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Viral Synergism in Transgenic Plants

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BARD Final Scientific Report
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Project Title: VIRAL SYNERGISM IN TRANSGENIC PLANTS

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VIRUS RESISTANCE, RISK ASSESSMENT

Abbreviations used in the report, in alphabetical order:

CMV, GFP, GUS, HC-Pro, PCR, PPV, PTGS, PVX, PVY, SAAT, TMV, WMV-II, ZYMV

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Appendix GF

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Publication Summary (numbers)

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

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted				0
Issued (allowed)				0
Licensed				0

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Visits/Meetings	1	2	2	5
Sabbaticals				0
Postdoctorates				

- **Cooperation**, briefly explain whether synergistic, complementary or supportive.
Cooperation was synergistic and complementary _____

Final Scientific Report: Abstract

Transgenic plants expressing viral genes offer novel means of engendering resistance to those viruses. However, some viruses interact synergistically with other viruses and it is now known that transgenic plants expressing particular genes of one virus may also mediate synergy with a second virus. Thus, our specific objectives were to (1) determine if transgenic plants resistant to one virus showed synergy with another virus; (2) determine what viral sequences were essential for synergy; and (3) determine whether one of more mechanisms were involved in synergy. This project would also enable an evaluation of the risks of synergism associated with the use of such transgenic plants. The conclusions deriving from this project are as follows:

- There is more than one mechanism of synergy.
- The CMV 2b gene is required for synergistic interactions.
- Synergy between a potyvirus and CMV can break natural resistance limiting CMV movement.
- Synergy operates at two level – increase in virus accumulation and increase in pathology –independently of each other.
- Various sequences of CMV can interact with the host to alter pathogenicity and affect virus accumulation.
- The effect of synergy on CMV satellite RNA accumulation varies in different systems.
- The HC-Pro gene may only function in host plant species to induce synergy.
- The HC-Pro gene is a host range determinant of potyviruses.
- Transgenic plants expressing some viral sequences showed synergy with one or more viruses. Transgenic plants expressing CMV RNA 1, PVY Nib and the TMV 30K gene all showed synergy with at least one unrelated virus.
- Transgenic plants expressing some viral sequences showed interference with the infection of unrelated viruses. Transgenic plants expressing the TMV 30K, 54K and 126K genes, the PVY Nib gene, or the CMV 3a gene all showed some level of interference with the accumulation (and in some cases the pathology) of unrelated viruses.

From our observations, there are agricultural implications to the above conclusions. It is apparent that before they are released commercially, transgenic plants expressing viral sequences for resistance to one virus need to be evaluated for two properties:

- Synergism to unrelated viruses that infect the same plant. Most of these evaluations can be made in the greenhouse, and many can be predicted from the known literature of viruses known to interact with each other. In other cases, where transgenic plants are being generated from new plant species, the main corresponding viruses from the same known interacting genera (e.g., potexviruses and cucumoviruses, potyviruses and cucumoviruses, tobamoviruses and potexviruses, etc.) should be evaluated.
- Inhibition or enhancement of other resistance genes. Although it is unlikely that plants to be released would be transformed with HC-Pro or 2b genes, there may be other viral genes that can affect the expression of plant genes encoding resistance to other pathogens. Therefore, transgenic plants expressing viral genes to engender pathogen-derived resistance should be evaluated against a spectrum of other pathogens, to determine whether those resistance activities are still present, have been lost, or have been enhanced!

BARD Final Scientific Report: Achievements

The significance of the main scientific achievements is as follows:

1. There is more than one mechanism of synergy. The recent discovery by others of various viral genes besides the *Cucumber mosaic virus* (CMV) 2b gene and the potyvirus HC-Pro gene being involved in suppressing cellular mechanisms of post-transcriptional gene silencing (PTGS), supports our conclusions that there are subtle as well as specific differences in synergy between different classes of virus, as well as host effects on the synergy.
2. The CMV 2b gene is required for synergistic interactions. Again, this fits with recent observations made by others, who have shown that the CMV 2b gene and the *Potato virus Y* (PVY) HC-Pro gene both suppress PTGS, although the CMV 2b appears to interfere with the establishment of PTGS and the HC-Pro gene interferes with the maintenance of PTGS.
3. Synergy between *Zucchini yellow mosaic virus* (ZYMV) and CMV can break natural resistance limiting CMV movement. This raises the possibility that infection by a potyvirus or CMV could not only derepress a silenced transgene (resulting in the gain of susceptibility in a plant resistant to a different virus, or restoration of a transgenically-silenced, undesirable metabolic function), but could also inhibit natural resistance genes to other pathogens.
4. Synergy operates at two level – increase in virus accumulation and increase in pathology –independently of each other. In the various mechanisms involved in synergy, the straight increase in viral accumulation itself does not cause increases in pathology, and increases in pathology, by the interaction of two viruses, does not in itself lead to increases in their titers. The work also shows that there may be connections between the mechanisms involved in synergy and those involved in tolerance to plant viruses.
5. Various sequences of CMV can interact with the host to alter pathogenicity and affect virus accumulation. This suggests that viruses have more than one factor interacting with the host to induce pathology, and also more than one factor that may activate or inhibit host response genes involved in limiting virus infection.
6. The effect of synergy on CMV satellite RNA accumulation varies in different systems. The role of the satellite RNA is similar to the role of host anti-viral response genes; i.e., to

reduce the virus titer and pathogenicity. This may suggest a link between satellite RNAs and host anti-viral response genes.

7. The HC-Pro gene may only function in host plant species to induce synergy. The ability of the HC-Pro to shut off plant response genes appears to be limited to plant species that are hosts for the corresponding potyvirus.
8. The HC-Pro gene is a host range determinant of potyviruses. The correlation of HC-Pro activity in host species indicates that the function or lack thereof of the HC-Pro determines whether a plant will host infection (to varying extents) by a given potyvirus. Most potyviruses have a narrow host range, but there are over 100 known potyviruses, together encompassing a very broad range of agricultural and horticultural plants.
9. Transgenic plants expressing some viral sequences showed synergy with one or more viruses. Transgenic plants expressing CMV RNA 1, PVY NIb and the TMV 30K gene all showed synergy with at least one unrelated virus. This again confirms that more than just HC-Pro and 2b genes are involved in countering host responses limiting infection.
10. Transgenic plants expressing some viral sequences showed interference with the infection of unrelated viruses. Transgenic plants expressing the TMV 30K, 54K and 126K genes, the PVY NIb gene, or the CMV 3a gene all showed some level of interference with the accumulation (and in some cases the pathology) of unrelated viruses. This shows that unexpected benefits can also arise from transgenes generated to inhibit infection by one virus. Thus, expression of both the NIb gene of PVY and the TMV 54K gene, which show complete resistance to the homologous viruses, TMV and PVY, would also produce some resistance to *Potato virus X* (PVX), and not show synergy with any of the above viruses or with CMV.

The agricultural implications of the above achievements relate to additional evaluations that need to be done with transgenic plants prior to their commercial release. That is, transgenic plants expressing viral sequences for resistance to one virus need to be evaluated for two properties:

1. Synergism to unrelated viruses that infect the same plant. Most of these evaluations can be made in the greenhouse, and many can be predicted from the known literature of viruses known to interact with each other. In other cases, where transgenic plants are being generated from new plant species, the main corresponding viruses from the same known interacting genera (e.g., potexviruses and cucumoviruses, potyviruses and cucumoviruses, tobamoviruses and potexviruses, etc.) should be evaluated.

2. Inhibition or enhancement of other resistance genes. Although it is unlikely that plants to be released would be transformed with HC-Pro or 2b genes, there may be other viral genes that can affect the expression of plant genes encoding resistance to other pathogens. Therefore, transgenic plants expressing viral genes to engender pathogen-derived resistance should be evaluated against a spectrum of other pathogens, to determine whether those resistance activities are still present, have been lost, or have been enhanced! All three situations were observed in this study, and none were predicted beforehand.

While it is impossible to estimate the benefits to agriculture of these recommendations, in the short run, failure to do so will have negative consequences, in the long run.

The cooperation resulted in the objectives being promoted at three international meetings (risk assessment meetings in Budapest in 1997 and Braunschweig in 1998, an EMBO meeting near Madrid in 1997) and one US national meeting (ASV meeting in Amherst in 1999). The potential effects of synergy are now recognized in the risk assessment community, and the fact that there is more than one mechanism of synergy is also recognized in the plant pathology community. The cooperation also enabled the technology transfer between the two labs, as well as access to instrumentation and specializations particular to the two labs.

Final Scientific Report

I. Synergism in Transgenic Plants Expressing TMV and CMV Sequences.

A. TMV-Transgenic Tobacco Infected with PVX, CMV or PVY.

Tobacco transgenic for the TMV 54K (replicase) gene and resistant to infection by TMV did not show any synergy (in either pathology or increased virus titer) with PVX. In the case of tobacco transgenic for the TMV 30K (movement) gene, a slight reduction in PVX accumulation was observed, as well as a reduction in the pathology induced by PVX, while in tobacco transgenic for the 126K (replication) gene, there was a 2-4-fold reduction in PVX accumulation with no effect on PVX-induced pathology. We also confirmed that double inoculation with PVX and TMV gave rise to synergy of both pathology and increased PVX accumulation (ca. 8-fold), with no increase in TMV accumulation. Thus, while TMV induced increases in PVX levels in doubly infected plants, transgenic plants expressing individual TMV genes did not induce synergy with PVX, and in some cases the transgenic TMV genes showed interference with PVX accumulations.

We extended the original goal of this section to examine the effect of the TMV transgenes on two other viruses: CMV and PVY. In doubly inoculated plants, TMV and PVY induced a synergy in pathology, but with no changes in virus titers, while in TMV transgenic plants PVY showed a synergy in pathology and an increase in virus accumulation (ca. 4-fold) in TMV 30K-transgenic tobacco, and no change in pathology, but a ca. 4-fold decrease in PVY levels in TMV 54K- and 126K-transgenic tobacco. By contrast, plants doubly infected with TMV and CMV showed a strong synergy of pathology with a ca. 16-fold increase in CMV levels, while in TMV transgenic plants CMV showed a 2-4-fold increase in CMV levels and an increase in pathology and virus movement in TMV 30K-transgenic tobacco, no effects in TMV 54K-transgenic tobacco, and a 2-4-fold decrease in CMV levels with no change in pathology in TMV 126K-transgenic tobacco. Thus, the transgenic plants expressing functional TMV movement protein showed synergy, while those expressing the TMV 54K or 126K replicase proteins showed no synergy, and either no effects or an interference in virus titre. Therefore, it appears that either more than one gene product of TMV or sequences not represented by the known genes products are required to induce synergy with PVX.

B. CMV-Transgenic Tobacco Infected with PVX, PVY or TMV.

We expanded the number of viruses tested in the transgenic tobacco plants expressing CMV RNA 1 (not resistant to CMV infection) CMV RNA 2 (full-length and resistant as well as full-length and susceptible, and two resistant lines containing truncated RNA 2), and the CMV 3a movement protein gene (5' half of RNA 3; susceptible to CMV infection). Besides PVX, PVY and TMV were also tested in transgenic plants, since we had confirmed that PVY and CMV as well as PVX and CMV, or TMV and CMV induced synergy. Again, three types of observations were made: All but four combinations showed no effect (i.e., the transgene neither induced synergy nor interference with the inoculated virus); two combinations showed synergy (PVY and TMV in full-length CMV RNA 1-transgenic tobacco) and two combinations showed interference (PVX and TMV in CMV 3a-transgenic tobacco).

C. PVY-Transgenic Tobacco Infected with CMV, PVX and TMV.

This section was not in the original proposal, but was done to round out the study, since transgenic tobacco expressing the PVY N1b gene and showing either resistance or susceptibility to PVY were available, and we had confirmed that PVY interacted synergistically with CMV, PVX and TMV. In this case, three types of observations were made: no effect (CMV); interference (to PVX in a PVY-resistant line; i.e., an increase in pathogenicity and an 8-fold reduction in virus titer in the line resistant to PVY and no effect in the PVY-susceptible line); and synergy (an increase in pathogenicity induced by TMV in the PVY-resistant line). Thus, a transgene exhibiting resistance to its homologous virus was able to interfere with infection of one unrelated virus (PVX) and increase the pathogenicity of another unrelated virus (TMV).

The ability of synergy to break resistance to the homologous virus was also examined. PVX and PVY as well as TMV and PVY were inoculated to transgenic plants resistant and susceptible to PVY infection. In the susceptible plants, typical synergy was observed, while in the resistant plants, synergy was not observed; only the symptoms of the virus unrelated to the transgene were induced. Thus, other viruses interacting synergistically with PVY do not break replicase-mediated resistance to PVY.

The observations on interference of PVX infection in transgenic tobacco expressing the PVY N1b gene and showing resistance to PVY were extended in the two ways: (1) Another transgenic line expressing the PVY N1b gene and showing resistance to PVY was tested for interference with PVX infection. This verified that the observations made previously were reproducible and were not due to somaclonal variation. (2) Samples were analyzed at different times post-inoculation, and showed that PVX accumulation in the PVY-

Nlb transgenic line was reduced, but eventually reached a similar level as in a non-resistant line.

II. Molecular Genetics of Synergism: ZYMV and CMV.

A. Generation of ZYMV-Transgenic Plants.

We decided to generate transgenic *Nicotiana tabacum* and *N. benthamiana* expressing ZYMV sequences to test for synergism with CMV in parallel with the production of transgenic cucurbits. Production of transgenic *Nicotiana* spp. are faster, so the results obtained in transgenic tobacco or *N. benthamiana* could provide an indication of the expression of the ZYMV genes (P1, HC-Pro and P3).

By PCR manipulation we generated a construct that contains the three genes P1, HC-Pro and P3 between the 5' and the 3' ZYMV non coding region (NCR). The ZYMV sequence was cloned under the 35S promoter and NOS terminator into the binary vector pBINPLUS. We screened R0 transgenic tobacco and *N. benthamiana* for the ZYMV sequence and we found that about 90% of the Kanamycin resistance plant were harboring this fragment. A total of 42 independent transformed lines were produced for tobacco and for *N. benthamiana*. All the plants were tested in the R0 generation by PCR for the presence of the gene of the HC-Pro of ZYMV, and R1 seeds collected from all these lines. Four independent R1 lines from each *Nicotiana* species were screened for HC-Pro protein expression, and one line from each species was identified that expressed the protein.

A new regeneration method was devised for *Cucurbita pepo*. This method produces plants by direct regeneration, which has not been reported previously in the literature for this species. After explant initiation on Murashige and Skoog (1962) medium, explants began to regenerate in standard growth room conditions. Regeneration varied between 50 to 90%, depending on the experiment and the variety. Elongation medium with 1mg/l gibberellin and 0.1mg/l benzyl adenine was more effective at shoot elongation than with only benzyl adenine for cv. True French and was therefore selected for further use. Similar results were obtained with other cultivars. The regeneration method functioned for all of the *C. pepo* varieties tested (cvs. Ma'ayan, Baraquet, Goldy and True French). Many plants were produced which grew well in the greenhouse.

Transformation of *C. pepo* by co-cultivation with *Agrobacterium tumefaciens* was attempted several times, both by conventional leaf disk type of inoculation, and by sonication-assisted *Agrobacterium* transformation (SAAT). We used transformation vectors bearing either reporter genes GUS-intron and the green fluorescent protein (GFP).

Experiments were performed either with selection on kanamycin-containing medium, or without selection by the screening for reporter gene activity of the population of regenerant plants. We found that a leaf disk type of inoculation with *Agrobacterium* was not very successful, but much larger areas of GUS-intron activity could be produced in explants by SAAT. Using SAAT we obtained no plants on selection with kanamycin. However, by screening for GUS-intron activity we found several chimeric plants out of hundreds of regenerants examined, expressing areas of GUS-intron activity in non-transformed tissues. Such chimeric plants had GUS-intron activity in large patches in the stem or leaves, or in the pith of a petiole. We now optimizing particle bombardment to reach the population of cells which regenerates new plants.

We decided to generate transgenic cucumber lines carrying the ZYMV HC-Pro genes, because of the results obtained with the cucumber cv. Delila (a CMV tolerant variety –see below). Transformation of cucumber was effected via the organogenetic pathway. We have followed basically the protocol published by Chee (Plant Cell Repts 9: 245-248), with minor modifications adopted throughout the program. We have used several commercial cultivars in this work: Bet Alpha, Delila, Kfir, Ilan, Prat, Sarig and others. The plants will be evaluated.

B. Preparation and Characterization of Antisera to ZYMV-Encoded Proteins.

To examine the expression of the HC-Pro of ZYMV in the transgenic plants we needed an antibody specific to the HC-Pro protein. Thus, we cloned the HC-Pro-gene into the pET expression vector. The expressed protein was injected into rabbits and the serum tested for immunogenicity of response to HC-Pro. This antiserum was not suitable, so we injected the purified native HC-Pro protein, and obtained an HC-Pro-specific antiserum.

C. Synergy Between ZYMV and CMV in Transgenic Plants vs. Double Infected Plants.

Mixed infection of the two viruses, ZYMV with CMV, led to the collapse and death of squash seedlings, and produced severe symptoms in cucumber and melon varieties, compared to infection with each virus separately. We also found an enhancement of symptom severity on squash and melon after mixed infection of the potyvirus *Watermelon mosaic virus II* (WMV-II) with CMV. The enhancement of symptoms also appears when ZYMV was co- inoculated with different strains of CMV such as: LS-CMV, Fny-CMV, K-CMV, or Is-CMV (local Israeli strain), and also where CMV contained satellite RNA. Interestingly, the sequence of inoculation (ZYMV before or after CMV) did not affect the

synergism: In both cases severe symptoms appeared on squash at about the same time. These results are different from those published in the PVY/PVX synergism system. A more detailed analysis of the synergistic interactions between CMV and ZYMV in doubly infected plants is given below, under "III. The Kinetics of Synergism."

The evaluation of synergy between CMV and transgenic plants expressing ZYMV genes was done in tobacco and *N. benthamiana*. *N. benthamiana* is a host for CMV and some strains of ZYMV, while tobacco is a host only for CMV. In order to evaluate synergism between the transgenes (the P1, HC-Pro and P3 genes) and CMV, a HC-Pro expressing line of tobacco and of *N. benthamiana* was inoculated with Fny-CMV. No difference in symptoms or CMV viral titer was observed between the infected transgenic line and control plants, and no difference was observed in the accumulation of CMV (measured by western blot). The non-synergistic interaction of CMV in the tobacco and *N. benthamiana* plants could be due to low level of HC-Pro expression, or to the host specificity of the potyvirus. It is possible that a host factor exists in cucurbits that interacts with the ZYMV synergistic effect, and that this factor is lacking in *Nicotiana* species.

For a future test of the latter possibility, transgenic tobacco plants were generated that expressed the GUS gene. From 300 plants showing strong GUS activity, 12 were found to be silenced for GUS activity. The silenced GUS gene was shown to be partially reactivated by inoculation with PVY in plants from two of these lines. After confirmation of this observation, plants of these lines will be crossed with the ZYMV HC-Pro lines, to determine whether PVY HC-Pro, but not ZYMV HC-Pro is able to derepress a silenced transgene.

III. Kinetics of Synergism: ZYMV and CMV.

A. Effect Of Synergism on the Level of Virus Accumulation.

1. In Squash: The accumulation of CMV in squash rapidly reached very high levels. Therefore, it was difficult to study the difference in accumulation in mixed infections. We found that the size of the inoculated plant was important to distinguish between CMV RNA accumulation in mixed vs. single infections. After mechanical inoculation of squash with 4 leaves, about 5 to 10 times the level of CMV(+)RNA and (-)RNA accumulated in mixed infections than occurred with a single infection of CMV alone at 10 d.p.i. In experiments with double infections, the plants were stunted and collapsed about 3-4 weeks p.i. The ratio of (-) strand to (+) strand was maintained during mixed infections compared to single infections with CMV, even though the total CMV RNA level increased considerably in the mixed infection. These results contrast with those published concerning the synergism

between PVY/PVX in tobacco. The level of accumulation of either ZYMV or WMV-II RNA and coat protein was the same in double infections and single infections for either potyvirus. We also found that double infection by ZYMV and CMV containing a satellite RNA did not affect the synergism, and the symptoms as well as the accumulation of both CMV RNA and coat protein were similar to what was observed in double infection without satellite RNA.

2. In Cucumber: In order to compare more precisely the level of CMV and ZYMV RNA and coat protein accumulation in double infections vs. single infections, we tested a cucumber cultivar (*C. sativus* L. cv. Delila) tolerant to CMV. This cultivar is symptomless to CMV and the virus accumulated very slowly and to a low level compared to the susceptible variety (cv. Bet-Alfa). Double infections of cucumber cv. Delila showed similar symptoms to infection with ZYMV alone, while on cv. Bet-Alfa symptoms were very severe, similar to those observed in squash. The cultivar Delila was then used as the host plant for studying the mechanism of synergism between potyviruses and CMV at the molecular level. With cv. Delila, we could test the synergism effect between the two viruses without the severe affect on plant symptoms. In mixed infection of tolerant cucumber (Delila), the level of (+) and (-) strands of CMV RNA and the coat protein were about 5 to 10 times higher than with CMV alone, while ZYMV RNA and coat protein levels remained the same. In a few cases, the level of CMV alone was undetectable in the tolerant cucumber, but after double infection (with ZYMV or WMVII) the level of CMV was very high. The ratio of (+) to (-) RNA was the same in mixed infections vs. single infections.

B. Effects of Synergism on Virus Movement.

It is apparent that the major effect of synergy in squash is on an increase in CMV accumulation as a consequence of increased replication. However, in cucumber, the effects of synergism appear to alter both CMV replication and CMV movement. The latter is especially true in the CMV tolerant cultivar Delila, where CMV movement is severely impeded by the natural resistance gene(s), and this resistance is overcome by co-infection with ZYMV. Thus, it may be that the ZYMV HC-Pro gene interferes with the expression of the natural resistance genes inhibiting CMV movement, but does not interfere with the genes blocking the development of symptom responses to CMV infection. This shows that the genes for tolerance to CMV operated at several levels, and one can separate the effects of synergy of replication from synergy of pathology.

To further study the effects synergy on pathology and virus movement, we inserted the five CMV genes into the ZYMV-AG (attenuated virus) full length clone. The two constructs harboring the 1a and 2a genes were found to be unstable within the ZYMV-AG vector (cloning difficulties). The ZYMV constructs expressing the MP, CP and 2b genes individually were found to be infectious and stable in the ZYMV genome, and the CP and MP proteins were detected by western blot analysis. The chimeric virus with the coat protein of CMV showed mild symptoms, similar to the attenuated ZYMV virus, as also occurred when a reporter gene such as the GFP was expressed from ZYMV. The chimeric virus with the CMV movement protein showed more severe symptoms than the control, and the level of ZYMV was approximately the same as the control. Interestingly, ZYMV expressing the 2b produced severe symptoms, similar to those of CMV on sensitive cucumber (cv. Bet Alpha). However, such CMV symptoms were also produced on the CMV-tolerant cucumber variety Delila. This last result indicates that when the 2b gene is expressed from the ZYMV clone the symptom level is much greater than when plants are co-inoculated with both viruses. Possibly this is due to the expression of the 2b protein continuously with the ZYMV in all infected cells, or at a higher level, and the HC-Pro of ZYMV suppresses the plant's defense responses. This also suggests that the suppression of viral symptom expression is not linked to the resistance to CMV. These results all indicate some interference between CMV genes and symptom development as well as ZYMV accumulation.

IV. Kinetics of Synergism: PVX with TMV and CMV.

Infection of TMV and CMV or CMV and PVX stimulated the level of (+) CMV RNA, and the level of (-) CMV RNA to similar extents. That is, there was not a disproportional stimulation of (-) CMV RNA, as was observed for (-) PVX RNA in the case of infection by PVX and PVY. This is, however, similar to the situation of CMV in combination with CMV or WMV-II in cucurbit species (see above). Thus, the kinetics of synergy are different for viruses belonging to different genera and/or for various unrelated virus combinations.

Infection of various plants was examined with CMV in combination with PVX or ZYMV, to determine whether the CMV 2b gene was involved in the synergistic interactions involving other viruses. In tobacco, Fny-CMV with a truncated 2b gene in combination with PVX, did not show the synergy observed by infection with wt Fny-CMV and PVX, while PVX expressing the Fny-CMV 2b gene did show this synergy. Similarly, in squash, there was little difference between infection by CMV with a truncated 2b gene alone vs. together

with ZYMV. Thus, in both hosts, with viruses that are either stimulated by co-infection with CMV (PVX) or not (ZYMV), the CMV 2b gene is a major determinant of the synergy reaction. In the case of tobacco and PVX, the 2b gene is probably responsible for the synergy, while in the case of squash and ZYMV, the 2b gene is required for the synergy, even though the ZYMV HC-Pro gene is probably the major activator of the synergy with CMV.

As a follow-up to the observations that the HC-Pro gene of a potyvirus may show host-species specificity in its ability to induce synergy, two additional experiments were done: (1) *N. benthamiana* were infected by PVX expressing the ZYMV HC-Pro gene. This virus did not show synergism of either pathogenicity or virus accumulation. (2) *N. benthamiana* and tobacco plants were infected by PVX expressing the HC-Pro gene of *Plum pox virus* (PPV). PPV infects *N. benthamiana* but not tobacco. While there was no synergism exhibited by tobacco infected with PVX expressing the PPV HC-Pro gene, *N. benthamiana* infected by the same virus showed synergy and died. Thus, the ability of HC-Pro to express synergism is related to the ability of the virus providing the HC-Pro gene to infect that plant. That is, the HC-Pro gene is a host-range determinant factor.

Scientific Implications:

The scientific implications of the results described here are as follows:

1. There is more than one mechanism of synergy. Synergy between ZYMV and CMV was not affected by the order or inoculation, in contrast to synergy by PVX and PVY. Moreover, the ratio of (+) to (-) CMV RNA was not altered by co-infection with ZYMV, in contrast for PVX during co-infection of PVX with PVY.
2. The CMV 2b gene is required for synergistic interactions. This fits with recent observations made by others, who have shown that the CMV 2b gene and the PVY HC-Pro gene both inhibit cellular mechanisms of post-transcriptional gene silencing (PTGS), although the CMV 2b appears to interfere with the establishment of PTGS and the HC-Pro gene interferes with the maintenance of PTGS.
3. Synergy between a potyvirus and CMV can break natural resistance limiting CMV movement. This was shown in the CMV tolerant cucumber cultivar Delila.
4. Synergy operates at two level – increase in virus accumulation and increase in pathology – independently of each other. Synergy between TMV and PVY in tobacco occurred only at the level of an increase of pathology. Similarly, synergy of CMV accumulation occurred in cucumber cv. Delila, without an increase in pathology.

5. Various sequences of CMV can interact with the host to alter pathogenicity and affect virus accumulation. This was shown using a symptomless ZYMV vector expressing various CMV genes.
6. The effect of synergy on CMV satellite RNA accumulation varies in different systems. In contrast to the inhibition of satellite RNA accumulation observed during synergy between PVY and CMV in tobacco, the satellite RNA level was stimulated by ZYMV in squash.
7. The HC-Pro gene may only function in host plant species to induce synergy. The ZYMV HC-Pro gene did not induce synergy in plants that are either non-hosts (tobacco) or limited hosts (*N. benthamiana*) for ZYMV.
8. The HC-Pro gene is a host range determinant of potyviruses. PPV HC-Pro gene did not induce synergy in a non-host of PPV (tobacco), but did in a host of PPV (*N. benthamiana*).
9. Transgenic plants expressing some viral sequences showed synergy with one or more viruses. Transgenic plants expressing CMV RNA 1, PVY Nib and the TMV 30K gene all showed synergy with at least one unrelated virus.
10. Transgenic plants expressing some viral sequences showed interference with the infection of unrelated viruses. Transgenic plants expressing the TMV 30K, 54K and 126K genes, the PVY Nib gene, or the CMV 3a gene all showed some level of interference with the accumulation (and in some cases the pathology) of unrelated viruses.

Impact on Agriculture and the Environment:

From our observations, it is apparent that before they are released commercially, transgenic plants expressing viral sequences for resistance to one virus need to be evaluated for two properties:

1. Synergism to unrelated viruses that infect the same plant. Most of these evaluations can be made in the greenhouse, and many can be predicted from the known literature of viruses known to interact with each other. In other cases, where transgenic plants are being generated from new plant species, the main corresponding viruses from the same known interacting genera (e.g., potexviruses and cucumoviruses, potyviruses and cucumoviruses, tobamoviruses and potexviruses, etc.) should be evaluated.
2. Inhibition or enhancement of other resistance genes. Although it is unlikely that plants to be released would be transformed with HC-Pro or 2b genes, there may be other viral genes that can affect the expression of plant genes encoding resistance to other pathogens. Therefore, transgenic plants expressing viral genes to engender pathogen-derived

resistance should be evaluated against a spectrum of other pathogens, to determine whether those resistance activities are still present, have been lost, or have been enhanced! All three situations were observed in this study, and none were predicted beforehand.

Publications:

Gal-On, A., Meiri, E., Elman, C., Gray, D.J. and Gaba, V. (1997). Simple hand-held devices for the efficient infection of plants with viral-encoded constructs by particle bombardment. *J. Virol. Methods* 64: 103-110.

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Gal-On, A., Meiri, E., Raccach, B. and Gaba, V. (1998). Recombination of engineered defective RNA species produces infective potyvirus in planta. *J. Virol.* 72: 5268-5270.

Kadouri, D., Peng, Y-H, Wang, Y., Singer, S., Huet, H, Raccach, B. and Gal-On, A. (1998). Affinity purification of HC-Pro of potyviruses with Ni^{2+} -NTA resin. *J. Virol. Methods* 76: 19-29.

Three other papers are in preparation and will be submitted in the near future: One dealing with the evaluation of synergy in various transgenic tobacco plants; one dealing with the characterization of the parameters of synergy between CMV and potyviruses in cucurbit species; and one dealing with the differential effects on tolerance to CMV by ZYMV in cv. Delila. Other papers, dealing with the (1) expression of various CMV genes in ZYMV and their effects on ZYMV accumulation as well as pathogenicity, and (2) the host-specific effects of viral HC-Pro genes on suppression of PTGS, will have to wait completion of the work on those topics. This work will continue in both labs.

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