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Mitotic Protoplasts and Their Infection with Tobacco Mosaic Virus RNA Encapsulated in Liposomes

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ABSTRACT

M-phase and S-phase protoplasts were prepared from tobacco cells in suspension culture after a high degree of synchronization using aphidicolin, a specific inhibitor for eukaryotic DNA polymerase . When TMV-RNA was introduced into these protoplasts mediated by REV liposomes, 37% of M-phase and 26% of S-phase protoplasts were infected as determined by the fluorescent antibody technique. After the 24 hr interval between the introduction of TMV-RNA into protoplasts and the determination of infection, half of the infected mitotic protoplasts formed dumbell-shaped daughter cells. The significance of synchronized protoplasts in genetic engineering of plant cells is discussed in reference to the delivery of DNA into the nucleus.

Abbreviation: LS medium, Linsmaier and Skoog medium; PEG, polyethylene glycol; REV, reverse-phase evaporation vesicles; TMV, tobacco mosaic virus.

INTRODUCTION

Although there are many reports on the synchronization of plant cells (see review of Komamine et al. 1978), the degree of synchronization is not satisfactory and the highest mitotic index so far reported was about 35% (Eriksson 1966). Recently a high degree of synchchronization was reported with HeLa cells using an antibiotic, aphidicolin which specifically inhibits eukaryotic DNA polymerase (Pedrali-Noy et al. 1980). We found that aphidicolin was very effective for inducing high synchronization of plant cells in liquid culture (Okada et al. in prep.). In this communication we report that protoplasts were isolated from M-phase and S-phase of the synchronized cells which were successfully infected with TMV-RNA encapsulated in liposomes.

MATERIALS AND METHODS

Tobacco BY-2 cells of Nicotiana tabacum L. cv. Bright Yellow 2 were cultured in a modified LS medium as reported previously (Linsmaier and Skoog 1965; Nagata el al. 1981) For synchronization 10 ml of 7-day-old suspension culture was transferred to 95 ml of modified LS medium containing 5 mg/l aphidicolin (kindly provided by Imperial Chemical Industries PLC, Macclesfield, Cheshire, England). After the cells were cultured in the medium containing aphidicolin for 24 hr, they were collected and washed 4 times with fresh LS medium. These cells were resuspended in fresh modified LS medium and cultured further. Cells were harvested at appropriate times and stained with propionic orcein to determine mitotic index. Protoplast preparation was started at 1 hr and 8 hr of culture using a combination of pectolyase Y23 (Seishin Pharmaceutical Ltd., Nihonbashi, Tokyo, Japan) and Cellulase Onozuka RA (Yakult Pharmaceutical Ind. Ltd., Takarazuka, Hyogo, Japan) according to Nagata et al. (1981). As enzyme treatment of 1 hr was sufficient for complete removal of cell walls, protoplasts obtained corresponded to the cells at 2 hr and 9 hr after aphidicolin treatment, respectively, TMV-RNA was encapsulated in liposomes of phosphatidylserine and chloresterol according to Nagata el al. (1981). Two micromoles of liposomes were added to 3 x 10^6 protoplasts. To this mixture was added equal volumes of 25% PEG 1540 (Koch-Light Lab. Ltd., Cionbrook, Buchs, England) dissolved in the protoplast culture medium and incubated for 15 min. After incubation the polymer was removed by washing with high pH-high Ca medium of Keller and Melchers (1973). The protoplasts were cultured as described previously (Nagata et al. 1981). After 24 hr of culture the number of infected protoplasts was determined by staining with fluorescent antibody against TMV according to Fukunaga et al. (1981).

RESULTS AND DISCUSSION

Changes in the mitotic index of BY-2 cells after aphidicolin treatment is shown in Fig. 1. The first peak of the mitotic index was observed at 9 hr after the removal of aphidicolin, while the second one was observed at 22 hr of culture. The values in Fig. 1 were low estimates, because cells in the early prophase were difficult to identify and ambiguous cases were not counted as M-phase. However, the identity of M-phase protoplasts was no difficulty, because they were completely composed of single cells, and the absence of cell wall facilitated the staining with the present procedure of propionic orcein. Thus, mitotic index of the protoplasts was counted to be 20-30% higher than that for the cells at the corresponding time, which presumably reflected the actual mitotic index. Protoplast preparation was started at 1 hr and 8 hr after aphidicolin treatment.

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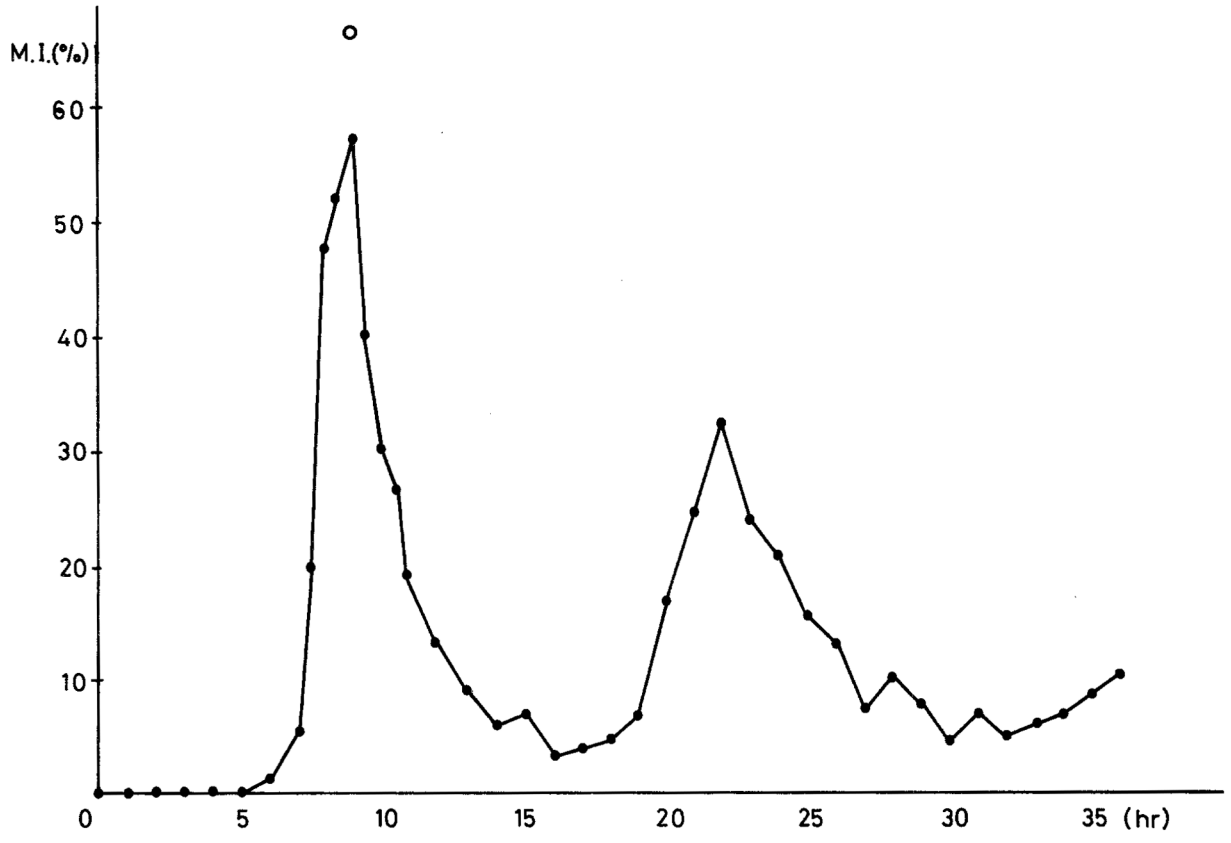
Fig. 1. Change of the mitotic index of tobacco BY-2 cells after aphidicolin treatment. After 24 hr treatment of aphidicolin, tobacco BY-2 cells were washed well to remove the chemical and cultured in the fresh medium. Mitotic index was determined at 1 hr intervals by staining with propionic orcein. A white circle at 9 hr shows the mitotic index of protoplasts obtained after treatment with Pectolyase Y23 and Cellulase Onozuka RS for 1 hr.



Since the enzyme treatment took nearly 1 hr, the protoplasts represented cells at 2 hr and 9 hr of culture. The protoplast preparation obtained at 9 hr of culture was denoted as "M-phase" protoplasts, because 67% were at the mitotic phase (Fig. 1). The preparation obtained at 2 hr was denoted as "S-phase" protoplasts. The rationale for this is that during aphidicolin treatment the cell cycle is known to be arrested at the border of G1/S (Pedrali-Noy et al. 1980) and after release of this arrest the cells start to resynthesize DNA. Thus these protoplasts should be predominantly in S-phase. Detailed analysis of M-phase and S-phase protoplasts are reported elsewhere (Okada et al. in press).

Both M-phase and S-phase protoplasts were subjected to inoculation with TMV-RNA encapsulated in liposomes according to Nagata el al. (1981) and the infection percentage was determined by staining with the fluorescent antibody technique after 24 hr of culture. About 37% of the M-phase and 26% of S-phase protoplasts were infected. Although there was some fluctuation of these values, the infection percentage of M-phase protoplasts was always higher than that of the S-phase. As there was a time difference of 24 hr between the infection of protoplasts and the determination of infection, more than half of the infected mitotic protoplasts formed dumbell shaped daughter cells (Fig. 2). However, such dumbell-shaped infected cells were observed rarely (ca. 1%) in the cells which were infected during S-phase. This is probably because the infected cells in S-phase could not go through DNA synthesis to mitosis perturbated by the active propagation of virus particles in the cells. Furthermore, it is of interest that in 10% of the infected population of M-phase protoplasts only half of the dumbell-shaped daughter cells was infected (Fig. 3). At the moment there are several ways to interpret this phenomenon, but it is worthwhile for study further why the infection was confined to one of two daughter cells, although both cells were interconnected with plasmodesmata.

In this study highly synchronized cells were obtained after aphidicolin treatment and the observed mitotic index of 60-70% was the highest so far reported for plant cells (Komamine et al. 1978). M-phase and S-phase protoplasts obtained by an enzyme treatment from these synchronized cells were successfully infected with TMV-RNA encapsulated in liposomes. Actually this is the first report of the preparation of protoplasts from synchronized cells and their application for experimental study. Furthermore, it should be stressed that this synchronization procedure using aphidicolin did not cause apparent genetic anomalies which were observed with other synchronization procedures (Eriksson 1966) and these protoplasts formed colonies at high frequency (Nagata unpublished observation).

Thus the implication of the potential of this system is important, because M-phase protoplasts can now be obtained for experimental

Fig. 2. Fluorescence micrograph of mitotic protoplasts infected with TMV-RNA encapsulated in REV liposomes. After 24 hr of culture the cells were stained with fluorescent antibody against TMV. Note that two daughter cells were infected. Magnification x 630.

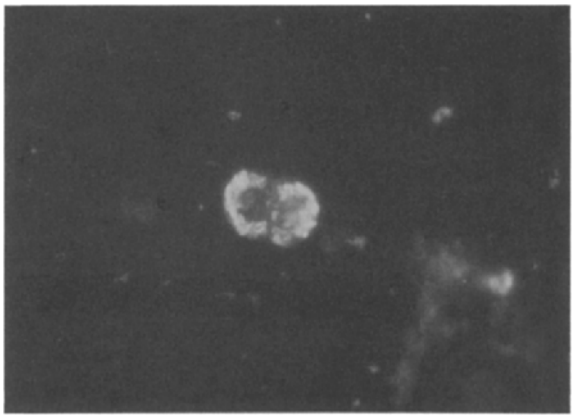
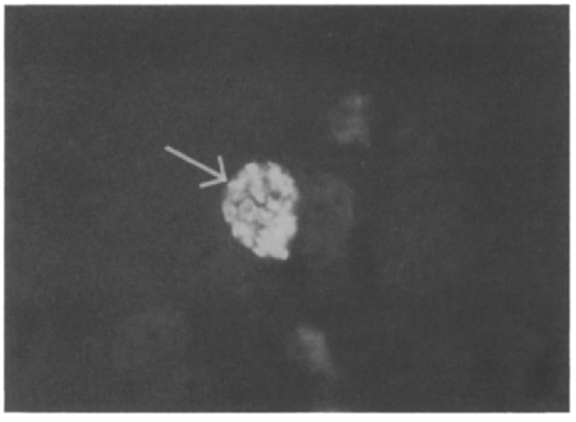


Fig. 3. Fluorescent micrograph of mitotic protoplasts infected with TMV-RNA encapsulated in REV liposomes. This specimen was treated in the same manner as that for Fig. 2. Note that only one of two daughter cells (arrow) was infected. Magnification x 630.



manipulation. As was shown previously (Fukunaga et al. 1981; Nagata el al. 1981) delivery of TMV-RNA into protoplasts mediated by liposomes was carried out in almost all viable protoplasts for multiplication of TMV particles to occur, the introduction of RNA into the cytoplasm was sufficient. These observations and the results reported in this paper may have relevance to achieve transformation of cells by foreign DNA. In liposome-mediated introduction of DNA into plant protoplasts the transfer of DNA from the cytoplasm to the nucleus may be hampered by the nuclear membrances.

The importance of the direct introduction of DNA into the nucleus was reported by Capecchi (1980), who showed increased frequency of transformation by microinjection. This approach is laborious and handling of plant protoplasts with a microinjection apparatus was much more difficult than that of animal cells (Nagata unpublished observation). The M-phase protoplasts do not retain the nuclear membranes and they may be the material of choice for the introduction of foreign DNA into the nucleus. We are currently trying to introduce liposome-encapsulated Ti plasmid from Agrobacterium tumefaciens into M-phase protoplasts to obtain transformation of plant cells at high frequency.

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