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Translational Enhancement by the 5′ Leader of Tobacco Mosaic Virus and Soybean Glycinin Gene in Transgenic Tobacco Plants

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Abstract: To increase the expression of a foreign protein in transgenic plant, the benefits of 5′-untranslated leader sequences of tobacco mosaic virus (TMV) RNA or soybean glycinin gene, *Gy2*, fused to a protein coding sequence were exploited. pGA643-derived plasmid contains 35S promoter of cauliflower mosaic virus, protein coding sequence of maize 10 kDa zein (*10kZ*) and *Gy2* terminator. The leader from *Gy2* or TMV RNA was inserted between the promoter and the coding sequence in each construct. The recombinant DNAs were introduced into tobacco plants by *Agrobacterium* mediated leaf disc transformation method. Although the transgene without the leader had more transcripts than the others, mRNAs containing the leader were translated more efficiently. It might be due to difference in the length of 5′-untranslated sequence and context surrounding the AUG codon, but could be sequence specific rather. These results suggest that the leader sequences of *Gy2* and TMV play important roles as an enhancer in translational control of foreign gene in transgenic tobacco plant(Received April 22, 1995; accepted June 21, 1995).

Introduction

Transformation and expression of a foreign gene in plant cells has become a major tool to obtain improved plant varieties of agricultural or commercial interest.[1-2] For successful improvement of plants, the transferred foreign gene should be well-translated as well as well-transcribed. Cauliflower mosaic virus (CaMV) 35S and 19S promoter, strong constitutive one, has been widely used for the successful expression of a range of heterologous genes in transgenic plants.[3] But all of transgenes under the control of strong promoter can not be expressed as intended. In order to shoot these troubles, post-transcriptional regulation including translational control should be counted.[4]

It was recently known that the 5′-untranslated leader sequences of plant viral RNAs can act as efficient enhancer for heterologous mRNA translation *in vitro* and *in vivo*, as well as in prokaryotic and eukaryotic systems.[5-6] When the natural leader sequences of a barley and a human gene were replaced with alfalfa mosaic virus RNA 4 leader sequence, there was 35-fold increase in mRNA translational efficiency in the rabbit reticulocyte and wheat germ systems.[7] Similar constructs were made including the uncapped mRNAs for two vertebrate genes and the bacterial GUS gene with or without the 67-nucleotide Ω sequence at 5′-terminal which was derived from the untranslated region of TMV RNA, and they were tested in the rabbit reticulocyte, wheat germ, and *E. coli* systems.[8] The TMV leader sequence enhanced translation of almost all kinds of mRNA in all of these systems.[8] The leader of TMV RNA was known to have two motifs, eight base direct repeat and (CAA)n motif, for translational enhancement.[9] Translational enhancement by the leader of CaMV was also observed.[10] However, the major factor of the enhancement seems to be due to a low dependence of the cap structure, cap binding protein[11] and initiation factors.[12]

The 5′-untranslated leader sequences of genes for highly abundant protein, such as a storage protein, were expected to have positive functions in the enhancement of gene expression. *Gy2*, one of the genes for major soybean storage protein, is synthesized during embryogenesis and reaches maximum at the mid-maturation stage.[13-15] Even though the tissue-specific expression of seed protein genes was known to be regulated mostly on the level of transcription,[16] it is possible that the efficient translation contributes to high level of gene expression.

In this report, we studied the effect of the TMV lead-

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er as well as *Gy2* leader for an efficient expression of transgene in transgenic tobacco plants. Transformed tobacco plants were analysed by Northern blot and immunoblot to compare the relative amount of mRNA with expressed protein. Our results demonstrate that the leaders used in recombinant transgenes can enhance the levels of foreign protein in tobacco plants. We show that this enhancement is primarily due to increased translation of the mRNA.

Materials and Methods

Strains, plasmids, and culture media.

Agrobacterium tumefaciens 5922 and PC2760 for plant transformation and *Nicotiana tabacum* var. *Xanthi* for a recipient strain of transformation were used.[17] *Escherichia coli* MC1061 [F^-, *araD139*, Δ(*ara, leu*)*7696*, Δ*lacY74*, *galU*$^-$, *galK*$^-$, *hsdR*$^-$, *strA*], plasmid pUC18 and pGA643 were used for DNA manipulation. LB medium for *E. coli.* culture[18] and MS medium for tobacco cell culture[19] were used.

Enzymes and chemicals.

Klenow fragment, calf intestinal alkaline phosphatase, restriction enzymes and T4 DNA ligase were purchased from IBI and NEB. dNTPs were from Pharmacia. RNase A, proteinase K, agarose and MS medium were from Sigma Chemical Co. [α-^{32}P]dATP, nitrocelluose membrane, nylon membrane and ECL detection kit were from Amersham. Coomassie protein assay reagent was (23200 X) from PIERCE.

Construction of transgenes.

The following derivatives of pGA643,[20] an effective binary vector for heterologous gene expression in plant cells, were constructed (Fig. 1). We used a cDNA clone of 10 kDa zein, one of maize storage protein, as a coding gene, which was kindly provided by Dr. J. Messing.[21] In addition to *10kZ* coding sequence, recombinant plasmids contain CaMV 35S promoter and the fragment from 3′-noncoding region of *Gy2*, to ensure adequate termination of a transgene. In pCa Ω10G, a shorter form of leader sequence (Ω*) of the TMV U1 strain[9] was inserted between 35S promoter and *10kZ* coding sequence. In Ω*, 8 nt at 5′ end of Ω was accidentally deleted during the construction. In pCag10G, the leader sequence (g) of *Gy2*, was inserted instead of Ω*.[22] Fig. 1A shows the nucleotide sequences of the 5′-upstream region of recombinant plasmids. The map of the transgene contsructs are shown in Fig. 1B.

Transformation and regeneration of tobacco plants.

Tobacco plant (*N. tabacum* var. *Xanthi*) was transformed by leaf disc method with *Agrobacterium*,[23] and cultured at 28℃ with a 15 hrs-photoperiod. Callus was induced from *Agrobacterium*-infected leaf discs cultured on a semisolid [0.8% (w/v) agar] MS medium[19] with 0.5 mg/*l* 6-benzylaminopurine (BAP), 2.0 mg/*l* 1-naphthalene acetic acid (NAA), 3% (w/v) sucrose, pH 5.7. Kanamycin (200 mg/*l*) was used to select transformants. Rooting was carried out by transferring kanamycin-resistant shoot on a semisolid MS medium containing 0.1 mg/*l* NAA. After 1 month, the plantlets were transplanted to a vermiculite mixed soil.

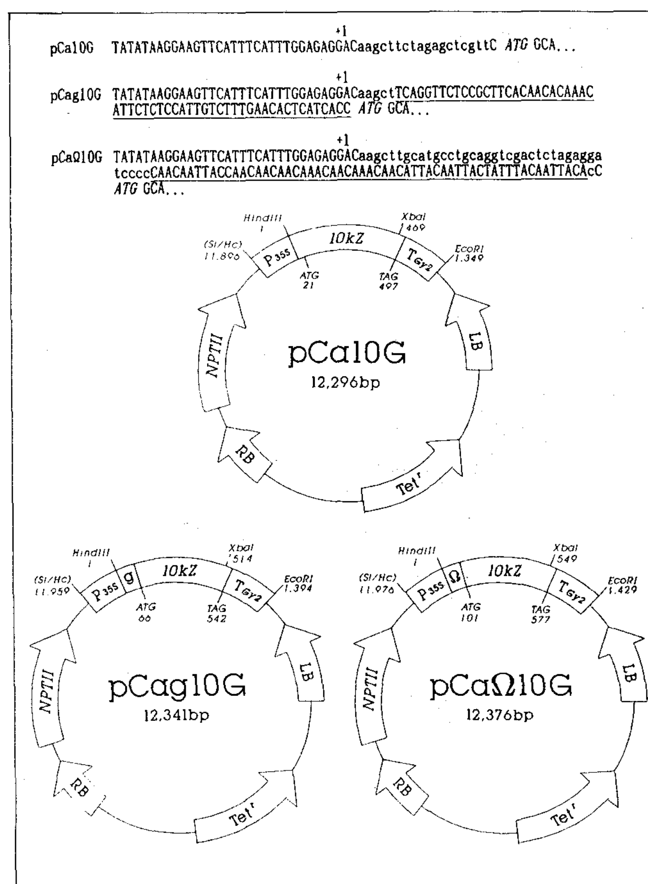

Fig. 1. Structure of pGA643-derived plasmid. A. The 5′-upstream sequences of chimeric *10kZ* in each transgene. The sequences from pGA643 linker are shown in lower case letter, the leaders are underlined, and the first ATG in italics. The predicted transcriptional start site is indicated +1. B. Plasmid map of each transgene. They contain CaMV 35S promoter (P_{35S}) from pGA643, 10 kDa zein coding region (*10kZ*) and soybean glycinin gene, *Gy2*, terminator (T_{Gy2}). The leaders from *Gy2*[21] and TMV RNA[9] is inserted between the promoter and the coding region (in pCag10G and pCaΩ10G). NPTII, neomycin phosphotransferase; Tet^r tetracyclin resistance gene; RB, right border; LB, left border.

Preparation of DNA.

Plasmid DNAs were prepared as described by Sambrook.[18] Genomic DNA was prepared by the precedure

of Shure with minor modification.[24]

PCR and Southern blot analysis.

PCR was carried out[25-26] in a volume of 100 μl with 150 ng of upstream primer (5′-ACACTTGATACATGTG-CCTG-3′) taken from the NOS terminator region of *npt* II gene,[27] 150 ng of downstream primer (5′-GCGGACG-TTTTTAATGTACT-3′) from the left border region of pTiT37,[28] 500 ng of template DNA, 2.5 units of *Taq* DNA polymerase, and a reaction buffer [10 mM Tris/pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM dNTPs (dGTP, dATP, dTTP, dCTP)]. Three-step amplification in Pharmacia LKB gene ATAQ controller was performed, and an initial denaturation step for 5 mins at 94℃ followed by 30 cycles of 1 min at 50℃; 3 mins at 72℃; 1 min at 94℃, followed by a final cycle of 1 min at 50℃; and 10 mins at 72℃. Phenol-extracted mixture precipitated by ethanol, separated on agarose gel, transferred onto nylon membrane, and hybridized[18] with a radiolabeled probe.[29]

Isolation and analysis of poly(A)$^+$ RNA from tobacco seeds and leaves.

Poly(A)$^+$ RNA was isolated by phenol extraction and oligo(dT)-cellulose affinity chromatography from leaves and immature seeds of tobacco collected 15 to 18 days after pollination.[18] It was separated on agarose gel with 10% formaldehyde, transferred onto nylon membrane, and hybridized[18] with a radiolabeled probe.[29] Differences in RNA accumulation were quantitated by using a Toyo digital densitometer.

Purification of 10 kDa zein and preparation of antibody.

The zein-2 fraction of maize storage protein were prepared from maize kernals[30] and separated by 15% SDS-PAGE. The band of 10 kDa was sliced, extracted with extraction buffer (100 mM Tris-HCl/pH 8.0; 100 mM NaCl; 0.5% SDS; 1% β-mercaptoethanol), adjusted to 10 % trichloroacetic acid, and precipitated for 12 hs at 4℃. After centrifugation for 10 mins at 10,000 g, the pellet was resuspended in TE buffer (50 mM Tris-HCl/pH 8.0; 10 mM EDTA).

One half mg of purified 10 kDa zein was mixed with equal volume of Freund's adjuvant and injected into rabbit (NewZealand White) 3 times at the interval of 2 weeks. Serum was processed by the method of Garvey.[31]

Extraction and analysis of proteins from tobacco seeds and leaves.

Dry, mature tobacco seeds were homogenized in extraction buffer (5% SDS, 10% glycerol, 5% β-mercaptoethanol and 250 mM Tris-HCl/pH 6.8). The homogenates were boiled for 5 min, centrifuged for 3 min, and their concentration was determined with Coomassie protein assay reagent™ according to the protocol suggested by supplier.[32] 100 ng of total proteins were loaded on SDS-PAGE gel and analysed by immunoblot technique[33] using antibodies prepared against 10 kDa zein and goat anti-rabbit 2nd antibody labelled with peroxidase. ECL detection reagents were used as a substrate for peroxidase. Differences in protein accumulation were quantitated by using a Toyo digital densitometer.

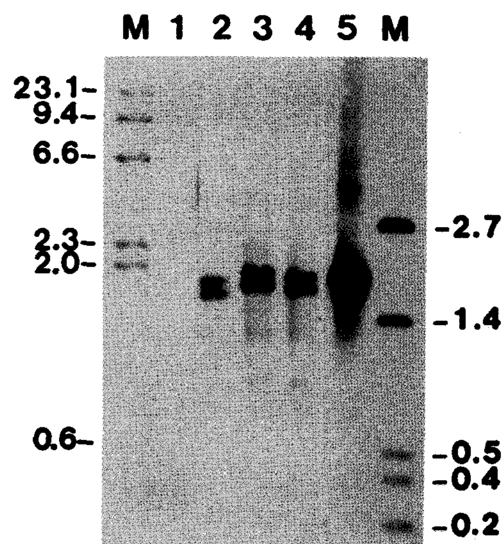

Fig. 2. Presence of transgenes in transformed tobacco plants. PCR was carried out[25,26] in a volume of 100 μl with 150 ng of T_{NOS} primer[27] and BL primer,[28] 500 ng of template DNA, 2.5 units of *Taq* DNA polymerase, and a reaction buffer. Three-step amplification was performed, and an initial denaturation step for 5 mins at 94℃ followed by 30 cycles of 1 min 50℃; 3 mins 72℃; 1 min 94℃, followed by a final cycle of 1 min 50℃; and 10 mins 72℃. Phenol-extracted mixture precipitated by ethanol, separated on agarose gel, transferred onto nylon membrane, and hybridized[18] with 32P-labeled EcoRI-fragment of p10kZ. Lane M, size marker (λ/*Hin* dIII); lane 1, amplified genomic DNA isolated from non-transgenic tobacco plant; lane 2-4, amplified genomic DNAs isolated from transgenic tobacco plants transformed with pCa10G, pCag10G and pCaΩ10G, respectively; lane 5, amplified plasmid pCa10G; lane M, size marker (pUC18/*Eco*RI+*Hin*fI).

Results

Recombinant genes are transferred into transformed tobacco plants

To test the presence of transgene in regenerated tobacco plants, genomic DNAs isolated from leaves were amplified by PCR. Amplified DNAs were separated on 0.8% agarose gel, and transferred onto nylon membrane. The membrane was hybridized with ^{32}P-labeled 450 bp *Eco*RI-fragment of *10kZ* (Fig. 2). There were specific signals only from genomic DNAs of transgenic tobacco

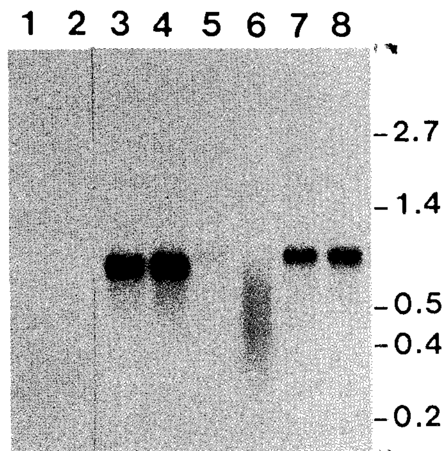

Fig. 3. Northern blot analysis for transgene expression. Poly(A)$^+$ RNAs were isolated from leaves and seeds of transgenic and non-transgenic tobacco plants through phenol extraction and oligo(dT) cellulose chromatography. One g of poly(A)$^+$ RNAs were separated on 1.4% formaldehyde agarose gel, transferred to nylon membrane, and hybridized with ^{32}P-labeled EcoRI-fragment of p10kZ. Lane M, λ/*Hind*III size marker; lanes 1 and 2, poly(A)$^+$ RNAs isolated from non-transgenic tobacco seeds and leaves, respectively; lanes 3 and 4, poly(A)$^+$ RNAs isolated from seeds and leaves of transformed tobacco with pCa10G respectively; lanes 5 and 6, poly(A)$^+$ RNAs isolated from seeds and leaves of transformed tobacco with pCag10 G, respectively; lanes 7 and 8, poly(A)$^+$ RNAs isolated from seeds and leaves of transformed tobacco with pCa 10G, respectively; lane M, pUC18/*Eco*RI+*Hin*fI size marker.

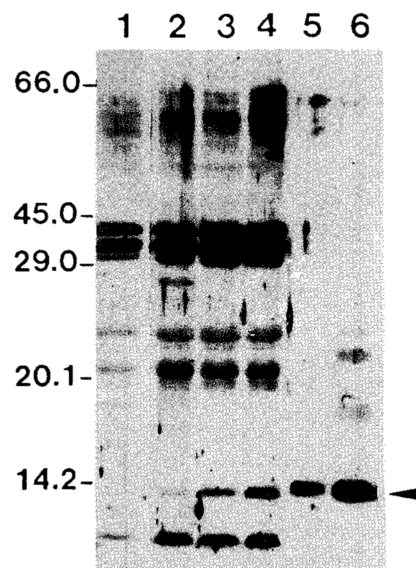

Fig. 4. Expression of 10 kDa zein (*10kZ*) protein in seeds of transgenic tobacco plants. A dry, mature tobacco seeds were extracted with the buffer (5% SDS, 10% glycerol, 5% -mercaptoethanol and 250 mM Tris-HCl/pH 6.8). The protein concentration was determined with Coomassie protein assay reagent™[32] Seed proteins (100 ng) from each construct were separated on 15% SDS-PAGE, transferred to nitrocellulose membrane, and probed with an antibody against *10kZ*. Lane 1, seed protein isolated from non-transgenic tobacco; lane 2~4, seed proteins isolated from tobacco transformed with pCa10G, pCag10G and pCaΩ10G, respectively; lane 5, purified 10 kDa zein protein; lane 6, zein-2 subfraction extracted from maize kernal.[30]

plants at about 1.6~1.7 kb as expectedly (Fig. 2, lane 2~4) suggesting the presence of transgenes. It is likely that the doublet shown from amplified DNA is due to the non-specific amplification of transgene. The amplified products of pCag10G and pCaΩ10G have slightly larger size than that of pCa10G because of the presence of leader sequences. The amplified products from the genomic DNAs of transgenic tobacco (Fig. 2, lane 2~4) showed similar results as that of plasmid pCa10G (Fig. 2, lane 5). These results demonstrate that each transgene was transferred into tobacco plants.

Transgenes are transcribed in transgenic tobacco plants

Fig. 3 shows Northern blot analysis of poly(A)$^+$ RNAs from leaves and seeds of transgenic and non-transgenic tobacco plants. ^{32}P-labeled 450 bp *Eco*RI-fragment of *10kZ* hybridized only to mRNAs from seeds and leaves of transgenic tobacco plants but no signal was detected from non-transgenic plant. The signal bands were around 880~960 nt as expectedly (Fig. 3 lane 3-8). The transcripts from each transgene are supposed to contain 21-101 nt of leader sequence (21 nt for pCa10G; 66 nt for pCag10G; 101 nt for pCa 10G; Fig. 1A), 450 nt of coding sequence, 210 nt of 3′-untranslated region (polyadenylation signal appears 210 nt downstream from translation stop codon, TAG) and about 200 nt of poly(A)$^+$ tail. Therefore, we could confirm that all transgenes were transcribed into mRNA in transgenic tobacco plants. The relative level of transcription, however, was rather variable. pCa10G transformant gave the highest and pCag10G the lowest. Especially there was quite severe degradation of mRNA in pCa10G especially from leaves. It was observed quite consistently, even though we don't know the exact nature at this moment.

Leader sequences enhance translation of *10kZ* in transgenic tobacco plants

Immunoblot analysis was carried out with an antibody for *10kZ* to investigate the effect of leaders on the translational efficiency. The antibody reacted only to the protein from tobacco plants transformed with pCa10G, pCag 10G and pCaΩ10G (Fig. 4, arrow head) demonstrating transformation and expression of transgene. The size of signal bands was pretty much the same as that of *10kZ* even though there were severe backgrounds. Because the antibody for *10kZ* is not monoclonal, it is likely to be due to a cross-reactivity of anti-*10kZ* antibody to tobacco seed proteins as noticed with maize seed protein (Fig. 4, lane 6). When equal amounts of total protein

Table 1. Relative expression level of transgenes in seeds of transgenic tobacco plants by transcription and translation.

Descriptions—Constructs	Cal0G	Cagl0G	CaΩ10G
Quantity of *10kZ* mRNA[a]	10.0	0.5	3.5
Quantity of *10kZ* protein[b]	0.8	2.7	4.5
Specific quantity of *10kZ* protein[c]	0.08	5.4	1.29
Relative translational enhancement	1.0	67.5	16.1

[a,b]densitometric unit, [c]b/a.

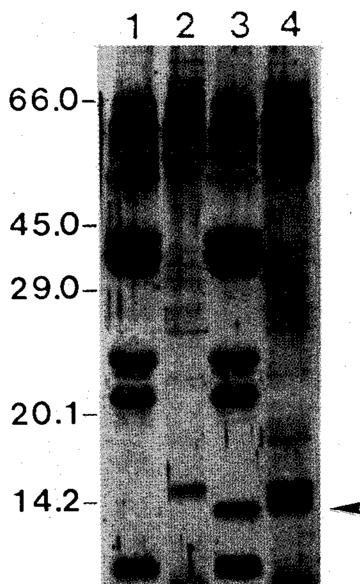

Fig. 5. Expression of 10 kDa zein (*10kZ*) protein in seeds and leaves of transgenic tobacco plants. The experiment was done according to the procedure in Fig. 4. Lanes 1 and 2, protein isolated from seeds and leaves of non-transgenic tobacco, respectively; lane 3 and 4, protein isolated from seeds and leaves tobacco transfromed with pCaΩ10G, repectively.

were loaded on gel, the intensity of the *10kZ* specific band increased in the order of pCal0G, pCagl0G and pCaΩ10G transformant (Fig. 4, lane 2~5). Transgene without the specific leader sequences (pCal0G) shows extremely low level of translation. However, pCagl0G and pCaΩ10G transformant show much higher level of protein expression than pCal0G transformant. Considering the relative level of mRNA in those transformant, these results suggest that the translation of *10kZ* mRNA is enhanced by the leader sequences, g and Ω*. Ω* from TMV could be more effective enhancer on the translation of foreign gene in tobacco plant, the natural host for TMV. Nevertheless, considering the relative amount of mRNAs and expressed proteins in transgenic tobacco plants measured by densitometric scanning of each band, enhancement by glycinin leader is the most pronounced (Table 1).

Translational enhancement by Ω* leader shows no tissue specificity

To find the tissue specificity of translational enhancement by Ω*, protein level of *10kZ* between seeds and leaves was analysed by immunoblot (Fig. 5). Only the proteins from seeds and leaves of tobacco transformed with pCaΩ10G showed signal by antibody against *10kZ* (Fig. 5, lane 3~4, arrow head). There is not much difference in the intensity of band between seeds and leaves of transgenic tobacco plant of pCaΩ10G, when equal amounts of total protein were loaded in gel (Fig. 5, lane 3~4). It demonstrates that Ω* leader sequence has no tissue-specific effect on the enhancement of translation as 35S promoter on transcription.

Discussion

Using Northern blot and immunoblot analysis, we have identified the effects of the 5′ untranslated leader sequences of TMV RNA and *Gy2* in transgenic tobacco plants. The comparison between the level of *10kZ* protein and mRNA reveals that glycinin and Ω* leader-bearing mRNA were translated much more efficiently than control without leader sequence (Table 1). Especially glycinin leader-bearing mRNA seems to be more efficiently translated than others, although pCaΩ10G containing Ω* leader was the highest in the quantity of *10kZ* protein expressed. It could reflect the fact that glycinin mRNA is translated very actively in soybean seed. We concluded that each leader sequences enhance the expression of transgene, *10kZ*, in the level of translation. Moreover, Ω* leader was demonstrated to have an effect on translational enhancement of *10kZ* mRNA in non-tissue specific manner in transgenic tobacco plants.

Like Shine-Dalgarno sequence in prokaryotes, it has been thought to be some sequence elements for translaional modulations in eukaryotic mRNA. Although there is no widespread consensus sequence in the 5′-untranslated leader of mRNA in eukaryotes, the scanning model [12,34] of Kozak predicted that translation could be mainly regulated by the primary sequence or context surrounding the AUG codon, secondary structure both upstream and downstream from the AUG codon, and by the length of leader. Several researches have shown that specific leader sequences have positive roles on the expression of foreign proteins in protoplast.[35-36] We demonstrate that these roles are due to the efficient translation of mRNA in transgenic plants. Fig. 3 and 4 show that mRNA transcribed from pCal0G, which has only 21 nt of 5′-untranslated region, had been translated less efficiently, compared to mRNAs from pCaΩ10G and pCagl0G containing relatively longer leader sequence of 66 nt and 101 nt, respectively. Short leader might impair the recognition of the first AUG codon.[37-39] It has been reported

that a purine in position −3 and a G in position +4 have the strongest effects on modulating translation.[40−41] There is pyrimidine, U, in position −3 in pCa10G which is less favorable context. Instead, Cag10G and pCaΩ10G have a purine in −3 position and a G in position +4 as are in optimum context, which might result in higher translation level than in the pCa10G. Secondary structure within a leader might reduce translational efficiency.[42−43] However, the leader sequences of each construct have little secondary structure (<−9 kcal/mol) as predicted by DNASIS program. Therefore, secondary structures within the leaders used in this study don't seem to contribute significantly. It was reported that the $(CAA)_n$ motif and the direct repeats of ACAAUUAC sequence in Ω are essential for the enhancement of translation.[9] A combination of one copy of the 8 base direct repeat and 25 base of repeated $(CAA)_n$ motif was identified as the core regulatory element, although the $(CAA)_n$ is more critical.[9] Also, there was CACAA sequence repeated twice in the leader of glycinin, which was similar to the $(CAA)_n$ motif in Ω*. Our results also demonstrate that these motifs have non-tissue specific effect in leaves and seeds because the expression levels in both tissues of pCaΩ10G transformant are almost the same (Fig. 5, lane 3∼4).

In general, transgenes under the control of CaMV 35S promoter were expressed in seeds and leaves. We also observed that transgenes under the control of CaMV 35S promoter transcribed in seeds and leaves. Unexpectedly, there was significant variation in transcription level among each transgene under the control of CaMV 35S promoter in transgenic tobacco plants. Transgene without the specific leaders (pCa10G) have several-fold more transcripts than the transgenes with them. It suggests that 5′-untranslated leader sequences could have negative effect on the transcription or the stability of transgene mRNAs. At this moment, the reason of the effect is not clear.

In conclusion, we confirm that the 5′-untranslated leader sequences of TMV and soybean glycinin gene, *Gy2*, enhance the translation of the foreign gene in transgenic plants. This effect could be directly due to the sequence motif containing multiples of CAA sequence. In order to ensure adequate expression of a transgene, 5′-untranslated leader sequences as well as promoters should be seriously selected. Furthermore, if a coding gene be heterologous to transgenic plant, the beneficial effect of the leader would be ever greater.[44]

Acknowledgement

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담배 모자이크 바이러스와 대두 Glycinin 유전자의 5′ Leader Sequence를 이용한 외래 유전자의 전이효율 증진

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초록 : 형질전환 식물체에서 외래 단백질의 발현을 높이기 위하여, 외래 단백질 유전자를 TMV RNA 또는 *Gy2* 5′-untranslated leader 부위와 재조합시킨 plasmid들을 재조하였다. pGA643에서 유래된 이들 plasmid에는 cauliflower mosaic virus의 35S promoter와 *Gy2* terminator를 포함하고 있고, 외래 유전자로는 옥수수의 10 kDa zein (*10kZ*) 유전자를 사용하였다. *Gy2* 또는 TMV RNA의 leader 부위는 promoter 부위와 단백질 coding sequence 사이에 삽입하였다. Agrobacterium을 매개로 하는 leaf disc 형질전환법을 이용하여 재조합 단백질들을 담배에 도입하였다. Leader를 포함하지 않은 도입 유전자의 전사 효율이 leader를 포함하는 도입 유전자들 보다 높았지만, leader를 포함한 mRNA들이 보다 효율적으로 전사되었다. 이는 5′-untranslated sequence의 길이와 AUG codon 주위의 context에 의한 영향보다는 이들 leader sequence의 특이적 염기서열에 의한 영향이 큼을 의미한다. 이러한 결과는 형질전환된 담배에서 외래 유전자의 전이효율을 조절하는 데 있어서 *Gy2*와 TMV의 leader sequence들이 enhancer로서 역할을 하고 있음을 의미한다.

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