FINAL PROJECT REPORT

Project Title: Finding the Achilles' heel of a new virus infecting stone fruits

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Cooperators: N/A

Other funding sources

Agency Name: USDA-APHIS Center for Plant Health Science and Technology
Amt. requested: $50,013 was received in FFY 2014 to determine the incidence of the new luteovirus-like virus in the foundation program of the CPCNW.
Notes: WSU is including this information on other funding available for the support of similar research undertaken by the faculty member (K. Eastwell). 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

Total Project Funding: $83,178

Budget History:

Item	2015	2016	2017
Salaries	$8,143	$8,469	$8,808
Benefits	$3,339	$3,472	$3,611
Wages	$0	$0	$0
Benefits	$0	$0	$0
Equipment	$0	$0	$0
Supplies	$15,935	$15,689	$15,712
Travel	$0	$0	$0
Plot Fees	$0	$0	$0
Miscellaneous	$0	$0	$0
Total	$27,417	$27,630	$28,131

Objectives

This project had four major objectives, looking at different aspects of the viruses' biology in an attempt to determine whether these two viruses were a significant threat to the cherry industry in the PNW, and if so identify areas where control measures could be applied.

Obj. 1. *Determine if aphids are vectors of the newly discovered virus, and which aphid species in particular can transmit the virus to adjacent trees.*

Obj. 2. *Identify the relevant members of the host range that may be a reservoir of the virus in the fruit producing region of the cherry industry.*

Obj. 3. *Observe the development of symptoms on cherry cultivars that are critical to the cherry industry*

Obj. 4. *Develop a robust assay system for the detection of this virus*.

Significant Findings

Obj. 1. This objective was completed.

Obj. 2. This objective was completed

Obj. 3. This objective was completed.

Obj. 4. This objective was 75% completed.

- Both viruses have relatively limited distribution in Washington orchards: *Nectarine stem pitting-associated virus* was detected in 1.8% of the trees tested (8 out of 450), whereas *Prunus virus F* was detected in 11.7% (51 out of 450).

- Both viruses show low titer and scattered distribution in sweet cherry trees, suggesting that they are poorly adapted this species. Similarly, neither virus was observed to produce disease symptoms on sweet cherry.

- Neither virus was found to infect bitter cherry (*Prunus emarginata*) chokecherry (*P. virginiana*) suggesting that these two species are not reservoirs for further infection.

- Neither virus was found to be transmitted by the green peach (*Myzus persicae*) or black cherry (*M. cerasi*) aphids in this study, in part due to low virus titer and distribution.

- Real-time PCR assays were developed for these two viruses, allowing sensitive and accurate detection.

RESULTS & DISCUSSION

This project focused on examining the biological impact and risk of spread of two newly reported viruses. The first, *Nectarine stem pitting-associated virus* (NSPaV), is luteovirus first observed in imported nectarine cultivars in California in 2013 (Bag et al. 2015), that was, as the name suggests, associated with stem pitting disease symptoms. The second virus, *Prunus virus F* (PrVF) was originally detected in a sweet cherry tree in a commercial orchard in Grant county, Washington State, that showed leaf spot symptoms associated with cherry necrotic rusty mottle disease (Villamor et al. 2017). Deep sequencing of this tree indicated the presence of *Cherry necrotic rusty mottle virus*, as well as *Apple chlorotic leaf spot virus*, *Cherry virus A*, and *Prune dwarf virus*. In addition to these known viruses, a novel fabavirus was identified, which was named, in the absence of an associated disease symptom, *Prunus virus F* (Villamor et al. 2017).

Given that the cherry industry is at present dealing with the little cherry disease complex, the emergence of additional pathogens is unwelcome, therefore this project was proposed to carry out a brief risk assessment for NSPaV and PrVF. Four objectives were studied, three examining the biology of these two viruses: host range, symptom expression, and vector transmission, and a final objective to develop diagnostic assays for future detection and diagnosis.

Host range

When these two viruses were found to be present in Washington state, samples were collected to determine their incidence in five sweet cherry producing counties (Table 1). NSPaV occurred only in eight out of 450 samples tested, and only in Grant county. PrVF, on the other hand, was detected 51 out of the 450 samples, from orchards in Yakima, Grant and Chelan counties.

Table 1. Occurrence of NSPaV and PrVF within cherry production regions of Washington State.

County	NSPaV positive samples	PrVF positive samples	Total samples
Benton	0	0	75
Douglas	0	0	25
Grant	8	21	63
Chelan	0	13	175
Yakima	0	17	112
Total	**8 (1.8%)**	**51 (11.3%)**	**450**

Given the incidence of both viruses in major cherry producing regions of the state, a two-fold approach was taken. Previous research had shown that NSPaV was graft transmissible to *P. avium* and to the indicator species *P. tormentosa* (Villamor et al. 2016), while PrVF was graft transmissible to *P. avium*, and the interspecific hybrid rootstock Krymsk-6 (*P. cerasus x (P. cerasus x P. maackii*). In addition, PrVF was detected on Gisela rootstocks with parentage consisting of combinations of either *P. cerasus, P. canescens*, or *P. avium*. Given these observations, a greenhouse trial was established to see whether either virus could readily infect Bing cherry on two common rootstocks, Mazzard and Gisela-6. These trees were subsequently inoculated with either NSPaV or PrVF as a

single infection, or with both NSPaV and PrVF in a double infection in April 2016. Eighteen months later the plants were sampled and sectioned for virus presence and symptom expression. Sequential sections of the taproot, the stem of the rootstock below the graft union, the stem of the scion above the graft union, and terminal shoot and leaf tissue were collected and screened by virus-specific real-time PCR for virus presence (Table 2). We found that NSPaV had a very patchy distribution in both scion host combinations, with no systemic (found throughout the plant) infection observed. PrVF distribution was more consistent, although it was rare to find a systemic infection in either host.

Table 2. Distribution of NSPaV and PrVF in Bing on Mazzard and Gisela-6 rootstocks as determined by real-time PCR.

	Bing on Mazzard				Bing on Gisela-6			
Virus	**Scion Shoot**	**Scion Stem**	**Rootstock Stem**	**Rootstock Taproot**	**Scion Shoot**	**Scion Stem**	**Rootstock Stem**	**Rootstock Taproot**
NSPaV	0/3	0/1	1/3	1/3	0/5	0/5	1/5	1/5
PrVF	1/3	1/3	1/3	1/3	2/5	4/5	3/5	2/5
NSPaV w/PrVF	0/3	0/3	0/3	1/3	1/5	1/5	0/5	0/5
PrVF w/NSPaV	1/3	1/3	1/3	0/3	4/5	1/5	1/5	2/5

Both viruses accumulated to a very low titer in either scion-rootstock combination (Figure 1). This, combined with the scattered distribution and generalized root-biased tropism suggests that that they are poorly adapted to commercial *Prunus* spp., and are unable to establish a full systemic infection.

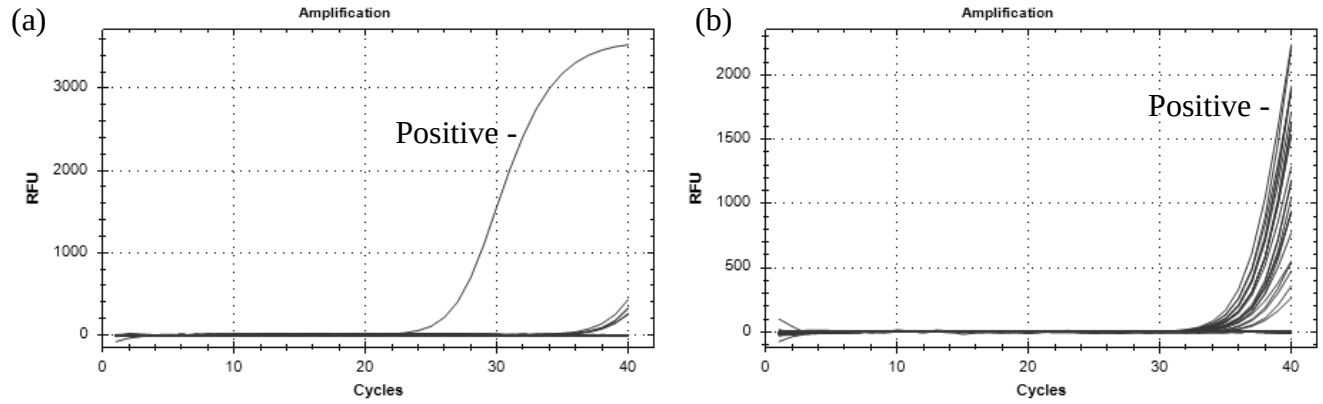

Figure 1. Titer of (a) NSPaV, and (b) PrVF in Bing scion material on either Mazzard or Gisela-6 rootstock as determined by real-time PCR. The positive control sample is marked.

In addition to examining the ability of these two viruses to infect commercial cherry, we also performed and experiment to see whether they could infect common, wild cherry species. *P. emarginata* (bitter cherry) and *P. virginiana* (choke cherry) were graft-inoculated with bark patches from NSPaV (in the 2015 and 2016 growing seasons) or PrVF (2016 only) infected sources, and tested for virus presence. No virus was found in either host during the 2015/2016 seasons by endpoint PCR, nor was either virus detected using the newly developed real-time PCR assays during the 2017

season. These data would suggest that both viruses have a limited host range within the *Prunus* genus, and that the likelihood of spread into, or from, wild cherries is remote.

Symptom expression

One of the major questions posed in this study was whether these two recently discovered viruses, NSPaV and PrVF, are potentially pathogenic to cherry. No foliar or trunk symptoms were observed on infected field plants that could definitely be associated with either of these two viruses; the trees did exhibit overall poor growth and slightly reduced fruit size, which may be attributed to infection by another virus or pathogen, or to poor orchard management. In order to confirm that neither virus affected commercially significant cherry cultivars, we established a greenhouse trial with Bing scions on Mazzard or Gisela-6 rootstock as described earlier. As can be seen in Table 3, no distinctive symptoms that could be attributed to infection by either pathogen were observed, nor were there any abnormalities to distinguish them from the uninoculated negative controls.

Table 3. Symptoms observed in Bing on two different rootstocks after infection with NSPaV and/or PrVF.

Host	Symptoms Observed	Inoculum		
		NSPaV	PrVF	NSPaV + PrVF
Bing on Mazzard	Foliar	0/3	0/3	0/3
	Stem Pitting	0/3	0/3	0/3
Bing on Gisela-6	Foliar	0/5	0/5	0/5
	Stem Pitting	0/5	0/5	0/5

These data would suggest that neither virus is a significant pathogen of cherry. However, we advise caution as this was not an exhaustive study of a) all potential scion and rootstock combinations, b) was conducted under greenhouse conditions, and c) did not examine potential interaction with other more prevalent viral pathogens of cherry, such as *Prune dwarf virus* or *Little cherry virus 2*. On the other hand, as discussed earlier, both viruses appear to be poorly adapted to cherry cultivars, and thus have difficulty establishing a systemic infection. Maladaptation is important as it reduces the likelihood of disease symptoms occurring in isolation; this may however change should interaction with a co-infecting virus occur.

Vector transmission

The third aspect of these two viruses examined was their vector transmissibility. Both belong to genera, *Luteovirus* and *Fabavirus* respectively, whose members have been shown to be aphid transmissible; as vector type is usually a common factor linking related viruses, aphid were used in this study. Insect transmission of both virus was attempted using black cherry (*Myzus cerasi*) or green peach (*M. persicae*) aphids. Neither species was able to transmit NSPaV to either sweet cherry (*P. avium*) or peach (*P. persica*) seedlings, despite using both 24hr and 72hr virus acquisition periods.

Similarly, both species failed to transmit PrVF to sweet cherry seedlings; peach plants were not used for PrVF transmission as so far it has only been detected in sweet cherry. For PrVF we used a short acquisition period as fabaviruses are transmitted a non-persistent manner in which long feeding times can reduce the efficiency of virus transmission. Screening of a small sample of green peach aphids after the acquisition period by PCR was negative, suggesting no virus was acquired. These data may indicate neither species of aphid is appropriate vector for NSPaV or PrVF, or more simply, that these two viruses are not vectored by aphids. However, the titer of these two viruses in cherry, as reported earlier, suggests another possibility. While endpoint PCR was performed prior to transmission to ensure that the plants were virus positive, subsequent real-time PCR results suggest that the titer of each was simply too low for the aphids to successfully acquire enough virus to transmit. From the Ct values we may estimate that the average tissue section has between 1-10 virus copies present, whereas most successfully aphid transmitted viruses maintain titers of 100-1000 fold higher. Based on this result we conclude that these two viruses are unlikely to be transmitted to neighboring trees or orchards at a significant frequency.

Detection

Finally, during the course of this study two additional genomes each for NSPaV and PrVF were generated via high-throughput sequencing. These sequences, combined with publically available genomes allowed us to build an alignment to examine genetic diversity within these two viruses, and identify regions of the genome suitable for assay design. Also taken into consideration was the extremely low titer of both of these viruses in cherry, the decision was taken to design real-time PCR assays, as this platform provides greater speed and sensitivity than endpoint PCR.

Taqman-based real-time assays were designed against the GP3 fusion protein (bases 3104-3194) of the NSPaV genome, and against the RNA1 polyprotein (bases 3140-3266) of PrVF; PrVF RNA2 showed considerable divergence between isolates and was not considered for assay design. Both real-time PCR assays were optimized for reaction time, annealing/extension temperature, and magnesium concentration against known positives held in the CPCNW collection. Unfortunately due to the paucity of extant NSPaV, and to a lesser extent, PrVF isolates, the two assays have not been completely validated; this will be completed prior to publication. The assays were found to be highly sensitive, detecting trace amounts of their respective target viruses in infected tissue during the host range and cherry virus symptom (Figure 1) experiments described earlier.

Summary

This study, on the biology and detection of two recently discovered viruses infecting stone fruits, *Nectarine stem pitting-associated virus*, and *Prunus virus F*, revealed that neither virus is widespread in the Washington cherry industry, and unlike the Western X phytoplasma for example, do not infect wild cherry relatives and thus appears to lack reservoir species. While both viruses could infect commercial cherry varieties, they appear to have significant difficulty doing so, exhibited through scattered distribution in the plant and low virus titer. These factors likely contributed to a lack of successful aphid transmission in this study, and suggest that its ability to spread may be limited. Similarly, neither virus was observed to produce visible symptoms on Bing on two different rootstocks, indicating that they do not, unlike *Little cherry virus 2*, present a significant threat to the Washington cherry industry, with the caveat that this was not an exhaustive study on all possible scion-rootstock combinations or stresses (environment, coinfection with other viruses, etc) that could induce disease. Finally, through this study we were able to produce a Taqman-based real-time PCR assay for each virus, allowing sensitive and accurate detection and continued monitoring.

Literature Cited

Bag, S., Al Rwahnih, M., Li, A., Gonzalez, A., Rowhani, A., Uyemoto, J. K., & Sudarshana, M. R. (2015). Detection of a new luteovirus in imported nectarine trees: a case study to propose adoption of metagenomics in post-entry quarantine. *Phytopathology*, *105*(6), 840-846.

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EXECUTIVE SUMMARY

This project focused on examining the biological impact and risk of spread of two newly reported viruses infecting stone fruit, *Nectarine stem pitting-associated virus* (NSPaV), and *Prunus virus F* (PrVF). Four objectives were proposed, three examining the biology of these two viruses: host range, symptom expression, and vector transmission, and a final objective to develop diagnostic assays for future detection and diagnosis.

We found that while both viruses are present in Washington state, neither is widespread in commercial cherry orchards, with incidences of 1.8 and 11% for NSPaV and PrVF respectively, and is limited to select counties. While both viruses can infect sweet cherry species, virus titer is low and both exhibit a scattered distribution in plants. Interestingly, neither virus was able to infect the wild cherry relatives *Prunus emarginata* (bitter cherry) and *P. virginiana* (choke cherry), suggesting that these are not reservoir species, reducing the risk of spread. Stunting and reduced fruit size in the field could not be conclusively associated with these two viruses and no symptoms were observed on Bing cherry on either Mazzard or Gisela-6 rootstock in greenhouse experiments, indicating that these not likely significant pathogens of cherry, though should continue to be monitored. Aphid transmissibility of these two viruses was not observed experimentally using two different species, the black cherry (*Myzus cerasi*) and green peach (*M. persicae*) aphids. This is likely due to low virus titer in the source plants, but may also indicate that another species is the vector. Finally, through this study we were able to produce a Taqman-based real-time PCR assay for each virus, allowing sensitive and accurate detection and continued monitoring.

In summary, based on this data neither virus appears to be an emerging threat to the cherry industry, with no identified reservoir species, and a limited ability to spread. Using the tools developed in this project, continued monitoring of the incidence of these two viruses is encouraged, as is scouting and visual inspection to confirm that no symptoms that can be associated with these viruses are emerging. There are two areas that deserve further investigation however, to see whether either of these viruses interacts with economically significant pathogens of cherry, such as *Little cherry virus 2* or *Prunus necrotic ringspot virus*, and to further examine their ability to cause disease of a wider range of scion and rootstock combinations, and under different environmental conditions.