Title of Report: Interim progress report for CDFA agreement number 17-0430-000-5A.

Title of Project: Survey and analysis of grapevine leafroll-associated virus-3 genetic variants and application towards improved RT-qPCR assay design.

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Introduction

Grapevine leafroll-associated virus-3 (GLRaV-3; genus *Ampelovirus*; family *Closteroviridae*) is the most important virus pathogen of grapevine and the main etiological agent of grapevine leafroll disease (Burger et al., 2017). The long-distance spread of GLRaV-3, caused by the movement of infected vines, can be controlled effectively if clean stock is made available to growers. The economic benefits from the provision of GLRaV-3 certified virus-free planting stock is valued at $53.5 million annually for the north coast of California alone (Fuller et al. 2013). However, the control and management of GLRaV-3 in planting stock depends on accurate identification of the virus.

To date, designing a sensitive and robust GLRaV-3 RT-qPCR has been complicated by the fact that GLRaV-3 is genetically highly diverse. Recent studies based on genome wide phylogenetic analysis demonstrated that the species can be divided into eight distinct subclades (Groups I-VIII) and four supergroups (Supergroups A-D) (Maree et al. 2015). Assay design has also been hindered by incomplete sequence data in the GenBank. No complete genome sequences exist for group IV and V isolates (Maree et al. 2013) and the partial sequence data available for New Zealand variants (Chooi, et al. 2013a; Chooi et al. 2013b) was not included in the most recently designed GLRaV-3 assays by Bester et al. (2014), which opens the possibility of missing such isolates employing the current standard detection test. We hypothesize that additional diverse isolates exist and propose that a more complete characterization of GLRaV-3 diversity is a prerequisite for the design of a reliable RT-qPCR assay that detects all known variants.

Multiple studies have demonstrated that high-throughput sequencing (HTS) is a very useful new research tool for detecting viruses present in grapevines independent of high sequence identity (reviewed in Hadidi et al. 2016), such innovative technology is being used as routine diagnostic tool at the Foundation Plant Services (FPS). As a national and international grapevine importation center, FPS provides virus testing services. In this research project we screen different grapevine populations via Enzyme-linked immunosorbent assay (ELISA) and different RT-qPCR assays targeting single GLRaV-3 variants, and later analyze select vines using HTS. Similar methodology was employed during a preliminary work, where we reconstructed the complete genomes of four new GLRaV-3 variants. Two variants are closely related to "GH24", the divergent isolate first identified in S. Africa (Maree et al. 2015). The other two are similar to GLRaV-3f, a variant identified in Napa Valley (Sharma et al. 2011). These latter two sequences are only ±75% similar to isolates in group VII and VIII and 80% similar to those in supergroup A (Maree et al. 2015), indicating that they may represent a new GLRaV-3 subgroup. Thus, new GLRaV-3 isolates (including divergent variants) are being sequenced, incorporating additional genetic data into a more complete characterization of GLRaV-3 genetic variation, which was used for designing an improved GLRaV-3 RT-qPCR assay.

Objectives

The overall goal of this research project was to design a reliable and robust RT-qPCR assay that detects all known variants of GLRaV-3. The specific objectives were:

1. Screen select grapevine populations for new variants of GLRaV-3.
   1,872 samples were collected from grapevine populations with a historically high incidence of GLRaV-3. These populations included the USDA National Clonal Germplasm Repository (NCGR) in Winters, CA, the Davis Virus Collection at UC-Davis, the FPS pipeline of foreign and domestic introductions, 16 selected vineyards in the main grape-growing areas of California (Napa, Sonoma, San Luis Obispo and Central Sierra), and samples provided by international collaborators (South Africa, New Zealand and Australia). Of the 1,872 samples, 1,148 (61%) samples tested positive for GLRaV-3 using the ELISA assay. Such samples corresponded to domestic selections or international plants originating from Israel, Croatia, Portugal, Italy, Hungary, Canada, Japan, Turkmenistan, Spain, South Korea, France and Greece. On the other hand, all the samples (1,872) are being tested by five strain-specific GLRaV-3 assays (common isolate, "e", "f", "NZ2", "NdA" and "GH24"). We deduct that the large number of samples analyzed resulted in a very rich representation of GLRaV-3 isolates and based on the detection assays (ELISA and RT-qPCR individual assays) we will identify candidates for HTS analysis.

2. Incorporate new genetic data into a more complete characterization of genetic variation across the GLRaV-3 genome to inform assay design.
   In addition to the samples employed during this research project, from 2017 to date, FPS has identified 386 GLRaV-3-positive samples. This detection was conducted using the ELISA kit, strain-specific GLRaV-3 assays or HTS; in the last case, 9 samples were determined GLRaV-3-positive by HTS analysis. Further work is in progress to fully characterize those 9 isolates and later incorporate their sequences to publicly available viral databases.

3. Construct improved assays utilizing multiple primers sets for detecting all existing GLRaV-3 variants.
   A new GLRaV-3 RT-qPCR assay, called FPST, was designed using publicly available GLRaV-3 sequences as well as our own divergent GLRaV-3 variants that were sequenced at FPS. The process began with multiple alignments and identifying regions with low sequence diversity that were suitable for assay design. In this case, FPST targeted the highly conserved 3' terminal region of the virus genome.

4. Empirically test and validate proposed assay designs using GLRaV-3 positive controls.
   The FPST assay was empirically tested and validated using single isolate positive controls, representing all the GLRaV-3 groups. When compared with previous GLRaV-3 assays, FPST was the one RT-qPCR assay that detected ALL variants obtained to date (Table 1). Later, all the collected samples (1,872) were tested using the FPST assay, identifying 1,148 (61%) samples with GLRaV-3. From a comparative perspective, all the samples testing positive by ELISA previously, tested positive by the FPST assay. The 100% match between the results suggests that both assays have similar efficiencies.

Table 1. Test results of divergent GLRaV-3 variants from FPST and individual GLRaV-3 RT-qPCR assays. The results demonstrate the challenge of designing a RT-qPCR assay to a genetically diverse genome. Until FPST was designed, variant-specific assays led to false negative test results.

GLRaV-3 Isolate	Original Source	Location in CA	GLRaV-3 RT-qPCR Assays						
			Original assay	"e"	"f"	"NZ2"	"GH24"	"NdA"	FPST
Common	Various countries	Widespread in vineyards throughout CA	POS	NEG	NEG	NEG	NEG	NEG	POS
"e"	Napa Valley	Primarily Napa Valley	NEG	POS	NEG	NEG	NEG	NEG	POS
"f"	Napa Valley	Five vineyards in regions throughout CA	NEG	NEG	POS	NEG	NEG	NEG	POS
"NZ2"	New Zealand	Santa Barbara	NEG	NEG	NEG	POS	NEG	NEG	POS
"GH24"	South Africa	FPS virus collection	NEG	NEG	NEG	NEG	POS	NEG	POS
"NdA"	Italy	FPS virus collection	NEG	NEG	NEG	NEG	NEG	POS	POS

5. Disseminate research progress and results.
   Preliminary results have been presented during growers' meetings organized by the UC Cooperative Extension, and scientific meetings (meetings listed in the presentations related to the project section). A press release and a peer-reviewed scientific article are being prepared, which will be shared with growers and researchers. Additionally, the novel detection tool will be shared with diagnostic labs involved in the grapevine industry in the US and around the world.

Publications and Presentations Related to the Project

- Current Issues in Vineyard Health course on December 5, 2017.
- California Department of Food and Agriculture (CDFA) annual meeting on February 7, 2018.
- Current Wine and Winegrape Research course on February 21, 2018.
- Abstract submitted to 19th Conference of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICGV) on April 9-12, 2018.

Research Relevance Statement

FPS works closely with CDFA to register and certify grapevines and is the source of foundation planting material for California nurseries. FPS developed a sensitive and robust GLRaV-3 RT-qPCR assay, which detected all the known variants of the virus. This state of the art tool will be used in routine diagnostic tests reducing the risk of GLRaV-3 spreading through infected vines.

Layperson Summary of Project Accomplishments

We prescreened (ELISA and RT-qPCR individual assays) of select grapevine populations followed by HTS to identify additional genetically diverse GLRaV-3 isolates, generate a representative collection, and use this information to design a highly reliable RT-qPCR assay. Consequently, a newly designed and validated RT-qPCR assay (FPST), successfully detected all known variants of GLRaV-3.

Status of Funds
Expenditures for this project for the reporting period (October 2017 to February 2018) totaled $72,336.06 (including liens). The balance of $91,936.94 is expected to be spent in full by the project end date of June 30, 2018.

Intellectual Property
We do not anticipate that our published assay designs will be subject to the policies of the University of California for managing intellectual property.

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