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Increase in Cucumber Mosaic Virus Concentration in Japanese Radish Plants Co-infected with Turnip Mosaic Virus

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Abstract

Dual infection of turnip mosaic virus (TuMV) and cucumber mosaic virus (CMV) causes a severe mosaic disease in Japanese radish plants. This paper deals with the interactions between these viruses in radish plants. TuMV caused mild mosaic symptoms of Japanese radish plants by itself, but CMV did not. Plants inoculated with both viruses displayed more severe mosaic symptoms than plants inoculated only with TuMV. Indirect ELISA tests indicated that CMV accumulation was significantly enhanced in the presence of TuMV, but TuMV levels were little affected by co-infection with CMV. The enhanced accumulation of CMV in plants infected with TuMV as well as CMV was evident in systemically infected leaves rather than the inoculated cotyledons and this suggested that systemic transport and spread of CMV in radish plants were enhanced in the presence of TuMV.

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Key words: synergism, cucumber mosaic virus, turnip mosaic virus.

INTRODUCTION

In fields on the dunes in Niigata Prefecture, a severe mosaic disease of Japanese radish plants (*Raphanus sativus* L.) is very prevalent. The disease is generally associated with two aphid-born viruses, turnip mosaic virus (TuMV) and cucumber mosaic virus (CMV)[11]. Both viruses often infect radish plants together causing a severe mosaic disease. TuMV is readily detected in plants with symptoms, whether or not they also contain CMV. However, although CMV is often found in radishes with TuMV it has rarely been found in plants in the field as a single infection[13,14]. It is well known that combinations of unrelated plant viruses often react synergistically, leading to enhanced concentrations and more severe symptoms in plants with multiple infections[4,8,10]. In this study both viruses were inoculated to radish plants, either alone or together, and the plants were then assayed to determine their relative virus contents by indirect ELISA. The aims were to investigate whether TuMV assisted CMV multiplication and transport when both viruses were present together in radish plants. In this paper we report on a very significant increase in the concentration of CMV in radish plants also inoculated with TuMV.

MATERIALS AND METHODS

Viruses and plants. Both viruses used in this study were isolated from Japanese radish plants. The CMV isolate was obtained from a severely diseased plant that was also infected with TuMV. A sap extract from this plant was first inoculated to tobacco (*Nicotiana tabacum*

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cv. Ky-57) and the systemically infected upper leaves free from TuMV were then used to passage the virus through single local lesions in broad bean (*Vicia faba*). The CMV isolate obtained in this way was then maintained in tobacco.

The TuMV isolate was obtained from a plant not contaminated with other viruses. After a single-lesion transfer on *Chenopodium amaranticolor*, the isolate was propagated in turnip (*Brassica campestris* cv. Yorii).

In all experiments, Japanese radish seedlings (cv. Miyashige-sobutori) were used as test plants and maintained in a greenhouse.

Inoculation. In preliminary experiments we found that our CMV isolate did not readily infect radish plants when inoculated mechanically. Thus, concentrated, purified virus preparations were used for all inoculations. The purified virus pellets were resuspended in 0.01 M phosphate buffer (pH 8.0). The final concentration of CMV and TuMV in all the inocula were adjusted to 1 mg/ml and 100 μg/ml, respectively. These virus solutions were inoculated to both cotyledons of young radish seedlings, either alone or together. Initial inoculations were first done when the cotyledons were fully expanded and the first true leaf was commencing to emerge. For sequential inoculations, the second virus was challenge-inoculated to newly opened first and second leaves 10 days after the first inoculation. All the inoculations were repeated at least twice.

Purification. Purified preparation of CMV were obtain from infected tobacco using the method described by Lot *et al.*[9] while TuMV was purified from the infected turnip leaves by the procedure of Choi *et al.*[5]

Sampling. Inoculated cotyledons and newly opened upper leaves were taken from individual test plants sacrificed at various intervals after inoculation and weighed (8 to 10 plants per treatment). A cork borer was used to cut a leaf disc (1 cm diameter) from each cotyledon and leaf; the discs were then stored at −20 C pending enzyme immunoassay.

Indirect enzyme-linked immunosorbent assay. Separate antisera against CMV and TuMV were produced in rabbits by a series of injections of the purified viruses emulsified with Freund's complete adjuvant. The final titers of the CMV and TuMV antisera were estimated by ring interface test to be 1/1,024 and 1/512, respectively.

The gamma-globurin fractions were purified from these antisera according to the methods of Clark and Adams[6]. The procedure of non-precoated indirect ELISA was used to determine virus content[12]. Each disc was ground 1:1,000, w:v in carbonate coating buffer (pH 9.6) and the homogenate applied to two wells of polystylene microtiter plates (Dynatech Lab. Inc.). The anti-CMV IgG and anti-TuMV IgG preparations were used at concentrations of 0.5 μg/ml and 1.0 μg/ml respectively, and an affinity-purified goat anti-rabbit IgG-alkaline phosphatase conjugate (Herix Biotech Ltd.) was diluted 1,000-fold before use. The reactions were stopped 30 and 60 min after incubating with substrate when assaying CMV and TuMV, respectively. The ELISA absorbance values were measured with a Corona MTP-12 spectrophotometer at 405 nm. Under these conditions, CMV and TuMV could be detected in purified preparations at concentrations down to 1∼2 ng/ml and 5∼10 ng/ml, respectively.

RESULTS

Occurrence of viruses in radish fields

The results of tests for virus infections in radish plants from the fields are shown in Table 1. Leaf samples were collected from plants showing severe, mild and no symptoms, and assayed for both CMV and TuMV by ELISA. TuMV was detected in all plants displaying mosaic symptoms irrespective of their severity and whether or not they contained CMV. On the contrary, CMV was only ever detected in plants that were also infected with TuMV. Most of the severely diseased plants (94%) contained both CMV and TuMV while the majority of plants with mild symptoms were infected only with TuMV. Thirty per cent of the symptomless plants

Table 1. Distribution of viruses in the radish fields

Symptoms [a]	Viruses detected				
	None	CMV alone	TuMV alone	CMV and TuMV	Total number of plants [b]
M	0	0	3 [c]	47	50
m	0	0	39	11	50
0	35	0	15	0	50

a) Leaf samples were collected from radish plants which showed severe mosaic (M), mild mosaic (m) and no symptoms (0).
b) Samples were collected from 50 plants in each symptom category.
c) Values represent the number of samples in which the respective viruses were detected. Those samples whose ELISA values exceeded 0.100 were regarded as positive[11], because ELISA values of the control (healthy plants) were between 0.02 and 0.07 under the same condition.

Table 2. Relative concentrations of each virus in both singly and doubly infected radish leaves

ELISA for	Plants [a] inoculated	Days after inoculation				
		5	10	15	20	25
CMV	singly	0.180 [b]	0.404	0.041	0.021	0.088
	doubly	0.115	0.795	1.478	0.722	1.256
TuMV	singly	0.147	0.836	0.865	0.647	0.853
	doubly	0.147	0.862	0.997	0.834	1.033

a) Viruses were inoculated simultaneously, and assayed for the inoculated cotyledons 5 days, a mixture of the cotyledons and 1st and 2nd leaves 10 days, and a mixture of upper 3 leaves including the top one 15, 20 and 25 days after inoculation, respectively.
b) Number represents the ELISA value which is averaged from 8 replicates.

were also infected with TuMV.

Symptoms on radish plants

Typical mosaic symptoms developed on all the plants inoculated with TuMV and those inoculated with both TuMV and CMV within 7~10 days after inoculation. The plants first developed vein clearing of the upper leaves 4~7 days after inoculation followed by a systemic mosaic. Symptoms were more severe on plants inoculated with both viruses than on those only infected with TuMV. In the later stages of infection, severe mosaic symptoms accompanied with leaf curling were usually observed on the plants infected with both viruses while the symptoms on plants infected with TuMV alone became very mild and often obscure. Plants inoculated only with CMV did not develop symptoms, in agreement with the results of our preliminary experiments[11].

Changes in virus content of inoculated plants

The results of ELISA tests to determine the relative levels of CMV and TuMV in inoculated plants over time are shown in Table 2. Samples were taken at five-day intervals after the plants were inoculated. The results indicated that the accumulation of CMV was enhanced to a remarkable degree when the plants were also infected with TuMV. In plants inoculated only with CMV, few virus could be detected in leaf samples taken 15 days or later after inoculation. On the contrary, there was little difference in the TuMV content between plants infected with one or both viruses although, in the later stages of infection, the ELISA values for TuMV were slightly higher for the plants infected with both viruses than for those inoculated with TuMV alone. Similar results were obtained in a series of sequential inoculation experiments (Table 3). Either pre-infection or challenge with TuMV led to large increases in the CMV content

Table 3. Effects of TuMV-preinfection or challenge-inoculation on transition of CMV concentration in the infected radish leaves

Plants [a] inoculated with	Days after inoculation					
	5	10	15	20	25	30
TuMV 10 days after CMV	0.285 [b]	0.422	0.040	0.879	0.786	0.369
CMV singly	0.309	0.488	0.092	0.083	0.067	0.025
CMV 10 days after TuMV	—	—	0.451	0.880	0.834	0.457
CMV singly	—	—	0.094	0.032	0.034	0.020

a) In sequential inoculation, the second virus was challenged to newly opened 1st and 2nd leaves 10 days after the first inoculation. Then, CMV was assayed for the inoculated cotyledons 5 days, a mixture of the cotyledons and 1st and 2nd leaves 10 days, and a mixture of upper 3 leaves including the top one 15, 20, 25, and 30 days after the first inoculation, respectively.

b) Number represents ELISA value which is averaged from 8 replicates.

of radish plants whereas few CMV was recovered from newly opened upper leaves of plants inoculated with this virus alone.

Relative virus content in cotyledon and individual leaves

Another set of experiments was designed to investigate the virus content of individual leaves at different positions. Samples were collected from the inoculated cotyledons and the first three true leaves at intervals up to 15 days after inoculation. The results showed significant enhancement of CMV accumulation in the systematically infected leaves of plants that had also been inoculated with TuMV (Fig. 1). The enhancement was first detected in the first true leaf five days after inoculation. Presence or absence of TuMV had relatively little effect on the CMV content of inoculated cotyledons.

In plants infected only with CMV, the content of CMV in the three uninoculated upper leaves was relatively low even though significant amounts of virus were present in the inoculated cotyledons. This suggested that transport of virus from inoculated cotyledons to the upper leaves was being retarded in the plants infected only with CMV.

The content of TuMV in the cotyledons and leaves of plants inoculated with one or both viruses was studied in a similar manner. There was little difference in TuMV content between plants infected with TuMV alone and plants inoculated with both viruses (Fig. 2). Large amounts of TuMV accumulated in the systemically infected leaves of both sets of plants. However there was slightly more TuMV in the plants that were also infected with CMV.

Enhancement of CMV accumulation in systemically infected leaves of plants containing TuMV was further examined using CMV inocula at various concentrations. Radish cotyledons were inoculated with purified CMV preparations containing between 10 and 2,000 μg of virus per ml. Half the plants were also inoculated simultaneously with TuMV (100 μg/ml). All plants were grown-on in a growth chamber at 25 C with a 16 hr photoperiod. One week later, all the inoculated cotyledons, the petioles of the cotyledons and first true leaves were harvested individually and tested by ELISA for their virus content. The results are shown in Fig. 3. Consistently high ELISA absorbance values for CMV were recorded for plants also infected with TuMV regardless of the CMV concentration in the inoculum. However in plants inoculated with CMV alone, there was a response to virus concentration in the inoculum. When highly concentrated inocula were used ($>$100 μg/ml) the CMV content of the cotyledons was similar to that in plants also inoculated with TuMV. However the virus content of the petioles of the cotyledons and the first true leaves were still considerably depressed by comparison. Use of inocula containing less than 100 μg/ml and no TuMV led to very low levels of CMV accumulation in the plants.

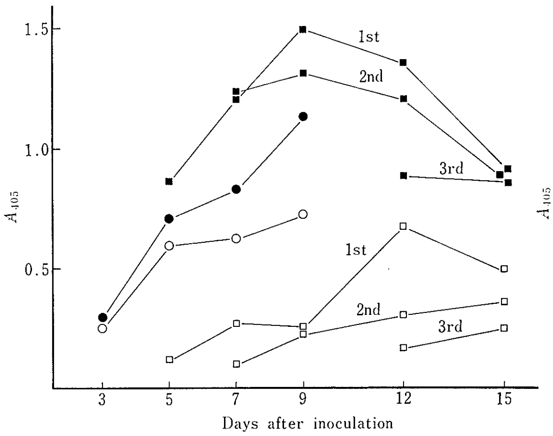

Fig. 1. Relative CMV contents of cotyledons (circles) and upper leaves (squares) at different positions on plants inoculated with CMV alone (open) and with both CMV and TuMV (close).

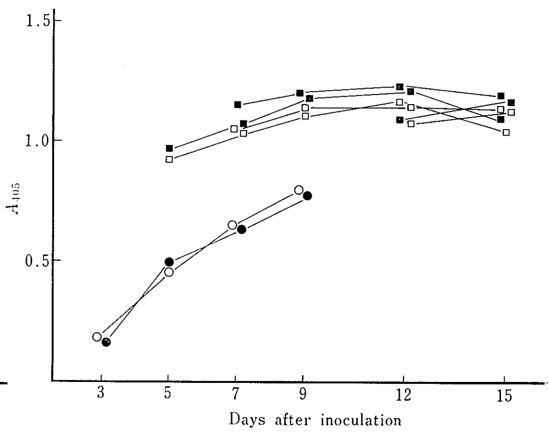

Fig. 2. Relative TuMV contents of cotyledons (circles) and upper leaves (squares) at different positions on plants inoculated with TuMV alone (open) and with both TuMV and CMV (close).

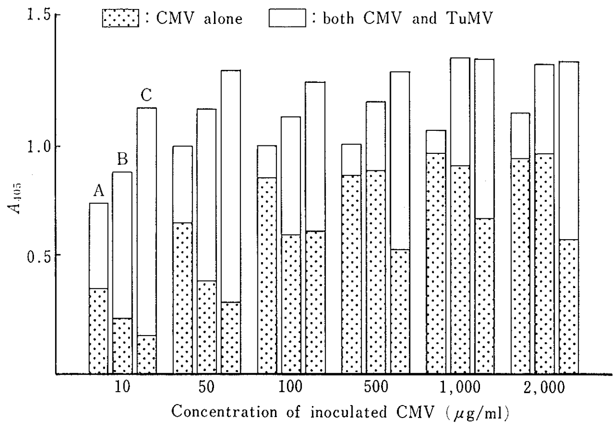

Fig. 3. Relative content of CMV in Japanese radish plants 7 days after inoculation, when inocula were varied with respect to CMV concentration. Data are presented for the content of inoculated cotyledons (A), petioles of the inoculated cotyledons (B), and the first true leaves (C) and represent the means from ten plants.

DISCUSSION

Japanese radish is the most widely grown root vegetable in Japan, and the mosaic disease that affects it is common in crops. Although it is well known that TuMV and CMV are the main causal agent of this disease, there have been few comparative studies on the occurrence, effects and interactions of single and multiple infections[13,14]. Tsuchizaki *et al.*[13] reported that CMV was rather difficult to recover from the systemically infected leaves of Japanese radish plants inoculated only with CMV although it was regularly recovered when TuMV also infected plants. In our experiments it was also rather difficult to recover CMV from the upper uninoculated leaves of plants whose cotyledons had been infected with CMV alone, even when concentrated preparations containing as much as 1 mg/ml were used as inocula. However, the CMV content was consistently enhanced as shown in Tables 2 and 3, when the plants were also infected with TuMV, irrespective of whether infection with this latter virus took place at either time they were inoculated with CMV, or earlier or later. Plants infected with both viruses showed more severe symptoms than those infected only with TuMV, probably due to the high concentration of CMV. On the contrary, TuMV concentration was little affected by co-infection with CMV.

The significant enhancement of CMV accumulation in plants also infected with TuMV was evident in the upper systemically infected leaves. The virus content of systemically infected leaves on plants inoculated only with CMV was consistently low and virus multiplication tended to be delayed (Fig. 1). Similar results were also obtained in studies on CMV and TuMV in another host, turnip, (cv. Yorii, data not shown). However, enhancement of CMV accumulation in infected turnip leaves also infected with TuMV was not as great as that in radish plants because the virus content in the leaves of turnip plants infected only with CMV was relatively high compared to that in radish plants inoculated with CMV. In addition, turnip leaves infected with CMV displayed mild chlorotic spots.

It appeared that long distance transport of virus from the cotyledons to the upper ones was retarded in radish plants inoculated only with CMV, suggesting that systemic spread of CMV in the infected radish plants was enhanced in the presence of TuMV. There have been many report on similar phenomena in which multiplication or distribution of one virus was enhanced by co-infection with another unrelated virus. For example, multiple infection of barley with tobacco mosaic virus (TMV) and brome mosaic virus led to systemic spread of TMV in barley plants[7]. In potato plants infected with potato virus X (PVX) and potato virus Y (P-VY), the PVX concentration increased significantly even though the PVY concentration did not[10]. Furthermore, potato leaf roll virus invaded the mesophyll parenchyma cells of infected plants when they also contained PVX[1] or PVY[3]. Atabekov *et al.*[2] suggested that these types of phenomena might be ascribed to enhanced rates of the virus transport in the presence of second virus that was spreading systemically at a rapid rate. They also assumed that movement between the mesophyll and phloem tissues of the host plants was important for the long distance transport of the dependent virus that were generally defective in this area, but which could be complemented for when present together with the helper virus and thus permit systemic spread of the dependant virus. In our experiments, we found significantly higher levels of CMV in the petioles of cotyledons inoculated with both viruses (Fig. 3), and this fact support their suggestion. Although little is known about the mechanism of virus transport, our results suggest that the improved transport of CMV from the inoculated cotyledons to the upper leaves through the petioles of the Japanese radish plants co-infected with TuMV was responsible for the higher levels of CMV found in those plants inoculated with both viruses.

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和 文 摘 要

佐野義孝・小島 誠：カブモザイクウイルスと重複感染したダイコンにおけるキュウリモザイクウイルス濃度の増大について

カブモザイクウイルス(TuMV)とキュウリモザイクウイルス(CMV)の重複感染はダイコンに激しいモザイク病を引き起こす。ダイコンにおける両ウイルス間の相互作用について比較・考察を行った。TuMVは，単独感染した場合でもダイコンに軽微なモザイク症状を引き起こしたが，CMV単独の感染植物は無病徴であった。また，これら2種のウイルスを接種された植物は，TuMVの単独感染の場合よりもさらに激しいモザイク症を呈した。間接ELISAにより，ダイコン葉におけるCMVの増殖量はTuMVの存在により高まることが示されたが，一方，TuMVの濃度はCMVとの重複感染による影響をほとんど受けなかった。TuMVと重複感染したダイコンにおけるCMV濃度の増加は，接種葉よりも全身感染した上位葉において顕著に観察されたことから，CMVのダイコン葉における全身的な移行と拡散がTuMVの存在により助長されることが示唆された。