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

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This is an ongoing project from a prior year Agreement.

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 and *Prunus* fruit tree virus and viroid real-time reverse transcription quantitative PCR (RT-qPCR) assays and update or design new assays if current assays are inadequate or absent. 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 virus-like agents 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 and *Prunus* tree populations for targeted viruses and viroids to compile a representative set of isolates, evaluate current published assays (if available), 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/probes 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 detection in pome and *Prunus* fruit trees, which will contribute to maintaining the highest quality nursery stock.

Scope of Work

Describe the goals and specific objectives of the proposed project and summarize the expected outcomes. If applicable, describe the overall strategy, methodology, and analyses to be used. Include how the data will be collected, analyzed, and interpreted as well as any resource sharing plans as appropriate. Discuss potential problems, alternative strategies, and benchmarks for success anticipated to achieve the goals and objectives.

Project's Benefit to Nursery Industry

New advances in real-time 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 fruit tree virus and viroid real-time RT-qPCR assays and update or design new assays if current assays are inadequate or absent. 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 Plant Services (FPS) that harmonizes with other state's pome industries. In that sense, FPS is working closely with the Clean Plant Center Northwest (CPCNW) 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 virus-like agents present in a variety of crops. In this project we will screen select pome and *Prunus* tree populations for viruses and viroids using HTS to develop the most robust real-time RT-qPCR assays. The CDFA has recently adopted real-time qPCR-based methods for detecting some grapevine viruses. We anticipate the continued shift to real-time qPCR 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 viruses and other pathogens in California orchards. The development of a robust, sensitive and reliable detection method with a broad-range detection capacity is very much desirable and needed for large scale virus/viroid testing. This method will tremendously help the clean stock programs and the fruit tree industry for early detection of the viruses and other pathogens in their material with lower cost and in a shorter time.

Objectives

Objective 1: Evaluate currently available real-time RT-qPCR assays and screen select fruit tree populations for targeted pathogens to compile a representative set of isolates.

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: *Evaluate currently available real-time RT-qPCR assays and screen select fruit tree populations for targeted pathogens to compile a representative set of isolates.*

Plum pox virus (PPV) is a highly destructive pathogen of *Prunus* fruit trees and currently quarantined in the US. A recently published study (Hajizadeh et al., 2019) described the high divergent diversity of PPV, such diversity complicates its detectability. Fotiou et al. (2019) developed a "universal" assay for PPV, thus, we will further investigate and *in silico* validate the detection capacity of this assay.

This funding cycle (2020-2021), we will collect additional 40 pome or *Prunus* samples (for a total of ca. 250 samples in this project) with allegedly infection by the targeted pathogens. Subsequently, these samples will be HTS analyzed to verify the

infection status and identify the involved pathogen. Of special interest is getting material infected by nectarine virus M
(NVM), peach mosaic virus (PcMV), cherry rusty mottle associated virus (CRMaV), tomato ringspot virus (ToRSV), tobacco ringspot virus (TRSV) and apple rubbery wood viruses 1 & 2 (ARWV 1 & 2), due to the limited number of isolates identified so far. We are planning to obtain this infected material through the USDA-APHIS program located in Beltsville, MD. The PI of this project holds a USDA-APHIS permit to move infected fruit tree material in the US.

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 HTS data, generated during this funding cycle year, will be analyzed using the FPS' in-house bioinformatics pipeline (i.e. 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 data 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.*

New real-time RT-qPCR assays for apple green crinkle associated virus (AGCaV), citrus concave gum-associated virus (CCGaV), citrus virus A (CiVA), ARWV1 & 2 (specific assays) will be constructed using a multistep process (Figure 1). Thus, multiple primers and/or probes will be used to cover known genetic diversity of the pathogen

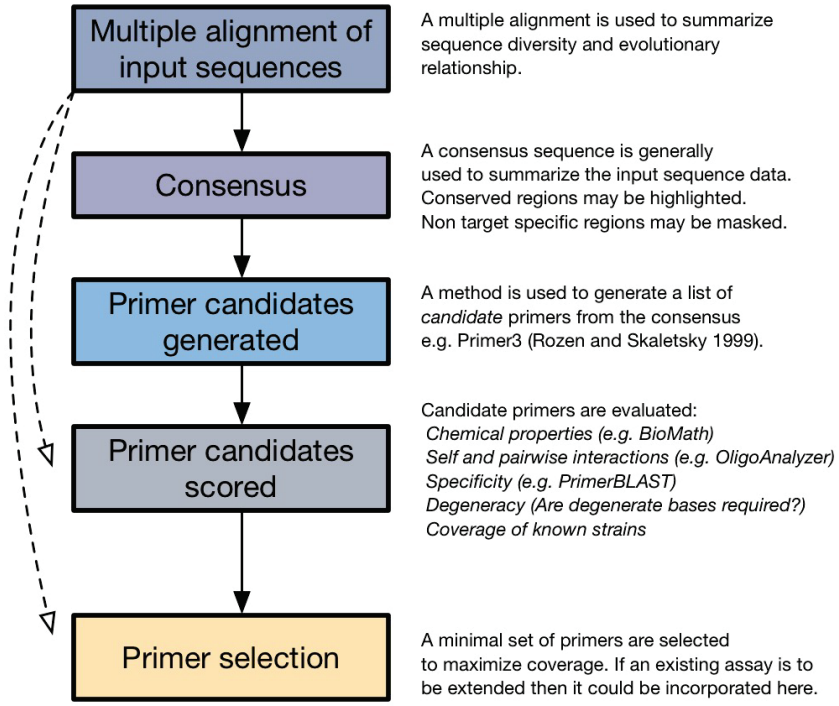

Figure 1. Generic schematic for PCR detection assay design used by programs and web servers (e.g. Gadberry 2005; Duitama et al. 2009).

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

Newly developed assays for AGCaV, CCGaV, CiVA and ARWV1 & 2 will be tested against a representative number of positive and negative samples (i.e. virus-free plants or plants infected by unrelated viruses) to make sure that each system has specificity and a broad range of detection capacity as expected. These samples were obtained from different pome and

Prunus populations, including FPS, CPCNW and USDA-NCGR in Winters, California. Additionally, we will work closely with scientists at the USDA-APHIS program located in Beltsville, MD to obtain more infected material.

Replicating the survey conducted at the USDA-NCGR *Prunus* germplasm collection to further validate the assays for *Prunus* pathogens (see previous reports), this year, we will conduct a field survey in different pome fruit tree-growing areas in California. Such survey will be conducted in collaboration with growers, farm advisors and other stakeholders; we will sample from multiple commercial orchards and nurseries. The goal is to challenge the newly designed assays for pome pathogens.

Objective 5: *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.