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The Pro/Hel Region Is Indispensable for Packaging Non-Replicating Turnip Yellow Mosaic Virus RNA, but Not Replicating Viral RNA

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Turnip yellow mosaic virus (TYMV) is a spherical plant virus that has a single 6.3 kb positive strand RNA. The genomic RNA has a tRNA-like structure (TLS) at the 3′-end. The 3′-TLS and hairpins in the 5′-untranslated region supposedly serve as packaging signals; however, recent studies have shown that they do not play a role in TYMV RNA packaging. In this study, we focused on packaging signals by examining a series of deletion mutants of TYMV. Analysis of encapsidated viral RNA after agroinfiltration of the deletion constructs into *Nicotiana benthamiana* showed that the mutant RNA lacking the protease (Pro)/helicase (Hel) region was not encapsidated by the coat proteins provided in trans, implicating that a packaging signal lies in the Pro/Hel region. Examination of two Pro$^-$Hel$^-$ mutants showed that protein activity from the Pro/Hel domains was dispensable for the packaging of the non-replicating TYMV RNA. In contrast, the mutant TYMV RNA lacking the Pro/Hel region was efficiently encapsidated when the mutant TYMV was co-introduced with a wild-type TYMV, suggesting that packaging mechanisms might differ depending on whether the virus is replicating or not.

INTRODUCTION

Turnip yellow mosaic virus (TYMV), a positive strand RNA virus, is a type member of tymoviruses and mainly infects *Cruciferae* plants. The TYMV genome is a single 6.3 kb RNA with an ^{m7}GpppG cap at the 5′-end and a tRNA-like structure (TLS) at the 3′-end. TYMV is an icosaheral virus composed of a single coat protein that constitutes about 65% of the virus by weight (Dreher, 2004). The 3′-TLS enhances the translation of TYMV RNA (Matsuda and Dreher, 2004; Matsuda et al., 2004). Two proteins, whose open reading frames (ORFs) extensively overlap, are translated from the genomic RNA (gRNA). ORF-206, which encodes domains of methyltransferase (Mtr), NTPase/helicase (Hel) and polymerase (Pol) activities, is the only coding region required to support RNA replication in single cells (Weiland and Dreher, 1989). It is produced as a polyprotein and self-cleaved to yield 141- and 66-kDa proteins, the latter of which has Pol activity. p69 from the second ORF is required for virus movement within the plant and is also a suppressor of gene silencing (Chen et al., 2004). The coat protein is not expressed from the gRNA, but produced from a single subgenomic RNA (sgRNA) generated during replication.

Upon infection, TYMV induces invagination of outer membranes chloroplasts, forming replication vesicles. TYMV assembly is postulated to occur around the vesicle neck as replicated viral RNAs are extruded (Matthews, 1991). Currently, it is not known exactly how TYMV RNA is specifically packaged with the coat proteins. In eukaryotic RNA viruses, the most thoroughly characterized packaging signal is that of Tobacco mosaic virus (TMV), a helical plant RNA virus in which the specific interaction between the coat protein and a 69-nucleotide (nt) region of the movement protein ORF leads to the specificity of virion assembly (Rao, 2006; Zimmern, 1977). Among plant RNA viruses with icosahedral symmetry, sequence elements that act as specific packaging signals have been characterized only for a few viruses, such as Turnip crinkle virus (TCV) and Brome mosaic virus (BMV) (Rao, 2006).

In TYMV, packaging of the gRNA was proposed to be initiated at two hairpins in the 5′ untranslated region (5′-UTR). Hellendoorn et al. (1996) observed remarkable similarity among five tymoviruses in the secondary structure of the 5′-UTR that contains a symmetrical internal loop consisting of C-C or C-A mismatches, despite large sequence differences. TYMV gRNA has two such hairpin structures in the 5′-UTR that become more stable at low pH due to protonated C residues participating in C-C and C-A base pairing (Bink et al., 2002; Hellendoorn et al., 1996). Several passages of infection of the hairpin mutants with altered internal loops generated revertants with a wild-type phenotype, which had restored C-C or C-A mismatches, showing the importance of the mismatches (Hellendoorn et al., 1997). When the role of the two 5′-UTR hairpins was directly addressed using the mutants lacking both or one of the two hairpins, however, it was found that the two hairpins were not required for packaging (Shin et al., 2009).

3′-TLS has also been suggested to serve packaging signals. In BMV, gRNA1 (B1) and gRNA2 (B2), encoding replication factors, are packaged into two separate virions, whereas gRNA3

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(B3) and its subgenomic coat protein mRNA (B4) are co-packaged into a third virion (Rao, 2006). All the three gRNA and one sgRNA contain a highly conserved 200-nt 3′-UTR sequence that can be folded to mimic TLS. TLS was found to be required for the packaging of four BMV RNAs (B1, B2, B3, and B4) *in vitro* (Choi et al., 2002). Previously, we examined whether or not the 3′-TLS of TYMV RNA also played a role in packaging using an *Agrobacterium*-mediated transient in vivo expression system. The result showed that TLS was not needed for TYMV RNA encapsidation (Cho and Dreher, 2006).

Overall, the packaging signals of TYMV RNA have not been identified. In this study, we have examined a series of deletion variants for the presence of *cis*-acting packaging signals in TYMV RNA. We obtained rather conflicting results in that the Pro/Hel region is necessary for the packaging of non-replicating TYMV RNA, but it is dispensable for the packaging of replicating viral RNA.

MATERIALS AND METHODS

DNA constructs

To prepare the deletion constructs TYΔMtr-MB, TYΔMtr-NB, and TYΔPrH-BN, the sequence between unique restriction sites was removed from a wild-type construct TY^W (Cho and Dreher, 2006) and replaced with the linker made by annealing the following oligonucleotides: 5′-AATTGAACCATGGCTAGCG-3′ and 5′-GATCCGATCCGCTAGCCATGGTTC-3′ for TYΔMtr-MB; 5′-CAT-GGAAGCTTCTAGAGG-3′ and 5′-GATCCCTCTAGAAGCTTC-3′ for TYΔMtr-NB; 5′-GATCCAAGCTTCTAGAGG-3′ and 5′-CTAGCCTCTAGAAGCTTG-3′ for TYΔPrH-BN. To construct TYΔPoC-NS, the region from nt-6093 to the 3′-end of TY^W was PCR-amplified using the following primers: 5′-CGAGCTAGC-CCAAACTCCTCATCTCCA-3′ and 5′-GGAGA-ATTCTCTAGA-TGGCTCTCCCTTAG-3′. The amplified sequence was then used to replace the DNA between the *Nhe*I and *Xba*I sites of the TY^W construct.

TYΔPol and TYΔPHP were derived from an expression vector TY-V^{2tymo} (Shin et al., 2008b). The DNA sequence comprising a tymobox, coat protein ORF, and TLS was amplified by PCR. During the PCR, an extra tymobox and several restriction sites (*Sna*BI, *Eco*RI, and *Spe*I sites) were incorporated into the vector. This modified sequence was then used to replace the sequence between the *Nhe*I and *Xba*I sites of the TY^W. In the resulting construct, TYΔPol, the DNA between *Bam*HI and *Eco*RI sites was removed and replaced with a linker made with the following oligonucleotides to produce TYΔPHP: 5′-GATC-CAAGCTTCTAGAGG-3′ and 5′-AATTCCTCTAGAAGCTTG-3′. The frameshift mutant TYm-FS^{Bam} was generated by digesting the wild-type construct TY^W with *Bam*HI and then by ligating again after filling-in reactions. In ΔMtr-L^{stop}, the region between *Mfe*I and *Bam*HI sites was removed and replaced by a linker composed of the following oligonucleotides: 5′-AATTGAACCA-TGGCTTAGTGAG-3′ and 5′-GATCCTCACTAAGCCATGGT-TC-3′. 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 by Hwang et al. (2009). 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°C. For the encapsidation assay, the leaf sample was ground with 4 volumes of 0.1 M sodium phosphate buffer, pH 7.0. The homogenate was clarified by the addition of 0.2 volumes chloroform, centrifuged briefly, and stored at 4°C until use.

RNA analysis

The ribonuclease protection assay for encapsidated RNA and Northern analysis were performed as described (Cho and Dreher, 2006). Briefly, in the encapsidation assay, the leaf extract was incubated with RNase A (5 μg/ml final concentration) for 1 h at 37°C and for an additional 1 h in the presence of proteinase K and SDS (100 μg/ml and 0.5% final concentrations, respectively). The surviving RNA, representing encapsidated RNA, was then extracted with acidic phenol and chloroform, and precipitated with ethanol.

Using 1% agarose gel electrophoresis, 1 or 2 μg of the total RNAs and equivalent amounts of the RNA samples from the encapsidation assay were size-fractionated and transferred onto Hybond N^+ membranes (GE Healthcare). The blots were hybridized with a DNA probe representing the coat protein ORF (nt-5641 to nt-6231), the Mtr domain (nt-74 to nt-1769), or the Pro domain (nt-1671 to nt-3312). The probe DNA was amplified by PCR and labeled with digoxigenin (DIG) using DIG-dUTP. The blots were developed by chemiluminescent immunodetection of DIG (Roche Molecular Biochemicals).

Virion analysis from leaf extracts

Virions in 20 μl of the leaf extract were separated by electrophoresis on a 1% agarose gel. For virion RNA analysis, 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 (GE Healthcare) by capillary transfer using 20X SSC, and analyzed by Northern blot hybridization as described above. For capsid protein analysis, the agarose gel was treated with 0.1 M Tris buffer (pH 7.5). The virions on the gel were transferred to Hybond ECL nitrocellulose membranes (GE Healthcare). The membrane was exposed to anti-TYMV rabbit antiserum and subsequently to a goat anti-rabbit HRP conjugate (Bio-Rad). The membrane was developed by the chemiluminescent detection method using luminol (Millipore).

RESULTS

The Pro/Hel region is required for packaging non-replicating TYMV

Previous experiments have shown that either the 3′-TLS or 5′-UTR is not required for the packaging of TYMV RNA (Cho and Dreher, 2006; Shin et al., 2009). To determine the signals necessary for packaging, we prepared a series of TYMV constructs having various internal deletions (Fig. 1). The deletion constructs were prepared by removing the sequence between the unique restrictive enzyme sites from the wild-type TYMV construct TY^W, followed by inserting either a short linker or a PCR-amplified DNA with deletions. The mutant construct was introduced into *N. benthamiana*, using the *Agrobacterium*-mediated T-DNA transfer system. Ten days after the agroinfiltration, the infiltrated leaf was collected for analysis.

To check whether the deletion mutant RNA was encapsidated, we carried out an RNase protection assay previously developed for studying encapsidated RNAs in leaf extracts (Cho and Dreher, 2006). Briefly, the extracts were treated with RNase A prior to de-proteinization of RNAs and analyzed by Northern blot hybridization. Controls for the RNase protection encapsidation assay are shown in Fig. 2A, demonstrating the susceptibility of free RNA (lanes 2 and 5) and resistance of the encapsidated virion RNA (lanes 3 and 6) to the RNase treatment.

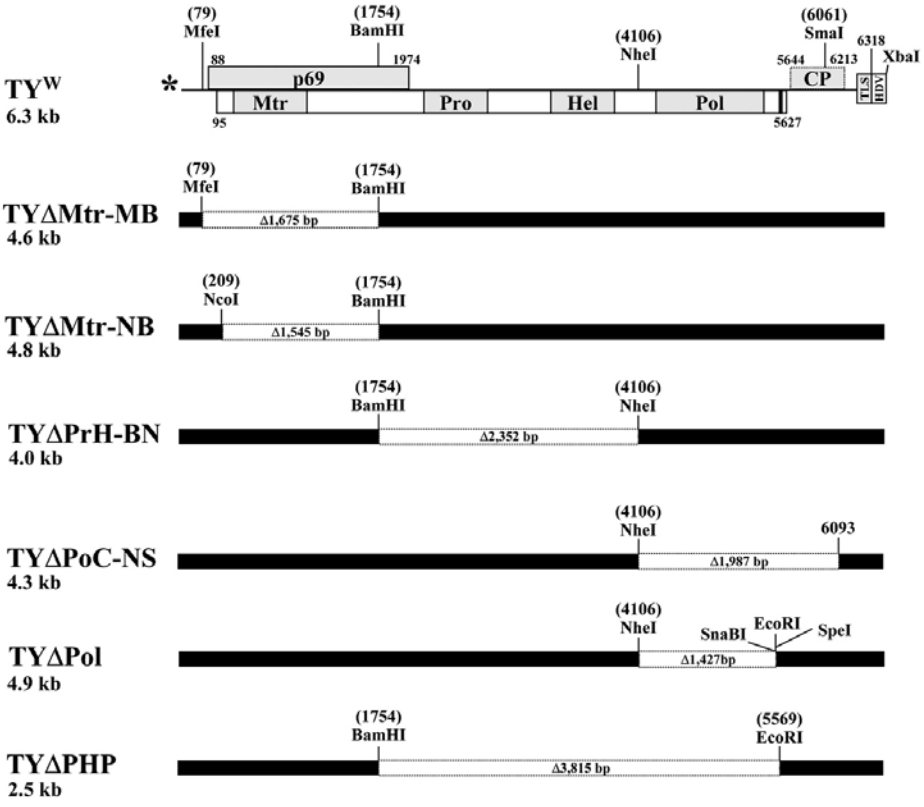

Fig. 1. TYMV deletion constructs. TY^W represents a wild-type TYMV construct. Deletion constructs TYΔMtr-MB, TYΔMtr-NB, TYΔPrH-BN and TYΔPoC-NS were prepared by removing the sequence between the unique restriction sites from the TY^W construct, and then by replacing with a linker or PCR-amplified sequence (see "Materials and Methods"). The positions of the restriction sites and those of the beginning and end of three open reading frames are indicated in the maps by the nucleotide numbers. TYΔMtr-MB and TYΔMtr-NB constructs show a deletion of 1.7 kb and 1.5 kb sequences, respectively, which include the methyltransferase (Mtr) domain of p206. TYΔPrH-BN has a deletion of a 2.4 kb sequence that contains the protease (Pro) and helicase (Hel) domains. TYΔPoC-NS construct has a deletion of a 2.0 kb sequence near the 3′-end of the genome that includes the polymerase (Pol) domain of p206 and ~3/4 of the coat protein (CP) open reading frame. TYΔPol and TYΔPHP were derived from a TYMV expression vector TY-V^{2tymo}. The TYΔPol mutant has a deletion of a 1.5 kb sequence of the polymerase domain. TYΔPHP mutant has a deletion of a 3.8 kb sequence comprising protease, helicase, and polymerase domains.

The total RNA of the leaf and the RNA protected from the RNase treatment were examined using DIG-labeled coat protein ORF DNA as probe. As expected, the deletion mutant TYMVs did not replicate; hence, coat proteins were not generated by the deletion constructs. To see whether the deletion mutant RNA was encapsidated, the coat protein construct CPpA, which expresses TYMV coat proteins under the influence of the 35S promoter (Cho and Dreher, 2006), was co-infiltrated into the plant along with the deletion construct. TY^R is a non-replicating mutant due to a point mutation in the Pol domain (Weiland and Dreher, 1993), and was included as the control. It had been demonstrated to be encapsidated by coat proteins provided in trans (Cho and Dreher, 2006). The RNase protection assay showed that the mutant RNAs with deletion of a 1.7 kb sequence from *Mfe*I to *Bam*HI (ΔMtr-MB) and a 1.5 kb sequence from *Nco*I to *Bam*HI (ΔMtr-NB) were efficiently encapsidated (Fig. 2B, lanes 10 and 11). Deletion of either the Pol domain (ΔPol) or the Pol domain plus ~3/4 of the coat protein ORF (ΔPoC-NS) did not affect the encapsidation, either (Fig. 2B, lanes 13 and 14). In contrast, encapsidated RNA was not detected when there was deletion of either a 2.4 kb sequence from *Bam*HI to *Nhe*I (ΔPrH-BN) (Fig. 2B, lane 12) or a 3.8 kb sequence comprising ~ 2/3 of the p206 ORF (ΔPHP) (Fig. 2B, lane 15). To see if the results were reproducible, we repeated the agroinfiltration experiments 6 times, and two representative results are shown in Fig. 2B.

We also examined virions in leaf extracts. In this experiment, virions were separated on a non-denaturing agarose gel, and virion RNAs were liberated from capsids by alkaline treatment and visualized by Northern analysis. Under this condition, the virion RNA in the leaf extract was resistant to RNase treatment and migrated in a single predominant band that co-migrated with the purified intact virions (Fig. 3A, lane 3). Under the same non-denaturing condition, deproteinized viral RNA migrated in several diffused bands (Fig. 3A, lane 2). Thus, the presence of encapsidated virion RNA is also demonstrated by this assay.

Single bands of virion RNAs were also observed in the extracts prepared from the leaves agroinfiltrated with ΔMtr-MB, ΔMtr-NB, ΔPoC-NS, and ΔPol constructs (Fig. 3B, lanes 3, 4, 6 and 7). However, such virion RNA bands were not detected in the cases of ΔPrH-BN and ΔPHP mutants (Fig. 3B, lanes 5 and 8). We also used the probe derived from the sequence corresponding to the Mtr or Pro domain, and we obtained the same results. The band intensity of the ΔPoC-NS RNA (Fig. 3B, lane 6) was relatively weak when a coat protein probe was used, compared to when either the Mtr or Pro probe was used. This is due to the fact that the majority of coat protein ORF is deleted in the mutant ΔPoC-NS. Analysis of viral capsids by western blot showed that TYMV capsids were present in all leaf extract preparations, including ΔPrH-BN and ΔPHP mutants (Fig. 3C). Since the mutant viral RNAs are all detected in total RNA preparations by Northern analysis (Fig. 3D), this reiterates that the region between the *Bam*HI and *Nhe*I sites, corresponding to the Pro/Hel domain, is necessary for TYMV RNA packaging.

Pro/Hel region is dispensable for packaging when the viral RNA is replicating

We then examined what would happen when the mutant con structs were co-introduced into plants along with the wild-type TYMV construct. Total RNA analysis showed that the TYMV

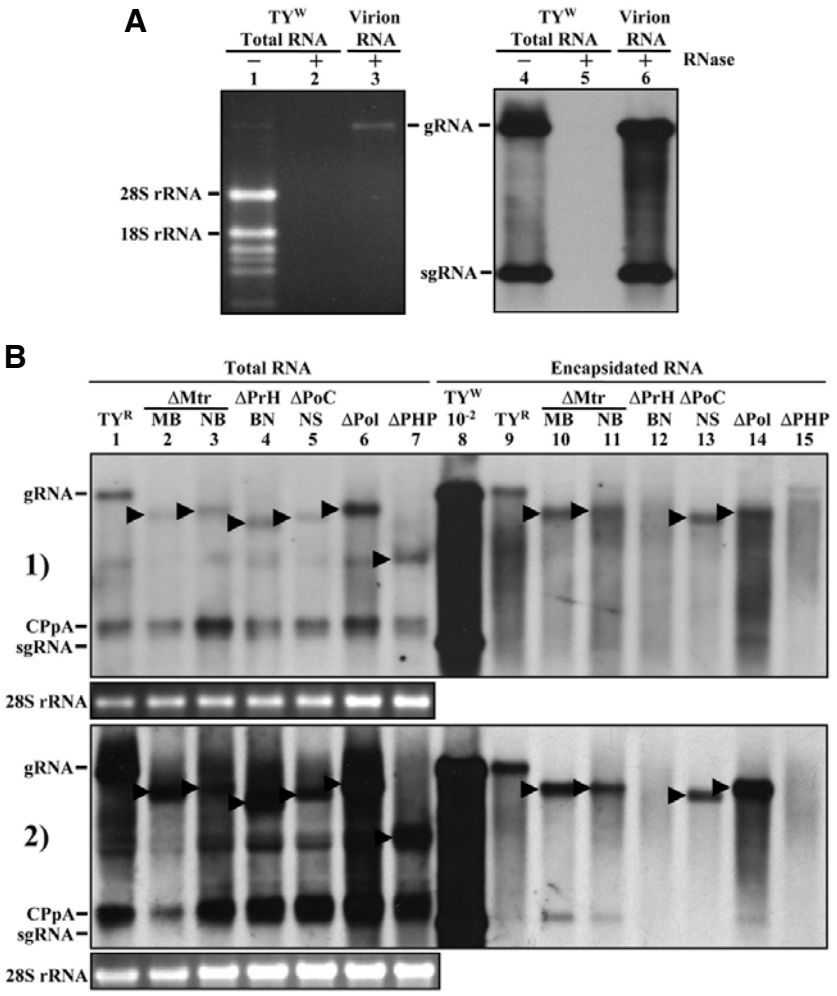

Fig. 2. Encapsidation of deletion mutant RNAs by coat proteins provided in trans. (A) An agarose gel and a Northern blot demonstrating the efficacy of the RNase protection assay. Total RNA (lane 2) and extract (lane 3) from the leaf agroinfiltrated with TY^W was treated with RNase, and then deproteinized by phenol extraction. A total RNA sample without the RNase treatment was also included (lane 1). The RNA samples were electrophoresed on a 1% agarose gel and analyzed by Northern blot hybridization, using a DIG-labeled coat protein ORF DNA as the probe (lanes 4-6). (B) Northern blot analysis of encapsidation of deletion mutant RNAs. RNA samples from two independently infiltrated leaves were analyzed on duplicate Northern blots (panels 1 and 2). Deletion mutant constructs were agroinfiltrated into *N. benthamiana* plants along with the coat protein construct CPpA. Ten days after agroinfiltration, the total RNA was extracted from the agroinfiltrated leaf, and the leaf extracts were subjected to the RNase protection assay to examine encapsidation of the RNA. Total RNAs and equivalent amounts of the RNA samples from the encapsidation assay were size-fractionated by agarose gel electrophoresis, and examined by Northern blot analysis as described above. Mutant genomic RNAs are indicated by arrowheads. The lower panels show the 28S rRNA section of the agarose gel after electrophoresis and ethidium bromide staining, shown as a loading control.

deletion mutants except the ΔMtr mutants co-replicated along with the wild type (Fig. 4). This result suggests that some replication elements essential for replication are lacking in the ΔMtr mutants. This finding is very informative since replication elements other than the 3′-end -CCA (Deiman et al., 1998; Yoshinari et al., 2000) and 5′-UTR (Shin et al., 2008a) have not been reported.

When encapsidation of deletion mutant RNAs was examined by the RNase protection assay, it was observed that both ΔPrH-BN and ΔPHP RNAs were encapsidated as efficiently as the wild-type TYMV RNA (Fig. 4, lanes 9 and 12 in panel 1, and lanes 11 and 14 in panel 2). Other deletion mutants were also encapsidated well. Here, the sgRNA was also efficiently encapsidated, unlike the coat protein mRNA produced from an independent expression vector (see Fig. 2B). Overall, these results show that ΔPrH-BN and ΔPHP mutant RNAs are encapsidated when all the TYMV proteins are present, whereas they are not when only coat proteins are provided.

An explanation for these rather conflicting results could be that efficient TYMV RNA packaging requires the protein activities that are missing in ΔPrH-BN and ΔPHP mutants. These activities would of course occur when wild-type TYMV would be co-introduced into the plant along with the mutants. If this is the case, the deletion mutants other than the ΔPrH-BN and ΔPHP would have produced truncated but functional p206 proteins in terms of protease and helicase acitivities. TYΔPoC and TYΔPol mutants could have produced such a protein, since the deleted region is beyond the Pro and Hel domains. In the case of TYΔMtr mutants, the linkers used were in-frame with the p206 reading frame. Thus, a functional protein having protease and helicase activities might have been produced from the TYΔMtr mutants. We then verified this hypothesis.

Packaging of non-replicating TYMV RNA does not require either protease or helicase activity

We constructed two $Pro^{-}Hel^{-}$ mutants (Fig. 5A). One such mutant, $TYm\text{-}FS^{Bam}$, was constructed by introducing a frameshift mutation at the *Bam*HI site of the wild-type construct TY^W. In this mutant, the reading frame is changed and a stop codon appears at nt-1972 instead of nt-5627. The other mutant, $TY\Delta Mtr\text{-}L^{stop}$, is identical to TYΔMtr-MB, except that the linker contains two consecutive in-frame stop codons.

These mutant constructs were introduced into *N. benthamiana* plants along with the CPpA construct. Total RNA analysis showed that the mutants were not replicating (lanes 2 and 3 in Figs. 5B and 5C). This was not surprising because protease and helicase activities are essential for TYMV replication (Dreher, 2004). Northern analysis of the RNA samples from the RNase protection assay showed that both $Pro^{-}Hel^{-}$ mutants were efficiently encapsidated (lanes 5 and 6 in Figs. 5B and 5C). Analysis of virions in the leaf extracts also showed the presence of virion RNAs co-migrating with intact TYMV virions

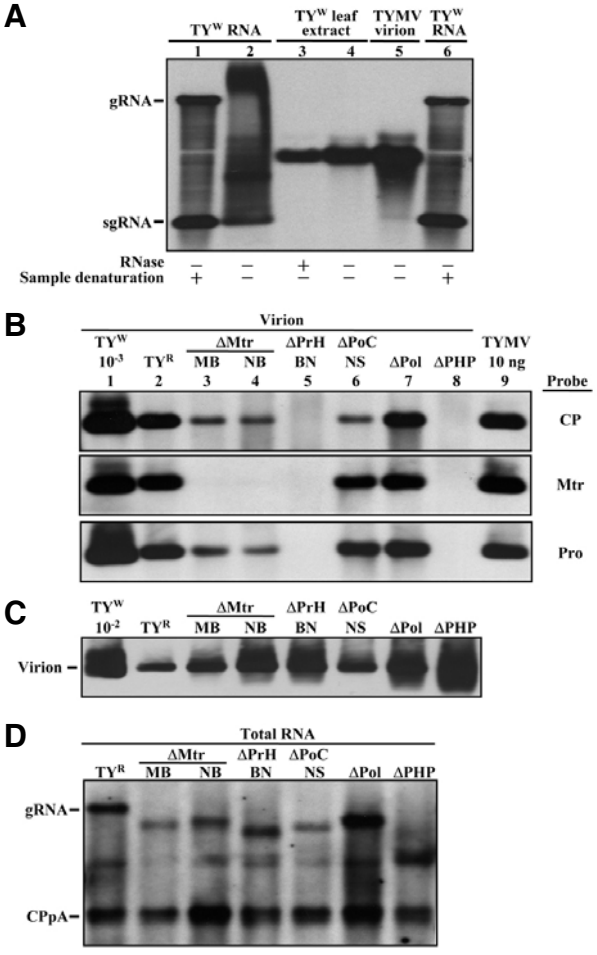

Fig. 3. Verification of encapsidation by analysis of virions in leaf extracts. (A) Controls for virion analysis. The virions in leaf extracts, with or without RNase treatment, were electrophoresed on a 1% agarose gel, blotted onto a Hybond N^+ nylon membrane after denaturation with NaOH, and examined by hybridization with a DIG-labeled coat protein DNA probe (lanes 3 and 4). Total RNA from the leaf agroinfiltrated with TY^W, either with or without sample denaturation (lanes 1, 2, and 6), and purified TYMV virions (lane 5) were included as controls. (B) Northern analysis of virions in leaf extracts. Extracts from the leaves agroinfiltrated with various deletion constructs were examined by agarose gel electrophoresis and Northern blot analysis as described. The probe used was the DIG-labeled DNA representing the coat protein ORF, the Mtr domain, or the Pro domain. TY^W represents the extract from the leaf agroinfiltrated with TY^W. Purified TYMV virion was included as the control (lane 9). (C) Western analysis of virions. The virions were examined using an anti-TYMV coat protein antibody, after agarose gel electrophoresis and blotting to a Hybond ECL membrane. (D) Analysis of total RNA. Total RNA extracted from the agroinfiltrated leaves was size-fractionated by 1.0% agarose gel electrophoresis and examined by Northern blot analysis using the DIG-labeled coat protein DNA as the probe.

(bottom panels in Figs. 5B and 5C), suggesting that both TYm-FS^{Bam} and TYΔMtr-L^{stop} mutant viral RNAs were packaged into virions.

Overall, these results show that the non-replicating TYMV RNA is encapsidated by coat proteins as long as the Pro/Hel region is present in the viral RNA and that the packaging does not require viral proteins other than coat proteins. This is the first report suggesting that the packaging of TYMV RNA requires a sequence element in the viral RNA. We also observed that the packaging of replicating TYMV RNA does not need the sequence element in the Pro/Hel region. These seemingly conflicting results would give us an important clue to the mechanism of TYMV RNA packaging.

DISCUSSION

A previous study showed that TY^R, a non-replicating gRNA, was efficiently encapsidated by coat proteins provided in trans. In contrast, coat protein RNAs produced from an expression vector independent of viral replication were not encapsidated, even if the sequence of the coat protein RNA was identical to that of sgRNA (Cho and Dreher, 2006). The behavior of coat protein RNA was somewhat strange because TYMV sgRNA encoding the coat protein is encapsidated as efficiently as the gRNA when the coat protein RNA is produced during replication (Cho and Dreher, 2006; Shin et al., 2008b; 2009). In this study, we obtained the same results.

This rather unexpected behavior of TYMV sgRNA can be explained by assuming that the affinity of coat protein to sgRNA is far low compared to the affinity to gRNA and that the sgRNA can be encapsidated only when there is abundant sgRNA. Previous experiments, however, showed that this was not the case. When the 5′-UTR of TYMV RNA was deleted or modified, the mutant still replicated, but the replication of both gRNA and sgRNA decreased by up to 1,000-fold. Under the circumstances, the sgRNA was encapsidated as efficiently as the gRNA (Shin et al., 2009).

Overall, these results suggest that the packaging of sgRNA, but not gRNA, is coupled to replication, and that the packaging of non-replicating TYMV RNA requires a *cis*-acting sequence element. In this study, we have shown that the Pro/Hel region is required for the packaging of non-replicating TYMV RNA, further supporting this idea. The requirement of the Pro/Hel region also explains why TYMV CP RNA is not encapsidated when it is not coupled to replication.

Replication-coupled packaging has also been observed in other viruses. In BMV, transient expression of BMV RNAs and coat protein subunits in *N. benthamiana* leaves showed that expression of a functional viral replicase is essential for specific viral RNA packaging (Annamalai and Rao, 2005). It was also shown that efficient packaging of subgenomic coat protein mRNA was functionally coupled to translation of the coat protein from replication-derived mRNA (Annamalai and Rao, 2006). Flock house virus (FHV) is a bipartite, positive strand RNA insect virus that encapsidates its two genomic RNAs in a single virion; here, neither RNA1 (which encodes the viral replicase) nor RNA2 (which encodes the capsid protein) was packaged efficiently when the capsid protein was supplied in trans from nonreplicating RNA. However, the capsid protein synthesized in cis from replicating RNA2 packaged RNA2 efficiently, suggesting that capsid protein translation from replicating RNA2 is required for the specific packaging of the FHV genome (Venter et al., 2005).

In this study, we have shown that a specific interaction exists between TYMV RNA and coat protein when non-replicating RNA is encapsidated. The specificity seems to disappear when packaging is coupled to replication, which is an unexpected observation. Considering that the selective packaging of viral genome is crucial to virus propagation, however, it is likely that there is an alternative mechanism to selectively encapsidate

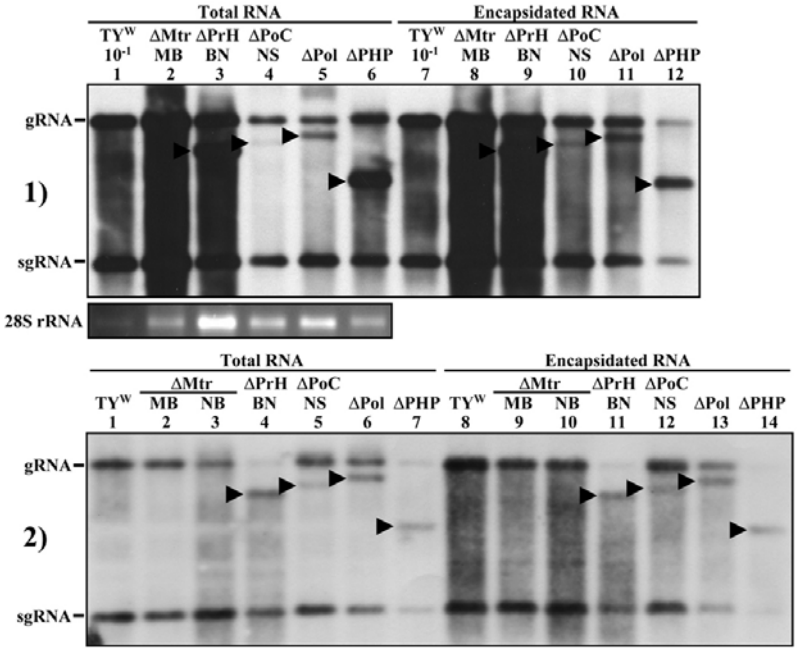

Fig. 4. Replication and encapsidation of deletion mutant TYMV RNAs when wild-type TYMV is present. The deletion constructs were introduced into *N. benthamiana* plants along with the wild-type construct TY^{W}. Seven days after agroinfiltration, the leaves were collected for analysis of total and encapsidated RNA samples. The samples from two independently infiltrated leaves were analyzed by Northern blot hybridization and are represented in panels 1 and 2. The blot in panel 2 was exposed to an X-ray film for a shorter time. Encapsidation assay and Northern analysis were done as described in Fig. 2. The genomic RNAs derived from deletion constructs are indicated by arrowheads.

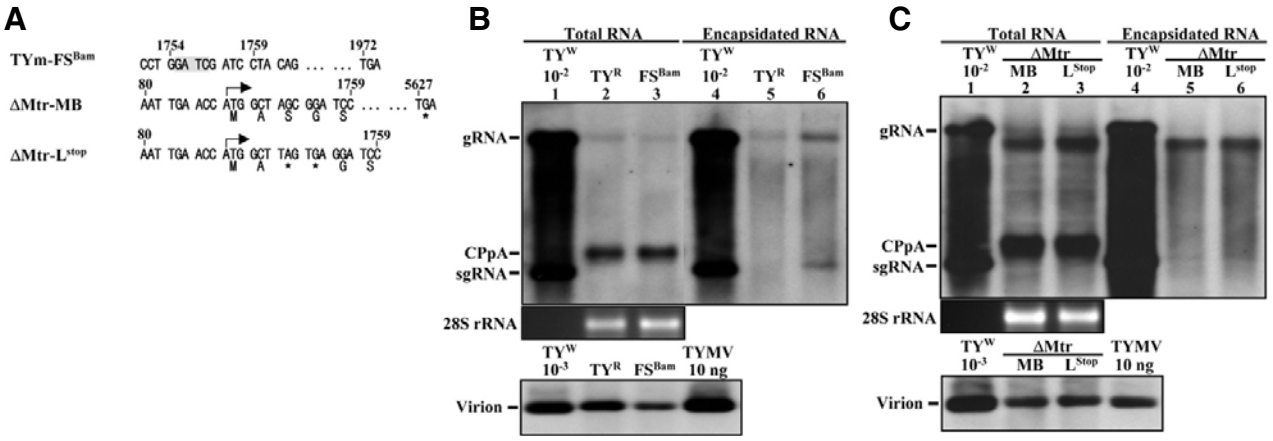

Fig. 5. Encapsidation of Pro^{-}Hel^{-} mutants. (A) Constructs of Pro^{-}Hel^{-} mutants. In TYm-FS^{Bam}, a frameshift mutation was introduced at the *Bam*HI site. The extra nucleotides added by this manipulation are shaded. A stop codon is encountered at nt-1972 rather than at nt-5269 in this mutant. ΔMtr-L^{stop} is identical to TYΔMtr-MB, except that two consecutive stop codons (asterisks) are present in the linker. (B) Analysis of encapsidation of TYm-FS^{Bam}. *N. benthamiana* plants were co-inoculated with the Pro^{-}Hel^{-} mutant and CPpA. Encapsidation assay and Northern analysis were performed as described in Fig. 2. The bottom panel represents the result of the Northern blot analysis of RNAs of the virions in leaf extracts. The virion RNA analysis was performed as described in Fig. 3. (C) Analysis of encapsidation of ΔMtr-L^{stop}. Northern analysis of total RNA, RNase-resistant RNA, and the virion RNA was carried out as described above.

TYMV viral RNA when it is coupled to replication. It is still possible that the packaging of TYMV gRNA and sgRNA produced from replicating RNA requires some *cis*-acting elements, different from those needed for non-replicating viral RNAs. A candidate region for the element would be coat protein ORF or TLS, which is common to both gRNA and sgRNA.

The other mechanism could be selective recruitment of viral RNA to a replication site. All positive strand RNA viruses replicate their RNA genomes in vesicles derived from intracellular membranes (Ahlquist, 2006). The replication vesicles are formed on outer membranes of chloroplasts in the case of TYMV (Dreher, 2004). To form such replication complexes, viral and host replication proteins as well as viral RNA must be localized to the replication vesicle. The mechanisms of localization and viral RNA recruitment have been defined for only a few positive-strand RNA viruses. BMV protein 1a (Chen et al., 2001; Janda and Ahlquist, 1998), FHV replicase protein A (Van Wynsberghe et al., 2007) and tombusvirus replication protein p33 (Panavas et al., 2005) have been shown to direct their own localization. These proteins interact with viral RNA templates and recruit to the replication sites (Chen et al., 2001; Pogany et al., 2005; Van Wynsberghe and Ahlquist, 2009).

In TYMV, the multifunctional protein p206 is cleaved to produce p141 and p66, both of which are co-localized to the site of replication. p141 is localized to the chloroplasts by itself and is able to induce clumping (Prod'homme et al., 2003). Localization

of p66 depends on the presence of p141. The domain in p141 that interacts with p66 was mapped to the Pro domain (Jakubiec et al., 2004). Although the function of TYMV helicase has not been elucidated in detail, a Hel$^-$ mutant with a point mutation does not replicate (Weiland and Dreher, 1993). The Hel domain is presumed to be the site of interaction with the template RNA, as in the case of the NTPase/helicase domain of BMV 1a (Wang et al., 2005). The detailed mechanisms of TYMV RNA recruitment, however, remain to be elucidated in future studies.

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