ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2007, **39**(6): 431–437 **CN** 31-1940/Q

Synergy between Cucumber Mosaic Virus and Zucchini Yellow Mosaic Virus on *Cucurbitaceae* Hosts Tested by Real-time Reverse Transcription-Polymerase Chain Reaction

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Abstract Cucumber mosaic virus (CMV) and zucchini yellow mosaic virus (ZYMV) are two principal viruses infecting cucurbitaceous crops, and their synergy has been repeatedly observed. In our present work, a real-time reverse transcription-polymerase chain reaction procedure was established to study the accumulation kinetics of these two viruses in single and combined infections at the molecular level. The accumulations of open reading frames (ORFs) for 1a, 2a, 3a and coat protein (CP) of CMV and CP of ZYMV were tested. In the single infection, CMV-Fny ORFs accumulated to their maxima in cucumber or bottle gourd at 14 d post-inoculation (dpi), and gradually declined thereafter. ZYMV-SD CP ORF reached maximal accumulation at 14 and 28 dpi on cucumber and bottle gourd, respectively. However, when co-infected with CMV-Fny and ZYMV-SD, the maximal accumulation levels of all viral ORFs were delayed. CMV-Fny ORFs reached their maxima at 21 dpi on both hosts, and ZYMV-SD CP ORF reached maximal accumulation at 21 and 28 dpi on cucumber and bottle gourd, respectively. Generally, the accumulation levels of CMV-Fny ORFs in the co-infection were higher than those in the single infection, whereas the accumulation of ZYMV-SD CP ORF showed a reverse result.

Keywords cucumber mosaic virus; genomic RNA; real-time RT-PCR; relative quantification; synergy; zucchini yellow mosaic virus

Cucumber mosaic virus (CMV), the type species of the genus *Cucumovirus* (family *Bromoviridae)*, is a virus of many economically important crops worldwide and found to be able to infect over 1000 plant species from 85 families, including dicotyledons, monocotyledons, herbage and woody plants, horticultural crops and wild plants [1]. This virus was first reported on cucumber plants, and *Solanaceae*, *Fabaceae* and *Cucurbitaceae* including cucumber (*Cucumis sativus*) and bottle gourd (*Lagenaria siceraria*) are the crops most often affected. CMV induces a variety of symptoms depending on virus strains and plant hosts, but it mostly causes systemic mosaic, stunt, leaf and fruit deformation, and brings obvious loss of yield. CMV has a tripartite, positive-sense RNA genome with five major open reading frames (ORFs), including 1a, 2a, 2b, 3a and coat protein (CP) [2–7]. The quantitative determination of CMV genome RNAs has been described in our previous report [8], and the absolute amounts of them are found to be different between virions.

Zucchini yellow mosaic virus (ZYMV) is another important virus infecting *Cucurbitaceae*. Since it was first reported in Italy, ZYMV has become a global farm crop virus [9]. It transmits with aphids between plants at a high speed and has been reported as causing destructive epidemics on *Cucurbitaceae* crops in Italy, France, UK, Australia, Egypt, USA, and also some African and Asian countries [10]. This virus causes mosaic, yellowing, shoestringing, stunt, and fruit and seed deformation, and

Received: October 24, 2006 Accepted: March 14, 2007
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DOI: 10.1111/j.1745-7270.2007.00292.x

brings obvious loss of yield. As a potyvirus, ZYMV has filamentous virus particles of approximately 750 nm containing a single positive-sense RNA genome [11]. The genomic RNA of ZYMV encodes a single polyprotein, which is proteolytically cleaved by self-encoded proteases into functional proteins including CP, the helper component protease (HC-Pro) that also has gene silencing suppressor activity, the cylindrical inclusion protein that assists virus movement, the combined viral protein genome-linked (VPg)-protease protein, RNA replicase and other protein products with unclear functions [10].

It is very common that plants are infected by two or more viruses in natural conditions [12]. Different interactions among viruses have been observed, such as synergism, helper-dependence, cross-protection, replacement, mutual suppression, and a mixture of antagonistic and synergistic interactions. Certain pairs of virus co-infection have been used to induce symptoms more severe and to present higher virus accumulation than expected when they interact in an additive manner. In the case of CMV and ZYMV co-infection, severe reduction of host output, and even a rising incidence of diseases and prevalence of new diseases, has been observed by Zhang *et al.* [13]. The synergic interaction between CMV and ZYMV has been reported in the last 20 years [14–18]. Fattouh detected the mean ratio of CP of CMV and ZYMV from complexly inoculated zucchini squash (*Cucurbita pepo*) by enzyme-linked immunosorbent assay, and found that CP of CMV was higher than that of ZYMV. Using the molecular hybridization method, Wang *et al.* detected that the amounts of CMV RNAs and its CP increased in cases of complex infection [16,17].

However, all those traditional approaches have their limitations and disadvantages, and could not precisely quantify the changes of virus accumulation kinetics in the course of single or co-infection. Fluorescence-based, real-time reverse transcription-polymerase chain reaction (RT-PCR) has been regarded as the most reliable technology for mRNA detection, RNA quantification and gene expression analysis [19]. Compared with conventional approaches, it has higher sensibility, higher efficiency, and less contamination during the assay process and can give more dependable results [20,21].

In our present work, we establish a real-time RT-PCR procedure for detecting genes of CMV-Fny and ZYMV-SD and relatively quantifying the changes in the kinetics of virus RNA accumulation. This will help us to understand the mechanism of virus replication, and analyze the relationship between virus and host in antivirus engineering research, crop breeding and farming manipulation.

Materials and Methods

Plants and viruses

Seedlings of cucumber (*C. sativus* cv. Jinyou 1) and bottle gourd (*L. siceraria* cv. Yonghu 2) were grown in pots and maintained in growth chambers between 22 ºC and 28 ºC, with a 16 h light and 8 h darkeness cycle. Twelve seedlings for each treatment were grown for pot-test, and three were sampled for real-time PCR detection. In parallel to this test, three cultivars of bottle gourd (*L. siceraria* cv. Yonghu 2, 15, 31) were grown in the field and used for the disease index test, so that there were 30 plants for each treatment. Isolates, CMV-Fny and ZYMV-SD, were inoculated and maintained on seedlings of tobacco and bottle gourd respectively, then biologically assayed for their infectious virility. Seedlings of two- to three-true leaf stage were used for viral infection assays. For the single infection assays, CMV and ZYMV were mechanically inoculated on the two cotyledons of the seedlings; and for the co-infection assays, CMV and ZYMV were separately inoculated on the two cotyledons of the seedlings. The total amount of inocula in the co-infection was equal to that in the single infection. Seedlings inoculated with ddH_2O were used as the mock treatment.

RNA extraction and real-time RT-PCR

Four disks, approximately 0.1 g in weight, were sampled from upper uninoculated leaves at a 7 d interval from 7 to 35 days post-inoculation (dpi). The first and second leaves above the inoculated cotyledons were used for the first sampling, and the third and fourth ones were used for the second sampling, continuing in the same way until the fifth sampling. Our experiment lasted for 35 d, when the seedlings were at the 9- to 11-true-leaf stage. Total RNA was extracted from these disks using Trizol reagent (TaKaRa, Dalian, China), treated with DNase I (TaKaRa), and stored at –80 ºC until used.

Primers used for PCR amplifying of 18S rRNA and CMV-Fny ORFs 1a, 2a, 3a and CP have been described previously [8]. A pair of primers (5′-terminal primer CATCGAGGTTGTTTGGTCTTGA and 3′-terminal primer GCAGTGTGCCGTTCAGTGTCT) were selected for amplifying ZYMV-SD CP ORF, expecting a 66 bp fragment product. A two-step amplification protocol was used for real-time RT-PCR as previously reported [8] and reverse transcription was carried out in a 10 μl reaction mixture, including 1 μg total RNA.

Data analysis

Data were analyzed with ABI PRISM 7000 sequence detection system software (Applied Biosystems, California, USA) as our previous report [8]. To carry out the relative quantification, 18S rRNA was used as an internal control to normalize the amount of CMV and ZYMV ORFs between treatments. The *C*t values of CMV-Fny 1a, 2a, 3a and CP, and ZYMV CP ORFs were compared directly with 18S rRNA *C*t and results were expressed through ratios of the target-specific signal to the internal reference. Relative quantification was carried out by the $-\Delta\Delta Ct$ method. Thus, the fold changes in CMV and ZYMV (target gene) relative to the 18S rRNA was determined by **Equation 1**:

$$\text{Fold change}=2^{-\Delta\Delta Ct} \quad 1$$

where $\Delta\Delta Ct=(Ct_{\text{Target}}-Ct_{18S})_{\text{Time }x}-(Ct_{\text{Target}}-Ct_{18S})_{\text{Time }0}$. Time x was any time point and time 0 was the minimum expression time of each gene. Relative quantification of gene expression and the logarithm of relative quantification using the $2^{-\Delta\Delta Ct}$ method was considered the gold approach [22].

Disease index

Symptoms on hosts were observed at 7, 14, 21, 28 and 35 dpi and rated according to symptom expression: grade 0, no obvious symptom; grade 1, light systemic mosaic or spot; grade 2, typical systemic mosaic symptom on leaves; grade 3, severe mosaic but without obvious malformation of the whole plant; grade 4, severe mosaic with necrosis or plant dwarfing, or dead. Based on host symptoms, the disease index was calculated as **Equation 2** [23]:

$$DI=\frac{\sum n_x\times \text{grade}_x}{n_{\text{total}}\times \text{grade}_{\text{hightest}}}\times 100\% \quad 2$$

in which, DI is the diseach index, grade_x is a certain grade, n_x is the number of plants of this grade, $\text{grade}_{\text{highest}}$ is the highest grade, and n_{total} is the total number of plants.

Results

Assessment of symptoms and synergic interaction

Seedlings of both cucumber and bottle gourd infected with CMV-Fny had systemic mosaic, foliage distortion, and flowering retardation. However, the symptoms on bottle gourd were much more severe than those on cucumber. ZYMV-SD also induced severe symptoms on cucumber and bottle gourd, with systemic mosaic, yellowing, plant dwarfing and wilting, and clearly deformed leaves and fruits. It also produced more severe symptoms on bottle gourd than on cucumber. The two *Cucurbitaceae* crops co-inoculated with CMV-Fny and ZYMV-SD produced much more severe symptoms, including deformation, necrotic lesion, wilting and even death of the whole plant.

The severe symptoms showed that these two viruses were synergic on the *Cucurbitaceae* crops. Statistical data showed that, at 28 dpi, the mean disease index of three cultivars of bottle gourd in the single infection of CMV-Fny and ZYMV-SD were 34.98% and 37.12%, respectively. However, the mean disease index increased to 55.28% on the cultivars co-infected with these two viruses, which was 1.58-fold of that in CMV-Fny single infection and 1.50-fold of that in ZYMV-SD single infection. The disease indices for bottle gourd (Yonghu 2) in the single or co-infection at different infection stages are listed in **Table 1**.

Table 1 Disease index with single or complex infection on bottle gourd (Yonghu 2)

Inoculum	Disease index (%)				
	7 dpi	14 dpi	21 dpi	28 dpi	35 dpi
CMV-Fny	17.11	18.42	27.10	33.66	38.42
ZYMV-SD	34.21	36.84	38.63	37.60	40.00
Fny+SD	38.33	48.33	50.01	53.75	51.67

Three cultivars of bottle gourd (*Lagenaria siceraria*) were tested in this study, but only the data tested for Yonghu2 are shown as similar results were detected on the other two cultivars. Thirty seedlings for each treatment were tested in the field experiment. CMV, cucumber mosaic virus; dpi, days post-inoculation; ZYMV, zucchini yellow mosaic virus.

Specificity of real-time RT-PCR

In order to confirm the specificity of each selected primer pair, virtual real-time RT-PCR was carried out for each ORF. The amplification curves were smooth and of good reproducibility (**Fig. 1**). Each homogeneous RT-PCR produced a single, sharply defined melting curve with a narrow peak (**Fig. 2**). The amplified products were also confirmed by agarose gel electrophoresis (**Fig. 3**).

Accumulation kinetics for CMV ORFs in the single or co-infection

The relative amounts of CMV ORFs 1a, 2a, 3a and CP

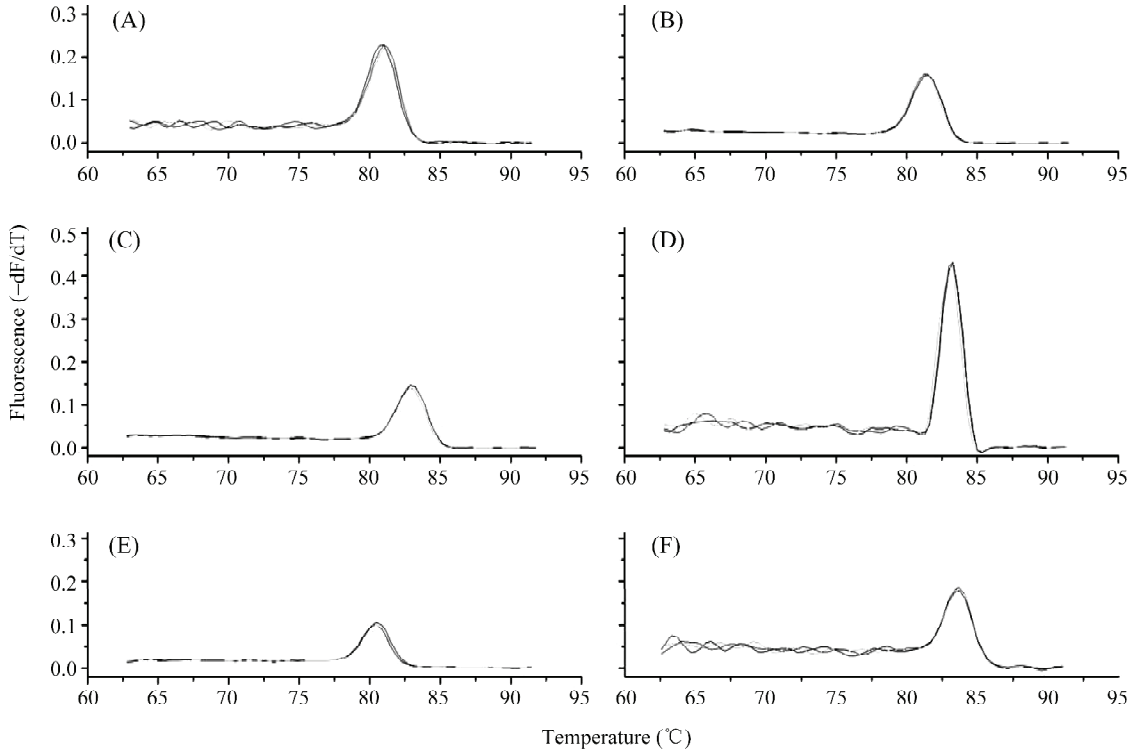

Fig. 2 Dissociation curves analyzing cucumber mosaic virus (CMV-Fny) and zucchini yellow mosaic virus (ZYMV-SD) open reading frames (ORFs)

(A) CMV-Fny 1a ORF. (B) CMV-Fny 2a ORF. (C) CMV-Fny 3a ORF. (D) CMV-Fny CP ORF. (E) ZYMV-CP ORF. (F) 18S rRNA .

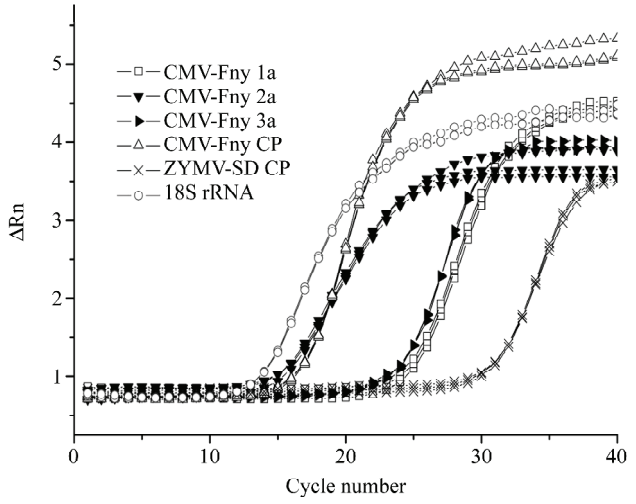

Fig. 1 Amplification curves of each detected open reading frame in cucumber plants infected by a complex of cucumber mosaic virus (CMV-Fny) and zucchini yellow mosaic virus (ZYMV-SD)

A software program calculated a ΔRn using the equation $\Delta Rn=Rn^{+}-Rn^{-}$, where Rn^{+} was the fluorescence emission of the product at each time point, and Rn^{-} was the fluorescence emission of the baseline. Three repeats were assayed for each treatment.

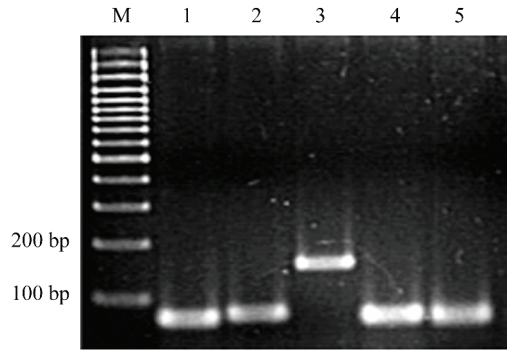

Fig. 3 Agarose gel electrophoresis of reverse transcription-polymerase chain reaction products for open reading frames (ORFs) of cucumber mosaic virus (CMV-Fny) and zucchini yellow mosaic virus (ZYMV-SD)

M, marker; 1, CMV-Fny 1a ORF; 2, CMV-Fny 2a ORF; 3, CMV-Fny 3a ORF; 4, CMV-Fny coat protein ORF; 5, ZYMV-SD coat protein ORF.

on cucumber (Jinyou 1) seedlings infected with CMV-Fny alone were quantified by real-time RT-PCR. For CMV single infection, the accumulation of each CMV ORF on

cucumber plants was hardly detected at 7 dpi, but their accumulation reached a peak rapidly at 14 dpi, and declined gradually thereafter (**Fig. 4**). The accumulation of each CMV ORF on cucumber plants co-infected with CMV-Fny and ZYMV-SD was much higher than that in CMV single infection at 7 dpi. The accumulation increased gradually to maximum at 21 dpi, then steadily declined in the following weeks (**Fig. 4**). In general, the accumulation ratios of each ORF between the single and co-infection reached the maximum levels at 7 dpi. Co-infection of CMV and ZYMV stimulated the early stage replication of CMV RNAs and postponed the decline of their accumulation levels. At 35 dpi, the relative amounts of CMV-Fny ORFs 1a, 2a, 3a and CP in the co-infection were 1.48-, 2.24-, 2. 51- and 0.99-fold of those in the single infection, respectively (**Fig. 4**).

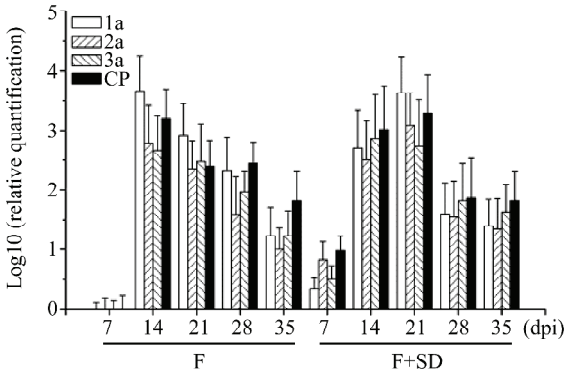

Fig. 4 Time courses of the relative amounts of cucmber mosaic virus (CMV-Fny) open reading frames (ORFs) 1a, 2a, 3a and coat protein (CP) detected from cucumber leaf tissue

Time courses of the relative amounts of CMV-Fny ORFs 1a, 2a, 3a and CP detected from cucumber leaf tissue in single infection or co-infection set the logarithm (relative quantification) of CMV-Fny 1a, 2a, 3a, CP ORFs on cucumber plants at 7 dpi as zero. Data were mean of base-10 logarithm of relative quantification values from three plants; error bars represented the standard error. F, CMV-Fny; SD, zucchini yellow mosaic virus ZYMV-SD.

When the accumulation of the CMV ORFs 1a, 2a, 3a and CP on bottle gourd was assayed, we found that ZYMV influenced CMV accumulation obviously. Early stage replication of CMV ORFs was also stimulated in the co-infection on bottle gourd at 7 dpi. The accumulation of CMV ORFs in the single infection increased rapidly to a maximum level at 14 dpi. However, the maximum accumulation of CMV ORFs in the co-infection was reached at 21 dpi, which was much higher than that in the single infection at 14 dpi. The accumulation of each CMV ORF declined gradually 21 d later. At 35 dpi, the relative amounts of CMV-Fny ORFs 1a, 2a, 3a and CP in the co-infection were 15.14-, 4.90-, 3.16- and 2.57-fold of those in the single infection, respectively (**Fig. 5**). Taken together, co-infection of ZYMV increased the CMV replication and/or accumulation of CMV in both hosts, especially in bottle gourd.

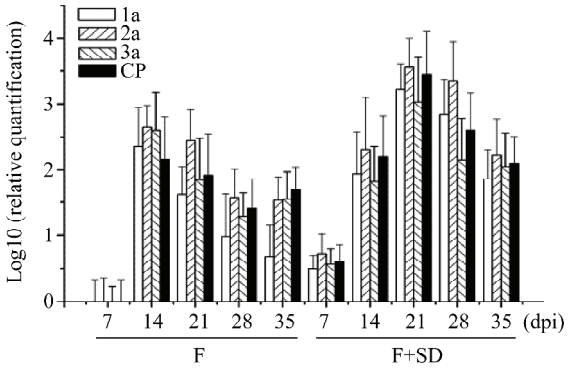

Fig. 5 Timecourses of the relative amounts of CMV-Fny ORFs 1a, 2a, 3a and CP from bottle gourd

Timecourses of the relative amounts of CMV-Fny ORFs 1a, 2a, 3a and CP detected from bottle gourd in single infection or co-infection. Set the logarithm (relative quantification) of CMV-Fny 1a, 2a, 3a and CP ORFs on bottle gourd at 7 dpi as zero. F and SD presented CMV-Fny and ZYMV-SD, respectively. Data were mean of base-10 logarithm of relative quantification values from three plants; error bars represented the standard error.

Accumulation kinetics of ZYMV CP ORF in single or co-infection

During our testing period, the relative amounts of ZYMV CP ORFs increased rapidly, and reached their maximum in the single infection on cucumber at 14 dpi, but reached their maximum in the co-infection at 21 dpi. On bottle gourd, the accumulation of ZYMV CP ORF reached the maximum level in both single infection and co-infection at 28 dpi, but the relative amount of ZYMV CP ORF in the co-infection was clearly lower than that in the single infection (**Fig. 6**). At 35 dpi, the relative amounts of ZYMV-SD CP ORF in the co-infection were 90% and 22% of those in the single infection on cucumber and bottle gourd, respectively. Collectively, co-infection of CMV reduced the replication and/or accumulation of ZYMV in both hosts.

Discussion

Based on our previous quantitative determination of

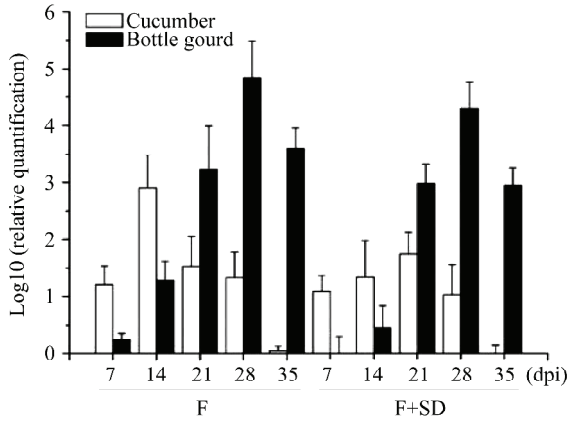

Fig. 6 Timecourses of the relative amounts of zucchini yellow mosaic virus (ZYMV-SD) coat protein (CP) open reading frames in single infection or co-infection on cucumber and bottle gourd

On cucumber plants, set the logarithm (relative quantification) of cucumber mosaic virus (CMV) and ZYMV complex inoculation at 35 dpi as zero. On bottle gourd plants, set the logarithm (relative quantification) of ZYMV single inoculation at 7 dpi as zero. Data were mean of base-10 logarithm of relative quantification values from three plants; error bars represented the standard error. F, CMV-Fny; SD, ZYMV-SD.

relative amounts of CMV ORFs, the accumulation kinetics of CMV-Fny and ZYMV-SD in single or co-infection on cucumber and bottle gourd plants was tested. It had been reported that when potyvirus co-infected the cucurbit with other viruses, the accumulation of the other viruses increased, however, the accumulation of potyvirus decreased [14]. In the case of CMV and ZYMV co-infection, CMV CP accumulation was approximately 2.03-fold higher than in single infection, but the accumulation of ZYMV CP did not change significantly [18]. However, in this work, the accumulation of ZYMV CP ORF was found to be greatly suppressed in the co-infection plants (**Fig. 6**). Similar results have been reported on cucumber and zucchini squash plants tested by Northern blotting [16,17]. It was found that the accumulation of CMV-Fny RNAs increased on both hosts. Ratios of CMV ORFs increased in the synergic interaction when host plants were co-infected with ZYMV. In our experiment, the total RNAs extracted from leaf tissues reached different levels in field tests and pot-tests, and results of real-time PCR detection for field samples at 14 dpi presented similar results to that of pot-test of each treatment. These results presented the first determination of different ORFs of CMV increased in the co-infection. All of the above findings supported the conclusion that CMV could be enhanced but ZYMV could be suppressed in the co-infection. In particular, a very significant enhancement of CMV-Fny was present in co-infected bottle gourd plants, which reached 15.14-fold (1a ORF) that of single infection, and suppression of ZYMV was also detected.

After inoculation, the virus replicated in the host tissues, then the host experienced three stages: latently infected, infectious, and post-infectious [13]. In our present work, the accumulation of CMV RNA always reached a peak at 14 dpi on two cucurbit hosts that were similar to those involved in the "cycling" described by Gal-On *et al*. [26]. We also found that the maximum accumulation on co-infected hosts appeared earlier than in hosts with single infection. This indicated that virus long-distance movement was stimulated in the co-infection (**Figs. 4** and **5**).

Focusing on the synergy of the two viruses, cross-protection has been used to control the large-scale happening and mass epidemic viruses. An attenuated strain of ZYMV-WK was used widely as a bio-control agent in many areas in Europe and Japan [27–29], but its interaction with CMV as an epidemic virus in the same ecological position has not been mentioned. Kosaka *et al.* also used many attenuated strains of ZYMV to control viral infection successfully and enhance resistant ability against the virus on cucumber and other plants [30,31]. But, according to our present work, the accumulation of CMV increased rapidly in the co-infection. We also found that co-infection yielded lower accumulation of ZYMV. Therefore, using attenuated CMV as a bio-control agent would be more promising than attenuated ZYMV, as CMV occurred more prevalently on *Cucurbitaceae* crops [23]. Our results suggest that ZYMV, as a bio-control agent, should be used with care when CMV exists as another principal virus, and an attenuated CMV could be safer than ZYMV. Our experiments have not yet included challenge inoculations of severe strains of viruses, but our conclusion is valuable for agricultural strategy in the use of bio-control agents.

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Edited by
Minghua XU