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Aminoacylation identity switch of turnip yellow mosaic virus RNA from valine to methionine results in an infectious virus

(RNA virus replication/tRNA-like structure/RNA pseudoknot/tRNA identity/anticodon)

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ABSTRACT **The turnip yellow mosaic virus genomic RNA terminates at its 3′ end in a tRNA-like structure that is capable of specific valylation. By directed mutation, the aminoacylation specificity has been switched from valine to methionine, a novel specificity for viral tRNA-like structures. The switch to methionine specificity, assayed *in vitro* under physiological buffer conditions with wheat germ methionyl-tRNA synthetase, required mutation of the anticodon loop and the acceptor stem pseudoknot. The resultant methionylatable genomes are infectious and stable in plants, but genomes that lack strong methionine acceptance (as previously shown with regard to valine acceptance) replicate poorly. The results indicate that amplification of turnip yellow mosaic virus RNA requires aminoacylation, but that neither the natural (valine) specificity nor interaction specifically with valyl-tRNA synthetase is crucial.**

The genomes of a number of positive-strand RNA plant viruses contain 3′-terminal structural elements that resemble transfer RNAs. These tRNA-like structures (TLSs) are substrates in interactions with aminoacyl-tRNA synthetases, translational elongation factors EF-Tu and EF-1α, and (CTP, ATP):tRNA nucleotidyltransferase (1, 2). Viral RNAs are known to be aminoacylated *in vivo* during the infection cycle (3). Three aminoacylation specificities have been reported for viral RNAs—valine, tyrosine, and histidine—typified by turnip yellow mosaic virus (TYMV), brome mosaic virus, and tobacco mosaic virus RNAs, respectively (1, 2).

The major identity elements that determine specific aminoacylation of TYMV RNA with valine are located in the anticodon loop of the viral TLS (Fig. 1; ref. 4), as they are for *Escherichia coli* $tRNA^{Val}$ (5, 6). Mutation of these identity elements results in drastic loss of valine acceptance (4). Such mutant genomic RNAs show defective replication, and we have observed a correlation between valylation assayed *in vitro* and viral accumulation in plant cells (7, 8). The presence of identity elements that confer efficient valine acceptance is thus a clear requirement for plant infectivity in the TYMV system. Based on investigations with brome mosaic virus (9, 10) and more recently with TYMV (11, 12), the chief role of the TLS is thought to be in promoting minus-strand synthesis, the first stage of the replication cycle, rather than in promoting genome stability or translational expression (10, 12).

Our previous studies on the role of aminoacylation in the replication cycle (7, 8) could not distinguish between two roles for the identity elements, either a requirement for efficient interaction with valyl-tRNA synthetase (ValRS) to sequester this host protein into a minus-strand initiation complex, or a requirement that a viral RNA becomes valylated to be an efficient template for replication. To address this question, we were interested to determine the effect of altered aminoacylation specificity on the replication of TYMV RNA. The ability to switch the identities of *E. coli* tRNAs between valine and methionine with sequence changes in the anticodon alone (5) suggested that an identity switch to methionine should also be feasible for TYMV RNA. We report here that the aminoacylation specificity can indeed be changed from valine to methionine, and that the resultant genomes are highly infectious.

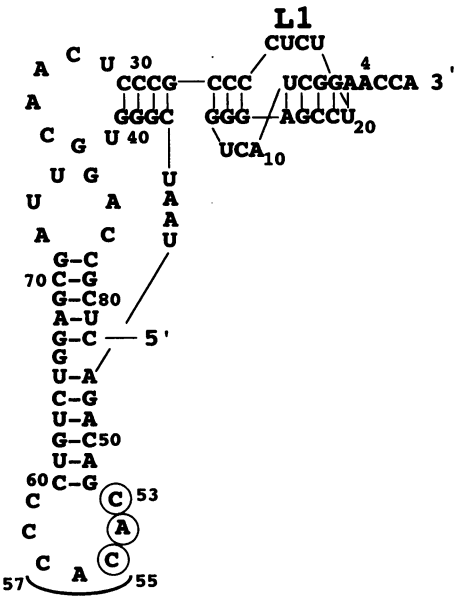

FIG. 1. tRNA-like structure of TYMV RNA. The 3′ 82 nucleotides of the 6.3-kb genomic RNA are drawn in the three dimensional conformation most resembling canonical tRNA. The anticodon triplet is underlined (nucleotides 57–55) and the 3 nucleotides mutated in the anticodon loop are circled. Loop L1 of the pseudoknot forming the aminoacyl acceptor stem is marked. Nucleotides are numbered from the 3′ end.

MATERIALS AND METHODS

Plant Material and Virus Stocks. Turnip (*Brassica rapa* cv. Just Right) and Chinese cabbage (*Brassica pekinensis* cv. Spring-A1) were grown in an environmentally controlled chamber at 21°C under 16-hr daylength. pTYMC is a cDNA-based plasmid clone from which infectious RNA can be prepared by *in vitro* transcription (13). TYMC is the cloned Corvallis strain of TYMV (14). Anticodon mutants TYMC-U55/A53 and TYMC-U55/C54/A53 have been described (7).

TYMC and mutant derivative viruses were purified from leaves by precipitation with polyethylene glycol according to

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Abbreviations: TLS, tRNA-like structure; TYMV, turnip yellow mosaic virus; ValRS, valyl-tRNA synthetase; MetRS, methionyl-tRNA synthetase; EF, elongation factor.

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the procedure of Lane (15). Virion RNAs were purified by double extraction with phenol/chloroform (1:1), followed by ethanol precipitation.

Construction of Mutant Viral RNAs. Mutations in the acceptor stem pseudoknot of the TLS were produced via PCR-mediated oligonucleotide-directed mutagenesis. Deoxyoligonucleotides that were complementary to TLS nucleotides 1–35, but had internal deletions, were used in PCR together with an upstream primer containing the T7 RNA polymerase promoter. These primers generated transcriptional templates (16) encoding the pseudoknot L1 deletions in combination with a CCCAUCA anticodon loop (*Hin*dIII-linearized pTYMC-U55/C54/A53 was used as the PCR template). Transcription of the amplification products yielded RNAs [264 nucleotides long for the wild-type (wt) sequence] with correct 3′-CCA termini directly compatible with aminoacylation (16, 17). Such RNAs representing 3′ fragments of the viral RNA are referred to as TY-wt, TY-U55/C54/A53 (L1 = UU), etc.

The same mutations were transferred into the genome-length clone pTYMC by subcloning mutant *Sma*I^{6062}–*Hin*dIII$^{3'}$ fragments. These mutant sequences were generated by PCR on the same template as above, using an upstream oligonucleotide priming at nucleotide 6014 on the TYMC genome, and downstream mutagenic primers analogous to those used above, except that a *Hin*dIII site was positioned at the 5′ end of the primer (this positions a *Hin*dIII site immediately adjacent to the 3′ end of the genomic sequence). A control set of mutants containing the L1 mutations only was constructed by PCR as above, except that linearized pTYMC was used as the PCR template. Genomic RNAs capped at their 5′ ends were made with T7 RNA polymerase from DNA linearized with *Hin*dIII as described (13).

Preparation of tRNA^{Met} Transcripts. A gene corresponding to the sequence of wheat germ tRNA^{Met} (18) was constructed by annealing two synthetic deoxyoligonucleotides, a 57-mer comprising the sequence of a T7 promoter fused to nucleotides 1–40 of the tRNA, and a 46-mer comprising the antisense strand of nucleotides 32–77. The annealed oligomers were made double stranded with T7 DNA polymerase (United States Biochemical), and the product was cloned into the *Sma*I site of pUC18, generating a *Bst*NI site coincident with the 3′ end of the tRNA sequence. Transcripts with 5′ monophosphate termini were made from *Bst*NI-linearized plasmid DNA with T7 RNA polymerase in the presence of GMP (19). The transcripts have the anticodon loop sequence CUCAUAA; the modified tRNA has the anticodon loop sequence C-U-(2′-*O*-methylcytidine)-A-U-{*N*-[(9-ribofuranosyl purin-6-yl)*N*-methylcarbamoyl]threonine}-A (18).

Methionylation Studies. RNAs were aminoacylated at 30°C by a methionyl-tRNA synthetase activity that was partially purified from wheat germ (4) and used at 0.13 μg protein/ml. Buffer IV used for methionylation assays contained 30 mM Hepes·KOH (pH 7.5), 100 mM KOAc, 2.5 mM $Mg(OAc)_2$, 1.5 mM ATP, and 10 μM [^{35}S]methionine (12 or 30 Ci/mmol; 1 Ci = 37 GBq). Kinetic parameters were determined by assaying initial rates over RNA concentrations between 5 and 1000 nM, and analyzing data on double-reciprocal Lineweaver–Burk plots with the ENZFITTER program (BioSoft, Milltown, NJ). Assays were conducted as previously described (4).

Inoculation of Protoplasts and Plants, and Analysis of Viral Products. Protoplasts (4×10^5) released from turnip leaves were inoculated with 5 μg of transcript RNAs (20) and incubated for 40 hr at room temperature, under constant illumination. At least three independent transcription products corresponding to each viral construct were assayed in at least two independent experiments with separately prepared protoplasts to obtain average accumulation data.

Three to 4-week-old Chinese cabbage plants were inoculated with either lysates of infected protoplasts (20) or genomic transcripts (2.5 μg) in 50 μl of 50 mM glycine, 30 mM K_2HPO_4 (pH 9.2), 1% (wt/vol) celite, and 3 mg/ml bentonite. Virions were passaged by inoculating seedlings with purified virions (5 μg) in 50 μl of 0.1 M potassium phosphate (pH 7.0) and 1% (wt/vol) celite.

Total cellular RNA was isolated from protoplasts, glyoxalated, and subjected to quantitative Northern blot analysis as described (12). The hybridization probe consisted of a minus-sense *in vitro* transcript complementary to nucleotides 5708–5988 in the coat protein coding region of the TYMV genome. Coat protein accumulation was monitored by Western blot analysis with detection by enhanced chemiluminescence (ECL, Amersham; ref. 12).

The TLS regions of progeny virion RNAs obtained from infected plants were sequenced after polyadenylylation, reverse transcription, and PCR amplification as described (12).

RESULTS

TYMV RNAs with Methionine Identity. We had previously shown that a TYMV RNA variant with the two anticodon loop mutations C55 → U and C53 → A, which result in a CCCAUAA anticodon loop similar in sequence to that of higher plant tRNA^{Met} (18), is virtually devoid of valine identity (4) and is unable to amplify in plant cells (7). Preliminary experiments with wheat germ methionyl-tRNA synthetase (MetRS) indicated that this RNA, as well as a second mutant with a CCCAUCA anticodon loop, had acquired a high level of methionine identity when assayed in low ionic strength nonphysiological buffers. Thus, V_{max}/K_M values for methionylation with wheat germ MetRS in 25 mM Tris·HCl (pH 8.0), 2 mM $MgCl_2$, 0.1 mM spermine, 1 mM ATP, and 10 μM methionine were 0.31 and 0.88 for TY-U55/A53 and TY-U55/C54/A53 RNAs, respectively, relative to that for a wheat germ tRNA^{Met} transcript. Clearly, as for *E. coli* (5) and yeast (21) MetRS, the methionine identity elements recognized by wheat germ MetRS are concentrated in the anticodon loop. Surprisingly, however, we discovered that methionylation of the viral RNAs, but not of tRNA^{Met}, was inefficient in a higher ionic strength (IV) buffer (Table 1, column L1 = CUCU) designed to approximate conditions found *in vivo* (conditions that give similar translational fidelity in initiation codon usage between *in vitro* translation and *in vivo* expression; ref. 22). This indicated that TYMC-U55/A53 and TYMC-U55/C54/A53 RNAs would probably not be methionylated *in vivo*, and that these were ambiguous mutants for studying the effect of altered aminoacylation.

The acceptor stem pseudoknot (Fig. 1) seemed the most likely location in the TYMV TLS where additional mutations would permit a full switch to methionine acceptance under physiological conditions. The pseudoknot is a feature unique to the viral RNA, distinguishing it structurally from tRNA^{Met}.

Table 1. Kinetics of methionylation of TYMV RNA mutants and tRNA^{Met} by wheat germ MetRS in physiological buffer conditions

		TY-U55/C54/A53				
	tRNA^{Met}	L1 = CUCU	L1 = CUU	L1 = CU	L1 = UU	L1 = C
K_M, nM	161	103	97	73	70	62
V_{max}	34.1	0.4	1.7	2.0	4.2	9.1
V_{max}/K_M	0.21	0.004	0.02	0.03	0.06	0.15
Rel. V_{max}/K_M	1.00	0.017	0.083	0.13	0.28	0.70

An unmodified tRNA^{Met} transcript with a 5′-GMP terminus was prepared from a cloned synthetic gene representing wheat germ tRNA^{Met} (18). The mutant TYMV RNAs (TY-U55/C54/A53 and its derivatives with shortened L1 loops) corresponded to the 3′ 264 nucleotides of the TYMV genome (17). L1 = CUCU refers to the wild-type L1 sequence. Aminoacylation reactions were in IV buffer at 30°C. V_{max} values are in arbitrary units.

It is also at the end of the acceptor stem, where at least one aminoacyl-tRNA synthetase (glutaminyl-tRNA synthetase from *E. coli*) imposes significant structural alterations on its substrate during catalysis (23). High ionic strength conditions could make a structural rearrangement involving the pseudoknot energetically unfavorable, but this should be mitigated by destabilizing the pseudoknot through sequence changes. Mans *et al.* (24) have shown that shortening pseudoknot loop L1 (Fig. 1) decreases but does not abolish pseudoknot stability; RNA with L1 as short as a single C could be valylated (24).

Four derivatives of the triple mutant TY-U55/C54/A53 RNA, which methionylated at slightly higher rates than the U55/A53 double mutant (see above), were constructed, and the kinetics of their methionylation by wheat germ MetRS were compared with that for higher plant $tRNA^{Met}$ (unmodified transcript) (Table 1). Stepwise L1 shortening resulted in progressively increased V_{max}, with little change in K_M. The most actively methionylating mutant (TY-U55/C54/A53/L1 = C) had a $V_{max}/K_M = 0.7$ relative to $tRNA^{Met}$. Thus, with a combination of mutations in the anticodon loop and the 3′-pseudoknot, it has been possible to achieve a switch from valine to highly efficient methionine charging for TYMV RNA in physiological buffer conditions.

Infectivity of TYMV RNAs with Methionine Identity. The combined anticodon and pseudoknot mutations represented in Table 1 were transferred into genomic RNAs to permit analysis of their effect on viral replication. In parallel, a control set of genomic RNAs carrying the pseudoknot mutations in the wild-type anticodon context (i.e., RNAs with valine identity) was studied. Viral accumulations in turnip protoplasts were assayed by detection of coat (virion) protein in Western blots and of the 6.3-kb genomic RNA in Northern blots. Infectivity in Chinese cabbage plants was followed by visually scoring disease symptoms and by determining virus yield by direct virion purification. Mutants TYMC-U55/C54/A53 (L1 = CUU, CU, and UU), which could be methionylated with V_{max}/K_M values ≥0.1 relative to $tRNA^{Met}$ (Table 1), were found to accumulate to levels within 3- to 4-fold of wild type in protoplasts and in plants (Fig. 2). The shortest L1 mutation (L1 = C) was not compatible with a high level of replication, in either the valine or methionine identity background. And, as previously reported (7), TYMC-U55/C54/A53 RNA with a wild-type L1 (L1 = CUCU), which was expected to become at best poorly methionylated *in vivo* (Table 1), accumulated to very low levels in protoplasts, and was not infectious in plants. In contrast, RNAs with L1 = CUU, CU, or UU were infectious, whether they had valine or methionine identity, although the accumulations of viral products from genomes capable of methionylation were lower than for the corresponding RNAs with valine identity (Fig. 2). The systemic symptoms of the methionylatable mutants in Chinese cabbage plants were indistinguishable from wild type, but were delayed in their appearance.

Analysis of progeny virion RNAs isolated from plants inoculated with RNAs with methionine identity verified that RNAs capable of methionylation had replicated, excluding the possibility that there had been reversion to wild-type or pseudo wild-type (valylatable) genotypes. All progeny virion RNAs, even after several passages, could be methionylated *in vitro*, under both low and high ionic strength conditions, comparably to corresponding transcript RNAs (Fig. 3), but could not be valylated (not shown). Direct sequence analysis of the TLS after reverse transcription–PCR from second passage virion RNAs showed that a methionine-specific anticodon loop sequence was retained in all cases, although there were minor sequence changes in some instances. The following sequence changes were observed: progeny RNA from one plant inoculated with TYMC-U55/C54/A53 (L1 = UU) was unchanged; progeny RNAs from four plants inoculated with TYMC-U55/C54/A53 (L1 = CU) had acquired the changes L1 = UU in one plant, C54 → A (wild type) in two plants, and no change

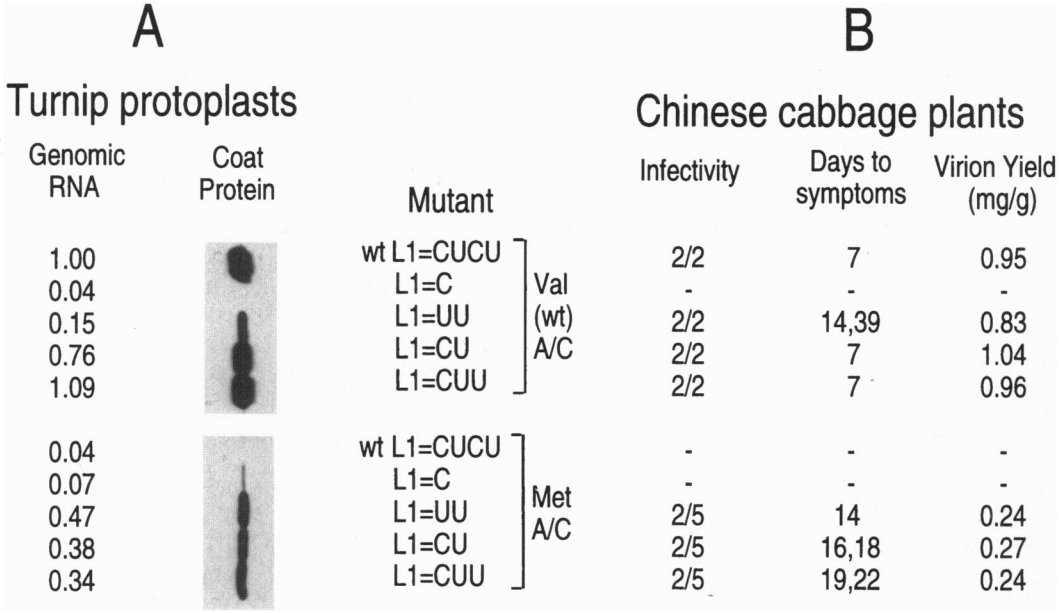

FIG. 2. Replication of valine- and methionine-charging TYMV variants in protoplasts and plants. The mutants identified in the central column have the same L1 pseudoknot loop mutations in either the wild-type TYMC context, indicated as Val A/C (A/C, anticodon), or the TYMC-U55/C54/A53 context, indicated as Met A/C. The wild-type (wt) sequence of loop L1 is CUCU. (*A*) Turnip protoplasts were inoculated with 5′-capped genomic transcript RNAs, and viral products were analyzed in extracts of protoplasts harvested 40 hr after inoculation. Genomic RNA accumulations were quantitated relative to wild type via Northern blots, and coat (virion) protein accumulations were studied with Western blots. (*B*) Infectivity in Chinese cabbage plants was tested by inoculation with lysates from protoplasts analyzed in *A*. The yield of virions, prepared from symptomatic, noninoculated leaves, is reported (mg virus/g fresh weight of leaf). The results of visual observation of the progress of infection are also given: the Infectivity column reports the number of plants visibly infected/number of plants inoculated, and the Days to symptoms column reports the number of days from inoculation to symptom appearance (numbers separated by commas reflect different timing in the two symptomatic plants). Errors in RNA and virion yield are about 20%.

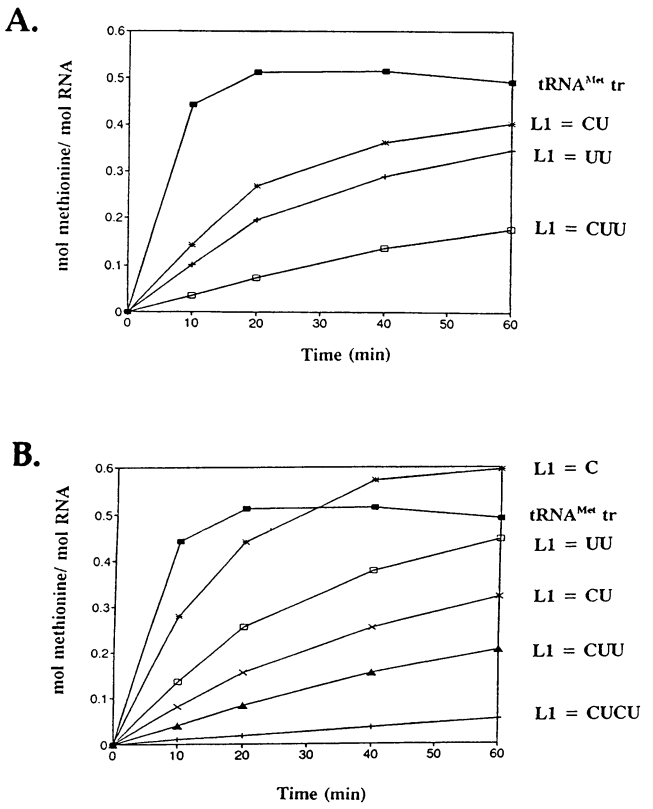

FIG. 3. Methionylation *in vitro* of progeny virion RNAs. In *A*, progeny virion RNAs (0.1 μM) recovered from plants inoculated with TYMC-U55/C54/A53(L1 = CU, UU, and CUU) RNAs were aminoacylated in IV buffer, with $tRNA^{Met}$ transcript as a control. In *B*, the full set of L1 derivatives of TY-U55/C54/A53 transcript RNA were methionylated under the same conditions.

in the fourth plant that yielded far less virus; progeny RNAs from four plants inoculated with TYMC-U55/C54/A53 (L1 = CUU) had acquired no changes in two cases, and an A68 → G mutation in the other two.

DISCUSSION

Methionine: A Novel Aminoacylation Specificity for Pseudoknotted Viral RNAs. Our experiments have altered the aminoacylation specificity of a viral TLS, creating a specificity (methionine) that has not been observed among natural viral RNAs. The valine to methionine identity switch expands to four the number of aminoacyl-tRNA synthetases that are known to be capable of efficiently aminoacylating a viral TLS, and both classes (25) of this enzyme family are represented (class I, valine, tyrosine, and methionine; class II, histidine). The pseudoknotted acceptor stem design of the plant viral TLSs therefore does not necessarily limit aminoacylation to the three naturally occurring specificities, and viral TLSs with previously unknown aminoacylation specificities may be discovered in the future. However, the need to introduce mutations into the acceptor stem pseudoknot to achieve efficient methionylation under physiological conditions suggests that pseudoknots of different stability or design may be appropriate for aminoacylation by different aminoacyl-tRNA synthetases.

In the case of charging by wheat germ MetRS, the TYMV RNA pseudoknot appears to be a structural impediment to catalysis, rather than the site of identity or of recognition elements. The improved catalysis resulting from a shortened loop L1 and its expected pseudoknot destabilization, is almost exclusively due to improvement in V_{max} rather than K_M (Table 1). The relevant effect of the L1 mutations may be to destabilize the terminal base pair (A5–U20) and/or permit a hair-pinning of the 3′-ACCA terminus. Such effects are known from the *E. coli* glutaminyl-$tRNA^{Gln}$/glutaminyl-tRNA synthetase cocrystal (22) and appear to control the efficiency of *E. coli* initiator $tRNA^{Met}$ as a substrate for methionyl-tRNA transformylase (26, 27). Similar requirements have not previously been reported for MetRS.

Strong methionine identity elements are clearly located in the anticodon loop. Nucleotides 55 and 53 (3′ anticodon nucleotide and 3′ nucleotide of the anticodon loop; Fig. 1) together control the valine and methionine identities in TYMV RNA in a mutually exclusive way (ref. 4 and Table 1). This distribution of methionine identity elements in the anticodon loop is similar to that in the yeast system (21) and distinct from the situation in *E. coli*, where the 3′ nucleotide of the anticodon loop does not appear to be involved in distinguishing valine and methionine identities (5).

Aminoacylation and the Replication of TYMV RNA. The primary motivation for altering the aminoacylation specificity of TYMV RNA was to test whether the valine specificity, or an interaction with ValRS, was crucial in the TYMV system. Previous studies had already shown that mutations that decreased the valine acceptance of TYMV RNA result in decreased viral amplification (7). We have now observed that viral amplification can be rescued in nonvalylatable mutants by mutations that confer efficient aminoacylation with methionine. The resultant genomes are infectious to plants (Fig. 2). These results demonstrate that the normal valine specificity of TYMV RNA is not crucial. Further, since MetRS and ValRS are highly diverged enzymes (28), and since MetRS is dimeric (29) while ValRS is monomeric in plants (30), it seems inconceivable that MetRS could replace ValRS in specific interactions with the replicational machinery. Thus, we conclude that the requirement for the presence of strong identity elements, either valine or methionine, in replicating TYMV RNA is due to a requirement for aminoacylation.

Since the role of the TYMV TLS is most likely in replication (11, 12), it appears that the efficient recruitment of a genomic RNA as a template for replication in the TYMV system requires that the RNA be aminoacylated. Aminoacylation with either valine or methionine is acceptable. The requirement for aminoacylation may be via interaction of a viral replication protein with the 3′ terminus, or alternatively, it may reflect a need for interaction with elongation factor EF-1α, which binds aminoacylated tRNAs and is known to bind valylated TYMV RNA (31). Interestingly, EF-Tu, the bacterial homologue of EF-1α, is a subunit of the replicase of the RNA bacteriophage Qβ (32), and an analogous role for EF-1α in the replication of viruses such as TYMV has been postulated (1). However, EF-1α has not been detected in partially purified extracts with TYMV replicase activity (31, 33), suggesting that if EF-1α is indeed involved in the replication of TYMV RNA, it may not be as an integral subunit of the replicase. Indeed, the role of aminoacylation, and the EF-1α interaction that may be expected to follow from aminoacylation, may conceivably be transient, perhaps playing a role in the initial specification of a genomic RNA as a template, which might then undergo multiple rounds of replication without the requirement for aminoacylation or EF-1α interaction.

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