

Genome Size Constraint in Replication and Packaging of Turnip Yellow Mosaic Virus

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Turnip yellow mosaic virus (TYMV) is a spherical plant virus that has a single 6.3 kb positive strand RNA as a genome. Previously, we have made the recombinant TYMV construct containing a 0.7 kb eGFP gene or a 1.8 kb GUS gene. The genomic RNAs from these constructs were efficiently encapsidated. To examine in more detail whether size constraint exists for replication and packaging of TYMV, we have inserted into the TY-GUS an extra sequence derived from either eGFP or GUS. We also made a recombinant containing RNA1 sequence of Flock house virus. These TYMV recombinants were introduced into *Nicotiana benthamiana* leaves by agroinfiltration. Northern blot analysis of the viral RNAs in the agroinfiltrated leaves showed that the genomic RNA band from the recombinant TYMV became weaker as longer sequence was inserted. The result also showed that the efficiency of genomic RNA encapsidation decreased sharply when an extra sequence of 2.2 kb or more was inserted. In contrast, the recombinant subgenomic RNA containing an extra sequence of up to 3.2 kb was efficiently encapsidated. Overall, these results show that size constraint exists for replication and encapsidation of TYMV RNA.

Key Words: TYMV, Plant RNA virus, Size constraint, RNA packaging, Replication

INTRODUCTION

Turnip yellow mosaic virus (TYMV), a spherical positive-strand RNA virus, is a type member of tymoviruses and infects mainly *Cruciferae* plants (1). The TYMV virion is an icosahedron, which is composed of 180 identical coat proteins (2). The genomic RNA (gRNA), which is 6.3 kb in size, contains three open reading frames (ORFs). The biggest ORF produces a replication protein, p206, which is cleaved by its own protease activity into p140 and p66. The latter is a RNA polymerase. A 0.7 kb subgenomic RNA (sgRNA) encoding coat protein (CP) is produced during

replication. The sgRNA is also packaged into virions, either packaged separately or co-packaged along with the gRNA (3).

In many spherical RNA viruses, there is size constraint in the RNA that can be encapsidated (4). Spherical plant RNA viruses have various sizes of genomes, however, the sizes of the virions are in the range of 25 to 35 nm. In these viruses, the capsid volume is typically a prime factor to determine the upper limit of the packaged RNA. Turnip crinkle virus (TCV), whose genome is 4,054 nt, clearly shows the example; the RNA larger than 4,600 nt is not encapsidated even though the RNA has an appropriate packaging signal (5). In contrast, Cucumber mosaic virus

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(CMV) was reported to package the RNA of 6,500 nt that is about twice as large as the CMV gRNA (3,300 nt). In the case of Brome mosaic virus (BMV), the virion becomes larger as bigger RNA is encapsidated (4).

In this study, we have investigated how much extra sequence can be inserted into the TYMV genome without compromise on RNA replication and packaging. To address the issue, we have used a previously developed TYMV expression vector, which contains an extra tymobox, sub-genomic RNA promoter, and a multiple cloning site. The expression vector, designated TY-V^{2tymo}, was used to express foreign genes, such as eGFP and GUS. The recombinant TYMV constructs produced gRNAs that were 0.8 and 1.9 kb bigger than the wild-type TYMV gRNA. The recombinant gRNAs were observed to be efficiently encapsidated (6).

Here, we have prepared recombinant TYMV constructs whose inserts are bigger than 1.9 kb, and have investigated to what extent the virus can accommodate the recombinant RNA without harmful effects. The results show that the RNA packaging becomes inefficient as the insert is bigger than or equal to 2.2 kb. The results also show the tendency that the replication of the recombinant viral RNA becomes weaker as bigger extra sequence is inserted.

MATERIALS AND METHODS

DNA constructs

TY-V^{2tymo}, TY-GUS, and TY-eGFP constructs have been described previously (6). The construct TY-GUS-eGFP was made by inserting an eGFP gene into the *SpeI* site (downstream of the GUS gene) of the TY-GUS. The eGFP gene was PCR-amplified using the following primers: upstream primer eGFP-F-*SpeI* (5'-GAC ACT AGT ATG GTG AGC AAG GGC GAG -3'; *SpeI* site is underlined) and downstream primer eGFP-R (5'-CAG ACT AGT CTA TTT GTA TAG TTC ATC CA -3'; *SpeI* site is underlined). To make TY-GG constructs, the GUS sequence between nt #6 and nt #1512 of the GUS ORF was PCR-amplified. The amplified DNA was designed to have *EcoRI* recognition sequence at the 5'-end and *AscI* and *SpeI* recognition

sequences (GGCGCGCC ACTAGT) at the 3'-end. The PCR product was inserted into the *EcoRI/SpeI* sites of the TY-V^{2tymo}. The resulting construct was designated TY-GUS (1.6), since the size of the extra sequence in this construct was 1595 nucleotides when counting all the inserted sequence. The *AscI* and *SpeI* sites of the TY-GUS(1.6) were then used to insert 0.4 kb (nt #24 to nt #423 of GUS ORF), 0.6 kb (nt #24 to nt #623), 0.8 kb (nt #24 to nt #823) or 1.2 kb (nt #24 to nt #1246) GUS sequence, to produce TY-GG(2.0), TY-GG(2.2), TY-GG(2.4) and TY-GG(2.8).

To make the construct TY-FHV, the eGFP sequence in TY-eGFP was replaced with a 3.2 kb Flock house virus (FHV) sequence from pFHV-wHDV. The pFHV-wHDV was prepared using the cDNA for FHV RNA1, which was a kind gift from Dr. Miller at the University of Michigan.

Plant material

Agroinfiltration of the *Agrobacterium tumefaciens* harboring various TYMV constructs into *Nicotiana benthamiana* was carried out as described previously (7). Agroinfiltration of all the constructs was accompanied by agroinfiltration of a binary vector supporting the expression of the RNAi suppressor p19 of Tomato bushy stunt virus (8). Seven days after agroinfiltration, the infiltrated leaves were collected. For RNA and protein extraction, the leaf samples were frozen in liquid nitrogen immediately after collection and stored at -80°C.

Analysis of viral RNA

Total RNA was isolated from frozen leaf samples using Easy-Red (iNtRON Biotechnology, Sungnam, Korea). The ribonuclease protection assay for encapsidated RNA and Northern analysis were performed as described (7). Briefly, in the encapsidation assay, the leaf extract was incubated with RNase A (5 µg/ml final concentration) for 1 h at 37°C and for an additional 1 h in the presence of proteinase K and SDS (100 µg/ml and 0.5% final concentrations, respectively). The surviving RNA, representing encapsidated RNA, was then extracted with phenol and chloroform, and precipitated with ethanol.

RNA samples were resuspended in 48% formamide

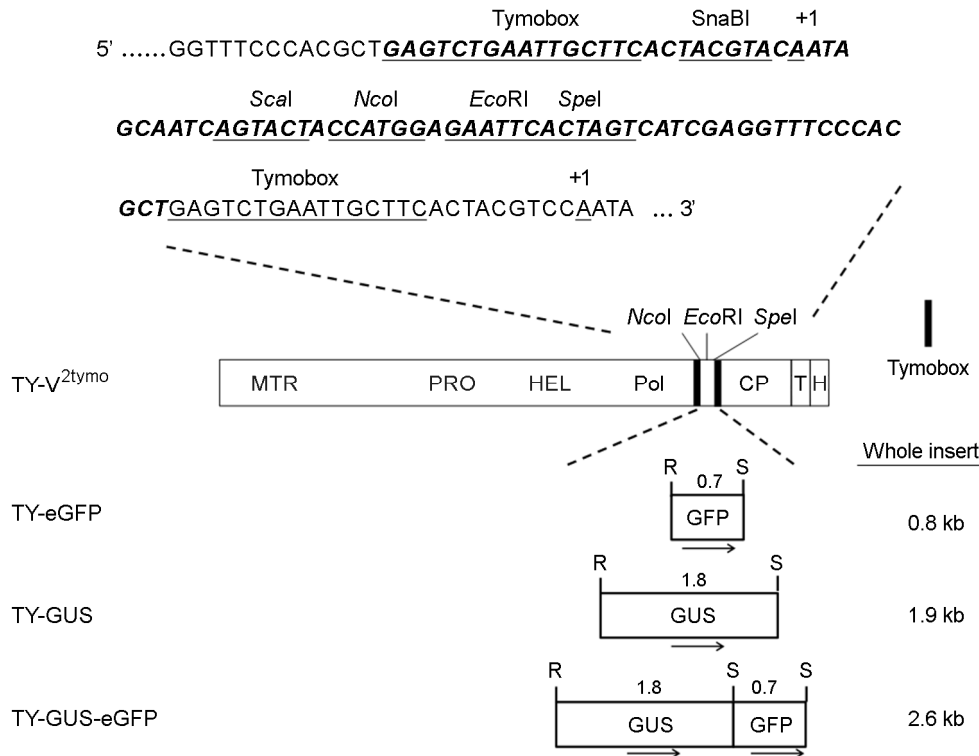


Figure 1. Recombinant TYMV constructs. TY-V^{2tymo} is an expression vector that has an extra tymobox, a subgenomic RNA promoter, and a multiple cloning site (6). The methyltransferase (MTR), protease (PRO), helicase (HEL), and polymerase (POL) domains of TYMV genome are indicated. T and H represent the tRNA-like structure and hepatitis delta virus ribozyme, respectively. The inserted sequence is shown in bold and italic letters. Transcription start site is indicated by +1. TY-eGFP, TY-GUS, and TY-GUS-eGFP were prepared by inserting the foreign sequence into the *EcoRI*/*SpeI* sites of TY-V^{2tymo}. R and S represent *EcoRI* and *SpeI* restriction sites, respectively. The sizes of the whole insert are shown on the right. The arrows below the inserted genes represent the direction of transcription. The numeral above the inserted sequence indicates the size of the gene.

solution containing 10 mM EDTA and incubated at 65°C for 10 min before electrophoresis on 1% agarose gel. RNAs were transferred to Hybond N⁺ membranes (GE Healthcare), and the blots were hybridized with a digoxigenin (DIG)-labeled DNA probe hybridizing to the TYMV CP ORF. The blots were developed by chemiluminescent immunodetection of DIG (Roche Molecular Biochemicals, Basel, Switzerland). The band intensity was quantified using the image analysis software ATTO CS Analyzer 2.0.

RESULTS

The gRNAs of TY-eGFP (7.1 kb) and TY-GUS (8.2 kb) are 0.8 kb and 1.9 kb bigger, respectively, than the wild-type TYMV gRNA. As mentioned earlier, those recombinant

gRNAs were observed to be efficiently encapsidated into the virions (6). We were curious whether or not the TYMV virions can accommodate bigger RNAs. We were also interested in whether viral RNA replication would be influenced by the insert size. To address this issue, we made a recombinant TYMV construct containing both GUS and eGFP genes. An additional eGFP sequence was PCR-amplified, digested with *SpeI*, and inserted into the *SpeI* site at the 3'-end of the GUS gene (Fig. 1). The eGFP sequence could have been inserted in two different orientations. Although it was previously shown that the replication and RNA packaging was not influenced by the addition of an eGFP gene (6), the insertion of eGFP sequence into the TYMV genome in opposite orientation might affect the replication and encapsidation of the viral RNA. Thus, we

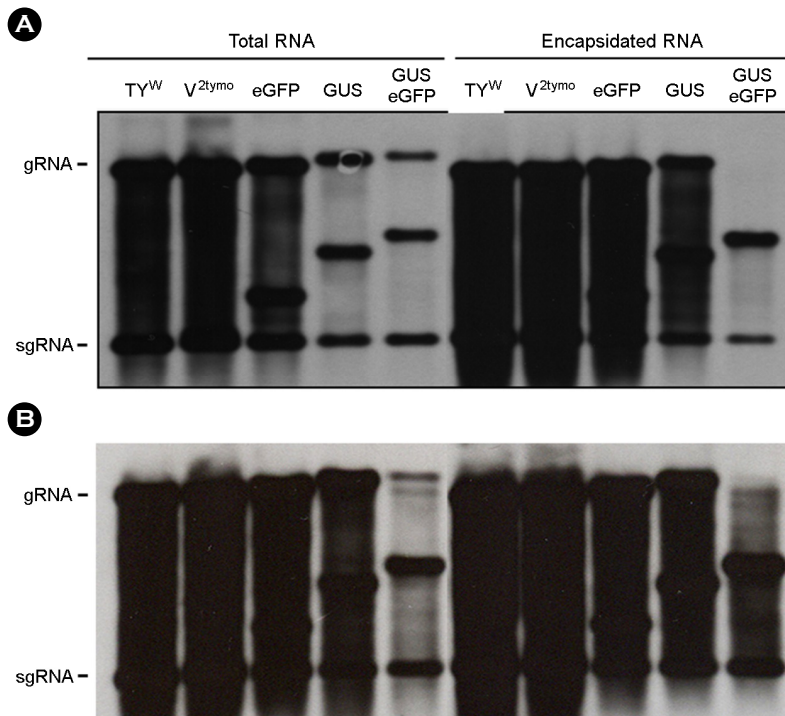


Figure 2. Replication and packaging of recombinant TYMV constructs. Recombinant TYMV constructs were agroinfiltrated into *N. benthamiana* plants. A week after agroinfiltration, total RNA was extracted from the agroinfiltrated leaf. For encapsidation assay, leaf extracts were treated with RNase A (5 µg/ml), followed by phenol/chloroform extraction. Experiments were repeated at least two times. RNA samples were size-fractionated by agarose gel electrophoresis and analyzed by Northern blot hybridization using a DIG-labeled CP ORF DNA as a probe. Two representative blots are shown (A and B). The positions of genomic RNA (gRNA) and sub-genomic RNA (sgRNA) are indicated.

chose the construct where the additional eGFP gene was in sense orientation. The construct was designated TY-GUS-eGFP. The size of the insert was 2.6 kb. The TYMV constructs including the TY-GUS-eGFP, TY-GUS and TY-eGFP were introduced into *N. benthamiana* by agroinfiltration.

Seven days after the inoculation, total RNA and leaf extracts were prepared from the infiltrated leaves. Total RNA and encapsidated RNA samples were analyzed to examine whether or not replication and RNA packaging of the recombinant TYMVs were influenced by the insertion. To examine the packaging efficiency of the recombinant TYMV RNA, the leaf extract was treated with RNase A and then the protected RNA was extracted with phenol/chloroform. These RNA samples were examined by Northern analysis using as probe the DIG-labeled DNA encoding the CP. The result shows that replication tends to be reduced as the insert size increases (Fig. 2). Replication of the TY-eGFP was comparable to that of the wild-type TYMV construct TY^W and TY-V^{2tymo}. However, in the cases of TY-GUS and TY-GUS-eGFP, whose inserts were 1.9 kb

and 2.6 kb, respectively, it was apparent that replication was suppressed. Nevertheless, two sgRNAs were produced as expected in all recombinant constructs. Larger sgRNA represents the recombinant sgRNA that contains the foreign sequence as well as the CP ORF. In all constructs, the recombinant sgRNA was produced as much as the genuine sgRNA.

Northern analysis of the encapsidated RNA samples protected from RNase digestion showed that the gRNA of the TY-GUS-eGFP was absent after the RNase A treatment (Fig. 2). Repeated experiments showed that gRNA band sometimes appeared in the TY-GUS-eGFP 'encapsidated' RNA sample, however, the gRNA band was much weaker compared to those of sgRNAs. The result clearly showed that 2.6 kb insertion could hamper the encapsidation of the recombinant gRNA.

To confirm the size effect observed in TY-GUS-eGFP, we prepared constructs with inserts whose sizes were similar to that of TY-GUS-eGFP. For this purpose, we first made a variant TY-GUS construct containing about 1.5 kb sequence from the GUS gene, where *AscI* recognition

sequence was introduced at the 3'-end. Since the size of the total extra sequence inserted into this construct was 1,595 nucleotides, the construct was designated TY-GUS(1.6). We have inserted additional sequence of GUS (0.8 kb and

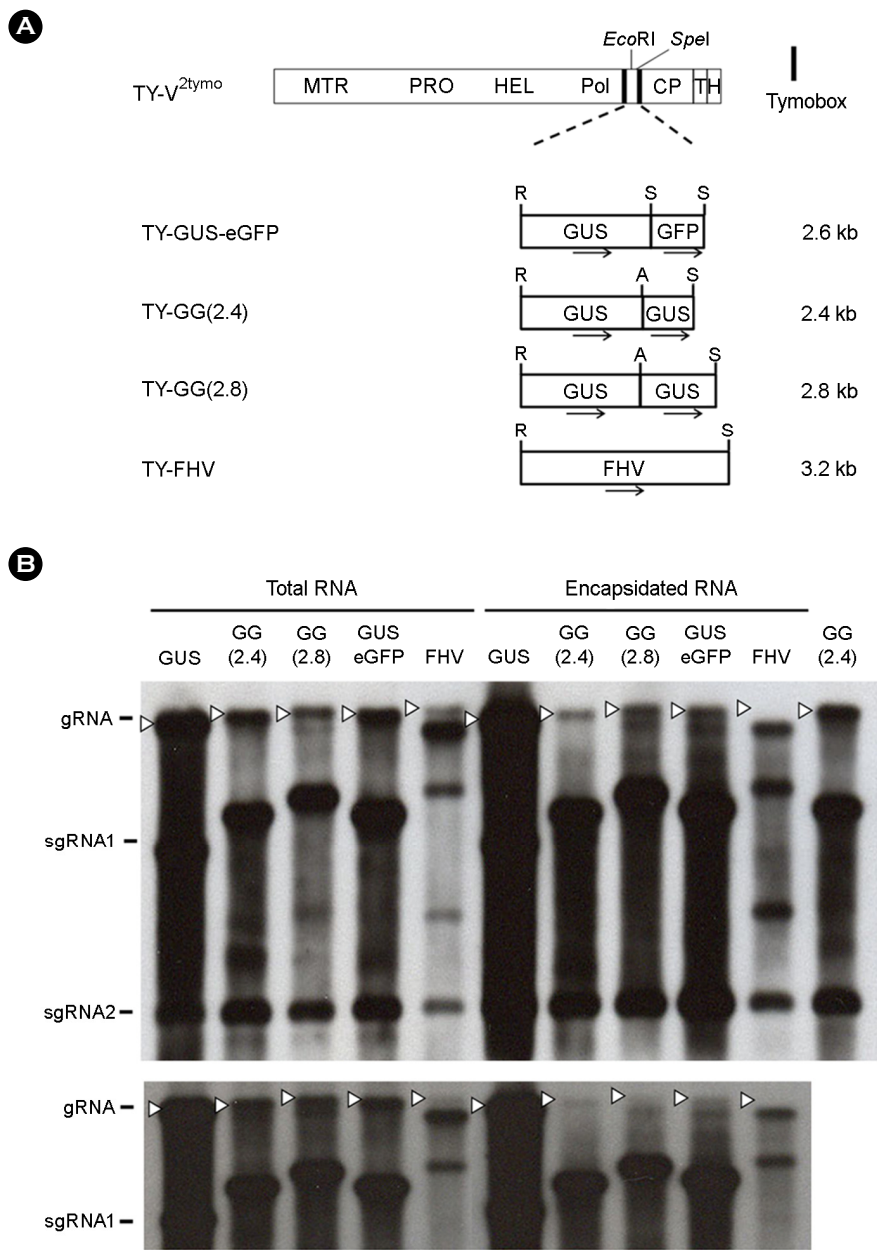


Figure 3. Replication and packaging of the recombinant TYMV whose insert is equal to or larger than 2.4 kb. (A) TYMV constructs. TY-GG(2.4) and TY-GG(2.8) constructs were prepared by inserting 0.8 kb and 1.2 kb extra GUS sequence, respectively, into the *Ascl/SpeI* sites of the TY-GUS(1.6), where *Ascl* recognition sequence was introduced at the 3'-end of the insert (see MATERIALS AND METHODS for details). TY-FHV construct contains a RNA1 sequence of Flock house virus. The sizes of the inserts are represented on the right of the constructs. Orientation of the inserted gene sequences are indicated by arrows. (B) Northern analysis of the replication and packaging of the recombinant TYMV RNAs. Total RNA and encapsidated RNA samples were analyzed by Northern blot hybridization, as described in Fig. 2. Two representative blots are shown. The bottom panel represents an upper part of the gel showing the gRNAs and the larger sgRNAs. The rightmost sample in the top panel is a total RNA sample from TY-GG(2.4), which was included as a blotting control. gRNAs are indicated by white arrowheads.

1.2 kb) into the TY-GUS(1.6) to have TY-GG(2.4) and TY-GG(2.8) constructs (Fig. 3A). In these constructs, the two copies of the GUS sequences were designed to have same orientations so that double-stranded RNA eliciting RNAi would not be produced. Another construct was TY-FHV that contained the FHV RNA1 sequence. TY-FHV construct has a 3.2 kb insert.

When total RNAs were examined after agroinfiltration of these constructs, it was observed that the level of the gRNA from the variant TYMV s was far lower than that of TY-GUS (Fig. 3B). In the case of wild-type TYMV, gRNA is produced as much as the sgRNAs. The same was observed in the TY-eGFP and TY-GUS constructs that produce an additional sgRNA (Fig. 2). This is expected because both

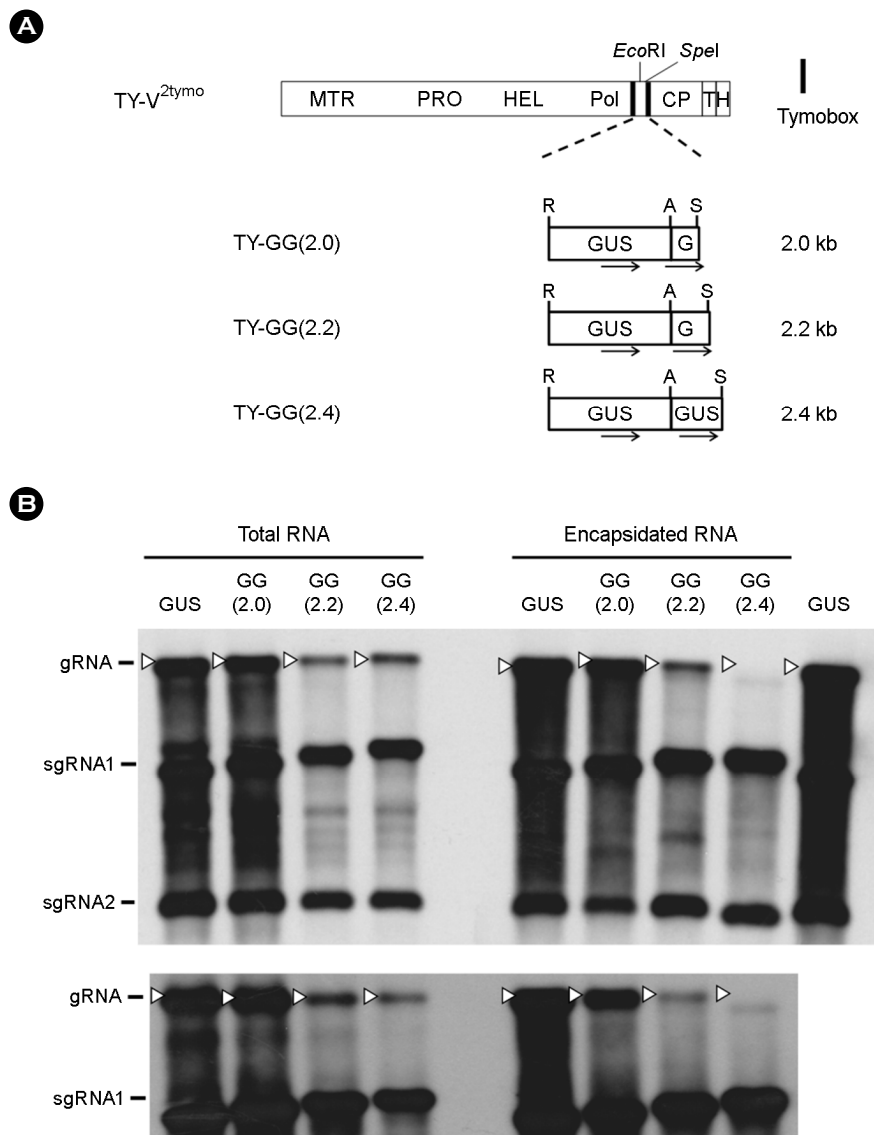


Figure 4. Replication and packaging of the recombinant TYMV whose insert is smaller than 2.4 kb. (A) TYMV constructs. TY-GG (2.0) and TY-GG(2.2) constructs were prepared by inserting 0.4 kb and 0.6 kb extra GUS sequence, respectively, into the *Ascl/SpeI* sites of the TY-GUS(1.6). (B) Northern analysis of the replication and packaging of the recombinant TYMV RNAs. Total RNA and encapsidated RNA samples were analyzed by Northern blot hybridization, as described in Fig. 2. The rightmost TY-GUS sample in the top panel was included as a blotting control. gRNAs are indicated by white arrowheads.

gRNA and sgRNA are generated using the same template, (–)-strand gRNA. The 1:1 ratio of gRNA to sgRNA, however, was not observed in those two TY-GG constructs. The band intensities of gRNAs in total RNA samples relative to those of larger sgRNA species were in the range of 0.2 to 0.4.

In the case of TY-FHV, suppressed replication was even more conspicuous. In the TY-FHV construct, gRNA (top band) and two sgRNAs (3rd and 5th bands from the top) were observed as anticipated. Additionally, two more RNA bands were also observed. Those bands, whose identities are not clear, are thought to be by-products of FHV replication, since it was reported that homo- and hetero-dimers of FHV RNAs were frequently generated during FHV replication (9).

When encapsidated RNA was examined, it was evident that the proportion of gRNA in the encapsidated RNA is far lower in the TY-GG and TY-FHV constructs, compared to the gRNA of TY-GUS (Fig. 3B). When comparing to the total RNA samples, the intensity of the gRNA in the 'encapsidated' RNA samples is much weaker relative to that of the larger sgRNA (gRNA bands are indicated by arrowheads in Fig. 3B). In the case of TY-GG(2.4), for example, the relative band intensity of the encapsidated gRNA to that of the larger sgRNA was about 0.05, whereas the relative intensity of the gRNA in total RNA sample was about 0.4. For TY-FHV, the encapsidated gRNA was negligible. This result indicates reduced efficiency of the gRNA packaging in the TY-GG and TY-FHV constructs. The experiments were repeated at least two times with similar results. The inefficiency was conspicuous in the case of TY-FHV gRNA; the gRNA band was barely detected in the encapsidated RNA sample. Except for the gRNA, those RNAs from the TY-FHV were efficiently encapsidated.

The results obtained so far suggest that insertion of up to 1.9 kb does not affect RNA packaging. However, insertion of 2.4 kb or more could have detrimental effect on gRNA packaging. To assess the effect of genome size more closely, we made two more constructs whose inserts are 2.0 kb and 2.2 kb (Fig. 4A). The two constructs, designated TY-GG(2.0) and TY-GG(2.2), were made by inserting additional

sequence of GUS into the TY-GUS(1.6). Analysis of total RNA samples of these constructs showed that the gRNA from the TY-GG(2.0) accumulated as much as the TY-GUS (Fig. 4B). The gRNA and the two sgRNA maintained 1:1:1 ratio. In contrast, insertion of 2.2 kb sequence was observed to suppress accumulation of the recombinant gRNA, much like the TY-GUS-eGFP and other constructs having inserts bigger than 2.4 kb. The result of the encapsidation assay also showed that the packaging of the recombinant gRNA from TY-GG(2.2) decreased abruptly, contrasting with the gRNAs from TY-GG(2.0) and TY-GUS.

Overall, the insert size that does not compromise the replication and encapsidation of viral RNA seems to lie between 2.0 and 2.2 kb. In contrast to the gRNAs, sgRNAs from all these constructs, including the largest sgRNA from TY-FHV (3.9 kb), were efficiently encapsidated. This again shows that the main determinant of recombinant RNA packaging is not the sequence but the size of the insert.

DISCUSSION

There has been only limited information on how TYMV RNA is packaged into virions. In many spherical plant RNA viruses, the CP has an N-terminal arginine-rich motif (ARM), which interacts with RNA (4). TYMV CP does not have the ARM at the N-terminal. The TYMV CPs are postulated to get partially assembled around the exit of the replication vesicles, which are formed on the outer membranes of chloroplasts. The CPs then fully assemble with the TYMV RNA as the RNA exits out of the vesicles (10). TYMV CPs can also self-assemble without viral RNAs. Therefore, significant amounts of empty virions, about 20~30% of total virions, are typically observed in TYMV-infected tissues. Another feature unique to TYMV is that the TYMV virion contains spermidines (1).

Packaging mechanisms for TYMV RNAs have also been elusive. TYMV gRNA has two hairpin structures in the 5'-untranslated region (5'-UTR) (11). Previous studies reported that the 5'-UTR hairpins could serve as packaging signals (12, 13). However, recent studies show that the hairpins and other 5'-UTR sequences are not required for

TYMV RNA packaging (14). It was also revealed that the 3'-tRNA-like structure (TLS), which was reported to serve as a signal for BMV RNA packaging (15), did not serve as a packaging signal for TYMV RNA (7). To make it more complicated, recent studies with deletion constructs showed that the region corresponding to the Pro and Hel domains was necessary for packaging of non-replicating TYMV RNA, although it was observed that the Pro/Hel region was dispensable for packaging of replicating TYMV RNA (16).

In this study, we have focused to examine whether TYMV has genome size constraint on RNA packaging. The results in Fig. 2 and 3 show the tendency that the gRNA level becomes lower as a longer sequence is inserted into the TYMV genome. The sgRNA levels were not as much influenced as the gRNAs. The far lower gRNA levels compared to those of sgRNAs could be ascribed to inefficient gRNA synthesis. The afore-mentioned TYMV 5'-UTR including the two stem-loop structures have been reported to act as replication enhancers (14, 17). Therefore, if the insertion had caused incomplete synthesis of (–)-strand gRNA, some of the (–)-strand gRNAs would have lacked the replication enhancers, resulting in decreased (+)-strand gRNA synthesis. sgRNA synthesis would not have been affected in that case, since the sgRNA promoter, tymobox, resides in the middle of the (–)-strand gRNA. Alternatively, the low level of –gRNA could be due to the inefficiency of gRNA packaging. When a recombinant gRNA with large insert is not packaged into virions, the gRNA is more prone to degradation by RNases.

Overall decrease in replication could be due to RNAi. Although precautions were taken to avoid the production of double-stranded RNA, RNAi could have been elicited as the inserted sequence became longer. Previously, it was observed that insertion of foreign sequences suppressed the replication of the TYMV recombinants. The inhibition of replication was relieved by co-expression of RNAi suppressors (6). Replication of TY-GUS was more suppressed than TY-eGFP, indicating that RNAi could have been influenced by either length or sequence of the insert.

The results obtained in this study show that gRNAs larger than 8.3 kb are not efficiently encapsidated. One can

think of the possibility that the inserted sequence could have somehow interfered with the formation of a correct secondary or tertiary structure that serve as a packaging signal. The other explanation could be that the size of the RNA is the key determinant for restricting the RNA encapsidation. Considering that the TYMV virion is 28~30 nm in size, which is not big for a 6.3 kb genome, it would not be easy for the TYMV virion to accommodate RNAs that are much bigger than the wild type. The dramatic decrease in packaging efficiency of the gRNA from TY-GG(2.2) compared to that of the TY-GG(2.0) or TY-GUS favors the latter possibility.

Overall, when comparing with other spherical plant viruses, TYMV virions look more flexible than TCV virions, however, they are not as flexible as CMV or BMV. As far as gRNA packaging is concerned, insertion of more than 2.0 kb (32% of TYMV genome) restricts the encapsidation of the gRNA. In contrast, the recombinant sgRNA containing as large as 3.2 kb insert is efficiently encapsidated. This feature might be exploited to encapsidate a recombinant TYMV sgRNA without packaging gRNA.

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