Journal of Bacteriology and Virology 2013. Vol. 43, No. 1 p.54 – 63
http://dx.doi.org/10.4167/jbv.2013.43.1.54

Original Article

Read-through Mutation in the Coat Protein ORF Suppresses Turnip Yellow Mosaic Virus Subgenomic RNA Accumulation

Hyun-Il Shin[2] and Tae-Ju Cho[1*]

[1]Department of Biochemistry, Chungbuk National University, Cheongju; [2]Division of Malaria and Parasitic Diseases, National Institute of Health, Korea Centers for Disease Control and Prevention, Cheongwon-gun, Korea

We have previously observed that a sequence in coat protein (CP) ORF of Turnip yellow mosaic virus (TYMV) is required for efficient replication of the virus. The sequence was predicted to take a stem-loop structure, thus termed SL2. While examining various SL2 mutants, we observed that all the modifications resulting in extension of translation beyond the CP ORF significantly suppressed subgenomic RNA accumulation. The genomic RNA level, in contrast, was not affected. Introduction of an in-frame stop codon in the CP ORF of these constructs restored the level of subgenomic RNA. Overall, the results suggest that the read-through makes the subgenomic RNA unstable.

Key Words: TYMV, Read-through, sgRNA stability

INTRODUCTION

Turnip yellow mosaic virus (TYMV) is an icosahedral virus and is a type member of the family *Tymoviridae*. It has a single 6.3 kb plus-strand RNA as a genome (1). The TYMV genomic RNA (gRNA) contains three open reading frames (ORFs) encoding the replication protein (p206), movement protein (p69), and coat protein (CP). p206 is a multifunctional protein and has several domains indicative of methyltransferase, protease, helicase, and polymerase activities. After synthesis, p206 is self-cleaved into two pieces; p141 and p66. CP is not expressed from the gRNA but from the subgenomic RNA (sgRNA), which is produced in the process of gRNA replication. A distinct structural feature of TYMV RNA is a tRNA-like structure (TLS) at the 3'-end. The TLS serves as a translational enhancer (2, 3).

TYMV infects mainly *Cruciferae* plants, including Chinese cabbage and turnip. The site of viral replication is vesicles formed by invagination of the outer membranes of chloroplasts (4). The replication proteins, p141 and p66, are co-localized to the replication vesicles. p141 is able to localize to the replication vesicle on its own, and induce chloroplast clumping. p66, which is a RNA-dependent RNA polymerase (RdRP), requires p141 for its localization to the vesicles (5). The domain of p141 that interacts with the RdRP was mapped to the proteinase domain (6). It is not known how TYMV gRNA is recruited to the replication complex.

Previous studies have reported that 5'-UTR plays an important role in TYMV replication. TYMV gRNA contains two hairpins in the 5'-UTR. A distinctive feature of the hairpins is that the stem-loop structures contain C-C or C-A

Received: December 18, 2012/ Revised: February 14, 2013/ Accepted: February 18, 2013
*Corresponding author: Tae-Ju Cho. Department of Biochemistry, Chungbuk National University, Cheongju 361-763, Korea.
Phone: +82-43-261-2309, Fax: +82-43-267-2306, e-mail: tjcho@chungbuk.ac.kr
**This study was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2012-0007588) and by a Chungbuk National University research grant in 2011.

mismatches (7). These mismatches were supposed to be protonatable and implicated in replication and encapsidation (8). The role of the 5'-UTR in replication has been confirmed in recent studies, although the role of the 5'-UTR including the two hairpins in encapsidation was disproved (9). TYMV replication proceeds in two steps. First, minus-strand RNA is synthesized using the TYMV gRNA as a template. Then, plus-strand gRNA and sgRNA are synthesized on the minus strand. The sgRNA synthesis is thought to be controlled by a 16-nt-long RNA sequence, called tymobox, that overlaps the 3'-end of p206 ORF and is conserved throughout the tymoviruses (1). The 5'-UTR is presumed to act as a replication enhancer when plus-strand RNA is synthesized using the minus-strand RNA as a template. Search for the replication elements promoting minus-strand RNA synthesis has been made by *in vitro* transcription studies using purified TYMV RdRP preparations. The results of the *in vitro* transcription studies have shown that synthesis of minus-strand RNA was found to be predominantly controlled by a -CCA "initiation box" at the 3'-end of the RNA, which needs to be non base-paired and readily accessible (10, 11, 12). It was unexpected that the short -CCA sequence was a sole requirement for efficient minus-strand RNA synthesis, since template selection of only viral RNAs seemed to be hardly achieved by such a short sequence.

Previously, we have made efforts to develop an expression vector based on TYMV. The TYMV vector contains an extra tymobox, a subgenomic RNA (sgRNA) promoter, and a cloning site upstream of the CP ORF, which is near the 3'-end of TYMV genome. When a foreign gene encoding eGFP or GUS was inserted into the cloning site, two sgRNAs were produced, as expected (13). The foreign gene was contained in a bigger sgRNA. The recombinant sgRNA was produced as abundantly as the genuine sgRNA. However, the amount of the proteins from the foreign gene was not as much as anticipated. In a hope to improve the protein production, we developed a second expression vector, where an extra tymobox and cloning sites were inserted downstream of the CP ORF. However, we observed that the viral RNA replication of the recombinants was greatly suppressed (14). The recombinant TYMV barely replicated. This suggested presence of a replication element in the CP ORF, the distance of which to the 3'-end was critical. Deletion mutant analysis indicated that a sequence between nt-6139 and nt-6181, which was predicted to take a stem-loop structure, was important in promoting TYMV replication (15).

Subsequent examination of various mutant constructs of the potential replication element resulted in unexpected findings. We report here that extension of translation beyond the CP ORF inhibited sgRNA accumulation. Curiously, gRNA level was not affected. The results obtained in this study might be explained by ribosomal behavior at the 3'-end of the TYMV RNAs.

MATERIALS AND METHODS

DNA constructs

TY-eGFP2 and TY-eGFP2+CP_{6203} constructs have been described previously (15). The construct TYΔCP2 was made by replacing the sequence between *Sma*I and *Eco*RI sites of the TY-eGFP2+CP_{6203} with the linker ΔCP2, which was composed of two oligonucleotides: 5'-CCGGGTCACTAG-TACGCGTG-3' and 5'-AATTCACGCGTACTAGTGAC-3'. In TYΔCP2+SL2, a linker containing the wild-type SL2 sequence was inserted between the *Sma*I and *Eco*RI sites of the TYΔCP2. Other TYΔCP2 variant constructs (TYΔCP2-2, TYΔCP2-3, and TYΔCP2-2FS) and TYΔCP2+SL2 variants (TYΔCP2+m1, TYΔCP2+m2, TYΔCP2+m3, TYΔCP2+m4, TYΔCP2+m1', TYΔCP2+m2', TYΔCP2+m3', TYΔCP2+ m4', TYΔCP2+m2'-AC, and TYΔCP2+m2'-CC) were prepared likewise. The oligonucleotides for the linkers used to make these constructs are summarized in Table 1. 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 described previously (16). Agroinfiltration of the TYMV constructs was accompanied by agroinfiltration of a binary vector supporting the

Table 1. Linkers used in this study

Linker	Sequence of oligonucleotides
ΔCP2	5' CCGGGTCACTAGTACGCGTG 3'
	3' CAGTGATCATGCGCACTTAA 5'
SL2	5' CCGGGCCGCATCGACCTGCATAATAACTGTATCAGGAACTCTCTCGATGCACG 3'
	3' CGGCGTAGCTGGACGTATTATTGACATAGTCCTTGAGAGAGCTACGTGCTTAA 5'
mSL2	5' CCGGGCCGCTAGTACATGCATTATAACTGTATCAGGAACTCTCTCGATGCACG 3'
	3' CGGCGATCATGTACGTAATATTGACATAGTCCTTGAGAGAGCTACGTGCTTAA 5'
m1	5' CCGGGCCTGCATAATAACTGTATCAGGG 3'
	3' CGGACGTATTATTGACATAGTCCCTTAA 5'
m2	5' CCGGGGCATCGACCTGCATAGAATATCAGGAACTCTCTCGATGCG 3'
	3' CCGTAGCTGGACGTATCTTATAGTCCTTGAGAGAGCTACGCTTAA 5'
m3	5' CCGGGGCATCGACCTGATAATAACTGTATCAGGAACTCTCTCGATGCG 3'
	3' CCGTAGCTGGACTATTATTGACATAGTCCTTGAGAGAGCTACGCTTAA 5'
m4	5' CCGGGGCATCGACCTGCATAATAACTGTATCAGGATCGATGCG 3'
	3' CCGTAGCTGGACGTATTATTGACATAGTCCTAGCTACGCTTAA 5'

expression of Tomato bushy stunt virus (TBSV) p19 (RNAi suppressor). Seven to ten 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℃.

Analysis of viral RNA and coat protein

Total RNA was isolated from frozen leaf samples using Easy-Red (iNtRON Biotechnology, Sungnam, Korea). RNA samples were resuspended in 48% formamide solution containing 10 mM EDTA and incubated at 65℃ for 10 min before electrophoresis on 1% agarose gel. RNAs were transferred to Hybond N^+ membranes (GE Healthcare, Chalfont St. Giles, England), and the blots were hybridized with a digoxigenin (DIG)-labeled DNA probe hybridizing to the TYMV CP ORF (16). The blots were developed by chemiluminescent immunodetection of DIG (Roche Molecular Biochemicals, Basel, Switzerland).

CP protein expression was examined by 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot analysis. Leaf samples (0.1 g) were ground in 200 μl of 2X sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 2% 2-mercaptoethanol, 10 μg/ml bromophenol blue) and boiled for 5 min before analysis. Western blot detection used anti-TYMV rabbit antiserum in conjunction with horseradish peroxidase-conjugated goat anti-rabbit IgG and chemiluminescent detection method using Luminata™ Forte (Millipore, Bedford, MA, USA).

RESULTS

Examination of SL2 variants

Previous deletion mutant analysis showed that the sequence between nt-6139 and nt-6181, predicted to form a stem-loop with an asymmetric bulge, was critical to the replication of TYMV (15). The stem-loop structure was termed SL2. When the sequence between nt-6067 and nt-6202 was deleted from wild-type TYMV (TYΔCP2), sgRNA and its product CP were hardly detected. Replication was recovered to 5~10% of wild type, when the sequence representing the SL2 was inserted into the TYΔCP2 (15). The partial recovery is thought to be ascribed to the non-functional CP, since 32 amino acids are missing in the CP produced from the TYΔCP2+SL2. The location and pre-

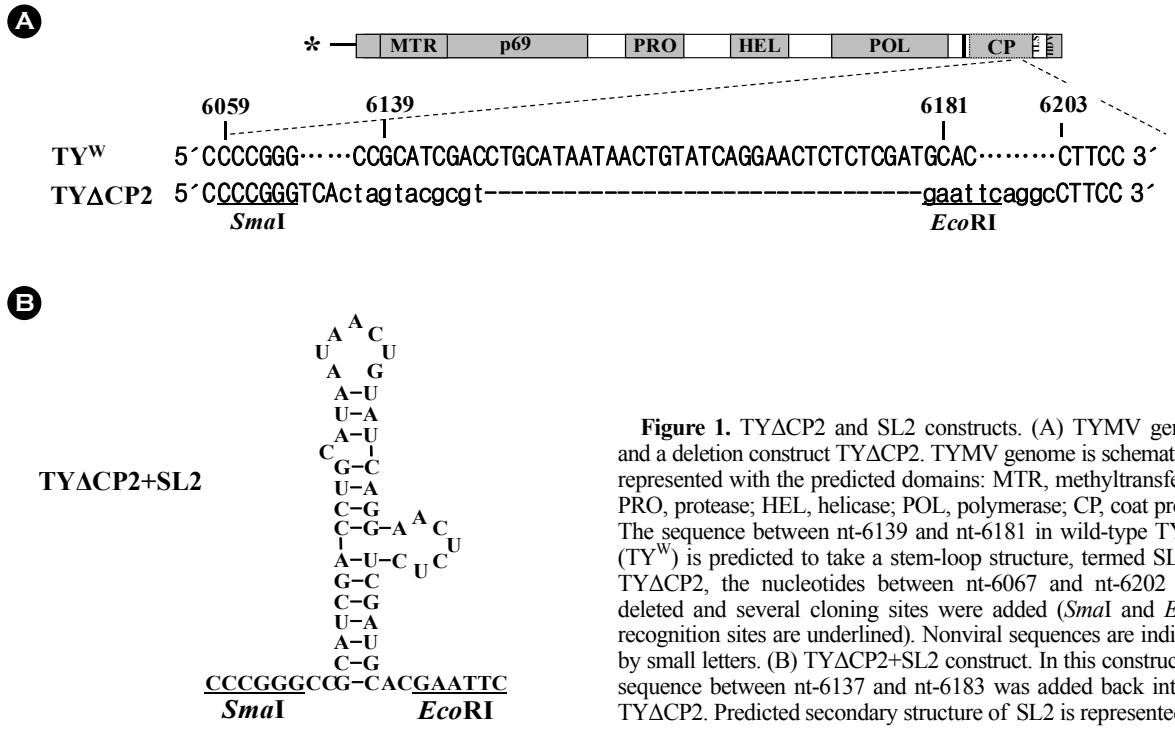


Figure 1. TYΔCP2 and SL2 constructs. (A) TYMV genome and a deletion construct TYΔCP2. TYMV genome is schematically represented with the predicted domains: MTR, methyltransferase; PRO, protease; HEL, helicase; POL, polymerase; CP, coat protein. The sequence between nt-6139 and nt-6181 in wild-type TYMV (TY^W) is predicted to take a stem-loop structure, termed SL2. In TYΔCP2, the nucleotides between nt-6067 and nt-6202 were deleted and several cloning sites were added (*Sma*I and *Eco*RI recognition sites are underlined). Nonviral sequences are indicated by small letters. (B) TYΔCP2+SL2 construct. In this construct, the sequence between nt-6137 and nt-6183 was added back into the TYΔCP2. Predicted secondary structure of SL2 is represented.

dicted secondary structure of the SL2 are depicted in Fig. 1.

To examine which part of the SL2 is important, the SL2 was further modified (Fig. 2A). In TYΔCP2+m1 construct, the bottom half including the side bulge was removed. In TYΔCP2+m2, the terminal loop was replaced with a GAAA tetraloop, since it was known that loops are often involved in RNA-RNA interaction, which is important for enhancing or suppressing the replication (17). In TYΔCP2+m3, the non-paired C was deleted. Non-paired nucleotides in the stem-loop structure sometimes serve as a target for replication proteins. For example, the replication of tombusvirus requires highly specific binding of the p33 replication protein to a conserved sequence within the TBSV gRNA, termed the p33 recognition element (p33 RE). The critical part of the p33 RE is a C · C mismatch (18). In TYΔCP2+m4, the side bulge was replaced with a single A nucleotide. Analysis of replication of these mutants showed that gRNA level was relatively unaffected in these mutants, while sgRNA level was substantially reduced (Fig. 2B). CP was barely detected, either. Here we see the breakdown of the 1:1 molar ratio of gRNA and sgRNA. This is unexpected, since 1:1 ratio has always been observed in wild-type and recombinant TYMVs.

It was noted that CC and AC sequence adjacent to the SL2 were missing in the TYΔCP2+m1 to TYΔCP2+m4 constructs, when compared to the wild type and the TYΔCP2+SL2. To see whether this difference had any influence on the replication of variant TYMVs, the TYΔCP2+m1~TYΔCP2+m4 constructs were additionally modified to have the CC/AC sequences (designated TYΔCP2+m1'~TYΔCP2+m4'. See Fig. 3A) and their replication was examined (Fig. 3B). The result showed that sgRNA level was restored in the TYΔCP2+m1'~TYΔCP2+m4' constructs. Insertion of just CC/AC into the TYΔCP2 (TYΔCP2-2) gave similar enhancing effect. In the TYΔCP2-2 construct, gRNA level increased. Moreover, both sgRNA and CP production were observed (Fig. 3C). This indicates that the CC/AC could have exerted a minimal replication-enhancing activity.

Examination of read-through effect

An alternative explanation could be that the results

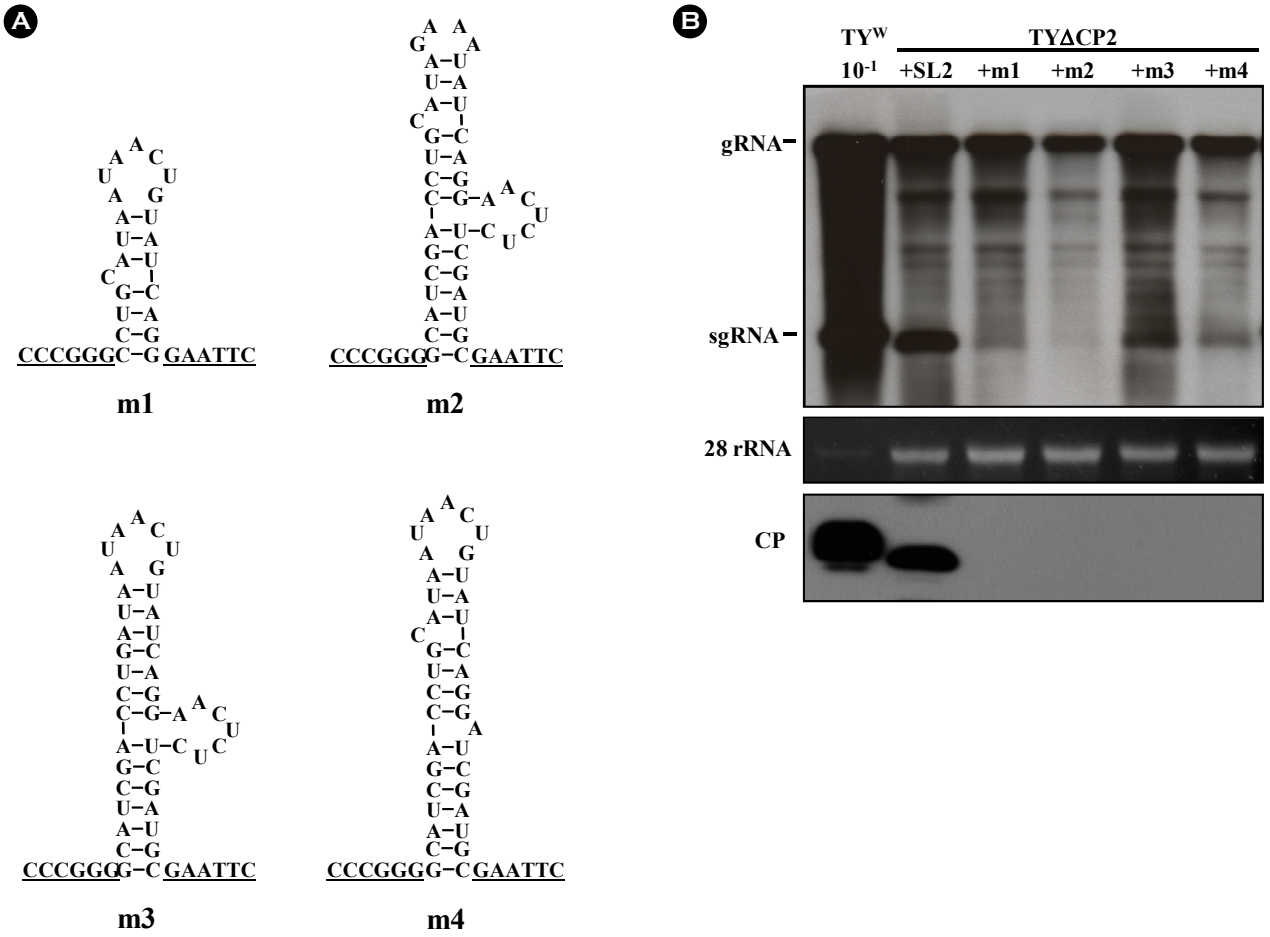


Figure 2. Influence of SL2 modifications on TYMV replication. (A) Variant SL2 constructs. TYΔCP2+m1 lacks the bottom half of the stem. In TYΔCP2+m2, the terminal loop was replaced with a GAAA tetraloop. In TYΔCP2+m3, the non-paired C in the upper stem was deleted. In TYΔCP2+m4, the side bulge was replaced with a single A. (B) Northern and Western analysis of the SL2 mutants. Seven days after agroinfiltration of *N. benthamiana* leaf with various TYMV constructs, total RNA was extracted from the leaf. 1 μg or 0.1 μg (10^{-1}) of total RNA was size-fractionated in the 1% agarose gel and examined by Northern blot analysis, using the DIG-labeled probe representing the CP ORF. The blots were developed by chemiluminescent immunodetection of DIG. The panel below the Northern blot represents a gel stained with ethidium bromide. In Western blot analysis of coat protein expression (bottom panel), 1 μl of 1:10 diluted (10^{-1}) or undiluted leaf extract was loaded and electrophoresed in 12.5% SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane. Coat protein was detected using anti-TYMV coat protein rabbit antibody and anti-rabbit HRP conjugate. The membrane was developed by a Luminata^{TM} Forte (Millipore) using luminol as the substrate.

shown in Fig. 2 and 3 were due to ribosome behavior at the 3'-end of viral RNAs. It was noted that the TYΔCP2+m1~TYΔCP2+m4 constructs would have extension of reading frame beyond the CP ORF (Fig. 4). In these constructs, reading frames were shifted at or after the site of modification. In the resulting two alternative reading frames, translation could continue up to the 3'-end, since no stop codons are encountered. For example, 35 amino acids could be additionally added to the C-terminus of CP, compared to the TYΔCP2+SL2. In contrast, the TYΔCP2+m1'~TYΔCP2+m4' variants had a stop codon at or before the original stop codon of the CP ORF. As a result, all the TYΔCP2+m1~TYΔCP2+m4 constructs would potentially allow ribosomes to reach the 3'-end of RNA, while the TYΔCP2+m1'~TYΔCP2+m4' constructs would not.

To see whether the read-through indeed influenced on the sgRNA accumulation, stop codons were introduced into the TYΔCP2+m1~TYΔCP2+m4 constructs (Fig. 5A). These constructs were prepared by deleting one or two nucleotides after the *Eco*RI site, resulting in introduction of in-frame

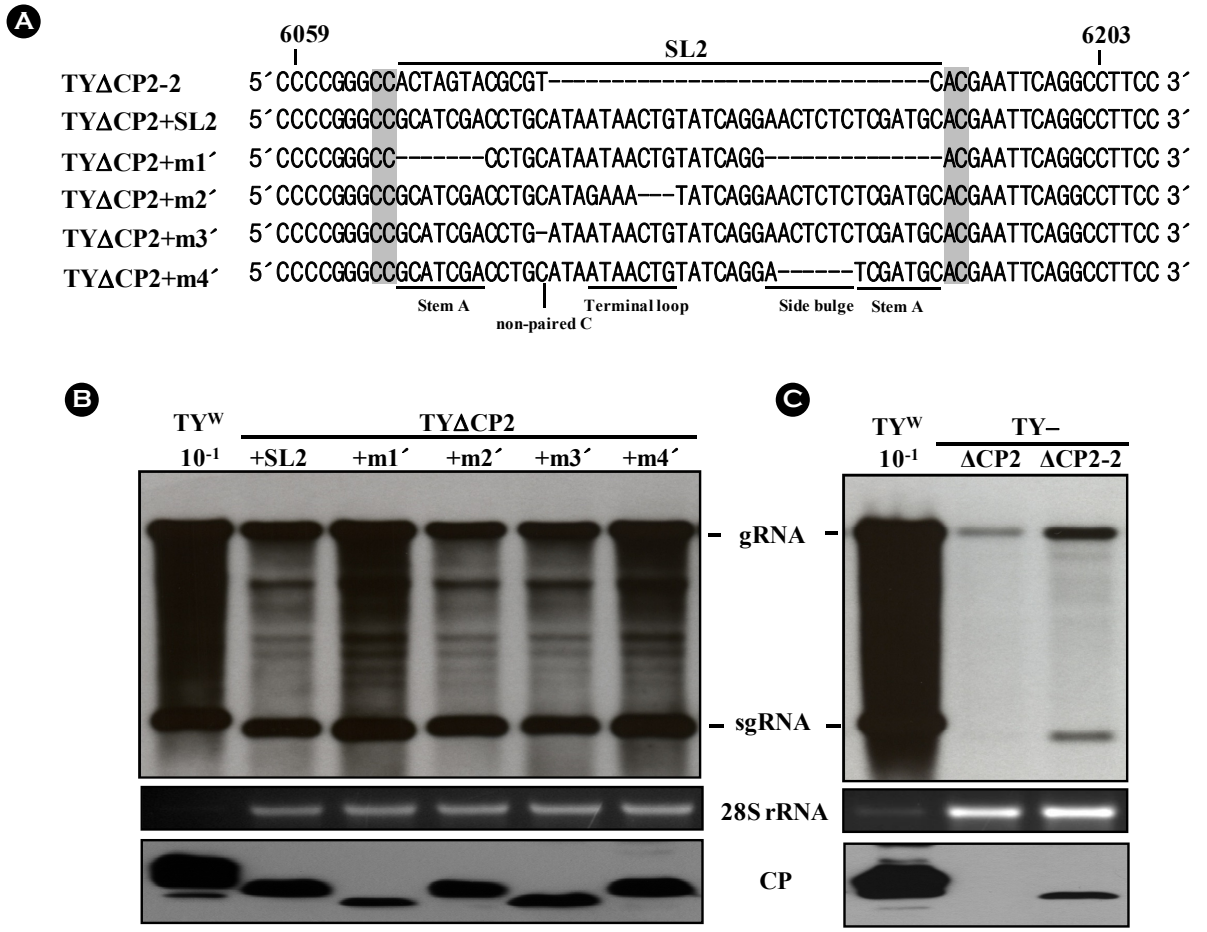


Figure 3. Effect of the sequence adjacent to the SL2. (A) Alignment of the constructs derived from TYΔCP2. As in TYΔCP2+SL2, TYΔCP2+m1'~TYΔCP2+m4' constructs have CC and AC nucleotides upstream and downstream of the SL2 sequence. In contrast, the CC and AC nucleotides are missing in TYΔCP2+m1~TYΔCP2+m4 constructs (see Fig. 2A). In TYΔCP2-2, the CC and AC nucleotides were added into the TYΔCP2. (B) Replication of the TYΔCP2+m1'~TYΔCP2+m4' constructs containing the CC/AC. (C) Replication of the TYΔCP2-2 containing CC/AC. Northern and western analyses were done as described in Fig. 2.

stop codons. These constructs were termed TYΔCP2+m1^{stop}~TYΔCP2+m4^{stop}. Analysis of the replication of these constructs showed that sgRNA level was restored (Fig. 5B). This suggests that the read-through beyond the CP ORF indeed suppressed the sgRNA accumulation. In TYΔCP2+m1^{stop}, the level of CP was not consistent with that of sgRNA. This inconsistency could be due to altered conformation of the mutant CP. In other words, the mutant CP might have been rapidly degraded or barely recognized by the antibody used in Western blot analysis.

The effect of read-through could have also been observed in other constructs. TYΔCP2+SL2-2 lacks CC/AC sequence, which causes read-through (Fig. 6A). In this construct, gRNA level was about the same as the TYΔCP2+SL2. However, the level of sgRNA was far lower than the TYΔCP2+SL2. The protein product of sgRNA, CP, was not detected either (Fig. 6B). In the case of TYΔCP2+mSL2-2, where the CC/AC is lacking and mutations were introduced with intent to disrupt the stem-loop structure of the SL2, replication was hardly observed, as in the TYΔCP2+mSL2 construct (15). Thus, read-through effect could not be tested, although the low level of replication again demonstrated the importance of secondary structure of the SL2. In TYΔCP2-2-FS, one nucleotide is deleted in the linker sequence between *Sma*I and *Eco*RI, compared to TYΔCP2-2. This also leads to read-through. In this construct, gRNA level was

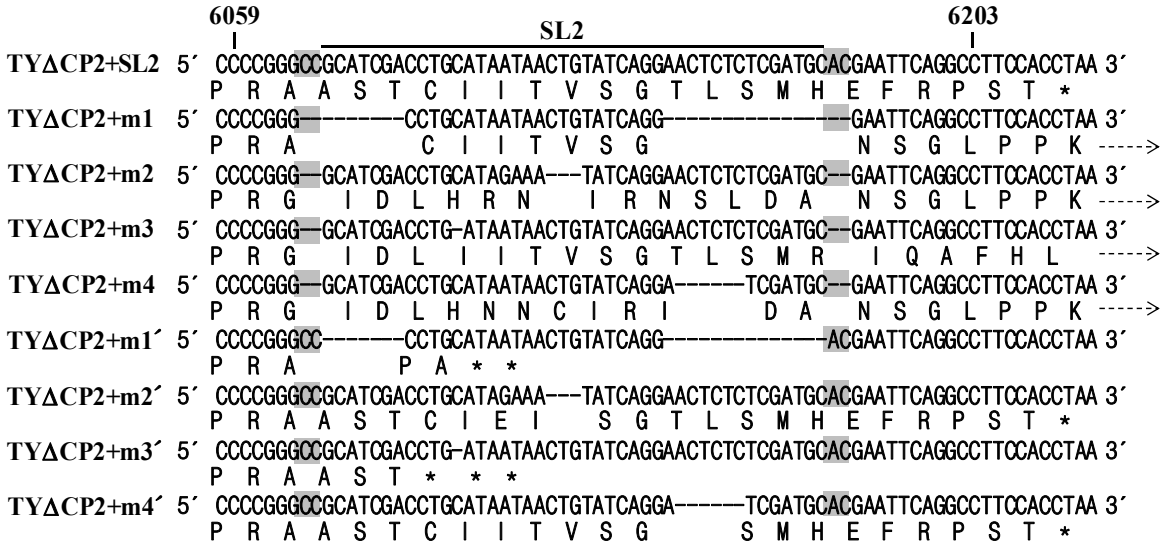
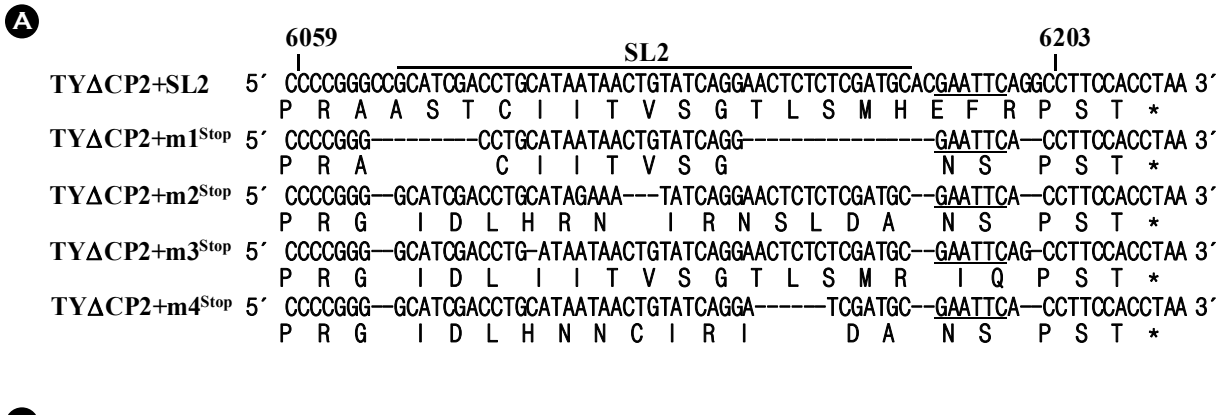


Figure 4. Alignment of the predicted translation products from TYΔCP2-derived constructs. Nucleotide and predicted amino acid sequences of the TYΔCP2+SL2, TYΔCP2+m1~TYΔCP2+m4, and TYΔCP2+m1'~TYΔCP2+m4' constructs are shown. Stop codons are indicated by asterisks. In TYΔCP2+m1~TYΔCP2+m4 constructs, translation would be extended beyond the CP ORF up to the 3'-end. Broken arrows indicate read-through. 35 amino acids could be additionally added to the C-terminus of CP, compared to the TYΔCP2+SL2.



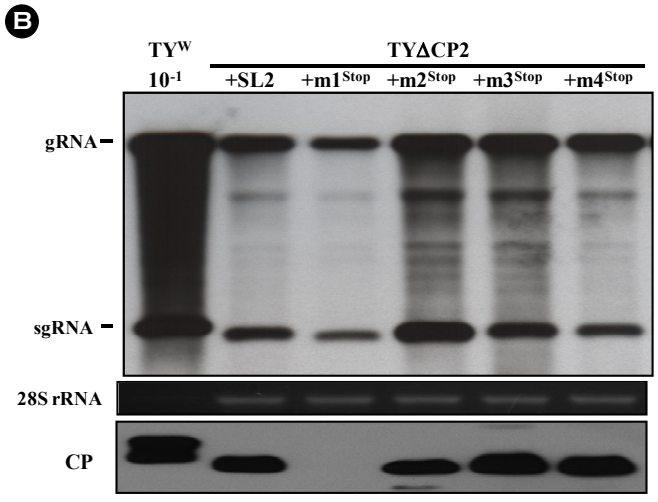


Figure 5. Effect of in-frame stop codons on sgRNA accumulation. (A) Alignment of the TYΔCP2+m1~TYΔCP2+m4 constructs having in-frame stop codons. In TYΔCP2+m1^{stop}~TYΔCP2+m4^{stop} constructs, one or two nucleotides after the *Eco*RI site (underlined) were removed to have an in-frame stop codon as in TYΔCP2+SL2. (B) Replication of the TYΔCP2+m1^{stop}~TYΔCP2+m4^{stop} constructs. Northern and western analyses were done as described in Fig. 2.

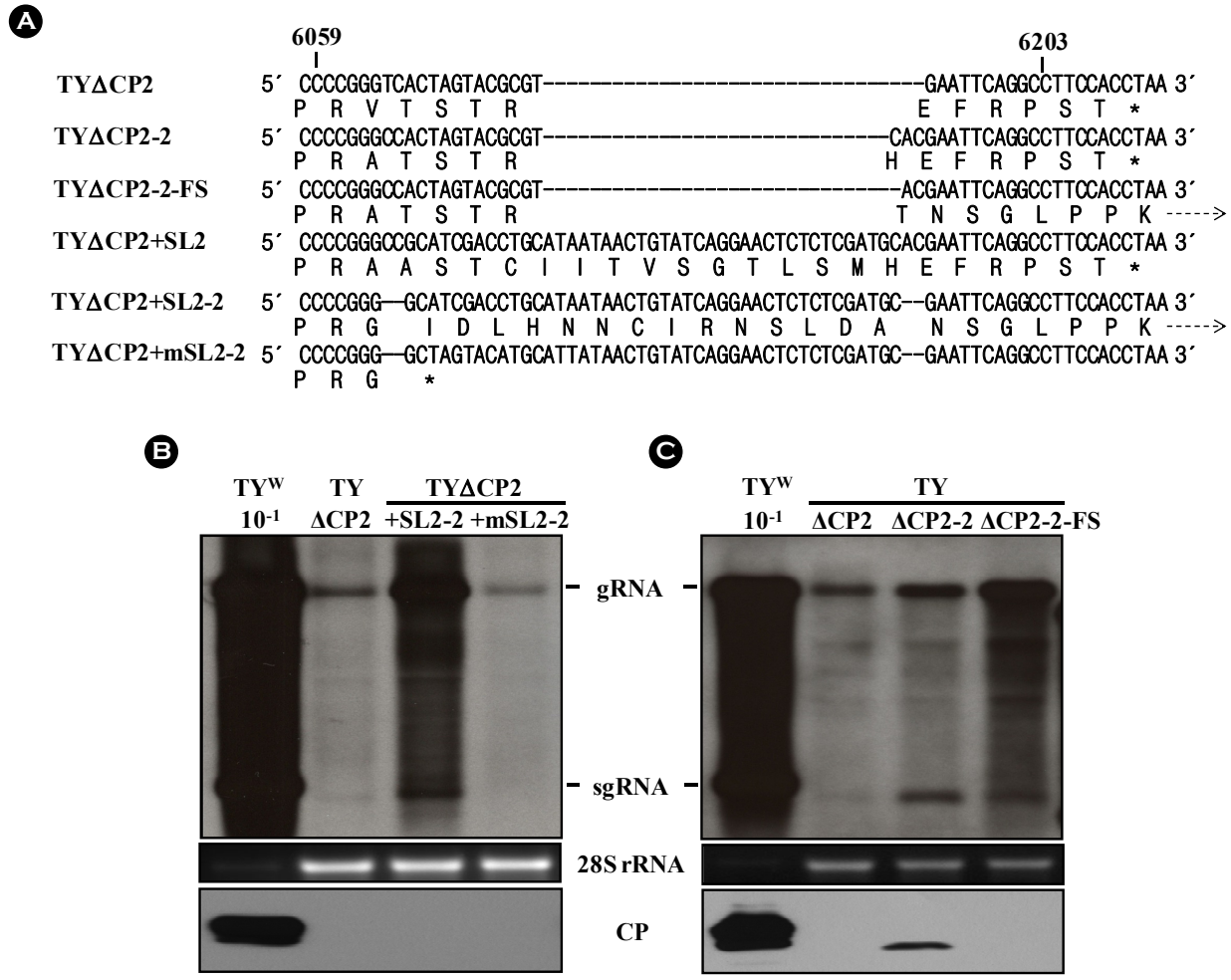


Figure 6. Effect of read-through mutations on the replication of TYΔCP2 and TYΔCP2+SL2 variants. (A) Alignment of various TYΔCP2 and TYΔCP2+SL2 variants and their expected translation products. TYΔCP2-2-FS has one nucleotide deletion, compared to the TYΔCP2-2. This deletion results in frameshift and read-through beyond the CP ORF, as in TYΔCP2+m1'~TYΔCP2+m4' constructs. TYΔCP2+SL2-2 has a four-nucleotide (CC/AC) deletion, also resulting in read-through beyond the CP ORF. In TYΔCP2+mSL2-2, the four nucleotide (CC/AC) deletion leads to premature termination. Broken arrows indicate read-through. (B) Replication of TYΔCP2+SL2-2 and TYΔCP2+mSL2-2 constructs. (C) Replication of TYΔCP2-2-FS. Total RNAs from the agroinfiltrated with the constructs were analyzed by Northern blot hybridization, as described in Fig. 2. Bottom panels represent the results of the western analysis of CP.

higher than the TYΔCP2-2. However, the level of sgRNA was lower than the TYΔCP2-2 (Fig. 6C). Thus, the cases of the TYΔCP2+SL2-2 and TYΔCP2-2-FS constructs also show that read-through beyond the CP ORF resulted in suppression of sgRNA accumulation.

DISCUSSION

It is interesting to see that read-through beyond the TYMV CP ORF leads to sharp decrease in sgRNA level. It is also interesting that gRNA level was generally not affected by the read-through mutations. This unexpected result may have been related to ribosome behavior at the 3'-end of TYMV sgRNA. TYMV RNAs, both gRNA and sgRNA, have a 5' ^{m7}GpppG cap, like cellular mRNAs. However, TYMV RNA has no poly(A) tail. Instead, it has an aminoacylatable TLS at the 3'-end. The TLS is an excellent mimic of tRNA^{Val}. It serves as efficient substrates for wheat germ valyl-tRNA synthetase and CCA nucleotidyltransferase as is tRNA^{Val} (19). The translation elongation

factor eEF1a binds the valylated RNA to form a valyl-RNA:eEF1a:GTP complex (20). The TLS has translational enhancing activity, and full enhancing activity relies on the ability of the TLS to be aminoacylated and bound by eEF1a (19).

Efficient translation of eukaryotic mRNAs is thought to occur in a closed loop format. As for TYMV, it has been postulated that the eEF1a binding at the 3'-TLS is involved in closed loop formation through interaction with the cap-binding complex (21). However, direct evidence has not been provided for this model. Here, the results obtained in this study seem to support the model that eEF1a acts as a molecular bridge between the 5'- and 3'-ends of TYMV RNAs.

In the constructs used in this study, all the modifications were done within the CP ORF. Thus, eEF1a binding to variant TYMV RNAs would be the same as to wild-type viral RNAs. In TYΔCP2+m1~TYΔCP2+m4 constructs and other constructs showing reduced sgRNA level, however, translation can be extended to the 3'-end of the sgRNA. In this case, ribosomes might have detached eEF1a bound at the 3'-TLS. This would make the sgRNA susceptible to degradation. The gRNA was presumed to be unaffected, because the CP ORF on gRNA was not translated. Therefore, in the case of gRNA, the eEF1a could manage to bind to the 3'-end irrespective of the modifications, and protect the gRNA by forming closed loops. In the cases of wild type and other variant constructs showing normal sgRNA levels, the sgRNAs would have been protected likewise, since ribosomes would fall off before the TLS.

Is there a possibility that the mutations described in this study could have affected sgRNA synthesis rather than sgRNA degradation? The role of TYMV CP in sgRNA production, however, is not likely, considering the following observation: extension at the C-terminal CP all resulted in decrease in sgRNA level, whereas truncation of the C-terminal led to normal sgRNA production. Since either extension or truncation of the C-terminal part of CP is thought to end up with disruption of CP, same results would have been expected in both cases. The actual results were contrary to the expectations. Moreover, the hypothesis hardly explains the observation that the mutations in CP ORF influenced only on the sgRNA level.

The issue on which part is important in the SL2 has not been resolved in this study (Fig. 3). The unexpected result might have been due to an unusual structural feature of the SL2. The SL2 structure might have consisted of two stem-loop structures. In the upper and lower stem-loop structures, the stem is 7 bp-long in both structures (when omitting the non-paired C in the upper structure), and the terminal and side loops all consist of 7 nucleotides. This redundancy model may explain the result shown in Fig. 3, because one of the two structures in the SL2 was intact in m1'-m4' constructs. Alternatively, SL2 might simply function as a spacer whose sole function is to maintain an important but yet unidentified replication element in a particular secondary (or non base-paired) structure.

REFERENCES

1) Dreher TW. Turnip yellow mosaic virus: transfer RNA mimicry, chloroplasts and a C-rich genome. Mol Plant Pathol 2004;5:367-75.
2) Matsuda D, Dreher TW. The tRNA-like structure of Turnip yellow mosaic virus RNA is a 3'-translational enhancer. Virology 2004;321:36-46.
3) Matsuda D, Bauer L, Tinnesand K, Dreher TW. Expression of the two nested overlapping reading frames of Turnip yellow mosaic virus RNA is enhanced by a 5' cap and by 5' and 3' viral sequences. J Virol 2004; 78:9325-35.
4) Matthews REF. Tymovirus group. In Plant Virology. 3rd ed. San Diego: Academic Press; 1991. p. 231-9.
5) Prod'homme D, Jakubiec A, Tournier V, Drugeon G, Jupin I. Targeting of the turnip yellow mosaic virus 66K replication protein to the chloroplast envelope is mediated by the 140K protein. J Virol 2003;77:9124-35.
6) Jakubiec A, Notaise J, Tournier V, Héricourt F, Block MA, Drugeon G, *et al*. Assembly of turnip yellow mosaic virus replication complexes: Interaction between the proteinase and polymerase domains of the replication proteins. J Virol 2004;78:7945-57.
7) Hellendoorn K, Michiels PJ, Buitenhuis R, Pleij CW.

Protonatable hairpins are conserved in the 5'-untranslated region of tymovirus RNAs. Nucleic Acids Res 1996; 24:4910-7.
8) Hellendoorn K, Verlaan PW, Pleij CW. A functional role for the conserved protonatable hairpins in the 5' untranslated region of turnip yellow mosaic virus RNA. J Virol 1997;71:8774-9.
9) Shin HI, Tzanetakis IE, Dreher TW, Cho TJ. The 5'-UTR of Turnip yellow mosaic virus does not include a critical encapsidation signal. Virology 2009;387:427-35.
10) Deiman BA, Koenen AK, Verlaan PW, Pleij CW. Minimal template requirements for initiation of minus-strand synthesis *in vitro* by the RNA-dependent RNA polymerase of turnip yellow mosaic virus. J Virol 1998; 72:3965-72.
11) Deiman BA, Verlaan PW, Pleij CW. *In vitro* transcription by the turnip yellow mosaic virus RNA polymerase: a comparison with the alfalfa mosaic virus and brome mosaic virus replicases. J Virol 2000;74: 264-71.
12) Singh RN, Dreher TW. Specific site selection in RNA resulting from a combination of nonspecific secondary structure and -CCR- boxes: initiation of minus strand synthesis by turnip yellow mosaic virus RNA-dependent RNA polymerase. RNA 1998;4:1083-95.
13) Shin HI, Kim IC, Cho TJ. Replication and encapsidation of recombinant Turnip yellow mosaic virus RNA. BMB Rep 2008;41:739-44.
14) Shin HI, Cho TJ. A sequence in coat protein open reading frame is required for Turnip yellow mosaic virus replication. J Bacteriol Virol 2011;41:109-16.
15) Shin HI, Cho TJ. Characterization of a replication element in the coat protein ORF of Turnip yellow mosaic virus. J Bacteriol Virol 2012;42:49-55.
16) Cho TJ, Dreher TW. Encapsidation of genomic but not subgenomic turnip yellow mosaic virus RNA by coat protein provided in trans. Virology 2006;356:126-35.
17) Miller WA, White KA. Long-distance RNA-RNA interactions in plant virus gene expression and replication. Annu Rev Phytopathol 2006;44:447-67.
18) Pogany J, White KA, Nagy PD. Specific binding of tombusvirus replication protein p33 to an internal replication element in the viral RNA is essential for replication. J Virol 2005;79:4859-69.
19) Dreher TW. Functions of the 3'-untranslated regions of positive strand RNA viral genomes. Annu Rev Phytopathol 1999;37:151-74.
20) Dreher TW, Uhlenbeck OC, Browning KS. Quantitative assessment of EF-1alpha.GTP binding to aminoacyl-tRNAs, aminoacyl-viral RNA, and tRNA shows close correspondence to the RNA binding properties of EF-Tu. J Biol Chem 1999;274:666-72.
21) Dreher TW, Miller WA. Translational control in positive strand RNA plant viruses. Virology 2006;344:185-97.