**Final Scientific Report
Cover Page**

Project Number:

IS-4314-10C

Date of Submission of the report:

June/30/2014

Project Title:

Role of the viral AlkB homologs in RNA repair

Investigators

Principal Investigator (PI):

Munir Mawassi,

Co-Principal Investigators (Co-PIs):

V.V. Dolja

Institutions

ARO, Israel

Oregon St. U.

Keywords: Grapevine virus A, Grapevine leafroll associated virus-2, Grapevine leafroll associated virus-3, RNA silencing, Viral RNA suppressors.

Abbreviations commonly: GFP, green fluorescent protein; GLRaV-2, *Grapevine leafroll associated virus-2*; GLRaV-3, *Grapevine leafroll associated virus-3*, GVA, *Grapevine virus A*; ORF, open reading frame; RSS, RNA silencing suppressor.

Budget: IS: \$135,000

US: \$135,000

Total: \$270,000

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

Final Scientific Report

Summary

AlkB proteins that repair DNA via reversing methylation damage are conserved in a broad range of prokaryotes and eukaryotes including plants. Surprisingly, AlkB-domains were discovered in the genomes of numerous plant positive-strand RNA viruses, majority of which belong to the family *Flexiviridae*. The major goal of this research was to reveal the AlkB functions in the viral infection cycle using a range of complementary genetic and biochemical approaches. Our hypotheses was that AlkB is required for efficient replication and genetic stability of viral RNA genomes. The major objectives of the research were to identify the functions of GVA AlkB domain throughout the virus infection cycle in *N. benthamiana* and grapevine, to investigate possible RNA silencing suppression activity of the viral AlkB, and to characterize the RNA demethylation activity of the mutated GVA AlkB *in vitro* and *in vivo* to determine methylation status of the viral RNA. Over the duration of project, we have made a very substantial progress with the first two objectives. Because of the extreme low titer of the virus particles in plants infected with the AlkB mutant viruses, we were unable to analyze RNA demethylation activity and therefore had to abandon third objective. The major achievements with our objectives were demonstration of the AlkB function in virus spread and accumulation in both experimental and natural hosts of GVA, discovery of the functional cooperation and physical interaction between AlkB and p10 AlkB in suppression of plant RNA silencing response, developing a powerful virus vector technology for grapevine using GLRaV-2-derived vectors for functional genomics and pathogen control in grapevine, and in addition we used massive parallel sequencing of siRNAs to conduct comparative analysis of the siRNA populations in grape plants infected with AlkB-containing GLRaV-3 versus GLRaV-2 that does not encode AlkB. This analysis revealed dramatically reduced levels of virus-specific siRNAs in plants infected with GLRaV-3 compared to that in GLRaV-2 infection implicating AlkB in suppression of siRNA formation. We are pleased to report that BARD funding resulted in 5 publications directly supported by BARD, one US patent, and 9 more publications also relevant to project. Moreover, two joint manuscripts that summarize work on GVA AlkB (led by Israeli PI) and on viral siRNAs in grapevine (led by US PI in collaboration with University of Basel) are in preparation.

Final Scientific Report

Final Scientific Report

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged		3	2	5
Submitted, in review, in preparation	2*			2
Invited review papers		2		2
Book chapters				
Books				
Master theses			1	1
Ph.D. theses			2	2
Abstracts			1	1
Not refereed (proceedings, reports, etc.)				

*in preparation

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Israel:

Master students: Ifat Edan
 PhD: Sabrina Haviv, Cathy Dayan-Glick
 Post Doc.: M. Muruganantham (from India)

US:

Master students:
 PhD:
 Post Doc.: Agueda Renovell Ferrer, Kristin Kasschau

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings				
Longer Visits (Sabbaticals)				

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted				
Issued (allowed)		1		1
Licensed		1		1

Final Scientific Report

Achievements

In our project we proposed to address functions of the viral AlkB domains in virus biology and pathology using GVA and two grapevine leafroll viruses as models. Our specific objectives were: 1) to identify the functions of GVA AlkB domain throughout the virus infection cycle in *N. benthamiana* and grapevine; 2) to investigate possible RNA silencing suppression activity of the viral AlkB (alone and in combination with p10); and 3) to characterize the RNA demethylation activity of the mutated GVA AlkB *in vitro* and *in vivo* to determine methylation status of the viral RNA. Over the duration of project, we have made a very substantial progress with the first two objectives. Because of the extreme low titer of the virus particles in plants infected with the AlkB mutant viruses, we were unable to analyze RNA demethylation activity and therefore had to abandon third objective. The major achievements with the first two objectives were: i) demonstration of the AlkB function in virus spread and accumulation in both experimental and natural hosts of GVA; ii) discovery of the functional cooperation and physical interaction between AlkB and p10 AlkB in suppression of plant RNA silencing response. In addition, we developed a powerful virus vector technology for grapevine using GLRaV-2-derived vectors for functional genomics and pathogen control in grapevine. Furthermore, we used massive parallel sequencing of siRNAs to conduct comparative analysis of the siRNA populations in grape plants infected with AlkB-containing GLRaV-3 versus GLRaV-2 that does not encode AlkB. This analysis revealed dramatically reduced levels of virus-specific siRNAs in plants infected with GLRaV-3 compared to that in GLRaV-2 infection implicating AlkB in suppression of siRNA formation. We are pleased to report that BARD funding resulted in 5 publications directly supported by BARD, one US patent, and 9 more publications also relevant to project. Moreover, two joint manuscripts that summarize work on GVA AlkB (led by Israeli PI) and on viral siRNAs in grapevine (led by US PI in collaboration with University of Basel) are in preparation. Below we describe activities performed and experimental outcomes achieved in this project. In Appendix, we provide more detailed descriptions of the experimental data.

ARO:

To examine the AlkB role in virus infection and to test the possibility of functional cooperation of p10 and AlkB in RNA silencing suppression, we introduced alanine-scanning mutations into the AlkB-coding region to replace three types of the AlkB residues: i) catalytic residues invariant among all AlkB homologues including viral ones; ii) residues specifically conserved in all viral, but not cellular AlkB domains; iii) non-conserved amino acid residues (see Appendix for details). The GVA AlkB-mutants were assembled into the pCambia2301 binary vector between two CaMV 35S promoters and a terminator sequences. The GVA AlkB-mutants were tested for infectivity in *N. benthamiana* plants, using agroinfiltration. All mutants were infectious to *N. benthamiana* plants, though some exhibited delayed symptom appearance. GVA accumulation was tested using real-time PCR and immunoblot analyses. Results indicated that the *AlkB-H1* and *AlkB-R2* mutant GVAs accumulated to significantly lower levels compared to GVA WT or other mutants (see Appendix).

We have also developed a method for inoculation of genetically-modified GVA to grapevine (*Vitis vinifera*). The GVA-cDNA, cloned into the binary vector pCambia2031 under the CaMV 35S promoter, was mobilized to *Agrobacterium* and introduced into young grape plantlets maintained in tissue culture through drenching of roots (agro-drenching). Inoculated plantlets were transferred to soil and examined for virus infection for periods of up to 12 months. This method resulted in infection rate ranging from 50 to 100%. GVA accumulation in mature grapevine plants was tested using nested and real-time PCR. The obtained results showed that mutating the AlkB motif (*H1*, *R2* and *E*) affected GVA accumulation in *V. vinifera* young plantlets and mature plants (see Appendix).

Final Scientific Report

According to our working model, viral AlkB could suppress the plant RNA silencing defense to facilitate viral replication and accumulation. Previously, it was reported that GVA p10 possesses RNA silencing suppressor (RSS) activity in *N. benthamiana* plants (Chiba *et al.*, 2006; Zhou *et al.*, 2006). Our work further showed that the p10 activity was significantly enhanced upon expression of GVA minireplicon (Haviv *et al.*, 2012) suggesting that expressed of replicon-encoded ORF1 supplied an enhancer of p10 RSS activity. Therefore, we tested possible involvement of AlkB in suppression of RNA silencing. The AlkB ORF was cloned into a binary vector pCAMBIA2301 and co-infiltrated with or without p10 and GFP reporter into *N. benthamiana* leaves. The experimental approaches included monitoring GFP fluorescence using the confocal laser-scanning microscopy and MICA (see Appendix), as well as measuring the levels of GFP siRNA. The results showed that AlkB does enhance the RSS activity of p10 and therefore implicated AlkB into RNA silencing suppression. This conclusion was further supported by experiments in which the AlkB mutations H1 and R2 suppressed the synergistic effect of AlkB on RSS activity of p10.

Since AlkB increases the p10 RSS activity, we examined whether these two proteins interact each with the other, using the yeast two-hybrid system. We found that p10 is a self-interacting protein also capable of binding AlkB. Because the tested AlkB mutants did not affect AlkB:p10 interaction, we concluded that this interaction involves protein areas other than catalytic center.

In conclusion, work by Israeli lab established a significant role of AlkB in GVA infection both in an experimental host *N. benthamiana* and in a natural host grapevine. Furthermore, our data strongly suggested that the mechanism whereby AlkB contributes to virus accumulation in infected plants involves enhancement of the p10 activity in suppression of RNA silencing, as well as p10:AlkB interaction. These experimental outcomes allowed us to advance a working model according to which the synergistic functional interaction between AlkB and p10 results in demethylation of either viral RNA or siRNA by AlkB that, in turn, attenuates the host RNA silencing response and promotes virus infection. This model will be tested in our future research.

US

The original tasks for the US lab were to engineer the GVA AlkB ORF into the genome of GLRaV-2 that is not known to encode this protein, and to investigate the resulting effects on virus infection and virus-induced gene silencing (VIGS). To achieve this goal, we generated a GLRaV-2-based gene expression vector and developed a technique for agroinfection of grapevine using vacuum infiltration of the whole micropropagated plants. We further characterized the pattern of systemic infection using a GFP-expressing GLRaV-2 variant (GLRaV-2:GFP) and found that after initial replication in the bark phloem cells, virus invades leaf veins, roots, and both phloem and mesocarp cells of the berries. The GLRaV-2 vector exhibited remarkable genetic stability with some plants expressing GFP after 3 years post inoculation. Most importantly, we have established GLRaV-2 as a powerful VIGS vector capable of efficient silencing of the grapevine endogenous genes exemplified by *PDS* and *Chl I*, each required for chlorophyll biosynthesis. These results were published in *J. Virol.* (Kurth *et al.*, 2012) and highlighted in the Spotlight section of this journal.

Upon the discovery by the Israeli lab of the functional cooperation between AlkB and p10, it became clear that expressing AlkB alone using GLRaV-2 vector is unlikely to result in significant biological effect. Thus, we refocused on the following experimental approaches: i) genome-wide investigation of siRNAs in grapevine infected by GLRaV-2 and AlkB-containing GLRaV-3 (collaboration with Dr. M. Pooggin, University of Basel); ii) generation of the GLRaV-2 VIGS variants targeting GLRaV-3 to evaluate virus control potential of the VIGS vectors and to address the virus-virus interactions in relation to RNAi induction; iii) genomic characterization of an emerging virus GLRaV-7. In addition, we worked on the virus evolution pathways and virus-host interactions

Final Scientific Report

in grapevine involving collaborations with Dr. E.V. Koonin (National Center for Biotechnology Information) and Dr. C. Ritzenthaler (University of Strasbourg). Although these latter collaboration projects are not technically a part of the BARD proposal, they are relevant to a broader research impact of within a field of virology.

The initial phase of our siRNA project involved development of a new technology of massive parallel sequencing of viral siRNAs in several virus-plant models including grapevine. This work showed that both RNA and DNA viruses induce abundant formation of siRNAs whose sequences densely cover the sense and antisense orientations of the viral genomes. It was also found that the complete genomes of DNA viruses from *Caulimoviridae* and *Geminiviridae* families can be reconstructed by deep sequencing and *de novo* assembly of viral siRNAs using bioinformatics tools. Furthermore, we utilized this approach to reconstruct an emerging single-stranded DNA virus with circular genome (distantly related to geminiviruses) and two viroids associated with economically important red blotch disease of grapevine. Our findings showed that deep siRNA sequencing allows for *de novo* reconstruction of any DNA or RNA virus genome, as well as for studying the mechanisms of siRNA biogenesis and RNAi-based antiviral defense. This work has been recently published in PLoS ONE (Seguin *et al.*, 2014).

More recently, using this technology, we have compared the siRNA populations induced during grapevine infections with AlkB-encoding GLRaV-3 and evolutionarily related, but possessing no AlkB, GLRaV-2. Strikingly, we found that the size of GLRaV-3-specific siRNA population was dramatically smaller than that of GLRaV-2-specific population. The majority of GLRaV-3 siRNA reads per genome position was below 20, whereas the GLRaV-2 siRNA reads were at the 200 level. Likewise, the 'hot spot' areas of GLRaV-3 siRNAs along virus genome were around 40 reads with only one exceeding 80, whereas such hot spots in GLRaV-2 genome were ~500 with one spot exceeding 1,200 reads (see Appendix). These data suggest that the GLRaV-3-expressed AlkB domain suppresses induction and/or amplification of virus-specific siRNAs, likely via demethylation of either viral genomic RNA or siRNAs. In contrast, as we have shown earlier, the closterovirus suppressors of RNA silencing such as GLRaV-2 p24 act via sequestration of the siRNAs (Chapman *et al.*, 2004).

We have also generated four GLRaV-2 VIGS variants targeting GLRaV-3 genes encoding viral RNA replicase, capsid protein, as well as 5'- and 3'-UTR. Each of these variants was introduced to grapevine using agroinfection. Pilot experiments have shown that the control vector with no VIGS inserts does not prevent superinfection with GLRaV-3 providing adequate experimental model. Evaluation of the VIGS efficiency in controlling mealybug transmission of GLRaV-3 will be completed this fall in collaboration with Dr. R. Almeida (UC Berkeley). Completion of this new aim has a potential to provide powerful virus control strategy for grapevine and contribute to understanding the role played by AlkB and RNAi suppressors in mixed viral infections.

In collaboration with Dr. A. Rowhani (UC Davis), we used deep sequencing analysis of a virus-infected, asymptomatic grapevine to reveal a virome containing five RNA viruses and a viroid including the novel GLRaV-7 genome that represents a new virus genus (Al Rwahnih *et al.*, 2012).

In conclusion, we have achieved major breakthroughs in developing a GLRaV-2-based gene expression and VIGS vector technology for grapevine and in understanding population structure of the virus-induced siRNAs in plants infected by AlkB-encoding and AlkB-less viruses by applying cutting-edge small RNA sequencing and bioinformatics approaches. We have also characterized two emerging grapevine viruses, GLRaV-7 and a geminivirus-like DNA virus. Future application of the generated knowledge will contribute to developing VIGS vectors controlling important grapevine pathogens. We also hope to continue our collaboration with Israeli lab to reveal the molecular mechanism whereby viral AlkB counteracts plant RNA silencing defense.

Final Scientific Report

Details of Cooperation:

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

The Israeli and US lab were in constant contact discussing experimental outcomes and designs of the future experiments via e-mail. This was especially significant in developing the optimal agroinoculation techniques for GVA and GLRaV-2 models, and for the mutagenesis experiments. The US PI has visited Israel for the BARD Eilat meeting on RNA interference in 2010 when the PIs met and discussed the strategy of the research. The planned visit of the Israeli PI to US was cancelled due to visa complications.

Although so far the BARD-supported research was published separately, we are currently working on two joint manuscripts that will be submitted later this year. One of them will present most significant outcomes of the functional characterization of AlkB mutants, a study led by the Israeli PI. Another work is centered on the data on siRNA populations in plants infected by grapevine viruses that either do or do not encode AlkB. In the future, we are fully committed to continuing our collaboration on uncovering the mechanisms whereby AlkB contributes to virus amplification and counterdefence. Our amended and refined working model for these studies is firmly based on the novel data and conclusions that followed from BARD-funded project.

Final Scientific Report

List of Publications:

US

Directly supported by BARD award:

- Seguin, J., Rajeswaran, R., Malpica-Lopez, N., Martin, R.R., Kasschau, K., Dolja, V.V., Otten, P., Farinelli, L., and Pooggin, M.M. (2014) *De novo* reconstruction of consensus master genomes of plant RNA and DNA viruses from siRNAs. *PLoS ONE*, 9(2): e88513.
- Maree, H.J., Almeida, R.P.P., Bester, R., Chooi, K.M., Cohen, D., Dolja, V.V., Fuchs, M.F., Golino, D.A., Jooste, A.E.C., Martelli, G.P., Naidu, R.A., Rowhani, A., Saldarelli, P., Burger, J. (2013) Grapevine leafroll-associated virus 3. *Front. Microbiol.* **4**:82. (21pp).
- Dolja, V.V. and Koonin, E.V. (2013) The closterovirus-derived gene expression and RNA interference vectors as tools for research and plant biotechnology. *Front. Microbiol.* **4**:83 (10pp).
- Kurth, E.G., Peremyslov, V.P., Prokhnevsky, A.I., Kasschau, K.D., Miller, M., Carrington, J.C., and Dolja, V.V. (2012) Virus-derived gene expression and RNAi vector for grapevine. *J. Virol.* **86**, 6002-6009.
- Martelli, G.P., Abou Ghanem-Sabanadzovich, N., Agranovsky, A.A., Al Rwahnih, M., Dolja, V.V., Dovas, C.I., Fuchs, M., Gugerli, P., Hu, J.S., Jelkmann, W., Katis, N.I., Maliogka, V.I., Melzer, M.J., Menzel, W., Minafra, A., Rott, M.E., Rowhani, A., Sabanadzovich, S., Saldarelli, P. (2012) Taxonomic revision of the family Closteroviridae with special reference to the grapevine leafroll-associated members of the genus Ampelovirus. *J. Plant Pathol.* **94**, 7-19.
- Al Rwahnih, M., Dolja, V.V., Daubert, S., Koonin, E.V., Rowhani, A. (2011) Genomic and biological analysis of *Grapevine leafroll-associated virus 7* reveals a possible new genus within the family *Closteroviridae*. *Virus Res.* **163**, 302-309.
- US Patent: V.V. Dolja and V.P. Peremyslov "Closterovirus Vectors and Methods". No. 8,415,147 Issued April 9, 2013; OSU Ref. No. 06-57; Klarquist Ref. No. 245-79793-10.

Additional relevant publications

- Koonin, E.V. and Dolja, V.V. (2014) Virus world as an evolutionary network of viruses and capsid-less selfish elements. *Microbiol. Mol. Biol. Rev.* **78**, 278-303.
- Dolja, V.V. and Krupovic, M. (2013) Accelerating expansion of the viral universe. Editorial overview. *Curr. Opin. Virol.* **3**, 542-545.
- Koonin, E.V. and Dolja, V.V. (2013) A virocentric perspective on the evolution of life. *Curr. Opin. Virol.* **3**, 546-557.
- Koonin, E.V. and Dolja, V.V. (2012) Expanding networks of RNA virus evolution. *BMC Biol.* **10**:54 (<http://www.biomedcentral.com/1741-7007/10/54>).
- Dolja, V.V. and Koonin, E.V. (2012) Capsid-less RNA viruses. In: *eLS* (Encyclopedia of Life Sciences). John Wiley & Sons Ltd, Chichester.
- Dolja, V.V. and Koonin, E.V. (2011) Common origins and host-dependent diversity of plant and animal viromes. *Curr. Opin. Virol.* **1**, 322-331.
- Amari, K., Lerich, A., Schmitt-Keichinger, C., Dolja, V.V., and Ritzenthaler, C. (2011) Tubule-guided cell-to-cell movement of a plant virus requires class XI myosin motors. *PLoS Pathog.*, 7(10):e1002327.

Final Scientific Report

Israel:

Directly supported by BARD:

Haviv S, Moskovitz Y, Mawassi M. (2012). The ORF3-encoded proteins of vitiviruses GVA and GVB induce tubule-like and punctate structures during virus infection and localize to the plasmodesmata. *Virus Res.* 163:291-301.

Haviv S., Iddan Y., Goszczynski D.E., Mawassi M. (2012) The ORF5 of Grapevine virus A is involved in symptoms expression in *Nicotiana benthamiana* plants. *Annals of Applied Biology* 160: 181-190.

Additional relevant publications

Meng B, Venkataraman S, Li C, Wang W, Dayan-Glick C, Mawassi M. (2013). Construction and biological activities of the first infectious cDNA clones of the genus Foveavirus. *Virology* 435:453-62.

du Preez J, Stephan D, Mawassi M, Burger JT. (2011). The grapevine-infecting vitiviruses, with particular reference to grapevine virus A. *Arch Virol.* 156:1495-503.

Final Scientific Report

Appendix

Description of the research activities and results

ARO (these data are on preparation to be submitted in an ARO-US joint publication):

AlkB mutant's infectivity and replication

To examine the role of AlkB in virus infection and replication, seven Alanin-scanning mutations were introduced into the AlkB-coding region to replace three types of the AlkB residues (Fig. 1b): (i) three catalytic residues invariant among all AlkB homologues including viral ones (H1, D, H2); (ii) three residues specifically conserved in all viral, but not cellular AlkB domains (Q, R1, R2); (iii) one non-conserved residue (E). Replacement of H1/D to Alanin abolished de-methylation activity of the viral AlkBs (van den Born *et al.*, 2008). The GVA mutant DNAs were cloned into the pCambia2301 binary vector between two CaMV 35S promoters and a terminator.

Figure 1: GVA point mutations in AlkB Sequence. (a) Schematic diagram of wild type GVA clone (pGVA-549). The GVA cDNA, was cloned into the pCambia2301 binary vector between two CaMV 35S promoters and a terminator. Boxes represent open reading frames (ORFs); MP, Movement Protein; CP, Coat Protein; p10, the RSS encoded by ORF5. (b) GVA AlkB sequence. Blue bold (H1, D, H2) represents conserved amino acids in all organisms. Red bold (Q, R1, R2) represents conserved amino acids in viruses. Black bold (E) represent a non-conserved amino acid (control). Each of the 7 amino acids was modified to Alanin, to create mutant clones: pGVA-541 (H1), pGVA-542 (D), pGVA-543 (E), pGVA-544 (Q), pGVA-545 (H2), pGVA-546 (R1) and pGVA-547 (R2).

The clones were introduced into *N. benthamiana* plants, via *Agrobacterium tumefaciens* transformation, using Agro-Infiltration method. GVA inoculated *N. benthamiana* plants exhibited photo-bleaching symptoms and the leaves were much smaller. In order to reveal changes in viral symptoms, we compared between the mutants and the wild type GVA inoculated plants. Two mutants delayed in viral symptoms appearance: *AlkB-H1* (blue) and *AlkB-R2* (red), 6 days and 10 days, respectively (Fig. 2). All plants were symptomatic 20 days post inoculation. Sequence analysis of all mutants found each of the seven mutations in the viral genomes. To examine a possible role for AlkB in GVA replication, we extracted total RNA and protein from the first symptomatic leaf of each tested plant. RNA samples were analyzed by Real-Time PCR (Fig. 3a) and protein samples were analyzed by western-blot (Fig. 3b). Compared to WT-GVA, the expression of *AlkB* gene in *AlkB-H1* and *AlkB-R2* mutants was much lower (70% and 6%, respectively) (Fig. 3a), as well as the titer of the

Final Scientific Report

virus which was tested by CP specific antibody (Fig. 3b). These results indicate a linkage between RNA replication and virus accumulation in GVA.

Figure 2: Viral symptoms appearance in GVA Agro-inoculated *N. benthamiana* plants. Plants were inoculated with infective clones of GVA: wild type GVA (WT-GVA) and mutated clones at the AlkB sequence (AlkB-E, H1, H2, D, R1, R2, Q). Daily symptoms observations were performed. Each average calculated using data from 7 plants. $P < 0.001$

Figure 3: GVA replication in the first symptomatic leaf of Agro-inoculated *N. benthamiana* plants. Plants were inoculated with infective clones of GVA: wild type GVA (WT-GVA) and the mutants *AlkB-H1/R2*. Total RNA and proteins were extracted from the first symptomatic leaf of each plant and analyzed using Real Time PCR and Western-blot methods. (a) Real Time PCR analysis using AlkB specific primers. (B) Western-blot analysis using GVA-CP specific antibody.

Further experiments were made to examine the effect of the AlkB mutations on GVA's replication dynamics. The clones *AlkB-H1*, *R2*, *E* and GVA-WT were introduced to *N. benthamiana* plants, using Agro-Infiltration method. Total RNA and proteins were

Final Scientific Report

extracted from leaves tissue (two leaves above the inoculated leaves) at 3, 6, 10, 13, 17 and 20 days post inoculation (DPI). Only after 6 days GVA replication (Fig. 4a) and titer (Fig. 4b) could be observed. All three mutants exhibited low levels of RNA and protein during the entire experiment (even at 20 DPI) compared to GVA-WT, especially *AlkB-H1*.

Figure 4: GVA replication and protein levels in Agro-inoculated *N. benthamiana* plants. Plants were inoculated with wild type GVA clone (WT), or with mutants (*AlkB-H1*, *R2*, *E*). Total RNA and proteins were extracted (from leaves tissue) at 3, 6, 10, 13, 17 and 20 days post inoculation (DPI). **(a)** AlkB gene expression levels were analyzed by Real-Time PCR using specific primers. **(b)** GVA protein levels were analyzed by Western blot, using CP specific antibody.

AlkB mutation affects GVA accumulation in *V. vinifera* plants

Virus infection of micro propagated *V. vinifera* plantlets was achieved by using a an agro drenching technology. Two-weeks-old *V. vinifera* cv. Prime plantlets, which had been cultured on a solid WPM medium, were detached and their roots were wounded and inserted into tubes with filter papers immersed in an MS liquid medium. The plantlets were inoculated with a suspension of *Agrobacterium* carrying the GVA-WT, *AlkB-E*, *AlkB-H1* or *AlkB-R2* clones. Ten days later, the plantlets were transferred to fresh MS liquid media, where they were kept for additional 10 days. Then, plantlets were transferred to fresh media supplemented with carbenicillin for other 10 days. At the end of this period (30 days), the plantlets were sampled for subsequent analyses. Half of the inoculated plantlets were examined for virus infection via nested-PCR. We found that, 85-100% of the tested plantlets were successfully inoculated (Fig. 5a, c, g); excluding of the plantlets inoculated with *AlkB-H1*, where only 50% of the tested plantlets were found to be infected (Fig. 5e). The other half inoculated plantlets were transferred to pots and maintained in the greenhouse. The mature inoculated plants were examined for virus accumulation 3, 6, and 12 months post inoculation, using nested-PCR. GVA infection was detected only in plants inoculated with the wild type virus (WT-GVA) (Fig. 5b). The mutant GVA variants could not be detected in

Final Scientific Report

the mature grapevine plants (Fig. 5d, f, h). Since we could not detect accumulation of GVA mutants in mature grapevine plants, the further analyses have been applied to only plantlets. In order to examine the effect of the AlkB mutations on GVA replication in the natural host plant, we extracted total RNA from grapevine plantlets 30 days post inoculation followed by cDNA and Real-Time PCR analyses (Fig. 6). The results obtained from this experiment supported our previously results. The AlkB mutants (*H1*, *R2* and *E*) suppressed GVA replication in *V. vinifera* plantlets (Fig. 6) and mature plants (Fig. 5d, f, h), as well as in *N. benthamiana* plants (Fig. 3 and 4).

Figure 5: GVA accumulation in Agro-inoculated grapevine plants. Plantlets were inoculated with infective clones of wild type GVA (WT-GVA) and mutants (*AlkB-E*, *H1*, *R2*), using the Agro-drenching method. Total RNA was extracted from plantlets 30 days post inoculation or from mature plants 12 months post inoculation. Nested PCR was performed to detect GVA accumulation in stem tissue, using AlkB specific primers. (a) Wild type GVA detection in plantlets, and (b) in mature plants. (c) Detection of GVA carrying the *AlkB-E* mutation in plantlets, and (d) in mature plants. (e) Detection of GVA carrying the *AlkB-H1* mutation in plantlets, and (f) in mature plants. (g) Detection of GVA carrying the *AlkB-R2* mutation in plantlets, and (h) in mature plants. L, DNA ladder; 1-8, number of tested plants.

Final Scientific Report

Figure 6: GVA replication in Agro-inoculated grapevine plantlets. Plantlets were inoculated with cloned wild type GVA (WT-GVA) and or GVA mutants (*AlkB-E*, *H1*, *R2*), using the Agro-drenching method. Total RNA was extracted from plantlets 30 days post inoculation to examine virus replication using Real-Time PCR analysis with GVA-CP specific primers.

GVA AlkB involved in RNA silencing suppression in *N. benthamiana* plants

Viral AlkB proteins repair RNA damage by oxidative demethylation (van den Born *et al.*, 2008). It is believed that methylation cause viral RNA degradation via plant RNA silencing mechanism (Mette *et al.*, 2000; Matzke and Birchler, 2005). Thus, viral AlkB could suppress the plant RNA silencing mechanism to enable viral replication and accumulation. It has been reported that GVA p10, encodes by ORF5, possesses a RNA silencing suppressor (RSS) activity in *N. benthamiana* plants (Chiba *et al.*, 2006; Zhou *et al.*, 2006). Yet we found that this RSS activity is enhanced by expression of GVA minireplicon (Haviv *et al.*, 2012). This minireplicon contains the 5'-UTR and the 3'-UTR and ORF1 sequence. Since the GVA ORF1 contains the AlkB sequence, we conducted experiments to examine whether the motif is involved in RSS activity. We cloned the AlkB sequence into a binary vector (pCambia2301) and co-infiltrated it with p10 into *N. benthamiana* leaves, and reported the results 7 days post infiltration via monitoring the green fluorescence of GFP using the confocal laser-scanning microscope (Fig. 7a) and MICA analyses (Fig. 7b). In addition we examined the levels of GFP siRNA (Fig. 7c). We found higher fluorescence levels of GFP in the leaves infiltrated with AlkB plus p10 (Fig. 7a-3), compared to leaves infiltrated only with p10 (7a-2). GFP alone has faded 7 days post infiltration (Fig. 7a-1). These results indicate that AlkB enhanced the RSS activity of p10 and therefore it can be involved in RNA silencing suppression. For further analyzing this hypothesis, we tested RSS activity of the AlkB mutants (H1, R2 and E) with p10. We found that the mutations H1 and R2 decreased the RSS activity of p10 (Fig. 7a-5, a-6), whereas the mutation E did not (Fig. 7a-4). Taken together, the mutations *H1* and *R2* delayed GVA symptoms appearance (Fig. 2), affected GVA replication and accumulation (Fig. 3, 4), as well as suppressed RSS activity of p10 in *N. benthamiana* (Fig. 7). The mutation *E* had no effect on GVA symptoms appearance (Fig. 2) or on p10 RSS activity (Fig. 7), but it affected GVA replication and accumulation both in *N. benthamiana* (Fig. 4) and grapevine plants (Fig. 5).

Final Scientific Report

Examination of protein interaction between AlkB and p10

To examine protein-protein interaction, we used the Yeast Two Hybrid System. We found that p10 exhibited self-protein-protein interaction. Since GVA AlkB is involved in enhancing p10 RSS activity, we intended to examine whether this activity involves protein-protein interaction. We cloned the p10 into a “prey” vector (pJG4-5) and the AlkB into a “bait” vector (pEG202), following by transformation into yeast cells. Our results showed protein-protein interaction between AlkB and p10 (Fig. 8).

Figure 7: RNA silencing suppressor (RSS) activity analysis of AlkB and p10 in *N. benthamiana* plants. (a) Confocal microscopy images of GFP fluorescence levels in *N. benthamiana* leaves, 7 days post co-Infiltration with a binary vector expressing: (1) GFP ; (2) GFP+p10; (3) GFP+p10+AlkB; (4) GFP+p10+AlkB-E; (5) GFP+p10+AlkB-H1; (6) GFP+p10+AlkB-R2. **(b)** MICA image analysis of GFP fluorescence levels. **(c)** GFP siRNA accumulation in *N. benthamiana* leaves, which were co-infiltrated with a binary vector expressing: (1) Empty vector (negative control); (2) GFP; (3) GFP+p10; (4) GFP+p10+AlkB; (5) GFP+p10+AlkB-E; (6) GFP+p10+AlkB-H1; (7) GFP+p10+AlkB-R2. EtBr, etidium bromide stained RNA gel as an equal loading control.

Final Scientific Report

Figure 8: Protein interaction between AlkB and p10 using Yeast Two-Hybrid System. (A) Positive control interaction (pJG4-5 + pSH17-4). (B) Negative control interaction (pJG4-5 + pRFHM1). (C) Protein interaction between AlkB and p10. (D) No protein interaction between the whole Replicase and p10.

Final Scientific Report

US

Because large part of our research directly funded by the BARD award has been published (Kurth *et al.*, 2012; Al Rwahnih *et al.*, 2012; Seguin *et al.*, 2014), we do not include corresponding data in this Appendix.

However, in a figure below, we present some of the siRNA population data briefly discussed in our Report. These data show the results of deep sequencing of the siRNA libraries derived from the grapevine plants infected either with GLRaV-3 (A) or GLRaV-2 (B). The heights of the blue (positive-sense) and red (negative-sense) lines corresponds to the number of specific siRNAs derived from the cognate, 25-nucleotides-long region of the virus genome. The genome maps of each virus are shown to scale below the siRNA data. Please, note the strikingly distinct scales of the siRNA reads, up to 80 and 40 for the sense and antisense siRNAs for GLRaV-3, respectively, versus 550 and 1,250 for those in GLRaV-2 siRNA population. These data prompted us to advance a hypothesis according to which the demethylation activity of the viral AlkB acts to suppress either induction or amplification of the virus-specific siRNAs generated by plant defense response. We plan to test this hypothesis in our future collaboration activities with the Israeli lab.

A

GLRaV-3-induced siRNAs in a naturally-infected Pinot Noir grapevine

B

GLRaV-2-induced siRNAs in a naturally-infected Pinot Noir grapevine