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FINAL REPORT

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**Etiology of the Rugose Wood Disease of Grapevine
and Genetic Manipulation of the Associated Vitiviruses**

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Project Title: Etiology of the Rugose Wood Disease of Grapevine and Genetic Manipulation of the Associated Vitiviruses

Investigators

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Signature
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Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings			1	1
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Description of Cooperation:

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Submitted				
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Final Scientific Progress Report

Abstract

Rugose wood (RW) complex is a group of grapevine diseases, which spread in most vine growing areas. Mostly, vines are infected with multiple viruses, and the composition of the mixture can have profound effects on disease expression, generally, involving synergistic effects on symptom expression. The *Vitivirus* members, *Grapevine virus A* (GVA) and *Grapevine virus B* (GVB), are probably of the most common components of RW complex, though, additional elongated-phloem-associated viruses were also implicated. Because of the complexity of RW, the role of GVA and GVB in the etiology of the disease has not been determined conclusively. Our research aimed to develop the tools that allow study the etiology of the rugose wood disease.

The Israeli laboratory engineered an infectious cDNA of GVA tagged with GFP reporter gene into pCAMBIA vector between the CaMV 35S promoter and a transcriptional terminator. When this clone was examined by agroinfection of *Nicotiana benthamiana* plants, high levels of GFP expression were detected by means of confocal microscopy. This places us in a unique position to address problems of GVA biology and pathology and to apply this GVA vector for evaluation of viral gene effects on virus replication, accumulation, spread in infected vines, host range determination and symptomatology. In this research we aimed to construct, as well, a stable infectious clone of GVB. Using GVA and GVB infectious clones we planned to develop a strategy to infect grapevine plants that enables us to examine effects of single- and mixed-infection of grapevines with GVA and GVB.

By the end of the project we have achieved:

1. Cloning and sequencing the entire genome of a GVB isolate
2. Assembly of a full-length and infectious GVB clone.
3. Confirm infectivity of the GVB clone
4. Develop efficient method to infect micropropagated grapevine plantlets with each of the cloned GVA and GVB.
5. Apply VIGS in *N. benthamiana* and grapevine plants, providing a system that can be used for functional genomics.
6. Examining gene functions of GVA p10 and GVB p14 in suppression of RNA silencing and pathogenesis.

These outcomes of our experiments are expected to provide information on the genetics of these poorly characterized viruses and their role in RW disease.

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Achievements

Cloning and sequencing of GVB genome:

In this project we took an advantage of the GVB 94/971 isolate which was isolated from corky bark-diseased grapevine plants. Plants of *N. benthamiana* were used to propagate and maintain this isolate under greenhouse conditions. The full sequence of GVB 94/971 was determined and deposited in the GenBank databases under the accession number EF583906.

Construction of a full-length and infectious GVB clone:

The full-length cDNA of GVB 94/971 was assembled and cloned into pUC57 vector under the T7 RNA polymerase promoter to generate pGVB21. The DNA of pGVB21 was used for *in vitro* transcription. Freshly prepared *in vitro* transcripts were used for inoculation of *N. benthamiana* protoplasts and plants. Replication of GVB21 was confirmed by Northern and Western blot analyses. For inoculation via agro-infiltration the cDNA of GVB21 was inserted into a binary vector, pCAMBIA2031 with double 35S promoters and a terminator. The resulting clone, pCAM-GVB33, was used to infect *N. benthamiana* and *N. occidentalis* leaves by means of the Agroinfiltration. Infection of pCAM-GVB33 was confirmed by monitoring symptoms development and by Northern and Western blot analyses.

Thus, the cloned GVB RNA was found to be infectious in plants. It is now routinely maintained in herbaceous plants by means of plant-to-plant passages.

Examining vitiviruses-mediated gene silencing:

- VIGS in *N. benthamiana*

The vector GVA-118, which was developed in the Israeli lab, was designed to express the foreign gene through the MP-sgRNA promoter. A 500 nts product of the PDS gene of *N. benthamiana* (NbPDS) was cloned into the GVA vector, followed by assembly of the construct into pCAMBIA2301. When tested, plants inoculated with the GVA-NbPDS started to display phenotypes that were confirmed by hybridization and RT-semi-quantitative PCR analyses to be associated with the silencing of the endogenous PDS gene. The photobleaching symptoms were prominent in the midrib and lateral veins. Quantification analysis revealed that the mean level of PDS mRNA in the photobleached veins was 85% lower than that in the green leaf veins.

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Attempts to infect *Vitis vinifera* plants with GVA and GVB:

It proved difficult to inoculate mature grapevine plants using the *Agrobacterium*-mediated procedures. Several inoculation procedures were tested to achieve plant infection with cloned GVA or GVB clones, including stem-slashing, rubbing or biolistic bombardment. However, none of the inoculated plants became infected.

The next strategy was to examine agroinfection of micro-propagated *Vitis vinifera* cv. Prime plantlets, using an agrodrenching method. Two-week-old *V. vinifera* cv. Prime plantlets were detached and their roots were wounded and inserted into tubes with filter papers immersed in an MS liquid medium containing a suspension of *Agrobacterium* carrying the cloned GVA or GVB. Ten days later, the plantlets were transferred to fresh liquid media supplemented with cefotaxime. The agrodrenched plantlets were examined for virus infectivity by means of RT-PCR and electron microscopy 28 days after the agrodrenching (dpd). When applied this inoculation protocol, we found that approximately 50% of the tested plantlets (>200) became infected by 28 dpd, as indicated by RT-PCR analysis.

GVA or GVB-infected plantlets were selected to be transferred and maintained under greenhouse conditions. Plants were examined for GVA and GVB infection once per a month and were found to be infected with GVA and GVB, respectively.

For conclusion, in this BARD project we ended with *V. vinifera* plants which are infected with pure population of GVB or GVA. These plants will enable us in the near future to examine the role of GVA and GVB and their mixed infection in RW and disease development.

- VIGS in *V. vinifera*

The feasibility of the agrodrenching technology for infecting micropropagated plantlets led to the examination of VIGS in *V. vinifera*, as well. A fragment of the PDS gene was amplified from *V. vinifera* cv. Prime and inserted into the GVA vector, followed by assembly into pCAMBIA2301. A suspension of *Agrobacterium*-carrying pGVA-VvPDS was used to agrodrench micropropagated *V. vinifera* plantlets. Infection of the inoculated plantlets was confirmed by RT-PCR. Plantlets inoculated with GVA-VvPDS began to display photobleaching symptoms that were attributed to silencing of the endogenous gene PDS at 14-20 dpd. These symptoms were exhibited by 40-60% of the inoculated plantlets, and appeared mainly in leaf veins and at leaf margins. VIGS and decrease in the level of PDS mRNA in photobleached leaves of *V. vinifera* plantlets were confirmed by means of hybridization and RT-semi-quantitative PCR analyses.

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Analyses revealed that the PDS mRNA levels in the photobleached leaves were, on average, 70% lower than those of the green leaves.

In conclusion, VIGS considered being a powerful tool for studying the functions of host genes. Until recently, the only VIGS vectors available were mainly suitable for use in annual plants. To the best of our knowledge, the GVA-derived VIGS vector is the first to be used for silencing the endogenous PDS genes in both *N. benthamiana* and *V. vinifera* plants. Unlike in most of the known VIGS vectors, silencing of the PDS gene in *N. benthamiana* via the GVA-derived VIGS vector is apparently restricted to the leaf veins, which could limit the use of this tool for genes essentially confined to the veins.

Application of silencing vectors to the woody grapevine plant would enable high-throughput analysis of gene function in this important crop. The ability to induce VIGS depends on the ability of the virus vector to infect the plant tissue, but it is difficult to infect grapevine with cloned GVA by means of the standard *Agrobacterium*-based delivery strategies. The results indicate that we have overcome these inoculation difficulties by adapting the agroinfiltration method for the inoculation of micropropagated *V. vinifera* plantlets and for silencing the endogenous PDS gene through the GVA vector, thereby providing a method and a tool for reverse genetic studies of a variety of processes occurring in micropropagated *V. vinifera* plants. In addition, this research project resulted in the generation of grapevine plants infected with a pure population of GVA derived from a cloned infectious cDNA sequence. This development is of major importance for the investigation of molecular events associated with viral infections responsible for disease establishment and the development of disease symptoms.

Studying viral gene functions:

The availability of GVA and GVB infectious clones enabled us to design experimental analysis to study their gene expression and functions in infected plants. Each of ORFs 2, 3, 4, and 5 of GVB were cloned into pET28 expression vector, under the T7 RNA promoter. Viral proteins were expressed into BL21 *E. coli* cells. Proteins were recovered by SDS-PAGE, and polyclonal antibodies were prepared.

When tested for specificity, with Western analyses using total proteins extracted from GVB-infected *N. benthamiana* plants, antibodies raised against ORF3 and 4 products reacted with the MP and CP of GVB respectively. However, the Western analyses failed to detect specific reactions with antibodies raised against ORF2 and ORF5 products.

ORF5 products of GVA and GVB as suppressors of gene silencing:

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Our work suggested that GVA p10 and GVB p14 are involved on pathogenesis and suppression of RNA silencing. To identify components that are involved in suppression of RNA silencing, we used the GFP-tagged GVA-minireplicon which was developed in the Israeli lab. This mini-GVA replicon was generated by deleting the sequences of ORF2, ORF3 (the MP gene), ORF4 (CP gene) and most ORF5, retaining the sequences of ORF1 and the 5' and 3' UTR (essential for replication). Agroinfiltration of the minireplicon into leaves of *N. benthamiana* revealed, at 7dpi, only a few cells with green fluorescence due to activation of the RNA gene silencing response. Yet, co-infiltration of the minireplicon with GVA p10 resulted in 20 folds increase of higher green fluorescence, suggesting enhancement of activity of p10 as an RNA silencing suppressor. When the GVB ORF5 product (p14) was examined for its ability to function as RSS with the presence of the GVA-minireplicon, similar results and conclusions were obtained. GVB p14 possesses a strong RSS activity with the presence of the minireplicon. Taken together, these results suggested that ORF1 acts as an element, which still needs to be determined, for enhancement of GVA p10 OR GVB p14 activities as RSS.

Description of the Cooperation:

The principle investigators were in contact via email. While attending the ICVG meeting in Stellenbosch in South Africa, the PI and the co-PI have met and discuss the results and planned the experiments of the project.

Information regarding GVA, GVB and Vitiviruses and also regarding primers, clones and antibodies of GVA and GVB was exchanged.