Development and validation of real time quantitative PCR* assays *for the detection of fruit tree viruses

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Project Summary/Abstract

Briefly describe the long-term objectives for achieving the stated goals of the project.

This project will evaluate the broad-range detection capacity of currently available pome fruit and Prunus fruit tree virus, viroid and phytoplasma assays and design new quantitative PCR (qPCR) assays if current assays are inadequate. High throughput sequencing (HTS) will be used to screen select pome and Prunus tree populations for targeted pathogens. The California Department of Food and Agriculture (CDFA) is currently working to update the Pome Fruit Tree Registration and Certification regulations in order to create regulations that are harmonized with other state's pome industries. Current detection methods for viruses (and other pathogens) identified as targeted viruses by the pome fruit working group, in addition to primary Prunus viruses, will be investigated. Our objectives are to screen select pome fruit and Prunus tree populations for targeted viruses, viroids and phytoplasma to compile a representative set of genome sequences (as complete as possible) and evaluate current published primers, incorporate new genetic data into a more complete characterization of genetic variation across the targeted pathogens to inform assay design, construct improved assays utilizing multiple primers sets for detecting all existing targeted pathogen variants, empirically test and validate proposed assay designs using positive controls, and disseminate research progress and results. The overarching goal of this work is to design the most robust assays for virus, viroid and phytoplasma detection of pome fruit and Prunus fruit trees which will contribute to maintaining the highest quality nursery stock.

Project's Benefit to Nursery Industry

This project is ongoing, we are in year 2 of 3, what is next starting on July 1, 2019 to June 30, 2020 is as follow:

- Complete the initial validation of all assays.

Disease Agent	Assay Updated or Generated	Initial Validation
Cherry rasp leaf virus (CRLV)		
Tobacco ringspot virus (TRSV)		
Pear decline Phytoplasma	Not Modifications*	Completed
Pear blister canker viroid (PBCVd)		
Cherry green ring mottle virus (CGRMV)		
Cherry leafroll virus (CLRV)		

*Phytoplasma assay did not need modifications.

- Large-scale validation of detection assays (share with CPCNW at Processer).
- Screen more fruit tree samples, including samples from commercial orchards.

Objectives	2019-2020
Screen for targeted viruses	X
Virus characterization	X
Construct improved assays	X
Test assay designs	X
Disseminate results	X

New advances in qPCR have significantly improved the detection of pathogens, allowing quick, more sensitive and precise quantification compared to conventional PCR. This work will evaluate the broad-range detection capacity of currently available pome fruit and Prunus virus, viroid and phytoplasma assays and design new qPCR assays if current assays are inadequate. This work is especially timely as the development of these more robust pome fruit pathogen detection assays concurs with recent changes to the CDFA Pome Registration and Certification Program in anticipation of creating a program at Foundation Pfant Services (FPS) that harmonizes with other state's pome industries. In that sense, FPS is working closely with the Clean Plant Center Northwest (CPCNW; Washington State University) in Prosser, WA to standardize the testing process of new domestic and foreign fruit tree introductions.

HTS is a very useful new research tool for detecting viruses and other pathogens present in a variety of crops. In this project we will prescreen select pome and Primus tree populations for viruses, viroids and phytoplasma and analyze select trees using HTS to develop the most robust qPCR assays. The CDFA has recently adopted qPCR-based methods for detecting some grapevine viruses. We anticipate the continued shift to qPCR-based methods given their potentially higher sensitivity. Any assays we develop as a result of this project will be made available to CDFA and private commercial diagnostic labs and will augment the production of certified propagation material and the effective control of fruit tree pathogens in California orchards. The development of a robust, sensitive and reliable generic detection method with a broad-range detection capacity is very much desirable and needed for large scale virus, viroid and phytoplasma testing.

This method will tremendously help the clean stock programs and the fruit tree industry for early detection of pathogens in their material with lower cost and in a shorter time.

Objectives

Objective 1: Screen select pome and Prunus tree populations for targeted pathogens to compile a representative set of genome sequences (as complete as possible), and evaluate current published primers.

Objective 2: Incorporate new genetic data into a more complete characterization of genetic variation across the targeted pathogens to inform assay design.

Objective 3: Construct improved assays utilizing multiple primers sets for detecting all existing targeted pathogen variants.

Objective 4: Empirically test and validate proposed assay designs using positive controls.

Objective 5: Disseminate research progress and results.

Work Plans and Methods

Objective 1: *Screen select pome and Prunus tree populations for targeted pathogens to compile a representative set of genome sequences (as complete as possible), and evaluate current published primers.*

In the first year of this project (funding cycle 2018/2019), 60 pome or Prunus samples were collected and screened for the presence of targeted pathogens (Table 1) using HTS. These samples were originated from the National Clonal Germplasm Repository (NCGR) in Davis, CA and Corvallis, OR and the CPCNW. Consequently, 47 pome or Prunus samples tested positive for targeted pathogens and selected as controls to validate proposed assay designs. In the cases of tomato ringspot virus (ToRSV), Arabis mosaic virus (ArMV) and tobacco ringspot virus
(TRSV) were unable to obtain isolates infecting pome or Prunus trees, but isolates infecting grapevine were included as positive controls; such grapevines are part of the FPS pipeline of new introductions and they were previously analyzed by HTS.

During this fiscal year, we will collect additional 40 pome or Pruhus samples (for a total of 100 samples) and later analyze them by HTS. Of special interest is getting isolates of ToRSV, ArMV and TRSV infecting fruit trees. We will contact collaborators in Canada and France to import plant material carrying targeted pathogens including the previously mentioned viruses. The PI of this project owns an import permit to bring infected fruit tree material to the US. Subsequently, all the pome and Prunus samples will be subjected to HTS to determine infection status and identify the involved pathogens.

Table 1. Targeted pathogens of fruit trees.

Disease	Disease Agent	Host
Apple chlorotic leaf spot	Apple chlorotic leafspot virus (ACLSV)	Pome, Prunus
Apple mosaic	Apple mosaic virus (ApMV)	Pome, Prunus
Apple stem grooving	Apple stem grooving virus (ASGV)	Pome
Apple stem pitting, Pear stem pitting, Pear stony pit disease	Apple stem pitting virus (ASPV)	Pome
Flat apple disease	Cherry rasp leaf virus (CRLV)	Pome, Prunus
Tobacco ringspot	Tobacco ringspot virus (TRSV)	Pome
Apple union necrosis	Tomato ringspot virus (ToRSV)	Pome, Prunus
Pear decline	Pear decline Phytoplasma	Pome
Apple scar skin/Dapple apple disease	Apple scar skin viroid (ASSVd)	Pome
Pear blister canker	Pear blister canker viroid (PBCVd)	Pome
Cherry green ring mottle	Cherry green ring mottle virus (CGRMV)	Prunus
Cherry leafroll	Cherry leafroll virus (CLRV)	Prunus

Little cherry	Little cherry virus 1 (LChV-1); Little cherry virus 2 (LChV-2)	Prunus
Prune dwarf	Prune dwarf virus (PDV)	Prunus
Prunus necrotic ringspot	Prunus necrotic ringspot virus (PNRSV)	Prunus
Arabis mosaic	Arabis mosaic virus (ArMV)	Prunus

As conducted in the first year of this project, published assays of fruit trees will be first analyzed *in silica* to investigate their detection capacity; thus, to avoid duplicated and unnecessary work. Primers involved in those assays will be compared against de sequencing data available at the GenBank or at the FPS database using bioinformatics programs. In that sense, Katsiani et al (2018) just published a new assay for little cherry virus 1 (LChV-1), which will be investigated.

Objective 2: *Incorporate new genetic data into a more complete characterization of genetic variation across the targeted pathogens to inform assay design.*

All the new sequencing data (i.e. HTS), generated during this fiscal year, will be analyzed using the FPS' in-house bioinformatics pipeline; we have recently finished validating an implementation of a local UNIX server based high throughput viral meta-genomics pipeline. Subsequently, near-complete genomes of targeted pathogens will be deposited in GenBank; thus, incorporating new genetic 9ata into a more complete characterization of genetic variation across the targeted pathogens.

Objective 3: *Construct improved assays utilizing multiple primers sets for detecting all existing targeted pathogen variants.*

During the first year of this project (funding cycle 2018/2019), ten different detection assays for targeted pathogens were developed (new assays) or updated via a multistep process (Figure 1). Briefly, in order to generate reliable qPCR assays, sequence data available at GenBank or generated at FPS was aligned using a custom script developed in house. Conserved regions among variants and suitable for detection assays were considerably variable in such it become impossible to design a single pair of PCR primers or a single real-time probe (MGB probe) to detect the full range of virus, viroid or phytoplasma isolates/strains; consequently, multiple primers and/or probes were used in single reverse transcription (RT)-qPCR reactions (Table 2). In several occasions, the sequence alignment revealed primers/probes already included in previously published assays, but such assays were updated with extra primers or probes for highly divergent variants of the pathogen (Table 2).

Remaining qPCR assays for LChVl, TRSV, cherry leafroll virus (CLRV), cherry rasp leaf virus (CRLV), cherry green ring mottle virus {CGRMV), pear blister canker viroid (PBCVd) and pear decline Phytoplasma will be constructed upon program approval and funding.

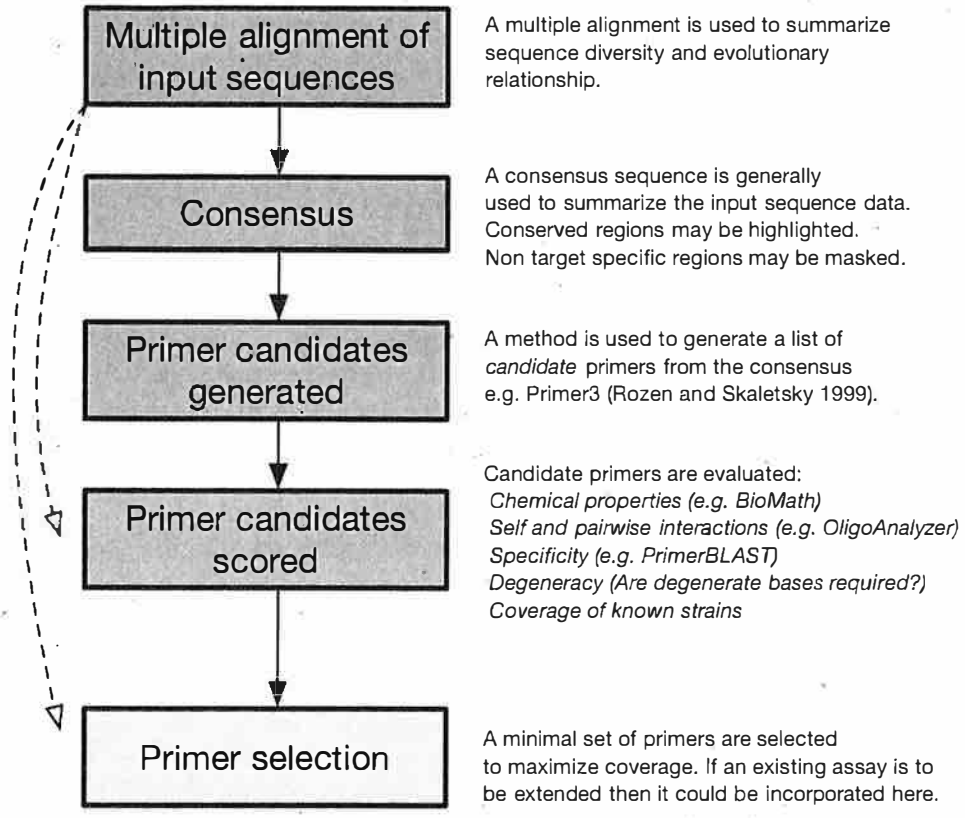

Figure 1. Generic schematic for PCR detection assay design used by programs and web servers (e.g. Gadberry 2005; Duitama et al. 2009). Extensions for HTS data primarily affect the multiple alignment which is used as a representation of sequence diversity at multiple points during the process.

Similar to analysis conducted on previously published assays (Objective 1), detection assays generated during this project will be analyzed *in silico* using bioinformatics tools to determine if the proposed assay can cover or not all the genetic diversity of targeted pathogens. Based on the previous analysis, assays may be updated with additional primers or probes.

Table 2. Designed or updated assays for detection of fruit tree pathogens.

Pathogen	Type of Primer	# of Primers	5' Fluorophore	Target Region	Reference
ToRSV	Forward	2		CP/Polyprotein	Osman et al. 2014 (Updated)
	Reverse	2			
	Probe	1	FAM		
ArMV	Forward	2		RNA2/P2/CP	This study
	Reverse	1			
	Probe	3	FAM		
ApMV	Forward	4		CP	Osman et al. 2014 (Updated)
	Reverse	2			
	Probe	2	FAM		
ACLSV	Forward	1		CP	Osman et al. 2017 (Updated)
	Reverse	3			
	Probe	1	FAM		
PDV	Forward	4		CP	Osman et al. 2014 (Updated)
	Reverse	2			
	Probe	2	FAM		
PNRSV	Forward	4		CP	This study
	Reverse	2			
	Probe	2	FAM		
ASSVd	Forward	5		Viroid genome	This study

	Reverse	4			
	Probe	2	FAM		
ASPV	Forward	5		CP	This study
	Reverse	5			
	Probe	2	FAM		
ASGV	Forward	2		MP/Polyprotein	This study
	Reverse	1			
	Probe	1	FAM		
LChV2	Forward	2		RDRP	This study
	Reverse	1			
	Probe	2	FAM		

Objective 4: *Empirically test and validate proposed assay designs using positive controls.*

All the assays (Objective 3) will be tested against a representative number of positive and negative samples to make sure that each system has specificity and a broad range of detection capacity as expected. These samples will be obtained from different pome and Prunus collections, including the NCGR in California and Oregon, and the CPCNW in Washington. We will work closely with scientists responsible of these collections to identify fruit trees with a previously known infection by targeted pathogens; infection determined by an alternative testing method (i.e. ELISA or conventional PCR). In the case of contradicting results (i.e. ELISA positive and qPCR negative), samples will be prioritized for HTS analysis. Consequently, HTS will not only reveal the real infection status of the sample, but also any fail in the proposed detection assay.

Objective S: *Disseminate research progress and results.*

We will communicate opportunities, progress, and results of this project to growers, stakeholders, and scientific peers. We will use growers' meetings organized by the UC Cooperative Extension, symposiums, and scientific meetings to accomplish our objective. Information will also be disseminated in printed form through news articles, research reports, and peer-reviewed scientific papers. In addition, for broader dissemination of information at the national level, results will be shared with the National Clean Plant Network and the CPCNW.