

## Replication of Recombinant Flock House Virus RNA Encapsidated by Turnip Yellow Mosaic Virus Coat Proteins in *Nicotiana benthamiana*

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It was previously observed that recombinant flock house virus (FHV) RNA1 was efficiently packaged into turnip yellow mosaic virus (TYMV), provided that the TYMV coat protein (CP) sequence was present at the 3'-end. FHV RNA encapsidated by TYMV CPs also had a four-nucleotide extension at the 5'-end. Since even a short extension at the 5'- and 3'-ends of FHV RNA1 inhibits replication, we examined whether the recombinant FHV RNA is indeed capable of replication. To this end, we introduced constructs expressing recombinant FHV RNAs into the plant *Nicotiana benthamiana*. Northern blot analysis of inoculated leaves suggested abundant production of recombinant FHV RNA1 and its subgenomic RNA. This demonstrated that recombinant FHV RNA with terminal extensions at both ends was competent for replication. We also showed that the recombinant FHV RNA can express the reporter gene encoding enhanced green fluorescent protein.

**Key Words:** Turnip yellow mosaic virus, Flock house virus, RNA replication, Reporter gene expression

### INTRODUCTION

RNA has emerged as a viable therapeutic molecule, especially for cancer immunotherapies, infectious disease vaccines, and protein replacement therapies (1, 2). Unlike DNA, RNA does not integrate into chromosomes; therefore, insertional mutagenesis will not occur. RNA does not need to enter into the nucleus for its expression. Recently, plant viruses have been investigated as vehicles to deliver RNA into animal cells because they are safe to use and large quantities can be obtained with ease. Smith *et al.* (3) prepared pseudovirus particles in which Semliki Forest virus (SFV) RNA was encapsidated by tobacco mosaic virus (TMV) coat proteins (CPs). The SFV RNA contained a TMV origin of

assembly, and the pseudovirus particles were shown to express reporter genes when transfected into baby hamster kidney cells. More recently, hybrid virus-like particles were prepared *in vitro* through an assembly of cowpea chlorotic mottle virus (CCMV) CPs in the presence of Sindbis virus RNA containing a CCMV packaging signal (4). When mammalian cells were transfected with the hybrid viral particles, they were shown to replicate and express reporter genes.

We aimed to tap the potential of utilizing the natural encapsidation machinery of plant viruses to package therapeutic RNA molecules. We have used the turnip yellow mosaic virus (TYMV), as it was previously shown that foreign genes can be encapsidated into TYMV virions, provided that it is expressed in the form of TYMV subgenomic RNA (sgRNA) (5). TYMV is a spherical plant virus that has

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a monopartite genome of 6.3 kb positive-sense RNA (6). TYMV is the type species of the genus *Tymovirus*, family *Tymoviridae*. Previously, it was also observed that the flock house virus (FHV) RNA1 was packaged into TYMV virions when the FHV RNA had the TYMV CP sequence at its 3'-end (7). Here, FHV RNA1 was employed to amplify reporter genes. FHV, a member of the *Nodaviridae* family, is a positive strand RNA virus of insect origin. FHV has the ability to infect a wide variety of cells, including mammalian, yeast, and plant cells (8~10), in addition to insect cells. It has a bipartite genome. RNA1 is 3.1 kb and encodes the RNA-dependent RNA polymerase (RdRP). FHV RNA1 can replicate independently of RNA2, which encodes for the capsid protein (11). RNA3, a sgRNA of RNA1, has two overlapping open reading frames (ORFs) for B1 and B2 proteins. B2 is known as a potent RNAi suppressor (12).

Ball (8) reported that even short terminal extensions of FHV RNA nearly abolish replication. In particular, this work indicated that 5'-terminal extension was detrimental to RNA1 replication. One nucleotide (G) addition did not inhibit replication; however, two and 10 nucleotide additions decreased replication by 80% and 98%, respectively. Notably, 3'-terminal extension was less crucial compared to the 5'-terminal extension. Nevertheless, 12 nucleotide additions at the 3'-end resulted in more than 40% decrease in replication. Recombinant FHV RNA1 encapsidated in TYMV virions has a four-nucleotide extension at the 5'-end and a rather long heterologous sequence (around 800 nucleotides) at the 3'-end. The effect of a long foreign sequence at the 3'-end on FHV RNA1 replication has not been investigated.

Herein, we assessed whether recombinant FHV RNA encapsidated in TYMV can replicate and express the reporter gene encoding enhanced green fluorescent protein (eGFP). To this end, we first examined whether recombinant FHV RNA1 was able to replicate and express eGFP in the plant *Nicotiana benthamiana*. We showed that FHV RNA and eGFP were abundantly produced. Overall, our findings show the potential that TYMV can be used to deliver recombinant FHV RNA that can replicate and express foreign genes upon delivery to host cells.

## MATERIALS AND METHODS

### DNA constructs

The construct pFHV-wtH2 containing FHV RNA1 and hepatitis delta virus (HDV) ribozyme sequences was prepared using the cDNA for FHV RNA1, which was a cordial gift from Dr. Miller at the University of Michigan. The FHV and HDV sequences in the pFHV-wtH2 construct were inserted between the *StuI* and *KpnI* sites of the pCass2 vector (13). The sequence between the *HindIII* and *SpeI* sites, which contains double 35S promoters of cauliflower mosaic virus (CaMV) and FHV sequence, was then transferred into the pCB302-3 vector (14), producing the pCB FHVwtH2 construct. The sequence between the *BamHI* and *SpeI* sites of pCB FHVwtH2 was replaced with the sequence in the construct TY-FHV(eGFP), which was described previously (7). The resulting construct was designated as pCB FHV(eGFP)-wHDV. The TYMV CP sequence was added to the 3'-end of the insert by replacing the sequence between the *MluI* and *XbaI* sites of pCB FHV(eGFP)-wHDV with the sequence in the TY-FHV(eGFP) construct, yielding pCB FHV(eGFP)-CP-0. The RNA produced from the pCB FHV(eGFP)-CP-0 construct would have 14 additional nucleotides at the 5'-end. The 5'-end of the FHV sequence was then modified to make its RNA product same as the RNA encapsidated in TYMV. The sequence between the *StuI* and *BamHI* sites was amplified using the following two primers: forward primer (5'-pAATAGTTTGAAACAAATAAAACAG-3') and reverse primer (5'-CTCAAATTTGGATCCTCCA-3'; the *BamHI* recognition site is underlined). The PCR product was digested with *BamHI* and was used to replace the sequence between the *StuI* and *BamHI* sites of pCB FHV(eGFP)-CP-0. The resulting construct was designated as pCB FHV(eGFP)-CP.

The pCB FHV-CP construct was derived from pCB FHV(eGFP)-CP. The 3'-terminal part of the FHV RNA1 sequence of pCB FHVwtH2 was PCR-amplified using the following two primers: forward primer (5'-CGTGCAGGGACCTCGACAACCTGCA-3'; the *FspI* recognition site is underlined) and reverse primer (5'-CGACTAGTACCTCTGCC-

CTTTCGGGCT-3'; the *SpeI* recognition site is underlined). The PCR product, approximately 270 bp, was used to replace the sequence between the *FspI* and *SpeI* sites of pCB FHV (eGFP)-CP. The pCB FHV(eGFP) $\Delta$ HDV-CP construct was derived from the TY-FHV(eGFP) $\Delta$ HDV-CP construct (7).

### Plant material

Agroinfiltration of *Agrobacterium tumefaciens* harboring various constructs into *Nicotiana benthamiana* was carried out as previously described (15). 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°C. For the encapsidation assay, the leaf sample was ground with four 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°C until further use.

### Analysis of RNA

Total RNA was isolated from frozen *N. benthamiana* leaf samples using Easy-Red (Intron, Seoul, Korea). The ribonuclease protection assay for encapsidated RNA in TYMV virions and northern blot analysis were performed as previously described (15). RNA samples were size-fractionated by electrophoresis on 1% agarose gel, and were transferred onto Hybond N<sup>+</sup> membranes (GE Healthcare Life Sciences, Pittsburgh, PA, USA). The blots were hybridized with a DIG-labeled DNA probe representing the TYMV CP ORF, the FHV sgRNA, or both.

### Western analysis of eGFP expression

Leaf samples (0.1 g) for protein analysis were ground in 200  $\mu$ l of 2  $\times$  SDS-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and were boiled for 5 min. Proteins were separated by 12.5% SDS-PAGE followed by western blot analysis. eGFP was detected using rabbit anti-eGFP antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in conjunction with HRP-conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA, USA). The blot was developed by a chemiluminescent detection method, using Luminata<sup>TM</sup>

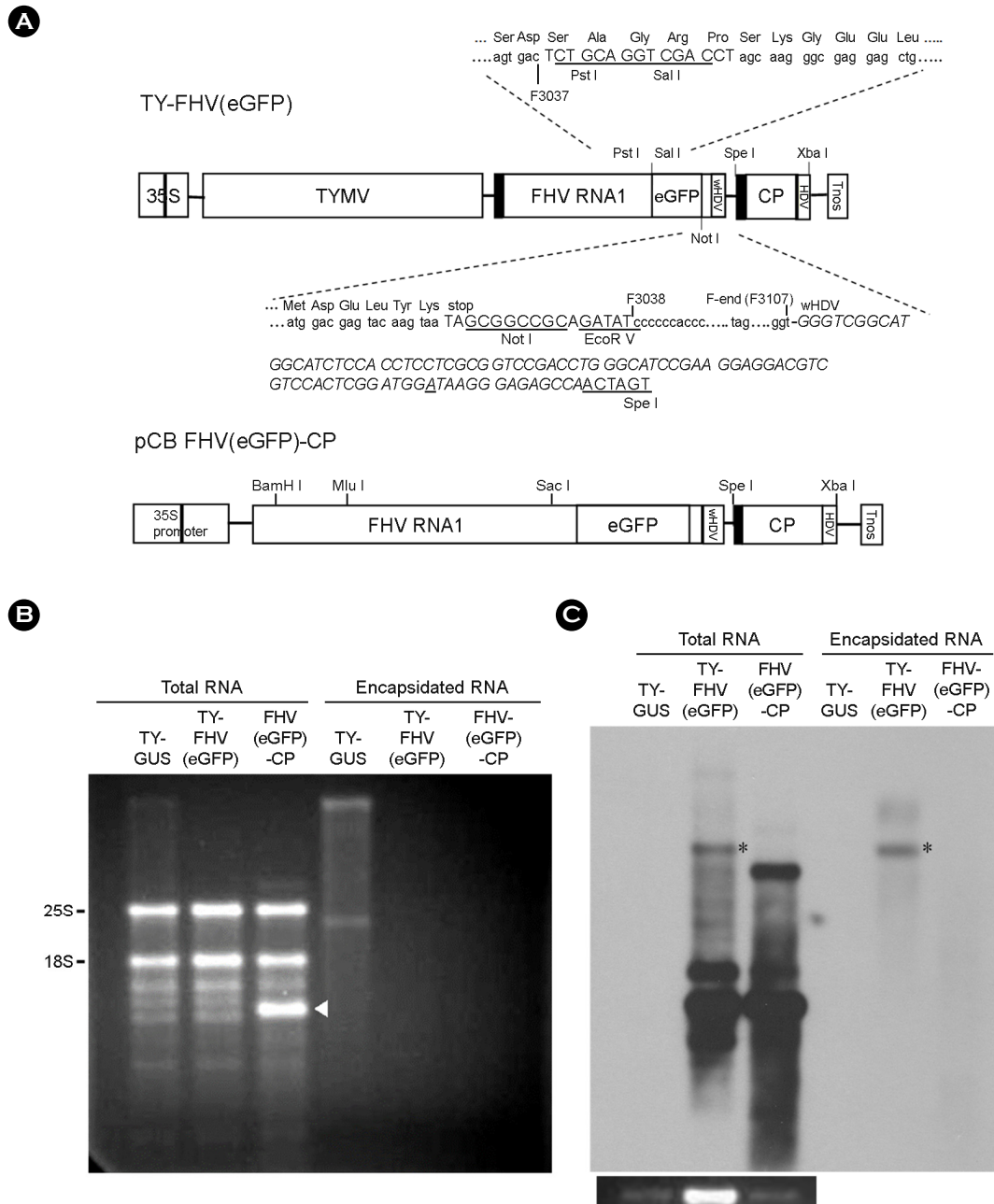
Forte (Millipore, Bedford, MA, USA).

## RESULTS

### Replication of TYMV CP-encapsidated FHV RNA in *N. benthamiana*

Previously, we have shown that FHV RNA can be packaged into TYMV when expressed as TYMV sgRNA (7). Fig. 1A shows the TY-FHV construct used to express FHV RNA1. FHV RNA1 is produced under the influence of an additional TYMV tymobox, a sgRNA promoter. The tymobox is upstream of the stop codon of the TYMV RdRP ORF. Thus, when FHV RNA is expressed from the sgRNA promoter, it would have a four nucleotide (5'-AATA-3') extension at the 5'-end, which is inevitable during expression of the FHV sequence as a TYMV sgRNA while maintaining the RdRP ORF intact. The recombinant FHV RNA also had a TYMV CP sequence at the 3'-end. The FHV sequence was followed by a weak version of the HDV ribozyme sequence (16), which was inserted in a hope to slowly cut the 3'-terminal TYMV CP sequence off the FHV-CP RNA. We assumed that HDV activity was needed to make the FHV-CP RNA replicable; however, strong HDV activity at this position would lead to removal of replication elements present at the 3'-terminal part of TYMV RNA. Hence, we used a weak version of HDV. The eGFP gene was included as a reporter near the 3'-end of the FHV genome, in-frame with the B2 ORF of FHV RNA1. Since the insertion site is downstream of the stop codon of the FHV replicase ORF, the eGFP gene insertion does not influence FHV replicase activity.

The observation that FHV-CP RNA, but not FHV RNA without the CP sequence, was packaged into TYMV virions led us to examine whether recombinant FHV RNA could replicate when it was delivered to plant or animal cells. As mentioned earlier, we were concerned whether TYMV CP-encapsidated FHV RNA replicates. To address this issue, we prepared a construct designated as pCB FHV(eGFP)-CP (Fig. 1A) to mimic the FHV RNA that is packaged in TYMV virions. The FHV RNA was expressed under the influence of double CaMV 35S promoters.



**Figure 1.** Replication and encapsidation of recombinant FHV RNA1. (A) DNA constructs. Preparation of TY-FHV(eGFP) was described previously (7). Briefly, FHV RNA1 sequence was placed downstream of the tymobox (black box), a sgRNA promoter. eGFP sequence was inserted into the B2 ORF of the FHV genome. The ribozyme sequence derived from the HDV is italicized. The nucleotide A that makes the ribozyme weak is underlined. A portion of the TY-FHV(eGFP), the DNA comprising the FHV RNA1 sequence, eGFP gene, HDV ribozyme, and TYMV CP sequence, was placed downstream of double 35S promoters of CaMV in pCB 302-3, yielding the pCB FHV(eGFP)-CP construct (for details, see MATERIALS AND METHODS). (B) Agarose gel electrophoresis. Seven days after agroinfiltration of *N. benthamiana* leaves with various FHV constructs, total RNA was extracted. Encapsidated RNA samples were prepared by incubating leaf extracts with RNase A, followed by phenol/chloroform extraction and ethanol precipitation. RNA samples were size-fractionated in a 1% agarose gel and stained with EtBr. 25S and 18S rRNAs are indicated. (C) Northern blot analysis of FHV RNA replication. 500 ng of total RNA and equivalent amounts of encapsidated RNA were size-fractionated in a 1% agarose gel and examined by northern blot analysis, using the DIG-labeled probe targeting FHV sgRNA. The blots were developed by chemiluminescent immunodetection of DIG. The panel below the northern blot shows 25S rRNA stained with EtBr. Agroinfiltration and northern blot analysis were carried out in triplicate, and a representative blot is shown. TY-GUS construct containing a  $\beta$ -glucuronidase gene (5) was included in this experiment as a negative control, since the FHV probe does not hybridize to the TY-GUS RNA.

When the pCB FHV(eGFP)-CP construct was introduced into *N. benthamiana*, an RNA species was copiously produced (arrowhead in Fig. 1B). When total and encapsidated RNA samples from agroinfiltrated leaves were analyzed by northern blot analysis using a probe targeting FHV sgRNA, it was observed that FHV RNA was abundantly produced in leaves inoculated with the pCB FHV(eGFP)-CP construct (Fig. 1C). The FHV RNA produced from pCB FHV(eGFP)-CP was 10 times more abundant than FHV RNA from TY-FHV(eGFP) (notably, the pCB FHV(eGFP)-CP RNA sample used for loading was 1/10 of the TY-FHV(eGFP) RNA sample). The results clearly show that the FHV(eGFP)-CP RNA generated self-replicating FHV RNAs, as evidenced by the abundant and small RNA bands representing FHV sgRNA. The expected FHV(eGFP)-CP RNA, however, was not clearly seen in the pCB FHV(eGFP)-CP RNA sample. As reported earlier (7), FHV RNA with the TYMV CP sequence at the 3'-end (marked by an asterisk) was encapsidated into TYMV virions.

#### Analysis of FHV RNA replication products

To confirm the presence of FHV(eGFP)-CP RNA in the pCB FHV(eGFP)-CP sample, northern blot analysis was performed again using five times more RNA, where TYMV CP sequence was used as a probe (Fig. 2A). In the TY-FHV(eGFP) sample, two sgRNAs were observed, though the presence of genomic RNA (gRNA) was not evident. In the pCB FHV(eGFP)-CP sample, only one RNA band representing the FHV(eGFP)-CP RNA was observed. In the RNase protection assay, the RNA from the pCB FHV(eGFP)-CP construct was not protected (Fig. 2B), as expected, because there were no viral coat proteins.

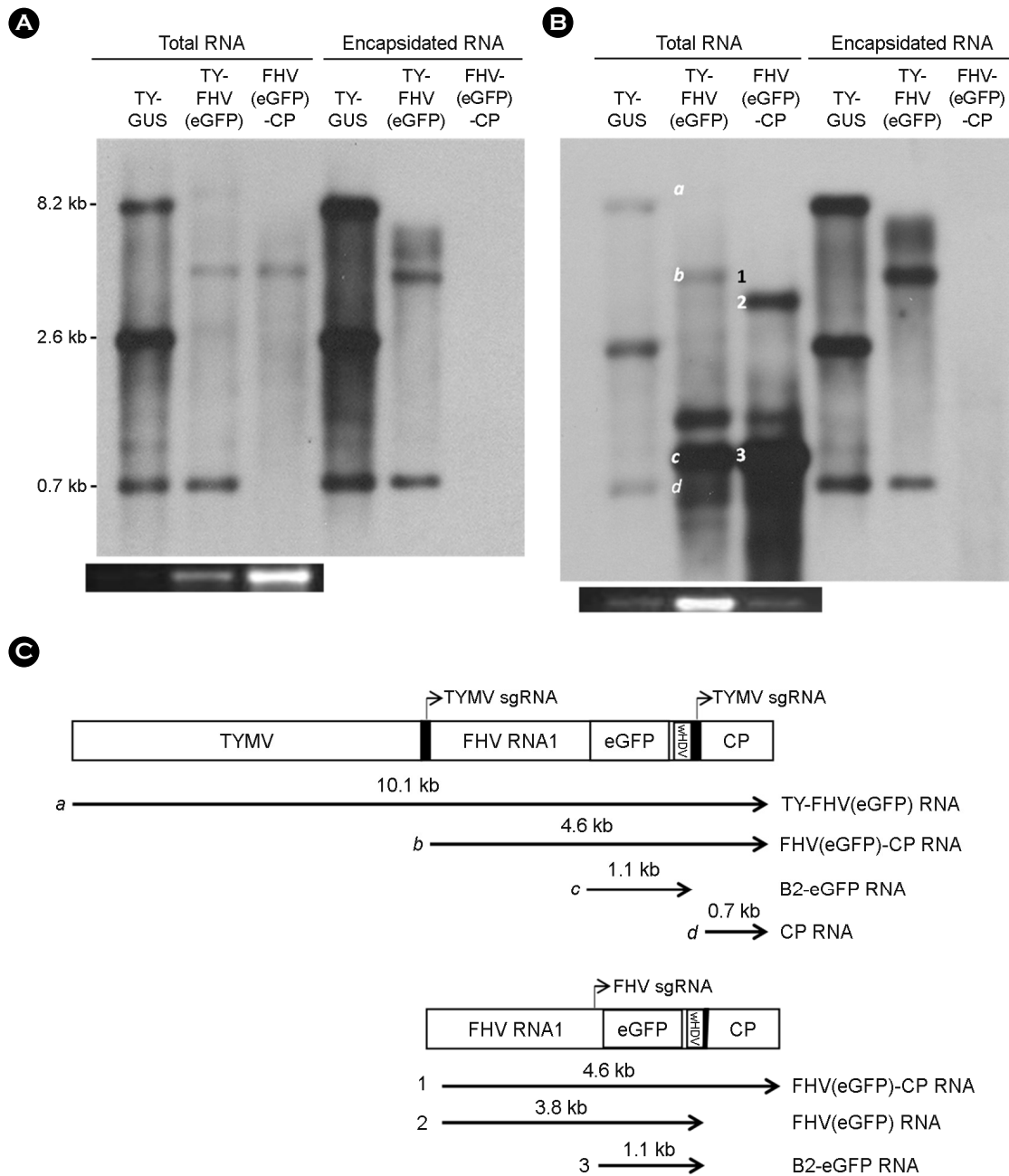
To identify the RNAs produced from the two constructs, we performed northern blotting once again using probes targeting the TYMV CP and FHV sequences (Fig. 2B). Here, all the identifiable bands anticipated from the TY-FHV(eGFP) and pCB FHV(eGFP)-CP samples are labeled. The four RNA bands from TY-FHV(eGFP) are labeled *a* to *d*. Based on the RNA size and the results of hybridization with the CP and FHV probes, the interpretation is shown in Fig. 2C. RNA species *a* was not clearly visible but is most

likely a gRNA, approximately 10.1 kb in size. The weak intensity indicated that the replication of this large TYMV gRNA species is not efficient. Probably, the inserted sequence might have interfered with the recognition of replication elements present at the 5'-end of the gRNA, thereby preferentially inhibiting production of gRNA. RNA species *b* represents recombinant TYMV sgRNA (4.6 kb) containing the FHV sequence. RNA species *c* and *d* represent recombinant FHV sgRNA (1.1 kb) and TYMV sgRNA (0.7 kb), respectively. Additional bands represent the products generated during FHV RNA replication, since those bands were hybridized with the FHV probe and not the TYMV probe. Presumably, these RNA species are FHV RNA homo- and heterodimers that are frequently produced during FHV RNA replication (17).

Three RNA bands from the pCB FHV(eGFP)-CP sample were identifiable and labeled #1 to #3 (Fig. 2C). The #1 RNA species, which is the same as RNA species *b* (Fig. 2B), is FHV(eGFP)-CP RNA; #2 is recombinant FHV RNA1 containing the eGFP sequence (3.8 kb); and #3 is the same as RNA species *c* and represents recombinant FHV sgRNA (1.1 kb). Thus, these results demonstrated that recombinant FHV RNA1 with the TYMV CP sequence at the 3'-end replicates and is capable of producing RNA1 and RNA3.

#### Reporter gene expression

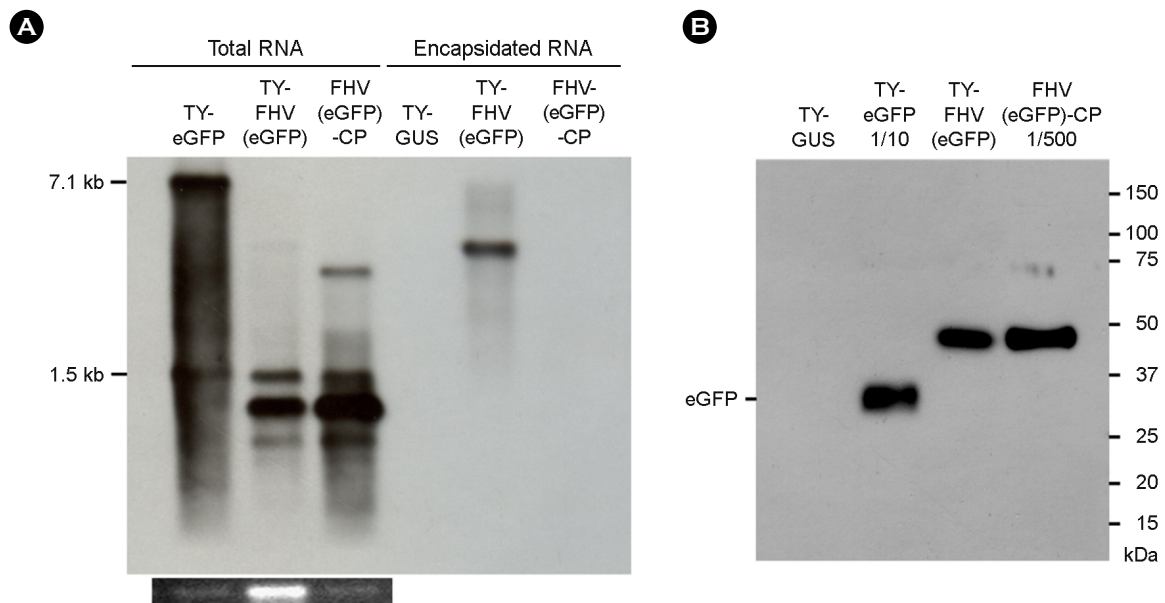
The eGFP gene was inserted in-frame near the 3'-end of B2 ORF of the FHV RNA1. Thus, eGFP was expected to be produced as a fusion protein of B2-eGFP from recombinant FHV RNA3 (bands #3 and *c* in Fig. 2B). Fig. 3A shows that the recombinant FHV RNAs were also detected with an eGFP probe. Western blot analysis of eGFP showed that the recombinant eGFP protein was abundantly expressed in leaves inoculated with the constructs (Fig. 3B). Interestingly, there was a detected discrepancy between the level of RNA and the amount of eGFP protein. eGFP synthesis from TY-FHV(eGFP) was lower than expected; the level of eGFP RNA was comparable to or more than eGFP RNA from the TY-eGFP sample; however, the amount of eGFP protein in the TY-FHV(eGFP) sample was about 10 times less than eGFP in the TY-eGFP sample. In contrast, the



**Figure 2.** Analysis of replication products. Northern blot analysis of FHV RNA replication (A & B) was done as described in Figure 1C, using the DIG-labeled probe targeting the TYMV CP sequence (A) or a mixture of probes targeting the FHV sgRNA and TYMV CP probes (B). Experiments were performed in triplicate, yielding similar results. C) Schematic representation of the FHV RNA replication products. The sgRNA promoter tymobox is represented as a black box. Four RNA species (*a*, *b*, *c*, and *d*) from TY-FHV (eGFP) and three species (1, 2, and 3) from pCB FHV (eGFP)-CP, which were identified by the size and the results of hybridization, are depicted below the blots. TY-GUS RNA was included as a size marker.

amount of eGFP in the pCB FHV(eGFP)-CP sample was much more abundant than that expected from the level of

RNA detected. Thus, eGFP RNA translation seems to be repressed when both TYMV and FHV are replicating. These



**Figure 3.** Analysis of eGFP expression. (A) Northern blot analysis of eGFP expression. Total or encapsidated RNAs were examined as described in Figure 1, using the DIG-labeled probe targeting the eGFP gene. (B) Western blot analysis of eGFP expression. 10  $\mu$ l of leaf extracts was electrophoresed in a 12.5% SDS-polyacrylamide gel, and the proteins were transferred to nitrocellulose membrane. For TY-eGFP and FHV (eGFP)-CP, diluted samples (1:10 and 1:500 dilution, respectively) were used. eGFP was detected using an anti-eGFP antibody and anti-rabbit HRP conjugate. Experiments were performed in triplicate.

results may indicate that the translation of eGFP RNA is differentially influenced by the replicating virus. In this context, TYMV replication seems to have a more negative effect on the translation of eGFP RNA as compared to FHV replication.

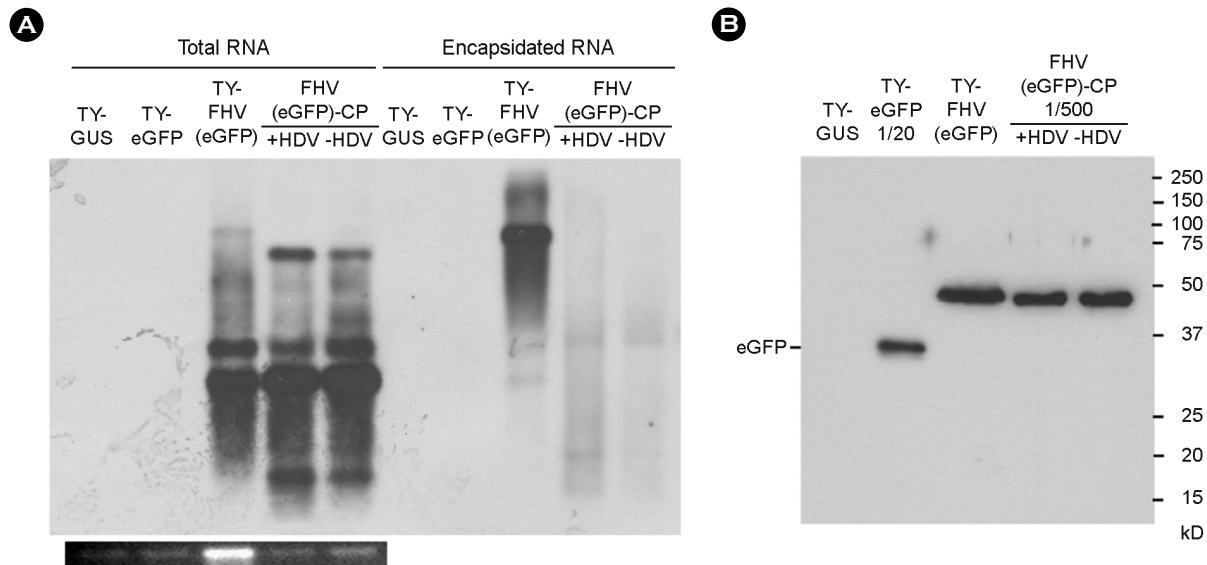
#### Effect of hepatitis delta virus ribozyme sequence

Previously, it was observed that TY-FHV $\Delta$ HDV, which lacks the hepatitis delta virus (HDV) ribozyme sequence, produced FHV RNA products at a level similar to TY-FHV (7). This suggests that cleaving off the TYMV CP sequence is not necessary for FHV RNA replication. To examine whether it is also true for the pCB FHV(eGFP)-CP construct, we made the pCB FHV(eGFP) $\Delta$ HDV-CP construct where the HDV sequence following the FHV RNA sequence was deleted. Northern blot analysis of the replication of this construct in *N. benthamiana* showed that the RNA products from the construct lacking HDV were similar to those from the construct having the HDV ribozyme sequence (Fig. 4A).

Western blot analysis of eGFP expressed from the pCB FHV(eGFP) $\Delta$ HDV-CP construct also showed that the amount of eGFP was comparable to that of the eGFP protein from the pCB FHV(eGFP)-CP construct (Fig. 4B). This illustrated that the HDV ribozyme sequence is not necessary for the replication of the recombinant FHV RNA. This result suggested that the FHV replicase can recognize the 3'-terminal sequence of FHV RNA1, even though the FHV sequence is followed by a rather long heterologous sequence.

## DISCUSSION

In this study, we have shown that the TYMV CP sequence at the 3'-end of FHV RNA does not affect replication, which is a crucial insight given that the 3'-terminal CP sequence is essential for FHV RNA to be packaged into TYMV virions. In the FHV-CP RNA, the FHV 3'-terminal sequence serving as a replication element resides far from the 3'-end, but recombinant FHV RNA still successfully replicated. This



**Figure 4.** Replication of recombinant FHV RNA1 lacking HDV ribozyme sequence. (A) Northern blot analysis of FHV RNA1 replication. Total or encapsidated RNAs were examined as described in Fig. 1, using the DIG-labeled probe targeting the FHV sgRNA. (B) Western blot analysis of eGFP expression. 10  $\mu$ l of leaf extracts was electrophoresed in a 12.5% SDS-polyacrylamide gel, and the proteins were transferred to nitrocellulose membrane. For TY-eGFP, the protein sample was diluted to 1:20. For FHV(eGFP)-CP and FHV(eGFP) $\Delta$ HDV-CP, the samples were diluted to 1:500. eGFP was detected as described in Figure 3B. Experiments were performed in triplicate.

indicated that the FHV 3'-terminal sequence, which is internally located in FHV-CP RNA, was recognized by the FHV replicase. Presumably, the TYMV CP sequence did not interfere with recognition of FHV replication signals by FHV replicase, possibly by forming an independent secondary or tertiary structure.

Although the effect of terminal nucleotide extension has not been systematically examined, previous studies have shown a tendency that the 5'-end is more crucial than the 3'-end. Annamalai *et al.* (18) observed that addition of 17 and 33 nt sequences at the 3'-ends of FHV RNA1 and RNA2, respectively, did not significantly affect FHV RNA replication in *N. benthamiana*. As mentioned earlier, Ball (8) also reported that addition of nucleotides at the 5'-end of FHV RNA1 affected replication more than nucleotide addition at the 3'-end. In FHV RNA2 and RNA3 replication, it was reported that replication was greatly influenced by 5'-end nucleotide addition (19, 20). Nevertheless, these observations were not consistent with our results. Figures 1 and 2 show that four-nucleotide addition at the 5'-end did not

seem to inhibit FHV RNA replication in *N. benthamiana*. We also examined FHV RNA replication in *N. benthamiana* using the FHV(eGFP)-CP-0 construct (see MATERIALS AND METHODS), in which the RNA product was expected to have 14 additional nucleotides at the 5'-end. This variant replicated at similar levels as RNA having four additional nucleotides at the 5'-end. The discrepancy between previous reports and our results may imply that local sequence context is important.

This study shows that recombinant FHV RNA encapsidated by TYMV coat proteins can replicate and express reporter genes even though the FHV RNA has heterologous sequences at both ends. Thus, it demonstrates one of the effective ways of packaging therapeutic and self-replicable RNA molecules into a bio-compatible carrier using a natural packaging mechanism.

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