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Project Title:

Development of virus induced gene silencing tools for functional genomics in grapevine

Investigators

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Abbreviations commonly: GFP, green fluorescent protein; GRSPaV, *Grapevine rupestris stem pitting-associated virus*; GVA, *Grapevine virus A*; ORF, open reading frame; VIGS, virus-induced gene silencing.

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Detailed description of the research activities and results

The aim of our project was to engineer each of *Grapevine virus A* (GVA) and *Grapevine rupestris stem pitting-associated virus* (GRSPaV) into VIGS vectors to be used for functional genomics in grapevine in Israel and Canada, respectively. The objectives of our proposed research were:

1. Construction of GVA- and GRSPaV-VIGS vectors.
2. Development of efficient methods for grapevine inoculation.
3. Functional genomics in *V. vinifera*.

The research is designed to establish VIGS systems based on two different viruses to be used for functional genomics in grapevine. The first and second objectives do address construction of such VIGS systems based on GVA and GRSPaV and establish efficient strategies to inoculate grapevine plants with these viral vectors. The use of two different viral systems enables each party involved to work with their local viruses. The third objective addresses the application of these VIGS vectors in silencing endogenous grape genes.

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Israel:

Construction of GVA-based VIGS vector:

In the first year of the project we developed a GVA vector, which contained a heterologous MP-sgRNA promoter derived from a distinct GVA isolate. This vector was found to be efficient for expression of the foreign gene up to six plant-to-plant passages, which probably might not be adequate for experiments with grapevine plants, where virus would persist for a long time. Therefore, to improve the stability and efficiency of the GVA vector, we swapped the heterologous MP-sgRNA promoter and constructed GVA vectors in which the foreign sequences are transcribed through a promoter obtained from the *Grapevine virus B* (GVB), a closely related virus belongs to same genus *Vitivirus*. For this, we chose the promoter of the GVB CP-sgRNA and swapped it with the duplicated promoter in the earlier GVA vector. The GVA-GVB vector was then examined as an expression vector in *N. benthamiana* plants, using the GFP reporter gene. We tested the stability of GVA-GVB vector, and the GFP sequence in inoculated plants generated through several plant-to-plant passages. We found that the foreign gene was stable in the GVA-GVB vector for more than ten plant-to-plant passages. Infection and stability of the GVA variants were carried out by examining viral RNA accumulation by means of RT-PCR and hybridization analyses, as well as by employing confocal laser scanning microscopy to determine GFP expression. The GVA-GVB vector was used for this research project to express *V. vinifera* endogenous genes for studying VIGS as it will be described.

Inoculation of V. vinifera:

Because mechanical inoculation of grapevines with GVA is problematic, we employed a much more efficient agro-infection procedure. The GVA-GVB vector (tagged with GFP) was cloned into a binary vector (pCAMBIA2031) under the CaMV 35S promoter. The GVA-GVB-GFP in the binary vector was introduced into *Agrobacterium*. Next we applied strategies involving drenching of roots of young grape plantlets maintained in tissue culture (agro-drenching). Infectivity of plantlets was evaluated by RT-PCR and confocal microscopy. We are encouraged by our experiments with *V. vinifera*, which revealed efficient infection rate in the inoculated plantlets which reached up to 80%. During the second and third year of the project, we continued on improving the protocol to infect *V. vinifera* through roots inoculation method by introducing some modification for the inoculation protocol and shortened the procedure to achieve mature plants from the inoculated plantlets (see Figure A below).

Figure A: RT-PCR screening of 9 grapevine mature plants inoculated with GVA-GVB based vector. Lane PC, positive control.

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Functional genomics in V. vinifera:

A GVA-vector carrying a DNA fragment containing 500 nts of grapevine PDS gene has been constructed in our laboratory and found to be effective in silencing PDS in micropropagated grapevine plantlets. In the light of this result, we cloned several endogenous genes derived from *V. vinifera*, such as *VvDELLA1*, 2, & 3 that control expression of Gibberellins in grapevine and the genes *VvANR*, *VvLARI* (which were reportedly related to the quality of wine; its color, flavor, mouth-feel and aroma), *VvUFGT*, *VvmybA1*, *VvPP2C1*, *VvPP2C2*. Each of these genes was introduced into the GVA-GVB vector. The resultant vectors were then use to infect grapevine plantlets as described above. Analysis of silencing of these genes in inoculated plantlets was conducted at the molecular levels using semi-quantitative and real-time PCR assays in the second and the third years of the project.

To examine effect of silencing *VvLARI* gene, total RNA was extracted from plantlets inoculated with GVA-GVB vector carrying *VvLARI* fragment and used it for cDNA synthesis with the primer 18dT, followed by a semi-quantitative PCR assay using primers specific to *VvLARI*. As a control, we performed a semi-quantitative PCR procedure using cDNA performed for plantlets inoculated with GVA-GVB vector lacking the *VvLARI* fragment. The results showed remarkably less amplification of the plant *VvLARI* mRNA from the plantlets inoculated with GVA-GVB vector carrying *VvLARI* fragment (Lanes 1-3 in the Figure B below) than those plantlets inoculated with GVA-GVB vector lacking the *VvLARI* fragment (Lanes 4-5).

Figure B: Semi-quantitative PCR analyses conducted for mature grapevine plantlets inoculated with GVA-GVB vector carrying VvLARI fragment (Lanes 1-3) and for plantlets inoculated with GVA-GVB vector lacking the VvLARI fragment (Lanes 4-5)

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Quantitative studies measuring the reduction of mRNA levels in silenced plants: VIGS have been reported to reduce mRNA levels up to 99.5%. However, levels of reduction vary and may depend on the expression level of the endogenous gene. For instance, highly expressed genes may be more efficiently silenced than genes that are expressed at low or moderate levels. To quantify mRNA levels we applied real-time PCR assays. To this end, and to examine the effect of silencing *VvDELLA1*, 2, & 3 that control expression of Gibberellins in grapevine, total RNA was extracted from plantlets inoculated with GVA-GVB vector carrying three fragments corresponding to the *VvDELLA1*, 2, & 3 genes and used it for cDNA synthesis with the random primer, followed by PCR and Real-time PCR assays using primers specific to one of the *VvDELLA1*, 2, & 3 genes. As a control, we performed these assays using cDNA performed for plantlets inoculated with GVA-GVB vector lacking the *VvDELLA1*, 2, & 3 fragments.

The results showed remarkably less amplification of the plant *VvDELLA3* mRNA from the plantlets inoculated with GVA-GVB vector carrying the three fragments (Figure C below).

Figure C: Quantification of DELLAs mRNA levels in *plantlets* infected with GVA. Grapevine plantlets were infected with GVA virus vector inserted with DELLAs genes (1+2+3) and grown in culture conditions for two months before transferred to greenhouse. Plantlets infected with virus only used as control (549-4). RNA extraction was conducted 30 days post infection (dpi). Real -Time PCR was carried out on samples (experimental and control) of three plant each (n=3). Rotor-Gene Q Software was used for data analysis ($\Delta\Delta C_T$). The results above demonstrate one of two experiments showing similar pattern.

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To examine effect of silencing *VvDELLA1*, 2, & 3 in mature grapevine, grapevine plantlets which were infected with GVA virus vector carrying fragments of the three *VvDELLA1*, 2, & 3 genes were selected and transferred to greenhouse. Plants were tested for phenotype appearance by monitoring growth, and measuring their sizes. Also, total RNA was extracted from these plants and used for cDNA synthesis with the random primer, followed by PCR and Real-time PCR assays using primers specific to one of the *VvDELLA1*, 2, & 3 genes. As a control, we performed these assays using cDNA performed for plants inoculated with GVA-GVB vector lacking the *VvDELLA1*, 2, & 3 fragments.

The results showed remarkably less amplification of all the plant *VvDELLA1*, 2, & 3 mRNAs in plants inoculated with GVA-GVB vector carrying the three fragments (Figure D below).

*Figure D: Quantification of *VvDELLA1*, 2, & 3 mRNA levels in mature grapevine plants infected with GVA carrying fragments of the *VvDELLA1*, 2, & 3 genes. RNA extraction was conducted four months post infection. Real-Time PCR was carried out on samples (experimental and control) of three plants each (n=3). Rotor-Gene Q Software was used for data analysis ($\Delta\Delta C_T$). The results above demonstrate one of two experiments showing similar pattern.*

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Interestingly, mature grapevine plants, which were inoculated with the GVA vector carrying fragments of the endogenous *VvDELLA1*, 2, & 3 genes, show larger foliage than plants inoculated with GVA without the endogenous genes. Figure E below shows photos of such plants 6 months after inoculation. Figure F summarize the effect of silencing the endogenous *VvDELLA1*, 2, & 3 genes on the growth of mature grapevine plants.

*Figure E: examining the effect of silencing the endogenous *VvDELLA1*, 2, & 3 genes on the growth of mature grapevine plants. MS: left side: Mock inoculated plants; right side: plants inoculated with GVA vector carrying endogenous *VvDELLA1*, 2, & 3 sequences*

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Examining the effect of silencing endogenous DELLA genes 1, 2, & 3 on tallness of grapevine plants

Figure F: examining the effect of silencing the endogenous *VvDELLA1*, 2, & 3 genes on the growth of mature grapevine plants. MS: Mock inoculated plants; 549-4: plants inoculated with GVA vector that does not include endogenous *VvDELLA1*, 2, & 3 sequences; DEL1+2+3: plants inoculated with GVA vector carrying endogenous *VvDELLA1*, 2, & 3 sequences

Summary:

- In this research we have assembled GVA vectors that were able to express foreign genes or sequences through a viral sgRNA promoter derived from the closely related virus GVB.
- We have developed efficient protocol to infect grapevine plantlets with GVA-derived clones through roots.
- We assembled various GVA vectors carrying various endogenous sequences derived from *VvLAR1* and *VvDELLA1*, 2, & 3 genes.
- Using semi-quantitative PCR assay, we showed silencing of the endogenous *VvLAR1* gene in plants inoculated with GVA vector carrying *VvLAR1* sequences.
- Using real-time quantitative PCR assay, we showed silencing of the endogenous *VvDELLA1*, 2, & 3 genes in plants inoculated with GVA vector carrying *VvDELLA1*, 2, & 3 sequences.
- We showed phenotypic effect of silencing of the endogenous *VvDELLA1*, 2, & 3 genes in plants inoculated with GVA vector carrying *VvDELLA1*, 2, & 3 sequences.

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Canada:

U. Guelph

In the research grant proposal, we had identified three objectives for the research on GRSPaV (please see the research proposal for details of the objectives). In the past three years, we have made good progress with the first two objectives. We are pleased that some of the work conducted in this research was published in the high-impact journal of the field, *Virology* (Meng et al., 2013. *Virology*, 435, 453-462). Because of the lack of systemic infection in herbaceous experimental systems and the long time and the low frequency of GRSPaV in reaching systemic infections in grapevine, we did not see any benefit in pursuing the third objective as originally planned. Below is a summary of what we have achieved in the past three years on our part of the project involving GRSPaV.

I. Construction of full-length viral cDNA clone of GRSPaV and two GFP-tagged variants in the binary vector pCambia. A schematic diagram for the three viral constructs is shown in Fig. 1.

At the start of this project, we had made a full-length viral clone based on a pUC18 based vector for rub-inoculation of plants. However, we discovered that rub-inoculation is very inefficient. Because of the high efficiency of infection through agrobacteria inoculation, we sought to subclone the full-length clone pRSP-28 into the binary vector, pCambia-1390. Because this is a binary vector, it can replicate in both *E. coli* for cloning and subsequently manipulations and in *Agrobacterium tumefaciens* for inoculation based on agro-infiltration. Briefly, the DNA fragment corresponding to the 35S promoter sequence and the first 1420 bp of the viral cDNA was PCR amplified from pRSP-28 with primers 35S-Xba and Bam1420. After digestion with XbaI and SpeI (there is a SpeI site in the viral cDNA sequence that is flanked by the BamHI site), the PCR product was cloned into pCambia-1390, resulting in intermediate plasmid pCamRSP5'-3. Restriction site for SmaI was introduced into the above plasmid via site-directed mutagenesis, resulting in plasmid pCamRSP5'Sm-3. Subsequently, the cDNA fragments flanked by SpeI and SmaI were released from pRSP-28 through restriction digestion and cloned into pCamRSP5'Sm-3, resulting in the full-length clones pRSP28-2_(Cam).

To create pRSP-GFP2_(Cam), the recognition sites for BamHI, AgeI and KpnI were introduced before the start codon of ORF5 (encoding the capsid protein) via site-directed mutagenesis using an intermediate plasmid containing the cDNA fragment that corresponds to the 3' half of the viral genome. A DNA fragment of 97 bp containing the putative promoter for subgenomic RNA for the CP gene was obtained from a distinct strain, GRSPaV-BS, through PCR and inserted into the KpnI site. The EGFP sequence was amplified via PCR and inserted into the BamHI site. Subcloning was then conducted to produce pRSP-GFP2_(Cam). In this construct, the GFP expression is under the transcriptional control of the native promoter for the CP gene, which would produce a non-fused GFP as well as all viral proteins required for the replication of the virus upon infection.

To create pRSP-CP:GFP_(Cam), the restriction site for BamHI was first introduced right before the stop codon of ORF5 through site-directed mutagenesis using an intermediate plasmid containing the 3' half of the viral genome as described above. Subsequently, the eGFP sequence was inserted into the BamHI site. Upon subcloning, we produced the recombinant viral clone, pRSP-CP:GFP_(Cam). Unlike clone pRSP-GFP2_(Cam), this construct would express a fusion protein between CP and GFP where GFP was fused to the C-terminus of the CP.

The availability of these three binary vector based full-length clones laid the foundation for downstream assays for the infectivity of these clones in both grapevine and herbaceous

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experimental hosts. Because the GFP-tagged clones offer the advantage of the autofluorescent reporter GFP, we focused the infectivity assays using these two GFP-tagged viral clones.

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II. Infectivity assays of full-length GRSPaV clones in grapevine using three

inoculation methods. The positive results from agro-infiltration of *N. benthamiana* clearly showed us that the viral clones are infectious (see section III below). This prompted us to conduct infectivity assays using the natural host, grapevine. It is extremely difficult to inoculate woody plants such as grapevine plants. To explore the potential effectiveness of alternative methods for inoculation of grapes, we attempted four methods: (1) rub-inoculation of seedling grapevine plants with purified plasmid DNA; (2) vacuum infiltration of plantlets generated from green cuttings of *V. rupestris* “St. George”; (3) agro-infection of plantlets derived from in vitro tissue culture; and (4) biolistic bombardment of grapevine plants derived from green cuttings. The first two methods were conducted at the University of Guelph. Because the third method has not been established in my laboratory at the University of Guelph, it was performed through collaboration in the laboratories of Dr. Mawassi at the Volcani Center, Israel and Dr. Stobbs at AAFC, Vineland, Ontario. We have found that only the third method was effective in initiating infection through delivery of viral clones. However, based on the data that was obtained, it seems that pRSP-GFP2_(Cam) took a long time (six months) to cause systemic infection and that the percentage of systemically infected grapevine plants was very low. It is possible that the manipulations to the viral clone (insertion of the GFP gene and the subgenomic promoter from the distinct strain GRSPaV-BS) might have caused the defects in either virus genome replication or movement. Dr. Mawassi’s laboratory conducted another round of inoculation experiment toward the end of 2012. However, no positive results have been obtained. To test the feasibility of biolistic bombardment for infectivity assays, we have initiated this experiment during summer 2012 in collaboration with Dr. Stobbs’ laboratory. Initial test did not reveal presence of the virus through RT-PCR. To increase virus titer, the inoculated plants were put to dormancy in cold storage and will be tested again this year.

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Inoculate grapevine plants were observed for expression of GFP with fluorescence microscopy, Western blotting with anti-GFP antibodies, and RT-PCR using virus specific primers. So far, conclusive data were obtained in grapevine plants that were inoculated with the third method. After 30 days post infection, GFP was clearly detected in roots of plantlets derived from two varieties, 'Thompson' (Fig. 3A) and 'Prime' (Fig. 3C). RT-PCR test confirmed the presence of viruses in the same tissue where GFP was detected by fluorescence microscopy (Fig. 3B). As to the rub-inoculated grape seedling, we detected a faint amplification product in only some of the non-inoculated systemic leaves with RT-PCR and western blotting (Fig. 3C and data not shown). No evidence of infection was obtained in any of the grape plants that were inoculated through vacuum infiltration through fluorescence microscopy and western blotting.

III. Infectivity assays of GRSPaV clones in herbaceous experimental plants.

III-1. Infectivity assay through fluorescence microscopy: Taking advantage of the autofluorescent GFP tag, we assayed for the infectivity of the recombinant viral clones tagged with GFP. Because the GFP expression can only occur from a subgenomic RNA, which can only be produced upon successful replication of the virus, expression of GFP would be an indication of successful replication of the full-length viral clone. To this end, we delivered the pRSP-GFP2_(Cam) and pRSP-CP:GFP_(Cam) into *A. tumefaciens* strain EHA105 through electroporation. Agrobacterium cultures containing the introduced viral clones were infiltrated using a needle-less syringe into leaves of *N. benthamiana*. GFP expression was assayed by observation of green fluorescence using confocal laser scanning microscopy and Western blotting using anti-GFP antibody that is commercially available. As a positive control, we also used a GFP-tagged PVX infectious viral clone. As shown in Fig. 2, pPVX-GFP produced clear GFP in cells of upper leaves of *N. benthamiana* (Fig. 2A). In leaves of *N. benthamiana* infiltrated with pRSP-GFP2_(Cam) and pRSP-CP:GFP_(Cam), we also observed clear green fluorescence starting at 2 days post infiltration. Interestingly, at later time points, agro-infiltrated cells produced rather large globular bodies (Fig. 2B). To ensure reproducibility, we repeated the experiments two more times and observed similar results.

To corroborate this data, we conducted western blotting using anti-GFP antibody. As shown in Fig. 2C, the expected GFP protein band was detected in the leaves of *N. benthamiana* at 1, 2, 3, and 7 days post-infiltration (Fig. 2C, lane 2-5). We also observed that the amount of GFP increased with time (Fig. 2). As expected, strong GFP bands were detected in extracts from leaves *N. benthamiana* infiltrated with pPVX-GFP (Fig. 2C, lanes 7-9). GFP was not detected in leaves of *N. benthamiana* infiltrated with buffer (Fig. 2C, lane 1).

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III-2. Infectivity assay through electron microscopy: We further verified infectivity of our viral clones through visualization of virus particles through transmission electron microscopy. Extracts were made from tissue taken from infiltrated leaves of *N. benthamiana* as well as from upper, non-infiltrated leaves, followed by EM observation. Filamentous virions of the expected size of 700 nm were consistently observed in infiltrated leaves. To verify that these virions were specific for GRSPaV, immunogold labeling was performed, followed by electron microscopy. As shown in the figure below, these virions are clearly labeled with gold particles, confirming that they are of GRSPaV. We have noticed that these virions exhibit various length, likely due to the tendency of breakage as reported earlier (Petrovic et al., 2003). In some cases, we have observed possible dismantling of virions. Light fixation of leaf extract with glutaraldehyde appears to stabilize virions. Also, these virions seem to be slightly thinner compared to those of PVX. Together, these observations confirmed the earlier finding that GRSPaV virions were unstable (Petrovic et al., 2003). The successful observation of GRSPaV virions from infiltrated leaves suggests that the full-length viral clone is indeed infectious, at least in the infiltrated leaves of *N. benthamiana* plants. Transmission electron microscopy of extracts derived from leaves infiltrated with pRSP-GFP2_(Cam) also revealed the presence of virions that are indistinguishable from those produced by pRSP28-2_(Cam). We have noticed that the number of virions present in *N. benthamiana* leaves infiltrated with pRSP-GFP2_(Cam) is much lower than that from *N. benthamiana* leaves infiltrated with pRSP28-2_(Cam), suggesting that the heterologous subgenomic promoter introduced upstream of the CP gene in pRSP-GFP2_(Cam) is not as efficient as the native subgenomic promoter. These data suggest that the GFP-tagged viral clone, pRSP-GFP2_(Cam), is infectious in leaves of *N. benthamiana* upon agro-infiltration. Interestingly, no virus particles were ever observed in extracts derived from upper, non-infiltrated leaves. This suggests that the virus is not able to cause systemic infections in this host.

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III-3. Infectivity assay through Northern blotting: Based on the genomic structure of GRSPaV, it is expected that viral proteins corresponding to ORFs 2-5 will be expressed from three subgenomic RNAs. To further demonstrate the infectivity of the viral clones, we conducted Northern blotting using radioisotope-labeled riboprobe targeting the 3' terminal 430 nucleotides of the viral genome. A series of Northern blot hybridization experiments were conducted to find out the optimal conditions for the Northern blot hybridization, which include different temperatures for hybridization and washing, exposure time, the amount of total RNA to be loaded, and the conditions for transfer of RNA from agarose gel to nitrocellulose membrane. We were able to detect some signals corresponding to the genomic RNA and perhaps even the subgenomic RNA for the viral capsid protein. However, due to the non-specific hybridization of the riboprobe with plant ribosomal RNAs and the low levels of viral RNAs, a definitive conclusion cannot be made at this stage. This suggests that the amount of both genomic and subgenomic RNA produced were too low to be detected.

IV. Search for a systemic experimental host for GRSPaV. Because woody plants in general take a long time to establish and as such will not be the best experimental system for biological research, we wanted to find a herbaceous plant species that may support infection by GRSPaV, both locally and systemically. Once such a plant is identified, we would be able to quickly test all experimental systems before we move on to performing the investigations on grapevines. To this end, plants of the following four species were inoculated with viral clones pRSP28-2_(Cam) and pRSP-GFP2_(Cam) through agro-infiltration: *N. occidentalis* 37B, *N. clevelandii*, *N. benthamiana*, and transgenic *N. benthamiana* expressing the movement protein of *Red clover necrotic mosaic virus* (RCNMV). Table 1 shows the growth stage and number of plants that were inoculated with buffer, or the viral clones. These plants are among the most susceptible systemic hosts for a large number of plant viruses. For example, *N. occidentalis* 37B successfully supported systemic infection of *Apple stem pitting virus*, the type member of the *Foveavirus* genus (Koganezawa, H. & Yanase, H. 1990). It has been shown that the movement protein of RCNMV can complement movement of diverse viruses including PVX and *Tobacco mosaic virus* (Dr. Steve Lommel North Carolina State University, personal communication; Morozov et al., 1997; Giesman-Cookmeyer et al., 1995). At 24 dpi, total RNAs were isolated from the young leaf on top of each of the plants and subjected to RT-PCR using virus-specific primers RSP21 and RSP22 or PDS853F and PDS1352R that are specific to the plant gene phytoene desaturase (PDS) as an internal control (for information on PDS and the PDS primers, see Section IV below). While RT-PCR using PDS primers produced strong amplification of the expected DNA, GRSPaV-specific amplification product was not detected in any of the samples (Table 1). In a separate experiment, strong viral suppressor of RNA silencing was co-infiltrated with these viral clones in a hope to facilitate viral replication and movement. However, systemic infection was not established in *N. benthamiana* plants. These results suggest that GRSPaV was unable to move long distance to cause systemic infection in upper, non-inoculated leaves in any of these four *Nicotiana* species.

Table 1. Agro-infiltration of four commonly used herbaceous plant species for viral cDNA clones of GRSPaV. For each plant, 2 leaves (1 partially expanded and 1 fully expanded) were infiltrated with either infiltration buffer or with agrobacterium suspension containing either pRSP28-2_(Cam) or pRSP-GFP2_(Cam).

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Plant species	Growth Stage	Number of Plants Inoculated with:			RT-PCR Result	
		Buffer	pRSP28-2 _(Cam)	pRSP-GFP2 _(Cam)	PDS	GRSPV
<i>Nicotiana benthamiana</i>	6	1	4	4	+	-
<i>N. benthamiana</i> -MP	6	1	4	4	+	-
<i>N. occidentalis</i> 37B	6-7	1	4	4	+	-
<i>N. clevelandii</i>	8-9	1	4	4	+	-

Together, these data suggest that the full-length viral clones, pRSP28-2_(Cam) and pRSP-GFP2_(Cam) are infectious in inoculated leaves of several species of *Nicotiana*. To our surprise and contrary to our expectation, neither clone is able to cause systemic infection in any of these plant hosts tested. This may suggest that GRSPaV, as an ancient virus native to grapevine, is unable to produce systemic infection in herbaceous plants. This further demonstrates the uniqueness of viruses infecting grapevine or many other woody perennials.

V. Development of a grapevine protoplast-based system for the study of GRSPaV.

Due to the lack of efficiency of initiating viral infection in grapevine using the infectious cDNA clones, we sought to develop alternative systems for the study of molecular and cellular aspects of GRSPaV replication and infection. To this end, we established a highly effective methodology to obtain protoplasts from a wild species, *Vitis labrusca*. We conducted a systemic experimentation to find out the best conditions for transfection of infectious cDNA clones of GRSPaV into protoplast. Two transfection methods were tested: protoplast electroporation and polyethylene glycol (PEG)-mediated transfection. We found that both methods are effective for the expression of GFP as a C-terminal fusion to the capsid protein of GRSPaV (used as a positive control for experimentation). Preliminary experiments also demonstrate that pRSP-GFP2_(Cam) is infectious in these protoplasts as judged by green fluorescence due to GFP expressed from the viral clone. Interestingly, the GFP signal is weak, suggesting the level of expression from this viral clone may be insufficient. We will conduct further experiments this summer to fully realize the potential of the grapevine protoplast system for the molecular and cellular biology study of GRSPaV. Such an experimental system would be highly useful to the grapevine virology community for investigation of other grapevine viruses.

OVERALL SUMMARY: In the past three years, we have conducted a number of research activities related to this project. We feel that we have made excellent progress with the project. We have made two full-length viral clones of GRSPaV using the binary vector pCambia-1390, one wildtype and the other tagged with GFP. We have shown that both viral clones are infectious in the natural host grapevine as well as in several experimental host species. This conclusion was based on several lines of evidence, including the visualization of virus particles, time course observation of green fluorescence due to expression of GFP, western blotting as well as northern blotting. We have tested the feasibility of four inoculation methods and concluded that one of these methods (i. e., agrobacterium-mediated inoculation of tissue cultured plantlets) is effective. Effectiveness of biolistic bombardment as an inoculation method has yet to be evaluated. We also attempted to discover a herbaceous experimental host for the study of the molecular and cell biology of GRSPaV. Unfortunately, none of the tested experimental plants is able to support systemic infection by GRSPaV. Lastly, we have also established a grapevine protoplast system. The full potential of this system for the study of GRSPaV and other grapevine viruses awaits to be revealed. This research project has also provided excellent opportunity for the training of highly qualified personnel as it supported training of a Postdoctoral Fellow, a research associate, and a couple of project students. We are very pleased that part of the work has been published in the prestigious journal, *Virology* (Meng, B., Venkataraman, S., Li, C., Wang, W., Dayan-Glick,

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C. & Mawassi, M. (2013). Construction and biological activities of the first infectious cDNA clones of the genus *Foveavirus*. *Virology* 435, 453-462). Importantly, this is the first cDNA clone ever constructed for GRSPaV or for the genus *Foveavirus*, of which GRSPaV is a member.

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Plant material:

- a. **Tissue culture:** Softwood cuttings of *Vitis rupestris* St. George certified free of Rupestris stem-pitting associated virus (RSPaV) were obtained from Dr. Jay Subramanian, University of Guelph, Vineland campus. *In vitro* tissue cultures were established from these cuttings using plant material at different stages of growth sampled from different tissues. St. George was chosen since it is a sensitive indicator variety for RSPaV. While tissue cultures could be initially established, their growth slowed and cultures later browned and died. An array of different media modifications was tried, including adjustments to the media (salts, iron, hormones, and agar concentrations), lighting, temperatures and growth cabinet humidity. Despite rigorous surface disinfection of the plant material, low level contamination persisted and could not be overcome by antibiotics. A review of the scientific literature has reported similar difficulties by other researchers with this Rupestris variety. For this reason, green shoots propagated from virus certified material were used.
- b. **Green cuttings:** Virus-certified St. George cuttings were successfully rooted and leaf material was tested by Reverse transcriptase polymerase chain reaction assay (RT-PCR) and verified for the absence of RSPaV. The material was subsequently propagated for use in inoculation tests. Other grape varieties selected from a former nuclear virus-tested block on the Jordan research farm were tested for use in the study using the direct real time polymerase chain reaction assay (DRT-qPCR). Samples were taken from different parts of the vines to determine the best sampling location. From May-mid August, 2012, plants of 7 varieties tested negative for RSPaV: Baco noir, Cabernet sauvignon, C. franc, Chardonnay, Pinot gris, P. noir, and Vidal. Wood from the mother plants was collected and propagated in the greenhouse and used for the plasmid introduction studies. However, when retested in late 2012, a few of the plants propagated from each of the mother plants from the field block tested positive for RSPaV and have been flagged in the study.
- c. **Infected Control Plant Material:** Rupestris stem pitting associated virus (RSPaV) positive dormant wood cuttings (accession 1050-02) were obtained from the Centre for Plant Health, CFIA, Saanichton, B.C. Also acquired were RSPaV cuttings of H59 and H22 from Dr. B. Meng. Rooted plants were established from these for use as positive controls. All plants tested positive for RSPaV by DRT-qPCR. Assay methodology provided by Dr. Annette Nassuth (U of G) was modified to optimize RSPaV detection.

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Introduction of the plasmid into grapevine. A number of different approaches were used to introduce the vector into grapevine:

- a. **Ballistic gun:** The BioRad Helios gene gun and BioRad's Biolistic PDS/He particle delivery system was used on young plantlets of RSPaV-free St. George as well as Chardonnay and Vidal. Three different plasmid vectors were supplied by Baozhong Meng, University of Guelph, for introduction into the plants. Vectors included (1) pRSP28-2 (CAM), a wild type with cDNA virus with Cambia binary vector, (2) pRSP-GFP-cDNA virus with a binary vector, and (3) pPVX-GFP with a herbaceous system binary vector. A total of 64 plants were injected.
- b. **Insertion of plasmid vector into stems or leaves.** Holes (1.5mm in diameter) were drilled into stems of 27 green shoot propagated St. George and actively growing Agrobacterium/vector culture scrapped from agar plates and packed into the holes and sealed with parafilm. In further experiments 20 plants, petiole and leaf vein tissue was pressure inoculated using a syringe inserted into the foliar or petiolar tissues. Inoculated plants were transferred to a greenhouse and grown out.
- c. **Mechanical inoculation:** Twenty-four virus-certified St. George rooted cuttings were mechanically inoculated by rubbing the plasmid vector on carborundum dusted leaves using a Q-tip. Leaves on an additional 21 plants were superficially injured with razor blades which had been dipped in the vector suspension. In an additional 22 plants, leaves were gently rubbed with fine diamond grit (400 mesh) sandpaper presoaked in the plasmid vector. Plants were transferred to the greenhouse and grown out.
- d. **Unrooted cutting dipping.** Ninety St. George cuttings were submerged in plasmid suspension and the bottom ends trimmed with pruners. Cuttings were dipped in Seradix™ rooting compound and moved to a mist bench for rooting.
- e. **Sonication inoculation.** Leaves on 12 plants were immersed in vector suspension and sonicated (Microson Ultrasonic Cell Disrupter ML2000, 65 watts rms, probe ca. 1 cm from leaf surface) for 45 sec. . Plants were subsequently potted and grown out in the greenhouse.

Results of Plasmid introduction trials. Field sampling of known RSPaV infected vines indicated that basal leaves closest to the trunk most consistently tested RSPaV positive. Virus was also detected in younger canes, shoots and leaves. RSPaV was found in highest titer in the larger veins of the leaves. Virus was consistently detected in root samples throughout the growing season. All tests were done using DRT-qPCR. While virus could be detected early in the growing season, several of the PCR negative tested vines tested PCR positive in late summer.

Following the various plasmid introduction methods, testing of the vector inoculated grapevines failed to detect the presence of the plasmid at 4 and 12 wk post plasmid introduction. Some plants, inoculated later in the summer were not tested before putting into dormancy. Plants were put into a cycle of dormancy in winter 2013 and will be retested in June 2013. As with many viruses, a dormancy cycle may be necessary for detection of the plasmid. Green fluorescent protein vector treated plants were examined under fluorescence microscopy, but no fluorescence was detected.

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Probe and primers have been ordered to complete the testing of the PVX inoculations. PVX infected material has been isolated as positive controls for the tests.

Design of a Universal one-tube Reverse transcriptase real-time single primer/probe set PCR assay.

Virus free St. George negative for RSPaV and were used in developing the real time PCR assay protocol. Grape leaf or root exudate was used for development of the assay. We selected an amplicon sequence which was the most conserved region of all eight of the RSPaV strains listed in the NCBI database. From this sequence, primers and a probe were constructed to confirm the universality of the assay.

The direct real time real time PCR method (DRT-qPCR), as described earlier for plum pox virus detection (1), was adapted and optimized for RSPaV detection utilizing a direct plant extraction buffer (DiPEB) eliminating the need for RNA extraction from the plant samples. The DiPEB consists of a 10mM sodium phosphate buffer, pH 8.0 amended with 50 mM NaCl, 20 mM MgCl₂, 0.25% (v/v) Tween 20, and adjusted to pH 7.2 Grape foliar tissue (0.3-0.5g) for testing was homogenized in 3-5ml of ELISA extraction buffer, centrifuged (4,000g for 8 min) and diluted 10 fold with DiPEB and 2ul used as a template for DRT-qPCR. To each sample 23ul of DRT master mix, 1x RT-PCR, 5 U reverse transcriptase (NEB) 1.5U Taq polymerase, 2mM MgCl₂, 0.8mM dNTP's, 0.15pm of each primer NG(U)1:

5'ACTTTCCACGCCTTATATATT 3' and NG(U)-2:

5'GATTTC AAGGCTGGGTTA 3', and 2.5pM of probe NG(U)-3

5'FAM/CCTGTTCGCTGGAATACCGTACTAATA/31ABkFQ/-3' was added to a total volume of 25ul. After transcription reaction for 20 min at 42°C and 3 min at 95°C for the initial Taq activation, PCR reaction mixtures were incubated in a thermocycler (CFX96, Bio-Rad Laboratories, Mississauga, Ontario Canada) for 42 cycles for 10 s at 95°C followed by 10 s for 55°C and 6 s at 72°. Fluorescence from FAM reporter was detected at 505-537 nm wavelength. (1) W-S. Kim, L.W. Stobbs, S.M. Lehman, D. James, and A.M. Svircev. Can. J. Plant Pathol. 30:: 308-317.

Results of DRT-qPCR tests. All oligos prepared against the different known strains of RSPaV tested positive for RSPaV using the probe and primer developed at this site. The DRT-qPCR assay was found to be as sensitive and accurate as the standard qPCR assay using spin column RNA extraction. Depending on availability and access to wood infected with these different strains, the viruses will be tested using the designed probe and primer.

Incidence of RSPaV in feral grape.

Feral grape cuttings were collected from 18 winery and ancillary sites across the Niagara region from mid July to late August 2012. In total, 50 samples were assayed by DRT-qPCR for the presence of RSPaV. No feral grapevines were found to be RSPaVpositive. Feral grape comprised a mixture of *Vitis labrusca* and *riparia* spp.

Next Steps:

1. All plasmid inoculated vines, following a period of cold induced dormancy, will be tested by PCR for presence of the plasmid integration.
2. GFP inoculated plants will be examined by fluorescence microscopy.

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3. Testing will be completed for the real time PCR assay of the known RSPaV strains and results published.
4. Any grapevine material testing positive for plasmid will be inoculated with RSPaV to assess efficacy of plasmid gene silencing.

Description of the Cooperation:

Cloned cDNA constructs including were exchanged between the Israeli, and the Canadian laboratories.

More details of collaboration are described in the report.

A manuscript describing the collaborative work of the Canadian and the Israeli labs was published.