April 14, 2021

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

<u>Does Not Contain Confidential Business Information</u>

Dear Bernadette Juarez,

RE: <u>Request for Confirmation of Exemption from Regulations Under 7 CFR Part 340 for Nonbrowning Arctic® Apple Event HCR835</u>

Okanagan Specialty Fruits Inc. respectfully seeks confirmation from Biotechnology Regulatory Services (BRS) that Nonbrowning Arctic® apple Event HCR835 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 v. 06/18/2020:

<u>Requester's Information and Contact</u>:
    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

<u>Description of the plant</u>:
The recipient of the nonbrowning trait in this application is an apple plant. Specifically, the parental cultivar Honeycrisp (HCR), 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 HCR835 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

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 HCR835 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.

HCR835 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 HCR835.

In HCR835, as in the antecedent organisms GD743 and GS784, 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 HCR835 which is typical of all Arctic® apple cultivars. The nptII selection marker permitted the identification of HCR835 during the transformation process. The level of NptII protein in HCR835 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.

Although a plant-trait-MOA (Suppression of PPO causing non-browning in apple) is the same in HCR835 as in the antecedent organisms GD743 and GS784, an improved gene delivery system was used for the development of HCR835 by using a binary vector named VOSF compared to binary vector GEN-03 used for the development of GD743 and GS784. 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.

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

Characteristic	Candidate HCR835 Apple	Antecedent GD743 Apple	Antecedent GS784 Apple
Organism	Apple	Apple	Apple
Cultivar	Honeycrisp	Golden Delicious	Granny Smith
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	GEN-03	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 HCR835 will offer growers, packers, processors, wholesalers, retailers, food service and consumers a nonbrowning variant of the popular Honeycrisp 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,
*   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 HCR835 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.
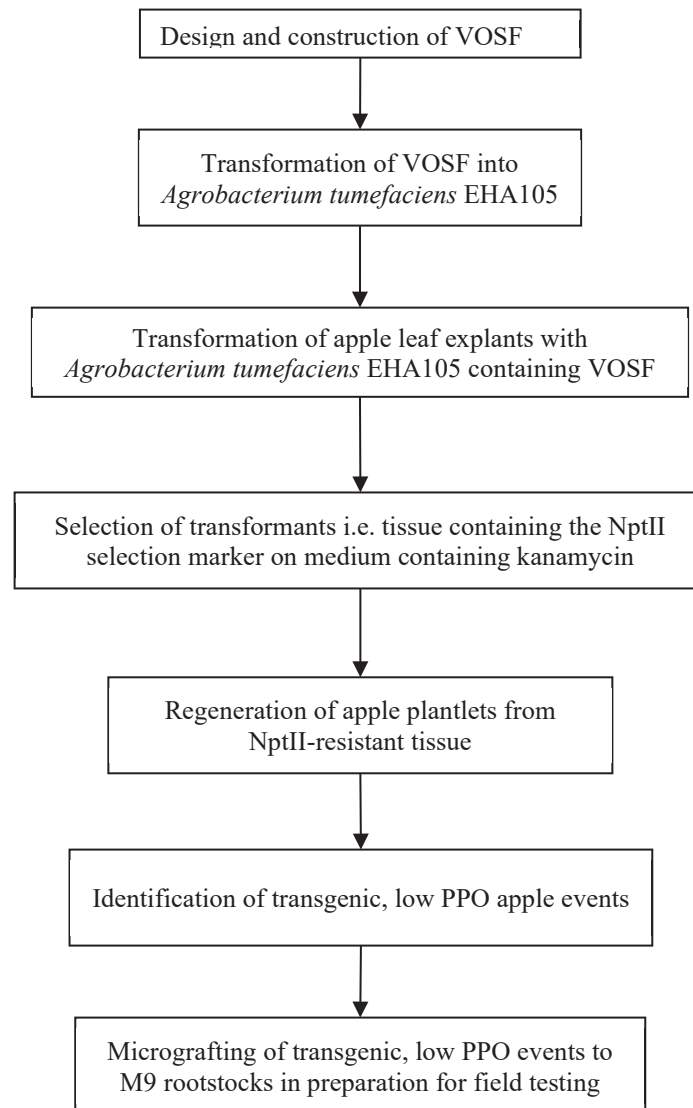
A more in-depth description of the above is given in Exhibit-4. Included there is also a comparison of the transgenes from VOSF and the antecedent GEN-03 (Figure 2: Exhibit-4).

[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 HCR835 was the same as used to develop antecedent organisms GD743 and GS784. 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 HCR835, GD743 and
GS784

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│     Design and construction of VOSF  │
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│        Transformation of VOSF into   │
│    Agrobacterium tumefaciens EHA105  │
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│    Transformation of apple leaf explants with │
│  Agrobacterium tumefaciens EHA105 containing VOSF │
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┌─────────────────────────────────────────┐
│  Selection of transformants i.e. tissue containing the NptII │
│     selection marker on medium containing kanamycin │
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                  ▼
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│     Regeneration of apple plantlets from │
│            NptII-resistant tissue    │
└─────────────────────────────────────┘
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│  Identification of transgenic, low PPO apple events │
└─────────────────────────────────────┘
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│   Micrografting of transgenic, low PPO events to │
│  M9 rootstocks in preparation for field testing │
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c)   Function of the modified gene or genetic element

HCR835 was produced by Agrobacterium-mediated transformation of the apple cultivar
Honeycrisp (HCR) 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 HCR835 apple. The antecedent organisms, GD743 and GS784

were also generated by Agrobacterium-mediated transformation with a vector (GEN-03) containing the same functional elements.

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

To describe the insertions that create HCR835 from Honeycrisp we employ a unique mapping strategy based on long read Oxford Nanopore 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 HCR835 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 any read potentially arises from the VOSF vector. To do this we use BBDuk to identify any read that contains 21 bp of 100% identity to the VOSF vector (10,643 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 efficiently reduces our reads pool from >1,000,000 reads to just over 4,639 reads allowing us to predict the structure and location of the insertions using less computer resources.

This reads pool 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 HCR835 from the parent cultivar. For more in-depth narrative and data of this process see Exhibit-3.

Our reads selection process for HCR835 ultimately identified 8 reads from a total of 1,187,212 reads (~ 14,996 Megabases) that were used to map the junctions. The genomic locations of the insertions for 8 reads are shown in Exhibit-3 via Figures 4 and 5. We also performed a whole genome sequencing of HCR835 using Illumina platform and analyzed short-read data (data not shown) by same strategy as described for long-read data. We were able to verify the insertions of 8 reads (data not shown) as revealed by Nanopore long read data, with no evidence of any additional insertions.

When mapping with 21X overall coverage (10.5X 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 5 reads per junction. We find it improbable that we would miss this many chimeric reads in our mapping process. In addition to HCR835, 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 HCR835 has revealed evidence of insertion in chromosome 12. (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).

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 comprises 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 HCR835 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 HCR835 is similar to Arctic® apple events GD743 and GS784 (described in petition 10-161-01p) in terms of transgene family, trait and MOA. 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.

HCR835 was produced by Agrobacterium-mediated transformation of the apple cultivar Honeycrisp (HCR) 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 HCR835 apple. The antecedent organisms, GD743 and GS784 were also generated by Agrobacterium-mediated transformation with the GEN-03 vector resulting in suppression of PPO.

In HCR835, 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 nonbrowning phenotype typical of all Arctic® apple cultivars. The nptII selection marker permitted the identification of HCR835 during the transformation process. The level of NptII protein in HCR835 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 HCR835 apple, described in this request for confirmation, was transformed using the same PPO genes and functionally similar binary vector as events GD743 and GS784, yielding the same nonbrowning phenotype, and does not meet the definition of a regulated article under 7 CFR Part 340.
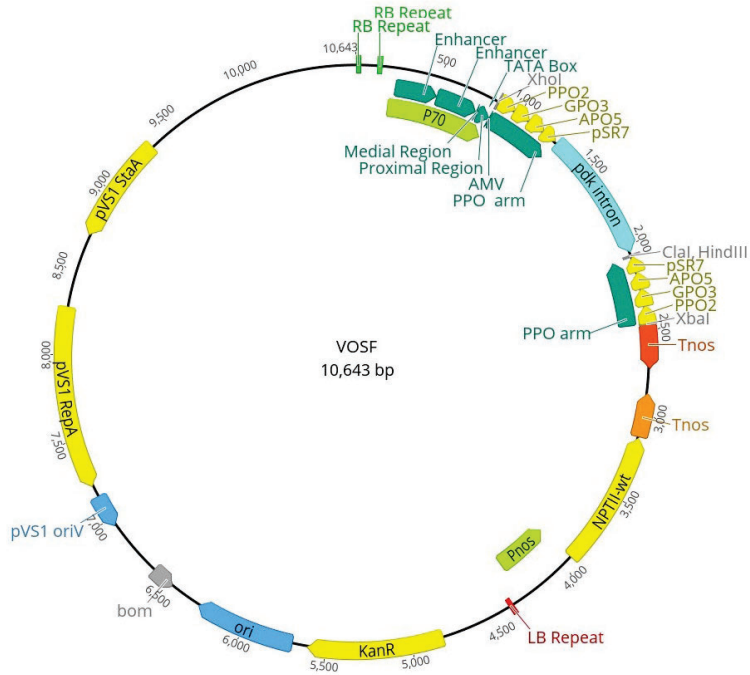
Sincerely,
Okanagan Specialty Fruits Inc.

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 HCR835. 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 HCR835

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).

Figure 2: Similarity of VOSF and GEN-03

Following LASTZ alignment shows how all the critical elements of the tDNA in VOSF were taken directly from the GEN-03 vector previously described in 10-161-01p.

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.

van Engelen, F. A. *et al.* (1995) 'pBINPLUS: an improved plant transformation vector based on pBIN19', *Transgenic Research*, 4, pp. 288–290.

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.

Rothstein, S. J. *et al.* (1981) 'Genetic Organization of Tn5', *CSH Symp Quant Biol 45*, 1, pp. 99–105.

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 HCR835

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

Transformation of VOSF into *Agrobacterium tumefaciens* EHA 105:
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 and used for plant transformation.

Transformation of apple leaf explants with *Agrobacterium tumefaciens* EHA 105 containing VOSF:

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 VOSF vector at a density of 3×10^8 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 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, low PPO apple events:

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 HCR835 and HCR 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 HCR835 or HCR 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 HCR835 and the control HCR done over three subcultures (n = 3).

We found that PPO activity was reduced by 98% in HCR835 relative to its control (Table 2). The standard deviation for HCR835 is very small, consistent with the uniformly low PPO activity in Arctic® apple events. The standard deviation for the HCR control is larger, as is sometimes seen in wildtype tissue samples that vary widely in PPO activity.

Table 2: PPO Activity in HCR835 – Tissue Culture Leaves

Cultivar	Mean Specific Activity	S^1	n^2	PPO Suppression[3]
HCR835	27	16	6	98.92 %
HCR	1494	168	2	

[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 HCR – Mean Specific Activity of HCR835) / Mean Specific Activity of HCR)*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 HCR835 and HCR were collected, snap frozen in liquid nitrogen and stored at -80ºC until processing. Leaf samples were measured for PPO activity. The data in Table 3 is a summary of our greenhouse screening for PPO activity in HCR835.

We found that PPO activity was reduced 95% in HCR835 relative to its control (Table 3). 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 HCR835 under greenhouse conditions.

Table 3: PPO Activity in HCR835 – Greenhouse Leaves

Cultivar	Mean Specific Activity	S^1	n^2	PPO Suppression[3]
HCR835	81	23	5	95.34 %
HCR	1739	270	5	

[1] S = standard deviation
[2] n = number of samples per cultivar
[3] PPO Suppression = ((Mean Specific Activity of HCR – Mean Specific Activity of HCR835) / Mean Specific Activity of HCR)*100

The event HCR835 identified through this PPO screening process and was advanced to field trial for confirmation using a controlled bruising assay.

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

Micrografting was done according to the method of (Lane et al., 2003).

<u>References</u>
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 [Genomic DNA by Ligation (SQK-LSK109); Version: GDE_9063_v109_revS_14Aug2019]. Briefly, 1.5 µg 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 (Beckman Coulter Inc.) with 70 % Ethyl alcohol and then used for adaptor ligation (SQK-LSK109; Oxford Nanopore Technologies). The DNA library was cleaned up using AMPure XP beads with Long Fragment Buffer. The quality of the DNA library was confirmed using Tape Station (Agilent Technologies). Finally, 750 ng of the prepared library was loaded into a flow cell (FLO-MIN106D Version R9; Oxford Nanopore Technologies) for sequencing with Oxford Nanopore MK1C sequencer.

Sequencing Coverage and Quality

The HCR835 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 21x 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 10.5x. 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)	1,187,212
Nucleotides	14,995,998,270
Genome Size (HFTH1)	708,540,000
Coverage	21

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

The reads were mapped to the most recent and high quality apple reference available, 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 22.06x to 29.063x with an average of 23.792x. The amount of the reference genome that was covered by at least one sequence read (Percent of Ref Seq), 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 5: Mapping HCR835 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	136,337	977,814,997	22.077	74.9%	95.3%
2	38,449,405	175,153	1,188,458,886	22.676	74.9%	95.3%
3	37,138,690	152,112	1,116,898,812	23.615	75.5%	94.2%
4	31,012,745	126,759	936,779,856	25.367	76.3%	99.1%
5	47,891,858	198,711	1,439,776,803	25.438	81.8%	95.4%
6	35,567,198	134,674	1,042,780,692	23.005	75.8%	98.3%
7	35,934,761	139,600	1,039,642,883	24.118	76.8%	98.3%
8	31,511,015	126,947	925,196,916	23.507	76.0%	98.3%
9	34,800,404	144,012	1,046,824,621	23.015	76.0%	96.7%
10	43,815,736	180,711	1,298,100,584	22.06	76.0%	96.7%
11	42,456,296	173,817	1,301,451,152	24.041	75.9%	97.7%
12	32,285,079	129,878	969,684,533	23.559	75.8%	97.5%
13	44,866,511	177,760	1,417,642,905	29.063	71.0%	98.6%
14	31,515,206	120,707	940,664,113	23.823	76.7%	97.7%
15	56,644,392	227,367	1,684,165,833	23.392	76.2%	97.6%
16	41,670,059	176,544	1,226,961,542	22.266	76.3%	96.0%
17	33,998,825	135,244	1,003,648,365	23.445	76.2%	95.6%
00	7,992,922	59,748	546,992,472	198.179	83.6%	68.5%

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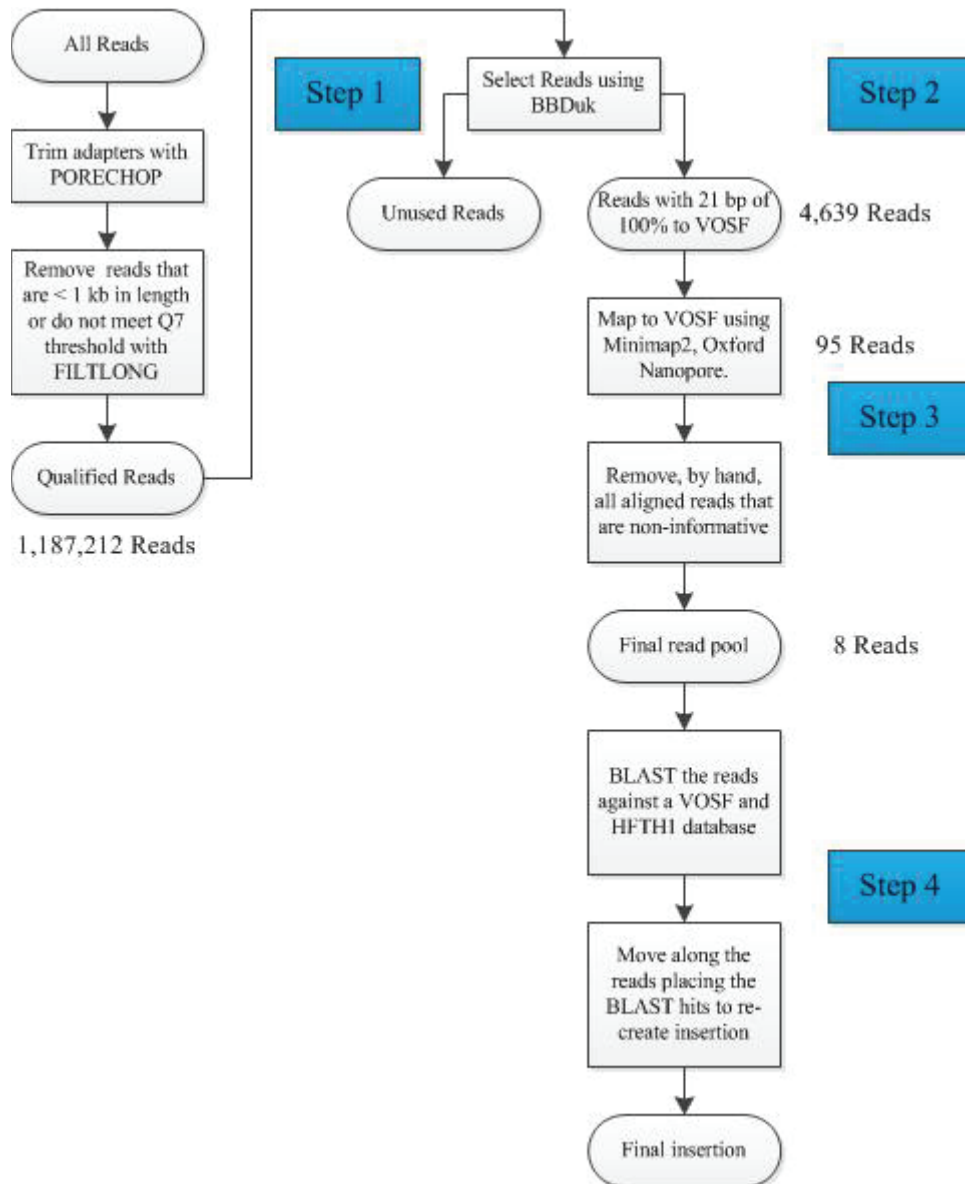
Non-informative reads are those that either: Have a full linear alignment within the transgene of VOSF or align only to a single copy of one the sub-units of the PGAS gene.

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 FILTLONG command. 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 HCR835 1,187,212 reads were left for final analysis.

Step 2: Select Reads using BBDuk

Qualified HCR835 reads (1,187,212) were selected using BBDuk to identify reads which include 21 bp with 100% identity to the VOSF vector[1]. The filtered read pool 4,639 reads) is expected to include three types of reads: (i) reads that map to the VOSF 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 3: Reads Mapping to the VOSF Vector

To reduce the pool to the final informative ones, 4,639 BBDuk selected reads were mapped to the VOSF vector using the Minimap2 assembler with the Oxford Nanopore data type. A total of 95 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 criteria were noted and put into a separate, final, pool (a total of 8 reads).

Step 4: Mapping the insertion

To identify the insertion site within the apple genome, the 8 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.

T-DNA Genomic Location

The junction mapping process (Steps 1 – 4, above) identified two apple/vector junctions co-located in chromosome 12 (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.

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[1] BBDuk.sh in-Cleaned_HCR835.fasta out=umatched.fasta outm=readsmatchingVOSF.fasta ref=VOSF.fasta k=21 stats=stst.txt

Figure 4: Chromosome 12 Insertion - Left Side
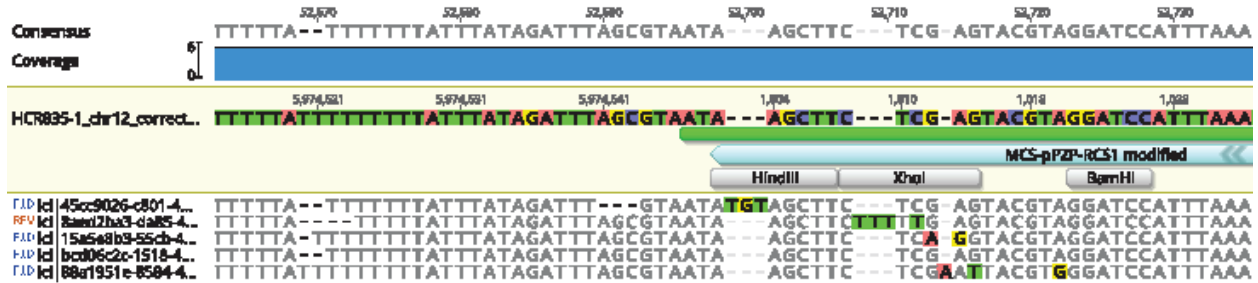
A 60 bp fragment crossing the junction with mapped reads is provided as evidence of the junction. This figure shows chromosome 12 (Chr12:5,974,615…5,974,646) attached to the RB of VOSF (VOSF:162…193).

## Figure 5: Chromosome 12 Insertion - Right Side
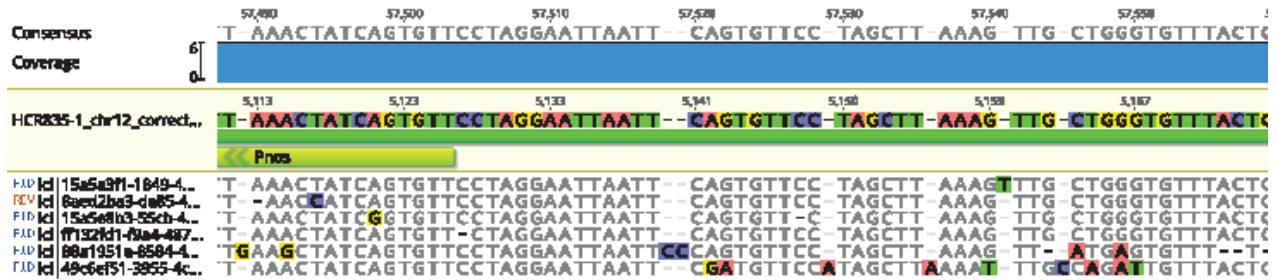
A 60 bp fragment crossing the junction with mapped reads is provided as evidence of the junction. This figure shows the LB of VOSF (VOSF:4,273…4,304) attached to chromosome 12 (Chr12:5,974,664…5,974,695).

The genomic location of the insertion in chromosome 12 (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: HCR835 - Chromosome 12 - Genomic Location
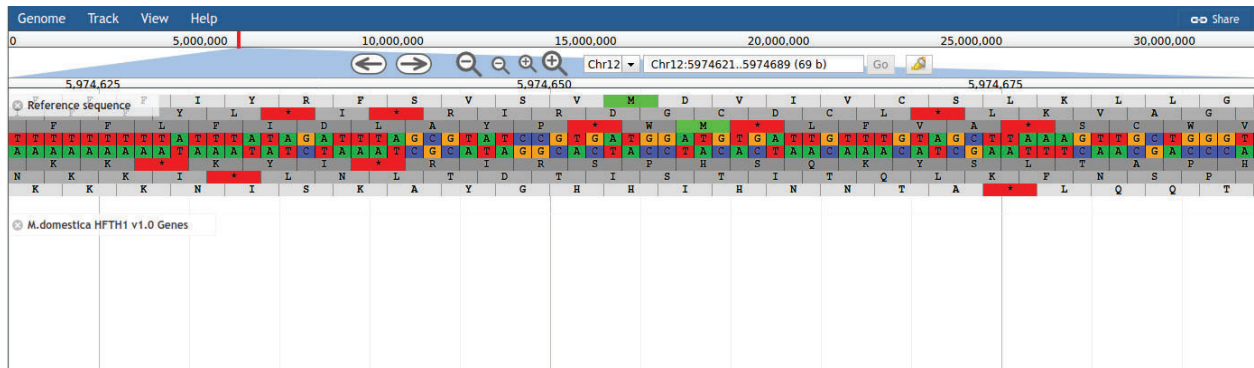
A screenshot covering the insertion site in HCR835 Chromosome 12 (Chr12:5,974,621…5,974,689) showing no predicted genes or transcripts present at the insertion site (JBrowse, GDR).

Figure 7: Coverage of the VOSF in HCR835
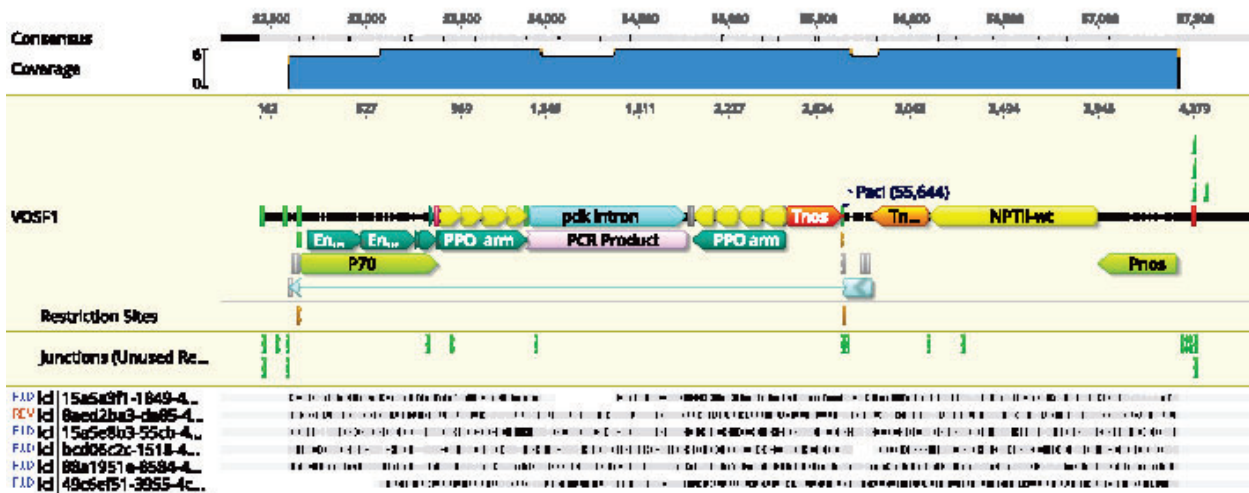
A total of 8 reads mapped to the VOSF binary vector using Minimap2 reveal which regions of the T-DNA were included in HCR835. Here only from the right border (RB) to left border (LB) is represented in the reads. The sequencing coverage was used to predict the approximate copy number (one insertion for HCR835).

Predicted Insertion Maps: Figure 8: Chromosome 12 Insertion

This Insertion comprises a complete intact copy of the T-DNA from right border (Junction_1) to left border (Junction_2) with chromosome 12.

Open Reading Frames

The inserted DNA, including the genomic region flanking each insertion, was analyzed for putative open reading frames (ORFs) using Geneious Prime. 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 VOSF vector sequence plus unique junction sequences created in HCR835 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 73 unique ORFs were identified that are derived from the 10643 bp VOSF binary vector, including backbone. A single unique ORF was identified over junction 1 in the HCR835 event.

To assess the potential allergenicity of inserted sequences, the deduced amino acid sequences of these 74 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.[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 74 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[2] 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 74 ORFs, 25 derived from VOSF none from HCR835 produced hits within Geneious. No instances of "toxin" or "toxic" were found in the 25 .txt alignment files generated online using the NCBI tool.

Minimal Potential for Unintended Consequences

[1] AllergenOnline (http://www.allergenonline.org/databasehelp.shtml, accessed 200505).
[2] NIH-NLM-NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed 200505).

Okanagan Specialty Fruits has chosen to use long read next-generation sequencing and a unique mapping approach to describe the insertions that create HCR835 from Honeycrisp 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 HCR835 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 HCR835 from its parent cultivar Honeycrisp.

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Exhibit-4: Development of VOSF and Structural Differences with GEN-03

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) that 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 *nptII* selection marker in GEN-03 and VOSF differ in the following ways: (i) GEN-03 comprises the mutant *nptII* coding sequence[2] (Yenofsky, Fine and Pellow, 1990). VOSF comprises the wildtype *nptII* coding sequence (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.

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[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).

[2] Compared to the sequence of the normal gene (Beck *et al.*, 1982), a single change at nucleotide position 2096 was found, where the guanosine is replaced with a thymidine residue, converting codon 182 from GAG (glutamate) to GAT (aspartate).

The PPO suppression transgene in the GEN-03 vector is a sense transgene. Since double strand RNA (dsRNA) is the trigger for RNA interference (RNAi) the sense suppression transgene must be rearranged on insertion to create a double stranded RNA product. In all Arctic apple cultivars designed to date, T-DNA insertion mapping enabled by next-generation sequencing (NGS) has revealed multiple copies of the T-DNA region with at least two copies forming an inverted repeat with the PPO suppression genes pointing towards each other, most often with one of the associated terminator (Tnos) sequences missing. To summarize, the consequences of using a sense transgene are three-fold. First, in order to induce RNAi we end up selecting for events that comprise multiple T-DNA insertions, rearranged to create a dsRNA trigger. Second, because this complex rearrangement is required, only around 1 in 10 transgenic events result in a product that is PPO suppressed. Third, molecular characterization of our insertions is complex.

In the VOSF vector, we have used shorter versions of the same PPOs used in GEN-03, and have rearranged the sequences into an intron-containing hairpin RNA (ihpRNA) structure. The ihpRNA is designs to produce dsRNA meaning that a single copy, intact insertion of the 4390 bp T-DNA could induce PPO suppression. This will simplify molecular characterization. In theory, up to 100 % of the transgenic events will be suppressed. The PPO suppression sequences are under control of the same double-enhanced CaMV 35s promoter and nopaline synthase terminator as in GEN-03. The structural details of VOSF are provided in Exhibit-1. Below we provide the major structural differences between VOSF and GEN-03:

VOSF versus GEN-03

Element	VOSF	GEN-03
Right Border (RB)	Double RB	Single RB
Selection Marker	wildtype *nptII*	mutant *nptII*
Phenotype	nonbrowning	nonbrowning
Mode of Action	suppression of PPO	suppression of PPO
Suppression Type	ihpRNA	sense
Target Genes	PPO Gene Family	PPO Gene Family
Sequences	90 - 100 bp	400 - 460 bp
Insertion Structure	single copy, intact	multicopy, rearranged
Insertion Size	4390 +/- 100 bp	~19 – 80 Kb

The selection marker is under control of Pnos in both VOSF and GEN-03. A 383 bp fragment has been removed from between the nptII coding sequence and Tnos in VOSF

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