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 Jong-term objectives for achieving the stated goals of the project.

Executive Summary

This project will evaluate the broad-rarige detection capacity of currently available pome fruit and Prunus fruit tree virus 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 viruses. The California Department of Agriculture 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 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 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 *bf* genetic variation across the targeted viruses to inform assay design, construct improved assays utilizing multiple primers sets for detecting all existing targeted virus variant, 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 detection of pome fruit 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 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 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 virus 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.
High throughput sequencing (HTS) is a very useful new research tool for detecting viruses present in a variety of crops. In this project we will prescreen select pome and Prunus tree populations for viruses and analyze select trees using HTS to develop the most robust qPCR assays. The California Department of Food and Agriculture (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 viruses 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 testing. This method will tremendously help the clean stock programs and the fruit tree industry for early detection of the viruses in their material with lower cost and in a shorter time.

Objectives

Objective 1: Screen select pome fruit and Prunus tree populations for targeted viruses 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 viruses to inform assay design.

Work Plans and Methods

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

1.1 Obtain fruit tree virus isolates.
This objective will create a new resource comprised of targeted fruit tree virus (Table 1} isolates obtained from screening symptomatic fruit tree material.

We will obtain material, using Dr. Al Rwahnih's USDA-APHIS permit to move infected plant material, previously identified positive for these viruses from diverse resources. These resources include, but are not limited to: The National Clonal Germplasm Repository (NCGR}, Davis and Corvallis, Oregon, which contains pome and fruit trees collected from around the world; Clean Plant Center Northwest, Washington State University Prosser, Washington; and from the positive control collection at the Canadian Food Inspection Agency.

In addition, we will target other common/ newly described pome and fruit tree viral agents including: American plum line pattern virus (APLPV), Cherry necrotic rusty mottle virus (CNRMV), Cherry virus A {CVA}, Hop stunt viroid (HSVd}, Plum bark necrosis stem pitting associated virus {PBNSPaV), Peach latent mosaic viroid (PLMVd}, Peach Rosette Mosaic Virus
(PRMV}, Apple latent spherical virus (ALSV), Tobacco necrosis virus (TNV), Tomato bushy stunt virus (TBSV}, Apple proliferation phytoplasma, Apple fruit crinkle viroid (AFCVd}, Apple rubbery wood associated virus 1 and 2 (ARWaVl and 2), and apple-associated luteovirus (AaLV}.

Total nucleic acid (TNA) will be extracted from leaf petioles and/or cambial scraping of wood collected from the virus-positive or suspected virus-positive fruit tree sources and conventional molecular assays will be used to identify or confirm virus-infected material.

Table 1. Targeted viruses of fruit trees.

Disease	Disease Agent	Host
Apple chlorotic leaf spot	*Apple chlorotic leafspot virus* (ACLSV)	Pome, Prunus
Apple mosaic	*Apple mosaic virus* (ApMV, TApMV)	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

1.2 HTS sequencing and assembly methodology.
Total nucleic acid (TNA} extracts will be prepared from leaf petioles or bark tissue as described by Al Rwahnih et al. (2015}. Briefly, approximately 0.2 g of tissue was homogenized using a Homex grinder (Bioreba, South Bend, IN} and TNA extracts were prepared using a MagMAX™-96 viral RNA isolation kit (Ambion, Austin, TX) as per manufacturer's protocol. Aliquots of TNA samples from source trees were subjected to ribosomal RNA (rRNA) depletion and complementary DNA (cDNA) library construction using a TruSeq Stranded Total RNA with Ribo-Zero Plant kit (lllumina, San Diego, CA). Sequencing was performed on the lllumina NextSeq 500 platform.

1.3 Evaluate published primers.
We will evaluate already published PCR detection systems for their virus detection capacity (number of viruses and virus isolates that they can detect). These primers will be thoroughly evaluated for their specificity, efficiency, and the broad-range detection capacity of currently available fruit tree virus assays. If the detection system passes all the quality control measures it will be confirmed for routine use in virus detection. If it fails quality control measures, we will proceed with developing a new and improved assay.

This objective will begin in the first year.

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

Analyze the sequences and identify viruses in the samples:

The resulting sequence reads will be demultiplexed and adapter trimmed using the lllumina pipeline and bcl2fastq software. We have found.that additional filtering stringency at this step reduce crosstalk between samples with negligible reduction in yield. Reads from each of the samples will be used to generate de-nova meta-genome assemblies using FPS' in-house bioinformatics pipeline (We have recently finished validating an implementation of a local UNIX server based high throughput viral meta-genomics pipeline}. Meta-genome assembly of RNA sequence will be accomplished using a best in class assembler for this purpose SPADES with parameters supporting the separation of variant sequences in the case of multiple virus and multiple variant infections. Subsequent annotation of viral genome sequences will be done using BLASTN for highly related nucleotide virus sequences and TBLASTX for sequences so divergent as to be most easily related through their functionally conserved protein sequences (Altschul et al., 1997). Partial and full-length sequences of variants obtained will be utilized for assay development. Local and global multiple sequence alignments will be constructed to support assay development. Upon completion of the assay, all related sequence information will be deposited in Genbank.

This objective will begin in the first year.

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

3.1 Primer/probe design
Sequence data generated by HTS (in objective 2) will be combined with the available sequences in the GenBank for the conserved gene of all targeted viruses in each viral species will be aligned by using the pileup program (Figure 1). PCR primers and probes of approximately 20-30 nucleotides long will be designed from regions on the genome with 100% consensus among different sequences.

3.2 Degenerate primers/probes.
If regions with 100% consensus were not found on the pile up sequences, then based on the sequence variation among different viral species and strains, degenerate primers and probes will be designed in which the primers/ probes sequences match the full pile up sequences in this region.

3.3 Multiple primers/probes
If the sequences on the conserved genes are highly variable in such it become impossible to design a single pair of PCR primer or a single real-time probe to detect the full range of viral species and strains in the designated genus, then we will attempt to design multiple primers and probes to be use in single RT-qPCR reaction. This plan will require more thorough evaluation to make sure that the interaction between primers, probes and primers/probes are minimal and their effect on RT-qPCR amplification is negligible.

Figure 1.