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BARD

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Suppression of Potyvirus Infection by a Closterovirus Protein

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Project Title: Suppression of Potyvirus Infection by a Closterovirus Protein

Investigators

Principal Investigator (PI): Valerian V. Dolja

Institutions

Oregon State University

Co-Principal Investigators (Co-PIs): Amit Gal-On Agricultural Research Organization

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Collaborating Investigators:

Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

Virus vector, Leader proteinase, Virus resistance

Abbreviations commonly used in the report, in alphabetical order:

BYV, beet yellows virus; L-Pro, leader proteinase; TEV, tobacco etch virus; ZYMV, zucchini yellow mosaic virus

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Signature
Principal Investigator

for Signature
Authorizing Official, Principal Institution

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)				
Submitted, in review, in preparation		1		1
Invited review papers				
Book chapters				
Books				
Master theses		1	1	2
Ph.D. theses		1	1	2
Abstracts	1			1
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	3	1	5
Longer Visits (Sabbaticals)				

Description of Cooperation:

The PI visited ARO in Israel for two weeks early into collaboration period in order to develop detailed experimental plan. One of the coPIs (Amit Gal-On) visited OSU in the US in order to conduct joint experiments, as well as to install the device for efficient plant inoculation. In addition, the PI and co-PI have met during the EMBO Workshop (Spain, 1997) and twice during American Society for Virology meetings in 1999 and 2001 (Amherst, MA and Madison, WI, respectively).

Patent Summary (numbers)

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

Abstract

The plant virus family *Potyviridae* is the largest and most destructive of all plant viruses. Despite the continuous effort to develop resistant plant varieties, there is a desperate need for novel approaches conferring wide-range potyvirus resistance. Based on experiments with the tobacco etch potyvirus (TEV)-derived gene expression vector, we suggested approach for screening of the candidate resistance genes. This approach relies on insertion of the genes into a virus vector and evaluation of the phenotypes of the resulting recombinant viruses. The genes which suppress infection by the recombinant virus are selected as candidates for engineering transgenic resistance.

Our analysis of the TEV variants expressing proteins of the beet yellows closterovirus (BYV) revealed that one of those, the leader proteinase (L-Pro), strongly and specifically interfered with the hybrid TEV infection. Since closterovirus L-Pro is evolutionary related to potyviral helper component-proteinase (HC-Pro), we suggested that the L-Pro interfered with HC-Pro function via a *trans*-dominant inhibitory effect. Based on these findings, we proposed to test two major hypotheses. First, we suggested that L-Pro-mediated suppression of potyvirus infection is a general phenomenon effective against a range of potyviruses. The second hypothesis stated that the suppression effect can be reproduced in transgenic plants expressing L-Pro, and can be utilized for generation of resistance to potyviruses. In accord with these hypotheses, we developed two original objectives of our proposal: A) to determine the range of the closterovirus-derived suppression of potyviral infection, and B) to try and utilize the L-Pro-mediated suppression for the development of transgenic resistance to potyviruses.

In the first phase of the project, we have developed all major tools and technologies required for successful completion of the proposed research. These included TEV and ZYMV vectors engineered to express several closteroviral L-Pro variants, and generation of the large collection of transgenic plants. To our satisfaction, characterization of the infection phenotypes exhibited by chimeric TEV and ZYMV variants confirmed our first hypothesis. For instance, similar to TEV-L-Pro_(BYV) chimera, ZYMV-L-Pro_(LJYV) chimera was debilitated in its systemic spread. In contrast, ZYMV-GUS chimera (positive control) was competent in establishing vigorous systemic infection. These and other results with chimeric viruses indicated that several closteroviral proteinases inhibit long-distance movement of the potyviruses upon co-expression in infected plants.

In order to complete the second objective, we have generated ~90 tobacco lines transformed with closteroviral L-Pro variants, as well as ~100 lines transformed with BYV Hsp70-homolog (Hsp70h; a negative control). The presence and expression of the transgene in each line was initially confirmed using RT-PCR and RNA preparations isolated from plants. However, since detection of the transgene-specific RNA can not guarantee production of the corresponding protein, we have also generated L-Pro- and Hsp70h-specific antisera using corresponding synthetic peptides. These antisera allowed us to confirm that the transgenic plant lines produced detectable, although highly variable levels of the closterovirus antigens.

In a final phase of the project, we tested susceptibility of the transgenic lines to TEV infection. To this end, we determined that the minimal dilution of the TEV inoculum that is still capable of infecting 100% of nontransgenic plants was 1:20, and used 10 plants per line (in total, ~2,000 plants). Unfortunately, none of the lines exhibited statistically significant reduction in susceptibility. Although discouraging, this outcome prompted us to expand our experimental plan and conduct additional experiments. Our aim was to test if closteroviral proteinases are capable of functioning *in trans*. We have developed agroinfection protocol for BYV, and tested if co-expression of the L-Pro is capable of rescuing corresponding null-mutant. The clear-cut, negative results of these experiments demonstrated that L-Pro acts only *in cis*, thus explaining the lack of resistance in our transgenic plants. We have also characterized a collection of the L-Pro alanine-scanning mutants and found direct genetic evidence of the requirement for L-Pro in virus systemic spread. To conclude, our research supported by BARD confirmed one but not another of our original hypotheses. Moreover, it provided an important insight into functional specialization of the viral proteinases and generated set of tools and data with which we will be able to address the molecular mechanisms by which these proteins provide a variety of critical functions during virus life cycle.

Achievements

A major result of our collaboration is development of a novel approach for the screening of the candidate genes for transgenic resistance to plant viruses. Using gene vectors derived from two potyviruses, ZYMV (Israel) and TEV (US), we demonstrated that expression of some proteins leads to specific inhibition of recombinant virus spread in infected plants. In particular, we discovered that the leader proteinases of such closteroviruses as BYV, LIYV, and CTV exert an inhibitory effect onto long-distance spread of ZYMV and TEV. Since neither GUS, nor other closterovirus proteins of the mol. wt similar to that of the L-Pro markedly affected systemic infection by corresponding ZYMV or TEV chimeras, we concluded that this effect is specific and is due to interference of the closteroviral L-Pro with the HC-Pro function in systemic spread. This approach of candidate gene screening is widely applicable to all viruses which were developed into gene expression vectors. Obviously, it can be used not only with the virus-derived, but also with the plant-derived genes such as candidate R genes conferring virus resistance. Engineering of the foreign genes into virus vectors and testing the resulting phenotypes is much faster than generating and screening transgenic plants. Because of that, the approach developed in our project can be strongly recommended for preliminary characterization of the candidate resistance genes.

In a second phase of our project, we attempted at developing transgenic resistance to potyviruses using closteroviral L-Pro gene identified as promising candidate in a first phase. All of the ~200 transgenic tobacco lines were developed in Israel and screened for the transgene expression and resistance to TEV in the US. Unexpectedly, none of the tested transgenic lines exhibited statistically significant virus resistance. This fact pointed to an important limitation of the 'screening for resistance by co-expression approach': not every protein capable of inhibiting the infection of the virus vector is able to confer transgenic resistance. Among several possible explanation of this phenomenon, two appear to be the most plausible. First, the level of foreign protein expression achieved by using virus gene vector is normally higher than that found in transgenic plants. Due to that, the lack of virus resistance in transgenic plants may be attributed to insufficient accumulation of the effector protein. This problem, however, is intrinsic to transgenic technology in general. Due to positional effects and gene silencing, the levels of foreign transgene expression are normally low to moderate. As a consequence, any protein-based transgenic resistance should rather rely on candidate proteins that are biologically active at the low expression levels.

An alternative explanation for the lack of transgenic resistance to a potyvirus conferred by closterovirus L-Pro is that L-Pro acts *in cis* rather than *in trans*. Although this was not planned

under the original objectives, we designed a novel experimental system in order to test this possibility (see Figs. 1 and 2 in the Appendix). We have engineered a cDNA copy of mini-BYV genome into binary vector suitable for agroinfection of plants. This mini-BYV variant possessed genes encoding L-Pro, viral replicase, and p21, an enhancer of genome amplification. In addition, it was tagged via insertion of GUS gene. Preliminary experiments demonstrated efficient agroinfection and accumulation of GUS activity that provided a sensitive measure of RNA replication. Next we generated a mini-BYV variant in which the complete L-Pro ORF was deleted (a null mutant) and an additional binary vector that would express wild type L-Pro from an autonomous mRNA (Fig. 2B). As expected, deletion of the L-Pro resulted in a dramatic, ~1,000-fold reduction in GUS activity detected post agroinfection. Interestingly, simultaneous expression of the wild-type L-Pro with this null mutant did not rescue its replication, thus indicating that the L-Pro functions only *in cis*, but not *in trans*. This result provided important information concerned with the mechanism of L-Pro action and explained why L-Pro was inactive in conferring transgenic resistance to potyviruses.

Our original explanation of the suppression of potyvirus systemic spread by co-expressed closteroviral L-Pro was based on assumption that L-Pro interferes with the HC-Pro function in long-distance transport. To further substantiate this idea, we directly addressed the possible L-Pro function in systemic infection. To this end, we generated 24 alanine-scanning mutations throughout the L-Pro sequence and characterized the resulting phenotypes. We found that the majority of these mutants were competent in RNA amplification in protoplasts and cell-to-cell movement in inoculated leaves. The systemic infectivity of these mutants was tested using agroinfection. It was found that four mutants exhibited significant, 2-week delay in colonization of the upper leaves, as well as only limited spread of infection within these leaves. Strikingly, three alanine-scanning mutations resulted in complete arrest of the systemic infection (Fig. 3 in the Appendix). These data provided direct genetic evidence of the L-Pro function in closterovirus long-distance transport and supported our assumption of the similarity in the functional profiles of closteroviral L-Pro and potyviral HC-Pro.

An additional important outcome of our collaboration has to do with applications to biotechnology. Expression of the foreign proteins by TEV and ZYMV vectors is achieved via polyprotein synthesis and processing by two potyviral proteinases, HC-Pro and NIa, which cleave at the foreign protein extremities *in cis* and *in trans*, respectively. However, only some plant viruses encode proteinases that are capable of processing foreign proteins. To increase the utility of polyprotein processing approach for virus vectors that do not encode suitable proteinases, we developed a novel prototype of the self-processing foreign protein. This prototype represents a

GUS reporter fused to a small (~20 kDa), papain-like proteinase domain derived from the TEV HC-Pro. The resulting GUS-Pro reporter ORF was tested in a context of BYV genome via its insertion immediately downstream from the start codons in several BYV ORFs (Fig. 1). It was expected that the formation and translation of the recombinant subgenomic RNA will result in efficient self-processing and release of the GUS-Pro. The authentic virus product encoded downstream from the GUS-Pro should also be produced in a virtually wild type form. To our satisfaction, the recombinant BYV variants were infectious and capable of producing both fully processed, enzymatically-active GUS-Pro, and free BYV protein. These collaborative experiments conducted in US during the visit of one of the co-PIs (Amit Gal-On) provided a proof of a concept and largely expanded applicability of the polyprotein processing approach for the virus vectors that do not normally produce proteinases.

In conclusion, this project generated important information concerned with the complex and partially overlapping functional profiles of the potyviral HC-Pro and closteroviral L-Pro. Both of these leader proteinases are required for polyprotein processing, efficient amplification of the viral RNA genomes, and virus long-distance transport. Moreover, we found that co-expression of the HC-Pro and L-Pro in particular host plants results in gross debilitation of the potyviral transport. This result is important for understanding mixed virus infections that are common in agriculture. For instance, sugar beets are frequently co-infected by BYV and beet mosaic potyvirus. Most important applied aspects of our work include development of a novel approach for the screening of the candidate genes for transgenic resistance to plant viruses and expansion of the utility of self-processing proteins in plant biotechnology. An armamentarium of virus vectors, transient expression cassettes, and transgenic plant lines generated in a course of our collaboration will be of paramount importance for further work in the Israeli and US labs aimed at both fundamental and applied aspects of potyviral and closteroviral biology.

Publications: Results of the early phase of the project were jointly presented at the EMBO Workshop in Spain (see corresponding Abstract in Appendix). American lab currently prepares for publication a paper by C.-W. Peng and V.V. Dolja entitled "Leader proteinase of the *Beet yellows virus* functions in long-distance transport" that was in part supported by the BARD grant. Finally, results of our project were incorporated into the following theses:

Hagiwara, Y. (1999) Regulation of gene expression in the beet yellows virus. M.S. thesis, Oregon State University, Corvallis, Oregon.

Peng, C.-W. (2002) Multiple functions of the leader proteinase in a closterovirus life cycle. Ph.D. thesis, Oregon State University, Corvallis, Oregon.