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5′-Leader of a Photosystem I Gene in *Nicotiana sylvestris*, *psaDb*, Contains a Translational Enhancer*

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Messenger RNA primary structures responsible for translational efficiency of a photosystem I gene, *psaDb*, of *Nicotiana sylvestris* were studied using a transgenic tobacco system. The entire 5′-leader (23 base pairs) with the first four amino acid codons of the protein coding region was fused in frame with the β-glucuronidase (GUS) gene under the control of the 35 S promoter of cauliflower mosaic virus (CaMV). This construct (CaMV::*psaDb*-GUS′) was introduced into tobacco. GUS activity and GUS mRNA levels were determined for individual transformants, revealing that the insertion of the *psaDb* sequence greatly enhanced the GUS activity relative to GUS mRNA abundance. The GUS activity/GUS mRNA was 14 times higher in the CaMV::*psaDb*-GUS′ transformants than in the control CaMV::GUS′ transformants. The high GUS activity/GUS mRNA of the CaMV::*psaDb*-GUS′ transformants was reduced 20-fold when 13 bases within the *psaDb* leader were altered. These 13 bases are common to the leaders of an *Arabidopsis* ferredoxin gene and the *psaDb* gene of *N. sylvestris*. Since GUS proteins encoded by these chimeric GUS genes have identical amino acid sequences, these results indicate that the 5′-leader of the *psaDb* mRNA contains a translational enhancer element.

Translation is a potential step to regulate gene expression, but its significance is quite poorly understood compared with transcriptional regulation (1). In eukaryotes, the most extensively studied cases involving translational regulation are the mammalian genes for ferritin and 5′-aminolevulinate synthase, whose products are involved in the storage and use of iron, respectively. Messenger RNAs of these genes have an iron responsible element within their untranslated leader (2). When iron is depleted, this element is bound by iron responsible element-binding protein (3), which depresses translation (2, 4–7).

Messenger RNAs of some plant viruses are translated with greater efficiency than host mRNAs. This phenomenon is ascribed to their leader sequences. This is true for tobacco mosaic virus, alfalfa mosaic virus RNA 4, brome mosaic virus RNA 3, potato virus X, and tobacco etch virus (8). In these cases the viral leaders contain translational enhancers; however, the underlying mechanisms remain to be elucidated. As for cellular mRNAs, there have been few reports on translational enhancer elements to date. In this study, we examined the 5′-portion of *psaDb* mRNA (see below) and found that its 5′-leader greatly enhances the translational efficiency of a chimeric β-glucuronidase (GUS)[1] reporter gene *in vivo*.

psaDb is a nuclear gene for a photosystem I (PSI) subunit in *Nicotiana sylvestris* (9), and its product, a PSI-D subunit, is known to associate with ferredoxin, which is a soluble electron carrier reduced by PSI. Recently, the untranslated leader of the *Arabidopsis* ferredoxin gene, *fedA*, was shown to enhance the expression of a reporter gene in a transient expression assay, but it is not known whether this enhancement occurs at translation or not (10). In this study, we found that the *fedA* and *psaDb* leaders share common motifs and that mutations introduced into these motifs abolished the translational enhancer activity of the *psaDb* leader.

EXPERIMENTAL PROCEDURES

Materials—*N. sylvestris* is a diploid ancestor of *Nicotiana tabacum*, which is an amphidiploid (11). The two species have highly homologous genomes. Constructs containing fragments from an *N. sylvestris* gene, *psaDb* (9), were transformed into *N. tabacum*.

Construction of Chimeric GUS Genes—Four fusion genes (see Figs. 1 and 6) were used in this study. 1) pBI121 contains the CaMV::GUS construct (12). 2) For CaMV::*psaDb*-GUS′ two oligonucleotides (5′-CTAGACTTCTCTCAATCCAACTTTTCTATGGCCATGGCAC-3′ and 5′-GATCGTGCCATGGCCATAGAAAAGTTGGATTGAGAGAAGT-3′) were annealed and inserted into the *Xba*I-*Bam*HI site of pBI221. After sequencing the introduced region, an *Eco*RI-*Hin*dIII fragment containing the resultant chimeric gene was introduced into a binary vector, pBI101, in place of the original promoterless GUS gene of pBI101 (12). 3) For CaMV::GUS′ two oligonucleotides (5′-CTAGATGGCTATGGCTC-3′ and 5′-GATCGAGCCATAGCCAT-3′) were annealed and introduced into pBI101 as for CaMV::*psaDb*-GUS′ described above. 4) For CaMV::*psaDb*LM-GUS′ two oligonucleotides, (5′-CTAGACTTCGAGACCTCACCAGGGTCTATGGCCATGGCAC-3′ and 5′-GATCGTGCCATGGCCATAGACCCTGGTGAGGTCTCGAAGT-3′) were annealed and introduced into pBI101 as described above.

Ti-Mediated Gene Transfer and GUS Assays—Binary vectors containing the chimeric GUS genes mentioned above were mobilized into *Agrobacterium tumerfaciens* LBA4404, and leaf discs of *N. tabacum* cv. Petit Havana SR1 were transformed (13). Plants were grown at 25 °C under continuous light. From the regenerated transformants (M_0 generation), fully developed leaves of 3–6 cm in Magenta culture boxes (Magenta GA-7, Sigma) were harvested and frozen in liquid N_2, ground to fine powder, and stored at −80 °C. A part of the powdered tissue was used for GUS assays (12), and the rest was used for RNA extraction. Protein concentration was determined according to Bradford (14) and used to normalize measured GUS activities.

RNA Analysis—Total RNA was extracted from the powdered tissue by the acid guanidium thiocyanate-phenol-chloroform method (15), followed by three phenol/chloroform extractions and LiCl precipitation. A GUS gene-specific primer (5′-GTCGAGTTTTTTGATTTCACGGGTTGGGGTTTCTA-3′) was labeled at the 5′-end with ^{32}P and used for primer extension analysis as described previously (9). Extension prod-

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[1] The abbreviations used are: GUS, β-glucuronidase; PSI, photosystem I; CaMV, cauliflower mosaic virus; UTR, untranslated region.

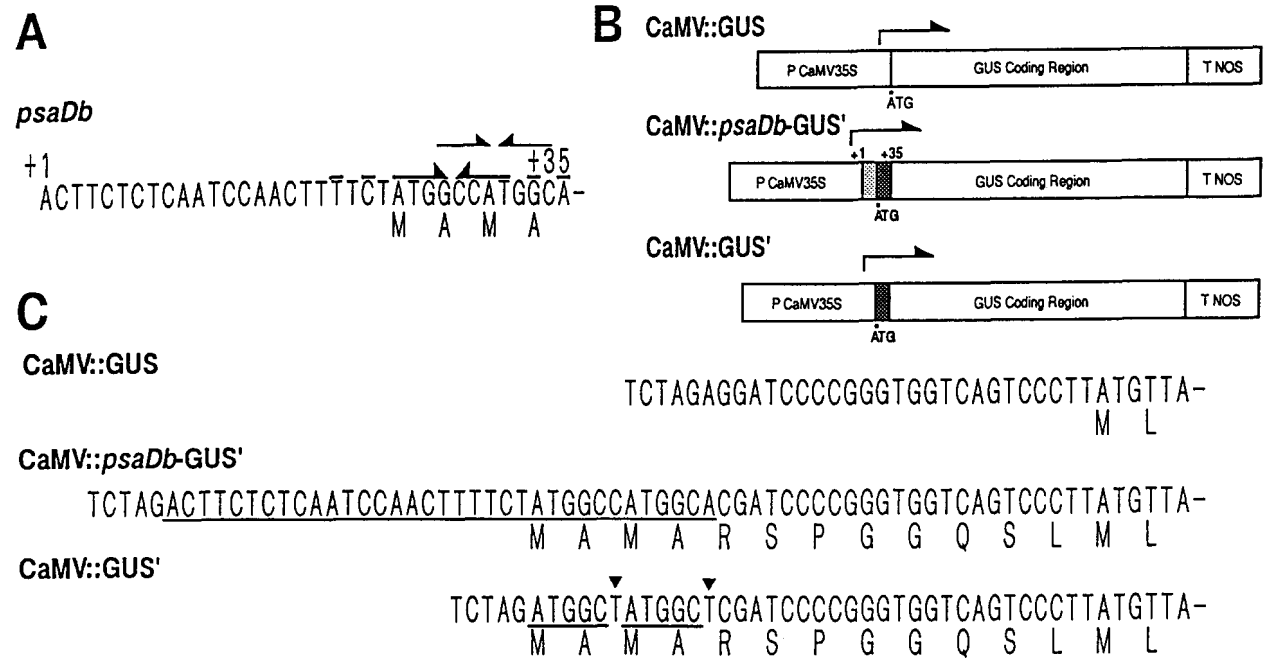

FIG. 1. **Constructs of the chimeric GUS genes.** *A*, the 5′-terminal sequence of the *psaDb* mRNA. The transcription start site is shown as *+1*. *Arrows* indicate palindromic sequences. *B*, schematic representation of the chimeric GUS genes. *Arrows* indicate the transcription start sites. CaMV::GUS represents the original GUS reporter gene of pBI121 (12). The *psaDb* fragment shown in *A* was inserted into the untranslated leader of CaMV::GUS to form CaMV::*psaDb*-GUS′. CaMV::GUS′ is a control gene identical to CaMV::*psaDb*-GUS′ except for the lack of the 23-base pair *psaDb* leader sequence and the two base substitutions as shown in *C*. *C*, nucleotide sequences of the chimeric GUS genes downstream of the *Xba*I sites (TCTAGA), aligned with the N-terminal sequences of the encoded proteins. The *underlined* portion shows the inserted *psaDb* sequence. *Arrowheads* indicate the base substitutions to alter the palindromic sequences.

ucts were electrophoresed in 8 M urea, 6% polyacrylamide gel, and the radioactivity of each band was analyzed using a Fujix BAS2000 Imaging Analyzer (Fuji Photo Inc., Japan).

RESULTS

Experimental Strategy—psaDb mRNA has a 23-base untranslated leader (Fig. 1*A*). In this study, we examined the possible involvement of this leader in translational regulation of *psaDb* mRNA by use of a transgenic tobacco system. The entire 5′-leader with the initial four codons of *psaDb* (Fig. 1*A*) were introduced into the untranslated leader of the original CaMV::GUS construct of pBI121 to obtain a chimeric GUS gene named CaMV::*psaDb*-GUS′ (Fig. 1, *B* and *C*). As a result of this insertion, the GUS protein gained 12 amino acids at its N terminus (Fig. 1*C*, CaMV::*psaDb*-GUS′), which might alter the specific activity or turnover rate of the protein. We refer to this modified GUS protein as GUS′. In order to assess the effect of the inserted *psaDb* sequence on GUS′ gene expression, we prepared a control GUS′ construct, CaMV::GUS′, whose protein coding region has the same nucleotide sequence as that of CaMV::*psaDb*-GUS′ except for the sixth and the twelfth nucleotides from the initiator ATG (Fig. 1*C*, *arrowheads*). These nucleotides were substituted in CaMV::GUS′ to alter the palindromic sequences at the translation initiation site but did not affect the amino acid sequence. These three types of chimeric genes, CaMV::GUS, CaMV::*psaDb*-GUS′, and CaMV::GUS′, were introduced into tobacco, and the resultant transformants were regenerated.

5′-Terminal 35 Bases of psaDb mRNA Enhance Translational Efficiency in Vivo—The steady-state GUS activity in leaves was determined for individual transgenic plants grown under continuous light (Fig. 2). In comparison with the control transformants (CaMV::GUS′), GUS activity was much higher in the transformants having the *psaDb* sequence (CaMV::*psaDb*-GUS′). This enhancement of GUS activity may have been caused by internal

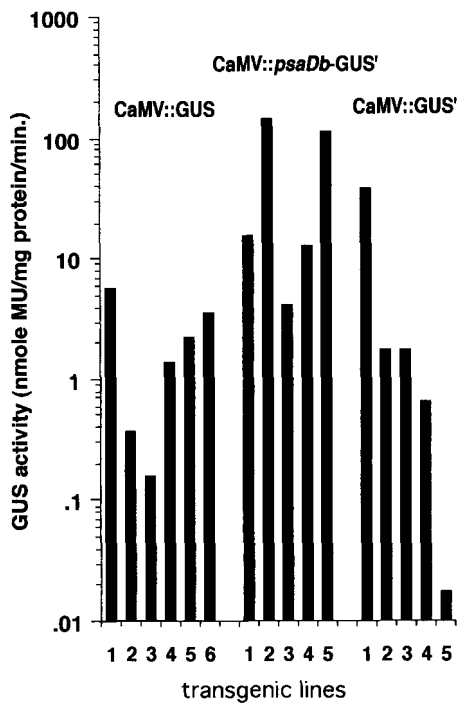

FIG. 2. **Insertion of the *psaDb* sequence elevates GUS activity in transgenic tobacco.** Mature leaves of individual transgenic lines were assayed for GUS activity. Note the logarithmic scale.

cis-elements for transcriptional activation, stabilization of the transcripts, and/or elevation of translational efficiency. For the purpose of comparing the translational efficiencies among these transgenic plants, we determined the steady-state mRNA levels of the chimeric GUS genes by primer extension analysis (Fig. 3).

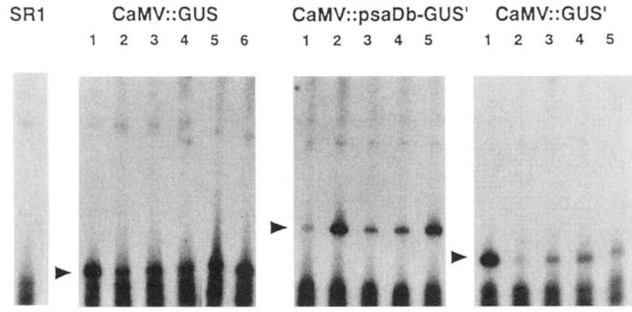

FIG. 3. **mRNA level of the chimeric GUS genes in transgenic tobacco.** Total RNAs prepared from an untransformed plant (SR1) and from the transformants indicated were subjected to primer extension analysis. *Numbers* indicate transgenic lines as in Fig. 2. *Arrowheads* indicate the bands of chimeric GUS mRNAs, which are absent from the untransformed plant (*SR1*).

The lengths of the extension products were different according to the lengths of the inserted fragments (Fig. 3). GUS activities of the individual transformants were divided by their respective mRNA levels (Fig. 4, *GUS activity/mRNA*), to examine the translational efficiency of the given GUS mRNAs. This value was 14 times higher in CaMV::*psaDb*-GUS′ than in CaMV::GUS′. Thus, the inserted *psaDb* leader of 23 bases and/or the palindromic sequences at the translational initiation site (Fig. 1, *A* and *C*) greatly enhances the translation of GUS′ messages.

5′-Leader Motifs Are Shared between psaDb of N. sylvestris and fedA of Arabidopsis—Recently, Casper and Quail (10) reported that the 5′-untranslated region (UTR) of the *Arabidopsis* ferredoxin gene, *fedA*, enhances gene expression. They fused the 5′-UTR of *fedA* to a luciferase reporter gene under the control of the *fedA* promoter, and the expression of this fusion gene was examined by a transient expression assay using *Arabidopsis* seedlings. When the 5′-UTR was deleted, luciferase activity was reduced 25-fold. The apparent similarity of the 5′-UTRs of *psaDb* and *fedA* in enhancing the reporter activity of chimeric genes led us to speculate that the translation of both genes may be enhanced by similar mechanisms. Therefore, the 5′-leader sequences of *psaDb* and *fedA* were compared (Fig. 5). These leaders share two common motifs, and these motifs cover more than half of the *psaDb* leader. We designate these 5′-leader motifs LM1 (TCTCAA) and LM2 (CAACTTT).

Substitution of LM1 and LM2 within CaMV::psaDb-GUS′ Greatly Decreases Translational Efficiency—In order to clarify whether LM1 and LM2 really operate as translational enhancers, these two motifs within the CaMV::*psaDb*-GUS′ construct were altered to form CaMV::*psaDb*LM-GUS′ (Fig. 6*A*). This construct with leader mutations was introduced into tobacco, and its GUS activity/GUS mRNA was determined for each transformant. As shown in Fig. 6*B*, mutations in LM1 and LM2 reduced the translational efficiency of the *psaDb*-GUS′ message 20-fold. This indicates that LM1 and/or LM2 are integral parts of the translational enhancer.

DISCUSSION

This study clearly showed that the 5′-terminal 35 bases of the *psaDb* mRNA contain a translational enhancer element (Fig. 4), and that base substitution within the 5′-leader abolished this enhancer activity (Fig. 6). These results lead us to conclude that the 5′-leader of *psaDb* contains a translational enhancer element. This element enhances translation as much as 20-fold (Fig. 6). Since translational enhancer elements have rarely been documented in eukaryotic cellular messages, characterization of this element deserves much attention. The primary question is how this element activates translation. In recent years, cap-independent translation initiation mediated

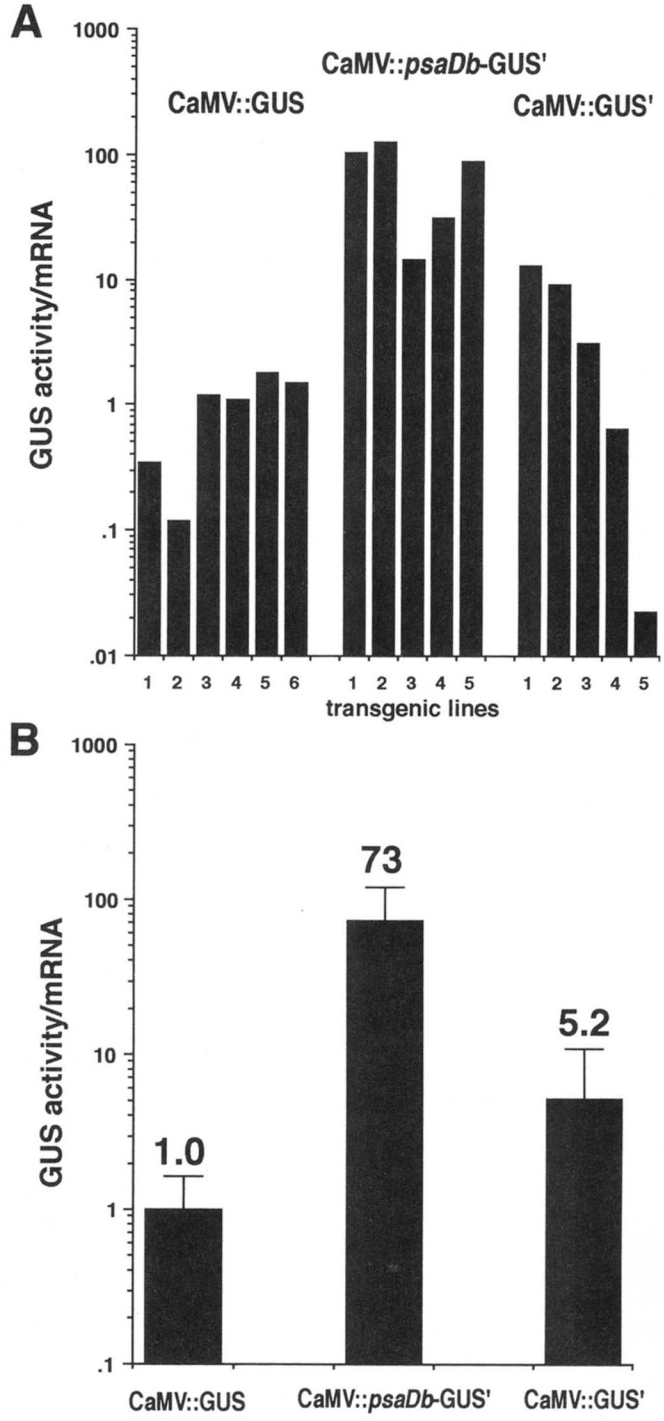

FIG. 4. **The ratio of GUS activity to GUS mRNA.** The average value of the CaMV::GUS transformants is presented as 1.0. *A*, the GUS activity/GUS mRNA was determined for each transformant. *Numbers* indicate transgenic lines as in Figs. 2 and 3. *B*, average values and standard deviations for each of the chimeric constructs are presented.

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                                        LM1     LM2
N.sylvestris psaDb                      ACTTCTCTCAATCCAACTTTTCT ATG
A.thaliana fedA TAATCCTCAAAAATCTCAACTTTATCTCCCAAAACACAAAACAAAAAAAA ATG
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FIG. 5. **Comparison of the entire 5′-leader sequences of the *psaDb* gene of *N. sylvestris* and the *fedA* gene of *Arabidopsis*.** Two conserved motifs designated as LM-1 and LM-2 are *underlined*.

by the 5′-leader has been reported in poliovirus (16), encephalomyocarditis virus (17), the human immunoglobulin heavy-chain binding protein (BiP) gene (18), and *Antennapedia* of

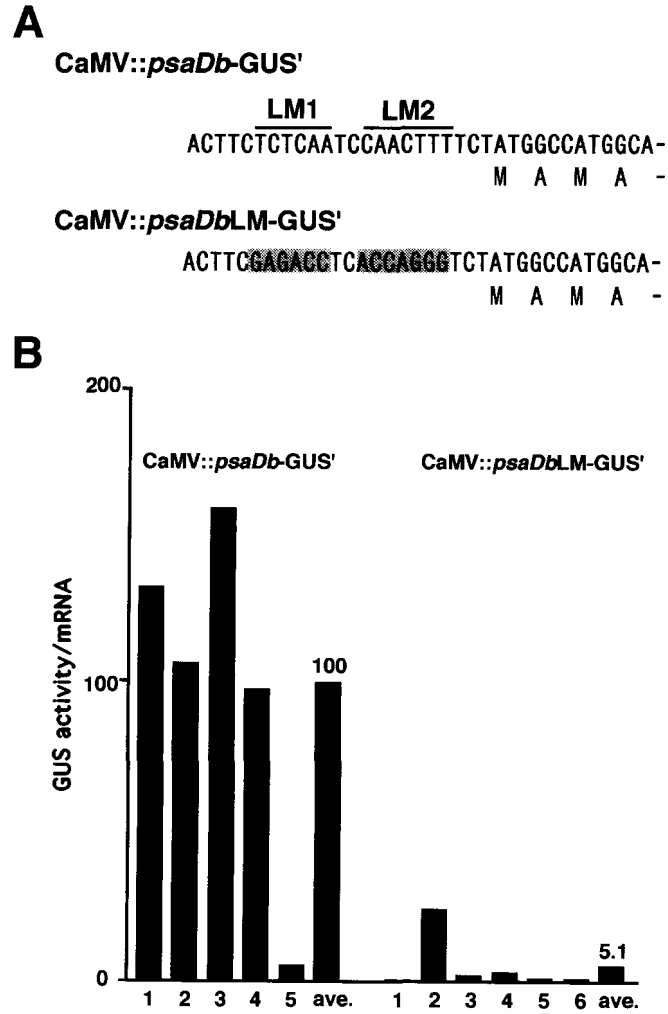

FIG. 6. **Substitution of LM1 and LM2 decreases translational efficiency.** *A*, the indicated 5'-leader motifs LM1 and LM2 in the CaMV::*psaDb*-GUS' construct were altered (*shaded boxes*) to generate a new chimeric construct, CaMV::*psaDb*LM-GUS'. *B*, GUS activity/GUS mRNA values were determined for individual transformants of CaMV::*psaDb*-GUS' and CaMV::*psaDb*LM-GUS', and the averages are shown.

Drosophila (19). In these cases, an internal ribosomal entry site within the leader is a determinant for the rate of translation initiation. However, in contrast to the short *psaDb* leader of 23 bases, these leaders are generally very long (several hundred nucleotides or more). Whether the translational enhancer of the *psaDb* leader exerts its function in a cap-dependent or independent manner should be examined. Computer analysis of only the 35-base *psaDb* sequence using an energy-minimizing algorithm indicates that it does not form a stable secondary structure (data not shown). It is possible, though unlikely, that the *psaDb* leader sequence in the chimeric CaMV::*psaDb*-GUS' construct interacts with the GUS coding sequence to form a secondary structure of functional significance. To study this, a chimeric construct using a reporter gene other than GUS is currently being examined.

This study revealed that the *psaDb* leader shares common motifs, LM1 and LM2 (Fig. 5), with the leader of the *Arabidopsis* ferredoxin gene, *fedA*. The *fedA* leader was reported to enhance the expression of a chimeric reporter gene 25-fold in a transient expression assay, but it is not known if this enhancement was exerted transcriptionally or post-transcriptionally (10). Judging from the result that substitution of LM1 and LM2 in the *psaDb* leader abolished the translational enhancer activity (Fig. 6), LM1 and/or LM2 are integral parts of the translational enhancer. Therefore, it is likely that LM1 and/or LM2 in the leaders of both *psaDb* and *fedA* enhance translation. Since the *psaDb* product (a PSI-D subunit) is the binding site of ferredoxin on PSI's surface (20), it is reasonable that *psaD* and *fedA* are co-regulated, in this case at translation. Further dissection of the LM1 and LM2 motifs in respect to their enhancer activity and to their possible interactions with cellular proteins may provide insight into translational regulation in plant cells.

The GUS activity/GUS mRNA value was 5-fold higher in the CaMV::GUS' construct than in the original CaMV::GUS construct (Fig. 4). This difference is probably, at least in part, due to the two ATG codons in the 12 amino acids introduced at the N terminus of the protein, since the number of N-terminal methionines is known to affect translational efficiency (21, 22). In plant genes, AACAATGGC is highly conserved at the translation initiation site, and a G residue at the +4-position (relative to the A of the ATG) is especially conserved (23, 24). In this respect, CaMV::GUS' has a better context (ATGG) than the original CaMV::GUS (ATGT), and this may be another contributing factor for the noted difference in translational efficiency. In addition, the 12 amino acids introduced at the GUS' N terminus may slightly alter the protein stability and/or enzyme activity. However, though worthy of study, this difference in translational efficiency between CaMV::GUS' and CaMV::GUS is not the main concern of this paper; the enhancer activity of the *psaDb* leader is.

Last, we mention the applicability of the *psaDb* enhancer to plant gene technology. In transgenic tobacco, apparent GUS activity of the CaMV::*psaDb*-GUS' construct was about 2 orders of magnitude higher than that of the CaMV::GUS construct (Fig. 2). For what we have examined, the expression of the CaMV::*psaDb*-GUS' construct showed no apparent organ

specificity.[2] Thus, the CaMV::*psaDb*-GUS′ construct could be used instead of CaMV::GUS for a 100-fold increase of expression. Furthermore, the *psaDb* leader itself could be used to increase expression levels for a wide variety of plant vectors.

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