

Modification of *Turnip yellow mosaic virus* coat protein and its effect on virion assembly

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Turnip yellow mosaic virus (TYMV) is a positive strand RNA virus. We have modified TYMV coat protein (CP) by inserting a c-Myc epitope peptide at the N- or C-terminus of the CP, and have examined its effect on assembly. We introduced the recombinant CP constructs into *Nicotiana benthamiana* leaves by agroinfiltration. Examination of the leaf extracts by agarose gel electrophoresis and Western blot analysis showed that the CP modified at the N-terminus produced a band co-migrating with wild-type virions. With C-terminal modification, however, the detected bands moved faster than the wild-type virions. To further examine the effect, TYMV constructs producing the modified CPs were prepared. With N-terminal modification, viral RNAs were protected from RNase A. In contrast, the viral RNAs were not protected with C-terminal modification. Overall, the results suggest that virion assembly and RNA packaging occur properly when the N-terminus of CP is modified, but not when the C-terminus is modified. [BMB Reports 2013; 46(10): 495-500]

INTRODUCTION

Turnip yellow mosaic virus (TYMV), a positive strand RNA virus and the type member of tymoviruses, infects mainly *Cruciferae* plants. TYMV has a relatively simple genome. A single 6.3 kb genomic RNA contains three open reading frames, two of which are overlapping. Coat protein (CP) is produced from the sole subgenomic RNA (1). TYMV virions are non-enveloped 28-30 nm T=3 icosahedrons which are composed of a single 20 kDa coat protein. The structure of TYMV has been extensively studied by electron microscopy (2, 3) and X-ray crystallographic analysis (4, 5). TYMV was one of the

first characterized icosahedral viruses (6). The virions are very stable, predominantly due to hydrophobic protein-protein interactions (4, 7), so that empty capsids coexist with infectious viruses in infected tissue.

Viruses, especially non-enveloped viruses, are considered to be a versatile platform which can be used to display peptides, proteins, or various labels. Recently, plant viruses have been increasingly used as bionanoparticles for medicine (8, 9), mainly because plant viral particles can be inexpensively produced from infected plants with high yields, and because they are safe to use. One promising application is the utilization of plant viruses as a nanoparticle for the multivalent display of antigenic peptides (10, 11). An ordered array of antigenic peptides or proteins is known to induce stronger immune responses compared to single antigenic molecules. Plant viruses have also shown promise for biomedical applications, such as intravital vascular imaging (12, 13), drug loading (14), and cancer-targeting (15).

Compared with other plant viruses, TYMV has some unique advantages (16). One such advantage is that TYMV is stable from 4°C to RT for months and 60°C for several hours. It can withstand a wide pH range, up to 50% organic solvent and a variety of reaction conditions. TYMV virions can be chemically modified. Barnhill *et al.* (16) reported that approximately 60 lysines per TYMV particle are reactive toward N-hydroxysuccinimide ester reagents. In addition, about 90 to 120 carboxyl groups are exposed on the surface. The reactive carboxyl groups can be modified to display short chain ethylene glycol polymers and peptides by Cu(I) catalyzed azide-alkyne cycloaddition reaction, as what is possibly the best example of "click chemistry" (17).

The surface of viral particles can also be modified by genetic engineering. N- and C-terminal residues have been the major targets for such engineering. Hayden *et al.* (18) replaced 10 selected parts of TYMV CP with those of *Belladonna mottle tymovirus* (BeMV). When inoculated, 6 of the 10 recombinants were viable, and gave rise to symptoms in Chinese cabbage. The most successful replacement was the one at the N-terminal region. Sastri *et al.* (19) modified the N-terminus of the coat protein of *Physalis mottle virus*, another member of the genus tymovirus, and showed that the N-terminus can be extended by 41 amino acid residues without affecting the assembly, when

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expressing the mutant coat protein in *E. coli*. Such N-terminal extension has not been engineered for TYMV CP. As far as C-terminal modifications are concerned, Hayden et al. (18) reported that the mutant where C-terminal 9 amino acid residues were replaced with BeMV did not produce systemic symptoms or local lesions. In contrast, Bransom et al. (20) reported that a C-terminal mutant containing extra five amino acids was viable, and was able to spread systemically, although particles produced by the C-terminal mutant were less stable. Thus, only limited information has been accumulated on how much modification can be tolerated by TYMV CP.

In this study, we have taken those experiments further by fusing a c-Myc epitope peptide to the N- or C-terminus of TYMV CP, and have examined the effect of extension on virion assembly. We also examined whether the modification had any influence on viral RNA replication and packaging in plants, which had not been examined extensively.

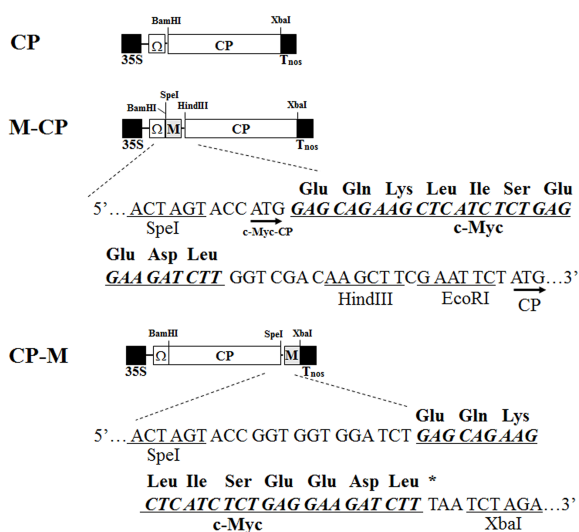


Fig. 1. CP constructs. Construct CP/pA (CP) produces a CP mRNA with a 5'-UTR that includes the Ω translational enhancer from the *Tobacco mosaic virus* and a poly(A) tail at the 3'-end. In pCBm-M-CP (M-CP) and pCBm-CP-M (CP-M) constructs, a c-Myc (M) epitope tag sequence was inserted at the N- and C-terminus of the CP, respectively. The c-Myc sequence is underlined and the amino acid sequence is indicated. In M-CP, the c-Myc peptide is fused to the N-terminus of TYMV CP via a linker consisting of seven amino acids (Gly-Arg-Gln-Ala-Ser-Asn-Ser). In CP-M, the c-Myc peptide is linked to the C-terminus of TYMV CP via a peptide of four amino acids (Gly-Gly-Gly-Ser). These constructs were prepared using the binary vector pCB302-3. 35S represents the 35S promoter of the *Cauliflower mosaic virus*, and Tnos is the transcription terminator of the nos gene.

RESULTS AND DISCUSSION

Effect of N- and C-terminal modification on the assembly of TYMV CP

To examine the assembly of the modified CP of TYMV, the CP/pA (CP) construct (21), which produces wild-type TYMV CP, was engineered. The modified versions of the CP/pA, designated pCBm-M-CP (M-CP) and pCBm-CP-M (CP-M), were prepared by inserting a c-Myc epitope at the N- or C-terminus of the CP (Fig. 1). The M-CP has the c-Myc tag peptide at the N-terminus, followed by a linker peptide consisting of seven amino acids and TYMV CP. In the CP-M, CP was extended at the C-terminus with 14 amino acids, consisting of a linker peptide of four amino acids and the c-Myc tag.

These CP constructs were introduced into *Nicotiana benthamiana* leaves using the *Agrobacterium*-mediated T-DNA transfer system (21). Seven days after agroinfiltration, total RNA and leaf extracts were prepared from the infiltrated leaves. Total RNA was examined by Northern analysis, using the DIG-labeled DNA encoding the CP as a probe. The result of the Northern analysis is shown in Fig. 2A. The wild-type TYMV construct TY^W (21) and the wild-type CP construct were included as controls. Analysis of the total RNA showed that the mRNA levels of CP, M-CP, and CP-M were about the same (Fig. 2A). Those CP mRNAs were observed to be a little larger than the TYMV sgRNA, which encodes CP. This is because the

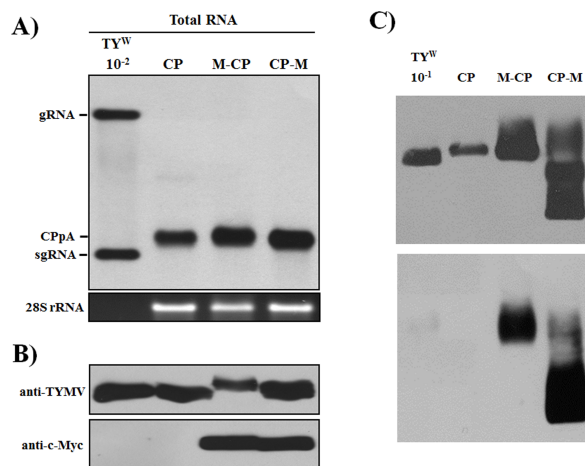


Fig. 2. Expression and assembly of modified CPs. (A) Northern analysis of CP mRNA expression. Seven days after agroinfiltration of the CP constructs into *Nicotiana benthamiana*, leaf samples were collected for total RNA preparation. Total RNAs were size-fractionated on a 1% agarose gel and were analyzed by Northern blot hybridization using the DIG-labeled CP DNA probe. (B) Western analysis of CP. CP was detected using anti-TYMV antiserum or anti-c-Myc epitope antibody after SDS-PAGE and blotting to a nitrocellulose membrane. (C) Analysis of CP assembly. Virions in leaf extracts were examined by Western analysis using an anti-TYMV antibody (top panel) or an anti-c-Myc epitope antibody (bottom panel), after non-denaturing agarose gel electrophoresis.

mRNAs from CP, M-CP and CP-M are polyadenylated, whereas the TYMV sgRNA is not. Analysis of the CP by Western blot after SDS-polyacrylamide gel electrophoresis (PAGE) also showed that the M-CP and CP-M mRNAs were all translated efficiently (Fig. 2B). The modified CP from the M-CP and CP-M could also be detected by anti-c-Myc tag antibody.

TYMV CP can self-assemble without viral RNAs. Thus, empty virions (20-30%) are usually found in infected leaves (1). We examined if the TYMV CP assembly occurred properly with the N- or C-terminal modification. For this purpose, the virions in leaf extracts were separated on a non-denaturing agarose gel and were visualized by Western analysis. The empty virions produced from the CP construct migrated in a single predominant band that co-migrated with the wild-type virion (TY^W). Such a virion band was also observed in the case of M-CP, suggesting that the N-terminal extension has no detrimental effect on CP assembly. In the case of CP-M, the anti-TYMV CP antibody detected a band that migrated faster than wild-type virions (Fig. 2C). The result indicates that the CP modified at the C-terminus did not assemble correctly into virions.

The results obtained in this study are consistent with the previous report by Sastri *et al.* (19), who showed that the N-terminus of the coat protein of the *Physalis mottle virus* can be extended by 41 amino acid residues without affecting the assembly in *E. coli*. As far as the C-terminal modification is concerned, the result obtained in this study conflicts with the re-

sult obtained by Bransom *et al.* (20) who reported that the C-terminal extension was viable. Perhaps, the modification by Bransom *et al.* was too small (5 amino acids) to influence the assembly.

Influence of CP modification on TYMV replication and packaging

For closer examination of the CP mutants, we examined the influence of the modifications on the replication and packaging of the TYMV. For this purpose, we have made TYMV constructs containing the modified CP ORF (Fig. 3A). To make the TY-M-CP construct, the CP ORF in a TYMV expression construct was replaced with the M-CP. The TY-CP-M construct was made using a TYMV construct where the SpeI site was introduced into the 3'-end of the CP ORF. Since a pseudoknot structure around the CP ORF stop codon was reported to interact with the 3'-TLS and to be essential for the translation of TYMV RNAs (22), we inserted the pseudoknot sequence following the c-Myc sequence so that the interaction between the pseudoknot and TLS was not interrupted.

Fig. 3B shows that replication of the TY-CP-M was strongly suppressed. Previously, we have observed that replication was nearly abolished when a foreign sequence was inserted between the CP ORF and the 3'-TLS (23). The same result was obtained when the sequence between nt-6139 and nt-6181, which is proximal to the end of CP ORF, was deleted (24), suggesting that a cis-acting element required for efficient replication exists near the end of CP ORF. In the TY-CP-M, the insertion was presumed to disturb some interactions involving the replication element.

To see whether the viral RNAs were encapsidated, we carried out an RNase protection assay. The result shows that the mutant viral RNAs of TY-M-CP were packaged as efficiently as the wild type. In the case of TY-CP-M, however, the viral RNAs were barely packaged. This again shows that the CP modified at the N-terminus assembles correctly into virions, but that the CP modified at the C-terminus does not.

To confirm the result, we performed virion RNA analysis after electrophoresis of the virions in leaf extracts on a non-denaturing agarose gel (25). In this experiment, the viral RNA was liberated by alkaline treatment after the electrophoresis on an agarose gel. The viral RNA was blotted to a nylon membrane, and was detected with a DIG-labeled probe DNA. The virion analysis showed that the TY-M-CP produced a band at the position corresponding to the TYMV virion (Fig. 4A). However, such a band was not detected in the case of TY-CP-M. Analysis of CP by Western blot after SDS-PAGE showed that coat proteins were produced as expected in all constructs (Fig. 4B). This shows that the gRNA and sgRNA of TY-CP-M are not packaged, unlike the viral RNAs from other constructs. This result is consistent with the result shown in Fig. 3B. We also examined virions in leaf extracts by Western analysis after agarose gel electrophoresis. As shown in Fig. 4C, TY-M-CP produced a band co-migrating with the wild-type virion (TY^W). However, in the case of TY-CP-M, the major band was ob-

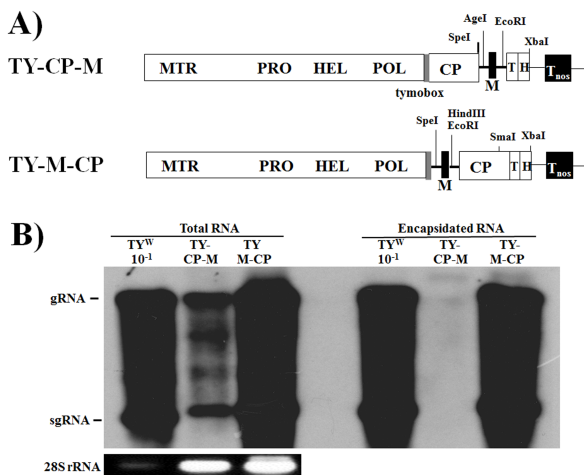


Fig. 3. Replication and RNA packaging of the recombinant TYMV expressing modified CPs. (A) TYMV constructs with modified CPs. The methyltransferase (MTR), protease (PRO), helicase (HEL), and polymerase (POL) domains of TYMV genome are indicated. M, T and H represent the c-Myc tag, tRNA-like structure, and HDV, respectively. (B) Northern analysis of viral RNAs. Total RNA was prepared from the *Nicotiana benthamiana* leaves which were collected 7 days after agroinfiltration. For the encapsidation assay, leaf extracts were prepared and were subjected to the RNase protection assay. Northern analysis of total RNA and encapsidated RNA was performed as described in Fig. 2A.

served to move faster, again indicating that the majority of the CP modified at the C-terminus did not assemble properly. Virions with the expected size and morphology could also be observed by electron microscopy in the leaf extracts prepared from the leaf agroinfiltrated with TY-M-CP (Fig. 4D). The results showed that the assembly and packaging of TYMV is influenced by C-terminal modification but not by N-terminal modification, indicating that the C-termini of TYMV CP are critically involved in virion assembly.

The crystal structure of TYMV has been solved to 3.2 Å resolution (4). The data show that the C-termini of CPs are on the exterior, while the N-termini appear to be on the interior. The crystal structure is inconsistent with an immunological study showing that N-terminal residues 1-12 are immunoreactive (26). Canady *et al.* theorized that this inconsistency was caused by a movement of the N-terminal segment, from the interior of a virion to the exterior, through an opening at the apex of the pentamer clusters. As shown in Fig. 2C and 4C, the c-Myc peptide fused to the N-terminus of CP was reactive with anti-TYMV antiserum and the anti-cMyc tag antibody, after native agarose gel electrophoresis and blotting to a nitrocellulose membrane. Thus, there seems to be a good possibility that the c-Myc tags are exposed to the exterior of the virions. The results in Fig. 2C and 4C are also consistent with a previous immunological study which predicted the C-terminal

residues 183-189 to be on the exterior surface of the virion (27) and with the virion crystal structure (4).

Overall, the results obtained so far suggest that the N-terminal segment of TYMV CP is rather flexible and can be modified without compromise with virion assembly and viral RNA packaging. The nature and scale of the possible modifications which can be tolerated at the N-terminus of TYMV CP remain to be elucidated.

MATERIALS AND METHODS

DNA constructs

The CP/pA construct, where CP ORF was inserted into the *Bam*HI/*Xba*I sites of pCB302-3, has been previously described (21). To make the construct pCBm-M-CP, the sequence corresponding to CP ORF, 3'-TLS and HDV was PCR-amplified with the following primers: 5'-GTACGGATCCAAGAATTCTATGGA AATCGACAAAGA-3'; 5'-GGAGAATTCTCTAGATGGCTCTCC CTTAG-3' (*Bam*HI, *Eco*RI, and *Xba*I restriction recognition sites are underlined). The PCR product was digested with *Bam*HI and *Xba*I, and was cloned into a modified pCB302-3, where a 1.2 kb *Kpn*I fragment, including part of the bar gene, was deleted and the *Kpn*I recognition sites were removed. Then, a c-Myc tag linker was inserted into the *Bam*HI/*Eco*RI sites of the construct. The linker was prepared with the following oligonucleotides: 5'-GATCCAAGTACTAGTACCATGGAGCAGAAGCTCAT CTCTGAGGAAGATCTTGGTTCGACAAGCTTCG-3'; 5'-AATT CGAAGCTTGTGACCAAGATCTTCCTCAGAGATGAGCTTC TGCTCCATGGTACTAGTTGGATC-3' (the nucleotide sequence corresponding to the c-Myc tag is underlined). In the resulting construct, pCBm-M-CPTH, the sequence between *Hind*III and *Xba*I sites was replaced with CP DNA containing CP ORF plus a short 3'-untranslated region (18 nt). The resulting construct, designated pCBm-M-CP, does not have the 3' tRNA-like structure (TLS) or hepatitis delta virus (HDV) ribozyme sequence. The *Spe*I/*Xba*I fragment of the pCBm-M-CPTH was inserted into the *Spe*I/*Xba*I sites of a TYMV expression construct that has the same cloning sites as the TY-V^{2tymo} (28) except that the *Spe*I site is additionally present between the *Scal* and *Nco*I sites (see also Fig. 3A). The resulting construct was designated TY-M-CP.

The TY-CP-M construct was made by deleting the eGFP sequence from TY-eGFP₂₆₂₀₃ (23), followed by the insertion of a c-Myc tag linker into the *Spe*I/*Eco*RI sites of the construct. The linker was prepared with the following oligonucleotides: 5'-CTAGTACCGGTGGTGGTCTCTGAGCAGAAGCTCATCTCT GAGGAAGATCTTTG-3'; 5'-AATTCAAAGATCTTCCTCAGAG ATGAGCTTCTGCTCAGAACCACCACCGGTA-3' (the nucleotide sequence corresponding to the c-Myc tag is underlined). The CP ORF and the C-terminal c-Myc tag sequence was amplified by PCR, and was used to replace the CP ORF in the pCBm-M-CP. The resulting construct was designated pCBm-CP-M. After these manipulations, the resulting constructs were verified by sequence analyses.

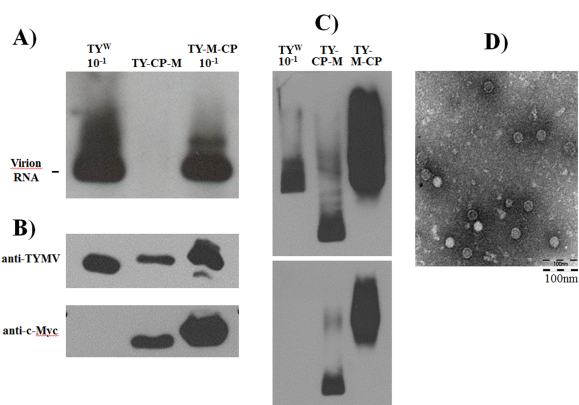


Fig. 4. Virion assembly of the recombinant TYMV expressing modified CPs. (A) Northern analysis of virions. The virions in leaf extracts were electrophoresed on a 1% non-denaturing agarose gel and were then treated with 0.2 N NaOH. The liberated virion RNA was blotted onto a nylon membrane and was examined by hybridization with a DIG-labeled CP DNA probe. (B) Western analysis of CP. CP in leaf extracts was analyzed by Western blot after SDS-PAGE, as described in Fig. 2B. (C) Western analysis of virions. After non-denaturing agarose gel electrophoresis, the virions were transferred to a nitrocellulose membrane, and were probed with anti-TYMV CP antibody or with anti-c-Myc tag antibody. (D) Electron micrograph of ca. 30 nm particles prepared from leaves agroinfiltrated with TY-M-CP construct. A 100-nm size bar is shown at the bottom right corner.

Plant material

Agroinfiltration of the *A. tumefaciens* harboring various CP and TYMV constructs into *Nicotiana benthamiana* was carried out as described previously (21). 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 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°C until use.

Analysis of RNA

Ribonuclease protection assay for encapsidated RNA and Northern analysis were performed as previously described (21). Briefly, in the encapsidation assay, leaf extracts were incubated with RNase A ($5\ \mu\text{g}/\text{ml}$ at the final concentration) for 1 h at 37°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 DNA probe representing the coat protein ORF (nt-5641 to nt-6231).

Analysis of virions in leaf extracts

Virions in leaf extracts were separated by electrophoresis on a 1% agarose gel. For the analysis of virion RNA, the gel was treated with 0.2 N NaOH and then with 0.1 M Tris buffer (pH 7.5). The virion RNAs in the gel were then transferred onto Hybond N⁺ membranes by capillary transfer using 20X SSC, and were analyzed by Northern blot hybridization. For capsid protein analysis, the agarose gel was treated with 0.1 M Tris buffer (pH 7.5), but not with NaOH. After transfer of virions to a Hybond ECL membrane, the membrane was exposed to anti-TYMV rabbit antiserum or anti-cMyc tag antibody (Santa Cruz Biotechnology, CA, USA), and subsequently to goat anti-rabbit HRP conjugate (Bio-Rad, Hercules, CA, USA). The membrane was developed by the chemiluminescent detection method using LuminataTM Forte (Millipore, Bedford, MA, USA).

For virion observation, immunosorbent electron microscopy was carried out as previously described (21). Briefly, carbon-coated gold electron microscopy grids (Electron Microscopy Sciences, Hatfield, PA, USA) were coated with TYMV antiserum prior to incubation with leaf extracts. The samples were negatively stained with 2% ammonium molybdate solution.

Analysis of coat protein after SDS-PAGE

Coat protein (CP) was examined by 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot analysis, as described (29). Leaf samples (0.1 g) were ground in 200 μl of 2X sample buffer and were boiled for

5 min. Western blot detection used anti-TYMV rabbit antiserum or anti-cMyc tag antibody in conjunction with HRP-conjugated goat anti-rabbit IgG and a chemiluminescent detection method using LuminataTM Forte.

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