Time Course Analysis of Immunolocalization of Coat Protein and Replicase Proteins in Protoplasts Inoculated with Tobacco Mosaic Virus

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Abstract

The localization of coat protein and putative viral replicase proteins (130 kDa and 180 kDa) of tobacco mosaic virus (TMV) in tobacco protoplasts was examined at different post-inoculation intervals, using specific antibodies and protein A-gold complex. Gold labels specific to antiserum against virus particle were first detected 4 hr postinoculation (p. i.) in small areas of the cytoplasm, where virus particles were not initially observed. At 6-15 hr p.i. when virus multiplication was active, virus particles were accumulated and formed a paracrystalline array in the cytoplasm. Specific labels were found only on the crystalline aggregates and in the cytoplasm near the aggregates. Many virus aggregates were observed in each section of protoplasts at this stage. At 24 hr p.i., larger aggregates of virus particles were heavily labelled, while areas of the cytoplasm, where no virus particles were evident, were labelled scarcely. Nuclei, plastids and mitochondria were free of labels throughout the infection stages. Gold labels for 130 kDa protein were first detected on small TMV-specific inclusions in the cytoplasm at 4 hr p.i.: Thereafter, these inclusions grew both in number and size, and occurred frequently in the vicinity of virus aggregates. Labels were localized on the inclusions but not on virus aggregates throughout the infection stages. Those inclusions have dense labels and grew a little further in size, though virus replication at this stage declined to a low level. Gold labels for 180 kDa protein on the inclusions and other parts of protoplasts at each stage were observed similarly as the labels for 130 kDa protein. These observations indicate that replication of genomic RNA, synthesis of coat protein and subsequent virus assembly do take place at limited areas in the cytoplasm, where virus particles appeared and formed crystalline aggregate at later stages.

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Key words: tobacco mosaic virus, replicase genes, coat protein, immunogold electron microscopy.

INTRODUCTION

Tobacco mosaic virus (TMV) is a rod-shaped virus with a length of 300 nm and a diameter of 18 nm, made up of about 2,100 subunits of a single coat protein species and a single-stranded messenger-sense genomic RNA. This genomic RNA is about 6,400 nucleotides long and encodes at least four proteins; three non-structural proteins (130 kDa, 180 kDa, and 30 kDa) and 17.5 kDa coat protein^{5,11)}.

Establishment of *in vitro* transcription of infectious RNA from full-length cDNA of TMV^{2,7,10)} enabled us to reveal the functions of these viral proteins by reverse-genetic approaches. The techniques

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of making transgenic plants in parallel but in part contributed to the clarification of functions. 130 kDa and 180 kDa proteins are considered to be components of TMV replicase⁶⁾. The 30 kDa protein functions in cell-to-cell movement of the virus in plant tissues^{3,8)}, presumably through plasmodesmata^{1,17)}. The coat protein is used for encapsidation of the newly synthesized genomic RNA into virus particles and also involved in long-distance movement, like from one leaf to another^{14,16)}.

Previously we have investigated the temporal profiles of synthesis of these TMV-encoded proteins and corresponding mRNA in protoplasts which were synchronously inoculated with viral RNA^{19,20)}. The protoplast system has several advantages over the intact plant system. First, many cells are able to be simultaneously inoculated with virus at a high frequency. Secondly, protoplasts from suspension culture cells have relatively small vacuoles and rather abundant cytoplasm. Vacuoles in cells of intact plant tissues usually occupy a large fraction of cell volume and leave a small fraction for cytoplasm between vacuoles and along with the cell membrane. Use of the protoplast system provides an opportunity to look more closely the events occurring in the cytoplasm after virus infection. In this paper, we demonstrate intracellular localization of the TMV-encoded proteins (130 kDa, 180 kDa and coat proteins) in protoplasts as a function of time after inoculation by using specific antibodies and protein A-gold complex, and discuss the spatial relation of virus replication and virus assembly sites.

MATERIALS AND METHODS

Virus. The Japanese common strain of TMV (OM), which was propagated in *Nicotiana tabacum* L. cv. Xanthi, was used. TMV RNA was prepared from purified virus as described previously¹⁶⁾.

Antisera. Anti-TMV serum was produced by injecting rabbits with virus preparations purified by differential and sucrose density gradient centrifugations. Antibody titer against purified TMV was 1/1024 in the microprecipitin test. Western blot analysis showed that the antiserum is also highly reactive to monomeric coat protein subunits (data not shown)¹⁴⁾.

Antisera to 130 kDa and 180 kDa proteins were raised separately against fusion proteins (composed of β -galactosidase and either 130 kDa protein or 180 kDa protein) which were produced in *E. coli*¹²⁾.

The antiserum to 130 kDa protein reacts with both 130 kDa and 180 kDa proteins, while the antiserum to 180 kDa protein reacts only with 180 kDa protein¹²⁾. Both antisera react rarely with host proteins¹²⁾.

Viral RNA inoculation of protoplasts. Protoplasts which were prepared from suspension culture tobacco cells (*Nicotiana tabacum* L. cv. BY-2) were inoculated with TMV-RNA by the electroporation method as described previously²⁰⁾. Inoculated protoplasts were cultured in modified MS medium supplemented with 0.4 M mannitol¹⁸⁾ at a concentration of 2×10^5 protoplasts/ml.

Immunolocalization with antibodies and protein A-gold complex. Protoplasts were collected in 15-ml tubes at 4, 6, 8, 12, 15, 18, 24 or 48 hr postinoculation (p.i.) by a brief centrifugation and then fixed with 2.5% glutaraldehyde in 0.1 M Na phosphate (pH 7.4)–0.4 M mannitol, for 2 hr at room temperature. Fixed protoplasts were treated with 0.1 M NH_4Cl for 30 min, rinsed with 0.1 M Na phosphate (pH 7.4), and then dehydrated in a series of ice cold ethanol (30% to 100%)⁹⁾. Protoplasts were transferred to polyethylene capsule tubes (BEEM, USA) and packed by a brief centrifugation. The dehydrated samples were infiltrated with Lowicryl K4M (Polysciences, USA) at -20°C for 24 hr and embedded by UV-polymerization for 48 hr at -20°C . Ultrathin sections were cut from embedded samples using a glass knife and mounted on formvar-coated 200-mesh nickel grids.

The grids were floated on drops of 1% bovine serum albumin in PBS (10 mM Na phosphate, pH 7.2, 150 mM NaCl) for 30 min at room temperature, and then were placed on drops of antiserum at 1 : 400–1 : 1000 dilutions for 2 hr. The specimens were washed with PBS four times for 10 min each and treated with protein A-gold complex (15 nm gold particles, EY laboratory, USA) for 1 hr at room temperature. After rinsing with PBS and then with distilled water, sections were stained with uranyl acetate for 10 min and then with lead citrate for 3 min. Specimens were examined under a JEM-100C electron microscope (JEOL, Ltd., Japan).

RESULTS

Immunogold labelling for coat protein

Specific gold labels for coat protein were first observed in restricted areas of the cytoplasm in protoplasts at 4 hr postinoculation (Plate I-1 and 2). There were no characteristic features in cytoplasm around the areas with labels, although endoplasmic reticulum (ER) was often observed in their vicinity (Plate I-2). At this stage, virus particles were not discernible in the areas or in any other portions of the cells.

At 6 hr p.i., labelled areas of the cytoplasm varying in sizes were found frequently in protoplasts (Plate I-3 and 4). Labelled areas especially larger ones had virus particles, some of which were arranged close each other and had an intense immunolabelling (Plate I-3, 4 and 5). Gold labels were often found in the cytoplasm just adjacent to virus aggregates (Plate I-5), where no virus particles were observed, indicating that the areas of cytoplasm can be nascent accumulation/assembly sites of coat protein.

During the period of 6 to 12 hr p.i., crystalline virus aggregates in the cytoplasm grew larger with advance of postinoculation time. Gold labels were found exclusively in virus aggregates or in the neighboring cytoplasm at the later stage of infection as 12 hr p.i. (Plate II-6). At 24 hr p.i., much larger aggregates of virus particles were observed in the cytoplasm and had intense labels (Plate II-7). At this stage, gold labels were scarce in the cytoplasm where no virus particles were observed (Plate II-7). At 48 hr p.i., virus aggregates occupied a substantial fraction of cytoplasm and were heavily labelled. Labels were observed also in the cytoplasm apart from viral crystalline aggregates. In some protoplasts, labels were noted more frequently in the cytoplasm at this stage (Plate II-8).

Granular inclusions in which 130 kDa and 180 kDa proteins were detected by immunogold labelling in our earlier report¹³⁾ appeared in the vicinity of crystalline virus aggregates. The inclusions were not labelled by antiserum to TMV (Plate II-7).

The nuclei as well as plastids or mitochondria were free of labels for coat protein throughout the infection stages (Plate I-5, Plate II-6, 7 and 8).

Virus particles were occasionally observed scattering in the vacuoles at 48 hr p.i. These virus particles might have dispersed from cytoplasm into the vacuoles due to incidental breakages of the tonoplast during the fixation or embedding process of protoplasts.

Sections from noninoculated protoplasts that were incubated with antiserum to TMV or sections from TMV-infected protoplasts that were treated with preimmune serum had few labels due to non-specific bindings.

Immunogold labelling for 130 kDa or 180 kDa protein

When sections of protoplasts at 4 hr p.i. were treated with antiserum to 130kDa protein, gold labels were observed on infection-specific small electron-dense structures in the cytoplasm (Plate III-9). These novel electron-dense structures are considered to correspond to a premature form of the granular inclusions which we have previously reported in TMV-infected tobacco leaf cells¹³⁾. At 6 hr p.i., inclusions grew further both in number and size (Plate III-10).

At 12 hr p.i., many heavily labelled inclusions varying in size and shape occurred in the cytoplasm. Larger inclusions were irregular in shape. Smaller inclusions seemed to be newly formed (Plate III-11). These inclusions were observed usually in the vicinities of virus aggregates. Labels for 130kDa protein were not noted in virus aggregates throughout the infection stages. At 18 or 24 hr p.i., inclusions were even larger in size and elliptic rather than irregular in shape (Plate IV-12 and 13). These inclusions contained fibrillar structures and often had small electron translucent vacuole-like areas within them, but no surrounding membranes. These inclusions had intense labels (Plate IV-12 and 13). At 48 hr p.i., inclusions were elliptic in shape, and had intense labels (Plate V-14 and 15).

Throughout the time-course analysis for 130 kDa protein, gold labels were not observed to be associated with the other organelles such as nuclei, plastids, mitochondria and vacuoles.

When the sections of TMV-inoculated protoplasts were treated with antiserum to 180 kDa protein, which reacts to 180 kDa protein but not to 130 kDa protein, gold labels were observed only on the

granular inclusions which were tagged with antiserum to 130 kDa protein. However, the number of labels on the inclusions was less than that observed when sections were labelled for 130 kDa protein (Plate VI-16) as described earlier¹³⁾.

Specific labelling for either 130 kDa or 180 kDa proteins was observed neither on sections of mock-inoculated protoplasts nor on sections of TMV-infected protoplasts treated by preimmune serum, except a few scattering labels that seemed to be non-specific.

DISCUSSION

In TMV-infected protoplasts, gold labels for 130 kDa and 180 kDa proteins were observed only in granular inclusions in the cytoplasm. These proteins are involved in the synthesis of genomic RNA⁶⁾ and presumably also in subgenomic RNAs²¹⁾. Combining available informations, it can be imagined that subgenomic mRNA for coat protein was first synthesized within or around the granular inclusions. It has been shown that TMV accumulation in protoplasts was completely inhibited by cycloheximide but not by chloramphenicol¹⁵⁾. This fact indicates that 80 S ribosomes in the cytoplasm are used for virus protein synthesis but not 70 S ribosomes in organelles. In TMV-infected protoplasts, viral mRNAs moved to 80 S ribosomes to form large polyribosomes, where a large amount of coat proteins is produced. During the stage of vigorous virus replication, gold labels for coat protein were found on crystalline virus aggregates and nearby cytoplasm where no virus particles were observed, indicating that the cytoplasm adjacent to crystalline virus aggregates with gold labels is the main site for coat protein synthesis in TMV-infected protoplasts. Encapsidation of genomic RNA into virions could occur at the periphery or inside the virus aggregates. Consistent with this idea, the putative area, where genomic RNAs are synthesized/replicated, is in or around the granular inclusions which are located near crystalline virus aggregates. Newly formed virus particles are incorporated into crystalline aggregates. During the later course of infection in protoplasts when virus multiplication slowed down, coat protein antigens were found also in the cytoplasm apart from virus aggregates. These antigens could be regarded as free coat protein which were synthesized in excess than the amount necessary for encapsidation of genomic RNA.

Our observation showed that 130 kDa and 180 kDa proteins which are responsible for the RNA synthesis were detected exclusively in the granular inclusions. In our protoplast system, the synthesis of TMV-RNA (genomic RNA) and subgenomic mRNA for the 30 kDa and coat proteins is first observed at 2-4 hr p.i., reached to the maximum level at 6-8 hr p.i.¹⁸⁾ and then declined. The granular inclusions were smaller in size at the early stage of infection when virus multiplication was vigorous. And the inclusions grew larger after the synthesis of TMV-RNA declined considerably. These observations indicate that only a small proportion of 130 kDa and 180 kDa proteins observed in inclusions are used actually in RNA synthesis whether or not the RNA replication site is the inclusions. Probably, most of the 130 kDa and 180 kDa proteins are functional at the early stages but they are accumulated as a biologically inactive entity and form dense granules. This situation coincides with the model postulated for cowpea mosaic virus (CPMV)⁴⁾, where non-structural proteins involved in replication of CPMV-RNA is able to synthesize only one RNA molecule each and thereafter accumulate in the cytoplasm as inactive molecules.

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和 文 摘 要

細川大二郎・渡辺雄一郎・岡田吉実：タバコモザイクウイルスの外被蛋白質および非構造蛋白質(130 kDa および 180 kDa 蛋白質)のタバコプロトプラストにおける局在の経時的観察

タバコモザイクウイルス(TMV)の外被蛋白質および非構造蛋白質(130 kDa および 180 kDa 蛋白質)のタバコプロトプラストにおける局在をプロテイン A-金コロイド法を用いて検討した。外被蛋白質の抗体では、金粒子の標識が接種 4 時間後に最初に細胞質の一部に認められた。その後、この初期の金粒子の標識部位は細胞質に数カ所認められるようになり、やがてこの部位には TMV 粒子が観察された。TMV 粒子は結晶配列の集塊となり、集塊の大きさが急速に

増加したが、この期間、金粒子はウイルス粒子の集塊とその近傍にのみ観察された。ウイルスの増殖が定常期に達する時期(接種24時間後)には金粒子の標識はウイルス粒子の認められない細胞質にも広く観察されるようになった。核、プラスチド、ミトコンドリアにはいずれの時期にも金粒子の標識は認められなかった。130 kDa 蛋白質の抗体では、金粒子の標識が接種4時間後に細胞質の形成初期の顆粒状封入体に認められた。その後、この封入体は数を増し、ウイルス粒子の集塊の近傍に観察され、形態は不定型から楕円形へと変化し、その大きさが増加したが、金粒子の標識はこの封入体にのみ認められた。180 kDa 蛋白質の抗体では、金粒子は130 kDa 蛋白質の認められた封入体にのみ観察されたが、金粒子の数はかなり少なかった。130 kDa 蛋白質及び180 kDa 蛋白質はTMVのRNAの複製酵素あるいはその構成成分であると考えられているので、以上の結果は、ウイルスRNAの複製、外被蛋白質の合成、ウイルス粒子の形成などのTMVの増殖の過程が、細胞質の限られた部位で起こることを示していると考えられた。

Explanation of plates

Plate I Electron micrographs of TMV RNA-infected tobacco protoplasts labelled with antibody to TMV and protein A-gold. Bars represent 0.5 μm . ER : endoplasmic reticulum ; P : plastid ; V : virus particle.

- 1 and 2. Part of protoplasts at 4 hr p.i. showing gold labels in restricted areas in the cytoplasm and nearby endoplasmic reticulum.
- 3-5. Part of protoplasts at 6 hr p.i. showing gold labels in small aggregates of virus particles and in the cytoplasm near the aggregates.

Plate II Electron micrographs of TMV RNA-infected tobacco protoplasts labelled with antibody to TMV and protein A-gold. Bars represent 0.5 μm (6 and 8) and 1 μm (7). I : granular inclusion ; N : nucleus ; No : nucleolus ; M : mitochondrion ; P : plastid ; V : virus particle.

6. Part of a protoplast at 12 hr p.i. showing gold labels on an aggregate of virus particles and on cytoplasm next to the aggregate.
7. Part of a protoplast at 24 hr p.i. showing dense gold labels on aggregates of virus particles and labels scattered on the cytoplasm. A nucleus, plastids, mitochondria and granular inclusions are free of labels.
8. Part of a protoplast at 48 hr p.i. showing gold labels on an aggregate of virus particles and on the cytoplasm apart from the virus aggregate.

Plate III Electron micrographs of TMV RNA-infected tobacco protoplasts labelled with antiserum to 130 kDa protein and protein A-gold. Bars represent 0.5 μm (9 and 10) and 1 μm (11). I : granular inclusion ; P : plastid ; V : virus particle ; Va : vacuole.

9. Part of a protoplast at 4 hr p.i. showing gold labels on a small electron-dense structure (nascent granular inclusion) in the cytoplasm.
10. Part of a protoplast at 6 hr p.i. showing gold labels on a granular inclusion.
11. Part of a protoplast at 12 hr p.i. showing gold labels on granular inclusions near aggregates of virus particles in the cytoplasm.

Plate IV Electron micrographs of TMV RNA-infected tobacco protoplasts labelled with antiserum to 130 kDa protein and protein A-gold. Bars represent 0.5 μm (12) and 1 μm (13). I : granular inclusion ; P : plastid ; V : virus particle ; Va : vacuole.

12. Part of a protoplast at 18 hr p.i. showing gold labels on granular inclusions.
13. Part of a protoplast at 24 hr p.i. showing dense gold labels on a elliptic granular inclusion.

Plate V

- 14 and 15. Electron micrographs of TMV RNA-infected tobacco protoplasts at 48 hr p.i. labelled with antiserum to 130 kDa protein and protein A-gold showing elliptic granular inclusions and surrounding large aggregates of virus particles in the cytoplasm. Bars represent 1 μm . I : granular inclusion ; N : nucleus ; M : mitochondrion ; P : plastid ; V : virus particle.

Plate VI

16. Electron micrograph of a TMV RNA-infected tobacco protoplast 48 hr p.i. labelled with antiserum to 180 kDa protein and protein A-gold showing gold labels on a granular inclusion. Bar represents 1 μm . I : granular inclusion ; P : plastid ; V : virus particle.

Plate I

Plate II

Plate III

Plate IV

Plate V

Plate VI