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Characterization of the EMCV-IRES Mediated Bicistronic Translation in Plant Cells

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Abstract

Internal ribosome entry sites (IRES) were first identified as sequences in viral genomes that allow cap-independent translation initiation. The IRES sequence from the *encephalomyocarditis* virus (EMCV) is able to direct internal translation initiation that allows co-expression of multiple genes from one mRNA. Although EMCV-IRES has also been demonstrated to be functional in transgenic plants, the characteristics of the EMCV-IRES in plant cells have not been fully defined. Using a transient expression system by microprojectile bombardment and quantitative reporter gene assay, we have analyzed EMCV-IRES activity in plant cells. We show that the spacing between the IRES sequence and the second cistron is crucial for IRES-dependent translation efficiency. However, the order of cistrons is not an important factor for the EMCV-IRES-dependent translation in plant cells. Studies in different plant species reveal species specificity for EMCV-IRES activity. Analysis of transgenic tobacco plants suggested that the EMCV-IRES-dependent translation is suppressed in roots.

Key words: *encephalomyocarditis* virus (EMCV), gene expression, internal ribosome entry site (IRES), luciferase, translation.

Abbreviations

CaMV, cauliflower mosaic virus; DLRA, dual-luciferase reporter assay; EMCV, *encephalomyocarditis* virus; Fluc, firefly luciferase; IRES, internal ribosome entry site; nt, nucleotide; ORF, open reading frame; Rluc, *Renilla* luciferase; TMV, tobacco mosaic virus.

Introduction

Most eukaryotic mRNAs are translated in a cap-dependent mechanism in which the 40S ribosomal subunit is recruited to the 5′cap of mRNA. However, uncapped mRNAs of Picorna viruses are translated in a cap-independent manner. Internal ribosome entry site (IRES) elements were identified from the long 5′UTRs of picornaviral RNAs and shown to mediate this cap-independent translation (Jackson *et al.*, 1990). The translation of these RNAs is initiated when 40S ribosomal subunit recognizes the IRES sequences. IRES sequences have also been shown to be present in 5′UTRs of other viruses, hepatitis C virus (Tsukiyama-Kohara *et al.*, 1992; Wang *et al.*, 1993) and insect picorna-like virus(Sasaki and Nakashima, 1999; Wilson *et al.*, 2000), and some cellular mRNAs, eIF4G (Gan and Rhoads, 1996; Gan *et al.*, 1998) and cMyc (Nanbru *et al.*, 1997, 2001). It has been shown that the IRES-mediated translation also requires canonical translation initiation factors and functions in parallel with cap-dependent translation in host cells (Hellen and Sarnow, 2001).

The IRES element from the *encephalomyocarditis* virus (EMCV), one of the picornaviruses, has been studied in detail and utilized for bicistronic expression in animal cells. The EMCV-IRES is functional both in animal cells *in vivo* and in rabbit reticulocyte lysate *in vitro*. The discovery of IRES elements within the 5′UTRs of mRNAs of yeast endogenous transcription factors TFIID and HAP4 suggested that yeast cells also have a cap-independent translation initiation mechanism (Iizuka *et al.*, 1994). Although the transcription/translation mechanism in yeast is believed to be similar to that in mammalian cells, the EMCV-IRES is not functional in living yeast cells (Evstafieva *et al.*, 1993). These results suggest the existence of different

regulatory mechanisms in various cell types in which EMCV-IRES-dependent translation is involved.

In plants, 5′UTRs of genomic RNA and subgenomic RNA I_2 of crucifer infecting *tobamovirus* (crTMV) are able to function as IRES, and initiate internal translation in tobacco leaves (Ivanov *et al.,* 1997; Skulachev *et al.,* 1999). Recently, it was shown that the EMCV-IRES sequence is able to promote translation from a bicistronic construct in plants (Urwin *et al.,* 2000). These results suggest an existence of cross-kingdom cap-independent translation initiation mechanisms in eukaryotes.

Although the bicistronic vector containing the EMCV-IRES has been shown to translate two proteins simultaneously in transgenic tobacco (Urwin *et al.,* 2000), no further studies including detailed quantitative analysis have yet been described in plant cells. In this study, using two reporter genes, *Renilla* luciferase (Rluc) and firefly luciferase (Fluc), we conducted a dual-luciferase reporter assay (DLRA) that allows quantitative measurement of IRES activities. The function of the EMCV-IRES was assessed in various plant cells using a transient assay and transgenic tobacco system. In addition, we also investigated the influence of the coding sequences on the efficiency of the EMCV-IRES-based bicistronic vector.

Materials and Methods

Plasmid construction

An Rluc fragment was excised by *Nde*I-*Xba*I from pRL-null (Promega) and inserted into *Nde*I-*Nhe*I sites of pSP-luc+ (Promega). The DNA fragment containing Rluc and Fluc coding sequence was excised by *Xba*I blunt-*Nhe*I and cloned into *Sac*I blunt-*Xba*I sites of pBI221 (Clontech) to create pR-F. The EMCV-IRES sequence was amplified by PCR with the three primer sets [*Bgl*II-IRES: 5′-ggagatctTGCAGGTCGAGCATGCATCTAG-3′, IRES-*Avr*II(-1): 5′-ggcctaggTGTGGCAAGCTT ATCATCGTGT-3′, IRES-*Avr*II(0): 5′-ggcctagg TTGTGGCAAGCTTATCATCGTGT-3′ and IRES-*Avr*II(+1): 5′-ggcctaggGTTGTGGCAAGC TTATCATCGTGT-3′] using pIRESneo (Clontech) as template. In all three cases, *Bgl*II-IRES corresponding to the 568nt region upstream the ATG-10 of EMCV-IRES was used as a forward primer. For reverse primers, IRES-*Avr*II(-1), IRES-*Avr*II(0) or IRES-*Avr*II(+1) containing the ATG-10 were used. PCR products were digested with *Bgl*II and *Avr*II, and then inserted into *Bgl*II-*Avr*II sites within the intercistron of pR-F to create a series of pR-EI-F vector.

To make the pR-spacer-F, a 734bp DNA fragment was excised from 221-EGFP-C1 plasmid (Morohashi *et al.,* 2003) by *Nco*I-blunt-*Bgl*II and then inserted into *Avr*II-blunt-*Bgl*II sites within intercistron of pR-F. To make pF-R, a *Bgl*II-*Eco*RI fragment from pSP-luc+ was cloned into *Bgl*II-*Eco*RI sites of pRL-null. The resulting plasmid was digested by *Bgl*II-*Xba*I and inserted into *Bgl*II-*Xba*I sites of pSP-luc+ to create pSP-Rluc. Then the *Nhe*I-*Eco*RI fragment from pSP-Rluc containing an Rluc coding sequence was inserted into *Xba*I-*Eco*RI site of pSP-luc+ to create pSP-Fluc-Rluc. The *Xba*I blunt-*Nhe*I fragment from pSP-Fluc-Rluc was cloned into SacI blunt-*Xba*I sites of pBI221 to create pF-R. The R-EI-F(0) PCR product digested with *Bgl*II and *Avr*II was inserted into *Bgl*II-*Avr*II sites within the intercistron of pF-R to create pF-EI-R(0). Plasmid vectors for Agrobacterium-mediated transformation were constructed by excising the *Hin*dIII-*Eco*RI fragment and cloned into *Hin*dIII-*Eco*RI site of pBI121 vector.

Transient expression assay

Tobacco leaves (*Nicotiana tabacum* cv. SR1), onion epidermal cells, spinach leaves and five-day-old tobacco BY-2 cells were used for transient assays. The microprojectile bombardment and reporter gene assays were conducted as described previously (Matsuo *et al.,* 2001).

Analysis of transgenic plants

Leaves and roots of transgenic tobacco plants were homogenized in passive lysis buffer (Promega). The homogenate was clarified by centrifugation and then used for the luciferase assay with a DLRA kit (Promega). The soluble protein concentration in 1/10-diluted cell lysate was measured with a DC protein assay kit (Bio-Rad).

Results

In order to examine the efficiency of the EMCV-IRES in various plant cells, we constructed a series of vectors for transient assay by microprojectile bombardment. The 5′ UTR of the EMCV RNA genome comprises 834 nucleotides and the IRES element has been localized to between nucleotides 260 and 836. The EMCV-IRES contains 11 AUG triplets and the 11th AUG codon (AUG-11) is used as the authentic initiation site (Kaminski *et al.,* 1990). Since the EMCV-IRES used in this study is a truncated version that has no AUG-11 sequence, it is expected that the 10th AUG codon (AUG-10) that contains a favorable context sequence can be

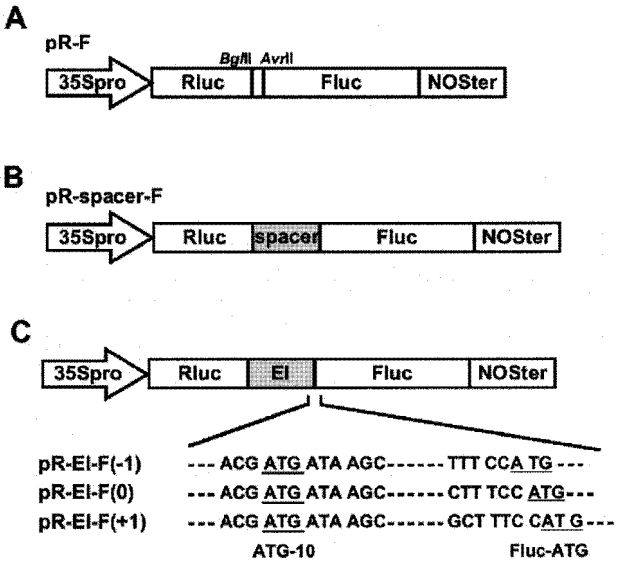

Fig. 1 Schematic diagram of bicistronic expression vectors.

Reporter genes are transcribed under control of the CaMV35S promoter and the nos terminator. The locations of putative initiation codon within EMCV-IRES, ATG-10 and the Fluc-ATG for each construct are shown. The detailed construction procedure is described in the text.

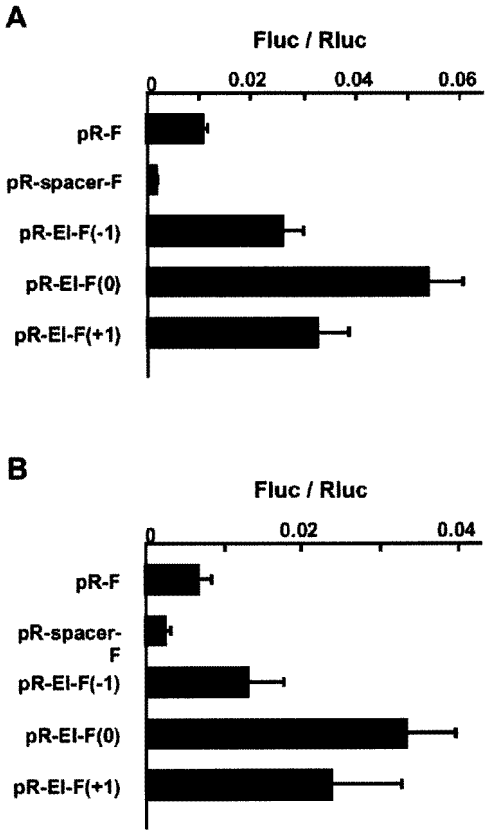

Fig. 2 Transient expression experiments in tobacco leaves (A) and tobacco BY-2 cells (B).

Plasmids were introduced into plant cells by microprojectile bombardment. Fluc and Rluc activities were measured with a DLRA system. Translation efficiency of the second cistron is shown as Fluc/Rluc value. Values are mean ± SE for three independent measurements. The absolute values for Rluc activity were between 10^4 and 10^5 U per assay.

used as an initiation site (Kozak, 1989). To examine the position effect of the downstream ORF relative to the AUG-10, we made a construct containing the CaMV 35S promoter driving expression of a bicistronic construct comprising Rluc and Fluc open reading frames separated by the EMCV-IRES to create pR-EI-F. Three derivatives of this construct were assembled, pR-EI-F(-1), pR-EI-F(0) and pR-EI-F(+1), so that the initiation codon for the Fluc coding region (Fluc-AUG) was positioned -1, 0 (in-frame) and +1 relative to the AUG-10 of the EMCV-IRES (**Fig. 1C**). Transcription from these constructs generates a single mRNA encoding two reporter proteins, Rluc and Fluc. In a further construct, pR-F, two open reading frames were connected without a spacer sequence (**Fig. 1A**). A final construct, pR-spacer-F was assembled with a 700 bp spacer sequence inserted between the two ORFs (**Fig. 1B**). The spacer sequence and the EMCV-IRES contain multiple stop codons that inhibit ribosome scanning and read-through translation of the mRNA. With these plasmid constructs, the expression level of the second cistron was compared with that from the pR-F. In this study, we quantified the activities of each luciferase reporter genes by the DLRA system (Matsuo *et al.*, 2001).

The five plasmid constructs shown in **Fig. 1** were subjected to a microprojectile bombardment-mediated transient expression assay using both tobacco leaves and tobacco BY-2 suspension cells. Samples were assayed by the DLRA 24 hours after bombardment. As shown in **Fig. 2**, a drastic increase of the Fluc/Rluc ratio was detected from the samples bombarded with plasmids containing the EMCV-IRES insert. However, consistent with the previous report, Fluc activity was barely detectable in samples bombarded with pR-spacer-F containing the 700 bp spacer insertion (Attal *et al.*, 2000). Comparison of the relative position of Fluc-ATG to the ATG-10 of the EMCV-IRES showed that the relative Fluc activity of pR-EI-F (0) is significantly higher than those of pR-EI-F (-1) and pR-EI-F (+1) in both tobacco leaves and BY-2 cells.

We investigated the influence of tissue- and cell type-specific regulation on the EMCV-IRES mediated translation efficiency by a transient expression assay using four plant cell types, tobacco leaves, tobacco BY2 suspension cells, onion epider-

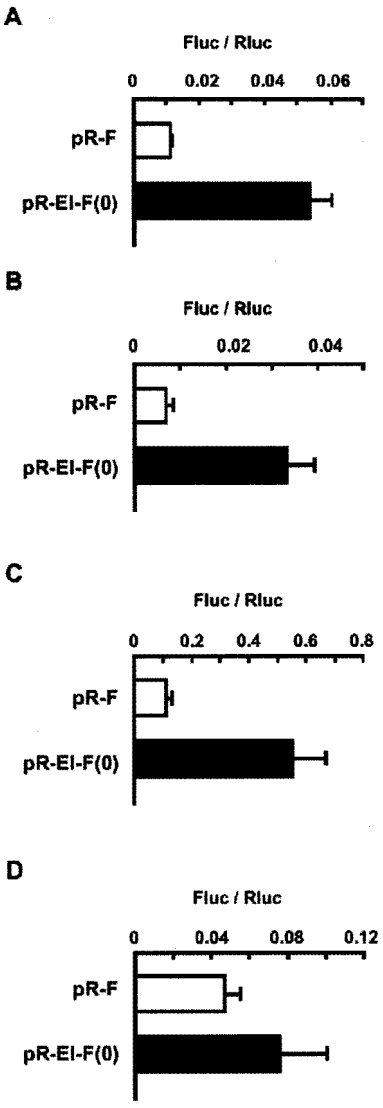

Fig. 3 Transient expression experiments in various plant cells.

(A) tobacco leaves, (B) tobacco BY-2 cells, (C) onion epidermal cells, (D) spinach leaves.

Black and white bars show Fluc/Rluc value of bicistronic vectors with or without EMCV-IRES, respectively. Values are mean ± SE for three independent measurements. The absolute values for Rluc activity were between 10^4 and 10^5 U in all assays.

mal cells and spinach leaves. Results obtained by the microprojectile bombardment of the pR-EI-F (0) shown in **Fig. 3** demonstrate that the EMCV-IRES is able to enhance the translation efficiency of the downstream cistron in the cell types tested. Although it is clear that the EMCV-IRES is active in onion and spinach cells, the relative Fluc/Rluc ratio differed with the cell type. As shown in **Fig. 3**, a higher Fluc activity was detected from onion epidermal cells, on the other hand, the EMCV-

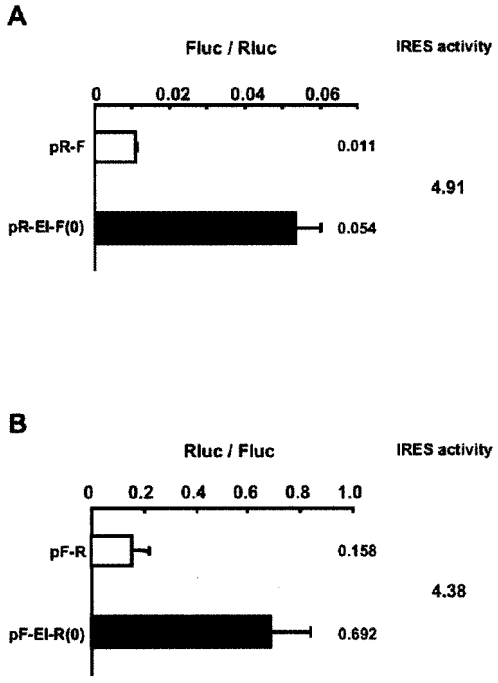

Fig. 4 The EMCV-IRES activity is insensitive to the positional exchange of two reporter genes in plant cells.

Bicistronic vectors, using either Rluc (A) or Fluc (B) as first cistron, were transiently expressed in tobacco leaves. The EMCV-IRES activity is shown as fold increase of relative activity by the EMCV-IRES insertion. Values are mean ± SE for three independent assays. The absolute values for first cistron were between 10^4 and 10^5 U for Rluc and between 10^3 and 10^4 U for Fluc.

Table 1 Activities of the EMCV-IRES mediated translation in transgenic tobacco. Leaves from indepenndent lines were used for DLRA assay.

Construct	line	Fluc activity[1)]	Rluc activity[1)]	Fluc / Rluc ($x10^{-4}$)
pR-F	1	1.279	5795	2.2
	2	0.647	2771	2.3
	3	1.439	3879	3.7
pR-EI-F(0)	1	10.83	2377	45.6
	2	238.8	593.9	4021
	3	387.0	1656	2337

[1)] Luciferace activities are the average of photon counts per second by the luminometer.

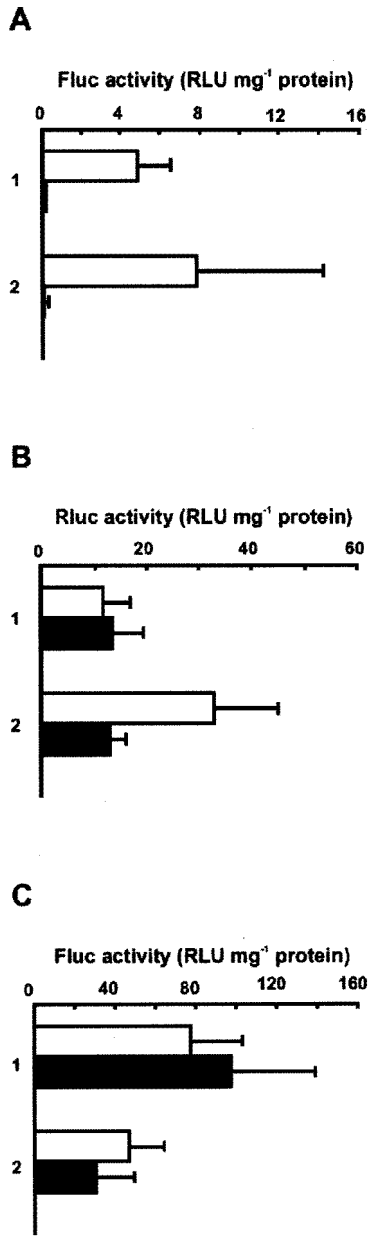

Fig. 5 Reporter activities in transgenic tobacco leaves and roots.

Activities of the IRES-mediated Fluc activity derived from pR-EI-F(0) (A) and cap-dependent Rluc activity derived from pR-EI-F(0) (B) were measured in two independent pR-EI-F(0) transgenic tobacco plants. Activities of the cap-dependent Fluc were measured in monocistronic 35S::Fluc transgenic tobacco plants (C). Luciferase activities per soluble protein in extracts from each tissue are shown. White and black bars show activity in leaves and roots, respectively. Values are mean ± SE for three independent assays.

IRES mediated enhancement of Fluc activity of spinach leaves was relatively low. In both onion and spinach, the in-frame construct pR-EI-F(0) promoted the highest efficiency of Fluc internal initiation of translation (data not shown).

Previous studies on functional characterization of the EMCV-IRES in plants exploited GFP with Fluc as the second cistron in a bicistronic construct (Urwin *et al.*, 2000). However, these data were qualitative and not quantitative. In order to obtain quantitative data, we used Rluc and Fluc reporters for transient assays of the EMCV-IRES bicistronic vector. However, the use of Fluc as first cistron in such constructs has been reported in animal cell to negatively affect the EMCV-IRES mediated translation initiation of second cistron (Hennecke *et al.*, 2001). In order to examine the possible involvement of sequence within the coding region of reporter genes in translation efficiency, we tested the effect of Rluc and Fluc on the EMCV-IRES activity by exchanging the first with the second cistron. A transient assay by microprojectile bombardment on tobacco leaves (**Fig. 4**), onion epidermal cells and tobacco BY-2 cells (data not shown) showed no significant difference in EMCV-IRES activity as estimated by the fold increase by the insertion of IRES sequence. These results suggest that the activity of EMCV-IRES mediated translation initiation is independent of the sequence of reporter genes. These results also suggested that the coding region of Fluc and Rluc do not contain sequences that regulate the EMCV-IRES activity in the assay system exploited in this study.

Although a previous study on EMCV-IRES in plant was carried out using a transgenic tobacco system, no quantitative assays were presented. To obtain quantitative data for EMCV-IRES efficiency, we generated transgenic tobacco plants harboring pR-EI-F(0) and pR-F by *Agrobacterium*-mediated transformation (McCormick *et al.*, 1986). Using mature leaves obtained from three independent transgenic lines of each construct, we investigated the expression levels of each reporter gene (**Table 1**). In three independent transgenic lines tested, Rluc activity was slightly reduced by the presence of EMCV-IRES, however, Fluc expression efficiency as estimated by Fluc/Rluc ratio increased 10-1000 times by the EMCV-IRES insertion.

Fig. 5 shows a comparison of Rluc and Fluc activity in leaves and roots of pR-EI-F(0) transgenic tobacco plants. In all lines tested, Fluc activities of roots were lower than that of leaves and barely detectable by the dual luciferase assay (**Fig. 5A**). Although slightly reduced levels were observed, Rluc activity in roots was comparable to that in leaves. The expression pattern of Rluc in plants transformed with pR-EI-F(0) was similar to that of Fluc in plants harboring 35S::Fluc (**Fig. 5 B, C**).

These results are consistent with the previous observation that the IRES-mediated Fluc expression is dramatically reduced in hypocotyls and roots (Urwin *et al.,* 2000), but provides quantification of the differences.

Discussion

A previous study clearly demonstrated that the EMCV-IRES is able to initiate internal translation in plants (Urwin *et al.,* 2000). However, these studies relied on non-quantitative *in vivo* imaging data and the relative expression level of the second cistron directed by the EMCV-IRES insertion was not determined. In this study we used Fluc and Rluc for quantitative assay of the EMCV-IRES activity with the DLRA system. Also, we have exploited a transient expression by microprojectile bombardment for the rapid and reproducible assay for the evaluation of the EMCV-IRES-mediated translation efficiency.

As a part of our efforts to define the precise requirements of IRES activity, we have shown that a single base pair insertion or deletion between the IRES and the downstream ORF that shifts the reading frame by +/- 1 resulted in a dramatic change in the IRES activity. These data demonstrate the importance of the relative position of 3' AUG of the IRES and the initiation AUG of the second ORF for optimal efficiency of the IRES-mediated translation enhancement. Similarly, it is possible that the relative position of the first ORF to the IRES sequence is also an important factor when considering the efficiency of IRES-mediated translation. The effect on the relative position of the first ORF to the IRES sequence remains to be investigated.

The composition and arrangement of ORFs have been reported to be important factors for the IRES-dependent translation of the second cistron (Hennecke *et al.,* 2001). In this study, however, we found no evidence of the influence of the coding sequence on the EMCV-IRES-mediated translation efficiency as demonstrated by similar rations of activity irrespective of which ORF was first and which was second. The difference in result of the influence of coding sequence composition on IRES activity from previous studies (Hennecke *et al.,* 2001) and our observations may be due to the difference in assay system and the IRES sequence. Also, it is possible that differences in plasmid construction for the IRES-mediated bicistronic vector influence the efficiency of IRES-mediated translation. In our experiments, we constructed an IRES-ORF fusion to base pair precision in an optimal design by PCR that allows efficient translation of the second ORF. In the only previous study of EMCV-IRES activity in plant cells, the plasmid constructs were made by using restriction enzyme sites that may result in "out-of-frame" constructs with reduced efficiency of the second ORF translation (Urwin *et al.,* 2000). Further study will be required for the elucidation of mechanisms by which IRES efficiency is regulated.

Although the EMCV-IRES has been shown previously to be active in transgenic tobacco (Urwin *et al.,* 2000), other previous studies indicated that the EMCV-IRES is not functional in the wheat germ extract system *in vitro* (Skulachev *et al.,* 1999; Matsuo *et al.,* unpublished). These results suggest that the EMCV-IRES activity may vary with the plant cell type or species. The results of transient expression experiments showed that the EMCV-IRES is functional in all four plant cells tested, indicating that the EMCV-IRES-mediated translational enhancement of transgenes can be applicable to a wide range of plant species. Although the Fluc/Rluc ratio derived from pR-EI-F(0) was higher in onion epidermal cells than in tobacco leaves, BY-2 cells or spinach leaves, the differences in IRES activity might depend on the property and configuration of the reporter gene in different plant cells. A previous study indicated that the Fluc activity is less stable than the Rluc activity (Matsuo *et al.,* 2001). Since the stability of Fluc varies with the cell type, relatively high Fluc activity in onion epidermal cells in this assay system may be due to the difference in cell type-dependent Fluc stability. Further analysis will be required for the elucidation of the cell-type specificity in the EMCV-IRES-mediated translational enhancement.

We have shown that the translation efficiency of downstream ORF increased more than 1000 times by the insertion of the EMCV-IRES in transgenic tobacco plants (Table 1). On the other hand, the effect of the EMCV-IRES insertion appeared to be rather modest in the transient assay by microprojectile bombardment. As shown in **Fig. 4**, the observed translational enhancement of the downstream ORF by insertion of the EMCV-IRES was up to five-fold. The relatively low efficiency of the EMCV-IRES activity in the transient assay system is mainly due to the relatively high expression levels of downstream ORF in pR-F. These results therefore suggest that the internal initiation or leaky scanning occur more often in the transient assay system exploited in this study compared to the transgenic plant harboring an identical promoter-reporter construct.

Data obtained by the analysis of transgenic plants indicate that the activity of Rluc in pR-EI-F(0) and Fluc in 35S::Fluc are essentially similar suggesting

the absence of tissue specificity in the cap-dependent translation efficiency. Insertion of the EMCV-IRES element does not appear to influence the efficiency of cap-dependent translation because the expression level of the first ORF, Rluc, is unaffected by the presence of IRES sequence. However, low levels of Fluc activity in roots of transgenic plants harboring pR-EI-F(0) suggest that the EMCV-IRES-mediated internal translation is not efficiently initiated in root tissue (**Fig. 5**). These results suggest the possibility of tissue-specific regulation of the EMCV-IRES activity in transgenic tobacco.

Such spatiotemporal variations in IRES-mediated translation have been studied in transgenic mice by comparing the IRES from fibroblast growth factor 2 (FGF-2) mRNA and the EMCV-IRES using dual luciferase bicistronic vectors. Although the IRES from FGF-2 mRNA exhibited strong tissue specificity, the EMCV-IRES activity was high in most adult organs and no spatiotemporal variations were observed (Creancier *et al.*, 2000). It is interesting to note that the EMCV-IRES shows tissue specificity in plant cells but not in its original host cells. Further studies on the EMCV-IRES mediated translational enhancement in plant cells should provide insight into mechanisms of spatiotemporal regulation of translation in plant gene expression and development.

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