FINAL PROJECT REPORT

Project Title: Evaluating a universal plant virus microarray for virus detection

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Total Project Request: **Year 1:** $35,165 **Year 2:** $34,584 **Year 3: N/A**

Percentage time per crop: Apple: 50% Pear: 10% Cherry: 18% Stone Fruit: 22%

Other funding sources

WSU is including this information on other funding available for the support of similar research undertaken by the faculty member proposing this research. These resources are listed to identify other support granted for this research and are not included as a commitment of cost-share by the institution.

Agency Name: **National Clean Plant Network (NCPN)**
Amt. requested/awarded: $49,902 (Sept 2011 to Sept 2012)
Notes: Support was provided for a Master's student working on apple green crinkle disease and a Ph.D. student investigating the etiology of cherry viruses. This is part of a larger comprehensive grant from the NCPN to the WSU Clean Plant Center - Northwest.

Agency Name: **WTFRC Cherry Research**
Amt. requested/awarded: $44,522 (2011); $ 46,303 (to February 2012)
Notes: Whereas the major focus of WTFRC Project Number CH-10-108 is the management of *Cherry leaf roll virus* and related viruses in the orchard, a small portion of the funds (ca. 10%) are directed to characterization of the complete genomes of members of the virus family *Betaflexiviridae* that infect cherry.

WTFRC Collaborative expenses: None

Total Project Funding: $69,749 from WTFRC

Budget History:

Item	Year 1:	Year 2:	Year 3: N/A
Salaries	$13,464[1]	$14,003[1]	
Benefits	$5,655[1]	$5,881[1]	
Wages			
Benefits			
Equipment			
Supplies	$13,250[2]	$14,700	
Travel	$2,796[3]		
Plot Fees			
Miscellaneous			
Total	**$35,165**	**$34,584**	

Footnotes:

1. Salary and benefits are requested for 0.33 FTE Postdoctoral research position to perform the molecular analysis.
2. Laboratory supplies including the printing of micro-array slides, sample RNA extraction and purification, and next generation sequencing and data analysis.
3. Travel for one co-PI to participate in a 3-day workshop in Beltsville, MD on the application and interpretation of the microarray chip data for the diagnosis of plant viruses.

OBJECTIVES

This project evaluated the effectiveness of contemporary technologies for the detection of viruses found in fruit trees. The most appropriate technology is being pursued for the detection and rapid identification of viruses associated with diseases of fruit trees, and for delivery of virus-tested fruit tree cultivars to the industry in an efficient and safe manner.

SIGNIFICANT FINDINGS

- Both the Universal Plant Virus Microarray (UPVM) and deep sequencing technologies require careful interpretation of raw data, particularly if the presence of previously uncharacterized pathogens is suggested.
- Frequent occurrence of multiple viruses in a single fruit tree was documented.
    - Deep sequencing effectively resolved complex mixtures of viruses in tissue samples, including multiple strains of the same virus in a single sample.
    - Accurate interpretation of UPVM data from samples with multiple infections was limited.
- Deep sequencing identified virus sequences in samples without any prior knowledge of viruses that may be present, and can reveal previously uncharacterized viruses.
    - New hosts of two known viruses were identified.
    - Five potentially new virus species were identified including a DNA-containing virus.
        - Requires further investigation for confirmation of virus identification and association with disease.
        - Previously unreported viruses found in fruit trees from the U.S.A., Spain, Israel, New Zealand and Brazil.

RESULTS & DISCUSSION

This investigation compares emerging technologies with existing methods for the detection and identification of viruses. Proper virus identification is crucial for proper disease management in growers' blocks. Although there are few alternatives available once an otherwise productive tree has become infected with virus, correct identification of the pathogen will allow growers to make economically sound decisions about tree removal and replanting, and about measures that can be taken to minimize further spread of the virus to adjacent plantings.

At the commencement of this project, the use of microarray technology appeared to offer an efficient path forward for rapid plant virus diagnosis. The Universal Plant Virus Microarray (UPVM) had been developed by USDA for the floriculture industry and its application to perennial crops was investigated. Preliminary trials in 2010 quickly revealed that this technology and the associated computer software were unable to reliably and correctly identify viruses present when more than one virus occurred in a sample; it is common for fruit trees to be infected with several different virus species. In the summer of 2012, a new software package was developed by the laboratories advancing the UPVM. To evaluate the potential of this new software to overcome the above limitation, a scientist from the Dr. Claude Fauquet group at the Danforth Plant Science Center worked in our facility for two weeks preparing additional samples for analysis by the UPVM. RNA was extracted from sixty fruit trees derived from 22 cherry, 20 apple, six plum, five peach, four pear, two apricot and one quince trees. Included in these samples are trees affected by diseases with unknown etiology. Among the diseases included are apple rubbery wood, apple green crinkle, Stayman blotch, green Newton, apple rough skin and Bisbee internal bark necrosis. All of these diseases are graft-transmissible suggesting a virus may be the causal agent. The UPVM of the 60 samples and related positive control samples are still being analyzed at the Danforth Center using the UPVM and the updated version of the T-predict software. Results will be compared to reverse transcription polymerase chain reaction (RT-PCR) analysis for the presence of specific viruses.

The initial difficulty experienced with the UPVM in resolving complex virus populations led the project to re-evaluate its potential for addressing the desired objective of a reliable method to identify viruses present in fruit trees. Concomitantly, during the first few months of this project, deep sequencing became much more accessible and the cost of that technology declined significantly. The project therefore investigated deep sequencing as a viable alternative to microarray methods.

Deep sequencing is a procedure that allows researchers to look at the entire genetic composition of a plant sample, including any viruses or microorganisms that might be associated with the tissue sample. Since deep sequencing looks at all genetic information in the sample simultaneously and indiscriminately, prior knowledge of the presence of specific disease agents is not required. This is the underlying power of the technology. The ability of deep sequencing to correctly detect pathogens in fruit tree tissue was evaluated by comparing results with those obtained by virus-specific RT-PCR.

The results obtained from 68 deep sequencing reactions illustrate the powerful potential of this method. In general, results from deep sequencing were in agreement with the results obtained by conventional RT-PCR. Only two samples yielded RT-PCR results that suggested that a virus was present that was not detected by deep sequencing. Apple sample 237.15 was tested twice. In both cases, RT-PCR suggested that *Apple stem grooving virus* was present whereas no *Apple stem grooving virus* sequences were detected by deep sequencing. Similarly, cherry sample 8863 yielded a band in the RT-PCR that suggested it was infected with *Prune dwarf virus* whereas no *Prune dwarf virus* sequences were detected by deep sequencing. In both of these cases, the RT-PCR amplification products will need to be sequenced to verify that the product is derived from the indicated virus and not gratuitous amplification of host or contaminating sequences.

In direct contrast to this observation, deep sequencing revealed many more viruses than were detected by current standard RT-PCR protocols. Deep sequencing identified viruses in 16 samples that were not detected by RT-PCR. Important observations from the deep sequencing project are summarized below:

1. Grower sample number 8863 was taken from an orchard that exhibited rapid decline in sweet cherry production over the past four years. The symptoms observed in the orchard resembled those typical of little cherry disease, but repeated attempts to detect *Little cherry virus 2* by RT-PCR in samples taken from that orchard yielded negative results. In contrast, deep sequencing revealed the presence of a virus that appears to be a unique variant of *Little cherry virus 2*. Since deviation of this sequence from published *Little cherry virus 2* sequences is significant, further research is being conducted to determine if it is a variant of *Little cherry virus 2* or a new virus species related to *Little cherry virus 2*.
2. *American plum line pattern virus* sequences were detected in five pome fruits originating from the U.S.A., Israel and New Zealand. *American plum line pattern virus* has a wide host range, but has not been reported in *Malus* or *Pyrus* species. The contiguous sequences indicative of this virus were fairly short, so additional testing is necessary to confirm the association of this virus with these new hosts.
3. *Cherry virus A* was recently discovered in *Prunus* species and is causing some concern in those fruit tree species where it is suspected of increasing the severity of disease caused by other viruses. In this project, *Cherry virus A* sequences were detected in six apple trees from the U.S.A., Israel and New Zealand. In parallel to the observation of *American plum line pattern virus* sequences in pome fruits, further analyses are required to confirm the association between the deep sequencing results and the presence of the virus in host tissue.
4. Nine samples were tested by RT-PCR using the TriFoCap primer set (Foissac *et al.*, 2005). This test is a broad spectrum test that will detect the presence of viruses that are members of the genera *Trichovirus, Foveavirus* or *Capillovirus*. In all nine samples where the TriFoCap assay was

positive, at least one virus expected to react with this assay was detected. This provided support for the use of the broad spectrum TriFoCap assay. However, the TriFoCap assay will not identify the specific virus species present; that additional information would need to be obtained by further RT-PCR analyses or by sequencing.

5. Apple green crinkle disease can be a serious disease that can render a crop unmarketable. The disease is difficult to diagnose since the etiological agent is unknown and the disease only appears under certain environmental conditions. Adding to the uncertainty in diagnosing apple green crinkle disease is that feeding on young apple shoots by rosy apple aphids (*Dysaphis plantaginea*) can lead to fruit deformation that resembles apple green crinkle disease. The saliva injected into the plant by the aphid is translocated to the fruit where the fruit symptoms develop. Deep sequencing was being explored as a tool to confirm the association of a specific virus to the disease. In a single tree sample number 119.65 that exhibited apple green crinkle-like symptoms, the suspected plant virus could not be detected by RT-PCR or by deep sequencing. However, in leaf samples from this tree, deep sequencing did reveal the presence of *Rosy apple aphid virus* (genus: *Densovirus)*, a virus that replicates in aphids and is present in the salivary material secreted by aphids. Although this virus is not thought to replicate in plants, its presence in this tissue sample confirms that rosy apple aphids had infested the tree and that the symptoms were likely the result of that feeding rather than by apple green crinkle disease. Thus, deep sequencing resolved an incongruity obtained from RT-PCR analysis.

6. A virus sequence that is similar to *Citrus leaf blotch virus* (genus: *Citrivirus*) was detected in two plum samples from Israel. This virus is known to induce stem pitting in citrus hosts, and no insect vector is known. The complete genome of this virus was obtained by both deep sequencing and RT-PCR so there is no doubt that the sequences are viral in nature. However, neither the incidents of this virus in stone fruits nor the impact of the virus on fruit quality and yield are known.

7. Partial genomic sequences of four additional new viruses were revealed by deep sequencing. Although the complete genomes were not obtained in this project, the segments of virus-specific sequence were quite large, and thus provide convincing evidence that novel viruses are present. The virus sequences are related to four different virus genera, and members of each genus are known to be insect transmitted. Additional sequencing will be needed to confirm that all of these virus-like sequences are associated with viruses and to confirm the identity of the viruses.

These results provide a measure of the power of deep sequencing to reveal the presence of virus sequences with no prior knowledge of the pathogens present at the initiation of the test. Because several of these viruses were previously undescribed, no virus-specific assay system existed to detect them. Even within a virus species, considerable sequence variation can occur. If sequence differences occur at specific nucleotides, they could render the RT-PCR assay unreliable. This is exemplified by the detection of a virus sequence related to *Little cherry virus 2* in a symptomatic tree. The detailed analysis provided by deep sequencing suggests that a distinct variant of *Little cherry virus 2* could be responsible for the decline of the orchard. This relationship had remained hidden for three seasons because of the inability of the available RT-PCR assays to detect the virus.

The presence of a virus sequence in a particular plant does not necessarily mean that the virus is pathogenic. Although most viruses rob vital metabolites from the tree and thus reduce growth to some degree, information about more serious effects cannot be predicted. Obtaining virus sequences via deep sequencing is just the first step from which we can determine the biology of the associated viruses including its mode of transmission and its impact on production. Fortunately, the results of deep sequencing provide the sequence information that is necessary to build other testing formats that allow such investigations to proceed. The utility of this technology is clearly demonstrated by this preliminary assessment.

EXECUTIVE SUMMARY

Project Title: Evaluating a Universal Plant Virus Microarray for virus detection

The object of this project was to investigate the utility of contemporary laboratory tools for the reliable detection of plant viruses. The two technologies investigated were the use of microarrays and deep sequencing. Although both strategies have the potential to detect pathogens without prior detailed knowledge of the viruses present, deep sequencing emerged as the preferable method. The use of a previously developed microarray would detect and identify previously uncharacterized viruses to the genus level; however, the technique was unable to unravel the individual viruses that could exist as complexes in fruit trees. Moreover, characterization of the virus(es) required reliance on additional sequencing reactions. For these reasons, deep sequencing provided much more precise information about the virus(es) present in a given sample. In this assessment of the application of deep sequencing to 68 samples, several new viruses and virus variants were identified. The deep sequencing results provided an important foundation for further investigation of the viruses detected. The sequence data can be used directly for development of virus-specific assays that could be used in studies of virus host range and vectors. The economic impact of each virus must be determined. This is a combination of the virus impact on fruit quality and quantity, and the ability to move quickly to adjacent fruit trees. These important questions of epidemiology are beyond the immediate goal of virus detection.