Localization of Tobacco Mosaic Virus in Tobacco Callus Tissue

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Abstract

Tobacco callus was raised from a stem of tobacco mosaic virus (TMV)-infected tobacco plant (cv. Bright Yellow). By means of successive transfers of green colored compact peripheral tissue, intermediate tissue between green and translucent tissue, and translucent soft inner tissue of the callus respectively on Murashige and Skoog's medium, the following 3 types were developed. Callus A; dark green colored callus composed of compactly arranged small cells. Callus B; green colored callus composed of compact peripheral tissue and translucent inner tissue. Callus C; translucent and soft callus composed of loosely arranged large cells.

TMV concentration in tobacco callus declined by repeated transfers of translucent soft tissue, while it was maintained at a high level when green colored compact tissue was successively transferred. The distribution of TMV in relation to cell arrangement in the tobacco callus was investigated by means of fluorescent antibody technique. TMV antigen in tobacco callus was unevenly distributed, that is, it was frequently localized in compactly arranged small cells around tracheid-like cells, whereas it was rarely observed in soft tissue where large cells were loosely arranged. The decline of TMV concentration in the translucent soft callus was thought to be due to the rapid propagation of healthy cells escaped the virus infection.

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Introduction

Kassanis4) reported that tobacco mosaic virus (TMV) concentration in cultured tumorous tissues remained constant for many years, and Raychaudhuri and Mishra8) also described similar results with chilli mosaic virus. These results suggest that the virus concentration will be maintained at a constant level for many years under some suitable conditions. On the other hand, some results suggesting uneven distribution of TMV in callus tissues have been reported. Hildebrandt and Riker3) reported that among clones originated from single TMV-infected callus some lost the virus completely during successive transfers while others maintained it at a high level. Chandra and Hildebrandt1) and Hansen2) also confirmed the same phenomenon. Thus, it is well known that large differences in TMV concentration arise among tobacco callus tissues during successive transfers even if they were raised from the same tobacco plant infected systemically. Reinert9) reported that the change of virus activity in callus tissues was different with the kinds of virus, viz., some viruses were maintained for long period but others declined rapidly.

The explanation for the mechanisms of the virus decline in callus tissues is lacking. To approach this problem, the distribution of TMV in relation to cell

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arrangement in the different types of callus tissues was investigated by means of fluorescent antibody technique. TMV concentration in each type of callus tissue was also bioassayed periodically.

**Materials and Methods**

*Tobacco callus tissue.* Murashige and Skoog\(^7\) medium (free from Edamin, added with 20 g/l of sucrose and 6 g/l of agar) supplied with 1 mg/l of *a*-naphthalene acetic acid (NAA) and 0.2 mg/l of kinetin was used for tissue culture. The pH was adjusted with either 1 N NaOH or 1 N HCl to 5.7-5.8. Thirty ml of the medium was allotted to each 100 ml Erlenmyer's flask, and was autoclaved at 120 C for 10 min. Tobacco callus was raised from a TMV-infected tobacco (cv. Bright Yellow) stem and was cultured for about 50 generations (each generation: 30 days) on the medium under 3,000 lux of the continuous white fluorescent light at 25 C. About 100 mg of the tissue taken aseptically was used for every transfer. The following three stocks (Fig. 2) were developed from the original callus by successive transfers as shown in Fig. 1. Callus A: Dark green colored callus composed of compact peripheral tissue and moderately compact inner tissue, which was produced by repeated transfers of green colored peripheral tissue of the callus. Callus B: Green colored callus composed of compact peripheral tissue and translucent, soft inner tissue, which was produced by successive transfers of the intermediate tissue between the green peripheral and translucent inner tissues. Callus C: Translucent, soft and friable callus which was produced by repeated transfers of the translucent tissue of the callus.

Infectivity assay. *Nicotiana glutinosa* was used for bioassay of TMV concentration in the callus. The plants grown in 12 cm pots in a greenhouse for 60-80 days were moved to a 25 C air-conditioned greenhouse a week before inoculation, and thier upper and lower leaves were cut off three days before inoculation, leaving four fully expanded leaves. Unless otherwise mentioned, a small piece taken from
the peripheral tissue of each callus was weighed and homogenized with mortar and pestle, and was suspended in appropriate volume of distilled water. In the case of the peripheral tissue of callus A and B, 500-fold (W/V) diluted suspension was commonly used as inoculum. Inoculation was made by cotton swabs on half leaves of *N. glutinosa* dusted with 400 mesh carborandum. Purified TMV, the concentration of which was expected to produce 50–150 local lesions on a half-leaf, was simultaneously inoculated on the opposite half-leaves as a control. Sixteen leaves were used for each sample. Local lesions were counted three days after inoculation. All the experiments were repeated at least three times.

**Fluorescent antibody method.** The anti-TMV serum was prepared from a rabbit given an intramuscular injection of 50 mg TMV emulsified with Freund's incomplete adjuvant and an intravenous injection of 10 mg TMV a month later. The antiserum showed the titer of 1/512 in precipitin tube test. Immunoglobulin G was obtained from the antiserum by ammonium sulfate precipitation. Conjugation of the protein with fluorescein isothiocyanate (FITC) was made by the method of Marshall *et al.*\(^5\), except that FITC added was 1% of the protein and agitation was 4 hr. The mixture was passed through Sephadex G-25 column to separate conjugated globulin from uncoupled dye. The conjugate was then fractionated through diethyl-amino-ethyl (DEAE) cellulose column. The fraction which was estimated to be 1.5 F/P molar ratio by optical density at 280 and 495 nm was used. The callus sections (20 μm thick) prepared by cryostat were dried on a glass slide which had been smeared with Mayer's albumen and covered with a drop of the globulin-FITC conjugated solution. The section was incubated for an hour in a moist chamber at 37°C. The sections were then washed several times in PBS (0.01 M phosphate buffer, pH 7.0, containing 0.85% NaCl) and mounted with buffered glycerin.

Stained specimen were observed under a light-microscope (TIYODA FM-200 A). To distinguish the fluorescence by TMV antibody-FITC conjugate from natural one, sections from healthy callus tissues were also treated with the conjugate as a control.

**Results**

**Growth of callus tissue**

Generally, the callus tissues grew slowly at the beginning, rapidly between 10th and 30th day and again slowly afterwards. The mean fresh weights of ten callus tissues at 30th day after the transfer were 3.9 g in callus A, 7.5 g in callus B and 10.6 g in callus C. Water contents of these callus tissues at the same day were 91.0% in the peripheral tissue of callus A, 93.6% and 97.7% respectively in the peripheral and inner tissues of callus B, and 97.9% in callus C.

**Histological observations**

Frozen sections of each callus tissue were prepared by cryostat. Most areas in the peripheral tissue of callus A were composed of compactly arranged small cells and many tracheid-like cells (Fig. 3). In the peripheral tissue of callus B, various cells different in size were observed, that is, some areas were composed of compactly arranged small cells and others loosely arranged large cells (Fig. 4). Most areas in the inner tissue of callus B were composed of loosely arranged large cells (Fig. 5). The tissue of callus C was composed of large cells showing little cell associations (Fig. 5). Modes of cell length of the small (Fig. 3) and large cell (Fig. 5) groups, when calculated by the formula;

\[
L = \sqrt{\text{length of long axis} \times \text{length of short axis}}
\]
Fig. 2. Different types of tobacco callus. From left to right, callus A, B, and C, respectively. The upper row shows external appearance of each callus. The lower row shows internal appearance of each callus.

Fig. 3. Section of peripheral tissue of callus A. Scale indicates 500 μm.

Fig. 4. Section of peripheral tissue of callus B. Scale indicates 500 μm.

Fig. 5. Section of callus C. Scale indicates 500 μm.

were 20 μm and 95 μm, respectively.

**TMV concentration in callus tissues**

As shown in Table 1, TMV concentration in the peripheral tissue of callus A was about 1.5 fold of that in the peripheral tissue of callus B. In callus B, TMV concentration was always higher in the peripheral tissue than in the inner tissue. TMV was not detected in callus C even when undiluted sap was inoculated.

*Decline of TMV concentration by serial transfers of translucent inner tissue of the callus*

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>Stock</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>Peripheral tissue</td>
</tr>
<tr>
<td>1</td>
<td>138&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>143</td>
</tr>
<tr>
<td>3</td>
<td>183</td>
</tr>
</tbody>
</table>

| a) Figures indicate relative concentrations of TMV. |
| b) Number of lesions produced on 16 half-leaves. |
| c) Crude undiluted sap was used as inoculum. |
Since significant difference in TMV concentration was found between peripheral and inner tissues of callus B, different tissues about 100 mg each were transferred successively (Fig. 1) and TMV concentrations were compared at the end of each generation to see if the difference of TMV concentration was amplified or to see how long does it take to get virus-free callus from TMV infected callus. With repeated transfers of the inner tissue, translucent tissue gradually became predominant in the callus, and the whole body was occupied by the translucent and friable tissue at the end of the 6th generation. Growth of the translucent callus was about two times greater than that of the green callus. As shown in Table 2, TMV concentration in the callus grown by serial transfers of the translucent tissue was high at the beginning, but it markedly decreased and could not be detected at the end of the 6th generation. When peripheral green tissue was used for every transfer, TMV was maintained till the 6th generation. Histological observation of the TMV diminished translucent callus at the end of the 6th generation revealed that whole part of the callus was composed of loosely arranged large cells (Fig. 5).

Table 2. Decline of TMV concentration in tobacco callus by repeated transfers of translucent inner tissue of callus B

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>No. of transfer&lt;sup&gt;a&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>100&lt;sup&gt;b&lt;/sup&gt; (3439)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>100 (4614)</td>
</tr>
<tr>
<td>3</td>
<td>100 (5176)</td>
</tr>
</tbody>
</table>

<sup>a</sup>) Cultures were transferred every 30 days.
<sup>b</sup>) Each figure indicates relative concentration of TMV.
<sup>c</sup>) Number of lesions produced on 16 half-leaves.

TMV-free callus was also developed from callus A in the same manner, but 2–4 more generations were needed in this case. As shown in Table 3, almost similar results were obtained with newly raised callus tissues.

Table 3. Decline of TMV concentration in newly raised callus tissues by repeated transfers of translucent tissue

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>No. of transfer&lt;sup&gt;a&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Translucent tissue</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Green tissue</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
</tr>
</tbody>
</table>

<sup>a</sup>) Cultures were transferred every 30 days.
<sup>b</sup>) Figures indicate relative TMV concentrations.
<sup>c</sup>) Number of lesions produced on 16 half-leaves.

**Fluorescent antibody staining**

Green or yellow green fluorscense caused by TMV-antiserum-FITC conjugate showing the presence of TMV antigen was often observed in the peripheral tissue.
of both callus A and B, but no such fluorescence was observed in callus C and healthy control. Tracheid-like cells showed light blue auto-fluorescence, and chlorophyll red one.

TMV antigen was unevenly distributed and large masses of cells showing FITC specific fluorescence was observed mainly in the compactly arranged small cell areas adjacent to the tracheid-like cells (Fig. 6A). Even in the large cells, FITC specific fluorescence was occasionally detected when they were located adjacent to compactly

Fig. 6. A: Fluorescent antibody reaction in peripheral tissue of callus B. Scale indicates 200 μm. B. Fluorescent antibody reaction in inner tissue of callus B. Scale indicates 200 μm.
Dots in the figures express the specific fluorescence. Strength of the specific fluorescence was expressed by dot density. Central part of each cell in compact tissue showed strong fluorescence, but dots were reduced in number to show cell arrangement. Tracheid-like cells showing non-specific blue fluorescence are shown by thick line.
arranged small cells containing TMV. The antigen was rarely seen in the isolated large cells (Fig. 6B). Most of the small cell clusters were infected even if they were completely separated from each other among loosely arranged large cells. TMV concentration was compared between the small cell clusters and large cell tissues in callus B. As shown in Table 4, infectivity was always higher in the small cell clusters than in the large cell tissues, supporting the results obtained by fluorescent antibody method.

Table 4. TMV concentrations in small cell clusters isolated from translucent inner part of callus B

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>Translucent inner part</th>
<th>Green peripheral part</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Small cell cluster</td>
<td>Large cell tissue</td>
</tr>
<tr>
<td>1</td>
<td>100*(2) (2468)*</td>
<td>27</td>
</tr>
<tr>
<td>2</td>
<td>100 (1901)</td>
<td>39</td>
</tr>
<tr>
<td>3</td>
<td>100 (6026)</td>
<td>26</td>
</tr>
<tr>
<td>4</td>
<td>100 (1716)</td>
<td>43</td>
</tr>
<tr>
<td>5</td>
<td>100 (1310)</td>
<td>87</td>
</tr>
</tbody>
</table>

a) Figures indicate relative concentrations of TMV.
b) Number of lesions produced on 16 half-leaves.

**Discussion**

Mori *et al.* observed the localized distribution of TMV antigen in callus tissue by fluorescent antibody method, but they made no mention of its relation to cell arrangement. Fluorescent antibody experiments carried out in parallel with histological observations (Fig. 3, 4, 5) and biological assays (Table 1, 4) showed that TMV concentration was high in the green colored compact callus consisting of compactly arranged small cells, while it was low in the translucent soft callus consisting of loosely arranged large cells. TMV disappeared from the callus tissue when loose tissue was monthly transferred for several generations (Table 2). By the fluorescent antibody method it was shown that TMV antigen was frequently localized in the compactly arranged small cells around tracheid-like cells (Fig. 6A), while it rarely found in the loosely arranged large cells (Fig. 6B). The results mentioned above support the idea that TMV may move readily through vascular element, and from cell to cell, in the compact callus tissue. In the loose and friable callus, however, the movement of TMV seems to be difficult, because vascular elements were not differentiated and the cells are less associated each other. The disappearance of TMV in the callus tissue composed of loosely arranged large cells is probably due to rapid propagation of TMV-free cells which has escaped from the virus infection in TMV-infected callus.

As is well known, callus tissue is composed of heterogeneous cells. The results presented here clearly showed that the virus concentration in callus tissue closely related to tissue structure or cell arrangement. Furthermore, the structure and cell arrangement in callus tissues were shown to change easily by cultural conditions. Some discrepancies found in the previous reports might be explained by localized distribution of virus concerned. Why virus-free cells exist in the systematically infected tissues, and how virus-free cells appear in the virus-infected callus tissues are fundamental problems which should be made clear in future.
The presence of virus-free tissues in systemically infected plants is very important from practical points of view. Virus-free plants have been grown from systemically infected plants by means of the meristem tip culture. The results presented here suggest that virus-free callus tissue developed by means of successive transfers will be a suitable material for producing virus-free plants in future. In other words, virus-free plants may be produced in mass scale from virus-free friable callus tissue.

The author wishes to express his most sincere thanks and appreciation to ex-Professor Dr. Z. Hidaka, and Professor Dr. S. Wakimoto, Kyushu University, for their continuing encouragement and guidance.

Literature cited


和文摘要

タバコのカルス組織におけるタバコモザイクウイルスの局在性

大村敏博

タバコモザイクウイルス（TMV）に罹病したタバコ（ブライトエロー）の茎から起こした培養組織をMurashige and Skoogの培地を用い、緑色で黒い表面組織、表面と内部の中間部分および透明で軟かい内部をそれぞれ独立培養することにより下記の3種類のカルスを得た。カルスA，密に接続した小細胞からなる緑色のカルス。カルスB，緑色で黒い表層組織と透明で軟かい内部の組織からなるカルス。カルスC，ルーズな大細胞からなる透明で軟かいカルス。

カルス中のTMV濃度は透明で軟かい内部を継代培養すると低下したが、緑色で黒い表面の組織を継代したときは高濃度に保たれた。カルス中のTMVの分布と組織の状態との関係を蛍光抗体法を用いて観察した結果、カルス中のTMV抗原は偏在しており、導管様細胞のまわりの密に接続した小細胞の集団にしばしば分布していたが、ルーズな大細胞からなる組織にはあまり認められなかった。透明で軟かいカルス組織を継代することによってTMV濃度が低下する原因はウイルス感染からまぬかれた健全な細胞が急激に増殖するためであろうと考えられる。