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BARD Project Number: US 3623-04

Date of Submission of the report: March 25, 2008

Project Title: Functional analysis of virus and host components that mediate potyvirus-induced diseases

Investigators

Principal Investigator (PI):
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Iowa State University

Volcani- ARO

Volcani- ARO

Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.
Helper component, RNA silencing, micro RNA, disease, symptoms

Abbreviations commonly used in the report, in alphabetical order:

A – alanine; dpi – days post inoculation; F – phenylalanine; FRNK – amino acid Phenylalanine-Arginine-Asparagine-Lysine; HC-Pro – helper component protease; I – isoleucine; K – lysine; miRNA – micro RNA; N – asparagine; R – arginine; siRNA – short interfering RNA; TuMV – turnip mosaic virus; Y – tyrosine; ZYMV – zucchini yellow mosaic virus

Budget: IS: \$165,000

US: \$160,000

Total: \$325,000

3/20/08

Signature
Principal Investigator

3/20/08

Signature
Authorizing Official, Principal Institution

Marva K. Ruther
Post-Award Administrator
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Iowa State University

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged	1	1	1	3
Submitted, in review, in preparation	2	1		3
Invited review papers				
Book chapters		1		1
Books				
Master theses				
Ph.D. theses		1	1	2
Abstracts		3	4	7
Not refereed (proceedings, reports, etc.)				

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

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1	2		3
Longer Visits (Sabbaticals)				

Description Cooperation:

The US and IS collaborators communicated results and research strategies regularly by phone/SKYPE and email. The IS group focused on the ZYMV/cucurbit interaction and the US group focused on the TuMV/Arabidopsis interaction. These are parallel and complementary studies. The US/IS collaborators together completed construction of a squash and melon microarray and performed microarray hybridization and scanning at Iowa State University in July/August of year 2. This work involved the travel of Mr. Yoel Shibolet (the Ph.D. student currently working on the project in the IS laboratory) to Iowa State University to utilize expertise and equipment that is available for microarray studies. This cooperation involved the synthesis of PCR products from melon and squash SSH libraries (IS), the spotting of the PCR products onto glass slides (US), and hybridization, scanning, and analysis of the resulting data (US/IS). Details on the progress of this portion are presented in the Second Year Scientific Report. In addition, Dr. Whitham and Dr. Gal-On met in the US at the meeting of the American Phytopathological Society in Austin, TX where they discussed research results and plans. Dr. Whitham visited Volcani in the 3rd year to discuss results and exchange information and protocols on miRNA and siRNA assays and microarray analyses, to write the Shibolet et al manuscript, and to outline their new BARD proposal.

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Patent Summary (numbers)

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

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Abstract

The mechanisms underlying the development of symptoms in response to virus infection remain to be discovered in plants. Insight into symptoms induced by potyviruses comes from evidence implicating the potyviral HC-Pro protein in symptom development. In particular, recent studies link the development of symptoms in infected plants to HC-Pro's ability to interfere with small RNA metabolism and function in plant hosts. Moreover, mutation of the highly conserved FRNK amino acid motif to FINK in the HC-Pro of *Zucchini yellow mosaic virus* (ZYMV) converts a severe strain into an asymptomatic strain, but does not affect virus accumulation in cucurbit hosts. The ability of this FINK mutation to uncouple symptoms from virus accumulation creates a unique opportunity to study symptom etiology, which is usually confounded by simultaneous attenuation of both symptoms and virus accumulation. Our **goal** was to determine how mutations in the conserved FRNK motif affect host responses to potyvirus infection in cucurbits and *Arabidopsis thaliana*. Our **first objective** was to define those amino acids in the FRNK motif that are required for symptoms by mutating the FRNK motif in ZYMV and *Turnip mosaic virus* (TuMV). Symptom expression and accumulation of resulting mutant viruses in cucurbits and *Arabidopsis* was determined. Our **second objective** was to identify plant genes associated with virus disease symptoms by profiling gene expression in cucurbits and *Arabidopsis* in response to mutant and wild type ZYMV and TuMV, respectively. Genes from the two host species that are differentially expressed led us to focus on a subset of genes that are expected to be involved in symptom expression. Our **third objective** was to determine the functions of small RNA species in response to mutant and wild type HC-Pro protein expression by monitoring the accumulation of small RNAs and their targets in *Arabidopsis* and cucurbit plants infected with wild type and mutant TuMV and ZYMV, respectively.

We have found that the maintenance of the charge of the amino acids in the FRNK motif of HC-Pro is required for symptom expression. Reduced charge (FRNA, FRNL) lessen virus symptoms, and maintain the suppression of RNA silencing. The FRNK motif is involved in binding of small RNA species including microRNAs (miRNA) and short interfering RNAs (siRNA). This binding activity mediated by the FRNK motif has a role in protecting the viral genome from degradation by the host RNA silencing system. However, it also provides a mechanism by which the FRNK motif participates in inducing the symptoms of viral infection. Small RNA species, such as miRNA and siRNA, can regulate the functions of plant genes that affect plant growth and development. Thus, this binding activity suggests a mechanism by which ZYMV HC-Pro can interfere with plant development resulting in disease symptoms. Because the host genes regulated by small RNAs are known, we have identified candidate host genes that are expected to play a role in symptoms when their regulation is disrupted during viral infections.

As a result of this work, we have a better understanding of the FRNK amino acid motif of HC-Pro and its contribution to the functions of HC-Pro, and we have identified plant genes that potentially contribute to symptoms of virus infected plants when their expression becomes misregulated during potyviral infections. The results set the stage to establish the roles of specific host genes in viral pathogenicity. The potential benefits include the development of novel strategies for controlling diseases caused by viruses, methods to ensure stable expression of transgenes in genetically improved crops, and improved potyvirus vectors for expression of proteins or peptides in plants.

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Achievements

The FRNK motif of HC-pro is required for symptoms and for the suppression of RNA silencing. The FRNK motif in ZYMV (IS) and TuMV (US) was extensively mutated to test the symptom expression and accumulation of the resulting mutant viruses in cucurbits and *Arabidopsis*, respectively. Adjacent to the FRNK motif there is generally an “R” in cucurbit-infecting potyviruses (FRNKR), whereas in most of the other potyviruses, such as TuMV, there is an “I” (FRNKI). By mutating this motif, we were able to uncouple symptoms from ZYMV accumulation, but in TuMV, symptoms and virus accumulation were always tightly coupled. Results for TuMV in *Arabidopsis* and *N. benthamiana* were strikingly similar, indicating that effect of the mutations was independent of the host. The TuMV^{FINKI} and ^{FINKR} mutants were severely attenuated compared to wild type TuMV^{FRNKI} virus. The TuMV^{FRNKR} mutation gave an intermediate accumulation level with mild symptoms, but it was excluded from the newly emerging tissues and stems, unlike the wild type virus. In contrast to TuMV, the FRNKR to FRNKI mutation in ZYMV did not affect virus infectivity and symptom severity, while the FINKI double mutation had a restricted area of infection and squash plants recovered from the virus after three weeks. We propose that the FRNKI to FINKI mutation in TuMV made a dramatic charge change in this area which prevents infectivity. However in ZYMV, the FINK mutation is less severe due to the R amino acid following the FRNK motif. This conclusion was supported by additional mutations such as FKNK, which conserves charge, that were as infectious as wild type, whereas mutations such as FRNA and FRNL, which reduce charge, attenuated infection.

We found a strong positive correlation between the ability of an HC-Pro mutant to suppress silencing and support viral infection. We compared the suppressor activity between the severe ZYMV^{FRNK} and the attenuated mutant ZYMV^{FINK} and found that the HC^{FRNK} and HC^{FINK} proteins have similar suppressor activities. This is in contrast to TuMV in which the HC^{FINK} protein loses silencing suppressor activity - consistent with the severely reduced ability of this mutant virus to infect plants. ZYMV^{FINK} mutants can maintain their silencing suppressor activity and systemically infect squash at an early stage at a similar level to ZYMV^{FRNK}, but not cause symptoms. Whereas, TuMV^{FINK} is nearly dead and possesses no silencing suppressor activity. The inability of TuMV^{FINK} to suppress gene silencing was further investigated, and we found that the TuMV HC^{FINK} mutant protein could not accumulate in plants, because the mRNA encoding the mutant HC-Pro became a target for

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RNA silencing. This silencing was reversed by co-expression with wild type HC-Pro or another silencing suppressor such as TBSV P19.

Effect of the FRNK motif on small RNA metabolism and function. Because the ZYMV HC^{FRNK} and HC^{FINK} both were able to suppress gene silencing, but the FINK mutant was attenuated for symptoms, we developed the working hypothesis that wild type and mutant HC-Pro affect the function of small RNAs in host plants differently. The accumulation of miRNA and siRNA and their targets was examined in plants infected with wild type and mutant ZYMV. Major differences were found between the level of accumulation of miRNA and miRNA* in infected and non-infected plants and also differences between plants inoculated with the mild ZYMV^{FINK} and severe ZYMV^{FRNK}. In general, miRNA and miRNA* levels were higher in virus-infected plants, and especially in ZYMV^{FRNK}-infected plants, compared to uninfected plants. miRNA are small regulatory RNA molecules produced by the plant. The miRNA* is the complementary strand that is normally degraded very rapidly, but our results show that viral infection prevents their degradation. Major differences in miRNA* expression were found between ZYMV^{FRNK}- and ZYMV^{FINK}-infected plants. These observations support the hypothesis of the involvement of the FRNK motif with miRNA/miRNA* accumulation which may be associated with symptom elicitation.

The higher accumulation of miRNA/miRNA* in ZYMV^{FRNK}-infected plants, as compared to ZYMV^{FINK}-infected plants, led us to examine differential smRNA binding by ZYMV-HC-Pro mutant proteins. We found that wild-type ZYMV HC-Pro binds miRNA/miRNA* duplexes *in vitro*. This binding presumably stabilizes such duplexes *in vivo*. The importance of the FRNK box charge was demonstrated by the result that the charge-preserving ZYMV-HC^{FKNK} mutant displayed severe symptoms and bound smRNA with an affinity similar to ZYMV-HC^{FRNK}. In contrast, ZYMV-HC^{FRNA} and ZYMV-HC^{FINK} mutants permitted virus replication and displayed mild symptoms, but had weaker miRNA/miRNA* duplex binding activity. Thus, the FRNK box is a potential point of contact with smRNA duplexes and is essential to HC-Pro to sequester miRNA and directly influence symptom development.

Based on these data, we propose that symptoms are, in part, a result of viral interference with miRNA-mediated gene regulation in young, developing leaves. We propose that, in very young leaves, the sequestration of miRNA/miRNA* duplexes by HC-Pro renders most of the miRNAs inactive, which causes increased levels of mRNAs that are normally regulated by

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miRNAs or other endogenous small RNAs. Because the levels of these mRNAs are critical to development, the plants develop disease phenotypes. The lower affinity of the HC^{FRNK} mutants for miRNA/miRNA* duplexes allows sufficient populations of free miRNAs to be maintained in young leaves permitting normal development. Thus, the decreased accumulation of miRNA and miRNA* levels observed by the ZYMV^{FRNK} virus as compared to the ZYMV^{FRNK} virus may account for the abrogation of symptoms.

Identification of plant genes associated with virus disease symptoms. A major goal of this work was to identify host genes that are candidates for mediating symptoms. The previous section describes how miRNAs and their target genes are differentially affected by mutant and wild type HC-Pro. The genes that are regulated by the miRNAs are known, though most have not been studied with respect to virus symptoms. We have used this information to develop a list of host genes that can be tested for roles viral pathogenicity. The genes of greatest interest to us are those regulated by miR159, miR166, miR168, miR319, and TAS3-D7. Their target genes regulate plant development through their roles as transcription factors, RNA silencing components, and hormone signaling. In addition, we found through microarray analysis that TuMV downregulates a large number of genes involved in photosynthesis and chloroplast biosynthesis and cell wall expansion. The downregulation of these genes is consistent with the chlorotic and stunted growth phenotypes of TuMV-infected plants. Interestingly, we have found that these genes are downregulated by TuMV by a mechanism that is not affected by HC-Pro, but dependent on certain components of the RNA silencing system. As expected, this work has led to the identification of numerous plant genes that are candidates for contributing to potyvirus symptoms. These genes are the subjects of future studies to determine their regulation and roles in potyviral pathogenicity.

List of publications and patents to date (* indicates corresponding author)

1. Whitham, S. A.*, Yang, C., and Goodin, M. M. (2006). Global impact: Elucidating plant responses to viral infection. *Mol. Plant Microbe. Interact.* 19:1207-1215
2. Shibolet, Y. M., Haronsky, E., Leibman D., Arazi T, Whitham, S. Wassenegger, M., Gaba V. and Gal-On, A.* (2007). The conserved FRNK box in plant viral suppressor of gene silencing HC-Pro is required for small RNA binding and mediates symptom development. *Journal Virology* 81:13135-13148.

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Appendix:

I. Abstracts presented at meetings and workshops (7 total)

II. Joint journal publication:

Shiboleth, Y. M., Haronsky, E., Leibman D., Arazi T, Whitham, S. Wassenegger, M., Gaba V. and Gal-On, A.* (2007). The conserved FRNK box in plant viral suppressor of gene silencing HC-Pro is required for small RNA binding and mediates symptom development. *J. Virology* 81:13135-13148.

III. US book chapter:

Carr, T. and Whitham, S. A. (2007). An emerging model system: *Arabidopsis* as a viral host plant. In *Viral Transport in Plants. Springer book series: Plant Cell Monographs*. Edited by E. Waigman and M. Heinlein. Springer-Verlag GmbH, Heidelberg, Germany. pp. 159-183

IV. IS journal publication:

Gal-On, A. (2007). Zucchini yellow mosaic virus: insect transmission and pathogenicity — the tails of two proteins. *Mol. Plant Pathol.* 8:139–150

V. US journal publication:

Whitham, S. A.*, Yang, C., and Goodin, M. M. (2006). Global impact: Elucidating plant responses to viral infection. *Mol. Plant Microbe. Interact.* 19:1207-1215