Advancing our knowledge on the detection, sampling and epidemiology of grapevine Pinot gris virus

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Project Summary/Abstract

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

Executive Summary

Grapevine Pinot gris virus (GPGV) was first discovered in 2012 by high-throughput sequencing (HTS) in Italian Pinot gris vineyards. In 2015, this new member of the genus *Trichovirus* (family *Betaflexiviridae),* was first reported in the US in a study of the collections at Foundation Plant Services (FPS, University of California-Davis) in Davis, California. Later, GPGV was also reported by a private virus testing laboratory, which was the first detection of GPGV in the Napa Valley vineyards. In California, GPGV has been identified in symptomatic and symptomless vines from different varieties. The relationship between GPGV infection and symptoms remains complex. Characterized California GPGV isolates share close homology with asymptomatic reference isolates and when symptoms were observed in GPGV-positive vines, those vines were also infected with other viruses or viroids. Thus, the primary goal of this proposed research is to advance our knowledge of the newly reported GPGV in California. We will investigate the virus distribution in grapevine tissues
(including canes) over time and the possibility of alternative hosts in California vineyards; in addition, we will investigate the option of a molecular assay with the capacity to distinguish between virulent and latent GPGV variants and test a recently released ELISA kit against different GPGV isolates. Consequently, the proposed research will address detection and biology of an emerging grapevine viral disease in California and will support the development of management strategies.

Project's Benefit to Nursery Industry

GPGV is a recently identified pathogen of grapevines in California. The virus was likely introduced to California with material historically imported from Europe. The extent and economic impact of GPGV infection in California has not been fully addressed, due to its very recent identification and consequent shortfall of information about it. GPGV has been reported infecting different red and white cultivars around the world. In Italy, the GPGV-associated disease led to economic losses through reduction in number, weight, and quality of berries. On the other hand, the relationship between GPGV infection and disease symptoms appears to be complex and is not yet fully understood. Initially, biological and molecular assays suggested the existence of symptomatic and asymptomatic GPGV strains. Later, a phylogenetic study sorted GPGV isolates in different clades (i.e. A, Band C), clades Band C mainly include isolates from plants with symptoms, and the corresponding GPGV variants should be considered virulent, while the clade A mainly includes isolates from symptomless grapevines, and thus the corresponding GPGV variants should be considered latent.

In 2016, Dr. Al Rwahnih at FPS obtained a one-year fund from the American Vineyard Foundation (AVF) to conduct the research project "Molecular characterization and improved detection of Californian isolates of GPGV". To gain a better understanding of the incidence and distribution of GPGV, field surveys were conducted throughout grape-growing regions in California; thus, GPVG was found infecting many varieties of grape, but only in the Napa County. Most of these vines were asymptomatic (Figure lA), and when symptoms were observed in GPGV-positive vines (Figure 18), HTS revealed the presence of other viruses including grapevine fanleaf virus (GFLV) (Table 1). Subsequently, all the sequenced California GPGV isolates shared close homology with previously characterized asymptomatic reference isolates. During a routine inspection in 2018, the California Department of Food and Agriculture (CDFA) identified a Negro Amaro vine displaying

GPGV-like symptoms (Figure lC) in an increase block com·mercial nursery. In contrast with our previously discerned etiology, this plant was infected with GPGV and grapevine yellow speckle viroid 1 (GYSVd-1), but free of GFLV (Table 1) Further investigation is needed to understand the relation between GPGV presence and symptoms expression in California.

Figure 1. (**A**) Asymptomatic vine infected with GPGV; (**B**) symptomatic Chardonnay vine infected with GPGV, GYSVd-1 and GFLV; (**C**) symptomatic Negro Amaro vine infected with GPGV and GYSVd-1.

Table 1. Selected grapevines samples analyzed by HTS and positive for GPGV.

Sample	Variety	Location	Infection	Symptoms Display
102	Unknown	Commercial vineyard	**GFLV**, GRSPaV, GRBV, **GYSVd-1**, **GPGV**	Symptomatic
103	Chardonnay	Commercial vineyard	GLRaV-3, GRSPaV, GVA, GRBV, HSVd, **GYSVd-1**, **GPGV**	Asymptomatic

104	Chardonnay	Commercial vineyard	GRSPaV, GSyV-1, GAMV, **GYSVd-1**, HSVd, **GPGV**	Asymptomatic
105	Chardonnay	Commercial vineyard	GRSPaV, **GFLV**, GAMV, **GYSVd-1**, HSVd, **GPGV**	Symptomatic
106	Chardonnay	Commercial vineyard	GLRaV-3, GRSPaV, **GFLV**, GRBV, **GYSVd-1**, HSVd, **GPGV**	Symptomatic
123	Cabernet sauvignon	Commercial vineyard	GRSPaV, GSyV-1, **GYSVd-1**, HSVd, **GPGV**	Asymptomatic
335	Negro Amaro	Nursery increase block	**GYSVd-1**, **GPGV**	Symptomatic

As discussed above, diagnosis of GPGV based on specific symptoms can be challenging because a) in some cases the virus appears to be asymptomatic, and b) in other cases, it occurs as a mixed infection with other symptomatic viruses or virus-like agents. Until very recently, sequence-based assays were the only method used for GPGV diagnosis. During our previously funded AVF project, we developed a reverse transcription quantitative PCR (RT-qPCR) assay for the detection of GPGV. Recently, a commercial serological assay (ELISA kit) was released. As part of this project, we will further investigate the reliability of this serological assay, comparing the generated results against our RT-qPCR assay. Lastly, we will investigate the feasibility of developing an assay with the ability to distinguish between virulent and latent GPGV variants. Any tool we develop as a result of this project will be made available to CDFA and private diagnostic labs and will facilitate the production of certified propagation material and the effective control of GPGV in California vineyards.

Since its first report in Italy, some progress has been made on understanding the epidemiology of GPGV, but additional research is needed. During a preliminary study, we evaluated the optimal time of year for sampling/ testing using leaf petioles. As a result, we found that virus titer of GPGV is higher in May/June compared to later in the season. To complement this initial work, we will investigate the distribution (i.e. virus titers) of GPGV in canes and other tissues, and how this relates to seasonal variations. The uneven distribution of virus in different types of plant tissue can affect the testing results. Finally, we will work closely with our collaborators in Italy (Pasquale Saldarelli and Elisa Angelini) to avoid any duplication of the work.

Observations in Europe h ave shown that GPGV can infect alternative hosts, including herbaceous plants and other woody hosts. In this project, we will explore the possibility of new sources of GPGV infection with special interest on free-living *Vitis* spp. located in riparian areas adjacent to vineyards in California.

Lastly, disseminating information is essential to share the latest knowledge on GPGV. Raising awareness on the potential effect of GPGV to grape production and communicating strategies for the reduction of its impact are major components of this proposal. As such, this research addresses one of the primary concerns identified by the Fruit Tree, Nut Tree and Grapevine Improvement Advisory Board (IAB), which involves disease management.

Objectives

Objective 1: Screen select grapevine populations for GPGV to compile a set of diverse isolates and investigate the possibility of a variant-specific assay.

Objective 2: Empirically test and validate detection assays using positive controls.

Objective 3: Investigate potential reservoirs of GPGV in wild plants from riparian areas adjacent to vineyards.

Objective 4: Determine distribution of GPGV in in canes and other grapevine tissues over time.

Objective 5: Disseminate research results to farm advisors and growers.

Work Plans and Methods

Objective 1: *Screen select grapevine populations for GPGV to compile a set of diverse isolates and investigate the possibility of a variant-specific assay.*

Previously, we tested samples from Napa, Sonoma, Santa Barbara, San Luis Obispo, Monterey, San Joaquin, Fresno, Merced, Madera, and San Benito Counties. Since GPGV has, thus far, only been detected from Napa County; we will continue to sample from vineyards in Napa to improve our understanding the virus epidemiology and determine the possible source of virus infection. In addition, this year we plan to collect more samples from grape-growing regions in California, including the North Coast and Central Sierra regions. We will collaborate with farm advisors, private testing laboratories, and private .consultants to identify commercial vineyards where the presence of the virus has been established or has been suggested. Overall, we plan to collect and test around 500 samples with geographically diverse provenance.

Stored total nucleic acid (TNA) samples (1,206) from the USDA National Clonal Germplasm Repository (NCGR) in Winters, CA will also be analyzed for GPGV presence; such samples were collected during a previous study about grapevine leafroll-associated virus 3 and represent different grapevine selections originated from around the world (Diaz-Lara et al., 2018). Lastly, we,will contact international collaborators to obtain GPGV-infected plant material, including symptomatic and asymptomatic isolates of the virus. We will obtain this material using Dr. Al Rwahnih's USDA-APHIS permit to move infected plant material from diverse resources.

Virus presence will be analyzed in samples of TNA isolated from leaf petiole tissue (in the case of newly collected samples) using the MagMax Plant RNA Isolation kit (Thermo Fisher) and tested by our developed RT-qPCR assay. Plants determined positive for GPGV will be analyzed by conventional PCR using the primers described bv. Morelli et al (2014), which amplify the end of the movement protein and the beginning of the coat protein genes. Later, PCR products will be direct sequenced and compared against the GenBank database to identify virulent and latent variants. Consequently, this objective will create a resource comprised of diverse GPGV isolates, which will be used to challenge the different detection methods (Objective 2).

Aiming to further improve the existing GPGV diagnosis, we will investigate the possibility of a novel molecular assay to distinguish between virulent and latent variants. This will involve the design of variant-specific PCR primers and optimized PCR conditions. In this process, all available GPGV sequences in the GenBank or FPS will be aligned to recognize conserved regions suitable for detec;tion assay and characteristic of a single variant (i.e. virulent variant vs. latent variant). Later, these primers will be evaluated for their specificity in silica using different bioinformatic programs. Primer pairs that show the best performance will be selected for further analysis (Objective 2). Additionally, we will evaluate different real-time PCR technologies including probe-based assays and DNA intercalating dye-based. Consequently, a real-time RT-PCR with the capacity to distinguish between virulent and latent GPGV variant will be the final product. *Continuation of this project is contingent upon program approval and funding.*

Objective 2: *Empirically test and validate detection assays using positive controls..*

In this objective, the variant-specific detection assay will be tested against a representative number of GPGV isolates to make sure that this method has robustness and reliability as expected. Briefly, we will use collected grapevine samples (i.e. infected and healthy plants) during the survey (Objective 1) to verify the efficiency of the detection assay: Thus, the new real-time RT-PCR assay will be employed to screen such grapevine samples and results compared with the initial test. Additionally, we will evaluate their amplification efficiency and specificity using appropriate software.

Likewise, we will challenge the recently released GPGV ELISA kit (Bioreba) against several positive and negative samples, virulent and latent variants. Selected samples with contradicting results (i.e. PCR positive, ELISA negative or vice versa) will be futher analyzed by HTS:thus incorporating new genetic data into a more complete characterization of genetic variation across the GPGV.

Objective 3: *Investigate potential reservoirs of GPGV in wild plants from riparian areas adjacent to vineyards.*

To determine the extent to which free-living *Vitis* spp. harbor GPGV and serve as a reservoir for the virus, we will survey five different riparian habitats in close proximity to vineyards with GPGV infection. These woody hosts will be assessed for symptoms and leaf petioie tissue will be tested by our developed RT-qPCR for the presence of GPGV and other common viruses. Sampled plants will be marked and GPS coordinates will be recorded to facilitate resampling. Any GPGV-positive plant will be further characterized by HTS. *Continuation of this project is contingent upon program approval and funding.*

Objective 4: *Determine distribution of GPGV in canes and other grapevine tissues over time.*

To develop an optimal sampling strategy for GPGV, we will investigate the spatiotemporal fluctuations in virus titer. We hypothesize that distribution of GPGV in infected grapevines varies over time and tissue type. GPGV-positive plants located in a commercial vineyard in Napa County, CA and th.e Davis Virus Collection (DVC, University of California-Davis) will be sampled periodically during a year. Tissues collected will include mature expanded leaves from the basal and middle part of the shoot, emerging leaves and canes during the dormant season. Subsequently, plant material will be analyzed with the real-time qPCR assay described by Moran et al (2018) to determine absolute quantification of virus titer.
Continuation of this project is contingent upon program approval and funding.

Objective 5: *Disseminate research results to farm advisors and growers.*

Research results will be communicated to stakeholders at growers' meetings organized by UCCE and at symposiums. Information will also be disseminated through newsletters, journals, reports and peer-reviewed scientific articles. In addition, results will be shared with the Grape Clean Plant Network for dissemination of information at the national level. Eventually, we will produce an easy-to-read fact sheet for interested parties.

Project Management

The project leader will be responsible for all the required planning, executing the plans, data analysis, and preparing the reports for IAB. The Co-Pl will help with original planning and disseminating research results to stakeholders. Progress will be assessed based on the data obtained and the data comparison between the newly developed system with the systems which were developed previously for GPGV testing, as well as data from the field trials and alternative hosts for the virus. We expect to successfully accomplish the objectives of this project within the timeframe if fully funded.

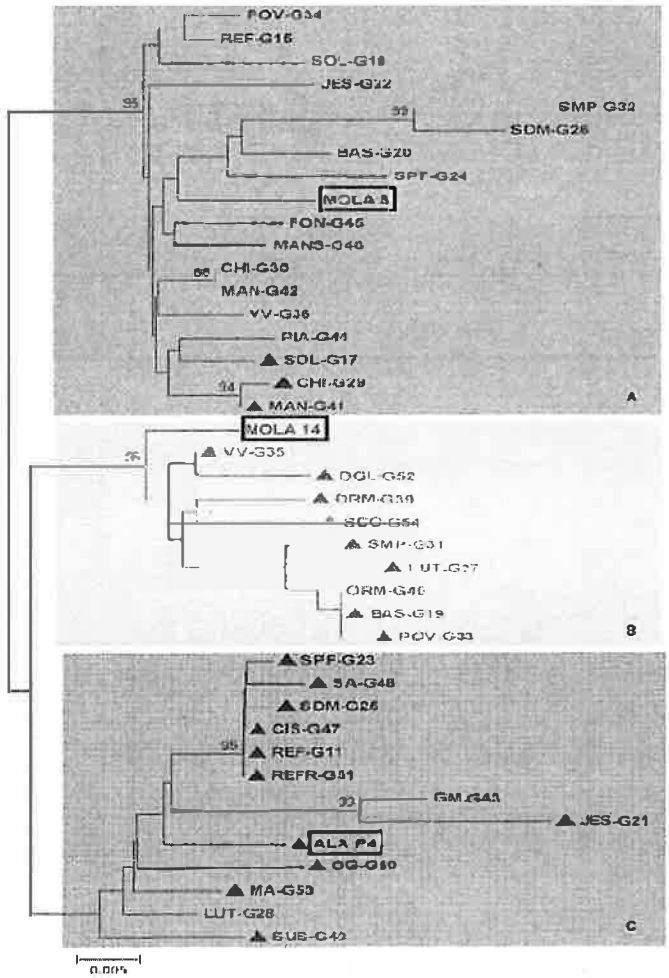
Literature Review

GPGV is a new member of the genus *Trichovirus* in the family *Betaflexiviridae,* first discovered only 6 years ago in Italy by HTS of Pinot gris *(Vitis vinifera)* vines. Italian strains of the virus showed chlorotic mottling, leaf deformation and stunting symptoms (Giampetruzzi et al. 2012). In the province of Trentino (Italy), the GPGV-associated disease led to economic losses through reduction in number, weight, and quality of berries. A three-year field study there showed that vines affected by GPGV had fewer canes and lower numbers and weights of bunches (Malossini et al., 2015). Similar symptoms were also reported from other Italian regions in several wine and table grape varieties such as Chardonnay, Traminer, Pinot noir, Black Magic, and Supernova. Bertazzon et al (2016) showed that GPGV recently appeared in Northeast Italy and has been present in some European countries for at least 10 years before its characterization. The results also suggest that GPGV was limited to a few countries before 2005 and that it spread more widely throughout Europe after 2010.

GPGV has now been reported in symptomatic and asymptomatic plants of other cuitivars in many European and Mediterranean countries. Outside of Europe, GPGV was found in China (Fan, et al., 2016), Korea (Cho et al., 2013), Canada (Poojari et al., 2016, Xiao et al., 2016), the United States (Al Rwahnih et al., 2016; Angelini et al., 2016), Brazil (Fajardo et al., 2017), Pakistan (Rasool et al., 2017) and Australia (Wu and Habili 2017).

In 2015, GPGV was identified in California during a study of the grapevine collection at FPS. Of 2,014 vines screened from the collection, including 23 vines of Pinot gris, only one asymptomatic vine cultivar Touriga Nacional was positive for GPGV. This is believed to be the first reported detection of GPGV in the US (Al Rwahnih et al. 2016). Based on this finding, FPS has added GPGV to the list of viruses included in the 2010 protocol (http://fps.ucdavis.edu/grape2010.cfm). Subsequently, FPS tested its entire foundation blocks {8,481 vines) and all imported and local selections in the pipeline for this virus. This testing res.ulted in detecting GPGV in several imported accessions from Canada, Greece and the Republic of Georgia, and eight vines located at the foundation blocks. Interestingly, GPGV was detected in a grapevine rootstock breeding selection originally propagated from seedlings. Additionally, GPGV was reported by a private virus testing laboratory from four separate vineyards in Napa Valley, California. Seven vines tested positive for GPGV, including selections of Cabernet Sauvignon, Cabernet franc and Chardonnay. This is believed to be the first reported detection of GPGV in the Napa Valley vineyards (Angelini et al., 2016). FPS has also been testing stored TNA samples which had been collected from a selection of vineyards from Napa Valley and elsewhere (Arnold et al., 2017), and a number of these samples have tested positive for the virus. To date, all the characterized California GPGV isolates shared close homology with the asymptomatic reference isolates and when symptoms were observed in GPGV-positive vines, those vines were also infected with other viruses or viroids.

As described above, the relationship between GPGV infection and disease symptoms appears to be complex and is not yet fully understood. Previous studies suggested that GPGV isolates can be classified in symptomatic and asymptomatic;



additionally, the presence or lack or a stop codon in the movement protein (MP) gene (i.e. 369-aa vs. 375-aa) was associated with this classification (Saldarelli et al., 2015). A survey of different cultivars from the Trentino region in Italy showed widespread distribution of GPGV, which was linked with symptomatic (79%) but also with symptomless (21%) vines (Saldarelli et al., 2015). Quantitative analyses of GPGV titer revealed great variability in the viral content of both symptomatic and asymptomatic plants. Generally, GPGV quantity in symptomatic vines was higher than in asymptomatic plants (Bianchi et al., 2015). More recently, Bertazzon et al (2017) proposed a new genetic classification (Figure 2) of GPGV isolates with a slightly different correlation among symptoms: clades B and C mainly include isolates from plants with symptoms, and the corresponding GPGV variants should be considered virulent, while the clade A mainly includes isolates from symptomless grapevines, and thus the corresponding GPGV variants should be considered latent. Finally, co-infections involving GPG\(and other viruses are frequently reported (Saldarelli et al., 2015).

Figure 2. Evolutionary relationships between GPGV isolates in a genomic portion spanning the end of the movement protein and the beginning of the coat protein genes. The shaded regions named A, B and C identify the main clades. Triangles indicate isolates originating from plants with symptoms (Bertazzon et al., 2017).

Knowledge about transmission mechanisms for GPGV were recently revealed. GPGV was transmitted by grafting to *V. riparia* Michx. and *V. vinifera* L. (Saldarelli et al., 2013 and 2015). Attempts to transmit GPGV mechanically to herbaceous plants including *Nicotiana occidentalis* and *Chenopodium quinoa* have been unsuccessful. Several studies concluded that *Colomerus vitis* may acquire GPGV and transfer the virus to healthy grapevines, making the mite a vector for natural GPGV transmission (Malagnini et al., 2016). This vector infests grapevines wherever they are grown and it has been reported in California.

Researchers in Italy identified symptomatic GPGV-positive herbaceous hosts (Figure 3) residing in vineyards (Gualandri, et al, 2016). This is an important epidemiological finding as, C. *vitis,* the only potential GPGV vector to date, is not known to feed on any plants other than grapes. The researchers amplified the whole genome of GPGV from total RNA from the herbaceous hosts. During the last congress of the International Council for the Study of Virus and Virus-Like Diseases of Grapevine (Santiago, Chile; April 2018), GPGV was reported to infect *Ascelpias syricaca, Fraxinus* spp., *Rubus* spp. and *Rosa* spp. (Demian et al., 2018). In the case of grapevine red blotch virus (GRBV), Perry et al (2016) and Bahder et al (2016) revealed the importance of riparian habitats with free-living *Vitis* spp. for virus cycle.

Figure 3. Virus-related symptoms observed on herbaceous plants. C. *album* L. (a) and *Silene latifolia* (b) plants showing chlorotic and mottling spots, respectively (Gualandri et al., 2016).

Until very recently, PCR-based assays were the only method used for GPGV diagnosis. In the past, we developed a RT-qPCR assay for the detection of GPGV. In 2018, Moran et al published a new real-time RT-PCR method for absolute quantification of GPGV, improving even more the reliability of diagnostics for the virus. The development of qPCR-based methods leads to superior sensitivity, speed, reproducibility, and limited risk of contaminations compared to conventional PCR (Osman et al., 2017): Recently, Poignavent et al (2018) developed polyclonal antibodies against GPGV and a commercial ELISA kit was released, both events are very promising for the GPGV diagnosis.

Since its discovery in 2012, some progress has been made on understanding the epidemiology of GPGV, but further research is needed. During a preliminary study, we evaluated the optimal time of year for sampling/testing using petioles. As a result, we found that virus titer is higher in May/June compared to later in the season, making GPGV a "spring virus". Setiono et al {2018) studied the distribution of GRBV in its host, identifying a variable distribution in the plant (i.e. highest virus titers in the petioles of fully expanded leaves and significantly reduced levels of virus in the shoot extremities). Regardless of the sensitivity of the detection method employed, without the correct sampling strategy, the efficacy of any diagnostic approach will be compromised (Setiono et al., 2018). In California, most growers and private testing laboratories use canes for virus detection, because of the senescent nature of grapevine.