Characterization of Synergy Between *Cucumber mosaic virus* and Potyviruses in Cucurbit Hosts

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ABSTRACT

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Mixed infections of cucurbits by *Cucumber mosaic virus* (CMV) and potyviruses exhibit a synergistic interaction. Zucchini squash and melon plants coinfecting by the potyvirus *Zucchini yellow mosaic virus* (ZYMV) and either Fny-CMV (subgroup IA) or LS-CMV (subgroup II) displayed strong synergistic pathological responses, eventually progressing to vascular wilt and plant death. Accumulation of Fny- or LS-CMV RNAs in a mixed infection with ZYMV in zucchini squash was slightly higher than infection with CMV strains alone. There was an increase in CMV (+) strand RNA levels, but no increase in CMV (–) RNA3 levels during mixed infection with ZYMV. Moreover, only the level of capsid protein from LS-CMV increased in mixed infection. ZYMV accumulated to

similar levels in singly and mixed infected zucchini squash and melon plants. Coinfection of squash with the potyvirus *Watermelon mosaic virus* (WMV) and CMV strains increased both the Fny-CMV RNA levels and the LS-CMV RNA levels. However, CMV (–) strand RNA3 levels were increased little or not at all for CMV on coinfection with WMV. Infection of CMV strains (LS and Fny) containing satellite RNAs (WL47-sat RNA and B5*-sat RNA) reduced the accumulation of the helper virus RNA, except when B5*-sat RNA was mixed with LS-CMV. However, mixed infection containing ZYMV and the CMV strains with satellites reversed the suppression effect of satellite RNAs on helper virus accumulation and increased satellite RNA accumulation. The synergistic interaction between CMV and potyviruses in cucurbits exhibited different features from that documented in tobacco, indicating there are differences in the mechanisms of potyvirus synergistic phenomena.

Infection of plants by multiple viruses is a common phenomenon (11). Double or mixed infection of plants often results in intensified symptom severity and higher virus accumulation, a phenomenon referred to as synergy (26,33). A notable increase in host symptoms during double infection compared with single infection was found in several studies involving combinations of potyviruses and viruses belonging to other genera. Combinations of such viruses leading to synergy include the potyviruses *Potato virus Y* (PVY), *Tobacco vein mottling virus*, or *Tobacco etch virus* (TEV) together with the potexvirus *Potato virus X* (PVX) in tobacco (8,33,37,38); *Maize dwarf mosaic virus* with the machlovirus *Maize chlorotic mottle virus* in maize (15); *Soybean mosaic virus* with two comoviruses, *Bean pod mottle virus* and *Cowpea mosaic virus* in soybean (1,6); PVY with the polerovirus *Potato leafroll virus* in *Nicotiana clevelandii* (3); *Zucchini yellow mosaic virus* (ZYMV) with either the polerovirus *Cucurbit aphid-borne yellow virus* in muskmelon (4) or the cucumovirus *Cucumber mosaic virus* (CMV) in cucumber (28); and *Turnip mosaic virus* with CMV in radish (34). In most of these interactions, the accumulation of the potyvirus did not increase, but the accumulation of the other virus did increase (1,15,30,33,37). In some cases, mixed infection with a potyvirus did not exhibit synergy, as in the cases of *Peanut mottle virus* with either *Tomato spotted wilt virus* (19) or *Bean pod mottle virus* (BPMV) (1). Finally, there are reports of enhancement (21) or reduction (28) of potyvirus accumulation in mixed infection with another virus.

The best characterized of the potyvirus-associated synergistic interactions is during double infection of tobacco with PVX and PVY. The doubly infected tobacco plants showed increases in accumulation of PVX compared with plants infected by PVX alone, with no increase in the accumulation of PVY (16,17,33,37). The increase in accumulation of PVX levels during double infection was due to an increase in the number of particles per cell, rather than to an increase in the number of infected cells (16). Moreover, in the doubly infected plants, there was a disproportionate and prolonged increase in the accumulation of the (–) strand RNA of PVX compared with the (+) PVX RNA in both plants and protoplasts, indicating enhancement of PVX replication rather than increased movement (37). Replication of the potyvirus genome was not required for the enhanced accumulation of either PVX or other viruses such as *Tobacco mosaic virus* or CMV, because the synergy of these viruses could be mediated by expression of either the 5'-proximal one-third of the potyviral genome in transgenic plants (37) or the P1/helper component proteinase (HC-Pro) gene of TEV from a PVX-based vector (30). Furthermore, mutations in the central coding region of the HC-Pro gene abolished the PVX–potyvirus interactions, indicating direct involvement of the potyviral HC-Pro in the synergistic response (35). In the case of synergistic interactions in transgenic plants expressing the TEV HC-Pro gene, the enhancement of CMV accumulation also resulted in a suppression of CMV satellite RNA accumulation (30).

Much less is known about the synergistic interactions occurring in cucurbit species doubly infected by CMV and potyviruses. Infections of cucurbit species by either potyviruses such as ZYMV or *Watermelon mosaic virus* (WMV) or by CMV are very common and cause considerable damage worldwide in severe epidemics in cucurbit fields, either in single or double infections (18,24). Enhancement of the CMV titer upon double infection with ZYMV

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was observed in cucumber plants (28,29). Ultrastructural analysis of cucumber leaves doubly infected by ZYMV and CMV showed that both types of virus particles were present in the same cell. Moreover, in protoplasts derived from infected plants, CMV accumulated to a considerably higher level in doubly infected cells than in singly infected cells (28). In infected plants, the number of cells infected by CMV was higher in double infection than in single infection, indicating that the enhancement of CMV levels during synergy was due to both an increase in the number of CMV particles per cell, as well as the number of CMV-infected cells (29).

The genome organizations of CMV and potyviruses are unrelated. CMV contains a tripartite, positive sense RNA genome expressing five genes: three from the genomic RNAs 1 to 3 and two from subgenomic RNAs 4 and 4A (9,27). CMV strains may support satellite RNAs, which attenuate CMV-induced symptoms (14). By contrast, potyviruses such as ZYMV and WMV contain monopartite, positive sense RNA genomes, encoding a single polyprotein that is cleaved to 9 or 10 mature proteins (31). Moreover, whereas any individual potyvirus has a very narrow host range, the collective host range of the large number of known potyviruses is very broad (36). By contrast, the strains of CMV have a collective host range of over 1,000 species (27). Thus, one can only measure the interactions between CMV and either ZYMV or WMV in cucurbit species. The molecular mechanisms involved in the interactions between CMV and cucurbit-infecting potyviruses are not understood, and neither are the effects on these interactions with host species, potyvirus species, CMV strains, or CMV satellite RNAs. Therefore, an analysis of the effect of these variables on potyvirus-CMV interactions was undertaken to compare the situation with the better understood potyvirus-PVX system in tobacco. Additionally, the analysis would ascertain the effects of coinfection with a potyvirus on CMV satellite RNA accumulation in a cucurbit host, where satellite RNAs do not suppress CMV accumulation as effectively as in solanaceous plants (14).

MATERIALS AND METHODS

Plant genotypes, virus strains, and inoculations. Zucchini squash (*Cucurbita pepo* cv. Ma'ayan) and melon (*Cucumis melo* cv. Arava) plants were grown in a temperature-controlled greenhouse at 25°C with a photoperiod of 14 h. Zucchini squash seedlings were used as source plants to maintain the viral cultures for inoculation of ZYMV-AT (2), Fny-CMV and LS-CMV (40), and WMV (2). Inoculations of test plants were carried out mechanically by rubbing cotyledons after dusting with Carborundum. Inocula were extracted by grinding leaves in distilled water. Double inoculation was performed by preparing extracts from equal fresh weights of young leaves of source plants and mixing the extracts prior to inoculation. In mixed infections, squash or melon plants were inoculated with Fny-CMV+ZYMV-AT or LS-CMV+ZYMV-AT. When WMV was employed in mixed infections, zucchini squash plants were inoculated with Fny-CMV+WMV or LS-CMV+WMV. In mixed infections with satellite RNAs (23), zucchini squash plants were inoculated with a mixture of satellite RNA and either Fny-CMV or LS-CMV. The combinations applied were: WL47-sat RNA/Fny-CMV+ZYMV-AT; WL47-sat RNA/LS-CMV+ZYMV-AT; B5*-sat RNA/Fny-CMV+ZYMV-AT; and B5*-sat RNA/LS-CMV+ZYMV-AT. Mock inoculations and individual inoculations with Fny-CMV, LS-CMV, and ZYMV-AT or WMV were employed as controls.

Total RNA extraction and northern blot hybridization analysis. Total RNAs were extracted from three leaf disks (one per leaf; 9-mm diameter) collected from the second true leaves of three plants with Tri-reagent solution (Molecular Research Center, Inc., Cincinnati) according to the manufacturer's instructions. RNA samples were equalized for electrophoresis in denaturing gels used for northern blot hybridization analyses, which were performed according to Maniatis et al. (25), except as noted below. The RNAs in the gels were blotted onto Hybond-N⁺ membranes (Amersham, Piscataway, NJ) and fixed by exposure to UV for 2 min. Prehybridization with Rapid-hyb buffer (Amersham)

Fig. 1. The infection symptoms of Zucchini yellow mosaic virus (ZYMV) and Cucumber mosaic virus (CMV) alone or together in zucchini squash cv. Ma'ayan. Plants were A, mock-inoculated or inoculated with B, Fny-CMV, C, LS-CMV, D, ZYMV-AT, E, Fny-CMV plus ZYMV-AT, or F, LS-CMV plus ZYMV-AT. Plants were photographed at 18 days postinoculation. Bar in each panel stands for the radius of the pot (8 cm).

was performed according to the manufacturer's instructions. Plus-sense CMV RNAs were detected by hybridization with ^{32}P -labeled RNA transcripts either complementary to the 3' terminal 200 nucleotide (nt) of CMV RNA3 (13) or to the central 888 nt of CMV RNA3. The 888-nt fragment was subcloned into pGEM-T (Promega, Madison, WI) from pFny309 (32), and (-) sense RNA transcripts were synthesized in vitro with SP6 RNA polymerase (Fermentas, Vilnius, Lithuania). Minus-sense CMV RNAs were detected by hybridization with ^{32}P -labeled RNA transcripts identical to the 3' terminal 200 nt of CMV RNA3 synthesized in vitro with T7 RNA polymerase (Fermentas) according to Gal-On et al. (13). CMV satellite RNA was detected with a ^{32}P -labeled RNA transcript complementary to the Is-CMV satellite RNA. The original Is-CMV satellite cDNA clone (12) was subcloned into the pGEM-T vector, and (-) sense satellite RNA transcripts were synthesized in vitro with T7 RNA polymerase. RNA was quantified by scanning the blot and analyzing the sum intensity of each RNA3 band using the Biomax program (Kodak, Rochester, NY).

Protein analysis: western blot and enzyme-linked immunosorbent assay. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis, three leaf disks (9-mm diameter; ≈ 100 mg total fresh weight) were collected from each of the second true leaves of three plants. Total proteins were extracted by grinding the pooled samples in 500 μl of sample buffer (0.5 ml of Tween 20 per liter and 20 g of polyvinylpyrrolidone per liter in phosphate-buffered saline [PBS]) and boiling for 5 min in a 1:1 mixture of ground sample plus 2 $\times$ SDS-PAGE loading buffer (25). The boiled material was centrifuged for 5 min at $10,000 \times g$, and 10 μl of supernatant for each sample was

fractionated by SDS-PAGE on a 12.5% polyacrylamide gel. The fractionated proteins were electro-blotted onto nitrocellulose membranes (Amersham), blocked with nonfat dry milk, and probed with rabbit antisera specific to LS-CMV capsid protein (1:1,000 dilution) or ZYMV-AT capsid protein (1:2,000 dilution). Antibodies against purified ZYMV and CMV virions were raised locally. The membranes were then incubated with goat anti-rabbit immunoglobulin G (IgG) conjugated to alkaline phosphatase (1:4,000 dilution), and the viral capsid proteins were visualized by incubating the membranes with the chromophore substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate toluidinium as described by Maniatis et al. (25). For enzyme-linked immunosorbent assay (ELISA), three leaf disks (9-mm diameter) were collected from each of the second and third true leaves of each test plant and were ground with 500 μl of either sample buffer (for ZYMV) or ELISA coating buffer (for CMV) (2). The concentration used (≈ 100 mg of total tissue per 500 μl of buffer) was optimal for the analysis of ZYMV by ELISA. Direct double-antibody sandwich ELISA for ZYMV (2) and indirect ELISA for CMV were performed with the above antisera according to Clarke and Adams (7). The absorbance at 405 nm was measured with an ELISA reader (Anthos Labtech Instruments, Austria).

RESULTS

Synergistic symptom responses in cucurbits coinfecting by ZYMV and CMV. ZYMV and each of the two CMV strains (LS-CMV, a member of subgroup II, and Fny-CMV, a member of subgroup IA) individually caused severe diseases in zucchini squash

Fig. 2. *Cucumber mosaic virus* (CMV) RNA accumulation in zucchini squash and melon plants after infection with CMV alone, *Zucchini yellow mosaic virus* (ZYMV) alone, or CMV plus ZYMV. Total RNAs extracted at 8 days postinoculation from leaf disks sampled from the second true leaves (one disk each from three different plants were extracted together) of infected **A**, zucchini squash or **B**, melon plants inoculated with either Fny-CMV (F) or LS-CMV (LS), in the presence or absence of ZYMV-AT (AT), were analyzed by denaturing agarose gel electrophoresis and northern blot hybridization. Lane H contains total nucleic acid extracted from mock-inoculated plants. Lane AT contains total nucleic acids extracted from plants infected with ZYMV-AT alone. **A**, ^{32}P -labeled RNA probes used for detecting CMV RNAs were complementary to either the central part of CMV RNA3 and its subgenomic derivative RNA4. **B**, (upper panel) the 3' end of the (+) CMV RNAs 1 to 4, or **B**, (middle panel) the 5' end of the (-) CMV RNAs 3 to 4, or CMV RNAs 1 to 4 indicated adjacent to the panel. rRNA was stained with methylene blue to give an indication of total RNA loading (bottom panel).

(Fig. 1) and melon (data not shown). Each virus typically induced severe symptoms after 10 to 14 days, consisting of yellow-green mosaic leaf deformation and stunting. The symptoms caused by each virus were distinguishable. Necrosis was not induced by ZYMV or either of the CMV strains tested on any of the plants, even by 30 days postinoculation (dpi). Zucchini squash (Fig. 1) and melon (data not shown) plants coinfecting by ZYMV and either Fny-CMV or LS-CMV displayed strong synergistic responses in symptom expression compared with plants infected by those viruses alone. Coinfection by ZYMV and either of the CMV strains led to necrosis of the newly emerging leaves, progressing to vascular wilt and plant death at 14 to 18 dpi (Fig. 1) (data not shown).

Changes in CMV RNA and capsid protein accumulation profiles associated with synergy in cucurbits. At 6 to 10 dpi, prior to the onset of necrosis, leaves showing mosaic symptoms were analyzed to evaluate the synergistic effect on the accumulation of viral RNAs in doubly infected plants. CMV RNAs accumulated to a high level in both singly and doubly infected zucchini squash plants (Fig. 2A). There was a small, but reproducible, increase in the level of (+) CMV RNAs accumulating after coinfection with ZYMV (Fig. 2A). The level of (+) CMV RNA increased slightly in plants infected by ZYMV plus Fny-CMV versus Fny-CMV alone, as seen in the accumulation of CMV RNA3 (Fig. 2A). However, a similar level of Fny-CMV capsid protein accumulated in mixed infection with ZYMV and single infection by CMV (Fig. 3). Interestingly, a great difference in accumulation of (+) LS-CMV RNAs and capsid protein can be observed in plants coinfecting with ZYMV and LS-CMV versus LS-CMV alone (Figs. 2A and 3). The level of ZYMV did not show any increase in singly versus doubly infected plants (Table 1), which was repeated in many similar experiments in different cucurbit hosts.

Similar results were obtained after coinfection of ZYMV and Fny-CMV in melon (Fig. 2B, upper panel). However, the levels of

accumulated (–) Fny-CMV RNAs in doubly infected versus singly infected melon plants appeared unchanged (Fig. 2B, middle panel). The levels of ZYMV remained constant in singly versus doubly infected melon plants, as also observed in zucchini squash (Table 1).

Synergistic interactions in zucchini squash infected by WMV and CMV. The Israeli isolate of WMV causes milder disease symptoms (without green blisters) on zucchini squash than the ZYMV isolate (2). Thus, coinfection of plants by CMV plus WMV allowed the synergistic effects on zucchini squash to be tested with less distortion of the plants. Additionally, it was possible to determine if the extent of the synergistic enhancement of CMV accumulation (on coinoculation with WMV) was similar to that observed by coinfection of CMV with ZYMV. Zucchini squash plants coinfecting by WMV and either LS-CMV or Fny-CMV also showed more severe symptoms than such plants infected by any of these viruses alone or by ZYMV alone (data not shown). Additionally, the squash plants remained alive (data not shown). Moreover, zucchini squash plants coinfecting by Fny-CMV and WMV accumulated approximately threefold more (+) CMV RNAs than plants infected by Fny-CMV alone (Fig. 4, upper panel). In the case of the (–) Fny-CMV RNAs, the level of increased accumulation in doubly infected versus singly infected plants was minimal compared with that of (+) RNAs (Fig. 4, middle panel). Coinfection of zucchini squash plants by LS-CMV and WMV slightly stimulated accumulation of (+) LS-CMV RNAs (Fig. 4, upper panel), whereas the accumulation of (–) LS-CMV RNAs was not enhanced (Fig. 4, middle panel). Thus, there were reproducible differences in the extent of increased accumulation of CMV RNAs due to coinfection with WMV (Fig. 4) and in the relative increase in accumulation of (+) versus (–) CMV RNAs (Fig. 4, upper versus middle panel), depending on the CMV strain. There were also CMV strain-specific differences in the extent of increased (+) CMV RNA accumulation induced by ZYMV (Fig. 2, upper panel) versus WMV (Fig. 4, upper panel).

Synergistic interaction between ZYMV and Fny-CMV in sequentially inoculated squash plants. It was demonstrated that the order of virus inoculation by PVX/PVY was critical for synergy in tobacco (17). The effect of the order of virus inoculation on enhancing CMV accumulation in mixed infection of zucchini squash plants was examined. All previous experiments used a mixture of viruses during coinoculations (CMV and either ZYMV or WMV). In this experiment, zucchini squash plants were first inoculated with ZYMV-AT and challenged 4 days later with Fny-CMV. The level of the CMV (+) RNAs in the sequentially mixed infection was enhanced by fivefold 6 days after the second inoculation compared with the single infection (Fig. 5, upper panel). The reciprocal inoculation of Fny-CMV 4 days prior to inoculation with ZYMV-AT yielded a similar result (data not shown). ZYMV/CMV synergy thus occurred irrespective of the order of virus inocula.

Effect of ZYMV-induced synergy on CMV satellite RNA accumulation in zucchini squash. Prior to analyzing the effects of coinfection with ZYMV on satellite RNA levels with two different strains of CMV, the effects of satellite RNA on CMV RNA accumulation was examined with two strains of CMV and

Fig. 3. Accumulation of Fny-*Cucumber mosaic virus* (CMV) (striped bar) and LS-CMV (open bar) in systemically infected zucchini squash at 8 days postinoculation. The level of accumulation of CMV strains was determined by quantitative double-antibody sandwich enzyme-linked immunosorbent assay using antiserum specific to LS-CMV (1:1,000). The value for each histogram represents the average of sample duplicates prepared from three leaf disks of the second leaves (one per leaf) of three plants. Bars stand for standard deviation.

TABLE 1. Accumulation of *Zucchini yellow mosaic virus* (ZYMV) in zucchini squash and melon plants infected with ZYMV alone or with *Cucumber mosaic virus* (CMV)

Host	Inoculum ^a					
	None	ZYMV-AT	Fny-CMV	Fny-CMV+ZYMV-AT	LS-CMV	LS-CMV+ZYMV-AT
Squash	0	0.869 ± 0.195	0	0.910 ± 0.065	0	0.962 ± 0.070
Melon	0	0.530 ± 0.098	0	0.505 ± 0.037	0	0.592 ± 0.023

^a Plants were mock-inoculated or inoculated with ZYMV-AT, Fny-CMV, LS-CMV, Fny-CMV+ZYMV-AT, or LS-CMV+ZYMV-AT. Samples were collected at 8 days postinoculation using three leaf disks collected from the second leaves (one disk per leaf) of three plants. The levels of ZYMV were measured by enzyme-linked immunosorbent assay (optical density = 405 nm) with an antiserum specific to ZYMV-AT capsid protein (1:2,000). Values are mean ± standard deviation ($n = 3$).

two satellite RNAs. The accumulation of (+) CMV RNA in zucchini squash plants infected by Fny-CMV plus either of two satellite RNAs (WL or B5*) was decreased compared with the levels observed in zucchini squash plants infected by Fny-CMV alone (Fig. 6A, middle panel). This was also true for plants infected by LS-CMV plus WL-sat RNA versus LS-CMV alone (Fig. 7A, middle panel). However, for LS-CMV plus B5*-sat RNA, the (+) CMV RNAs accumulated to a higher level than in plants infected by LS-CMV alone (Fig. 7A, middle panel). Thus, the suppressor effect of satellite RNA on CMV RNA accumulation depends on the satellite RNA and the helper virus (Figs. 6A and 7A).

To determine whether the accumulation of CMV satellite RNA was affected by ZYMV in a cucurbit host, zucchini squash plants were infected by CMV containing satellite RNAs with or without ZYMV. In zucchini squash plants doubly infected by ZYMV and CMV containing satellite RNA, the levels of accumulated (+) CMV RNAs increased irrespective of the nature of the CMV strain or the satellite RNA, although each of these factors affected

the extent of the increase (Figs. 6A and 7A). Thus, in contrast to the situation in solanaceous plants infected by CMV plus satellite RNA and a potyvirus (30), zucchini squash plants infected by ZYMV plus CMV strains representing two of the major subgroups showed increases in both the levels of CMV RNAs and the satellite RNAs rather than suppressing CMV satellite RNA accumulation.

Effect of satellite RNA on capsid protein accumulation in zucchini squash. Analysis of the effects of double infection by ZYMV and CMV (plus satellite RNA) on the accumulation of CMV capsid protein showed that double infection increased the accumulation of CMV capsid protein to levels similar to that observed during infection by CMV alone. This effect was the same regardless of the CMV strain examined (Figs. 6B and 7B). The satellite RNA depressed the level of CMV capsid protein, but ZYMV-induced synergy reversed this effect (Figs. 6B and 7B). However, double infection resulted in little, if any, change in the level of ZYMV capsid protein (Figs. 6B and 7B).

DISCUSSION

Synergy between potyviruses and CMV was examined in two cucurbit hosts (zucchini squash and melon) using two strains of CMV (representing subgroups IA and II) and two potyviruses (ZYMV and WMV). There were slight differences observed in the effects of synergy on the levels of (+) and (-) CMV RNA accumulation due to the nature of the potyvirus and the CMV strain, as well as the nature of the host. However, the nature of the host (Fig. 2A versus Fig. 2B and C) apparently had less of an effect than either the nature of the CMV strain (Figs. 2 and 4) or the potyvirus (Fig. 2 versus Fig. 4). In all cases, synergy in pathology was correlated with a slight to moderate increase in the accumulation of the (+) CMV RNA (Figs. 2 and 4 to 7). In-

Fig. 4. *Cucumber mosaic virus* (CMV) RNA accumulation in zucchini squash plants infected with CMV alone or with *Watermelon mosaic virus* (WMV). Total RNAs extracted at 8 days postinoculation from three leaf disks collected from the second leaves (one disk from each of three different plants were extracted together) of three plants inoculated with Fny-CMV (F), LS-CMV (LS), WMV (W), Fny-CMV plus WMV (F+W), or LS-CMV plus WMV (LS+W) were analyzed by denaturing agarose gel electrophoresis and northern blot hybridization. Lane H contains total nucleic acid extracted from mock-inoculated plants. 32 P-labeled RNA probes specific for CMV RNAs were complementary to either the 3' end of (+) CMV RNAs 1 to 4 (upper panel) or the 5' end of (-) CMV RNAs 1 to 4 (middle panel). The electrophoretic positions of CMV RNAs 3 and 4, or CMV RNAs 1 to 4, are indicated adjacent to the panels. rRNA was stained with methylene blue to indicate total RNA loading (bottom panel).

Fig. 5. Analysis of Fny-*Cucumber mosaic virus* (CMV) RNAs in zucchini squash cv. Ma'ayan. Plants were mock-inoculated (H), or inoculated with *Zucchini yellow mosaic virus* (ZYMV)-AT (AT) alone, or first with ZYMV-AT and 4 days later were challenge inoculated with Fny-CMV (AT/F) or Fny-CMV alone (F). Samples were taken 6 days after the last inoculation. The presence of CMV (+) RNAs (upper panel) were examined by northern blot hybridization with 32 P-labeled RNA probe complementary to the 3' 200 nucleotide of (+) Fny-CMV RNAs 1 to 4. The electrophoretic positions of CMV RNAs 1 to 4, are indicated adjacent to the panels. rRNA was stained with methylene blue to give an indication of total RNA loading (bottom panel).

Fig. 6. RNA and capsid protein accumulation in zucchini squash plants infected by Fny-Cucumber mosaic virus (CMV) plus satellite RNA in the presence or absence of Zucchini yellow mosaic virus (ZYMV). Zucchini squash plants were inoculated with Fny-CMV containing either the WL47-sat RNA (F/WL) or the B5*-sat RNA (F/B5*) alone or with ZYMV-AT (AT). **A**, Total nucleic acids from mock-inoculated (H) or infected plants were extracted at 8 days post-inoculation (dpi) from three leaf disks collected from the second leaves (one per leaf) of three plants, and the RNAs were analyzed by denaturing agarose gel electrophoresis and northern blot hybridization. Satellite RNAs (upper panel) and CMV RNAs (lower panel) were detected using ³²P-labeled RNA probes specific for (+) satellite RNA sequences or CMV RNA3 sequences, respectively. The electrophoretic positions of satellite RNA and CMV RNAs 3 and 4 are indicated. rRNA was stained with methylene blue to give an indication of total RNA loading (bottom panel). **B**, Total proteins were extracted at 8 dpi, and the proteins were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and were immunoprobed by western blot analysis using antisera specific to either the CMV capsid protein (upper panel) or the ZYMV capsid protein (lower panel). The positions of the capsid proteins are indicated (CP), and the sizes of relevant molecular weight marker (M) in kilodaltons are indicated adjacent to the panels.

terestingly, no significant change was found in the level of the (–) CMV RNA3 (Figs. 2 and 4). The relatively small increase of Fny-CMV accumulation in mixed infection with ZYMV in zucchini squash and melon (Fig. 2) compared with the greater increase of CMV in mixed infection of LS-CMV (Fig. 2A) and WMV (Fig. 4) could be due to two factors. These two factors associated with Fny-DMV infection are (i) rapid viral accumulation, and (ii) rapid appearance of symptoms. Together in mixed infection with ZYMV, these produce rapid cell death, progressing quickly to plant death. These two factors may have resulted in less of an effect on the CMV titer due to the viral synergy compared with the slower dual infection of LS-CMV and WMV. There was a severe intensification in disease symptom development induced by the synergistic infection, regardless of whether the level of (+) CMV RNA increased slightly (for Fny-CMV in zucchini squash or melon in the presence of ZYMV) or moderately (for Fny-CMV in zucchini squash in the presence of WMV). There was no preferential increase in the level of (–) RNA, as was observed for PVY-mediated synergy of PVX in tobacco (37). Similarly, TEV stimulated a much larger increase in (+) CMV RNA accumulation in tobacco than we observed in cucurbits, although this may have been due to a number of factors, including the strains of CMV or potyviruses used and sampling procedures (30,38).

There may be significant differences in how potyvirus-mediated synergy is manifested in tobacco versus in cucurbits. This is supported by the observation that double infection by ZYMV and CMV in cucumber resulted in more cells being infected as well as an increase in CMV levels per infected cell (28). By contrast, double infection by PVX and PVY in tobacco resulted in an increase in PVX levels per infected cell (37), but not an increase in the number of infected cells (16). Moreover, in the case of

infection by PVX and PVY, the order of inoculation affected the synergy (17,33), whereas in the case of infection by ZYMV and CMV, the order did not (our data, 28). Because CMV (5) and PVX (39), as well as potyviruses (22), harbor genes to suppress host defenses, the different responses between PVX/PVY synergy and CMV/ZYMV synergy to the order of inoculation might result from the differences in suppression of posttranscriptional gene silencing by CMV 2b and PVX p25 (5,20,22,39). Alternatively, the differences observed in synergy in these two plant families may relate to effects of these viruses on host defense mechanisms in cucurbit hosts versus in solanaceous hosts. For example, it is known that the absence of the CMV 2b protein shows different effects in promoting systemic infection in tobacco versus cucumber (10,20), suggesting differences in the effect of the 2b protein on the defense mechanisms.

Differences in the manifestations of synergy involving TEV in tobacco and ZYMV in zucchini squash were also observed in the way in which the level of satellite RNA of CMV was affected by synergy and in the way in which synergy affected the interaction between CMV and its satellite RNAs. In contrast to what was observed in tobacco (30), infection of zucchini squash by ZYMV and CMV plus satellite RNA did not suppress the accumulation of satellite RNA, but rather further stimulated the accumulation of both the satellite RNA and the helper virus (Figs. 6 and 7). The relationship between CMV accumulation and satellite RNA accumulation is usually antagonistic; i.e., increases in satellite RNA accumulation lead to reduction in CMV RNA accumulation (14). Thus, the effect of the coinfecting potyvirus may act on disengaging the link between satellite RNA accumulation and its ability to suppress CMV RNA accumulation, or in parallel, independently enhance the levels of CMV and its satellite RNA accumulation.

Fig. 7. RNA and capsid protein accumulation in zucchini squash plants infected by LS-Cucumber mosaic virus (CMV) plus satellite RNA in the presence or absence of Zucchini yellow mosaic virus (ZYMV). Zucchini squash plants were inoculated with LS-CMV containing either the WL47-sat RNA (LS/WL) or the B5*-sat RNA (LS/B5*) alone or with ZYMV-AT (AT). **A**, Total nucleic acids from mock-inoculated (H) or infected plants were extracted at 8 days postinoculation (dpi) from three leaf disks collected from the second leaves (one per leaf) of three plants, and the RNAs were analyzed by denaturing agarose gel electrophoresis and northern blot hybridization. Satellite RNAs (upper panel) and CMV RNAs (lower panel) were detected using 32 P-labeled RNA probes specific for (+) satellite RNA sequences or CMV RNA3 sequences, respectively. The electrophoretic positions of satellite RNA and CMV RNAs 3 and 4 are indicated. rRNA was stained with methylene blue to give an indication of total RNA loading (bottom panel). **B**, Total proteins were extracted at 8 dpi, and the proteins were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and were immunoprobed by western blot analysis using antisera specific to either the CMV capsid protein (upper panel) or the ZYMV capsid protein (lower panel). The positions of the capsid proteins are indicated (CP), and the sizes of relevant molecular weight markers (M) in kilodaltons are indicated adjacent to the panels.

This effect appears to be peculiar to either cucurbit species, or zucchini squash in particular, because it was not observed in synergistic interactions occurring in tobacco plants, which normally support much higher levels of satellite RNA accumulation than cucurbit species (14). Thus, it is conceivable that the role of satellite RNA may be to suppress the effects of CMV-encoded inhibitors of plant defense responses, resulting in a reduction of CMV levels and an attenuation of CMV-induced symptoms. However, in double infection with a potyvirus, the attenuation of CMV accumulation may be negated by potyviral-encoded proteins interfering with host defense responses against CMV. This could result in an increase in CMV accumulation, which then results in a further increase in satellite RNA accumulation. It is noteworthy that the effects of satellite RNA on CMV accumulation and pathogenicity resemble the effects of deleting the 2b gene from CMV (10). Therefore, it would be interesting to determine the effects of CMV satellite RNA on CMV 2b-induced suppression of silenced transgenes, and the role of the CMV 2b gene on potyvirus-induced synergy. Such analyses may provide further evidence for a linkage between the roles of satellite RNA and the CMV 2b gene in synergy and in antagonizing or bolstering host defense responses.

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