Effect of Co-Infection with Systemic Viruses on the Localization of Tobacco Mosaic Virus in Cucumber Cotyledons

Hidetoshi UEKUSA*, Makoto ISHII*, Tohru TERAOKA*, Daijirou HOSOKAWA* and Minoru WATANABE*

Abstract

When cucumber cotyledons that had been inoculated with tobacco mosaic virus (TMV) were challenge-inoculated with cucumber green mottle mosaic virus (CGMMV), the multiplication of TMV and the size and number of starch-lesions in the cotyledons remained the same as in single inoculation with TMV. Inoculation with CGMMV before TMV-infection, however, decreased the amount of TMV and the number of starch-lesions. The same results were obtained when cucumber mosaic virus (CMV) was inoculated before or after TMV. When cucumber cotyledons were inoculated with zucchini yellow mosaic virus (ZYMV) 4 days before TMV inoculation or jointly inoculated with ZYMV and TMV, TMV concentration increased roughly 7 and 4 times, respectively. In this case, TMV was widely distributed in horizontal and vertical directions in the cotyledon tissues, but eventually localized. In cucumber cotyledons co-infected with ZYMV and CGMMV, CGMMV concentration was not affected by ZYMV infection. Factors involved in the localization of TMV are discussed based on these observations.

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Key words: tobacco mosaic virus, cucumber, localization, starch-lesion, double-infection.

INTRODUCTION

Evidence has shown that cell-to-cell movement of a virus in plants is mediated by its own coding protein. The ability of a virus to spread from one cell to another is believed to be one of the primary factors in determining its host range. Malyshenko and his co-workers demonstrated that transport of a virus lacking the ability to spread from cell to cell in a host plant could be complemented by another virus which has the ability to spread systemically in the plant.

Tobacco mosaic virus (TMV) is restricted to small areas around the primary infection sites of cucumber leaves without obvious lesions. The area infected with TMV, however, appears as a starch-lesion when the leaves are decolorized by ethanol and stained by iodine. Cucumber leaves are also systemically infected with cucumber green mottle mosaic virus (CGMMV), cucumber mosaic virus (CMV) and zucchini yellow mosaic virus (ZYMV).

In order to characterize the mechanism(s) of TMV localization in cucumber leaves, we therefore attempted to determine the effects of a double infection with CGMMV, CMV or ZYMV on TMV multiplication and distribution in these leaves.

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MATERIALS AND METHODS

Plants and viruses. Viruses used in this study were a Japanese common strain of TMV, a cucumber strain of CGMMV, a yellow strain of CMV, and ZYMV. The inocula of TMV, CGMMV and CMV were prepared by differential centrifugation methods from tobacco, cucumber and tobacco leaves, respectively. The inoculum of ZYMV was prepared from cucumber leaves with systemic symptoms by homogenizing them in 1/15 M phosphate buffer (pH 7.2).

Cucumber (Cucumis sativus L. cv. Choujitsu-ochiai), grown in a pot filled with soil mixture (sterilized soil : parlight : barmiqulight = 2 : 2 : 1) and kept in a greenhouse, was used as the host plant. Liquefied fertilizer was applied every 3 days. Cotyledons, 8 to 10 days-old, were used for inoculation.

Determination of virus content in cucumber cotyledons. The concentrations of TMV, CGMMV and CMV in cucumber cotyledons were determined using enzyme-linked immunosorbent assay (ELISA) according to the procedure of Clark and Adams. Antisera to each virus were prepared in rabbits by immunizing them with viruses purified by sucrose density gradient centrifugation. Since TMV-antiserum and CGMMV-antiserum were antigenically related, antisera specific to each virus were prepared by absorption with the other virus. The examined leaves were ground in 9 volumes (w/v) of phosphate buffered saline containing 0.05% Tween 20 (PBS-Tween, pH 7.2). The homogenate was centrifuged at 10,000 × g for 15 min and the supernatant was used for the determination of virus content.

Starch-lesion. Starch-lesions on cucumber cotyledons were determined after the cotyledons had been kept overnight in the dark at 25°C to deplete them of excessive starch. Chlorophyll was removed with 70% ethanol in an 80°C water bath. The starch-lesions were stained by placing the cotyledons in I-KI-lactic acid mixture. After washing them with water, the number of starch-lesions was counted and diameter of each lesion was measured using an optical microscope.

Fluorescent antibody staining of leaf cross sections. Cross sections of cucumber cotyledons were prepared by the paraffin embedding method of Sainte-Marie. The cotyledons were cut into pieces 1 × 1 cm, fixed in 95% ethanol overnight and dehydrated in 100% ethanol for 1 hr at 4°C (4 times). The leaf pieces were soaked in xylene for 2 hr at 4°C (4 times), and embedded in paraffin for 2 hr at 53-57°C (4 times). The paraffin blocks were cut into 10-25 μm thicknesses using a microtome. The sections were mounted on slide glasses smeared with Mager’s albumin, air-dried and stored at 4°C. Cross sections were stained by the indirect immunofluorescence method. The first antibody was the same as those used in ELISA tests, and FITC-labeled anti-rabbit goat IgG (Capell) was used as the second antibody.

RESULTS

Double infection with TMV and CGMMV

When cucumber cotyledons were initially inoculated with TMV on the upper side, and then 2 or 4 days later were reinoculated with CGMMV on the lower side, or were simultaneously inoculated with

<table>
<thead>
<tr>
<th>Sequence of inoculation</th>
<th>Starch-lesion</th>
<th>Virus concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Diameter</td>
</tr>
<tr>
<td>Charged with CGMMV 4 days after TMV</td>
<td>97.4 (%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>95.6 (%)</td>
</tr>
<tr>
<td>Charged with CGMMV 2 days after TMV</td>
<td>96.8</td>
<td>96.0</td>
</tr>
<tr>
<td>Simultaneous inoculation with TMV and CGMMV</td>
<td>86.2</td>
<td>95.3</td>
</tr>
<tr>
<td>Charged with TMV 2 days after CGMMV</td>
<td>79.3</td>
<td>105.2</td>
</tr>
<tr>
<td>Charged with TMV 4 days after CGMMV</td>
<td>37.0</td>
<td>124.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> The diameter and number of starch-lesions as well as TMV and CGMMV contents are expressed as a percentage of that found in control cotyledons (mock-inoculation). Cotyledons were sampled at 144 hr after TMV inoculation. Virus contents were measured using ELISA.
Table 2. Diameter and number of starch-lesions, and concentrations of TMV and CMV in cucumber cotyledons doubly infected with both viruses

<table>
<thead>
<tr>
<th>Sequence of inoculation</th>
<th>Starch-lesion</th>
<th>Virus concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Diameter</td>
</tr>
<tr>
<td>Challenged with CMV 4 days after TMV</td>
<td>87.0 (%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>104.4 (%)</td>
</tr>
<tr>
<td>Challenged with CMV 2 days after TMV</td>
<td>108.3</td>
<td>102.4</td>
</tr>
<tr>
<td>Simultaneous inoculation with TMV and CMV</td>
<td>115.2</td>
<td>98.6</td>
</tr>
<tr>
<td>Challenged with TMV 2 days after CMV</td>
<td>97.4</td>
<td>93.5</td>
</tr>
<tr>
<td>Challenged with TMV 4 days after CMV</td>
<td>36.3</td>
<td>92.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> The diameter and number of starch-lesions as well as TMV and CMV contents are expressed as a percentage of that found in control cotyledons (mock-inoculation). Cotyledons were sampled at 144 hr after TMV inoculation. Virus contents were measured using ELISA.

Table 3. TMV or CGMMV concentration in cucumber cotyledons co-inoculated with ZYMV

<table>
<thead>
<tr>
<th>Sequence of inoculation</th>
<th>Relative virus concentration (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>TMV (%)</th>
<th>CGMMV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Challenged with TMV 4 days after ZYMV</td>
<td>754.2 (%)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Challenged with CGMMV 4 days after ZYMV</td>
<td>—</td>
<td>92.4 (%)</td>
<td>—</td>
</tr>
<tr>
<td>Challenged with TMV 2 days after ZYMV</td>
<td>417.8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Challenged with CGMMV 2 days after ZYMV</td>
<td>—</td>
<td>93.9</td>
<td>—</td>
</tr>
<tr>
<td>Mixed inoculation with TMV and ZYMV</td>
<td>404.8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Simultaneously inoculated with TMV and ZYMV</td>
<td>99.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Simultaneously inoculated with CGMMV and ZYMV</td>
<td>—</td>
<td>92.8</td>
<td>—</td>
</tr>
<tr>
<td>Challenged with ZYMV 2 days after TMV</td>
<td>110.8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Challenged with ZYMV 2 days after CGMMV</td>
<td>—</td>
<td>100.0</td>
<td>—</td>
</tr>
</tbody>
</table>

<sup>a</sup> Relative virus concentration was expressed as a percentage of the amount found in control cotyledons (mock-inoculation). Cotyledons were sampled at 144 hr after TMV or CGMMV inoculation. Virus concentration were measured using ELISA.

these two viruses on the upper side and the lower side, respectively, the amounts of TMV and CGMMV and the diameter and number of starch-lesions were not significantly different compared to those in the cotyledons infected with either of the virus alone.

When cucumber cotyledons were initially inoculated with CGMMV on the lower side, and then 2 or 4 days later were secondarily inoculated with TMV on the upper side, the number of starch-lesions and the amount of TMV decreased, but there was no change in lesion diameter or the amount of CGMMV compared to control (Table 1).

**Double infection with TMV and CMV**

When cucumber cotyledons were initially inoculated with TMV on the upper side, and then 2 or 4 days later were reinoculated with CMV on the lower side, or were simultaneously inoculated with TMV and CMV on the upper and the lower sides, the amounts of the two viruses and the diameter and number of starch-lesions were not significantly different compared to that in the cotyledons infected with only one of the viruses.

When cucumber cotyledons were initially inoculated with CMV on the lower side, and then 2 or 4 days later were reinoculated with TMV on the upper side, the number of starch-lesions and the amount of TMV decreased, but there was no change in the diameter of the lesions or the amount of CMV compared to control (Table 2).

**Double infection with TMV or CGMMV and ZYMV**

In cucumber cotyledons secondarily inoculated with TMV four days after initial inoculation with ZYMV, TMV accumulation increased 7-fold over the control cotyledons inoculated with TMV alone. In cotyledons secondarily inoculated with TMV two days after initial inoculation with ZYMV, or jointly inoculated with the two viruses, TMV concentration also increased about 4-fold in comparison with...
Fig. 1. Effect of ZYMV infection on concentration of TMV in cucumber cotyledons when TMV was inoculated 4 days after ZYMV. (○) Initial mock-inoculation and secondary inoculation with TMV. (●) Initial inoculation with ZYMV and secondary inoculation with TMV. TMV concentration was measured using ELISA.

TMV inoculation alone. In these cotyledons, there was no change in the diameter of starch-lesions although they decreased in number. However, when cotyledons were simultaneous inoculated with TMV and ZYMV on the upper and the lower side, or were initially inoculated with TMV and then 2 or more days later were reinoculated with ZYMV, no difference in TMV accumulation was observed (Table 3).

The time course of TMV accumulation was examined in cucumber cotyledons challenge-inoculated with TMV 4 days after ZYMV inoculation. The accumulation curve in the challenge-inoculated cotyledon began to increase rapidly 24 hr after TMV inoculation compared to that of the control cotyledons infected with TMV alone. Thereafter, although TMV concentration in the latter cotyledons reached its maximum level 48 hr after its inoculation, the concentration in the former cotyledons continued to increase rapidly until 72 hr after TMV inoculation (Fig. 1).

Double infection of CGMMV with ZYMV had no effect on the concentration of CGMMV (Table 3).

**Distribution of TMV in tissue co-infected with ZYMV**

TMV distribution was examined using the immunofluorescent antibody technique in cotyledon tissues in which the TMV amount was increased due to double infection with ZYMV. Its distribution in the cotyledons initially inoculated with ZYMV and secondarily reinoculated with TMV was more remarkably spread in horizontal and vertical directions than that was found when TMV was inoculated alone following mock-inoculation with a phosphate buffer, or when initially inoculated with TMV and secondarily inoculated with ZYMV. TMV localization was eventually established and no systemic distribution of TMV was observed (Plate I-B, C).

The relationship between TMV-infected areas and starch-lesions was investigated by iodine staining the cross-section previously stained with a fluorescent antibody. All areas where TMV was tightly localized were stained dark brown by the iodine (Plate I-D). However, when TMV was widespread because of the initial inoculation with ZYMV, no iodin-stained areas were seen (Plate I-E, F).

**DISCUSSION**

Wu and his co-workers reported that the number of starch-lesions formed by TMV infection in cucumber leaves reduced when there was previous infection with CGMMV\(^{15}\). They speculated that the reason for this was either the inhibition of starch-lesion formation on CGMMV-infected leaves, or a
decrease in infectivity because of aging of the leaves caused by the infection. In these experiments, when cotyledons infected with CGMMV were reinoculated with TMV, the amount of TMV and the number of starch-lesions were reduced. The same results were obtained in co-inoculation of CMV and TMV. Even in secondarily inoculated with TMV after initial mock-inoculation with a phosphate buffer, the number of starch-lesions and the amount of TMV decreased. In cotyledons in which lesion number decreased, the diameter of the starch-lesions formed was the same. Thus, the changes in the physiological state of cotyledons initially inoculated with CGMMV or CMV is believed to involve reduction of the infection sites of TMV, rather than inhibition of multiplication and spread of TMV in the infected tissues.

In contrast, when cucumber cotyledons were initially inoculated with TMV and secondarily with CGMMV or CMV, virus content was similar to that in cotyledons infected with each virus alone. The zones around necrotic local lesions produced by the viruses are usually resistant to challenge inoculation (localized acquired resistance). This resistance is found against not only initially inoculated virus but several other viruses, demonstrating a lack of specificity. Findings from these experiments were that there was no reduction in accumulation of the challenge-inoculated virus when cotyledons were initially inoculated with TMV and subsequently with CGMMV or CMV. Therefore, although the resistance can block the advance of TMV infection, it is unlikely that the resistance is induced in zones around the TMV-infected areas.

There are many examples of the transport-deficient viruses in plants being complemented by another related or unrelated virus. The spreading virus has been suggested to provide the movement protein to the transport-deficient virus, thus facilitating its movement. In these experiments, TMV was not shown to spread systemically in cucumber cotyledons by double inoculation with CGMMV, a member of the same tobamovirus that TMV belongs to, or with CMV (an unrelated virus). The multiplication rate and distribution in cucumber cotyledons were enhanced, however, in the presence of ZYMV; but localization of TMV was ultimately established and there was no systemic infection. With ZYMV reinoculation following final localization of TMV (2 days or more after TMV-inoculation) this enhancement of TMV did not occur. It is thus unlikely that inactivation of TMV-coded movement protein is responsible for TMV localization in the cotyledons. In summary, cucumber cotyledon resistance to TMV is not preexisting, but is induced by some response of the plant after infection, and this response is delayed by double infection with ZYMV. This assumption coincides with previous reports on cucumber cotyledon treated with actinomycin D.

In the previous paper, we reported that the accumulation of TMV in cucumber cotyledons continued to increase for some period after localization of TMV (starch-lesions) has been established. This response may thus indicate an obstruction of the transportation of TMV around TMV infection sites.

Literature cited
Plate I

Explanation of plate

Plate I
A-C: Cross sections of cucumber cotyledons stained with fluorescent antibody. Observation was made with a fluorescence microscope. Areas containing virus antigen (TMV) show yellow-green fluorescence.
D-F: Cross sections of cucumber cotyledons stained with iodine stain after staining with fluorescent antibody. Observation was done using an optical microscope.
A and D are the same cross sections of cucumber cotyledons initially mock-inoculated and secondarily inoculated with TMV. B, C, E and F are cross sections of cucumber cotyledons inoculated with TMV 4 days following ZYMV inoculated. B and E, C and F are the same cross sections.