Annual Report to:

AMERICAN VINEYARD FOUNDATION, VITICULTURE CONSORTIUM, and CALIFORNIA COMPETITIVE GRANT PROGRAM IN VITICULTURE AND ENOLOGY

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Project Title:

Grapevine Fleck virus: Molecular Detection and Investigation of Strain Variability

Principal Investigator:

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Objectives and Experiments Conducted to Accomplish Objectives:

The objective of this project is to investigate the strain diversity among different grapevine fleck virus isolates by partial cloning and sequencing of their genome. Availability and use of this information is crucial for developing a sensitive and reliable reverse transcriptase-polymerase chain reaction (RT-PCR) assay for the detection of this virus in grapevine tissues. Specific objectives include:

1. Identify fleck isolates collected from different areas and determine their reactivity in ELISA test.

2. Clone and partially sequence 5 to 10 most diverse isolates and compare their sequences. This sequence information will reveal the degree of differences among these isolates.

3. Develop RT-PCR for identifying individual strains of the virus (design and use specific primers for each strain) as well as more universal primers that could detect all or groups of strains at a time. The latter primers will be used in virus detection assays.

Experimental Procedures:

Objective 1: To identify GFKV strains or viruses associated with fleck disease, about 47 different fleck isolates were tested on *Vitis rupestris* cv St. George, a field biological indicator, and by ELISA. Twenty three of these isolates (which originated from 7 different countries) were selected based on ELISA data for sequence analysis and determination of their relationship.

Objective 2: The sequence information for the coat protein region of GFKV was obtained from our colleague in Bari, Italy. A pair of PCR primers was designed to flank the coat protein gene of this virus. Several attempts at designing different pairs of primers failed to amplify this region. High GC content of the virus genome (which results in extensive secondary structures that inhibit RT-PCR) was the suspected reason for failure in our attempts. Subsequently, we decided to develop a nested RT-PCR protocol for the amplification of the coat protein region of the genome. In this protocol, a pair of primers with a higher annealing temperature was designed and used in the primary RT-PCR reaction. This PCR product (672 nt long) was then diluted 15 fold and 2 microliters were then used in a second round of PCR using primers that were internal on the first PCR product and amplifying a 592 nt long product. Using this protocol, we were able to amplify near full length coat protein gene of GFKV. Total of 23 vines were selected and tested by this procedure and only 17 of them produced the desired product. The PCR products were then ligated to pCR2.1 vector and cloned into competent *E. coli* cells according to manufacturer's recommendations (Invitrogen). The resulting colonies were cultured and DNA was purified and screened by restriction digestion using EcoRI restriction enzyme. Selected clones were sequenced by automated sequencing facility at UC Davis. The sequences were analyzed using GCG computer software program.

Objective 3: We have optimized the conditions for sample preparation and PCR. We also investigated the use of nested RT-PCR in detection of GFKV. This method is reported to be more sensitive than standard RT-PCR. In this method, first set of primers was used to amplify a target sequence of the gene. Subsequently, part of the amplified product was diluted and added to a fresh PCR cocktail containing new set of primers which were designed to internally amplify from the original PCR product. We have designed a very efficient pair of primers for nested RT-PCR. We are currently in the process of optimizing this method. In addition, we also have focused on simplifying sample preparation and optimizing RT- PCR conditions for the detection of GFKV. We have used this simplified sample preparation method in our routine testing of grapevine materials. In this sample preparation method, grape tissue is homogenized in ELISA extraction buffer (a carbonate-based buffer containing PVP40, BSA, and Tween 20) at a ratio of 1:20 to 1:40 of sample:buffer. Subsequently, 2 microliters of this homogenate are added to 25 microliters of GES buffer (glycine buffer containing EDTA and NaCl) and boiled at 95C for 5-10 min. Finally 2 microliters of this preparation are added to RT-PCR cocktail and amplified.

Summary of Research by Objectives:

Objective 1: Among all ELISA tested plants, 23 plants with varying readings in ELISA (a range from low to high readings) were selected for coat protein sequence analysis. These plants were the representative of GFKV isolates from seven different countries.

Objective 2: The nested PCR primers successfully amplified the coat protein gene of GFKV from 17 isolates out of the 23 plants which were originally selected for this experiment. The 6 virus isolates which did not amplify may have different sequences in the primer-binding region

of the gene. However, after sequencing and sequence analysis, we found that the coat protein gene sequences of these 17 GFKV isolates were fairly homogeneous. They were from 95% to 99.5% similar to each other at the nucleotide sequence level. We will test additional samples from more diverse sources (especially those which did not amplify in our first attempt) to determine if different strains of GFKV exist.

Objective 3: The genomes of viruses in this plant virus group have a high GC content (about 75% compare to 45-50% for other RNA plant virus groups), which causes strong bonding in secondary RNA structure. To succeed with RT-PCR, the secondary structure of the target sequence needs to be eliminated. There are ways to eliminate these structures including high temperature and use of certain chemicals, but both treatments are harmful to the enzymes used for making the cDNA templates for the RT-PCR amplification. Our repeated efforts failed using optimum temperature (required for annealing the primers to the template) to make the cDNA template for RT-PCR amplification. However, in collaboration with our colleague in the Center for Plant Health in Sidney, B.C. we could overcome this problem and successfully amplified a piece of GFKV genome by RT-PCR (Fig. 1).

We have designed nested primers to use in nested RT-PCR for the detection of GFKV. Our preliminary data (Fig. 2) indicate that this method is at least ten times more sensitive than regular RT-PCR. From two samples represented in this Figure, regular RT-PCR could weakly detect virus in sample #1 and not in sample #2. When the RT-PCR product from the first reaction was diluted and run again with nested primers very strong band (representative of the nested RT-PCR product) was observed.

Our future goal will include to: 1) sequence the coat protein gene of more isolates, especially those which failed in our first attempt. According to a report from Italy (3) a range of different viruses related to GFKV are present in grapevine, some of which are latent in the host. However, to develop a reliable PCR method for the detection of this virus it is crucial to identify all these related strains and design primers for their detection accordingly. 2) Nested RT-PCR may have a great potential for the detection of GFKV, especially when the titer of virus in the grapevine host is low. We are planning to optimize the conditions for this method and develop a one tube reaction procedure.

Publications or Reports:

1. Golino, D.A., Sim, S., and Rowhani, A. 2000. Identification of the latent viruses associated with young vine decline in California. Abstract and presentation in the "13th Meeting of the International Council for the Study of Viruses and Virus-Like Diseases of the Grapevine". 12-17 March 2000. Adelaide, South Australia.

2. Rowhani, A., Biardi, L., Johnson, R., Saldarelli, P., Zhang, Y.P., Chin, J., and Green, M. 2000. Simplified sample preparation method and one-tube RT-PCR for grapevine viruses. Abstract and presentation in the "13th Meeting of the International Council for the Study of

Viruses and Virus-Like Diseases of the Grapevine". 12-17 March 2000. Adelaide, South Australia.

3. Sabanadzovic, S., Abu-Ghanem, N., Castellano, M.A., Digiaro, M., and Martelli, G.P. 2000. Grapevines host a family of grapevine fleck virus-like viruses. 13th Meeting of the International Council for the Study of Viruses and Virus-Like Diseases of the Grapevine". 12-17 March 2000. Adelaide, South Australia.

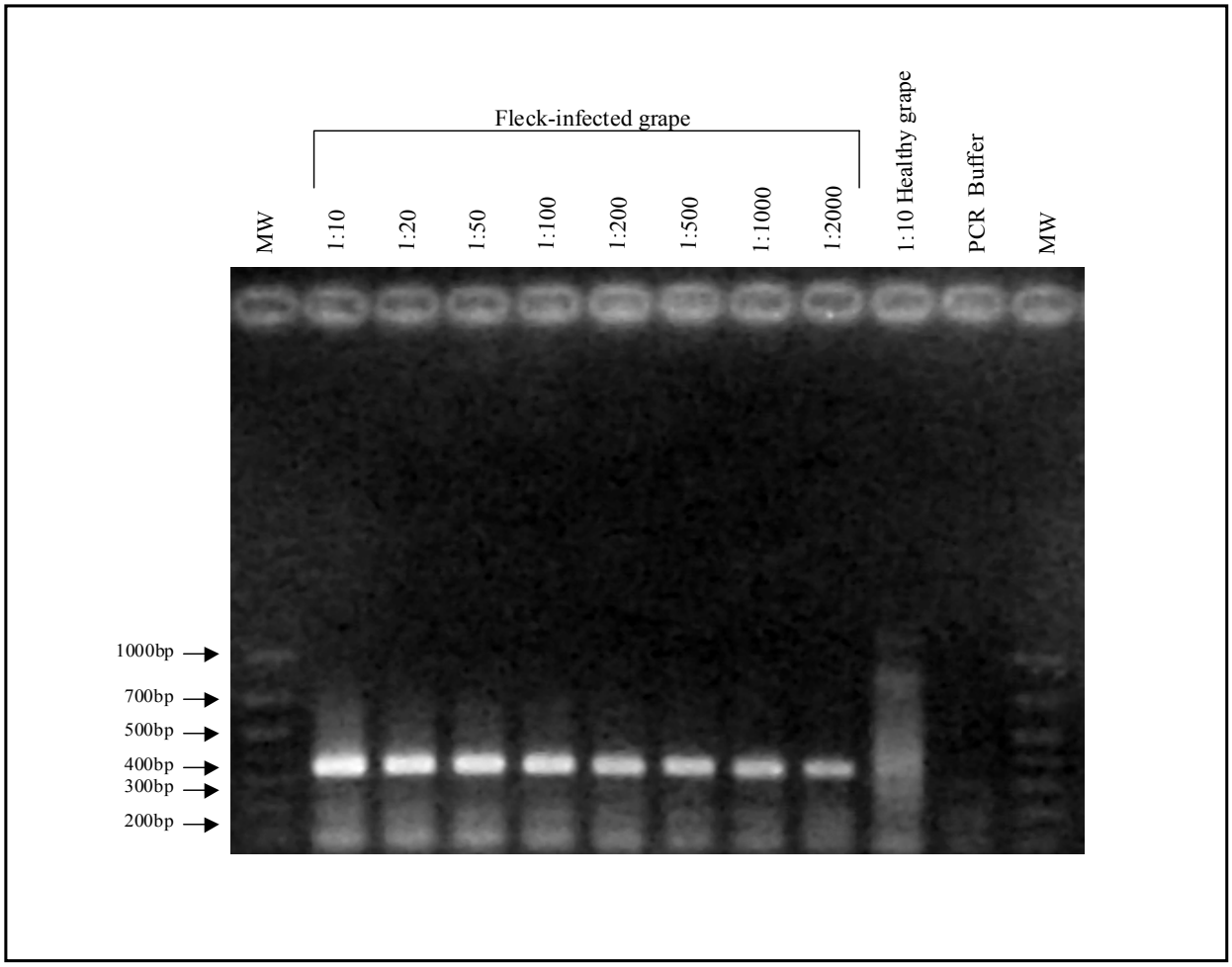
General Summary of Current Years Results:

- Specific PCR primers were designed to amplify the coat protein gene of GFKV to use in sequence comparisons of different isolates. Amplification by regular PCR was attempted two times and failed. Finally we designed another pair of primers, internal to the first set and used in nested RT-PCR and successfully amplified the majority of the genome (592 nt out of 672 nt for the full length of the coat protein gene). This nested RT-PCR was tried on 23 different GFKV isolates and it was successful on 17 of them. The sequence comparison among these 17 isolates revealed that they were quite homogeneous and had similarities between 95% to 99.5%.

- We have found that nested RT-PCR is more sensitive than regular PCR. Although it requires an additional step to complete, but in our preliminary experiments it was shown to be tens of times more sensitive than regular RT-PCR. Based on its sensitivity and reproducibility, the nested RT-PCR will have a great potential for use in testing grape materials for virus detection.

Figure 1

Gel analysis of RT-PCR products. Petiole samples from infected and healthy grapevines were prepared as described. Infected sample was serially diluted to 1:2000. A one-tube PCR reaction was developed that allows for quick processing of samples and minimal sample handling. The PCR products were resolved on a 1.4% agarose gel in 1X TAE buffer at 80V for 60 minutes. The gel was stained with an ethidium bromide solution and photographed using the Kodak DC120 digital camera and software.



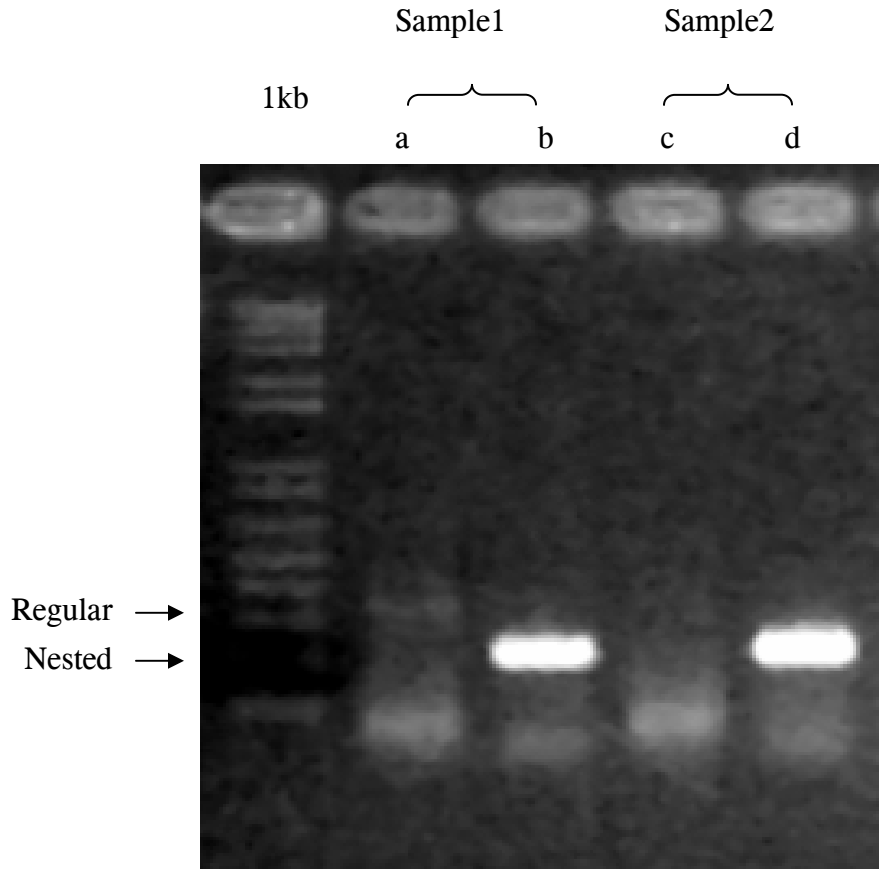


Fig. 2. A comparison between regular and nested RT-PCR for the detection of GFkV. The results from two different samples (samples 1 and 2) are shown in the picture. Lanes a and c are the results from regular PCR, which only sample 1 shows a weak band. Lanes b and d are the results from nested PCR.