

Replication and encapsidation of recombinant Turnip yellow mosaic virus RNA

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Turnip yellow mosaic virus (TYMV) is a positive strand RNA virus that infects mainly *Cruciferae* plants. In this study, the TYMV genome was modified by inserting an extra subgenomic RNA promoter and a multiple cloning site. This modified TYMV was introduced into *Nicotiana benthamiana* using a *Agrobacterium*-mediated T-DNA transfer system (agroinfiltration). When a gene encoding β -glucuronidase or green fluorescent protein was expressed using this modified TYMV as a vector, replication of the recombinant viruses, especially the virus containing β -glucuronidase gene, was severely inhibited. The suppression of replication was reduced by co-expression of viral silencing suppressor genes, such as tombusviral p19, closteroviral p21 or potyviral HC-Pro. As expected, two subgenomic RNAs were produced from the recombinant TYMV, where the larger one contained the foreign gene. An RNase protection assay revealed that the recombinant subgenomic RNA was encapsidated as efficiently as the genuine subgenomic RNA. [BMB reports 2008; 41(10): 739-744]

INTRODUCTION

Turnip yellow mosaic virus (TYMV) is a positive strand RNA virus that infects mainly *Cruciferae* plants, such as Chinese cabbage and turnip. TYMV replicates to very high yields in infected plants and can be purified by relatively simple procedures (1, 2). TYMV consists of 180 identical copies of a 20-kDa coat protein (CP) arranged in a T = 3 icosahedron of diameter 28 nm. The virion has pentameric and hexameric protein aggregates that protrude from the surface and form deep valleys at the quasi three-fold axes (3). TYMV has a single 6.3 kb genomic RNA (gRNA) with an ^{m7}GpppG cap at the 5' end and a tRNA-like structure (TLS) at the 3' end (4). Two proteins, p206 and p69, are translated from the gRNA. The multifunctional protein p206 is self-cleaved to yield two proteins

p140 and p66, where the latter is an RNA-dependent RNA polymerase. p140 contains domains indicative of methyltransferase, protease, and NTPase/helicase activities. p69 is produced from an open reading frame (ORF) that extensively overlaps with the p206 ORF. p69 is required for viral movement within the plant (5) and is also a suppressor of gene silencing (6). A single subgenomic RNA (sgRNA) is produced during replication, which serves as the mRNA for CP expression.

Plant RNA viruses have been used as a vector to express heterologous proteins in plants (7). Recently, plant viruses have also been exploited for the production of virus-like particles (VLPs) for vaccination. Antigens displayed on VLPs are known to be more immunogenic than soluble antigen molecules, due to the highly ordered representation of antigen. Tobacco mosaic virus (TMV) has been successfully employed for this purpose (8, 9). Another emerging application of plant viruses is their potential use as a vector for genetic medicine. TMV was recently used to deliver a vaccine gene to mice (10). Considering growing safety concerns associated with using DNA viruses or retroviruses, whose genome is incorporated into host genome, RNA viruses, especially plant RNA virus, has become the vector of choice for safe genetic delivery. Another merit for (+) strand RNA viruses is that it does not have to enter the nucleus for gene expression. Furthermore, plant viruses can be obtained by simple procedures at a yield of more than 1 mg per gram of tissue.

In this study, we have tested the potential use of TYMV as an RNA delivery vector. Specifically, we wanted to determine if a foreign gene could be expressed as a TYMV sgRNA and whether the recombinant sgRNA would be encapsidated. Synthesis of TYMV sgRNA occurs by internal initiation of transcription on a promoter, called tymobox, located on the minus strand RNA (11). Recent Northern analysis of TYMV virions have revealed that equal numbers of gRNA and sgRNA are produced and that the TYMV sgRNA is encapsidated as efficiently as the gRNA (12). So far, however, relatively little information has been accumulated on exactly how the TYMV RNA is packaged. In this study, we developed a TYMV expression vector that contained an extra tymobox promoter and showed that both the recombinant TYMV gRNA and sgRNA produced from this vector were efficiently packaged.

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RESULTS AND DISCUSSION

TYMV expression vector construct

Subgenomic RNA synthesis of TYMV is controlled by a 16 nt-long RNA sequence, called 'tymobox', that overlaps the 3' end of p206 ORF and is conserved throughout the tymoviruses (13). Schirawski et al. (14) reported that a second, shorter conserved sequence just downstream of the tymobox served as an initiation box. To develop TYMV as an expression vector, we modified a wildtype TYMV replicon construct TY^W (12). The TYMV RNA is expressed under the influence of a dual 35S promoter, and the 3' end is processed by the hepatitis delta virus (HDV) ribozyme (15), which is attached to the end of TYMV. An extra tymobox and a multiple cloning site were inserted into the TY^W through conventional PCR and cloning procedures (see MATERIALS AND METHODS), yielding TY-V^{2tymo} (Fig. 1A). The duplicated sequence in TY-V^{2tymo} was nt 5581 to 5636 of the TYMC genome. A SnaBI site was introduced by substituting nt 5623 C with A, which has been shown to not affect sgRNA production (14).

ScaI, NcoI, EcoRI, and SpeI sites were also inserted to serve as a multiple cloning site. In this construct, a heterologous gene would be expressed from the larger sgRNA, while CP would be produced from the smaller sgRNA. To see if the extra sequence affected the replication of the modified TYMV, *Nicotiana benthamiana* plants were agroinfiltrated with *Agrobacterium tumefaciens* harboring the expression vector TY-V^{2tymo}. Analysis of the total RNA from agroinfiltrated leaf

showed that the modified TYMV was replicating as efficiently as the wildtype (Fig. 1B). Western blot analysis indicated that CP expression from the modified TYMV was as efficient as the wildtype (Fig. 1C).

Replication of the recombinant TYMV containing a heterologous gene

To examine the utility of the TYMV-based vector, the genes encoding β-glucuronidase (GUS) and enhanced green fluorescent protein (eGFP) were cloned into the TY-V^{2tymo} expression vector to produce TY-GUS and TY-eGFP, respectively. When the TY-GUS construct was introduced into *N. benthamiana* by agroinfiltration, replication of the recombinant virus was severely suppressed (Fig. 2A). This inhibition of viral replication could potentially be due to RNA silencing elicited in the plant in response to the recombinant TYMV. In higher plants, RNA silencing is thought to serve as an adaptive, antiviral defense system in plants (16, 17). Plant viruses, in turn, have evolved a wide range of mechanisms to overcome RNA silencing, one of which is virus-encoded silencing suppressors (17).

To determine whether the inhibition of recombinant TYMV replication would be relieved by viral RNA silencing suppressors, we introduced the TY-GUS construct into *N. benthamiana* along with potyviral HC-Pro from *Tobacco etch virus* (18), closteroviral p21 from *Beet yellows virus* (19), or tombusviral p19 from *Tomato bushy stunt virus* (20). Using this strategy, the replication of the recombinant TYMV recovered to the wildtype level when one of the viral RNAi suppressors was co-expressed (Fig. 2A). Northern analysis of total RNA

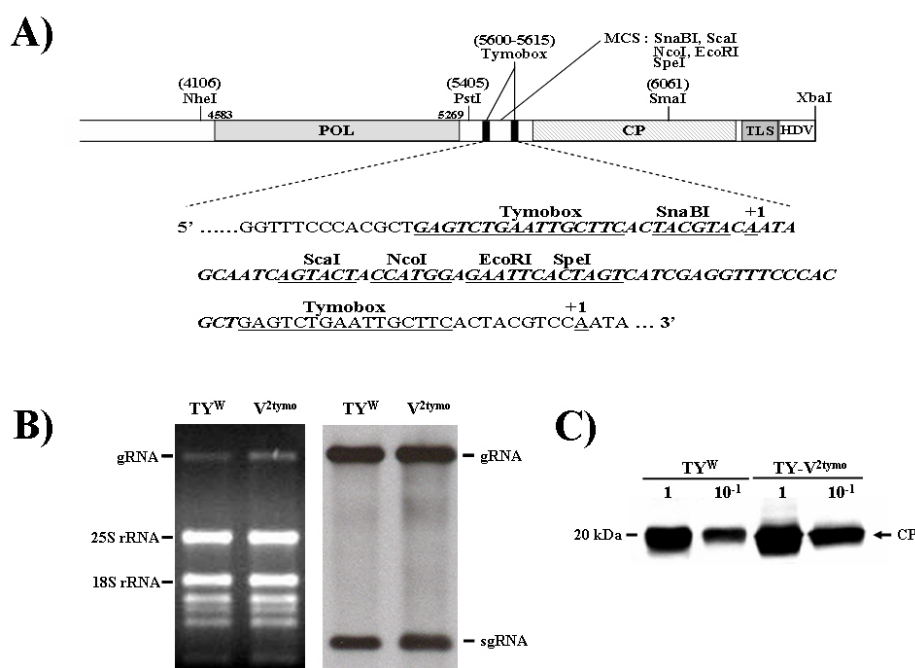


Fig. 1. TY-V^{2tymo} expression vector. A) Sequence of the vector. The extra sequence is shown in bold italic. Tymbobox and restriction recognition sequences are underlined. The transcription start sites are indicated by +1. B) Replication of TY-V^{2tymo}. Seven days after agroinfiltration of *N. benthamiana* leaf with TY^W or TY-V^{2tymo} (V^{2tymo}), total RNA was extracted from the leaf. 1.5 μg of the total RNA was size-fractionated in a 1.0% agarose gel (left panel) and analyzed by Northern blot hybridization using a DIG-labeled DNA probe that represents the coat protein (CP) ORF (nt 5641 to 6231). The RNA hybridization to the probe was visualized by chemiluminescent detection of DIG (right panel). The positions of gRNA and sgRNA are indicated. C) Western analysis of CP expression. 1 μl of undiluted (1) or 1 : 10 diluted (10⁻¹) leaf extract was loaded and electrophoresed in 12.5% SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane. The membrane was incubated with anti-TYMV CP rabbit antibody followed by anti-rabbit HRP conjugate. TYMV CP was detected by coloration with 4-chloro-1-naphthol.

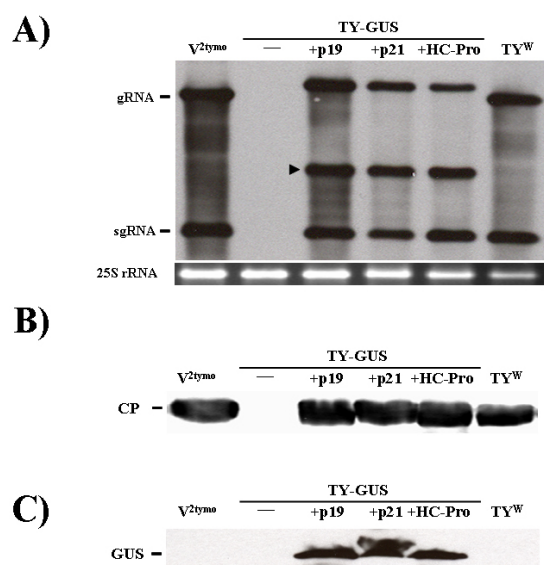


Fig. 2. Replication of TY-GUS. A) Influence of viral RNAi suppressors. The TY-GUS construct was introduced into *N. benthamiana* along with one of the viral silencing suppressors: tombusviral p19, closteroviral p21, or potyviral HC-Pro. “–” indicates that none of the viral silencing suppressors were co-expressed. The samples from the leaf agroinfiltrated with TY^W or TY-V^{2tymo} were shown as controls. 250 ng of the total RNA extracted from the infiltrated leaf was analyzed as described in Fig. 1. The sgRNA that were larger than the authentic sgRNA is indicated by an arrowhead. B) CP expression of the recombinant TY-GUS. 10 μ l leaf extract was analyzed by Western blot as described in Fig. 1C. C) GUS expression. 10 μ l leaf extract was analyzed by Western blot using anti-GUS antibody. The membrane was developed by a chemiluminescent detection method using luminol as the substrate.

from inoculated leaves showed that two sgRNAs were produced from the recombinant TYMV as expected, one of which was larger than the genuine CP sgRNA. The two sgRNAs were produced in a 1 : 1 ratio, suggesting that the extra inserted ty-mobox was fully functional. Analysis of CP expression also confirmed that the recombinant TYMV replicated as efficiently as the wildtype (Fig. 2B). Western analysis using an anti-GUS antibody showed that GUS was expressed at the translation level (Fig. 2C). The TY-eGFP construct also showed some degree of inhibition in replication, which was similarly reduced by viral silencing suppressors (Fig. 3A and 3B). eGFP expression at the protein level was also confirmed by Western analysis using an anti-GFP antibody (Fig. 3C).

Although TYMV has its own silencing suppressor, p69, the TYMV suppressor evidently did not work for the recombinant TYMV. This may have occurred because the p69 does not function properly in *N. benthamiana*, which is not a natural host for TYMV. Alternatively, this could be due to the fact that p69 suppresses gene silencing by a mechanism that is different from those of p19, p21, and HC-Pro. One of the most characterized viral suppressors is the tombusviral p19, which binds to short double-stranded RNA (dsRNA) specifically; hence, in-

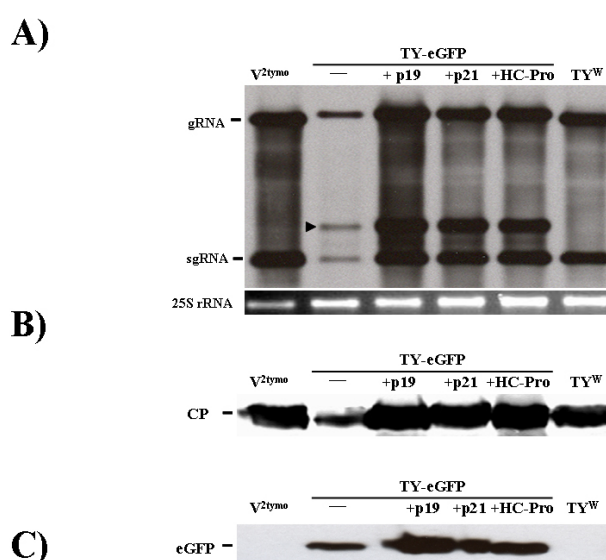


Fig. 3. Replication of TY-eGFP. A) Influence of viral RNA silencing suppressors. TY-eGFP was co-expressed with the viral suppressor p19, p21, or HC-Pro, and analyzed as described in Fig. 2. The larger sgRNA is indicated by an arrowhead. B) CP expression of the recombinant TY-eGFP. TYMV CP was examined as described in Fig. 2B. C) eGFP expression. 10 μ l leaf extract was analyzed by Western blot using anti-GFP antibody, as described in Fig. 2C.

hibits the formation of the active RNA-induced silencing complex (21). Recently, closteroviral p21 and potyviral HC-Pro, like tombusviral p19, were reported to form complexes specifically with 21nt dsRNA (22). TYMV p69 has been shown to efficiently suppress silencing that is induced by sense transgenes like the other three viral suppressors (6). However, it does not affect inverted repeat-induced silencing, whereas p19 and HC-Pro suppress the inverted repeat-induced silencing (23). Chen et al. (6) also observed that p69 promoted the miRNA pathway. On the basis of these observations they proposed that p69 inhibits a cellular function that leads to dsRNA production. Therefore, the ineffectiveness of p69 could be due to the presence of an extensive dsRNA structure or a structure resembling miRNA precursors in the recombinant TYMV RNA.

Encapsidation of recombinant TYMV RNA

To determine if recombinant TYMV RNAs containing heterologous sequence could be efficiently encapsidated, we performed an RNase protection assay, where leaf extracts were treated with RNase A prior to deproteinization with phenol. The extract from the leaf agroinfiltrated with TY^W was also subjected to the RNase protection assay, as a control. Examination by agarose gel electrophoresis showed that only the encapsidated viral RNA was protected from RNase treatment (Fig. 4A). We also tested a leaf extract mixture that contained added purified viral RNA. Northern analysis of the sample after the RNase protection assay showed that none of the

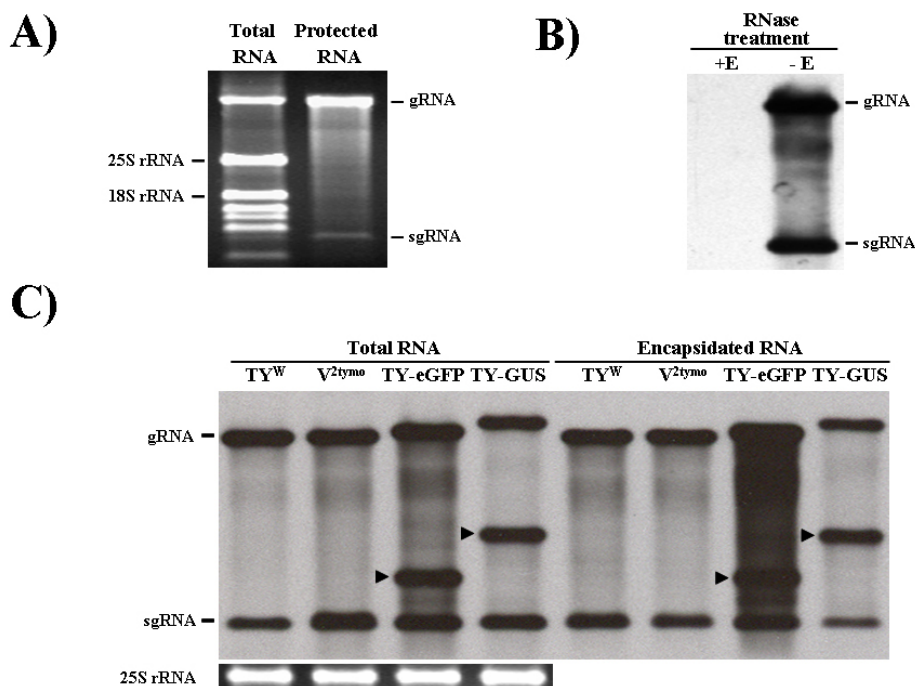


Fig. 4. Encapsidation of recombinant TYMV RNAs. A) An agarose gel demonstrating the efficacy of the RNase protection assay. 1.5 μ g of total RNA from the leaf agroinfiltrated with the wildtype construct (TY^W) and the equivalent RNA sample obtained from the RNase protection assay were size-fractionated in 1% agarose gel and stained with ethidium bromide. B) Northern analysis of the non-encapsidated RNA sample that was subjected to the RNase protection assay. 0.2 μ g of the purified viral RNA was added to 200 μ l of the leaf extract prepared with a healthy, uninoculated leaf, and then the mixture was treated with RNase A. The sample was examined by Northern blot hybridization. “–” and “+” represent before and after RNase A (E) treatment. In the case of the “–” sample, 1 : 10 diluted mixture was loaded. C) Northern analysis of TY- eGFP and TY-GUS. The recombinant constructs were introduced into *N. benthamiana* with the viral suppressor p19. Seven days after agroinfiltration, 250 ng of total RNA and equivalent amounts of RNAs from the RNase treatment were analyzed by Northern blot hybridization, as described in Fig. 1. The recombinant sgRNAs are indicated by an arrowhead.

non-encapsidated RNA was detected (Fig. 4B). The extract prepared from the leaf agroinfiltrated with the TY-GUS or TY-eGFP was then treated with RNase and examined by Northern blot hybridization. The result showed that all the TYMV gRNA and sgRNA containing the foreign sequence were encapsidated as efficiently as the genuine TYMV RNA (Fig. 4C). This demonstrates that a foreign sequence can be inserted into the TYMV genome without adversely affecting RNA packaging.

Currently the exact mechanism of TYMV RNA packaging is not known, but the principle that governs viral RNA packaging, in general, is an interaction between CP and a specific signal on RNA (24). Among spherical plant RNA viruses, evidence for the existence of a packaging signal was first demonstrated for *Turnip crinkle virus* (TCV), where a bulged hairpin loop of 28 nt at the 3'-end of the TCV CP-coding region was found to be the most essential element of the packaging core (25). Subsequently, bipartite packaging signals were reported in *Brome mosaic virus* (BMV) (26). In the case of TYMV, two hairpins at the 5' untranslated region (UTR) of TYMV gRNA have been implicated as packaging signals, since deletion or modification of the 5'-UTR hairpins resulted in delay of symptom development and poor encapsidation (27, 28). However, the mutant RNA lacking most of the 5'-UTR including the two hairpins was still packaged into virions, although more empty virions were produced (27). Thus, the two 5'-UTR hairpins appear to be important but not essential for the packaging of TYMV RNA. In the case of BMV, which has a TLS at the 3' ends of all three gRNAs and one sgRNA, TLS was reported to

be required for encapsidation of RNA3 (29). However in the case of TYMV, the mutant TYMV gRNA lacking TLS was found to be packaged as efficiently as the wildtype TYMV (12). Thus, the packaging signals for TYMV RNA remain elusive.

Rod-shaped viruses, such as TMV, can accommodate RNAs larger than wildtype RNA. However, spherical plant viruses, which are typically 25-35 nm in diameter, seem to have some physical constraints for the size of RNA they can package (24). TCV, for example, can not package RNAs larger than the wild-type genome even when they contain the appropriate packaging signals (25). Apparently, the TYMV virion has the capacity to accommodate RNAs up to 8 kb, since the size of the GUS gene is ca. 1.8 kb. Therefore, it may be possible to insert a gene as large as 7 kb into the cloning site of TY-V^{2tymo}. In such a construct, the recombinant sgRNA may only be packaged into virions whereas the recombinant gRNA containing the large insert will likely be excluded from packaging.

MATERIALS AND METHODS

DNA constructs

The DNA containing the extra tymobox, CP ORF and hepatitis delta virus (HDV) ribozyme sequence was prepared by two sequential PCR amplification. The first PCR cycle was done using the following two primers: upstream primer TymoSES1 (CTTCACTACGTACAATAGAATTCAGTACATCGAGGTTCCAC; TYMV sequence (nt 5581 to 5596 of TYMC) is in italic, *Sna*BI, *Eco*RI, and *Spe*I sites are underlined) and downstream primer HDV-R29 (GGAGAATTCICTAGATGGCTCTCCCTTAG;

HDV sequence is in italic, *Xba*I site is underlined). The PCR product was used as a template in the second PCR, where the following two primers were used: upstream primer TymoSES2 (CGAGCTAGCTGAGTCTGAATTGCTTCACTACGTACAATAG; tymbobox sequence is in bold, *Nhe*I and *Sna*BI sites are underlined). An approximate 900-bp PCR product was digested with *Nhe*I and *Xba*I, and then inserted into the TY^W deletion construct where the DNA comprising the polymerase (pol) domain, CP ORF and HDV sequence was removed. This construct lacked the pol domain, thus it was designated as TYdPol. The pol domain was then amplified by PCR and inserted back into the construct after confirming the sequence. The resulting construct was designated as TY-V^{2tymo}.

The enhanced green fluorescent protein (eGFP) sequence was PCR-amplified from a plasmid containing an eGFP gene (GenBank Accession # U76561) as a template with the following two primers: eGFP-F (GACGAATTCAGTGAGCAAGGGCG-AGGAG; eGFP sequence is in bold, *Eco*RI site is underlined) and eGFP-R (ACTGATCATTACTTGTACAGCTCGTCCAT; eGFP sequence is in bold, *Spe*I site is underlined). The β -glucuronidase (GUS) sequence was similarly PCR-amplified from a plasmid containing a GUS gene (GenBank Accession # U12639). The PCR products were digested with *Eco*RI and *Spe*I, and inserted into the TY-V^{2tymo} vector.

Agroinfiltration and Northern analysis of viral RNA

Agroinfiltration of the TYMV constructs into *Nicotiana benthamiana* was carried out as described previously (12). Total RNA was isolated from frozen leaf samples using Trizol (Invitrogen). For the encapsidation assay, leaf samples (0.1-0.5 g) were ground with 400 μ l of 0.1 M sodium phosphate buffer (pH 7.0) per 0.1 g tissue, clarified by centrifugation in a microfuge, and treated with 5 μ g/ml RNase A at 37°C for 1 h. After removal of RNase through proteinase K treatment, samples were extracted twice with phenol/chloroform, followed by ethanol precipitation. RNA samples were resuspended in 48% formamide solution containing 10 mM EDTA and incubated at 65°C for 10 min before electrophoresis on 1% agarose gel. RNAs were transferred to Hybond N⁺ membranes (Amersham) by capillary transfer using 20X SSC. Northern blots were hybridized with a DNA probe representing the CP ORF (nt 5641 to 6231). The probe DNA was amplified by PCR and labeled with digoxigenin (DIG) using DIG-dUTP, as described by Wang et al. (30). The blots were developed by chemiluminescent immunodetection of DIG (Roche Molecular Biochemicals).

Western analysis of CP, GUS, and eGFP expression

Leaf extract samples for protein analysis were prepared as described previously (12). Proteins were separated by 12.5% SDS-polyacrylamide gel electrophoresis and then electroblotted to Amersham Hybond ECL membrane for detection with anti-TYMV rabbit antiserum, anti-GFP antibody (Santa Cruz Biotechnology) or anti-GUS antibody (Invitrogen). After

the membrane was exposed to the goat anti-rabbit HRP conjugate (Bio-Rad), it was developed either by the colorimetric HRP detection method using 4-Cl-1-naphthol (Bio-Rad) or by the chemiluminescent detection method using luminol (Millipore).

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