The Infectivities of Turnip Yellow Mosaic Virus Genomes with Altered tRNA Mimicry Are Not Dependent on Compensating Mutations in the Viral Replication Protein†

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Five highly infectious turnip yellow mosaic virus (TYMV) genomes with sequence changes in their 3′-terminal regions that result in altered aminoacylation and eEF1A binding have been studied. These genomes were derived from cloned parental RNAs of low infectivity by sequential passaging in plants. Three of these genomes that are incapable of aminoacylation have been reported previously (J. B. Goodwin, J. M. Skuzeski, and T. W. Dreher, Virology 230:113–124, 1997). We now demonstrate by subcloning the 3′ untranslated regions into wild-type TYMV RNA that the high infectivities and replication rates of these genomes compared to their progenitors are mostly due to a small number of mutations acquired in the 3′ tRNA-like structure during passaging. Mutations in other parts of the genome, including the replication protein coding region, are not required for high infectivity but probably do play a role in optimizing viral amplification and spread in plants. Two other TYMV RNA variants of suboptimal infectivities, one that accepts methionine instead of the usual valine and one that interacts less tightly with eEF1A, were sequentially passed to produce highly infectious genomes. The improved infectivities of these RNAs were not associated with increased replication in protoplasts, and no mutations were acquired in their 3′ tRNA-like structures. Complete sequencing of one genome identified two mutations that result in amino acid changes in the movement protein gene, suggesting that improved infectivity may be a function of improved viral dissemination in plants. Our results show that the wild-type TYMV replication proteins are able to amplify genomes with 3′ termini of variable sequence and tRNA mimicry. These and previous results have led to a model in which the binding of eEF1A to the 3′ end to antagonize minus-strand initiation is a major role of the tRNA-like structure.

The turnip yellow mosaic virus (TYMV) genome is a 6,318-nucleotide (nt)-long positive-strand RNA with a capped 5′ end and a tRNA-like structure (TLS) at the 3′ end. The TLS is an efficient and specific mimic of tRNAVal in its capacity as a substrate for valylation (in its -CCA 3′-adenylation by [CTP, ATP]:tRNA nucleotidyltransferase (in its -CC3′ form), a substrate for 3′-adenylation by eEF1A, and in the ability of the valylated RNA to form a tight complex with eEF1A·GTP (formerly known as EF-1α·GTP) (5).

In a series of studies, we have investigated the role of the TLS and its tRNA mimicry by studying the infectivity and replication of various mutants with altered tRNA-like properties. Point mutations of the valine identity nucleotides in the anticodon loop, resulting in the loss of the ability of the RNA to be valylated in vitro, also result in the loss of infectivity in plants and of virus amplification in protoplasts (20). TYMV RNAs with mutations that switch the aminoacylation from valine to methionine, but not closely related mutants that are incapable of aminoacylation, are infectious to plants and amplify to about half the level of wild-type RNAs in protoplasts (7). These experiments indicated that efficient viral amplification requires the genomic RNA to be capable of aminoacylation, without a specific requirement for valylation or interaction with valyl-tRNA synthetase.

In other experiments, chimeric TYMV genomes in which the native TLS was replaced with heterologous termini were used to probe the role of tRNA mimicry. Those genomes bearing valylatable TLSs derived from other tymoviruses were infectious to plants, but chimeric genomes carrying tRNAVal or the TLSs from tobacco mosaic virus or brome mosaic virus were not (18), indicating that a generic tRNA-like element is not sufficient for viral amplification.

The chimeric viruses with the most unexpected properties were TYMC-H, -XX, and -YY, which were highly infectious to plants despite the inability of their genomic RNAs to be aminoacylated (9). TYMC-H RNA has a 3′ end derived from erysimum latent tymovirus that is structurally divergent from the TYMV TLS and that has little tRNA character (Fig. 1) (5). TYMC-XX and -YY RNAs have a TLS derived from tobacco mosaic virus RNA modified to contain short TYMV sequences in the anticodon and acceptor stem (Fig. 1) (9). All three genomes derived their high infectivity as a result of serial passaging in Chinese cabbage plants, during which time adaptive mutations presumably became fixed. We have previously reported the mutations appearing within the heterologous 3′ sequences (9). We now address whether the increased infectivities acquired by these viruses during passaging are attributable to the mutations acquired within the 3′-terminal region of the genome, or whether mutations elsewhere, such as in the open reading frame (ORF) encoding the essential replication polyprotein p206, are responsible.

We find that much of the increased infectivity of each virus is in fact a function of the modified sequences in the 3′ untranslated region (3′-UTR) and not the result of mutation in the TYMV replication proteins that might optimize interaction...
with the heterologous termini. Adaptation of two other chimeric genomes to high infectivity upon serial passaging is shown to be associated with mutation of the movement protein encoded by ORF-69 and not with changes in the heterologous TLS. These studies have led to a new model for the role of the tRNA mimicry of TYMV RNA and provided well-characterized variant genomes that can be used to test that model.

MATERIALS AND METHODS

Virus stocks and plant material. pTYMC, a full-length cDNA clone of TYMV (22), and the chimeric genomic clones pTYMC-EMV (18) and pTYMC-US5/C54/A53/L1=UU) (7) have been described previously. The plant-adapted viruses TYMC-XX, TYMC-YY, and TYMC-H were described by Goodwin et al. (9).

Chinese cabbage plants (Brassica pekinensis cv. Spring A-1) were grown and infected as described previously (9). Due to discontinued availability of Spring A-1, a closely related hybrid cultivar, W-R60 Green, was used for protoplast isolation and transfection experiments.

Virions from plant tissues were prepared by polyethylene glycol precipitation (12), with additional purification by pelleting through a 40% sucrose cushion (80,000 rpm at 4°C for 30 min in a TL100.2 rotor; Beckman Instruments), followed by banding (centrifugation as above at 16°C for 2 h) in a preformed CsCl gradient (1.6 to 1.23 g/ml) made in virion storage buffer (50 mM sodium acetate [pH 4.5], 1 mM EDTA, 1 mM MgCl2). Virions were finally concentrated in Centricon 100 devices (Amicon Corp.) to 10 to 50 mg/ml and stored at 4°C.

In vitro transcription and inoculation of plants and protoplasts. Genomic RNAs (5' capped) were transcribed with T7 RNA polymerase from plasmid DNAs linearized with HindIII as described (22). Twelve-day-old Chinese cabbage plants were inoculated with 5 μg of genomic RNA transcripts or with 1 μg of virion RNA in inoculation buffer (50 mM glycine, 30 mM K2HPO4 [pH 9.2], 1% [wt/vol] celite, 3 mg of bentonite per ml). Protoplasts (5 × 106) released from Chinese cabbage leaves were inoculated with 5 μg of transcript RNAs or 2 μg of virion RNAs and held for 40 h under dim fluorescent light prior to harvest (22).
Detection of viral products. TYMV infection in plants was monitored by direct adsorption double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) essentially as described (2). In brief, serial dilutions of extracts from infected leaves were adsorbed on ELISA microtiter plates. Viral antigen was detected by incubation with anti-TYMV serum followed by alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) and colorimetric development using the alkaline phosphatase substrate p-nitrophenyl phosphate (Sigma). Amounts of viral antigen were calculated using calibration curves obtained from standard virus dilutions on the same microtiter plates.

Western blot detection of coat protein accumulation was carried out using enhanced chemiluminescence detection (ECL; Amersham) (9), exposure to X-ray film, and quantitation via densitometry with reference to calibration standards run in parallel. Total cellular RNA was isolated from protoplasts or infected plants, glyoxylated, and subjected to Northern blot analysis using Zeta-Probe nylon membranes (Bio-Rad) and alkaline transfer (22). Membranes were probed in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% sodium dodecyl sulfate (SDS)–0.2 mg of polyanetholesulfonic acid per ml–50% formamide at 65°C for 16 h. The 32P-labeled minus-strand riboprobe was synthesized using T7 transcriptase (Life Sciences, Inc.). For sequencing other regions of the genome, RNA by dideoxy sequencing with avian myeloblastosis virus (AMV) reverse transcriptase (Life Sciences, Inc.) was conducted with viral RNA now shown to possess this TLS mutation C6 (nt 6318) were amplified by PCR using native Pfu DNA polymerase (18). The EMV TLS can be valylated as efficiently as that from TYMV, but the valylated RNA forms substantially weaker ternary complexes with eEF1A·GTP than does valyl-TYMV RNA (Kv values of ca. ∼60 and 2 nM, respectively) (5). Both of these cloned RNAs replicated quite well in protoplasts (Table 2) but had markedly attenuated infectivity and pathogenicity in plants (Table 1), with only 40% of inoculated plants becoming infected and with symptoms appearing after a substantial delay (7, 18).

To obtain plant-adapted derivatives of TYMC-EMV and TYMC-Met with higher infectivity, these RNAs were serially passaged through Chinese cabbage plants. After six and two passages, infections initiated with TYMC-EMV and TYMC-Met RNA, respectively, had evolved the highly infectious progeny designated TYMC-E and TYMC-M, respectively (Table 1). These plant-adapted progeny viruses produced symptoms with a delay of only 1 to 2 days that were similar to those of the wild-type TYMC, although early symptoms were less pronounced. Virion yields were 0.18 and 0.11 mg/g (fresh weight) of infected leaf material for TYMC-E and TYMC-M infections, respectively, similar to the virion yields reported previously for TYMC-XX, -YY, and -H infections (9). Sequencing of the 3′-UTRs of TYMC-E and -M RNAs failed to identify any sequence changes from the original inoculum (not shown). High infectivities of TYMC-XX, -YY, and -H RNAs are largely due to the mutations acquired in their TLSs. To determine whether the TLS mutations identified in Fig. 1 as having arisen in TYMC-XX, -YY, and -H RNAs during passage in plants were responsible for the high infectivities of these RNAs, the 3′-terminal region from each progeny RNA was cloned into the wild-type genomic cDNA clone pTYMC. After RT-PCR, fragments downstream of the SmalI restriction site were subcloned to replace the 257-nucleotide SmalI wild-type fragment of pTYMC. The entire subcloned fragments were sequenced to ensure the absence of mutations outside the heterologous TLS domain. The genomic RNAs generated by transcription of the resulting clones were designated TYMC-XX1, -YY1, and -H1 (Table 1). In the case of TYMC-YY, the silent C6150→U mutation within the coat protein ORF was found to be present, and this mutation was included in TYMC-YY1/U6150 RNA (Table 1).

In a series of plant and protoplast inoculations with 5′-capped transcript genomic RNA, the infectivities and replication levels of the recloned variants were compared with those of the plant-adapted progeny virion RNAs and wild-type TYMC RNA (Tables 1 and 2). TYMC-XX1 and TYMC-H1 transcripts produced infections in all inoculated Chinese cabbage plants, and initial symptoms appeared on average only half a day later than those from inoculation with TYMC transcripts (Table 1). Symptom intensities were noticeably less after inoculation with the chimeric RNAs than TYMC RNA until full symptom expression was reached, at which time all infected plants were strongly symptomatic. These infectivities represent a great improvement over the infectivities of the progenitor TYMC-TYV and TYMC-ELV RNAs (Table 1). The timing and severity of symptom development after inoculation with TYMC-XX1 and -H1 transcript RNAs were very similar to those resulting from infection with the plant-adapted virion RNAs TYMC-XX and TYMC-H (Table 1).
TABLE 1. Infectivity of chimeric TYMV genomes and symptom appearance in Chinese cabbage plants

<table>
<thead>
<tr>
<th>TYMC genome</th>
<th>Properties of cloned progenitor genomea</th>
<th>Properties of plant-adapted progeny virusb</th>
<th>Properties of cloned progeny genomesd</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time to initial symptoms in plants (dpi)</td>
<td>Infectivity: no. symptomatic/ no. inoculated</td>
<td>Vein clearing (dpi)</td>
</tr>
<tr>
<td>WT</td>
<td>7–8</td>
<td>6/6</td>
<td>7.7</td>
</tr>
<tr>
<td>-TYV</td>
<td>43</td>
<td>1/6</td>
<td>7.9</td>
</tr>
<tr>
<td>-XX</td>
<td>45</td>
<td>1/6</td>
<td>8.9</td>
</tr>
<tr>
<td>-YY1</td>
<td>16–61</td>
<td>5/6</td>
<td>8.7</td>
</tr>
<tr>
<td>-EMV</td>
<td>16–31</td>
<td>2/5</td>
<td>9.2</td>
</tr>
<tr>
<td>-E</td>
<td>14</td>
<td>2/5</td>
<td>8</td>
</tr>
</tbody>
</table>

a Symptom appearance in noninoculated leaves. Note that at early times of symptom appearance, symptom intensity was less for all chimeric genomes than for TYMC-WT, wild type. ND, not done.
b Data taken from references 9 and 18 for initial cloned chimeric or mutant constructs. dpi, days postinfection.
c Properties of virus sequentially passaged until high infectivity was attained (all inoculated plants visibly infected within 2 days of symptom appearance on plants inoculated with the wild type) except TYMC (progeny of TYMC transcripts, amplified in Chinese cabbage); inoculations with 1 µg of virus.
d Properties of cloned chimeric TYMC genomes reconstructed by incorporating 3' mutations identified in plant-adapted viral RNAs; inoculations with 5 µg of 5'-capped genomic transcripts. Values are the averages for all the plants whose infectivities are reported in the rightmost column.
e Plant-adapted viruses (9).
f Product of cloning the 3' sequence of TYMC-XX RNA shown in Fig. 1 in place of the TYMV TLS; differs from TYMC-TYV RNA by the U98-C TLS mutation.
g Product of cloning the 3' sequence of TYMC-YY RNA shown in Fig. 1 in place of the TYMV TLS; differs from TYMC-TYV-BP RNA by the U88-C TLS mutation and the addition of C2.
h Differences from TYMC-Y1 by inclusion of the C6150-U substitution present in TYMC-YY, a silent mutation in the coat protein coding region.
i This sequence differs from TYMC-YY1 by inclusion of the C6150-U substitution present in TYMC-YY, a silent mutation in the coat protein coding region.
j Product of cloning the 3' sequence of TYMC-H RNA shown in Fig. 1 in place of the TYMV TLS; differs from TYMC-ELV-BP RNA by the C6-U TLS mutation and the deletion of a C adjacent to the 3'-A.
k The sequence shown in Fig. 1 for the heterologous EMV-derived RNA has been corrected from that reported (18): the sequence joining the TYMV and EMV sequences is slightly different (5'-UAAUU and not 5'-UUAG as previously stated), and the native EMV U43 is present, not A43 as previously reported. The reported properties of TYMC-EMV RNA (18) are correct.
l Plant-adapted virus resulting from 2 sequential passages in Chinese cabbage, using sap inoculum for the first 4 passages and 5 µg of virus for the latter two; 5/5 infectivity was reached after 4 passages. No sequence changes were found in the EMV-derived TLS.
m Previously named TYMC-U55/CS4/A53(L1-UU) (7).

A reproducible difference in the timing and severity of symptom progression was observed between inoculation with TYMC-YY1 and -YY1/U6150 RNAs (Table 1). TYMC-YY1/U6150 RNA produced infections similar to those described above for TYMC-XX1 and -H1, approximating the infections of the plant-adapted TYMC-YY RNA. Infection with TYMC-YY1 proceeded slightly more slowly (Table 1), showing milder early symptoms. Nevertheless, both cloned RNAs were able to infect all inoculated plants and were far more infectious and pathogenic than their progenitor, TYMC-TYV-BP RNA.

The yields of viral products were monitored in both infected plants and protoplasts (Table 2); representative Western and Northern blots detecting coat protein and viral genomic and subgenomic RNAs, respectively, are shown in Fig. 2. Inoculation of protoplasts with TYMC-XX1 and TYMC-YY1/U6150 RNAs resulted in accumulations of coat protein and genomic RNA about half that of wild-type TYMC infections; accumulation resulting from inoculation with TYMC-YY1 RNA was 30 to 40% of wild type, and accumulations from TYMC-H1 RNA were 20 to 25% of wild type (Table 2 and Fig. 2A and B). These cloned RNAs thus support a conclusion of increased viral replication in protoplasts relative to their respective progenitor RNAs, an increase of some 2.5- to 5-fold from that supported by TYMC-TYV and -TYV-BP RNAs and 1.5-fold from TYMC-ELV RNA (Table 2).

The accumulations of coat protein and genomic RNA in noninoculated leaves showing mature symptoms were about half that of wild-type infections in plants inoculated with TYMC-XX1, -YY1, -YY1/U6150, and -H1 RNAs (Table 2), indicating the ability of all the cloned RNAs to generate highly productive infections. The accumulations after inoculation with the cloned transcripts were 60 to 80% of those in plants inoculated with plant-adapted TYMC-H, -XX, and -YY RNAs (Table 2). As also suggested by the milder early symptoms produced by the cloned as opposed to plant-adapted RNAs, some mutations that contribute to viral dissemination in planta may be present outside the 3'-UTR of the plant-adapted TYMC-XX, -YY, and -H RNAs. Nevertheless, our results
TABLE 2. Amplification of cloned and plant-adapted chimeric TYMV in plants and protoplasts

<table>
<thead>
<tr>
<th>TYMV genome</th>
<th>Amplification of cloned progenitor genome in protoplasts</th>
<th>Amplification of plant-adapted progeny virus</th>
<th>Amplification of cloned progeny genomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rel. accum. of coat protein</td>
<td>Rel. accum. of genomic RNA</td>
<td>Rel. accum. of coat protein</td>
</tr>
<tr>
<td>WT (transcript)</td>
<td>1.00</td>
<td>1.00 ± 0.03</td>
<td>1.00 ± 0.06</td>
</tr>
<tr>
<td>WT (virion)</td>
<td>1.00</td>
<td>1.00 ± 0.16</td>
<td>1.00 ± 0.05</td>
</tr>
<tr>
<td>-TYV</td>
<td>0.08</td>
<td>0.08 ± 0.07</td>
<td>0.55 ± 0.08</td>
</tr>
<tr>
<td>-XX</td>
<td>1.01 ± 0.10</td>
<td>0.54 ± 0.04</td>
<td>0.43 ± 0.03</td>
</tr>
<tr>
<td>-XX1</td>
<td>0.58 ± 0.07</td>
<td>0.53 ± 0.03</td>
<td>0.42 ± 0.07</td>
</tr>
<tr>
<td>-TYV-BP</td>
<td>0.14</td>
<td>0.97 ± 0.06</td>
<td>0.68 ± 0.10</td>
</tr>
<tr>
<td>-YY</td>
<td>0.09 ± 0.06</td>
<td>0.65 ± 0.09</td>
<td>0.25 ± 0.08</td>
</tr>
<tr>
<td>-YY1/yy150</td>
<td>0.14</td>
<td>0.69 ± 0.03</td>
<td>0.86 ± 0.11</td>
</tr>
<tr>
<td>-ELV</td>
<td>0.69 ± 0.03</td>
<td>0.86 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>-H</td>
<td>0.52 ± 0.03</td>
<td>0.65 ± 0.09</td>
<td>0.25 ± 0.08</td>
</tr>
<tr>
<td>-E</td>
<td>0.35 ± 0.02</td>
<td>0.26 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>-Met</td>
<td>0.69 ± 0.03</td>
<td>0.86 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>-M</td>
<td>0.52 ± 0.03</td>
<td>0.65 ± 0.09</td>
<td>0.25 ± 0.08</td>
</tr>
</tbody>
</table>

a Relative levels of genomic RNA detected in extracts made from turnip protoplasts 40 h postinfection determined by Northern blotting; data taken from references 7, 9, and 18.
b Relative accumulations (Rel. accum.) in Chinese cabbage plants, cv. Spring A-1, inoculated with 1 μg of virion RNA. Viral coat protein levels were determined by ELISA; accumulation after inoculation with TYMC virion RNA was 3.34 ± 0.16 mg of viral coat protein per g of fresh leaf tissue. Genomic RNA levels were determined via Northern blots. Averages from at least three analyses of pooled leaves from five to six plants harvested 15 days postinfection are presented.
c Inoculation of 0.5 × 10^6 to 1 × 10^6 Chinese cabbage cv. W-R60 Green protoplasts with 1 μg of virion RNA. Coat protein levels and genomic RNA levels were determined by Western and Northern blotting, respectively; averages are from at least three independent inoculation experiments.
d Chinese cabbage plants, cv. Spring A-1, inoculated with 5 μg of 5'-capped genomic transcripts. Viral coat protein levels were determined by ELISA; accumulation after inoculation with TYMC transcript RNA was 2.74 ± 0.19 mg of coat protein per g of fresh leaf tissue. Genomic RNA levels were determined via Northern blots. Averages from at least three analyses of pooled leaves from five to six plants harvested 15 days postinfection are presented.
e Inoculation of 0.5 × 10^6 to 1 × 10^6 Chinese cabbage cv. W-R60 Green protoplasts with 5 μg of 5'-capped genomic transcripts. Extracts were made at 40 h postinfection, and coat protein and genomic RNA levels were determined by Western and Northern blotting, respectively; averages are from at least three independent inoculation experiments.

Clearly demonstrate that the small number of mutations acquired in the 3'-UTR during plant adaptation and fixed in TYMC-XX1, -YY1, and -H1 RNAs are responsible for most of the increased replication and infectivities of these RNAs.

Coding mutations in the movement protein ORF are present in some of the plant-adapted genomes. While the TLS mutations acquired in plants were primarily responsible for the plant adaptation of the RNAs just discussed, no such mutations were associated with the plant adaptation of TYMC-E and -M RNAs. To discover what type of mutations did account for the increased infectivities of these RNAs, all of the TYMC-E RNA and much of TYMC-M RNA were sequenced. Since the data in Table 2 suggested that additional mutations contributing to maximal symptom development in plants could be present in these RNAs, a substantial portion (about half) of TYMC-E Y RNA and small segments, including the 5'-UTRs, of TYMC-XX and -H RNAs were also sequenced. The regions sequenced and the mutations found are reported in Fig. 3.

Complete sequencing of TYMC-E RNA revealed only three mutations. All three were within the p206 coding region, but none altered the amino acid sequence of p206. The two mutations that were also in the overlapping ORF-69 (movement protein coding region) resulted in the amino acid substitution lysine 336 → glutamate and threonine 366 → serine (Fig. 3). The plant-adapted TYMC-E RNA did not replicate to higher levels in protoplasts than its progenitor TYMC-EMV RNA (Table 2). This is consistent with the absence of mutations affecting the viral replication protein p206 and from the 5'- and 3'-terminal regions of the genomic RNA. We speculate that plant adaptation was the result of improved movement protein function.

Less extensive regions of TYMC-M RNA were sequenced. A mutation resulting in a substitution in the movement protein was also identified in this RNA: phenylalanine 401 → serine. A second mutation, affecting the p206 replication polyprotein, was also found, an arginine 673 → cysteine substitution in a domain of unknown function between the methyltransferase-like and protease domains (Fig. 3). As for TYMC-E RNA, the plant adaptation of TYMC-M RNA was not associated with increased RNA replication in protoplasts (Table 2), but rather with improved ability to establish infection in plants.

About half of TYMC-YY RNA was sequenced, including all of ORF-69, revealing another mutation affecting the movement protein but silent with regard to p206, tyrosine 601 → histidine (Fig. 3). The silent C6150 → U mutation in the coat protein ORF has been discussed above. The limited sequencing of TYMC-XX and -H RNAs revealed a U27 → C mutation in the 5'-UTR of TYMC-XX RNA (Fig. 3). The mutations identified in the partially sequenced genomes identify other mutations that could contribute to full plant adaptation.
**DISCUSSION**

Wild-type TYMV replication proteins are able to amplify genomes with 3’ termini of variable sequence and tRNA mimicry. The results summarized in Tables 1 and 2 for TYMC-XX1, -YY1, and -H1 RNAs demonstrate clearly that TYMV genomes with wild-type 5’ noncoding regions and encoding wild-type proteins can be highly infectious despite the presence of a heterologous 3’ TLS that is incapable of significant aminoacylation (9). By subcloning into TYMC RNA the XX, YY, and H TLS sequences incorporating a small number of mutations acquired during passaging and adaptation to high infectivity in plants, we have shown that the acquired mutations were responsible for most of the improved infectivity and replication generated during passaging. No sequence changes in the TYMV replication proteins are thus required in order to replicate the TYMC-XX1, -YY1, and -H1 genomes, which have very different 3’ sequences and overall 3’ structure compared to TYMC RNA (Fig. 1). Partial sequencing of the plant-adapted genomes (Fig. 3) has shown that mutations are present outside the 3’ noncoding region, and these mutations appear to contribute to maximally efficient viral spread and symptom development in plants (Table 1). One such example is provided by the more vigorous symptom development of infections resulting from inoculation with TYMC-YY1/U6150 RNA compared with TYMC-YY1 RNA (Table 1), although the C6150U mutation also directly affects viral RNA accumulation (Table 2) by an unknown mechanism.

By plant adaptation through serial passaging of TYMC-EMV and TYMC-Met RNAs, producing the highly infectious TYMC-E and TYMC-M RNAs, respectively (Table 1), we have shown that TYMV genomes with two further variations in tRNA mimicry can be highly infectious. The EMV-TLS (Fig. 1) present in TYMC-EMV and -E RNAs can be efficiently valylated, but the valylated RNA is more weakly bound by eEF1A·GTP than is the valylated TYMV TLS (5). The modified TYMV TLS present in TYMC-Met and TYMC-M RNAs (Fig. 1) is capable of efficient aminoacylation with methionine in place of the usual valine (7). Although the initial TYMC-EMV and TYMC-Met RNAs were infectious in Chinese cabbage (Table 1) and replicated moderately well in protoplasts (Table 2), sequential passaging was performed to see whether improved amplification and infectivity could be achieved and whether such improvements were associated with mutations in the 3’ TLS or in the replication protein coding regions. While enhanced infectivities were achieved (Table 1), replication in protoplasts was not improved (Table 2), and no sequence changes were accumulated in the 3’ TLS during passaging of either RNA (Fig. 3). Complete sequencing of the plant-adapted TYMC-E RNA showed that only three mutations were acquired. Two of these altered the movement protein sequence, while the third was a silent mutation in ORF-206 (Fig. 3). Movement protein mutations were also found in the plant-adapted TYMC-M and TYMC-YY RNAs, suggesting that alteration of the movement protein resulted in improved viral spread and symptom development for several of the plant-adapted viruses. The only coding change in the TYMV replication protein coding regions identified in this study was a mutation in a region of unknown function present in TYMC-M RNA (Fig. 3). Note that TYMC-M RNA does not show superior replication in protoplasts over TYMC-Met RNA (Table 2).

Our studies have thus shown that the wild-type TYMV replication proteins are able to replicate genomic RNAs with very variable 3’ sequences and tRNA-like properties, although some decreases in viral RNA accumulation were observed (Table 2). The only sequence common to the TLSs shown in Fig. 1 is the 3’ CCA terminus. Clearly, the TYMV TLS does not represent a unique structural element recognized by the viral replication complex en route to minus-strand synthesis.
This insight corroborates the recent conclusion derived from in vitro studies with the TYMV RNA-dependent RNA polymerase that the initiation of minus-strand synthesis is largely controlled by the CCA initiation box present at the 3' terminus (3, 17). The finding that RNA synthesis in vitro is not dependent on features within the TLS other than the terminal CCA separates tRNA mimicry from RNA synthesis. The same separation has emerged from the present studies of infectious genomes with various degrees of tRNA mimicry. We can thus reject the possibility that the tRNA mimicry of TYMV RNA is a requirement for minus-strand synthesis, for instance, by serving to recruit host tRNA-associating proteins, such as eEF1A, to act as transcription factors during minus-strand synthesis (10).

Model suggesting that a major role of the tRNA mimicry of TYMV RNA is to permit negative regulation of minus-strand synthesis. If the tRNA mimicry of TYMV RNA is not crucially involved in promoting the mechanics of minus-strand initiation, what role does it play? The fact that point mutations in the valine identity elements in the TYMV RNA anticodon loop that abolish valylation result in the almost complete abrogation of viral amplification (20) indicates that tRNA mimicry does play a critical role in the TYMV lifecycle. We can thus reject the possibility that the tRNA mimicry of TYMV RNA is a requirement for minus-strand synthesis, for instance, by serving to recruit host tRNA-associating proteins, such as eEF1A, to act as transcription factors during minus-strand synthesis (10).

Model suggesting that a major role of the tRNA mimicry of TYMV RNA is to permit negative regulation of minus-strand synthesis. If the tRNA mimicry of TYMV RNA is not crucially involved in promoting the mechanics of minus-strand initiation, what role does it play? The fact that point mutations in the valine identity elements in the TYMV RNA anticodon loop that abolish valylation result in the almost complete abrogation of viral amplification (20) indicates that tRNA mimicry does play a critical role in the TYMV lifecycle. Two possible roles could be in promoting 3'-end integrity and RNA stability. Evidence has been presented that host [CTP, ATP]: tRNA nucleotidyltransferase acts as a telomerase to maintain intact CCA 3' termini of brome mosaic virus RNAs (14), and that function is also likely for TYMV RNA. However, point mutations in the anticodon would not alter interaction with this host enzyme (16), indicating the existence of another crucial role for tRNA mimicry. RNA stability could be promoted by stable association of the 3' end with a protein, such as eEF1A, but our observation that significant levels of radiolabeled full-length double-stranded genomic RNAs accumulate for genomes with point mutations in the valine identity elements (20) suggests that the loss of aminoacylatability does not greatly destabilize viral RNA.

We propose that a major role of the tRNA mimicry of TYMV RNA is to permit negative regulation of the access of the replicase to the minus-strand initiation site. This would readily be accomplished by eEF1A·GTP bound to valylated TYMV RNA, since this complex is very stable (Kd = 2 nM) (5) and the protein directly contacts the 3' terminus and aminoacyl moiety (8). We have already observed that TYMV RNA-dependent RNA polymerase is sensitive to the conformational presentation of the RNA template and seems to lack the capacity to unwind the template prior to initiation (17). In DNA transcription parlance, our model suggests that the TLS acts as an operator sequence that is bound by the repressor protein eEF1A·GTP to negatively regulate minus-strand initiation. We have recently articulated this model elsewhere (4), and an analogous repressive role has been eloquently argued for the coat protein of alfalfa mosaic virus (13). The latter model differs from our model for TYMV in invoking a viral rather than host protein as the repressor.

What might the role of such negative regulation be? Negative regulation at late times (shut-off) of minus-strand synthesis has long been a feature of the positive-strand RNA virus replication cycle, particularly for animal viruses. The mechanism of shut-off of minus-strand synthesis is not well understood but, in the case of Sindbis alphavirus, is believed to be due to a shift in the form of the viral polymerase during the infection (19).
Although it has not been established that minus-strand synthesis is turned off in the course of a TYMV infection, this might be accomplished by eEF1A \( \cdot \) GTP binding. However, this would require some way to increase the availability of eEF1A \( \cdot \) GTP or increase its binding ability at the appropriate stage of the infection. It is unclear at present how this might come about.

Repression of minus-strand synthesis may alternatively be an early function, designed to permit adequate translational expression before an RNA is converted into an active replicon. This may be an especially important function for viruses with strong coupling between translation and replication, as is the case with TYMV (21). In such systems, the first translational cycle of an inoculum genomic RNA could lead to the channeled delivery of the newly formed replication proteins to the 3′ end, followed by immediate minus-strand initiation and conversion of the mRNA into a replicon. Such events could result in insufficient production of viral proteins to sustain a full-blown infection capable of overriding host defenses. The binding of eEF1A \( \cdot \) GTP would delay the premature conversion of viral RNAs from messenger to replicon function. Since binding is tight but not irreversible, some RNAs will nevertheless undergo this transition, permitting both translation and replication to proceed early in the infection. As the concentration of replicase increases, an increasing proportion of viral RNAs will be recruited into active replication. According to this model, mutation of the valine identity elements would lead to loss of the ability to bind eEF1A \( \cdot \) GTP, interfering with viral amplification but not necessarily minus-strand synthesis, just the result observed (20).

If eEF1A \( \cdot \) GTP serves as the repressor in the case of wild-type TYMV RNA, what proteins could function in this capacity for the variant genomes characterized in this study? eEF1A \( \cdot \) GTP is a candidate repressor for some of these genomes: TYMC-Met and -M RNAs can be efficiently aminoacylated and thus unable to interact with GTP or increase its binding ability at the appropriate stage of the infection. Although it is not clear how the variant genomes characterized in this study? 

The variant TYMV RNAs studied here will provide a powerful resource for testing the above model for the role of tRNA mimicry and for identifying the repressor proteins involved. This will lead to an understanding of the long-obscure role of viral tRNA mimicry and perhaps introduce a new paradigm for the role of host proteins that interact with viral RNAs: a negative, repressive role, rather than the positive roles that have usually been considered (11).

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