BMB
Reports

BMB Rep. 2014; 47(6): 330-335
www.bmbreports.org

Replication and packaging of Turnip yellow mosaic virus RNA containing Flock house virus RNA1 sequence

*Hui-Bae Kim, Do-Yeong Kim & Tae-Ju Cho**
Department of Biochemistry, Chungbuk National University, Cheongju 361-763, Korea

Turnip yellow mosaic virus (TYMV) is a spherical plant virus that has a single 6.3 kb positive strand RNA as a genome. In this study, RNA1 sequence of Flock house virus (FHV) was inserted into the TYMV genome to test whether TYMV can accommodate and express another viral entity. In the resulting construct, designated TY-FHV, the FHV RNA1 sequence was expressed as a TYMV subgenomic RNA. Northern analysis of the *Nicotiana benthamiana* leaves agroinfiltrated with the TY-FHV showed that both genomic and subgenomic FHV RNAs were abundantly produced. This indicates that the FHV RNA1 sequence was correctly expressed and translated to produce a functional FHV replicase. Although these FHV RNAs were not encapsidated, the FHV RNA having a TYMV CP sequence at the 3′-end was efficiently encapsidated. When an eGFP gene was inserted into the B2 ORF of the FHV sequence, a fusion protein of B2-eGFP was produced as expected. [BMB Reports 2014; 47(6): 330-335]

INTRODUCTION

Turnip yellow mosaic virus (TYMV) represents a type species of the genus *Tymovirus* (1). TYMV is a plant virus that infects mainly *Cruciferae* plants, including turnip and Chinese cabbage. TYMV has a monopartite positive-strand RNA genome of 6.3 kb that 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 an RNA polymerase. Prod'homme *et al*. (2) reported that the p66 is transported to replication sites, the outer membrane of chloroplasts, with the help of p140. Coat protein (CP), whose ORF resides at the 3′-end of the genomic RNA (gRNA), is expressed on a subgenomic RNA (sgRNA) that is produced during replication. The ORF overlapping with the p206 ORF encodes p69, which acts as a movement protein and RNAi suppressor (3).

In this study, we have examined whether TYMV can accommodate a gene that can be amplified, as a first step towards the development of TYMV as a gene delivery vector. Although there are a lot of technical hurdles on the way to the development of plant viruses as gene delivery vectors, plant viruses have recently been considered as an alternative nanoparticle to deliver drugs or therapeutic genes to human cells (4). Previously, we have shown that foreign genes, eGFP and GUS, can be inserted into TYMV genome with relatively little effect on replication (5). The foreign genes are expressed as sgRNAs under the influence of an extra tymobox, a sgRNA promoter. In this study, we engineered the TYMV genome so that it contained a bigger insert, an RNA1 sequence of Flock house virus (FHV). FHV is a bipartite, positive-strand RNA insect virus, whose 4.5 kb genome is known to be one of the smallest genomes among animal RNA viruses (6). The 3.1 kb RNA1 encodes an RNA-dependent RNA polymerase, and the 1.4 kb RNA2 encodes a capsid precursor protein. A subgenomic 0.4 kb RNA (RNA3) is produced from the RNA1. The RNA3 contains two ORFs encoding B1 and B2 proteins, the latter of which is known to suppress RNA silencing (7). A remarkable feature of FHV is that the virus has the ability to cross the kingdom barrier in infection. Although FHV virions are infectious only to insect, FHV RNA can replicate in mammalian (6), plant (8), and yeast (9) cells when transfected into the cells. FHV RNA1 can replicate autonomously when transfected into permissive cells in the absence of either RNA2 or capsid proteins (10).

In the recombinant TYMV constructs described in this study, the FHV RNA1 sequence was produced as a sgRNA of TYMV; the recombinant sgRNA has the FHV sequence at the 5′-end, and the TYMV CP ORF at the 3′-end. The results show that self-replicating FHV RNA is produced from the recombinant TYMV sgRNA, and that the eGFP gene inserted into the FHV genome is correctly expressed. The results also show that not all the RNAs produced during the replication of TYMV are encapsidated, contrary to previous studies. Nevertheless, it was possible to encapsidate the FHV RNA into the TYMV virions. The information obtained in this study might be useful when there is a need to amplify a therapeutic gene that is delivered to a target animal cell in the form of RNA.

*Corresponding author. Tel: +82-43-261-2309; Fax: +82-43-267-2306; E-mail: tjcho@chungbuk.ac.kr

http://dx.doi.org/10.5483/BMBRep.2014.47.6.187

Received 16 August 2013, Revised 17 September 2013, Accepted 26 September 2013

Keywords: Encapsidation, FHV RNA1, Flock house virus, Recombinant virus, TYMV

ISSN: 1976-670X (electronic edition)

RESULTS AND DISCUSSION

DNA constructs

Before making the TY-FHV construct, we cloned the FHV RNA1 sequence in a plasmid, and the 3′-terminal part was modified so that several cloning sites and a weak version of HDV (wHDV) followed the FHV sequence (Fig. 1A). The cloning sites (*Pst*I, *Sal*I, *Not*I, *Eco*RV) were inserted near the 3′-end of B2 ORF. The DNA between *Sna*BI and *Spe*I sites of the plasmid pFHV-wHDV was used to replace the DNA in TY-eGFP. In the resulting construct TY-FHV, a recombinant sgRNA would be produced from the first tymobox (Fig. 1B).

The recombinant TYMV sgRNA would have FHV RNA1 sequence at the 5′-end, and TYMV CP ORF at the 3′-end. This sgRNA would serve as an mRNA for translation of FHV replicase. However, the RNA did not seem to be a good template for FHV replicase, because the 3′-end of the RNA would be very different from the authentic FHV RNA1. That is why we decided to put HDV ribozyme right after the FHV RNA1 sequence; the 3′ TYMV CP sequence could be cleaved off the recombinant sgRNA, leaving only FHV RNA1 sequence. Since the cleavage in turn would seriously interfere with TYMV RNA replication, we used a weak variant of HDV. The ribozyme variant contains A at the #75 position instead of C, which has 0.02% activity of the wild type HDV ribozyme (11). The recombinant sgRNA (sgRNA1) would have four extra nucleotides (AATA) at the 5′-end, compared to the authentic FHV RNA1. The extra nucleotides were inevitable, since the sequence around the *Sna*BI site constituted the sgRNA promoter and thus could not be changed (12). We also prepared a construct lacking the wHDV sequence (TY-FHVΔHDV) and the construct containing an eGFP gene in the FHV genome (TY-FHV(eGFP)). The eGFP gene was inserted into the *Sal*I/*Not*I sites in the FHV B2 ORF (see Fig. 1A).

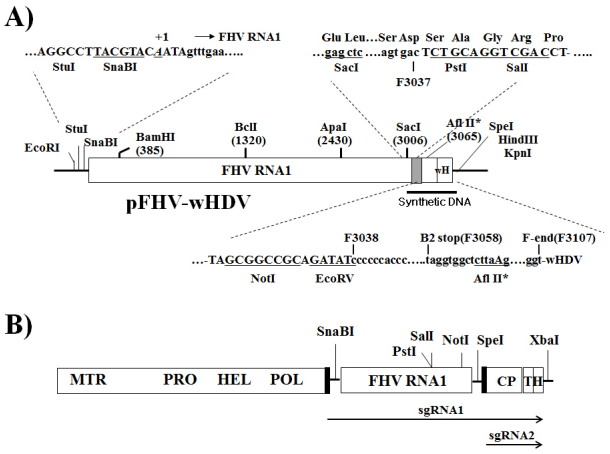

Fig. 1. FHV constructs. (A) pFHV-wHDV construct. In this construct, the cDNA of FHV RNA1 is contained in a plasmid vector. The 3′-terminal portion, the sequence between *Sac*I and *Spe*I sites, was replaced with a synthetic DNA that contains several restriction recognition sites (grey box,) and a ribozyme sequence derived from hepatitis delta virus (HDV). In the HDV ribozyme sequence, the 75th nucleotide C was replaced with A, to make it have weaker ribozyme activity (11). The sgRNA transcription start site is indicated by +1. Downstream of the B2 stop codon, an *Afl*II site was introduced by replacing one nucleotide (capital letter). (B) TY-FHV construct. The DNA between *Sna*BI and *Spe*I sites of the pFHV-wHDV was used to replace the eGFP gene of TY-eGFP (5). The FHV sequence is placed downstream of the tymobox (black box), a subgenomic RNA (sgRNA) promoter. The two sgRNAs produced from this construct are depicted by arrows.

Replication of TY-FHV constructs and FHV RNA production

The TY-FHV constructs were introduced into *Nicotiana benthamiana*, using the *Agrobacterium*-mediated T-DNA transfer system. Seven days after the agroinfiltration, the infiltrated leaf was collected for RNA extraction. Northern blot analysis of the total RNA using TYMV CP probe showed that the RNA bands from the TY-FHV constructs were weaker than those from TY-GUS (Fig. 2A). Considering that the replication of TY-GUS was about 10-20% of the wild type TYMV construct TY^{W} (5), it

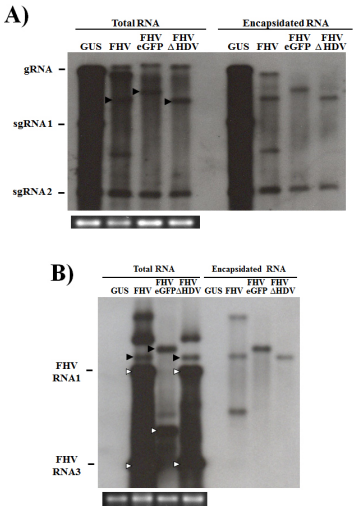

Fig. 2. Replication and RNA packaging of TY-FHV constructs. Seven days after agroinfiltration of *N. benthamiana* leaf with various TY-FHV constructs, total RNA was extracted from the leaf. The leaf extract was treated with RNase A, and then the viral RNA protected from the RNase action was prepared by phenol extraction. 1 μg of total RNA and equivalent amounts of encapsidated RNA were size-fractionated in the 1% agarose gel and examined by Northern blot analysis, using the DIG-labeled probe representing the (A) TYMV CP ORF, or (B) FHV sgRNA. The blots were developed by chemiluminescent immunodetection of DIG. The panel below the Northern blot represents a gel stained with ethidium bromide. In panel A, gRNA (8.1 kb), sgRNA1 (2.5 kb), and sgRNA2 (0.7 kb) of TY-GUS are marked on the left. The recombinant sgRNAs from TY-FHV constructs are indicated by black arrow-heads. In panel B, putative FHV RNA1 (3.1 kb) and sgRNA (0.4 kb or 1.1 kb) are indicated by white arrow-heads. The RNA bands marked by black arrow heads are the same as in panel A.

seemed that TY-FHV RNAs replicate rather poorly. TY-FHV constructs produced several unexpected RNA bands. Besides the gRNA and two sgRNAs (the larger ones are indicated by black arrow heads), one or two extra RNA species were observed. RNase protection assay showed that the two sgRNAs were efficiently encapsidated. In contrast, the packaging of the gRNA seemed to be inefficient.

We examined whether the TYMV CP was cleaved from the recombinant TYMV sgRNA that contained both FHV and TYMV CP ORF. If the TYMV CP sequence was cut off, the product RNA might have been able to replicate by the FHV replicase. Since the FHV RNA could not be detected by the TYMV CP probe, we performed the hybridization again, with the FHV probe representing the FHV sgRNA (RNA3). The result is shown in Fig. 2B. Here, several additional bands appeared. In particular, we observed the RNAs that were presumably representing the FHV RNA1 and RNA3 (indicated by white arrow heads). The 'FHV RNA1' was thought to be generated by cleaving the TYMV CP from the recombinant sgRNA1. The strong intensity of the RNA bands and the presence of FHV sgRNA (FHV RNA3) suggest that the FHV RNAs were products of FHV replication. Curiously, however, the same RNA bands were also observed in the case of TY-FHVΔHDV, indicating that FHV RNA1 could have been produced from any FHV-CP RNAs and have replicated efficiently irrespective of the presence of 3′-terminal TYMV CP.

Previously, Ball (6) reported that extended sequences beyond the ends of the authentic FHV RNA1 inhibited RNA replication. 12 additional nucleotides at the 3′-end lowered the RNA yield by about half. Extension of 26 and 43 nucleotides at the 5′- and 3′ends, respectively, abolished the replication. Annamalai *et al*. (13), in contrast, reported that 33 nucleotide extension at the 3′-end could support the replication of FHV RNA. Our result indicates that the FHV replicase correctly recognized the 3′-terminal sequence of the FHV RNA1 on the FHV-CP RNA and terminated (or initiated) the transcription right at the sequence. Probably, the 3′-terminal sequence of the FHV RNA1 of the FHV-CP RNA, even in the presence of TYMV CP sequence at the 3′-end, could have taken the right secondary or tertiary structures to be recognized by the FHV replicase. The 5′-end of the FHV-CP RNA has a 4-nucleotide extension. This extension apparently did not inhibit the translation and replication of the FHV RNA.

The RNA band corresponding to the FHV RNA1 was not observed in TY-FHV(eGFP). Interestingly, however, the RNA band representing the recombinant FHV sgRNA containing eGFP gene (marked by white arrow head) was observed. We do not know how the FHV sgRNA was generated. It might be that the inserted sequence (eGFP) somehow inhibited the production of FHV RNA1 but not the production of FHV sgRNA during the transcription reaction using the (-)-strand of FHV RNA as a template.

To identify all the RNAs produced from the TY-FHV constructs, we performed the Northern blot hybridization again using a mixed probe of TYMV CP and FHV sequences. The results are shown in Fig. 3A. Here, all the bands from TY-FHV and TY-FHV(eGFP) are labeled. The eight recognizable RNA bands from TY-FHV are labeled 1 to 8. Based on the size and the hybridization results with CP and FHV probes, five out of the eight RNA species could have been identified. The interpretation is represented in Fig. 3B. #1 RNA would be a gRNA whose size is 9.4 kb. The weak intensity might indicate that the production of (-)-strand RNA is frequently incomplete due to the long size. Or, the inserted sequence could have interfered with the recognition of replication elements present at the 5′-end, thereby inhibiting the production of gRNA. #4 RNA band would represent the recombinant TYMV sgRNA (3.8 kb) containing FHV and CP sequences. #5 RNA species would be the FHV RNA1 (3.1 kb). #7 and #8 RNA bands

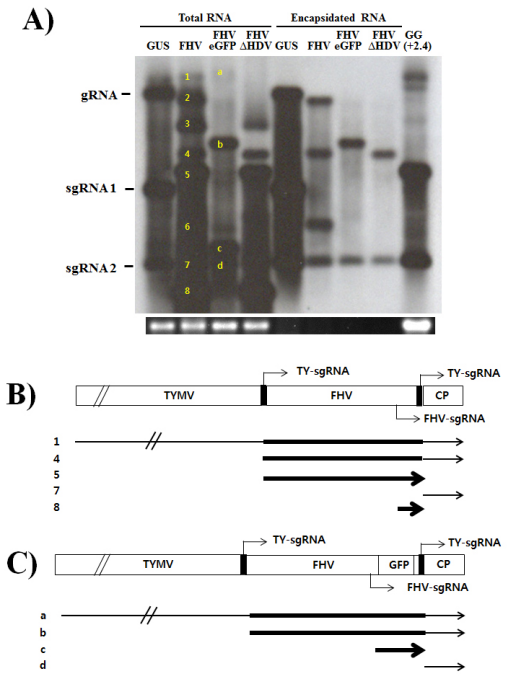

Fig. 3. Northern analysis, using a mixture of TYMV CP and FHV probes. (A) Northern blot hybridization was carried out, as described in Fig. 2, except that the probe used was a mixture of TYMV CP and FHV sgRNA sequences. The RNAs produced from TY-GUS, i.e. gRNA (8.1 kb), sgRNA1 (2.5 kb), and sgRNA2 (0.7 kb), are marked on the left. Eight RNA species from TY-FHV are marked 1 to 8. Four RNA species from TY-FHV(eGFP) are marked a to d. The rightmost sample TY-GG(2.4), which contains a 2.4 kb insert sequence (two copies of partial GUS sequences), was included as a blotting control. (B) Schematic representation of the RNA species from TY-FHV. The sgRNA promoter tymobox is represented as a black box. Five RNA species, which are identified by the size and the results of hybridization to two different probes, are depicted below the construct. (C) Schematic representation of the RNA species from TY-FHV(eGFP). Four RNA species are schematically drawn below the construct. The FHV sequences are indicated in thick lines.

would represent the TYMV sgRNA (0.7 kb) and the FHV sgRNA (0.4 kb), respectively. As for the three RNA bands (#2, #3, and #6), we do not know what those are and how they were produced, although #2 and #3 RNA species are thought to be the products of recombination.

Four RNA bands from TY-FHV(eGFP) were labeled as a to d, and the interpretation of the RNAs is represented in Fig. 3C. RNA species a would represent a gRNA, whose size is 10.2 kb. RNA b would be a TYMV sgRNA containing the FHV sequence (4.6 kb). RNA c is thought to be a recombinant FHV sgRNA (1.1 kb) containing the eGFP gene. RNA d would be the TYMV sgRNA, whose size is 0.7 kb.

Packaging FHV RNA into TYMV virion, and expression of the eGFP reporter

We were interested in whether or not the RNA containing FHV sequence could be encapsidated into TYMV virions. Figures 2 and 3 show that the FHV-CP RNAs (band 4 and band b) were as efficiently encapsidated as the authentic TYMV sgRNA. The gRNAs from the TY-FHV constructs were found to be not well packaged. The inefficiency of RNA packaging could be due to the large size of the RNA. Recently, we have observed that the efficiency of gRNA becomes low when the insert is larger than 2.4 kb.

So far, it has been observed that the gRNAs and sgRNAs produced during TYMV replication are all encapsidated. Here, the rule was broken. As for the sgRNAs produced from TY-FHV constructs, those RNAs seemed to be efficiently encapsidated into TYMV virions as long as the RNAs contain the TYMV CP sequence. The RNAs lacking the TYMV sequence, for example FHV RNA1 and FHV sgRNA, were not encapsidated. This observation can be explained by assuming that a packaging signal resides in the TYMV CP sequence. Previous studies, however, have shown that the CP sequence has nothing to do with viral RNA packaging. Deletion of 5′ 448 nucleotides (about three-quarters) of the CP ORF and tymobox did not affect the RNA packaging (14). We also observed that the recombinant TYMV RNA with either deletion of 3′ quarter of CP ORF or deletion of 3′-end tRNA-like sequence was efficiently encapsidated. Therefore, the inefficient RNA packaging of the FHV RNAs could not be ascribed to the absence of TYMV CP sequence.

An alternative explanation could be that the replication sites of TYMV and FHV are different. TYMV RNA replication occurs on the outer membrane of chloroplasts (1). In contrast, FHV replicates on outer mitochondrial membranes (15). Thus, the FHV-CP RNA would be produced within the spherules on chloroplasts, and encapsidated by the TYMV CPs as they exit from the spherules. Some of the FHV-CP RNAs would serve as an mRNA for translation of FHV replicase. The replicase would then translocate to mitochondria, along with FHV-CP RNA, and replicate FHV RNAs there. Considering this scenario, the FHV RNA1 and FHV sgRNA produced on mitochondria would not have the chance to be encapsidated by the TYMV CPs that are partially assembled around the spherules on chloroplasts.

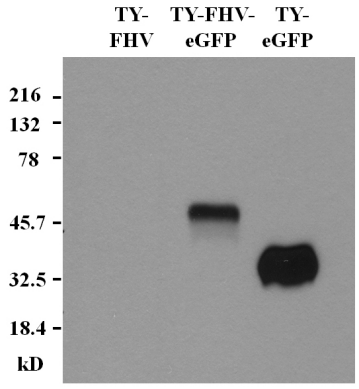

Fig. 4. Western analysis of eGFP expression in TY-FHV(eGFP). 10 μl of leaf extract was loaded, and electrophoresed in 12.5% SDS-polyacrylamide gel. TY-eGFP was included as a control. The proteins were transferred to a nitrocellulose membrane. eGFP was detected using anti-GFP rabbit antibody and anti-rabbit HRP conjugate. The membrane was developed by a Luminata™ Forte (Millipore), using luminol as the substrate.

The RNA corresponding to the band c was thought to represent the FHV sgRNA containing the eGFP reporter gene. The insertion site was at the 3′-terminal part of the B2 ORF, which is present near the 3′-end of RNA1 (Fig. 1A). This insertion site was reported to be productive in expression of eGFP (16). In addition, the insertion of the eGFP gene was such that a fusion protein of B2-eGFP would be generated. To see if such a fusion protein was produced, we examined the leaf extract prepared from the leaves inoculated with TY-FHV(eGFP), by Western analysis using an anti-eGFP antibody. The result is shown in Fig. 4. The detected band was about 47 kDa in size, which was expected for the B2-eGFP fusion protein.

In conclusion, the results show that FHV RNAs can be encapsidated in the form of FHV-CP RNA. The results also show that FHV RNA replication occurs and that FHV sgRNA is produced. The reporter gene expressed as an FHV sgRNA is translated correctly. Although it remains to be seen whether the FHV(eGFP) sgRNA can indeed be generated from the FHV(eGFP)-CP RNA, this shows the feasibility that a gene can be amplified with the help of FHV RNA1 in animal cells, once it is delivered to the cell in the form of RNA.

MATERIALS AND METHODS

DNA constructs

To make the construct TY-FHV, the eGFP sequence in TY-eGFP (5) was replaced by the 3.2 kb DNA, between the *Sna*BI and *Spe*I sites of pFHV-wHDV (Fig. 1A). The FHV-wHDV was prepared by using the cDNA for FHV RNA1, which was a kind gift from Dr. Miller at the University of Michigan. The DNA between *Sac*I and *Spe*I sites of the pFHV-wHDV was a synthetic DNA, which contains several cloning sites (*Pst*I, *Sal*I, *Not*I,

*Eco*RV) and a ribozyme sequence derived from Hepatitis delta virus (HDV). The nucleotide C at the position #75 was changed to A so that it has weaker ribozyme activity (11).

TY-FHVΔHDV was prepared by replacing the 3′-terminal part (the sequence between *Fsp*I and *Spe*I) of the FHV-wHDV with the DNA lacking the HDV sequence, which was PCR-amplified using the following two primers: upstream primer FHV(+)2838 (5′ CGT GCG CAG GGA CCT CGA CAA CCT GCA 3′; *Fsp*I restriction recognition site is underlined), and downstream primer FHV(-)3107 primer (5′ CGA CTA GTA CCT CTG CCC TTT CGG GCT 3′; *Spe*I recognition site is underlined). To make the construct TY-FHV(eGFP), a PCR-amplified eGFP gene was first inserted into the *Sal*I and *Not*I sites of the pFHV-wHDV. The two primers used in the PCR are as follows: upstream primer eGFP-F-SalI (5′ GCC GTC GAC CTA GCA AGG GCG AGG AGC TG 3′; *Sal*I recognition site is underlined), and downstream primer eGFP-R-NotI (5′ CAG GGC GGC CGC TAT TAC TTG TAC TCG TCC A 3′; *Not*I recognition site is underlined). The DNA between *Sna*BI and *Spe*I of the resulting construct was then used to replace the sequence between the *Sna*BI and *Spe*I sites of the TY-FHV. After these manipulations, the resulting constructs were verified by sequence analyses.

Plant material

Agroinfiltration of the *Agrobacterium tumefaciens* harboring various TYMV constructs into *Nicotiana benthamiana* was carried out as previously described (17). 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 were stored at -80^{o}C. For the encapsidation assay, the leaf sample was ground with 4 times its volume of phosphate buffer (pH 7.0). The homogenate was clarified by the addition of 0.2 volumes of chloroform, centrifuged briefly, and was stored at 4^{o}C, until use.

Analysis of RNA

Ribonuclease protection assay for encapsidated RNA and Northern analysis were performed as previously described (18). Briefly, in the encapsidation assay, leaf extracts were incubated with RNase A (5 μg/ml at the final concentration) for 1 h at 37^{o}C, and for an additional 1 h in the presence of proteinase K and SDS before phenol extraction. Total RNAs and equivalent amounts of the RNA samples from the encapsidation assay were size-fractionated by electrophoresis on a 1% agarose gel, and were transferred onto Hybond N^{+} membranes (GE Healthcare, Chalfont St. Giles, England). The blots were hybridized with a DIG-labeled DNA probe, representing the TYMV coat protein ORF, or the FHV sgRNA.

Western analysis of eGFP expression

Leaf samples (0.1 g) for protein analysis were ground in 200 μl of 2X sample buffer and were boiled for 5 min. Proteins were separated by 12.5% SDS-polyacrylamide gel electrophoresis followed by Western blot analysis, as described (19). eGFP was detected using rabbit anti-GFP antibody (Santa Cruz Biotechnology, CA, USA) in conjuction with HRP-conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA, USA). The blot was developed by a chemiluminescent detection method, using a Luminata™ Forte (Millipore, Bedford, MA, USA).

ACKNOWLEDGEMENTS

This study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2010-0021868).

REFERENCES

1. Dreher, T. W. (2004) Turnip yellow mosaic virus: transfer RNA mimicry, chloroplasts and a C-rich genome. *Mol. Plant Pathol.* **5**, 367-375.
2. Prod'homme, D., Jakubiec, A. Tournier, V., Drugeon, G. and Jupin, I. (2003) Targeting of the Turnip Yellow Mosaic Virus 66K replication protein to the chloroplast envelope is mediated by the 140K protein. *J. Virol.* **77**, 9124-9135.
3. Chen, J., Li, W. X., Xie, D., Peng, J.R. and Ding, S.W. (2004) Viral virulence protein suppresses RNA silencing-mediated defense but upregulates the role of microRNA in host gene expression. *Plant Cell* **16**, 1302-1313.
4. Yildiz, I., Shukla, S. and Steinmetz, N. F. (2011) Applications of viral nanoparticles in medicine. *Curr. Opin. Biotechnol.* **22**, 901-908.
5. Shin, H.-I., Kim, I.-C. and Cho, T.-J. (2008) Replication and encapsidation of recombinant Turnip yellow mosaic virus RNA. *BMB Rep.* **41**, 739-744.
6. Ball, L. A. (1995) Requirements for the self-directed replication of Flock house virus RNA1. *J. Virol.* **69**, 720-727.
7. Li, H., Li, W. X. and Ding, S. W. (2002) Induction and suppression of RNA silencing by an animal virus. *Science* **296**, 1319-1321.
8. Selling, B. H., Allison, R. F. and Kaesberg, P. (1990) Genomic RNA of an insect virus directs synthesis of infectious virions in plants. *Proc. Natl. Acad. Sci.* **87**, 434-438.
9. Price, B. D., Ruekert, R. R. M. and Ahlquist, P. (1996) Complete replication of an animal virus and maintenance of expression vectors derived from it in Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci.* **93**, 9465-9470.
10. Johnson, K. L. and Ball, L. A. (1999) Induction and maintenance of autonomous Flock house virus RNA1 replication. *J. Virol.* **73**, 7933-7942.
11. Perrotta, A. T., Shih, I. and Been, M. D. (1999) Imidazole rescue of a cytosine mutation in a self-cleaving ribozyme. *Science* **286**, 123-126.
12. Schirawski, J., Voyatzakis, A., Zaccomer, B., Bernardi, F. and Haenni, A. L. (2000) Identification and functional analysis of the turnip yellow mosaic tymovirus subgenomic promoter. *J. Virol.* **74**, 11073-11080.
13. Annamalai, P., Rofail, F., DeMason, D.A. and Rao, A.L.N.

(2008) Replication-coupled packaging mechanism in positive-strand RNA viruses: Synchronized coexpression of functional multigenome RNA components of an animal and a plant virus in *Nicotiana benthamiana* cells by agroinfiltraion. *J. Virol.* **82**, 1484-1495.
14. Shin, H.-I., Kim, H. Y. and Cho, T.-J. (2010) The Pro/Hel Region is indispensable for packaging non-replicating Turnip Yellow Mosaic Virus RNA, but not replicating viral RNA. *Mol. Cells* **29**, 463-469.
15. Miller, D. J., Schwartz, M. D. and Ahlquist, P. (2001) Flock house virus RNA replicates on outer mitochondrial membranes in Drosophila cells. *J. Virol.* **75**, 11664-11676.
16. Price, B. D., Roeder, M. and Ahlquist, P. (2000) DNA-directed expression of functional flock house virus RNA1 derivatives in Saccharomyces cerevisiae, heterologous gene expression, and selective effects on subgenomic mRNA synthesis. *J. Virol.* **74**, 11724-11733.
17. Cho, T.-J. and Dreher, T. W. (2006) Encapsidation of genomic but not subgenomic Turnip yellow mosaic virus RNA by coat protein provided in trans. *Virology* **356**, 126-135.
18. Shin, H.-I., Chae, K.-H. and Cho, T.-J. (2013) Modification of Turnip yellow mosaic virus coat protein and its effect on virion assembly. *BMB Rep.* **46**, 495-500.
19. Shin, S. Y., Kim, C. G. and Lee, Y. H. (2013) Egr-1 regulates the transcription of the BRCA1 gene by etoposide. *BMB Rep.* **46**, 92-96.