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Elements regulating *Potato leafroll virus* sgRNA1 translation are located within the coding sequences of the coat protein and read-through domain

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Translation of viral proteins from subgenomic RNAs (sgRNAs) is a common strategy among positive-stranded RNA viruses. Unlike host mRNA, sgRNA of *Potato leafroll virus* (PLRV) does not possess a cap at its 5′ end nor a poly(A) tail at the 3′ terminus, both of which are known to be crucial for translation of RNA in eukaryotic cells. Here, we demonstrate, that in wheat germ extract (WGE) truncation of the sgRNA1 5′ UTR increases translation efficiency, as it has previously been observed in rabbit reticulocyte lysate (RRL), whereas removal of the 3′ UTR does not affect translation. We also describe two regulatory elements located within the coding sequence of the coat protein (CP) gene and its read-through domain (RTD) and are responsible for regulation of *in vitro* translation of the PLRV sgRNA1. The first element is composed of the purine sequence AAAGGAAA located between the AUG codons of the CP and 17K genes. Deletion of this domain or its substitution by pyrimidines reduced by half the translation of both genes, whereas deletion of the RTD resulted in a 3.6-fold reduction in translation efficiency. This is the first report of translation regulatory elements of plant viruses located within a coding region.

Keywords: polerovirus, subgenomic RNA, *in vitro* translation

INTRODUCTION

Potato leafroll virus (PLRV) is a type member of genus *Polerovirus*, family *Luteoviridae*. PLRV virions encapsidate a non-polyadenylated, single-stranded (ss) positive-sense RNA molecule of nearly 6 kb in length with a genome-linked protein (VPg) at the 5′ end (Mayo *et al.*, 1982). Analysis of the nucleotide sequence revealed the presence of eight ORFs organized in two gene clusters (Fig. 1). Those two clusters are separated by an intergenic region (Mayo *et al.*, 1989; Mayo & Ziegler-Graff, 1996). To express its genes PLRV employs a variety of mechanisms. The ORFs in the first cluster (ORF0, ORF1 and ORF2) are translated from the genomic-length RNA (gRNA), whereas ORFs designating the second cluster are expressed from sgRNA1 (ORF3, ORF4 and ORF5) and sgRNA2 (ORF6 and ORF7). The fact that PLRV sgRNA1 is an exact copy of genomic non-polyadenylated RNA poses the question of how this RNA substitutes for the major cytoplasmic functions associated with poly(A), i.e., control of mRNA stability and modulation of translation efficiency. Viruses that lack a cap and/or a poly(A) tail must have developed alternative strategies for the regulation of their translation. Particular attention has been directed towards non-coding elements with respect to their influence on gene expression. Leader sequences (LS), also called 5′ UTRs, have multiple effects on the translation of downstream-located genes and on mRNA stability (Gallie & Walbot, 1992; Nicolaisen *et al.*, 1992;

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Abbreviations: CP, coat protein; gRNA, genomic RNA; IRES, internal ribosome entry site; LS, leader sequence; ORF, open reading frame; RRL, rabbit reticulocyte lysate; RTD, read-through domain; PCR, polymerase chain reaction; PLRV, Potato leafroll virus; sgRNA, subgenomic RNA; UTR, untranslated region; WGE, wheat germ extract.

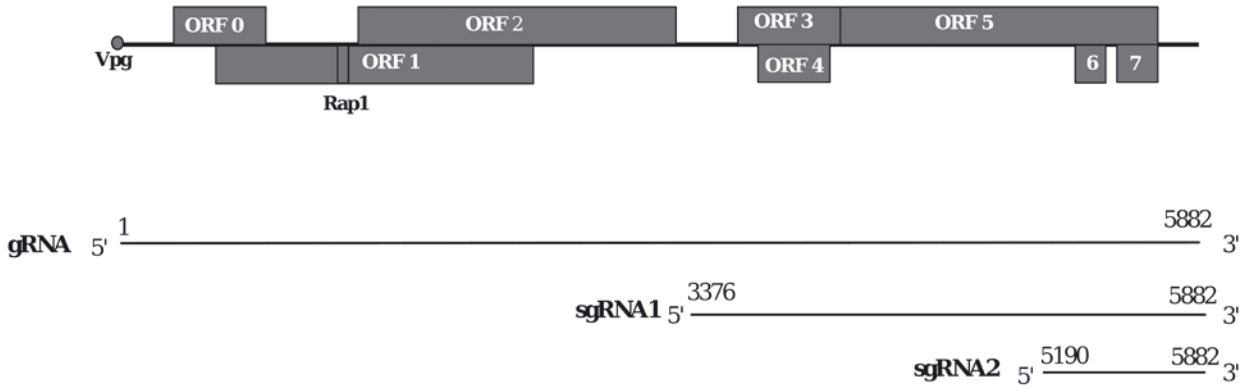

Figure 1. Schematic representation of PLRV genome and viral RNAs of positive polarity.
ORF, open reading frame; 6, 7, ORF6 and ORF7; Vpg, viral protein genome-linked; Rap1, replication-associated protein 1. Nucleotide numbers according to the PLRV sequence (accession number X74789).

Pooggin & Skryabin, 1992; Zelenina *et al.*, 1992; van Vossen *et al.*, 1993; Johnston & Rochon, 1996; Turner *et al.*, 1999). The 5′ leaders of several plant viral mRNAs, including members of the tobamoviral, potyviral, comoviral and luteoviral families, are responsible for conferring cap-independent translation (reviewed by Kneller *et al.*, 2006).

The role of the 3′ UTR on translation efficiency and viral RNA stabilization has also been studied in detail *in vitro* and *in vivo* and demonstrated for human and animal viruses, such as HCV and rotavirus (Vende *et al.*, 2000; Bradrick *et al.*, 2006), as well as for plant viruses such as *Tomato bushy stunt virus* (TBSV), *Satellite tobacco necrosis virus* (STNV) and *Turnip crinkle virus* (TCV) (Danthinne *et al.*, 1993; Wu & White, 1999; Qu & Morris, 2000). In the case of *Barley yellow dwarf virus* PAV isolate (BYDV-PAV) belonging to the genus Luteovirus of family *Luteoviridae*, a highly efficient cap-independent translation of gRNA and sgRNA1 is conferred by a 109-nt BYDV-PAV cap-independent translation element (BTE) located in the 3′ untranslated region (UTR) (Wang & Miller, 1995; Wang *et al.*, 1997; Guo *et al.*, 2000). As it was shown in wheat germ extract (WGE) system, BTE mimics the 5′ cap and can be functionally replaced by a cap analogue, but not by the poly(A) tail (Wang *et al.*, 1999). The presence of the above regulatory elements has also been proposed for other members of the genus Luteovirus, e.g., *Bean leafroll virus* (BLRV) (Domier *et al.*, 2002).

For PLRV the implication of the non-coding regions as determinants of translation efficiency has also been investigated. The influence of both UTRs on protein expression of gRNA and sgRNAs has been demonstrated (Juszczuk *et al.*, 1997; 2000). Analysis of protein expression in rabbit reticulocyte lysate (RRL) showed that the sgRNA1 LS appears to be a translation silencer of the genes located downstream, and it was proposed that the role of the 5′ UTR is to maintain a proper ratio of the proteins synthesized. Furthermore, *in vitro* experiments revealed that, at least in RRL, there is no translation enhancer element within the 3′ UTR. However, it could not be excluded that the 3′ UTR-located elements of PLRV are not active in the experimental system used, as it was demonstrated for BYDV (PAV) (Wang & Miller, 1995). This suggests that PLRV has elaborated as yet unknown mechanisms to modulate translation and RNA stability. Therefore, it seemed interesting and important to investigate the elements modulating the translation efficiency.

In this study we demonstrate that two regions of the coding sequence of the CP gene and its readthrough domain may modulate the translation from sgRNA1 in wheat germ extract.

MATERIALS AND METHODS

Templates for *in vitro* transcription. Two plasmids were used for amplification of PCR-generated templates for *in vitro* synthesis of RNA transcripts. Plasmid pJF, containing the full-length cDNA of the PLRV genome fused to the T7 RNA polymerase promoter (Sadowy *et al.*, 1998) and psgRNA1, a pUC118 derivative, carrying viral cDNA corresponding to sgRNA1 (unpublished). All primers used for amplification of viral cDNA fragments are listed in Table 1. In description of all plasmids and transcripts the letters p and t, respectively, were given at the beginning of their names. The numbering of nucleotides corresponds to the PLRV RNA sequence (accession number X74789). Using the pJF plasmid as a template and sg5end and sg3 primers, a PCR product corresponding to the sgRNA1 was amplified and designated sg (nt 3376–5882). The Δ3′ (nt 3376–5738) PCR product corresponding to sgRNA1

Table 1. Description of oligonucleotides used in this study.

Primer	Sequence[a] (5′-3′)	Location[b]	PCR template Construct[c]
sg3	ACTACACAACCATGTAAGAGGATCTTGGC	5854–5882	sg, Δ3′, ΔLS, ΔVTE, mVTE, ΔSL
sg5end	*CCCAAGCTTAATACGACTCACTATA***GA**CAAAAGAACACTGAAGGAGCT-CAC	3376–3400	sg, Δ3′, ΔRTΔ3′, ΔVTE, mVTE, ΔSL
T7ΔLS_NEW	*TTAATACGACTCACTATA***G**GCGATCAATTGTTAATG	3574–3590	ΔLS, ΔLSΔ3′
sg3Δ_1	TTTCCTCCCTTGGAATGGCTTTTCAGC	5712–5738	Δ3′, ΔLSΔ3′
sg3Δ_2	CAGGCTCTGATCCGGAGTCTA	4215–4236	ΔRTΔ3′
Δ SLU	TACTTCAGTTCGTCAGCGAGGC	3934–3955	psgΔSL
Δ SLD	CATCCTTGAATGCCGGG	3875–3891	psgΔSL
VTE_L	**AGA**AACCACGACCGTACTCATTA	3586–3605	psg_mVTE
VTE_U	**TT**AAATGTCAATGGTGGTGTACAA	3611–3632	psg_mVTE
RBP_U1	AATGTCAATGGTGGTGTACAACAA	3612–3635	psgΔVTE
RBP_L	CACGACCGTACTCATTAACAATTGA	3578–3602	psgΔVTE

[a]The sequence of the T7-promoter is presented in italics, nucleotides not corresponding to the PLRV RNA sequence are in bold. [b]Position in the PLRV genome. [c]Names of *in vitro* transcription PCR-derived templates or names of constructed plasmids with the sequence corresponding to sgRNA1 with modification. Numbering corresponds to PLRV genome (accession number X74789).

with truncated 3′ UTR region was synthesized as above, but using sg5end and sg3Δ_1 primers. The ΔLS (nt 3574–5882) PCR product bearing the last 14 nucleotides of LS was synthesized by PCR with T7ΔLS_NEW and sg3 primers. The ΔLSΔ3′ (nt 3574–5738) template lacking the 3′ UTR and the LS was obtained with T7ΔLS_NEW and sg3Δ_1 primer pair. With primers sg5end and sg3Δ_2 and pJF as a template, the ΔRTΔ3′ (nt 3376–4236) PCR product was synthesized. This template was truncated at position 4236, and does not possess the coding region for the RTD of the viral CP. All PCR products served as templates for *in vitro* transcripts synthesis that are schematically presented in Fig. 2.

Three psgRNA1 plasmid derivatives, psgmVTE, psgΔVTE and psgΔSL, bearing modifications within the CP protein gene were constructed as follows. To delete the 42 nt (3892–3935) corresponding to the stem-loop structure region within the CP coding sequence (structure not shown), the psgRNA1 template was linearized with ΔSLD and ΔSLU primers. To modify the purine-rich region located downstream from the AUG codon of the CP gene, two different constructs were prepared. Using the primer pair VTE_L and VTE_U, purines of the putative regulatory sequence AAAGGAAA (nt 3606–3613) were partially substituted with pyrimidines (TCTTTAAA). Partial (nt 3603–3611) deletion of the purine-rich region was introduced in a PCR reaction with the RBP_L and RBP_U1 primers. Prior to religation and cloning, the PCR products were incubated with *Pfu* polymerase in the presence of nucleotides to generate blunt ends and then phosphorylated with T4 kinase at the 5′ ends. To verify the presence of the desired modifications, DNA of selected clones was sequenced. Derived plasmids psgΔVTE, psgmVTE and psgΔSL served as templates for PCR reactions with the sg5end and sg3 primers. Two more PCR products truncated at nucleotide position 4236 and with deletion or substitution of the purine-rich region were synthesized using sg5end and sg3Δ_2 primers and psgΔVTE and psgmVTE plasmids. These products served as templates for *in vitro* synthesis of transcripts tΔVTE, tmVTE, tΔSL, tΔVTEΔRT and tmVTEΔRT (Fig. 2).

In vitro* transcription.** *In vitro* transcription of PCR generated templates was performed using the RiboMAX Large Scale RNA Production System-T7 (Promega), in the presence of 1 mM m7(5′)Gppp(5′)G cap analogue (Promega) according to the manufacturer's protocols. After completion of the *in vitro* transcription reaction, the DNA template was removed by digestion with RQ-DNase (Promega) according to the manufacturer's instructions. To separate capped transcripts from unincorporated m^7G-pppG (cap-analogue) and ribonucleotides, the mixture was purified on a Sephadex G-50 (Pharmacia) column by centrifugation for 5–8 min at 1000 × ***g according to the instructions of the supplier.

Translation *in vitro*. For *in vitro* translation 0.08 pmol of each transcript, 5 μl of WGE (Promega) and 10 μCi of [^{35}S]L-methionine (Amersham) were

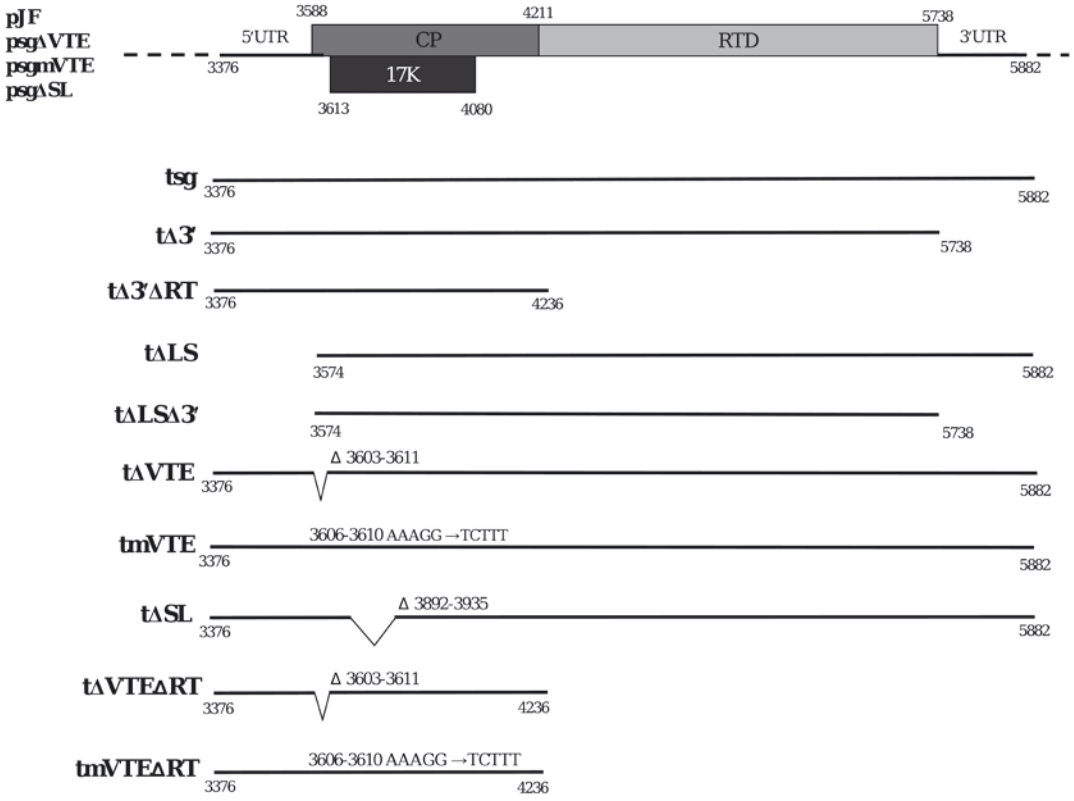

Figure 2. Schematic representation of transcripts used for translation of PLRV sgRNA1 in experiments characterizing the role of coding and non-coding regions.
UTR, untranslated region; CP, coat protein; RTD, read-through domain; 17K, 17 kDa protein. Black lines represent transcripts corresponding to viral RNA. Deletions are indicated by broken line. Numbering of nucleotides according to the PLRV sequence (accession number X74789).

combined in 10 μl final volume and incubated at 25°C for two h according to the manufacturer's protocol. The protein products were analyzed by separation on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) and subsequent autoradiography.

Quantitative analysis of *in vitro* synthesized radioactive protein. Bands corresponding to viral CP and MP were excised from dried SDS/PAGE gels and subjected to radioactivity measurements in scintillation fluid (Orgscynt, BioCare) using a scintillating counter (Pharmacia).

RESULTS AND DISCUSSION

The role of non-coding sequences of sgRNA1 in the synthesis of CP and 17K proteins

To investigate the role of non-coding regions of sgRNA1 in the translation of the CP and 17K proteins in WGE, transcripts corresponding to sgRNA1 of PLRV but lacking almost the entire 5′ UTR (ΔLS) or having a truncated 3′ UTR (Δ3′) were used as templates for *in vitro* translation reactions in the presence of [^{35}S]methionine. Translation of both tΔLS and tΔ3′ in WGE (Fig. 3A, lanes 4 and 2, respectively) yielded two major proteins of 23 kDa, corresponding to the CP, and 17 kDa, corresponding to the 17K protein. The quantification of the radioactivity of the bands excised from the gel showed that the sgRNA 5′ UTR has an inhibitory effect on CP and 17K expression since removing most of the subgenomic 5′ UTR resulted in a 2.74-fold increase in translation efficiency (Fig. 3B). This is consistent with previous findings in RRL (Juszczuk *et al.*, 2000); however, the excess of viral proteins produced in the WGE is half that in RRL. Quantification of protein products showed that deletion of the 3′ UTR leads to an increase in the level of translation to 1.2 (Fig. 3B). To test the influence of deletions of both 5′ UTR and 3′ UTR, translation of tΔLSΔ3′ was examined. In this case, the level of proteins synthesized was similar to that obtained by translation of tΔLS (Fig. 3A, lane 5). The 3′ UTR of the PLRV sgRNA1 seems not to exhibit a major regulatory function on the translation of proteins from gRNA1 (Fig. 3A, lane 2) since the amount of viral proteins synthesized from the tΔ3′ templates is slightly higher than that from the sub-

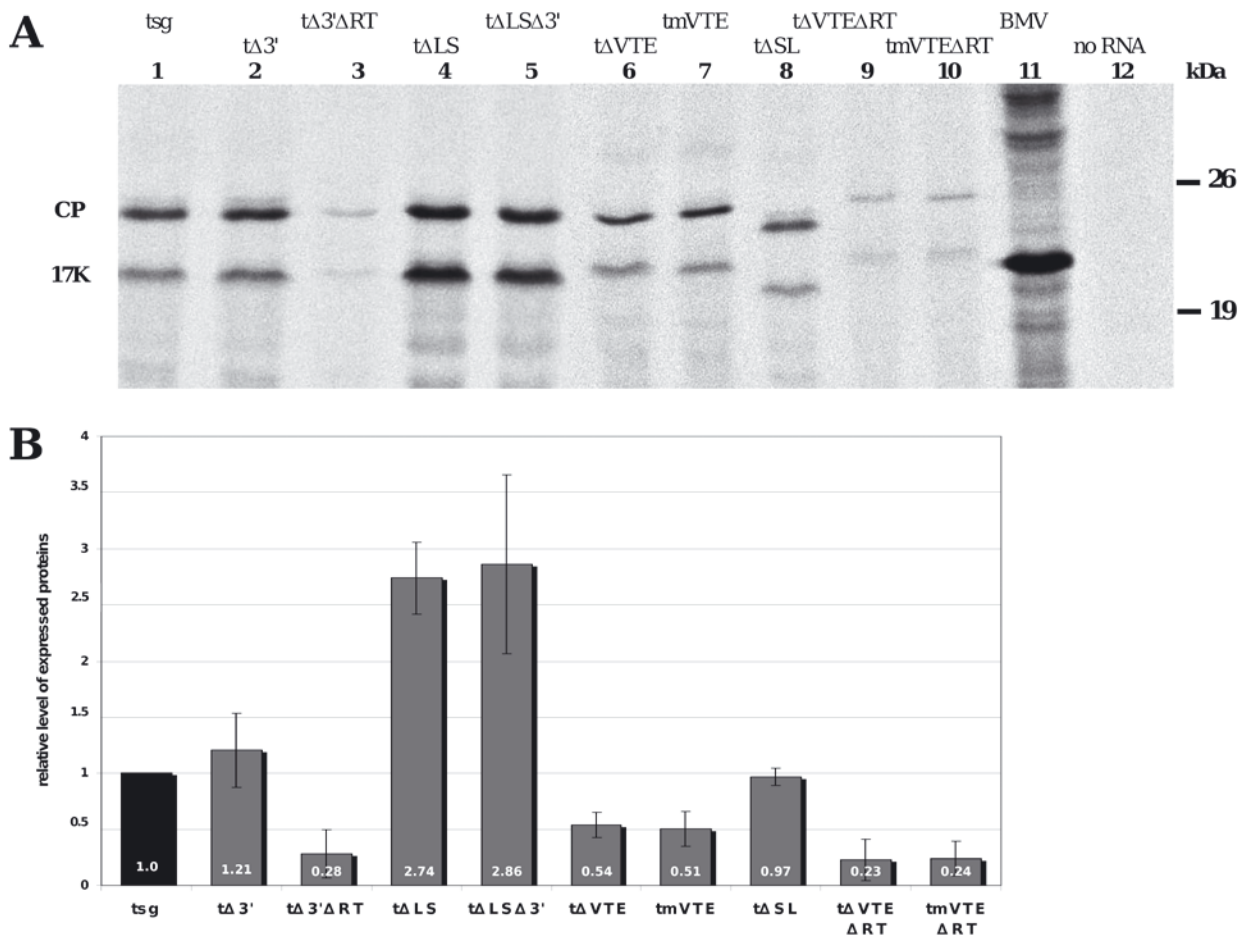

Figure 3. Effect of sequence modifications on translation of sgRNA1 *in vitro*.
A. Autoradiogram of wheat germ translation products of modified transcripts of PLRV sgRNA1. Lane 1, tsg, transcript corresponding to sgRNA1; lanes 2–5, transcripts with truncated UTRs (Fig. 2); lanes 6–10, transcripts with modifications within the coding sequences of sgRNA1; lane 11, BMV control RNA; lane 12, no RNA. CP, coat protein of PLRV; 17K, 17 kDa protein of PLRV. Molecular mass marker bands are indicated (Fermentas Cat. no. SM0441). B. Quantitative representation of total proteins synthesized in wheat germ extracts from particular transcripts. The diagram represents relative levels of *in vitro* synthesized proteins. The amount of proteins synthesized from tsg was taken as 1. The data presented are the average of four independent experiments.

genomic template without modifications (Fig. 3B). Characterizing the influence of the sgRNA1 3′ UTR in RRL (Juszczuk *et al.*, 1997) it was suggested that the 3′ UTR of PLRV does not influence the translation of the viral CP and 17K proteins. Analyzing the results obtained in WGE we can conclude that the 3′ UTR present in transcripts corresponding to sgRNA1 displays a slight inhibitory effect (Fig. 3B). Our results are in opposition to the finding made for BYDV (PAV), a member of the same family as PLRV but from another genus. In BYDV (PAV) the BTE, located in the 3′ UTRs of genomic and subgenomic RNA, was identified (Wang & Miller, 1995; Wang *et al.*, 1997). This element, together with the 5′ UTRs of gRNA and sgRNA1 of BYDV (PAV), plays a significant role in initiation and selective control of cap-independent translation (Wang *et al.*, 1999; Guo *et al.*, 2000).

The influence of coding regions of sgRNA1 on the synthesis of CP and 17K proteins

Coding sequences known to moderate translation of positive-stranded RNA viruses have been identified only for a few viruses. In hepatitis A virus, the coding sequence located downstream of the AUG codon enhances translation of viral gRNA by contributing to the internal ribosome entry site (IRES) function (Graff & Ehrenfeld, 1998). Translation of Sindbis virus mRNA was also demonstrated to be affected by sequences located downstream of AUG (Frolov & Schlesinger, 1994). It was demonstrated that hairpin structures are involved in efficient translation and accumulation of Sindbis virus structural proteins (Frolov & Schlesinger, 1996).

A lack of translation enhancers within the non-coding regions of PLRV sgRNA1 led us to

search for regulatory elements embedded in the coding sequences. Within the PLRV P1 coding region that is essential for viral multiplication an ORF encoding 5-kDa replication-associated protein 1 (Rap1) has been identified (Jaag *et al.*, 2003). It was also found that Rap1 translation is regulated by an IRES, with an unusual structure and location within ORF1. Core structural elements for this internal ribosome entry include a conserved AUG codon and a downstream GGAGAGAGAGG motif with inverted symmetry**.** This finding suggests the possible presence of a similar unusual translation regulatory sequence for ORFs expressed from sgRNA1. Sequence analysis of the sgRNA1 revealed the presence of a purine-only region AAAGGAAA (nt 3606–3613) just downstream of the AUG codon of the CP gene.

Translation of tΔ3′ΔRT, tΔVTE, tmVTE and tΔSL yielded two major proteins of 23 kDa and 17 kDa (Fig. 3A, lanes 3, 6, 7 and 8, respectively). Quantitative analysis of the proteins synthesized from tΔSL showed that the amount of CP and 17K proteins is retained at the same level as for tsg (Fig. 3B). This suggests that the stem-loop structure predicted within the CP gene has no influence on sgRNA1 translation efficiency. In contrast, quantification analyses indicate a strong dependence of the level of sgRNA1 protein expression on the presence of the read-through domain sequence. The absence of almost the entire sequence of ORF5 (nt 4236–5738) reduced the level of sgRNA1 protein synthesis to 0.28 (Fig. 3B). Taking into account the data (Juszczuk *et al.*, 2000) in which deletion of the ORF5 sequence from nucleotides 4457 to 5739 had no influence on the translation level, it can be concluded that the translation regulatory element present within the ORF5 sequence is located between nucleotides 4236–4457 (numbering on the basis of the PLRV genome).

Changes within tΔVTE and tmVTE, independently of the type of change, resulted in an about 2-fold decrease in translation efficiency. Replacing purines with pyrimidines in tmVTE caused a significant decrease in CP and 17K synthesis. A similar result was obtained by deletion of the six nucleotides AAAGGA (nt 3606–3611) from the purine-only region in tΔVTE. Given these data, the possibility of the participation of the purine-only sequence in translation regulation cannot be excluded. Deletion of AAAGGA in tΔVTE dramatically changed the distance between the AUG codons of the CP and 17K genes. Surprisingly, the ratio of the CP and 17K proteins in the case of tΔVTE translation remained unchanged and was similar to that of tsg (Fig. 3A, lanes 6 and 1, respectively). This observation suggests that the model proposed by Dinesh-Kumar and Miller (1993) to explain the enhancement of translation initiation of the BYDV sgRNA at the CP AUG by pausing of ribosomes at the 17K AUG does not agree with what is observed for PLRV CP translation. Translation of tΔVTEΔRT and tmVTEΔRT (Fig. 3A, lanes 9 and 10, respectively) also yielded two major proteins of 23 kDa and 17 kDa, but with a nearly 5-fold decreased efficiency compared to tsg. These results suggest that the regulatory elements within the RTD and purine-only region are independent and may act *via* unrelated mechanisms.

Our results indicate that translation of PLRV sgRNA1 in WGE is regulated by elements located within the coding sequence of the CP gene and RTD. The most significant effect, nearly 3.6-fold reduction in translation, was observed after removal of the entire coding sequence for the RTD. Taking into account the data obtained by Juszczuk and coworkers (1997) in which deletion of the ORF5 sequence from nucleotides 4457 to 5739 had no influence on the translation level, it can be concluded that the translation regulatory element present within the ORF5 sequence is located between nucleotides 4236–4457 (numbering as for the PLRV genome). The reduction in translation by to one half of that of sgRNA1 in transcripts with modified or partially deleted purine-only region between the AUGs of CP and 17K genes suggests also the presence of an additional element modulating protein expression from sgRNA1 in WGE. This is the first report where the role of coding sequences in translation of a plant RNA virus has been demonstrated, although the mode of action of these elements remains unclear.

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