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Review

Novel features in the tRNA-like world of plant viral RNAs

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Abstract. tRNA-like domains are found at the 3′ end of genomic RNAs of several genera of plant viral RNAs. Three groups of tRNA mimics have been characterized on the basis of their aminoacylation identity (valine, histidine and tyrosine) for aminoacyl-tRNA synthetases. Folding of these domains deviates from the canonical tRNA cloverleaf. The closest sequence similarities with tRNA are those found in valine accepting structures from tymoviruses (e.g. TYMV). All the viral tRNA mimics present a pseudoknotted amino acid accepting stem, which confers special structural and functional characteristics. In this review emphasis is given to newly discovered tRNA-like structures (e.g. in furoviruses) and to recent advances in the understanding of their three-dimensional architecture, which mimics L-shaped tRNA. Identity determinants in tRNA-like domains for aminoacylation are described, and evidence for their functional expression, as in tRNAs, is given. Properties of engineered tRNA-like domains are discussed, and other functional mimicries with tRNA are described (e.g. interaction with elongation factors and tRNA maturation enzymes). A final section reviews the biological role of the tRNA-like domains in amplification of viral genomes. In this process, in which the mechanisms can vary in specificity and efficiency according to the viral genus, function can be dependent on the aminoacylation properties of the tRNA-like domains and/or on structural properties within or outside these domains.

Key words. Plant RNA viruses; tRNA-like structures; pseudoknot; aminoacylation identities; RNA replication.

The intriguing tRNA-like structures

The discovery of RNA domains, differing in folding from a tRNA cloverleaf but mimicking a canonical tRNA function, such as aminoacylation [1, 2], was a puzzling surprise, especially when it was shown that these structures are not involved in translation [3, 4]. The first characterized tRNA-like domains were found at the 3′ extremities of genomic RNAs from plant viruses (reviewed in [5]), and it is now accepted that one of their roles lies in replication (see below). Today, many mimics recapitulating structural and functional features of tRNAs have been discovered in nature, all participating in specific metabolic pathways other than ribosome-dependent protein synthesis [6]. Often, these mimics are substrates of aminoacyl-tRNA synthetases and are involved in processes such as translational regulation of gene expression [7, 8], intron-splicing events [9] and tagging of abnormal proteins for proteolysis [10]. Although the biological significance of these mimicries is not well understood, one can hypothesize that their origin is ancient [6] and that their study sheds light on evolutionary links between translation, replication and other metabolic pathways. In this review we will restrict our focus to tRNA-like structures found in genomic RNAs from plant viruses and emphasize the newest structural and functional data.

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New viruses and new viral genera possessing tRNA-like structures

In the 1970s and 1980s, the discovery of tRNA-like structures in plant viruses followed the discovery of their aminoacylation properties. This was the case with TYMV RNA, first shown to be specifically valylated [1, 2] before its structural mimicry with tRNA was elucidated (fig. 1A, B). This was also the case with the tRNA-like structures of BMV [13] (fig. 2) and TMV [15] (fig. 3) RNAs (for reviews see [5, 16–18]). A more rational search of such structures came later with the development of sequencing facilities and of software allowing genome analysis and RNA structure prediction, based notably on similarities with all or part of already known tRNA-like structures [19–22]. This reversed procedures for tRNA-like searches, and when viral RNA genomes were sequenced, tRNA-like structures could be identified before any functional study. This was the case for new members of the tymovirus genus such as ELV [23], where a structure partially resembling that of the TYMV tRNA-like domain was found, or for the furoviruses and the related pomoviruses, where a series of tRNA-like structures, also resembling that of TYMV, could be predicted (e.g. [24–27]). In the case of tobamoviruses, tRNA-like structures that strikingly mimic that of TMV could be predicted for ORSV-cy [28] and cr-TMV [29]. The modeling approach combined with phylogenic comparisons also

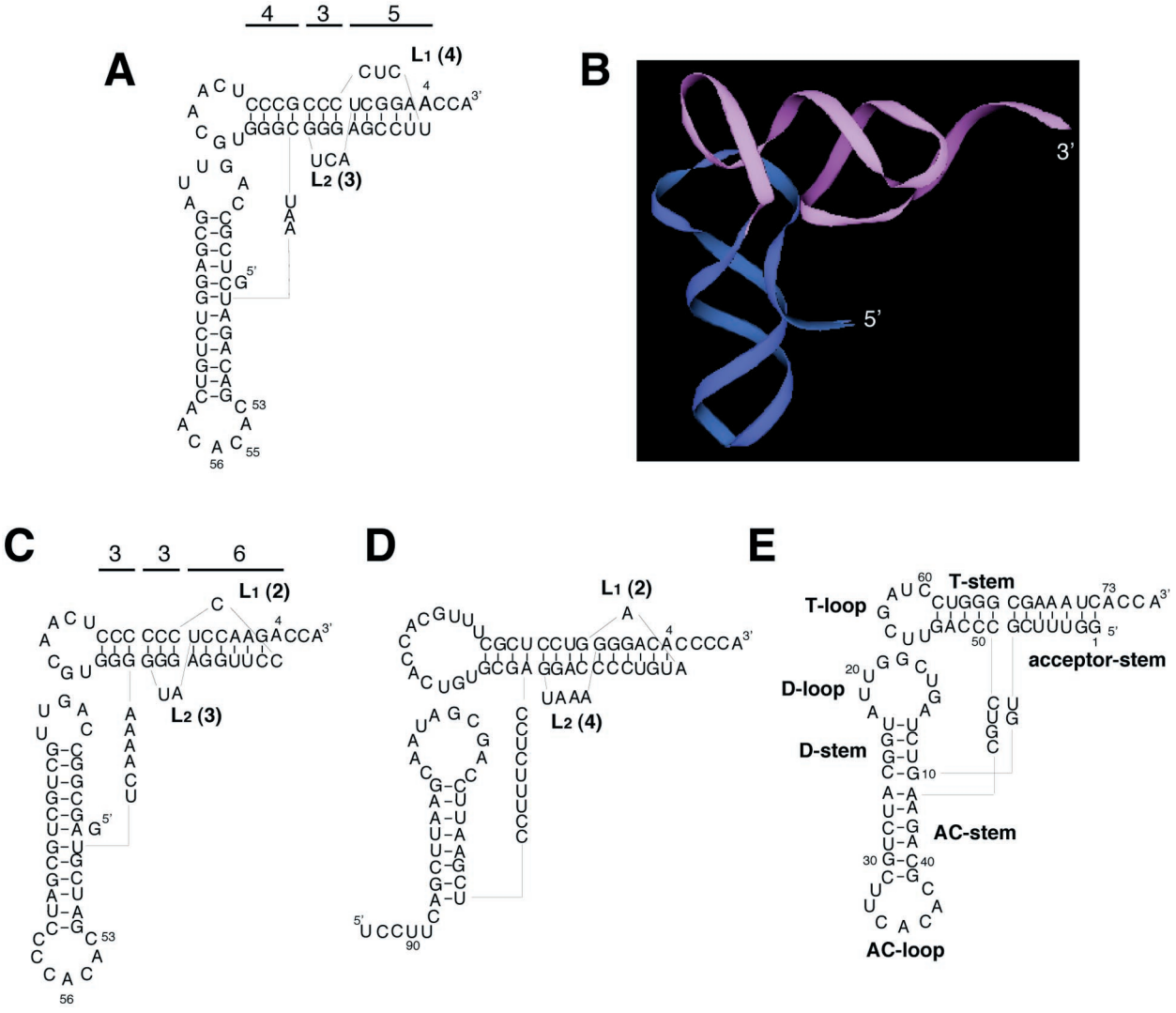

Figure 1. The tRNA-like structures from two tymoviral RNAs and one furoviral RNA and comparison with canonical tRNA. (*A*) Secondary and (*B*) tertiary model of the valylatable tymoviral TYMV tRNA-like structure; (*C*) 2D models of the valylatable furoviral PMTV2 tRNA-like structure and (*D*) of the non-valylatable tymoviral ELV 3′-end RNA; (*E*) folding of canonical yeast $tRNA^{Val}$ (for sequence data on tRNAs see [11]). The length of the L_1 and L_2 loops is indicated in brackets. Note that the first nucleotide of L_1 loop faces the discriminator position 4. The acceptor helix of the valylatable tRNA-like structures (*A* and *C*) is formed by the stacking of three helical segments whose number of base pairs is explicitly given on the top of the molecules. The 3D model in (*B*) has been generated by DRAWNA [12].

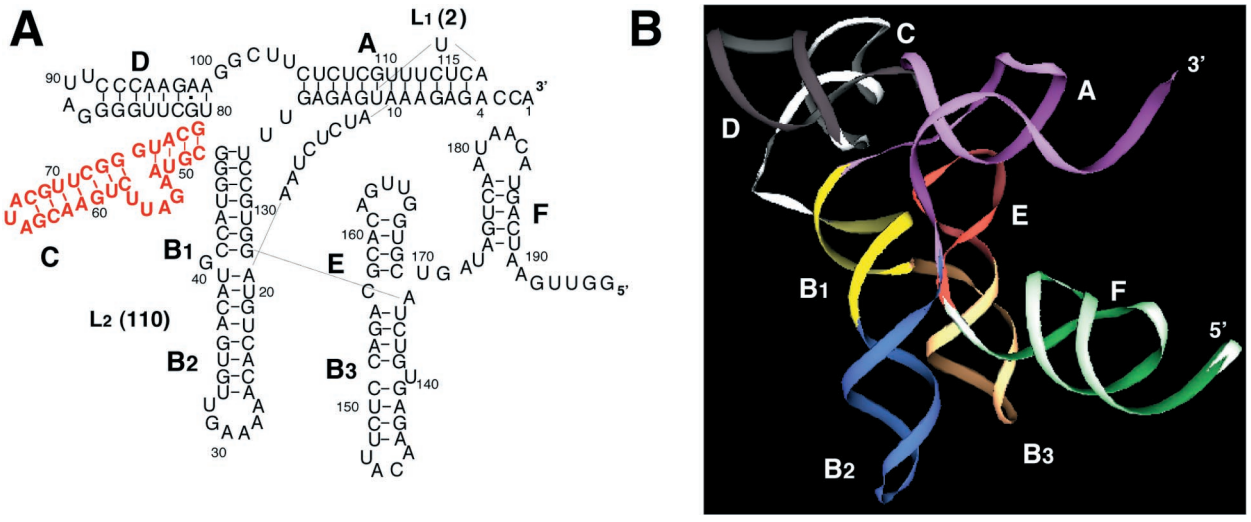

Figure 2. (*A*) Secondary model of the tyrosylable BMV tRNA-like structure. Length of the L_1 and L_2 loop is indicated in brackets. Note that the first nucleotide of the L_1 loop faces the discriminator position 4. The different domains (A–F) are explicitly indicated. Domain C, for which a high-resolution NMR structure is available [14], is highlighted in red (see text for details). (*B*) Tertiary model of the BMV tRNA-like structure generated by DRAWNA [12].

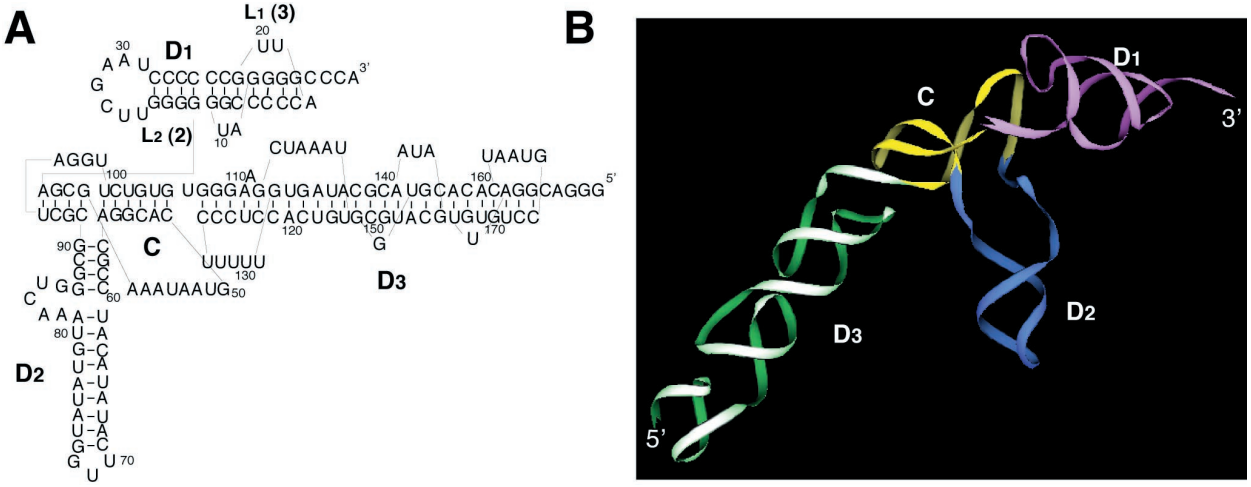

Figure 3. (*A*) Secondary model of the histidylable TMV tRNA-like structure. The length of the L_1 and L_2 loop is indicated in brackets. Note that the first nucleotide of the L_1 loop faces the discriminator position 4. The different domains (C–D3) are explicitly given. (*B*) Tertiary model of the TMV tRNA-like structure generated by DRAWNA [12].

led to a new perception of the structure of the AMV RNA 3′ end [30]. Long considered as formed by a series of hairpins, this RNA can also adopt a conformation that includes a pseudoknot very close to that found in the tRNA-like structure of bromoviral RNAs. These findings are of importance, because the increased number of RNAs possessing similar properties may permit a better understanding of the tRNA mimicry. According to this view, the common and differential functional properties of the tymoviral and the newly discovered furoviral tRNA-like structures have been discussed [31, 32]. Further, based on partial sequence similarities of the 3′ end of the STMV RNA with the 3′ end of TMV RNA and of phylogenetic relationships between RNAs from the tobamoviridae genus, a secondary structure for the 3′ noncoding region of STMV RNA could be proposed [33]. Such structural and functional comparisons will certainly increase with the growing number of tRNA-like structures that remain to be discovered in viral RNAs.

New secondary structures

All the known RNAs encompassing tRNA-like domains originate from seven different plant virus genera, namely the tymo-, tobamo-, tobra-, bromo-, cucumo-, hordei- and

furoviruses. In this section, emphasis is given only to the newly discovered functional tRNA-like structures, namely those present in RNAs from furoviruses or furo-like viruses (pomoviruses and pecluviruses) and in STMV RNA. To facilitate understanding of these tRNA mimics, we summarize the structural background concerning canonical tRNAs. These molecules fold into a characteristic L-shape with the amino acid acceptor end and the anticodon at both extremities of the fold. The amino acid acceptor branch (one branch of the L) is composed by the stacking of 12 bp (7 from the acceptor stem plus 5 from the T-stem) and the anticodon branch, consisting of 10 bp originating from the stacking of the D-stem and the connecting 26–44 pair (5 bp in total) over the anticodon stem (5 bp) (see fig. 1E, and [34]). Sequences of nearly all tRNA-like domains known to date can be arranged in two-dimensional (2D) folds reminiscent of the L-shaped architecture of tRNA [35, 36], but with deviations from the standard molecule. The first model of a tRNA-like structure was that from the 3′ end of TYMV RNA [37], and it revealed unambiguously the expected L-shaped architecture (fig. 1A, B). In all these RNAs, the presence of a pseudoknot is required to build the equivalent of the amino acid acceptor branch [38]. Thus, for example in TYMV RNA, the stacking of the two helical segments of the pseudoknot (5 and 3 bp) and of a third segment (4 bp) closed by a T-loop mimic leads to a structure in which the separate helical segments form a single continuous helix mimicking the 12 bp of the amino acid acceptor branch found in tRNA [39].

Seven secondary structures from the 3′ end of furo-, pomo- and pecluviral RNAs (SBWM1, SBWM2, PMTV2, BSBV3, PCV1, PCV2, IPCV1) were proposed [32]. Most of them share structural properties with canonical tRNAs and with the TYMV tRNA-like structure. The similarities are illustrated in figure 1C, which depicts the proposed secondary structure of PMTV2. Notably, in the pecluviral RNAs, one finds an insertion of about 40 nucleotides (nts) in the acceptor branch of the tRNA-like domain that generates two unpaired nucleotides in the stem proximal to the T-loop mimic [32]. Although many amino acid acceptor-like branches are of 12 bp, the number of base pairs present in each helix differs and is imposed by the presence of the pseudoknot. These considerations also hold true for the tymoviral tRNA-like structures. Indeed, when the amino acid acceptor-like branch is built of three helical segments of 5, 3 and 4 bp, loop L_1 is composed by 3 or 4 nts, whereas it is only of 2 residues when helices are of 6, 3, and 3 bp in length [31, 32] (compare figs. 1A and C). In addition, the majority of these molecules share a 12 bp anticodon branch. The only exception is the IPCV1 tRNA-like structure, with a 13 bp anticodon branch. No classical T- and D-loops are identified in these tRNA-like structures, suggesting that other tertiary interactions stabilize them in a tRNA fold [31, 32]. Another striking fact concerns ELV RNA, which differs widely from other tymovirus RNAs by the absence of an identifiable anticodon domain and by a longer acceptor arm of 14 bp (fig. 1D).

Concerning the RNA from STMV, a secondary structure of its 3′-terrminal sequence was proposed that mimics the fold of the homologous region of TMV RNA [33]. This tRNA-like fold is followed by a stretch of pseudoknots. It contains a continuous helix similar to the amino acid accepting branch made of only 11 bp and an equivalent of the anticodon branch of 13 bp closed by three nucleotides. Further, the similarities of the ORSV-cy [28] and cr-TMV [29] RNAs with the tRNA-like structure of TMV RNA strongly suggest a tRNA mimicry in those viral RNAs, though existence of a tRNA-like fold awaits experimental verification. A partial mimicry restricted to a pseudoknotted amino acid acceptor branch, as found in the secondary structures of bromo-, cucumo- and hordeiviral RNAs, likely exists at the 3′ end of alfamo- and ilarvirus RNAs.

The structural characteristics of the tRNA-like domains found in furoviruses and in STMV are summarized in table 1.

Novel 3 D models

In 1987, a three-dimensional (3D) model of the 3′-terminal 86 nts of TYMV RNA was established by computer modeling [40] based on partial sequence homologies with tRNA and probing data [37, 41]. This was the first precise 3D model of a viral tRNA-like structure that emphasized an L-shaped structure of the 3′ end of this RNA, strikingly similar to that of canonical tRNAs. Structural features of the TYMV tRNA-like structure were largely described in several reviews [16–18] and will not be further discussed here. Since 2D structures of several RNAs from tymo- and furoviruses are known, it would be of great interest to verify if they fit in the 3D model established for the TYMV RNA.

In the last decade, 3D computer models were proposed for the tyrosylable tRNA-like domain from BMV RNA [42] and for the histidylable domain from TMV RNA [43]. In contrast to TYMV, the structural mimicry of these domains with tRNA is not clear at either the secondary or the tertiary level. No cloverleaf could be drawn with both RNA sequences, and tRNA features such as anticodon domains or anticodon triplets were difficult to identify. Concerning the 3′ end of BMV RNA, three tentative models of folding were proposed that differed by the relative orientation of the different helices and by the assignments of the arms [38, 44, 45]. Based on new systematic probing data, a refined model of the last 201 nts of BMV RNA was generated by computer modeling [42] (fig. 2). Although this newer model is composed of eight

Table 1. Summary of structural characteristics of the 3′ end of RNAs from furo- and tobamoviruses discovered since 1995.

RNAs	Virus genera	Size of tRNA-like structure	Base pairs in the acceptor branch (1)	Base pairs in anticodon branch (2)
SBWMV1	furovirus	82 nts	12 (5 + 3 + 4)	11 (5 + 1 + 5)
SBWMV2	furovirus	82 nts	12 (5 + 3 + 4)	12 (5 + 1 + 6)
BSBV3	furovirus	84 nts	12 (5 + 3 + 4)	12 (5 + 1 + 6)
PMTV2	pomovirus	82 nts	12 (6 + 3 + 6)	12 (6 + 1 + 5)
PCV1	pecluvirus	124 nts	10 + 2[a] (5 + 3 + 2 + 2[a])	12 (5 + 1 + 6)
PCV2	pecluvirus	124 nts	10 + 2[a] (5 + 3 + 2 + 2[a])	13 (6 + 1 + 6)
IPCV1	pecluvirus	124 nts	10 + 2[a] (5 + 3 + 2 + 2[a])	13 (6 + 1 + 6)
STMV	satellite virus	110 nts[c]	11 (4 + 3 + 4)	13[b]

(1) Indicates the number of base pairs in the amino acid acceptor branch; in parenthesis is given their distribution in the three stems forming the acceptor branch (from the CCA-end to the T-loop mimic).
[a] Insertion of about 40 nts in stem 3 near the T-loop mimic leading to a 12 bp acceptor branch with a gap of 2 nts in stem 3.
(2) Indicates the number of base pairs in the antidocon branch; in parenthesis is given their distribution in the D- and anticodon-stems, including the mimic of pair 26–44.
[b] Anticodon branch has a less regular structure.
[c] Functional experiments were performed on the 188-nts-long molecule; but taking into account the structural data, the actual tRNA-like domain is about 110 nts [33].

intricate structural domains (A, B_1, B_2, B_3, C, D, E, F), an overall mimicry with the L-shaped architecture of tRNA was found, with domain A mimicking the acceptor branch and domain B_3 being a plausible mimic of the anticodon branch [42]. However, the unambiguous assignment of an anticodon branch was difficult, since no hairpin of the BMV RNA contains either a 7-nt anticodon loop or a tyrosine anticodon. Recently, studies of point and deletion mutants combined with footprinting experiments specified the role of hairpin B_2 as an anticodon domain [46]. This is in contrast to B_3 previously defined as being the anticodon domain. Complementary structural investigations by nuclear magnetic resonance (NMR) spectroscopy have been performed on hairpin C [14]. This structure, which directs synthesis of the viral RNA (see below), is composed of two discrete domains separated by an internal loop and is closed by a compact 5′–AUA–3′ triloop from which the 5′-adenosine is exposed out in solution (fig. 2A).

The same modeling approach was used to bring insight into the structure of the 3′-terminal domain of TMV RNA. The secondary structure of this RNA was first proposed by Rietveld et al. [47] and showed three structural domains (D_1, D_2 and D_3) connected by a central core (C) (fig. 3). Modeling of the 3′-terminal 182 nts of TMV RNA indicates a mimicry with the L-shape of canonical tRNAs which is achieved by the pseudoknotted domain D_1, analogous to the amino acid acceptor branch, and by the anticodon branch, mimicked by domain D_2 [43]. Tertiary folding of the central core of the molecule is formed by a pseudoknotted three-way junction and imposes the structure and the orientation of the entire tRNA-like domain. This architecture is valid for the 3′ noncoding regions of tobamoviral RNAs as well as for the tRNA-like domain of the STMV [33]. Likewise, it would be interesting to study the ORSV-Cy and cr-TMV RNAs, members of the tobamovirus genus, that are proposed to bear a 2D structure similar to that of TMV RNA.

Engineered tRNA-like structures

Viral tRNA-like structures are composed of two independent structural domains that mimic the L-shape of tRNA and also contain external domains. This opens the possibility to engineer a minimal and compact structure derived only from the amino acid acceptor branch. Small helices or minihelices recapitulating the acceptor domain of canonical tRNAs were commonly used to study tRNA identity [48, 49]. Indeed, such molecules have the ability to be recognized by tRNA-specific proteins (reviewed in [50, 51]). In the tRNA-like field, minimal RNA helices mimicking the amino acid acceptor branch of TYMV [52, 53], TMV [43] and BMV [54] RNAs were synthesized by in vitro transcription. These molecules, whose size varies from 34 to 42 nts (fig. 4), present a pseudoknot that is crucial for their folding. Particular interest should be given to minihelices derived from the BMV tRNA-like acceptor branch that contain a novel RNA motif designated ‘resected pseudoknot’ (i.e. missing loop L2, one of the two connecting loops in pseudoknots) [54]. Resected pseudoknots are formed by annealing a circular RNA with an oligoribonucleotide of 10 residues terminating with a $-CCA_{OH}$ 3′ end (fig. 4C). More details concerning the functional properties of these small RNA molecules are given below.

Pseudoknotted domains

As mentioned before, pseudoknotting is a type of RNA folding that was discovered when trying to construct an

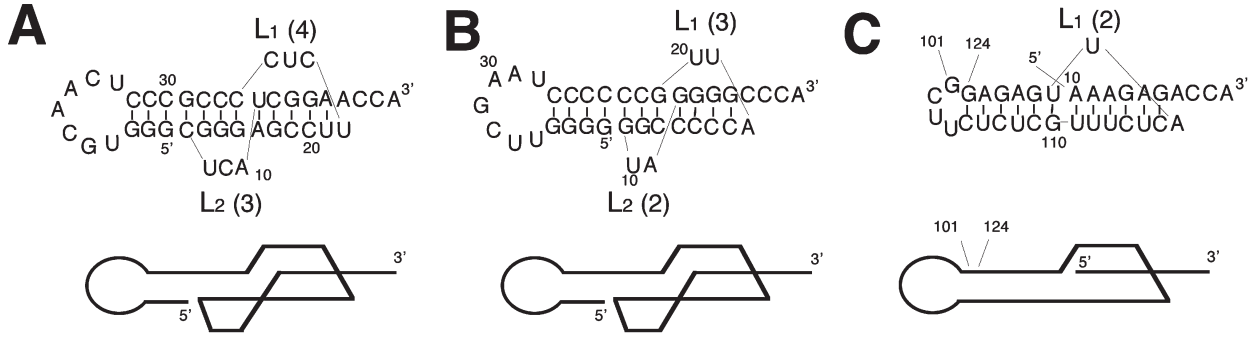

Figure 4. Minimalist RNA structures and corresponding schematic sketches derived from the amino acid acceptor domain of tRNA-like structures from (*A*) TYMV RNA, (*B*) TMV RNA and (*C*) BMV RNA. Numbering is as in the entire tRNA-like structure. Notice the folding of BMV minihelix formed by annealing of a short oligonucleotide (10 nts) with a circular RNA of 24 nts. Length of the L_1 and L_2 loops is indicated in brackets. Note that the first nucleotide of the L_1 loop faces the discriminator position 4.

amino acid acceptor branch of the TYMV tRNA-like similar to that of canonical tRNA [37]. After this discovery, it became apparent that this motif occurs in the folding of many viral RNAs and other types of RNA molecules where it fulfills significant structural and functional roles. This has been discussed by several authors [55–59]. The presence of this tertiary folding in an RNA is mainly revealed after phylogenetic comparisons and enzymatic or chemical structure probing [47, 60, 61]. Besides their architectural role, pseudoknots permit the exposure of nucleotide determinants for protein recognition. Prediction programs that include the formation of pseudoknots in complex RNA molecules using thermodynamic parameters and free energy values are now available [19–22, 62].

Recently, NMR spectroscopy and X-ray crystallography investigations have improved understanding of the peculiar pseudoknot folding (for a review, see [63]). Noticeably, the structure of a 44 nt-long RNA hairpin which recapitulates the T-arm and acceptor arm of the tRNA-like structure of TYMV RNA was solved by NMR methods [64, 65]. It gives emphasis to the unexpected conformation of the pseudoknot loops, which cross the A-helix of the acceptor branch. Loop 1 crosses the major groove of the helix thanks to only two nucleotides, and loop 2 spans the minor groove and interacts closely with the opposing helix through hydrogen bonds to a central adenosine.

Functional mimicries of tRNA-like structures

The functional mimicry of tRNAs by plant viral tRNA-like structures was discovered on the basis of their aminoacylation properties [1, 2, 5, 66], and was further extended to other tRNA-specific functions (reviewed e. g. in [18]). Recent investigations have included additional viral RNAs belonging to the tymo- and furovirus genera, and have increased our comprehension of aminoacylation mimicry. These studies have shown that the degree of tRNA mimicry is not uniform toward all functions of tRNA. Indeed, a tRNA-like structure can efficiently mimic one function of a tRNA but not the others. On the other hand, the functional mimicry of a tRNA can be divergent within a single genus, and even between the genomic RNAs of a given virus [31, 32, 67, 68]. We summarize below the functional properties of tRNA-like structures with regard to their tRNA mimicry.

Interaction with tRNA maturation enzymes

The interaction of tRNA-like structures with tRNA maturation enzymes, as first discovered for nucleotidyl-transferase [69], RNase P [70] and modification enzymes [71], is well known (reviewed e. g. [18]). Adenylation appears to be a common characteristic and was found for BMV, TMV, TYMV and several other tymoviral RNAs, and in some cases is even as efficient as in tRNAs. Only one of the tRNA-like structures tested possesses a weak capacity to be adenylated; it is the ELV RNA, whose structure deviates largely from canonical tRNAs (fig. 1 D). The adenylation properties of the tRNA-like structures probably reside in features present in the acceptor branch (e. g. sequences clustered in the T-like loop, and a pseudoknot). This is confirmed by the specific, although weak, adenylation capacity of the AMV RNA, the 3′ extremity of which can adopt the pseudoknotted conformation of bromovirus RNAs [30].

RNase P processes a lesser variety of tRNA-like structures. That from *Escherichia coli* effectively cleaves the TYMV, CMV and EMV tRNA-like structures at a position equivalent to that in tRNAs, but not the BMV and TMV RNAs. The nature of the nucleotide upstream of the terminal $-CCA_{OH}$ sequence and of the first base pair of the acceptor stem may be implicated in the differential recognition of the tRNA-like structures by RNase P (for a review on RNase P, see [72]).

Plant viral tRNA-like structures are devoid of modified residues. This could mean that these structures are not

recognized by tRNA modification enzymes in vivo. However, in vitro experiments done on the TMV tRNA-like structure showed that this RNA can be to some extent a substrate for modification enzymes [71, 73]. More recently, TYMV RNA has been shown to be a good substrate of modification enzymes in vitro [74] and in vivo, in oocytes from *Xenopus laevis* [75]. These observations show that plant viral tRNA-like structures are potentially appropriate_substrate mimics for tRNA modification enzymes, and thus can become tools to study the specificity of these proteins.

Interaction with aminoacyl-tRNA synthetases

Only three aminoacylation specificities have so far been found for tRNA-like structures from plant viruses, namely valine, histidine and tyrosine (table 2).

Valine identity

Knowledge concerning valine identity mainly relies on studies on the aminoacylation properties of the TYMV tRNA-like structure (see [18]). Interestingly, it has been shown that the same residues located at equivalent positions in the viral RNA and $tRNA^{Val}$ are important for valylation in both contexts. These are A_{56}, A_{55}, C_{53} and A_4 in the TYMV context, mimicking the valine identity nucleotides from the anticodon loop A_{35}, A_{36}, C_{38} and the discriminator residue A_{73} in $tRNA^{Val}$ [76, 77] (table 2 and fig. 5A). To further investigate the valylation properties of tRNA-like structures, this mimicry has been studied in different structural contexts. For instance, valylation properties predicted for furoviral or furoviral-like RNAs (SBWM1, SBWM2, PMTV2, BSBV3, PCV1, PCV2, IPCV1) on the basis of their secondary structure (see above) have been experimentally confirmed [32]. Sequence comparisons of the tRNA-like structures from four tymoviruses (TYMV, KYMV, ELV and EMV), the above seven furoviruses, and a tobamovirus (SHMV) have been made. They reveal that only 4 nts are strictly conserved in the efficiently valylated molecules: the four valine identity nucleotides identified in the anticodon loop and at discriminator position [31, 32] (table 2). Interestingly, three of the tRNA-like structures have a less-efficient valylation capacity: SHMV RNA for which the discriminator analogue is a C residue; PCV2 RNA, which lacks the central anticodon analogue; and ELV RNA for which the anticodon domain is missing (fig. 1D). The first RNA has a weaker valylation efficiency as compared with the other RNAs, and the two last RNAs are not valylated at all. Such results are in agreement with the predominance in valine identity of the anticodon nucleotides over the discriminator residue. An investigation of the valylation properties of the TYMV RNA was based on in vitro selection of molecules derived from the viral tRNA-

Table 2. Nucleotides specifying valine, tyrosine and histidine identities in tRNAs [50] and viral tRNA-like frameworks.

RNAs	Valine identity nucleotides	Tyrosine identity nucleotides	Histidine identity nucleotides
tRNAs			
$tRNA^{Val}$	A_{35}, C_{36}, C_{38}, A_{73}		
$tRNA^{Tyr}$		A_{73}, C_1–G_{72}, G_{35}, U_{35}, A_{36}	
$tRNA^{His}$			G_{-1}–A_{73}, G_{34}, U_{35}
Tymovirus tRNA-like structures			
TYMV	A_{56}, C_{55}, C_{53}, A_4		U_{21}–A_4
KYMV	A_{56}, C_{55}, C_{53}, A_4		U_{21}–A_4
SHMV	A_{56}, C_{55}, C_{53},		U_{21}–A_4
EMV	A_{56}, C_{55}, C_{53}, A_4		not histidylated
ELV	not valylated		U_{21}–A_4
Furovirus tRNA-like structures			
SBWM1	A_{56}, C_{55}, C_{53}, A_4		A_{21}–C_4
SBWM2	A_{56}, C_{55}, C_{53}, A_4		U_{21}–C_4
BSBV3	A_{57}, C_{56}, C_{54}, A_4		A_{21}–C_4
PCV1	A_{98}, C_{97}, C_{95}, A_4		A_{21}–C_4
PCV2	not valylated		A_{21}–C_4
IPCV1	A_{98}, C_{97}, C_{95}, A_4		U_{21}–C_4
PTMV2	A_{55}, C_{54}, C_{52}, A_4		not histidylated
Bromovirus tRNA-like structures			
BMV		A_4, C_{116}–G_5	A_{117}–A_4
Tobamovirus tRNA-like structures			
TMV			A_{18}–C_4, G_{72}, U_{71}
STMV			A_{19}–C_4, G_{72}, U_{71}

Note that numbering of the tRNAs is from 5′ to 3′, and for convenient usage, that of tRNA-like structures from 3′ to 5′. In a same column, functionally equivalent nucleotides in tRNA and tRNA-like structures are aligned.

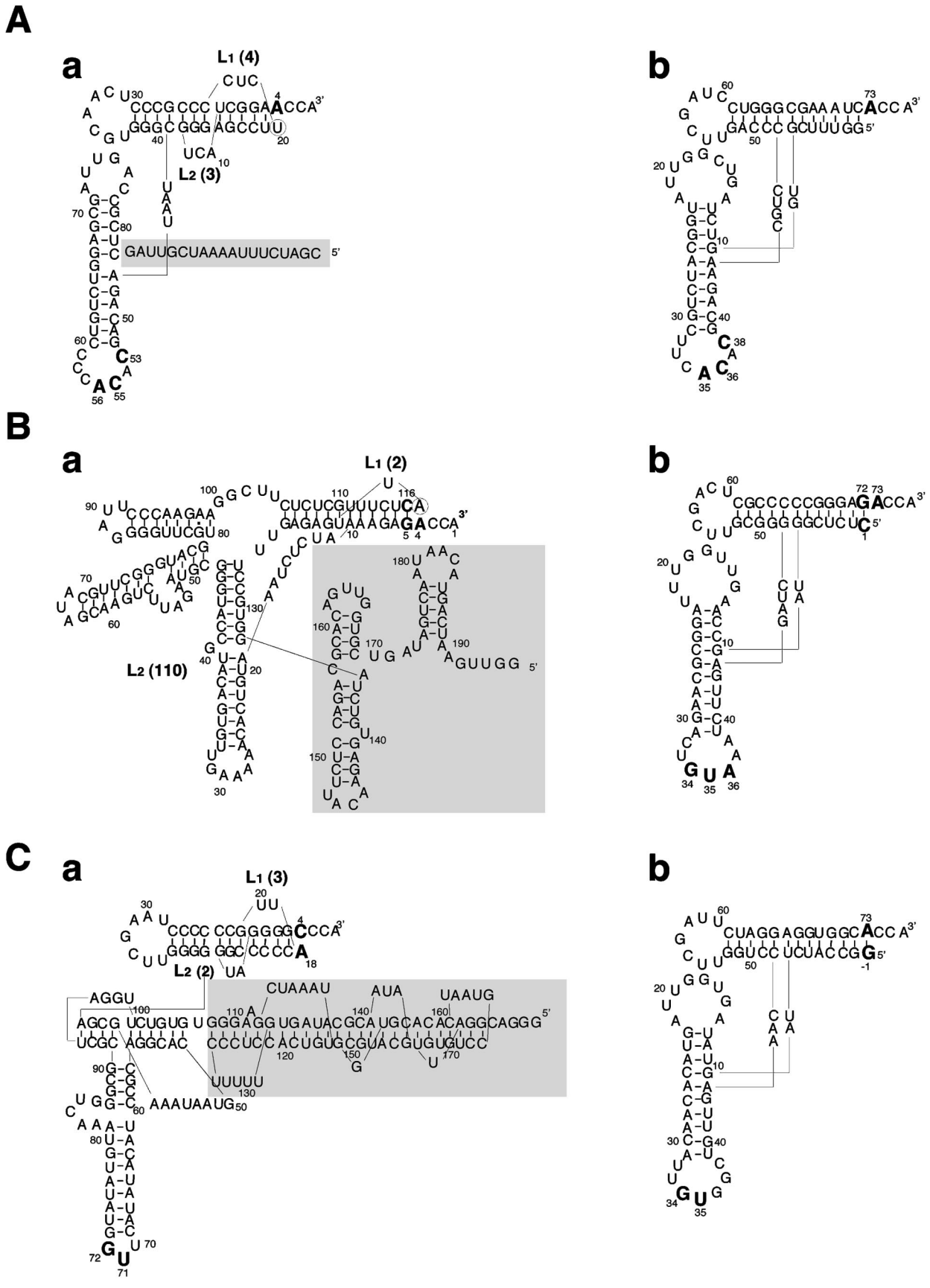

Figure 5. Comparison of the folding of valine (*A*), tyrosine (*B*) and histidine (*C*) accepting RNAs with tRNA-like structures (from TYMV, BMV and TMV RNAs in (*Aa, Ba, Ca,* respectively) and yeast tRNAs specific for valine, tyrosine and histidine in (*Ab, Bb, Cb,* respectively) (for sequence data on tRNAs see [11]). Identity nucleotides [50] for valylation (*A*), tyrosylation (*B*) and histidylation (*C*) are given in bold. The first nucleotide of the L_1 loop responsible for the histidylation properties of the TYMV and BMV RNA are encircled. Regions of the tRNA-like structures dispensable for efficient aminoacylation are boxed in light grey. Numbering of tRNAs starts at their 5′ end and of tRNA-like structures at their 3′ end.

like structure randomized in the anticodon loop and in loop L_1 [78]. Results first confirmed that the three anticodon identity nucleotides are necessary to confer valine identity to the TYMV RNA. Moreover, in agreement with a previous study based on mutants of the TYMV L_1 loop [79], these experiments established that the length, but not the sequence, of loop L_1 is important for efficient valylation. The in vitro selection method further highlighted the absence of a functional relationship between the anticodon loop and the pseudoknot, suggesting that both domains act independently. Altogether, the conclusions from this investigation favor the view of a strict conservation of the identity rules for recognition of different types of RNA substrates by aminoacyl-tRNA synthetases, whatever the structural scaffold in which the identity nucleotides are embedded [6, 50].

Tyrosine identity

Viruses belonging to the bromo-, cucumo- and hordeivirus genera possess tyrosylable RNAs. The tRNA-like structures present at their 3′ extremity all share a particularly intricate structure, as highlighted in the case of the BMV tRNA-like structure (fig. 2). In this structure, residues are found mimicking the yeast major tyrosine identity elements, namely the discriminator residue A73 and the first base pair C_1–G_{72} of the accepting stem (table 2 and fig. 5B) [46, 80]. Strikingly, however, the BMV tRNA-like structure possesses neither a canonical 7 nts anticodon loop nor the tyrosine anticodon triplet GUA, which is known to play a role in yeast tyrosine identity. Nevertheless, recent experiments showed that the yeast TyrRS needs the B_2 hairpin, oriented perpendicularly to the acceptor branch, to anchor the tRNA-like structure and to efficiently catalyze the tyrosylation reaction [46].

Histidine identity

STMV and TMV are the two members of the tobamoviruses for which histidylation properties have been studied. Histidine identity in yeast depends essentially on the presence of an additional residue at the 5′ extremity of the tRNA in combination with the discriminator residue [53] and weakly on the anticodon residues G_{34} and U_{35} [33]. Thus, residues analogous to these nucleotides should be found in the histidylable tRNA-like structures. This assumption was verified for the STMV and TMV tRNA-like structures [33, 43] (fig. 5C). Further, histidylation of minimalist structures confirmed the predominant role of the acceptor stem identity nucleotides (an extra base pair) over the anticodon ones [43]. The efficient aminoacylation of these structures shows further that the presence of an extra base pair is more important for histidine identity than is the nature of the nucleotides since they possess a C:A instead of a G:A extra base pair in yeast $tRNA^{His}$ (table 2 and fig. 5C). These data agree with the observation that the phosphate group of nucleotide –1 is mainly responsible for the specificity of histidylation in classical tRNAs [81].

Multiple identities

According to the structural models of the pseudoknots present in plant tRNA-like structures, with the first nucleotide of loop L_1 facing discriminator residue (fig. 5), it was predicted that these residues in the pseudoknot should mimic the extra base pair of $tRNA^{His}$ and consequently that all tRNA-like structures should have histidine identity. This assumption was verified in vitro with the TYMV and BMV tRNA-like structures. Moreover, a mutational analysis done on minisubstrates derived from the TYMV and BMV tRNA-like structures confirmed that their histidylation specificity is mostly due to the formation of this extra base pair [52, 54, 82, 83] (table 2 and fig. 5). Further, the histidylation capacity of tRNA-like structures has been extended to the other RNAs from the tymo-, furo-, pomo- and pecluvirus genera [31, 32]. However, two of them have a decreased level of histidylation, i.e. the EMV and the PMTV2 tRNA-like structures, due to their peculiar L_1 loop composed of only two residues which may not allow a precise stacking of the first nucleotide of this loop over the final base pairs of the acceptor arm.

Altogether, it appears that the common feature of all tRNA-like structures is their potential to be recognized by HisRS. Thus it can be hypothesized that the potential of pseudoknots to mimic the –1 residue of $tRNA^{His}$ could have been a way used in evolution to screen for aminoacylatable RNAs. This could also mean that the primordial specificity of the viral tRNA-like molecules was for histidine. In a further step, additional specificities requiring more elaborate identity sets were added, and as a result, the histidine identity may have become hidden in certain contemporary plant viral tRNA-like structures [83]. The BMV tRNA-like structure has also been shown to possess valylation capacity [83], likely due to the presence of the valine identity residue A at the discriminator position. All these relaxed aminoacylation specificities of the tRNA-like structures further support the view that the tRNA mimics obey the same identity rules as do canonical tRNAs. However, the strength of the additional histidine and valine identities is weak compared with their dominant identity, so that it seems unlikely that these properties have in vivo implications in contemporary systems [84]. More likely, they are functional remnants of the evolutionary history of these molecules.

Only three aminoacylation identities mimicked?

Since only three aminoacylation specificities were found to date in the RNAs of the seven virus genera possessing

tRNA-like structures, one can question the reasons for this low spectrum of tRNA mimicry. Is the nature of the amino acid important for replication, or are the three synthetases the only ones able to interact with tRNA-like structures possessing a pseudoknot? These are still open questions. Notably, the three amino acids valine, tyrosine and histidine present no strong chemical similarities, except for a weak relatedness in hydrophobicity, nor do the three synthetases belong to the same class of synthetases [85, 86] or interact with tRNA in a similar fashion. An initial answer to the question of the specificity spectrum of viral tRNA-like structures comes from RNA engineering. Exchanging the valine anticodon by a methionine anticodon in the TYMV tRNA-like structure, in other words, exchanging the valine to methionine identity determinants, led to efficient charging by MetRS [79]. This shows that other synthetases may be able to aminoacylate tRNA-like structures. Moreover, in allowing the identity switch of a tRNA-like structure, as for classical tRNAs, this work adds further support to the universal character of tRNA identity rules [50, 87].

Interaction with elongation factor but not ribosome

To study the route followed by aminoacylated tRNA-like structures after the attachment of the amino acid, other tRNA-recognizing factors were considered. It was shown in vitro that *E. coli* EF-Tu · GTP has a high affinity for the aminoacylated TMV and TYMV tRNA-like structures, in the same range as for aminoacylated tRNAs [88, 89] and medium affinity for the BMV tRNA-like structure [90]. Recently, it was shown that the valylated TYMV RNA is also efficiently recognized by the eukaryotic counterpart of EF-Tu · GTP, EF-1α · GTP [91]. The dissociation constant of wheat germ EF-1α · GTP for the valylated TYMV tRNA-like structure and for a control molecule, lupine valyl-tRNA^{Val}, are in the same range [91]. This property of TYMV RNA has been extended to other tymoviruses and also to furo- and furo-like viruses [31, 32]. However, four tRNA-like structures (those from EMV, IPCV1, PCV1 and PTMV2 RNAs) bind with a lower affinity to EF-1α · GTP (their k_Ds are at least 10-fold higher). These structures have a particular arrangement of the 12-bp acceptor stems due to the connections of the pseudoknot L1 and L2 loops (see above). Indeed, their acceptor stems are composed of a combination of 3, 3 and 6 bp and not of 4, 3 and 5 bp as in the other tymo- and furovirus RNAs. Since EF-1α · GTP interacts with tRNAs at the level of their acceptor stems (reviewed in [84]), it seems likely that this type of acceptor stem architecture leads to a weaker affinity for EF-1α · GTP.

To date, no interaction of plant viral tRNA-like structures with the ribosome or with ribosomal subunits has been detected. The tRNA-like structures may thus not participate in the translation process, and indeed protein synthesis assays that would be dependent on tRNA-like structures have been unsuccessful [3, 5, 92].

Biological roles of viral tRNA-like domains

The numerous tRNA-specific structural and functional properties of the 3′ ends of plant viral genomes raise questions about the role of these domains in viral amplification or in any other process related to the viral life cycle. This question was raised after the first demonstration of aminoacylation properties of the TYMV genomic RNA in the early 1970s and has been discussed in a number of reviews [4, 5, 16, 18, 84]. Initially, it was considered that the aminoacylated viral RNA could be involved in protein synthesis as is the case for classical tRNAs. However, this was shown not to be the case, as quoted above. A further idea emerged when it became clear that the aminoacylated RNA is recognized by elongation factor EF-Tu, which was known at that time to be a subunit of phage Qβ replicase and opened the possibility that the tRNA properties of the viral RNA were the basic support for initiation of viral replication [4]. This possibility has been investigated with various approaches on different viral RNAs and, indeed, has found experimental support. Before summarizing the present knowledge on the contribution of the 3′-tRNA-like domains to viral amplification, the important aspects of viral infection will be recalled. This will help evaluating all possible contributions of the genome 3′ end.

Positive-strand viral RNAs have two major functions in viral amplification. First, they are messenger RNAs encoding virus-specific proteins such as coat protein, viral replicase subunits, helicases and movement proteins that need to be translated by the host translational machinery. Second, they are templates for transcription into minus-strand RNA copies, the minus strands becoming themselves templates for synthesis of numerous plus-stranded RNAs, which will then be encapsidated and form the genome of the progeny virions. The possible theoretical roles of the 3′-untranslated tRNA-like regions in viral replication include minus-strand promotion, provision of a telomere, regulation of access to the minus-strand origin and possibly packaging of the viral RNA into coat protein (reviewed in [84]). The contribution of the 3′ end to messenger functions of the viral genomic RNA may concern stability, modulation of translational expression and targeting of RNA to specific subcellular sites [84]. In addition to these functions, there are regulatory aspects which might be considered, such as noninterference between translation and replication of a single genomic RNA molecule (no clash between the ribosome reading the RNA from its 5′ to its 3′ end, with the replicase reading the genome from its 3′ to its 5′ end). Finally it should

be considered that the initial plus-strand genome needs to be transcribed into only a limited number of minus-strand copies, whereas these minus-strand copies will be transcribed a large number of times into new plus-strand copies. Thus, although very important for the initial steps of viral amplification, there is only a limited need for replicative properties of the 3′-end tRNA-like domain.

A number of the above-listed possibilities have been explored experimentally for specific tRNA-like structures, from TYMV, BMV or TMV genomes. In what follows, the results will be summarized in considering (i) those functions linked to the aminoacylation properties of the 3′-end domains, (ii) those functions linked to structural tRNA-like properties of these domains and (iii) those functions linked to specific features, not related to tRNA properties.

Roles of the tRNA-like domains linked to their aminoacylation properties

The aminoacylation properties of the different viral genomes can vary both in specificity and efficiency not only according to the viral genus considered but also within a given genus (see above). The extreme situation to be noticed is the valylation capacity of the tRNA-like domain of PCV RNA1 and complete absence of valylation of RNA_2 of this virus [32]. Both RNAs share a very similar tRNA-like end; RNA_2 however, is missing an essential anticodon valine identity element. Thus, the role of aminoacylation may not be the same for all viruses and within a given virus, for all RNAs.

The contribution of the valylation, and more generally aminoacylation properties of the TYMV RNA to viral replication, has been investigated in much detail. One approach consisted in analysis of mutational effects within the tRNA-like domain on viral amplification in protoplasts and in plants. This led mainly to the conclusion that progressive loss in aminoacylation is directly correlated to progressive loss in virus amplification. In other words, aminoacylation is an important property for genome amplification, but the amino acid esterified at the 3′ end of the RNA does not have to be valine [93, 94]. The tRNA-like functional properties are thus directly linked to viral replication, and the tRNA-like domain contains promoter signals for replication. Further mutational analyses, including the creation of chimerical tRNA-like sequences, led to the conclusion that the main function of the TYMV tRNA-like structure is other than to act as a generic tRNA-like element and showed that the remainder of the genome is coadapted [67, 95]. Another approach consisted in the purification of the TYMV replicase, an RNA-dependent RNA polymerase (RdRp), which made it possible to investigate the contribution of the tRNA-like domain to replication in vitro [96–99]. There was the surprise. Although the enzyme specifically amplifies the TYMV RNA, it can use alternative substrates to initiate replication, including various truncated versions of the tRNA-like structure, tRNA-like variants with a disrupted pseudoknot, shortened fragments up to the 28 3′ nucleotides and artificial substrates including CC(A/G) stretches. It has been concluded that the tRNA-like domain of TYMV does not contain promoter signals for minus-strand synthesis, but only the initiation site for this event, namely the $-CCA_{OH}$ 3′-terminal sequence [96]. Initiation of replication takes place opposite the penultimate C [96]. These results call for a link with the aminoacylation properties of the viral RNA.

A very interesting proposal, compatible with all the present knowledge accumulated on TYMV and other tymoviral genomic RNAs, was proposed recently [84]. Although aminoacylation is crucial in the specific case of the natural TYMV tRNA-like structure, its functions can be taken over by a terminus that lacks aminoacylation but possesses some 'offsetting attributes'. The tRNA-like domain likely plays a dual role. It is the initiator site for minus-strand synthesis, but also – and to a far more important level – it plays the role of a repressor towards minus-strand synthesis. Indeed, aminoacylation, and in particular interaction with elongation factor EF-1α, blocks access to viral replicase. This negative regulation of minus-strand synthesis may serve to delay the switch from translation to replication, or promote a switch from negative- to positive-strand synthesis.

Purification of highly template dependent RNA-dependent RNA polymerase from BMV-infected barley [100] enabled investigation of the role of the tRNA-like domain in replication. The contribution of the aminoacylation potential of the BMV genomic RNAs to viral amplification was explored [101, 102]. In this case, it appears that aminoacylation of RNAs 1 and 2, but not 3, is important [103]. However, as is the case for TYMV RNA, very short nonaminoacylatable, RNA fragments are sufficient to promote replication [104]. In particular, one internal helix containing a knob and extended artificially by a $-CCA_{OH}$ end, was shown to be of major importance [14]. Thus, it is very likely that the aminoacylation properties of BMV RNAs are not involved in promoting minus-strand synthesis [84].

Roles of the tRNA-like domains linked to their structural properties

tRNA-like domains of viral genomes are highly structured and contain a number of tRNA-specific domains. This is especially true in the case of TYMV where the mimicry with canonical tRNAs is most obvious (see above). The case of TMV deviates to some extent from a classical tRNA, with extra-large dimensions of the anti-

codon branch of the 3D L-shape (fig. 3). Some furoviruses have an additional domain between the two branches of the L. The most divergent and complicated structures remain those of the tyrosylatable tRNA-like structures, with a major difficulty in assigning an anticodon domain (figs 2 and 5). Whenever present, specific domains were shown to be involved in tRNA aminoacylation properties. Thus, for example, valine identity elements were assigned within the anticodon loop of the TYMV tRNA-like structure, and the 3′-pseudoknotted structures are absolute requirements for any functional property.

The unifying feature of all tRNA-like structures remains the presence of the $-CCA_{OH}$ end. This accessible structural element, physically well separated from the rest of the structure, is indeed a major functional domain. It not only allows aminoacylation and further binding of the elongation factor, but is also the initiation site for replication. Moreover, recognition by tRNA nucleotidyltransferase of this extremity (and likely of some upstream domains) helps to maintain an intact 3′ end of the viral RNA. Nucleotidyl-transferase activity has thus been considered as a telomerase activity [105, 106] and it has been suggested that the tRNA structural properties are of major importance in distinguishing the specific $-CCA_{OH}$ sequence from the 3′ end from internal –CCA-sequences, and allowing distinctive access to this sequence by protein partners.

Roles of the 3′-end untranslated domain, not related to tRNA properties

The contribution of the tRNA-like domains to the messenger functions of the viral genomes has been investigated. It was demonstrated that the TMV tRNA-like domain can substitute very effectively for the 3′-poly (A) ends of GUS or luciferase reporter genes, and even more, enhance their translational efficiency [107, 108]. The effects of the tRNA-like domain also include improved stability and synergic action with the 5′-capped end of the RNA. Interestingly, whereas the BMV tRNA-like domain also displays these kinds of properties, the 3′ ends from TYMV and AMV RNAs do not [108]. In fact, the properties of the 3′ end of TMV RNA are not due to its folding into a tRNA-like structure, but to the presence of a row of pseudoknots upstream of the tRNA-like domain [107] (see also fig. 5C).

Concluding remarks

The presence of tRNA-like structures with tRNA-like properties at the 3′ end of a number of plant viral genomic RNAs has been and is still intriguing. However, the detailed investigation of tRNA properties combined with the studies of replicative properties of full-length viral genomes with variable 3′-end sequences and structures in protoplasts and plants provided insight to a number of questions. Access to purified viral replicases specific for TYMV and BMV RNAs led to important new insights as to the role of the 3′ ends in viral amplification, and in particular modulated the general conceptual point of view. Instead of promoters for minus-strand synthesis, the role of tRNA-like domains are likely restricted to initiation sites. From a major contribution as positive regulators they are now mainly considered as repressors of minus-strand synthesis. The contribution of tRNA-like domains to the messenger properties of the viral genomes has been confirmed. It is now very interesting to compare the tRNA-like domains present at the 3′ end of the plus-strand RNAs with the sequences and structures present at the very 3′ end of the minus-strand copies of the genomes. Indeed, whereas the former domains are involved both in replication and translational properties, the latter are likely only involved in intensive initiation of replication.

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