**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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| **Principal Investigator:** | **Co-Principal Investigator:** | **Cooperator:** |
| Maher Al Rwahnih | Deborah Golino | Kristian Stevens |
| Foundation Plant Services | Foundation Plant Services | Foundation Plant Services |
| University of California | University of California | University of California |
| Davis, CA, 95616 | Davis, CA, 95616 | Davis, CA, 95616 |
|  |  |  |
| **Cooperator:** | **Cooperator:** | **Cooperator:** |
| Vicki Klaassen | Adib Rowhani | Hans J Maree |
| Foundation Plant Services | Foundation Plant Services | Stellenbosch University |
| University of California | University of California | South Africa |
| Davis, CA, 95616 | Davis, CA, 95616 |  |
|  |  |  |
| **Cooperator:** | **Cooperator:** | **Cooperator:** |
| Monica Cooper | Lynn Wunderlich | Rhonda Smith |
| Cooperative Extension | Cooperative Extension | Cooperative Extension |
| University of California | University of California | University of California |
| Napa, CA 94559 | Placerville, CA 95667 | Santa Rosa, CA 95403 |
|  |  |  |
| **Cooperator:** | **Cooperator:** |  |
| John Preece | Alfredo Diaz-Lara |  |
| National Clonal Germplasm Repository | Foundation Plant Services |  |
| University of California |  |
| Davis, CA, 95616 | Davis, CA, 95616 |  |

**Period of the Grant:** July 2017 to June 2018.

**Objectives**

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

1. Incorporate new genetic data into a more complete characterization of genetic variation across the GLRaV-3 genome to inform assay design.
2. Construct improved assays utilizing multiple primers sets for detecting all existing GLRaV-3 variants.
3. Empirically test and validate proposed assay designs using GLRaV-3 positive controls.
4. Screen select grapevine populations for new variants of GLRaV-3.
5. Disseminate research progress and results.

**Background**

Grapevine leafroll-associated virus 3 (GLRaV-3) is the main etiological agent of grapevine leafroll disease (GLD), one of the most important virus diseases of grapevine which is distributed worldwide. The long-distance spread of GLRaV-3, caused by the movement of infected vines, can be controlled effectively if GLRaV-3 is accurately identified and virus-tested clean stock is made available to growers. In turn, accurate GLRaV-3 identification and the production of a large amount of tested planting stock require a high throughput testing method that is sensitive and specific for GLRaV-3. As the source of all California Registered or Certified grapevines, Foundation Plant Services (FPS), has met the need for reliably detecting GLRaV-3 in large sample numbers by focusing on the development of reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) assays.

RT-qPCR assays detect viruses by amplifying a small section of the virus genome to levels that can be easily detected. Amplification is achieved by identifying regions of the genome that are unique to any given virus yet are conserved among all the genetic variants of that particular virus. While identifying conserved regions across genetic variants can be difficult with any RNA virus, due to its relatively high mutation rate, GLRaV-3 has an exceptional number of highly diverse variants. Recent studies based on genome-wide phylogenetic analysis demonstrated that GLRaV-3 variants can be divided into eight distinct groups, six of which have been identified in California (CA). This level of genetic diversity makes it almost impossible to identify a conserved region common to all isolates for design of a single qPCR-based assay. Up to now, FPS has dealt with this problem by designing variant-specific assays. However, it isn’t feasible to test large numbers of vines using different assays. In addition, the California Department of Food and Agriculture (CDFA), which works closely with FPS on the grapevine certification process, recently adopted RT-qPCR assays as the standard tool for virus detection.

In 2017, Dr. Maher Al Rwahnih and collaborators obtained funds from the Pierce’s Disease and Glassy-winged sharpshooter Research Board to conduct this research project. As part of this study, a new GLRaV-3 RT-qPCR assay was generated, such assay could overcome the genetic diversity of the virus detecting all GLRaV-3 variants characterized to date. Additionally, the near complete genomes of 37 new GLRaV-3 isolates, including 3 divergent variants, were reconstructed using high-throughput sequencing (HTS) and later deposited in publicly available databases.

**Work Performed and Accomplishments Achieved during the Grant Period**

* The new GLRaV-3 RT-qPCR assay was designed using publicly available GLRaV-3 sequences as well as our own GLRaV-3 isolates that were sequenced at FPS. The design involved a total of 43 GLRaV-3 sequences, including 23 sequences available in GenBank and 20 partial sequences (above 16,000 nucleotides) generated at FPS and representing different genetic variants. Later, a multiple alignment was used to identify regions with low sequence diversity that were suitable for assay design. In this case, the new RT-qPCR targeted the 3’ terminal region of the virus genome, originating the name of FPS Terminal or FPST assay.
* The FPST assay has been 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.
* To further test the new assay, a total of 2,452 samples were obtained from grapevine populations with a historically high incidence of GLRaV-3 or with observable GLD symptoms. These populations included the USDA National Clonal Germplasm Repository in Winters, CA, the Davis Virus Collection at UC-Davis, the FPS pipeline of foreign and domestic introductions, selected vineyards in the main grape-growing areas of CA (Napa, Sonoma, San Luis Obispo, Fresno, Monterey, Central Coast, Coachella Valley, North Coast, San Joaquin Valley and Central Sierra region), and samples provided by collaborators in Australia, New Zealand, Spain and South Africa.
* Of the 2,452 samples, 1,378 (56%) samples tested positive for GLRaV-3 using the FPST RT-qPCR assay. These samples corresponded to domestic or foreign grape selections originating from 46 countries. The large number of samples analyzed resulted in a very rich geographic representation of GLRaV-3 variants.
* Further verification of the FPST assay was obtained by testing the above 2,452 samples with a new GLRaV-3 enzyme-linked immunosorbent assay (ELISA) kit that was developed using funds from the Fruit Tree, Nut Tree, and Grapevine Improvement Advisory Board, managed by CDFA. This new ELISA kit has detected all known GLRaV-3 variants characterized to date. The side-by-side comparison indicated that all samples testing positive by the FPST RT-qPCR assay also tested positive by the new ELISA kit. The 100% match between the results suggests that both assays have similar efficiencies.
* The FPST assay is being shared with different diagnostic labs in the US (including the CDFA) and around the world. Consequently, the novel detection tool can be used in routine diagnostic tests reducing the risk of GLRaV-3 spreading through infected vines due to false negative test results. Finally, results of this project have been shared with growers and researchers involved in the grapevine industry during local and international meetings.

Moving forward, we will continue to test more samples and challenge the FPST RT-qPCR assay against possible new divergent variants of GLRaV-3. In addition, we will characterize more GLRaV-3 variants using HTS. The availability of more complete GLRaV-3 genome sequences will aid in further characterizing the genetic diversity covered by the assay which will be updated upon finding any new divergent variants. We cannot predict if the FPST assay will detect all the GLRaV-3 variants found in the future, however, the improved assay detects all known variants to date.