Replication of double-stranded RNA in mycovirus from the plant pathogenic fungus, *Fusarium solani*

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Abstract

A mycovirus (named FusoV) from the plant pathogenic fungus *Fusarium solani* possessed two types of double-stranded (ds) RNA genome, designated M1 and M2. RNA-dependent RNA polymerase activity was detected in FusoV particle fractions. An in vitro RNA polymerase reaction using purified FusoV particles that was supplemented with NTPs revealed the synthesis of single-stranded (ss) RNA species and a subsequent formation of dsRNAs having the same size as M1 and M2. The ssRNA species synthesized in the first stage were proved to be of positive polarity (coding strand) for both M1 and M2 by dot blot hybridization analysis. These results suggest that FusoV genomic dsRNA replicates in a conservative manner.

Keywords: *Fusarium solani*; Double-stranded RNA; In vitro replication; RNA-dependent RNA polymerase

1. Introduction

Mycoviruses or virus-like particles (VLP) have been found in over 100 fungi from more than 60 genera [1]. Most of them are nonenveloped isometric particles whose diameters are between 30 and 70 nm. They contain dsRNA genome which is often divided into two to four segments and one major polypeptide for capsid protein. A mycovirus, named FusoV, was isolated from *Fusarium solani* f. sp. *robiniae* SUF704, a pathogen of *pseudorobiniae* (*Robinia pseudoacacia* L.), which is a nonenveloped isometric particle (30 nm in diameter) comprising two segments of dsRNA genomes, M1 (1.9 kb) and M2 (1.7 kb), and a major capsid protein of 44 kDa [2]. A nucleotide sequence analysis of cDNAs synthesized from FusoV genomic dsRNAs revealed that M1 and M2 encoded RNA-dependent RNA polymerase and capsid protein, respectively (manuscript in preparation).

In vitro replication of dsRNAs has been examined to clarify the mode of replication and transcription of viral genomic dsRNA in its fungal host. A detailed mechanism of viral replication was reported for the L-A virus of the yeast *Saccharomyces cerevisiae*. In L-A virus, positive (+) stranded ssRNAs were synthesized from dsRNA template within viral particles (transcription), subsequently extruded from viral particles, and then used for the synthesis of viral protein (translation) in host cytoplasm. Finally, the newly synthesized (+) strands were packaged into new viral particles and functioned as templates for nega-
tive (−) strand synthesis to form new dsRNA (replication) inside the particles.

In this paper, we describe a replicase and a transcriptase activity associated with Fusov particles and propose a replication mechanism for the Fusov dsRNA genome.

2. Materials and methods

2.1. Strains, plasmids and culture condition

The culture condition of Fusarium solani f. sp. robiniae SUF704 and SUF704-f, a Fusov-free strain obtained by a single spore isolation, was described previously [2]. Escherichia coli JM105 was used for propagation of plasmids, and MV1184 was used as a host for the M13K07 helper phage propagation to obtain ssDNA. Plasmids pFV101 and 201 contained a part of M1 cDNA (1645 bp) in pUC118 with an opposite orientation. Similarly, pFV301 and 401 contained M2 cDNA (456 bp).

2.2. In vitro replication

Fusov particles used for in vitro replication reaction were purified through sucrose gradient centrifugation and CsCl density gradient centrifugation, as described previously [2]. The Fusov containing fraction was dialyzed against 15 SSB (15% sucrose, 10 mM Tris-HCl pH 6.8, 5 mM 2-mercaptoethanol) and used for in vitro replication according to the method of Barroso and Labarere [6]. 100 μl of Fusov fraction was mixed with 200 μl of reaction mixture (100 mM Tris-HCl pH 7.5, 10 mM magnesium acetate, 1 mM each of ATP, CTP, GTP and 0.5 mM UTP ([α-32P] 12.6 MBq/μmol), 200 units/ml Ribonuclease inhibitor (Takara), 50 μg/ml actinomycin D, 5 mM 2-mercaptoethanol), and then incubated at 30°C for various times. The reaction was terminated by extraction with 1 vol. of phenol/chloroform (1:1) and 1 vol. of chloroform/isoamyl alcohol (49:1). Total RNA was precipitated with 0.5 vol. of 7.5 M ammonium acetate and 3 vol. of cold ethanol (−20°C), washed with 99% ethanol, and then dissolved in 10 μl of diethylpyrocarbonate treated water. The ssRNA species synthesized was separated from total RNA by precipitation with 2 M LiCl at 0°C for 1 h. The synthesized ss and dsRNAs were fractionated by electrophoresis in 1.0% agarose gel with TAE buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA), and then detected by autoradiography after fixing the gel with 7% trichloroacetic acid.

2.3. Dot blot hybridization analysis

ssDNAs corresponding to each strand of M1 and M2 cDNAs were obtained from four plasmids, pFV101, 201, 301 and 401, propagated in E. coli MV1184 using M13 helper phage K07. Each ssDNA (200 ng) was blotted on a GeneScreen Plus membrane (Dupont) using a Bio-Dot apparatus (Bio-Rad). The 32P-labelled ssRNA synthesized during in vitro replication was obtained by LiCl precipitation and used as a hybridization probe. Hybridization was performed as described by Church and Gilbert [7], though with a slight modification. Prehybridization and hybridization were performed at 42°C for 2 and 16 h, respectively, in a solution consisting of 50% formamide, 0.5 M sodium phosphate buffer (NaPB) pH 7.2, 3.5% SDS, 1% BSA, and 1 mM EDTA. The membrane was washed with 40 mM NaPB pH 7.2, 1 mM EDTA, 5% SDS at 42°C for 5 min, and then with 40 mM NaPB pH 7.2, 1 mM EDTA, 1% SDS for 5 min.

2.4. Northern hybridization analysis

Total RNA was prepared from F. solani SUF704 as described by Sims et al. [8]. To detect the ssRNA species associated with the Fusov genome, total RNAs were fractionated by native 1.0% agarose gel electrophoresis with TAE buffer and transferred onto GeneScreen Plus membrane. The strand-specific probe was synthesized by the Multiprime DNA labelling system (Amersham) using ssDNAs of M1 and M2 cDNAs as templates. The hybridization condition was described above.

3. Results and discussion

3.1. In vitro replication of Fusov genomic dsRNA

When the Fusov particle fraction was used for in vitro replication, synthesis of 32P-labelled RNA
Fig. 1. Detection of in vitro replication products by agarose gel electrophoresis. Newly synthesized RNA species that incorporated \( \alpha^{-32}P \)UTP added as a substrate were detected by autoradiography. The position of the purified M1 and M2 dsRNAs is indicated by an arrow. Lane 1, in vitro replication products of purified Fusov; lane 2, supernatant fraction after LiCl precipitation; lane 3, pellet fraction after LiCl precipitation; lane 4, in vitro replication products of crude extract of \( F. \ solani \) SUF704-f (FusoV-free strain); lane M, \( ^{32} \)P-labelled \( \lambda \) HindIII size marker.

This RNA polymerase activity was confirmed as associated with Fusov particles for two reasons. First, the polymerase activity was resistant to actinomycin D, an inhibitor of fungal host DNA-dependent RNA polymerase which may contaminate the Fusov fraction. Second, this RNA polymerase activity was not detected in in vitro replication reactions using crude extracts from \( F. \ solani \) SUF704-f (FusoV-free strain) obtained by single spore isolation [2] (Fig. 1, lane 4). The synthesized RNA was composed of at least two species, since two separate bands were fractionated on 1.0% native agarose gel electrophoresis. The faster migrating band was confirmed to be dsRNA M1 and M2 from its electrophoretic mobility similarity to purified M1 and M2 and from its solubility in 2 M LiCl (Fig. 1, lane 2). This electrophoresis system did not separate M1 and M2 dsRNA due to poor resolution. This band was proved to consist of dsRNA due to its resistance to digestion by RNase A in 2 \( \times \) SSC (0.3 M NaCl, 0.03 M sodium citrate) (data not shown).

The RNA species corresponding to the slower migrating band was separated by precipitation with 2 M LiCl (Fig. 1, lane 3), and was sensitive to digestion by RNase A in 2 \( \times \) SSC (data not shown), indicating that this RNA species was ssRNA. To determine whether this ssRNA product of in vitro replication was related to either strand of M1 and M2 dsRNAs, dot blot hybridization against each strand of M1 and M2 cDNAs was achieved using the synthesized \( ^{32} \)P-labelled ssRNA as a probe. The probe hybridized to ssDNAs prepared from pFV201 and 401, which corresponded to the \((-\) strand of M1 and M2 cDNAs respectively, showing that the ssRNA product was a mixture of \((\pm)\) strands from both M1 and M2 dsRNA (Fig. 2). A time-course experiment of in vitro replication showed that most of the replication products in the early reaction stage (until 10 min) were ssRNA, and subsequently synthesis of dsRNA occurred (Fig. 3). The viral dsRNA replication has been classified into two types [3]. One is a conservative replication that is observed in yeast L-A virus and mammalian reo- and rotavirus [3-5]. The other is a semiconservative replication found in bacteriophage \( \phi 6 \) and mycoviruses from the genera \( Aspergillus \) and \( Penicillium \) [3,9,10]. The difference between the two lies in the first step of dsRNA replication, that is, synthesis of the \((\pm)\) strand from dsRNA (transcription). In semiconservative replication, the newly synthesized transcript \((\pm)\) strand displaces the \((\pm)\) strand of the parental dsRNA template, and the parental \((\pm)\) strand is released from the viral particle. On the other hand, in conservative replication, the newly synthesized \((\pm)\) strand is released from the viral particle and the parental \((\pm)\) strand remains in dsRNA form within the particle. The dsRNA synthesis observed in in vitro replication, that is, the initial synthesis of \((\pm)\)

Fig. 2. Dot blot hybridization analysis using LiCl-precipitated fractions from in vitro replication products as a probe. Hybridization membrane was blotted with ssDNAs corresponding to either strand of M1 and M2 cDNAs; pFV101, \((\pm)\) strand of M1 cDNA; pFV201, \((-\) strand of M1 cDNA; pFV301, \((\pm)\) strand of M2 cDNA; pFV401, \((-\) strand of M2 cDNA.
Fig. 3. Time-course analysis of in vitro replication products. RNA species, synthesized by in vitro replication reaction for various times, were fractionated by agarose gel electrophoresis and then detected by autoradiography. The faster migrating band was a dsRNA species and the slower migrating band was a ssRNA species (see Section 3). The lane number is the reaction time (min). Lane M was ³²P-labelled λ HindIII size marker.

strand ssRNA (transcription) and the subsequent accumulation of dsRNA coupled with (-) strand synthesis (replication), suggested that FusoV dsRNA replication proceeds in a conservative manner.

3.2. FusoV associated ssRNA in fungal cells

The in vitro replication reaction using purified FusoV particles suggested that genomic dsRNA replicated in a conservative manner. To confirm that this type of replication actually occurred in a fungal host cell, total RNAs extracted from SUF704 cells were analyzed by Northern hybridization to detect FusoV-related RNA species. The probes used were four kinds of ³²P-labelled ssDNA, which were complementary to strands of either M1 or M2 dsRNA (see Section 2). In a preliminary experiment, purified dsRNAs M1 and M2 were fractionated through denatured agarose gel (1.2%) containing 2.2 M formaldehyde, transferred onto nylon membrane (GeneScreen Plus, Dupont), and hybridized with each of the four probes. Hybridization signals appeared at the position of the dsRNAs with all four probes, indicating that dsRNA was denatured to ssRNA in this condition (data not shown). When dsRNA was electrophoresed in native agarose (1.0%) gel, no signal was detected with any of the four probes (Fig. 4, lane D). It is likely that dsRNA did not dissociate at all in this electrophoresis condition. Northern hybridization was achieved for total RNAs fractionated by native agarose gel, which would only allow detection of ssRNA species related to M1 and M2 dsRNAs. Hybridization signals were obtained only when ssDNA synthesized from either pFV201 or pFV401 [(+) strand of M1 and M2 cDNAs] was used as a probe (Fig. 4). This indicated that the (-) strands from both M1 and M2 dsRNAs, which were not synthesized in vitro replication, accumulated in fungal host cells. This (-) strand accumulation has not so far been reported in mycovirus dsRNA replication. It may be postulated that newly synthesized (+) strands released from virus particles were used as a template for (-) strand synthesis by viral encoding RNA-dependent RNA polymerase which existed in a free form in the cytoplasm of the fungal host. Accumulation of the resulting (-) strands could be explained if they were not packaged into a new

Fig. 4. Northern hybridization analysis of FusoV-related ssRNA species in host cells. Strand specific probes used were synthesized using ssDNA of M1 and M2 cDNAs as a template (see Section 2). Total RNA and purified M1 and M2 dsRNAs were fractionated through native agarose gel (1.0%) and blotted onto the nylon membrane (GeneScreen Plus), creating a condition that eliminated hybridization signals from dsRNAs. The probes used for hybridization were indicated at the top of each autoradiograph (+) and (-) indicate the positive and the negative strand, respectively. Lane T, total RNA extracted from F. solani SUF704; lane D, purified dsRNA.
virus particle, or if the packaged (−) strand did not function as a template for double-strand synthesis (replication). The clarification of the mechanism for (−) strand accumulation accompanied by Fusov viral dsRNA replication in fungal host cells must await further investigation.

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References


