

Multiplication and Translocation of Tobacco Mosaic Virus Microinjected into Callus Cells of TMV-resistant Line CMT-IR03 Selected from Tobacco Tissue Culture

Hideyoshi TOYODA*, Yoshinori MATSUDA**, Kazuyuki CHATANI***
and Seiji OUCHI*

One of our objectives in plant biotechnology is to select useful genetic variations, so-called somaclonal variation¹⁾, induced in plant tissue cultures for improving important agricultural traits such as disease and insect resistance to breed new cultivars of major crop plants^{2,3)}. In our previous work, we selected the tobacco mosaic virus (TMV)-resistant line (CMT-IR03) from axillary bud-derived calli of tobacco plants and succeeded in regenerating intact plants resistant to TMV⁴⁾. The genetic analysis of the resistant line indicated that the resistance was dominantly and heterozygously induced during callus cultures⁴⁾. However, the molecular mechanism for the resistance of this line has not been elucidated. In our laboratory, some techniques for callus culture and microinjection have been improved for cytological analysis of the resistance expression in host cells against viral infection⁵⁾. The progress of these investigations enabled us to examine multiplication and cell-to-cell movement of the virus by directly introducing viral particles into single cells⁶⁾ of cell-aggregates^{7,8)} of callus tissues of host plants. With these newly developed methods, the present study was designed to analyze the resistance expression in callus cells derived from the TMV-resistant CMT-IR03 line.

TMV (strain OM) was purified from systemically infected tobacco leaves (*Nicotiana tabacum* cv. Bright Yellow) by four cycles of differential centrifugation (10,000 × g, for 20 min. and 100,000 × g, for 90 min.)⁹⁾, and the resultant pellet of TMV was dissolved in 0.01 M phosphate buffer (pH 6.7) and stored at -20°C. An inoculum solution was prepared to contain 0.1 µg TMV/µl in 0.01 M phosphate buffer and sterilized through membrane filtration before use.

Friable callus tissues were induced from axillary buds of the CMT-IR03 line and the parental cultivar Bright Yellow, according to the method described previously⁹⁾. Specifically, axillary buds were isolated from the plants five to six weeks after sowing, surface-sterilized with 70% EtOH and 1% sodium hypochlorite, and rinsed with sterile water. The tissues were placed on a medium (MS) of Murashige and Skoog¹⁰⁾ supplemented with 0.5 µg/ml 2,4-D and 0.01 µg/ml kinetin, adjusted to pH 5.6, and solidified with 0.8% agar. The cultures were incubated at 26°C under continuous illumination of 4,000 lux. The callus that proliferated from axillary buds was subcultured at intervals of 2 weeks for three months. Then the friable callus tissues obtained were suspended in a liquid MS medium (without agar) and dissociated into single cells or cell aggregates by gentle shaking (80 strokes/min.). Single cells and cell-aggregates were separated by filtration with a stainless steel sieve of different pore sizes and embedded into an agar-solidified MS medium, using the plate culture system reported previously^{8,11)}. For microinjection of callus cells, a culture

* Faculty of Agriculture and **Institute for Comprehensive Agricultural Science, Kinki University, 3327-204 Nakamachi, Nara 631, Japan

*** Present address: Plant Biotech Division, Daiichi Engei Plantech, Shizuoka 410-13, Japan

plate was constructed; the flat central area was surrounded by the ring agar bed and overlaid by a cover solution (MS medium without agar). The flat central area was prepared by pouring a mixture of 1 ml each of cell suspension ($2-3 \times 10^3$ cells/ml) and melted MS agar medium (1.6%) into the central hole of the ring bed. The location of cells embedded in a central flat area was recorded on the basis of the grid lines scored at the bottom surface of the Petri dish. Callus cells were pre-incubated for 2 days and the cells showing an active cytoplasmic streaming (CS) were selected as target for microinjection of TMV. The injection of tobacco callus cells was conducted under a stream of sterile air with the aid of an Olympus Injectoscope IMT-YF II, a phase contrast microscope for injection. The injection pipette containing an inoculum solution was inserted into cytoplasm of target cells and kept for 10 sec. The inoculum solution was pushed out by pressing silicon oil in a tube linked with the pipette. For the injection of cell-aggregates, the cell positioned at the edge of the target aggregate was injected and other constituent cells were kept uninjected. These cells were incubated for 4 days, and all of constituent cells were re-injected with fluorescein isothiocyanate (FITC)-conjugated anti-TMV antibody by a pricking microinjection method described previously⁸⁾.

As pointed out in the previous paper⁵⁾, the present method for microinjection enabled us to precisely introduce viral particles into target callus cells and to easily check the survival of injected cells by observing cytoplasmic streaming. In the present study, the survival rates were considerably high, more than 80% in both the CMT-IR03 and control parent lines (Fig. 1-A). After the survival of TMV-injected cells was carefully checked, only the cells maintaining CS were regarded as successfully injected ones and used for the following fluorescent antibody staining. Fig. 1-B shows the multiplication of TMV injected into single callus cells derived from CMT-IR03 and control tobacco plants. The present study indicated that almost all (more than 90%) of successfully injected cells were positively stained with fluorescent antibody, and that there was no difference in the rates of viral multiplication between the calli of CMT-IR03 and control parental line. These results suggest that the CMT-IR03 line is not equipped to suppress the viral multiplication *per se*

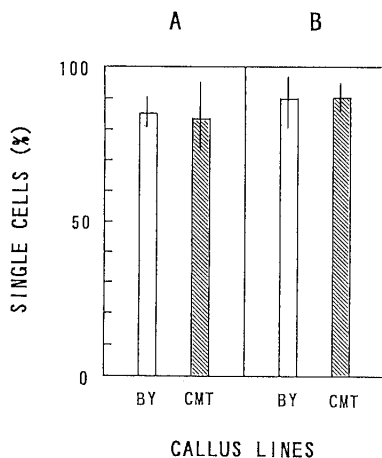


Fig. 1 Survival rates of TMV-injected single callus cells of the TMV-resistant line (CMT-IR03) and parental tobacco plants (BY) (A) and multiplication of TMV in injected cells (B). Single callus cells were embedded in an agar-solidified MS medium, microinjected with TMV and cultured for 4 days. Before fixation, the maintenance of cytoplasmic streaming in the cells was microscopically examined to determine their survival rates. The fixed cells were stained with a FITC-labelled anti-TMV antibody. The data were given as means of five separate experiments.

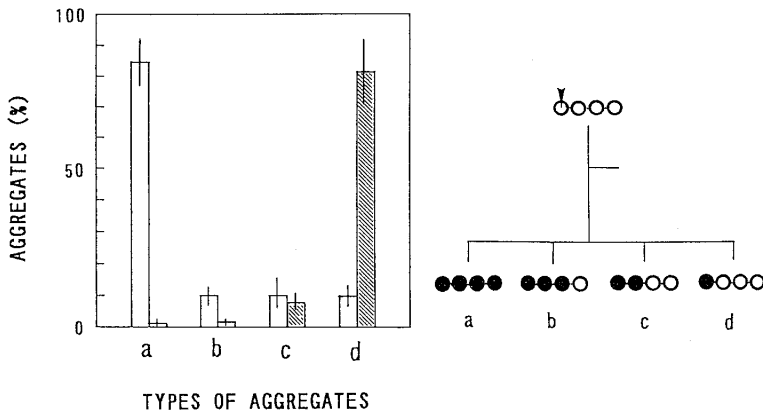


Fig. 2 Multiplication of TMV microinjected into aggregate-constituent cells of calli derived from the TMV-resistant line (CMT-IR03) (shaded column) and parental tobacco plants (open).

Four-cell-aggregates were embedded in an agar-solidified MS medium and their outmost constituent cells were injected with TMV and incubated for 4 days. The aggregates whose constituent cells maintained cytoplasmic streaming were stained with the FITC-labelled anti-TMV antibody. The aggregates were classified into 4 types (a-d) on the basis of fluorescence appearance in the constituent cells. Arrow represents the cell microinjected with TMV and closed circle shows the cells positively stained with the fluorescent antibody. The data were given as means of five separate replications.

in the callus cells into which TMV was directly introduced. In the following experiment, therefore, we have examined whether the movement or translocation of virus could possibly be suppressed in this resistant line.

Our previous work demonstrated that TMV stably multiplied in callus tissues obtained from systemically infected tobacco plants⁹⁾. In addition, the virus microinjected into cells of tomato callus aggregates was found to translocate easily to non-injected adjacent cells of the aggregates⁸⁾. With this microinjection method, we examined the resistance appearance in callus aggregates of tomato cultivar ('Zuiko') carrying Tm-2^a gene¹²⁾ for suppression of viral translocation, and indicated that this cultivar in fact inhibited the movement of TMV among aggregate-constituent cells⁷⁾. Thus, callus cell-aggregates were found to be useful material for cytologically analyzing the viral movement in a multicellular system of higher plants. In the present study, TMV was microinjected into the cell located at the edge of 4 cell-aggregate (**Fig. 2**), implying that the positive staining of non-injected adjacent cells definitely indicates the translocation and multiplication of the virus. After 4 days of injection, the aggregates were examined for their survival rates by checking CS in all constituent cells. In this experiment, the survival rates of the aggregates were considerably high in both lines (84.2 ± 0.3 and $80.8 \pm 3.3\%$ in the CMT-IR03 and the parental line, respectively). These living aggregates were selected and reinjected into all constituents of the aggregates with FITC-labelled antibody. As shown in **Fig. 2**, TMV-injected aggregates were classified into four types (a-d) on the basis of fluorescence detection in their constituent cells.

First, the viral multiplication was investigated among injected cells of each aggregate. The rates were 80.1 ± 2.2 and $84.2 \pm 0.3\%$ in the CMT-IR03 and parental calli, respectively, and comparable to the multiplication efficiency in TMV-injected single cells as discussed above. These results support the previous inference that the CMT-IR03 calli would not suppress the multiplication of virus which had been introduced into the host cells. On the other hand, a significant difference was detected in the frequency of fluorescence staining in constituent cells of the aggre-

gates between two lines. In callus aggregates of control parent line, more than 80% of them showed the fluorescence in all of the constituent cells (type-a). On the contrary, the majority of the aggregates of CMT-IR03 line showed the type-d, indicating that there was no multiplication in most of non-injected adjacent constituent cells of the aggregates (**Fig. 2**). Since the survivability of these constituent cells were confirmed before they were stained with fluorescent antibody, it was clear that the lack of multiplication was due to the expression of resistance mechanism operating in the CMT-IR03 cells, but not to cell death. Moreover, the multiplication was detected in these constituent cells when TMV was directly injected into them (data not shown). Thus, the present study strongly suggests that the resistance mechanism accumulated in the CMT-IR03 line of tobacco is the suppression of the intercellular translocation of the virus in the plant tissue.

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