

Pseudouridine and ribothymidine formation in the tRNA-like domain of turnip yellow mosaic virus RNA

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ABSTRACT

The last 82 nucleotides of the 6.3 kb genomic RNA of plant turnip yellow mosaic virus (TYMV), the so-called 'tRNA-like' domain, presents functional, structural and primary sequence homologies with canonical tRNAs. In particular, one of the stem-loops resembles the TΨ(pseudouridine)-branch of tRNA, except for the presence of a guanosine at position 37 (numbering is from the 3'-end) instead of the classical uridine-55 in tRNA (numbering is from the 5'-end). Both the wild-type TYMV-RNA fragment and a variant, TYMV-mut G₃₇U in which G-37 has been replaced by U-37, have been tested as potential substrates for the yeast tRNA modification enzymes. Results indicate that two modified nucleotides were formed upon incubation of the wild-type TYMV-fragment in a yeast extract: one Ψ which formed quantitatively at position 65, and one ribothymidine (T) which formed at low level at position U-38. In the TYMV-mutant G₃₇U, besides the quantitative formation of both Ψ-65 and T-38, an additional Ψ was detected at position 37. Modified nucleotides Ψ-65, T-38 and Ψ-37 in TYMV RNA are equivalent to Ψ-27, T-54 and Ψ-55 in tRNA, respectively. Purified yeast recombinant tRNA:Ψsynthases (Pus1 and Pus4), which catalyze respectively the formation of Ψ-27 and Ψ-55 in yeast tRNAs, are shown to catalyze the quantitative formation of Ψ-65 and Ψ-37, respectively, in the tRNA-like 3'-domain of mutant TYMV RNA *in vitro*. These results are discussed in relation to structural elements that are needed by the corresponding enzymes in order to catalyze these post-transcriptional modification reactions.

INTRODUCTION

One characteristic feature of the single-stranded RNA genomes of plant viruses, as well as of some animal viruses, is the occurrence of a sequence at their 3'-ends that can behave as and fold into a tRNA-like structure. This tRNA mimicry was initially discovered because of the ability of the viral RNA genomes to be aminoacylated *in vitro* at their terminal CCA-ends by specific

aminoacyl-tRNA synthetases of various origins (*Escherichia coli*, yeast, wheat germ, *Xenopus laevis*). It was later demonstrated that such tRNA-like domains were also recognized by several other tRNA-specific enzymes or factors, such as the (ATP, CTP):tRNA nucleotidyl-tRNA transferase, translation elongation factors (EF-Tu and EF-1α) from *E. coli* or wheat germ, *E. coli* peptidyl-tRNA hydrolase and *E. coli* or *Bacillus subtilis* RNase P (reviewed in 1–3).

The first evidence that tRNA-like domains of plant viral RNA can also interact with and thereby be modified by the tRNA-modification machinery was given by Dudock and co-workers (4,5). They demonstrated that, upon incubation in *E. coli* cell extracts, one of the uridines in tobacco mosaic virus (TMV) RNA was slowly but quantitatively modified into 5-methyluridine (m⁵U, ribothymidine, also abbreviated as T). This uridine methylation occurred at a position in a loop of TMV-RNA that resembles that of T found in the characteristic TΨ(pseudouridine)-loop of tRNAs. Enzymatic formation of a small amount of 5-methylcytosine (m⁵C) was also demonstrated upon incubation of the tRNA-like domain of the TMV-RNA with purified tRNA(cytosine-5-) methyltransferase of HeLa cells, but in this case the exact position of the methylated cytosine was not determined (6). A 5-methylcytosine modification in the *in vitro* transcript of the rat brain identifier (ID) sequence, which also possesses a tRNA-like structure at its 3'-end, was demonstrated upon incubation of the RNA transcript in a HeLa cell extract (7). In the latter case, the methylated cytosine was located at a position of the rat ID sequence where a m⁵C is frequently found in most eukaryotic tRNAs (position 49 at the beginning of the tRNA TΨ-stem; 8). On the other hand, the TYMV-RNA fragment, as extracted from the infected plant cells, was reported not to contain detectable amounts of modified nucleosides (9).

In the present work the sequence corresponding to the last 82 nucleotides (nt) of the 6.3 kb genomic RNA (positive-strand RNA) of turnip yellow mosaic virus (TYMV) was tested as a substrate for the yeast tRNA-modification enzymes. This fragment has a 3D structure very similar to that of canonical tRNA, except that it contains a pseudoknot in its acceptor branch, an extended anticodon stem and unusual D-loop/T-loop interactions (10–13). A T7-transcript of this TYMV-RNA fragment as well as of a variant bearing a G mutated into a U in the loop corresponding to the TΨ-loop in tRNAs, were incubated in a yeast

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extract or with two purified recombinant yeast tRNA- Ψ synthases (Pus1 and Pus4) catalyzing respectively the formation of Ψ in the anticodon branch (position 27 of tRNA) and in the T Ψ -loop (position 55 of tRNA). The enzymatic formation of T as well as Ψ at two different locations of these two TYMV-tRNA-like fragments is demonstrated.

MATERIALS AND METHODS

Enzymes and chemicals

Radiolabeled [α - 32 P]nucleotide triphosphates (400 Ci/mmol) were from Amersham (UK). Tris, dithiothreitol (DTT), nucleoside triphosphates, spermidine, *Penicillium citrinum* nuclease P₁, *Aspergillus oryzae* RNase T₂ were from Sigma (St Louis, MO, USA), restriction enzyme *Mva*I from MBI Fermentas (Vilnius, Lithuania), RNasin from Promega (Madison, WI, USA), S-adenosyl-L-methionine (AdoMet) from Boehringer Mannheim (Germany), 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (CMCT) from Aldrich (USA). Chemically synthesized deoxyoligonucleotides were purchased from MWG-Biotech (Germany). They were used without further purification. Bacteriophage T7 RNA polymerase was purified according to a previously published procedure (14) using an overproducing strain kindly provided by Dr Studier (Brookhaven, NJ, USA). Avicel thin-layer cellulose plates (type F1440) were from Schleicher & Schuell (Dassel, Germany). All other chemicals were from Merck Biochemicals (Darmstadt, Germany).

Plasmids and preparation of radiolabeled RNA transcripts

Plasmid pTYAlu encoding the tRNA-like domain of wild-type TYMV RNA (82 nt) was a gift of Dr T. Dreher (15). To improve the transcription yield, this plasmid contains a non-viral hexanucleotide extension (GGGAGA) at the 5'-end immediately downstream of the T7 promoter. A variant of this plasmid (pTYAlu-mutG₃₇U) was constructed by site-specific mutation of a guanine at position 37 (numbering from the 3'-end of the RNA transcript) in loop II (Fig. 1A) into an uracil in order to create the sequence GUUC that is a characteristic feature of the so-called T Ψ -loop of all sequences of precursor tRNAs. This variant was obtained using Amersham Site-Directed Mutagenesis Kit according to (16). *In vitro* transcription using T7 RNA polymerase and purification of the radiolabeled transcripts of the TYMV tRNA-like domain and its variant (mut G₃₇U) by electrophoresis on urea gels were performed as described elsewhere (17).

Source of enzymes and enzyme assays

Yeast S100 extract was prepared as described earlier (17). Purified recombinant yeast multisite-specific tRNA: Ψ synthase (Pus1) (18) and tRNA: Ψ -55 synthase (Pus4) (19) were obtained as described in earlier papers, respectively in (20) and (19). Both recombinant proteins bear a His₆-tag on their N-terminals and were purified to homogeneity on a Ni²⁺-NTA-agarose column (Qiagen GMBH, Hilden, Germany), followed by chromatography on Mono-Q HR5/5 column (Pharmacia, Uppsala, Sweden).

Enzymatic formation of modified nucleotides in the RNA transcripts was tested in a standard reaction mixture containing 100 mM Tris-HCl, pH 8.0, 10 mM MgSO₄, 2 mM DTT, 0.1 mM EDTA, 100 mM NH₄-acetate, 20 μ M AdoMet, 8 U RNasin and

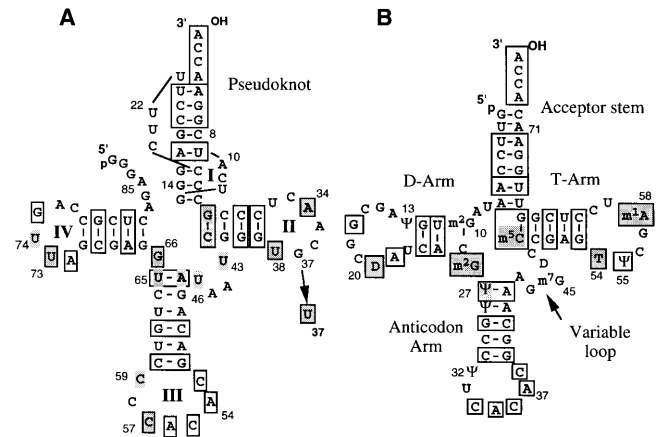


Figure 1. Analysis of potential post-transcriptional modification sites within the TYMV tRNA-like domain. (A) Sequence and secondary structure of the 3'-end of TYMV-RNA including the pseudoknotted acceptor stem. Numbering of nucleotides is from the 3'-end. Only the sequence GGGAGA at the 5'-end are non-viral encoded nucleotides. Boxed nucleotides are common to yeast tRNA^{Val} (anticodon CAC) (B). Shaded nucleotides are potential modification sites according to the modification pattern of yeast tRNA^{Val} (B) and to the consensus modification pattern of all yeast tRNAs sequenced so far (24). The point mutation (G37→U37) is indicated by an arrow. (B) Cloverleaf structure of yeast tRNA^{Val} with naturally occurring modified nucleotides. Numbering of nucleotides is conventionally from the 5'-end. Sequence homologies of tRNA^{Val}(CAC) with the TYMV tRNA-like structure are boxed. Modification sites in yeast tRNA^{Val} which correspond to potential modification sites in the tRNA-like structure are shaded.

50–100 fmol (1–2 nM final concentration) 32 P-labeled RNA substrate in a total volume of 50 μ l. The reaction was initiated by the addition of S100 yeast extract (0.5–1 mg protein/ml final concentration) or purified recombinant yeast Pus1 or Pus4 (1 μ g/ml final concentration). Prior to the addition of enzyme and AdoMet, the reaction mixture was heated for 3 min at 65°C and slowly cooled for correct tRNA-like domain annealing. After incubation at 30°C for the indicated period of time (Table 1 and 2, also figure legends), samples were treated and analyzed for the presence of modified nucleotides as described previously (17). The relative amount of 32 P-labeled modified nucleotides, as compared to the total amount of 32 P-labeled non-modified nucleotides, was measured by counting the radioactivity in the corresponding spots obtained after chromatography of nuclease P1- or RNase T2-RNA hydrolysates on a thin-layer cellulose plate. Quantification was performed using a PhosphorImager counter (Molecular Dynamics, Sunnyvale, CA, USA) and ImageQuant software. The accuracy of this method was found to be about ± 0.1 mol of modified nucleotide per mol RNA.

Mapping of Ψ residues in TYMV transcripts modified by Pus1 and Pus4

Mapping of Ψ residues in the transcripts of TYMV and TYMVmut modified *in vitro* by recombinant Pus1p and Pus4p have been performed using CMCT/RT approach (21). About 10 μ g of T7 transcript were incubated for 1 h with the equal amount (10 μ g) of Pus1p or Pus4p correspondingly. The transcript was purified by polyacrylamide gel electrophoresis and subjected to CMCT modification as described previously (18). Unmodified transcripts were used as controls. Reverse transcription was

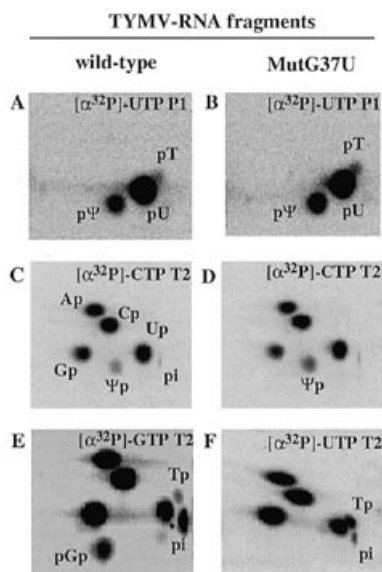


Figure 2. Modified nucleotides formed in TYMV tRNA-like domain (wild-type and mutG₃₇U) when incubated at 30°C for 90 min in S100 yeast extract. The figure shows selected autoradiograms of 2D-t.l.c. plates of 3'- and 5'-phosphate nucleotides obtained after complete digestion of the radiolabeled RNA with nuclease P1 or RNase T2 (as indicated inside each panel). The chromatographic system has been described previously (17). Identification of nucleotides was made by comparison with references maps (as in 51).

performed using 0.5–1.0 μg of CMCT-modified RNA and the 5'-³²P-labeled oligonucleotide (TGGTTCGGATGACCCTC-GG), complementary to 19 3'-nt of TYMV.

RESULTS

Potential sites of nucleoside modification in the tRNA-like domain of TYMV-RNA

Figure 1A shows the secondary structure of the 82 nt of the 3'-domain of the TYMV-RNA, extended at its 5'-end by a non-viral sequence of 6 nt (GGGAGA). This so-called tRNA-like domain contains four well-organized stem and loop regions, three of which (stem-loops II, III and IV) have close structural and sequence resemblance with those of yeast tRNA^{Val} (anticodon CAC, Fig. 1B). A major difference occurs in region 1–27 of the TYMV-RNA, including stem-loop I. However, as first suggested by Pleij and co-workers (22,23), reviewed in (2), the GGG sequence of loop I and complementary nucleotides CCC from the single strand region between stems I and II, base-pair in a Watson–Crick mode, thus forming a characteristic pseudoknotted structure allowing a general mimicry of the acceptor arm of tRNA. Recent NMR studies performed on the 44 nt 3'-fragment of TYMV RNA fully confirm the existence of this pseudoknotted structure (13). More subtle differences between TYMV and tRNA^{Val} concern the presence of a UGC sequence in loop II instead of the UUC (TΨC) sequence present in the TΨ-loop of all tRNAs, a 4 bp stem II instead of 5 in the TΨ-stem of tRNA, the absence of two conserved G-residues in loop IV, and an anticodon stem of 6 bp instead of 5 in canonical tRNAs. A single-stranded region (4 nt), equivalent to the variable region of tRNAs, is also present.

Sequence comparison between yeast tRNA^{Val} and the wild-type TYMV tRNA-like structure highlights 39 common 'parental' residues (boxed in Fig. 1A and B). Out of these 39 identical parent bases, six are modified in yeast tRNA^{Val} and thus are likely potential modification sites in TYMV-RNA. They are D-20.2 (corresponding to U-73 in the TYMV-fragment), m²G-26 (corresponding to G-66), Ψ-27 (U-65), m⁵C-49 (C-42), T-54 (U-38) and m¹A-58 (A-34) (shaded in Fig. 1B). After mutation of G-37 to U-37 in the TYMV-RNA fragment (mutG₃₇U), an additional potential site of modification is created (corresponds to Ψ-55 in tRNA^{Val}).

Taking into account the type and location of modified nucleotides found in *Saccharomyces cerevisiae* cytoplasmic tRNAs (33 sequences altogether) (24), and comparing with the nucleotide sequence of the TYMV-RNA fragment (Fig. 1A), five additional potential modification sites should be considered in the viral RNA (shaded in Fig. 1A). These are U-43 equivalent to position 48 in tRNAs, modified to D-48 in tRNAs bearing U-48 (16 cases out of a total of 28), U-46 which corresponds to Um-44 in few tRNAs (4 out of 8), C-57 to m⁵C-34 or Cm-34 in only two yeast tRNAs, respectively tRNA^{Leu}(anticodon m⁵CUA) and tRNA^{Trp}(anticodon CmCA), C-59 to m³C-32 (4 out of 11) or Cm-32 (3 out of 11) in few tRNAs and U-74 to D-20.1 in all yeast tRNAs bearing an U-20.1. Thus, altogether, one can consider that there are 11 (12 in the G₃₇U variant) potential targets for yeast tRNA-modification enzymes in the tRNA-like structure of TYMV-RNA (shaded in Fig. 1A).

Ψ and T are formed in the TYMV tRNA-like domain upon incubation with yeast S100 extract

To test whether TYMV-RNA can be modified *in vitro* by the yeast modification-machinery, the transcripts, each radiolabeled with one of the four [α-³²P]triphosphate nucleotides, were incubated in yeast extract and analyzed for their modified nucleotides content.

When the P1-hydrolysates of either wild-type or mutant transcripts internally labeled with [α-³²P]ATP, -CTP or -GTP were analyzed by chromatography on 2D-thin-layer plates, no spots corresponding to modified derivatives of AMP, GMP and CMP were found. However, when the [α-³²P]UTP-radiolabeled transcripts were used, the presence of [5'-³²P]ΨMP and, to a lesser extent, of [5'-³²P]TMP was detected (Fig. 2A and B).

From the tRNA mimicry of the 3'-end domain of TYMV-RNA and the presence of the corresponding enzymatic activities in yeast S100 extract (25), one would expect that more modified nucleotides than just Ψ and T may occur. The absence of modified nucleotides in loop III (positions 54 and 57) is not surprising due to the fact that the homologous residues (positions 37 and 34) in yeast tRNA^{Val} are not modified (Fig. 1B). The absence of m²G-66 in the TYMV-RNA fragment, corresponding to m²G-26 in yeast tRNA^{Val}, can be rationalized on the basis of the absence of identity elements for m²G/m²G-26 formation in yeast tRNAs, which are known to include two G-C pairs in the D-stem and 5 nt in the variable loop (26), reviewed in (27). Despite the fact that transcripts of several yeast tRNAs (specific for Asp, Phe and Ser) are substrates for the yeast enzymes catalyzing *in vitro* formation of dihydrouridines (25) (H. Grosjean, unpublished results), none of the uridines in loop IV (positions 73 and 74) and in position 43, was converted into dihydrouridines. The reason why Um-46 in TYMV-RNA was not formed is probably related to the fact that

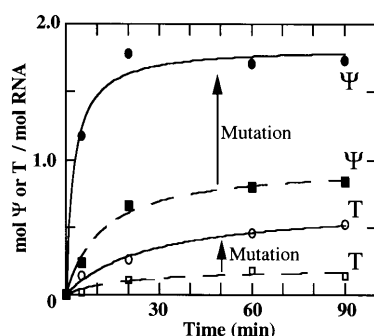


Figure 3. Time course for the formation of Ψ (filled symbols) and T (open symbols) in the tRNA-like domain of TYMV-RNA upon incubation at 30°C in the presence of S-adenosylmethionine and yeast S100 extract. Dashed lines are for the wild-type TYMV-RNA fragment, while the continuous line corresponds to TYMV-RNA mut $G_{37}U$. Values were obtained from estimation of the relative radioactivity in the corresponding spots as detected in Figure 2 for different incubation times. Results obtained from the P1 or T2 hydrolysates are essentially the same (Table 1).

the yeast enzyme catalyzing the ribose methylation at position 44 of tRNAs requires a long variable extra arm for recognition (24). The tRNA(adenosine, N^1) methyltransferase catalyzing the formation of m^1A-58 in tRNAs is able to work on yeast tRNA transcripts under our *in vitro* conditions (H. F. Becker, unpublished results). Therefore, the absence of m^1A-34 in the TYMV transcript upon incubation in yeast extract most probably reflects some kind of hindrance due to the pseudoknotted structure in the TYMV-RNA. Finally, despite the presence of active tRNA: m^5C -methyltransferase in S100 yeast extract (as tested using tRNA^{Phe} and tRNA^{Asp} transcripts; 25,28), no formation of m^5C-42 in TYMV RNA (corresponding to m^5C-49 in tRNAs) was detected.

Figure 3 shows the time course of Ψ and T formation in the wild-type TYMV fragment (dashed lines) and in the $G_{37}U$ variant (solid lines). Table 1 lists the yield of modified nucleotides obtained after 90 min incubation of the transcripts with S100 yeast extract. About 1 mol of Ψ per mol tRNA and only a small amount (0.2–0.3 mol) of T were formed in the wild-type TYMV-fragment, whereas in the $G_{37}U$ variant, 1.7 mol of Ψ and 0.5–0.7 mol of T per mol RNA appeared under the same experimental conditions. The fact that >1 mol of Ψ was formed

in the TYMV-mut $G_{37}U$ indicates that two different uridines were modified.

Autoradiographies presented in Figure 2C–F and quantitative data in Table 1 reveal that the 3'-nucleotide adjacent to both Ψ MPs in the wild-type and the $G_{37}U$ mutant is a CMP (Fig. 2B and C), while the 3'-nucleotide adjacent to TMP is a GMP in the wild-type TYMV fragment (Fig. 2E) and a UTP in the $G_{37}U$ mutant (Fig. 2F). Therefore, the Ψ formation in TYMV and TYMVmut occurs in sequence UC and formation of T in TYMV and TYMVmut takes place in UG and UU sequence, respectively. Thus the nucleotide-38 in both the wild-type and $G_{37}U$ mutant is the most probable candidate for a T formation. As far as the Ψ MP are concerned, one most probably originates from the isomerization of the uridine at position 65 in the wild-type TYMV-RNA fragment (corresponding to $\Psi-27$ in yeast tRNA^{Val}) and the second Ψ MP in the variant TYMV-RNA fragment obviously originates from the mutated U-37 (corresponding to $\Psi-55$ in yeast tRNA^{Val}). This conclusion fits with the quantitative data showing that >1 mol/mol RNA are formed only in the mutated TYMV-RNA fragment (Table 1).

Purified yeast Pus1p and Pus4p catalyze formation of Ψ in tRNA-like domain of TYMV-RNA

To confirm the data obtained with the S100 yeast extract, the same radiolabeled RNA transcripts tested above were now each incubated with one of the two purified recombinant yeast tRNA: Ψ synthases, namely Pus1p which catalyzes the *in vitro* formation of $\Psi-27$ and $\Psi-28$ in several yeast tRNAs lacking intron (18,29) and Pus4p which catalyzes the site-specific formation of $\Psi-55$ in all yeast tRNAs (19).

As shown in Figure 4, Pus1p catalyzes the quantitative formation (1 mol/mol RNA) of a Ψ in TYMV RNA. This modification is 5'-adjacent to a cytosine (as revealed by nearest neighbour analysis with [^{32}P]CTP-labeled transcript, Table 2) in both the wild-type and TYMV-mut $G_{37}U$. One such uridine (U-65) is located at the junction between arm III and arm IV (Fig. 1A). This position indeed corresponds to $\Psi-27$ occurring naturally in yeast tRNA^{Val} (Fig. 1B). The other Ψ synthase, Pus4p, catalyzes the quantitative formation of Ψ (1 mol/mol RNA) in the $G_{37}U$ -variant of the TYMV-RNA fragment but not in the wild-type fragment (Fig. 4B). Therefore, and in combination with the fact that the 3'-neighbouring nucleotide is a C (Table 2), modification occurs at position U-37, adjacent to C-36. This position is homologous to $\Psi-55$ in canonical tRNA.

Table 1. Modified nucleotides formed after incubation of the TYMV tRNA-like substrates with yeast S100 extract supplemented with AdoMet

	Modified nucleotides	[α - ^{32}P]ATP		[α - ^{32}P]UTP		[α - ^{32}P]CTP		[α - ^{32}P]GTP	
		P1	T2	P1	T2	P1	T2	P1	T2
TYMV wild-type	Ψ	0.0	0.0	0.8	0.0	0.0	0.7	0.0	0.0
	T	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.3
TYMV mut $G_{37}U$	Ψ	0.0	0.0	1.7	0.0	0.0	1.7	0.0	0.0
	T	0.0	0.0	0.5	0.7	0.0	0.0	0.0	0.0

After incubation, the radiolabeled RNA was hydrolyzed by nuclease P1 or RNase T2 as indicated in Materials and Methods. Identification of the modified nucleotides in the hydrolysates was performed by 2D-t.l.c. as shown in Figure 2. All time courses correspond to incubation for 5, 20, 60 and 90 min at 30°C. Quantitative analysis of the radioactivity in each spots of the t.l.c. was performed using a PhosphorImager. Only the data for the 90 min incubation time are given. Modification yield is expressed as mol of modified nucleotide per mol RNA, with an accuracy of 0.1 mol/mol RNA.

Table 2. Modified nucleotides formed after incubation of the TYMV tRNA-like substrates with purified recombinant yeast Pus1p and yeast Pus4p

	Modified nucleotides	[α - 32 P]ATP		[α - 32 P]UTP		[α - 32 P]CTP		[α - 32 P]GTP		
		P1	T2	P1	T2	P1	T2	P1	T2	
Pus1p										
TYMV wild-type	Ψ	0.0	0.0	<u>1.0</u>	0.0	0.0	<u>1.0</u>	0.0	0.0	
TYMV mutG ₃₇ U	Ψ	0.0	0.0	<u>1.0</u>	0.0	0.0	<u>1.0</u>	0.0	0.0	
Pus4p										
TYMV wild-type	Ψ	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
TYMV mutG ₃₇ U	Ψ	0.0	0.0	<u>1.0</u>	0.0	0.0	<u>1.0</u>	0.0	0.0	

Experimental conditions were the same as in Table 1, except that yeast S100 extract was replaced by purified Pus1p or Pus4p.

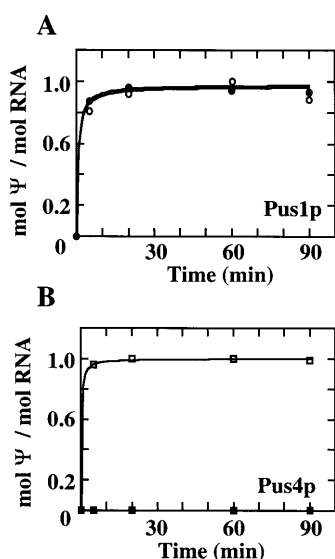


Figure 4. Time course for the formation of Ψ in the TYMV tRNA-like structure incubated at 30°C with purified recombinant yeast tRNA modification enzyme. (A) Multisite-specific tRNA: Ψ synthase (Pus1p); (B) tRNA: Ψ -55 synthase (Pus4p). Filled symbols are for TYMV-RNA (wild-type); open symbols are for TYMV-RNA (mutG₃₇U).

Direct evidence for the formation of Ψ -65 and Ψ -37 in the TYMV and TYMVmut transcripts incubated with Pus1p and Pus4p was obtained by chemical mapping using CMCT/RT approach. The results of reverse transcription are presented in Figure 5A and B. In both cases, the full length of the TYMV transcripts was explored, but only the part bearing Ψ residues is presented in Figure 5. The strong stops in reverse transcription patterns of enzymatically modified TYMV transcripts demonstrate the appearance of Ψ residues at positions 65 and 37 upon the incubation with recombinant Pus1p and Pus4p, respectively.

DISCUSSION

The genomes of numerous positive-strand RNA plant viruses contain 3'-terminal structural domains that mimic the 3D-architecture of tRNA. Based on sequence comparisons, chemical and enzymatic probing and structural modelling, 3D-models for tRNA-like terminal structures of various plant RNAs have been

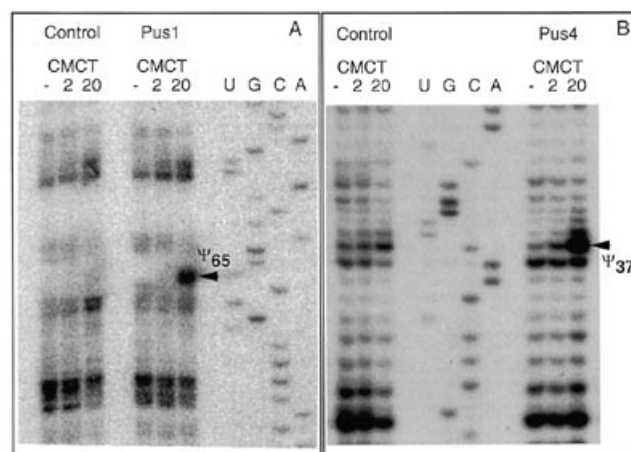


Figure 5. Mapping of Ψ residues in TYMV and TYMVmut transcripts modified *in vitro* by recombinant Pus1p (A) and Pus4p (B). The CMCT-treatment (control, 2 min, 20 min) and reverse transcription were performed as described in Materials and Methods. Unmodified T7 transcripts of TYMV and TYMVmut treated as control samples are presented on the left. Sequencing ladders (lines UGCA) are shown. The arrows point out the strong stops corresponding to CMC-modified Ψ .

proposed (reviewed in 3). These models account for most of the observed properties of these tRNA-like structures, such as their aminoacylation by specific aminoacyl-tRNA synthetases and/or their interactions with several other proteins or enzymes usually working on tRNAs (reviewed in 2,3).

Among the different tRNA-like structures modelled so far, that of the TYMV-RNA is the most closely related to the canonical 'L'-shaped tRNA architecture (12,13,22,30,31). One can therefore expect that it may serve as substrate for the tRNA modification enzymes that are present in the cytoplasm of eukaryotic cells.

In the present work, we demonstrate that the *in vitro* transcript corresponding to the tRNA-like domain of wild-type TYMV-RNA is an excellent substrate for the yeast multisite-specific tRNA: Ψ synthase (Pus1p) and a poorer substrate for the yeast tRNA:uracil-54 methyltransferase (RUMT). Yet, changing only 1 nt in loop II of the wild-type TYMV-RNA fragment (Fig. 1A), in order to generate a loop sequence in the mutated RNA that now becomes identical to a T Ψ -loop in canonical tRNAs, not only allowed the almost quantitative enzymatic formation of ribo-

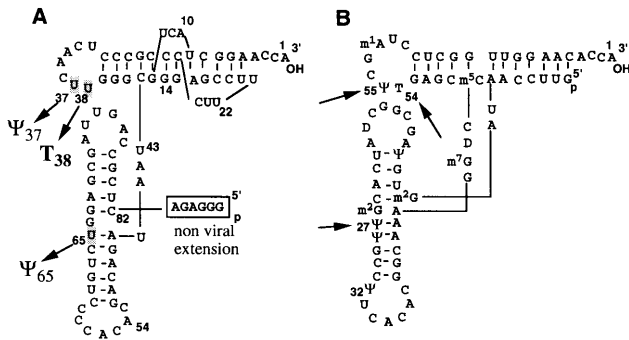


Figure 6. Localization of the target nucleotides for yeast-tRNA modification enzymes within TYMV RNA. Putative L-shaped structures are shown for TYMV tRNA-like fragment (mutG₃₇U) (A) and yeast tRNA^{Val} (B). Modification sites are indicated by arrows. In yeast tRNA^{Val} (fully matured sequence) arrows point out the three modified nucleotides that also occurred in plant RNA transcripts.

thymidine at position 38, but also allowed the 3'-adjacent mutated U-37 of the viral RNA to become fully isomerized to Ψ-37 by the yeast tRNA:Ψ-55 synthase (Pus4p). Figure 6A shows where these modified nucleotides occur on a schematic representation of the 'L' folding of the TYMV-RNA fragment as compared to the similar folding of tRNA^{Val} (Fig. 6B).

The enzymatic formation of both T-54 and Ψ-55 in tRNAs does not require an intact 3D-architecture, but only a stem-loop RNA fragment bearing a characteristic structure stabilized by reverse Hoogsteen base pair (28,32–36). Likewise, enzymatic formation of Ψ-27 in several yeast tRNAs is catalyzed by a multisite-specific tRNA:Ψ synthase (Pus1p) which does not require a 3D-architecture. Indeed, yeast Pus1p not only catalyzes *in vitro* the formation of Ψ within the 3D-core of a tRNA molecule (at positions 27 and/or 28), but also in other RNA fragments that obviously do not have the characteristic tRNA 'L'-shape conformation, such as a minisubstrate of tRNA composed of a yeast tRNA^{Ile} anticodon stem extended by its 60 nt long natural intron (18,29,37; reviewed in 38). Therefore, the fact that Ψ-37, T-38 and Ψ-65 are formed *in vitro* in the tRNA-like fragment of TYMV-RNA cannot be taken as an argument of perfect 'global' tRNA mimicking, but rather as the indication that 'local' subdomains of both the tRNA and the plant genomic TYMV-RNA fragment are sufficiently similar (in terms of sequence, conformation and/or dynamic properties) in order to be recognized and modified by the same set of modification enzymes (see also discussion in 33). A precedent exists with the *in vitro* formation of ribothymidine in a loop of a transcript corresponding to a fragment of *E.coli* 16S-ribosomal RNA catalyzed by the *E.coli* RUMT. However, the efficiency of this ribothymidine formation at position 788 in rRNA was much lower than T-54 formation in a stem-loop fragment originating from a tRNA (39).

The enzymatic formation of T-38, even at a low level, in the wild-type TYMV-RNA fragment bearing a G-37 in loop II (Fig. 1B) was not expected. Indeed, using as substrate a 19mer oligonucleotide mimicking the TΨ-arm of yeast tRNA^{Asp}, we showed that the presence of a G-55, 3'-adjacent to U-54 (as in loop II of the TYMV-RNA), completely abolishes the enzymatic formation of T-54 catalyzed by the yeast tRNA:uracil-54 methyltransferase. However, the efficiency of methylation was also dependent on the length of the stem supporting the T-loop as

well as on other parts of the tRNA molecule (28). The same conclusion was reached for *E.coli* RUMT catalyzing the formation of T-54 in *E.coli* tRNAs (40). Therefore, with the tRNA-like TYMV-RNA fragment, it appears that the nucleotide identity requirement at the position 3'-adjacent to the methylated target U-38 is less stringent than when a short RNA minihelix bearing a smaller number of potential contacts with the enzyme is used as substrate. However, mutation of G-37 to U-37, thus creating a loop II identical to that of TΨ-loop in yeast tRNA^{Val}, allowed an almost quantitative formation of both T-38 and Ψ-37. This last result is in accordance with those reported by Dudock and co-workers for tobacco mosaic virus (TMV) RNA (4,5). Upon incubation of the genomic TMV-RNA in an *E.coli* cell extract the quantitative formation of a ³H-radiolabeled T residue (almost 1 mol/mol RNA) at position 34 from the 3'-end of tRNA-like domain was demonstrated. Most probably the U-33, 3'-adjacent to the T-34 in TMV-RNA was also modified to Ψ-33, but its formation was not detected using [³H]methyl incorporation. Interestingly, beside the efficient formation of [³H]T-34 in TMV RNA, Dudock and co-workers also mentioned the enzymatic formation of a small amount of ³H-product having the 2D-thin layer chromatography (t.l.c.) mobility characteristic of m⁷Gp. However, the exact location of this minor modified nucleotide within the TMV-RNA genome was not explored (5).

The absence of m⁵C-42 in the incubated TYMV-RNA fragment is noteworthy. This result is not related to the absence or low activity of the corresponding m⁵C-methyltransferase in the yeast extract as this activity can be detected using yeast tRNA^{Phe} or yeast tRNA^{Asp} transcripts (25,28). Also, the microinjection of the TYMV-RNA fragment into the cytoplasm of *X.laevis* oocyte, revealed the quantitative formation of m⁵C-42 at a position that is homologous to the m⁵C-49 in several yeast tRNAs (H. Brulé, H. Grosjean, R. Giegé and C. Florentz, *Biochimie*, submitted). Interestingly, enzymatic formation of m⁵C in TMV-RNA (at an undetermined position; 6) and in the *in vitro* transcript of the rat brain ID sequence, which also possesses a tRNA-like structure at its 3'-end (7), were demonstrated in a HeLa cell extract. Therefore, the lack of m⁵C in the TYMV-RNA fragment incubated in a yeast extract could reflect differences in identity requirements for the yeast tRNA(cytosine-5) methyltransferase in comparison with the same enzyme from the higher eukaryotes.

In summary, we have shown that the tRNA-like domain of the TYMV-RNA genome can serve as substrate for a few tRNA modification enzymes from eukaryotic cells but that the occurrence and the efficiency of such reactions depends on the identity requirements of the tRNA modification enzymes of the host cells. Despite earlier work showing that no detectable amount of modified nucleotide is present in TYMV-RNA extracted from the plant cell (9), we demonstrate here that such potentiality exists. It might well be that in the plant cells, where the virus is overproduced, the amounts of modified nucleotides are lower than in our *in vitro* experimental conditions. What could be the consequences of such nucleotide modifications on the biological function and/or the replication of the viral genome remains to be determined.

The presence of a tRNA-like structure within an RNA molecule is not limited to plant viral genomes, but also found in several other RNAs, such as at the 5'-end of defective-interfering RNA of Sindbis virus (41), in the leader region of certain prokaryotic polycistronic messenger RNAs (42–44), in the so-called ID sequence present in the intron of vertebrate brain-specific

mRNAs (7,45), in group I intron catalytic core (46), as well as in the 5'- and 3'-end sequences of bacterial 10Sa RNA, also known as tmRNA which functions as both a tRNA and an mRNA on the ribosome (47,48; reviewed in 49). Recent work of Felden and co-workers (50) clearly demonstrated the presence of both T and Ψ residues in the part of *E.coli* tmRNA sequence corresponding to TΨ-loop of tRNA. For the other tRNA-like molecules this question about the presence and location of modified residues still remains open. It would be interesting to know which nucleotide(s), if any, in these different types of tRNA-like structures could be modified post-transcriptionally and what may be the importance of these modifications on the conformation as well as on the function of the RNA.

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