**Note**

Mutational Analysis of the Potato Virus Y 5' Untranslated Region for Alteration in Translational Enhancement in Tobacco Protoplasts

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The 185 nucleotide 5' untranslated region (5'UTR) of potato virus Y ordinary strain (PVYO) showed translation-enhancing activity on the β-glucuronidase (GUS) gene in tobacco protoplasts. Mutational analysis of the 5'UTR was done to find sequence motifs necessary for the enhancement. Deletions within the 1-130 nucleotide region of 5'UTR stimulated the GUS expression in some cases, while the GUS activity declined with deletions in the 131-185 nucleotide region. The results indicated that the last 55 nucleotides of PVYO 5'UTR might play the much important role in the translational enhancement in plant cells.

**Key words:** potato virus Y; 5' untranslated region; translational enhancement; mutational analysis

Plant viral 5' untranslated regions (5'UTRs) have been shown to enhance translation in vitro or in planta (reviewed in refs. 1 and 2). One of the best studied ones is the omega (Ω) sequence of tobacco mosaic virus (TMV). In Poyytivirus genus, fusion of the 5'UTR from tobacco etch virus (TEV) to a reporter gene encoding β-glucuronidase (GUS) enhanced protein expression 8- to 21-fold in vitro and in vivo. Similarly, it was found that 5'UTRs of pea seed-borne mosaic virus (PSSMV) and turnip mosaic virus (TuMV) enhanced translation of GUS in vivo and/or in vitro.

Although the function of 5'UTR of potato virus Y n strain (PVYN) in translation initiation has been studied in vitro, there were no reports on the in vivo function of PVY 5'UTR. Besides, the specific functional domains or motifs in 5'UTRs of potyviruses have not been identified.

This study was done to analyze the properties of 5'UTR of potato virus Y ordinary strain (PVYO). To investigate whether this 5'UTR functions as a translational enhancer, as well as whether specific nucleotide motifs are required for this activity, the 5'UTR and its derivatives were fused to the GUS reporter gene and their effects on translation of downstream GUS in tobacco protoplasts were examined.

The complete nucleotide sequence of the genomic RNA of PVYO was analyzed in our laboratory (M. Hidaka et al., in preparation). The 5'UTR of PVYO consists of 185 nucleotides (nt) and has 96% identity to that of PVYN (Fig. 1A). Multiple alignments of the 5'UTR sequences of seven PVY strains7-11 showed conservation of several motifs such as UUUCG pentanucleotide blocks9 and 'Box a' and 'Box b' motifs.11 However, the functions of these motifs have not been discovered yet. The CAA tri-nucleotide blocks, which are the elements of the (CAAn) region in the TMV Ω sequence, were also found. Formation of two stem-and-loop structures were predicted by computer analysis.

![Fig. 1. Multiple Sequence Alignment of PVY-O 5'UTR with Other PVY Strains and Its Predicted Secondary Structure.](image)

The 5'UTR sequences of PVY-O, PVY-I (PVYO), PVYn, PVYc (PVYC), PVYb (PVY-B), and PVYJ, and PVYmm11 are aligned in panel A. Dashes indicate gaps inserted to improve sequence alignments. 'Box a' and 'Box b' are indicated by shadowed boxes. UUUCG motifs are in open boxes. CAA sequences are indicated by bold face. In panel B, the secondary structure of the PVY-O 5'UTR region (1st) predicted by MFOLD programs (Michael Zuker) is shown. The stability of this structure is calculated to be -49.4 kJ/mol. 'Box a', 'Box b', and UUUCG motifs are indicated by bold face. The viral initiation codons are underlined in both panels.

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containing 5'UTR and ATG, *i.e.*, the XbaI–BamHI fragment of pVH185.

The plasmids pENGUS-1, pENGUS-2, pENGUS-3, pENGUS-4, pENGUS-5, pENGUS-6, and pENGUS-7, which have deletions corresponding to the 5'UTR at 1–16, 19–25, 34–46, 68–75, 85–89, 136–158, and 176–188 (including the viral initiation codon) nt positions, respectively, were obtained as follows: After site-directed mutagenesis of pVH185, the deletion-containing fragments were cloned into XbaI/BamHI-digested pGUS. The 1–130 or 131–183 nt regions of 5'UTR were amplified from pVH185 by PCR and were also cloned into XbaI/BamHI-digested pGUS to generate pENGUS-8 and pENGUS-9, respectively.

Each resulting plasmid was introduced into tobacco (*Nicotiana tabacum* Samsun NN) protoplasts by electroporation and the cells were then cultured for 2 days. Fluorometric analysis of GUS activity expressed transiently from the plasmids in tobacco protoplasts was done essentially as described.1,2

As shown in Fig. 2B, the GUS activity expressed from pENGUS (19,100 U) was 24-fold higher than that from pGUS (805 U). Although we have not analyzed the amounts and stability of mRNAs transcribed from both plasmids, we believe that this dramatically higher GUS activity of pENGUS reflects the translation-enhancing activity of the PVY-O 5'UTR.

Compared to pENGUS, deletion of the first 16 nucleotides (pENGUS-1) stimulated translation of GUS. Deletions of the conserved 'Box a' (pENGUS-2) and 'Box b' (pENGUS-4) did not show significant changes on translation of GUS. GUS activity was increased upon the deletion of the mid-part of the first stem-and-loop structure (pENGUS-3) and deletion of the first UUUCUA (pENGUS-5), which is also in the mid-part of the first stem-and-loop structure, significantly stimulated GUS activity. Interestingly, the deletion of the second and third UUUCUA repeats (pENGUS-6) slightly reduced GUS activity. When the viral AUG codon and its upstream region were deleted (pENGUS-7), the GUS activity decreased considerably. pENGUS-8, which contains only the 3-terminal 55 nt of the PVY-O 5'UTR, did not change the GUS activity. On the contrary, the translation of GUS from pENGUS-9 having the first 130 nt of 5'UTR was reduced significantly.

In this study, we could not conclusively define the responsibility of the motifs conserved in PVY-O 5'UTR to the translational enhancement because deletion of each motif did not exhibit loss-of-function. Functional redundancy may exist within PVY-O 5'UTR like TMV.6

Levis *et al.* showed that the first 16 nt of 5'UTR have an essential function in initiation of translation in vitro.6 They therefore suggested that this region might be an attachment site for ribosomes. If this observation holds also for the *in planta* system, then deletion of this region might result in the loss of translational initiation and enhancement. However, our results clearly showed that the first 16 nt were completely dispensable for translational initiation and enhancement. At present, the reasons for these contradictory results are unclear.

Nicolaisen *et al.* showed that the shortened 5'UTR (having initial 83 nt of 143 nt 5'UTR) of PShMV increased translation compared to the intact 5'UTR and suggested the absence of a stable secondary structure plays a major role in determining the ability of 5'UTRs to enhance translation.40 The stimulation of GUS translation caused by pENGUS-3 and pENGUS-5 could be explained in the same way because computer analysis suggested the disruption of the first stem-and-loop structure by these deletions. On the other hand, disruption of the second stem-and-loop structure (pENGUS-6 and pENGUS-8) did not cause significant change of GUS activity. As the stem part of this structure is short, this structure may not be a great hindrance to ribosomes.
Truncating the first 130 nt of 5′UTR, which leaves only the rest 55 nt of 5′UTR (pENGUS-8), did not change the GUS activity. However, the partial or complete deletions of the 3′-terminal 55 nt of 5′UTR, pENGUS-6 and pENGUS-9, respectively, significantly reduced GUS activity. This indicates that the 3′-terminal 55 nt region of PVY-O 5′UTR is very important in the translational enhancement. This phenomenon is similar to the findings of Carrington and Freed that the functional region of TEV 5′UTR (143 nt) is contained within the last 63 nt. We can suggest a possibility that the local sequence context around the viral initiation codon or the sequence just upstream of the initiation codon is important. The reports suggesting that ribosomes bound to an internal site within the 5′UTRs of TuMV and PVYn may help to reveal the importance of this region.

Although the mechanisms of translational enhancement are not well understood, specific motifs, or binding domains for plant host translation factors or ribosomes, as well as the secondary configuration of 5′UTR may be involved in this process.

References