

In vitroにおけるBMV RNA合成に及ぼす外被蛋白質の阻害作用

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著者名	堀越, 守 三瀬, 和之 古澤, 巖 獅山, 慈孝
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Further Studies on Inhibitory Effect of Brome Mosaic Virus Coat Protein on the Viral RNA Synthesis *in vitro*

Mamoru HORIKOSHI*, Kazuyuki MISE*, Iwao FURUSAWA*
and Jiko SHISHIYAMA*

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We previously reported that brome mosaic virus (BMV) coat protein inhibited the viral RNA synthesis *in vitro*¹⁾. In attempt to understand the inhibitory effect of BMV coat protein on BMV RNA synthesis *in vitro*, the 3' terminal 200 nucleotides of genomic RNA 3 (3' T-200GR) which shows template activity for the BMV replicase was prepared. *In vitro* RNA synthesis using 3' T-200GR as template was inhibited by BMV coat protein and 3' T-200GR was not encapsidated by the coat protein. These results suggested that the inhibition of *in vitro* RNA synthesis by BMV coat protein was due to the competition of coat protein and replicase for their binding site in the RNA sequence.

The replicase isolated from brome mosaic virus (BMV)-infected barley leaves can synthesize replicative forms of BMV *in vitro* using BMV RNA as template. Using this system, we have shown that the coat protein of BMV inhibits the viral RNA synthesis *in vitro*¹⁾. In that study, we hypothesized that the mechanism of inhibition is either that template RNAs are encapsidated by the coat protein or that the replicase competes with coat protein for the binding site in RNA sequence. In this paper, the effect of coat protein on RNA synthesis *in vitro* was investigated using 3' terminal 200 bases of genomic RNA 3 (3' T-200GR) prepared from cDNA of genomic RNA 3.

BMV replicase was isolated from BMV-infected barley leaves as described previously¹⁾ and BMV coat protein was prepared by the method of Hiebert *et al.*²⁾ The standard reaction mixture for replicase assay contained 50 mM Tris-HCl, pH 8.2; 10% glycerol; 10 mM KCl; 15 mM MgCl₂; 1 mM EDTA; 2 µg actinomycin D; 50 µg each of ATP, GTP, CTP; 2 µCi [³H]UTP (50 Ci/mmol); 120 units RNasin; 4 mM 2-mercaptoethanol, 5 µg BMV RNA or the same molar 3' T-200GR (0.45 µg) and 40 µl of replicase in a final volume of 130 µl. The mixtures were incubated at 30 C for 30 min and the radioactivity was measured by the paper disc assay³⁾. For analysis of the products of *in vitro* RNA synthesis, 25 µCi of [³²P]UTP (3,000 Ci/mmol) was used in place of [³H]UTP in the reaction mixture described above. ³²P-labeled RNA synthesized *in vitro* was extracted with SDS-phenol and electrophoresed in a 1.2% agarose gel in 40 mM Tris-acetate, pH 8.0, containing 1 mM EDTA. After electrophoresis at 100 V for 3.5 hr, the gel was stained with ethidium bromide, photographed, dried, and exposed to X-ray film at -80 C for 2 days.

Preparation of 3' terminal 200 bases of genomic RNA 3 was carried out as follows. A cDNA of the 3' region of BMV RNA 3 (containing nucleotide 1917 to 2117) was generated by reverse transcriptase with an oligonucleotide primer [d(GACCTGGTCTCTTTTAGA)]. The primer was complementary to 14 nucleotides at the 3' end of the viral RNA and contained a *Tth*III

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

1) Horikoshi, M., Nakayama, M., Yamaoka, N., Furusawa, I. and Shishiyama, J. (1987). *Virology* 158: 15-19. 2) Hiebert, E., Bancroft, J.B. and Bracker, C.E. (1968). *Ibid.* 34: 492-508. 3) Okuno, T. and Furusawa, I. (1979). *Ibid.* 99: 218-225.

restriction site. RNA was degraded by NaOH treatment and second strand was synthesized with the Klenow fragment of DNA polymerase I. The *Sac*I (nucleotide 1480) fragment of BMV cDNA corresponding to the 3' region of BMV RNA 3 was cloned into the plasmid pUC9 at the *Pst*I site, using oligo(dG)-oligo(dC) tailing method. The resultant plasmid, pBT3, was digested with *Hind*III (nucleotide 1917) and *Pst*I. Digestion products were electrophoresed through a 1% low melting point agarose gel, and a band (about 200 bp) was extracted. The fragment containing the cDNA sequence corresponding to 3' terminal 200 nucleotide of BMV RNA 3 and a *Tth*III site was ligated into the *Hind*III/*Pst*I site of transcriptional vector pSP64. The recombinant plasmid was named pBSP3. All recombinant DNA and cloning techniques mentioned above were essentially according to Maniatis *et al.*⁴⁾ The 3' terminal genomic RNAs preserving accurate 3' terminus were synthesized from *Tth*III-linearized pBSP3 DNA as described⁵⁾. The reaction took place at 37 C for 2 hr and incubated for another 15 min at 37 C in the presence of DNase. The mixture was extracted with phenol-chloroform (1 : 1) and then with chloroform. The RNA was recovered by sodium acetate-ethanol precipitation with carrier tRNA. The pellet was dissolved in diethylpyrocarbonate (DEPC)-treated water and then sub-

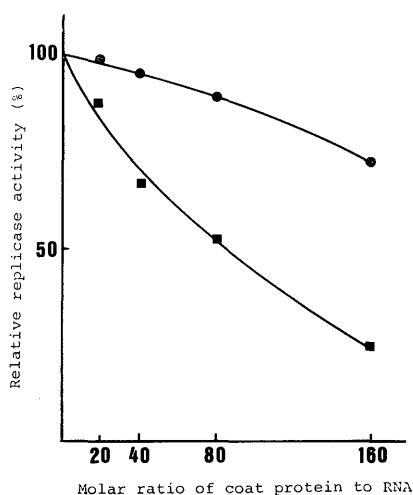


Fig. 1. Effect of BMV coat protein on BMV RNA synthesis *in vitro*. Various molar ratios of coat protein were added to the reaction mixture containing BMV RNA or 3'T-200GR and incubated for 60 min at 30 C. RNA synthesis activity was measured as the incorporation of ³H-UMP into an acid insoluble fraction. Template RNA: ● BMV RNA, ■ 3'T-200GR. When BMV RNA or 3'T-200GR was added to the reaction mixture in the absence of coat protein, incorporation of ³H-UMP was 5,194 or 2,736 cpm, respectively.

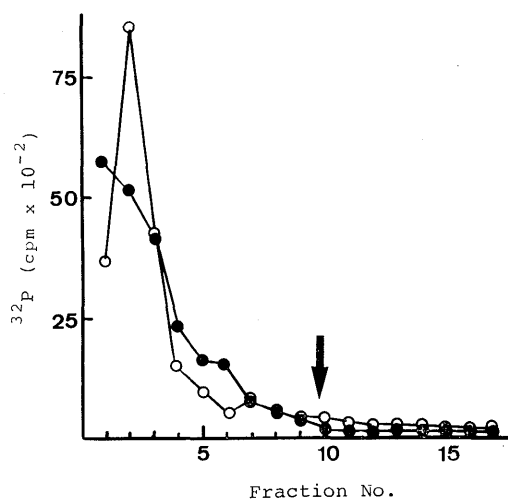


Fig. 2. Sucrose density gradient analysis of BMV coat protein-3'T-200GR complex formed in reaction mixture without enzyme fraction. The reaction mixture containing 0.45 μ g of ³²P-labeled 3'T-200GR and 16 μ g of coat protein was incubated for 15 min and then centrifuged at 45,000 rpm for 90 min at 4 C in a Hitachi RPS-50 rotor through a 10~40% sucrose gradient in 10 mM Tris-HCl, pH 7.4, 10 mM KCl and 5 mM MgCl₂. After centrifugation and fractionation, radioactivity of each fraction was measured. The position of virions (arrow) was determined from absorbance at 260 nm. ●, 3'T-200GR; ○, 3'T-200GR + coat protein.

jected to further purification on a NENSORB column (Du Pont Comp. USA) followed by ethanol precipitation. The amount of RNA synthesized was estimated from the incorporation of radioactivity.

When BMV RNA or 3' T-200GR was added to the *in vitro* RNA synthesis system as template, double-strand RNAs of the expected molecular weights were synthesized by replicase isolated from BMV-infected barley leaves (data not shown). When BMV coat protein was added to this system, RNA synthesis was inhibited in proportion to the amount of BMV coat protein (Fig. 1). The inhibition by the coat protein on RNA synthesis was more effective with 3' T-200GR than with BMV RNA. The coat protein binds to several internal sequence in BMV RNA⁶⁾, therefore, we have hypothesized that the relative ratio of coat protein per one binding site in 3' T-200GR was higher than that in BMV RNA when the same molar amount of coat protein was added to each reaction mixture. This might be the cause of the greater inhibitory effect of coat protein on 3' T-200GR than on BMV RNA (Fig. 1).

To investigate whether 3' T-200GR are encapsidated by coat protein, 0.45 μ g of ³²P-labeled 3' T-200GR and 16 μ g of coat protein were added to the reaction mixture without enzyme fraction, and incubated for 15 min at 30 C. The mixture was analyzed by 10–40% sucrose density gradient centrifugation. The complex of coat protein and 3' T-200GR showed slightly higher sedimentation value than that of 3' T-200GR alone, but no radioactivity was detected in the position of virions (Fig. 2). Although the binding site of coat protein was supposed to be in the sequence of 3' T-200GR because RNA synthesis on 3' T-200GR was inhibited by the coat protein *in vitro*, 3' T-200GR was not encapsidated by the coat protein. We have shown previously that *in vitro* RNA synthesis was completely inhibited even if BMV RNA is not fully encapsidated¹⁾. The results in this paper, suggest that the inhibition of *in vitro* RNA synthesis by coat protein is due to the competition of coat protein and replicase for their binding site in the RNA sequence.

和 文 摘 要

堀越 守・三瀬和之・古澤 巖・獅山慈孝：In vitro における BMV RNA 合成に及ぼす外被蛋白質の阻害作用

われわれはすでに BMV RNA レプリカーゼによる *in vitro* BMV RNA 合成が、BMV の外被蛋白質によって阻害されることを報告した。今回は BMV RNA 3 の cDNA から鋳型活性のある 3' 端 200 塩基の RNA (3'-T200GR) を作製し、外被蛋白質による *in vitro* RNA 合成に対する阻害作用を調べた。その結果、3'-T200GR を鋳型とした RNA 合成は BMV 外被蛋白質によって阻害されたが、3'-T200GR は外被蛋白質によってウイルス粒子として再構成されなかった。これらの結果から、*in vitro* BMV RNA 合成の阻害は RNA 鎖中のレプリカーゼ結合部位をレプリカーゼと外被蛋白質が競合した結果であると推察した。

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