

クロロプラストにおけるブロムモザイクウイルス RNA の複製

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著者名	中山,政治 堀越,守 三瀬,和之 山岡,直人 朴,杓允 古沢,巖 獅山,慈孝
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Replication of Brome Mosaic Virus RNA in Chloroplasts*

Masaharu NAKAYAMA**, Mamoru HORIKOSHI**, Kazuyuki MISE**, Naoto YAMAOKA***,
Pyonyun PARK****, Iwao FURUSAWA** and Jiko SHISHIYAMA**

Abstract

The replication site of brome mosaic virus (BMV) RNA was investigated in BMV-infected barley cells. Subcellular fractions were separated from protoplast lysates by differential centrifugations and Percoll gradient centrifugation. The activity of RNA synthesis was assayed in the each fraction in the presence of actinomycin D. The RNA dependent RNA polymerase activity was detected in a purified chloroplast fraction and the activity per chlorophyll increased with purification of chloroplasts. The existence of RNAs hybridized with cDNA of BMV RNA was detected in the purified chloroplasts in the dot blot assay. Replicative forms were synthesized in the *in vitro* RNA synthesis system adding chloroplasts isolated from BMV-infected cells instead of enzyme fraction. By the microautoradiographic observation, the silver grains were observed on the thylakoid when the purified chloroplasts isolated from BMV-infected cells were added to the reaction mixture and incubated in the presence of actinomycin D and ³H-UTP. These results strongly suggested that at least BMV RNA can replicate in chloroplasts of BMV-infected barley cells.

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Key words : BMV, RNA replication site, chloroplasts.

Introduction

The brome mosaic virus (BMV) replicase has been extracted from the so-called membrane fraction of infected barley cells⁶⁾. The enzyme had a high template specificity and synthesized double stranded viral RNA *in vitro*^{1,6,9,11)}. However, the location of this enzyme in infected cells is still unclear. To detect the site of replication of BMV RNA, the synthesis activity of the replicative form can be used as a marker.

In cucumber mosaic virus (CMV)⁷⁾, and cowpea mosaic virus (CPMV)-infected cells³⁾, the viral RNA-replicase complexes were extracted from membrane fractions and could synthesize double stranded viral RNA in the absence of added template RNA. Watanabe and Okada¹⁵⁾ detected the synthesis of double stranded viral RNA in a 2,500 × g fraction of extracts of tobacco mosaic virus (TMV)-infected tobacco cells. Lafleche *et al.*⁸⁾ proposed that the outer membrane of chloroplasts is the site of replication of

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** Laboratory of Plant Pathology, Faculty of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606, Japan 京都大学農学部

*** Laboratory of Plant Pathology, Faculty of Agriculture, Mie University, Tsu-city 514, Japan 三重大学農学部

**** Department of Medicine, University of Teikyou, Itabashi-ku, Tokyo 173, Japan 帝京大学医学部

turnip yellow mosaic virus (TYMV) RNA.

In this paper, the site of replication of BMV RNA was investigated.

Materials and Methods

Preparation of subcellular fraction. The first leaves of barley (*Hordeum vulgare* L. cv. Goseshikoku) seedlings were inoculated with purified BMV (1 mg/ml) using Carborundum as an abrasive. Six to 8 days after inoculation, the second and third leaves showing mosaic symptoms were harvested and the adaxial epidermis of these leaves was dusted with Carborundum and brushed with a silicon sponge cap. After washing with water, the leaves were floated on an enzyme solution which consisted of 1% cellulase and 0.5 M mannitol, adjusted to pH 5.8 and incubated for 3 hr at 30 C with occasional shaking. The protoplast suspension was filtered through 4 layers of cheese cloth and protoplasts were collected and washed by centrifugation for 2 min at $100\times g$. Also, protoplasts isolated from mock-inoculated leaves were used as a control. Protoplasts were resuspended with 70 ml of buffer A [10 mM 2(N-morpholino)ethanesulfonic acid containing 1 mM EDTA, 1% bovine serum albumin (BSA) and 0.33 M mannitol pH 6.5]. The suspension was passed through a nylon mesh (20 μ m pores) with a Pasteur pipette to lyse the protoplasts. The protoplast lysate was centrifuged for 10 min at $120\times g$. The resultant pellets were resuspended with 1 ml of buffer B (50 mM Tris-HCl, pH 8.0, 1 mM 2-mercaptoethanol, 3 mM EDTA and 0.44 M mannitol) and used as a crude nuclear fraction. The supernatant was centrifuged for 20 min at $3,000\times g$ and $30,000\times g$. Both pellets were resuspended with 1 ml of buffer B and used as crude chloroplast and membrane fractions, respectively. For further purification of the chloroplasts, the $3,000\times g$ pellets were resuspended with 1.5 ml of buffer B. The suspension was applied to a discontinuous gradient of 10, 40 and 70% Percoll and centrifuged for 20 min at $10,000\times g$. Purified chloroplasts were obtained from a band between the 10 and 40% Percoll layers.

In vitro RNA synthesis. The reaction mixture (120 μ l) of the *in vitro* RNA synthesis system consisted of 50 mM Tris-HCl pH 8.2, 10 mM KCl, 15 mM $MgCl_2$, 1 mM EDTA, 2 μ g actinomycin D, 50 μ g each of ATP, CTP, GTP, 2.5 μ Ci 3H -UTP (50 Ci/mmol), 4 mM 2-mercaptoethanol and 50 μ l of each fraction obtained by differential and Percoll gradient centrifugations. Template RNA was not added to the reaction mixture. The mixtures were incubated for 60 min at 30 C and the trichloroacetic acid insoluble radioactivity was measured by the paper disc assay method previously described⁹⁾.

Measurement of chlorophyll content. To each fraction (200 μ l) obtained by differential centrifugation and Percoll gradient centrifugation was added 3 volumes of acetone. The mixtures were shaken vigorously and centrifuged for 2 min at $10,000\times g$, and then the supernatant was analyzed by absorbance at OD₆₄₅ and OD₆₆₃ with a spectrophotometer. The following equation was used for determination of chlorophyll concentration²⁾.

$$\text{Chlorophyll (mg/ml)} = (20.2 \times \text{OD}_{645} + 8.02 \times \text{OD}_{663}) / 20$$

Electrophoresis and autoradiography. Fifty μ l of each fraction obtained by dif-

ferential and Percoll gradient centrifugation was added to the *in vitro* RNA synthesis system and incubated for 60 min at 30 C in the presence of actinomycin D and 25 μ Ci α - 32 P-UTP (3,000 Ci/mmol). The 32 P-labelled products were subjected to electrophoresis in 1.2% agarose gels in 50 mM Tris-acetate pH 8.2, containing 1 mM EDTA. After electrophoresis for 4 hr at 100 volts, the gel was dried and exposed to X-ray film for 2 days. Double stranded BMV RNAs were co-electrophoresed as a marker⁹⁾.

Microautoradiography. Five hundred μ l of purified chloroplast fraction was added to the *in vitro* RNA synthesis system and incubated for 60 min at 30 C in the presence of actinomycin D and 2.5 μ Ci 3 H-UTP. After incubation the mixture was placed on ice and glutaraldehyde was added to a final concentration of 2%. After 6 hr at 0 C, the chloroplasts were collected by centrifugation for 20 min at 5,000 $\times g$ and washed three times with cold 50 mM phosphate buffer pH 7.2. The specimens were postfixed with 1% OsO₄ for 2 hr at 0 C. After washing with the same buffer, chloroplasts were wrapped in lens paper, dehydrated through an ethanol series, placed in propylene oxide and embedded in a Spurr low viscosity medium (Polyscience Inc.). Ultrathin sections were stained with uranyl acetate for 30 min. The surfaces of sections were coated with carbon, covered with Sakura autoradiographic emulsion Type NR-H2 and exposed for 6 weeks at 4 C.

Hybridization. The cDNA copies of BMV RNAs were cloned by the method of Gubler and Hoffman⁴⁾. The d (TGGTCTCTTTTAGAG), which is complementary to 15 nucleotides at the 3' end of the viral RNA, was used as the primer for first-strand DNA synthesis. After second-strand synthesis, double strand cDNA was cloned into pUC9 by oligo (dG)-oligo (dC) tailing⁴⁾. Transformation of *E. coli* JM83 was carried out as described by Hanahan⁵⁾. Plasmids containing BMV cDNA were prepared by the alkali lysis method of Maniatis *et al.*¹⁰⁾. The plasmid DNA was labelled with 32 P-dCTP by nick-translation (nick-translation kit, Amersham). The 32 P-labelled cDNA hybridized with each BMV RNA separated by formaldehyde-agarose gel electrophoresis¹⁰⁾.

To examine whether BMV RNA exists or not in purified chloroplasts, 32 P-labelled cDNA of BMV RNA was used as a probe. Purified chloroplasts were treated with 2 ml of buffer B containing 10 μ g/ml RNase A for 30 min at 30 C and collected by centrifugation. SDS was added to the chloroplast suspension to give a 1% concentration and the mixture was shaken vigorously with an equal volume of water saturated phenol. The mixture was centrifuged for 10 min at 10,000 $\times g$. To the aqueous layer was added 2.5 volume of cold ethanol. RNA was co-precipitated with tRNA and suspended with 20 μ l of water. Nitrocellulose paper was equilibrated with 3 M NaCl/0.3 M trisodium-citrate (20 $\times$ SSC) for 10 min and dried under a lamp. Five μ l of RNA was spotted onto the paper and baked in an oven for 2 hr at 80 C. The RNA blots were prehybridized for 20 hr at 42 C. The prehybridization buffer contained 50% (v/v) formamide, 5 $\times$ SSC, 50 mM sodium phosphate pH 6.5, sonicated denatured salmon sperm DNA at 250 μ g/ml and 0.02% (w/v) each BSA, Ficoll and polyvinylpyrrolidone. The hybridization buffer contained 4 parts of the same buffer and 1 part of 50% (w/v) dextran sulfate. The nick-translated probes were denatured for 5 min at 100 C, cooled and added to the hybridization buffer, and the blots were hybridized for 20 hr at 42 C. The

blots were washed with four changes of $2\times$ SSC containing 0.1% SDS for 5 min each at room temperature and then washed with two changes of $0.1\times$ SSC containing 0.1% SDS for 15 min each at 50 C. The blots were exposed to Fuji X-ray film for 3 days at -70 C.

Results

In vitro RNA synthesis

The activity of RNA dependent RNA polymerase in each fraction prepared from mock inoculated and BMV-infected protoplast lysates was assayed by incorporation of ^3H -UMP into an acid insoluble fraction. The results indicated that the highest activity was obtained in the purified chloroplast fraction from infected protoplasts (Table 1). Both the crude nuclear ($120\times g$ pellet) and membrane ($30,000\times g$ pellet) fractions also showed considerable radioactivity, however, these fractions showed a deep green color, suggesting that they were contaminated with chloroplasts and degraded chloroplasts. The radioactivity of supernatant fraction also showed a high value because its volume was so much larger compared with others that the total radioactivity might have been overestimated.

To investigate whether BMV RNA was synthesized in a nucleus or not, the crude nuclear fraction was resuspended in a small volume of buffer B and centrifuged in a 30-90% Percoll gradient for 30 min at $10,000\times g$. *In vitro* RNA synthesis activity in each fraction was measured in the presence or absence of actinomycin D. As shown in Fig. 1, the RNA dependent RNA polymerase activity appeared only near the 40% Percoll region, while, the peaks of DNA dependent RNA polymerase activity appeared at both the 40% (chloroplast fraction) and 75% (nuclear fraction) Percoll region. That is, RNA-dependent RNA polymerase activity was not detected at 75% Percoll where DNA-dependent RNA polymerase activity was detected, suggesting that the replication of BMV RNA was not performed in a nucleus. As clearly shown in Table 2, the activity of RNA dependent RNA synthesis per unit of chlorophyll contents (mg) as assayed in the presence of actinomycin D increased with purification of chloroplasts.

Autoradiography

RNAs synthesized by crude nucleus, purified chloroplast and membrane fractions in

Table 1. RNA dependent RNA polymerase activity in subcellular fractions

Fraction	Incorporation of ^3H -UMP (Total cpm)	
	Mock-inoculated	BMV-infected
Crude nuclear	5,250	59,500
Purified chloroplast	4,860	128,250
Membrane	2,800	57,830
Supernatant	34,000	94,000

Fifty μl of each fraction was added to the reaction mixture of *in vitro* RNA synthesis system and incubated for 60 min at 30 C in the presence of actinomycin D. Values in this Table are from one experiment and are representative of those obtained in three separate experiments.

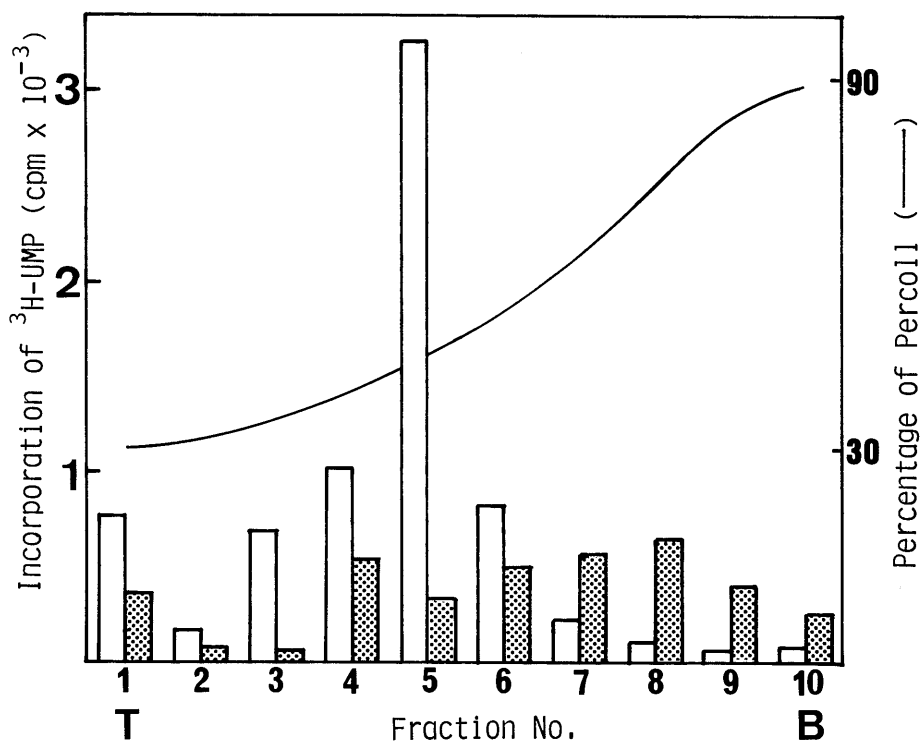


Fig. 1. The RNA synthesis in each fraction of crude nuclear fractionated by 30-90% Percoll gradient centrifugation.

□ : RNA dependent RNA polymerase activity (radioactivity in the presence of actinomycin D), ▨ : DNA dependent RNA polymerase activity [(radioactivity in the absence of actinomycin D)-(radioactivity in the presence of actinomycin D)]. T and B indicate the positions of top and bottom of tube, respectively.

Table 2. RNA dependent RNA polymerase activity in subcellular fractions per chlorophyll

Fraction	Incorporation of ³ H-UMP (Total cpm)	cpm/mg chlorophyll
Protoplast lysate	135,000	1,730
Crude chloroplast	31,260	2,140
Purified chloroplast	211,260	19,240

See the note of Table 1.

the presence of actinomycin D and ³²P-UTP were electrophoresed and autoradiographed (Fig. 2). When a purified chloroplast fraction was added to the reaction mixture of *in vitro* RNA synthesis, the synthesized RNAs were resistant to RNase in 2×SSC, and they migrated to the same position of replicative forms isolated from BMV-infected cells. And also in the case of other fractions their synthesis was detected (Fig. 2).

Microautoradiography

Chloroplasts purified by Percoll gradient centrifugation from mock-inoculated or

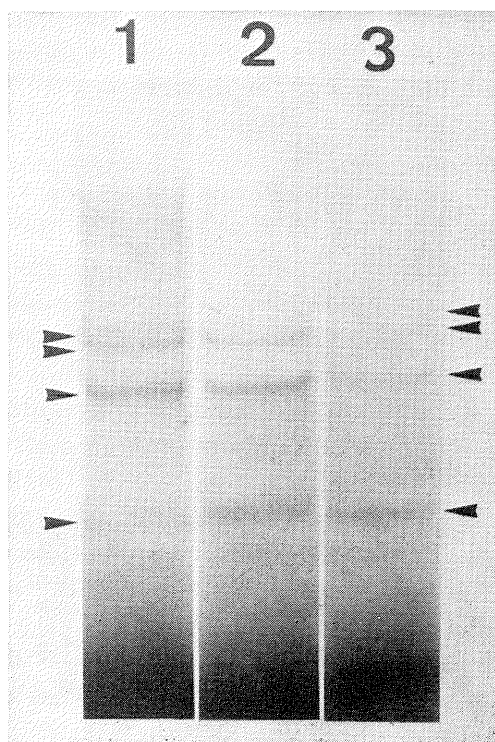


Fig. 2. Autoradiogram of polynucleotides products synthesized *in vitro*. Crude nucleus (lane 1), purified chloroplast (lane 2) and membrane fractions (lane 3) were used for an *in vitro* RNA synthesis in the presence of actinomycin D and α - ^{32}P -UTP as the labeled nucleotide. The reaction mixtures were incubated for 30 min at 30 C. Arrows indicate mobilities of replicative forms of BMV RNAs as markers.

BMV-infected protoplast lysate were added to the reaction mixture and incubated in the presence of actinomycin D and ^3H -UTP. Ultrathin sections for the mass of these chloroplast were subjected to microautoradiography. As shown in Fig. 3, although the most chloroplasts were degraded during incubation, the silver grains were associated with grana lamella. From observation of about 200 chloroplasts, the average number of silver grains per chloroplast of mock inoculated and BMV-infected cells was estimated 0.4 and 1.26, respectively. There was no contamination of any microorganella except for a small amount of mitochondria and the silver grains were not observed in background (Fig. 3B).

Hybridization

To detect the existence of BMV RNA in chloroplasts, a dot blot assay was used. Purified chloroplasts were treated with $10\text{ }\mu\text{g/ml}$ RNase A to avoid contamination of BMV RNA and then RNAs were extracted from the chloroplast fraction, blotted onto a nitrocellulose paper. The blots were hybridized with ^{32}P -labelled cDNA of BMV RNA. Only in chloroplasts isolated from BMV-infected cells hybridized RNAs were detected (Fig. 4).

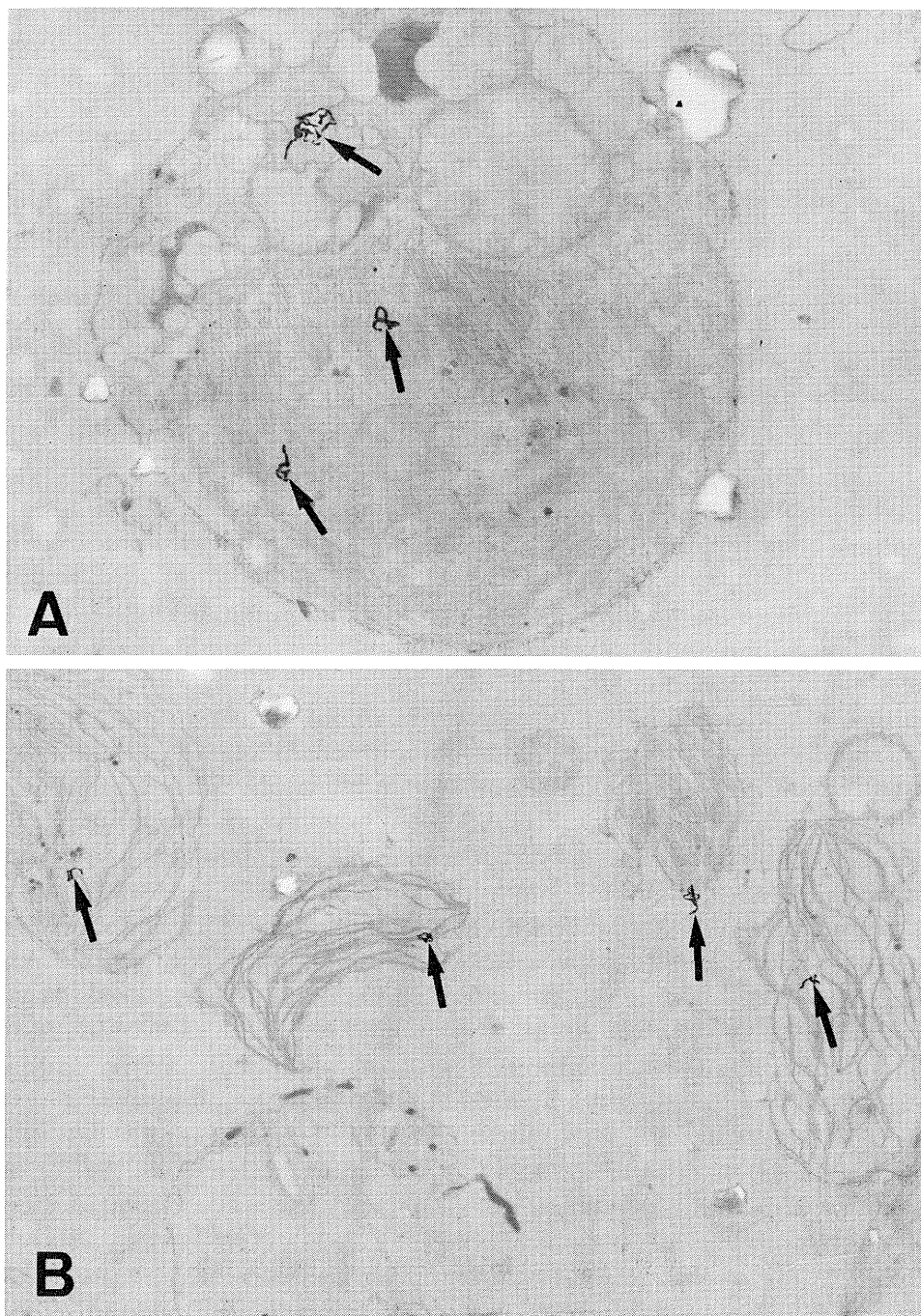


Fig. 3. Autoradiogram of ultrathin sections from purified chloroplast. Purified chloroplasts were used for an *in vitro* RNA synthesis in the presence of actinomycin D and ^3H -UTP as the labelled nucleotide.
(A) $\times 12,000$, (B) $\times 5,000$.

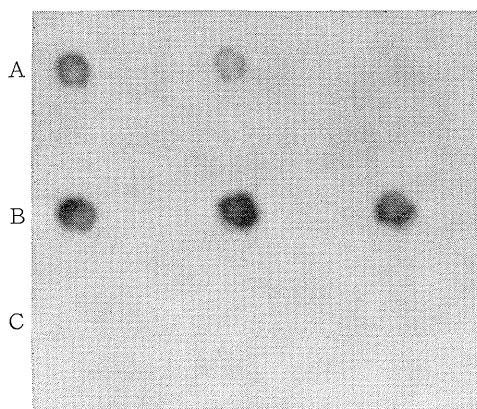


Fig. 4. A dot blot assay of BMV RNA. The total RNA was isolated from chloroplasts, heated to 100 C for 10 min, and quickly cooled. Five μ l of RNA solution was spotted onto the nitrocellulose paper. The dot blot was hybridized with nick-translated cDNA of BMV RNA and exposed to X-ray film for 72 hr at -70 C. (A) 1 ng, 200pg, 40pg of BMV RNA extracted from virions (from left to right), (B) 1, 1/2, 1/4 of total RNA in chloroplasts isolated from BMV-infected cells (10^4 cells), (C) from mock-inoculated cells (10^4 cells).

Discussion

The replication site of BMV RNA was investigated in BMV-infected barley cells and we concluded that the replication of viral RNA would be carried out in chloroplasts from the following evidences: (1) the highest activity of RNA dependent RNA polymerase was detected in a purified chloroplast fraction isolated from BMV-infected cells in the presence of actinomycin D, (2) replicative forms of BMV RNA were synthesized in purified chloroplasts, (3) RNA dependent RNA polymerase activity per chlorophyll was increased with purifying chloroplasts, (4) by microautoradiographic observation, silver grains were observed on the grana lamella when chloroplast fraction was added to the reaction mixture for *in vitro* RNA synthesis instead of an enzyme fraction and incubated in the presence of actinomycin D and ^3H -UTP. The number of silver grains per chloroplast isolated from BMV-infected cells was more than that from mock inoculated cells, (5) RNAs hybridized with cDNA of BMV RNA existed in purified chloroplasts.

From experimental results by the use of marker enzymes, such as succinate dehydrogenase, inosine-di-phosphatase, and glucose-6-phosphatase, as Nilsson-Tillgren *et al.*¹²⁾ did, we excluded the possibility that purified chloroplast fraction included Golgi apparatus and endoplasmic reticulum except small amount of mitochondria (data not shown). The fact was confirmed with the electromicroscopic observation (Fig. 3). Although the activity of RNA dependent RNA polymerase and the synthesis of replicative form were also detected in crude nuclear and membrane fractions (Table 1, Fig. 2), this may be resulted with contaminated chloroplasts, because these fractions showed deep green color.

Viral RNA-replicase complex has been detected in the so-called membrane fraction in CMV⁷⁾, CPMV³⁾, and BMV-infected⁶⁾ cell and could synthesize replicative forms *in vitro* without added template RNA, however, the location of viral RNA replication is still unclear. While in TYMV, it is known that TYMV RNA is synthesized in the outer membrane of chloroplast⁸⁾. Recently, Reinero and Beachy¹³⁾ reported that TMV coat protein was embedded inside or otherwise complexed with the thylakoid membrane of chloroplasts isolated from TMV-infected tobacco cells and suggested that the degree

of mosaic symptom depended on the amount of coat protein in the chloroplasts. Since the replicase of BMV was solubilized only in the presence of detergent, this enzyme might also be embedded in grana membrane.

In the case of plants showing mosaic symptom by viral infection, these results indicate that we should consider not only indirect effect by viral multiplication to chloroplasts but also active participation of chloroplast as a site of viral multiplication.

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和 文 摘 要

中山政治・堀越 守・三瀬和之・山岡直人・朴 杓允・古沢 巖・獅山慈孝：クロロプラストにおけるブロムモザイクウイルス RNA の複製

ブロムモザイクウイルス (BMV) RNA のオオムギ細胞中における複製場所について調べた。各 subcellular 画分は protoplast lysate から分画遠心分離とパーコール濃度勾配遠心分離によって得た。各画分における RNA 合成活性はアクチノマイシン D 存在下で測定した。RNA 依存 RNA 合成酵素活性は純化したクロロプラスト画分で特に高く、クロロフィル当りのその活性はクロロプラストを純化するにつれてさらに高くなった。純化クロロプラスト中に BMV RNA の cDNA とハイブリダイズする RNA が存在することをドットプロット法により確認した。BMV 感染細胞から純化したクロロプラストを RNA 依存 RNA 合成酵素の代りに *in vitro* RNA 合成系に加えると replicative form が合成された。BMV 感染細胞から純化したクロロプラストをアクチノマイシン D および ³H-UTP 存在下の *in vitro* RNA 合成系に加えて培養し、マイクロオートラジオグラフィを行ったところ、チラコイド上に銀粒子が観察された。これらの結果は BMV RNA が BMV 感染オオムギ細胞のすくなくともクロロプラストで複製できることを強く示唆している。