long Fragment Buffer. The quality and quantity of the DNA library was confirmed using TapeStation (Agilent Technologies) and Qubit fluorometer. Finally, 840 ng of the final library was loaded in a flow cell (FLO-MIN114; R10.4.1) for sequencing.

Sequencing Coverage and Quality

The CPI1013 read library was trimmed for quality below Q7 (80% base call certainty) and a minimum read length of 1 kb nucleotides. A theoretical sequencing coverage of 23x was calculated using a modification of the Lander/Waterman equation (Table 4). Apple is diploid so our insertions are expected to have a sequencing coverage of 11.5x. The Lander/Waterman equation used to calculate coverage assumes that the reads are distributed evenly about the genome 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 4: Calculation of Theoretical Sequencing Coverage

Descriptor	Value
Qualified Reads (Q7)	2,508,053
Nucleotides	16,727,261,717
Genome Size (HFTH1)	708,540,000
Coverage	23.6

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

The reads were mapped to the high quality apple reference, HFTH1 (Zhang *et al.*, 2019) available at the Genome Database for Rosaceae (Jung *et al.*, 2019), using Geneious Minimap2 assembler to determine actual sequence coverage (Table 5). Mean coverage ranged from 22.436x to 33.294x with an average of 26.035x. The amount of the reference genome that was covered by at least one sequence read (Percent of Ref Seq), ranged from 95.8% to 99.5% with an average of 97.565%. The predicted gap in our sequencing coverage (2.435%) corresponds to approximately 17,252,949 bp.

Table 5: Mapping CPI1013 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	32,944,118	207,950	1,001,452,444	24.805	76.2%	97.4%
2	38,449,405	261,684	1,202,851,116	25.696	77.3%	95.8%
3	37,138,690	242,216	1,144,854,463	25.056	76.4%	97.9%
4	31,012,745	208,620	944,745,515	25.414	77.3%	97.4%
5	47,891,858	312,151	1,533,300,813	33.294	84.3%	97.3%
6	35,567,198	216,442	1,088,958,475	26.135	77.6%	99.5%
7	35,934,761	229,221	1,086,864,951	26.102	78.2%	97.9%
8	31,511,015	203,889	935,679,703	24.713	77.4%	97.2%
9	34,800,404	220,183	1,049,838,0501	24.521	78.0%	97.0%
10	43,815,736	269,442	1,310,936,828	24.436	77.1%	96.0%
11	42,456,296	279,081	1,326,134,112	25.645	77.1%	97.3%
12	32,285,079	198,073	973,062,390	25.542	78.0%	98.6%
13	44,866,511	295,983	1,461,743,760	29.524	76.4%	99.1%
14	31,515,206	196,109	967,892,083	25.097	78.0%	97.9%
15	56,644,392	360,098	1,709,852,940	24.635	77.4%	98.2%
16	41,670,059	287,327	1,302,737,655	26.048	79.3%	98.0%
17	33,998,825	211,195	1,028,062,917	25.936	77.7%	96.1%
00	7,992,922	186,050	976,109,166	409.989	84.9%	74.9%

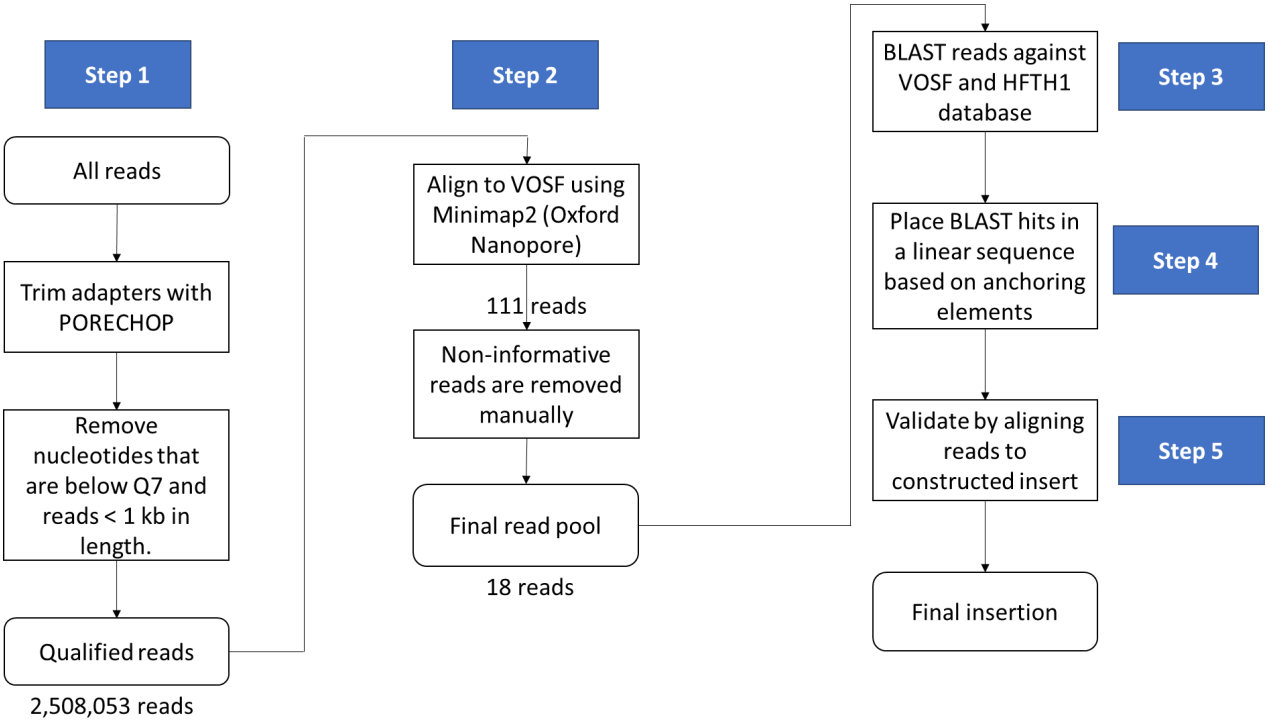
[1] Geneious (Prime) Minimap2, Oxford Nanopore.

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

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 3.

Figure 3: Workflow for Detection of T-DNA Insertions



Non-informative reads are those that either: Have a full linear alignment within the transgene of VOSF or align only to a single copy of one the sub-units of the PGAS gene.

Step 1: Cleaning the Initial Read Pool

Adapter sequence present in the raw read data is removed using the PORECHOP command from the command line. Second is removing the data that is of poor quality through the use of the BBDuk plugin within Geneious. This step removed reads that were shorter than 1000 bases in length and had an overall quality score lower than Q7 (80% accuracy). Statistics for the remaining data was verified with NANOPLOT, visually verifying that the data met the minimum quality and length. For CPI1013 2,508,053 reads were left for final analysis.

Step 2: Reads Mapping to the VOSF Vector

To reduce the pool to the final informative ones, 2,508,053 reads were mapped to the VOSF vector using the Minimap2 assembler with the Oxford Nanopore data type. A total of 111 reads mapped

to the VOSF vector. The alignment was then visually inspected to filter out non-informative reads defined as reads that either: mapped entirely and linearly in the tDNA or to a single sub-unit of one of the transgene copies. Reads that did not meet either criterion were noted and put into a separate, final, pool (a total of 18 reads).

Step 3-5: Mapping the insertion

To identify the insertion site within the apple genome, the 18 reads from Step 3 were BLAST against a custom database using the apple reference (HFTH1) which is available at the Genome Database for Rosaceae (Jung *et al.*, 2019) and one for the VOSF vector. This process revealed a read (8a3b1926-6be0-424b-bda1-1f85afdd552f) that was 10,594 base pairs in length that spanned the entire insertion.

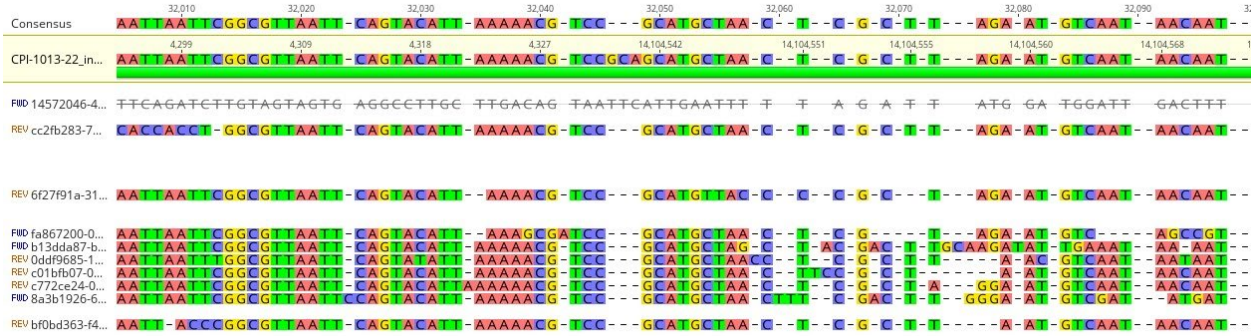
T-DNA Genomic Location

The junction mapping process (Steps 1 – 5, above) identified two apple/vector junctions co-located in chromosome 13 (Figure 4 and 5). This is consistent with an insertion sites. No other chimeric (vector to chromosome) reads were identified in the pool of final reads.

Figure 4: Chromosome 13 Insertion - Left Side

A fragment crossing the junction with mapped reads is provided as evidence of the junction. This figure shows chromosome 13 (Chr12:14,104,487…14,104,520) attached to the RB of VOSF (VOSF:4,379…4,336).

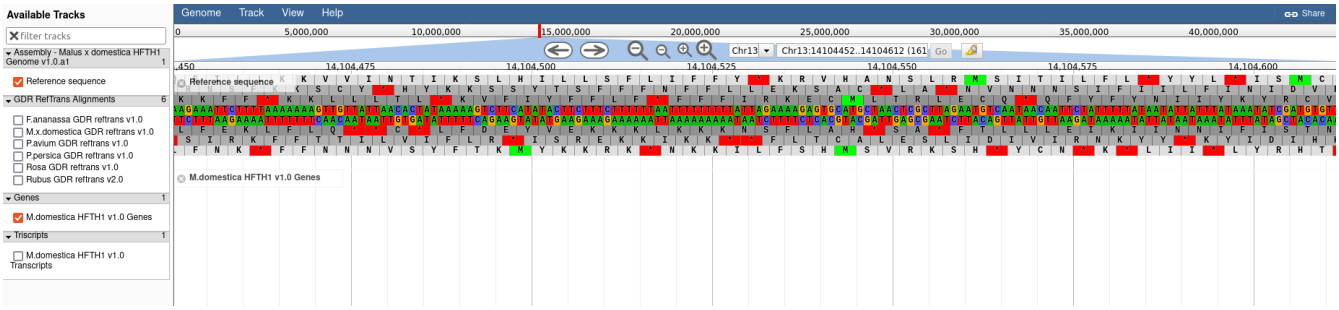
Figure 5: Chromosome 13 Insertion - Right Side



A fragment crossing the junction with mapped reads is provided as evidence of the junction. This figure shows the LB of VOSF (VOSF:4,294…4,334) attached to chromosome 13 (Chr13:14,104,541…14,104,573).

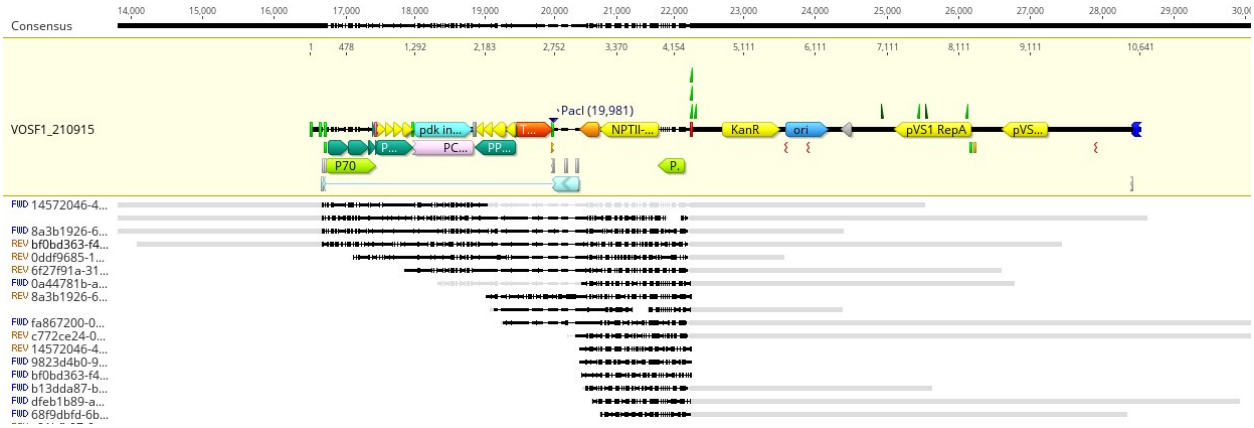
The genomic location of the insertion in chromosome 13 (Figure 6) is shown as evidence that the insertion does not disrupt any known endogenous gene. No predicted genes or transcripts are present in the annotation window for the *M. domestica* HFTH1 v1.0 reference.

Figure 6: CPI1013 - Chromosome 13 - Genomic Location



A screenshot covering the insertion site in CPI1013 Chromosome 13 (Chr13:14,104,452…14,104,612) showing no predicted genes or transcripts present at the insertion site (JBrowse, GDR).

Figure 7: Coverage of the VOSF in CPI1013



Reads mapped to the VOSF binary vector using Minimap2 reveal which regions of the T-DNA were included in CPI1013. Here only from the right border (RB) to left border (LB) is represented in the reads. The sequencing coverage was used to predict the approximate copy number of inserted elements. Coverage shows the presence of a second copy of the LB through to the Tnos (orange) element.

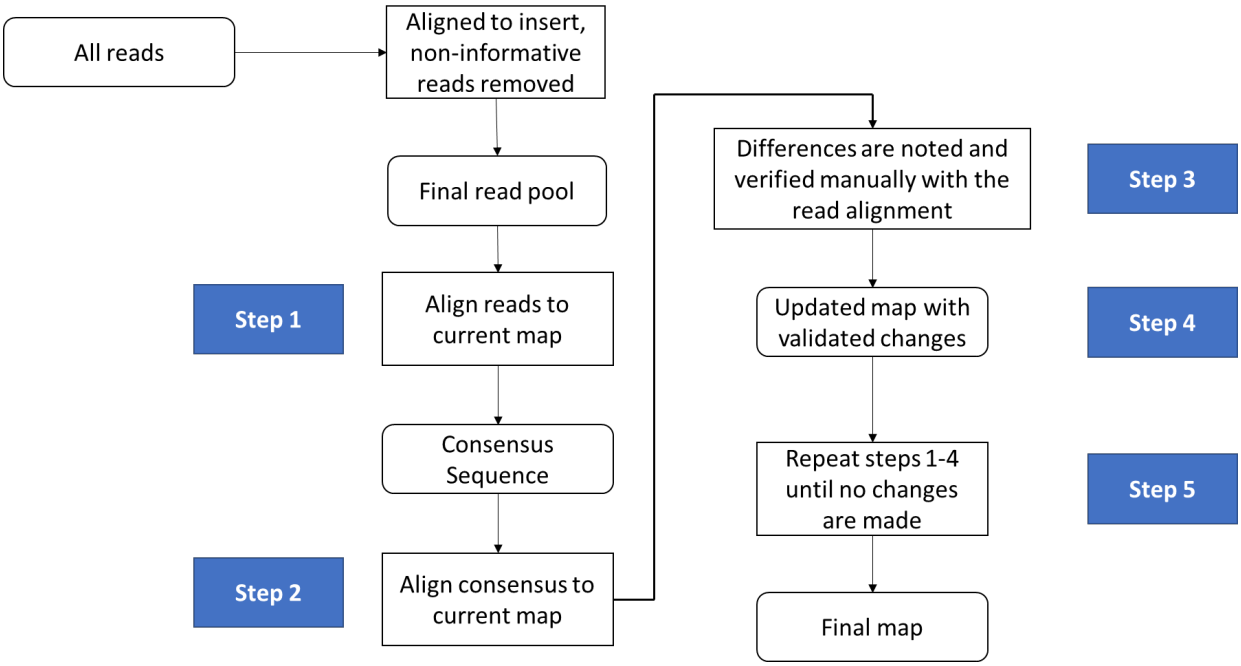
Figure 8: Predicted Insertion Maps, Chromosome 13 Insertion

This Insertion comprises a junction between chromosome 13 and the LB of VOSF. The Pnos, NPTII and Tnos elements are then followed by 90 base pairs of VOSF sequence around the LB. This then leads into the full RB to LB copy of the VOSF transgene that junctions with chromosome 13.

CPI1013 Insert Map Refinement

To better characterize the CPI1013 event the insertion map needed to be refined on a nucleotide level to be 100% accurate. Data for this purpose was generated as described above in "Sequencing Library Preparation and Whole Genome Sequencing".

Figure 9: Workflow for Refinement of the Insert Map



Step 0: Initializing the read pool

Three sets of HAC called reads (3,232,638 reads total) were aligned to the initial insert using Minimap2 with Geneious. Reads that mapped only to the HFTH1 reference portion of sequence in the map or only the PPO genes in the transgene were removed as being non-informative. The remaining reads were all extracted and pooled together. This provided 27 high accuracy reads that were used in the subsequent steps.

Step 1: Aligning reads to the current map

The final read pool was again mapped against the most recent version of the insert map using Minimap2. The consensus sequence over the span of the insert map was then extracted as a continuous string of nucleotides.

Step 2: Aligning the extracted consensus to the current map

The sequence that was generated in step 1 is aligned to the insert map using Minimap2.

Step 3: Noting and verifying differences in the alignment

All differences that exist between the current insert map and the consensus sequence have their positions and change noted. Each noted position and change is then verified by going back to the read alignment from step 1. Each read that overlaps the position is looked at to see if it validates the noted change in the consensus. Changes that were observed in the majority of reads (and not due to problematic sequencing) were then deemed to be valid.

Step 4: Updating the map with validated changes

The insert map is then updated with the changes that were deemed to be valid. Once all changes have been made to the insert map, this now becomes the current insert map.

Step 5: Iterate on steps 1-4

Steps 1 through 4 is then repeated with the same final read pool and the current map. Each time differences are noted and changes made if they are deemed valid. The process repeats until no new changes are made to the insert map in step 4.

Open Reading Frames

The inserted DNA, including the genomic region flanking each insertion, was analyzed for putative open reading frames (ORFs) using Geneious Prime. Any new 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, CTG or TTG) and continuing until the first termination codon (TAA, TGA, TAG). The entire VOSF vector sequence plus unique junction sequences created in CPI1013 were translated in all six reading frames and deduced amino acid sequences of greater than 29 amino acids (87 nucleotides) 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 95 ORFs were identified that are derived from the 7,774 nucleotide insert map for CPI1013 including 1,000 nucleotides from HFTH1 flanking either side of the insertion. These, translated generated a total of 95 potential proteins. 17 of these open reading frames were located strictly within the flanking HFTH1 and were removed from further analysis. Of the remaining 78, 28 were exact duplicates of other open reading frames and were removed from further analysis for a final list of 50 unique ORFs to be analyzed.

To assess the potential allergenicity of inserted sequences, the deduced amino acid sequences of these 50 ORFs were compared to entries in the Food Allergy Research and Resource Program (FARRP) Allergen Protein Database (Version 21). This version of the database contains 2233

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.[1]. 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 50 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 of hits then had each ORF individually aligned with the NCBI online blastp tool[2] returning up to 5000 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 50 ORFs, 27 derived from CPI1013 had initial hits using BLASTp. No instances of "toxin" or "toxic" were found in the 27 .txt alignment files greater than the threshold of 10^{-2} and does not meet the criteria for further investigation or concern.

Minimal Potential for Unintended Consequences

Okanagan Specialty Fruits has chosen to use long read next-generation sequencing and a unique mapping approach to describe the insertions that create CPI1013 from Cripps Pink 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 CPI1013 as part of the safety assessment of this enhanced apple cultivar. The whole genome sequencing analysis is 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. Insertions are not located within any predicted endogenous coding or regulatory region. There are no additional open reading frames introduced in the inserted sequence that align to known allergens.

Any additional insertion would potentially create 2 new ORFs at the site of insertion. 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 CPI1013 from its parent cultivar Cripps Pink.

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[2] NIH-NLM-NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed 230207).

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Exhibit-4: Development of VOSF

In the development of Arctic® apple cultivars, two different traits are transferred into popular conventional cultivars of apple. The first trait is resistance to the antibiotic kanamycin which enables the selection of transgenic events during the transformation process[1]. The second trait is the gene of interest (GOI), a polyphenol oxidase (PPO) suppression transgene, designed to reduce expression of the entire apple PPO gene family. The first binary vector that successfully embodied these traits was called GEN-03 (Map and details provided in 10-161-01). The GEN-03 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 Gene bank (Benson *et al.*, 2005). The VOSF is based on a universal binary vector VOSF0, which is based on pCAMBIA_0390, a derivative of pPZP200 (Hajdukiewicz, Svab and Maliga, 1994).

The universal binary vector was developed at OSF as follows. The binary vector backbone for VOSF0 was recovered from the binary vector TAG0 (James Thomson, USDA) with *AvrII-ScaI* restriction endonucleases. An expression cassette in Blue Heron pUC Kan Minus MCS vector (called EOSF) was synthetized at the Blue Heron Biotech (Bothell, USA) carrying the following elements: a nopaline synthase promoter (Pnos), wild type *nptII*, nopaline synthase terminator (Tnos)and a modified multiple cloning site (MCS) from pPZP-RCS1 (Goderis *et al.*, 2002) flanked by the restriction sites for *AvrII* (5') and *ScaI* (3') restriction endonucleases. The above-described expression cassette was excised from EOSF with *AvrII*-ScaI restriction endonucleases and ligated into the respective sites of TAG0 vector backbone resulting in VOSF.

The universal binary vector VOSF0 provides the following advantages over pBINPLUS:

- The origin of replication in VOSF0 (pVS1) supports higher plasmid copy (2.0 – 2.9 copies / cell) than the origin of replication in pBINPLUS (RK2) (1.0 copies / cell) which is correlated with higher transformation frequency in maize (Lee and Gelvin, 2007).
- A modified multiple cloning site derived from pPZP-RCS1 (Goderis *et al.*, 2002) which comprising 17 (hexanucleotide and octanucleotide) restriction sites and 4 homing endonuclease sites, which provides a better cloning flexibility compared to the previously developed pBINPLUS or pCAMBIA vectors.
- The wildtype *nptII* selection marker was used in VOSF (Beck *et al.*, 1982). The wildtype *nptII* provides higher kanamycin resistance and can improve transformation. Additionally, the *nptII* gene in GEN-03 contains 389 bp of sequence between the stop codon and the nopaline synthase terminator (Tnos). In the construction of VOSF, 383 bp of this sequence was removed, shortening the intervening region between the stop codon and terminator to 6 bp. The removal of intervening sequences between the promoter, coding sequence and terminator has been done in the pYBA vector series (i.e. Genbank Accession KU221171). This reduces the size of T-DNA to be transferred into the plant.

In the VOSF vector, we have used shorter versions of the same PPOs previously used in GEN-03, and have rearranged the sequences into an intron-containing hairpin RNA (ihpRNA) structure. The

[1] The nopaline synthase promoter (Pnos) drives the expression of the *nptII* coding sequence. We have found that the Pnos drives expression at a sufficient level in tissue culture to allow the selection of kanamycin resistant transgenic shoots. However, the amount of NptII protein in mature apple fruit is below the limit of detection by ELISA (Agdia) meaning that Pnos does not function (or functions poorly) in mature apple fruit). The NptII protein is found at a detectable level in apple leaves (ca. 4 – 5 ng NptII per g FW apple).

ihpRNA is designed to produce dsRNA meaning that a single copy, intact insertion of the 4390 bp T-DNA could induce PPO suppression. This simplifies molecular characterization. In theory, up to 100 % of the transgenic events will be suppressed. The PPO suppression sequences are under control of a double-enhanced CaMV 35s promoter and nopaline synthase terminator. The structural details of VOSF are provided in Exhibit-1.

VOSF

Element	VOSF
Right Border (RB)	Double RB
Selection Marker	wildtype *nptII*
Phenotype	nonbrowning
Mode of Action	suppression of PPO
Suppression Type	ihpRNA
Target Genes	PPO Gene Family
Sequences	90 - 100 bp
Insertion Structure	single copy, intact
Insertion Size	4390 +/- 100 bp

The selection marker is under control of Pnos3. A 383 bp fragment has been removed from between the nptII coding sequence and Tnos in VOSF

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