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Project Title:

Development of Polymerase Chain Reaction for Rapid Detection of Grapevine Leafroll Associated Viruses 1 through 5

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Objectives and Experiments Conducted to Accomplish Objectives:

The objective of this project is to develop fast, sensitive, and reliable reverse transcriptase-polymerase chain reaction (RT-PCR) methods for the detection of grapevine leafroll associated viruses (GLRaV) 1 through 5 in grapevine. The specific objectives will include:

1. Develop RT-PCR methodologies for the detection of GLRaVs 1 through 5.

    - Sequence fragments of viral genome for viruses that do not have such sequences already available. The sequence information is necessary for designing PCR primers.

    - Design and evaluate virus-specific and universal primers for detection of single and multiple leafroll associated viruses, respectively.

2. Develop a simple and reliable sample preparation method for grapevine tissue for testing large number of samples at a time.

3. Investigate strain diversity among leafroll associated virus types. This will aid in design of primers that can detect all strains.

4. Investigate and develop protocols for colorimetric RT-PCR assay.

Experimental procedures:

Objective 1: To develop RT-PCR methodology for the detection of GLRaVs 1 through 5, cDNA will be synthesized from the viral genome (derived from purified virus preparations or dsRNAs) and cloned. Cloned cDNAs will be sequenced using the facilities available at UC Davis. Oligonucleotide primers (20-30 bases) will be made from the sequences for RT-PCR use. For some of these viruses (e.g., GLRaV 2 and 3), the complete genome has been sequenced by other investigators and the information was stored in the GenBank, which is accessible to researchers through the internet. In these cases we will use the available information for designing PCR primers. The specificity of each primer pair in detecting the virus will be evaluated by testing against number of different virus isolates collected from different regions. The primer pairs which specifically amplify each viral genome and its different isolates and strains will be selected for virus detection application and field survey work. We also will investigate the use of nested RT-PCR in detection of these viruses. This method is reported to be more sensitive than regular PCR. In this method, our usual primers will be used to amplify a target sequence. Then part of the amplified product will be diluted and an aliquot will be added to a different PCR reagent containing new set of primers which are designed to internally amplify a segment from the original PCR product.

Objective 2: Grapevine tissues contain large amounts of polysaccharides, phenolic compounds and other substances that have been proven to make both nucleic acid extraction and virus detection difficult by PCR. Therefore, preparing samples from grapevine tissues for PCR usually is a time consuming process and may take hours to prepare. Some progress has been made in my laboratory to simplify this process. We are planning to develop a simple extraction methodology for grape tissue, which will enable us to process large number of samples per day. In this method, grape tissue will be 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. Then 2 microliters from this homogenate will be added to 25 microliters of GES buffer (glycine buffer containing EDTA and NaCl) and boiled at 95C for 5-10 min. Finally, 2 microliters from this preparation will be added to PCR cocktail and amplified.

Objective 3: We have gathered ELISA data from large number of different isolates on GLRaV 1 through 5. These ELISA data indicated that the OD405 reading values (reading was done by an ELISA machine) for different plants varied from very low and boarder line readings to very high readings. These data suggest the possibility of the occurrence of more than one strain of the virus in different plants. In order to develop a reliable RT-PCR methodology for the detection of a virus the designed primers must be able to detect all possible strains of that virus in different plants, otherwise there will be chances of getting false negative results from some samples. In this objective we propose to thoroughly test each set of PCR primers against different isolates and strains of selected virus. If necessary, clone and sequence part of the genome of these strains, which are not recognized by the primers and use the sequence information to design a more efficient and universal primers.

Objective 4: When RT-PCR data require gel electrophoretic analysis for interpretation, the technology is still cumbersome for large-scale sampling efforts. We have developed a much simplified colorimetric assay for the detection of some of the viruses attacking fruit and nut trees (7). In this protocol the RT-PCR products are analyzed in an ELISA plate and the resulting color development (for virus-positive samples) are evaluated and quantified by an ELISA reader. We will try to develop and apply this methodology for RT-PCR detection of GLRaVs 1 to 5 in grapevines. In colorimetric PCR, the microtiter plates were coated with streptavidine. After 1 hour incubation at 37C, the plates were washed and then incubated with virus-specific biotin-labeled detection probes (an oligonucleotide complementary to an internal site of PCR product for a given virus) and incubated for 30 min at 37C. After washing, the denatured PCR product labeled with Digoxygenin (PCR product was labeled by adding Digoxygenin-labeled dUTP in the PCR reaction) was added and incubated for 1.5 hr at 46C. Plates were washed and incubated with alkaline phosphatase-conjugated antibody prepared to Digoxygenin and incubated for 1 hr at 37C. Finally, the plates were washed and incubated at room temperature with colorimetric substrate for 1-2 hours. Color development was then analyzed by measuring absorbance at 405 nm with an ELISA plate reader.

Summary of Research by Objectives:

Objective 1: As it was reported in our 2000-2001 report, we have made specific detection PCR primers for GLRaVs-1 to 5. Universal primers that could detect GLRaVs-1, -2 and -4,-5 were designed. Nested PCR primers were also designed for all these five different GLRaVs and are being used for their detection in samples which regular PCR test results were inconsistent or weak and they worked quite well.

Objective 2: A simple sample preparation method for RT-PCR detection of grapevine viruses in grape tissue was developed. This simplified method is not any more complex or labor intensive than the method used in ELISA. To further simplify the process of sample analysis, we developed a one-tube RT-PCR method. In this method all the necessary reagents for reverse transcription and PCR amplification are added to the sample in one simple step. Once the reaction is complete, it can be loaded onto an agarose gel for electrophoresis and analysis. This one-tube (or one step) method requires less sample manipulation, therefore, significantly reduces chance of cross contamination. Also it reduces the time required for set up of the PCR reaction and preparation of the product for gel electrophoresis and analysis. In the past year we developed a sample blotting method for collecting samples in the field. In this method, a cross section is made on leaf petioles or young shoots and the cut area blotted on special type of nitrocellulose or nylon membrane and dried. Later in the laboratory each blot (representative of a sample) is cut and processed in a special buffer, then an aliquot from this processed sample is added to PCR reaction for amplification and detection. This is a very simple method for sample collection, it does not need any technical training and anybody can use the technique to collect the sample, blot it on the membrane in the field and send the blotted membrane to the laboratory for processing and testing. If necessary samples prepared by this method could be stored for a long time before processing for PCR detection.

Objective 3: In order to develop a reliable PCR detection methodology, it is very important to identify variations among different isolates (or strains) of each one of these GLRaVs and accordingly design PCR primers that could detect all diverse isolates. We maintain a collection of GLRaVs here in Davis and have evaluated the reactivity of number of these isolates selected from different types of GLRaVs by ELISA. In the past year we reported the results of our investigation for GLRaVs-2 and –3. In year 2001-2002 we focused on GLRaVs-1, -4 and –5. For GLRaV-1, 10 different isolates were selected and their coat protein gene were amplified, sequenced and compared. The coat protein of this virus is consisted of 680 nt. This comparison showed that these isolates were quite similar and the percent similarity among them ranged from 95-99% at the coat protein region. For GLRaV-5 we had access to only 5 isolates. We designed a pair of PCR primer that could amplify the coat protein. We used this pair of primer, amplified the coat protein gene and sequenced. This sequence consisted of 786 nt. Sequence comparison among these 5 isolates showed 95-99% homology indicating that these tested viruses were quite homogeneous too.

In this sequencing and sequence comparison experiments, we found 3 and 2 GLRaV-1 and GLRaV-2, respectively, which tested positive by PCR when the specific detection primers were used but we were not able to amplify and sequence their coat protein gene for comparison in this study. In a different test, we found 3 each of GLRaV-1 and –2 which were tested positive by ELISA and having quite high reading in this test but tested negative by PCR detection primers, and unable to amplify and sequence their coat protein gene for this study. The observations indicated that these viruses may have some significant differences in their sequences compared to the one which have been studied. Therefore, the sequences of available PCR primers do not completely match their sequences to amplify the targeted segment of their genome. However, we are planning to design number of different primers from the already known coat protein sequence and use these primers to amplify pieces of this gene for the above mentioned isolates, sequence and study them in more detail. If this method failed, we will use other strategies, e.g. to walk through the gene by using one of the already available detection primers or use the random priming and cloning method. It is quite crucial to know the extent of the genetic variation among these isolates in order to be able to design efficient and reliable PCR primers that could detect different strains of these viruses. For GLRaV-4, the coat protein sequence is not available yet. We attempted several times using different strategies to clone and sequence part or the genome of this virus including the coat protein gene but all our efforts failed. We used isolate LR106 in our investigation. The vine infected with this virus seemed to be singly infected with GLRaV-4. However, we tried to extract virus-specific dsRNA and use in our cloning and sequencing, but we could not obtain enough dsRNA for our experiment due to the low titer of virus in the host. We increased the amount of tissue used for this purpose, but again the efforts failed. Some preliminary data indicated that this virus is closely related to GLRaV-5, therefore, we tried to use the coat protein sequence information available for this virus and design degenerate PCR primers and use them for amplification of the coat protein of GLRaV-4. In this effort we obtained a PCR product from GLRaV-4 infected vine with expected size revealed in gel electrophoresis, but when we sequenced the product and compared it with other GLRaVs, it did not show any homology with any of these viruses. However, we are planning to continue our efforts in this regard.

Objective 4: For this objective, it is required to design biotinilated probes which will have homology with part of the PCR product. These probes will be designed and tested in the following year as soon as we gather more information in regards to GLRaVs-1 and -2 (when determine the variation among their different isolates as described in objective 3).

To further evaluate the reliability and sensitivity of PCR procedures, beginning fall of 1999 we started testing all quarantine and newly introduced grape selections (brought to FPMS for disease testing and elimination) by PCR for different viruses including GLRaV 1-5. The results from these tests were compared with other available detection methodologies including ELISA, and indexing on field indicator (Cabernet Franc) for leafroll disease. Comparison of different testing methodologies for 137 of these plants is summarized in Table 1. The ELISA system available for GLRaV-5 is not reliable and develops high background, therefore, we decided not to include this test. Overall, as it is shown in Table 1, PCR performed much better and detected larger number of plants infected with GLRaVs-1 to -5 especially in regards to GLRaVs-2 and 4.

Table 1: Comparison between ELISA and PCR in detecting GLRaVs-1 to 5 in grapevine tissue. Total of 137 vine were selected in this experiment and tested on Cabernet Franc for the presence of leafroll disease and 26 of them showed the symptom in the field. These samples were later tested by ELISA and PCR and the results are summarized in the table below.

TEST	LR-1**	LR-2	LR-3	LR-4	LR-5	TOTAL
ELISA	2	1	12	1	N/A	16
PCR	2	7	14	6	12	41

** LR= grapevine leafroll associated virus.

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. Routh, G., Zhang, Y.P., Saldarelli, P., and Rowhani, A. 1998. Use of degenerate primers for partial sequencing and RT-PCR-based assay of grapevine leafroll-associated viruses 4 and 5. Phytopathology 88: 1238-1243.
3. 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.
4. Rowhani, A., Chin, J., Zhang, Y.P., Biardi, L., and Golino, D.A. 1999. Simplified extraction method for sample preparation for PCR and development of a colorimetric detection

technique for analysis of PCR products of virus-infected grapevine. Am. J. Enol. Vitic. 50: 374.
5. Zhang, Y.P., and Rowhani, A. 2000. A strategy for rapid cDNA cloning from double-stranded RNA templates isolated from plants infected with RNA viruses by using Taq DNA polymarase. J. Virol. Methods 80: 59-63.

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Concise General Summary of Current Years Results

- Specific PCR primers for GLRaVs 1 through 5 were designed, tested and optimized. One step RT-PCR methodology was also developed and the reagents and testing conditions were optimized.

- A simple blotting methodology for sample collection in the field was developed. Leaf petioles or young shoots are cut and blotted on specific nylon or nitrocellulose membrane and then these membranes are brought to the laboratory for processing and virus detection. The advantages of this method are: no technical training is required, and if necessary, the samples can be stored for a long time before they are prepared and tested.

- In order to develop a reliable RT-PCR detection method, it is very important to identify variations among different isolates (or strains) of each one of these GLRaVs and accordingly design PCR primers that could detect all diverse isolates. In the past year, sequences from the coat protein gene from 10 and 5 different isolates of grapevine leafroll associated virus (GLRaV) -1 and -5, respectively, were compared. The number of nucleotides which were compared from the coat protein gene were 680 and 786 nt for GLRaV-1 and -5 respectively. All GLRaVs-1 isolates used in this study showed 95-99% homology in their compared sequences indicative of a homogeneous population. We had the same observation with GLRaV-5 with again 95-99% homology.

- Reliability and sensitivity of RT-PCR for the detection of GLRaVs-1 to -5 was compared with ELISA and with symptom expression of leafroll disease on its biological indicator. From 137 vines tested in this experiment, 26, 16, and 29, respectively tested positive on biological indicator, ELISA, and RT-PCR. The results indicate that the RT-PCR is more sensitive than either indexing on biological indicator host or ELISA. Counting RT-PCR test for GLRaV-5 in this experiment (did not have access to a reliable ELISA reagent for this virus), we found that total of 41 vines were tested positive by PCR which again outnumbers biological index on indicator (27 plants).