Characterization and Localization of the Viral RNA Replication Complex in Tobacco Plants Infected with Cucumber Mosaic Virus

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Abstract

Activity of membrane-bound RNA-dependent RNA polymerase (M-RDRP) from cucumber mosaic virus (CMV)-infected tobacco plants increased prior to virus multiplication and that of soluble enzyme (S-RDRP) increased rapidly after concentration of CMV has reached its maximum. *In vitro* products of M-RDRP was mainly double-stranded CMV RNAs as replicative forms. M-RDRP was suggested to be associated with endoplasmic reticulum (ER) by subcellular fractionation of the membrane-fraction using Percoll discontinuous gradient-centrifugation and electron microscopic observation. The ER-associated enzyme had viral RNAs as endogenous templates and was firmly integrated into membrane structure, even after treated with detergent or Mg^{++} -depletion or partially purified by Sepharose 4B chromatography. The purification procedures could effectively remove S-RDRP which synthesized small heterogeneous RNA *in vitro*. Joined with our previous study using CMV-infected tobacco protoplasts, it was suggested that S-RDRP is a host-encoded enzyme induced in leaf tissues but not in protoplasts as a consequence of virus infection process like "Pathogenesis-related proteins." We speculate that the CMV RNA replication complex is composed at least of translation products of viral RNAs, ER membranes and viral template RNAs.

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Key words: cucumber mosaic virus, RNA-dependent RNA polymerase, replicase, endoplasmic reticulum.

INTRODUCTION

Cucumber mosaic virus (CMV) is a plant virus belonging to Cucumovirus group which has a tripartite RNA genome¹⁵⁾. Infection with CMV has been reported to induce particularly high RNA-dependent RNA polymerase (RDRP) activity in cucumber cotyledons and in tobacco plants^{3,18)}. The purified soluble enzyme (S-RDRP) from CMV-infected tobacco plants was shown to be indistinguishable from the enzyme found in uninfected tobacco plants, indicating that S-RDRP is host-encoded, and was clearly different from that which was induced in cucumber plants on grounds of protein composition and other enzymological properties¹⁸⁾. In CMV-tobacco protoplast system, however, we recently reported that its activity was very low and remained almost unchanged upon infection with CMV, suggesting that S-RDRP is not involved in viral RNA replication¹⁴⁾. The report clearly demonstrated that the membrane-bound enzyme

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(M-RDRP) found in CMV-infected tobacco protoplasts synthesized *in vitro* plus-strand virus RNAs 1-4 as double stranded forms. The M-RDRP activity was also induced by inoculation only with CMV RNAs 1 and 2.

In the present study, we investigated the time course of the changes in activity of S- and M-RDRP in tobacco leaves after infection with CMV to compare with that in CMV-tobacco protoplast system¹⁴⁾ in order to know in more detail about the significance of both enzymes in virus RNA replication. In addition, localization of M-RDRP was investigated by subcellular fractionation and electron microscopy to know which organelle(s) M-RDRP would be associated with. Partial purification and analyses of *in vitro* products of the enzyme were also described.

MATERIALS AND METHODS

Plants, virus and RNAs. Tobacco plants (*Nicotiana tabacum* cv. Xanthi nc) were grown in an air conditioned greenhouse (22-28 C) and used for the preparation of the enzyme extracts and propagation of CMV (CMV-Y) from which satellite RNA was eliminated¹⁷⁾. CMV dsRNAs were purified from CMV infected tobacco plants according to the method of Morris and Dodds¹²⁾ and were used as markers for gel electrophoresis.

Fractionation of S- and M-RDRP. Tobacco leaves inoculated with CMV were harvested at daily intervals and stored frozen at -75 C before use. The infected leaves were homogenized using a mortar and pestle after adding per gram of plant material 2 ml of Buffer A (50 mM Tris-HCl, pH 8.0, 0.1 M NH₄Cl, 10 mM MgCl₂, 90 mM 2-mercaptoethanol, 1 mM EDTA and 15% glycerol). The homogenate was squeezed through Miracloth (Cal Biochem) and centrifuged at 1,000 × *g* for 10 min. A portion of the supernatant was set aside for assay of the concentration of CMV on cowpea leaves. The supernatant was centrifuged again at 20,000 × *g* for 20 min. Resultant supernatant was mixed with an equal volume of saturated (NH₄)₂SO₄, then the precipitate formed was collected by centrifugation at 10,000 × *g* for 20 min. The pellet dissolved in the extraction buffer (0.25 ml/g of starting leaves) was used as S-RDRP. The pellet after centrifugation at 20,000 × *g* was washed once with Buffer A. The final pellet resuspended in Buffer A (0.25 ml/g of starting leaves) was used as M-RDRP.

Subcellular fractionation of the M-RDRP. CMV-inoculated tobacco leaves (10 g) were harvested 3 days after inoculation and were homogenized using a mortar and pestle after adding 20 ml of buffer B (0.3 M mannitol, 20 mM Tris-HCl, pH 8.0, 0.1 M NH₄Cl, 3 mM MgCl₂, 0.1% BSA and 60 mM 2-mercaptoethanol). The homogenate was squeezed through Miracloth and centrifuged at 1,000 × *g* for 10 min. The supernatant was centrifuged at 20,000 × *g* for 20 min. The resultant pellet was resuspended in either Buffer B or one containing 3 mM EDTA instead of MgCl₂ and layered onto a discontinuous Percoll (Pharmacia) gradient (made of 60%, 45%, 28% and 5% Percoll in 0.25 M sucrose, 20 mM Tris-HCl, pH 8.0, 0.1 M NH₄Cl, 10 mM DTT, and 1 mM EDTA or 3 mM MgCl₂) prepared essentially according to Nishimura *et al.*¹³⁾ The tubes were centrifuged for 30 min at 16,000 rpm in a Beckman SW 41 rotor at 4 C. All procedures were done on ice or at 4 C.

To obtain a large amount of membrane-fraction, the infected leaves were homogenized directly in Buffer C (0.3 M mannitol, 50 mM Tris-HCl, pH 8.0, 0.1 M NH₄Cl, 90 mM 2-mercaptoethanol and 1 mM EDTA) using an electric blender. The homogenate was squeezed through Miracloth. The 20,000 × *g* pellet was obtained from the filtrate in the same manner as described above and referred to as crude M-RDRP. The pellet was fractionated using a large scale Percoll gradient containing 1 mM EDTA and a Beckman SW 25.2 rotor. After centrifugation on the gradient, the interface between 5% and 28% Percoll was collected and centrifuged at 100,000 × *g* for 1.5 hr. Precipitated membranes were resuspended in Buffer D containing 20 mM Tris-HCl, pH 8.0, 0.1 M NH₄Cl, 1 mM EDTA, 0.1 mM DTT and 15% glycerol (0.1 ml/g of starting leaves) and stored frozen at -75 C. The fraction was rich in endo-

plasmic reticulum¹³). Hence, it was termed an ER-fraction. It was notable that M-RDRP activity recovered in ER-fraction was very low if the starting leaves had been frozen before use (data not shown).

Electron microscopy. CMV-infected tobacco leaves were harvested 2–3 days after inoculation and protoplasts were prepared from the leaves as previously described²⁰. The protoplasts were suspended in a medium containing 0.3 M mannitol, 50 mM Tris-HCl, pH 8.0, 0.1 M NH₄Cl, 90 mM 2-mercaptoethanol, and either 1 mM EDTA or 3 mM MgCl₂, and were then ruptured through a syringe needle. Subcellular fractionation by the successive centrifugations followed by discontinuous Percoll gradient centrifugation was done as described above. Particulate components at the interfaces between 5 and 28% of Percoll, and 28 and 45% of Percoll were pooled separately. The samples were fixed with glutaraldehyde and OsO₄, then embedded in Spurr's low viscosity medium essentially according to Lord *et al.*¹⁰ Ultra-thin sections were cut, poststained in uranyl acetate and lead citrate, and examined on a JEM-100B electron microscope.

RDRP assay and extraction of synthesized products. The standard assay for S- and M-RDRP was carried out using [³H]-GTP as a radioactive precursor as previously described¹⁴. [³H]-RNA synthesized *in vitro* was extracted from large scale reaction mixtures (0.5–2 ml) containing twice concentration of [³H]-GTP and no exogenous template RNA. After inoculation, the reaction mixtures were made 1% with respect to sodium dodecyl sulfate (SDS) and treated with 50 µg/ml of Proteinase K at 37 C for 15 min. The RNA in the mixtures was extracted twice with one volume of water-saturated phenol containing 0.1% 8-hydroxyquinoline and collected by ethanol precipitation.

RESULTS

Time course of appearance of S- and M-RDRP activities

Studies on the time course of RDRP activity and CMV concentration were summarized in Fig. 1. Progeny CMV was detectable a day after inoculation and reached a maximum on day 3, while increase in M-RDRP activity preceded CMV multiplication and the activity reached a plateau on day 2. On the other hand, S-RDRP activity which required the addition of the exogenous template RNA for activity began to rise after CMV concentration had reached its maximum and increased rapidly in the later stages of infection. M-RDRP did not require addition of the template RNA for activity until 2–3 days after inoculation. M-RDRP activity increased again, coincided with increase in that of S-RDRP. The activity became to show template dependency to some extent, though showing no template specificity (data not shown).

Localization of M-RDRP

The membrane-fraction prepared from infected leaves 3 days after inoculation was fractionated by the discontinuous Percoll density-gradient centrifugation in the presence of Mg⁺⁺ or EDTA. Assay for RDRP over the gradient containing EDTA showed a major peak that was at the interface between 5% and 28% of Percoll (band A) which was white with pale yellowish green (Fig. 2A). On the other hand, when the gradient contained Mg⁺⁺, the sedimentation behavior of the enzyme activity changed drastically and a greater portion of the activity moved into the lower interface between 28% and 45% of Percoll (band B) which was white in color (Fig. 2B). Substantial activity showing template dependency was observed at the top of the gradients, which was considered to be S-RDRP contaminated in the membrane fraction or released from the membranes during the centrifugation.

Electron microscopic study revealed that the band A was predominantly typical segments of endoplasmic reticulum (ER) in character (Fig. 3A) and band B contained mainly mitochondria and other microbodies (Fig. 3B) when EDTA was included in the buffer system. On the other hand, in the presence of Mg⁺⁺, the band B also contained ER in addition to mitochondria and other microbodies (Fig. 3D). Lord *et al.*¹⁰ reported that density of the ER of castor bean in-

Fig. 1 Days after inoculation

Fig. 2

Fig. 1. Time course of the appearance of S- and M-RDRP activities in CMV-infected tobacco plants. S- (Δ , $\blacktriangle$) and M-RDRPs ($\circ$, $\bullet$) prepared from CMV-infected tobacco leaves which were harvested at daily intervals were assayed in the presence (Δ , $\circ$) or absence ($\blacktriangle$, $\bullet$) of exogenous CMV RNA as a template. Relative infectivity of CMV ($\square$) was determined by local lesion assay using cowpea leaves. Note the difference in scale between M and S-RDRP.

Fig. 2. Changes in the distribution of RDRP activity when the membrane-fraction was separated by centrifugation in a discontinuous Percoll density-gradient column containing 1 mM EDTA (A) or 3 mM $MgCl_2$ (B). Arrows indicate the positions of interface between different concentrations of Percoll. Enzyme activity was assayed in the presence ($\circ$) or absence ($\bullet$) of CMV RNA as a template.

Fig. 3. Electron micrographs of material recovered from the interfaces in Percoll density-gradient columns which had been centrifuged as shown in Fig. 2. (A) and (B) indicate materials at the interfaces between 5% and 28% and between 28% and 45% of Percoll, respectively, in the presence of EDTA. (C) and (D) indicate those at the corresponding interfaces in the presence of Mg^{++} . All pictures were taken at the same magnification.

creased by attachment of ribosomes to the membrane surface in the presence of Mg^{++} instead of EDTA. It is, therefore, suggested that the enzyme is associated with the membranous materials, possibly the ER.

Partial purification of ER-fraction

When the NP-40 treated ER-fraction was fractionated with gel-filtration using a Sepharose 4B column, M-RDRP activity was eluted at the void volume (Fig. 4) as described by Dorssers *et al.*²⁾ with cowpea mosaic virus (CPMV) replication complex. The activity was recovered in pellet after centrifugation at $100,000 \times g$ for 1.5 hr. The pellet was resuspended in Buffer D containing 0.1% Nonidet P-40 and the solution was referred to as NP-40 enzyme. This fraction was slightly turbid and free from green pigments. M-RDRP at the main peak did not require addition of exogenous template RNA for activity, indicating that the enzyme complex still had template RNAs even after treated with the detergent. A little RDRP activity showing template dependency was eluted at fractions 27 to 30, which would be S-RDRP released from the membranes.

Analysis of RNA products synthesized *in vitro*

Radioactive RNAs synthesized *in vitro* using various membrane-bound enzymes, which were prepared from the inoculated leaves 3 or 7 days after inoculation, were resolved in agarose gels. Three major RNA species, which comigrated with CMV dsRNA 1, 2 and 3, were observed under native condition (Fig. 5, lanes 1–8). In addition to the known viral RNA products, considerable incorporation of [3H]-GMP into small and polydisperse RNAs was observed especially when the crude membrane-fraction and ER-fraction prepared from the leaves 7 days after inoculation were used (lanes 3 and 4), which was the similar case with the results in the experiments using CMV-infected cucumber cotyledons⁶⁾. Electrophoresis under denaturing conditions revealed that four species of full-sized CMV RNA (RNA1–4) were synthesized *in vitro* on the endogenous template RNAs contained in the enzyme preparations (lanes 10–12). In a set of pulse-chase experiments, a smear of radioactivity upward from the replicative form (RF) bands was observed after pulse for 15 min, and then disappeared after chase for 45 min and faded into RFs (lanes 5, 6 and 9, 10). The pulse-labeled smear probably represent the replicative intermediate (RI). The total RNA products were found to be 69% resistant to RNase digestion in $2 \times SSC$. Reduction of the radioactivity by the RNase treatment should be due

Fig. 4. Purification of CMV RNA replication complex by Sepharose 4B column chromatography. ER-fraction (1.4 ml) prepared as described in the text was treated with 1% of a nonionic detergent, Nonidet P-40 for 1 hr with stirring and centrifuged at $20,000 \times g$ for 20 min. The supernatant was applied onto a Sepharose 4B column (0.9×50 cm) in Buffer D containing 0.1% Nonidet P-40 as described by Dorssers *et al.*²⁾ and eluted with the buffer at a flow rate of 1.9 ml/hr. Fractions (1 ml) were collected and assayed for RDRP activity in the presence (○) or absence (●) of CMV RNA as a template.

Fig. 5. Electrophoretic analyses of *in vitro* products of crude membrane, ER-fraction and NP-40 enzyme. RNA samples were electrophoresed in horizontal 1.5% agarose gels in 10 mM Na-phosphate, pH 7.0, under native (Lanes 1–8) or glyoxal-denaturing conditions (Lanes 9–12)¹¹⁾. The radioactive bands were detected by fluorography using EN³HANCE (NEN) and Kodak X-Omat R film which was pre-exposed according to Laskey and Mills⁸⁾. Products synthesized *in vitro* for 60 min in the presence of [³H]-GTP using crude membrane fraction (lanes 1 and 3) and ER-fraction (lanes 2 and 4), which were prepared from the leaves 3 days (lanes 1 and 2) and 7 days (lanes 3 and 4) after inoculation, were compared with each other under native condition. ER-fraction prepared from leaves 3 days after inoculation was incubated for 15 min (lanes 5 and 9), followed by further incubation for 45 min with excess amount of unlabeled GTP (100 times of the labeled GTP) (Lanes 6 and 10) or was incubated for 60 min (lanes 7 and 11). NP-40 enzyme was also incubated for 60 min (lanes 8 and 12).

to vanishing of single-stranded RNA tails in the partially double-stranded RNAs (RI) and, if any, single-stranded RNAs free from the endogenous template RNA. RNA-RNA hybridization test showed that the RNase-resistant RNA products represented plus-stranded CMV RNA (data not shown), as previously shown in the experiments using CMV-infected protoplasts system¹⁴⁾. Taken together, it is suggested that M-RDRP catalyses continuous plus-strand elongation on viral RI and the catalytic process terminates when RI is converted to the RF, as shown with CPMV replication complex²⁾ and with particulate fraction in CMV infected cucumber cotyledons⁶⁾. *In vitro* products of NP-40 enzyme (lanes 8 and 12) were shown to be essentially the same as those of ER-fraction enzyme (lanes 7 and 11) except that the low molecular weight RNAs were not detected, indicating that the characteristics of NP-40 enzyme had essentially unchanged upon the non-ionic detergent treatment.

DISCUSSION

RNA-dependent RNA polymerase in soluble form, which is increased in amounts by RNA virus infection, has no template specificity, and RNA synthesized *in vitro* by the enzyme is complementary to the RNA provided as the template. Furthermore, the size of the products is

usually much smaller than that of template RNAs^{5,7,9,16}). Thus, the question whether these enzymes are actually involved in viral RNA replication had remained controversial. For example, Jaspars *et al.*⁶) speculated that the host RNA polymerase would be involved in the initiation of the synthesis of CMV RNAs, because even after the particulate fraction was subjected to an extensive solubilization and washing treatment, a large amount of small, heterogeneous RNA, which was thought to be synthesized by the host enzyme, was still produced. However, the activity of S-RDRP was not induced at all in CMV infected tobacco protoplasts¹⁴) and the study reported here showed that the enzyme activity increased only after virus concentration had reached its maximum in CMV-infected tobacco leaves. These results show that the host enzyme is enhanced only at the tissue level as suggested with CPMV infected cowpea leaves and protoplasts²¹), and the induction of the enzyme production is a consequence of the virus infection process like the case such as "Pathogenesis-related proteins", rather than that the enzyme is involved in CMV replication. Indirect evidence¹⁹) showing that S-RDRP was not elicited by viral gene by experiments using cucumber cotyledons inoculated with separate CMV RNA(s) would support this speculation. Significance of the host-enzyme in plant physiology and physiological plant pathology is still obscure.

M-RDRP in the later stage of infection synthesized *in vitro* a large amount of small, heterogeneous RNAs in addition to the ds CMV RNAs (Fig. 5). This fact would coincide with the increase in the enzyme activity of the membrane-fraction which required the addition of exogenous template RNA for full incorporation of ³H-GMP into total RNAs synthesized *in vitro* (Fig. 1). A small amount of the small RNAs was also observed in the crude membrane or ER-fraction prepared from the leaves 3 days after inoculation, but not in NP-40 enzyme (Fig. 5), suggesting that S-RDRP was contaminating the membrane-fraction and could be released during purification procedures as shown in Fig. 2 and Fig. 4. In fact, according to our unpublished results, the enzyme released from the membrane-fraction by treatment with detergent or Mg⁺⁺-depletion²²) could be purified by column chromatography using Poly-U or Blue-Sepharose followed by glycerol gradient-centrifugation as described by Takanami and Fraenkel-Conrat¹⁸) and the purified enzyme was shown to have the same mobility as the soluble, host enzyme in SDS-polyacrylamide gel electrophoresis.

M-RDRP involved in CMV RNA replication was suggested to be associated with ER membranes by the fact that the behavior of M-RDRP coincided with that of ER in the density-gradient centrifugation in the presence or absence of Mg⁺⁺, and could not be solubilized or released from the membrane-structures by Mg⁺⁺-depletion nor detergent treatment. As to the association of CMV replication with cytoplasmic membranes, similar conclusion has been drawn in previous studies showing; (1) CMV and its satellite dsRNAs copurified with a size-homogeneous vesicular fraction¹) (2) Electron microscopic examination of CMV and tomato aspermy virus-infected leaf cells revealed the presence of membrane-bound vesicles associated with the tonoplasts⁴). Joined with our previous study¹⁴), we can speculate that CMV RNA replication complex comprises at least of ER membranes in addition to translation products of CMV RNA 1 and 2, and templates RNAs.

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和 文 摘 要

高浪洋一・新田直人・久保 進：キュウリモザイクウイルス感染タバコ葉における ウイルス RNA 複製複合体の性質と所在

キュウリモザイクウイルス (CMV) を接種したタバコ葉から膜結合 (M) ならびに可溶性 (S) の RNA 依存 RNA 合成酵素画分を調製し、それらの酵素活性とウイルス濃度の推移を調べた結果、M 活性はウイルス濃度の増加に先行して上昇し、S 活性はウイルス濃度が飽和した後に急上昇した。M 酵素の試験管内合成産物は主として複製型の 2 本鎖 CMV RNA であった。Percoll 不連続密度勾配遠心分画ならびに電子顕微鏡観察の結果から、M 酵素は細胞内小胞体に関係していることが示唆された。M 酵素は内在性鋳型としてウイルス RNA を含んでおり、界面活性剤や Mg^{++} イオン欠乏処理あるいは Sepharose 4B カラムクロマトグラフィーによる精製を行った後も、細胞内膜構造に強固に結合していた。これらの精製操作によって、試験管内で低分子の RNA を合成する S 酵素を M 酵素から除去することが可能であった。タバコプロトプラストを用いた実験の結果では S 酵素は CMV 感染によってもまったく誘導されず、この酵素は葉組織レベルでのみ誘導される宿主由来の酵素であり、PR 蛋白質と同様にウイルス感染の結果として生ずるものと考えられた。CMV RNA 複製酵素複合体は少なくともウイルス RNA の翻訳産物、宿主細胞の小胞体ならびに内在性鋳型ウイルス RNA により構成されると推察された。