Improved Detection and Evaluation of the Biological Significance of Grapevine Vitivirus

Maher Al Rwahnih, UC Davis

Project Summary/Abstract

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

Executive Summary

Currently, five different viruses are formally classified as vitiviruses (genus *Vitivirus,* family *Betaflexiviridae)* and known-to infect grapevine: grapevine virus A (GVA), grapevine virus B (GVB), grapevine virus D (GVD), grapevine ,virus.(GVE). and grapevinevirus. (GVF). Some of these vitiviruses are associated with the etiology of rugose wood (RW),disease grapevine and are vectored by mealybugs (family Pseudococcidae) and soft-scale insects (family Coccidae);. however, the main route of their transmission is by propagation using infected plant material. In addition, vitiviruses are·:frequently detected in coinfection with members of the family *Closteroviridae* (i.e. grapevine leafroll-associated ;viruses.1; 2 and 3), resulting in synergistic interactions that can lead to lethal effects in several scion and rootstock combinations. Through the application of high-throughout sequencing (HTS), five new viruses were discovered in grapevine during the last two years and have been proposed as members in the *Vitivirus* genus. These new viruses were tentatively named grapevine virus G (GVG), grapevine virus H (GVH), grapevine virus I (GVI), grapevine virus J (GVJ) and grapevine virus L (GVL). In contrast with other previously known vitiviruses (i.e. GVA, B and D), the biological significance of the novel vitiviruses remains largely unknown, including effects on vine performance and mechanisms of transmission. In 2018, a limited survey was launched to determine the prevalence of GVG, H, I, J and L in California; as a result, all five viruses were detected across different grapevine populations via conventional PCR. This project will update existing detection assays and in the case of the novel vitiviruses, design new reverse transcription quantitative PCR (RT-qPCR) assays to replace the current conventional PCR-based assays. This will increase the reliability and efficiency of vitivirus detection in grapevines. Additionally, the possibility of a generic (universal) assay with the capacity of detecting all the grapevine vitiviruses will be further investigated; such assay will represent an alternative to reduce the work associated with diagnosis. Another objective of this project is to investigate the biological significance of novel vitiviruses. We plan to conduct a field trial to determine if the novel vitiviruses (GVG, H, I, J and L) can cause RW-like symptoms (i.e. wood marking symptoms on the trunk) on commonly used indicator hosts LN 33, St. George, Kober SBB and Cabernet franc. Inoculated grapevines will be examined for wood symptom development periodically. Finally, information generated from this project will be shared with nurseries and diagnostic labs to prevent a future negative impact to the grapevine industry.

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

Previously known grapevine vitiviruses (GVA, 8, D, E and F) are widespread and have been reported from all major grapevine-producing regions of the world, with GVA especially being one of the most regularly detected viruses. The grapevine-infecting vitiviruses are reported to be associated with the RW complex, which includes several important diseases that result in modifications to the woody cylinder, cambium tissue, and bark of vines (Figure 1). Symptom development depends on the virus-host combination and on environmental conditions. GVA, GV8, GVD are putative agents of Kober 588 (V. berlandieri x V. riparia) stem grooving, corky bark in LN 33 (Couderc 1613 x V. berlandieri) and growth reduction in Freedom, respectively; however, the complete etiologies have not been resolved for any of these disease complexes. GVF was associated with graft incompatibility of Cabernet Sauvignon, resulting in death of plants. Lastly, there is no reported disease caused by GVE in grapevine. In contrast, the potential pathogenic role of new grapevine vitiviruses (GVG, H, I, J and L) is still unknown.

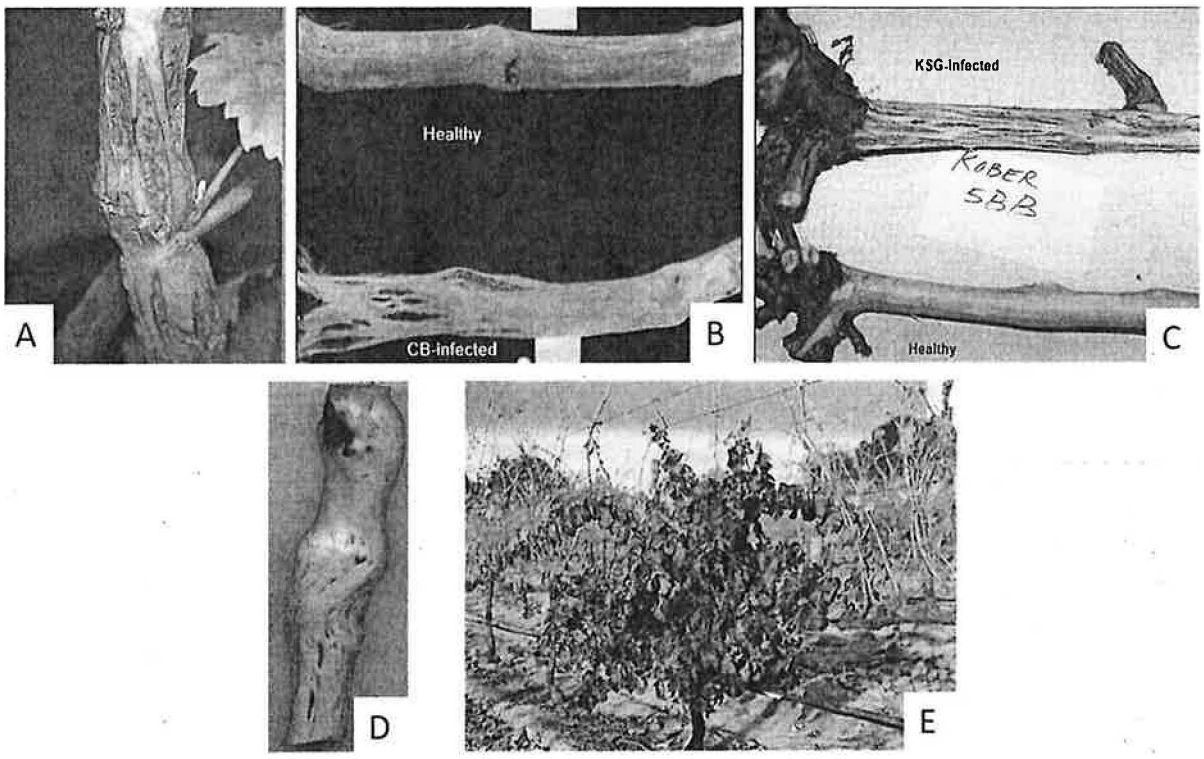

Figure 1. Grapevine disease symptoms putatively associated with vitivirus infection. **A**, Corky bark and swelling symptoms on young LN 33 vine; **B**, Corky bark symptoms on LN33 wood; **C,** Kober Stem Grooving symptoms on Kober 5BB wood; **D**,

Stem pitting and grooving on rootstock wood, under the grafting point associated with vitivirus infection; E, Shiraz disease symptoms on a Merlot vine (natural infection in the vineyard) at the end of the vegetative season. Nearby vines unaffected by the disease showed normal hardening of their canes and normal loss of foliage (Minafra et al., 2017).

Other viruses infecting grapevine are the grapevine leafroll-associated viruses (GLRaV; family *Closteroviridae),* which are frequently detected in coinfections with vitiviruses. Even when vitiviruses by themselves do not cause symptoms on common grapevine scion and rootstock combinations, a mixed infection of a vitivirus with a member of the family *Closteroviridae* may create a serious problem in the field, especially if one of the susceptible rootstocks has been used for propagation. Our observations in California have shown that Cabernet Franc and Chardonnay vines propagated on Freedom rootstock die within 1-2 years after inoculation when inoculated with a virus source containing GLRaV-1 or• GLRaV-3 and GVA. Reports from South Africa indicate that Shiraz disease is caused by a co-infection of GVA and GLRaV-3;

All the five-novel grapevine vitiviruses (GVG, H, I, J and L) were detected recently during an initial study using conventional PCR at Foundation Plant Services (FPS, University of California-Davis), which represents the first identification of such viruses in the US. New advances in qPCR have significantly improved the detection of pathogens, allowing quick, more sensitive and precise quantification compared to conventional PCR. This project will design new RT-qPCR assays for detection of novel vitiviruses. In the case of GVA, B, D, E, F, we will evaluate the broad-range detection capacity and specificity of currently available RT-qPCR assays, and update these assays if they are inadequate. Genetic diversity and recombination are common among viruses, which can compromise the reliability of PCR-based assays. Since FPS works closely with the California Department of Food and Agriculture (CDFA) to register and certify grapevines and is the source of foundation planting material for California nurseries, FPS just developed a new RT-qPCR assay for the reliable detection of GLRaV-3 (Project: "Survey and analysis of grapevine leafroll-associated virus-3 genetic variants and application towards improved RT-qPCR assay design") and currently is working on the update and development of new assays for the common Prunus and pome fruit viruses (Project: "Development and validation of real time quantitative PCR assays for the detection of fruit tree.viruses"). Consequently, we anticipate the continued shift to qPCR-based methods for virus detection given the improvement in accuracy and high throughput sample processing efficiency. Additionally, in this project we will further investigate the possibility of a generic (universal) assay involving degenerate PCR primers with the capacity of detecting all the grapevine vitiviruses; thus, simplifying the vitivirus diagnosis. Any assay we develop as a result of this project will be made available to CDFA and commercial diagnostic labs and will facilitate the production of certified propagation material and the effective control of vitiviruses in California vineyards.

As discussed previously, to date there is no scientific data available to indicate symptoms caused by new grapevine-infecting vitiviruses. The grapevine bio assay is a test for viruses based on a panel of grapevine selections that serve as indicator hosts. The bio assay detects those disease agents that are designated as of particular agronomic significance. The selections include Cabernet franc, which generates a diagnostic interveinal reddening and down-rolling of leaf margins as an indicator of leaf roll disease caused by members of the family *C/osteroviridae;* Kober SBB, which expresses stem grooving disease associated with infection by GVA; LN 33, which develops corky-bark symptoms associated with infection by GVB; and St. George *(V. rupestris),* which develops diagnostic leaf symptoms due to infection by grapevine fanleaf virus or grapevine fleck virus, and stem pitting symptoms from infection by grapevine Rupestris stem pitting-associated virus. We note that these are not the only grapevine viruses that can be detected with these indicator cultivars. For example, Cabernet franc is also an indicator for grapevine red blotch virus and St. George is an indicator for grapevine asteroid mosaic associated virus. Consequently, we plan to conduct a field trial to investigate symptomatology caused by GVG, H, I, J and Lon the four different indicator plants,. In these woody indicators, we will verify their infection status using the improved RT-qPCR assays (generated during the project) and assess their reaction to novel vitiviruses. Finally, results of this research will be shared with growers and other stakeholders involved in the grapevine industry.

Objectives

Objective 1: Construct new or improve individual RT-qPCR assays for all known grapevine vitiviruses.

Objective 2: Screen select grapevine populations for vitiviruses and validate improved RT-qPCR assays.

Objective 3: Construct a generic (universal) assay for all grapevine vitiviruses.

Objective 4: Empirically test and validate the universal assay using positive controls.

Objective 5: Evaluate the biological effects of GVG, H, I, J and L on the common grapevine indicators.

Objective 6: Disseminate research progress and results.

Work Plans and Methods

Objective 1: *Construct new or improve individual RT-qPCR assays for all known grapevine vitiviruses.*

To recognize conserved regions suitable for detection assays in novel vitiviruses, each virus will be examined by using an in-house bio informatics pipeline. Briefly, all the sequence data available at the GenBank or previously generated at FPS will be aligned. PCR primers and real-time probes (MGB) of approximately 20-30 nucleotides long will be designed from regions on the genome with 100% consensus among different virus isolates. If the sequences on the conserved regions are variable such that it becomes impossible to design a single pair of PCR primers or a single probe to detect the full range of isolates in the designated region, then we will attempt to design multiple primers and probes to be used in single qPCR reactions. In addition, primers and probe(s) for each novel vitivirus will be compared against the other *Vitivirus* species to check for low sequence similarity and consequently their specificity. Likewise, sequence data of GVA, B, D, E, F will be aligned independently. Later, currently available PCR primers will be evaluated for their detection and specificity *in silica* using different bio informatic programs. Finally, current RT-qPCR assays will be updated with extra primers or probes for highly divergent variants of the virus. *Continuation of this project is contingent upon program approval and funding.*

Objective 2: *Screen select grapevine populations for vitiviruses and validate improved RT-qPCR assays.*

Grapevine populations with a historical incidence of vitiviruses will be tested by the newly developed RT-qPCR assays (Objective 1) to make sur_e that each system has reliability as expected. The efficiency of each system will also be compared with the current (old) RT-qPCR assays in the case of GVA, B, D, E and F and conventional RT-PCR assays for GVG, H, I, J and L.

We will sample select grapevine populations with geographically diverse provenance. These populations include but are not limited to: The USDA National Clonal Germplasm Repository (NCGR) in Davis, CA; the FPS domestic and quarantine material; the University of California-Davis Virus Collection (DVC); and select commercial vineyards in California. We are planning to collect material from around 1,000 vines across all locations to screen for the presence of grapevine vitiviruses.

The NCGR contains approximately 10,000 grapevines collected from around the world. While we have used NCGR grapevines in previous studies, demonstrating that several grapevines are infected with GVH, the collection has never been systematically screened for all the vitiviruses. FPS has two grapevine populations with a very high incidence of viruses. The DVC collection includes nearly 800 grapevines that are primarily from domestic sources. The FPS pipeline includes over 400 grapevines that are maintained in pots as either quarantine material or domestic selections. Finally, we will screen select vineyards in main grape-growing areas of California, including the Napa, Sonoma, Lodi, and Central Sierra regions. Additionally, we will contact international collaborators to obtain vitivirus-infected plant material. We will obtain this material, using Dr. Al Rwahnih's USDA-APHIS permit to move infected plant material from diverse resources.

Total nucleic acids (TNA) will be extracted from leaf petioles and/or cambial scraping of wood collected from grapevines. Later, TNA extracts will be tested by either old and new assays for the presence of vitiviruses and results will be compared. Selected grapevines samples with contradicting results or producing high Ct values with the new RT-qPCR assays will be HTS analyzed. Likewise, grapevines infected by GVG, H, I, J and L will be analyzed by HTS to generate additional sequencing data and later deposited in GenBank. Consequently, we will incorporate new genetic data into a more complete characterization of genetic variation across vitiviruses to inform assay design and update our RT-qPCR assays if necessary.

Objective 3: Construct a generic (universal) assay for all grapevine vitiviruses.

The discovery of 10 different grapevine-infecting vitiviruses represent a tremendous advance into a better understandir;ig of genetic variation across the genus Vitivirus, but this also leads to an increase in work associated with diagnosis. Generic or universal assays to detect a group of viruses are valuable tools to improve laboratory efficiency. For example, a previous study resulted in a generic (universal) assay for detection of several grapevine-infecting nepoviruses (genus Nepovirus)
(Digiaro et al., 2007). we will further investigate the possibility of a universal assay with the capacity of detecting all the grapevine vitiviruses, and anticipate using degenerate PCR primers based on multiple genome-wide alignments using nucleotide and amino acid sequences to identify conserved regions among the different grapevine vitiviruses. Continuation of this project is contingent upon program approval and funding.

Objective 4: Empirically test and validate the universal assay using positive controls.

In this objective, the developed universal assay will be tested against a representative number of isolates of each vitivirus. Briefly, we will use different grapevine samples tested positive for vitiviruses during Objective 2; in addition, samples free of vitiviruses but positive for other virus species will be included. The generic assay will be employed to screen such grapevine samples and results compared with the initial tests (i.e. individual RT-qPCR assays), revealing the efficiency and robustness of the universal assay. Finally, if a discrepancy is observed between an individual assay and the generic assay, the involved sample will be prioritized for HTS analysis. Continuation of this project is contingent upon program approval and funding.

Objective 5: Evaluate the biological effects of GVG, H, I, J and L on the common grapevine indicators.

Selected grapevine accessions tested positive for novel vitiviruses (GVG, H, I, J and L) during Objective 2 will be graft inoculated into four woody indicators to determine the effects on indicator plants; additionally, vitivirus source plants will be examined for GLRaV to count any synergistic interaction. The panel of biological index hosts will include four grapevine selections: LN 33, St. George, Kober SBB and Cabernet franc. Six replicates of these four indicator plants will be bud chip inoculated from each test plant. Two bud chips will be grafted to each indicator plant, and the grafted plants will be maintained in the greenhouse for 1 month to allow the graft to heal. After 2 to 4 weeks of acclimatization in a shade house, the number of surviving buds will be counted; one viable bud out of two will be scored as successful bud take. Successfully grafted plants will be planted in the field. The grapevines in the field will be observed periodically for the next 18 months for symptom development. LN 33, St. George, Kober SBB, Cabernet franc plants will be sacrificed, the trunk bark will be removed to inspect and record the wood marking symptoms. Tests in which at least three plants are symptomatic will be scored as positive. The controls will include plants chip budded from GVA and GVB infected sources, a healthy source and non-chip budded plants. Starting the second year, leaf petioles will be collected from all grafted plants, TNA will be extracted from each sample and later analyzed by RT-qPCR to test for the presence of the viruses inoculated into each plant. This information will let us correlate the presence of the virus with the symptoms recorded. Continuation of this project is contingent upon program approval and funding.

Objective 6: *Disseminate research progress and results.*

An important aspect of this project will be to communicate opportunities, progress, and results 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.