Efficacy and Safety of RNAi-mediated Plum Pox Virus Resistance in ‘Honeysweet’ Plum.

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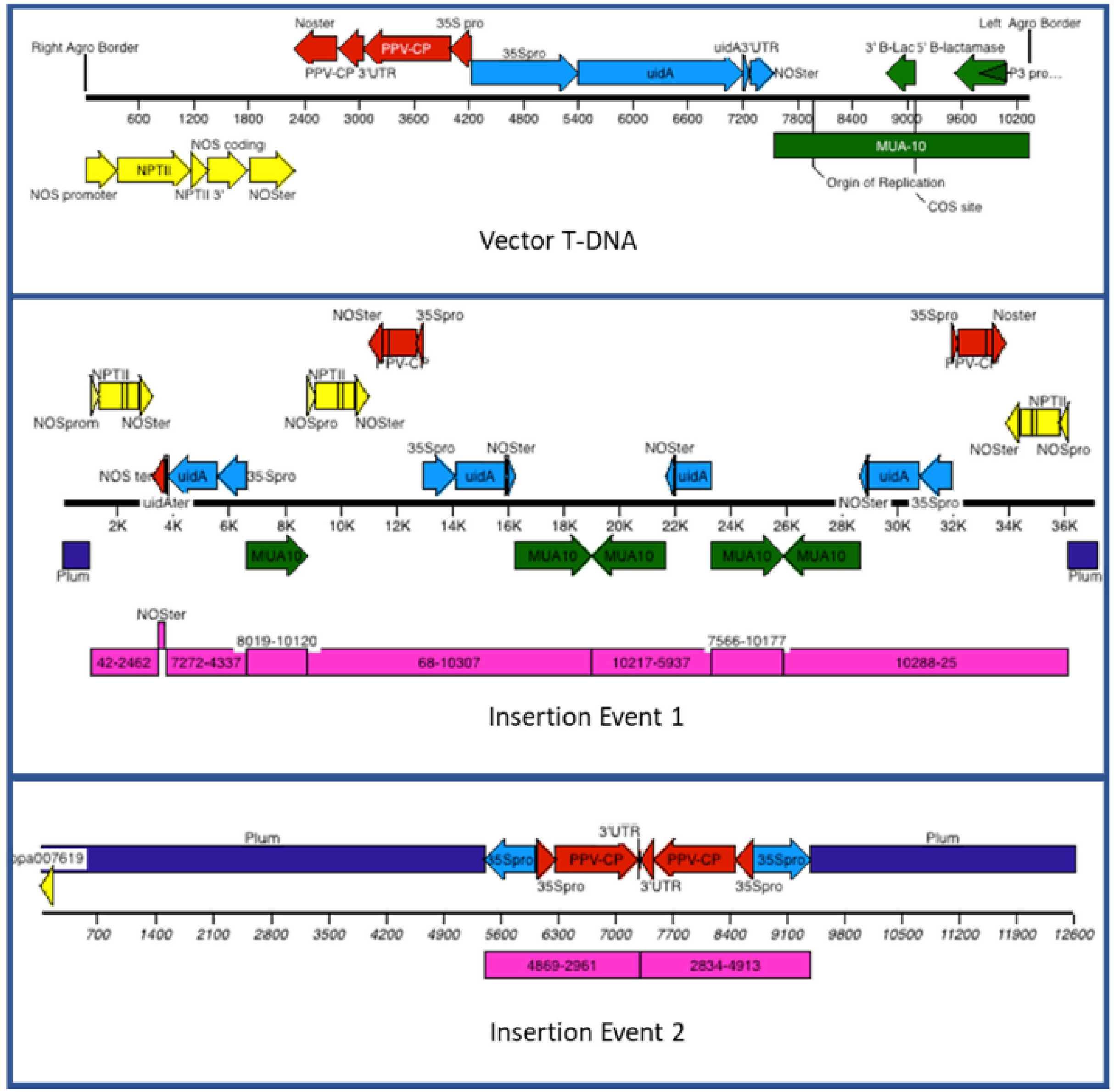


Fig. 1 Diagram of the initial transformation T-DNA and the rearrangements resulting at the insertion event 1 and insertion event 2. Each of the transgene regions is color coded with the NPTII gene, promoter, and terminator in yellow, the PPV-CP in red, the UIDA in blue, the MUA-10 in green, and plum in dark blue. The regions in pink represent the fragments of the initial T-DNA specified by the base numbers

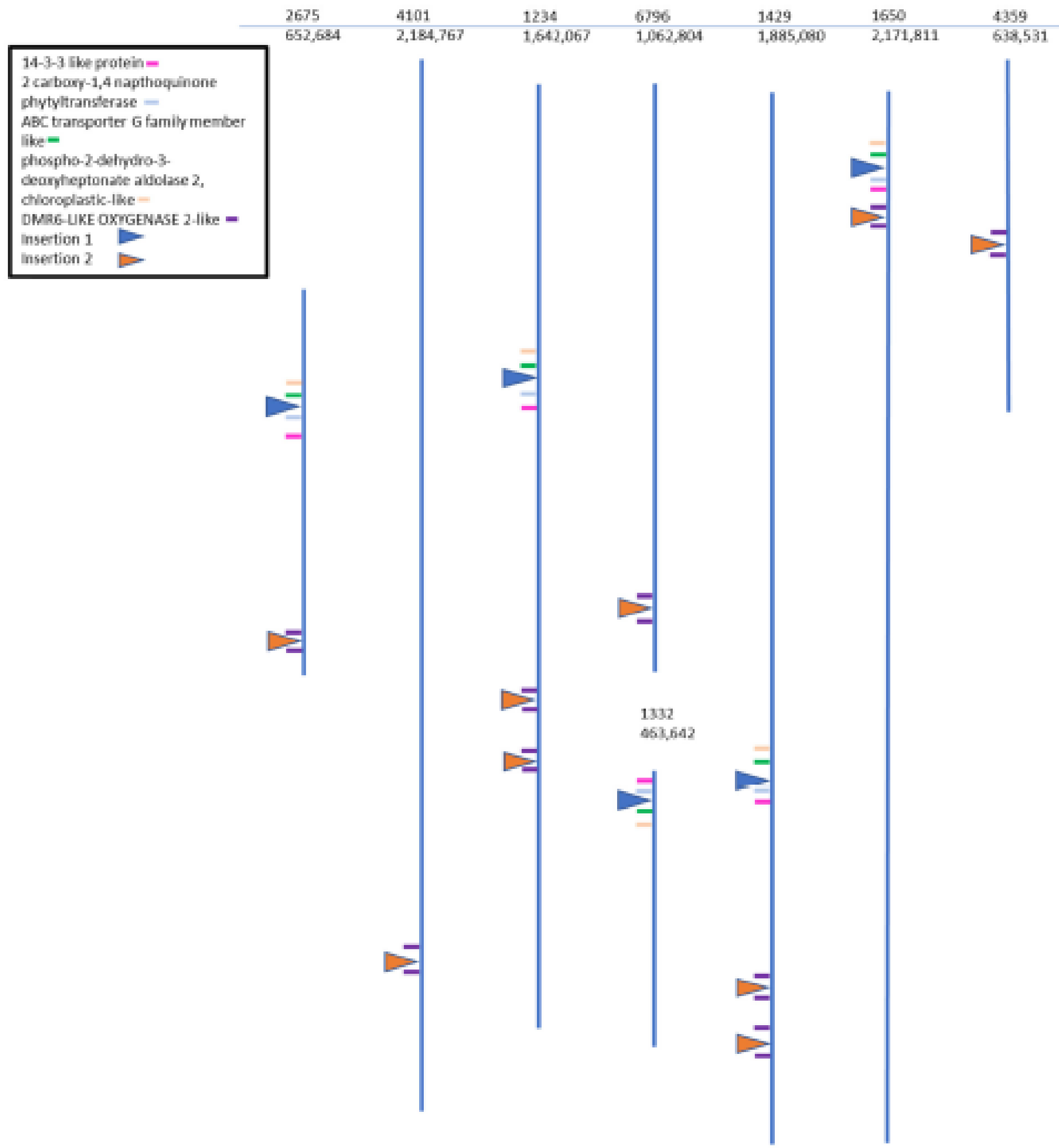


Fig. 2 Diagram of the plum scaffolds that contain sites that match the insertion events. Under each scaffold number is the size of the scaffold in bases. The insertion sites are marked with a triangle and the flanking genes with a bar. The key identifies each by color

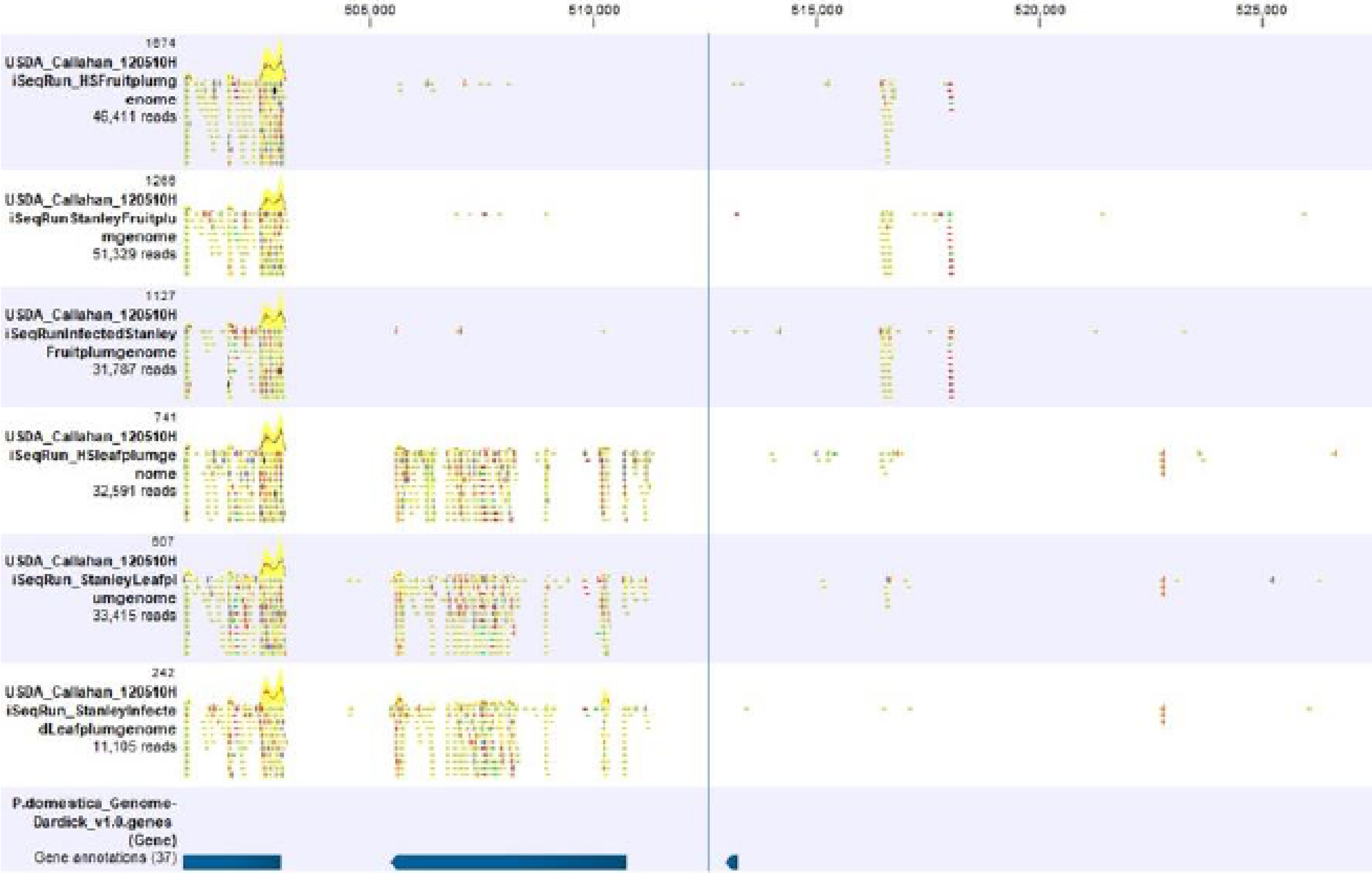
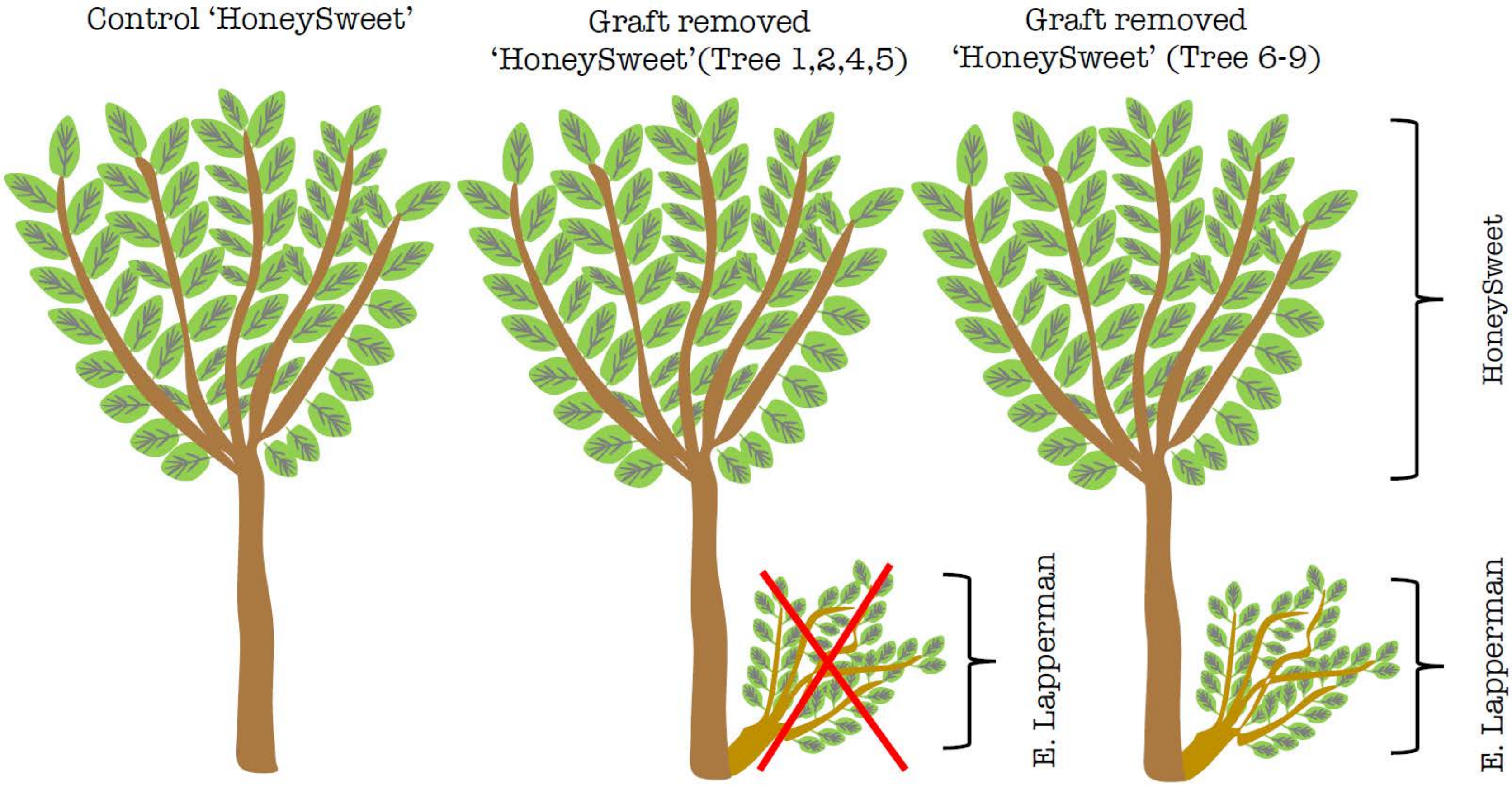


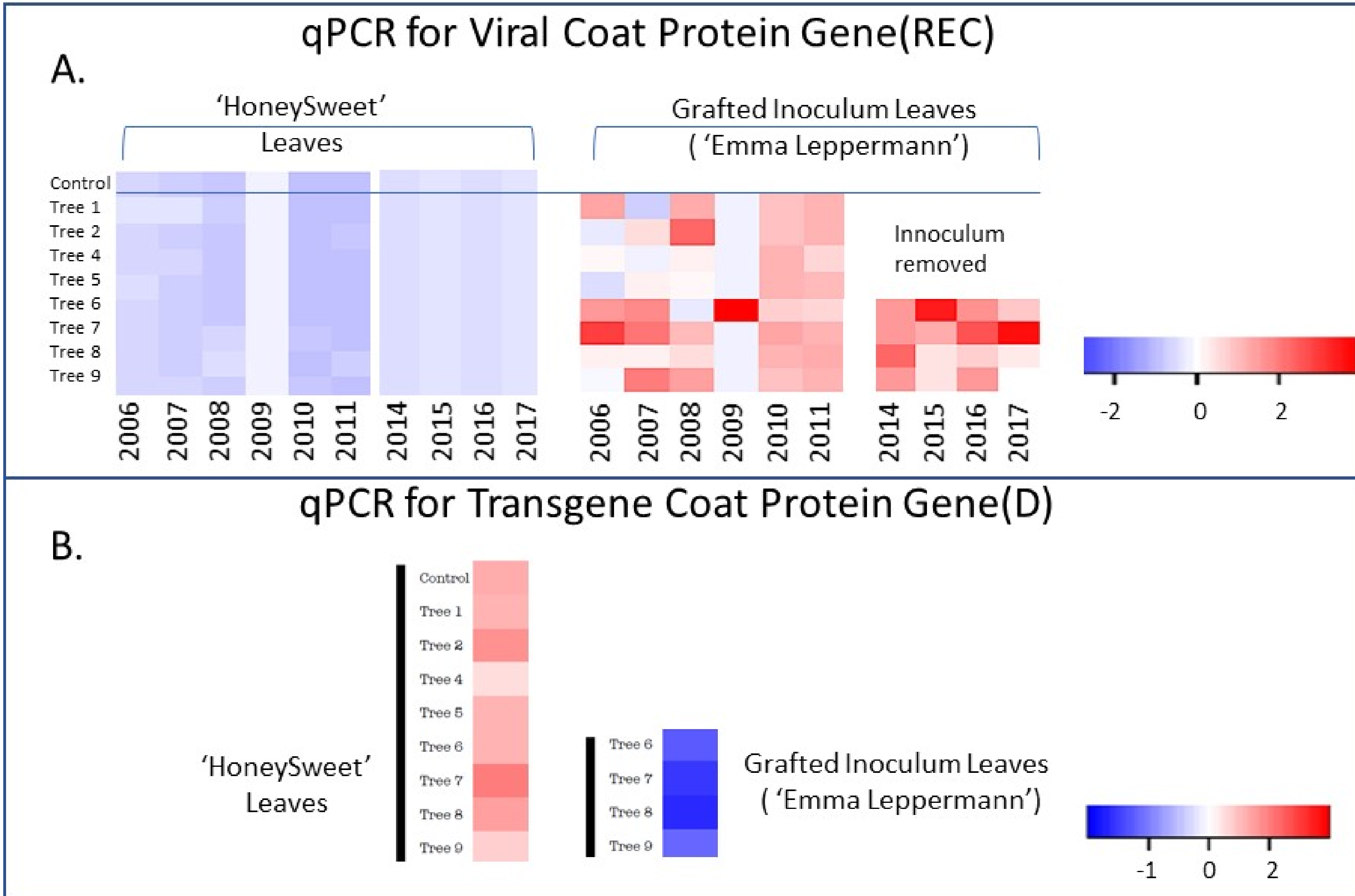
Fig. S4. RNA expression of genes flanking the site of insertion 1 in Scaffold 2675. No new transcripts were detected and no differences in expression patterns between 'HoneySweet', 'Stanley' and 'Stanley' infected with PPV. Each line represents a sequencing read with yellow representing a sequence that maps to more than one location, red and green are unique but either the positive or negative strand of DNA. The vertical line is the site of the insertion and the horizontal arrows on the bottom are predicted exons and represent the only predicted gene in the region presented.

Field Studies of siRNA Expression (*Two Datasets*)

Country	Varieties	PPV Status
U.S.	‘Honeysweet’ and ‘Stanley’	PPV Free
Czech Republic	‘Honeysweet’ and ‘Stanley’	Exposed to PPV
Spain	‘Honeysweet’ and ‘Stanley’	Exposed to PPV
Bulgaria	‘Stanley’	Exposed to PPV

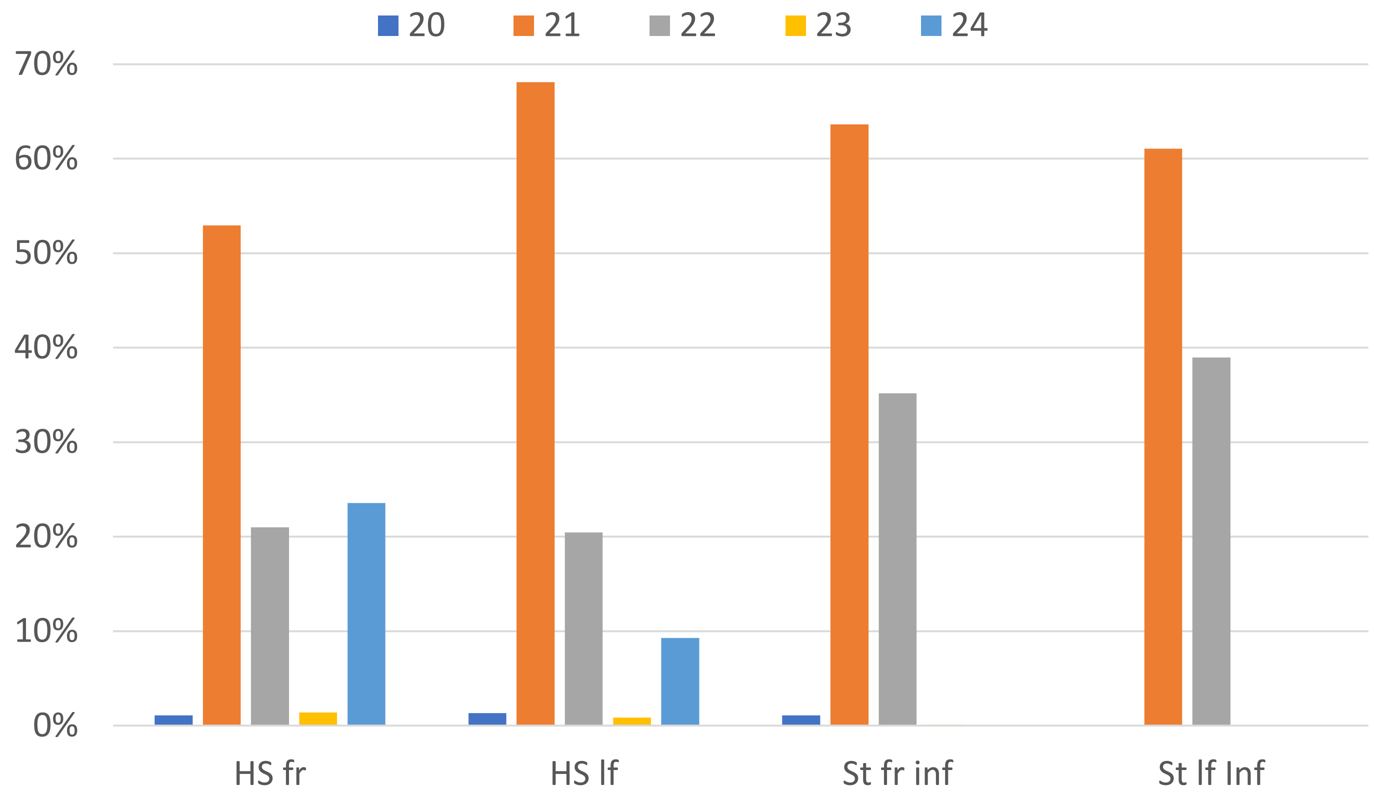
‘Honeysweet’ Trees Graft Inoculated with ‘Emma Lapperman’




qPCR for Viral Coat Protein Gene(REC)
A.
'HoneySweet' Leaves
Grafted Inoculum Leaves ('Emma Leppermann')
Control
Tree 1
Tree 2
Tree 4
Tree 5
Tree 6
Tree 7
Tree 8
Tree 9
Innoculum removed
2006
2007
2008
2009
2010
2011
2014
2015
2016
2017
-2
0
2
qPCR for Transgene Coat Protein Gene(D)
B.
'HoneySweet' Leaves
Control
Tree 1
Tree 2
Tree 4
Tree 5
Tree 6
Tree 7
Tree 8
Tree 9
Grafted Inoculum Leaves ('Emma Leppermann')
-1
0
2

Size distribution by % of sRNA matching PPV-CP in 'Honeysweet' vs PPV Infected 'Stanley'

B.



‘Honeysweet’ fruit contain significantly less siRNA than PPV infected fruit.

Table 1: Abundance of PPV and PPV-CP-derived sRNAs in non-transgenic ‘Stanley’, PPV-infected ‘Stanley’, and transgenic ‘HoneySweet’ C5 plums

sRNA Origin	‘Stanley’	PPV-Infected ‘Stanley’	‘HoneySweet’ C5
PPV	63[a] (0.001%)[b]	352,132 (35.2%)	3,126 (0.3%)
PPV-CP	10.98 (0%)	20,404 (2.0%)	3,065 (0.3%)

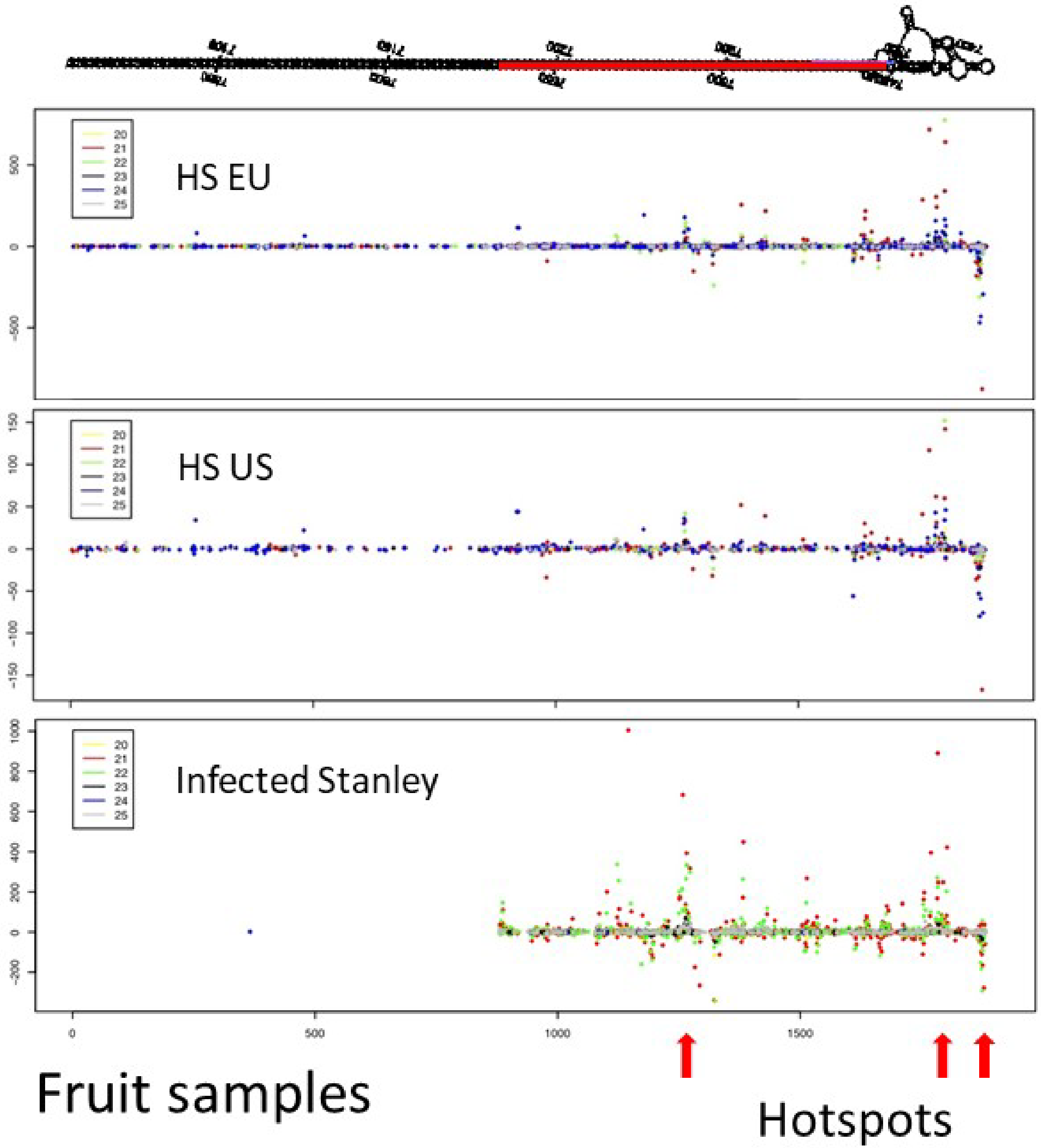
a. Values represent the reads per kilobase million (RPKM) calculated as the number of sRNAs (20-24 bases long) found in each sample divided by the total number of sRNAs that map to the peach genome and then multiplied by a million.
b. Values in parentheses are the percentage of total reads matching sequences derived from PPV or PPV-CP sequences.

Graft Inoculated Trees (Leaf)	HS Control	E. Leppermann Inoculum	HS Below Inoculum	HS Above Inoculum	HS Below Removed Inoculum	HS Above Removed Inoculum
CP-D	1948	9	3852	4394	3931	4910
GUS	371	0	436	433	437	523
NPTII	2	0	2	2	2	3
BLA	29	0	59	80	77	92
CP-REC	1	6172	4	5	5	6
3' UTR_1	0	1122	1	1	1	1
P1	4	24635	10	16	16	21
HC PRO	4	19595	8	13	13	16
P3	5	29266	12	20	19	24
6K1	1	3314	1	2	3	3
CI	7	42296	18	29	29	33
6K2	0	3245	1	2	2	3
NIa-VPG	2	7064	3	4	5	6
NIa-PRO	3	14880	6	12	11	13
NIb	4	18359	9	14	13	16
3' UTR_2	0	460	0	1	0	0

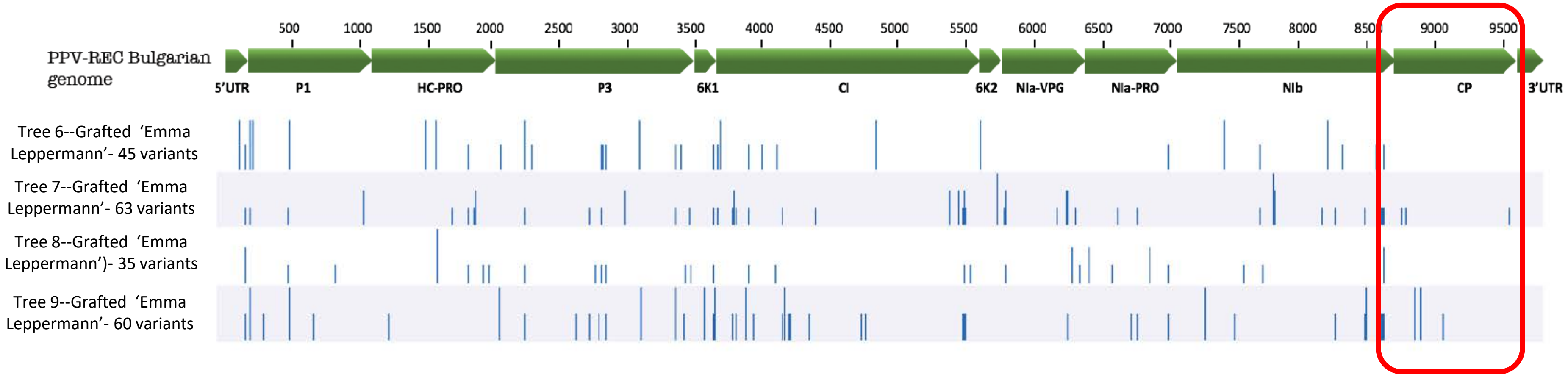
Multi-Location Trees	Leaf					Fruit				
	US HS	US Stanley	Spain Stanley	Bulgaria Stanley	EU HS	US HS	US Stanley	Spain Stanley	Bulgaria Stanley	EU HS
CP-D	1159	1	107	1	1628	138	3	1248	53	380
GUS	207	2	0	0	925	83	1	2	1	209
NPTII	5	0	0	0	9	2	0	0	0	3
BLA	72	0	0	10	272	43	0	1	0	91
CP-M	1	0	0	49	0	0	0	21	616	0
3' UTR_1	0	0	0	0	0	0	0	19	1	0
P1	1	1	378	194	0	0	1	3295	1930	1
HC PRO	1	1	333	172	0	0	0	2914	1596	1
P3	1	1	589	157	0	0	0	4178	1718	1
6K1	0	0	44	0	0	0	0	268	0	0
CI	1	1	393	50	0	0	0	3380	823	1
6K2	0	0	14	47	0	0	0	207	381	0
NIa-VPG	0	0	132	10	0	0	0	1187	173	1
NIa-PRO	0	0	206	0	0	1	0	2225	1	1
NIb	0	0	131	10	0	0	1	1600	164	1
3' UTR_2	42	2	3	9	93	14	1	152	269	51

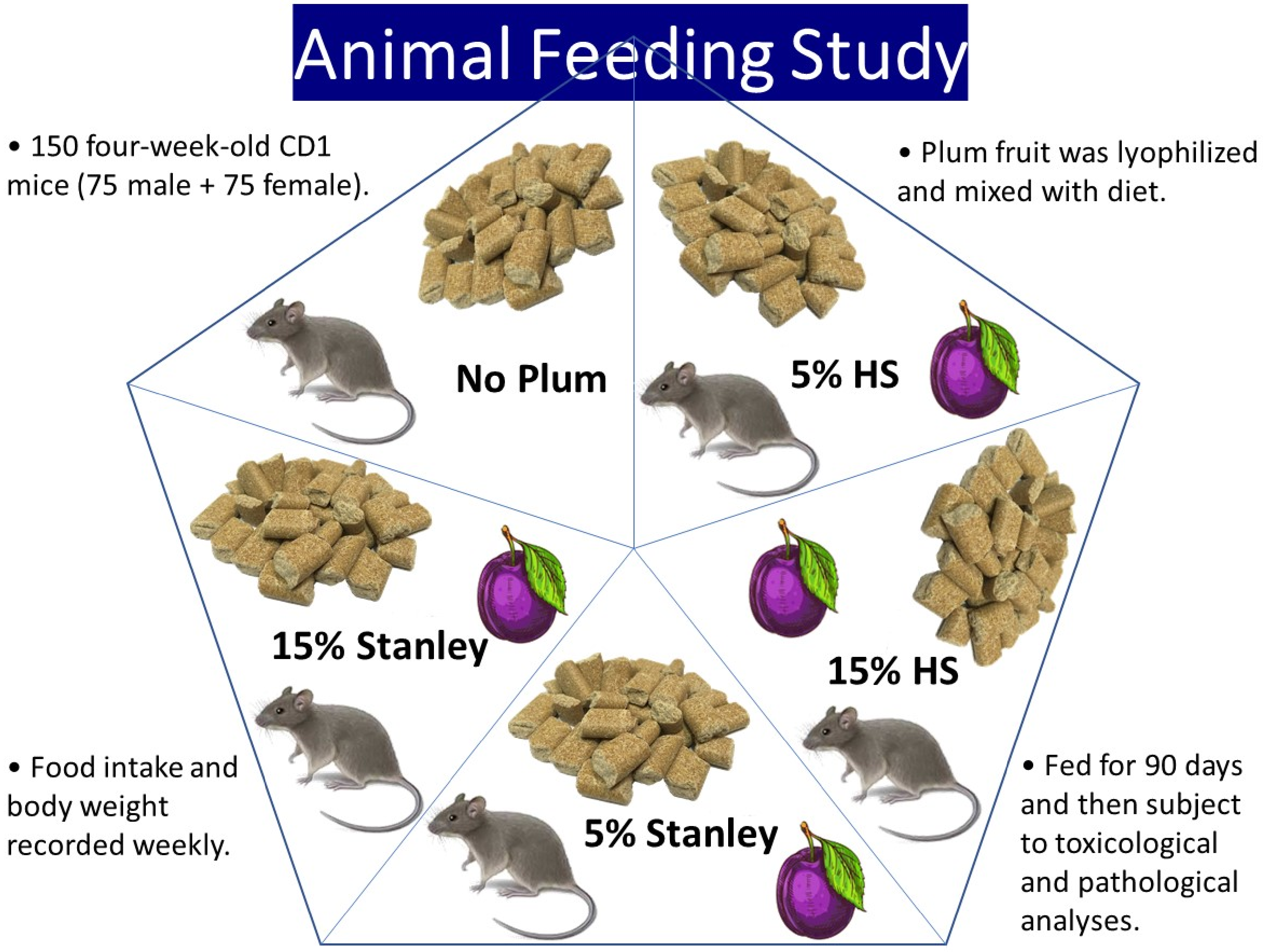
1	25	50	1000	4000

Scale for Reads/Million


HS EU
HS US
Infected Stanley
20
21
22
23
24
25
0
500
1000
1500
Fruit samples
Hotspots

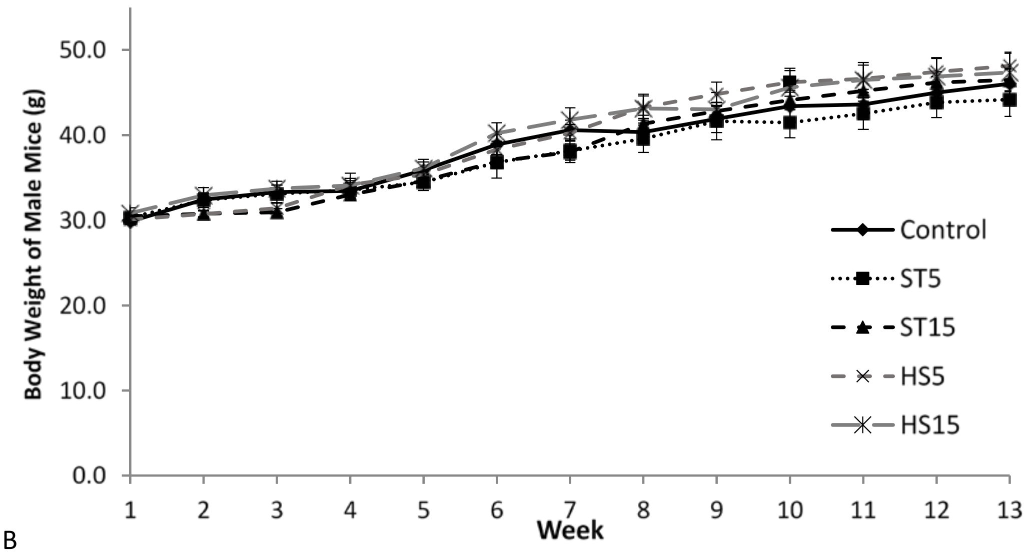
No Evidence for Viral Adaptation in PPV Infected Grafted Limbs.





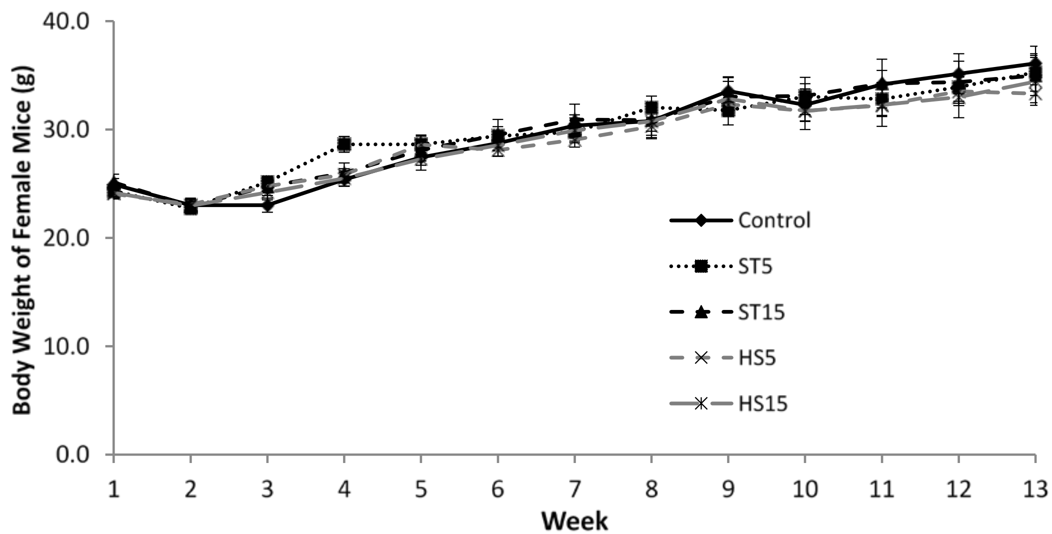
Animal Feeding Study
• 150 four-week-old CD1 mice (75 male + 75 female).
• Plum fruit was lyophilized and mixed with diet.
No Plum
5% HS
15% Stanley
15% HS
5% Stanley
• Food intake and body weight recorded weekly.
• Fed for 90 days and then subject to toxicological and pathological analyses.

A



50.0
40.0
30.0
20.0
10.0
0.0
Body Weight of Male Mice (g)
1 2 3 4 5 6 7 8 9 10 11 12 13
Week
Control
ST5
ST15
HS5
HS15

B



40.0
30.0
20.0
10.0
0.0
Body Weight of Female Mice (g)
1 2 3 4 5 6 7 8 9 10 11 12 13
Week
Control
ST5
ST15
HS5
HS15

Supplementary File 10. Total White Blood Cells and White Blood Cell Differentials for Female Mice

Parameter	Control	ST5%	ST15%	HS5%	HS15%	P-value	Normal Values
Total WBC (1x10^3)	9.19 ± 2.03	8.28 ± 1.30	8.11 ± 1.22	10.2 ± 1.22	7.96 ± 1.25	0.3109	1.8-10.7
Lymphocytes (%)	80.75 ± 1.44	81.91 ± 1.11	81.58 ±1.39	81.00 ± 1.21	77.83 ± 0.68	0.1346	55.8-91.6
Neutrophils (%)	18.17 ± 1.40^{ab}	15.92 ± 0.75^{b}	17.00 ± 1.30^{b}	18.25 ± 0.87 ^{ab}	20.67 ± 0.89^{a}	**0.0365**	6.6-38.9
Monocytes (%)	0.92 ± 0.34	2.25 ± 0.85	1.25 ± 0.33	1.17 ± 0.30	1.17 ± 0.34	0.3380	0-7.5
Basophils (%)	0.08 ± 0.08	0.17 ±0.11	0 ± 0	0 ± 0	0.33 ± 0.14	0.0572	0-2.0
Eosinophils (%)	0.17 ± 0.11	0 ± 0	0.17 ± 0.17	0.25 ± 0.13	0 ± 0	0.3935	0-3.9
Lymphocytes	7374 ± 1576	6792 ± 1071	6702 ± 1041	9778 ± 1348	6217 ± 976	0.2737	--
Neutrophils	1769 ± 488	1332 ± 220	1302 ± 195	2177 ±282	1642 ± 270	0.2828	--
Monocytes	47.75 ± 16	158 ± 47	96 ± 27	165 ± 42	84 ± 25	0.0727	--
Basophils	4.8 ± 10.7	0 ±0	0 ± 0	0 ± 0	32.6 ±15.0	0.0456	--
Eosinophils	12.3 ± 9.4	9.7 ± 9.7	9.7 ± 9.7	23.8 ± 12.6	0 ± 0	0.2361	--

Supplementary File 8. 'Honeysweet' Study Blood Chemistry for Females

Parameter	Control	ST5%	ST15%	HS5%	HS15%	P-value	Normal Range
CHOL (mg/dL)	100.33 ± 6.05	106.42 ± 7.27	111.50 ± 9.45	104.08 ± 5.58	113.83 ± 7.54	0.6911	--
TRIG (mg/dL)	116.08 ± 13.49	104.92 ± 11.51	98.83 ± 8.20	115.58 ± 12.86	108.08 ± 8.20	0.0782	--
ALT (U/L)	35.08 ± 8.39	38.58 ± 12.41	23.67 ± 2.04	32.75 ± 5.74	27.27 ± 3.64	0.6357	17-77
AST (U/L)	96.33 ± 16.12	103.75 ± 24.42	84.58 ± 5.63	101.75 ± 15.94	269.67 ± 180.05	0.4700	54-298
ALP (U/L)	43.92 ± 2.90	38.58 ± 2.35	45.42 ± 2.98	39.17 ± 2.77	37.75 ± 3.83	0.2730	35-96
GLU (mg/dL)	285.67 ± 19.95	297.83 ± 32.19	289.25 ± 21.29	300.00 ± 28.20	257.25 ± 24.68	0.7752	62-300
PHOS (mg/dL)	10.51 ± 1.89	9.99 ± 0.74	9.02 ± 0.32	8.65 ± 0.30	10.18 ± 1.34	0.7417	5.7-9.2
Ca (mg/dL)	9.11 ± 0.16	9.06 ± 0.14	9.13 ± 0.09	8.90 ± 0.06	9.07 ± 0.08	0.6389	7.1-10.1
TBIL (mg/dL)	0.12 ± 0.01	0.13 ± 0.02	0.11 ± 0.00	0.12 ± 0.01	0.22 ± 0.11	0.4963	0-0.9
TP (g/dL)	4.30 ± 0.08	4.16 ± 0.09	4.22 ± 0.06	4.15 ± 0.07	4.18 ± 0.05	0.5546	3.5-7.2
ALB (g/dL)	2.64 ± 0.06	2.60 ± 0.06	2.63 ± 0.03	2.53 ± 0.08	2.57 ± 0.04	0.5341	2.5-3.0
GLOB (g/dL)	1.63 ± 0.04	1.56 ± 0.04	1.59 ± 0.04	1.57 ± 0.04	1.59 ± 0.03	0.7974	1.0-4.2
AG Ratio	1.65 ± 0.03	1.68 ± 0.04	1.67 ± 0.04	1.66 ± 0.05	1.61 ± 0.05	0.7600	--
BUN (mg/dL)	19.17 ± 0.80	17.33 ± 0.92	17.33 ± 0.83	17.50 ± 1.11	19.08 ± 0.78	0.3515	8-33
CREAT (mg/dL)	0.20 ± 0.00	0.21 ± 0.01	0.20 ± 0.00	0.20 ± 0.00	0.20 ± 0.00	0.4521	0.2-0.9
Na (mEq/L)	142.64 ± 0.43	142.92 ± 1.02	143.50 ± 0.69	141.40 ± 1.01	143.36 ± 0.83	0.4355	140-160
K (mEq/L)	4.76 ± 0.14	5.68 ± 0.93	4.80 ± 0.08	5.03 ± 0.16	4.57 ± 0.18	0.4732	5-7.5
Cl (mEq/L)	111.18 ± 0.57	111.00 ± 0.98	111.50 ± 0.75	112.10 ± 0.59	110.82 ± 0.91	0.8243	88-110
Na/K ratio	30.20 ± 0.90	30.37 ± 0.73	29.99 ± 0.57	28.39 ± 1.01	31.90 ± 1.41	0.1820	--

CHOL=cholesterol; TRIG=triglycerides; ALT=alanine aminotransferase; AST=aspartate aminotransferase; ALP=alkaline phosphatase; GLU=glucose; PHOS=phosphate; Ca=calcium; TBIL=total bilirubin; TP=total protein; ALB=albumin; GLOB=globins; AG Ratio=albumin/globin; BUN=blood urea nitrogen; CREAT=creatinine; Na=sodium; K=potassium; Cl=chloride; Na/K ratio=sodium/potassium

Data are means ± SE; Glucose values do not represent normal fasting glucose due to anesthesia;

-- indicates no normal range for the mouse.

Mouse #116 in DP-15% ALT very high values; Results shown with and without #116

Supplementary File 11. Pathology Report Female Mice.

Tissue	Control	ST5%	ST15%	HS5%	HS15%	Chi Sq (P-value)
Heart	0/12 (0%)	0/12 (0%)	1/12 (8.3%)	0/12 (0%)	1/12 8.3%)	0.541
Aorta	0/11 (0%)	0/11 (0%)	0/10 (0%)	0/12 (0%)	0/9 (0%)	---
Lung	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	---
Trachea	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/11 (0%)	0/12 (0%)	---
Thyroid	0/8 (0%)	1/9 (11.1%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0.365
Thymus	0/12 (0%)	0/11 (0%)	0/12 (0%)	0/11 (0%)	0/12 (0%)	---
Lymph node	0/10 (0%)	0/11 (0%)	1/10 (10%)	0/12 (0%)	0/10 (0%)	0.357
Bone	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	---
Bone marrow	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	---
Skeletal muscle	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	---
Cerebrum-Gray	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	---
Cerebrum-White	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	---
Cerebellum	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	---
Meninges 1&2	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	---
Spinal Cord	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/11 (0%)	---
Peripheral Nerve	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	---
Eye-LENS	0/12 (0%)	0/12 (0%)	0/9 (0%)	0/12 (0%)	0/12 (0%)	---
EYE-UVEA	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/10 (0%)	0/11 (0%)	---
Eye-Retina	0/12 (0%)	0/12 (0%)	0/11 (0%)	0/11 (0%)	0/12 (0%)	---
Eye-Cornea	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	---
Mammary Gland	0/12 (0%)	0/11 (0%)	0/12 (0%)	0/11 (0%)	1/11 (9.1%)	0.4196
Ovary	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	---
Uterus	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	---
Vagina	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	---
Skin	0/12 (0%)	1/12 (8.3%)	1/11 (9.1%)	0/12 (0%)	1/12 (8.3%)	0.703
Liver*	3/12 (25%)	8/12 (66.7%)	2/12 (16.7%)	3/12 (25%)	5/12 (41.7%)	0.133
Kidney*	5/12 (41.7%)	5/12 (41.7%)	6/12 (50.0%)	4/12 (33.3%)	7/12 (58.3%)	0.569
Urinary bladder	1/12 (8.3%)	1/12 (8.3%)	0/11 (0%)	0/10 (0%)	0/10 (0%)	0.613
Spleen	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	---
Salivary glands	2/12 (16.7%)	2/12 (16.7%)	2/12 (16.7%)	2/12 (16.7%)	3/12 (25%)	0.796
Esophagus	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	---
Stomach	1/12 (8.3%)	2/12 (16.7%)	2/12 (16.7%)	4/12 (33.3%)	2/12 (16.7%)	0.614
Pancreas	1/12 (8.3%)	0/12 (0%)	1/11 (9.1%)	0/12 (0%)	0/12 (0%)	0.517
Duodenum	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	---
Jejunum	1/12 (8.3%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0.397
Ileum	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	---
Cecum	0/12 (0%)	0/12 (0%)	0/12 (0%)	1/12 (8.3%)	1/12 (8.3%)	0.541
Colon	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	---
Rectum	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	---
Adrenal glands*	4/12 (33.3%)	6/12 (50%)	3/12 (25%)	3/12 (25%)	4/12 (33.3%)	0.312

Data presented as number of animals with any abnormal pathology per tissue read.
ST=Stanley plum; HS=HoneySweet plum

BLAST Searched Mouse Genome for Potential Off-target Hits

		Raw	Normalized TPM					Match	Bases				
	Rank	reads	HSF	STF	HSL	STL	Length	Accession#	Matched	Gap	Start	Stop	Identity
AGAGGACACAGAGAGACACACC	17	1219	22.43	9.45	26.81	-	22	gi|315075272|ref|N	20	1	1	20	low density lipoprotein receptor-related protein 6 Lrp6
AGAGGACACAGAGAGACACAC	27	799	17.15	-	18.47	-	21	gi|315075272|ref|N	20	1	1	20	low density lipoprotein receptor-related protein 6 Lrp6
AGGGGAGTGTAGTGGTCTCGG	89	293	3.06	0.89	8.77	-	21	gi|153791795|ref|N	19	1	3	21	myoferlin Myof
GGGGAGTGTAGTGGTCTCGGT	149	170	6.65	1.52	1.05	-	21	gi|153791795|ref|N	20	1	2	21	myoferlin Myof
AGGGGAGTGTAGTGGTCTCGGT	150	169	1.37	-	5.57	-	22	gi|153791795|ref|N	20	1	3	22	myoferlin Myof
GGTGTGTCTCTCTGTGTCCT	211	103	4.27	1.07	0.39	-	20	gi|284004882|ref|N	18	0	1	18	tripartite motif-containing 66 Trim66
GGTGTGTCTCTCTGTGTCCT	211	103	4.27	1.07	0.39	-	20	gi|284004880|ref|N	18	0	1	18	tripartite motif-containing 66 Trim66 variant 1
GGTGTGTCTCTCTGTGTCCT	211	103	4.27	1.07	0.39	-	20	gi|284004878|ref|N	18	0	1	18	tripartite motif-containing 66 Trim66 variant 2
TAAAAATCAAAGGCATATCTG	139	187	0.63	1.96	5.96	-	21	gi|118130656|ref|N	19	0	3	21	DCN1 defectinve in cullin neddlyation 1 domain containing 5 Dcun1d5
AGAGCTCCGCAGTCTTGTTT	184	129	-	5.08	2.81	-	20	gi|158341626|ref|N	18	1	1	18	phospholipase B domain containing 2 (Plbd2)
AAATGACTTCAACGACACCCG	193	119	0.90	0.98	3.55	-	21	gi|22208851|ref|NM	19	1	1	19	olfactory receptor142 Olfr142
GCAACCTGACAGACTACAGCC	223	93	0.63	2.05	2.26	-	21	gi|118150646|ref|N	19	1	1	19	RNA exonuclease 1 homolog
GTTCCATTCTCTATGCACCAA	114	227	0.42	-	8.53	-	21	gi|142349713|ref|N	19	1	2	20	member RAS oncogen RAB9B
CTTTTAGACAAATTATGGCAC	155	161	-	2.85	5.03	-	21	gi|256985127|ref|N	19	2	2	20	CCR-4-NOT transcription complex, subunit4 (Cnot4) variant 3
CTTTTAGACAAATTATGGCAC	155	161	-	2.85	5.03	-	21	gi|256985125|ref|N	19	2	2	20	CCR-4-NOT transcription complex, subunit4 (Cnot4) variant 2
CATTTCTCAATGCTGCTGCCT	225	92	-	-	3.58	-	21	gi|291045287|ref|N	19	1	1	19	5' nucleotidase ecto (Nt5e
CATTTCTCAATGCTGCTGCCT	225	92	-	-	3.58	-	21	gi|141802310|ref|N	19	1	1	19	coiled-coil domain containing 68 Ccdc68

Myof - defects in muscle regeneration and angiogenesis.

Nt5e - calcification of joints and arteries and alterations in dendritic cell infiltration in tissues.

Lrp6 - Alzheimers, cancer and osteoporosis.

*** Found no evidence of any associated pathologies.**

Summary

- 'Honeysweet' has a complex multi-site insertion event that causes no measurable changes in flanking regions.
- siRNA expression in 'Honeysweet' is relatively stable under different environments over time.
- Viral infection of plums produces significantly higher levels of siRNA than 'Honeysweet'.
- A ten year study showed that PPV populations in limbs grafted to 'Honeysweet' show no evidence of adaptive evolution.
- A 90-day mouse feeding study did not reveal any measurable health effects of 'Honeysweet' plum consumption.

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