

Isolation of Virus-Free Protoplasts from TMV-Infected Tobacco Leaves, and Their Susceptibility to Reinoculation with TMV

Sadao KOBAYASHI*, Masahiko MIYAHARA**, Daijiro
HOSOKAWA***, Minoru WATANABE*** and
Takuji KOZAKA***

小林貞夫*・宮原真彦**・細川大二郎***・渡辺 実***・高坂渾爾*** : TMV 感染
タバコ葉からのウイルスフリー・プロトプラストの分離とその TMV 感受性

Mosaic symptoms on tobacco mosaic virus (TMV)-infected tobacco leaves consist of dark-green and light-green areas. Infectivity tests and electron microscopy so far reported have shown that TMV concentration is high in the light-green areas and low in the dark-green areas^{1,2,3,4,5}. It was also reported that the dark-green areas were resistant to re-inoculation with TMV^{3,5}. These results suggest that certain cells are infected with TMV whereas others contain little or no virus in the dark-green areas. In this study we attempted to isolate virus-free protoplasts from the dark-green areas, and to reinoculate them with TMV.

Xanthi tobacco seedlings of 5-6 leaf stage were inoculated with TMV (OM strain), and 3-6 weeks after the inoculation protoplasts were prepared from systemically infected leaves by the method of Kassanis and White⁶ with slight modifications. After peeling off the lower epidermis with a forceps, dark-green and light-green areas were cut out separately from the stripped leaves with a razor blade. These leaf pieces (ca. 1 g) were immersed, stripped side down, in a solution (10 ml) containing 0.5 M mannitol, 0.4 % (w/v) Macerozyme R-10 and 1.2 % (w/v) Cellulase Onozuka R-10, pH 5.6, and incubated at 25 C for 4-9 hr, occasionally with adequate shaking. Protoplasts thus obtained were washed with 0.5 M then with 0.6 M and finally with 0.7 M mannitol solution by centrifugation at $80 \times g$ for 3 min. The percentages of living protoplasts were between 70 % and 80 % in case of dark-green areas or healthy leaves, while between 65 % and 75 % in case of light-green areas. These protoplasts were inoculated with TMV by the method of Takebe and Otsuki⁷. Purified TMV was dissolved to 4 μ g/ml in 0.02 M potassium

* Present address: Faculty of Agriculture, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan
現在: 東京大学農学部

** Present address: Hokko Kagaku Kogyo Co., Ltd., Atsugi, Kanagawa 243, Japan 現在: 北興
化学工業開発研究所

*** Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183,
Japan 東京農工大学農学部

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citrate buffer, pH 5.2, containing 0.7 M mannitol and $12 \mu\text{g/ml}$ poly-L-ornithine. After the TMV solution was kept at 25 C for 20 min, it was mixed with an equal volume of the protoplast suspension, and then maintained at 25 C for 20 min. Then the protoplasts were washed three times in 0.7 M mannitol solution containing 0.1 mM CaCl_2 by centrifugation at $80 \times g$ for 3 min, and transferred into the incubation medium as described by Aoki and Takebe⁸⁾. Fluorescent antibodies were prepared as follows. Immuno- γ -globulin was isolated from a rabbit antiserum against TMV (titer 1/1024), and conjugated with fluorescein isothiocyanate, and then passed through a DEAE cellulose column (final titer 1/256). F/P molar ratio was between 1 and 2. The percentages of TMV-infected protoplasts were determined using fluorescent antibody technique. The stained preparations were examined under a Nikon fluorescence microscope.

The percentages of TMV-infected protoplasts isolated from dark-green and light-green areas in leaves at three different developmental stages were 18–26 % and 64–71 %, respectively (Table 1). This result indicates that most cells in dark-green areas contained little or no virus while majority of cells in light-green areas accumulated considerable amounts of virus at any observed stage of leaf development.

The following two experiments were conducted to know if the cells in the dark-green areas were resistant to reinoculation with TMV. The protoplasts isolated from dark-green and light-green areas were reinoculated with the same strain of TMV, and incubated for 72 hr at 28 C under continuous illumination by electric light (3000 lux). After the reinoculation, the percentage of TMV-infected protoplasts decreased slightly in the protoplasts from light-green areas because of the death of some protoplasts during the incubation. On the other hand, the percentage of TMV-infected protoplasts increased from 20 to 70 % in those from dark-green areas (Table 2). This result suggests that not less than 50 % cells remain virus-free in the dark-green areas, and that these virus-free cells are susceptible to TMV reinoculation. In the second experiment, half-leaves of TMV-infected tobacco leaves were reinoculated with a high concentration ($100 \mu\text{g/ml}$) of the same strain of TMV. The

Table 1. Percentage of TMV-infection in protoplasts isolated from dark-green and light-green areas in TMV-infected tobacco leaves

Expt. no.	Leaves for protoplast isolation	Percentage of TMV-infection ^{a)} in protoplasts from	
		Dark-green areas	Light-green areas
1	Upper leaves ^{b)}	21.6	71.2
	Middle leaves ^{c)}	19.1	69.1
	Lower leaves ^{d)}	21.1	67.5
2	Upper leaves	17.8	65.5
	Middle leaves	26.2	67.5
	Lower leaves	24.1	63.7

a) Percentage of TMV-infected protoplasts was determined by fluorescent antibody technique.

b) Young developing leaves.

c) Fully expanded leaves.

d) Matured old leaves.

Table 2. Percentage of TMV-infection before and after reinoculation with TMV in protoplasts isolated from dark-green and light-green areas in TMV-infected tobacco leaves

Protoplasts isolated from		Inoculation to protoplasts	Percentage of TMV-infected protoplasts	
Leaves	Areas		Before reinoculation	72 hr after reinoculation
TMV-infected leaves	Dark-green	Reinoculated with TMV	22.5	69.9
		Not reinoculated	—	19.0
	Light-green	Reinoculated with TMV	80.7	68.4
		Not reinoculated	—	70.0
Healthy leaves		Inoculated with TMV	0	55.3

Table 3. Percentage of TMV-infection in protoplasts isolated from dark-green and light-green areas in TMV-infected tobacco leaves after reinoculation with TMV

Protoplasts isolated from		Percentage of TMV-infected protoplasts ^{b)}
Leaves	Areas	
TMV-infected leaves reinoculated with TMV	Dark-green	32.6
	Light-green	75.4
TMV-infected leaves reinoculated with PB ^{a)}	Dark-green	29.0
	Light-green	70.0
Healthy tobacco leaves inoculated with TMV		54.2

a) 0.1 M phosphate buffer, pH 7.0.

b) Isolated 5 days after reinoculation.

opposite half-leaves were rubbed with 0.1 M phosphate buffer (pH 7.0). Five days after reinoculation, protoplasts were isolated from dark-green and light-green areas and the percentages of TMV-infection were determined. As a result, the percentages of TMV-infection showed no remarkable increase in dark-green areas as well as in light-green areas by the reinoculation (Table 3). The percentage of fluorescing protoplasts from the dark-green areas was less than 35 %, while more than 50 % cells became infected in leaves newly inoculated with TMV.

From these results it is concluded that not a few cells in the dark-green areas are virus-free and these cells seem to be resistant to reinoculation with TMV, but when isolated as protoplasts, they become susceptible to the virus. On the basis of the observation that tobacco plantlets regenerated from the dark-green areas showed a transitory resistance to TMV-infection, Murakishi and Carlson⁹⁾ suggested the presence of an inhibitory factor in these plantlets. Föglein *et al.*¹⁰⁾ reported that TMV-RNA synthesis was renewed in the protoplasts isolated from TMV-infected leaves of Xanthi tobacco in which virus synthesis

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had already slowed down or stopped. These findings may suggest a way of investigating the mechanism by which the TMV-resistant cells in the dark-green areas become susceptible when they are isolated as protoplasts.

The authors are indebted to Dr. K. Yora and Dr. Y. Doi, University of Tokyo, for helpful discussion of the results and proofreading.

(Received April 11, 1980)