Systemic movement of a movement-deficient strain of *Cucumber mosaic virus* in zucchini squash is facilitated by a cucurbit-infecting potyvirus

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Zucchini squash (*Cucurbita pepo*) is a systemic host for most strains of the cucumovirus *Cucumber mosaic virus* (CMV), although the long-distance movement of the M strain of CMV (M-CMV) is inhibited in some cultivars. However, co-infection of zucchini plants with M-CMV and the potyvirus *Zucchini yellow mosaic virus* strain A (ZYMV-A) allowed M-CMV to move systemically, as demonstrated by tissue-print analysis. These doubly infected plants exhibited severe synergism in pathology. Infection of zucchini squash by M-CMV and an attenuated strain of ZYMV (ZYMV-AG) showed a milder synergy in pathology, in which ZYMV-AG also facilitated the long-distance movement of M-CMV similar to that promoted by ZYMV-A. Variation in the extent of synergy in pathology by the two strains of ZYMV did not correlate with differences in levels of accumulation of either virus. Thus, the extent of synergy in pathology is at least in part independent of the resistance-neutralizing function of the potyvirus.

The systemic spread of plant viruses involves cell-to-cell movement into, through and out of various cell types of the vascular system, as well as long-distance transport through the sieve elements (reviewed by Lazarowitz & Beachy, 1999). Plants may have barriers that block systemic spread of viruses between any of the cell types in the vascular system. These barriers sometimes allow limited or reduced rates of movement (reviewed by Nelson & van Bel, 1998; Hull, 2002). In most cases, a reduced rate of cell-to-cell movement in the inoculated leaves results in a lack of systemic infection. An example of the latter involves the M strain of *Cucumber mosaic virus* (M-CMV), which cannot infect zucchini squash (*Cucurbita pepo*) systemically (Shintaku & Palukaitis, 1990). This block in long-distance movement has been mapped to specific sites in the viral capsid protein (CP) and was also associated with delayed cell-to-cell movement in the inoculated leaves (Wong et al., 1999).

In some cases, mixed infection by two viruses has been shown to overcome barriers to the cell-to-cell or long-distance movement of one of the viruses (reviewed by Atabekov & Taliansky, 1990; Nelson & van Bel, 1998; Hull, 2002). Moreover, in some mixed virus infections, there is an interaction between the two viruses, resulting in increased symptom severity and greater virus accumulation, a phenomenon referred to as synergism (reviewed by Hull, 2002). In synergism, it is generally recognized that one virus, which is not increased in accumulation (i.e. functioning essentially as a catalyst), acts as an up-regulator of the replication and/or movement of a unrelated virus, usually increasing the intensity of symptoms induced compared with single infection by either virus alone (Rochow & Ross, 1955; Calvert & Ghabrial, 1983; Poopol & Inouye, 1986a, b; Goldberg & Brakke 1987; Sano & Kojima, 1989; Vance, 1991; Anjos et al., 1992; Bourdin & Lecoq, 1994; Pruss et al., 1997). In most of the above cases of synergism, the ‘catalytic virus’ is a potyvirus. Synergism between potyviruses and CMV has been described in both tobacco and cucurbit plants (Poopol & Inouye, 1986a, b; Pruss et al., 1997; Wang et al., 2002). Zucchini squash and melon plants doubly infected with the potyvirus *Zucchini yellow mosaic virus* (ZYMV) and CMV have been shown to exhibit a severe synergistic pathological response and showed a strong increase in the level of accumulation of CMV positive-strand RNA and CP, with no increase in the accumulation of ZYMV (Wang et al., 2002). This was as true for a highly virulent strain (ZYMV-AT) as for an attenuated strain (ZYMV-AG) (Wang et al., 2002). Thus, we wanted to determine whether co-infection with ZYMV could overcome the barrier to the long-distance...
movement of M-CMV and whether the synergism of CMV accumulation and increased virulence also occurred in such doubly infected plants.

To determine whether ZYMV could neutralize the movement restriction of M-CMV in zucchini squash (C. pepo cv. Black Beauty), a severe isolate of ZYMV from Korea (strain A; Choi et al., 2002b) was co-inoculated with a pseudorecombinant CMV consisting of RNAs 1 and 2 of the Fny strain of CMV and RNA 3 of the M strain of CMV. This pseudorecombinant virus has previously been shown to exhibit the same limited movement in zucchini squash as M-CMV (Shintaku & Palukaitis, 1990; Wong et al., 1999). The CMV RNAs were generated from infectious transcripts of cDNA clones of these RNAs (Shintaku et al., 1992; Wong et al., 1999) and inoculated into tobacco plants. The systemically infected tobacco plants, showing bright yellow symptoms, were tested for the presence of the virus at 10 days post-inoculation (p.i.), by either Western blot analysis or RT–PCR using primers specific to the genus Cucumovirus (Choi et al., 1999). ZYMV-A was maintained in cucumber plants (Cucumis sativus cv. Baekdadaki). Inocula were prepared by grinding equal fresh weights of young leaves from these infected plants in 50 mM

Fig. 1. Symptoms of infection and immunological tissue-print analysis of singly and doubly infected zucchini squash leaves.
(a) Symptoms at 10 days p.i. observed in the upper leaves of zucchini squash plants inoculated with the M strain of Cucumber mosaic virus (M-CMV), Zucchini yellow mosaic virus strain A (ZYMV-A), ZYMV strain AG (ZYMV-AG), or combinations of M-CMV and either ZYMV strain (M+A, M+AG). (b) The second or third true leaf of zucchini squash plants infected with ZYMV and/or M-CMV was tissue-printed and probed for the presence of either CMV CP (left) or ZYMV CP (right) at 10 days p.i. H, healthy, mock-inoculated leaves.
phosphate buffer (pH 7) and either inoculating separately, or mixing the extracts just prior to inoculation of Carborundum-dusted squash cotyledons. The inoculated zucchini squash plants were maintained in a greenhouse at 26 °C with a photoperiod of 16 h.

ZYMV-A induced severe yellow-green mosaic and leaf malformation symptoms on zucchini squash plants 7–10 days p.i., while infection with M-CMV alone did not show any symptoms on the upper leaves (Fig. 1a). In contrast, zucchini squash co-infected with M-CMV and ZYMV-A showed severe systemic symptoms (Fig. 1a). Later in the course of infection, the symptoms in these doubly infected zucchini squash plants progressed to severe vascular wilt, stunting and plant death (not shown). These same results were obtained in each of three repeated tests.

To determine whether the synergism in pathology observed after double infection with M-CMV and ZYMV-A also led to neutralization of the barrier to the long-distance movement of M-CMV, systemically infected leaves were analysed by tissue printing and immunoprobing (Fig. 1b). For the tissue-print assays, the second or third true leaves of infected plants taken at 10 days p.i. were pressed onto nitrocellulose membranes, as described by Gal-On et al. (1994). The membranes were blocked with 5% non-fat skimmed milk and probed with polyclonal antiserum to either CMV CP (1:1000 dilution) or ZYMV CP (1:1000 dilution). The membranes then were incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase (1:7500 dilution) and were developed by incubating with Western Blot Substrate (Promega) according to the manufacturer’s instructions. Each experiment was carried out independently at least four times. The tissue-print assays of zucchini squash leaves doubly infected with ZYMV-A and M-CMV showed both ZYMV-A CP and M-CMV CP well dispersed in systemic tissues (Fig. 1b). In contrast, the upper leaves of zucchini squash inoculated with M-CMV alone did not show detectable levels of M-CMV (Fig. 1b), while such leaves of zucchini squash infected with ZYMV-A alone showed the presence of ZYMV-A CP (Fig. 1b).

To confirm the above conclusions, as well as to establish whether very low levels of M-CMV were present initially in the upper leaves of zucchini squash plants inoculated with M-CMV alone, RT–PCR analysis was carried out. One set of primers specific to and flanking the CP gene of CMV and another primer pair specific to and flanking the sequences encoding the ZYMV CP were used for RT–PCR. Gel analysis of RT–PCR products obtained from nucleic acids extracted from the upper leaves of plants doubly inoculated with M-CMV plus ZYMV-A showed the expected size band for the M-CMV CP gene (Fig. 2). No M-CMV CP gene-specific RT–PCR product was obtained using nucleic acids extracted from the upper leaves of plants doubly inoculated with M-CMV plus ZYMV-A showed the expected size band for the M-CMV CP gene (Fig. 2). No M-CMV CP gene-specific RT–PCR product was obtained using nucleic acids extracted from the upper leaves of plants inoculated with M-CMV alone, while the ZYMV-specific RT–PCR products were present in the samples inoculated with ZYMV-A, either singly or together with M-CMV (Fig. 2).
ZYMV-A itself induces quite severe symptoms in zucchini squash plants, in contrast to the attenuated strain ZYMV-AG, which induces only mild green mosaic symptoms in the first few leaves (Gal-On, 2000). Therefore, to examine the effect of the ZYMV pathogenicity determinants on the synergism in pathology in doubly infected plants, zucchini squash plants were co-inoculated with M-CMV and the attenuated ZYMV-AG. Zucchini squash co-infected with both M-CMV and ZYMV-AG also showed more severe symptoms (stunting, with mottle and mosaic) in newly emerging leaves than plants infected with ZYMV-AG alone (Fig. 1a, and data not presented). However, these symptoms were milder than those obtained after inoculation with ZYMV-A and M-CMV (Fig. 1a). Tissue-print assays showed that the CP of M-CMV could be detected in the upper infected leaves of the doubly infected plants (Fig. 1b). This was also confirmed by RT–PCR analysis of nucleic acids extracted from the systemically infected tissues (Fig. 2).

To ascertain whether the systemic movement of M-CMV was dependent on mutation of the CP CBP indirectly mediated by co-infection with ZYMV, total RNAs from zucchini squash plants systemically infected with both M-CMV and ZYMV were extracted and subjected to RT–PCR. The RT–PCR product was analysed by sequencing, either directly or after cloning into the pGEM-T Easy vector. The sequences revealed that the CP M-CMV had not mutated (data not shown), suggesting that ZYMV facilitated the long-distance movement of M-CMV by means other than by promoting the generation of movement in the elicitor of the resistance mechanism, the M-CMV CP.

To determine whether synergism mediated by ZYMV-A versus ZYMV-AG led to similar increases in the levels of accumulation of M-CMV in the systemically infected leaves, plant proteins were extracted and subjected to Western blot analysis. Total proteins were extracted from four leaf discs collected from the upper leaves of zucchini squash plants, as described by Choi et al. (2002a), and were separated by SDS–PAGE (Sambrook et al. 1989). After electrophoresis and electroblotting to nitrocellulose membranes (Micron Separation Inc.), the membranes were blocked and then probed with antisera to CMV CP or ZYMV CP, as described above for tissue printing. The immunoblots showed similar levels of accumulated M-CMV CP in the upper leaves of these doubly infected plants (Fig. 2). Although not quantitative as such, the RT–PCR results on similarly infected tissues supported this conclusion (Fig. 2).

To ascertain whether systemic movement of M-CMV had any effect on the level of accumulation of either ZYMV strain, systemically infected leaves showing yellow-mosaic symptoms (at 6–10 days p.i., prior to onset of plant death) were examined by Western blot analysis and ELISA for ZYMV. There was no increase in the levels of accumulation of either ZYMV strain, in singly versus doubly infected plants (Fig. 2 and data not shown). These results also indicated that CP accumulation of the two ZYMV strains was not correlated with symptom severity. Similar conclusions were drawn using melon or squash plants doubly infected with either ZYMV-A or ZYMV-AG, together with either Fny-CMV or LS-CMV; i.e. the levels of ZYMV were similar in singly vs. doubly infected plants (Wang et al., 2002), despite differences in pathology.

The above results also indicated that ZYMV-AG could either neutralize the barrier to the long-distance movement of M-CMV, or directly facilitate such movement, as for the more severe ZYMV-A strain. Since the HC-Pro protein of potyviruses has been shown to be a factor in long-distance movement (Cronin et al., 1995), changes in the ZYMV-AG HC-Pro protein associated with its attenuation (Gal-On, 2000) might have influenced its ability to interact synergistically with M-CMV. However, the above results indicated that synergistic interaction between CMV and ZYMV was not dependent on a particular genotype of ZYMV per se and that the effective long-distance movement of M-CMV was not in itself a major determinant of the extent of synergism in pathology.

Plant resistance to virus infection expressed in the form of restricted systemic movement of the virus has been described previously for various virus–host systems (Dufour et al., 1989; Goodrick et al., 1991; Nelson et al., 1993; Schaad & Carrington, 1996; Canto et al., 1997; Derrick & Barker, 1997; Winternant et al., 1997; Kaplan et al., 1997, 1998; Thompson & Garcia-Arenal, 1998; Wang et al., 1998; Ryabov et al., 1999). In most cases, the resistance appears to involve an inability of the virus to enter or exit the phloem, and specific cell types or locations of tissues associated with blockage of virus movement have been reported. The resistance to systemic infection by M-CMV in zucchini squash plants, which is overcome by infection with ZYMV, appears to be due to an inability of M-CMV to exit the sieve elements rather than a block in entry into the vasculature in the inoculated leaves (Haudenshield, 2001). It is not known how the delay in cell-to-cell movement of M-CMV (Wong et al., 1999) influences this block in systemic infection.

One possible mechanism by which ZYMV might neutralize the resistance to the systemic movement of M-CMV could be that the ZYMV-encoded HC-Pro, or one of the other ZYMV-encoded proteins involved in systemic movement, is directly able to help M-CMV move into new leaves. An alternative mechanism for systemic movement of M-CMV mediated by ZYMV could be via the ability of ZYMV to suppress the transcription of host factors involved in the formation of the barrier. The ability of HC-Pro to suppress gene silencing of reporter genes (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau & Carrington, 1998), as well as to neutralize a barrier against the systemic infection of Nicotiana tabacum by the potyvirus Plum pox virus (Sáenz et al., 2002), supports a role for HC-Pro in inhibiting the induction of host defence systems. This barrier may be one that is either specific to the movement of M-CMV, or not virus-specific, but which M-CMV is unable to suppress. A barrier specific to the movement of M-CMV
may be elicited by the CP of M-CMV (Wong et al., 1999). Mutation of the CP of Y-CMV or O-CMV at amino acid 129 from serine or proline, respectively, to leucine, as is present in M-CMV, has been shown to induce a host defence response in tobacco (Suzuki et al., 1995).

CMV has been reported to neutralize resistance against the potyvirus Pepper mottle virus (PepMoV) in pepper (Capsicum annuum cv. Avelar), allowing PepMoV to invade new systemic leaves (Guerrini & Murphy, 1999). In this case, there was no increase in accumulation of helper virus (CMV) (Guerrini & Murphy, 1999). Thus, it appears that when the movement-restricted virus is a potyvirus and CMV neutralizes resistance to the potyvirus, the potyvirus does not catalyse any increase in CMV accumulation. It will be interesting to know if this is a general or isolated phenomenon.

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References


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