Infection of Barley Protoplasts with Brome Mosaic Virus

T. Okuno, I. Furusawa, and C. Hiruki

Graduate Student and Assistant Professor, respectively, Laboratory of Plant Pathology, Faculty of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606, Japan; and Professor, Department of Plant Science, Faculty of Agriculture and Forestry, University of Alberta, Edmonton, Alberta, Canada, T6G 2E3.

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ABSTRACT

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High yields of protoplasts susceptible to brome mosaic virus (BMV) were obtained by incubating barley leaves, stripped of lower epidermis, in a mixture of 0.05% Macerozyme and 2% cellulase at 30 C for 2 hr. Multiplication of BMV was demonstrated by bioassay and fluorescent antibody staining. About 30% of the protoplasts were infected under optimal conditions. Poly-L-ornithine was not essential for infection, but it stimulated both the retention of virus particles by protoplasts and the infection of protoplasts with BMV. This is the first report of virus infection of protoplasts isolated from monocotyledonous species.

Additional key words: virus multiplication, *Hordeum vulgare*.

Protoplasts isolated from higher plants have been infected with many viruses including tobacco mosaic virus (TMV) and its RNA (1, 8, 20, 22, 24, 25), potato virus X (PVX) (17, 21), cucumber mosaic virus (CMV) (16), cowpea chlorotic mottle virus (CCMV) (10), brome mosaic virus (BMV) (11), cowpea mosaic virus (CPMV) (6), tobacco rattle virus (9), alfalfa mosaic virus (13), pea enation mosaic virus (PEMV) (12) and turnip yellow mosaic virus (TYMV) (18). Inoculation of tobacco mesophyll protoplasts with TMV resulted in synchronous multiplication in a majority of cells (24). This system has proved to be extremely useful for cytological, physiological and biochemical studies of virus infection and multiplication (14, 19, 24, 25). In the case of BMV, infection of tobacco mesophyll protoplasts was dependent on virus strain used. While an electrophoretic variant of BMV did multiply in tobacco protoplasts, a wild type BMV did not (11). We isolated protoplasts from detached leaves of barley (*Hordeum vulgare* L.) and were able to demonstrate BMV multiplication in the protoplasts for the first time. This report presents the results of our investigation.

MATERIALS AND METHODS

Plant.—Barley (*Hordeum vulgare* L. 'Goseshikoku') was grown in vermiculite in a controlled chamber at 20 C with a daily light period of 16 hr at a light intensity of about 10,760 lx (1,000 ft-c). They were watered with nutrient solution (Hyponex) every other day. Six- to 7-day-old seedlings were used as a source of protoplasts.

Virus growth.—Plants at the one-leaf stage were inoculated by rubbing Carborundum-dusted leaves with a piece of gauze moistened with inoculum suspension containing a standard strain of BMV (ATCC 66). Two wk after inoculation the infected leaves were harvested for purification.

Virus purification.—Purification of BMV was by the method previously described (7).

Preparation of protoplasts.—Four g of vigorously growing barley leaves were collected, the lower epidermis was stripped, and the remaining tissues were soaked in 50 ml of an enzyme solution pH 5.5 containing 2% cellulase (w/v) (Yakult Biochemicals, Tokyo), 0.05% Macerozyme (w/v) (Yakult Biochemicals, Tokyo) in 0.6 M mannitol. The incubation flask was shaken in a water bath at 30 C for 2 hr at a frequency of 80 excursions/minute. After incubation, the medium was filtered through a sheet of gauze to remove the upper epidermis and nondigested materials. The protoplasts thus obtained were collected by centrifuging for 3 min at 100 g and washed twice with 0.6 M mannitol solution by centrifuging for 3 min at 100 g. The yield of protoplasts was about 2×10^7/g of fresh leaf and more than 90% were apparently intact. A micrograph of isolated protoplasts is shown in Fig. 1.

Inoculation of protoplasts.—For inoculating protoplasts with BMV, the procedure was modified from the TMV-tobacco mesophyll protoplast system (23). Purified BMV was suspended at a final concentration of 100 μg/ml in 0.02 M potassium citrate buffer, pH 5.0, containing 2 μg/ml of poly-L-ornithine (MW 125,000) in 0.6 M mannitol. The solution was mixed with an equal volume of protoplast suspension (2×10^6 cells/ml) and shaken for 10 min at 25 C at a frequency of 80 excursions/minute. Protoplasts were separated from nonadsorbed virus particles by low-speed centrifugation and washed twice with 0.6 M mannitol solution containing 10 mM $CaCl_2$. Sedimented protoplasts were suspended in incubation medium containing 0.2 mM KH_2PO_4, 1 mM KNO_3, 1 mM $MgSO_4$, 10 mM $CaCl_2$, 1 μM KI, 0.01 μM $CuSO_4$, 0.7 M mannitol, and 500 μg/ml

cephaloridine, pH 5.3, and incubated in 10-ml portions at a concentration of 10^6 protoplasts/ml under continuous fluorescent light (1,000 lux).

Infectivity assay of protoplast extract.—Virus infectivity was assayed using a balanced assay design on *Chenopodium hybridum* leaves at the eight-leaf stage. At least eight half-leaves were used for a test inoculum. Protoplasts were collected by centrifuging for 3 min at 100 *g* and stored at −20 C. After thawing, the protoplasts were homogenized in 1 ml of 0.05 M acetate buffer, pH 5.0, containing 0.001 M $MgCl_2$, in a ground glass homogenizer for 1 min at 0 C. The resulting homogenate was used for inoculation without further dilution. Local lesions were counted 3 or 4 days after inoculation.

Fluorescent antibody staining.—Antibody to BMV was conjugated with fluorescein isothiocyanate (FITC) by the method previously reported (14). The conjugated globulin had a titer of 1/256. A fluorescein to protein molar ratio, estimated from absorbance at 495 nm and 280 nm, was 1.94. To check nonspecific staining, conjugated globulin was examined in the presence of an acetone powder preparation of noninoculated barley leaves. Protoplasts on a glass slide were fixed with 95% ethanol for 10 min. The specimen was then washed with phosphate-buffered saline (PBS). For staining, the protoplasts were covered with a drop of the conjugated globulin (1/4 dilution) on a glass slide which was placed in a moist chamber at 37 C for 1 hr. After being stained, the specimen was washed with PBS and then mounted with PBS containing 50% glycerine. A Zeiss fluorescence microscope was used to observe the stained specimens.

Labeled virus.—Labeled (^{32}P) BMV was prepared according to the method reported previously (2). Labeled BMV had a specific activity of 4.8 μCi/mg RNA.

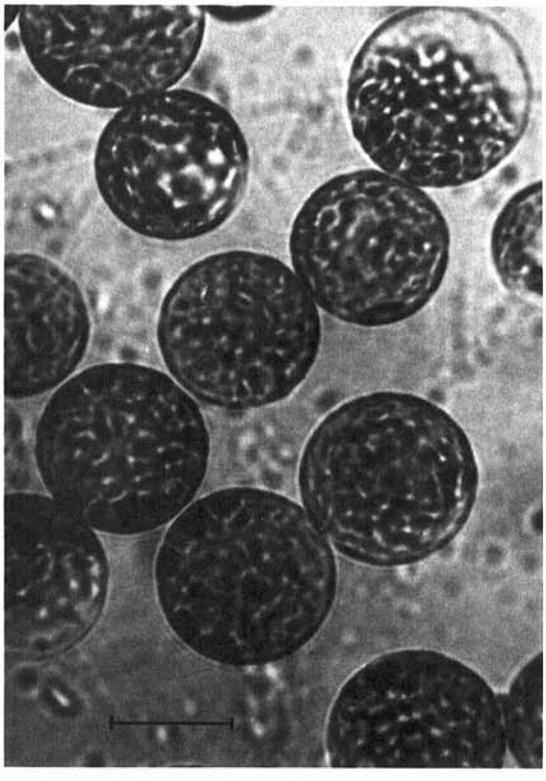

Fig. 1. Protoplasts isolated from barley mesophyll suspended in 0.6 M mannitol. The scale represents 25 μm.

RESULTS

Inoculation medium pH.—The infectivity of protoplasts inoculated at different pH values was assayed after 27 hr of incubation. Relatively high infectivity of BMV was obtained in a range from pH 4.7 to 5.7 (Fig. 2). At pH values above 5.7, a steep decline of BMV infectivity was noted. Under pH 4.7 or above pH 6.0, the protoplasts were degraded during incubation.

Concentration of poly-L-ornithine.—In the cases of viruses including TMV, CMV, PVX, CCMV, and tobacco protoplasts as a host, the infection is highly dependent on the presence of poly-L-ornithine (10, 16, 24). To investigate this point with barley protoplasts, 0 to 2 μg/ml poly-L-ornithine were added to the inoculation medium which contained 50 μg/ml BMV at pH 5.0. Virus infectivity and the fraction of protoplasts showing fluorescence were determined after 28 and 44 hr of incubation (Table 1). Poly-L-ornithine did not seem to be essential for infection of barley protoplasts with BMV. However, the infection was stimulated by increasing the concentration of poly-L-ornithine from 0 to 2 μg/ml (Table 1). On the other hand, the amount of virus particles retained by the protoplasts was approximately constant within a range of 0.1 to 1 μg/ml poly-L-ornithine (Table 2). When the concentration of poly-L-ornithine was increased to 10 μg/ml, retention of virus particles by the protoplasts was significantly increased. However, at this concentration, poly-L-ornithine, caused the aggregation of protoplasts.

Inoculum concentration.—Within a range of 1 to 50

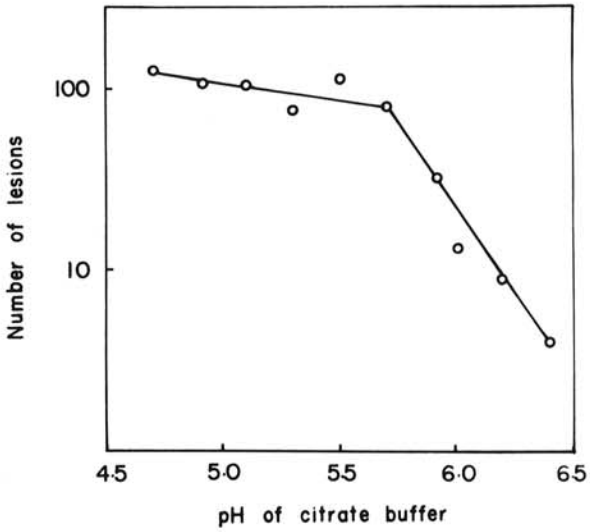

Fig. 2. Effect of pH of the inoculation medium on the infectivity of brome mosaic virus (BMV). Barley protoplasts were incubated with 50 μg/ml BMV for 27 hr in citrate buffer solutions of different pH values.

TABLE 1. Effect of poly-L-ornithine on infection of barley protoplasts with brome mosaic virus (BMV)

Expt. no.	Poly-L-ornithine (μg/ml)	Number of lesions incited by protoplast extract[a]	Protoplasts showing fluorescence (%)
1[b]	0	99.6 ± 20.0	-
	0.1	109.8 ± 26.3	-
	0.5	145.1 ± 45.0	-
	1	185.0 ± 45.4	-
2[c]	0	35.4 ± 22.9	10.2
	0.1	36.8 ± 24.5	10.5
	1	137.5 ± 89.8	25.7
3[c]	0	166.4 ± 52.4	-
	1	258.0 ± 117.4	-
	2	186.4 ± 64.8	

[a]Lesions incited on *Chenopodium hybridum* were counted.
[b]BMV infectivity was determined 28 hr after incubation.
[c]BMV infectivity and protoplasts showing fluorescence were determined 44 hr after incubation.

μg/ml BMV, the increase in BMV infectivity and protoplasts showing fluorescence was roughly proportional to the concentration of BMV in the inoculum used (Table 3). No significant increase in protoplasts showing fluorescence and BMV infectivity was obtained by increasing the virus concentration above 50 μg/ml. Protoplasts were inoculated with ^{32}P-labeled BMV in the presence or absence of poly-L-ornithine and washed twice with 0.6 M mannitol solution containing 10 mM $CaCl_2$. The washed protoplasts were then dried in vials and the amount of radioactivity was measured by a liquid scintillation counter. The higher the BMV concentration of inoculum used, the greater was the retention of virus particles by protoplasts (Table 4). The amount of virus particles retained by protoplasts was enhanced two- to threefold by adding 1 μg/ml of poly-L-ornithine to the medium.

Multiplication of BMV in protoplasts.—Extracts were obtained from inoculated protoplasts after 0 to 65 hr of incubation and inoculated to 10 half-leaves of *C. hybridum*. Although some infectivity was detectable immediately after incubation, there was no detectable infectivity 9 hr after incubation. Infectivity of BMV to protoplasts increased rapidly during the period between 16 hr and 24 hr; thereafter the increase was slow (Fig. 3).

Fluorescent antibody staining.—By using the fluorescent antibody staining technique (14), about 10 to 30% of the protoplasts were found to be infected 42 hr after incubation (Table 5). Yellow-green fluorescence was found in the protoplasts infected with BMV, first as small spots and a light network surrounding the chloroplasts followed often by the formation of fluorescing larger masses (Fig. 4-A). No such virus specific staining was evident when noninoculated protoplasts from barley mesophyll tissue were stained after 42 hr of incubation with labeled antibody specific for BMV antigen (Fig. 4-B).

Subsequently samples of the protoplasts were examined at various time intervals after inoculation. The first fluorescence due to BMV antigen was visible after 17 hr of incubation as weakly fluorescent specks. The appearance of BMV specific fluorescence coincided with the increase of virus infectivity in the protoplasts. The percentage of fluorescing protoplasts increased very rapidly from 4% to 18% between 17 and 27 hr of incubation and then rose more slowly to 22% at 42 hr of incubation (Table 5).

TABLE 2. Effect of poly-L-ornithine on retention of brome mosaic virus (BMV) by barley protoplasts[a]

Poly-L-ornithine (μg/ml)[b]	Virus retained		
	CPM[c]	(%)	Virus particles/ protoplasts
0	266	1.1	1.2×10^4
0.1	659	2.8	3.3×10^4
0.5	617	2.6	3.1×10^4
1.0	637	2.7	3.2×10^4
10.0	1,400	6.0	7.1×10^4

[a]Protoplasts (6.6×10^6) in 3 ml were mixed with 3 ml of inoculation medium containing 20 μg/ml of ^{32}P-BMV and poly-L-ornithine at a given concentration and incubated for 10 min at 25 C. Inoculated protoplasts were collected by centrifugation and washed with 0.6 M mannitol containing 10 mM $CaCl_2$.
[b]Concentration of poly-L-ornithine after mixing with protoplasts.
[c]The radioactivity of ^{32}P-BMV (10 μg/ml) added to each sample was 23,340 CPM.

DISCUSSION

The results presented in this paper demonstrate for the first time that barley, a monocotyledonous host of BMV, is a suitable source for protoplasts which may be infected with the virus after a brief incubation period. Approximately 30% of barley protoplasts that came into contact with BMV particles were infected using the procedure described in this paper. With regard to the conditions of infection, some modifications were made in this investigation for the barley protoplast system, although the basic technique was similar to those used for TMV, CMV, and PVX-tobacco mesophyll protoplast system (16, 17, 23). Firstly, an acidic medium was optimal for BMV infection at a range from 4.7 to 5.7 (Fig. 2). At pH ranges under 4.7 or over 6.0, the protoplasts tended to become degraded and detectable virus infectivity was low.

Therefore, pH 5.0 was used as a standard inoculating condition. Secondly, poly-L-ornithine was not essential for infection of barley protoplasts with BMV, although its presence resulted in increased levels of infection. These results confirm and expand the earlier findings that tobacco protoplasts were inoculable with BMV variant 5 in the absence of poly-L-ornithine and that they required high inoculum concentrations of 50 to 100 μg/ml for optimal virus infection (11). On the other hand, it is known that poly-L-ornithine is essential for infection of tobacco protoplasts with several viruses (1, 10, 16, 17, 24) and of *Brassica* protoplasts with TYMV (18). In two other virus-protoplast systems, namely CPMV in cowpea protoplasts (6) and PEMV in tobacco protoplasts (12), both of which involve multicomponent viruses, it is known that poly-L-ornithine is not essential in infection. A wild type of BMV did not multiply in tobacco protoplasts, even in the presence of poly-L-ornithine (11).

On the mechanism of the action of poly-L-ornithine, it has not yet been shown conclusively (25) whether it stimulates pinocytosis (15, 24), or induces plasmalemma lesions which presumably serve as binding sites of virus (4, 5). In the barley protoplast system, poly-L-ornithine was not essential for infection, but it stimulated infection with the BMV inocula in a range of 0.1 to 1 μg/ml (Table 1). Although approximately the same amount of virus particles was retained by the protoplasts in the same range of poly-L-ornithine concentrations, it substantially increased the amount of BMV attached to the protoplasts when the concentration of poly-L-ornithine was increased to 10 μg/ml (Table 2). It has been reported that only a small amount of the retained virus took part in the establishment of TMV infection in tobacco protoplasts (26). If this is the case with the BMV-barley protoplast

TABLE 3. Effect of brome mosaic virus (BMV) concentration on infection of barley protoplasts[a]

Expt. no.	BMV concentration (μg/ml)	Number of lesions[a]	Protoplasts showing fluorescence (%)
1	1	3.6 ± 1.3	-
	2	26.3 ± 14.8	-
	12.5	150.0 ± 17.6	-
	50	415.0 ± 182.8	-
	100	407.1 ± 151.0	-
2	12.5	-	6.4
	25	-	6.2
	50	-	19.5
	100	-	21.1

[a]BMV infectivity assays and counting of protoplasts showing fluorescence were done 42 hr after inoculation. Other procedures are in Materials and Methods.

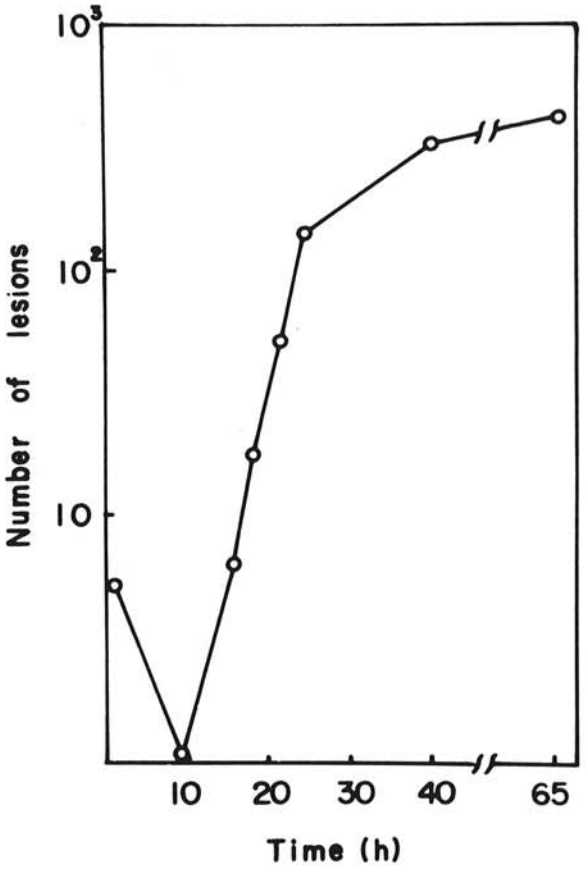

Fig. 3. Multiplication of brome mosaic virus (BMV) in barley mesophyll protoplasts. Leaves of *Chenopodium hybridum* were inoculated with the inoculum mixture contained BMV at a final concentration of 50 μg/ml at pH 5.1. For the composition of the inoculation medium, refer to the text.

TABLE 4. Effect of brome mosaic virus (BMV) concentration and poly-L-ornithine on retention of BMV by barley protoplasts[a]

Poly-L-ornithine (μg/ml)	BMV added		BMV retained		
	(μg/ml)[b]	CPM	CPM	%	BMV particles/ protoplasts
0	50	155,400	1,662	1.1	6.3×10^4
1	50	155,400	4,925	3.2	1.9×10^5
0	10	31,080	524	1.7	2.0×10^4
1	10	31,080	2,107	6.8	8.0×10^4
0	1	3,108	126	4.1	4.8×10^3
1	1	3,108	244	7.9	9.2×10^3

[a]Protoplasts (6.6×10^6) in 3 ml were mixed with 3 ml of inoculation medium containing ^{32}P-BMV at a given concentration in the presence or absence of poly-L-ornithine and incubated for 10 min at 25 C. Inoculated protoplasts were collected by centrifugation and washed twice with 0.6 M mannitol containing 10 mM $CaCl_2$.

[b]BMV concentration after mixing with protoplasts.

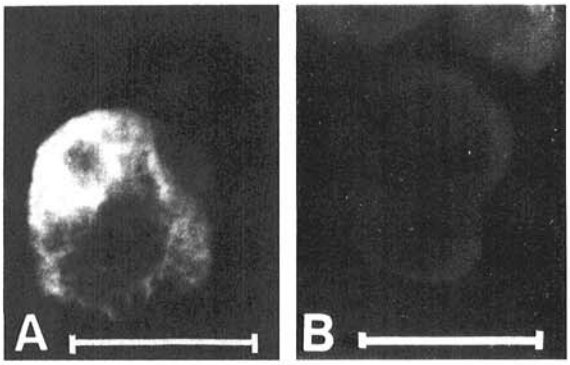

Fig. 4-(A,B). Fluorescence microscopy of barley protoplasts stained with labeled antibody specific for brome mosaic virus (BMV) antigen: **A)** infected with BMV. **B)** noninoculated. The scales represent 25 μm.

TABLE 5. The time-course examination of barley mesophyll protoplasts by the fluorescent antibody staining technique after inoculation with brome mosaic virus (BMV)

Time after inoculation (hr)	Protoplasts showing fluorescence (%)
0	0
17	4.0 ± 1.9
27	17.8 ± 7.1
42	21.6 ± 10.1

[a]Protoplasts were inoculated with 50 μg/ml BMV at pH 5.1.

system, then these results suggest that the infection of barley protoplasts was not simply dependent on the quantity of virus particles retained by the protoplasts. It seems that poly-L-ornithine plays some important role in effecting BMV infection of protoplasts or stimulating BMV multiplication in the protoplasts. It is known that the isoionic point of BMV is pH 7.9 (3) and this would suggest that BMV itself can act as a polycation at the pH range used in this investigation which would allow protoplasts to become infected in the absence of poly-L-ornithine. Therefore, additional role(s) of poly-L-ornithine, such as the above mentioned may be considered.

The method used in this investigation for the isolation of protoplasts was a one-step procedure (6) which involved incubation of young leaf tissue without the lower epidermis in a solution of Macerozyme and cellulase. This method is simpler and less time consuming (6) than the two-step procedure previously developed for the isolation of tobacco mesophyll protoplasts (23), and it provides high yields of relatively uniform barley mesophyll protoplasts. Young leaves of barley, one of the monocotyledonous species, contain mesophyll cells morphologically more uniform than those of species belonging to Solanaceae or Papilionaceae. Thus, the use of the one-step procedure does not lead to any serious complication in infection studies.

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