Volume 303, number 2,3, 169–172 FEBS 11091 June 1992


# The 5′ untranslated region from pea seedborne mosaic potyvirus RNA as a translational enhancer in pea and tobacco protoplasts

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Received 9 March 1992; revised version received 9 April 1992

We have exploited the transient expression of foreign genes introduced into plant protoplasts to investigate the effect of the pea seedborne mosaic potyvirus (PSbMV) 5′ untranslated region (5′UTR) on the level of gene expression in pea and tobacco protoplasts. The plant viral 5′UTRs were found to increase translation significantly in comparison to a plasmid containing no 5′UTR of viral origin. The enhancement effect of the 5′UTRs of PSbMV and tobacco etch potyvirus (TEV) was found to be similar in pea and tobacco protoplasts, indicating a host-independent role of the potyviral 5′UTRs in enhancing gene expression. Translational enhancement of the two potyviral 5′UTRs was similar to that of the 5′UTR of tobacco mosaic virus (TMV). This observation makes it attractive to use potyviral 5′UTRs as general translational enhancers in future genetic transformations of plants.

Pea seedborne mosaic potyvirus (PSbMV); Transient expression; 5′ untranslated region; *Nicotiana tabacum*; *Pisum sativum*

## 1. INTRODUCTION

Plant viral 5′ untranslated regions (5′UTRs) have been shown to enhance translation in plant cells [1–3]. One of the most efficient 5′UTRs investigated derives from tobacco mosaic virus (TMV). This 5′UTR consists of 68 nucleotides and enhances translation in fundamentally different environments including in vitro messenger-dependent extracts derived from *E. coli*, wheat germ and rabbit reticulocyte lysates, and in vivo in tobacco protoplasts and *Xenopus laevis* oocytes [2]. The TMV 5′UTR has a high content of A+U bases, and the predicted absence of stable secondary structures has been put forward as an explanation of the general enhancement effect [4].

In contrast to TMV RNA and most eukaryotic mRNAs, RNA molecules from poty- and picornaviruses lack a cap structure at their 5′ end which is believed to direct the entry of ribosomal subunits [5]. Internal entry of ribosomes has been suggested to take place in the animal picornaviruses to circumvent the need for a cap [6,7]. Similarly it was found that a 5′UTR from tobacco etch potyvirus (TEV) enhances translation in vitro independently of a cap structure [1].

We are working on coat protein-mediated resistance in pea against pea seedborne mosaic virus (PSbMV) and are interested in the ability of the PSbMV 5′UTR (143 nucleotides in size) to enhance translation in pea. A direct comparison of the relative efficiencies of different plant viral 5′UTRs in different host plants has not previously been done. In order to test for such possible plant species specificities of the 5′UTRs we have compared the effect of the PSbMV 5′UTR in protoplasts from pea and tobacco (host and non-host to PSbMV, respectively) with the effect of the 5′UTRs from TEV and TMV (both of which are infectious to tobacco but not to pea) on translation of the fused downstream reporter gene β-glucuronidase (GUS). This is also a test for the correlation between the host range of plant viruses and the effect on translation of the viral 5′UTR in the respective plant species.

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## 2. MATERIALS AND METHODS

### 2.1. *Plant material*

3-week-old pea (*Pisum sativum* L. cv. 'Fjord') plants grown in a greenhouse and sterile tobacco plants (*Nicotiana tabacum* L. cv. Samsun) were grown in darkness 3 days prior to protoplast isolation.

### 2.2. *DNA constructions*

The 5′UTR of PSbMV was cloned in pPS5 and pPS29 as described earlier [8]. The 5′UTR was assembled by opening pPS5 with *Sph*I and *Spe*I and inserting the *Sph*I/*Spe*I fragment of pPS29 to create pPSL1. pPSL1 was used as a template for introduction of an *Nco*I site at the AUG start codon using PCR and a 5′ add-on primer as described by Higuchi [9]. The primers used were the T7 primer which primes in the vector pGEM3Z next to the 5′ terminus of PSbMVs 5′UTR, and the 5′ add-on primer (5′-AGGATCCATGGTTTGATGAGCTTG-TTAC-3′) which primes just 5′ to the AUG start codon of PSbMV. The 5′ add-on primer contains an AUG start codon within the *Nco*I site (underlined). The PCR product contains two C's inserted between the PSbMV 5′UTR and the AUG start codon, as compared to the authentic PSbMV RNA.

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The transient expression vector pAGUS-1 [10] was digested with *Bam*HI, blunt-ended by treatment with mung bean nuclease, and, after phenol extraction, digested with *Nco*I. The 5'UTR of PSbMV was inserted into the vector fragment leading to pAGUS-PS (Fig. 1). This vector contains the authentic PSbMV 5'UTR except for the translation initiation site, where two C nucleotides have been inserted by PCR mutagenesis to create an *Nco*I site. The plasmid pAGUS-PS83, containing half of the PSbMV 5'UTR (nucleotides 1–83), was created by digesting pAGUS-PS with *Spe*I and *Nco*I, blunt-ending the restriction sites with Klenow enzyme and re-ligation of the vector (Fig. 1). pAGUS-TEV was constructed by digesting pRTL2–4G [1], a vector harbouring the TEV 5'UTR, with *Eco*RI followed by treatment with mung bean nuclease. After phenol extraction the DNA was digested with *Nco*I. The TEV 5'UTR fragment was isolated and ligated into the pAGUS-1 vector fragment leading to pAGUS-TEV, containing the authentic TEV 5'UTR except that two C nucleotides were inserted at the translation initiation site and that the 5'UTR contains an extra C nucleotide at its 5' end (Fig. 1). Except for the creation of an *Nco*I site at the translation initiation site of pAGUS-TN2 the 5'UTR of this vector is identical to the 5'UTR of TMV strain U1 [10] (Fig. 1). Plasmid DNA was introduced into *E. coli* using the method of Hanahan [11]. All vector constructions were checked by sequencing the relevant regions using the Sequenase ver. 2.0 kit (United States Biochemicals, USA).

2.3. *Protoplast isolation*

Pea mesophyll protoplasts were isolated essentially according to Glimelius [12]. Tobacco mesophyll protoplasts were prepared as the pea prototoplasts except that the enzyme solution was diluted with 0.5 vol. CPW9M containing 9% w/v mannitol [13].

2.4. *Polyethylene glycol-mediated transformation of protoplasts*

The protocol for PEG-mediated transformation of protoplasts was essentially as described by Negrutiu et al. [14].

2.5. *GUS assay and protein determination*

GUS assays were performed as described by Jefferson [15]. Protein content in each extract was measured using the protein assay kit from Bio-Rad Laboratories (Richmond, CA) with bovine serum albumin as standard.

2.6. *Software*

The 'Sequence Analysis Software Package' of the Genetics Computer Group, University of Wisconsin, was used. RNA secondary structure was analysed using the FOLD programme of Zuker and Stiegler [16].

## 3. RESULTS

pAGUS-1 [10] is a derivative of pBI 221 [15] in which the CaMV 35S promoter region −390 to −90 was duplicated to create a stronger promoter (CaMV35Se, Fig. 1). A duplication of CaMV 35S promoter sequences has previously been reported to cause significantly increased levels of transcription [17]. Furthermore, the sequence surrounding the translation initiation site has been changed from CTTATGT to ACCATGG in order to create an optimal context for initiation of translation [18]. Surprisingly our investigations show that there is no significant difference in GUS expression between pBI 221 and pAGUS-1 (data not shown) even though two putative improvements have been carried out in pAGUS-1 compared to pBI 221. Our data are supported by Skuzeski et al. [10] who observed only a small increase in GUS expression for pAGUS-1 compared to pBI 221 in tobacco protoplasts.

The transcription initiation site in pAGUS-1 starts at the G residue located 12 nucleotides upstream of the initiator codon AUG [10,19]. Assuming that only upstream sequences determine the transcription initiation site this means that the 5' end of the transcripts from the transient gene expression vectors, pAGUS-PS and pAGUS-TN2, are identical to the 5'UTRs of PSbMV and TMV strain U1, respectively, while the transcripts

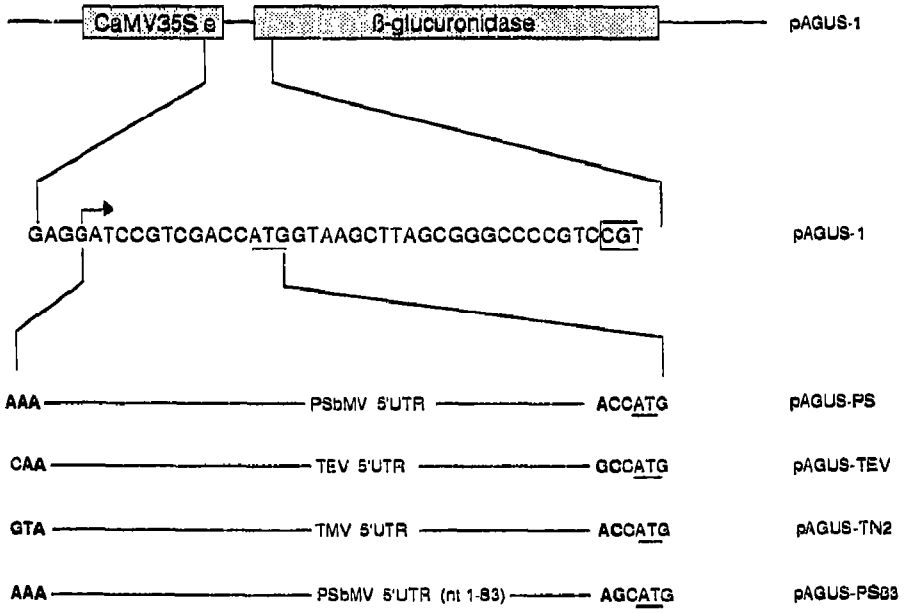


Fig. 1. pAGUS-1 and derived DNA constructs harbouring plant viral 5'UTRs. Only sequences surrounding transcription and translation initiation sites are shown. Bases printed in bold are of viral origin. The arrow indicates the transcriptional start site. The ATG start codon is underlined. The boxed CGT codon corresponds to codon 3 in wild-type GUS. For details see text. Partly from Skuzeski [10].

synthesized from pAGUS-TEV contain an extra C nucleotide at its 5' end which is not of viral origin.

The influence of the various 5'UTR constructs on GUS expression in tobacco and pea protoplasts is shown in Fig. 2. Each figure represents 3 individual experiments and each bar represents the average of double determinations. As can be seen from Fig. 2 the viral 5'UTRs enhance GUS expression relative to pAGUS-1 in all cases. The three plasmids pAGUS-PS, pAGUS-TN2 and pAGUS-TEV enhance GUS expression approximately 10 times compared to pAGUS-1, and this activity enhancement is observed both in pea and tobacco protoplasts.

pAGUS-PS83, which contains only the first 83 nucleotides of the authentic 143 nucleotide 5'UTR sequence of PSbMV, is the most efficiently expressed construct in both protoplast systems with a level of expression almost twice as high as for any of the other plant viral constructs (Fig. 2).

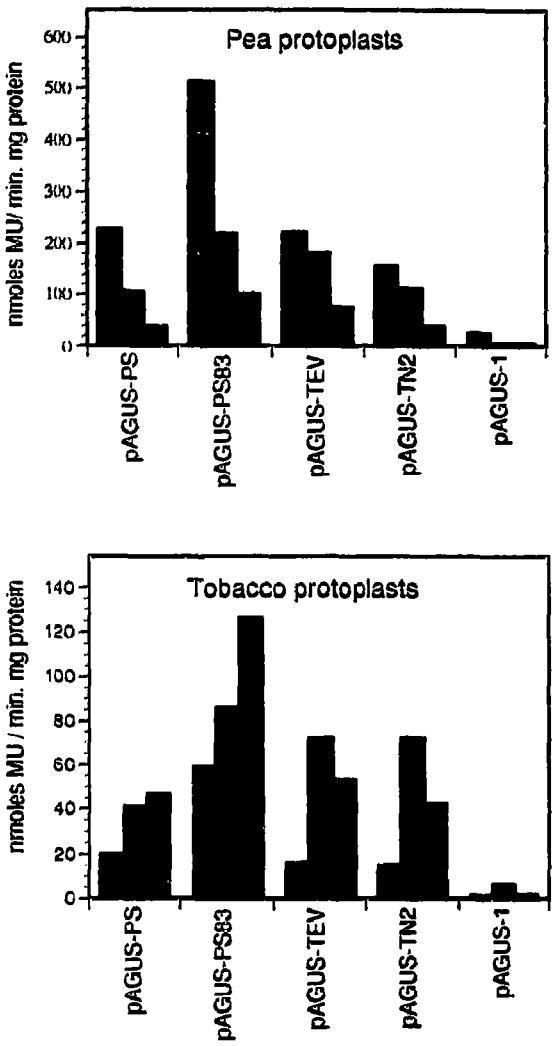


Fig. 2. The effect on GUS activity of various plant viral 5'UTRs in pea and tobacco protoplasts. The three types of bars represent three individual experiments. Each bar represents the average of double determinations. See text for details on plant viral 5'UTR plasmid constructs.

## 4. DISCUSSION

A major aim of this work was to determine whether there is a correlation between the ability of plant viral 5'UTRs to enhance translation in different plant species and the host range of the respective plant virus, or whether plant viral 5'UTRs can be used as general enhancer elements. We chose pea and tobacco protoplasts, as pea is a host for PSbMV but not for TMV or TEV [20,21], whereas tobacco is a host for TMV and TEV but not for PSbMV [22]. As can be seen from Fig. 2 the GUS expression pattern of the various DNA constructs is similar in pea and tobacco protoplasts, indicating that translation of the plant viral 5'UTRs does not play a major role in determining the host range of viruses in pea and tobacco, and hence, the plant viral 5'UTRs can be exploited as general enhancers of translation. These results corroborate the observation that host range is often determined at the level of cell-to-cell movement and is not solely manifested at the level of viral replication [23,24].

Interestingly Gallie et al. [2] reported that the turnip yellow mosaic tymovirus (TYMV) 5'UTR did not enhance translation in tobacco protoplasts. This failure to enhance translation could not be correlated to extensive secondary structure or other factors believed to influence translation. On that basis, it was suggested that the TYMV 5'UTR is very host dependent in its ability to enhance translation. This hypothesis needs, however, to be experimentally confirmed as protoplasts from other species, especially turnip, were not included in their investigations.

In pAGUS-PS83 only the first 83 bases of the PSbMV 5'UTR are present. This truncation caused a doubling in translational efficiency compared to the authentic PSbMV 5'UTR (Fig. 2). This indicates that length of the 5'UTR is not the sole determinant of translational enhancement. Furthermore Sleat et al. [4] showed that a non-viral 5'UTR of approximately the same length as the TMV 5'UTR did not enhance translation significantly in comparison with the TMV 5'UTR. The absence of secondary structure has been suggested to play a major role in determining the ability of mRNA 5'UTRs to enhance translation [3]. This may be put forward as an explanation of why the shortening of the PSbMV 5'UTR caused an enhancement of translation compared to the authentic PSbMV 5'UTR. A stem and loop structure predicted by computer analysis of the first 200 bases in the PSbMV genome was disrupted in pAGUS-PS83.

The enhancement of GUS expression caused by the viral 5'UTRs could theoretically be due to an increase in transcription efficiency, as well as an increase in translation efficiency. However, previous investigations

using mRNA transcripts, with or without viral 5'UTRs, introduced directly into protoplasts have indicated that the enhancement effect is engendered at the level of translation [1,2].

The 5'UTRs of the two potyviruses, PSbMV and TEV, were found to enhance GUS activity to a similar level as the 5'UTR of TMV in the two protoplast systems analyzed in this study. In contrast to TMV RNA, potyviral RNA molecules lack a cap structure at their 5' end. Instead the 5' end of potyviral RNA is linked to a virus-encoded protein, VPg. Enhancement of translation by the TEV 5'UTR has been found in vitro to be cap independent [1]. Thus, the possibility exists that the enhancement of translation observed for the two types of viral 5'UTRs is caused by different translational mechanisms.

We have cloned and characterized a translational enhancer from PSbMV. Together with the TEV 5'UTR we demonstrated it to be functional in a plant outside PSbMV and TEV's hostrange. We thus suspect that potyviral 5'UTRs are generally functional as enhancers of gene expression in dicots.

*Acknowledgements:* We thank Dr. James M. Skuzeski (The University of Utah, Salt Lake City, USA) for the plasmids, pAGUS-1 and pAGUS-TN2, and Dr. James C. Carrington (Texas A & M University, USA) for the plasmid, pRTL2-4G.

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