High Throughput Sequencing (HTS) as a Tool for Viral Pathogen Diagnosis and Expedited Release of Quarantined Propagative Plant Material

MAHER AL RWAHNIH
DIAGNOSTIC AND RESEARCH LABORATORY DIRECTOR, FOUNDATION PLANT SERVICES
Foundation Plant Services (FPS) Mission

- Produce, test, maintain and distribute elite disease-tested plant propagation material.
- Provide plant importation and quarantine services, virus testing and virus elimination.
- Coordinate release of UC-patented varieties.
- Link researchers, nurseries, and producers.
The Grapevine Registration & Certification Program is a voluntary program that provides for the testing of source vines for significant grape pathogens. Registered sources and certified nursery stock are then inspected by CDFA staff and maintained by the participant in a manner to protect them from exposure to regulated diseases.
California Registration and Certification Programs

Grapes  Fruit and Nut Trees  Strawberries
The Dangers of Samsonite Importation

Pierce’s Disease

Plum pox virus on stone fruit
National Grapevine Importation Program: Foreign Imports

- Housed at FPS
- Largest nationally-recognized program for importing grape selections into the US
- Serves as both an importation and quarantine facility
APHIS – Animal Plant Health Inspection Service

Plant Health (PPQ)

- APHIS’ Plant Protection and Quarantine (PPQ) program safeguards U.S. agriculture and natural resources against the entry, establishment, and spread of economically and environmentally significant pests, and facilitates the safe trade of agricultural products.
National Clean Plant Network (NCPN)

- A national network of clean plant centers, scientists, educators, state and federal regulators, and growers and nurseries.

- Focused on providing healthy planting stock of vegetatively propagated specialty crops to nurseries and growers.
Participating Crops

1. Fruit Trees
2. Grapes
3. Berries
4. Hops
5. Citrus
6. Sweet potatoes
7. Roses

www.nationalcleanplantnetwork.org
The NCPN Clean Plant Centers
START CLEAN

FPS

Registered Increase Blocks

Nurseries

Production Vineyards

Growers

STAY CLEAN
20 – 30 million grapevine plants sold per year trace back to FPS
New Grape Selection
- Foreign imports
- Domestic selections
- New cultivars

Disease Testing
- Tests positive
  - Retesting
- Tests negative
  - all tests negative

Disease Elimination Therapy
- Tissue culture
- Heat treatment

FOUNDATION

Provisional Foundation Vines

Professional Identification
- ID verified correct
- ID not correct
  - REMOVE

Registered Foundation Vines

To Nurseries
- Registered Stock

To Growers
- Certified Stock

Release time
2-6 years
Tests required by APHIS and CDFA for certification

Standard detection methods for a range of suspected ‘known’ viruses using a panel of specific tests: ELISA, RT-PCR and RT-qPCR

ELISA

RT-PCR

RT-qPCR

Biological Indexing: A broader techniques

Herbaceous Index

Woody Index
Can HTS replace the field indexing requirement??

We need to scientifically demonstrate the advantages of HTS over Biological Indexing

Run a side-by-side comparison
Comparison of Next-Generation Sequencing Versus Biological Indexing for the Optimal Detection of Viral Pathogens in Grapevine

Maher Al Rwahnih, Steve Daubert, Deborah Golino, Christina Islas, and Adib Rowhani

Department of Plant Pathology, University of California, Davis 95616. Accepted for publication 2 February 2015.

ABSTRACT

Al Rwahnih, M., Daubert, S. Golino, D., Islas, C., and Rowhani, A. 2015. Comparison of next-generation sequencing versus biological indexing for the optimal detection of viral pathogens in grapevine. Phytopathology 105:758-763. vines being tested. We compared the bioassay against next-generation sequencing (NGS) analysis of grapevine material. NGS is a laboratory procedure that catalogs the genomic sequences of the viruses and other pathogens extracted as DNA and RNA from infected vines. NGS analysis was found to be superior to the standard bioassay in detection
Estimated cost for conventional virus testing

<table>
<thead>
<tr>
<th>Assay</th>
<th>cost/selection</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR assays (37 pathogens)</td>
<td>$1,200</td>
</tr>
<tr>
<td>ELISA (4 pathogens)</td>
<td>$250</td>
</tr>
<tr>
<td>Herbaceous host indexing</td>
<td>$100</td>
</tr>
<tr>
<td>Woody host indexing</td>
<td>$350</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>$1,900</strong></td>
</tr>
</tbody>
</table>

Minimum release time: 2 – 3 years
With HTS testing option

- Cost $300 per selection
- Total testing time is 1 – 2 months

Main advantage:
- Detects ALL known and unknown viruses (all strains)
Summary

HTS analysis has advantages over the standard bioassay in:

- Detection of viruses of agronomic significance (including low titer viruses)
- Comprehensiveness
- Speed of analysis
- Discovery of novel, uncharacterized viruses
• **FPS has a new import permit** that allows the use of HTS analysis

• When HTS use is accepted for grapevine certification and registration in place of the current industry standard, growers will be able to start propagative increase and virus elimination programs with most new accessions **years earlier** than they can now.
The improved testing protocol

Provisional release time
- 2-4 months
- New Grape Selection
  - Foreign imports
  - Domestic selections
  - New cultivars
- Provisional Foundation Vines
- Professional Identification
  - ID verified correct
- Registered Foundation Vines
- To Nurseries
  - Registered Stock
- To Growers
  - Certified Stock

No virus-like agents detected with HTS

Final release time
- 2 years
- Disease Elimination
  - Therapy
    - Tissue culture
    - Heat treatment
- All tests negative
- Disease Testing
  - Retesting
    - Tests positive
- Foundation

*No virus-like agents detected with biological indexing*
What is next??

- Run side-by-side studies to accumulate further comparative data, until the replacement of the bioassay is accomplished.

- Coordinate with other countries, which are pursuing a similar protocol revision.
However, there are limitations...

1. Technical challenges

2. Establishment of biological significance
HTS technical challenges

- Sample preparation
- Libraries
- Sequencing
- Assembly
- Sequence Databases
- Annotation
- Read Mapping

UCDAVIS
UNIVERSITY OF CALIFORNIA
The use of HTS as a routine diagnostic tool

- Developing efficient sample preparation methods for large scale application.
- Developing bioinformatics algorithms to efficiently separate pathogen and host sequences

Validation.....Validation.... Validation!!!!!
Establishment of Biological Significance
Novel grapevine viruses discovered by HTS

- 2009 Al Rwahnih et al.: Description of Grapevine Syrah virus 1 (California, USA)
- 2011 Giampetruzzi et al.: Description of Grapevine Pinot gris-associated virus (Italy)
- 2011 Zhang et al.: Description of Grapevine vein clearing virus (Midwest USA), the first DNA virus found in Vitis.)
- 2012 Al Rwahnih et al.: Description of Grapevine virus F (California, USA)
- 2013 Al Rwahnih et al.: Identification of plant virus satellite. (California, USA)
- 2014 Maliogka and Katis: A putative badnavirus identified in vines affected by Roditis leaf discoloration (Greece)
- 2015 Al Rwahnih et al.: A putative reovirus identified in Cabernet Sauvignon vines (California, USA, Brazil) (Same as Summer Grape latent virus - Mississippi USA) Sabanadzovic et al., 2012
- 2016 Al Rwahnih et al.: A putative Fabavirus identified in Nagano Purple vines (South Korea, India)
- 2016 Al Rwahnih et al.: A novel monopartite geminivirus and its defective DNA molecule characterized from grapevine (Vitis vinifera L.). Grapevine geminivirus A (GGVA)
- 2016 Silva, et al.: Molecular characterization of grapevine enamolike virus, a novel putative member of the genus enamovirus (Brazil)
And many more novel grapevine viruses are currently in the pipeline....
Bioinformatic analysis cannot prove pathological causality

- Detection of a given pathogen sequence does not mean that pathogen is responsible for the disease.

- Koch’s postulates cannot be satisfied using only HTS-based data.
Establish the significance of HTS findings

- HTS discovery of novel viruses is based solely on genomics information!!!
- But **NO decision** can be made on the importance of a novel virus without information about its biological effects...
Biological effects are assessed by:

- Performing graft transmission
- Fulfillment of Koch’s postulates
- Spread and distribution studies
- Assessment of (symptoms) agronomic significance
A novel virus in a domestic selection: Grapevine red blotch virus (GRBV)

Fig. 1. Symptoms of grapevine red blotch disease on leaves of A, ‘Cabernet Franc’ clone 214 and B, ‘Cabernet Sauvignon’ clone 7 in fall. C, Red secondary and tertiary veins of a leaf from affected Cabernet Franc grapevine. D, Basal leaves on the shoots of a mature Cabernet Franc clone 214 grapevine showing red blotch symptoms in fall.

Association of a DNA Virus with Grapevines Affected by Red Blotch Disease in California

Maher Al Rwaehnih, Ashita Dave, Michael M. Anderson, Adib Rowhani, Jerry K. Uyemoto, and Mysore R. Sudarshana
GRBV: Chip bud inoculations

<table>
<thead>
<tr>
<th>Source vines</th>
<th>Tested</th>
<th>PCR +ve Petioles</th>
<th>PCR +ve Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Grapevines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cabernet franc</td>
<td>5</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Cab Sauvignon</td>
<td>5</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Zinfandel</td>
<td>5</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>b) Controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ungrafted</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Detection of *Grapevine red blotch virus* in graft-inoculated plants of Cabernet franc test plants inoculated with chip buds from source vines.
Fulfillment of Koch’s postulates

Red blotch

Grapevine red blotch virus

Plasmid vector

Healthy grape

Agrobacterium tumefaciens

Courtesy of Dr. Marc Fuchs
Grapevine red blotch-associated virus Is Widespread in the United States

B. Krenz, J. R. Thompson, H. L. McLane, M. Fuchs, and K. L. Perry

Grapevine red blotch-associated virus is widespread in California and U.S. vineyards.
M. R. SUDARSHANA (1), A. Gonzalez (1), A. Dave (1), A. Wei (2), R. Smith (3), M. M. Anderson (3), A. M. Walker (3)

Detection and genetic diversity of Grapevine red blotch-associated virus isolates in table grape accessions in the National Clonal Germplasm Repository in California
Maher Al Rwahnih, Adib Rowhani, Deborah A. Golino, Christina M. Islas, John E. Preece & Mysore R. Sudarshana

Grapevine Red Blotch-Associated Virus, an Emerging Threat to the Grapevine Industry
Mysore R. Sudarshana, Keith L. Perry, and Marc F. Fuchs
Regulation Updates

California, Oregon, Washington, and New York have added GRBV to the list of regulated viruses
Novel viruses in a foreign import:

Grapevine fabavirus (GFabV), a novel putative member of the genus Fabavirus

Grapevine geminivirus A (GGVA), a novel gemini-like virus
New introduction (quarantine material)

Two accessions of Japanese table grapes, introduced to the FPS from South Korea in 2013

A: chlorotic ringspots on NP
B: asymptomatic BB
C: chlorotic ringspots on Cab franc indicator graft inoculated with NP
D: leaf roll symptoms on Cab franc indicator graft inoculated with BB
HTS Results

Nagano Purple

- Novel Gemini-like virus: Grapevine geminivirus A (GGVA) and its defective DNA molecule (GGVA D-DNA)
- Novel Fabavirus (GFabV)
- GLRaV-3, GFkV, GSyV-1, and viroids
- GLRaV-2

Black Beet

- GGVA only with no defective DNA
- GFabV
- GLRaV-3, GFkV, GSyV-1, and viroids
- GVE, GRSPaV, GRVFV
Genome of Grapevine fabavirus (GFabV)

- GFabV shared 23-34% sequence identities with polyproteins RNA-1 and RNA-2 of Broad bean wilt virus 1
- GFabV only shared 40% with PrVF from Cherry
Fig. 3. Genome maps of the genomic A, and defective B, circular ssDNA molecules of Grapevine geminivirus A (GGVA). Arrows denote the virion-sense (V) and complementary-sense (C) geminiviral genes encoded by each molecule. IR, intergenic region sequences; V1, coat protein; V2, pre-coat protein; C1, replication-associated protein; C2, transcriptional activator protein; C3, replication enhancer protein; C4, host activator protein. C. The stem-loop in the IR of GGVA and GGVA D-DNA is depicted with the conserved geminiviral nonanucleotide sequence “TAATATTAC” shown in gray arc box (nucleotide position 2899 to 2). Number 1 (gray arrow) indicates position 1 in the viral genome corresponding to the predicted replication origin of the viral DNA.
### New Viruses, Next Steps…

- Communicate with Plant Protection and Quarantine (PPQ)
- Conduct a survey using new real time PCR assays
- Total of 1,262 vines tested

<table>
<thead>
<tr>
<th>Source</th>
<th>Number of plants tested</th>
<th>GFabV positive</th>
<th>Variety/Country of origin</th>
<th>GGVA positive</th>
<th>Variety/Country of origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCD Grapevine Virus Collection</td>
<td>585</td>
<td>0</td>
<td>N/A</td>
<td>3</td>
<td>Tamar/Israel</td>
</tr>
<tr>
<td>USDA NCGR</td>
<td>230</td>
<td>1</td>
<td>Bhokri/India</td>
<td>15</td>
<td>Koshu Sanjaku/Japan</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Muscat Angel/Japan</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Super Hamburg/Japan</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pione/Japan</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>J-167-045/China</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Kyoho/Japan</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Neo-muscat/Japan</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Longyan/China</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Scol. Kiralynoje/Hungary</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pearl of Zola/US</td>
</tr>
<tr>
<td>FPS New Introductions</td>
<td>250</td>
<td>0</td>
<td>N/A</td>
<td>4</td>
<td>BB/S.Korea, NP/S.Korea,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nehelescol/Japan, Shine</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Muscat, S. Korea</td>
</tr>
<tr>
<td>Commercial Vineyards</td>
<td>197</td>
<td>0</td>
<td>N/A</td>
<td>0</td>
<td>N/A</td>
</tr>
</tbody>
</table>
GGVA Relevance Study: NCGR Grapevine Germplasm Collection
Virus Elimination Therapy

Collect apical shoot tips

Excise meristem dome and 1-2 pairs of leaf primordia

Plants develop a shoot first, then root in 5 to 12 months
4 years after tissue culture treatment:

Cv. ‘Nagano Purple 0.1’
Tests results:
PCR: negative for all viruses
Bioassay: negative in all indictors

Cv. ‘Black Beet 0.1’
Test results:
PCR: positive only for GFabV
Bioassay: positive in Cv. ‘St George’
Indicator plants Cv. ‘St George’ grafted with Black Beet 0.1
### PCR results

<table>
<thead>
<tr>
<th>Virus</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grapevine fleck virus (GFkV)</td>
<td>NEG</td>
</tr>
<tr>
<td>Grapevine geminivirus A (GGVA)</td>
<td>NEG</td>
</tr>
<tr>
<td>Grapevine leafroll-associated virus 3 (GLRaV-3)</td>
<td>NEG</td>
</tr>
<tr>
<td>Grapevine rupestris stem pitting-associated virus (GRSPaV)</td>
<td>NEG</td>
</tr>
<tr>
<td>Grapevine virus E (GVE)</td>
<td>NEG</td>
</tr>
<tr>
<td>Grapevine fabavirus (GFbV)</td>
<td>POS</td>
</tr>
</tbody>
</table>

- Repeat the indexing in 2017
- HTS on TCE plant and St George indicator
- Infectious clone-Koch’s postulates
Open questions:

- Fulfill of Koch’s postulates
- Spread or vector
- Distribution studies
- Assessment of (symptoms) agronomic significance
Mixed infection

The most pathogenic viruses in a mixed infection cannot be distinguished from the rest by NGS data.

Grapevine syrah virus-1 (GSyV-1)
Grapevine vein clearing virus (GVCV)
Grapevine Pinot gris virus (GPGV)

mixed infection with:

GRVFV, GRSPaV, GLRaV-9
AGVd, GYSVd HSVd

GFLV, GRSPaV, ToRSV

GRVFV, GRSPaV, GSyV-1
GYSVd1, HSVd
Discovery of insignificant background viruses

- Cryptic viruses
- Latent viruses
- Asymptomatic viruses

Our main goal is to single out the viruses with high economic impact (regulated viruses)

Science & Society

*Omnics: Fulfilling the Promise*

**Virus discovery: are we scientists or genome collectors?**

Marta Canuti and Lia van der Hoek

Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and Immunity (CINIMA), Academic Medical Center (AMC), University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands
What do we need?

We are in need of standard protocols/minimal requirement for HTS use.

Establish a framework for the evaluation of risks posed by new virus discovered by HTS.
Other Challenges!!!

- Naming the novel viruses!!
- Releasing the sequences!!
- Publishing!!
THANK YOU