# The practical application of RNAi technology to provide safe, effective, and durable resistance to Sharka disease caused by *Plum pox virus*.

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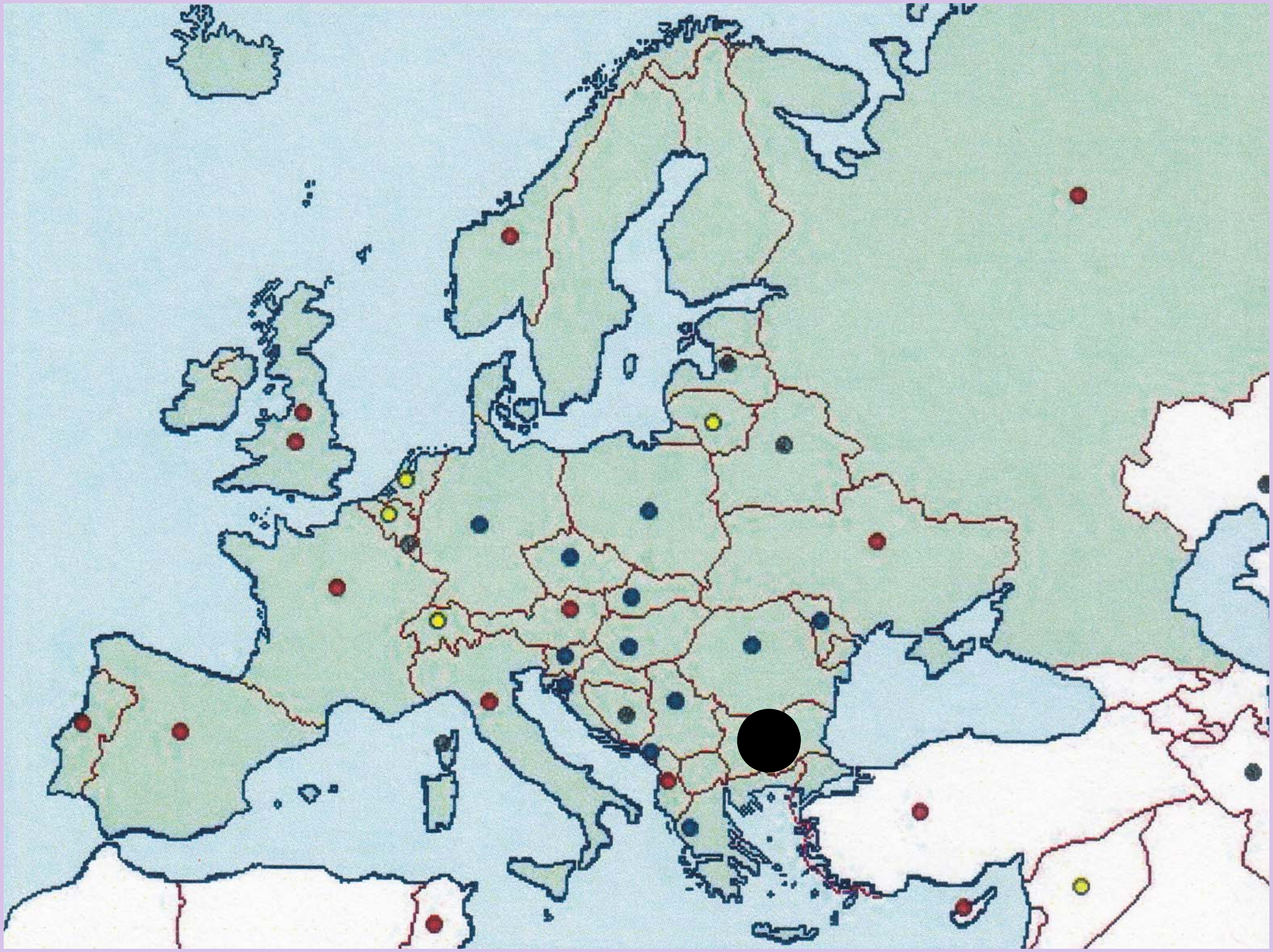
# International ‘HoneySweet’ Working Group

- ‘HoneySweet’ co-developed by U.S. and European team
- Field tested in Europe for over 17 years
- Solely the work of publicly-supported scientists, at public research institutions
- For the benefit of growers and consumers

# Sharka (*Plum pox virus*)

# PPV SPREAD – EUROPE



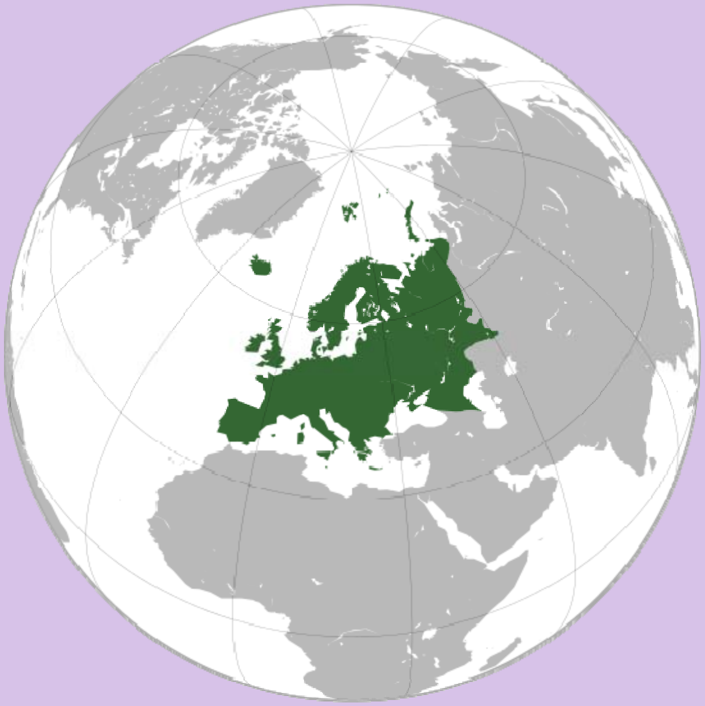
● widespread

● localized

○ occasional reports

http://www.cabi.org/isc/datasheet/42203

# PPV infection in selected European countries:



Bosnia-Herzegovina - **41%** of plum trees infected

Croatia - **51%** plum trees infected

Serbia - **58%** plums infected with PPV

Bulgaria - **62%** infection in plums

Romania – **69%** infection in plums

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Moldova - plum yield losses plum are **16-48%**

Greece – apricot production decreased from **35% to 13%** of world production due to PPV

Spain - **2.3 million** PPV-infected trees removed between 1989 and 2006 at a cost of over **63 M Euros**

2006 OEPP/EPPO 36 (2)
Zagrai et al UASMV 67 2010

# PPV control strategies:

Eradication (tree removal)

Quarantine

Tolerance (promotes virus spread)

Natural Resistance

- *P. domestica* plum – hypersensitivity (extreme sensitivity)
  multigenic, report of strain specificity

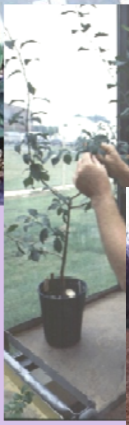
- *P. armeniaca* apricot - multigenic , strain specific?

- *P. dulcis* almond – multigenic? strain specific?

# TIMELINE FOR ‘HONEYSWEET’ DEVELOPMENT

Initial genetic engineering

1990



Greenhouse testing for resistance

1992-1995

U.S. field test

1995

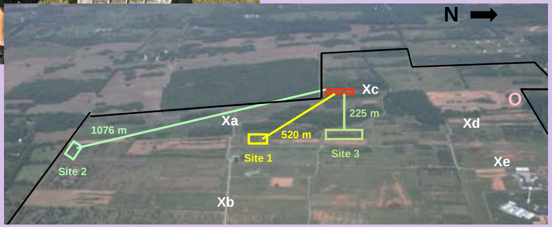
**European field tests since 1996**

Determination of the resistance mechanism

1995-2006

Field test risk assessments

1996-present



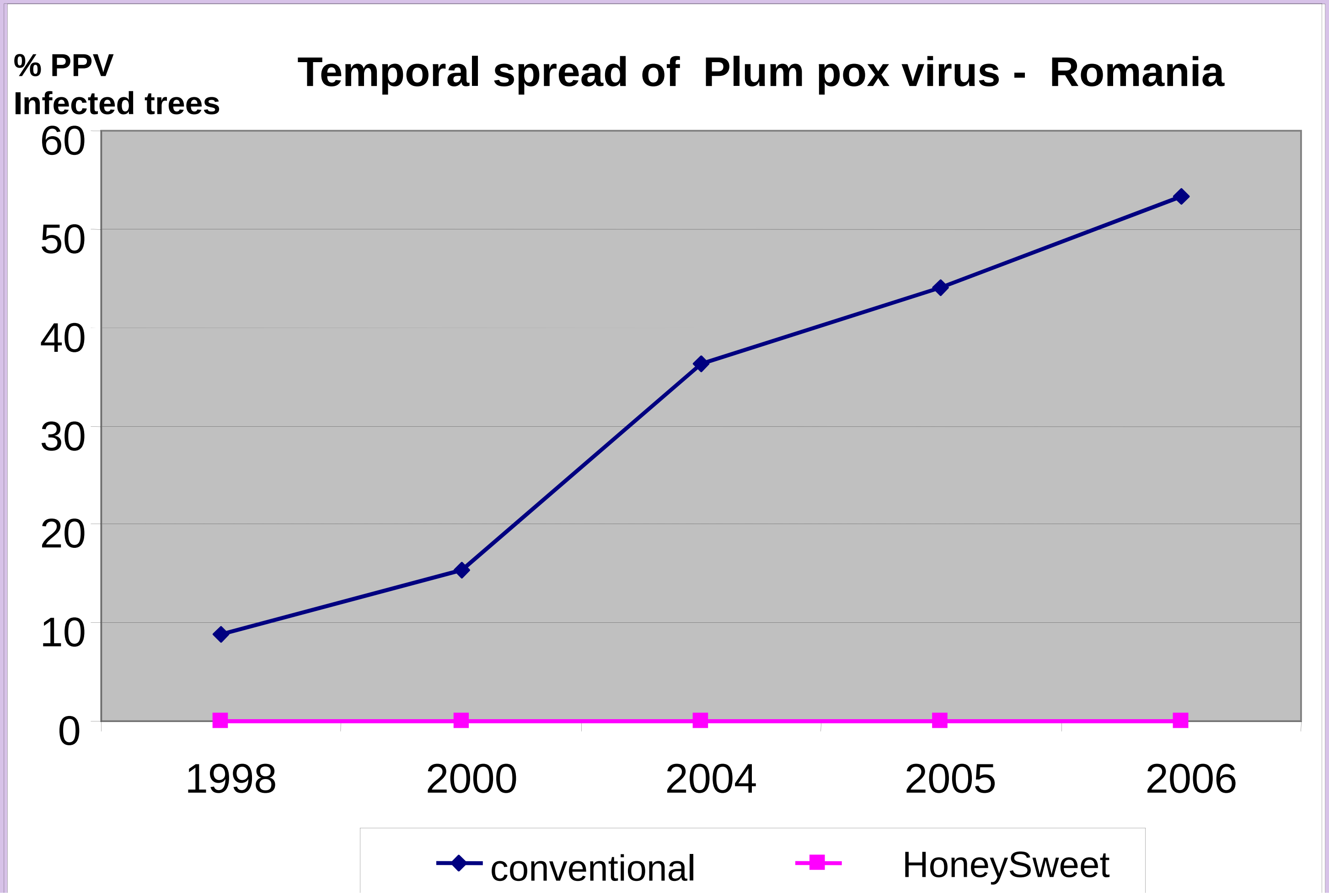
US

Regulatory submissions

2006-2007

# In over 17 years of field testing in 4 European countries (Czech Republic, Poland, Romania, Spain) no ‘HoneySweet’ trees have been naturally Infected with PPV by aphids

Romania example (courtesy of I. Zagrai)



# Results of Environmental Risk Studies

(all studies published in peer-reviewed journals)

**No virus recombinants**

**No effects on non-target insects**

**No effects on aphid populations**

**No effects on virus diversity**

**No breakdown of resistance in presence of other *Prunus* viruses**

**Gene flow is low**

# ‘HoneySweet’ is well-known in Europe and has been included in the following EU – funded projects:

- \- FP4-Bio4CT-960773: Risk assessment of genetic engineering woody plants expressing virus coat protein gene (1996-1999)
- \- FP5-QLK3-2002-024:Environnmental impact assessment of transgenic grapevines and plums on the diversity and dynamics of virus population (Transvir: 2003-2006)
- \- FP7-204429: Sharka containment in view of EU expansion (Sharco: 2008-2012)
- -FP7-269292: Intercontinental and temporal research studies on transgene engineered plants (Interest: 2011-2014)

# ‘HoneySweet’ plum

- It is highly resistant to all strains of PPV.

- No trees have ever been infected by aphids.

- Fruit quality is excellent – 21% sugar, large size, good productivity.

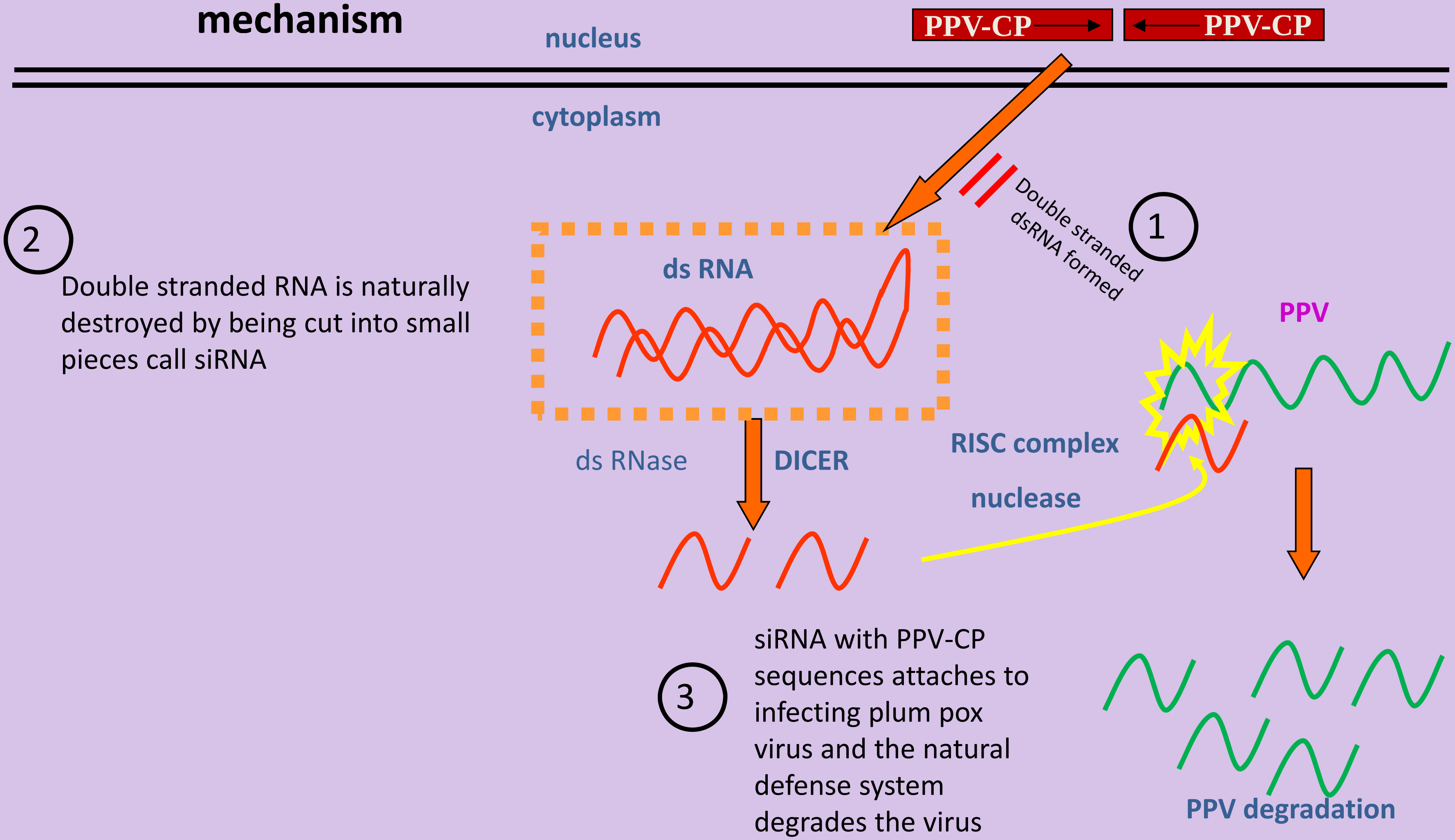
- The resistance is transferred as a single gene dominant trait through traditional cross hybridization.

**HoneySweet is fully approved for cultivation and consumption in the U.S. by APHIS, FDA, EPA**

# HONEYSWEET PPV RESISTANCE IS RNAi-BASED

## Pathogen derived resistance through RNA silencing
## A *natural* virus resistance mechanism

**An inverted repeat of the PPV-CP gene (hairpin) formed naturally, likely during Agro-mediated insertion**



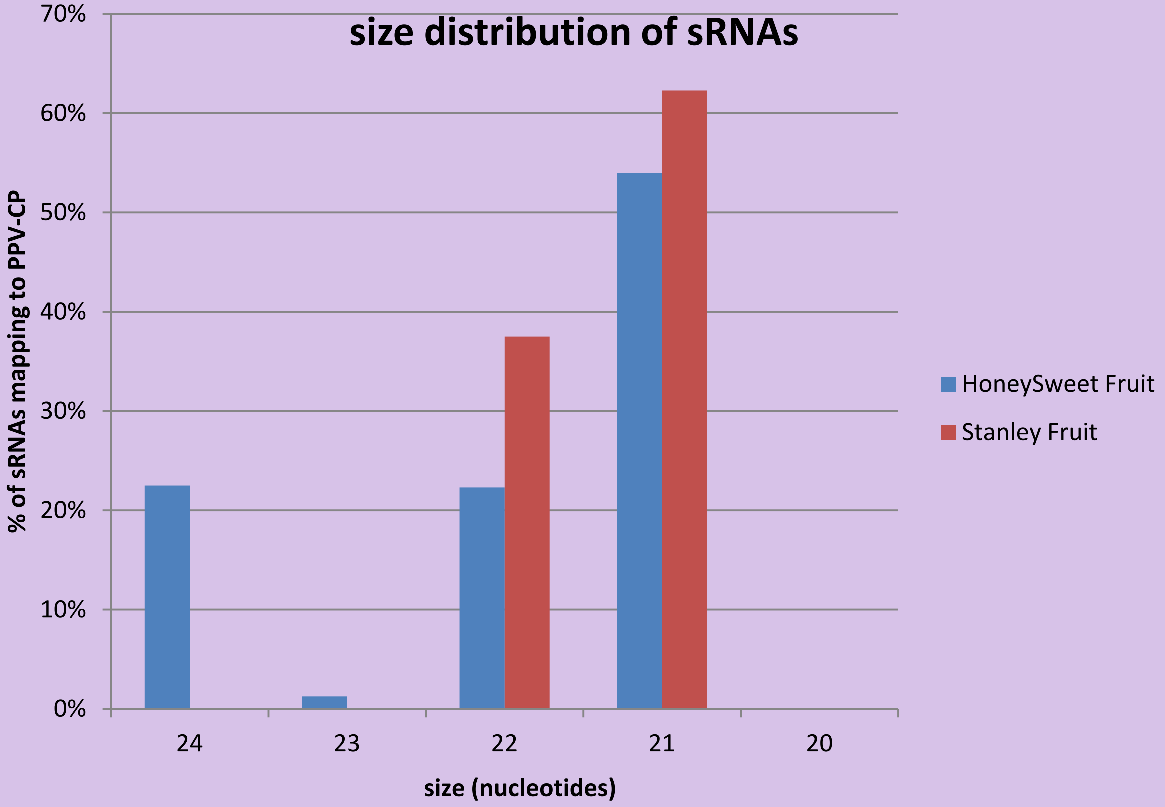
② Double stranded RNA is naturally destroyed by being cut into small pieces call siRNA

③ siRNA with PPV-CP sequences attaches to infecting plum pox virus and the natural defense system degrades the virus

# small RNA production in reaction to virus infection is not limited to HoneySweet

Hily et al. MPMI vol 18:794-799. 2005

## Control plants inoculated with PPV also produce siRNA in reaction to the virus.

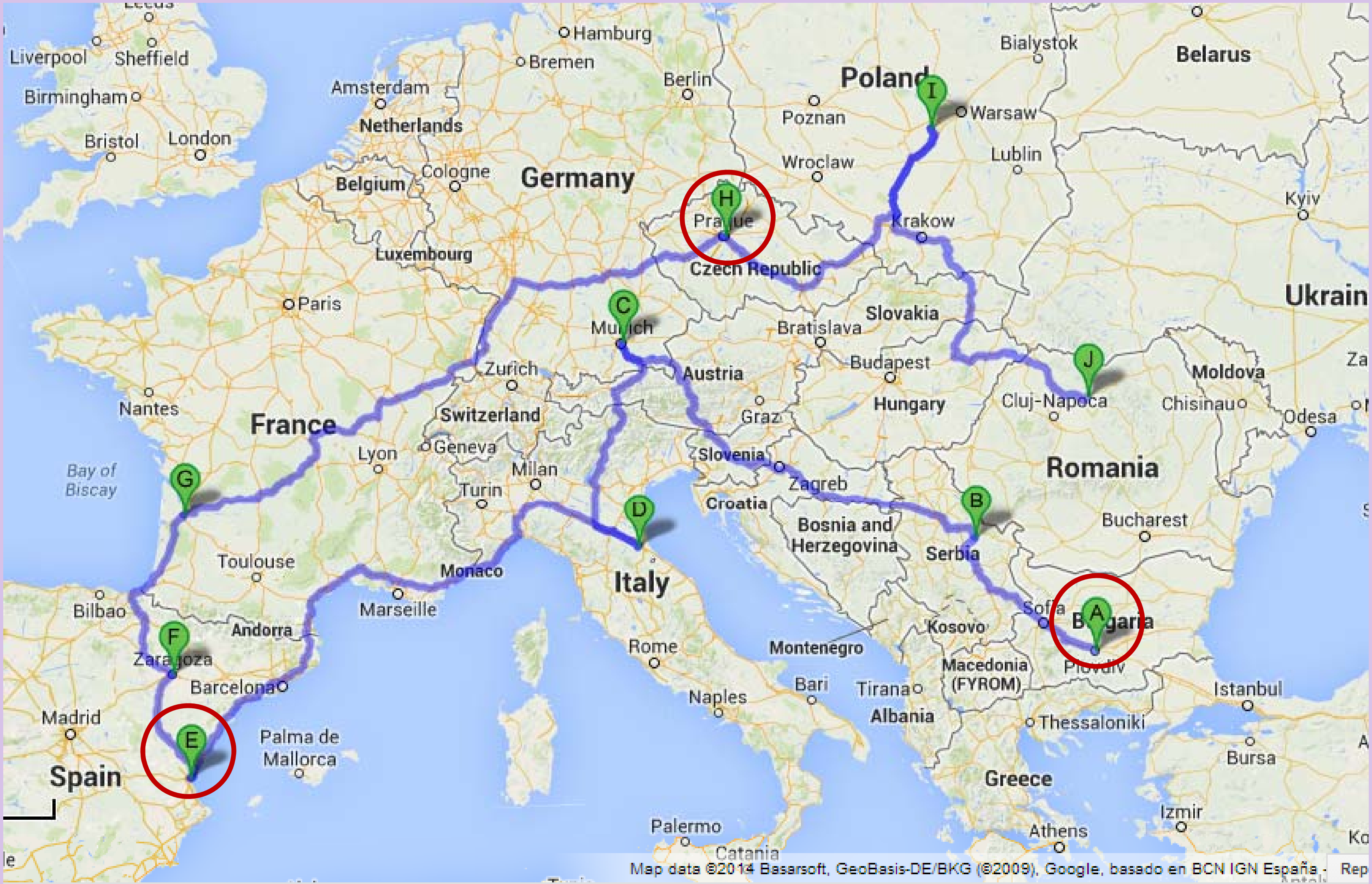


# In depth study of all mRNAs and small RNAs produced by 'HoneySweet' plum

*** Chris Dardick (USDA), Ann Callahan (USDA), Tadeusz Malinowski (Inhort) Michel Ravelonandro (INRA)**

- Next Gen sequencing technologies enable quantification of mRNAs and s RNAs via direct sequencing providing information about the sequence and abundance in a given tissue.

# Fruit collection locations in Europe for RNA and sRNA analyses, and compositional studies – Stanley infected, not infected and HoneySweet



**Bulgaria**, Serbia, Germany, Italy, **Spain** (2), France, **Czech Republic**, Poland, Romania (RNA studies from fruit collected in Spain, Czech Republic, and Bulgaria)

# Expression profiling study: mRNA and sRNA

***Ripe fruit and leaves were sampled from each tree- <u>36 samples</u>***

**HoneySweet**

- US Fruit and leaves (3 samples each: leaf and fruit)
- Czech Republic fruit and leaves (3 trees)
- Spain fruit and leaves (2 trees)

**Stanley**

- US Fruit and leaves (3 samples each: leaf and fruit)
- North Spain Fruit and leaves (3 trees)
- South Spain Fruit and leaves (2 trees) *PPV-D infected
- Bulgaria Fruit and leaves (2 trees) *PPV-M infected

# RNAseq and small RNAseq were performed on all 36 samples

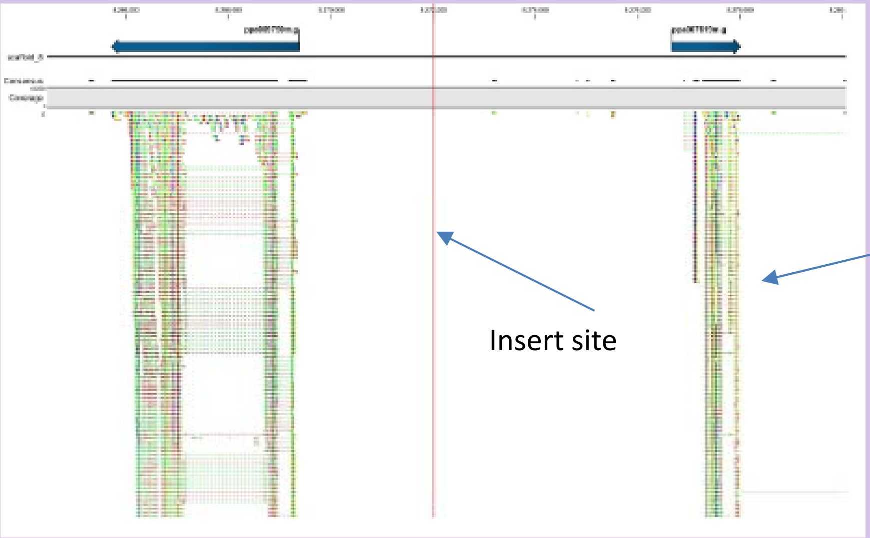
- 964,695,446 reads for mRNA transcripts
- 250,932,388 sRNA reads

Data was assembled to the reference peach genome and the transgene insertion sequences for analysis

# Transcript reads mapped to regions flanking hairpin insert show that the expression of genes flanking the gene insert was not altered.

Expression of genes flanking transgene insertion site

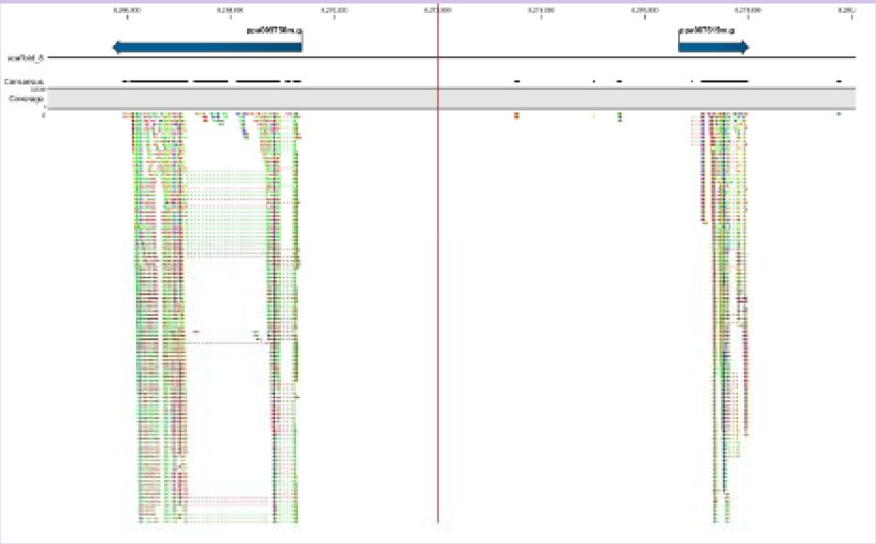
Stanley fruit



Aligned transcript reads

Insert site

HoneySweet fruit



Insertion site approx. position 8,272,00 on peach scaffold 8

# Summary of fruit RNA profiling

## mRNA - % of total reads matching PPV and/or transgene sequences

| | Stanley | Infected Stanley | HoneySweet |
|---|---|---|---|
| PPV all | 0.0004% | **2.0882%** | 0.0015% |
| CP alone | 0.0001% | 0.5812% | 0.0011% |
| UidA | 0.0000% | 0.0000% | **0.0174%** |
| NPTII | 0.0000% | 0.0000% | **0.0003%** |
| PBR322 | 0.0000% | 0.0000% | **0.0000%** |

## sRNA - % of total reads matching PPV and/or transgene sequences

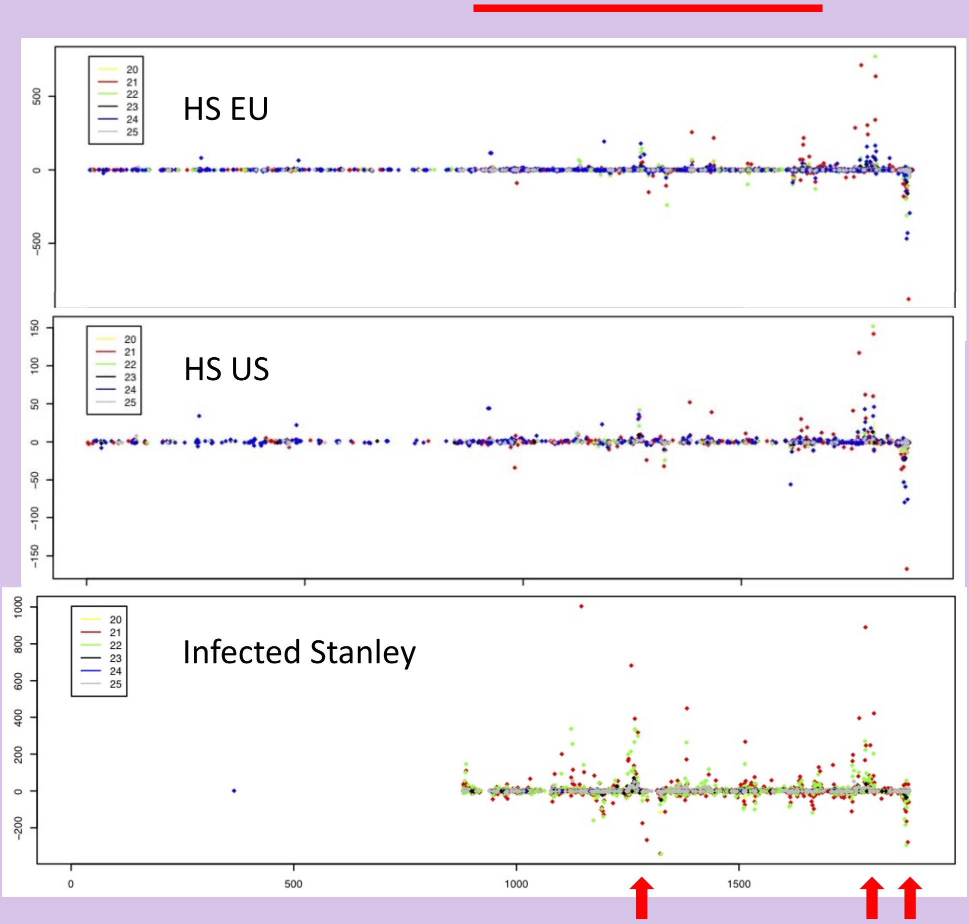
| | Stanley | Infected Stanley | HoneySweet |
|---|---|---|---|
| PPV all | 0.001% | **35.207%** | 0.306% |
| CP alone | 0.000% | 2.040% | 0.306% |
| UidA | 0.000% | 0.000% | **0.089%** |
| NPT | 0.000% | 0.000% | **0.004%** |
| pBR322 | 0.000% | 0.000% | **0.035%** |

35% of al reads were from the PPV genome

## Pattern of sRNA production is similar between HoneySweet and Infected Stanley

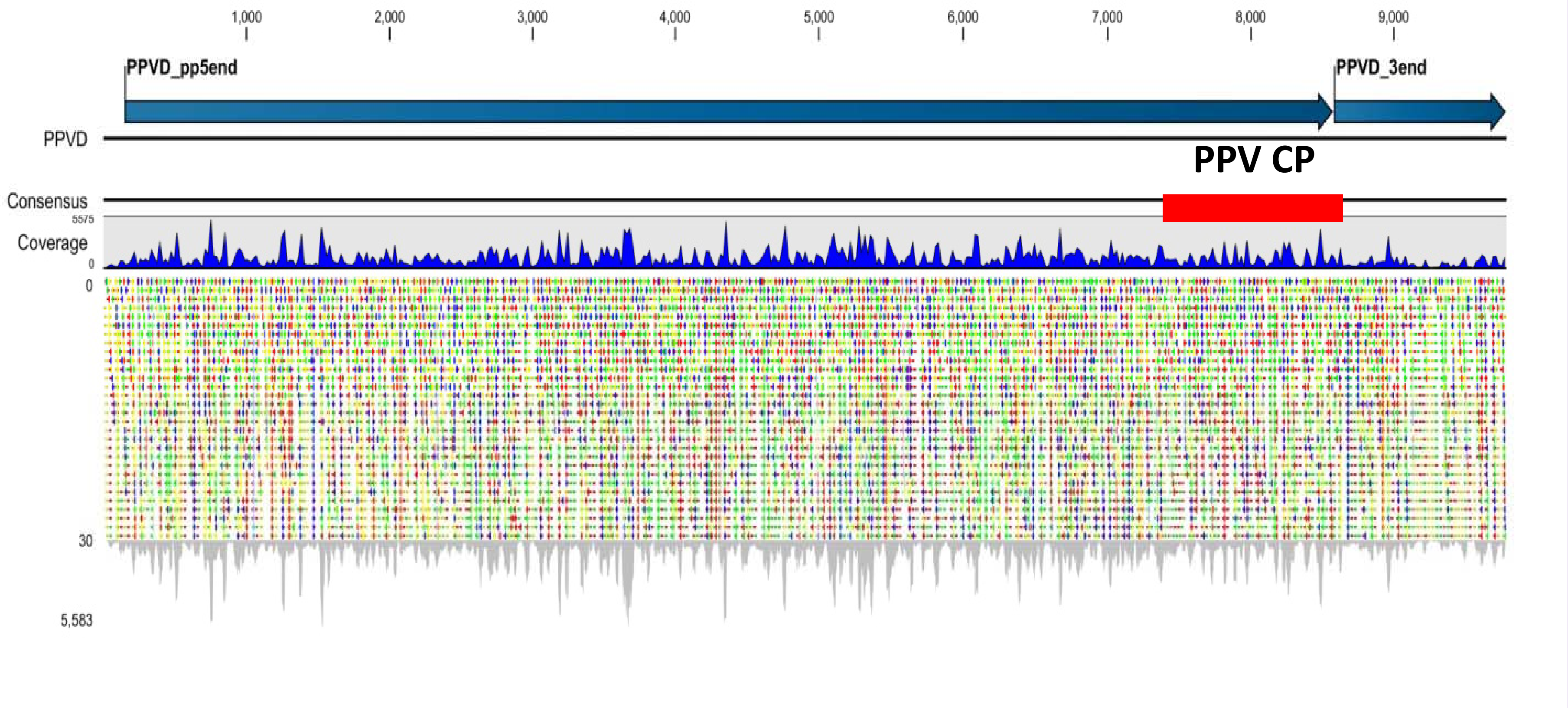
Representation of
Hairpin RNA
(CP region in red)

Fruit



Hotspots

# PPV infection in a tolerant, infected, marketable plum variety produces large amounts of sRNA

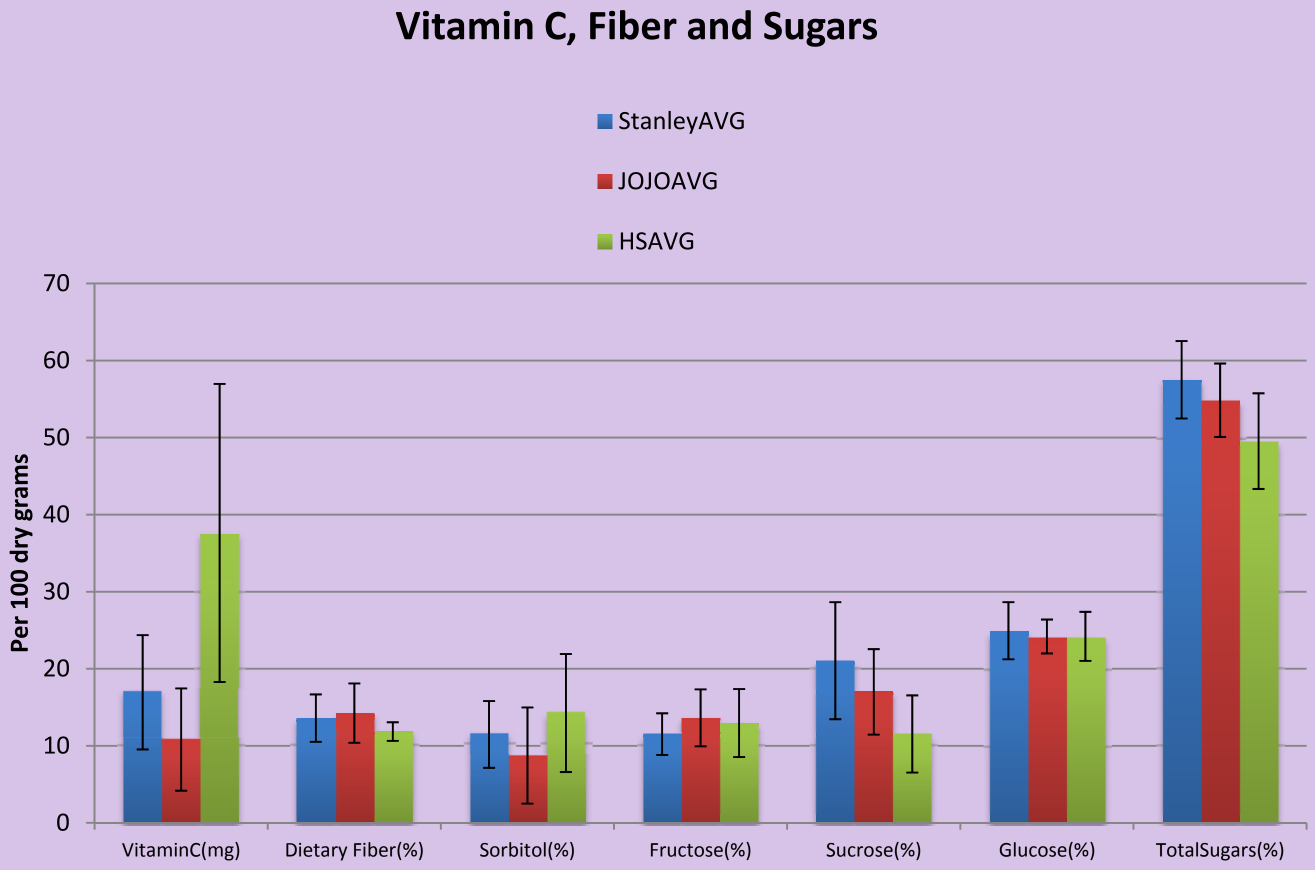


Stanley fruit

# We learned from mRNA and sRNA studies:

1) The ‘HoneySweet’ transgene insert does not cause detectable changes in transcription of flanking DNA and there is no evidence for new transcripts that span the transgene-plum borders.
2) Transgene small RNAs are produced at a relatively low abundance when compared to the millions of small RNAs produced naturally in the plant.
3) Virus infection leads to significantly more PPV-related small RNA production than produced by the PPV-CP transgene.
4) ‘HoneySweet’ produces no small RNAs that match human genes that are not already present in PPV-infected fruit (same results for mouse genes).

# Compositional Studies



Compositional studies from fruit collected from 10 European countries demonstrate that the composition of 'HoneySweet' fruit is within the range of the composition of plums currently marketed in Europe. The above graph shows results of a small, partial sample of the data collected on over 30 nutrient components examined from 40 plum varieties collected in Europe. This example shows data from the cultivars Stanley, Jojo, and HoneySweet for vitamin C, fiber, and sugars.

# RNAi technology for the development of Plum pox virus resistance as utilized in ‘HoneySweet’ plum demonstrates the efficacy, durability, and safety of the technology for the benefit of growers and consumers

HoneySweet plum