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Idiosyncratic Behaviour of tRNA-like Structures in Translation of Plant Viral RNA Genomes

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Tobacco mosaic virus (TMV) and Nemesia ring necrosis virus (NeRNV) belong to the Tobamoviridae and Tymoviridae families, respectively. Although their RNAs present different 5′-untranslated regions and different family-specific genomic organizations, they share common 3′-ends organized into three consecutive pseudoknot structures followed by a histidylatable tRNA-like structure (TLS). We investigate here whether the histidine residue becomes incorporated into viral proteins and if the TLSs of TMV and NeRNV play a role in viral translation. Our results indicate that, regardless of the genomic context, the histidine moiety does not become incorporated in proteins *via* ribosomal translation, and that disruption of the TLS in either viral RNA does not perturb the viral translation patterns. In the light of the present data and of previous results on tymoviral TLS^{Val} and bromoviral TLS^{Tyr} showing differential effects on translation, we suggest that the key role for the TLS in promoting translation initiation appears to be dictated by the TLS architecture and identity.

Keywords: histidylation; plant virus; protein synthesis; TMV; untranslated region

A number of plant viruses with mono- or multipartite (+)-stranded RNA genomes harbour instead of the standard poly(A) tail a tRNA-like structure (TLS) at their 3′-end. For most of these viruses, the essential role of these TLSs in RNA-dependent RNA replication has been shown convincingly.[1] However, in most cases this does not explain the high level of functional mimicry with canonical tRNAs. Indeed, these TLSs are known to be efficient substrates of tRNA-specific proteins, in particular aminoacyl-tRNA synthetases that aminoacylate their 3′-ends.[2–4] The most studied TLSs are those from turnip yellow mosaic virus (TYMV), tobacco mosaic virus (TMV) and brome mosaic virus (BMV) RNAs, which are representatives of three different amino acid specificities (valine,[5] histidine,[6] and tyrosine,[7] respectively). Three-dimensional models of these three TLSs are available,[8–10] and they emphasize an overall resemblance to the shape of canonical tRNAs, although the details of their folding patterns are different.[11]

Recently, it was shown that for the monopartite TYMV (Figure 1) the TLS plays a crucial role in translation initiation.[12] Specific incorporation of the TLS-attached valine residue at the N-terminal position of the viral polyprotein was observed, where one would expect a methionine residue donated by the canonical initiator tRNA. Disruption of the TLS led to complete abolishment of polyprotein translation with no effect on the synthesis of the other viral proteins. BMV, type member of the Bromoviridae family, has a tripartite genome with each RNA harbouring a bulky TLS of about 170 nt, which can be charged with tyrosine. In contrast with TYMV, no TLS-derived tyrosine incorporation into a viral protein product could be detected, but disruption of the TLS has severe adverse effects on viral translation.[13]

Abbreviations used: TLS, tRNA-like structure; TYMV, turnip yellow mosaic virus; TMV, tobacco mosaic virus; BMV, brome mosaic virus; NeRNV, Nemesia ring necrosis virus; UTR, 5′-untranslated region; UPD, upstream pseudoknot domain; ORF, open reading frame.

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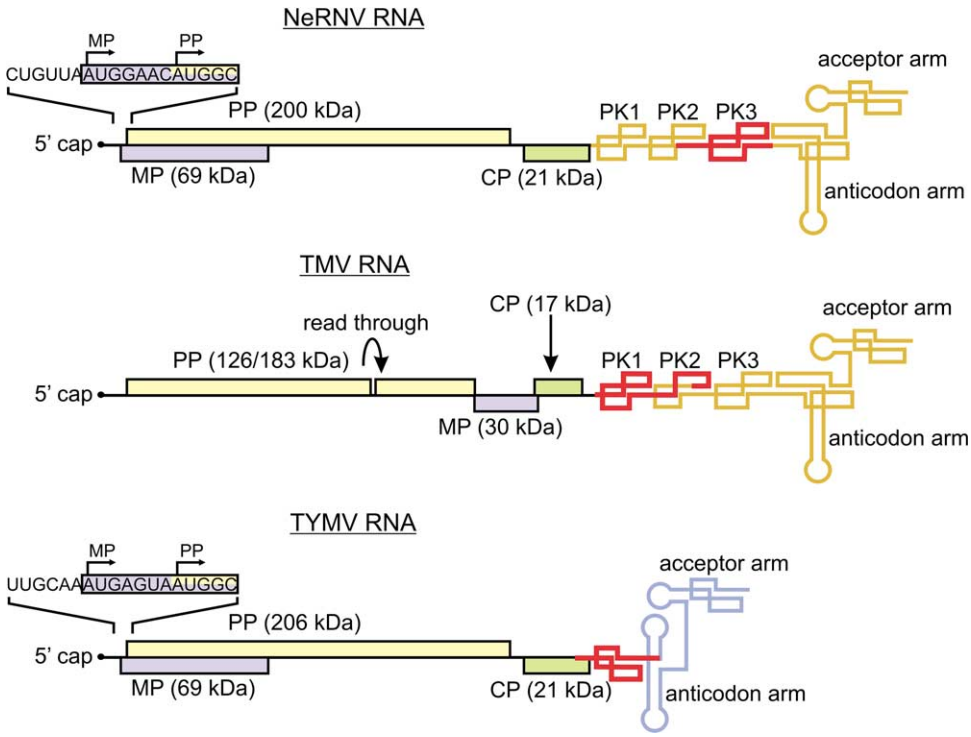

Figure 1. Genome organizations of NeRNV, TMV and TYMV RNAs. The viral-encoded proteins and their molecular mass are indicated (MP, movement protein; PP, replicase-containing polyprotein; CP, coat protein). For NeRNV and TYMV, the ORF encoding PP overlaps with the MP ORF (emphasis is given to the overlap). For TMV, the functional PP is synthesized by readthrough from the first ORF encoding a methyltransferase/helicase. For both NeRNV and TYMV, the CP, and for TMV both MP and CP, are translated *via* a different mechanism involving subgenomic RNAs. The 3′-UTRs are emphasized in the two-dimensional display and are not drawn to scale as compared to the entire genomic RNA. The red lines are the positions of oligonucleotide hybridization for RNase H cleavage; notice that in the case of TYMV, the oligonucleotide anneals partially to the CP-coding region. The different features of the 3′-UTRs, as well as the acceptor and anticodon arms together with the pseudoknot structures (PK), are indicated. The colouring of the 3′-UTRs highlights their structural relationships.

Here, we have investigated whether TLS^{His} can participate in translation, as was shown for TLS^{Val} and TLS^{Tyr}. For this purpose, we used two different plant viruses: the type member of Tobamoviridae, TMV, and the recently discovered Nemesia ring necrosis virus (NeRNV) that belongs to the Tymoviridae.[14] The latter is a chimera having a typical gene arrangement and 5′-untranslated region (UTR) of tymoviral RNAs (like TYMV), but a 3′-end folded into three consecutive pseudoknot structures (upstream pseudoknot domain, UPD) followed by a histidine-specific TLS as found only in tobamoviral RNAs[14] (see Figure 1). In other words, both TMV and NeRNV RNAs share a high level of similarity in their 3′-UTRs and strong differences in their 5′-ends. Specific histidylation occurs at the 3′-end of their TLSs,[6,14] and relies upon the presence of a nucleotide located within their pseudoknotted acceptor arm mimicking the G_{-1} histidine identity determinant of tRNA^{His}.[15] These different genome organizations and similar aminoacylation capacities allowed us to functionally dissociate the genomic environments of the viruses from the TLS^{His} properties in viral translation.

Search for histidine donation from viral His-RNA^{His} in protein synthesis

To search for an initiator role of TLS^{His} in viral translation, as was done for TLS^{Val} and TLS^{Tyr}, we first histidylated genomic TMV and NeRNV RNAs with [^{3}H]His in reactions catalyzed by yeast HisRS[16] (native RNAs were used instead of full-length, synthetic transcripts obtained by *in vitro* transcription, since the latter are inactive in *in vitro* translation). In both cases, up to 90% of the viral RNA molecules were histidylated. These [^{3}H]His-RNAs were used in cell-free, wheat-germ translation reactions as both templates and potential amino acid donors. As a positive control, an experiment using [^{3}H]Val-RNA^{TYMV} was performed in parallel. Figure 2 depicts the amounts of [^{3}H]His or [^{3}H]Val incorporation into TCA-precipitable material after incubation for 90 min. While only background levels of [^{3}H]His were observed in both TMV and NeRNV experiments, [^{3}H]Val was shown to be incorporated *via* a translation mechanism as demonstrated earlier.[12] From this experiment, we conclude that no TLS-mediated His donation occurs during

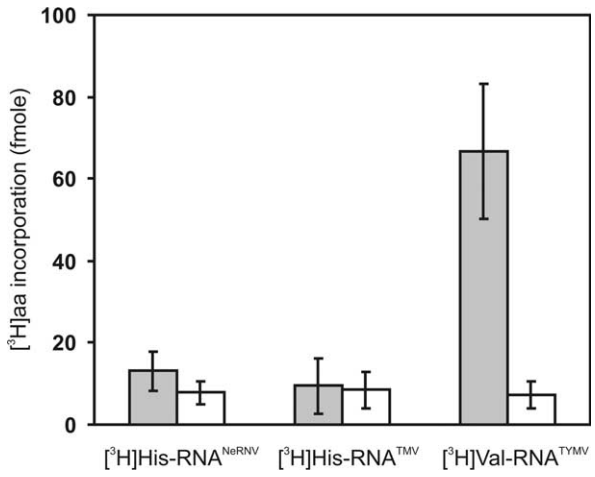

Figure 2. Comparison of amino acid incorporation into the viral protein products. [^{3}H]His-RNA^{NeRNV}, [^{3}H]His-RNA^{TMV} and [^{3}H]Val-RNA^{TYMV} were used at 50 nM as templates for *in vitro* translation in wheat-germ extract (Promega) in the presence of 1.1 mM unlabeled amino acids (grey bars) or in the presence of 1 mg/ml of cycloheximide (white bars). TMV RNA was a kind gift from P. Geoffroy; NeRNV and TYMV RNAs were isolated as described.[12,14] Valylation of RNA^{TYMV} was performed as described.[12] Histidylation reactions of RNA^{NeRNV} and RNA^{TMV} were conducted at 30 °C in an optimized medium for tRNA histidylation[16] (55 mM Tris–HCl (pH 7.5), 15 mM $MgCl_2$, 10 mM ATP, 2.5 mM glutathione, 30 mM KCl) with 10 μM L-[^{3}H]histidine and the required concentration of partially purified yeast HisRS). Incorporation of ^{3}H-labelled amino acids into protein products is expressed as fmol/100 μl reaction volume. The bars are averages of four independent experiments.

viral protein synthesis. In support of this statement, no incorporation of [^{3}H]His into protein products could be visualized by SDS-PAGE followed by autoradiography. To verify that biosynthesis of viral proteins is efficient, controls were conducted in the presence of [^{35}S]Met. Under such conditions, methionine incorporation into protein material occurs and thus verified that the three viral RNAs were active in translation (not shown).

Search for TLS^{His} participation in translation

Although we could not find any TLS-mediated His incorporation in proteins, the TLS^{His} structures themselves might be involved in translation. For TYMV, synthesis of the polyprotein was lost only after removal of the complete TLS.[12] For BMV, no TLS-mediated tyrosine incorporation was found, as in the present cases with TMV and NeRNV, even though TLS^{Tyr}, as such, was shown to be crucial for efficient translation of the viral proteins.[13]

To identify a putative other role of TLS^{His} in protein synthesis, we compared translation patterns using full-length RNAs and truncated viral RNA molecules deprived of their TLSs. Figure 3(a) shows the quantified and specific removal of the 3′ UTRs of the viral RNAs by means of deoxyoligonucleotide-directed RNase H cleavage. Removal of the TLSs from TMV and NeRNV RNAs had no detectable effect on translation efficiencies (Figure 3(b)). Since TLS cleavage was not complete, one could argue that the remaining, intact RNAs might be better competitors for recruiting ribosomes. Therefore, we also monitored translation of the viral RNAs at lower concentrations, ranging from 4.5–70 nM (the *in vitro* translation reactions were done with ~100 nM ribosomes), and we still could not detect any effect of TLS removal on TMV and NeRNV-RNA translation (not shown). Altogether, these experiments show the non-requirement of the TLS^{His} for viral translation. In a control experiment (Figure 3(b)), we show that deletion of the TLS from TYMV RNA abolishes polyprotein synthesis, a conclusion that confirms previous data.[12] This abrogated polyprotein translation could be restored by adding increasing concentrations of TLS^{Val}, whereas neither TMV nor NeRNV histidine-specific TLS was capable of doing so (not shown).

Idiosyncratic behaviour of TLSs in translation

We investigated the possible involvement of two TLS^{His} elements in translation initiation in their own and unique genomic contexts. This, and in order to uncouple the effect of TLS removal on translation from the effect on RNA replication, prompted us to perform these experiments *in vitro*. The 3′-UTR of TMV RNA has been reported to increase mRNA stability and translation efficiency of reporter mRNA constructs *in vivo*.[17,18] However, our results demonstrate that TLS^{His} does not participate in translation of TMV RNA, although we cannot exclude an ancillary function of TLS^{His} in the living cell. Because of the different genome organizations of TYMV and TMV RNAs with overlapping and non-overlapping open reading frames, respectively, the non-involvement of TLS^{His} in RNA^{TMV} translation could *a priori* be accounted for by the absence of such an overlap in the TMV genome. On the other hand, given the organizational resemblance between both TYMV and NeRNV RNAs, we expected a similar behaviour of both viral RNAs, regarding the participation of their TLSs in viral translation. However, this expectation was not fulfilled, since the NeRNV genome ensures TLS^{His}-independent polyprotein translation. We note that closer inspection of the TYMV and NeRNV RNAs reveals substantial sequence differences (45%).

To assess the putative involvement in TLS^{Val}-dependent polyprotein synthesis of the coding region of TYMV RNA, we made a construct composed of 548 nt from the 5′-end of TYMV RNA fused to 367 nt of its 3′-end (Figure 4). Removal of the internal coding region of TYMV

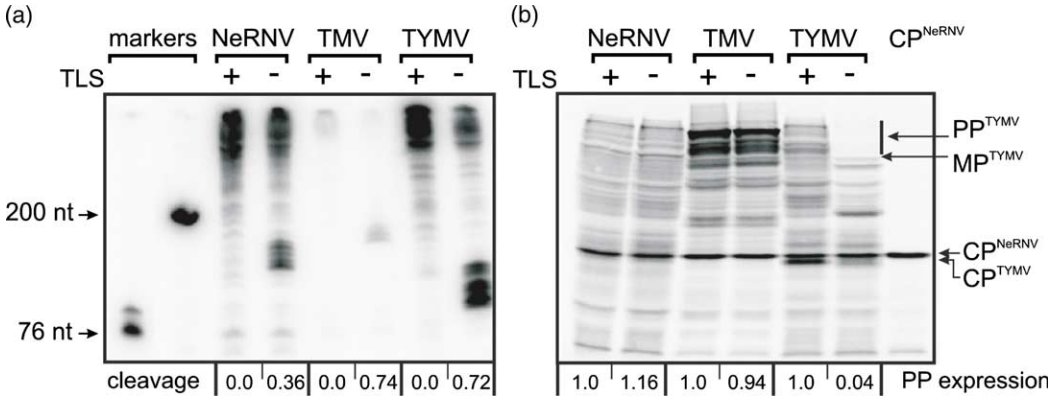

Figure 3. Effect of 3′-truncations on translation. (a) Autoradiogram visualizing RNA products after RN ase H treatment of the viral genomes. To follow the efficiency of TLS removal, viral RNAs were labelled at their 3′-ends with [α-^{32}P]ATP using yeast (ATP,CTP):tRNA nucleotidyl-transferase.[27] Subsequently, each 3′-labeled RNA (50 nM) was mixed with 1 μM RNA (subgenomic NeRNV RNA). Deoxyoligonucleotides (Invitrogen) complementary to 3′-TMV, TYMV or NeRNV RNA sequences (numbering in the 3′ to 5′ direction) 150–179, 86–122, 123–157, respectively, were annealed in a 100-fold molar excess to the (α-^{32}P)-labelled viral RNAs. Cleavage of the heteroduplexes and RNA purification was performed as described.[13] Control RNAs were treated as above but without the addition of deoxyoligonucleotides. The resulting RNAs with (+) or without (–) TLSs were separated by denaturing PAGE (8% (w/v) acrylamide) and visualized by phosphor imaging. Different size markers are shown (left), together with the quantification of the cleaved TLSs (bottom). The difference in RNA labelling is due to the fact that NeRNV and TYMV RNAs are encapsidated with a CCA3′ end, whereas isolated TMV RNA has a CC 3′-terminal sequence. The subgenomic RNA of NeRNV was synthesized by T7 transcription on a PCR fragment containing the CP gene and TLS^{His}. The PCR product was obtained by means of RT-PCR using primers 5′-GATTACGAATTCTAA TACGACTCACTATAGGGCTTCACTCATTTCAATAAC**ATG**GAAGAACTAAACCG-3′ (with CP start codon in bold) and 5′-TGGTTCCCTACCCGGGACAGGGGGAGATTCGAACTCC-3′. (b) Autoradiogram of an SDS-PAGE (12% (w/v) acrylamide) revealing the effects of TLS removal on the synthesis of viral proteins. Translation of the viral RNAs (50 nM) was done in wheat-germ extract, using [^{35}S]Met (0.40 μM, 43 TBq/mmol; MP Biomedicals, Irvine, CA) as described.[12] Full-length PP expression was normalized against CP^{NeRNV} (internal standard) and compared to normalized PP in the (+) lanes (bottom). In contrast to TMV and NeRNV, TYMV particles contain the subgenomic RNA for CP. As a consequence, CP^{TYMV} is visible in lanes loaded with TYMV translation products. Note that the reduced CP^{TYMV} synthesis in the last lane is due to the fact that the oligonucleotide for TLS^{Val} disruption anneals partially in the CP coding region. Cleavage efficiencies in (a) together with PP expressions in (b) are averages calculated from three independent experiments.

RNA abolishes the TLS^{Val} dependency of polyprotein translation. Instead, since mutation of either start codon increases translation of the other one, it is more likely that polyprotein becomes translated *via* a mechanism referred to as random ribosomal selection.[19] Apparently, shorter TYMV constructs do not use the authentic TLS-dependent translation process. This might explain why Dreher and co-workers did not find specific TLS^{Val}-induced translation when placing 5′ and 3′ regions of TYMV RNA around a luciferase reporter mRNA.[20] Instead, their constructs became translated *via* random ribosomal selection between the two different ORFs. Additionally, this implies that TLS^{Val}-mediated polyprotein synthesis is not (only) established by interactions of the 5′ and 3′-UTRs circularizing the TYMV RNA.[21,22]

In conclusion, it appears that the genome organization of a TLS-containing plant virus does not correlate with the amino acid donor function of its TLS in initiation of viral protein synthesis. In a more general perspective, when considering the behaviour of the three virus families, it appears that the phenomenon of TLS-mediated translation is inherent to the idiosyncratic architecture and amino acid identity of the TLSs. Indeed, TLS^{Val} and TLS^{Tyr} are active in TYMV and BMV RNA translation, respectively, whereas TLS^{His} is inactive in both tymoviral and tobamoviral RNA contexts. Dreher and colleagues made a large collection of synthetic hybrids composed of the genomic RNA of TYMV fused to heterologous TLSs.[23] The fittest viruses were obtained with TLSs from other valylatable tymoviruses. Interestingly, a TYMV construct in which the aminoacylation specificity of TLS^{Val} was changed to methionine, TLS^{Met}, yielded infectious virus.[24,25] Note that all the TLSs that interact with the ribosome and function in translation (TLS^{Val}, TLS^{Tyr} and TLS^{Met}) are those charged by class I aminoacyl-tRNA synthetases, whereas the inactive TLS^{His} is aminoacylated by a class II enzyme (both classes differ in the architecture and mechanism of the active site modules).[26] Whether this observation is fortuitous or has biological relevance remains an open question.

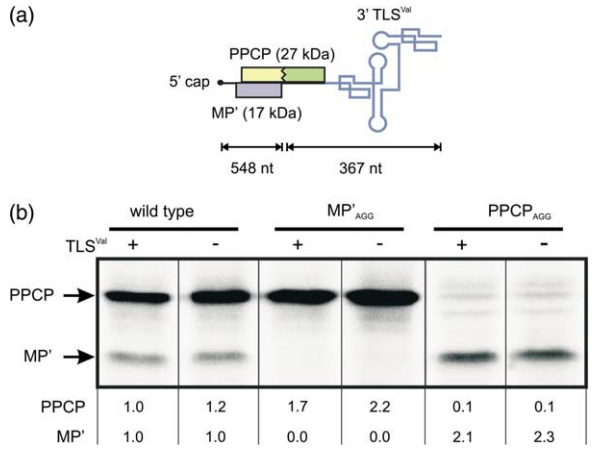

Figure 4. TLS^{Val} effect on translation of an abridged TYMV RNA. (a) Organization of the abridged TYMV RNA. This RNA was constructed by fusing the first 548 nt from the 5′-end with the last 367 nt of its 3′-end (the scheme is not drawn to scale). At the fusion point, two tandem stop codons were introduced into the MP frame, resulting in a sequence encoding MP′ of 17 kDa. In addition, the N-terminal 151 amino acid residues of PP became fused to the last 86 amino acid residues of CP, resulting in PPCP of 27 kDa. (b) Effect of TLS^{Val} on protein synthesis using three versions of abridged TMV RNAs. The Figure displays experiments conducted with wild-type RNA (short RNA fragment depicted in (a)), with MP'_{AGG} (short RNA mutated at the start codon for MP′) and with $PPCP_{AGG}$ (short RNA mutated at the start codon for PPCP). These RNAs were tested in the presence (+) or absence (−) of TLS^{Val} for *in vitro* translation in wheat-germ extract. In the case of −TLS, PCR products obtained with a nested 3′-primer excluding TLS^{Val} were used for *in vitro* transcription. Samples were separated by SDS-PAGE (15% (w/v) acrylamide) and quantified by phosphor imaging. In these experiments, the molar ratio of MP′ to PPCP is 1.5±0.3. The positions of PPCP and MP′ are indicated and their intensities, expressed as values relative to the wild-type construct in the presence of the TLS, are shown. Values are averages of three independent experiments.

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