BARD Final Scientific Report

Date of Submission of the report: _____ September 7, 2009

BARD Project Number: _____ IS-3784-05

Project Title: Role of RNA Silencing Suppression in the Pathogenicity and Host Specificity of the *Grapevine Virus A*

Investigators

Institutions

Principal Investigator (PI):

Munir Mawassi

A.R.O. The Volcani Center

Co-Principal Investigator (Co-PI):

Valerian V. Dolja

Oregon State University

Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.
Vitivirus, RNA silencing, grapevine viruses, plant protection.

Budget: IS: \$150,000 US: \$150,000 Total: \$300,000

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

BARD Final Scientific Report

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)	1	6	3 (pub.)	10
Submitted, in review, in preparation	1 (in prep)			1
Invited review papers		2		2
Book chapters				
Books				
Master theses			2	2
Ph.D. theses		1	2	3
Abstracts		10	5	15
Not refereed (proceedings, reports, etc.)			1	1

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

Mookkan Muruganantham (IS) _____

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1		3	4
Longer Visits (Sabbaticals)				

Description of Cooperation:

Patent Summary (numbers)

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

BARD Final Scientific Report

Final Scientific Progress Report

Abstract

RNA silencing is a defense mechanism that functions against virus infection and involves sequence-specific degradation of viral RNA. Diverse RNA and DNA viruses of plants encode RNA silencing suppressors (RSSs), which, in addition to their role in viral counterdefense, were implicated in the efficient accumulation of viral RNAs, virus transport, pathogenesis, and determination of the virus host range. Despite rapidly growing understanding of the mechanisms of RNA silencing suppression, systematic analysis of the roles played by diverse RSSs in virus biology and pathology is yet to be completed. Our research was aimed at conducting such analysis for two grapevine viruses, *Grapevine virus A* (GVA) and *Grapevine leafroll-associated virus-2* (GLRaV-2).

Our major achievements on the previous cycle of BARD funding are as follows.

1. GVA and GLRaV-2 were engineered into efficient gene expression and silencing vectors for grapevine. The efficient techniques for grapevine infection resulting in systemic expression or silencing of the recombinant genes were developed. Therefore, GVA and GLRaV-2 were rendered into powerful tools of grapevine virology and functional genomics.
2. The GVA and GLRaV-2 RSSs, p10 and p24, respectively, were identified, and their roles in viral pathogenesis were determined. In particular, we found that p10 functions in suppression and pathogenesis are genetically separable.
3. We revealed that p10 is a self-interactive protein that is targeted to the nucleus. In contrast, p24 mechanism involves binding small interfering RNAs in the cytoplasm. We have also demonstrated that p10 is relatively weak, whereas p24 is extremely strong enhancer of the viral agroinfection.
4. We found that, in addition to the dedicated RSSs, GVA and GLRaV-2 counterdefenses involve ORF1 product and leader proteases, respectively.
5. We have teamed up with Dr. Koonin and Dr. Falnes groups to study the evolution and function of the AlkB domain presents in GVA and many other plant viruses. It was demonstrated that viral AlkBs are RNA-specific demethylases thus providing critical support for the biological relevance of the novel process of AlkB-mediated RNA repair.

BARD Final Scientific Report

Achievements

Suppression of RNA silencing by GVA and GLRaV-2

Our BARD project resulted in a joint development of a novel, extremely sensitive, viral minireplicon-based assay for identification of the RNA silencing suppressors (Chiba et al., 2006). Using this assay, we showed that GVA p10 possesses consistent, albeit relatively weak, suppressor activity in *Nicotiana benthamiana*. Furthermore, we have demonstrated that p10 is a representative of a novel family of the Zn-ribbon containing viral RSSs. We have also showed that GLRaV-2 encodes an extremely strong RSS, p24 (Chiba et al., 2006). Most importantly, we have demonstrated that co-launching silencing suppressor with the viral replicon using agroinoculation dramatically improves the efficiency of agroinfection (Chiba et al., 2006). Taken together, this work paved the way for successful development of grapevine agroinfection described below.

As a continuation of this line of research, we have generated a GFP-tagged GVA-based minireplicon containing sequences of the ORF1 (Fig. 1A). We found that this minireplicon acts as a strong inducer of RNA silencing upon transient and transgenic expression in plants (Brumin et al., 2009). Strikingly, we have also found that the activity of p10 is ~100-fold stronger toward suppressing GVA minireplicon-induced silencing compared to that induced by heterologous closteroviral minireplicon (Fig. 3). This finding revealed that GVA suppression machinery involves a second component specified by ORF1. Because in addition to the typical replicase domains, ORF1 encodes AlkB domain, we suggest that AlkB is a potential co-factor in GVA-induced suppression of RNA silencing.

We have also revealed that p10 affects the symptom development on *N. benthamiana*. Using GVA variants harboring chimeric p10 derived from severe and mild GVA isolates, we have mapped the pathogenesis determinant to the N-terminal region of p10. Interestingly, we found that p10s of both severe and mild GVA strains possess similar RSS activity in the presence of the GVA-minireplicon indicating that p10 functions in counterdefense and pathogenesis are genetically separable.

We have examined p10 expression in the infected cells using a series of p10 mutants fused to GFP and FRET analysis. We found that: i) p10 is targeted to the nucleus; ii) molecules of p10 self-interact; iii) mutations within p10 that abolish this interaction also impair the RSS activity demonstrating that self-interaction is required for p10 function.

BARD Final Scientific Report

Infecting *V. vinifera* plants with genetically modified GVA and GLRaV-2

Initially, an ability of the GFP-tagged GVA clone to replicate in the grapevine cells was examined using a *V. vinifera* protoplast system (Fig. 4). Next, in a search for an efficient technique for launching viral cDNA into grapes, we explored a range of approaches involving agroinoculation. For GVA, an agrodrenching method was found to be the most optimal approach. Two weeks-old *V. vinifera* cv. Prime plantlets cultured on a solid rooting medium were detached, and their roots were wounded and immersed in the *Agrobacterium* suspension. The *Agrobacterium* was transformed with the binary vector carrying the cDNA of GVA. Ten days later, the plantlets were transferred to fresh media supplemented with cefotaxime. When the agrodrenched plantlets were examined for virus infection by RT-PCR assays 28 days post agrodrenching, ~50% of the tested plantlets (>200) became infected by GVA (Muruganahtham et al., 2009). The infection was demonstrated by virus-dependent GFP expression in the leaves (Fig. 2C), isolation of the typical GVA particles (Fig. 2E), and in-situ hybridization (Fig. 2D).

An alternative technique was developed for the GLRaV-2-derived vector. The *V. vinifera* (Sirah and Cabernet franc) plants were micropropagated for 4-6 weeks, removed and vacuum-infiltrated with agrobacterial suspension containing two bacterial strains. One strain was engineered to launch the GFP-tagged virus, and another – the GLRaV-2 silencing suppressor p24. Similar to GVA, ~50% of plants became infected 4 weeks post infiltration. The systemic infection was confirmed by the stable GFP expression in the phloem tissues of stems and leaves of the new growth (Fig. 6A-F). In addition, the RT-PCR analysis clearly showed that GFP expression was driven by the replicating viral RNA (Fig. 6G). Our ability to use GLRaV-2 vector for infecting both *N. benthamiana* and *V. vinifera* was used to reveal novel, host-specific functions of the GLRaV-2 leader proteases in virus invasion and counterdefence (Liu et al., 2009).

Collectively, these results demonstrated that we have developed GVA and GLRaV-2 into efficient vectors for the systemic gene expression in grapevine.

Virus-induced gene silencing (VIGS) in *N. benthamiana*

The GVA vector was designed to express the foreign gene through the MP-sgRNA promoter. A 500 nts-long fragment of the PDS cDNA of *N. benthamiana* (NbPDS) was cloned into the GVA vector. Upon agroinoculation with GVA-NbPDS, plants displayed the characteristic photobleaching phenotype that was also confirmed by hybridization and semi-quantitative RT-PCR analyses thus demonstrating silencing of the endogenous

BARD Final Scientific Report

PDS gene (Fig. 2A). The resulting mean level of PDS mRNA in the photobleached veins was only 15% of that in the control.

Using GFP-transgenic 16c line of *N. benthamiana*, we have also demonstrated that GFP-expressing GLRaV-2 vector acts as a strong inducer of GFP silencing (not shown).

VIGS in *V. vinifera*

The feasibility of VIGS for grapevine was tested via engineering a fragment of the *V. vinifera* PDS cDNA into the GVA vector. Plantlets inoculated with GVA-VvPDS exhibited the photobleaching symptoms attributed to silencing of the endogenous PDS gene 14-20 days post inoculation. As expected, these symptoms were present mainly in the leaf veins and at leaf margins (Fig. 2 B). The hybridization and semi-quantitative RT-PCR (Fig. 2 F) analyses revealed that the PDS mRNA levels in the photobleached leaves were ~30% of those of the normal leaves.

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. Application of silencing vectors to the grapevine will enable high-throughput analysis of gene function in this important crop. Here, we provided 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.

The experiments designed to determine if, similar to *N. benthamiana*, GLRaV-2 vector is an efficient inducer of VIGS in grapevine, are in progress.

AlkB proteins repair RNA damage

We have recently described the first functional characterization of AlkB proteins from three plant viruses including GVA (van den Born et al., 2008). The viral AlkB proteins efficiently reactivated methylated bacteriophage genomes when expressed in *E. coli* (Fig. 5B). Isolated AlkB proteins also displayed a robust, Fe²⁺- and 2-oxoglutarate-dependent, demethylase activity *in vitro*. Viral AlkB proteins preferred RNA over DNA substrates and thus represented the first AlkB proteins with such substrate specificity (Fig. 5C). Our results suggested a role for viral AlkB proteins in maintaining the integrity of the viral RNA genome through repair of deleterious methylation damage, and supported the notion that

BARD Final Scientific Report

AlkB-mediated RNA repair is biologically relevant. In addition, our phylogenetic study (Fig. 5A) indicated that viral AlkBs were acquired by flexiviruses similar to GVA from a plant pathogenic bacterium and then disseminated among diverse viruses via horizontal gene transfer.

AlkB mutants and infectivity

To directly examine the AlkB role in virus infection and to test the possibility of functional cooperation of p10 and AlkB in RNA silencing suppression, we initiated mutation analysis of AlkB in a context of the GFP-tagged GVA cDNA clone. Nine alanine-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; ii) three residues specifically conserved in all viral, but not cellular AlkB domains; iii) three non-conserved amino acid residues (blue, red, and black, respectively, in Fig. 1B). As we have shown, replacement of the catalytic residues fully abolishes demethylation activity of the viral AlkBs (van den Born et al., 2008). We assume that the residues specifically conserved in viral AlkBs are involved in the specificity toward RNA substrate and are therefore functionally important. Finally, non-conserved residues were chosen as control for the nonspecific sequence changes. Preliminary analysis of the mutant phenotypes in *N. benthamiana* showed that the two former classes of mutants exhibited slightly delayed systemic infection with marginally attenuated symptoms, whereas the mutants of the third type were similar to the wild type controls (data not shown). These results provide an important starting point for the planned in-depth analysis of the AlkB role in the viral infection cycle in *N. benthamiana* and *V. vinifera*.

Description of the Cooperation:

The principal investigators communicated via email on a regular basis. The US co-PI has visited Israel in the second year of the project to discuss the progress and to deliver seminars at Volcani and Weizmann Institute. Both the Israeli and the US investigators have met three times within the project duration at the international Congresses in South Africa, Italy, and Turkey. In these meetings, we have discussed the results of our collaboration and developed the principal concept for the current BARD proposal. Information regarding GVA and GLRaV-2 inoculation techniques, primers, clones, and other reagents have been exchanged.

BARD Final Scientific Report

Combined, the Israeli and US labs published 10 peer-reviewed papers and two invited reviews directly supported by the BARD since the project began in 2006 (selected publications are included).

- Liu, Y.P., Peremyslov, V.V., Medina, V., and Dolja, V.V. (2009) Tandem leader proteases of Grapevine leafroll-associated virus-2: Host specific functions in the infection cycle. *Virology* **383**, 291-299.
- Bertsch, C., Beuve, M., Dolja, V.V., Wirth, M., Pelsy, F., Herrbach, E., and Lemaire, O. (2009) Retention of the virus-derived sequences in the nuclear genome of grapevine as a potential pathway to virus resistance. *Biol. Direct* **4**:21.
- Muruganantham, M., Moskovitz, Y., Haviv, S., Horesh, T., Fenigstein, A., du Preez, J., Stephan, D., Burger, J.T., and Mawassi, M. (2009). Grapevine virus A-mediated gene silencing in *Nicotiana benthamiana* and *Vitis vinifera*. *J Virol. Meth.* **155**, 167-174.
- Brumin, M., Stukalov, S., Haviv, S., Muruganantham, M., Moskovitz, Y., Batuman, O., Fingstein, A., and Mawassi, M. (2009). Post-transcriptional gene silencing and virus resistance in *Nicotiana benthamiana* expressing a Grapevine virus A minireplicon. *Trans. Res.* **18**, 331-345.
- van den Born, E., Omelchenko, M.V., Bekkelund, A., Leihne, V., Koonin, E.V., Dolja, V.V., and Falnes, P. Ø. (2008) Viral AlkB proteins repair RNA damage by oxidative demethylation. *Nucleic Acids Res.* **36**, 5451-5461.
- Koonin, E.V., Wolf, Y.I., Nagasaki, K., and Dolja, V.V. (2008) The Big Bang of picorna-like virus evolution antedates the radiation of eukaryotic supergroups. *Nature Rev. Microbiol.* **6**, 925-939.
- Martelli, G.P., Adams, M.J., Kreuze, J.F., and Dolja, V.V. 2007. Family *Flexiviridae*: A case study in virion and genome plasticity. *Annu. Rev. Phytopathol.* **45**, 73-100.
- Peremyslov, V.V., and Dolja, V.V. (2007) Cloning of large positive-strand RNA viruses. *Curr. Protoc. Microbiol.* 6:16F.1.1-16F.1.25.
- Dolja, V.V., Kreuze, J.F., and Valkonen, J.P.T. 2006. Comparative and functional genomics of closteroviruses. *Virus Res.* **117**, 38-51.
- Koonin, E.V., Senkevich, T.G., and Dolja, V.V. (2006) The ancient virus world and evolution of cells. *Biol. Direct* **1**:29, doi:10.1186/1745-6150-1-29.
- Chiba, M., Reed, J.C., Prokhnevsky, A.I., Chapman, E.J., Mawassi, M., Koonin, E.V., Carrington, J.C., and Dolja, V.V. (2006) Diverse suppressors of RNA silencing enhance agroinfection by a viral replicon. *Virology* **346**, 7-14.
- Haviv, S., Galiakparov, N., Goszczynski, D., Batuman, O., M., Czosnek, H., Mawassi, M. (2006). Engineering the genome of Grapevine Virus A into a vector for expression of Proteins in herbaceous plants. *J. Virol. Meth.* **132**, 227-231.

B

GVA -QRYTAGGSIGF**H****A****D**DEPCYLPGGSVVTVNHLHG**E**ATFEVKENQSGK----- 697

GVA -EKKE**L**KDGDVYVMGPGM**Q**QTH**K****H**RVTSHTDGRCSIT**L****R**NKTVDYEAR**R**KGDEDS----- 756

Fig.1. A, Map of GVA genome with AlkB domain. MP, movement protein; CP, coat protein; p10, the RSS encoded by ORF5. **B**, Amino acids sequence of GVA AlkB. Blue, catalytic residues invariant among all AlkB; Red, residues conserved in viral but not in cellular AlkB; Bold black, non-conserved residues.

Fig. 2. Examining infectivity of GVA vector. **A**, Silencing of PDS gene in *Nicotiana benthamiana*. **B**, Silencing of PDS gene in *Vitis vinifera* plantlets (lower leaves, control). **C**, confocal microscope images showing GFP expression in inoculated plants. **D**, EM image of GVA particle in inoculated *V. vinifera*, bar: 200nm. **E**, in-situ hybridization with Cy3-labelled GVA probe showing accumulation of GVA in the phloem of mature *V. vinifera* plant 6 months post inoculation. **F**, semi-quantitative PCR of PDS gene in silenced *V. vinifera* compared to non-silenced and to EF-1 α gene.

Fig. 3. A, Schematic diagram of the GVA-GFP minireplicon; **B**, GFP expression in leaves infiltrated with GVA-GFP minireplicon at 7 days post infiltration; **C**, GFP expression in leaves infiltrated with GVA-GFP minireplicon plus p10 at 7 dpi.

Fig. 4. Examining infectivity of GVA vector in *Vitis vinifera* protoplasts using GVA variant expressing MP-GFP fused product. Note that the GVA MP is inducing production of tubule-like structures.

Fig. 5. (A) Phylogenetic tree of selected viral AlkBs rooted by proteobacteria outgroup (Prote; black). Blue, *Flexiviridae*; yellow, *Closteroviridae*; green *Potyviridae*; red, *Sadwavirus*. (B) Isolated His-tagged viral AlkB. (C) Activity of *E. coli* and GVA AlkB on various [3H]methylated RNA and DNA substrates.

Fig. 6. (A-C) Systemic infection of *N. benthamiana* by the recombinant GLRaV-2 engineered to express the ER-targeted GFP. (A) Initially inoculate epidermal cell showing labeled ER. (B) GFP expression in the veins of non-inoculated leaf. (C) GFP expression in the stem phloem. (D-E) *V. vinifera* infected by the GFP-expressing GLRaV-2. (D) Agroinfiltrated leaf. (E) GFP in the veins of the non-inoculated leaf. (F) GFP in the phloem cells in the inner bark surface. (G) RT-PCR amplification of two fragments of GLRaV-2 vector.